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Unwind and slow down: checkpoint activation by helicase and polymerase uncoupling

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Abstract

Phosphoinositide 3-kinase related protein kinases (PIKK) including ataxia-telangiectasia mutated (ATM), DNA-dependent protein kinase (DNA-PK), and ATM and Rad3-related (ATR) coordinate cellular responses to DNA damage. DNA-PK and ATM are primarily activated by double-strand breaks. The ATR kinase, in contrast, responds to numerous forms of genotoxic stress including intrastrand cross-links, oxidative damage, and polymerase toxins. At first glance, the disparate DNA structures that activate ATR impose a difficult biochemical challenge for damage sensing. However, recent data indicate that one DNA structure—single-stranded DNA (ssDNA) coated with a single-stranded DNA-binding protein (RPA)—is a common intermediate to activate ATR signaling in response to all of these genotoxic lesions. In this issue, Cimprich and colleagues (Byun et al. 2005) define how DNA damage and polymerase toxins use a common mechanism to generate the RPA–ssDNA needed to activate ATR. The method is the decoupling of helicase and polymerase activities at a replication fork.

Linking RPA-coated ssDNA to checkpoint kinase activation

The ATM and ATR protein kinases share many biochemical and functional similarities. Both are large protein kinases with significant sequence homology and a strong preference to phosphorylate serine or threonine residues followed by glutamine. Both target an overlapping set of substrates that promote cell cycle arrest. However, ATR is essential for the viability of replicating human and mouse cells while ATM is not (Brown and Baltimore 2000; Cortez et al. 2001). ATM functions in response to rare occurrences of double-strand breaks. ATR is activated during every S phase to regulate the spatial and temporal firing of replication origins, the repair of damaged replication forks, and to prevent the premature onset of mitosis (Nyberg et al. 2002; Shechter et al. 2004b). *ATM* mutations predispose carriers to develop cancer and are found in ~1% of people. *ATR* mutations are rare and probably only compatible with viability when heterozygous or hypomorphic. While the only clear link between *ATR* mutation and disease is in a few patients with the rare Seckel syndrome (O'Driscoll et al. 2003), disruptions in the ATR pathway do cause genomic instability, and the ATR signaling pathway is activated by most cancer chemotherapies. Thus, the mechanistic details of how ATR functions are being actively investigated by numerous laboratories.

One clue to how ATR might differ from ATM came from the identification of a protein (ATRIP) that selectively binds to ATR (Cortez et al. 2001). ATRIP and ATR are almost always found in a complex, and the stability of each of these proteins is dependent on the other. ATRIP is essential for all known ATR activities and deletion of ATRIP phenocopies deletion of ATR. Recently, a C-terminal domain of ATRIP that binds an N-terminal domain of ATR was identified (Ball et al. 2005; Falck et al. 2005). Interestingly, there are similarities in a small part of the ATR-binding domain of ATRIP to regions in the Ku80 and Nbs1 proteins that bind to

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their respective PIKK partners DNA-PK and ATM (Falck et al. 2005). Ku and Nbs1 help to localize DNA-PK and ATM to double-strand breaks (Dvir et al. 1992;Gottlieb and Jackson 1993;Carson et al. 2003;Kitagawa et al. 2004;Falck et al. 2005). Might ATRIP also serve as a recruitment module for the ATR kinase? Indeed, ATRIP is required for ATR localization to damage-induced nuclear foci (Ball et al. 2005), and ATRIP is required for ATR to bind DNA in *Xenopus* extracts (Kumagai et al. 2004).

A second important difference between ATR and ATM signaling should be appreciated. While ATM can be activated throughout the cell cycle, ATR activation is usually linked to ongoing DNA synthesis (Hekmat-Nejad et al. 2000;Lupardus et al. 2002;Stokes et al. 2002;Tercero et al. 2003). For example, in *Xenopus* extracts ATR associates with chromatin specifically during replication (Hekmat-Nejad et al. 2000) and inhibition of replication blocks ATR-dependent signaling (Lupardus et al. 2002;Stokes et al. 2002). In human cells, ATR activation after ultraviolet radiation (UV) also requires replication (Ward et al. 2004). This suggests that proteins or DNA structures that are specific to replicating cells may be needed to activate ATR. One possibility is that the replication fork itself is being sensed. A second possibility, and the one supported by the Cimprich data, is that a structure efficiently generated by replication enzymes (RPA–ssDNA) is sensed by ATR–ATRIP (Byun et al. 2005).

Several experiments indicate that RPA–ssDNA is essential for the activation of ATR checkpoint signaling. First, depletion of RPA from *Xenopus* extracts prevents ATR activation by topoisomerase inhibitors (Costanzo et al. 2003). Second, depletion of RPA from human cells with RNA interference prevents ATR–ATRIP localization to nuclear foci and phosphorylation of the ATR substrate Chk1 in response to UV radiation (Zou and Elledge 2003). Third, mutants in yeast RPA exhibit a checkpoint defect (Longhese et al. 1996;Kim and Brill 2001). Fourth, ATRIP can bind to RPA–ssDNA (Zou and Elledge 2003). Deletion of the RPA–ssDNA-binding domain on ATRIP prevents it from accumulating in nuclear foci that contain DNA lesions or stalled replication forks (Ball et al. 2005). Finally, RPA–ssDNA is required for the Rad17-dependent clamp loader to load a damage-specific clamp containing the human Rad9, Rad1, and Hus1 (9–1–1) proteins (Ellison and Stillman 2003;Zou et al. 2003). The 9–1–1 complex is essential for ATR checkpoint signaling. Thus, RPA–ssDNA serves a similar purpose for ATR as a double-strand break serves for ATM.

How to make RPA–ssDNA

If RPA–ssDNA is the DNA structure that activates the ATR–ATRIP checkpoint kinase, then how is it generated by diverse genotoxic stresses? Two hypotheses have been advanced. First, RPA–ssDNA is an intermediate in many DNA repair processes. For example, the repair of a UV-induced thymine dimer by nucleotide excision repair proceeds through nuclease and helicase steps that expose a ssDNA gap containing RPA. A second hypothesis is that the stalling of a DNA polymerase without the concomitant stalling of the DNA helicase could generate regions of RPA–ssDNA at a replication fork. Polymerase inhibitors, such as aphidicolin, activate ATR and cause extensive unwinding of plasmid DNA placed in a replication competent *Xenopus* egg extract (Walter and Newport 2000). DNA lesions that block DNA polymerases do not interfere with the viral T-antigen helicase in the SV-40 replication system (Cordeiro-Stone et al. 1999;Veaute et al. 2000). Is the eukaryotic replicative helicase also insensitive to DNA lesions? Cimprich and colleagues (Byun et al. 2005) now demonstrate conclusively that functional uncoupling of helicase and polymerase activities does occur and is required for checkpoint signaling in *Xenopus* extracts.

They begin by demonstrating that UV or cisplatin-damaged plasmids (containing intrastrand cross-links) are extensively unwound in replication-competent extracts. Importantly, unwinding is correlated with Chk1 phosphorylation. Chk1 is directly phosphorylated and

activated by ATR and is required for cell cycle arrest (Liu et al. 2000). The timing of Chk1 phosphorylation was slightly delayed compared with ssDNA formation, and as replication converted the unwound plasmid to duplex DNA, Chk1 phosphorylation disappeared. The authors then demonstrate that unwinding of the plasmid is dependent on the action of the MCM helicase. A fragment of the Rb protein that binds MCM7 and inhibits helicase activity (Sterner et al. 1998) blocks unwinding, as do neutralizing antibodies to CDC45, which functions as a cofactor for the MCM complex (Pacek and Walter 2004). Most importantly, when unwinding was inhibited, ATR no longer phosphorylated Chk1. Thus, the presence of the damaged DNA by itself is insufficient to generate a checkpoint signal. Instead, an interaction between the lesion and a replication fork is needed (Fig. 1).

These data indicate that the difference in the ability of the polymerase and helicase to use a damaged template provides the mechanism of generating the RPA–ssDNA required to activate the checkpoint. One implication of these results is that lesions that prevent the helicase from unwinding may not elicit an ATR-dependent checkpoint response. It would be interesting to determine whether an interstrand cross-link would block the helicase and/or elicit a checkpoint response in this system. Interstrand cross-linking agents such as mitomycin C, nitrogen mustard, and photoactivated psoralen do elicit ATR signaling when added to cultured cells (Lambert et al. 2003; Pichierri and Rosselli 2004); however, these agents also cause intrastrand cross-links, and their repair can proceed through an intrastrand cross-link intermediate, thus, it is unclear what is actually being sensed by ATR. It will also be important to examine how the specific placement of an intrastrand lesion on either the leading or lagging strand causes differences in the checkpoint response. Lagging-strand lesions may cause only small gaps of ssDNA instead of extensive unwinding because Okazaki fragment synthesis can reinitiate downstream of the lesion (Meneghini and Hanawalt 1976; Svoboda and Vos 1995). Unfortunately, since plasmids in *Xenopus* extracts initiate replication randomly, other systems will be needed to answer this question.

Another conclusion of the paper is that the amount of RPA–ssDNA generated determines the extent of Chk1 activation. Previous experiments in yeast had suggested that the number of active replication forks may determine a threshold required for ATR activation (Shimada et al. 2002). By performing careful molar and mass equivalent experiments with different size plasmids, Cimprich and colleagues (Byun et al. 2005) determined that the extent to which ssDNA is produced rather than the number of forks determines the amount of ATR activation in *Xenopus* extracts. It remains possible that there is a threshold to the ATR response, but perhaps in animal cells the threshold is reflected in different ATR signal strengths needed to activate different types of local or cell-wide checkpoint responses instead of a threshold for ATR activation per se. If one considers RPA–ssDNA as a ligand for a checkpoint receptor (analogous to a growth factor–receptor complex), then it is not surprising that there should be a dose-responsive activation of the checkpoint signal. The biochemical basis of this response may be the amount of ATR–ATRIP and/or 9–1–1 loaded onto the RPA–ssDNA.

Multiple receptors for the ssDNA–RPA ligand

If RPA–ssDNA is a ligand that activates ATR-dependent signaling, then what is the receptor for this ligand? ATRIP seems to be the likely candidate. It contains an N-terminal domain that is necessary and sufficient to bind to RPA–ssDNA, which is separable from its C-terminal ATR-binding domain (Ball et al. 2005). ATRIP binding to RPA–ssDNA promotes ATR association with DNA in vitro (Zou and Elledge 2003). ATRIP is also essential for ATR to localize to nuclear foci after DNA damage in vivo. Mutations in its N-terminal RPA-binding domain severely impair relocalization of ATRIP to DNA-damage-induced foci (Ball et al. 2005). Thus, ATRIP would appear to be at least one of the receptors for the RPA–ssDNA ligand.

However, recently published data indicate that a direct ATRIP–RPA–ssDNA interaction is neither sufficient for ATR–ATRIP localization to sites of DNA damage nor essential for ATR activation. ATR is not retained at or activated by stalled replication forks in Nbs1-deficient cells despite RPA–ssDNA accumulation (Stiff et al. 2005). ATR is also not retained at UV-induced lesions in H2AX-deficient cells, but it is activated and phosphorylates Chk1 in these cells (Ward et al. 2004). There are RPA–ssDNA-independent means of ATR–ATRIP association with DNA as well (Unsal-Kacmaz et al. 2002; Bomgardner et al. 2004; Peng and Chen 2005). Furthermore, while deletion of the N terminus of ATRIP prevents RPA–ssDNA binding and accumulation of ATRIP at sites of DNA damage, the mutant ATRIP protein still supports ATR-dependent Chk1 phosphorylation in cells (Ball et al. 2005). This result suggests that accumulation of the ATR–ATRIP complex in nuclear foci is separable from ATR activation. It should be noted that accumulation into immunofluorescently distinct foci is not a sensitive assay for recognition of DNA damage. Perhaps ATR–ATRIP can be recruited to the uncoupled replication fork without ATRIP binding to RPA–ssDNA, but the ATRIP–RPA–ssDNA interaction promotes retention and accumulation of ATR. These results should also be considered in relation to the observation that ATM activation is separable from its retention at double-strand breaks (Kitagawa et al. 2004; Falck et al. 2005). Similarly, there may be multiple steps to ATR activation, and the phosphorylation of different substrates probably requires different biochemical interactions.

There may be multiple receptors for RPA–ssDNA that participate in the checkpoint response. Cut5 (TOPBP1) participates in the loading of ATR onto RPA–ssDNA, and Cut5 loading is dependent on RPA after DNA damage (Parrilla-Castellar and Karnitz 2003). 9–1–1 complex loading by Rad17 also requires RPA–ssDNA but is independent of ATR–ATRIP (Ellison and Stillman 2003; Zou et al. 2003). Furthermore, RPA mutants that abolish interactions between RPA and replication factor C (RFC) in vitro cause checkpoint deficiencies in vivo (Kim and Brill 2001). Thus, there may be multiple receptors for the RPA–ssDNA ligand, and the original problem of how diverse DNA structures could activate a common signaling molecule seems to have been turned on its head.

A second signal for checkpoint activation

While RPA–ssDNA is essential for ATR signaling, it is not sufficient. Indeed, RPA–ssDNA by itself does not activate ATR in *Xenopus* extracts (Stokes et al. 2002). The Rad17-dependent loading of the 9–1–1 trimer at sites of DNA damage is essential for ATR to phosphorylate at least some of its substrates including Chk1 (Zou et al. 2002). In vitro, RPA–ssDNA and a recessed DNA strand end are required to stimulate 9–1–1 loading (Ellison and Stillman 2003; Zou et al. 2003). There are conflicting reports as to what type of end (3' or 5') is preferred by the Rad17 clamp loader. A recessed 3'-end generated by Pol α (a primer/template junction) is what is normally recognized by the RFC complex to load PCNA, whereas a recessed 5'-end would need to be generated by other enzymatic processing.

Cimprich and colleagues (Byun et al. 2005) also find that a second signal in addition to RPA–ssDNA is required for ATR to phosphorylate Chk1. High concentrations of aphidicolin or neutralizing antibodies to Pol α do not block unwinding of a damaged plasmid but do inhibit Chk1 phosphorylation. This is only true if the inhibitors are added prior to initiation of replication. Since these agents block the DNA synthesis but not RNA primase function of Pol α , extension of the RNA primer must be required for Chk1 phosphorylation. Importantly, ATR–ATRIP, RPA, and Pol α are still chromatin-associated under these circumstances, but the 9–1–1 complex is not. Thus, the second, Pol α -dependent signal promotes 9–1–1 loading.

The exact structure formed by Pol α that activates the checkpoint is still unclear. If the 9–1–1 complex is loaded at a recessed 3'-end in vivo, then perhaps all that is required is the DNA

extension at the primer–template junction. However, since a primed ssDNA template treated with MMS is insufficient to activate the checkpoint (Stokes et al. 2002), and recessed 5'-ends may be more efficient for 9–1–1 loading than recessed 3'-ends (Ellison and Stillman 2003), something more may be required. In addition, the amount of Pol α on chromatin increases in extracts treated with aphidicolin; therefore, multiple extended primers may be synthesized on the lagging strand, thereby providing a means to amplify the checkpoint signal (Michael et al. 2000).

Another question is why has the checkpoint evolved to require these two apparently independent events (ATR–ATRIP and 9–1–1 recruitment). Perhaps this two-step mechanism ensures that only ssDNA generated during specific types of activities can lead to Chk1-dependent cell cycle arrest. Yeast ATR is activated (as measured by yeast ATRIP phosphorylation) after DNA damage even in 9–1–1 mutants (Edwards et al. 1999). Are there other ATR substrates that do not require 9–1–1 loading? Mechanistically, why is 9–1–1 loading required for Chk1 phosphorylation? Does it recruit substrates to ATR? Structurally, the 9–1–1 complex resembles the PCNA clamp, so what is it clamping? Many additional checkpoint proteins including Claspin are also required for Chk1 phosphorylation. Thus, further research is required for a full mechanistic understanding of ATR signaling.

Other roles of the MCM complex in checkpoint signaling

One of the surprising results of the Cimprich study (Byun et al. 2005) is that an entire 10-kb plasmid is unwound by the MCM helicase after the polymerase is stalled. Replication of this size plasmid initiates from a single site in *Xenopus* extracts. Only an extra 100–200 bp of ssDNA are formed at a stalled replication fork after checkpoint activation in wild-type yeast cells (Sogo et al. 2002). Checkpoint deficiencies cause the length of the ssDNA tract to increase to between 500 and 800 bp. This suggests that one activity of the ATR checkpoint may be to inhibit ssDNA formation. The mammalian and *Xenopus* MCM complexes are phosphorylated by ATR (Cortez et al. 2004; Yoo et al. 2004). The function of the phosphorylation is unclear, but it is tempting to speculate that it could slow helicase activity to prevent the generation of long ssDNA tracts. Why then do the damaged plasmids in the *Xenopus* extracts completely unwind? Is this unique to this in vitro system or is the amount of ssDNA tolerated in animal cells larger than that in yeast?

In addition to being a substrate of ATR, the MCM complex also can physically bind to ATRIP and Rad17 (Cortez et al. 2004; Tsao et al. 2004). A C-terminal region of MCM7 mediates these interactions, which intriguingly is also the interaction domain for the Rb fragment that the Cimprich group (Byun et al. 2005) used to inhibit MCM activity. Partial depletion of MCM7 by RNA inhibition impairs ATR signaling, whereas depletion of other MCM subunits does not have this effect (Cortez et al. 2004; Tsao et al. 2004). In these experiments there is no detectable decrease in the rate of replication at the levels of MCM depletion achieved. While it is difficult to rule out that depletion of MCM7 impaired unwinding activity of the entire helicase complex in these experiments, the biochemical connection between MCM7, ATRIP, and Rad17 indicates that MCM7 may have a more direct role in checkpoint signaling.

Conclusions

The Cimprich data provide clear evidence that decoupling of helicase and polymerase activities to generate RPA–ssDNA is necessary but not sufficient for DNA damage and polymerase inhibitors to activate ATR in *Xenopus* extracts (Byun et al. 2005). It seems unlikely that this will be the only means of activating ATR. ssDNA gaps may activate ATR outside of S phase but probably rarely persist because of rapid repair activities (Costanzo and Gautier 2003; Stiff et al. 2005). ATR may also have some activity even at normal replication forks to regulate origin firing (Marheineke and Hyrien 2004; Shechter et al. 2004a). Nonetheless, Cimprich and

colleagues (Byun et al. 2005) have defined how decoupling of helicase and polymerase function through intrinsic differences in their substrate compatibilities provides a method of promoting checkpoint signaling in response to diverse genotoxins.

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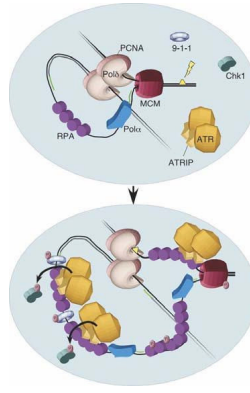


Figure 1.

A simplified model for how decoupling of the replicative helicase and polymerase generates RPA-ssDNA and promotes ATR activation. A DNA lesion does not block the MCM helicase from unwinding the DNA duplex, but it does stall the DNA polymerase. RPA binds to the single-stranded DNA that accumulates, and RPA-ssDNA promotes loading of the 9-1-1 and ATR-ATRIP complexes. The juxtaposition of these complexes allows ATR to phosphorylate Chk1, which then promotes cell cycle arrest. Other proteins at the replication fork are also phosphorylated by ATR, including the 9-1-1 complex, RPA, and the MCM complex. Many additional checkpoint proteins including Claspin, TopBP1/Cut5, and the Rad17 clamp loading complex are present at the stalled replication fork and promote Chk1 phosphorylation but are not placed in this diagram.