G2 damage checkpoints: what is the turn-on?

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Summary

Cells mount a coordinated response to DNA damage, activating DNA repair pathways and cell-cycle checkpoint pathways to allow time for DNA repair to occur. In human cells, checkpoint responses can be divided into p53-dependent and p53-independent pathways, the latter being predominant in G2 phase of the cell cycle. The p53-independent pathway involves a phosphorylation cascade that activates the Chk1 effector kinase and induces G2 arrest through inhibitory tyrosine phosphorylation of Cdc2. At the top of this cascade are the ATR and ATM kinases. How ATM and ATR recognize DNA damage and activate this checkpoint pathway is only beginning to emerge. Single-stranded DNA, a result of stalled DNA replication or processing of chromosomal lesions, appears to be central to the activation of ATR. The recruitment of replication protein A to single-stranded DNA facilitates the recruitment of several complexes of checkpoint proteins. In this context, ATR is activated and then phosphorylates the C-terminus of Chk1, activating it to enforce a block to mitotic entry.

Key words: DNA damage, Cell-cycle checkpoint control, ATR, ATM, Chk1

Introduction

For over a decade, we have understood the molecular control over the transition from G2 phase of the cell cycle into mitosis. In eukaryotic cells, with the exception of the budding yeast *Saccharomyces cerevisiae*, a well-characterized series of events regulates mitotic entry through the activity of the mitotic cyclin-dependent kinase (CDK) Cdc2. During interphase, Cdc2 forms a complex with accumulating B-type cyclins. These complexes are rapidly inactivated by the Wee1 family of nuclear tyrosine kinases through phosphorylation on tyrosine 15 (Y15). In vertebrates, threonine 14 is also phosphorylated by the dual-specificity Myt1 kinase, which, unlike the Wee1-family kinases, is located in the cytoplasm (Mueller et al., 1995). Myt1 should not be confused with Mik1, a Wee1-family member in the fission yeast *Schizosaccharomyces pombe* that is expressed predominately during S phase (Baber-Furnari et al., 2000; Christensen et al., 2000). When conditions are appropriate, Cdc2 becomes dephosphorylated by Cdc25 phosphatases, and the phosphorylation of key substrates by Cdc2 causes the cell to enter mitosis (Dunphy, 1994; Mueller et al., 1995).

To ensure accurate segregation of chromosomes, cells must prevent entry into mitosis in the presence of DNA damage. Attempts to segregate either broken chromosomes or chromosomes undergoing DNA repair are catastrophic: the failed mitosis generally leads to cell death or a level of genomic instability that leads to cell death or, if not, to tumorigenesis. Cells have therefore evolved G2 DNA damage checkpoint controls that prevent Cdc2 activation in the presence of DNA damage. These pathways are fairly well understood; how the damage is first detected has been less clear. Distinct DNA repair pathways repair different lesions in DNA (Fleck and Nielsen, 2004) and yet all forms of DNA damage commonly activate the same checkpoint. Is this checkpoint activated directly by signals emanating from lesions and, if so, have they a common feature? Or does the checkpoint engage once DNA repair or replication begins, sensing these processes themselves or a product of their action on different lesions? Interestingly, the replication checkpoint, which prevents mitotic onset until completion of DNA replication, uses many of the same components to prevent mitosis until completion of S phase. Finally, are any of these responses affected by the timing of the damage? Recent evidence suggests that one signal that leads to activation of the G2 DNA damage checkpoint emanates from single-stranded (ss)DNA bound by the ssDNA-binding protein replication protein A (RPA) and stimulates activation of the checkpoint effector kinase Chk1 by ATR. Here, we review these findings and discuss potential mechanisms leading to checkpoint activation.

Activating Chk1

The G2 DNA damage checkpoint has an architecture akin to other checkpoints: detectors, signal transducers, mediators and effectors (Fig. 1). As with the core regulation of the G2/M transition, this checkpoint appears to have ancient origins and is basically identical from fission yeast to humans (O'Connell et al., 2000). Its target is the serine/threonine protein kinase Chk1, which indirectly regulates Cdc2 by phosphorylating the proteins that regulate Y15 phosphorylation: Cdc25 phosphatases and Wee1-family kinases. Such phosphorylation alters the stability and/or localization of these proteins. Chk1 possesses an N-terminal catalytic domain and a C-terminal ~200 residue extension that is believed to act as a regulatory domain (Chen et al., 2000; Walworth et al., 1993). Chk1 activation is associated with its phosphorylation on C-terminal

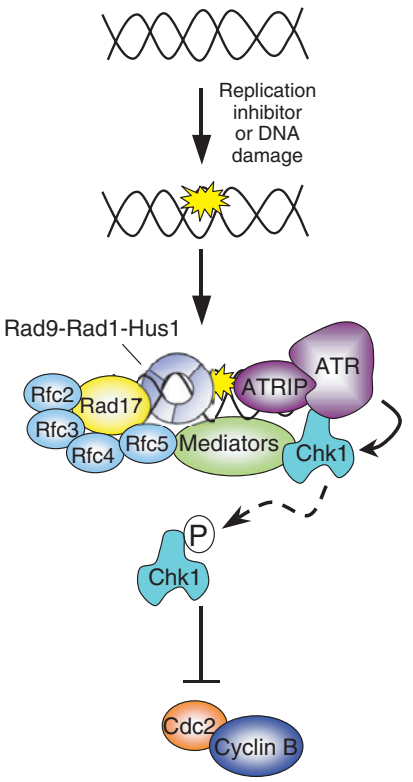

Fig. 1. The G2 DNA damage checkpoint. In response to DNA damage, or agents that block replication, checkpoint complexes consisting of (1) Rad17 and Rfc2-5, (2) Rad9, Rad1 and Hus1, and (3) ATR and ATRIP are recruited to the damaged region. Together with mediator proteins, ATR then activates Chk1 by phosphorylation to promote a blockade to Cdc2/Cyclin B activation, and thus cells are unable to enter mitosis.

residues (serines 317 and 345 in the human protein) (Liu et al., 2000; Zhao and Piwnica-Worms, 2001). This is catalyzed by ATM and ATR, which are large protein kinases that share sequence similarity to the phosphoinositide 3-kinase lipid kinases (PIKs), and are collectively referred to as the PIK kinases (PIKKs) (Bakkenist and Kastan, 2004). Note that ATM and ATR are physically and functionally distinct, and accumulating evidence points towards lesion- and cell-cycle-phase-specific use of either ATM or ATR in vertebrates. Thus, data pertaining to one of these PIKKs do not necessarily apply to the other. Here, we focus on ATR, which responds to a wide range of DNA-damaging agents; this is unlike ATM, which appears to respond specifically to double-stranded (ds)DNA breaks (Bakkenist and Kastan, 2004).

ATR interacts with another protein, ATRIP, which is phosphorylated by ATR following DNA damage and may regulate ATR activity (Cortez et al., 2001). For ATR to catalyze Chk1 phosphorylation, several additional proteins are required (O'Connell et al., 2000). These include the proliferating cell nuclear antigen (PCNA)-related complex of Rad9, Rad1 and Hus1 (commonly referred to as 9-1-1); and a replication factor-C (RFC)-related complex, in which Rad17 replaces the large Rfc1 subunit and forms a complex with the four smaller RFC subunits, Rfc2-Rfc5. During DNA replication, PCNA, which is a donut-shaped processivity factor or clamp for DNA polymerases, is loaded onto DNA by RFC, also known as a 'clamp loader' (Maga and Hubscher, 2003). Studies using a combination of purified and recombinant proteins have demonstrated that the 9-1-1 complex is loaded onto primed, ssDNA by the RFC-related Rad17-Rfc2-Rfc3-Rfc4-Rfc5 complex, presumably in an analogous manner (Bermudez et al., 2003; Ellison and Stillman, 2003; Majka and Burgers, 2003; Zou et al., 2003). These proteins localize to sites of DNA damage in intact nuclei, to which ATR and ATRIP independently localize (Kondo et al., 2001; Melo et al., 2001; Zou et al., 2002; Zou and Elledge, 2003).

Chk1 activation also requires other proteins, referred to as mediators. In fission yeast, two interacting BRCA1 C-terminal (BRCT)-domain proteins, Cut5 and Crb2, are required for Chk1 phosphorylation by the ATR homolog Rad3 (O'Connell et al., 2000). Recent work shows that phosphorylation of Rad9 by Rad3 leads to association of Rad9 with Cut5, and that this is necessary for Chk1 activation (Furuya et al., 2004). Furthermore, Crb2 binds specifically to phosphorylated Chk1 (Mochida et al., 2004), and Crb2 also associates with DNA damage foci (Du et al., 2003; Nakamura et al., 2004), although it is not clear whether the same pool of Crb2 is involved in both events. Human homologs of Cut5 (TopBP1) and Crb2 (53BP1) have been implicated in DNA damage responses (Wang et al., 2002; Yamane et al., 2003; Yamane et al., 2002), but whether similar mechanisms leading to Chk1 activation involve these proteins is not yet known. Another mediator protein, claspin, facilitates Chk1 activation in *Xenopus* egg extracts and in human cells (Chini and Chen, 2003; Kumagai and Dunphy, 2000; Lee et al., 2003). The yeasts contain a somewhat related protein, Mrc1, although this appears to be required for the activation of the Chk2 homolog, Cds1 (Alcasabas et al., 2001; Tanaka and Russell, 2001), a distinct checkpoint effector functioning primarily during delays in DNA replication. Vertebrates also contain another large mediator protein required for Chk2 activation, known as Mdc1, although this protein is not found in the yeasts (Goldberg et al., 2003; Lou et al., 2003; Stewart et al., 2003). BRCA1, a breast and ovarian tumor suppressor, has also been implicated in Chk1 activation in human cells (Yarden et al., 2002), although again a convincing BRCA1 homolog is not present in the yeasts.

Activating ATR

Ultraviolet (UV)-induced lesions such as thymidine dimers, or base alkylation induced by agents such as methyl methanesulfonate (MMS), are an impediment to DNA polymerases. Recent studies using human cells and nuclei added to *Xenopus* egg extracts suggest that these lesions require DNA replication to activate ATR-mediated Chk1 phosphorylation (Lupardus et al., 2002; Stokes et al., 2002; Ward et al., 2004). One model suggests that ssDNA generated at the stalled replication fork, perhaps coated by RPA, leads to the recruitment of checkpoint proteins and activation of Chk1 (Fig. 2). Is this model consistent with all published data, and is it the only mechanism for activation of ATR and/or Chk1?

Several observations are consistent with the idea that RPA-coated ssDNA can act as the checkpoint-activating signal. First, in budding yeast, ssDNA accumulates at double-strand breaks induced by the sequence-specific nuclease HO, which is normally involved in mating-type switching (Lee et al., 1998) and also at telomeres upon loss of function of the telomere-

binding protein Cdc13 (Garvik et al., 1995; Lydall and Weinert, 1995), both of which lead to activation of the checkpoint. Moreover, in mammalian cells and *Xenopus* egg extracts, RPA accumulates on chromatin following several types of genotoxic stress (Lupardus et al., 2002; Walter and Newport, 2000; Zou and Elledge, 2003), which suggests that ssDNA is formed. Second, the amount of ssDNA generated in yeast cells determines whether cells can adapt and resume division, albeit with unrepaired dsDNA breaks (Lee et al., 1998). Third, RPA function appears to be required for lesion detection by the ATR-ATRIP complex and downstream checkpoint signaling. Knocking down of RPA by RNA interference prevents ATR foci formation and reduces activation of Chk1 in mammalian cells (Zou and Elledge, 2003), and depletion of RPA from *Xenopus* egg extracts prevents the recruitment of ATR and Hus1 to chromatin during replication (You et al., 2002) or following replication arrest caused by aphidicolin treatment (Lee et al., 2003). Although these effects could be indirect and related to a failure to undergo DNA replication, one study has shown that RPA is needed for ATR to be recruited to ssDNA generated in a replication-independent manner (Costanzo et al., 2003). In addition, a mutation, *rfa1-t11*, in the large subunit of yeast RPA that allows DNA replication prevents full activation of the checkpoint and promotes more rapid adaptation following DNA damage (Kim and Brill, 2001; Pellicioli et al., 2001). This mutant also fails to recruit Ddc2 (ATRIP) to ssDNA in vivo (Zou and Elledge, 2003). Finally, both human and yeast RPA appear to be sufficient to mediate the binding of ATRIP/Ddc2 to ssDNA in vitro, whereas the RPA-t11 mutant protein complex is ineffective (Zou and Elledge, 2003).

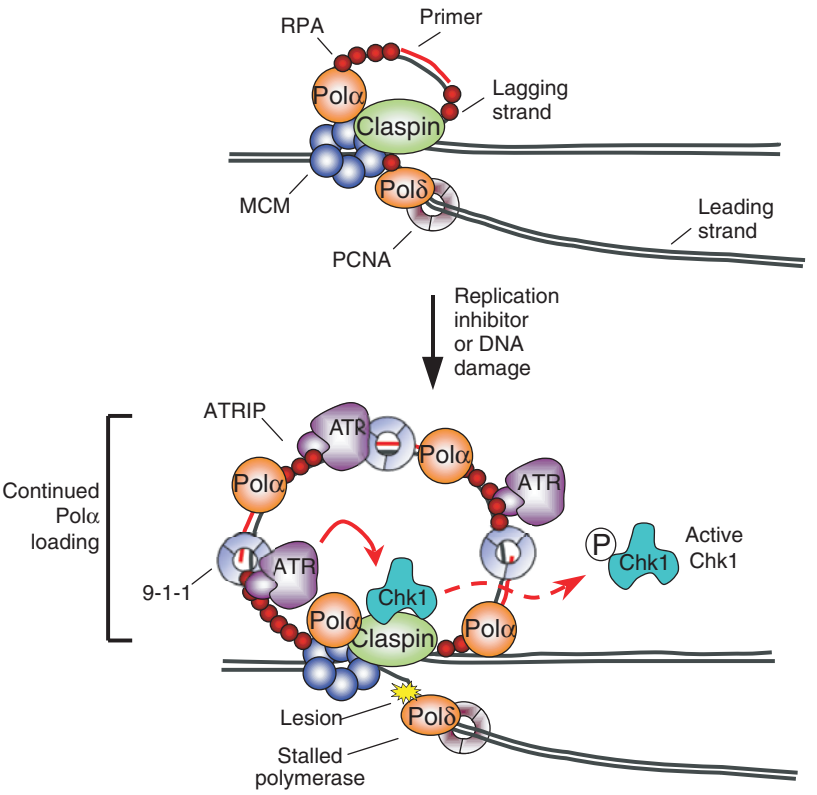

Fig. 2. Model for replication-dependent ATR activation. When a polymerase encounters a lesion (shown here on the leading strand), the polymerase is slowed while the minichromosome maintenance (MCM) helicase continues to unwind the duplex DNA. This leads to accumulation of single-stranded DNA on the leading and/or lagging strand and recruitment of RPA and polymerase α. The ATR-ATRIP complex and the 9-1-1 complex are then recruited to the ssDNA. Phosphorylation and activation of Chk1 follows and is mediated by claspin and/or additional mediator proteins.

Taken together, these observations make a very strong, although perhaps not definitive, case that RPA-coated ssDNA is crucial for checkpoint activation. However, it is not clear whether this is the only structure needed for checkpoint activation. Signaling to Chk1 requires the 9-1-1 complex, which is recruited to damage foci independently of ATR and appears to have requirements distinct from those of ATR-ATRIP for DNA binding, both in extracts and with purified proteins in vitro (Ellison and Stillman, 2003; You et al., 2002; Zou et al., 2003). Indeed, the loading of this complex onto primed ssDNA might suggest that a primer-template junction is required in addition to ssDNA. This would be consistent with the requirement for DNA polymerase α (Polα) activity in both *Xenopus* and *S. pombe*, although it is difficult to rule out the possibility that it is a step downstream of Polα/primase priming that is required (Bhaumik and Wang, 1998; Michael et al., 2000). One possibility is that RPA-coated ssDNA is sufficient to activate ATR but not Chk1. The possibility that ATR can be activated when Chk1 is not is suggested by the fact that, in *S. pombe*, phosphorylation of Rad26, the homolog of ATRIP, by Rad3 (ATR) occurs in the absence of the 9-1-1 complex and Chk1 phosphorylation.

Although significant quantities of RPA-coated ssDNA are generally present only during DNA replication, is activation of ATR and Chk1 restricted to S phase cells? Studies in *Xenopus* egg extracts and human cells indicate that ATR cannot be activated outside S phase following treatment with UV or MMS (Lupardus et al., 2002; Stokes et al., 2002; Ward et al., 2004). The generation of sufficient amounts of RPA-coated ssDNA by these agents might require the pausing of DNA replication. That is, the presence of a lesion during S-phase might act as a physical impediment to the advancing polymerase, causing the accumulation of ssDNA by uncoupling helicase and polymerase activities. In G1 and G2 phase, ATR activation might still require RPA-coated ssDNA to form, although through mechanisms different from those operating during S phase. For example, a dependence on ATR for ionizing radiation (IR)-induced checkpoint delay in G2 phase, and for Chk1 activation, has been demonstrated in human colon tumor HCT116 cells and mouse embryo fibroblasts (MEFs) lacking ATR (Brown and Baltimore, 2003; Cortez et al., 2001). Although IR induces dsDNA breaks, these are thought to be rapidly processed to ssDNA by exonucleases, including Mre11 (van den Bosch et al., 2003). In fission yeast, the ATR homolog Rad3 is clearly required for checkpoint responses during G2 phase, including those following UV irradiation (Bentley et al., 1996). Although fission yeast has an additional pathway of UV repair (UVDE) that involves

recombination that could generate ssDNA (Yonemasu et al., 1997), this pathway is not required for an efficient checkpoint. Repair by classical nucleotide excision also involves RPA (Araujo and Wood, 1999), and ssDNA generated during the repair process could in principle lead to Rad3 activation. Thus, ATR activation may not be confined to S phase in instances in which RPA-coated ssDNA can be generated by other mechanisms. It may, however, be optimal during S phase since amplification of the checkpoint-activating signal might be possible from stalled replication forks. It is also possible that different mediator proteins, or other fine-tuning events, are used outside S phase to activate Chk1.

Why recruit checkpoint proteins to sites of DNA damage?

In vitro binding assays and chromatin immunoprecipitation (ChIP) experiments have shown that ATRIP interacts with ssDNA when the latter is coated with RPA (Zou and Elledge, 2003). However, in experiments using kinase-dead forms of ATR, recruitment of ATR and RPA does not occur efficiently, and thus ATR activity is required for recruitment of ATR and RPA (Barr et al., 2003). ATR activity might be required to stabilize the association with chromatin, and RPA is indeed phosphorylated following DNA damage, probably by the PIKKs. Still, why go to the sites of DNA damage in the first place? Is localization of checkpoint proteins to sites of DNA damage necessary for the events leading to Chk1 activation, or are these proteins recruited to sites of DNA damage for other processes, including DNA repair?

Several observations suggest roles for checkpoint proteins in regulating genome integrity through mechanisms unrelated to Chk1 activation. The relative contribution of a checkpoint defect to the radiation sensitivity of a cell can be assayed by artificially delaying mitotic entry. This is easily achieved in fission yeast by use of a temperature-sensitive *cdc25* allele, *cdc25-22*. At non-permissive temperature (36°C), the mutant Cdc25 protein in these cells is inactive, and therefore the cells arrest in G2 phase with inactive, Y15-phosphorylated Cdc2. When shifted back to permissive temperature (25°C), the mutant protein is reactivated, Cdc2 is rapidly dephosphorylated and the cells move synchronously into mitosis with little loss of viability. Thus, following irradiation, checkpoint mutants can be prevented from entering catastrophic mitoses by inactivating the temperature-sensitive Cdc25, thus providing the time for DNA repair. In these assays, the sensitivity of cells lacking *chk1* to radiation can be largely overcome, which shows that the absence of a delay to mitosis is the primary defect in these cells, because restoring the delay by this protocol allows time for repair and successful passage through mitosis (Walworth et al., 1993). However, the degree of rescue is significantly lower in cells lacking the upstream components (Al-Khodairy and Carr, 1992), which suggests they play additional roles in the response to DNA damage, probably in events leading to its repair. Moreover, there are alleles of Rad26 (ATRIP) (Al-Khodairy et al., 1994) and Crb2 (53BP1) (Caspari et al., 2002) that have specific repair and/or DNA damage tolerance defects, and ATR-null MEFs released from the Polα inhibitor aphidicolin exhibit significant chromosome aberrations in the following mitoses (Brown and Baltimore, 2003). This also indicates a role for ATR in tolerance of replication arrest that is separable from cell-cycle progression. In fission yeast, all checkpoint proteins except Chk1 are required to maintain telomere length (Dahlen et al., 1998; Matsuura et al., 1999). Moreover, Rad17 and 9-1-1 (but not Chk1) are required for chromatin binding of the translesion synthesis (TLS) polymerase dinB (Kai and Wang, 2003). TLS is a damage tolerance mechanism by which specific polymerases can replicate past a lesion such as a thymidine dimer, although this does not repair the lesion *per se*. These observations clearly demonstrate Chk1-independent roles for the proteins in genome integrity.

A role in DNA repair is one explanation for the requirement for these proteins to be at the sites of damage, and may not be directly related to signaling through Chk1. However, showing this experimentally might be difficult, because the maintenance of the checkpoint arrest until completion of DNA repair could involve the sensing of active repair complexes on chromatin, and so separating these events may not be possible.

Maintaining the checkpoint signal

Once activated, Chk1 function is required until completion of DNA repair: early inactivation of Chk1 still results in catastrophic mitoses (Latif et al., 2004). Thus, once these complexes are assembled on damaged foci, sensing of persistent DNA damage must maintain an arrest. Experiments in fission yeast suggest that Rad3 (ATR) activity is dispensable for maintenance of checkpoint signaling (Bentley et al., 1996), although maintenance of the structure may be required. Furthermore, the checkpoint model relies on recruitment of RPA to damage foci and is generally discussed in the context of a dsDNA break. However, the repair of this break by recombination requires the replacement of RPA by Rad51 (Song and Sung, 2000). How this affects the association of checkpoint proteins and continued signaling is not known and is clearly something that must be resolved.

Conclusion and Perspectives

We are now approaching a detailed understanding of the spatial-temporal regulation of Chk1 activation by ATR. Clearly, the recruitment of multiple checkpoint proteins to RPA-coated ssDNA explains many situations in which ATR activates Chk1. What needs to be resolved is whether this is the only scenario in which these proteins are brought together in time and space to elicit a checkpoint signal or whether other structures, and perhaps other proteins, can also contribute in this signaling cascade. The relative contributions of ATM and ATR to different stimuli are beginning to be understood, but additional work needs to be done. Although ATM is more closely related to Tel1 in the yeasts, and ATR more related to Rad3/Mec1, the functional equivalency of these proteins is not absolute. Thus, genetic studies in the yeasts may not be as beneficial to sorting out details of signaling through ATM or ATR, which have proven very fruitful in extending our understanding of other events in checkpoint signaling. Given the central roles these proteins play in genome integrity, efforts to establish a clear model of how these events are controlled are extremely important, and this will no doubt continue to be the subject of intense study.

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