BubR1 blocks substrate recruitment to the APC/C in a KEN-box-dependent manner

Pablo Lara-Gonzalez, Maria I. F. Scott, Maria Diez, Onur Sen and Stephen S. Taylor*

Faculty of Life Sciences, University of Manchester, Michael Smith Building, Oxford Road, Manchester, M13 9PT, UK

*Author for correspondence (stephen.taylor@manchester.ac.uk)

Accepted 10 October 2011

Journal of Cell Science 124, 1–14

© 2011. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.094763

Summary

The spindle assembly checkpoint (SAC) is a signalling network that delays anaphase onset until all the chromosomes are attached to the mitotic spindle through their kinetochores. The downstream target of the spindle checkpoint is the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that targets several anaphase inhibitors for proteolysis, including securin and cyclin B1. In the presence of unattached kinetochores, the APC/C is inhibited by the mitotic checkpoint complex (MCC), a tetrameric complex composed of three SAC components, namely BubR1, Bub3 and Mad2, and the APC/C co-activator Cdc20. The molecular mechanisms underlying exactly how unattached kinetochores catalyse MCC formation and how the MCC then inhibits the APC/C remain obscure. Here, using RNAi complementation and in vitro ubiquitylation assays, we investigate the domains in BubR1 required for APC/C inhibition. We observe that kinetochore localisation of BubR1 is required for efficient MCC assembly and SAC response. Furthermore, in contrast to previous studies, we show that the N-terminal domain of BubR1 is the only domain involved in binding to Cdc20–Mad2 and the APC/C. Within this region, an N-terminal KEN box (KEN1) is essential for these interactions. By contrast, mutation of the second KEN box (KEN2) of BubR1 does not interfere with MCC assembly or APC/C binding. However, both in cells and in vitro, the KEN2 box is required for inhibition of APC/C when activated by Cdc20 (APC/C\(^{Cdc20}\)). Indeed, we show that this second KEN box promotes SAC function by blocking the recruitment of substrates to the APC/C. Thus, we propose a model in which the BubR1 KEN boxes play two very different roles, the first to promote MCC assembly and the second to block substrate recruitment to APC/C\(^{Cdc20}\).

Key words: Mitosis, Kinetochore, Spindle assembly checkpoint, Anaphase-promoting complex, Cdc20, Mad2

Introduction

The spindle assembly checkpoint (SAC) is a surveillance mechanism that delays anaphase onset until all the chromosomes have stably attached to spindle microtubules through their kinetochores (Musacchio and Salmon, 2007). The goal of the SAC is to inhibit the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that targets several anaphase inhibitors for proteolysis, including securin and cyclin B1. Once the SAC is satisfied, the APC/C becomes active, promoting the polypeptide chain and subsequent degradation of anaphase inhibitors such as securin and cyclin B1, thereby triggering anaphase onset and mitotic exit (Musacchio and Salmon, 2007).

Unattached kinetochores recruit numerous SAC components including Bub1, BubR1 (Mad3 in yeast), Bub3, Mad1, Mad2 and Mps1. These proteins in turn catalyse the release of a diffusible inhibitor that prevents APC/C activation (Musacchio and Salmon, 2007). It is now generally accepted that this inhibitor corresponds to the ‘mitotic checkpoint complex’, or MCC (Sudakin et al., 2001), which is comprised of Mad2, BubR1, Bub3 and Cdc20. Although Mad2 is essential for MCC formation, it can be present at sub-stoichiometric amounts with respect to the other proteins (Nilsson et al., 2008). Thus, it has been proposed that the main function of Mad2 is to promote the Cdc20–BubR1 interaction, after which Mad2 is released to create the final APC/C inhibitor (Nilsson et al., 2008; Kulukian et al., 2009; Westhorpe et al., 2011). Exactly how BubR1 then inhibits the APC/C remains to be determined.

BubR1 has a C-terminal kinase domain, but this does not appear to be required for SAC control; rather, it promotes the second function of BubR1 in mitosis, namely regulating kinetochore–microtubule interactions (Ditchfield et al., 2003; Harris et al., 2005; Lampson and Kapoor, 2005; Rahmani et al., 2009). Note that in yeast, Mad3 does not possess a kinase domain. In the N-terminus, BubR1 and Mad3 share a region of homology with Bub1 (Cahill et al., 1998; Taylor et al., 1998), and this TPR-like domain binds the kinetochore component Blinkin/KNL-1 (Bolanos-Garcia et al., 2005; Kiyomitsu et al., 2007; D’Arcy et al., 2010). In the central region, BubR1/Mad3 shares another short region of homology with Bub1, and this domain mediates binding to Bub3 (Taylor et al., 1998; Hardwick et al., 2000). The Bub3 binding site is also required for kinetochore localisation of BubR1 (Taylor et al., 1998). However, the requirement for Bub3 binding and/or kinetochore localisation in terms of SAC signalling is unclear (Malureanu et al., 2009; Elowe et al., 2010).

The Mad3 and BubR1 proteins also contain two KEN boxes; the first (KEN1) is in the extreme N-terminus, the second (KEN2) lies between the TPR domain and the Bub3 binding site. Because KEN boxes can act as APC/C degrons (Pfleger and Kirschner, 2000), these two sequences have attracted considerable attention. Indeed, these motifs are required for the APC/C-dependent degradation of both Mad3 and BubR1, but because this appears to occur in G1 or after checkpoint satisfaction, respectively, this mechanism does not seem to contribute to APC/C inhibition (King et al., 2007; Choi...
et al., 2009). By contrast, studies of yeast, flies and mammalian cells demonstrate that KEN1 is essential for inhibition of APC/C<sup>Cdc20</sup>, where it promotes SAC function by mediating the binding of BubR1 to the Mad2–Cdc20 subcomplex (Davenport et al., 2006; Burton and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008; Malureanu et al., 2009; Rahmani et al., 2009; Elowe et al., 2010). Several studies suggest that BubR1 has a second Cdc20 binding site, but the significance of this is undefined (Tang et al., 2001; Davenport et al., 2006; Malureanu et al., 2009; Elowe et al., 2010). The role of KEN2 is also not understood; although required for SAC function, its precise function remains unclear (Burton and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008; Elowe et al., 2010).

In checkpoint-arrested cells, BubR1 – as part of the MCC – is bound to the APC/C; however, when the SAC becomes satisfied, BubR1 dissociates (Morrow et al., 2005). Importantly, when engaged by the MCC, the APC/C recruits less substrate (Herzog et al., 2009). Two explanations could account for this: either MCC binding induces a conformational change in the APC/C that prevents substrate docking; or perhaps an MCC component acts as a pseudo-substrate, binding as a substrate would but without itself becoming ubiquitylated. Indeed, it was suggested that Mad3 does act as a pseudo-substrate in budding yeast (Burton and Solomon, 2007). This notion was based on the observation that Mad3 and a substrate, Hsl1, can compete for Cdc20 binding. However, it now appears that binding to Cdc20 alone is not sufficient for efficient ubiquitylation; rather, substrates need to bind to both Cdc20 and the APC/C (Eytan et al., 2006; Davenport et al., 2006; Malureanu et al., 2009). Two explanations could account for this: either MCC binding induces a conformational change in the APC/C that prevents substrate docking; or perhaps an MCC component acts as a pseudo-substrate, binding as a substrate would but without itself becoming ubiquitylated. Indeed, it was suggested that Mad3 does act as a pseudo-substrate in budding yeast (Burton and Solomon, 2007). This notion was based on the observation that Mad3 and a substrate, Hsl1, can compete for Cdc20 binding. However, it now appears that binding to Cdc20 alone is not sufficient for efficient ubiquitylation; rather, substrates need to bind to both Cdc20 and the APC/C (Eytan et al., 2006; Davenport et al., 2006; Malureanu et al., 2009). Two explanations could account for this: either MCC binding induces a conformational change in the APC/C that prevents substrate docking; or perhaps an MCC component acts as a pseudo-substrate, binding as a substrate would but without itself becoming ubiquitylated. Indeed, it was suggested that Mad3 does act as a pseudo-substrate in budding yeast (Burton and Solomon, 2007). This notion was based on the observation that Mad3 and a substrate, Hsl1, can compete for Cdc20 binding. However, it now appears that binding to Cdc20 alone is not sufficient for efficient ubiquitylation; rather, substrates need to bind to both Cdc20 and the APC/C (Eytan et al., 2006; Davenport et al., 2006; Malureanu et al., 2009). Two explanations could account for this: either MCC binding induces a conformational change in the APC/C that prevents substrate docking; or perhaps an MCC component acts as a pseudo-substrate, binding as a substrate would but without itself becoming ubiquitylated.

Based on the observations described above, a prevailing model suggests that the MCC is assembled from two sub-complexes, namely BubR1–Bub3 and Mad2–Cdc20, and that this process is critically dependent on first KEN box of BubR1 (Musacchio and Salmon, 2007). The MCC then binds the APC/C and inhibits substrate recruitment by a yet undefined mechanism. To delineate this mechanism, we established an RNAi-based complementation assay to define the domains of BubR1 required for SAC function. In parallel, we established an in vitro ubiquitylation assay to monitor inhibition APC/C<sup>Cdc20</sup>. Here, using these assays, we first clarify a number of conflicting observations in the literature regarding BubR1 structure and function. More importantly, we then show that the second KEN box promotes SAC function by blocking the recruitment of substrates to APC/C<sup>Cdc20</sup>.

Results
BubR1 binds to Mad2–Cdc20 and the APC/C through its N-terminal region
To assess which domains in BubR1 are important for SAC function, we generated HeLa cell lines stably transfected with different Myc-tagged BubR1 mutants (Fig. 1A). BubR1 ΔNT lacks the entire N-terminal region, including the TPR-like domain and the two KEN boxes; Δ42 contains a deletion in the Bub3 binding domain (Taylor et al., 1998); EK harbours a point mutation in the Bub3 binding domain (Harris et al., 2005; Elowe et al., 2010); and ΔKD lacks the entire kinase domain. Western blot analysis showed that upon tetracycline induction, all these mutants, with the exception of ΔKD, were expressed to similar levels as the endogenous protein (Fig. 1B and see also Fig. 2B). We next analysed their localisation in prometaphase by immunofluorescence. Wild-type Myc–BubR1 localised to kinetochores, as evidenced by co-staining with anti-centromere antibodies (ACAs) (Fig. 1C). Deletion of the N-terminal domain and the kinase domain did not affect this localisation pattern. By contrast, deletion or mutation of the Bub3 binding domain abolished kinetochore localisation (Fig. 1C). Thus, these data confirm that kinetochore localisation of BubR1 is mediated by the Bub3 binding site (Taylor et al., 1998).

Next, we evaluated which domains in BubR1 are important for the interaction with MCC components and the APC/C. Mitotically arrested HeLa cells were harvested, lysed and the BubR1 mutants immunoprecipitated using anti-Myc antibodies. Under these conditions, wild-type BubR1 and the ΔKD mutant were able to immunoprecipitate Bub3, Cdc20, Mad2 and the APC/C (Fig. 1D,E). As expected, the Δ42 and EK mutants were unable to interact with Bub3. These mutants also bound less Cdc20 and Mad2, and the interaction with the APC/C was similarly diminished (Fig. 1D,E), suggesting that Bub3 binding and/or kinetochore localisation is required for efficient MCC assembly. Deletion of the N-terminal domain abolished the interaction of BubR1 with Cdc20, Mad2 and the APC/C, although Bub3 binding was unaffected (Fig. 1D,E). These observations indicate that the N-terminus of BubR1 is essential for MCC assembly and strongly suggests that in the context of a mitotic cell, there might not be a second Cdc20 binding site downstream of the Bub3 binding region (Tang et al., 2001; Davenport et al., 2006; Malureanu et al., 2009). Indeed, when we characterised a BubR1 fragment encompassing only the N-terminal domain and the Bub3 binding site (N484) it bound to Cdc20 as efficiently as the wild-type protein (supplementary material Fig. S1).

BubR1 N-terminus is essential for the SAC
Next, we evaluated the consequences of these mutations on spindle checkpoint function. For this, we co-transfected the stable HeLa cell lines with plasmids encoding a BubR1 shRNA to repress the endogenous protein (Kops et al., 2004) and a GFP-tagged histone H2B to monitor chromosome segregation. Note that the shRNA plasmid typically reduced BubR1 levels by ~90% (Fig. 2A), and that the Myc-tagged BubR1 transgenes were rendered resistant to the siRNA encoded by the shRNA sequence (Fig. 2B). After 48 hours, cells were exposed to nocodazole and analysed by time-lapse microscopy to determine how long they remained arrested in mitosis. Whereas controls arrested for the duration of the experiment (>400 minutes), BubR1 depletion resulted in 50% of the cells exiting mitosis within ~30 minutes (i.e. $T_{50}$ ~30 minutes), indicating penetrant SAC override (Fig. 2C). This phenotype was largely rescued by expression of wild-type BubR1 ($T_{50}$ ~674 minutes). However, cells expressing the ΔNT mutant behaved similarly to BubR1-depleted cells, with a $T_{50}$ of ~32 minutes, confirming that the BubR1 N-terminus is essential for the SAC function (Fig. 2C). Interestingly, the Bub3 binding mutants elicited a partial rescue, restoring the $T_{50}$ to ~60 minutes, again suggesting that Bub3 binding and/or kinetochore localisation is required for efficient BubR1 function. Although the ΔKD mutant also gave a partial rescue, we suspect that this reflects the reduced expression level of this mutant (Fig. 2B).

We then evaluated the ability of these mutants to rescue mitotic timing during an unperturbed mitosis (Fig. 2D). Control cells spent on average ~55 minutes in mitosis, but BubR1-depleted cells exited within ~19 minutes. Expression of wild-type BubR1 and the ΔKD mutant restored mitotic timing to
~40 minutes. By contrast, cells expressing the ΔNT mutant exited mitosis in ~20 minutes, with similar kinetics as BubR1-depleted cells, again confirming the importance of the N-terminus in supporting SAC function. Interestingly, the Δ42 and EK mutants did restore mitotic timing, albeit not as well as the wild-type protein did, confirming that Bub3 binding and/or kinetochore localisation is indeed important in terms of the spindle checkpoint function of BubR1. Note that the N484 mutant could also rescue the SAC, both in the presence of nocodazole and during an unperturbed mitosis (supplementary material Fig. S1C,F,G).

Together, these results show that the N-terminus of BubR1 is essential for SAC function, most likely because it is required for the interaction(s) with Mad2, Cdc20 and the APC/C (Fig. 1D). Although Bub3 might not be required for MCC assembly and inhibition of Cdc20 in the APC/C in vitro (Tang et al., 2001; Fang, 2002; Kulukian et al., 2009), it is required in cells for maximal binding to MCC components and the APC/C (Fig. 1D). Indeed, BubR1 mutants that cannot bind Bub3 do rescue checkpoint function and mitotic timing, but only partially. The simplest explanation for this is that by binding Bub3, BubR1 is recruited to kinetochores, thereby increasing its local concentration at the site where Mad2–Cdc20 complexes are generated, thus facilitating efficient assembly of the MCC.

**BubR1 TPR domain promotes SAC function in a blinkin-independent manner**

To determine how the N-terminus of BubR1 promotes SAC function, we turned to the TPR-like domain, which has previously been implicated in SAC function through its interaction with blinkin/KNL-1 (Kiyomitsu et al., 2007). To test the significance of this domain, we generated four stable lines expressing two types of BubR1 TPR mutants (Fig. 3A,B). The first type, represented by A159W and F175G, are predicted
to disrupt the TPR structure and thus abolish blinkin binding (Kiyomitsu et al., 2007), whereas the second type, L126A and E161A/R165A, are predicted to disrupt blinkin binding without disrupting the TPR structure (D’Arcy et al., 2010). To test blinkin binding, we generated a novel anti-blinkin antibody suitable for immunoblotting (Fig. 3C) and immunofluorescence (supplementary material Fig. S2A). Importantly, all four mutants failed to bind blinkin (Fig. 3E), which is consistent with previous reports (Kiyomitsu et al., 2007; D’Arcy et al., 2010). Note however that all four mutants bound Bub3 as efficiently as the wild-type protein did (Fig. 3E). Moreover, all four TPR mutants localised to kinetochores (Fig. 3D). Interestingly, the A159W and F175G mutations, which disrupt TPR structure, also abrogated binding to Cdc20, Mad2 and the APC/C (Fig. 3E). By contrast, the L126A and E161A/R165A mutants bound Cdc20, Mad2 and the APC/C as efficiently as wild-type BubR1 did (Fig. 3E and supplementary material S2B). Consistent with these binding patterns, the A159W and F175G mutants failed to restore the mitotic timing defect induced by BubR1 RNAi (Fig. 3F,G). By contrast, the L126A and E161A/R165A mutants restored mitotic timing as efficiently as the wild-type protein. Taken together, these observations demonstrate that blinkin binding is not required for kinetochore localisation of BubR1, or for the ability of BubR1 to promote SAC function. Our data do show however that the integrity of the TPR domain is required not only for blinkin binding, as shown previously (Kiyomitsu et al., 2007), but also for the efficient binding of Mad2–Cdc20. As such, the TPR-like domain is essential for BubR1 function, but not for the reasons suggested previously (Kiyomitsu et al., 2007; D’Arcy et al., 2010).

BubR1 KEN boxes are required for the spindle checkpoint

We next turned our attention to the two KEN boxes within the BubR1 N-terminus, which have previously been shown to be important for BubR1 SAC function (Burton and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008; Malureanu et al., 2009; Rahmani et al., 2009; Elowe et al., 2010). Again, we generated HeLa lines stably transfected with Myc-tagged, tetracycline-inducible, RNAi-resistant BubR1 mutants in which either of the KEN boxes was deleted, namely D\textsubscript{K1} and D\textsubscript{K2}. These mutants were expressed at similar levels as the endogenous protein (Fig. 4A) and localised to kinetochores normally (supplementary material Fig. S3A). In immunoprecipitations, wild-type BubR1 and the two KEN mutants bound similar amounts of Bub3 (Fig. 4B and supplementary material Fig. S3B). However, deletion of the first KEN box abrogated the interactions with Cdc20, Mad2 and the APC/C. By contrast, the ΔK2 mutant bound to all these proteins as efficiently as the wild-type BubR1 did (Fig. 4B and supplementary material Fig. S3B), which is
consistent with the observations made with yeast Mad3 (Burton and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008). Using the BubR1 RNAi complementation assay, we next evaluated the functional importance of these motifs. Consistent with the fact that it is unable to interact with Cdc20, Mad2 and the APC/C, the BubR1\(\text{D}^K\) mutant could not rescue the SAC, either in the presence of nocodazole (Fig. 4C) or during an unperturbed mitosis (Fig. 4D). BubR1\(\text{D}^K\) had a weak ability to restore the SAC in the presence of nocodazole, with a \(T_{50}\) of \(~56\) minutes (Fig. 4C). It also restored mitotic timing during an unperturbed mitosis to \(~28\) minutes, which is lower than wild-type BubR1-expressing cells, which spent \(~40\) minutes from NEBD to anaphase onset (Fig. 4D). To probe the partial function of \(\text{D}^K\) in more detail, we analysed the rate of degradation of securin and cyclin B1. HeLa cells were co-transfected with plasmids encoding the BubR1 shRNA and either DsRed–securin (Holland and Taylor, 2006) or Venus–cyclin B1 (Garnett et al., 2009) and then imaged by time-lapse microscopy. As expected
control cells initiated the degradation of securin immediately upon metaphase (Fig. 4E,F). In cells depleted of BubR1, degradation initiated immediately after nuclear envelope break down (NEBD) (Fig. 4E,F). Whereas expression of wild-type BubR1 restored the initiation of securin degradation to metaphase, the BubR1 ΔK1 mutant had no effect, consistent with it being completely deficient in SAC. The ΔK2 mutant did delay the rate of securin degradation somewhat compared with BubR1 ΔK1, but nevertheless, initiation of degradation occurred immediately following NEBD (Fig. 4F).

Similar results were obtained when cyclin B1 degradation was measured (supplementary material Fig. S3C,D). Thus, as has been described previously (Burton and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008; Malureanu et al., 2009; Rahmani et al., 2009; Elowe et al., 2010), both KEN boxes of BubR1 are required for SAC function; however, their contributions are somewhat different. Whereas the first KEN box is essential for MCC assembly and APC/C binding, the BubR1 mutant lacking the second KEN box does assemble into the MCC and can bind the APC/C. However, despite binding the
APC/C as well as the wild-type protein did (Fig. 4B), BubR1 ∆K2 is largely checkpoint deficient, suggesting that at best, it can only weakly inhibit APC/C<sup>C<sub>Cdc20</sub></sup>.

Establishment of an assay to measure APC/C activity in vitro

We reasoned that analysing the ∆K2 mutant in more detail would provide novel insights into how the MCC actually inhibits the APC/C. To more directly probe the role of the second KEN box of BubR1 in APC/C inhibition, we analysed the BubR1 KEN mutants in an in vitro APC/C ubiquitylation assay. In contrast to previously described assays that used APC/C from Xenopus egg extracts and/or interphase cells (Tang and Yu, 2004; Herzog and Peters, 2005; Kraft et al., 2006), we wanted to establish an assay that more closely reflected the cell-based RNAi complementation assay described above; note that the APC/C undergoes extensive phosphorylation in mitosis, which contributes to its regulation (Kraft et al., 2003). Therefore, we aimed to isolate APC/C from mitotically arrested HeLa cells. However, we anticipated that a significant fraction of this APC/C would be bound by the MCC (Morrow et al., 2005). To address this, we reasoned that first immunodepleting BubR1 would allow us to then purify the remaining MCC-free APC/C, i.e. apo-APC/C (Herzog et al., 2009).

To isolate the APC/C, we raised an anti-Cdc27 antibody against a C-terminal peptide (Herzog and Peters, 2005). This antibody detected a single band of approximately 100 kDa (Fig. 5A), which migrated more slowly in mitosis, consistent with hyperphosphorylation (Kraft et al., 2003). Mass spectrometry revealed that this antibody could efficiently immunopurify the entire APC/C (Fig. 5B), and as anticipated, when we immunopurified APC/C from mitotically arrested HeLa cells, MCC proteins were bound (Fig. 5C). However, by first immunodepleting BubR1, we could obtain mitotic APC/C with very little MCC bound (Fig. 5C).

To activate the apo-APC/C in vitro, recombinant Cdc20 was generated (Fig. 5D) and assayed using the N-terminal 90 amino acids of cyclin B1 (CycB N90) as a substrate (Fig. 5E). Interestingly, APC/C that had not been subjected to BubR1 depletion had high basal activity and recombinant Cdc20 had only a minor stimulatory effect (Fig. 5F). We suspect that this reflects dissociation of BubR1 and Mad2 from purified MCC–APC/C complexes, leaving behind active APC/C<sup>Cdc20</sup>. Consistent with this idea, if BubR1 was first depleted, the resulting apo-APC/C had lower basal activity and addition of recombinant Cdc20 stimulated it markedly (Fig. 5F). Thus, prior removal of MCC-bound APC/C clearly provides a good source of mitotically phosphorylated apo-APC/C for in vitro assays. To reconstitute MCC-mediated inhibition, we then purified recombinant Mad2 and BubR1 (Fig. 5D). Once again, apo-APC/C had very low activity that was significantly stimulated by addition of Cdc20 (Fig. 5G). Moreover, although addition of either Mad2 or BubR1 in stoichiometric amounts with Cdc20 had little effect, in combination, they almost completely reverted the ability of Cdc20 to activate the APC/C, consistent with previous observations (Fang, 2002; Kulukian et al., 2009).

BubR1 KEN boxes are important for direct inhibition of APC/C<sup>Cdc20</sup> in vitro

Having established the in vitro ubiquitylation assay, we sought to determine the importance of the BubR1 KEN boxes in direct APC/C<sup>Cdc20</sup> inhibition. Although wild-type BubR1 was a potent APC/C<sup>Cdc20</sup> inhibitor when used in combination with Mad2, the ∆K1 mutant was largely ineffective (Fig. 6A,F). By contrast, BubR1 ∆K2 could partially inhibit APC/C<sup>Cdc20</sup> in the presence of Mad2 (Fig. 6A,F). Importantly, this is entirely consistent with the cell-based observations described above; whereas BubR1 ∆K1 is entirely checkpoint deficient, the ∆K2 mutant can restore mitotic timing to some extent (Fig. 4D).

It was previously suggested that the BubR1 N-terminal domain is sufficient to mediate inhibition of APC/C<sup>Cdc20</sup> (Malureanu et al., 2009). To test this, we generated a fragment of BubR1 containing the N-terminal 370 amino acids (BubR1 N370; Fig. 6B), and assayed its ability to inhibit the APC/C. In combination with Mad2, both full-length BubR1 and N370 were equally potent APC/C<sup>Cdc20</sup> inhibitors (Fig. 6C,F), confirming that this domain is indeed sufficient for APC/C<sup>Cdc20</sup> inhibition. Note that this domain lacks the Bub3 binding site, consistent with observations demonstrating that Bub3 is not essential for APC/C<sup>Cdc20</sup> inhibition in vitro (Tang et al., 2001; Fang, 2002; Kulukian et al., 2009). When we deleted the KEN boxes within BubR1 N370, we observed that ∆K1 totally failed to inhibit the APC/C<sup>Cdc20</sup>, whereas the ∆K2 mutant retained weak inhibitory activity (Fig. 6D,F). Similar results were obtained when securin was used as an APC/C substrate (Fig. 6E,F). Thus, these results show that in the context of either full-length BubR1 or in N370, APC/C<sup>Cdc20</sup> inhibition in vitro is completely dependent on the first KEN box and partially on KEN2.

BubR1 KEN boxes are required for blocking APC/C<sup>Cdc20</sup> substrate recruitment

Taken together, the cell-based data and the in vitro observations presented so far suggest the following: the BubR1 first KEN box is essential for MCC assembly and APC/C<sup>Cdc20</sup> binding, explaining why the ∆K1 mutant is unable to restore the SAC in BubR1-deficient cells, and why it cannot inhibit APC/C<sup>Cdc20</sup> in vitro. By contrast, BubR1 ∆K2 can assemble into the MCC and bind the APC/C, and consistent with its ability to partially inhibit APC/C<sup>Cdc20</sup> in vitro, it can partially restore mitotic timing in cells. To confirm that the cell-based binding data was indeed being recapitulated in the in vitro assay, we incubated MCC components with the APC/C for 45 minutes and then asked whether they were bound to the APC/C (Fig. 7A). Consistent with previous observations (Kulukian et al., 2009), Mad2 stimulated binding of BubR1 N370 to APC/C<sup>Cdc20</sup> (Fig. 7B, compare lanes 4 and 7). More significantly however, whereas BubR1 N370 ∆K1 did not bind APC/C<sup>Cdc20</sup>, the ∆K2 mutant bound as well as the wild-type protein (Fig. 7B, lanes 8 and 9; and supplementary material Fig. S4A), confirming that the in vitro assay does indeed reflect the cell-based observations. Note that even though we did not immunodeplete BubR1 for these assays before APC/C immunopurification, binding of BubR1 N370 and Mad2 to the APC/C was still dependent on the addition of recombinant Cdc20 (supplementary material Fig. S4B,C).

Finally, to understand why the ∆K2 mutant could not efficiently inhibit APC/C<sup>Cdc20</sup> despite the fact that it binds as well as the wild type, we analysed substrate binding. The APC/C was first incubated with MCC proteins, followed by addition of the CycB N90 substrate. The APC/C was then re-isolated and bound proteins analysed by western blot (Fig. 7A). Importantly, CycB N90 only bound the APC/C pre-incubated with Cdc20 (Fig. 7C,D; compare lanes 1 and 2). Note that this binding was dependent on the presence of the D-box in CycB N90.
Addition of BubR1 or Mad2 alone did not affect substrate binding, but in combination they prevented CycB N90 binding to APC/C Cdc20 (Fig. 7C,D; lane 6). Crucially, however, in the presence of Mad2, the BubR1 N370 ΔK2 mutant did not block substrate recruitment (Fig. 7C,D; lane 7). Similar results were obtained when we used securin as a substrate (Fig. 7E,F). Thus, it appears that although the second KEN box of BubR1 is not required for MCC assembly and APC/C binding, it is essential for blocking substrate recruitment to APC/C Cdc20.

**Discussion**

BubR1 is an essential component of the spindle checkpoint; by virtue of its Bub3 binding site, it is recruited to unattached kinetochores early during mitosis and, upon binding Cdc20–Mad2, forms the MCC, which is a potent inhibitor of the APC/C.

---

**Fig. 5. Measuring APC/C ubiquitylation activity in vitro.** (A) Immunoblot of HeLa cell lysates probed with the anti-Cdc27 antibody (RC27.3). (B) Colloidal Coomassie Blue gel of anti-Cdc27 immune complexes isolated from asynchronous HeLa cells indicating the APC/C subunits identified by mass spectrometry. The asterisk denotes a non-specific band. (C) Immunoblots of Cdc27 immune complexes isolated from mitotic extracts, with or without prior immunodepletion of BubR1. Membranes were blotted with antibodies against BubR1, Bub3, Mad2, Cdc20 and Cdc27. Dilutions of the control-depleted extract were included to estimate the immunodepletion efficiency. (D) Coomassie Blue gel of purified Cdc20, Mad2 and BubR1 proteins. The asterisk denotes a contaminant band. (E) Schematic showing the in vitro ubiquitylation assays. (F) Immunoblot of an APC/C ubiquitylation assay probed with anti-Myc antibodies to detect cyclin B1 N90 conjugates. Note that prior depletion of BubR1 reduces the basal activity of the APC/C. (G) Immunoblot of a ubiquitylation assay showing that only the combination of Mad2 and BubR1 can inhibit Cdc20-mediated activation of APC/C.
Exactly how the MCC inhibits APC/C activity is unknown. Here, using a cell-based RNAi complementation assay and an in vitro APC/C ubiquitylation assay, we report a structure–function analysis of BubR1, with particular emphasis on its two KEN boxes. Although BubR1 has already been subjected to several structure–function studies, there are a number of ambiguities in the literature and the mechanism by which BubR1 contributes to APC/C inhibition is still unclear. Based on our new findings, below we address these ambiguities and present a novel model explaining how BubR1 inhibits APC/C.

**How is the MCC assembled?**

The MCC, which is by definition composed of BubR1, Bub3, Cdc20 and Mad2, is a potent inhibitor of the APC/C (Sudakin et al., 2001). Although Mad2 might subsequently dissociate from the MCC (Nilsson et al., 2008; Westhorpe et al., 2011), in terms

---

**Fig. 6. BubR1 KEN boxes are important for APC/C inhibition.** (A) Ubiquitylation assay showing that, in the presence of Mad2, the BubR1 ΔK1 mutant cannot inhibit APC/C\(^{\text{Cdc20}}\). (B) Schematic showing the N370 fragment which encompasses the TPR-like domain and both KEN boxes; plus a Coomassie Blue gel showing purified BubR1 N370 proteins (arrow). The asterisk denotes a contaminant band. (C) Ubiquitylation assay showing inhibition of APC/C\(^{\text{Cdc20}}\) with either BubR1 FL or N370, in the presence of Mad2. (D,E) Ubiquitylation assays showing that in the presence of Mad2, BubR1 N370 ΔK1 does not inhibit APC/C\(^{\text{Cdc20}}\) whereas the ΔK2 mutant only elicits a partial inhibition. The assays were performed using CycB N90 (D) or securin (E) as APC/C substrates. (F) Bar graph quantifying the experiments in A, D and E. In each case, values were normalised to the activity of APC/C in the presence of only Cdc20 (100%) and represent the mean ± s.e.m. of at least two independent experiments each.
of assembly, the MCC is probably formed by the association of two subcomplexes, namely BubR1–Bub3 and Mad2–Cdc20. Whereas BubR1 and Bub3 appear to bind constitutively throughout the cell cycle (Taylor et al., 1998; Chen, 2002), the binding of Mad2 to Cdc20 is catalysed by unattached kinetochores during mitosis (De Antoni et al., 2005; Kulukian et al., 2009). Presumably therefore, upon generation of the Mad2–Cdc20 subcomplex, the BubR1–Bub3 subcomplex binds to form the MCC (Musacchio and Salmon, 2007). Data from both budding and fission yeast show that the N-terminal KEN box in Mad3, the fungi equivalent of BubR1, is essential for the interaction between Mad3 and Mad2–Cdc20 (Burton and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008). This simple picture is complicated by data from mammalian systems, which appear to suggest that BubR1 contains a second Cdc20 binding site in its C-terminal domain (Davenport et al., 2006; Malureanu et al., 2009; Elowe et al., 2010). However, our data are entirely consistent with observations from the yeast systems: when we deleted KEN1 from full-length BubR1, its binding to Mad2, Cdc20 and the APC/C was completely abolished (Fig. 4B). Note that at least some of the data supporting a second Cdc20 binding site come from experiments...
where the rescue constructs were grossly overexpressed with respect to the endogenous BubR1 (Malureanu et al., 2009). By contrast, in the system we describe here, our mutants were expressed at a level comparable with the endogenous protein (Fig. 4A). Whether overexpression accounts for other studies where Cdc20 binding is observed independently of KEN1 remains to be seen (Elowe et al., 2010). However, because Mad3 lacks the entire C-terminal region, and because the BubR1 N-terminus is sufficient to inhibit APC/C<sup>C<sub>Cdc20</sub></sup> in vitro (Fig. 6C), the relevance of a potential second Cdc20 binding site – if it exists – is questionable. Indeed, the study that originally identified this second site concluded that the binding of Cdc20 to this domain was Mad2 independent and did not play a role in the spindle checkpoint (Davenport et al., 2006). The emerging view is that the BubR1 C-terminal kinase domain is involved in regulating kinetochore–microtubule interactions independently of its role in the checkpoint (Harris et al., 2005; Rahmani et al., 2009; Elowe et al., 2010). Consistent with this notion, in our hands, the BubR1 AKD and N484 mutants appreciably rescued the SAC defect induced by BubR1 RNAi (Fig. 2C,D and supplementary material Fig. S1F,G), despite being expressed at low levels. In summary, synthesising all the available data from yeast, flies and mammalian cells, the most likely scenario is that the KEN box closest to the N-terminus of Mad3 or BubR1 is the key determinant in terms of Mad2–Cdc20 binding and as such plays an essential role in MCC assembly.

Another key feature of BubR1 is the Bub3 binding site. However, a region containing the TPR-like domain and the two KEN boxes, but lacking the Bub3 binding site, is sufficient to inhibit APC/C<sup>C<sub>Cdc20</sub></sup> in vitro (Fig. 5C). Consistently, full-length BubR1 can efficiently inhibit the APC/C<sup>C<sub>Cdc20</sub></sup> in vitro in the absence of Bub3 (Fig. 5G) (Tang et al., 2001; Fang, 2002; Kulukian et al., 2009). Because the Bub3 binding site is essential for kinetochore localisation of BubR1 (Taylor et al., 1998), these data suggest that kinetochore localisation of BubR1 might not be required for its checkpoint function, and indeed this argument was recently put forward (Malureanu et al., 2009). However, we show here that, in a cellular context, when BubR1 cannot bind Bub3, binding to Mad2–Cdc20 and the APC/C is compromised (Fig. 1D). Furthermore, the Bub3 binding mutants only very poorly restore checkpoint function (Fig. 2C). So, although Bub3 binding might not be essential for the physical interactions that result in MCC assembly and APC/C inhibition, in the cellular context, Bub3 binding is very important for maximal BubR1 function. Importantly, the Bub3 binding site and BubR1 kinetochore localisation domain are synonymous. Thus, it seems to us that the most plausible explanation to account for the existing data is that, through its ability to bind Bub3, BubR1 is recruited to kinetochores, thereby increasing its local concentration at the site where Mad2–Cdc20 complexes are being generated, thus promoting efficient assembly of the MCC.

Note, because the Bub3 binding site is important for kinetochore-based signalling, we suggest that it is not called the GLEBS motif, as this term implies Gle2/Rae1 binding (Wang et al., 2001), and although Bub3 might share extensive homology with Gle2/Rae1 (Taylor et al., 1998), there is no compelling evidence to suggest a functionally significant connection between BubR1 and Gle2/Rae1.

**How does the MCC inhibit the APC/C?**

Once assembled, the MCC somehow binds and inhibits the APC/C. As discussed above, Bub3 is not essential for APC/C<sup>C<sub>Cdc20</sub></sup> inhibition per se. Furthermore, recent observations suggest that following MCC assembly, Mad2 can be removed (Nilsson et al., 2008; Kulukian et al., 2009; Westhorpe et al., 2011), suggesting that once Cdc20 has been handed over to BubR1, Mad2 is also not essential for APC/C inhibition. Because Cdc20 is the APC/C activator being inhibited by the checkpoint, the key question in terms of understanding how the SAC works thus boils down to defining how BubR1 inhibits APC/C<sup>C<sub>Cdc20</sub></sup>. A key observation is that when the MCC is bound to the APC/C, less substrate is bound (Herzog et al., 2009), suggesting that the MCC, and by extension BubR1, somehow interferes with substrate recruitment. It was suggested that Mad3 acts a pseudosubstrate inhibitor for the APC/C (Burton and Solomon, 2007). This notion was based on the observation that budding yeast Mad3 could compete for binding to Cdc20 with the APC/C substrate Hsl1 in a KEN1-dependent manner. However, whether this reflects a bona fide pseudosubstrate mechanism is questionable because these assays were performed in the absence of the APC/C, and it has been shown that efficient substrate binding requires both the co-activator and the APC/C (Eytan et al., 2006; Matyskiela and Morgan, 2009). In other words, whether Mad3 can compete Hsl1 from the substrate-binding site of APC/C<sup>C<sub>Cdc20</sub></sup> was not demonstrated.

Significantly, several reports demonstrate that KEN2 is important for SAC function, but the mechanism underlying this was unclear (Burton and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008; Malureanu et al., 2009; Elowe et al., 2010). We confirm the importance of KEN2: in cells reconstituted with BubR1ΔK2, the SAC is effectively dead (Fig. 4E and supplementary material Fig. S3C). Strikingly however, this mutant assembles into the MCC and binds the APC/C as well as wild-type BubR1 does (Fig. 4B). Here, we define how the second KEN box mediates APC/C<sup>C<sub>Cdc20</sub></sup> inhibition: we show that BubR1 blocks substrate binding to the APC/C<sup>C<sub>Cdc20</sub></sup> and, moreover this depends on the second KEN box (Fig. 7C,E). We therefore propose a two-step mechanism to account for how BubR1 KEN boxes contribute to inhibition of APC/C<sup>C<sub>Cdc20</sub></sup> (Fig. 8). First, in a TPR-domain- and KEN1-dependent manner, BubR1 binds Cdc20–Mad2 subcomplexes as they are generated at the kinetochores to form the MCC. Second, this complex binds the APC/C and allows the second KEN box of BubR1 to engage with the APC/C to block substrate recruitment. In agreement with this, we observed that if a substrate is pre-bound to the APC/C, subsequent binding of BubR1 cannot displace it (supplementary material Fig. S4H), suggesting that binding and inhibition of the APC/C are two separate functions of BubR1.

Interestingly, recent structural studies have shown that substrates bind the APC/C at the interface between the co-activator and the APC/C subunit Apc10 (Buschhorn et al., 2010; da Fonseca et al., 2010). Therefore, it is tempting to speculate that KEN2 is positioned so that it engages with this same interface, thereby preventing substrate binding. This could explain why the MCC does not inhibit the degradation of prometaphase substrates, such as cyclin A and Nek2A, because Apc10 is not required for the recognition of these proteins as substrates (Izawa and Pines, 2011). Although this KEN2-mediated inhibition could reflect a bona fide pseudosubstrate mechanism, such a conclusion might be premature. Indeed, we cannot rule out the possibility that other mechanisms are at play. For example, cryo-electron microscopy analysis of the APC/C with and without the MCC bound show that the MCC proteins...
induce a relocation of Cdc20 within the APC/C (Herzog et al., 2009). Second, binding of Cdc20 to the APC/C is dependent on Cdc27 in metaphase, whereas in prometaphase it is independent of this subunit, presumably because of the presence of the MCC proteins (Izawa and Pines, 2011). Finally, despite the fact that deletion of KEN2 does not affect substrate recognition, the ΔK2 mutant can still partially inhibit APC/C_{Cdc20} in vitro (Fig. 6) and in cells (Fig. 4E,F). Thus, it is possible that binding of BubR1 to Cdc20 through KEN1 is responsible for some inhibition by changing the conformation of Cdc20, whereas KEN2 acts by inhibiting substrate binding. Nevertheless, further testing the role of the second KEN box of BubR1 might provide further insights into how the checkpoint prevents APC/C_{Cdc20} from ubiquitylating securin and cyclin B1, while targeting prometaphase substrates for proteolysis.

Closing remarks

The importance of BubR1 in terms of maintaining genome stability is highlighted by the observation that it is mutated in the human cancer predisposition syndrome mosaic variegated aneuploidy (Hanks et al., 2004). The primary role of BubR1 in maintaining the fidelity of chromosome segregation is to inhibit the APC/C_{Cdc20}. Although interfering with protein–protein interactions using small molecules has traditionally been difficult, modulating the BubR1-APC/C_{Cdc20} interaction might open up new opportunities for drug discovery, with antagonists driving cells out of an aberrant mitosis and agonists inducing a prolonged mitotic arrest, both of which can lead to cell death (Huang et al., 2009; Janssen et al., 2009).

Materials and Methods

Cell lines and RNAi

Stable HeLa cell lines were generated as described (Tighe et al., 2008) using a full-length BubR1 cDNA (Taylor et al., 1998) cloned into a pcDNA5/FRT/TO-Myc-based vector (Invitrogen) and mutated to render it RNAi resistant. The various BubR1 mutants (supplementary material Table S1) were generated by site-directed mutagenesis (Quikchange, Stratagene) or PCR amplification. To repress endogenous BubR1, HeLa cells were co-transfected in 12-well plates with GFP-histone H2B (Morrow et al., 2005) and pSUPER/BubR1 (Kops et al., 2004) plasmids using Lipofectamine 2000 (Invitrogen). Transgenes were induced with 1 μg/ml tetracycline then imaged by time-lapse microscopy 48 hours later (Morrow et al., 2005). To monitor degradation kinetics, cells co-transfected with securin–DsRed (Holland and Taylor, 2006) or cyclin-B1–Venus (Garnett et al., 2009) were imaged as described (Gurten et al., 2010). To repress Blinkin, cells were transfected with siRNAs (Dharmacon) (supplementary material Table S2) using Interferin (PolyPlus) and analysed 24 hours later.

Antibody techniques

The sheep anti-blinkin antibody was generated as described (Taylor et al., 2001) using amino acids 1319–1386 fused to GST. The anti-Cdc27 antibody was raised in a rabbit (Eurogentec) against a C-terminal peptide (Herzog and Peters, 2005) and affinity purified using Sulfolink columns (Pierce Biotechnology). All other antibodies are described in supplementary material Table S3. Immunoblotss were done as described (Tighe et al., 2004) and visualised using either film (Biomax MR; Kodak) or a Biospecturm 500 imaging system (UVP). Western blots were quantified using a VisionWorks LS software (UVP). Immunofluorescence was done as described (Tighe et al., 2004) and visualised using either film (Biomax MR; Kodak) or a Biospecturm 500 imaging system (UVP). Western blots were quantified using a VisionWorks LS software (UVP). Immunoprecipitations were performed as described (Holland et al., 2007).

Protein expression

BL21(DE3)pLysS E. coli cells were transformed with pET28a plasmids (Merck) encoding N- or C-terminal His-tag fusions, induced with 0.4 mM IPTG for 16 hours at 20°C, harvested then sonicated in lysis buffer [0.1% Triton X-100, 50 mM phosphate, pH 7.4, 300 mM NaCl, 10 mM imidazole, 0.4 mM PMSF and protease inhibitor cocktail (Roche)]. Soluble proteins were incubated with TALON beads (Clontech) for 1 hour at 4°C then after washing, proteins were eluted using 300 mM imidazole. For baculovirus expression, Cdc20 and full-length BUBR1 cDNAs were cloned into pBAC-2cp (Merck) and co-transfected into Sf9 cells with flashBAC Gold (GenWay Biotech) using Insect GenJuice (Merck). Following two rounds of amplification, 4 ml of baculovirus was used to infect 400 ml of Sf9 cells (~2x10^6 cells/ml) for 72 hours at 28°C. Cells were then collected and lysed [1% Triton X-100, 50 mM phosphate, pH 7.4, 150 mM NaCl, 10 mM imidazole, 0.4 mM PMSF and protease inhibitor cocktail (Roche)] and the His-tagged proteins purified as described above.

In vitro assays

HeLa cells (Taylor and McKeon, 1997) were treated with 0.2 μg/ml nocodazole for 16 hours and mitotic cells lysed (0.1% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA). To analyse degradation kinetics, cells were treated with 3.3 μM nocodazole (Sigma) and 20 μM MG132 (Sigma) for 2 hours. Immunoprecipitations were performed as described (Holland et al., 2007).

Fig. 8. Schematic explaining how the two KEN boxes in BubR1 promote MCC assembly and APC/C inhibition. The Cdc20–Mad2 subcomplex is generated at unattached kinetochores, and then binds the BubR1–Bub3 subcomplex in a KEN1-dependent manner to form the MCC. In turn, the MCC then binds the APC/C and inhibits substrate recruitment in a KEN2-dependent manner, possibly by occupying the substrate-binding pocket between Cdc20 and Apc10.
BubR1 blocks substrate binding to APC/Cdc20


