The PCNA–RFC Families of DNA Clamps and Clamp Loaders

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The proliferating cell nuclear antigen PCNA functions at multiple levels in directing DNA metabolic pathways. Unbound to DNA, PCNA promotes localization of replication factors with a consensus PCNA-binding domain to replication factories. When bound to DNA, PCNA organizes various proteins involved in DNA replication, DNA repair, DNA modification, and chromatin

Abbreviations: Pol α, δ, ε, DNA polymerase α, δ, ε; PCNA, proliferating cell nuclear antigen; RPA, single-stranded binding protein; RFC, replication factor C; Rfc2-5, complex of Rfc2, Rfc3, Rfc4, and Rfc5; RFC–Rad24, complex of Rfc2-5 and Rad24; RFC–Ctf18, complex of Rfc2-5 and Ctf18; RFC–Ctf18(7), complex of Rfc2-5, Ctf18, Dcc1, and Ctf8; Rad17/3/1, complex of Rad17, Mec3, and Ddc1; h9/1/1, complex of human Rad9, Rad1, and Hus1; ATPγS, adenosine 3-thio-triphosphate.

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modeling. Its modification by ubiquitin directs the cellular response to DNA damage. The ring-like PCNA homotrimer encircles double-stranded DNA and slides spontaneously across it. Loading of PCNA onto DNA at template–primer junctions is performed in an ATP-dependent process by replication factor C (RFC), a heteropentameric AAA^+ protein complex consisting of the Rfc1, Rfc2, Rfc3, Rfc4, and Rfc5 subunits. Loading of yeast PCNA (*POL30*) is mechanistically distinct from analogous processes in *E. coli* (β subunit by the γ complex) and bacteriophage T4 (gp45 by gp44/62). Multiple stepwise ATP-binding events to RFC are required to load PCNA onto primed DNA. This stepwise mechanism should permit editing of this process at individual steps and allow for divergence of the default process into more specialized modes. Indeed, alternative RFC complexes consisting of the small RFC subunits together with an alternative Rfc1-like subunit have been identified. A complex required for the DNA damage checkpoint contains the Rad24 subunit, a complex required for sister chromatid cohesion contains the Ctf18 subunit, and a complex that aids in genome stability contains the Elg1 subunit. Only the RFC–Rad24 complex has a known associated clamp, a heterotrimeric complex consisting of Rad17, Mec3, and Ddc1. The other putative clamp loaders could either act on clamps yet to be identified or act on the two known clamps.

I. Introduction

A recurring theme in DNA metabolism is that of proteins which adopt ring-like structures encircling the DNA. Proteins ranging from processivity factors in DNA replication to DNA helicases and nucleases have been shown to encircle the DNA (reviewed in *1*). Even though these proteins show no evolutionary relationship to each other and have widely varying enzymatic activities, or none at all, they all have reached the common goal of processive action on the DNA by encircling the nucleic acid they act on. Commonly, the proteins clamping around the DNA form homo- or heterooligomers and it is the quaternary structure of the oligomer that adopts the shape of a ring. However, exceptions exist; for example, the monomeric topoisomerase I and the nuclease FEN1 encircle the substrate DNA that they act on (*2–5*).

This review focuses on the eukaryotic processivity factor PCNA (proliferating cell nuclear antigen) and PCNA-interacting proteins, its associated clamp loader RFC (replication factor C), and related clamps and clamp loaders important for such disparate functions as the DNA damage checkpoint and the establishment of chromatid cohesion in eukaryotic cells.

II. The *E. coli* Paradigm for a Clamp–Clamp Loader System

The concept that a processivity factor could impose its function by encircling the DNA was established over 10 years ago by O'Donnell and coworkers. In an elegant biochemical study they showed that the β subunit of *E. coli* DNA polymerase III holoenzyme was sterically prohibited from dissociating off a circular double-stranded DNA molecule. Upon restriction endonucleolytic cleavage of the circular DNA, the β subunit dissociated by sliding off the ends—hence the name *sliding clamp* (6). Subsequent determination of the crystal structure of the β subunit beautifully confirmed these biochemical studies (7) (Fig. 1). The *E. coli* DNA sliding clamp is a homodimer that binds the DNA it encircles without sequence specificity. Obviously, geometric restrictions mediate stability of the clamp onto the DNA. By inference, any protein forming strong protein–protein interactions with the clamp also remains stably associated with the DNA. In the case of a DNA polymerase, this stable interaction enables processive DNA replication by the polymerase.

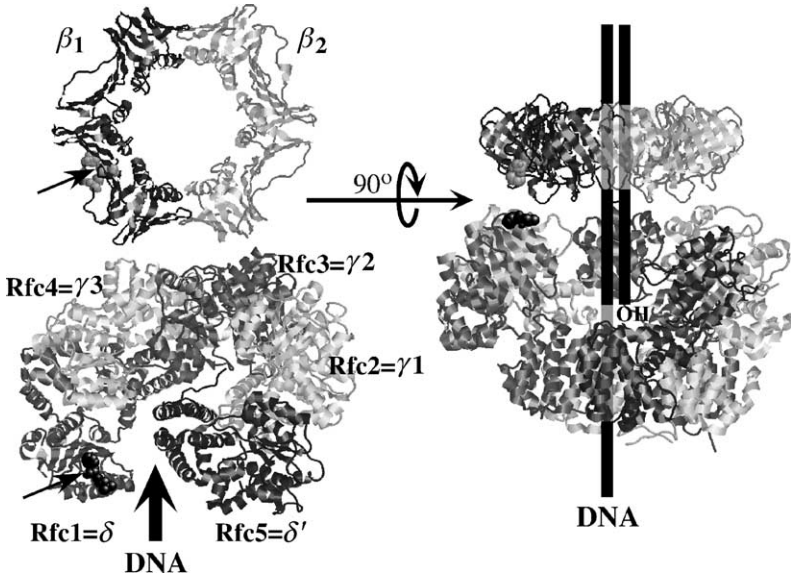

FIG. 1. Crystal structures of the β subunit and γ complex of *E. coli* DNA polymerase III holoenzyme. RasMol cartoon representations of the two structures are given (7, 12). The left panel is a top view of both structures and the right panel a side view after a 90° rotation of both structures. The proposed orientation of the substrate DNA is shown. Indicated as space-filling models (see arrows in left panel) are the hydrophobic amino acids of δ (L73, F74) that insert into a hydrophobic cavity of β (L177, P242, V247) and are thereby thought to cause ring opening (13). Structural homology at the subunit level between the γ complex and RFC is indicated.

The advantages of having a clamp–DNA polymerase complex for performing processive DNA replication, rather than a processive DNA polymerase because of its strong DNA-binding properties, is that the former complex in principle allows for facile release of torsional stress. As the DNA polymerase replicates DNA, it goes through a complete rotation every 10 nucleotides. For a replisome in which leading and lagging strands form a coordinated complex, and perhaps are even stably fixed in the cell in a replication focus, the threading of the DNA through the replisome would build up torsional stress in the leading DNA strand. Release of this torsional stress can be accomplished by temporary release of the DNA polymerase from the template–primer junction. However, because of its interactions with the clamp, connectivity of the polymerase with the DNA would be maintained. After release of torsional stress by rotation of the DNA within the cavity of the clamp, the polymerase could rebind to the primer terminus to continue DNA synthesis. It is perhaps for this reason that circular sliding clamps are found throughout nature from bacteriophages to human cells. Remarkably, their basic mode of action is very similar, although significant mechanistic differences exist between the various systems, as described later.

Formation of the protein ring around the DNA is an energetically driven process, which is carried out by a specific complex called the clamp loader. The clamp loader uses the energy of ATP to open up the clamp and load it around double-stranded DNA at template–primer junctions. The clamp loader for the β subunit sliding clamp is a complex consisting of the γ, τ, δ, δ', ξ, and χ subunits of DNA polymerase III holoenzyme (*8–10*). This complex is not only involved in clamp loading but also in coordinating the leading and lagging strands at the replication fork (*11*). However, the simplest form of the clamp loader that will efficiently load the β subunit in the test tube is the γ complex, a pentamer with the stoichiometry of one each of the δ and δ' subunits and three γ subunits (*12*). ATP binding is localized to each of the three γ subunits. The crystal structure of the pentameric γ complex together with that of a complex between the β and δ subunits provides clear insights in the mechanism of clamp loading (Fig. 1) (*13*). The core of the interface between the β and δ subunits involves three amino acids from δ, which form a plug that inserts into a hydrophobic pocket in β. Steric considerations show that only one δ subunit can be accommodated per β dimer. Although this insertion of δ is not at the dimer interface of β, the conformational changes accompanying the insertion of the hydrophobic plug are proposed to lead to long-distance changes that open the dimer interface proximal to the δ insertion site (*14*).

The clamp loader subunits belong to the class of the AAA^+ superfamily of proteins in which ATP binding and its hydrolysis is coupled to assembly and disassembly reactions (*15*). Like in other AAA^+ complexes, each subunit of the clamp loader that binds ATP has this ATP molecule localized at the interface with a neighboring subunit. This neighboring subunit positions an arginine

residue, which is part of an evolutionary conserved Ser-Arg-Cys motif (called sensor I motif, or the arginine finger), at the ATPase active site. The arginine residue promotes hydrolysis of bound ATP. In the $\delta'\gamma_3\delta$ complex, there is one arginine finger in each of the three γ subunits (*12*). The δ' subunit does not bind ATP, but it has an arginine finger which in the crystal structure of the complex is appropriately positioned to activate hydrolysis of the ATP bound to the γ_1 subunit (Fig. 1). During the termination phase of clamp loading, ATP hydrolysis is thought to proceed sequentially around the clamp loader ring; for example, δ' promotes hydrolysis of the ATP in γ_1, followed by γ_1-stimulated hydrolysis of the ATP in γ_2, and finally γ_2-stimulated hydrolysis of the ATP in γ_3.

III. The Eukaryotic Sliding Clamp PCNA

A. Structure of the Sliding Clamp

PCNA is the ortholog of the β subunit in eukaryotic cells. Despite very limited sequence conservation between β and PCNA at the amino acid level, the structures of the two proteins are remarkably alike. This structural similarity extends to all circular processivity clamps, including the sliding clamps from bacteriophages and PCNAs from archaeons (*7, 16–19*). A distinction between the eubacterial clamps and all other clamps is that the former are homodimers and clamps from bacteriophages, archaeons, and eukaryotes are heterotrimers. Each subunit of the *E. coli* β dimer is a 366-amino-acid protein containing three domains with similar fold and topology, whereas each subunit of the trimeric clamps is a protein of 227–261 (from T4 to human) amino acids containing two similar domains. Therefore, the complete circular assembly of each sliding clamp has a six-fold symmetry (Figs. 1 and 2).

Each of the six domains consists of a series of antiparallel β strands that forms a curved β sheet. Subunit–subunit interactions are also mediated by antiparallel β strands. Jointly, these six curved β sheets constitute a donut-like scaffold. Twelve α helices, two each per domain, are positioned on the inside of this scaffold in a direction roughly perpendicular to the face of the ring so that all twelve helices together form a flat cylindrical shape. Obviously, the PCNA ring has distinct front and back faces. On the front face is the C-terminal tail of each monomer and a long loop (the interdomain connector loop), which links the N- and C-terminal domains of each monomer. The front face of the ring has been shown to contact many DNA replication proteins and is oriented in the direction of DNA synthesis (*20*).

The inner cavity of the protein ring has a diameter of about 30 Å, which is much larger than that required to fit around double-stranded B DNA with a diameter of 20 Å. Even though PCNA has an overall negative charge, the inner surface of the ring has a positive electrostatic potential. As many as 10

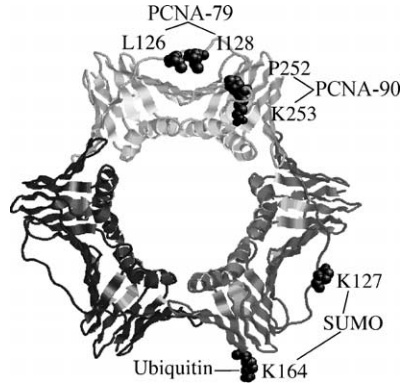

FIG. 2. RasMol cartoon representation of the structure of yeast PCNA (*16*). The three subunits are indicated in different shades of gray. Indicated in the subunit at twelve o'clock are the invariant amino acids (L126, I128) important for DNA-independent interactions between PCNA and proteins with a consensus PCNA-binding motif, and the invariant amino acids (P252, K253) important for DNA-dependent interactions of these proteins. Indicated in the subunit at four o'clock are modification sites on PCNA.

positively charged amino acids per subunit protrude from the α helices inward into the central cavity. These are counteracted by only three negatively charged amino acids. This ring of net positive charge could form favorable through solvent interactions with the negatively charged phosphodiester backbone of the double-stranded DNA.

The topological arrangement of the two domains in the PCNA monomer is such that the C-terminus of Domain 1 is separated from the N-terminus of Domain 2 by a distance of about 40 Å, which is bridged by a long crossover loop. This 16-amino-acid-long loop (residues 119–134), the interdomain connector loop, has only few interactions with the β sheet it passes across. Of particular interest in this loop are amino acids Leu126 and Ile128, which form an exposed hydrophobic cleft. Although in the loop there is little sequence conservation between PCNAs from various organisms, these two amino acids are invariant in all eukaryotic PCNAs. The cell cycle regulator p21 binds to PCNA and inhibits its activity (*21*). A 22-residue peptide derived from the C-terminus of p21 contains the sequence **Q**TS**M**TD**FY**, which is a motif conserved in virtually all PCNA-binding proteins (conserved residues in bold, see later). The crystal structure of human PCNA complexed with this p21 peptide shows extended interactions between the peptide and the interdomain connector loop (*17*). In this structure, the tyrosine and methionine of the PCNA-binding motif insert into the hydrophobic cleft formed largely by Leu126 and Ile128 of the interdomain connector loop, whereas the phenylalanine rests as a lid on top of this hydrophobic cluster. The hydrophobic pocket in human

PCNA that binds to the methionine and tyrosine of p21 is less exposed in the structure of yeast PCNA and may have been induced in human PCNA on binding of the p21 peptide. Except for this loop region, the structures of human and yeast PCNA overlap closely in all other regions of the molecule (*16, 17*).

B. Proteins Interacting with PCNA

An ever-increasing number of proteins that function in a variety of DNA metabolic pathways and cell cycle control have been shown to directly interact with PCNA. Table I lists only those PCNA-interacting proteins for which it has been shown that interaction with PCNA has functional consequences or that mutation of the PCNA-interaction motif shows phenotypic defects. The list of proteins that have been shown to interact directly with PCNA but for which the functional importance of this interaction has not yet been established is much larger; it is not included here, but reviewed elsewhere (*53–55*).

Although it is commonly accepted that functional PCNA–protein interactions occur in DNA-bound complexes in which PCNA encircles the DNA, there is strong evidence for the functional importance of binary PCNA–protein complexes (*37*). The N-terminal 20 amino acids of DNA ligase I serve as a replication focus targeting sequence. This short peptide sequence, which is both necessary and sufficient to target fluorescent markers to replication foci, contains the PCNA consensus binding motif **Q**xx[**I/L/M**]xx**F**[**F/Y**]. Mutation of the two phenylalanines to alanines abolishes both PCNA binding and targeting. With the exception of the catalytic subunit of DNA polymerase δ (Pol δ), all PCNA-binding proteins listed in Table I use this motif for interaction with PCNA. Therefore, PCNA may play a central role in recruitment and association of DNA replication proteins to replication foci. DNA repair proteins could similarly be localized to repair foci by association with PCNA.

The stimulation of DNA replication and repair enzymes by PCNA can generally be attributed to increased binding of these proteins to their DNA substrate through interactions with PCNA encircling the DNA. This simple model explains the PCNA-mediated increase in processivity by several DNA polymerases as well as their increased efficiency in the bypass of damage on the template DNA. It also explains the stimulation of several other DNA metabolic enzymes by PCNA (Table I). From the many studies of PCNA-binding proteins to date, a general theme for the functional significance of PCNA binding is emerging. On the one hand, there is a large class of consensus PCNA-binding proteins to which most interacting proteins appear to belong, exemplified by p21 and FEN1. These proteins may have a bifunctional interaction with PCNA. Whereas DNA-independent interactions promote protein localization, DNA-dependent interactions with PCNA promote increased protein binding to the substrate DNA. On the other hand, there are more specialized proteins, which include the clamp loader RFC and Pol δ, which bind at unique sites.

TABLE I
PCNA INTERACTING PROTEINS[a,b]

Protein	Effect of PCNA *in vitro*	Phenotype *in vivo* of motif mutation	References
Pol δ			22, 23
Catalytic/second subunit	Processivity, lesion bypass		35, 37
Pol32 (Cdc27)		Lethal, mutagenesis defect	24–26
Pol ε	Processivity		27
Pol κ	Catalytic efficiency, lesion bypass		28
Pol ι	Catalytic efficiency, lesion bypass		29
Pol η	Catalytic efficiency	Comparable to *rad30*-Δ	30, 31
Pol λ	Processivity, lesion bypass		32, 46
Pol ϕ	Processivity		33
FEN1	Stimulates nuclease	Partial *rad27* defect	34–36
DNA ligase I	Stimulates ligase	Mislocalization of ligase; defect in long-patch BER	37–39
MSH3		MMR defect	40
MSH6	MMR complex mobilization	MMR defect	40–42
MCMT	Stimulates methylation		43
APN2	Stimulates endonuclease		44
MYH		Mutator	45, 46
CAF-1	Chromatin assembly	Silencing defect	47, 48
P21	Inhibition of replication		49–51
ING1		No induction of apoptosis	52

[a]Only those proteins are listed for which it has been shown that interaction with PCNA has functional consequences or that mutation of the PCNA–interaction motif shows genetic defects.
[b]RFC and the SUMO and ubiquitin modification machineries are not included.

Other machineries that show more complex binding patterns are those involved in heterochromatic silencing and the SUMO- and ubiquitin-modification machinery for PCNA (47, 48, 56–59).

The knowledge that a large number of PCNA-binding proteins often participate in the same DNA metabolic pathway raises the question of how all these interactions are coordinated on the homotrimeric ring. For instance, during lagging strand DNA replication, multiple interactions exist between Pol δ and PCNA (see Section III.D), and, in addition, binding of both FEN1 and

DNA ligase to PCNA needs to be maintained for proper coordinated function of these three enzymes. Possible further complications in accommodating binding may exist if mismatch repair is tightly coupled to DNA replication, because Msh3, Msh6, and Mlh1 interact with PCNA (*60, 61*). An *in vitro* study of complex stability during Okazaki fragment maturation has shown that Pol δ and FEN1 form a stable and processive maturation complex with PCNA, whereas DNA ligase periodically enters this complex and performs ligation if a ligatable nick is present (*62*). In at least one archaeon, the problem of coordinating binding of the lagging strand maturation enzymes appears to have been solved by the evolvement of a PCNA heterotrimer, with one monomer showing preferential interactions with the DNA polymerase, the second monomer with FEN1, and the third monomer with DNA ligase (*22*).

C. FEN1 as a Model for PCNA-Interacting Proteins

FEN1 is a 5′-3′ flap exo-/endonuclease that plays an important role in multiple DNA metabolic processes, including Okazaki fragment maturation, nonhomologous end joining, and base excision repair. PCNA forms a complex with FEN1 and greatly stimulates its nuclease activity (*29*). Stimulation of the nuclease requires that PCNA encircle the substrate DNA. Kinetic studies indicate that PCNA does not guide FEN1 to the DNA cleavage site; rather, PCNA enhances FEN1 binding stability on the substrate DNA, allowing for greater cleavage efficiency (*63*). The crystal structure of FEN1 from *Pyrococcus furiosus* shows that its PCNA-binding motif (330**Q**ST**L**Q**S**W**F**337) protrudes like a finger from the otherwise globular structure of the protein, with the hydrophobic tryptophane and phenylalanine residing at the tip of the finger (*4*). This domain can easily be modeled into the hydrophobic pocket at the interdomain connector loop region of PCNA. Reports that FEN1 competes with p21 for binding to PCNA are consistent with this notion that FEN1 and P21 bind similarly to PCNA (*51, 64*). However, recent studies of PCNA–FEN1 interactions on the substrate DNA suggest that this coherent view of PCNA–protein interactions may have to be modified when PCNA encircles the DNA.

These conclusions derive from the study of complex stabilities of FEN1 with mutant yeast PCNAs on or off the DNA substrate (*36*). Mutation of the evolutionary invariant Leu126 and Ile128 residues in pcna-79 (IL126, 128AA) is expected to destroy the hydrophobic pocket in the interdomain connector loop and thereby eliminate FEN1 binding to PCNA. Conversely, the invariant amino acids Pro252 and Lys253 in the C-terminal tail localize approximately 15 Å distant from this hydrophobic pocket, and mutations of these residues in pcna-90 (PK252, 253AA) should not affect interaction with FEN1. As expected, binary interactions between FEN1 and pcna-90 were comparable to those in wild type, whereas FEN1 failed to interact with pcna-79. Surprisingly, however, the opposite result was obtained when stimulation of FEN1

nuclease activity by PCNA was measured; that is, pcna-79 was still very active in stimulating FEN1 nuclease activity, but pcna-90 stimulated very poorly. In agreement with these functional studies were complex stability studies which showed that no stable ternary DNA–FEN1–pcna-90 complex could be isolated, whereas a stable DNA–FEN1–pcna-79 complex could be isolated. These studies strongly indicate that when PCNA is loaded onto DNA, it undergoes structural changes, which reveal a new domain at the C-terminus for binding FEN1, and it is this domain that is essential for both PCNA–FEN complex formation onto the DNA and proper positioning of FEN1 for enzymatic activity. The lack of an observed interaction between FEN1 and the C-terminus of PCNA in the absence of DNA suggests that this binding domain only becomes exposed when PCNA encircles the DNA (*36*).

This model of distinct DNA-dependent interaction sites on PCNA extends to other proteins that have a consensus PCNA-binding motif. The yeast AP-endonuclease Apn2 forms a binary complex with PCNA by interactions with the interdomain connector loop, whereas the functional ternary DNA–Apn2–PCNA complex requires an intact C-terminal tail of PCNA (*44*). Similar conclusions were reached for interactions with a subunit of Pol δ (see later).

D. Multiple Specialized Interactions Between PCNA and Pol δ

The replication clamp PCNA was initially identified as a processivity factor for mammalian Pol δ (*65*). Soon afterward, this auxiliary factor was shown to be identical to a required factor for SV40 DNA replication (*66*). Pol δ from *S. cerevisiae* has three subunits of 125, 55, and 40 kDa (*67*). An additional small fourth subunit is found in the enzymes from *S. pombe* and human (*68–70*).

The catalytic subunit from human cells, p125, has been shown to interact directly with PCNA in studies by two groups (*71–73*). However, by multiple methods, including two-hybrid analysis, pull down experiments, farwestern analysis, and coimmunoprecipitation analysis, several other groups did not detect an interaction of PCNA with the large human subunit or the large yeast subunit (*67, 74–77*). Recently, an interaction was reported between the second subunit of Pol δ and PCNA (*75*). Again, studies by others with the human or yeast second subunit failed to detect such an interaction (*67, 72, 73, 77*). Furthermore, whenever an interaction has been reported, it has been a weak one at best. Neither of these two subunits has a consensus PCNA-binding motif.

The catalytic subunit and the second subunit of Pol δ form a stable heterodimeric complex. Regardless of the controversies regarding the binary PCNA–Pol δ binding studies, DNA synthesis by the two-subunit enzyme from all sources investigated is stimulated by PCNA, and, therefore, by implication this assembly must interact with PCNA on template-primer DNA (*78–82*). In fact, PCNA was originally purified by using a processivity assay with the bovine two-subunit enzyme (*65*). The synthetic activity of the recombinant human p125

subunit alone is not stimulated by PCNA; the second subunit is required for stimulation (*78, 79*). These observations suggest the importance of a PCNA-binding domain in the second subunit, or a requirement for this subunit to unmask a buried PCNA-binding site in the catalytic subunit, or the requirement for cooperative interactions between the two subunits in order to generate a functional PCNA-binding domain.

There is excellent agreement across all species that the third subunit of Pol δ, *S. cerevisiae POL32*, *S. pombe Cdc27*, or mammalian p68/p66, binds PCNA (*24, 26, 73, 74, 77, 83*). This subunit has a consensus PCNA-binding motif at the extreme C-terminus of the protein. Severe growth defects are associated with mutations in the PCNA-binding domain of *S. pombe* Cdc27 (*24, 25*). However, mutations in the PCNA-binding domain of *S. cerevisiae* POL32 show no growth defect and only a minor defect in the efficiency of mutagenesis (*26*). *In vitro*, in the absence of effector DNA, the PCNA-interaction domain of Pol32 is essential not only for binding of Pol32 to PCNA but also for binding of the three-subunit Pol δ to PCNA (*26*). However, this domain has minimal importance for processive DNA synthesis by the ternary DNA–PCNA–Pol δ complex. Rather, processivity is determined by PCNA-binding domains located in the catalytic (Pol3) and/or second (Pol31) subunits. Again, similar to that seen with FEN1 and Apn2, during DNA synthesis, interactions between the C-terminal domain of Pol32 and the C-terminal region of PCNA were observed. On the other hand, functional interactions of the other subunit(s) of Pol δ localize largely to the hydrophobic region near the interdomain connector loop of PCNA (*26*).

The tentative conclusion from all these PCNA-binding studies is that the interactions of PCNA with the large and the second subunit of Pol δ are extremely weak and difficult to map, whereas those with the third subunit are very easily revealed. In contrast, the functional interactions of PCNA with Pol δ at a template–primer, i.e., those that lead to increased processivity of the polymerase, are mainly derived from the responsible domain or domains on the first two subunits.

E. Modification of PCNA by Ubiquitin and SUMO

Recently, an extra layer of complexity in PCNA biology has been added by the discovery that this protein can undergo posttranslational modification (*58*). The *RAD6* postreplicative repair pathway uses several mechanisms to deal with DNA damage present during the S phase of the cell cycle, including Pol ζ-dependent mutagenesis and bypass replication by the *RAD30* (XPV) DNA polymerase η (reviewed in *84*). Two key players in this pathway are the *RAD6* and the *MMS2–UBC13* ubiquitin-conjugating enzymes. In response to DNA damage, PCNA is initially monoubiquitinated on Lys164 by *RAD6 and RAD18* (*30, 31*). Subsequent multiubiquitination via an unusual Lys63 linkage requires the *MMS2–UBC13* complex and *RAD5*.

Monoubiquitination of PCNA at Lys164 is essential for mutagenic DNA replication by DNA polymerase ζ and for bypass replication by Pol η. On multiubiquitination of Lys164, the efficiency of mutagenesis is reduced as lesions are channeled into a *RAD5*-dependent error-free pathway possibly involving template switching. Because *RAD6* and *RAD18* mutants are much more sensitive to DNA-damaging agents than is the ubiquitin-defective *pol30-K164R* mutant, it is clear that ubiquitination of PCNA is not the sole function of the RAD6 ubiquitin-conjugating enzyme.

SUMO modification of PCNA also occurs on Lys164, and secondarily on Lys127 (58). SUMO modification is cell-cycle dependent, although some increased modification on exposure to DNA-damaging agents is also observed. Competition between SUMO and ubiquitin for modification of Lys164 on PCNA regulates the efficiency of both spontaneous and damage-induced mutagenesis. Mutation of *SIZ1* which is essential for SUMO modification, results in increased levels of spontaneous and damage-induced mutagenesis (59).

IV. The Clamp Loader RFC

A. RFC Structure

RFC from eukaryotes is the heteropentameric complex that loads PCNA at template–primer junctions. A different nomenclature is used for the yeast and the human subunits of RFC. The ortholog of yeast Rfc1 in human is hRFC140, Rfc2 is hRFC37, Rfc3 is hRFC36, Rfc4 is hRFC40, and Rfc5 is hRFC38 (Table II). We use the yeast nomenclature throughout this review, recognizing that many contributions derive from studies with human RFC. All five *S. cerevisiae* RFC genes are essential for growth (85, 87, 88–92). The four small subunits from all organisms fall in a narrow range of 36 to 41 kDa. These four subunits form the core of the clamp loader complex and are also present in alternative clamp loader complexes (Section V). The large Rfc1 subunit

TABLE II
SUBUNITS OF *S. CEREVISIAE* AND HUMAN RFC

Yeast	Human	Activities of the separate subunit
Rfc1	p140	PCNA binding, DNA binding
Rfc2	p37	Template/primer binding, ATP binding
Rfc3	p36	PCNA binding, DNA binding, ATPase[a]
Rfc4	p40	PCNA binding, ATP binding
Rfc5	p38	

[a]ATPase activity was observed in yeast Rfc3 but not in human p36 (85, 86).

shows a large size variation between organisms: it is 77 kDa in *C. albicans*, 95 kDa in *S. cerevisiae*, and 128 kDa in humans. Most of the size variability in Rfc1 from different organisms is localized to a N-terminal domain, called motif I, which is unrelated to its function as a clamp loader (see later). All five subunits show strong sequence similarity to each other, to the archaeal clamp loaders, to the γ and δ subunits of the *E. coli* γ complex clamp loader, and to the gp44 clamp loader subunit of bacteriophage T4 (*90*). As members of the AAA^+ protein family [chaperone-like ATPases associated with a variety of cellular activities (*15*)], all five RFC subunits contain a stretch of around 220 amino acids in which seven homology regions (RFC boxes II–VIII) are localized (*90*). RFC boxes III and V contain nucleotide-binding sequences Walker A and Walker B, respectively (*93*). The C-termini of all RFC subunits are unique and are required for complex formation (*94, 95*).

Insights into the structure of the heteropentameric complex are derived from subunit–subunit interaction studies of both human and yeast RFC, from electron microscopy studies of human RFC, and from the crystal structure of the orthologous heteropentameric $\delta\gamma_3\delta'$ clamp loader complex from *E. coli* (*12, 96–100*). Pairwise subunit–subunit interaction studies have shown that Rfc2 interacts with Rfc5, Rfc2 with Rfc3, and Rfc3 with Rfc4. Two three-subunit complexes, i.e., Rfc2–Rfc3–Rfc4 and Rfc2–Rfc3–Rfc5, have been identified. Together with the finding that Rfc5 and Rfc2 were required to observe a complex between Rfc1 and the other Rfc subunits, these studies allow the conclusion that RFC should adopt a circular arrangement of interacting subunits as Rfc1–Rfc4–Rfc3–Rfc2–Rfc5. Electron microscopic studies of human RFC confirmed the ring-form arrangement of subunits, similar to that determined for the $\delta\gamma_3\delta'$ complex, suggesting a comparative assignment of the five Rfc subunits onto the $\delta\gamma_3\delta'$ structure as shown in Fig. 1 (*100*). The proposed functional homology to the wrench-stator-motor model proposed for the γ complex also allowed a tentative assignment for the function of each of the Rfc subunits in the PCNA loading process (*13*). According to this model, Rfc1 is similar to the δ wrench in the γ complex, which is proposed to open up the PCNA clamp. The Rfc2, Rfc3, and Rfc4 subunits bind and hydrolyze ATP and are similar to the three γ motor subunits in the complex. Like its functional ortholog δ', the Rfc5 subunit with an inactive ATP-binding domain is proposed to function as a stator that modulates the Rfc1–PCNA interactions.

B. RFC Binding to DNA

The best-recognized function of RFC is to load PCNA onto double-stranded DNA at template–primer junctions. The DNA recognition properties of RFC are embedded in the five-subunit enzyme and appear to be distributed over at least three subunits. The isolated Rfc2 subunit has been shown to preferentially bind primed single-stranded DNA in agreement with the established properties of

the complex (*85*). Yeast Rfc3 has an ATPase activity that is markedly stimulated by single-stranded, but not double-stranded, DNA, implying DNA binding by this subunit, and the human orthology preferentially binds primed single-stranded DNA (*85, 86*). Two DNA-binding domains have been identified in the large Rfc1 subunit (*95*). The N-terminal region of Rfc1 shows homology to prokaryotic DNA ligase and poly(ADP)-ribose polymerase (*101*). The isolated N-terminal domain of human Rfc1 has been shown to bind partially double-stranded DNA substrates in which at least one of the 5′-ends is phosphorylated and the phosphate is either recessed or at a blunt end, suggesting a possible functional significance of this domain at DNA ends (*102*). However, this domain is not essential for PCNA loading *in vitro* or *in vivo* (*95, 99, 103*). In fact, RFC containing Rfc1 with a truncated N-terminal domain showed increased clamp loading activity, which has been attributed to the elimination of competing DNA binding by this domain.

Studies of the function of RFC in DNA replication assigned a dual role for DNA binding by this clamp loader. First, DNA binding by RFC is associated with terminating primer synthesis for the lagging strand by DNA polymerase α-primase and displacement of Pol α (*104–107*). Second, binding by RFC to the template–primer junction serves to identify the loading site for PCNA. From these two functions, it is reasonable to assume that binding of RFC would perform two consecutive tasks in a coordinated fashion, called the polymerase-switch model, i.e., first to abrogate Pol α-mediated primer synthesis by dissociation of Pol α, followed by binding of PCNA and loading it at the site vacated by Pol α (*104*). However, studies with the yeast clamp loading system indicate that this mechanism does not operate, at least not in this simple form. These studies were only possible after the N-terminal DNA-binding domain of Rfc1, which is not required for clamp loading, was removed. The presence of this domain greatly complicated interpretations of DNA-binding studies with relation to clamp loading.

The multifaceted DNA-binding properties of RFC are illustrated in Fig. 3, which is a compilation based on DNA-binding studies of yeast RFC (*99, 108, 109*). Most of the questions relating to DNA and nucleotide binding were addressed by carrying out surface plasmon resonance (SPR) studies, which under well-controlled conditions can give kinetic and thermodynamic parameters comparable to that obtained in solution studies (*108, 110*).

Wild-type RFC binds strongly to template–primer DNA under near-physiological salt concentrations (125 mM NaCl) and only weakly to single-stranded DNA (*108*). At very low salt concentrations, binding of RFC to double-stranded DNA was also observed, although its stability on template–primer DNA was still much higher (*111*). Removal of the N-terminal domain of Rfc1 yielded a species of RFC called $RFC_{1\Delta}$, which is fully functional for PCNA loading, yet binds template–primer with much lower efficiency (Fig. 3).

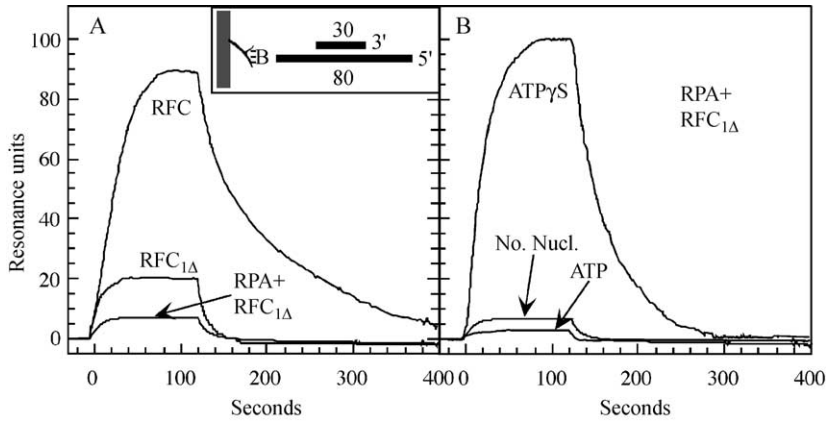

FIG. 3. Binding of RFC to DNA. The data are a compilation of three studies (*99, 108, 109*). SPR binding curves of 25 nM RFC or $RFC_{1\Delta}$ (RFC lacking the N-terminal domain of Rfc1, a DNA-binding domain unrelated to loading of PCNA) to a DNA chip were measured at 5 mM $MgCl_2$, 125 mM NaCl (Panel A). The curves in Panel B were measured in the same buffer containing either 100 μM ATPγS or 1 mM ATP.

Furthermore, in the presence of the eukaryotic single-stranded binding protein RPA, binding of $RFC_{1\Delta}$ to template–primer DNA is reduced even further. A remarkable change in binding properties is induced by ATP binding and its hydrolysis. In the presence of ATPγS, a nonhydrolyzable analog of ATP, strong binding of $RFC_{1\Delta}$ to template–primer DNA was observed, but with ATP present, the observed level of binding was even lower than that in the absence of the nucleotide cofactor. These results indicate that ATP binding to RFC induces a conformational change, which allows binding to DNA. However, hydrolysis of the bound ATP actively dissociates RFC from the DNA as binding with ATP is even lower than binding without nucleotide present (Fig. 3B). Therefore, nucleotide-induced binding of RFC to DNA appears to be a futile cycle (Fig. 5). Binding of human RFC to DNA was also stabilized by ATPγS, and nonspecific binding reduced by coating single-stranded DNA with RPA (*112*). Similarly, the ATP-bound form of the *E. coli* γ complex binds with high affinity to DNA, but hydrolysis of ATP converts it to a low-affinity state (*113*).

C. Loading of PCNA by RFC

At least three subunits of RFC—Rfc1, Rfc3 and Rfc4—show physical interactions with PCNA, and a molecular modeling study suggest that the Rfc5 subunit could also interact with PCNA (*86, 114–116*). The human three-subunit Rfc2–4 complex also binds PCNA (*95*). Both the yeast Rfc2-5 core complex and Holo-RFC show comparable ATPase activities that are equivalently stimulated by PCNA (*108*). However, ATPγS stimulates binding between the yeast Rfc2-5

core complex and PCNA, whereas ATP does not. Therefore, presumably hydrolysis of the bound ATP dissociates the PCNA–core complex (Fig. 5). This is in contrast to the PCNA–RFC complex, which is stably maintained by either ATP or ATPγS (*108, 117*). Therefore, it appears that hydrolysis of bound ATP releases PCNA from the Rfc2-5 core, but the PCNA-binding domain of Rfc1 stabilizes PCNA binding to RFC despite turnover of ATP. Perhaps, unlike Rfc2-5 the Rfc1 domain is not subject to allosteric control by ATP. The significance of this distinction may be of importance in the cell in that only RFC interacts productively with PCNA. The Rfc2-5 core binds PCNA poorly and actively dissociates bound PCNA to allow binding of PCNA to its proper clamp loader (Fig. 5).

One of the most important issues relevant to our understanding of the clamp loading mechanism is with regard to the order of events in this pathway. Thus, can a DNA-bound RFC complex recruit PCNA and load it, or, alternatively, is only a RFC–PCNA complex capable of productively binding DNA and loading of PCNA. The first mechanism (Model A in Fig. 4) is suggested in the polymerase-switch model, whereas the second mechanism (Model B) would be more in agreement with mechanistic insights derived from studies of the bacteriophage and *E. coli* clamp loaders (*118–120*). This question was addressed by carrying out surface plasmon resonance studies with yeast $RFC_{1\Delta}$. In a two-stage binding experiment, $RFC_{1\Delta}$, together with ATPγS, was first flowed across a chip containing primed DNA and PCNA and ATPγS were flowed across in the second stage. Simply put, if $RFC_{1\Delta}$ bound to DNA on the chip was able to load PCNA, an increase in signal would be observed when PCNA was flowed across the chip (A). In contrast, if $RFC_{1\Delta}$ had to dissociate from the DNA and form an ATPγS-mediated complex with PCNA prior to rebinding to the DNA on the chip, a decrease in signal would be observed, particularly if rebinding was inhibited by the presence of trap DNA during the second stage of the reaction (B). The latter prediction was experimentally verified, showing that only a PCNA–RFC complex can productively bind DNA in order to load PCNA. This conclusion does not exclude the possibility that transient binding of RFC to DNA may have functional importance, e.g., to abrogate primer synthesis by Pol α (*105, 107, 121*).

Figure 5 illustrates the pathway of PCNA loading by RFC. It includes the two abortive pathways in which ATP binding promotes complex formation whereas its hydrolysis causes complex dissociation; this is for binding of the Rfc2-5 core to PCNA, and of RFC to DNA.

D. ATP Usage of RFC During the Loading Cycle

Comprehensive mechanistic studies of the role of ATP in clamp loader interactions with the clamp and with DNA have been documented for the T4 and the *E. coli* system (*118–120, 122–124*). Given the structural similarities of the three model systems, one might expect that the mechanism of clamp loading in eukaryotes would be similar in detail to that in T4 and *E. coli*. However, there

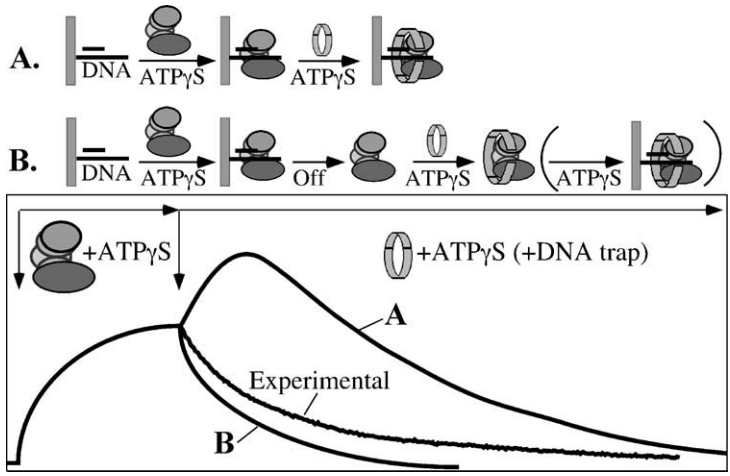

FIG. 4. A RFC–DNA complex is unable to recruit PCNA and load it. In this two-stage SPR experiment, RFC is flowed across a DNA chip in the first stage, followed by PCNA in the second stage. ATPγS is present in both stages. In the analysis, curve A is the simulated curve assuming that PCNA can bind to a RFC–DNA complex on the chip (Scheme A). Curve B is the simulated curve assuming that RFC has to dissociate from the DNA chip, then bind PCNA, and only then is able to rebind template–primer DNA on the chip (Scheme B). In simulated curve B, the second step, rebinding of the PCNA–RFC complex, has not been taken into account, and in the experimental setup, this step was minimized by the presence of a large excess of DNA trap during the second stage of the experiment (*109*). The experimental curve matches simulated curve B most closely.

are at least two reasons why there may be substantial differences. First, all subunits except *RFC5* have a consensus ATP-binding domain, suggesting the possible involvement of four ATPs in the reaction pathway. However, unlike the T4 clamp loader, these putative four ATP molecules would be localized in unique rather than identical subunits, likely assigning an unique function to each subunit and the ATP bound to it. ATP stoichiometry measurements have been fraught with difficulties in these complex systems. For instance, from the initial studies in the early 1980s until just a few years ago, two ATP molecules have been associated with loading of the β subunit by the γ complex (*120, 125*). It was only after the determination of the crystal structure of the $\delta\gamma_3\delta'$ complex that techniques were developed which could measure the three ATP molecules proposed to bind to each of the γ subunits (*12, 113*). And in the T4 system, differing results between two labs could only be reconciled after a detailed study of the quenching procedures used in rapid kinetic studies (*123, 124*).

Studies with clamp loaders from *E. coli* and eukaryotes have shown that except for the final step, ATPγS can carry out all steps of the clamp loading pathway, because these steps only require ATP binding and not its hydrolysis (*27, 117, 120, 122*). The final step, release of the loader from the DNA-bound

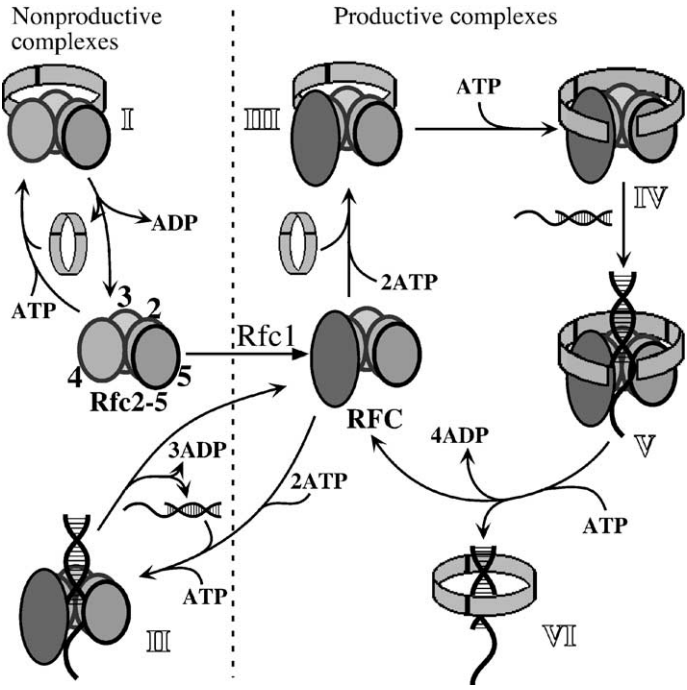

FIG. 5. Sequential mechanism for PCNA loading by RFC. The right section shows the multiple ATP-driven steps required for clamp loading. Complex III is equivalent to the weak complex observed in the absence of ATP. Complex IV is a very stable complex with the PCNA ring opened and requires three ATPs bound. Complex V is inferred from binding studies with ATPγS. Two nonproductive complexes are indicated in the left section: complex I between the Rfc2-5 core and PCNA and complex II between RFC and DNA. Formation of these complexes requires ATP binding, but its rapid hydrolysis dissociates the complexes. The Rfc2-5 core is inactive for clamp loading.

clamp, requires ATP hydrolysis, but even this requirement can be circumvented under certain conditions (*27, 126*).

Our studies of ATPγS binding to yeast RFC showed an interesting and striking departure from the prokaryotic mechanism (*109*). Whereas RFC alone bound two ATPγS molecules, three molecules of ATPγS were bound when either DNA or PCNA was present and four ATPγS molecules were bound when both PCNA and DNA were present. These binding stoichiometries suggest that each step on the reaction pathway by the eukaryotic clamp loader is propagated by binding of an additional ATP molecule (Figs. 5 and 6). Thus, although two ATPs can initially bind to RFC, the remaining two ATP-binding sites are either buried or have an extremely low affinity for ATP. Binding of PCNA to RFC-ATP_2 induces a conformational change, which makes one additional ATP-binding site available. On binding of DNA to the resulting PCNA–

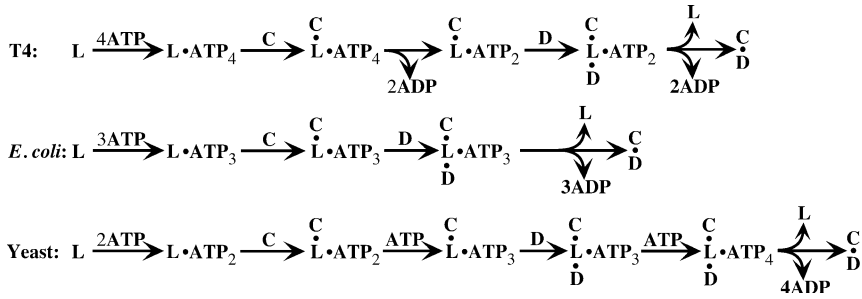

FIG. 6. ATP utilization by three clamp–clamp loading systems. L: clamp loader, C: clamp, D: DNA. See text for discussion.

$RFC–ATP_3$ complex, another conformational change in RFC makes one final ATP-binding site available. This fourth ATP needs to be bound for the loading process to proceed to completion (*109*). A comparison between ATP binding and utilization between the three types of clamp loader systems is shown in Fig. 6. This scheme not only highlights that clamp loading proceeds by an ordered mechanism requiring binding of the clamp to the loader prior to binding of primer–template DNA, but also indicates the marked stepwise regulation of the binding and usage of ATP molecules in the eukaryotic system. Such a regulation does not exist in prokaryotic and phage clamp loading systems and is therefore not *a priori* required. However, this stepwise mechanism should in principle not only permit editing of this process at multiple steps but also allow for divergence of the default process into more specialized modes. Indeed, multiple clamp loading systems have evolved in eukaryotes, each system consisting of a core containing the Rfc2, Rfc3, Rfc4, and Rfc5 subunits together with a separate large subunit (Fig. 7, see later). As these alternative clamp loaders likely have quite different functions and may even interact with different clamps, a stepwise binding mode of ATP to the clamp loader would provide a greater potential for regulation than the concerted ATP binding observed in prokaryotic systems.

Distinct differences between the three clamp–clamp loader systems have been observed with regard to the timing of hydrolysis of the bound ATP molecules. The T4 gp44/62 complex initially binds four ATPs, two of which are hydrolyzed on binding of the gp45 clamp and the other two to close the clamp onto the DNA (*119, 124*). The *E. coli* γ complex binds three molecules of ATP, but only hydrolyzes these rapidly and sequentially during closure of the clamp around the DNA and release of the loader (*113, 120*). Whether all four ATP molecules bound during PCNA loading by RFC are hydrolyzed on completion of clamp loading has not yet been established.

All four small subunits of RFC have a putative arginine finger, which promotes hydrolysis of the ATP bound in the neighboring subunit, but Rfc1 lacks

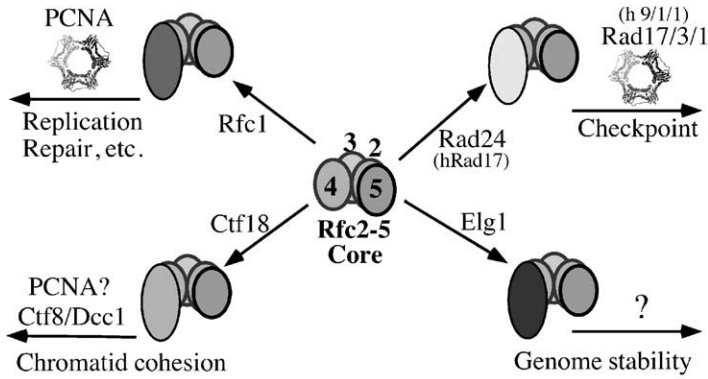

FIG. 7. Alternative clamp–clamp loader systems. Of these, the Ctf 18 complex forms a seven-subunit complex together with Ctf8 and Dcc1 (*127*). H9/1/1 and hRad17 are the *S. pombe*/ human orthologs of *S. cerevisiae* Rad17/3/1 and Rad24, respectively.

such a motif (see Section II). In the proposed arrangement of the Rfc subunits in Fig. 1 (see Section IV.A), sequential hydrolysis of the bound ATP on completion of clamp loading would proceed in a circular fashion, although the exact order, clockwise or counterclockwise in Fig. 1, remains to be established. Thus, the Rfc5 arginine finger would promote hydrolysis of the ATP bound to Rfc2, the Rfc2 arginine would activate Rfc3, the Rfc3 arginine would activate Rfc4, and finally the arginine finger in Rfc4 would activate hydrolysis of the ATP bound in Rfc1.

The realization that all steps in the clamp loading pathway are accompanied by sequential binding of ATP molecules does not address the function of these ATP-binding events or whether binding of all four ATPs and their eventual hydrolysis are required for clamp loading. Mutational studies of the ATP-binding sites of the Rfc subunits have shown that the ATP-binding site of Rfc1 is not required for the clamp loading function of RFC, whereas the ATP binding sites of Rfc2, Rfc3, and Rfc4 are essential for a functional clamp loader (*128*). These data agree well with the structural alignment between the γ complex and RFC, which assigns ATP-binding roles to only the Rfc2 (γ_1), Rfc3 (γ_2), and Rfc4 (γ_3) subunits (Fig. 1).

V. Alternative Clamps and Clamp Loaders

In the past few years, three additional clamp loaders or putative clamp loaders and one clamp have been identified in eukaryotic cells (Fig. 7). All these clamp loaders have the Rfc2-5 core in common with RFC. However, Rfc1 is replaced by a pathway-specific Rfc1-like protein, Rad24 for the DNA damage checkpoint, Ctf18 for the establishment of chromatid cohesion, and

Elg1 for an ill-defined pathway that functions in the maintenance of chromosome stability. All Rfc1 homologs are AAA^+ proteins, and there are still more proteins for which sequence comparison and protein threading algorithms show a strong relationship with Rfc1. These include proteins required for genome replication, genome stability, or recombination, such as Cdc6, Mgs1, and Rvb2, respectively. Whether these proteins are from complexes with the Rfc2-5 core still needs to be determined.

A. The DNA Damage Clamp and Clamp Loader

Genetic studies have shown that mutations in the *RAD17*, *MEC3*, or *DDC1* gene (*Rad1*, *Hus1*, and *Rad9*, respectively, in *S. pombe* and humans) lead to checkpoint defects (reviewed in *129–131*). The Rad17, Mec3, and Ddc1 proteins display sequence homology with each other and with PCNA (*116, 132*). These observations suggested that these three proteins could form a PCNA-like ring and function as a clamp involved in DNA damage checkpoint control. Indeed, electron microscopy studies of the human Rad17/3/1 complex (heterotrimer of the Rad17, Mec3, and Ddc1 proteins) showed a ring-like structure, whereas molecular modeling studies predicted a PCNA-like fold (*116, 133, 134*). The subunits of the complex are organized in a head-to-tail manner in which the N-terminus of Rad17 interacts with the C-terminus of Mec3, the N-terminus of Mec3 with the C-terminus of Ddc1, and the N-terminus of Ddc1 with the C-terminus of Rad17 (*135, 136*).

Similarly to the Rad17/3/1 clamp, the RFC–Rad24 clamp loader complex plays a part during the early stages of the checkpoint control response (reviewed in *131* and *137*). RFC–Rad24 may not recognize damaged DNA directly, but rather be attracted to an initially processed DNA lesion where it loads the Rad17/3/1 clamp. The large subunit of the clamp loader is proposed to contact the C-terminal region of human Rad1 (Rad17 in *S. cerevisiae*), whereas RFC3 and RFC5 may interact with C-terminal regions of Hus1 (Mec3) and Rad9 (Ddc1), respectively. Such an arrangement of interfaces predicts that during loading the clamp would open up between the Rad1 and Rad9 proteins (Rad17 and Ddc1) (*116, 138–140*).

Mutational analysis of the DNA damage checkpoint pathway have predicted that an exonuclease activity should function in this pathway, which is dependent on the *RAD24*, *RAD17*, and MEC3 genes (*141*). Studies with the Rec1 protein from *Ustilago maydis*, the ortholog of Rad17, have revealed an intrinsic 3′-5′-exonuclease activity (*142*). Similar activities have been reported for the human orthologs of Rad17 (Rad1) and Ddc1 (Rad9) (*143, 144*). However, other studies failed to detect an exonuclease activity, particularly in the heterotrimeric clamp assembly (*145, 146*). Because the documented nuclease activities of the individual checkpoint clamp proteins is very weak, the possibility exists that a cryptic nuclease is present in the clamp, which requires activation, perhaps by loading

onto DNA and/or posttranslational modification. Several checkpoint proteins, including Ddc1, are phosphorylated as part of the checkpoint response (*147*).

Rad17/3/1 and RFC–Rad24 interact in the absence of DNA (*134, 145, 146*). Similar to the PCNA–RFC complex, the formation of a stable Rad17/3/1–RFC–Rad24 complex was enhanced by ATP binding but not its hydrolysis. As PCNA has been shown to interact with the small subunits of RFC, it is not surprising that interactions between PCNA and RFC–Rad24 have been detected indirectly by the observation that PCNA stimulates the ATPase activity of RFC–Rad24 (*96, 86, 115, 145*). However, these interactions must be transient because a stable PCNA–RFC–Rad24 complex has not been identified.

In vitro studies have shown that RFC–Rad24, as well as its human ortholog RFC–hRad17, preferentially binds primed single-stranded DNA and gapped DNA. It also forms an ATP-dependent complex with the Rad17/3/1 clamp. This ATP-dependent clamp–clamp loader complex is active for loading the Rad17/31 clamp around partial duplex DNA in an ATP-dependent process (*145, 146*). On ATP hydrolysis, the Rad17/3/1 clamp is released from clamp loader and can freely slide across double-stranded DNA (*145*). The strong parallelism with the RFC–PCNA system suggests an analogous loading mechanism for both the yeast and human checkpoint clamp (Section IV.C). Thus, binding of ATP induces conformational changes in the clamp loader, which in turn allows clamp binding. Possibly, further ATP binding mediates clamp opening, followed by DNA binding. Finally, hydrolysis of the bound ATP would release the clamp from its loader. Consistent with this model are experiments which show that in the presence of ATPγS, the Rad17/3/1 clamp is prevented from sliding across the DNA (*145*).

The function of Rad17/3/1 and/or RFC–Rad24 at the site of the DNA lesion remains unknown. Both complexes are phosphorylated by the Mec1 protein kinase (ATR in human, a central regulator of the DNA damage checkpoint), and Mec1-dependent activation of the Rad53 (a protein kinase downstream in the checkpoint signal transduction cascade) was severely reduced in *RAD17*, *MEC3*, *DDC1*, and *RAD24* mutants. However, phosphorylation of other Mec1 targets seems not to be affected by the absence of Rad17/3/1 and/or RFC–Rad24 (*147–150*). Once loaded onto the DNA, the target for this clamp remains uncertain. Putative functions could involve activation of a cryptic exonuclease activity in the clamp itself or recruitment of other factors that could propagate the checkpoint response pathway.

B. The Chromatid Cohesion Clamp Loader

In eukaryotic cells, chromosomes stay topologically connected after DNA replication during the S phase until they are separated in the anaphase of the cell cycle (recently reviewed in *151* and *152*). Sister chromatid cohesion is essential for the equal segregation of replicated chromosomes to the daughter

cells. The establishment of the cohesion complex is tightly linked to DNA replication. Even though the cohesion complex persists from the S phase until the anaphase of mitosis, the activity of some proteins in this process is required only during the S phase of the cell cycle, suggesting that these proteins are important for the establishment of chromatin cohesion rather than being integral essential components of the cohesion complex (*153*).

S. cerevisiae Ctf18 (CTF: chromosome transmission fidelity), also called Chl12 (CHL: chromosome loss), is a Rfc1 homologue. Ctf18 is one of the proteins that functions in the establishment of chromatid cohesion (*127, 154*). *CTF18* deletion mutants are viable, but show increased rates of chromosome loss (*155*). A similar phenotype was seen with mutants in the *S. cerevisiae CTF8* and *DCC1* genes (*127*).

Ctf18, together with Ctf8 and Dcc1, forms a seven-subunit alternative clamp loader complex, RFC–Ctf18(7), with the Rfc2-5 core (*146, 127*). Isolation of a five-subunit RFC–Ctf18(5) complex, which lack the Ctf8 and Dcc1 subunits, shows that interactions between Ctf18 and Rfc2-5 are likely analogous to those of Rfc1 or Rad24 with Rfc2-5 (*156, 157*). The Ctf8 and Ddc1 subunits form a heterodimeric complex that binds to Ctf18 only (*127, 157*).

The realization that a clamp loader is involved in the establishment of chromatid cohesion immediately poses the question of which clamp this loader acts on. A search through the yeast database, using sequence comparison and threading algorithms, has not revealed any putative clamps except for those already known, i.e., PCNA and Rad17/3/1 (*158*). Possibly, the clamp used by RFC–Ctf18 is structurally quite different and therefore may not be detectable by using algorithms based on the structures of the known clamps. An alternative possibility that RFC–Ctf18 may act on one of the known clamps, has also been investigated. Human RFC–Ctf18 interacts with PCNA, but not with the h9/1/1 complex (*157, 159, 160*). As stated in Section V.A, because the Rfc2-5 core also interacts with PCNA, *a priori* this observation does not necessarily imply functional importance. However, recent studies of the human RFC–Ctf18 clamp loader do assign functional consequences to this interaction (*157*). Like the canonical RFC, both RFC–Ctf18(5) and RFC–Ctf18(7) load PCNA at a template–primer junction, and the PCNA loaded serves as a processivity factor for Pol δ. However, compared to RFC, this PCNA loading reaction by the cohesion clamp loaders is extremely inefficient and therefore not likely to specify a PCNA loading pathway under regular replication conditions.

Recent observations in our laboratory suggest the opposite, i.e., that RFC–Ctf18(5) and Rfc–Ctf18(7) can unload PCNA from DNA (Bylund and Burgers, unpublished results). Specificity for this reaction is provided by a requirement for hydrolyzable ATP, i.e., ATPγS will not substitute. As the unloading reaction proceeds fairly slowly as well, it is unlikely to function during Okazaki fragment synthesis, which requires rapid turnover of PCNA. However, unloading of

PCNA at selective sites at which replication is essentially complete could well be coupled to the assembly of a cohesion complex. It has been postulated that RFC–Ctf18 can act to perform a polymerase switch at the vicinity of cohesion sites, replacing the highly processive Pol δ with the less potent Pol σ, an enzyme that is also required for chromatid cohesion (*127, 149, 150, 155, 161*).

C. The Elg1 Clamp Loader Complex

Very recently, an additional AAA^+ protein, *S. cerevisiae* Elg1, has been shown to associate with the small subunits of RFC to form an isolatable RFC–Elg1 complex (*162, 164*). Yeast *ELG1* mutants show elevated levels of recombination and chromosome loss. Genetic interactions between *ELG1* and genes proposed to function in Okazaki fragment synthesis or in the restart of stalled replication forks suggest a function for Elg1 that is closely linked to the replication fork, presumably when it is stalled (*162, 164*).

The biochemical activities of RFC–Elg1 have not yet been determined. The subunit structure of the complex has been indirectly assessed from coimmunoprecipitation experiments, and it is possible that, like observed for the RFC–Ctf18 clamp loader, additional proteins associate with the basic five-subunit RFC–Elg1 complex (*162–164*). Coimmunoprecipitation experiments indicate that the RFC–Elg1 complex interacts with PCNA, but not with the Rad17/3/1 checkpoint clamp (*164*). Whether any functionality is associated with the interaction with PCNA still needs to be determined.

VI. Perspectives

At present, much uncertainty exists regarding the functions of the alternative clamp loaders, their interactions with known clamps, or the potential for interactions with as yet undiscovered alternative clamps. Research has focused on establishing genetic interactions between the different clamp loaders, and of each clamp loader in specific pathways, in the anticipation that it will more precisely define their function. A clearer view of their function in the cell would aid considerably in directing biochemical studies of these complexes.

Although it is well established from genetic studies that *RAD24*, *CTF18*, and *ELG1* function in separate pathways, there is also growing evidence for crosstalk between these pathways. For instance, genetic studies suggest a redundant function for *RAD24* and *CTF18* in the replication checkpoint (*156*). The replication checkpoint, which is distinct from the DNA damage checkpoint, slows down the S phase in response to nucleotide depletion, e.g., when hydroxyurea is present in the growth medium or at very low levels of damage (*165*). *RAD24* mutants are defective for the DNA damage checkpoint but not for the replication checkpoint, whereas *CTF18* mutants are defective

for neither. However, the double mutant is defective for the replication checkpoint, as it fails to slow down S phase progression in response to hydroxyurea. In fact, a *RAD24/CTF18/ELG1* triple mutant defective for all three alternative clamp loaders shows an even larger defect for the replication checkpoint (*164*).

However, a lack of understanding of the function and abundance of the Rfc2-5 core assembly may complicate these genetic studies. The alternative large clamp loader subunits, Rfc1, Rad24, Ctf18, Elg1, and possibly others, may compete for available Rfc2-5 core, and the elimination of one or more of these may upset the balance of the other assemblies, thereby contributing to the observed genetic phenotypes. These considerations indicate the importance of understanding the Rfc2-5 core assembly both in biochemical terms and as a possible separate entity inside the eukaryotic cell.

Acknowledgments

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