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Activation of ATR-related protein kinase upon DNA damage recognition

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Abstract

Chromosomes are constantly damaged by exogenous and endogenous factors. To cope with DNA damage, eukaryotic cells are equipped with three phosphatidylinositol 3-kinase-related kinases (PIKKs), such as ATM, ATR, DNA-PK. PIKKs are structurally related to phosphatidylinositol 3-kinase (lipid kinase) however possess protein kinase activities. The Mre11-Rad50-Nbs1 and the Ku complex interact with and activate ATM and DNA-PKcs at double-stranded DNA breaks (DSBs), respectively. In contrast, ATR responds to various types of DNA lesions by interacting with replication protein A (RPA)-covered single-stranded DNA (ssDNA). Several lines of evidence have established a model in which ATR is activated by interacting with ATR activating proteins including TopBP1 and ETAA1 at DNA lesions in humans, yet the interaction of ATR with RPA-covered ssDNA does not result in ATR activation. In budding yeast, the Mec1-Ddc2 complex (Mec1-Ddc2) corresponds to ATR-ATRIP. Similar to ATR, Mec1 activation is accomplished by interactions with Mec1 activating proteins, which are Ddc1, Dpb11 (TopBP1 homolog) and Dna2. However, recent studies provide results supporting the idea that Mec1^{ATR} is also activated by interacting with RPA-covered ssDNA tracts. These observations suggest that all the ATM, ATR, DNA-PK family proteins can be activated immediately upon DNA damage recognition.

Introduction

The DNA damage response consists of three different pathways controlled by the phosphatidylinositol 3-kinase-related kinases (PIKKs) [1–3]. These kinases include DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia telangiectasia-mutated (ATM), and ATM and Rad3-related (ATR). All PIKKs respond to DNA damage differently. ATM and DNA-PKcs are activated at double-stranded DNA breaks (DSBs) whereas ATR responds to various types of DNA lesions by interacting with single-stranded DNA (ssDNA) [4, 5].

The Mre11-Rad50-Nbs1 and the Ku complex recruit and activate ATM and DNA-PKcs at DNA ends, respectively [2, 6, 7] (Fig. 1). Therefore, the recruitment of ATM and DNA-PKcs is thus tightly coupled to the kinase activation. ATR forms a complex with a partner ATRIP

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and recognizes replication protein A (RPA) that binds ssDNA with a high affinity to localize at sites of DNA damage [4, 5, 8] (Fig. 1). Previous studies show that RPA-covered ssDNA (RPA-ssDNA) by itself does not stimulate kinase activity of ATR [4, 5]. Our recent studies of ATR homolog Mec1 support the idea that ATR family proteins can be activated upon DNA damage recognition [9] (Fig. 1).

Identification of ATR/Mec1 activating proteins

ATR activating proteins like TopBP1 and ETAA1 directly activate ATR-ATRIP at sites of DNA damage (Fig. 2). TopBP1 appears to engage with the Rad9-Rad1-Hus1 (9-1-1) complex at dsDNA-ssDNA junctions [4, 5]. ETAA1 interacts with RPA and localizes at stalled replication forks [10–12]. Both TopBP1 and ETAA1 directly activate ATR-ATRIP [10, 11]. In budding yeast, the Mec1-Ddc2 complex (Mec1-Ddc2) corresponds to ATR-ATRIP [13] (Fig. 2B). Mec1^{ATR}-Ddc2^{ATRIP} interacts with RPA-ssDNA to localize to sites of DNA damage [13]. The Ddc1^{Rad9}-Mec3^{Hus1}-Rad17^{Rad1} complex (the budding yeast 9-1-1 complex) recruits Dpb11 (TopBP1 ortholog) to the dsDNA-ssDNA junction [14, 15], where both Ddc1^{Rad9} and Dpb11^{TopBP1} stimulate Mec1^{ATR}-Ddc2^{ATRIP} kinase activity [16–20]. These observations have established the model of the Ddc1^{Rad9}-Dpb11^{TopBP1} pathway governing the Mec1^{ATR}-Ddc2^{ATRIP} checkpoint pathway throughout the cell-cycle. In comparison to the Ddc1^{Rad9}-Dpb11^{TopBP1} pathway, the Dna2 nuclease/helicase stimulates Mec1^{ATR} kinase activity and controls DNA damage and replication checkpoints in S phase [21]. All ATR/Mec1 activating proteins utilize the unstructured domains with aromatic amino acid residues [18, 19, 21, 22] suggesting that ATR/Mec1 activating proteins stimulate ATR/Mec1 through a similar protein-protein interaction mechanism.

The Mec1^{ATR} activating protein Dpb11^{TopBP1} also act as a checkpoint scaffold protein in budding yeast

In budding yeast, Mec1^{ATR} activates the downstream kinase Rad53 (Chk2 homolog) to enforce DNA damage checkpoint response [23, 24]. Signal transduction from Mec1^{ATR} to Rad53^{Chk2} requires checkpoint mediators, such as Rad9 and Mrc1 [25–28]. Mrc1 associates with components of the replication fork in S phase [29, 30] and specifically regulates the S phase DNA damage checkpoint [23, 27, 31].

The Rad9 checkpoint mediator controls checkpoint throughout the cell-cycle. The recruitment of Rad9 to sites of DNA damage is regulated through a Dpb11^{TopBP1}-dependent mechanism [32, 33] (Fig. 2). Dpb11^{TopBP1} acts as a scaffold protein that connects Ddc1^{Rad9} to the Rad9 checkpoint mediator [32, 34, 35]. The Ddc1^{Rad9}-Dpb11^{TopBP1} pathway therefore not only stimulates Mec1^{ATR} kinase activity but also promotes the recruitment of the Rad9 checkpoint mediator. Dpb11^{TopBP1} and the Rad9 checkpoint mediator correspond to Cut5/Rad4^{TopBP1} and Crb2, in fission yeast, respectively (Fig. 2). Similar to Dpb11^{TopBP1}, Cut5/Rad4^{TopBP1} acts as a scaffold for Crb2 recruitment in fission yeast [36, 37]. However, Cut5/Rad4^{TopBP1} plays a minor role in the activation of Rad3 (ATR/Mec1^{ATR} homolog) kinase in G2/M phase in fission yeast [38, 39]. Cyclin-dependent kinase (CDK) activity is important for coordinated execution of DNA end resection; that is,

extensive ssDNA generation occurs in G2/M phase [40]. Therefore, it has been suggested that a ssDNA-dependent mechanism can activate Rad3 in fission yeast in G2/M phase [38].

Separation-of-function mutation of Ddc2^{ATRIP} is defective in ssDNA- or RPA-ssDNA-mediated activation of the Mec1^{ATR}- Ddc2^{ATRIP} complex *in vitro*.

We have identified a separation-of-function *ddc2* mutation (*ddc2-S4*) that causes defects in Mec1^{ATR} activation but does not affect Mec1^{ATR} recruitment [41]. However, it remains to be elucidated how Ddc2^{ATRIP} controls Mec1^{ATR} activation at sites of DNA damage. To understand the significance of Ddc2^{ATRIP}-dependent Mec1^{ATR} activation, we further characterized the *ddc2-S4* mutation by carrying out genetic experiments. We found that the *ddc2-S4* mutation impairs Rad9 and Mrc1 phosphorylation after DNA damage [9], supporting the idea that the *ddc2-S4* mutation is defective in checkpoint activation throughout the cell cycle [9, 41]. The Ddc1^{Rad9}-Dpb11^{TopBP1} pathway and Dna2 contribute to Mec1^{ATR} activation in S phase [21]. We found that S-phase checkpoint signaling is more significantly defective in *ddc2-S4* mutants than in *ddc1*- and *dna2*-deficient mutants [9]. Thus, Ddc2^{ATRIP} controls Mec1^{ATR} activation independently of Ddc1/Dpb11 and Dna2 function [9]. We further examined the effect of *ddc2-S4* mutation on kinase activity of Mec1^{ATR}- Ddc2^{ATRIP} using an *in vitro* reconstitution system [9]. Whereas ssDNA stimulated Mec1^{ATR} activity, RPA did not, however, RPA promoted ssDNA-dependent Mec1^{ATR} activation. The Mec1^{ATR} - Ddc2^{ATRIP} complex containing Ddc2-S4 mutant (Mec1^{ATR}-Ddc2-S4) exhibited a basal kinase activity *in vitro*, but neither ssDNA nor RPA-ssDNA stimulated Mec1^{ATR}-Ddc2-S4 effectively [9]. These results suggest that Ddc2^{ATRIP} increases Mec1^{ATR} kinase activity at RPA-covered ssDNA tracts. Thus, like ATM and DNA-PKcs, ATR/Mec1 is activated immediately upon DNA damage recognition, supporting an evolutionary similarity in activation of ATM, ATR and DNA-PKcs (Fig. 1).

How does Ddc2^{ATRIP} mediate ssDNA- or RPA-ssDNA-dependent Mec1^{ATR} activation?

Although our results support a model in which Ddc2^{ATRIP} recognizes RPA-covered ssDNA and promotes Mec1^{ATR} activation at sites of DNA damage [9], the mechanism still remains to be understood.

It is unknown how Ddc2^{ATRIP} interacts with ssDNA or RPA to promote Mec1^{ATR} activation. Although the N-terminus of Ddc2 can interact with RPA [42, 43] (Fig. 3), it is dispensable for full Mec1^{ATR} function [42, 43], suggesting that other domains of Ddc2^{ATRIP} should be involved in RPA binding. Previous studies have uncovered that the KKRK region of Ddc2^{ATRIP} is critical for DNA binding *in vitro*, however, it remains to be determined whether the KKRK region is essential for ssDNA-dependent Mec1^{ATR} activation [44]. Moreover, it is formally possible that domains other than the KKRK region could contribute to ssDNA-dependent Mec1^{ATR} activation.

Ddc2^{ATRIP} interacts directly with DNA in an RPA-independent manner [9] (Fig. 3). Mec1^{ATR}-Ddc2 binds RPA even in the absence of ssDNA [9] (Fig. 3). Although RPA does

not activate Mec1^{ATR}, we cannot exclude the possibility that conformation change of RPA on ssDNA could stimulate Mec1^{ATR} kinase [9]. RPA binds ssDNA using multiple DNA binding domains and undergoes conformation change during ssDNA recognition [8, 45–47]. The Mec1^{ATR}-Ddc2^{ATRIP} complex can form a homodimer of Mec1^{ATR}-Ddc2^{ATRIP} heterodimer [48] (Fig. 3). RPA binds to ssDNA with a defined 5'→3' polarity [45]. It is therefore possible that the Mec1^{ATR}-Ddc2 homodimer complex undergoes conformation change when it interacts with RPA-covered ssDNA at sites of DNA damage (Fig. 3). It should be noted that RPA not only stabilizes ssDNA but also stimulates various repair processes *in vivo* [8, 49–53]. Like ATRIP and Ddc2^{ATRIP}, several DNA repair proteins interact with the N-terminus of the large subunit of RPA (Fig. 3). For example, Dna2, a Mec1^{ATR} activating protein, interacts with RPA [21, 54]. However, it remains to be determined how Dna2 competes with Ddc2^{ATRIP} for RPA binding.

Does ssDNA-dependent Mec1^{ATR} activation couple to Ddc1/Dpb11/Dna2-dependent Mec1^{ATR} activation?

We are still uncertain whether ssDNA-dependent Mec1^{ATR} activation is coupled to Ddc1/Dpb11/Dna2-dependent Mec1^{ATR} activation and how this coupling occurs. Mec1^{ATR}-Ddc2^{ATRIP} forms a dimer of heterodimers through multiple interfaces including the PIKK regulatory domain (PRD) [55]. PRD of ATR/Mec1 is proposed to interact with TopBP1 or Dpb11^{TopBP1}, and possibly other ATR/Mec1^{ATR} activating proteins. Interestingly, the PRD is closely positioned near the catalytic and activation loop at the kinase domain, thereby blocking kinase activity and substrate entry [48, 55]. However, the mechanism by which Dpb11^{TopBP1} accesses PRD of the Mec1^{ATR}-Ddc2^{ATRIP} complex to stimulate Mec1^{ATR} kinase activity still remains a mystery. The *ddc2-S4* mutation carries two substitution mutations (K263E, H382Y), where K263 contributes to Mec1^{ATR}-Ddc2^{ATRIP} homodimerization [48]. Thus, the *ddc2-S4* mutation may affect conformation changes of Mec1^{ATR}-Ddc2 homodimer upon ssDNA binding (Fig. 3). Mec1^{ATR} recognizes and phosphorylates Mec1^{ATR} activators (Ddc1^{Rad9}, Dpb11^{TopBP1} or Dna2) after DNA damage [56–58]. Conformation changes of the kinase domain upon ssDNA binding could therefore enhance binding of Mec1^{ATR} activators to the kinase domain and nearby PRD. Supporting this view, previous studies have shown that Mec1^{ATR} phosphorylates Dpb11^{TopBP1} and enhances the stimulating effect of Dpb11^{TopBP1} on Mec1^{ATR} kinase activity *in vitro* [20, 57]. In this model, ssDNA-dependent Mec1^{ATR} activation might be prerequisite for Dpb11^{TopBP1}-dependent Mec1^{ATR} activation (Fig. 3). It has been shown to activate Mec1^{ATR} more strongly in the presence of RPA and ssDNA [17].

Conclusion

A key feature of RPA is that, while binding ssDNA with high affinity, it must dissociate from and ‘hand-off’ ssDNA to other enzymes for further processing. RPA-ssDNA complexes serve as binding platforms in various DNA metabolic processes, and physical interactions between RPA and more than two dozen enzymes have been identified. Thus, dynamic interactions between ssDNA, RPA, Mec1^{ATR}-Ddc2^{ATRIP} and other RPA-binding proteins might be critical for efficient Mec1^{ATR} activation *in vivo*. ATR/Mec1 also controls

DNA replication and telomere elongation [59–63]. More detailed biochemical and structural studies will uncover how ATR/Mec1 triggers cellular checkpoint responses at RPA-covered ssDNA tracts.

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Figure 1. Activation of DNA-PKcs, ATM and ATR upon DNA damage recognition

A. DNA-PKcs interacts with Ku at DNA ends.

B. ATM interacts Mre11-Rad50-Nbs1 (MRN) complex at DNA ends.

C. ATR-ATRIP interacts with RPA-covered-ssDNA tracts.

Figure 2. Activators of ATR and ATR family proteins

TopBP1 or ETAA1 activates ATR in human cells. Ddc1^{Rad9}, Dpb11^{TopBP1} or Dna2 activates Mec1^{ATR} in budding yeast cells; Ddc1 and Dpb11 act throughout the cell-cycle whereas Dna2 plays a key role in S phase. Cut5/Rad4^{TopBP1} activates Rad3^{ATR} in fission yeast cells only in G1 phase.

Figure 3. Interactions among Mec1^{ATR}-Ddc2^{ATRIP}, RPA, Dpb11^{TopBP1}, Dna2, and ssDNA. Mec1^{ATR}-Ddc2^{ATRIP} interacts with ssDNA, RPA and RPA-ssDNA. ssDNA or RPA-ssDNA stimulates Mec1 kinase activities *in vitro*. Dpb11^{TopBP1} recognizes PRD near the C-terminus kinase domain of Mec1^{ATR}.
 A. Mec1^{ATR}-Ddc2^{ATRIP} can form a homodimer of Mec1^{ATR}-Ddc2^{ATRIP} heterodimer in the absence of RPA and ssDNA. Homodimerization of Mec1^{ATR}-Ddc2^{ATRIP} could block the access of Dpb11^{TopBP1} to Mec1^{ATR}.
 B. ssDNA interacts weakly with Mec1^{ATR}-Ddc2^{ATRIP} to stimulate Mec1^{ATR} activity, which might allow Dpb11^{TopBP1} to interact with PRD of Mec1^{ATR}.
 C. Mec1^{ATR}-Ddc2^{ATRIP} interacts with RPA. However, RPA does not stimulate Mec1^{ATR} activity. Homodimerization of Mec1^{ATR}-Ddc2^{ATRIP} could block the access of Dpb11^{TopBP1} to Mec1^{ATR}.
 D. Dna2 interacts with RPA-ssDNA. Dna2 interacts with RPA-ssDNA to stimulate Mec1^{ATR} activity.

D. Mec1^{ATR}-Ddc2^{ATRIP} interacts with RPA-ssDNA to stimulate Mec1^{ATR} activity. Dpb11^{TopBP1} might interact with the PRD of Mec1^{ATR}. However, Dna2 might compete with Mec1^{ATR}-Ddc2^{ATRIP} for binding to the N-terminus of Rfa1 (the large subunit of RPA).

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