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A new view of the spindle checkpoint

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Previous studies of the spindle checkpoint suggested that its ability to prevent entry into anaphase was mediated by the inhibition of the anaphase-promoting complex (APC) ubiquitin ligase by Mad2. Two new studies challenge that view by demonstrating that another checkpoint protein, BubR1, is a far more potent inhibitor of APC function.

The spindle assembly checkpoint is a quality control mechanism that prevents chromosome segregation errors. It acts to restrain cells from entering anaphase, the chromosome segregation step in mitosis and meiosis, until all replicated chromatids have formed proper attachments to a functional bipolar spindle. The checkpoint pathway transduces a signal generated by improperly attached kinetochores. This signal is presumably amplified and ultimately acts to halt the action of an enzyme required to drive cells into anaphase, the anaphase-promoting complex (APC*; otherwise known as cyclosome). It seemed as if the output of the spindle checkpoint was the best understood aspect of this pathway. Numerous studies suggested that an activated form of the Mad2 checkpoint protein generated specifically at unattached kinetochores binds and inhibits the actions of the APC (for review see Shah and Cleveland, 2000). However, two recent studies (Sudakin et al., 2001; Tang et al., 2001) indicate that Mad2 is not the whole story. Although these studies disagree on some conclusions, they strikingly agree on the most important issues. Another checkpoint protein, the BubR1 kinase, was found to bind and inhibit the APC and at least in vitro was a remarkably more potent inhibitor than Mad2 alone.

Studies of budding yeast revealed that the functions of six checkpoint proteins, Mad1–3, Bub1, Bub3, and Mps1, are required to prevent anaphase entry when spindle function is compromised (for review see Amon, 1999). These proteins appear conserved in all eukaryotic genomes sequenced to date with one key difference. In fungal species, Bub1 and Mad3 are sequence-related proteins. Both have similar NH_2 termini with defined Cdc20-binding (an APC activator) and Bub3-binding domains (Hardwick et al., 2000; Murray and Marks, 2001). Bub1 uniquely possesses a COOH-terminal protein kinase domain. All multicellular animal genomes examined also encode two members of the Bub1/Mad3 family; however, both possess COOH-terminal kinase domains. The kinase with the Mad3-like NH_2-terminus has been named BubR1. It has yet to be demonstrated that BubR1 is the functional ortholog of yeast Mad3, but this is the most reasonable presumption. Strikingly, localization studies have placed all six of the animal checkpoint proteins at kinetochores, and all but Mps1 are found with marked preference at those kinetochores that are improperly attached to spindle microtubules (Shah and Cleveland, 2000; Abrieu et al., 2001; Fisk and Winey, 2001).

Entry into anaphase requires the proteolytic destruction of certain cell cycle regulatory proteins. Most notable is securin (Pds1 in *Saccharomyces cerevisiae*), an anaphase inhibitor that acts by sequestering separin (Esp1) required for sister chromatid separation (Zachariae and Nasmyth, 1999). Securin and other proteins are targeted for destruction by the covalent addition of ubiquitin, catalyzed by the E3-type ligase activity of the APC. Attention was first focused upon Mad2 by the finding that it bound Cdc20, an activator of the APC that targets it to anaphase-specific substrates (Hwang et al., 1998; Kim et al., 1998). Subsequent studies showed that the interaction of Mad2 with Cdc20-bound APC inhibits its ubiquitin ligase activity (Li et al., 1997; Fang et al., 1998). The simple hypothesis that emerged from these studies is that Mad2 is converted to a diffusible inhibitory form during a transient association with unattached kinetochores. Support for this hypothesis came from the finding that Mad2 does in fact interact with unattached kinetochores in a dynamic fashion (Howell et al., 2000). Fluorescently labeled Mad2 repopulated photobleached kinetochores with a rapid recovery half time of ~24 s. Therefore, the role of other checkpoint components in this simple formulation would be upstream of Mad2, acting at the unattached kinetochore to convert Mad2 to its APC inhibitory form.

Well...not so fast, say these new studies. An association of BubR1 with Cdc20 and the APC had been observed previously (Chan et al., 1999; Wu et al., 2000), a finding inconsistent with the Mad2 lone inhibitor hypothesis. Both new studies report the purification of a complex from HeLa cells containing BubR1 and other checkpoint proteins. The complex characterized by Sudakin et al. (2001) and named the mitotic checkpoint complex (MCC) contains BubR1, Bub3, Cdc20, and Mad2 in near equal stoichiometry. Normalized to the amount of Mad2, the MCC was found to inhibit APC

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*Abbreviations used in this paper: APC, anaphase-promoting complex; MCC, mitogen checkpoint complex.

ubiquitination activity in vitro ~3,000-fold more effectively than purified recombinant Mad2 alone. Tang et al. (2001) examined the effects of the addition of individual recombinant protein preparations to an in vitro APC assay system. BubR1 added by itself was found to inhibit APC activity much more potently than Mad2 (K_I = 40 nM versus 2 μM for BubR1 and Mad2, respectively). Mutant forms of BubR1 defective for kinase domain function were found to inhibit APC as effectively as the intact form, a finding that calls attention to the absence of a kinase domain in the yeast homologue Mad3. Both studies indicate that the BubR1 complex inhibits APC stoichiometrically (as opposed to catalytically) by binding to APC and either holding Cdc20 in an inactive form or preventing the binding of free activating Cdc20.

Both groups concluded that the intracellular concentration of Mad2 is too low for it to act effectively as an APC inhibitor alone. Therefore, Mad2 must act in cooperation with other checkpoint proteins to exert its effects. Whether Mad2 acts in a complex with other checkpoint proteins, such as the MCC, or alone in a parallel and perhaps additive pathway of APC inhibition is not clear. A major disagreement between these two studies concerns the composition of the BubR1-containing complex. Sudakin et al. (2001) report that BubR1, Bub3, Cdc20, and Mad2 are present and equally represented in the inhibitory MCC. Associations between Mad3 and Bub3, Cdc20, and Mad2 have also been observed in *S. cerevisiae* (Hardwick et al., 2000). In contrast, the complex purified by Tang et al. (2001) contains equal amounts of only BubR1 and Bub3; Cdc20 is present at substoichiometric levels, and Mad2 does not appear to be present at all. Sudakin et al. (2001) show that the MCC contains only a small fraction of the total cellular Mad2 (<5%), and it is possible this did not remain associated through the Tang et al. (2001) purification protocol. On the other hand, the requirement or the role for Mad2 in MCC function has not been demonstrated yet (as has the requirement for BubR1). This difference between the two studies is certainly the result of the in vitro protocols applied to analyze this complex system and can be expected to be resolved with further study. The reader is reminded here that yeast genetic studies demonstrated that all six checkpoint proteins (Mad1–3, Bub1, Bub3, and Mps1) are required for the proper response in vivo and that double mutants do not seem to show additive effects (Alexandru et al., 1999). Although in vitro checkpoint reconstitution experiments have revealed important activities for individual proteins such as Mad2 and BubR1, we ultimately must provide an explanation for the combined actions of the entire set of checkpoint proteins in vivo.

Regulation of the inhibitory action of the BubR1 complex

A key question concerning an activity that inhibits APC function is how it is regulated in the cell cycle and under conditions of spindle damage. The most straightforward hypothesis is that improperly attached kinetochores will catalyze the formation of a diffusible APC inhibitory form. Sudakin et al. (2001) report provocative findings that suggest this may not be the case for the BubR1-containing MCC complex. They first characterized the activity of "mitotic" MCC, isolated from cells treated with a microtubule-depolymerizing drug and therefore expected to be activated for checkpoint function. Surprisingly, they subsequently found MCC with equivalent inhibitory activity and subunit composition in interphase cells, a stage in which mature kinetochores are not present and the spindle checkpoint should not be activated. Their further experimentation suggests that the ability of the MCC to inhibit the APC is not specified by the state of the MCC but is in fact a regulated property of the APC. APC isolated from mitotic cells was sensitive to MCC inhibition, but interphase APC was not. Although there are some differences, Tang et al. (2001) also report that the APC exhibits cell cycle stage-specific changes in its susceptibility to inhibition by pure BubR1. Sudakin et al. (2001) also show that the addition of chromosomes purified from cells arrested in mitosis could enhance inhibition of APC in vitro. This effect appeared to be mediated by an action of the mitotic chromosomes upon the APC as opposed to its activator Cdc20 or its inhibitor MCC. The chromosome effect may be a result of the reported ability of kinetochores to biochemically "remember" that they were not properly attached to the spindle despite cell-free purification (Campbell et al., 2000).

How might the APC's sensitivity to BubR1/MCC inhibition be regulated? The most plausible mechanism involves phosphorylation. Mitosis-specific phosphorylation of APC subunits is well documented, and preferential association of BubR1, Cdc20, and Mad2 to phosphorylated APC has been reported (Sudakin et al., 2001). Perhaps phosphorylation events required for inhibition are mediated by one of the spindle checkpoint kinases, Mps1, Bub1, or even BubR1. Another possibility is that the 95% of cellular Mad2 not associated with MCC participates in APC sensitization. Future studies must determine the mechanism of APC sensitization to inhibition in order to solidify this new view of checkpoint action.

The kinetochore motor connection

This new focus on BubR1 is of great interest for another reason. By virtue of its association with the kinetochore-based motor protein CENP-E, BubR1 has also been implicated in an early checkpoint step, leading to the generation of the prevent-anaphase signal. Proper bipolar spindle attachment in metaphase causes sister kinetochores to be placed under tension, since they are pulled towards opposite poles while still held together by cohesive forces. An undetermined aspect of either the failure of kinetochores to attach or their failure to be placed under bipolar tension (or both; Skoufias et al., 2001) is sensed by the checkpoint mechanism. Kinesin-related CENP-E is a kinetochore-based microtubule motor protein that has been implicated in kinetochore attachment to microtubules and/or tension generation. Interference with its function prevents proper metaphase chromosome alignment (Yao et al., 2000). CENP-E also participates in spindle checkpoint function. Depending on the experimental system, CENP-E function was found to either be required for the checkpoint response or required to prevent a BubR1-dependent checkpoint arrest (Chan et al., 1999; Abrieu et al., 2000; Yao et al., 2000). Localization of CENP-E to kinetochores requires the actions of two check-

point proteins, Mps1 and Bub1 (Abrieu et al., 2001; Sharp-Baker and Chen, 2001). Finally, CENP-E associates with BubR1 during M phase of the cell cycle (Chan et al., 1998; Yao et al., 2000). Thus, in addition to its newly demonstrated role in the output of the spindle checkpoint pathway, BubR1 may act at an early step as well. Although the functional nature of the BubR1–CENP-E interaction is not known, it is tempting to speculate that together they monitor an aspect of kinetochore attachment or tension. Besides missing the Bub1R-like kinase domain, yeast cells also do not posses a close homologue of CENP-E. Perhaps the BubR1 kinase domain, apparently not required for APC inhibition, functions with CENP-E in kinetochore-based checkpoint signal generation.

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Note added in proof. Studies by Guowei Fang (personal communication) similarly demonstrate that BubR1 is a more potent APC inhibitor than Mad2 and that Mad2 alone cannot inhibit at its physiological concentration. However, synergism between BubR1 and Mad2 with respect to APC inhibition was detected.

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