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Perspective

Clamping the Mec1/ATR Checkpoint Kinase into Action

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KEY WORDS

DNA replication, DNA damage, cell cycle checkpoint, clamp, checkpoint kinase

ABBREVIATIONS

PCNA	proliferating cell nuclear antigen
Rad17/3/1 or 9-1-1	checkpoint clamp
RPA	replication protein A
ATR	ataxia telangiectasia related protein
ssDNA	single-stranded DNA
NER	nucleotide excision repair
h	human
sc	*S. cerevisiae*
x	*Xenopus*

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ABSTRACT

The yeast checkpoint protein kinase Mec1, the ortholog of human ATR, is the essential upstream regulator of the cell cycle checkpoint in response to DNA damage and to stalling of DNA replication forks. The activity of Mec1/ATR is not directly regulated by the DNA substrates that signal checkpoint activation. Rather the signal appears to be transduced to Mec1 by factors that interact with the signaling DNA substrates. One of these factors, the DNA damage checkpoint clamp Rad17-Mec3-Ddc1 (human 9-1-1) is loaded onto gapped DNA resulting from the partial repair of DNA damage, and the Ddc1 subunit of this complex activates Mec1. In vertebrate cells, the TopBP1 protein (Cut5 in *S. pombe* and Dpb11 in *S. cervisiae*) that is also required for establishment of the replication fork, functions during replication fork dysfunction to activate ATR. Both mechanisms of activation generally upregulate the kinase activity towards all downstream targets.

INTRODUCTION

DNA damage resulting from internal or external insult constantly challenges cellular genome integrity. Analogous challenges are presented during DNA replication because of the presence of structural blocks or potential replisome dysfunction. Many DNA repair mechanisms exist to overcome these challenges and repair the damage. In addition, eukaryotic cells have several checkpoints that ensure an arrest of the cell cycle in order to provide an appropriate time-frame for DNA repair or for the completion of genome duplication.[1] Thus, the G_1/S checkpoint and G_2/M checkpoint ensure the intactness of the genome prior to proceeding with DNA replication and mitosis, respectively. Stalled replication forks activate the replication checkpoint. Determining the identity and activities of checkpoint factors that function in these pathways has been an area of intense investigation in the last two decades. Many checkpoint factors function in multiple checkpoint pathways, and partial redundancy of structurally related factors for a given pathway is not an uncommon occurrence. Here, we will focus on just two of these factors that have the capacity to activate a phosphorylation cascade: the yeast checkpoint clamp Rad17/3/1, the ortholog of human 9-1-1, and the replication and checkpoint protein TopBP1 (Cut5 in *S. pombe* and Dpb11 in *S. cervisiae*).[2,3] For a complete description of checkpoint mechanisms, the reader is referred to recent reviews (refs. 4–6).

The *S. cerevisiae* protein kinase Mec1 and its human ortholog ATR belong to the PIKK family of protein kinases. The founding member of this family is ATM, for ataxia telangiectasia mutated. Mutations in ATM lead to cancer predisposition and show a defect in checkpoint function in response to double-stranded breaks. Both ATR and Mec1 act early during checkpoint activation in response to damage in only one strand of the DNA such as UV-dimers and DNA gaps, and to stalling of the DNA replication fork. Mec1 has an associated subunit, Ddc2/Lcd1, that is essential for all known functions of the kinase, and the structure of the Mec1-Ddc2 complex is that of a heterodimer. The human ortholog of Ddc2 is ATRIP. Purified Mec1-Ddc2 and ATR-ATRIP show a very low protein kinase activity, and therefore, a reasonable assumption has been that the protein kinase is specifically activated as a regulated step during checkpoint function.

Several possible mechanisms for kinase activation present themselves. Could it be that the DNA substrates themselves activate Mec1? Mec1 and ATR are often called DNA damage sensor kinases. However, direct binding of these kinases to either normal or damaged DNA cannot be detected. Rather they associate with DNA through interactions with RPA, the heterotrimeric eukaryotic single-stranded DNA binding protein. In this regard, the alternative designation of Mec1/ATR as a transducer kinase is more

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appropriate. Could it be that RPA or the ssDNA-RPA complex activates Mec1? Although phosphorylation of the Rpa2 subunit of RPA by Mec1 or by ATR is enhanced when RPA is bound to ssDNA, this does not appear to represent the sought after kinase activation step. Phosphorylation of DNA-bound RPA is still very inefficient, and, more importantly, phosphorylation of other downstream targets is not enhanced by RPA-ssDNA. Possibly, binding of RPA to ssDNA induces a conformational change in Rpa2 that makes this subunit more accessible to the low state kinase activity of Mec1/ATR. Among the many downstream targets of Mec1/ATR is the yeast effector kinase Rad53, Chk1 and/or Chk2 in human, that mediates the global cellular responses ultimately resulting in cell cycle arrest, gene activation, increased DNA repair, and apoptosis. Last year, two activators of the transducer kinase Mec1/ATR were identified. These are the DNA damage checkpoint clamp Rad17/3/1 in yeast,[2] and the essential replication initiation and checkpoint protein TopBP1 in the *Xenopus* system[3] (Fig. 1). We will review these activating systems in more detail and then draw comparisons between them.

DNA DAMAGE CHECKPOINT IN YEAST

The DNA damage checkpoint is most simply understood in the G_1 phase of the cell cycle when the response is not complicated by damage at replication forks or issues relating to sister chromatid cohesion and chromosome segregation. The initiating steps of this checkpoint, as measured by phosphorylation of the effector kinase Rad53, minimally require Mec1-Ddc2, RPA, the checkpoint clamp Rad17-Mec3-Ddc1 (h9-1-1), the clamp loader Rad24-RFC (hRad17), an unknown nuclease, and the mediator Rad9. The latter is required for enhanced autophosphorylation of Rad53 and will not be considered further here.[7]

Dark repair of UV damage occurring during G_1 is almost exclusively accomplished by nucleotide excision repair (NER). The process of NER leads to a bimodal incision of the damaged strand resulting in a ~35 nt DNA gap to which the essential NER factor RPA is bound. In a seminal study, Giannattasio et al. determined that the actual processing of UV-induced damage by the NER system is required for activation of the DNA damage checkpoint.[8] These results indicate that an intermediate in NER forms the signal for the checkpoint. Could this signal be an RPA-coated gap? In order to address this central question we have studied the biochemical properties of the yeast checkpoint clamp and its loader, and their potential physical and functional interaction with Mec1.

The checkpoint clamp is a heterotrimeric toroidal complex that encircles DNA, much like its structural homolog PCNA (proliferating cell nuclear antigen). It consists of the Rad17, Mec3, and Ddc1 subunits (reviewed in ref. 9). The Rad17/3/1 clamp is the ortholog of the human 9-1-1 clamp, consisting of the Rad9, Rad1, and Hus1 subunits. Rad17/3/1 is loaded onto gapped DNA by its loader Rad24-RFC (hRad17-RFC) in an ATP-dependent manner. The DNA structure specificity for clamp loading has been a matter of disagreement (discussed in ref. 10). The clamp can be loaded onto partially double stranded DNA effectors with either a 5'- or a 3'-junction, provided the DNA is not coated with RPA. However, when the single-stranded DNA has been coated with RPA, 5'-loading strongly predominates (Fig. 1). Because of this specificity it has been suggested that the clamp may form a processive complex with a 5'-exonuclease in order to enlarge the gap which may be required for signal amplification.[11] In fact, the human 9-1-1 complex has been shown to interact with several DNA replication and repair factors including the flap endonuclease FEN1, DNA ligase I, DNA polymerase β, and MutY DNA glycosylase, but how these proteins function in the DNA damage checkpoint has not been determined.[12-15] Possibly, other DNA-damage response functions for the clamp exist, such as in mutagenesis.[16]

We investigated whether the clamp forms a functional complex with Mec1.[2] Indeed, when the Rad17/3/1 clamp was loaded by its loader onto naked gapped DNA, i.e., without RPA coating the ssDNA, Mec1 was able to functionally interact with this clamp, as shown by a 30–50 fold increase in the rate of phosphorylation of downstream targets. Among these targets was the effector kinase Rad53, the key downstream target of Mec1 in the DNA damage checkpoint pathway. However, phosphorylation of mammalian translation initiation protein PHAS-I, a commonly used general substrate for PIKKs, was also strongly enhanced, suggesting that the interaction between the loaded clamp and Mec1 led to a general activation of its kinase activity. Furthermore, activation of Mec1 was observed regardless whether the clamp was loaded onto a DNA substrate with a 3' junction or with a 5'-junction, indicating that the mere encircling of the DNA by the clamp sufficed for activation.

The role of RPA in Mec1 activation is less easily assessed. While activation of Mec1 per se did not require RPA, coating of the DNA by RPA increased the efficiency of activation. It is tempting to invoke a specific role for RPA, e.g., by recruiting Mec1 to the DNA through binding to its Ddc2 subunit. However, RPA also stimulates loading of the checkpoint clamp by its loader, which indirectly could cause more efficient activation of Mec1. Additional studies are required to sort out the multifarious roles of RPA in checkpoint activation in vitro.

While our studies clearly indicated that the DNA-loaded clamp activates Mec1, it did not address a possible function for the Rad24-RFC clamp loader in activation as the loader was absolutely required for clamp loading. In general, our clamp loading studies are carried out at physiological salt concentrations which impose high specificity on the process. However, when we carried out additional studies in low salt in order to mediate non-specific sliding of the clamp onto DNA ends, we were surprised to notice that not only could we dispense with the loader in order to activate Mec1, but even the DNA was dispensable. In fact, taking this one step further, we determined that just the Ddc1 subunit of the Rad17/3/1 clamp sufficed to activate Mec1 kinase activity, provided the studies were carried out at low salt (≤40 mM NaCl). The observed binding of Ddc1 to Mec1-beads was in complete agreement with this. The caveat of this simple activation scheme is that it is very salt sensitive. At physiological salt, Ddc1 no longer bound Mec1 and no longer stimulated its activity, and consequently, activation required full clamp loading by the loader onto the appropriate DNA substrate.

DNA REPLICATION CHECKPOINT IN *XENOPUS* EXTRACTS

Previous studies in *S. cerevisiae* and in *S. pombe* had indicated a specialized role for Dpb11 and Cut5 (Rad4), respectively, in the initiation of DNA replication and in the replication checkpoint.[17,18] Subsequently, similar roles were assigned to the mammalian homolog TopBP1.[19] Whether Dpb11/Cut5/TopBP1 also functions during the elongation phase of DNA replication is still a matter of debate (discussed in ref. 20). The dual role for Dpb11/Cut5/TopBP1 in DNA replication and the replication checkpoint might suggest that this protein could actually play an early role in the checkpoint as a damage sensor. Checkpoint activation studies in *S. pombe* have placed

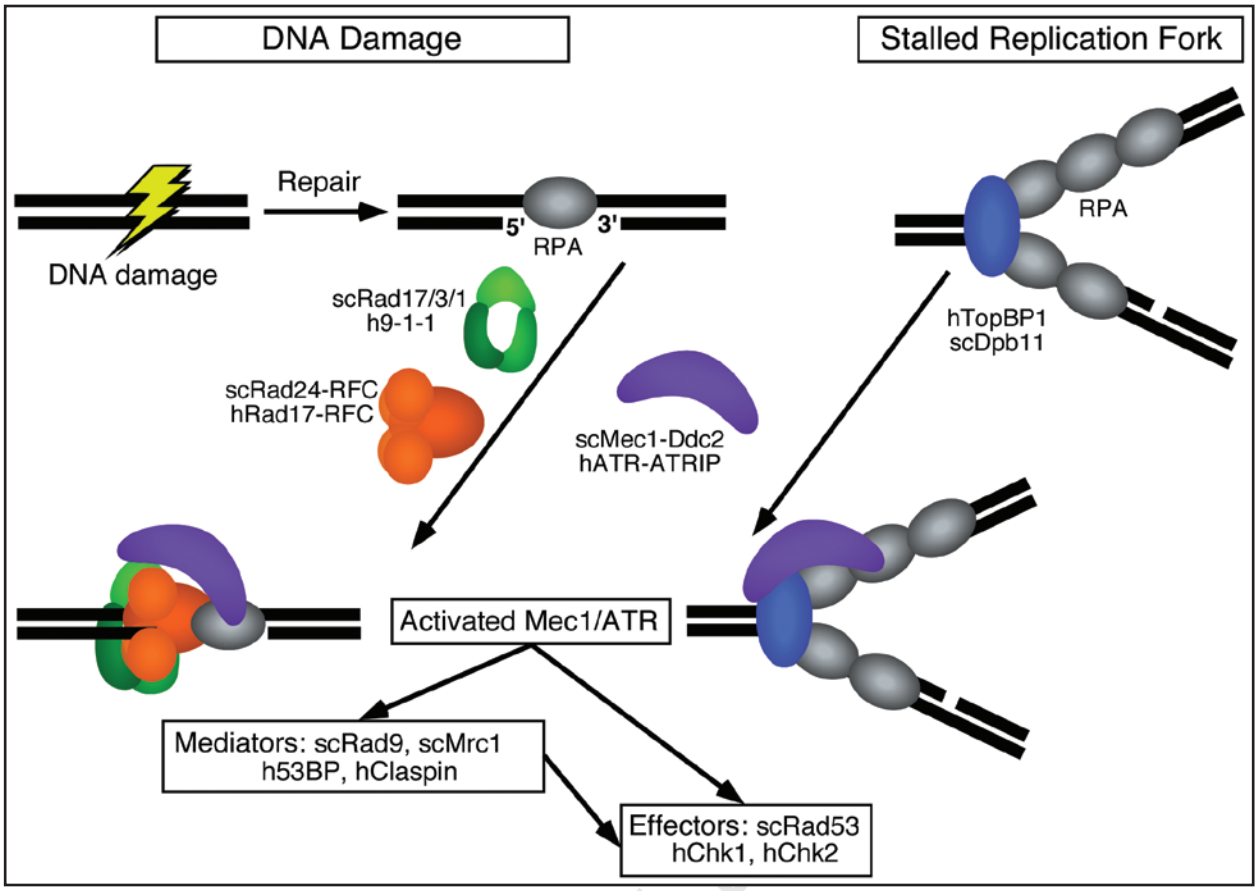

Figure 1. Two distinct pathways to activate Mec1/ATR. Left, activation by the checkpoint clamp in response to DNA damage; right, activation by TopBP1 in response to stalling of replication forks. The *S. cerevisiae* (sc) and human (h) proteins are shown.

Dpb11/Cut5/TopBP1 downstream of ATR, perhaps as a mediator.[21] However, these studies did not exclude an additional early function for this protein (reviewed in ref. 20).

The great advantages of the *Xenopus* extract system are that it allows for the study of DNA replication mechanisms and preserves the ability to respond to aberrant DNA structures to activate checkpoints. For instance, inclusion of aphidicolin, a DNA polymerase inhibitor, in the replication assay invokes the replication checkpoint that activates ATR, which in turn phosphorylates and activates the Chk1 effector kinase.[22] *Xenopus* TopBP1 is required for establishing the replication checkpoint.[23]

Kumagai et al. tested TopBP1 as a plausible activator of ATR in the *Xenopus* system.[3] Indeed, they showed that TopBP1 directly activates ATR kinase activity. For this purpose, they immunopurified xATR from uninduced egg extracts, and incubated it with recombinant TopBP1 and a phosphorylation target. Activated ATR showed greatly increased rates of phosphorylation of physiologically relevant substrates such as Chk1 and Mcm2, but also increased phosphorylation of the non-specific substrate PHAS-I. Surprisingly, this activation did not require the presence of any DNA substrate or RPA. How that requirement is bypassed in the purified kinase assay still needs to be determined.

TopBP1 contains multiple BRCT (BRCA1 C-terminal) domains that are known to mediate protein-protein interactions and function in the DNA damage response and DNA repair. These BRCT domains were not responsible for ATR activation. Rather, a ~300 amino acid domain situated between two BRCT motifs, and conserved in vertebrate cells, was sufficient to activate ATR. However, the function of this isolated small domain is misregulated, because gratuitous phosphorylation of Chk1 was observed in the absence of inducer when the domain was introduced into *Xenopus* extracts or overproduced in mammalian cell lines. Interestingly, a mutant form of TopBP1 with a mutation in the activating domain (W1138R) fully supported replication fork establishment when the mutant protein was added to a TopBP1-depleted extract, but failed to restore the checkpoint function of the depleted extract. These results indicate that the replicative and checkpoint functions of TopBP1 are specified in separable domains.

LESSONS FROM DIVERSE ORGANISMS

Do the two ATR-activating systems have common characteristics? Do they represent two parallel branches of checkpoint activation that are preserved in both organisms, or do they indicate fundamentally different pathways that have diverged from yeasts to vertebrates? In vitro, activation of yeast Mec1 by Ddc1 and of *Xenopus* ATR by TopBP1 appears to proceed similarly: the kinase activity towards all targets investigated is greatly enhanced. Both Ddc1-activated Mec1 and TopBP1-activated ATR show increased activity towards physiological targets such as Rad53/Chk1, and towards the non-specific kinase substrate PHAS-I. This suggests that the mechanism of activation is unlikely to be one in which the activator protein functions as an adaptor between the kinase and the substrate. The exceptions to this rule are Ddc2 and ATRIP, the regulatory subunits of Mec1

and ATR, respectively. Their phosphorylation is not enhanced upon Mec1/ATR activation in vitro,[2,24] nor does phosphorylation of Ddc2 or its *S. pombe* otholog Rad26 require an intact clamp or Dpb11/Cut5 in vivo.[25,26]

This all or none activation of Mec1/ATR suggests that to a first approximation the same downstream targets are phosphorylated regardless of the method of activation, i.e., through the clamp or through Dpb11/Cut5/TopBP1. Differentiation between the two pathways would then mainly come about through temporal and spatial positioning of the target proteins. Further complexity is brought about by the action of the other damage transducing kinase Tel1/ATM.

The strong structural and functional conservation of the checkpoint clamp in eukaryotes strongly suggests that the activation mechanism uncovered for yeast also applies to vertebrate organisms. Failure to observe activation of mammalian ATR in vitro by incubation of the kinase with the h9-1-1 clamp or with hRad9, the ortholog of Ddc1, could easily reflect an absolute requirement that the clamp be loaded onto effector DNA in order to interact with and stimulate ATR. To our knowledge, these latter types of studies with purified mammalian factors have not yet been carried out.

As discussed above, Dpb11/Cut5/TopBP1 is a conserved replication and checkpoint protein in eukaryotes. However, the ATR-activating domain identified in *Xenopus* TopBP1 is conserved only in vertebrates, and cannot be found in yeasts, fly or worm.[20] Possibly, another domain in Dpb11/Cut5 fulfills this function, or it is performed by an associated protein. Or is it possible that this type of activation does not exists in lower eukaryotes? In *S. pombe,* both the clamp and Cut5 are essential factors for the S-phase checkpoint suggesting the existence of a single checkpoint pathway in which both factors participate.[27] However, genetic studies in *S. cerevisiae* point to the existence of two parallel and partially overlapping S-phase checkpoint pathways, one with Dpb11 and one with the clamp.[28] In this organism, the existence of two separate activators of Mec1 does seem plausible. The roles of the clamp and of Dpb11/Cut5/TopBP1 in the S phase checkpoint may extend beyond that of the activation of ATR. Phosphorylated clamp subunit Ddc1/Rad9 interacts with Dbp11/Cut4.[28,29] This complex may function during normal S phase progression to sense stalling of the DNA replication fork.

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