Replication Protein A Directs Loading of the DNA Damage Checkpoint Clamp to 5′-DNA Junctions*

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The heterotrimeric checkpoint clamp comprises the Saccharomyces cerevisiae Rad17, Mec3, and Ddc1 subunits (Rad17/3/1, the 9-1-1 complex in humans). This DNA damage response factor is loaded onto DNA by the Rad24-RFC (replication factor C-like complex with Rad24) clamp loader and ATP. Although Rad24-RFC alone does not bind to naked partial double-stranded DNA, coating of the single strand with single-stranded DNA-binding protein RPA (replication protein A) causes binding of Rad24-RFC via interactions with RPA. However, RPA-mediated binding is abrogated when the DNA is coated with RPA containing a rpa1-K45E (rfα1-t11) mutation. These properties allowed us to determine the role of RPA in clamp-loading specificity. The Rad17/3/1 clamp is loaded with comparable efficiency onto naked primer/template DNA with either a 3′-junction or a 5′-junction. Remarkably, when the DNA was coated with RPA, loading of Rad17/3/1 at 3′-junctions was completely inhibited, thereby providing specificity to loading at 5′-junctions. However, Rad17/3/1 loaded at 5′-junctions can slide across double-stranded DNA to nearby 3′-junctions and thereby affect the activity of proteins that act at 3′-termini. These studies show a unique specificity of the checkpoint loader for 5′-junctions of RPA-coated DNA. The implications of this specificity for checkpoint function are discussed.

DNA damage elicits a broad range of cellular responses from DNA repair to inhibition of cell cycle progression. DNA damage checkpoints cause the arrest of cells at appropriate points in the cell cycle so that the integrity of the DNA can be restored (1). Although damage-induced checkpoints can be executed in many phases of the cell cycle, most of the progress in understanding the molecular details of this process has been made in the G1 phase of the cell cycle, because its execution is uncumbered by complications related to the progression of DNA replication or chromosome segregation. In G1, for the DNA damage checkpoint to be executed in response to UV irradiation, repair of damage needs to be initiated by the nucleotide excision repair machinery (2). This finding suggests that gaps made during the progression of nucleotide excision repair are targets for the checkpoint machinery.

Gaps in the double-stranded DNA contain three structural elements, a region of single-stranded DNA (ssDNA), a ss-ds DNA junction with a 5′-terminus (5′-junction), and a ss-ds DNA junction with a 3′-terminus (3′-junction). Although the ssDNA is a target for binding by the single-stranded binding protein RPA, the 5′- and 3′-junction can be targeted by a variety of proteins, including nucleases (to either junction), DNA polymerases (to 3′-junctions), and circular clamp proteins. The replication clamp proliferating cell nuclear antigen (PCNA) is uniquely targeted to 3′-junctions and in that capacity serves as a processivity factor for several DNA polymerases and for a large number of other proteins that participate in various DNA metabolic pathways (reviewed in Ref. 3). Much less is known about the PCNA-related checkpoint clamp Rad17/3/1, a heterotrimer composed of the yeast Rad17, Mec3, and Ddc1 subunits. This clamp is the ortholog of the human 9-1-1 clamp, consisting of Rad9, Rad1, and Hus1 subunits.

The final steps of the DNA damage checkpoint pathway resulting in inhibition of the cell cycle are well understood (reviewed in Refs. 4 and 5). However, molecular details are lacking regarding its initiation. Required for checkpoint initiation are the yeast ataxia telangiectasia-related protein kinase Mec1 and the checkpoint clamp Rad17/3/1 and its loader Rad24-RFC. Mec1 forms a heterodimer with Ddc2, and localization of Mec1/Ddc2 to DNA is mediated by interactions between Ddc2 and the eukaryotic single-stranded DNA-binding protein RPA (6). Loading of the clamp at sites of DNA damage proceeds independently of Mec1 localization (7, 8). The loader Rad24-RFC is a heteropentameric complex that has the four small Rfc2–5 subunits in common with the canonical clamp loader replication factor C (RFC), the loader of PCNA (9). DNA binding is specified by the clamp loader. Productive binding of RFC proceeds exclusively toward a 3′-junction. Successful loading of PCNA by RFC requires binding of ATP to the Rfc2, Rfc3, and Rfc4 subunits (10). ATP binding causes opening of the clamp-clamp loader complex, which then is capable of binding template-primer DNA. Hydrolysis of bound ATP causes closing of PCNA around DNA and release of RFC. Substitution of

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§ The abbreviations and trivial names used are: ssDNA, single-stranded; dsDNA, double-stranded DNA; RPA, replication protein A; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; Rad24-RFC, complex of Rfc2–5 and Rad24; Rad17/3/1, complex of Rad17, Mec3, and Ddc1; pol, DNA polymerase; SPR, surface plasmon resonance; nt, nucleotide(s); ATPγS, adenosine 5′-3′-(thio)triphosphate.

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the Rfc1 subunit by Rad24 (hRad17 in humans) endows this alternative complex with the capacity to load Rad17/3/1 onto DNA while abrogating loading of PCNA. Once loaded, the clamp has the ability to slide across double-stranded DNA, although less efficiently than does PCNA (11, 12).

Several studies have been carried out to determine the DNA substrate specificity for the checkpoint clamp and clamp loader from yeast and from human. We initially showed that yeast Rad24-RFC can load its clamp onto both 3′-junctions and 5′-junctions in an ATP-dependent reaction (12). Similar observations were made by Zou et al. (13). However, Ellison and Stillman conclude that 5′-junctions constitute the preferred substrate for loading of the human 9-1-1 clamp (14). It is important to understand the substrate specificity and what factors contribute to it, because this specificity determines whether the clamp binds target proteins, e.g. nuclease, that likely act at 5′- or 3′-junctions.

In an effort to find a reconciliation for the apparently contradictory results in DNA-loading specificity, we have carried out a comprehensive biochemical study of the substrate specificity for the yeast checkpoint clamp system. We have concluded that, although loading on naked DNA at both 5′- and 3′-junctions was observed, coating of the ssDNA with RPA strongly inhibited loading onto 3′-junctions, thereby endowing a unique specificity of the checkpoint loader for 5′-junctions.

EXPERIMENTAL PROCEDURES

Proteins, Yeast Strains, and DNA—The checkpoint factors and DNA polymerase δ (pol δ) were overproduced in yeast and purified as described previously (12, 15, 16). PCNA and RFC (a version lacking the N-terminal 172-amino-acid domain of Rfc1) were overproduced in Escherichia coli (17). The yeast Rpa1-K45E mutation, known as rpa1-t11 (18), was generated by tandem PCR with primers containing the desired mutation and using p11d-ctcRPA as the template, analogous to that described previously (19). The three-subunit protein complex (RPA-t11) was overexpressed in E. coli strain BI21 (DE3) and purified as described earlier (20). A truncation mutant of RPA lacking the N-terminal 180-amino-acid domain of Rpa1 (RPA-1Δ) was overproduced in E. coli and purified as described previously (21).

The oligonucleotides used in the SPR and replication assays were template oligonucleotide V21 (biont-5′-TTGGAATTCT-TTGTCCCCAAACTTAACCTATCTGACACATGCAGCTCAGATTAT-3′-biotin) and complementary oligos C21-1 (5′-junction, 3′-DS end, 5′-TG-TCAAAAAAAAAAAAAAGATTCAAAA-3′), C21-2 (3′-junction, 5′-DS end, 5′-ATTATACATTGACGCTGAGCAGCAGATG-3′), and C21-3 (3′-junction, 5′-9 nt gap, 5′-GCAGCAGACGAGTGAAGTCCGCTGTAAGTGGTGGTGCTGCGTCTG-3′). When used as primers for replication assays, oligos C21-2, C21-3, and C21-4 were labeled with T4 polynucleotide kinase and [γ-32P]ATP before annealing to the template V21. Streptavidin (3-fold molar excess over DNA) was then added to create biotin-streptavidin bumpers at the template ends. The 50-mer 5′- ATACGACTCTACTATAGGGCC- GAATGGGTTACCAGGGCCCCCCTCGAGGTCG-3′ or a derivative with terminal biotin moiety(ies) was hybridized to SS Bluescript DNA followed by the addition of a 3-fold molar excess of streptavidin.

Replication Assay—The 40-μl replication assays contained 25 mM Heps, pH 7.8, 70 mM NaCl, 8 mM MgAc2, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 100 μM each dNTPs, 1 mM ATP or ATPγS, 3.6 nM DNA, either V21/C21-2, V21/C21-3, or V21/C21-4 with the primer 5′-32P-labeled, and when present, 75 nM Rad17/3/1 and 30 nM Rad24-RFC. The reaction was pre-incubated for 2 min at 30 °C before the addition of 2 nM pol δ. Aliquots were taken at the indicated times, quenched in an equal volume of sequencing stop buffer, and analyzed by 8 M urea/16% PAGE. Quantitation was done by phosphorimaging.

DNA-Protein Interaction Analysis—Surface plasmon resonance (SPR) was performed in a BIacore X apparatus. The running buffer used in the analysis contained 25 mM Heps-NaOH, pH 7.8, 125 mM NaCl, 5 mM MgAc2, 1 mM dithiothreitol, 0.05% Tween 20, and 100 μg/ml bovine serum albumin. The DNA chips were prepared as described previously and contained ~50–100 resonance units of biotinylated DNA attached via streptavidin to an F1 chip (12). The interaction between Rad24-RFC (30 nM) and Rad17/3/1 (60 nM) with DNA was monitored at 20 °C by injecting, at a flow rate of 30 μl/min, 80 μl of the protein factors and 1 mM ATP over a DNA chip. Coating of the DNA with wild-type or mutant RPA was accomplished by injecting 80 μl of 100 nM RPA across the DNA chip. Regeneration of the chip was accomplished by a 10-μl injection of 1 mM NaCl, 0.02% SDS.

Gel Filtration Analysis of Clamp Loading—150 fmol of effector DNA was incubated with 250 fmol of Rad24-RFC and 2 pmol of [32P]Rad17/3/1 in buffer A (25 mM Heps-NaOH, pH 7.8, 75 mM NaCl, 8 mM MgAc2, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin) with 1 mM ATP at 23 °C for 3 min and then loaded onto a 5-ml BioGel A5m column equilibrated in buffer A. The radioactivity in each 6-drop fraction was determined in a scintillation counter.

RESULTS

Rad24-RFC Interacts with RPA—Previously, we used an SPR methodology to detect Rad17/3/1 loading onto partial duplex DNA (12, 15). These studies allowed us to make the following general conclusions with regard to clamp-clamp loader interactions and loading onto DNA. (i) Loading of Rad17/3/1 requires Rad24-RFC and ATP. (ii) In analogy with the canonical PCNA-RFC system, ATP binding to Rad24-RFC suffices for the formation of a high affinity clamp-clamp loader complex, clamp opening, and formation of a ternary complex with effector DNA, although hydrolysis of the bound ATP results in closure of the clamp around DNA and release of the loader. (iii) Under our standard reaction conditions containing 125 mM NaCl, the measured affinities of the clamp and the loader for each other and for DNA are in the 2–5 nM range. Therefore, we have carried out most of our current studies at 30 nM Rad24-RFC and 60 nM Rad17/3/1, which were saturating under all conditions.

A DNA substrate was used consisting of a 71-mer template and a 28-mer primer positioned flush at either end of the tem-
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![Diagram with labels A, B, C, D]

**FIGURE 1. Interaction between Rad24-RFC and RPA requires the N-terminal domain of Rpa1.** Proteins were flowed across a chip with the 5'-junction DNA substrate (V21/C21-1) as described under "Experimental Procedures." A, scheme of the assay. L, Rad24-RFC; C, Rad17/3/1. B, DNA was coated with RPA where indicated, and the clamp and/or loader together with ATP, except where indicated as − ATP, were injected at t = 0. The dashed line represents injection of buffer at t = 0 after coating with RPA. The hatched line indicates the dissociation phase of the C + L experiment fitted to first order kinetics. C, DNA was coated with wild-type or mutant RPA followed by injection of loader or RFC and ATP. D, DNA was coated with wild-type of mutant RPA as indicated followed by injection of clamp and loader and ATP.

Plate allowing ample ssDNA for binding by RPA. Biotin residues were incorporated at both ends of the template, thereby providing a double anchor to the streptavidin chip. We used a SPR chip containing flexible dextran linkers, anticipating that, on such a chip, both biotins on the template DNA would be able to bind accessible streptavids (Fig. 1A). Remarkably, although many different binding configurations can be envisaged with such a dual biotin arrangement, when the clamp and clamp loader together with ATP were flowed across a chip charged with 5'-junction DNA, the dissociation phase of the binding curve followed first order kinetics, suggesting that the majority of clamp-clamp loader complexes encounter DNA molecules that are in a similar environment (Fig. 1B).

Control experiments showed that only background signal was observed when any two factors were flowed across the chip containing naked DNA: Rad24-RFC with ATP, or Rad17/3/1 with ATP, or Rad24-RFC together with Rad17/3/1 but without ATP (Fig. 1B). However, when the DNA had previously been coated with RPA, strong binding was observed when Rad24-RFC alone was injected, regardless of whether Rad17/3/1 or ATP were also present. This strong binding signal was also observed when Rad24-RFC was flowed over RPA-coated 3'-junction DNA (data not shown). We reasoned that the observed signal resulted from direct interactions between RPA and Rad24-RFC, as had previously been reported for the human complex hRad17-RFC (13). This strong interaction was mediated through the Rad24 subunit because canonical RFC failed to elicit a signal when injected across a chip with RPA-coated DNA (Fig. 1C). Previously, interactions between RFC and RFA have also been reported, but these were likely too weak to give a positive signal under our assay conditions in buffer containing 125 mm NaCl (22, 23). The presence of the very strong loading-independent signal due to RPA-Rad24 interactions prevented us from determining the role of RPA in the loading process itself. Therefore, we tested mutant forms of RPA that could be expected to show a deficiency for interaction with Rad24.

As most RPA-interacting proteins mediate binding through the N-terminal region of the large Rpa1 subunit (24), we assessed whether mutant forms of RPA with mutations in that region would abrogate binding to Rad24-RFC. First, we tested a form of RPA lacking the N-terminal 180 amino acids of Rpa1 (RPA-1Δ). This RPA truncation mutant shows normal binding to ssDNA (21, 25). However, interaction with Rad24 was completely abrogated (Fig. 1C).

Next, we tested a mutant RPA containing the Rpa1-t11 mutation (K45E). The rfa1-t11 allele was originally isolated in a genetic screen for damage-sensitive Rpa1 mutants (18). The rfa1-t11 mutant shows defects in mitotic and meiotic recombination and double strand break repair and affects the adaptation of cells to irreparable double-stranded DNA breaks (18, 26, 27). The mutant does not measurably affect DNA replication, and the mutant RPA complex RPA-t11 binds ssDNA in a similar manner as wild-type (data not shown) (28). The mutant protein also shows reduced interactions with Ddc2, a subunit of the Mec1/Ddc2 checkpoint kinase (6). However, the rfa1-t11 mutant shows only very minor defects in the DNA damage checkpoint (data not shown) (6, 23, 29). Remarkably, in our SPR assay, no binding of Rad24-RFC to the mutant RPA (RPA-t11) was observed (Fig. 1C). These results suggest that the functionality of the DNA damage checkpoint is relatively insensitive to the strength of the RPA-Rad24 interactions.

When either of these two mutant RPAs was used in our checkpoint clamp-loading assay, we detected a robust signal that was dependent on the presence of Rad24-RFC, Rad17/3/1, and ATP (Fig. 1D and data not shown). Moreover, the kinetics of binding and dissociation were independent of the mutant RPA used to coat the DNA. Interestingly, when the DNA was coated with the interaction-defective mutants of RPA, the dissociation kinetics of the clamp-clamp loader were not greatly affected compared with those with naked DNA (koff = 0.018 s⁻¹ without RPA, 0.01 s⁻¹ with RPA-1Δ, and 0.011 s⁻¹ with RPA-t11). In contrast, dissociation of Rad24-RFC alone from DNA coated with wild-type RPA was very slow (koff < 0.001 s⁻¹). As expected, complex kinetics were observed when Rad17/3/1 was loaded by Rad24-RFC onto DNA coated with wild-type RPA, reflecting contributions due to RPA interactions with Rad24-RFC and to the stability of the loaded clamp.
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![Diagram](image)

**FIGURE 2. RPA restricts loading of Rad17/3/1 to 5'-junctions.** The assays were carried out as diagrammed in Fig. 1A. The chip contained either 5'-junction DNA (V21/C21-1; A and C) or 3'-junction DNA (V21/C21-2; B and D). The chips were coated with the indicated form of RPA followed at t = 0 by injection of Rad24-RFC (L) and Rad17/3/1 (C), with ATP (A and B) or RFC, and ATP and PCNA where indicated (C and D). Absolute response signals (in resonance units) were converted into relative response signals to correct for differences in absolute coupling of the 5'-junction DNA (50 resonance units) and the 3'-junction DNA (90 resonance units).

Thus, the use of RPA mutants allowed us to investigate clamp-loading kinetics on naked versus coated DNA.

**RPA Inhibits Loading of the Clamp at 3'-Junctions**—Having established a loading assay that allows us to investigate the role of RPA without interfering with binding to the clamp loader, we next investigated the role of the effector DNA. We used two template-primer substrates, with each primer providing one unique junction for loading, because the other side of the primer was situated flush against the terminal template biotin-streptavidin bumper. On the 5'-junction substrate, which was also used in the studies presented in Fig. 1, we observed robust loading of Rad17/3/1 in a clamp loader- and ATP-dependent manner. Coating of the DNA with RPA-1Δ or with RPA-t11 resulted in loading with almost the same efficiency (Fig. 2A). However, a radically different result was obtained with the 3'-junction substrate. Although loading of Rad17/3/1 on naked 3'-junction DNA proceeded with high efficiency and in a clamp loader- and ATP-dependent manner, loading on the same effector DNA coated with RPA-1Δ or RPA-t11 was completely abrogated (Fig. 2B). These results not only indicate that the 3'-junction of RPA-coated DNA is inaccessible to the checkpoint system but also that the double-stranded end of the DNA situated against the biotin-streptavidin bumper did not provide an entry site for loading. In a control experiment, we moved the primer downwands on the template so that a 9-nt gap was created between the 5'-end of the primer and the biotin-streptavidin. This gapped substrate (depicted in Fig. 4, right panel) was fully functional for checkpoint clamp loading, even when the DNA was coated with mutant RPA (data not shown).

Although RPA provides specificity for checkpoint clamp loading toward 5'-junctions, the exact opposite was observed for PCNA loading by RFC (Fig. 2, C and D). In these studies, we used a version of RFC that lacks the N-terminal 172 amino acids of Rfc1. This domain has been shown to bind phosphorylated 5'-junction DNA, yet this activity is dispensable for PCNA loading (17, 30, 31). Both nonspecific binding of RFC to 5'-junction DNA and inefficient loading of PCNA on this substrate was completely blocked by coating the DNA with RPA (Fig. 2C). On the other hand, although low-affinity binding of RFC to 3'-junctions was eliminated by RPA, functional loading of PCNA was not (Fig. 2D) (32).

The SPR technique has provided us with a solid and sensitive assay for checkpoint clamp loading, and the results allow for a rigorous comparison with previous analogous studies of PCNA loading by RFC. We wanted to confirm these results using more traditional clamp-loading assays in which primed circular DNA was used as substrate and clamp-DNA complexes were isolated by gel filtration. These assays could only be performed when the ssDNA was coated with RPA, because in its absence, clamp loading was both inefficient and nonspecific, likely because of the presence of large regions of ssDNA (data not shown). However, the assay allowed us to use wild-type RPA, as control experiments showed that very little clamp was associated with the wild-type DNA-coated DNA when either ATP or Rad24-RFC was omitted from the incubation or the ssDNA lacked a primer (Fig. 3) (15). Thus, unlike with the SPR assay, this assay allowed us to monitor complex formation on DNA coated with wild-type RPA.

The clamp was 32P-labeled for ease and accuracy of quantitation. The 3-kb SS Bluescript circle was primed with a 50-mer and loading of [32P]Rad17/3/1 monitored. Blocking of the 5'-end of the primer with biotin and streptavidin substantially reduced loading, whereas blocking of the 3'-end had no effect on the loading efficiency (Fig. 3). Blocking of both the 5' and 3'-ends yielded the same loading efficiency as did blocking of only the 3'-end. These data are consistent with our conclusion from the SPR studies, i.e. that loading to 3'-junctions is abrogated when the ssDNA is coated with RPA. They also suggest that the inactivity of the 3'-junction merely requires coating of the ssDNA, and either wild-type RPA, RPA-1Δ, or RPA-t11 can fulfill this function.

We also determined whether a DNA nick served as a loading site for the checkpoint clamp, but no activity at a nick was observed (Fig. 3). PCNA is loaded at a nick by RFC, as one would expect given its function in base excision repair (33). On the other hand, PCNA is not loaded at a nick by the alternative clamp loader Ctf18-RFC (25).

**After Loading at 5'-Junctions, the Clamp Can Slide to 3'-Junctions**—Previously, we have shown that Rad17/3/1 can slide across dsDNA (12). In the course of searches for enzymes that might be stimulated by the checkpoint clamp, we actually noted that Rad17/3/1 inhibited rather than activated pol. This inhibition did not appear to be specific, as the activity of E. coli DNA polymerase I, Klenow fragment, and that of T7 DNA polymerase was also inhibited by the checkpoint clamp (data not shown). Inhibition requires the clamp loader and ATP and therefore appears to represent the result of bona fide loading of
An alternative possibility would be that the mere occupancy of the clamp loading as the presence of the clamp on the dsDNA could be transparent to the polymerase. In a control reaction, ATP was replaced by the non-hydrolyzable analog ATP抵抗, very little inhibition of pol ε activity was observed (Fig. 4; compare lanes 18 and 19 with lanes 16 and 17), suggesting that the observed inhibition most likely originates from 5′-loading followed by sliding to the 3′-junction.

**DISCUSSION**

The replication clamp PCNA functions as a processivity factor for DNA polymerases and stimulates the activity of many other proteins in DNA metabolism, generally by increasing their residence time on the DNA. Based upon the structural and functional analogies between PCNA and Rad17/3/1, it has been assumed that the checkpoint clamp fulfills a similar function. In both S. pombe and S. cerevisiae, genetic studies suggest that the DNA damage checkpoint clamp and clamp loader are required for a robust mutagenic response (35–37). Interactions have been demonstrated between the clamp and DNA polymerase ε, the major mutagenic enzyme in the eukaryotic cell (37). However, these observations have not been verified in vitro; PCNA rather than Rad17/3/1 was shown to be a cofactor for translesion synthesis by pol ε (38).

Biochemical studies have identified two enzymes that are stimulated in vitro by the presence of the human 9–1–1 complex, the human ortholog of S. cerevisiae Rad17/3/1. The human 9–1–1 complex stimulates DNA polymerase β activity,
Directionality of the Checkpoint Clamp

![Diagram of checkpoint clamp configurations](image)

**FIGURE 5.** Polarity of the clamp at junctions depends on the site of loading. A, on naked DNA, Rad17/3/1 can be loaded at either junction and subsequently slide to a neighboring junction. B, RPA-coated DNA restricts loading to 5'-junctions, yet 3'-junctions can be functionalized through sliding. The binding orientation of RPA is from Refs. 42 and 43.

primarily by increasing the efficiency of primer recognition by pol β rather than the processivity of polymerization (39). The human checkpoint clamp also stimulates FEN1 activity (40). In these studies, the clamp was not loaded by the hRad17-RFC clamp loader but was allowed to slide onto the double-stranded ends of substrate DNA in a manner that was previously shown to also mediate RFC-independent loading of PCNA (41). This mode of clamp loading by diffusion onto dsDNA ends not only requires a large excess of clamp but also yields products with the clamp positioned in either orientation with regard to the primer-template junction. Therefore, it is not clear which side of the clamp is responsible for mediating the observed stimulation of FEN1 or pol β. For instance, is the increased primer recognition by pol β caused by interaction with the side of the clamp that the loader normally would position at the 3'-terminus or is it with the opposite side of the clamp that would be positioned there after loading at a 5'-junction followed by sliding across intervening dsDNA to the 3'-terminus (Fig. 5A)?

The studies described here show that Rad24-RFC is a dual polarity loader of Rad17/3/1 on naked DNA; however, loading of the clamp on 3'-junctions is inhibited when the DNA is coated with RPA (Fig. 2). These results are in agreement with those by Ellison and Stillman (14), who carried out their studies on RPA-coated DNA templates. In contrast, they do not support the conclusion from the Elledge group (13) that hRad17-RFC is a dual polarity clamp loader on RPA-coated DNA. However, in their experimental protocol, these authors used a concentration of RPA insufficient to coat the entire ssDNA region of the circular DNA (1 pmol of RPA/pmol of ssDNA circle). Therefore, naked 3'-junctions likely were present and permissive for clamp loading.

RPA binds ssDNA with a defined polarity (42, 43). The orientation of RPA is such that the N-terminal domain of Rpa1 will be at the 5'-end of the ssDNA and the C-terminal domain at the 3'-end. Consequently, the 5'-junction of primed ssDNA is closest to the N terminus, and the 3'-junction is closest to the C terminus of Rpa1. The mutant forms of RPA containing Rpa1-(ΔN1–180) or Rpa1-t11 (K45E) affect the N-terminal region of Rpa1, leaving the C-terminal region and its inhibitory effect on 3'-junction loading intact. In vivo, where RPA participates in replication and many repair processes, it is anticipated that RPA will bind to most DNA intermediates containing ssDNA, thereby restricting checkpoint clamp loading to 5'-junctions. For instance, during simple checkpoint activation in the G1 phase of the cell cycle, the ~30-nt DNA gap produced during the course of nucleotide excision repair would only allow loading of the clamp at 5'-junctions because of RPA binding at the gap. Therefore, under these conditions, the target protein for the checkpoint clamp would most likely be one that acts at 5'-junctions, e.g. a 5'-nuclease, and not a DNA polymerase that acts at 3'-junctions.

However, the studies in Fig. 4 suggest that additional checkpoint functionalities could exist when the DNA discontinuities are more complex. If multiple gaps are formed on the DNA, for instance at stalled DNA replication forks, loading of Rad17/3/1 at a 5'-junction could result in its presence at a neighboring 3'-junction because of its ability to slide across double-stranded DNA (Fig. 5B). And if RPA were not present at a gap, perhaps because the gap is too small for RPA binding, all four distinct clamp configurations are conceivable (Fig. 5A). Although the checkpoint clamp is not loaded at a nick (Fig. 3), robust loading was observed at a 9-nt gap.

The specificity for checkpoint clamp loading revealed in this study has certain predictive value. In the checkpoint pathway elicited by nucleotide excision repair of DNA damage (2), it is likely that the checkpoint will be functionalized by an enzyme that acts at 5'-junctions, perhaps an exonuclease. On the other hand, to understand checkpoint function in response to more complex forms of DNA damage, it is possible that 3'-junctions can also be of importance. In this regard, it will be important to determine how the polarity of the clamp at a given junction can affect its interactions with a target protein in the checkpoint pathway itself or in a pathway activated by the checkpoint.

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