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Turning off the G2 DNA damage checkpoint

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Abstract

In response to DNA damage, cells activate checkpoints to delay cell cycle progression and allow time for completion of DNA repair before commitment to S-phase or mitosis. During G2, many proteins collaborate to activate Chk1, an effector protein kinase that ensures the mitotic cyclin-dependent kinase remains in an inactive state. This checkpoint is ancient in origin and highly conserved from fission yeast to humans. Work from many groups has led to a detailed description of the spatiotemporal control of signaling events leading to Chk1 activation. However, to survive DNA damage in G2, the checkpoint must be inactivated to allow resumption of cell cycling and entry into mitosis. Though only beginning to be understood, here we review current data regarding checkpoint termination signals acting on Chk1 and its' upstream regulators.

Keywords

Checkpoint; Chk1; ubiquitin-dependent proteolysis; protein phosphatase; protein kinase

1. Introduction

Signal transduction pathways control cell fate decisions as finely tuned machines that activate effectors and show tight spatiotemporal regulation. Activating signals also generate negative regulators that feed back to dampen the initial signal. Examples of this include the MEK-dependent activation of MAPK-phosphatase to block the kinase cascade [1], the transactivation of Mdm2 by p53 to direct its own proteolysis [2], and the activation of the anaphase-promoting complex by Cdc2 phosphorylation to direct cyclin destruction [3]. By these types of mechanisms, the signals sent are reversible and allow cells to respond to ever changing stimuli.

The detection of DNA damage evokes two important responses to ensure cellular survival: lesion-specific repair pathways are activated and recruited to sites of DNA damage to affect their repair. To allow time for repair to complete, cell cycle checkpoints negatively regulate cyclin-dependent kinases (CDK) and thus delay progression through the cell cycle. Eukaryotic cells have two major DNA damage checkpoints. In higher organisms, a p53-dependent response can block cell cycle progression in G1 by transactivation of the Cdk2 inhibitor p21. p53 can also evoke an apoptotic response to DNA damage via the pro-apoptotic genes PUMA and NOXA [4]. A more ancient checkpoint, conserved from yeast to man, is activated in S- and G2-phases to ensure the mitotic CDK, Cdc2, remains inactive and the cells do not enter mitosis. This checkpoint culminates in the activation of Chk1, a protein kinase that modulates

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inhibitory tyrosine phosphorylation on Cdc2 via the kinase (Wee1) and phosphatase (Cdc25) that control Cdc2 [5]. However, once repair is completed, cell survival is reliant on Cdc2 being activated to allow mitosis to occur. Much has been learned regarding Chk1 activation (see below), but how is this checkpoint terminated?

2. Chk1 activation

Recent reviews present a detailed description of Chk1 activation and cite the primary sources of the data [5-7]. To conserve space, this is only briefly outlined here, and these reviews should be consulted for the primary citations. Chk1 kinase activity is induced by DNA damage. This increase in activity is dependent on its phosphorylation of C-terminal residues by the PI-3K-related protein kinases ATM and ATR. For this phosphorylation event to occur, there is a complex choreography of upstream regulators that interact with each other and with chromatin surrounding the initial lesion.

Whilst a diverse array of genotoxins will activate Chk1, current models of the ensuing activation of Chk1 center around the generation of single-stranded DNA (ssDNA), either as a direct result of a lesion (e.g. a stalled or collapsed replication fork), or from the processing of the initial lesion (e.g. exonuclease resection of double-stranded DNA breaks). Replication protein A (RPA) has a high affinity for ssDNA, and rapidly binds to it at the sites of DNA damage. RPA-coated ssDNA then acts as a landing pad for several checkpoint proteins. These include ATM and, perhaps more predominantly, ATR, which interact with RPA via their binding partners, the Mre11-Rad50-Nbs1 (MRN) complex, and ATRIP, respectively [8]. Independently, the Rad9-Rad1-Hus1 (9-1-1) complex, which structurally resembles PCNA, is loaded onto the RPA-coated ssDNA by a Replication Factor C-related complex, in which Rad17 replaces the RFC1 subunit. ATM/R phosphorylates MRN, ATRIP, and 9-1-1 subunits, and this promotes the recruitment of several BRCT-domain containing mediator proteins, including 53BP1 and TopBP1, which are also essential for Chk1 activation. At least in human cells, the activated pool of Chk1 comes from a chromatin-associated fraction that is released upon phosphorylation and activation [9]. Chk1 then serves to phosphorylate Wee1 and Cdc25 proteins, and this prevents Cdc2 activation.

This cascade thus requires the assembly of protein complexes, their recruitment to sites of DNA damage, the phosphorylation of several proteins, and a second wave of protein recruitment to simply activate a single protein kinase. Data from *S. pombe* suggests that Wee1 and Cdc25 are the only essential substrates of Chk1 with respect to signaling a G2 delay [10]. However, several other substrates of ATM and ATR have been described that affect DNA repair pathways. Among these are histone H2A in the yeasts, and the variant H2A, H2A-X in higher organisms [11]. The phosphorylated histones can span for 50-100kb either side of a double-stranded DNA break (DSB) and serves, at least in part, to recruit cohesin complexes to facilitate recombination [12].

3. Potential mechanism to terminate a Chk1-mediated signal

To date, no negative regulator of the checkpoint initiating signal has been described to attenuate the signal, though clearly Chk1 must be inactivated for cells to exit the checkpoint mediated cell cycle arrest, and indeed the inactivation of Chk1 is sufficient to promote entry into mitosis, even if repair is not completed [13]. In considering possible mechanisms of checkpoint termination, we envisage at least three mechanisms that are not mutually exclusive. Firstly, protein phosphatases could act to reverse the DNA damage induced phosphorylations, including those required to activate Chk1. Secondly, the activating phosphorylations could also serve as a mark for protein degradation, and thus a sustained checkpoint response would require continual signaling. Finally, independent pathways could antagonize the cell cycle arrest induced by checkpoint activation to promote mitosis. Although the fate of all protein

modifications promoted by DNA damage are not known, there is now experimental evidence for each of these inhibitory mechanisms, which is outlined in the following sections.

4. Reversal of activating phosphorylations

Whilst cells contain many protein kinases with exquisite substrate specificity, a relatively small number of protein phosphatases dephosphorylate phosphoproteins. As the substrate repertoire of these enzymes is diverse, it is technically challenging to demonstrate the dephosphorylation of a particular protein by a particular phosphatase. The catalytic subunits of these phosphatases are frequently regulated by interacting proteins, restricting their activity in space and time [14]. This regulation does not apply to experiments *in vitro*, where many unrelated phosphatases will dephosphorylate any number of substrates. So, when considering the role of protein dephosphorylation in checkpoint control, one must consider data that shows a protein is and must be dephosphorylated for cell cycle progression to ensue.

Dephosphorylation of Chk1

Work in our laboratory showed that, in fission yeast, the dephosphorylation of Chk1 is dependent on one PP1 isoform, Dis2. Dis2 is essential for timely recovery from a Chk1-dependent G2 cell cycle delay that correlates with persistent Chk1 phosphorylation, and Dis2 overexpression overrides the ability of the ATR homolog Rad3 to phosphorylate Chk1 [15, 16]. Thus, while Dis2 is not essential for viability, both the deletion and overexpression of Dis2 results in DNA damage sensitivity due to the opposing effects on Chk1 activity. Whilst Dis2 efficiently dephosphorylated Chk1 *in vitro*, so do Calf Intestine and Lambda phosphatases [17], highlighting the above discussed difficulty with *in vitro* dephosphorylation experiments. Importantly, Dis2 does not affect the phosphorylation status of other Rad3 targets, including Crb2 and Rad9 [16].

Using human cells, the Donehower laboratory showed that the PPM1D/Wip1 phosphatase interacts with Chk1, dephosphorylates Chk1 *in vitro*, and when overexpressed abrogates checkpoint responses. Moreover, siRNA to PPM1D leads to enhanced Chk1 phosphorylation in UV-irradiated cells, and together these data are consistent with PPM1D being important in Chk1 inactivation in human cells [18]. In this study, the authors also show that PP1 dephosphorylates Chk1 *in vitro*, but remarkably, PP2A, PP2B and PP2C did not. PPM1D is a target gene of p53, and indeed also dephosphorylated the ATM/R catalyzed phosphorylation of p53 on S15. However, cells lacking p53 do dephosphorylate Chk1 and recover from a G2 checkpoint arrest [19], and so in this context either PPM1D must dephosphorylate Chk1 independently of p53, or another phosphatase must substitute. Given the data in fission yeast, and the *in vitro* data presented in this paper, PP1 is a likely candidate.

In a separate study by the Piwinica-Worms laboratory [20], the inhibition of Chk1 activity with small molecule inhibitors was shown to promote the ATR-dependent phosphorylation of the inhibited Chk1 molecules, suggesting Chk1 may restrain its' own activity. In this case, the Chk1 inhibitors did not affect the phosphorylation of other ATR substrates. However, inhibitor-2, a PP1-specific inhibitor, did not affect Chk1 phosphorylation, and based upon sensitivity to the phosphatase inhibitor Fostriecin, the authors conclude that PP2A, PP4 and PP6 are the relevant phosphatases for Chk1.

That Chk1 is and needs to be dephosphorylated for termination of the checkpoint seems fairly clear. These studies do not give a unifying answer as to the phosphatase that is required for this. It is possible that the dephosphorylation of Chk1 can be achieved by any one of a number of phosphatases *in vivo*, and that the level at which this is controlled is either ATR activity, or some aspect of Chk1 itself, such as conformation or interaction with other proteins.

Dephosphorylation of other checkpoint proteins

Whilst there is plethora of phosphorylation events in the G2 DNA damage checkpoint, very little is known regarding the dephosphorylation of these proteins. If Chk1 phosphorylation requires sustained upstream signaling, then the targeting of upstream proteins is another means to inactivate Chk1. One result consistent with the model of sustained upstream signaling is that inactivation of the fission yeast mediator protein Cut5 (homolog of TopBP1) results in premature checkpoint termination [21]. However, similar experiments with the ATR homolog Rad3 show a sustained checkpoint response [22], so additional experiments are warranted to clarify this model.

As discussed above, histone H2A is another ATM/R substrate in the yeasts. In budding yeast, a phosphatase complex termed HTP-C, containing Pph3, Psy2 and a previously uncharacterized protein Yb1046w, has been shown to dephosphorylate H2A. Whilst cells lacking Pph3 or Psy2 are not particularly sensitive to Bleomycin treatment (which generates DSBs), under these conditions they do show strong synthetic genetic interactions with a range of DNA damage response genes [23]. Although phosphorylated H2A and H2A-X are common markers of DSBs, it is pertinent to note that the DNA damage sensitivity of non-phosphorylatable H2A in fission yeast is very mild [24], and that H2A-X nullizygous mice are viable [25].

In budding yeast, the major checkpoint effector kinase is Rad53, a distant relative of Chk2. Rather than arresting cells at the G2/M transition, Rad53 blocks the metaphase-anaphase transition through regulation of the Separase inhibitor Pds1 (Securin) [26-28]. Cells lacking Pph3 show persistent Rad53 activation, and thus a delay in recovery from a checkpoint arrest caused by an HO-endonuclease induced DSB [23]. Given this, it was somewhat surprising that *pph3Δ* cells were not Bleomycin sensitive, though an independent study identified Pph3 as a Rad53 phosphatase [29], and showed *pph3Δ* cells to be sensitive to the alkylating agents MMS, which can cause the generation of DSBs as an intermediate of repair. Two PP2C isoforms, Ptc2 and Ptc3, were previously shown to be required for Rad53 inactivation and checkpoint termination [30]. Whether these phosphatases are directly regulating Rad53 *in vivo*, or are indirectly affecting Rad53 via dephosphorylation of upstream proteins such as H2A is difficult to tease out, but nevertheless, it is clear that Rad53, like Chk1 in fission yeast and human cells, must be dephosphorylated for budding yeast cells to reenter the cell cycle.

5. Ubiquitin-dependent proteolysis of activated checkpoint proteins

An alternative fate of active phosphorylated checkpoint proteins is their proteolytic degradation. Phosphorylation can act as a mark for ubiquitination, a phenomenon extensively characterized for substrates of the Cullin-containing SCF E3 ubiquitin ligase [31]. Indeed one study in human cells showed that in the presence of the topoisomerase I poison Camptothecin (CPT), the phosphorylation of Chk1 on S345 by ATR precedes its ubiquitin-dependent proteolysis by an SCF ligase containing either the Cul1 or Cul4A Cullins [32]. Such degradation of Chk1 has not been reported in a number of other studies addressing Chk1 biochemistry, and so it is possible that this phenomenon is cell type specific, or specific to the replication associated DSBs produced by CPT.

Claspin is a checkpoint mediator protein originally identified by Dunphy and Colleagues in *Xenopus* by virtue of its association with Chk1 [33]. In *Xenopus* egg extracts, Claspin is essential for Chk1 activation by the polymerase alpha inhibitor aphidicolin. Under these conditions, Claspin is phosphorylated and this creates a docking site for the Polo-like kinase Plx1, and ensuing phosphorylation of Claspin by Plx1. Mutant, non-phosphorylatable Claspin accumulates on chromatin to very high levels. Under these conditions, Chk1 activity also remains high and hence the checkpoint remains engaged [34]. Several independent studies

identified that this accumulation of Claspin is due to a defect in its' ubiquitin-dependent proteolysis by SCF complexes containing the β -TrCP F-box protein [35-37].

As a mediator protein, Claspin acts to facilitate the phosphorylation of Chk1 by ATR. If degradation of a mediator leads to Chk1 inactivation, then one might expect that signaling to Chk1 continues in the presence of DNA damage, though is continuously abrogated by dephosphorylation and proteolysis. In this scenario, checkpoint termination would then simply require loss of the initiating signal. Whether other mediators such as 53BP1 and TopBP1 are similarly regulated has not been reported.

6. Checkpoint antagonists

As the effector of the G2 DNA damage checkpoint, Chk1 evokes a G2 cell cycle arrest via the regulation of Cdc25 phosphatases and Wee1 kinases. Evidence is beginning to accumulate that other signaling pathways can antagonize Chk1 at the level of these substrates.

In addition to the evidence that PP1 dephosphorylates Chk1, PP1 has been shown to dephosphorylate *Xenopus* Cdc25 on S287, the site of Chk1 phosphorylation [38]. As a consequence of Chk1-mediated phosphorylation of Cdc25 isoforms is their ubiquitin-dependent proteolysis, PP1 may be important in halting this process to allow Cdc25 to accumulate to levels required for CDK activation.

By contrast to the positive regulation of Wee1 isoforms by Chk1 phosphorylation of the non-catalytic domain, generally increasing their stability, there are several protein kinases known to negatively regulate Wee1 kinase activity. Two kinases in fission yeast, Cdr1 and Cdr2, were originally identified by the "changed division response"; when nitrogen becomes limiting, Cdr1 and Cdr2 inactivate Wee1 by phosphorylation of the catalytic domain, advancing the point of mitotic entry [39-43]. Under cycling conditions, deletion of *cdr1*, *cdr2* or both has only a minor impact on cell cycle progression. We identified dominant-negative mutations in *cdr1* that antagonize Chk1: these alleles suppress the DNA damage sensitivity of cells lacking Chk1, and gain-of-function for Cdr1 by itself causes checkpoint defects. Conversely, deletion of *cdr1* does not influence Chk1 function, explaining why dominant negative mutants were required for the phenotype [44]; that is, multiple Cdr-like kinases may be regulating Wee1 in response to various stimuli, and as negative regulators of Wee1, the cumulative effect of this is an antagonism of Chk1 as a positive regulator of Wee1. Although not seen in fission yeast, in *Xenopus* and budding yeast this pathway also impacts on Wee1 stability [45,46]. To date, the function of human Cdr kinases has not been reported, but given the high conservation of other G2/M cell cycle and checkpoint controls, it will be important to see if they too antagonize human Chk1.

7. Conclusions and perspectives

The G2 DNA damage checkpoint is a complex signaling pathway involving the assembly of several multi-protein complexes, and a phosphorylation cascade leading to Chk1 activation and Cdc2 inhibition. Much progress has been made in deciphering these signaling events, and although we still have much to learn about the molecular effects of the described biochemical events, the literature currently presents a detailed map of the events themselves, and the sequence by which they occur. By contrast, little is known as to how the checkpoint is terminated. Further, it is also not known what the cue for termination is; that is, what must be satisfied during DNA repair for the cell to begin to relive the brake? These events are more complicated to dissect due to the fact that the terminating machinery may not function exclusively in this response; that is, even with the known terminating events, other substrates for the phosphatases and ubiquitin ligases are known.

Much attention has been placed on Chk1 as a drug target in the treatment of human cancer. Many studies *in vitro* have shown that Chk1 dysfunction can sensitize cells to genotoxic therapies, and in doing so can synthetically reinstate responsiveness to treatments that tumors show resistance to, for example by loss of p53. Small molecule inhibitors of Chk1 are under clinical trial [47,48]. An alternative approach to inhibiting Chk1 signaling will be to prematurely terminate a Chk1-mediated signal. It is therefore important to augment our current and somewhat rudimentary knowledge of checkpoint terminating signals as a means to broadening the tools by which DNA damage checkpoint biology can be translated to the clinic.

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