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MAD3 Encodes a Novel Component of the Spindle Checkpoint which Interacts with Bub3p, Cdc20p, and Mad2p

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Abstract. We show that MAD3 encodes a novel 58-kD nuclear protein which is not essential for viability, but is an integral component of the spindle checkpoint in budding yeast. Sequence analysis reveals two regions of Mad3p that are 46 and 47% identical to sequences in the NH₂-terminal region of the budding yeast Bub1 protein kinase. Bub1p is known to bind Bub3p (Roberts et al., 1994) and we use two-hybrid assays and coimmunoprecipitation experiments to show that Mad3p can also bind to Bub3p. In addition, we find that Mad3p interacts with Mad2p and the cell cycle regulator Cdc20p. We show that the two regions of homology between Mad3p and Bub1p are crucial for these interactions and identify loss of function mutations within each domain of Mad3p. We discuss roles for Mad3p and its interactions with other spindle checkpoint proteins and with Cdc20p, the target of the checkpoint.

Key words: MAD3 • checkpoint • BUB3 • CDC20 • MAD2

Introduction

The spindle checkpoint delays the metaphase to anaphase transition in cells with defects in the interaction between kinetochores and microtubules of the mitotic spindle (Rieder et al., 1995; for review see Rudner and Murray, 1996; Wells, 1996). This delay allows misaligned or unattached sister chromatid pairs to form a bi-polar attachment to the spindle, thereby ensuring their accurate segregation during anaphase and cytokinesis (Nicklas, 1997).

Genetic screens in budding yeast, for mad (mitotic arrest defective)¹ and bub (budding uninhibited by benzimidazole) mutants, originally identified six components of the spindle checkpoint (Hoyt et al., 1991; Li and Murray, 1991). Sequence analysis and preliminary characterization has been reported for MAD1 (Hardwick and Murray, 1995), MAD2 (Chen et al., 1999), BUB1 (Roberts et al., 1994), BUB2, and BUB3 (Hoyt et al., 1991). Frog and human homologues of MAD2 and MAD1 have conserved their checkpoint functions and localize to unattached kinetochores in tissue culture cells (Chen et al., 1996, 1998; Jin et al., 1998; Li and Benezra, 1996). BUB1 encodes a protein kinase that binds to and phosphorylates Bub3p (Roberts et al., 1994), and mouse and fission yeast Bub1 homologues have been localized to unattached kinetochores (Bernard et al., 1998; Taylor and McKeon, 1997) in a Bub3-dependent manner (Taylor et al., 1998). In addition, it has been shown that the essential protein kinase encoded by MPS1 also has a spindle checkpoint function (Weiss and Winey, 1996), can phosphorylate Mad1p, and that its overexpression is sufficient to activate the spindle checkpoint (Hardwick et al., 1996). It has recently been reported by a number of groups that Bub2p is likely to function on a second branch of the spindle checkpoint pathway, which is quite distinct from that in which the Mad1, Mad2, Mad3, Bub1, and Bub3 proteins function (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). Bub2p and another component of this checkpoint branch, Byr4p/Bfa1p, have both been localized to spindle pole bodies in yeast (Fraschini et al., 1999; Li, 1999).

The molecular mechanisms by which spindle defects are monitored and send a signal that induces a cell cycle delay remain poorly understood (Hardwick, 1998). In insect spermatocytes, the lack of tension on kinetochores that have only attached to microtubules from one spindle pole appears to inhibit anaphase onset (Li and Nicklas, 1995). Whether the spindle checkpoint monitors tension in somatic cells remains unclear, however, it does not regulate Mad2 binding as this checkpoint protein is only detected on one or two kinetochores in cells treated with taxol, even though none of the kinetochores in these cells are under tension (Waters et al., 1998). The role of the spindle...
checkpoints proteins in unperturbed cell cycles is also uncertain, although the Mad2 protein (Gorbsky et al., 1998) and the Bub1 kinase (Taylor and M. K. Eon, 1997) do appear to control the timing of anaphase onset in normal cell division in animal cells.

The spindle checkpoint blocks sister chromatin separation by inhibiting the anaphase-promoting complex (A PC). Mad2p binds to Cdc20p, an essential activator of the A PC, and in fission and budding yeasts Cdc20p mutants that cannot be inhibited by the checkpoint fail to bind to Mad2p (Hwang et al., 1998; Kim et al., 1998). In vertebrates, components of the A PC have been found to control the timing of anaphase onset in normal cell division by inhibiting the anaphase-promoting complex (A PC). The spindle checkpoint blocks sister chromatin separation by inhibiting the anaphase-promoting complex (A PC).

Table I lists the strains used in this work, all of which are derivatives of W303 except the two-hybrid strains Y 187 and Y 190, and mad3-1 which is derived from S288c. Yeast media, growth conditions, stock solutions, and molecular techniques were as previously described (Guthrie and Fink, 1991; Hardwick and M. uray, 1995). M.1icrocolony assays were carried out as previously described (Li and M. uray, 1991).

### Cloning of Mad3

The Mad3 clone was isolated from a YCP50-based genomics library (Hardwick and M. uray, 1995). The mad3-1 mutant was transformed with this library, and after 2–3 d of growth on uracil-free plates, the Ur+ colonies were scraped off, diluted, and replated onto YPD plates containing 10 µg/ml benomyl. Plasmid DNA was prepared from benomyl-resistant colonies and individual plasmid isolates were tested for their ability to rescue mad3-1 (KH45). One plasmid, pKH502, was able to do this and could also rescue mad3-2 (KH160). Treatment with the Klenow fragment of DNA polymerase I to remove the overhanging bases after cutting at the unique

### Materials and Methods

#### Yeast Strains and Media

Table I lists the strains used in this work, all of which are derivatives of W303 except the two-hybrid strains Y 187 and Y 190, and mad3-1 which is derived from S288c. Yeast media, growth conditions, stock solutions, and molecular techniques were as previously described (Guthrie and Fink, 1991; Hardwick and M. uray, 1995). M.1icrocolony assays were carried out as previously described (Li and M. uray, 1991).

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The Journal of Cell Biology, Volume 148, 2000 872
Sacl site produced a clone (pKH504) that no longer rescued mad3 mutant. To confirm this that plasmaid contained the MAD3 gene rather than a suppressor, we inserted the flanking BamHII fragment into the URA3 integration vector pRS306 to produce pKH509. This plasmaid was linearized with HpaI and transformed into wild-type haploid cells (K H35). Transformants were then mated with K H45 (mad3-1) and K H160 (mad3-2), the resulting diploid strains were sporulated and 16 tetrads dissected from each cross. In all 32 tetrads the URA3 marker segregated away from bonemarrow sensitivity, demonstrating that we had cloned the MAD3 gene.

**Sequencing, Mapping, mad3 Gene Disruptions, Mutants, and Overexpression Constructs**

A flt subcloning fragments into pBluescript, we completely sequenced a 2.2-kb segment of DNA flanking the Saci site in pKH502 using a combination of the Sequenase II DNA sequencing kit (United States Biochemical) and the ABI prism cycle sequencing kit (Perkin-Elmer). This sequence contains a 1,548-bp ORF which has since been designated YJL013C in the Saccharomyces genome database.

To sequence the mad3-1 and mad3-2 mutant alleles, genomic DNA was prepared (Ward, 1990) and the mad3 locus amplified by PCR and then analyzed by cycle sequencing (PE Applied Biosystems). Each allele was sequenced multiple times on both strands.

Two mad3 gene disruptions were made (see Fig. 1): one, mad3Δ1, replaces the BglII-XbaI fragment (nucleotides 702–1161, amino acids 226–293) with a KpnI-BglII fragment containing the LEU2 gene (pKH515). The other, mad3Δ2, was made by PCR (pKH520) and replaces nucleotides 180–1441 (amino acids 40–88) with the URA3 gene (primer 1: CGGTAACCTCTAAATAGGCTTACAC, primer 2: GCCGAATTCTAATGTTTACTTCCAC, primer 3: CGGATCTTTAAGAATGAAATCA, primer 4: GATGACGCGGCCGCAATGGCTTATGAACGGGTTA, primer 5: GATGACGCGGCCGCAATGGCTTATGAACGGGTTA). The MAD3 overexpression construct (pKH512) inserts a COOH-terminal myc epitope (EQKLISEEDLN) and expresses CATAAGCGTTAATCGGACA) in pAS135.

**Preparation of Antibodies against Mad3p, Immunoblotting, and Immunofluorescence**

The MAD3 ORF was cloned into pGEX2T (Pharmacia) as a BamHI fragment. Yeast protein was overexpressed in Escherichia coli and affinity-purified using a GST fusion protein. The affinity-purified anti-Mad3p, anti-Bub1-GST fusion protein contains residues amino acids 176–515; pKH705 encodes amino acids 308–515; pKH706 encodes amino acids 417–515; pKH707 encodes amino acids 308–515; pKH708 encodes amino acids 417–515; pKH709 encodes amino acids 308–515. These constructs were made by PCR amplification of the MAD3 insertions, using either VENT polymerase or Bio-X-A cDNA polymerase (Boilone), followed by sub-cloning of the products into pAS1-CYH2 and H2, sequencing.

**Preparation of Antibodies against Mad3p, Immunoblotting, and Immunofluorescence**

**Results**

**Isolation and Sequence Analysis of MAD3**

To further our molecular analysis of the spindle checkpoint in budding yeast we isolated the MAD3 gene. The Mad3 protein is encoded by a yeast genomic library and plasmids were isolated that rescued the mutant's bonemarrow sensitivity. One plasmid with this property also rescued the mad3-2 allele. A fert restriction mapping, subcloning, and sequencing this plasmid was found to contain the open reading frame (ORF) YJL013C, which encodes Mad3p: creating a frameshift mutant in its coding sequence.
quence abolished its ability to complement mad3 mutants, and genetic mapping showed that it was allelic to mad3-1 and mad3-2 (see Materials and Methods).

We sequenced the mad3-1 and mad3-2 mutant alleles and found that they both contain single point mutations. In mad3-1 a mutation at nucleotide 1144 changes glutamate 382 to lysine, and in mad3-2 a mutation at nucleotide 261 introduces a stop codon in place of tryptophan 87 (see Fig. 1a).

BLAST searches with the Mad3p sequence revealed that the only protein in the budding yeast genome with significant homology to Mad3p is the previously identified spindle checkpoint protein Bub1p. Bub1p is a large 110-kD protein kinase with a COOH-terminal kinase domain, and the homology with Mad3p is towards its NH2 terminus. Fig. 1a indicates (in bold and underlined) the two regions of Mad3p with homology to sequences in the NH2-terminal half of Bub1p: amino acids 64–195 of Mad3p (homology region I) are 46% identical to amino acids 44–176 of Bub1p and amino acids 343–401 of Mad3p (homology region II) are 47% identical to amino acids 304–356 of Bub1p. Fig. 1b shows a Clustal alignment with sequences outside budding yeast and reveals that these regions of Mad3p have homology to sequences in the fission yeast and human homologues of Bub1 and also to a human protein that was identified as a Bub1/Mad3-related protein (Cahill et al., 1998; Chan et al., 1998; Taylor et al., 1998).

**MAD3 Encodes a Spindle Checkpoint Component**

To confirm that Mad3p has a spindle checkpoint function we made two gene disruption constructs. One, mad3Δ1, removes the COOH-terminal half of the protein by replacing amino acids 236–388 with the LEU2 gene and the other, mad3Δ2, is a more complete disruption which replaces amino acids 60–480 (82% of the MAD3 ORF) with the URA3 gene. Initially we tested the disrupted haploid strains using the two principal criteria for spindle checkpoint mutants: reduced ability to form colonies on benomyl-containing medium and an inability to delay cell division in response to spindle depolymerization. Fig. 2 shows that mad3Δ1 and mad3Δ2 strains have both of these phenotypes. A cin1 strain was used as a control to show the behavior of strains containing a structural microtubule defect: the cells were benomyl sensitive, and did not divide in the microcolony assay, in which individual cells were picked onto benomyl-containing media and observed for a number of hours and their cell divisions counted. The mad3 strains were also benomyl sensitive, and importantly they clearly continued to divide on the benomyl-containing plates (Fig. 2b). We also carried out FACS® analyses of synchronous mad3 and mad3,bub2 cultures treated with nocodazole (not shown). Our results were in agreement with a number of studies of the mad and bub mutants, where it has been shown that both branches of the spindle checkpoint must be inactivated for efficient DNA rereplication to occur (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999).

In addition, previously published work has shown that, like the other mad and bub mutants, mad3 mutants fail to maintain sister chromatid cohesion when treated with nocodazole and as a result die rapidly (Straight et al., 1996).
and that mad3 mutants interact with many mitotic mutants in the same way as the previously characterized mad1 and mad2 mutants (Hardwick et al., 1999). Taken together, these experiments clearly demonstrate that mad3 mutants have a very similar spindle checkpoint defect to that previously described for mad1 (Hardwick and Murray, 1995) and mad2 mutants (Chen et al., 1999).

To carry out biochemical analysis of Mad3p, we raised polyclonal antibodies to a bacterially expressed Mad3-GST fusion protein, and epitope-tagged Mad3p by adding a COOH-terminal myc epitope. Fig. 3 a shows that, after affinity purification, the polyclonal antibodies detected a polypeptide of 58 kD that was missing in mad3-2 and mad3-1 mutants and was still present in the mad3-1 strain. This immunoblot is consistent with our sequence analysis of the mad3 alleles and shows that our polyclonal antibodies are specific for Mad3p.

We used this antibody to analyze the abundance of Mad3p through the cell cycle (Fig. 3 c): wild-type yeast cells were synchronized in G1 with alpha-factor and then washed and released into rich growth media, either with or without the addition of nocodazole. Samples were taken every 20 min and analyzed by immunoblotting with anti-Mad3p, anti-Mad1p, and anti-Clb2p (a mitotic cyclin) antibodies. It is clear from this experiment that unlike Clb2p, which is absent in G1 and high in mitosis, the abundance of Mad3p does not alter during the cell cycle. In addition, unlike Mad1p which becomes hyperphosphorylated upon nocodazole treatment (Hardwick and Murray, 1995), there was no obvious posttranslational modification of Mad3p that could be resolved by SDS-PAGE, either during the cell cycle or upon checkpoint activation.
Our immunofluorescence analysis has failed to detect wild-type levels of Mad3p. However, strains containing a multi-copy vector expressing Mad3p-myc from the TPI promoter (pKH512) show a general nuclear localization for Mad3p when stained with either anti-myc or anti-Mad3p antibodies (Fig. 3b). As this plasmid has a 2-μm origin of replication there are widely differing levels of expression in the population, due to the wide variation in plasmid copy number. However, nuclear staining was observed at all detectable levels of expression suggesting that the protein is likely to be nuclear in wild-type cells. In some cells expressing high levels of Mad3p the antibodies clearly labeled more of the nucleus than was DAPI-stained. To confirm that the Mad3p staining was entirely nuclear we performed double label immunofluorescence experiments using anti-tubulin (not shown) and anti-Kar2p antibodies. The latter is a soluble protein of the endoplasmic reticulum and gave clear staining of the nuclear envelope, within which Mad3p was restricted (Fig. 3b).

**Mad3p Binds to Bub3p and Cdc20p**

Mad3p has two regions of homology with Bub1p, a protein that has been shown to bind to Bub3p (Roberts et al., 1994). Therefore, we wanted to test whether Mad3p could also interact with Bub3p. Initially, we used the two-hybrid assay to test whether Mad3p interacts with any of the known checkpoint components. Fig. 4a shows that full-length Mad3p interacts with both Bub3p and Cdc20p in the two-hybrid assay, but not significantly with Mad1p, Mad2p, Bub1p, or Bub2p. To determine which portion of Mad3p contains the binding sites for Bub3p and Cdc20p, a number of Mad3 deletion constructs were tested. Fig. 4b shows that homology region I of Mad3p, which contains the longer region of homology to Bub1p, is not necessary for its interaction with Bub3p, and that homology region II is necessary and sufficient for Bub3p binding. Conversely, Cdc20p was found to interact with fragments containing homology region I of Mad3p. Bub1p also contains a homologous region I and we next tested whether it could also interact with Cdc20p. In the two-hybrid assay homology region I of Bub1p does interact with Cdc20p: an average of at least three independent crosses. (b) Mapping the Bub3p and Cdc20p interaction domains of Mad3p. A series of Mad3p truncations was tested for Bub3p and Cdc20p interaction in the two-hybrid assay, identifying two distinct interaction domains.

Cdc20p interaction. These immunoprecipitation results are entirely consistent with the two-hybrid data and also show that neither of the mutations disrupts the entire structure of the protein. When taken together, they show that homology region I of Mad3p is needed to bind to Cdc20p, and that homology region II of Mad3p is necessary for Bub3p binding. What is the in vivo phenotype of these mad3 mutations? Fig. 5c shows that both the homology region I and the homology region II mutation lead to a benomyl-sensitive phenotype. Strains containing the region I mutation were as benomyl sensitive as a mad3 null mutant, and the region II mutation was almost as severe. This indicates that both the Bub3p and the Cdc20p interaction are important for Mad3p checkpoint function.

To test whether either region of homology was sufficient for the Bub3p or Cdc20p interaction, we performed in vitro binding experiments using bacterially expressed Mad3-GST fusion proteins. The purified fusion proteins were incubated with reticulocyte lysates within which either Bub3p or Cdc20p had been translated and radiolabeled, and then pulled down with glutathione agarose beads. Fig. 5d shows that radiolabeled Bub3p bound efficiently to the Mad3-GST fusion and that amino acids 176-409 of Mad3p (containing homology region II) were sufficient...
madr3p complexes were dependent on other checkpoint proteins (Chen et al., 1999). To determine whether the Mad3p complexes were dependent on other checkpoint components, we attempted to coimmunoprecipitate them from yeast strains specifically lacking a checkpoint component. To analyze the Mad3p–Bub3p association, we immunoprecipitated Mad3p from checkpoint mutant strains containing myc-tagged Bub3p. Immunoblotting these precipitates with anti-myc antibodies (Fig. 6 b) revealed that none of the known checkpoint proteins were required for the Mad3p–Bub3p interaction.

To analyze the Mad3p–Cdc20p and Mad3p–M ad2p interactions, we wished to perform a similar experiment, and for these interactions it was important to ensure that all strains arrested in mitosis. A strain mad and bub strains do not arrest well in nocodazole, we introduced a temperature-sensitive A PC mutation (cdc26Δ) into the checkpoint mutants. When shifted to 37°C, such strains arrest in metaphase due to an inability to degrade the anaphase inhibitor Pds1p (Hwang and Murray, 1997). These strains were grown to log phase and treated with nocodazole for 3 h at 37°C. Mad3p and Cdc20-HAP were then immunoprecipitated from native extracts. Fig. 7 shows the results of immunoblotting such immunoprecipitates for Mad3p, Mad2p, and Cdc20p. Fig. 7 a reveals that Cdc20-HAP was present in Mad3p immunoprecipitates at wild-type levels in bub2 extracts, but at reduced levels or was entirely absent in mad1, mad2, bub1, bub3, and mps1 extracts. A nti-M ad2p immunoblots revealed that a Mad3p–M ad2p complex was only detectable in wild-type and bub2
mutant strains. This is consistent with recent work showing that Bub2p lies on a quite different branch of the spindle checkpoint to the other Mad and Bub proteins (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). Fig. 7 b shows that the lack of a Mad3p–Mad2p interaction does not simply reflect the lack of a Cdc20p–Mad2p complex in the mutant extracts, as Mad2p could be detected in Cdc20p immunoprecipitates made from the same extracts. Wild-type levels of Mad2p–Cdc20p complex were detected in the mutant extracts, as Mad2p could be detected in Cdc20p immunoprecipitates made from the same extracts. Wild-type levels of Mad2p–Cdc20p complex were detected in mad3 and bub2 strains, confirming that Mad3p is not required for the formation of this complex (Hwang et al., 1998). The levels of Mad2p–Cdc20p were clearly reduced in the other checkpoint mutants. Mad1p could not be detected in either the Mad3p or the Cdc20p immunoprecipitates (not shown), showing that while it may aid in their formation it does not form a stable component of such complexes.

These experiments show that Mad3p was not required for Mad2p–Cdc20p complex formation, in fact in some experiments the Mad2p–Cdc20p complex was found at slightly higher levels in mad3 than in wild-type extracts. However, in both wild-type and bub2 strains, where nocodazole treatment would lead to an inhibition of Cdc20p-dependent APC activity, Mad3p was stably associated with both Cdc20p and Mad2p. We propose that it is this association of Mad3p with both Mad2p and Cdc20p that is crucial for inhibition of the APC and allows Mad3p to play its role in the spindle checkpoint.

Discussion
We have shown that MAD3 encodes a novel 58-kD com-

(a) Mad3p immunoprecipitations reveal that the Mad3p–Cdc20p complex was low in all checkpoint mutants, except for bub2, and was entirely absent in mad2. A Mad3p–Mad2p association could only be detected in wild-type and bub2 strains. (b) Cdc20p immunoprecipitations reveal that wild-type levels of Mad3p–Cdc20p were present in mad3 and bub2 extracts. The Mad2p–Cdc20p levels were reduced in all other mutants.
ponent of the spindle checkpoint that has significant homology to the NH$_2$-terminal region of the Bub1 protein kinase. Gene disruption experiments revealed that lack of Mad3p abolishes spindle checkpoint function, and mutational analyses indicate that two regions of Mad3p homology are critical for Mad3p's function. A number of approaches have been taken to show that homology region I of Mad3p is required for its interaction with Cdc20p, and that homology region II defines a Bub3p binding site.

**Checkpoint Function of Mad3p**

We used a number of assays to show that mad3 strains are spindle checkpoint defective. Mad3 strains show a similar benomyl sensitivity to mad1 and mad2 mutants. More importantly, in microcolony assays they show the same behavior as mad1 and mad2 and continue to divide in the presence of microtubule perturbations. This is quite unlike the behavior of wild-type cells or strains with structural microtubule defects, such as cin1 or tub mutants, which arrest in mitosis in response to microtubule depolymerization. Mutational analysis showed both homology regions to be important for checkpoint function. The region I mutant, which failed to bind to either Cdc20p or Mad2p (data not shown), behaved as a null mutant in benomyl sensitivity and microcolony assays. The region II mutant, which failed to bind Bub3p, is somewhat less benomyl sensitive. The reason for this is unclear and is currently under investigation.

Our immunofluorescence analysis revealed that Mad3p is nuclear in yeast, but the protein can only be detected when overexpressed. The only components of the budding yeast spindle checkpoint that have been localized at their wild-type expression level are Mad1p, Bub2p and Byr4p. Mad1p is found in a punctate nuclear pattern (Hardwick and Murray, 1995), and Bub2p and Byr4p are seen at spindle poles (Fraschini et al., 1999; Li, 1999). When they are overexpressed, Mad2p is found throughout the cell (Chen, R.-H., personal communication) and Bub1p and Bub3p are both nuclear proteins (Roberts et al., 1994). In vertebrate cells, homologues of Mad1, Mad2, Mad3 (Bub1R), Bub1, and Bub3 have all been shown to localize to kinetochores that have not captured microtubules (Chen et al., 1996, 1998; Li and Benezra, 1996; Taylor and Mckeon, 1997; Jin et al., 1998; Taylor et al., 1998) and the Mad1 and Mad2 proteins in yeast extracts (Hwang et al., 1995). The Mad2p interaction is essential for its checkpoint function. This could be because it is only as a part of a complex that certain checkpoint proteins are recruited to kinetochores (see below).

We have also shown that Mad3p can interact with Cdc20p which is the target of the branch of the spindle checkpoint that monitors kinetochore behavior. Two-hybrid analysis revealed a strong interaction between Mad3p and Cdc20p and also between Bub1p and Cdc20p. Deletion analysis suggested that the NH$_2$-terminal two-thirds of Mad3p, which contains homology region I, was required for this interaction. To test the importance of homology region I, we mutated conserved residues within it (G11S$_{159}$ > A A A A) and constructed a yeast strain containing only this mutant form of Mad3p. Immunoprecipitation analysis showed that the mutant Mad3p failed to bind well to Cdc20p and to Mad2p (data not shown), but that it still bound Bub3p. The resulting mad3 strains were benomyl sensitive indicating that the Mad3p–Cdc20p/Mad2p interaction is essential for its checkpoint function.

Stable complex formation between Mad3p and Cdc20p was previously shown to be dependent on the presence of the Mad1 and Mad2 proteins in yeast extracts (Hwang et al., 1998), which have themselves been shown to form a tight complex (Chen et al., 1999). We confirmed this result, although in certain experiments we found very low but detectable levels of the Mad3p–Cdc20p and the Mad2p–Cdc20p complex in nocodazole-treated mad1 mutant extracts. The Mad3p–Cdc20p complex was also found at low levels in bub1 and bub3 null strains and in mps1 ts strains at their restrictive temperature, but was never detected in the mad2 mutant. From this we conclude that Mad2p function is essential for a stable Mad3p–Cdc20p interaction, probably because it is itself part of the complex (see model in Fig. 8). The low levels of Mad3p–Cdc20p in the other mad/bub/mps1 strains could reflect a reduced stability of

**Mad3p Interacts with Other Checkpoint Components**

Bub1p and Bub3p form a complex in budding yeast, and amino acids 141–609 of Bub1p are sufficient for that interaction (Roberts et al., 1994). The homology between Mad3p and Bub1p prompted us to ask whether Mad3p could also bind to Bub3p. A combination of communoprecipitation, two-hybrid assays, and in vitro binding experiments showed that Mad3p does bind to Bub3p. We found that homology region I was not necessary for Bub3p binding and that a 95–amino acid Mad3p segment containing homology region II was sufficient for this interaction in the two-hybrid assay. Sequencing of the mad3-1 allele revealed that it contains a single point mutation (E382K) within homology region II, and communoprecipitation analyses show that this abolishes Bub3p binding. In vitro binding experiments showed that amino acids 176–409 of Mad3p (which lacks homology region I) were sufficient for Bub3p binding. Our results are in agreement with vertebrate experiments showing that homology region II of the human Bub1 and Mad3/Bub1-related proteins is required for their interaction with Bub3, and for their localization to the kinetochore (Taylor et al., 1998), suggesting that this region targets the recruitment of these proteins to kinetochores that lack bound microtubules.

We have shown by communoprecipitation that the Mad3p–Bub3p interaction is not cell cycle regulated and does not require the presence of the other known checkpoint proteins. We have obtained similar results for the Bub1p–Bub3p interaction (Brady, D.M., and K.G. Hardwick, manuscript submitted for publication), and we have previously reported such behavior for the Mad1p–Mad2p complex in budding yeast (Chen et al., 1999). Thus, there are a number of spindle checkpoint protein complexes that are formed constitutively. The region II mutation (mad3-1), and several mad1 mutations (Chen et al., 1999) show that formation of these complexes is required for checkpoint function. This could be because it is only as a part of a complex that certain checkpoint proteins are recruited to kinetochores (see below).
We have detected a Bub1p–Cdc20p complex in human cells, suggesting that Bub1p also binds Cdc20p, however, we have not been able to detect this complex in mouse cells (Wick, K.G., data not shown). Cdc20p-related protein Hct1p, but have found no evidence of a Bub1p–Hct1p complex in yeast. It is unclear why homology region I is so well conserved amongst species. The best candidates for this recruiting function are Mad2p, which has been shown to recruit Mad2p to kinetochores in Xenopus (Chen et al., 1998), and Bub3p which may recruit both Bub1p and Mad3p to kinetochores (Taylor et al., 1998). Bub2p does not appear to have a role to play in the formation of the Mad3p–Cdc20p/Mad2p complex, which is in agreement with its proposed role on a separate branch of the checkpoint pathway. Although we saw Mad2p associated with both Mad3p and Cdc20p, Mad1p does not appear to be a stable component of such complexes, as it was not detectable in either the Mad1p or the Cdc20p immunoprecipitation (Fig. 6a and data not shown). This is despite our previous observation that Mad1p and Mad2p form a very tight complex (Chen et al., 1999). These results suggest that Mad2p is present in at least two complexes: an association with Mad1p that is required to recruit Mad2p to kinetochores, and another association with Cdc20p and Mad3p. The Mad3p–Cdc20p/Mad2p complex does not require nocodazole treatment for its formation, as it is formed in cdc26 mutants arrested in metaphase at their restrictive temperature (data not shown). However, we have shown that the Mad3p–Cdc20p/Mad2p complex is the one through which Mad3p exerts its crucial role in inhibiting the metaphase-anaphase transition. This idea is supported by the observation that we were only able to detect significant levels of Mad3p in association with both Cdc20p and Mad2p in extracts from wild-type and bub2 cells, and they are the only cells in which a checkpoint arrest would be maintained.

Further in vitro binding experiments using recombinant proteins will be needed before the formation and interactions of the above checkpoint protein complexes can be fully understood. It has been argued from in vitro studies that tetrameric Mad2p when complexed with Cdc20p inhibits activation of the APC (Fang et al., 1998), and it will therefore be of particular interest to test the effect of Mad3p on the ability of Mad2p to inhibit Cdc20p function and thereby APC activity. It has recently been reported that hBub1 binds the APC in mitotic cells (Chen et al., 1999). We have tested whether Mad3p or Bub1p can interact with a component of the APC (Cdc23p), or with the Cdc20p-related protein Hct1p, but have found no evidence for such complexes by immunoprecipitation (Hardwick, K.G., data not shown).

It is unclear why homology region I is so well conserved between Mad3p and Bub1p. Our two-hybrid experiments suggested that Bub1p also binds Cdc20p, however, we have struggled to detect a Bub1p–Cdc20p complex by coimmunoprecipitation from wild-type cells (not shown). We have detected a Bub1p–Cdc20p complex in mad mutant extracts, where there is little if any Mad3p–Cdc20p, suggesting that Mad3p normally outcompetes Bub1p for interaction with Cdc20p. Further experiments have revealed that Bub1p forms a stable association with Mad1p in cells in which the spindle checkpoint has been activated (Brady, D.M., and K.G. Hardwick, manuscript submitted for publication). As there is no Mad3p or Cdc20p associated with the putative Mad1p–Bub1p/Bub3p complex we have proposed that it has a signaling function, acting upstream of Mad3p–Cdc20p/Mad2p.

Mad/Bub Proteins and Kinetochore Signaling

The components of the spindle checkpoint may function as large multi-protein complexes. Previous work has shown that Mad3p and Mad2p can be communoprecipitated with Cdc20p (Hwang et al., 1998), that Mad2p and Mad1p form a tight complex (Chen et al., 1999), and that Bub1p can be communoprecipitated with Bub3p (Roberts et al., 1994). Here we have confirmed the Mad3p–Cdc20p interaction and shown that Mad3p also interacts with Bub3p and Mad2p. Thus, six checkpoint components and a target of the spindle checkpoint have been shown to interact physically, suggesting that much of the checkpoint apparatus functions as one or more large multi-protein complexes.

Vertebrate homologues of Mad1, Mad2, Mad3, Bub1, and Bub3 bind to all kinetochores in cells that have been arrested in mitosis by microtubule polymerization inhibitors, and specifically localize to microtubule-free kinetochores during spindle assembly in normal cells (Chen et al., 1996, 1998; Li and Benezra, 1996; Taylor and C. Kwon, 1997; Chen et al., 1999, 1999; Jin et al., 1998; Taylor et al., 1998). The combination of the vertebrate and yeast results suggest a plausible pathway for the spindle checkpoint: microtubule-free kinetochores attract recruiting proteins, such as Mad1p and Bub3p and these in turn recruit other proteins (Mad2p [Chen et al., 1998], Mad3p, and Bub1p [Taylor et al., 1998]) which bind to and inhibit Cdc20p, thus preventing sister chromatids from separating. This scheme leaves several important questions: how do checkpoint components distinguish between kinetochores with and without bound microtubules, how does interaction of checkpoint components with the kinetochores lead to the inhibition of Cdc20p, how can a single microtubule or tension-free kinetochore inactivate the majority of the kinetochores?
Cdc20p in the cell, and do the other identified components of the checkpoint (Mps1p and Cdc55p) also participate in a kinetochore-bound signaling machine? A physical link between spindle checkpoint proteins and a kinetochore-bound motor protein was recently uncovered through the analysis of hBub1p (Chan et al., 1998, 1999), which we believe to be the human homologue of Mad3p. The kinesin motor, CENP-E, was found to interact with hBub1p, both in a two-hybrid screen and by coimmunoprecipitation. In addition, these proteins colocalized at kinetochores, particularly those that had yet to align at the metaphase plate. The hBub1 observations and our yeast biochemical studies suggest that Mad3p could also have a role to play in the recruitment of Cdc20p/Mad2p to kinetochores.

Recent results suggest that lesions in the spindle checkpoint play an important role in human cancer. Four cell lines derived from human colorectal cancers were found to carry mutations in the human homologue of Bub1 or the Bub1/Mad3-related gene (Cahill et al., 1998). These observations suggest that mutational inactivation of spindle checkpoint components is directly related to the chromosomal instability associated with colorectal and other cancers. The human Bub1/Mad3-related protein differs from budding yeast Mad3p by containing a COOH-terminal protein kinase domain. Although this feature gives it a similar overall structure to Bub1p, the protein kinase domain of the human Bub1/Mad3-related protein is clearly different to that of human and budding yeast Bub1 (Taylor et al., 1998). One explanation of these features is that the Bub1 and Mad3 genes are the product of an ancient gene duplication and that the protein kinase domain of yeast Mad3p has either been lost during evolution or separated into a different polypeptide.

We have recently identified a fission yeast Mad3 homologue (Hardwick, K.G., and D. Millband, unpublished data) and found that as budding yeast it lacks a protein kinase domain, but resolves this issue will require determining the structures of the Mad3p-related genes in other organisms.

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