Cyclin A and Nek2A: APC/C–Cdc20 substrates invisible to the mitotic spindle checkpoint

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Published in Biochemical Society Transactions 2010 Feb;38(Pt 1):72-7.
Summary

Active cyclin B1–Cdk1 (cyclin-dependent kinase 1) keeps cells in mitosis, allowing time for spindle microtubules to capture the chromosomes and for incorrect chromosome-spindle attachments to be repaired. Meanwhile, securin, an inhibitor of separase, secures cohesion between sister chromatids, preventing anaphase onset. The spindle checkpoint is a signalling pathway emerging from improperly attached chromosomes that inhibits Cdc20, the mitotic activator of the APC/C (anaphase-promoting complex/cyclosome) ubiquitin ligase. Blocking Cdc20 stabilizes cyclin B1 and securin to delay mitotic exit and anaphase until all chromosomes reach bipolar spindle attachments. Cells entering mitosis in the absence of a functional spindle checkpoint degrade cyclin B1 and securin right after nuclear-envelope breakdown, in prometaphase. Interestingly, two APC/C substrates, cyclin A and Nek2A, are normally degraded at nuclear envelope breakdown, even when the spindle checkpoint is active. This indicates that the APC/C is activated early in mitosis, whereas cyclin B1 and securin are protected as long as the spindle checkpoint inhibits Cdc20. Remarkably, destruction of cyclin A and Nek2A also depends on Cdc20. The paradox of Cdc20 being both active and inhibited in prometaphase could be explained if cyclin A and Nek2A are either exceptionally efficient Cdc20 substrates, or if they are equipped with ‘stealth’ mechanisms to effectively escape detection by the spindle checkpoint. In the present paper, we discuss recently emerging models for spindle-checkpoint-independent APC/C-Cdc20 activity, which might even have implications for cancer therapy.

Destruction of APC/C substrates directs cell division

Successful cell division critically depends on ubiquitin-dependent destruction of mitotic regulatory proteins [1,2]. In mitosis, the APC/C directs the destruction of mitotic cyclins and other key regulators in an ordered manner to direct progression through mitotic exit, sister chromatid separation and cytokinesis [3–5]. The multisubunit APC/C acts as an E3 enzyme that polyubiquitinates its substrates during mitosis and G1-phase. To be active, it requires the availability and binding of a WD40-domain activator protein, either Cdc20 or Cdh1 [6,7]. How the APC/C activators Cdc20 and Cdh1 ultimately promote APC/C activity is not completely clear, but they are thought to recognize D-box or KEN(Lys-Glu-Asn)-box destruction signals in substrates by their WD40 repeats. Interestingly, binding of D-box peptides to Cdh1 promotes the affinity of Cdh1 for the APC/C [8]. In addition, the N-terminal C-box, present in both Cdc20 and Cdh1, helps Cdc20 to bind and activate the APC/C [9]. Taken together, at the APC/C, binding of an APC/C substrate to Cdc20 might further stimulate APC/C activity.

In mitosis, the APC/C co-operates with Cdc20. Most evidence indicates that the other APC/C co-activator, Cdh1, is not activated in mitosis until cyclin B1 levels, and associated Cdk (cyclin-dependent kinase) 1 activity, fall sharply and inhibitory Cdh1 phosphorylations are removed. This does not happen until quite late in anaphase, when Aurora A, a strict APC/C–Cdh1 substrate, starts to be degraded [10–12] (Figure
Indeed, depletion of Cdc20 from mitotic cells stabilizes cyclin B1 and securin and stops cells in mitosis [13–15]. Loss of Cdh1 has no dominant effect on mitosis progression before late anaphase, yet causes premature S-phase entry and replication stress [10,16–18].

In a normal mitosis, the function of Cdc20 is strongly inhibited by the mitotic spindle checkpoint, a wait signal generated by any unattached kinetochores in prometaphase [19,20]. This ensures that Cdc20-dependent cyclin B1 and securin destruction is kept in check as long as required for the last chromosome to attach properly to the mitotic spindle. The checkpoint signal is generated by the action of two key proteins, Mad2 and BubR1, which co-operate in an incompletely understood manner with other spindle-checkpoint-relevant proteins (such as Mad1, Bub3, Bub1 and Mps1) to enforce the inhibition of Cdc20 [21].

With both Cdh1 being blocked by high cyclin B1–Cdk1 activity and Cdc20 inhibited by the action of the spindle checkpoint, it could be expected that the APC/C is not active early in mitosis and that all APC/C substrates remain stabilized from G2-phase at least up to metaphase. However, two robustly established prometaphase APC/C substrates, cyclin A and Nek2A, challenge this view (Figure 1). In the present paper, we discuss how the APC/C can target cyclin A and Nek2A early in mitosis, while the spindle checkpoint prevents the APC/C from processing cyclin B1 and securin.

**Activation of APC/C and Cdc20 at NEB (nuclear envelope breakdown)**

Cyclin A appears at the G1/S border, to form cyclin A–Cdk1 and cyclin A–Cdk2 kinase complexes that support S-phase and G2-phase progression [22]. Nek2A is a mammalian orthologue of the *Aspergillus* centrosomal kinase NIMA (never in mitosis in *Aspergillus nidulans*) required for the formation of correct mitotic spindles at mitosis onset [23]. The destruction of cyclin A and Nek2A starts within minutes of NEB [24–27], and in normally dividing cells, 80% of cyclinA and more than 50% of Nek2A is degraded before metaphase [24–27]. Cyclin A and Nek2A levels also decline to 20% of their G2-phase levels within 30–50 min when cells are kept in prometaphase by drugs that maintain the spindle checkpoint, whereas it takes 400–800 min for cyclin B1 and securin to reach such low levels in an active checkpoint [14,28,29].

Remarkably, upon significant depletion of Cdc20 from mammalian cells, cells arrest in mitosis with high levels of cyclin B and securin, but cyclin A is also stabilized [13,15]. Likewise, Nek2A destruction in *Xenopus* extracts not only is dependent on the APC/C, but also requires Cdc20 [27] (Figure 1). Indeed, we have confirmed that Nek2A is also a prometaphase Cdc20 substrate in mammalian cells (W. van Zon and R.M.F. Wolthuis, unpublished work). So how does Cdc20-dependent destruction of cyclin A and Nek2A evade inhibition by the spindle checkpoint?

First, we need to look at mechanisms by which the APC/C can be activated. The destruction pattern of cyclin A and Nek2A implies that relevant APC/C–Cdc20 activity
starts to rise as soon as cells are committed to mitosis. The APC/C-independent destruction at early mitosis of a strong interphase inhibitor of the APC/C, Emi1, has been suggested to play a role in cyclin A destruction, but there is debate whether loss of Emi1 really contributes to mitotic APC/C activation [30–32]. Another significant event early in mitosis, with kinetics of sites tested in good agreement with the onset of cyclin A and Nek2A destruction, is the phosphorylation of a large number of APC/C subunits [33–35]. The mitotic APC/C may be phosphorylated at no less than 70 different sites by mitotic kinases, particularly cyclin B1–Cdk1 and Plk1 [33,34,36]. The vastness of these modifications has complicated experimental progress towards revealing the true contribution of phosphorylation to APC/C activation, yet clearly suggests a significant impact on APC/C function.

Studies in yeast in which potential phosphoresidues of the phosphorylated TPR (tetratricopeptide repeat)-motif containing APC/C subunits, Cdc16, Cdc23 and Cdc27 (orthologous to APC6, APC8 and APC3) were replaced by nonphosphorylatable amino acids, the APC/C-12A mutant, have indicated that phosphorylated APC/C at least gains some activity in cells [35]. The cell cycle delay caused by the APC/C mutations was modest compared with that found in mutant B-type cyclin yeast strains, indicating that additional mitotic cyclin-dependent phosphosites may further direct APC/C activity [37]. In human cells, we also found an activating role of cyclin B1–Cdk1 activity in promoting mitotic exit, correlating with APC/C phosphorylation [34]. Perhaps Cdc20 function may also be supported by phosphorylation, as, interestingly, alanine replacement of four potential phosphoserine residues in yeast Cdc20 causes a metaphase delay in the background of an APC/C-12A mutant [37]. In conclusion, given its timing and scale, phosphorylation is a likely factor contributing to APC/C–Cdc20 activation in mitosis.

Phosphorylation might activate the APC/C by increasing the affinity for Cdc20, as was initially suggested on the basis of experiments in Xenopus extracts [38]. In human cells, however, we found that, even in G2-phase, some Cdc20 binds to the unphosphorylated APC/C. Although this interaction is indeed enhanced further at mitotic entry when the APC/C becomes phosphorylated, the modest increase in binding seems insufficient to us to explain the switch-like activation of APC/C–Cdc20 at prometaphase onset (W. van Zon and R.M.F.Wolthuis, unpublished work). In addition to a role in supporting Cdc20 binding, an interesting possibility is that APC/C phosphorylation increases the affinity for its mitotic substrates.

**Destruction of cyclin A and Nek2A in prometaphase**

Recent work indeed supports such a model for cyclin A. With the Pines laboratory (Gurdon Institute, Cambridge, U.K.), we proposed that cyclin A, by its Cdk partner, needs to bind a Cks protein to be directed to the APC/C in prometaphase [15]. We had found that, apart from reducing Cdc20 levels, depletion of the small Cdk subunits of the Cks family, Cks1 and Cks2, can also prevent cyclin A destruction in mitosis [15].
In *Drosophila*, a similar Cks-dependent mechanism seems to be required for cyclin A degradation in female meiosis [39]. It appears that Cks, by its anion-binding pocket that can bind phosphorylated cyclin–Cdk substrates such as the APC/C [40], facilitates recruitment of cyclin A–Cdk complexes when APC/C phosphorylation increases early in mitosis. Direct evidence for a role of Cks in docking cyclin A to the mitotic APC/C, independent of a cyclin-associated Cdk, is provided by recent work (Barbara di Fiore and Jon Pines, personal communication).

Could a similar mechanism of increased APC/C substrate capturing also be relevant for the prometaphase destruction of Nek2A? Nek2A destruction requires a unique extended D-box motif that is located at its C-terminus. Deletion studies revealed that, apart from the D-box motif, the last C-terminal amino acids, a methionine and an arginine residue, are particularly critical for the prometaphase destruction of Nek2A. Interestingly, a similar C-terminal dipeptide APC/C-interaction motif had been found previously in Cdc20, Cdh1 and the APC/C subunit APC10, and indeed Nek2A bound to the APC/C independently of Cdc20 by its C-terminal MR (Met-Arg) motif [26]. This shows that, whereas cyclin A–Cdk1 complexes depend on Cks as a bridging factor to be targeted to the APC/C, in analogy, Nek2A may use its MR motif for efficient targeting towards the APC/C. However, constitutive targeting to the APC/C does not explain how Nek2A destruction begins strictly at prometaphase. Perhaps APC/C phosphorylation could also contribute to increasing the affinity of Nek2A for the APC/C, but, interestingly, our initial observations show that Nek2A, in contrast with cyclin A, already binds the core APC/C complex precipitated from G2-phase cells (W. van Zon and R.M.F. Wolthuis, unpublished work). Possibly, loss of Emi1, or another critical event associated with NEB, may be needed to restrict Nek2A destruction to prometaphase onset.

A model in which enhanced recruitment to the prometaphase APC/C is critical for cyclin A and Nek2A destruction would also predict that cyclin A differs from, for instance, cyclin B1 in its ability to bind to the phosphorylated APC/C in prometaphase. Surprisingly, however, we found recently that cyclin B1–Cdk1 complexes bind firmly to APC/C–Cdc20 in prometaphase as well, in a manner similarly dependent on Cks and APC/C phosphorylation, at a time when the spindle checkpoint stabilizes cyclin B1 in cells (W. van Zon, J. Ogink, B. ter Riet, R.H. Medema, H. te Riele and R.M.F. Wolthuis, unpublished work). From these findings, a model emerges in which Cdc20-independent targeting of a substrate to the APC/C can support processivity in cells, but in itself is not sufficient to induce rapid processing and destruction in prometaphase.

Perhaps cyclin A and Nek2A destruction could effectively escape an activated spindle checkpoint if they were extremely efficient substrates, requiring much less Cdc20 than cyclin B1 destruction does. The observed docking of cyclin A–Cdk complexes and Nek2A to the APC/C could relax further the requirement for Cdc20 in such a model, so that only a few molecules of Cdc20 escaping checkpoint control in mitosis would be enough to initiate destruction in an efficient manner.
In contrast with this explanation, in experiments where Cdc20 was partially depleted from mitotic cells, cyclin A was quite effectively stabilized even under conditions that allowed cyclin B1 turnover [15]. This shows that loss of cyclin A is strongly dependent on Cdc20 and argues against a model in which cyclin A is simply a super-efficient APC/C–Cdc20 substrate in cells. Indeed, in vitro, cyclin A is also a poor APC/C substrate [41]. Inhibition of Cdc20 by the spindle checkpoint imposes the reverse, being highly permissive for cyclin A destruction, but protecting cyclin B1. Therefore it seems more likely that the Cdc20 molecules that are required for cyclin A destruction, and perhaps also for Nek2A destruction, are somehow invisible to the checkpoint, while other Cdc20 molecules are strictly kept in check for detecting or processing cyclin B1 and securin (Figure 2). Apparently, apart from increased binding to the core APC/C, an additional unique step may be required for the prometaphase APC/C substrates to be processed and this may relate to the way cyclin A and Nek2A bind Cdc20.
Can Cdc20-substrate binding in G2-phase prevent spindle checkpoint action?

To understand how prometaphase APC/C substrates may hide from the spindle checkpoint and learn how they differ from metaphase substrates, we have compared the ways by which cyclin A or cyclin B1 interact with Cdc20. Intriguingly, we found that, in contrast with cyclin B1, cyclin A already binds robustly to Cdc20 in G2-phase, and this requires a unique domain downstream of the D-box in the N-terminus of cyclin A, a region that is necessary, but not sufficient, for cyclin A degradation in prometaphase (W. van Zon, J. Ogink and R.M.F. Wolthuis, unpublished work, and [15,24,42]). In contrast, we found that the N-terminus or D-box region of cyclin B1 binds at best very weakly to Cdc20 even in mitosis (W. van Zon, J. Ogink, B. ter Riet, R.H. Medema, H. te Riele and R.M.F. Wolthuis, unpublished work). Yeast two-hybrid studies suggested that the unique cyclin A N-terminus associates directly with Cdc20 [43]. Taken together, a model emerges in which cyclin A requires a combination of signals for bivalent interaction with the APC/C for timely destruction, as both Cdc20 and the Cdk–Cks interaction are essential, but neither is sufficient ([15] and Barbara di Fiore and Jon Pines, personal communication).

Figure 2. Potential 'stealth' mechanisms by which prometaphase APC/C–Cdc20 targets escape spindle checkpoint control. Left-hand panel: schematic representation of WD40 protein Cdc20 (pink), cyclin A (red) that forms complexes with a Cdk (light blue) and a Cks protein (yellow), Nek2A that binds the APC/C by its C-terminal MR dipeptide (green), and the APC/C core complex (dark blue). Middle panel: in G2-phase, cyclin A forms complexes that are targeted to the APC/C by the Cdk subunits of the Cks family, as soon as the APC/C becomes phosphorylated in mitosis. Pre-mitotic complex formation between Cdc20 and cyclin A, in combination with efficient targeting to the APC/C by Cks helps cyclin A destruction to evade spindle checkpoint control (right panel). It can be envisaged that cyclin A and spindle checkpoint proteins compete for Cdc20 binding at the APC/C in mitosis (right-hand panel, question mark). Nek2A, the other well established APC/C–Cdc20 substrate that is degraded independently of the spindle checkpoint, is also targeted to the APC/C directly, but it is not known whether Cdc20 can bind in a checkpoint-independent manner to Nek2A, or whether Nek2A binding is regulated by APC/C phosphorylation (as indicated by question marks in the middle panel). See the text for further details and references.
Nevertheless, a multivalent interaction may actually be a more common mechanism in which substrates are processed by the APC/C [44–46] and in itself is again probably not sufficient to explain how Cdc20 functions while the spindle checkpoint is active. Possibly, the way cyclin A–Cdc20 complexes are formed interferes with the ability of checkpoint proteins to inhibit cyclin A-bound Cdc20. Competition could be enforced by the fact that many cyclin A–Cdc20 complexes are already present in G2-phase, before cells enter mitosis and the spindle checkpoint starts working. Combined with efficient Cdk- and Cks-dependent targeting of Cdc20–cyclin A to the timely phosphorylated APC/C, this could provide a rapid stealth mechanism by which cyclin A destruction sneaks by checkpoint control (Figure 2). To test such a model further, it would be quite interesting to see whether Nek2A could also bind Cdc20 in a way which might be competitive with the spindle checkpoint (Figure 2).

What is the function of prometaphase APC/C–Cdc20 activity?

Although non-degradable cyclin A can arrest cells in mitosis, normally mitotic exit is controlled by cyclin B1 destruction at metaphase and so it is unclear why cyclin A should be degraded during prometaphase. Cyclin A destruction may support the formation of a stable metaphase plate, although the observation that cells can reach metaphase alignment upon inhibition of the proteasome in mitosis argues against this idea [47].

Nek2A overexpression can also delay anaphase onset, perhaps through competition with other substrates or prolonging checkpoint activity [26]. Nonetheless, mutation of the D-box in Nek2A, which does not affect its kinase activity, delays destruction of Nek2A until post-anaphase, so active Nek2A cannot keep cells in mitosis [26]. Some evidence indicates a role for Nek2 inactivation in controlling human Hec1/Ndc80, a protein involved in kinetochore–microtubule attachments: whereas wild-type HsHec1 (human Hec1) fully complements the deletion of yeast Hec1, an hsHec1S165E mutant mimicking Nek2-dependent phosphorylation of HsHec1 gave only a partial rescue and caused increased chromosome segregation errors [48,49].

Possibly cyclin A-dependent kinase activity keeps cells in mitosis because cyclin A levels must decrease before cyclin B1 destruction can start. This may be the case if Cdc20 were rate-limiting for cyclin B1 destruction and were released from cyclin A before cyclin B1 and securin could be processed. We disfavour this idea, however, because we found no competition between cyclin A and cyclin B1 for Cdc20-binding in cells that entered mitosis in the presence of proteasome inhibitors and thus have G2-phase levels of both cyclin A and cyclin B1 [15]. This is consistent with the observation that cellular levels of APC/C and Cdc20 are severalfold higher than minimally required for efficient mitotic progression and substrate processing [14,15].

Furthermore, the mitotic arrest caused by impaired cyclin A destruction appears to be independent of spindle checkpoint activity, as human and Drosophila cells expressing non-degradable cyclin A stop in anaphase, when the checkpoint is
normally inactive [42]. Similarly, cells blocked in mitosis by a non-degradable cyclin B1 mutant arrest with high Cdk1 activity, but satisfy the spindle checkpoint, and degrade endogenous APC/C–Cdc20 substrates (W. van Zon and R.M.F. Wolthuis, unpublished work). Mutants of cyclin A that impair Cdk binding delay cyclin A destruction up to anaphase without delaying cells in mitosis [24,25,50], suggesting that persistent cyclin A–Cdk activity, like persistent cyclin B1–Cdk1 activity, can prevent mitotic exit and cytokinesis.

Interestingly in Saccharomyces cerevisiae, Clb5, a potential cyclinA orthologue which also controls S-phase progression, is degraded in mitosis in a spindle-checkpoint-independent manner as well [51]. Although the function of Clb5 destruction in yeast is unclear, it has been implicated in resetting the replication machinery and allowing preparations for S-phase re-initiation after cell division [51,52]. This suggests that prometaphase APC/C activity has a role in M–S-phase progression and in limiting DNA replication to once per cell cycle. We are currently analysing whether such a role for cyclin A destruction exists in human cells.

Anti-mitotic drugs, used in cancer therapy, can block cells in mitosis with high levels of cyclin B1 by maintaining spindle checkpoint activity. The fate of these cells varies significantly as cells either ‘slip out of mitosis’ owing to slow loss of cyclin B1 or die due to accumulating cell death signals [14,29]. The Mitchison group recently revealed that Cdc20 depletion causes a mitotic arrest that may be more prolonged as compared with the delay caused by spindle checkpoint drugs and inducing cell death more efficiently [14]. The essential role for APC/C–Cdc20 in the destruction of cyclin A and the ability of cyclin A to delay mitotic exit could perhaps contribute to a shift in cell fate from slippage to death. These observations may spark interest further in revealing the mechanisms that help the stealth substrates cyclin A and Nek2A disappear from the spindle checkpoint radar.

Acknowledgements

We thank Barbara di Fiore and Jon Pines (the Wellcome Trust and Cancer Research UK Gurdon Institute, Cambridge, U.K.) for sharing data before publication and fruitful discussions. W.v.Z. and R.M.F.W. are supported by a Vidi Grant from the Netherlands Organisation for Scientific Research (NWO). Work in the Wolthuis laboratory is supported further by grants from the Dutch Cancer Society [grant numbers KWF 2007-3789 and KWF 2008-4135].
FOUR Spindle checkpoint-independent APC/C-Cdc20 activity

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