How cyclin A destruction escapes the spindle assembly checkpoint

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Introduction

Ubiquitin-mediated proteolysis is fundamental to the proper control of mitosis. The anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase ensures the correct ordering of events by targeting specific proteins at specific times (Pines, 2006). The APC/C responds to the spindle assembly checkpoint (SAC) to restrict APC/C activity until metaphase, yet early substrates, such as cyclin A, are degraded in the presence of the active checkpoint. Cdc20 and the cyclin-dependent kinase cofactor, Cks, are required for cyclin A destruction, but how they enable checkpoint-resistant destruction has not been elucidated. In this study, we answer this problem: we show that the N terminus of cyclin A binds directly to Cdc20 and with sufficient affinity that it can outcompete the SAC proteins. Subsequently, the Cks protein is necessary and sufficient to promote cyclin A degradation in the presence of an active checkpoint by binding cyclin A–Cdc20 to the APC/C.
As a control, we used cyclin B1, lacking Cdc20 into prometaphase cells and found that this was Cdc20. To answer this, we injected purified cyclin A–GFP–Cdk2 binds to Cdc20 in G2 phase (Wolthuis et al., 2008), raising the We previously showed that some, but by no means all, cyclin A contributes to Cdc20 binding, but is not itself sufficient, and that both Cdc20 and Cks were required for its degradation (Wolthuis et al., 2008). However, we did not determine the mechanism by which cyclin A could be degraded in an SAC-resistant manner. To address this question, we first sought to identify the regions of cyclin A required to bind to Cdc20.

An extended sequence in the N terminus of cyclin A is required to target cyclin A for degradation in prometaphase (den Elzen and Pines, 2001; Geley et al., 2001; Jacobs et al., 2001). We generated several cyclin A deletion mutants (Fig. 1 A) tagged at the C terminus with the Flag epitope and a fluorescent protein (Venus; Nagai et al., 2002). As a control, we used cyclin B1, which is degraded in an SAC-sensitive manner (Clute and Pines, 1999). To ensure that the proteins were expressed at close to endogenous levels, we generated stable inducible HeLa cell lines in which a single copy of the plasmid was integrated into the genome at a flippase recognition target (FRT) site. The proteins were immunoprecipitated from prometaphase cells using an anti-Flag antibody and probed for Cdc20 and Cdk2. As expected, the N-terminal fragments lacking the cyclin box could not bind Cdk2 (Fig. 1, B and C).

Consistent with our previous findings (Wolthuis et al., 2008), cyclin A, but not cyclin B1, strongly bound to Cdc20, but neither the N-terminal fragment (1–98) nor the N-terminal fragment (124–137; Yu, 2007). Cyclin A competes with BubR1 for Cdc20 binding site on Cdc20, we tested the possibility that cyclin A could compete with the SAC complex for Cdc20 (note that by competition, we include mechanisms by which cyclin A could preferentially bind Cdc20 by inducing an allosteric change). Because we find only a minor fraction of Mad2 bound to Cdc20 (Nilsson et al., 2008), we tested whether cyclin A could displace BubR1. We immunoprecipitated Cdc20 from nocodazole-treated cells, added purified GST fusion proteins, and assayed their effect on the integrity of the Cdc20–SAC complex. Consistent with our competition hypothesis, excess cyclin A displaced BubR1 from Cdc20 (Fig. 2 B), and this depended on its ability to interact with Cdc20. Full-length cyclin A and the 1–165 fragment were both able to displace BubR1, whereas neither the 1–98 fragment of cyclin A nor the N terminus of cyclin B1 containing its D box (1–167) could do so (Fig. 2, C and D). We added a large excess (10–50-fold over Cdc20-coimmunoprecipitated BubR1) of the proteins in these in vitro reactions, but it is reasonable to suppose that the in vitro conditions do not recapitulate those in vivo and that competition in vivo may be more efficient.

To test whether cyclin A could compete with BubR1 for Cdc20 in vivo, we overexpressed cyclin A or cyclin A1–165 4–12-fold over endogenous and found that both greatly reduced the level of Cdc20 in BubR1 immunoprecipitates (Fig. 2, E and F). Thus, we conclude that both in vitro and in vivo, cyclin A is able to compete with the SAC proteins for binding to Cdc20, even when Cdc20 is already bound to the SAC complex.

Binding to Cdc20 is necessary but not sufficient for the proper timing of cyclin A degradation

Having identified a region of cyclin A sufficient to bind to Cdc20, regardless of whether Cdc20 was free or bound in an SAC complex, we asked whether this was sufficient to target a protein for destruction in prometaphase. We used Venus-tagged proteins to assay the precise timing of destruction in mitosis and compared full-length cyclin A, which is degraded as soon as cells begin prometaphase (Fig. 3 A; den Elzen and Pines, 2001; Geley et al., 2001; Wolthuis et al., 2008), with both the N-terminal fragments and a chimeric protein replacing the N terminus of cyclin B1 with that of cyclin A.

In support of our hypothesis, the N terminus of cyclin A conferred the ability to be degraded in an SAC-resistant manner on cyclin B1 (Fig. 3 B), whereas the cyclin A 1–98 fragment that could not bind Cdc20 was not degraded until anaphase (Fig. 3, C and D; note that data are plotted twice, once normalized.
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Cyclin A destruction escapes the checkpoint. Therefore, binding directly to Cdc20 is not in itself sufficient to confer SAC-resistant proteolysis. Thus, additional factors must contribute to the SAC-resistant degradation of cyclin A.

Cks1 is essential to mediate checkpoint-resistant degradation

To identify the missing component that confers SAC-resistant destruction, we focused first on the Cks protein because full-length cyclin A must bind to its Cdk partner and consequently to Cks to be degraded (Wolthuis et al., 2008). Moreover, the N terminus of cyclin A was sufficient to confer SAC-resistant to nuclear envelope breakdown (NEBD) and once to anaphase). In contrast, the 1–165 fragment that bound Cdc20 was degraded earlier, but notably, this was in metaphase not prometaphase (Fig. 3, E and F). Because metaphase equates to when the SAC has been satisfied, we tested whether this destruction was under the control of the SAC. We depleted Mad2 to inactive the SAC and found this advanced cyclin A (1–165) degradation to begin just after NEBD (Fig. 3, I and J), which is consistent with regulation by the SAC. In contrast, degradation of the 1–98 fragment was still delayed until anaphase (Fig. 3, G and H).

We conclude that although the 1–165 fragment can compete with BubR1 for Cdc20, it can only be degraded when the SAC is satisfied. Therefore, binding directly to Cdc20 is not in itself sufficient to confer SAC-resistant proteolysis. Thus, additional factors must contribute to the SAC-resistant degradation of cyclin A.

**Figure 1. The N terminus of cyclin A binds Cdc20.** (A) Schematic representation of cyclin A mutants. (B) Stable inducible HeLa FRT cell lines expressing cyclin B1, and the cyclin A mutants were synchronized in mitosis by a single thymidine block and released in the presence of nocodazole. To prevent cyclin A degradation, MG132 was added 2 h before collecting mitotic cells by shake off. Cells were lysed, and anti-Flag immunoprecipitates (IP) were probed for Cdc20, Cdk2, and Flag. (C) Cdc20 and Cdk2 levels in the anti-Flag immunoprecipitates (B) were quantified using an Odyssey scanner, corrected for the level of Flag-tagged protein, and normalized to the amount bound to wt cyclin A. Error bars indicate mean ± SEM from three experiments. (D) GST–cyclin A was purified from bacteria and incubated for 2 h at 4°C with His-Cdc20 purified from insect cells. Cyclin A was isolated on glutathione beads, and the beads and supernatants (SPN) analyzed by immunoblots were probed for Cdc20 and GST. Values are representative of seven experiments. Molecular mass markers are shown on the left (kilodaltons).
degradation on cyclin B1 (Fig. 3 B) that binds Cks via its Cdk1 partner. However, it was unclear whether the Cdk subunit itself contributed to the destruction of cyclin A.

Because we hypothesized that the role of Cks was to bind the APC/C, we predicted that we could bypass the Cdk and target the cyclin A 1–165 fragment for degradation even in the presence of an active SAC by fusing it to a Cks protein. In confirmation of this, a cyclin A (1–165)–Venus–Cks1 fusion protein was degraded just after NEBD (Fig. 4, C and D) with the same timing as wild-type (wt) cyclin A. Furthermore, fusing Cks1 to cyclin A (1–98) that could not bind Cdc20 had no effect; it was still degraded in anaphase (Fig. 4, A and B). Similarly, fusing Cks1 to the N terminus of cyclin B1 that cannot bind Cdc20 when the SAC is active did not change the timing of its destruction to prometaphase, although it made it a more efficient substrate (unpublished data; see van Zon et al. in this issue).

These results demonstrate that Cks1 is sufficient to confer SAC-resistant degradation on a cyclin A–Cdc20 complex, bypassing any requirement for Cdk binding or activity (Fig. S1, A and B). Thus, the primary reason why cyclin A has to bind to its Cdk to be degraded is to be targeted to the APC/C via the Cks protein.
Fusion to Cks1 promotes cyclin A ubiquitylation

If Cks does target cyclin A to the APC/C, one might predict that it should enhance cyclin A ubiquitylation. Therefore, we compared the in vitro ubiquitylation of cyclin A (1–165)–Venus (Fig. 4 E, lanes 1–9) and cyclin A (1–165)–Venus-Cks1 proteins (Fig. 4 E, lanes 10–19). Cks increased the processivity of cyclin A ubiquitylation because although the amount of mono- and oligoubiquitin species (one to five ubiquitins) was similar for the two substrates (Fig. 4 F), the proportion of polyubiquitin chains was higher for the Cks1 fusion (Fig. 4 G). Moreover, cyclin A (1–165)–Cks1 was clearly a better competitor in
Figure 4. **Cyclin A–associated Cks1 increases the efficiency of cyclin A ubiquitylation.** (A–D) Cells were injected in G2 phase with cyclin A (1–98)–Venus-Cks1 (A and B) or cyclin A (1–165)–Venus-Cks1 (C and D), and the fluorescence was measured through mitosis. Time is relative to NEBD (A and C) or anaphase (B and D). Error bars indicate mean ± SD of 19 cells from two experiments (A and B) or 24 cells from two experiments (C and D). (E) In vitro ubiquitylation assay of cyclin A (1–165)–Venus (lanes 1–9) or cyclin A (1–165)–Venus-Cks1 (lanes 10–19). Reactions were performed for the indicated time (shown in minutes) before analysis by SDS-PAGE and phosphoimaging. Control reactions are without APC/C (lanes 9 and 18) or E2 (lane 19). (F and G) Quantification of ubiquitin conjugates in E with one to five ubiquitin (F) and more than five ubiquitin (G) molecules were normalized to the total amount of ubiquitylated substrate. (F and G) Error bars indicate mean ± SEM from four experiments. (H) Ubiquitylation reactions for cyclin A (1–165)–Venus (lanes 1–4) and cyclin A (1–165)–Venus-Cks1 (lanes 5–8). 100 times excess of unlabeled cyclin A (1–165)–Venus (lanes 3 and 7) or cyclin A (1–165)–Venus-Cks1 (lanes 4 and 8) was added as a competitor at the beginning of the reaction. (I) Quantification of reactions in H. Numbers correspond to lanes in H. *, unmodified substrate; **, P < 0.01; ***, P < 0.05 (calculated using Student’s t test).
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in vitro APC/C-dependent ubiquitylation reactions than cyclin A (1–165; Fig. 4, H and I).

To test whether the Cks protein had to bind to the APC/C to promote cyclin A degradation in vivo, we mutated the anion-binding site of Cks1 (R20A; Watson et al., 1996). This mutant was severely disabled in its ability to bind to the APC/C, whereas binding to a Cdk was not affected (Fig. 5, A and B; and not depicted). When fused to cyclin A (1–165), Cks1 (R20A) was unable to confer SAC-resistant degradation; like the 1–165 fragment alone, the R20A fusion protein could only be degraded in metaphase once the SAC was satisfied (Fig. 5, C and D). Thus, the anion-binding site of Cks1 was essential to confer

Figure 5. Cks directly recruits cyclin A to the APC/C. (A) Purified His6-Cks1 and an anion-binding site mutant (R20A) and fusion proteins between cyclin A (1–165)–Venus and wt or (R20A) mutant Cks1 were immobilized on beads and incubated with extract from nocodazole-treated HeLa cells. Beads were analyzed by SDS-PAGE and immunoblotted for APC3, cyclin A, and Cks1. Molecular mass markers are shown on the left (kilodaltons). (B) APC3 binding to Cks1 (A) was quantified on an Odyssey scanner and corrected for the amount of Cks1. Values were normalized to wt Cks1. Error bars indicate mean ± SEM from three experiments. (C and D) Degradation of cyclin A (1–165)–Venus-Cks1 (R20A) measured as in Fig. 4 (A and B). Time is relative to NEBD (C) or anaphase (D). Error bars indicate mean ± SD of 28 cells from two experiments. (E) Working model. The cyclin A–Cdk–Cks complex binds to soluble or APC/C-associated Cdc20 by out competing the SAC complex. Recruitment to the APC/C is mediated by Cks interaction with phosphorylated APC/C.
SAC-resistant degradation on a cyclin A–Cdc20 complex. This result strongly indicates that Cks1 mediates the recruitment of cyclin A to a phosphorylated APC/C subunit. Several APC/C subunits are phosphorylated during mitosis (Kraft et al., 2003; Steen et al., 2008), of which APC3 is most likely to bind Cks1.

We conclude that two main factors contribute to the SAC-resistant degradation of cyclin A. First, cyclin A can bind directly to Cdc20 with sufficient affinity to displace SAC proteins. Second, the Cks protein targets a cyclin A–Cdk complex to the APC/C in a manner that allows Cdc20 bound to cyclin A to activate the APC/C.

Currently, we do not know exactly how cyclin A competes with the SAC proteins for Cdc20, in part because there is some disagreement over how the SAC proteins themselves bind Cdc20. We and others find that the majority of Cdc20 binds to an SAC complex with BubR1 and Bub3 but only a small fraction of Mad2, whereas others find that Mad2 forms a stoichiometric part of the complex (the reason for this discrepancy is unclear but may be attributable to the methods used to lyse the cells and isolate the SAC complexes or the methods used to calculate the amounts of protein in the complex; Nilsson et al., 2008; Herzog et al., 2009; Kulukian et al., 2009). However, there is general agreement that Mad2 is required for Cdc20 to bind to BubR1 in vivo; therefore, if cyclin A blocks access to the Mad2-binding site on Cdc20, either sterically or by inducing a conformational change, this would prevent BubR1 from binding. Alternatively, as suggested by our in vitro assays, cyclin A and BubR1 themselves might compete for Cdc20.

Because both cyclin A and cyclin B1 require Cdc20 for their degradation, is the difference between them simply that cyclin A has a greater affinity for Cdc20, or might it bind to Cdc20 in a different manner that allows SAC-independent destruction? We think the latter idea is unlikely because the SAC inhibits destruction of cyclin A (1–165) that binds Cdc20. Thus, our interpretation is that cyclin A can be degraded by either or both of two pathways (Fig. 5 E). Cyclin A can either compete for Cdc20 before being targeted to the APC/C by Cks, or a cyclin A–Cdk–Cks complex could bind to an APC/C to which Cdc20 is already bound as part of an SAC complex, and, once bound, the N terminus of cyclin A could displace the SAC proteins from Cdc20, thereby activating the APC/C.

Materials and methods
Cell culture and synchronization
HeLa cells were cultured in advanced DMEM (Invitrogen) supplemented with 2% fetal bovine serum. Cells were synchronized by a thymidine/aphidicolin protocol as previously described (Di Fiore and Pines, 2007). Cells were blocked with 2.5 mM thymidine (Sigma-Aldrich) for 24 h, released for 12 h, and blocked again with 5 µg/ml aphidicolin (Sigma-Aldrich) for 24 h. Cells were released into fresh medium. For prometaphase arrest, 0.1 µg/ml nocodazole (Sigma-Aldrich) or 100 nM taxol (Sigma-Aldrich) was added during release from a single thymidine block, and 12 h later, cells were either harvested by mitotic shake off or 10 µM MG132 (EMD) was added, and cells collected by mitotic shake off 2 h later. The HeLa-FRT cell lines (provided by S. Taylor, University of Manchester, Manchester, England, UK) were transfected using the FLIP-in system (Invitrogen) to generate stable inducible cell lines. Cells were induced with 1 µg/ml tetracycline (EMD) 12 h before harvesting.

Plasmids
Cyclin A and its mutants were cloned into a modified version of pCDNAs/FRT/TO (Invitrogen) containing Venus-Flag tag using the Gateway system. Fusions between cyclin A fragments and Cks1 were constructed in modified versions of the pEYPF-N1 plasmid, resulting in the YFP/Venus sequence expressed between cyclin A and Cks1. For the cyclin A–B1 fusion protein, cyclin A N terminus (aa 1–165) was fused to the C terminus of cyclin B1 (aa 171–433) in a modified version of pEYPF-N1 plasmid. For protein purification, pGEX (GE Healthcare), a modified version of pET30a (EMD) containing a strep tag, and pRSET (Invitrogen) vectors were used.

Microinjection and time-lapse imaging and analysis
For microinjection and time-lapse microscopy, the culture medium was replaced with Leibovitz’s L-15 medium (Invitrogen). Plasmids or recombinant protein were microinjected into G2 or prometaphase cells, respectively, using a semiautomatic microinjector (Eppendorf) on a microscope (DMIRE2; Leica). Differential interference contrast (DIC) and fluorescence images were captured every 3 min with a charge-coupled device camera (QuantEM 512B; Photometrics) using Slidebook software (Intelligent Imaging Innovations). A modified version of ImageJ software (National Institutes of Health) was used to quantify the fluorescence after background subtraction. DIC microscopy was used to monitor mitotic phases.

RNA interference
siRNA duplex against Mad2 (5’-GGAAGACUGGGACACAGU3’; Thermo Fisher Scientific), Cdc20 (5’-CGGAAGACCTGGCGTACA3’; Thermo Fisher Scientific), and control siRNA duplex against GAPDH (Applied Biosystems) were transfected at a final concentration of 100 nM with Oligofectamine (Invitrogen) according to the manufacturer’s protocols. Transfection was performed during synchronization 5 h after release from the thymidine block. For Cdc20, an extra transfection was performed before starting the synchronization protocol.

Protein expression and pull-downs
Recombinant proteins were induced with 0.5 mM IPTG in BL21 (DE3) at 25°C for 5 h and purified on glutathione Sepharose 4B beads (GE Healthcare), Streptactin matrix (IBA), or nickel beads (QiAGEN) according to the manufacturer’s protocols. Histagged Cdc20 was purified from baculovirus-infected SF9 cells as previously described (Nilsson et al., 2008). For GST pull-downs, GST proteins were immobilized on glutathione beads and incubated with purified Cdc20 in buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl2, 5% glycerol, and 1 mM DTT) with 10 µg BSA for 2 h at 4°C. For His6–Cks1 and cyclin A–Cks–strep–binding assays, purified proteins were coupled to nickel or Streptactin beads and incubated in buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, and 0.2% NP-40) for 2 h at 4°C with extracts from nocodazole-treated HeLa cells. For protein microinjection, cyclin A–GFP–His–Cdk2 complex was purified from baculovirus-infected insect cells using nickel beads, and the folding of the purified complex was assessed on a gel filtration column (Superdex 200; GE Healthcare).

Immunoprecipitation and competition experiments
Cells were lysed in 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.5% NP-40 plus protease inhibitor cocktail (Roche), and 10 mM microcinin (ECC- Products) for 20 min on ice. Cell lysates were clarified by a 12,000 g spin for 15 min at 4°C. Complexes were immunoprecipitated for 2 h at 4°C with anti-Flag (M2; Sigma-Aldrich) or anti-BubR1 (BD) antibody covaly with protein G ( Dynabeads; Invitrogen). After five washes in lysis buffer, proteins were eluted from beads by incubating for 5 min at 65°C in sample buffer. For the competition experiments, Cdc20 complexes were isolated using anti-Cdc20 (H7; Santa Cruz Biotechnology, Inc.) antibody. After the washes, beads with associated complexes were divided into several tubes, and 1–2 µl purified GST fusion protein was added to the beads and incubated in buffer A for 2 h at 4°C. The supernatant containing the unbound GST proteins and proteins released from the immunoprecipitated complexes were analyzed by immunoblotting. Beads were washed three times with buffer A, and the complexes still associated eluted in sample buffer for 5 min at 65°C.

Immunoblotting
After electrophoresis on 4–12% gradient Bis-Tris acrylamide gels, proteins were transferred to a PVDF membrane. Membrane saturation and all of the following incubation steps were performed in 3% low fat milk in 0.1% PBS-Tween, anti-Flag (M2; Sigma-Aldrich), anti-GST (B-14; Santa Cruz Biotechnology, Inc.), anti-cyclin A (Cancer Research UK), and anti-APC3 (BD) were used at 1:1,000. Anti-Cdc20 (Bethyl Laboratories), anti-BubR1 (Bethyl Laboratories and BD), and anti-Cdk2 (Koop, 2007) were used at 1:500. Anti-Cks1 (Invitrogen) was used at 1:1,500. Alexa Fluor 680 (Invitrogen)– and IRDye (800CW; LI-COR Biosources)-conjugated secondary antibodies were used at 1:5,000. The antibody signal was detected using the infrared imaging system (Odyssey; LICOR) for quantitative immunoblotting.
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