The role of the DNA sliding clamp in Okazaki fragment maturation in archaea and eukaryotes

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Abstract

Efficient processing of Okazaki fragments generated during discontinuous lagging-strand DNA replication is critical for the maintenance of genome integrity. In eukaryotes, a number of enzymes co-ordinate to ensure the removal of initiating primers from the 5'-end of each fragment and the generation of a covalently linked daughter strand. Studies in eukaryotic systems have revealed that the co-ordination of DNA polymerase δ and FEN-1 (Flap Endonuclease 1) is sufficient to remove the majority of primers. Other pathways such as that involving Dna2 also operate under certain conditions, although, notably, Dna2 is not universally conserved between eukaryotes and archaea, unlike the other core factors. In addition to the catalytic components, the DNA sliding clamp, PCNA (proliferating-cell nuclear antigen), plays a pivotal role in binding and co-ordinating these enzymes at sites of lagging-strand replication. Structural studies in eukaryotic and archaeal systems have revealed that PCNA-binding proteins can adopt different conformations when binding PCNA. This conformational malleability may be key to the co-ordination of these enzymes’ activities.

Introduction

DNA replication faces an inherent directionality problem: all replicative polymerases synthesize DNA in a 5'→3' direction, and yet duplex DNA is antiparallel, thus providing two template strands of opposing polarity as it is unwound. This problem is overcome by synthesizing one strand, the leading strand, while Pol δ (DNA polymerase) that Pol ε is largely responsible for replicating the leading strand, while Pol δ synthesizes the lagging strand [2,3]. The discontinuous nature of newly replicated lagging strands requires the co-ordinated action of numerous enzymes to replace the RNA primers used to initiate each Okazaki fragment, synthesize DNA to replace these primers and ligate the processed fragments into a continuous daughter strand, thus maintaining genome integrity. Whereas this process of Okazaki fragment maturation involves a relatively small complement of proteins in bacteria, studies over the last few years have revealed an additional complexity in eukaryotes and archaea and have begun to provide an insight into how the numerous activities required are co-ordinated.

FEN-1 (Flap Endonuclease 1) in Okazaki fragment maturation

In vitro reconstitution using purified proteins from Saccharomyces cerevisiae has shown that Pol δ is able to undergo ‘idling’ at the junction between two Okazaki fragments: repeated cycles of nucleotide addition and removal from the 3'-end of the nascent Okazaki fragment [4]. Continued synthesis by the polymerase at this junction has the effect of displacing the 5'-end of the downstream fragment, a process termed strand displacement, and idling has been proposed to prevent this process occurring unnecessarily. Strand displacement has the desirable effect of displacing initiating RNA from Okazaki fragments and rendering it suitable for cleavage. However, as Pol δ lacks an intrinsic 5'-3' nuclease, additional factors are required to facilitate this removal.

Eukaryotic FEN-1 is a structure-specific endonuclease capable of cleaving DNA or RNA at the base of a 5'-flap. The optimal substrate is a ‘double flap’ structure, with a 5'-flap and an overlapping single nucleotide 3'-flap [5]. A complex of an archaeal FEN-1 with DNA revealed the structural basis for this substrate specificity: a specific pocket on the surface of FEN-1 accommodates the single nucleotide 3'-flap in a manner probably important for the correct positioning of the sugar–phosphate backbone for cleavage [6].

The substrate preference of FEN-1 for 5'-flaps matches those produced by Pol δ during strand displacement, and when S. cerevisiae FEN-1 (Rad27) is included in in vitro assays, idling is no longer observed. Instead, a large number of mononucleotide products are released from the downstream primer [4]. It therefore appears that, although FEN-1 is active on larger flaps in vitro, its in vivo role is more likely to involve co-operation with Pol δ to repeatedly cleave...
mononucleotide products from the downstream primer as it is displaced. It is unclear how to reconcile this apparent Pol δ–FEN-1 coupling with the preferred double-flap substrate of FEN-1, which would require equilibration back upstream. It has been suggested that FEN-1 actually modulates the strand displacement properties of Pol δ and perhaps such an interaction would also permit branch migration of flaps [4]. Genetic studies in S. cerevisiae support a role for FEN-1 in Okazaki fragment maturation; although Rad27-null mutants are viable, they exhibit temperature-dependent growth arrest, sensitivity to DNA damage and loss of genome stability, indicative of defects in DNA replication.

Cleavage of flap structures by FEN-1 generates a nicked DNA substrate for subsequent ligation by DNA ligase I. DNA ligase I is specifically unable to ligate substrates containing RNA. The basis of this property derives from the close contacts of ligase encircling its substrate, preventing accommodation of an A-form RNA–DNA duplex [7]. This suggests that the removal of RNA primers by cycles of FEN-1 action could be ‘sensed’ by DNA ligase I, which can only act once a purely DNA duplex remains. However, in eukaryotes, initiating primers are synthesized by the Pol α–primase complex, which synthesizes ~30 nucleotides of DNA following the RNA primer [8]. Importantly, Pol α lacks the proofreading activity present in the other replicative polymerases and therefore this DNA must be removed in addition to the initiating RNA to maintain the fidelity of chromosomal DNA replication. Indeed, genetic interactions have demonstrated that Pol δ corrects errors produced by Pol α, consistent with strand-displacement synthesis on the lagging strand being utilized to remove regions of DNA synthesized by Pol α–primase [9]. This appears to be incompatible with a mechanism whereby the signal for ligation of Okazaki fragments is removal of RNA only. Indeed, regions of DNA significantly past the initiating RNA–DNA have been shown to be removed in vitro [10]. How this requirement for removal of low-fidelity DNA is balanced with prevention of wasteful nick translation too far into the previous Okazaki fragment is currently unclear.

**Alternative pathways for processing Okazaki fragments?**

Whereas the majority of cleavage products from in vitro Okazaki fragment maturation are small and consistent with a pathway involving Pol δ and FEN-1 only, observations from a partially reconstituted system suggest that some displaced flaps can ‘escape’ immediate cleavage as they are produced by Pol δ and become much longer [11]. In vivo, such ss (single-stranded) DNA flaps are rapidly bound by the ssDNA-binding protein RPA (Replication Protein A). Significantly, RPA has been shown to inhibit FEN-1 activity in vitro [12]. Evidence suggests a second nuclease, Dna2, may play a key role in such circumstances. Rad27 and S. cerevisiae Dna2 interact both physically and genetically [13]. Like FEN-1, Dna2 is a flap-structure-specific endonuclease, although, in contrast with FEN-1, Dna2 is unable to cleave RNA and is stimulated by RPA [14,15]. Dna2 can act in a sequential manner with FEN-1 to process displaced flaps in vitro; RPA binds long flaps and stimulates cleavage by Dna2 above their base. Shortening of the flap reduces the ability of RPA to bind, relieving the inhibition on FEN-1 and allowing it to cleave the final nucleotides of the flap, generating a ligatable nick [12]. In fact, displacement of RPA by Dna2 may even be independent of flap cleavage, highlighting the importance of FEN-1 access to short flaps [16]. As the initial site of Dna2 cleavage must occur in the DNA downstream of initiating RNA, this has been proposed as one potential mechanism by which all Pol α–primase nucleotides can be removed during maturation (Figure 1).

Whereas this two-nuclease pathway was originally proposed as the predominant mode of Okazaki fragment processing, more recent data suggest that it exists more as a back-up to one involving Pol δ and FEN-1 only. As discussed, the distributions of flap lengths measured in vitro indicate that the vast majority of flaps are of insufficient length for RPA binding [11], and furthermore, when using a dynamic strand-displacement system to measure flap cleavage, rather than a static flap substrate, RPA is far less inhibitory to FEN-1 cleavage, supporting the idea that FEN-1 is usually able to rapidly cleave the flap before RPA is able to bind [17]. The demonstration that, on fixed flap structures at least, FEN-1 appears to be able to displace the Dna2 protein further suggests that a selection pressure exists for FEN-1 to gain access to, and cleave, the flap before it becomes too long in length [18]. The recent demonstration that Dna2 is a key nuclease in the resection of double-strand breaks during
Co-ordination of Okazaki fragment maturation by PCNA (proliferating-cell nuclear antigen)

PCNA is a key component of the replication fork in eukaryotes and archaea, acting as a DNA sliding clamp to enhance the processivity of replicative polymerases by encircling DNA and tethering the polymerases to their template. PCNA is an analogue of the Escherichia coli β-clamp, and, strikingly, despite no apparent sequence homology, these proteins exhibit very similar architecture and overall dimensions (Figure 2) (reviewed in [29]). In addition, PCNA has been demonstrated to interact with a wide range of ‘client’ proteins involved in DNA replication, DNA repair and cell-cycle control [30]. A short consensus sequence for an interaction with PCNA, termed the PIP (PCNA-interacting protein) motif, has been identified and numerous structural studies have identified a conserved mode of interaction with PCNA, exemplified by the human p21–PCNA complex; the residues of the PIP motif form a β3 helix, positioning conserved hydrophobic and aromatic residues into a hydrophobic binding pocket formed on the surface of PCNA [31]. Each subunit of the PCNA trimer possesses one such binding site. Interestingly, proteins which bind the bacterial β-clamp also utilize a broadly similar interaction in which a shortened PIP-like motif adopts an extended peptide conformation and docks into a hydrophobic pocket formed by features on the surface of the β-clamp analogous to those on PCNA [32].

The binding of DNA replication proteins to PCNA is particularly pertinent to Okazaki fragment maturation, as some of the enzymes central to this process, e.g. Pol δ, FEN-1 and DNA ligase I, have all been shown to interact with PCNA [30]. Photobleaching experiments in mammalian cells have demonstrated that, whereas PCNA persists at DNA replication foci for extended periods of time, FEN-1 and DNA ligase I exhibit much more transient residence in these regions, invoking a model whereby PCNA acts as a stationary protein which a shortened PIP-like motif adopts an extended peptide conformation and docks into a hydrophobic pocket formed by features on the surface of the β-clamp analogous to those on PCNA [32].

This raises the intriguing possibility that the balance between PCNA and DNA replication proteins is significantly different in different organisms and that PCNA may function to stabilize processes involving only a subset of these proteins in some organisms. For example, in the crenarchaeon Pyrococcus horikoshii, RNase HII is utilized to a greater extent in vitro than in vivo, despite constitutive expression of a functional RNase HII protein [24]. Interestingly, whereas archaea possess DNA polymerases and orthologues of FEN-1, Dna2 is not broadly conserved in this domain of life. A Dna2 homologue from Pyrococcus horikoshii has been characterized and shown to possess nuclease and helicase activity, with the nuclease activity highly reduced on substrates with a 5′-RNA segment [26]. With regard to the limited phyletic distribution of Dna2 in archaea, it may be significant that Pyrococcus possesses a complex trimeric RPA [27]. In contrast, Sulfolobus, which lacks a Dna2 orthologue, has a simple monomeric ssDNA-binding protein that binds non-co-operatively to DNA with each monomer interacting with only four or five nucleotides [28]. The lack of universal conservation of Dna2 appears to be consistent with the primary pathway for Okazaki fragment processing involving DNA polymerase and FEN-1. Archaea, which possess all of the other core processing enzymes, may prove to be a useful model system in which to study the mechanisms of Okazaki fragment processing in more detail.
Figure 2 | Crystal structures of bacterial (E. coli), eukaryotic (human) and archaeal (S. solfataricus) sliding clamps

Intersubunit interfaces are indicated by lines. The expanded view of human PCNA shows the PIP motif (red) from p21 bound to a human PCNA subunit. The three distinct subunits of the S. solfataricus PCNA are shown in blue, red and yellow. Images were generated using PyMOL (DeLano Scientific; http://www.pymol.org) from PDB files 2POL, 1VYM and 2HII respectively.

affinity [42]. This has subsequently been shown to derive from the ability of PCNA to act as a fulcrum upon which XPF can exert force on its DNA substrate, distorting it into a suitable conformation for cleavage [43].

There is now significant evidence to suggest that simultaneous binding of multiple protein factors to a single PCNA ring can occur. In crenarchaeia such as S. solfataricus, PCNA is a heterotrimer, in contrast with the homotrimer of eukaryotes, and each subunit of PCNA has been demonstrated to possess specificity for the binding of a particular client protein involved in Okazaki fragment maturation; thus PCNA2 binds the presumed replicative PolB1, PCNA1 binds FEN-1 and PCNA3 binds DNA ligase I. These three factors have been shown be bridged by PCNA in vitro [41]. Structural studies have demonstrated this specificity for different client proteins to arise from subtle conformational differences in the hydrophobic pocket on the surface of the different PCNAs, enabling them to discriminate between PIP motifs of differing structure and sequence. Interestingly, binding to PCNA2, exemplified by PolB1, appears to utilize a shortened PIP motif which resembles that of a bacterial β-clamp-binding sequence [44].

Three molecules of human FEN-1 have also been co-crystallized with a single PCNA molecule, supporting the concept that, sterically at least, multiple proteins can be simultaneously accommodated on one PCNA ring [45]. Functional studies have also provided evidence for two different bacterial polymerases simultaneously binding the β-clamp [46], although this has been disputed [47]. The formation of a sliding-clamp-mediated processing complex on the lagging strand in this manner would be particularly advantageous given the model for primer degradation by repeated cycles of Pol δ and FEN-1 catalysis: the PCNA scaffold could allow rapid alternating access to the DNA template by both enzymes, without the need to recruit further molecules from solution. Co-localization of DNA ligase I would provide the further advantage of rapid generation of a covalent daughter strand following removal of initiator RNA–DNA.

In argument against simultaneous binding to PCNA, overexpression of DNA ligase I in vivo [48] or titration of DNA ligase I into reactions in vitro [49] has been shown to disrupt the binding of other client proteins. However, without the client protein specificity demonstrated by S. solfataricus PCNA, eukaryotic PCNA presents three identical binding sites, and thus it is certainly feasible that an anomalous excess of one client protein has the potential to displace others, even if simultaneous binding is possible in vivo where client protein concentrations may be more carefully balanced and regulated.

Conformational flexibility in PCNA-interacting proteins

Structural biology has now provided several clues as to how multiple proteins may be simultaneously associated with
a single PCNA. The three human FEN-1 molecules co-crystallized with PCNA all adopt different orientations with respect to PCNA, mediated by a flexible linker between the PIP motif and the catalytic core of FEN-1. Although two of the orientations are similar, one of them corresponding to a competent orientation to engage DNA, the third FEN-1 molecule forms extensive interactions with the side of PCNA, preventing access to its central pore through which DNA is proposed to pass. This orientation may correspond to a biologically relevant inactive ‘carrier’ conformation, in which FEN-1 is associated with PCNA, but unable to engage DNA, thus preventing interference with other active client proteins bound to PCNA (Figure 3). The existence of distinct active and carrier conformations may explain previous biochemical data showing that different regions of PCNA are responsible for binding of FEN-1 in solution and its stimulation on DNA; thus the PIP motif may be most important for recruiting FEN-1 to PCNA and holding it in a carrier configuration, while a different set of interactions may be responsible for maintaining FEN-1 in a catalytically active orientation.

Similar inactive conformations mediated by interactions with the side of the sliding clamp have been observed with the translesion DNA polymerases Pol IV of E. coli and Dpo4 of S. solfataricus, the former in good agreement with biochemical data demonstrating its ability to bind the β-clamp simultaneously with Pol III, without interfering with its activity. Structural changes in an archaeal polymerase upon interaction with its PCNA platform have also recently been shown to be important in switching between polymerase and exonuclease catalytic centres.

An even more striking conformational change has been observed between apparent carrier and active forms of DNA ligase I bound to PCNA. In the absence of its DNA substrate, DNA ligase I adopts an elongated configuration radiating out from the PCNA ring, which presumably allows other proteins to bind the ring and engage DNA. In the presence of its nicked DNA substrate, however, DNA ligase I undergoes a large rearrangement to completely encircle the DNA, a conformation which would appear to occlude the binding of other client proteins to PCNA.

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**Figure 3** | PCNA client proteins show conformational malleability

(A) Distinct conformers of PCNA–FEN-1. PCNA is shown in blue and FEN-1 is shown in green. The left-hand panel shows an inactive ‘carrier’ configuration. The right-hand panel shows FEN-1 positioned to interact productively with DNA. For clarity, only a single FEN-1 is shown; in the solved structure, however, all three PCNA subunits were engaged by FEN-1 molecules.

(B) Alternative conformations of DNA ligase I. The left-hand panel shows the structure of S. solfataricus DNA ligase I in an extended configuration, the right-hand panel shows human DNA ligase I in complex with DNA, revealing the domains of the ligase to encircle the DNA substrate. Images were generated with PyMOL (DeLano Scientific; http://www.pymol.org) using PDB files 1UL1 (human FEN-1–PCNA), 2HIV (S. solfataricus DNA ligase I), 1X9N (human DNA ligase I).
discussed, other proteins such as FEN-1 may remain bound to PCNA by adopting complementary carrier configurations locked to the side of the ring to accommodate active ligase. Alternatively, it is possible that the removal of other binding partners from PCNA intentionally ‘resets’ the ring, as the final stage in Okazaki fragment maturation.

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