Lesions in Many Different Spindle Components Activate the Spindle Checkpoint in the Budding Yeast *Saccharomyces cerevisiae*

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ABSTRACT

The spindle checkpoint arrests cells in mitosis in response to defects in the assembly of the mitotic spindle or errors in chromosome alignment. We determined which spindle defects the checkpoint can detect by examining the interaction of mutations that compromise the checkpoint (*mad1, mad2*, and *mad3*) with those that damage various structural components of the spindle. Defects in microtubule polymerization, spindle pole body duplication, microtubule motors, and kinetochore components all activate the *MAD*-dependent checkpoint. In contrast, the cell cycle arrest caused by mutations that induce DNA damage (*cdc13*), inactivate the cyclin proteolysis machinery (*cdc16* and *cdc23*), or arrest cells in anaphase (*cdc15*) is independent of the spindle checkpoint.

MITOSIS produces two daughter nuclei with identical genetic contents. To achieve this feat, cells have to delay chromosome segregation until all their chromosomes are correctly aligned on a bipolar spindle. Spindle assembly depends on the dynamic properties of microtubules nucleated by microtubule organizing centers (the spindle pole bodies in yeast), microtubule motors (reviewed in Hoyt and Geiser 1996; Stearns 1997), and the attachment of spindle microtubules to kinetochores [the complex of centromeric DNA and proteins that attaches chromosomes to microtubules (Figure 1), reviewed in Hyman and Sorger 1995; Allshire 1997]. Mutations or drugs that inhibit these functions lead to spindle defects that can cause aberrant chromosome segregation (reviewed in Hoyt and Geiser 1996). The spindle checkpoint guards against such errors by delaying the onset of chromosome segregation in cells whose spindles are defective (reviewed in Rudner and Murray 1996; Wells 1996).

The spindle checkpoint has been analyzed genetically in budding yeast. The *mitotic arrest deficient* (*mad*; Li and Murray 1991), *budding uninhibited by benzimidazole* (*bub*; Hoyt *et al.* 1991), *mps1* (Hardwick *et al.* 1996; Weiss and Winey 1996), and *cdc55* (Minshull *et al.* 1996) mutations inactivate the checkpoint and allow cells whose microtubules have been depolymerized to pass through mitosis. Experiments in yeasts and frog egg extracts suggest that the spindle checkpoint acts by preventing the activation of the cyclin proteolysis machinery (Minshull *et al.* 1994, 1996; Hardwick and Murray 1995; Chen *et al.* 1996; Li and Benezra 1996; Fang *et al.* 1998a; Hwang *et al.* 1998; Kim *et al.* 1998). This protein degradation system induces the exit from mitosis by activating a multiprotein complex [the cyclosome or anaphase-promoting complex (APC)] that catalyzes the ubiquitination of critical proteins, thus targeting them for degradation by the proteasome (Irniger *et al.* 1995; King *et al.* 1995; Sudakin *et al.* 1995). The key targets for ubiquitination are the B-type mitotic cyclins that associate with Cdc2/Cdc28 and proteins that are required for sister chromatid separation (Holloway *et al.* 1993; Funabiki *et al.* 1996; Yamamoto *et al.* 1996; Ciosk *et al.* 1998; Kumada *et al.* 1998).

To determine which lesions in the spindle the *MAD*-dependent spindle checkpoint detects, we combined the *mad1*, *mad2*, and *mad3* mutations with other mutations that affect spindle or function. These included mutations causing defects in microtubule polymerization (Huffaker *et al.* 1988; Hoyt *et al.* 1990; Stearns *et al.* 1990), spindle pole body duplication (Winey *et al.* 1991), kinetochore components (Cai and Davis 1990), or microtubule motors (Meluh and Rose 1990; Hoyt *et al.* 1992). These studies show that defects in microtubule polymerization, spindle pole bodies, microtubule motors, and kinetochores all arrest cells in mitosis by activating the spindle checkpoint.

MATERIALS AND METHODS

Media and strains: Media for yeast cultures and sporulation were as described (Sherman *et al.* 1974). Strains are listed in Table 1.

Genetic techniques: Standard techniques were used for

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TABLE 1

Yeast strains

Strain number	Relevant genotype
AFS34(W303-1a)	*MAT***a** *ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1*
RHC15.1	*MAT***a** *mad2::URA3*
KH123	*MAT***a** *mad1::HIS3*
KH143	*MAT***a** *mad1::URA3*
KH173	*MAT***a** *mad3::URA3*
RHC47	*MATα cbf1::LEU2*
ADR624	*MAT***a** *cdc23-1*
ADR1135	*MAT***a** *cdc23-1 mad1::HIS3*
ADR1140	*MAT***a** *cdc23-1 mad2::URA3*
ADR1143	*MAT***a** *cdc23-1 mad3::URA3*
ADR1168	*MAT***a** *cdc15-2*
ADR1163	*MAT***a** *cdc15-2 mad1::HIS3*
PHD1	*MAT***a** *cdc15-2 mad2::URA3*
PHD7	*MAT***a** *cdc15-2 mad3::URA3*
CM40	*MAT***a** *cdc13-1*
CM45	*MAT***a** *cdc13-1 mad1::URA3*
CM47	*MAT***a** *cdc13-1 mad2::URA3*
CM154	*MAT***a** *cdc13-1 mad3::HIS3*
CM106	*MAT***a** *mps2-1 ura3-52 leu2-3, 112*
CM77	*MAT***a** *mps2-1 mad1::URA3*
CM95	*MAT***a** *mps2-1 mad2::URA3*
CM107	*MAT***a** *mps2-1 mad3::URA3*
CM18	*MAT***a** *tub2-403 ura3-52 leu2-3,112 lys2-801*
CM30	*MAT***a** *tub2-403 mad1::URA3*
CM19	*MAT***a** *tub2-403 mad2::URA3*
CM228	*MAT***a** *tub2-403 mad3::URA3*
CM76	*MAT***a** *cdc20-1*
CM117	*MAT***a** *cdc20-1 mad1::HIS3*
CM119	*MAT***a** *cdc20-1 mad2::URA3*
CM123	*MAT***a** *cdc20-1 mad3::URA3*
CM132	*MATα kar3::LEU2*
AFS421	*MAT***a** *cin8::TRP1*

All strains are isogenic to W303 (*MAT***a** *ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1*, except for the *mps2-1* and *tub2-403* strains and their derivatives, which are isogenic to S288C. Only those aspects of the genotype that differ from those of W303 or S228C are listed.

yeast mating, sporulation, tetrad analysis, and α-factor treatment (Sherman *et al.* 1974).

Synthetic lethality: These experiments were performed in one of two ways, either by crossing a haploid lacking a microtubule motor to a *mad* strain, or by using gene disruption to create heterozygosity for loss of a microtubule motor gene in a strain already heterozygous for a *mad* mutation. The latter method is more cumbersome, but it eliminates the possibility that slowly growing strains, such as *kar3*, will have acquired suppressor mutations.

Cell death assays: The rate of cell death in *mad cdc* (cell division cycle) double mutants was determined as follows. A saturated culture was grown in YPD at 23°, and 0.1 ml was plated on a YPD plate and incubated for 2 days at 23°. Cells were collected by washing the plate with liquid YPD, diluted, and incubated at the nonpermissive temperature for the mutant. At the indicated times, appropriate dilutions of cultures were plated on YPD plates that were incubated for 3–5 days at room temperature before colony counting. This method was used because some of the mutants double so slowly at 23° that other methods of synchronizing the cells were ineffective. All figures are representative of experiments that were repeated at least three times.

RESULTS

We investigated the types of mitotic lesions that activate the *MAD*-dependent checkpoint by studying the interaction of *mad* mutations with mutations that cause defects in the execution of particular steps of mitosis (Figure 1). These include mutations that inhibit microtubule depolymerization, prevent spindle pole body duplication, remove centromere-binding proteins, and *cdc* mutations that arrest cells in mitosis at 37°.

We tested mitotic mutations in two classes, conditional lethal mutations and null mutations in nonessential genes. Figure 2 shows the rationale for these experiments. To determine the interaction of *mad* mutations with *cdc* mutations that cause arrest in G2 or mitosis, the properties of the *mad cdc* double mutant were compared with that of the *cdc* mutant alone. If the cell cycle arrest of the *cdc* mutant is independent of the spindle checkpoint, the *cdc mad* double behaves exactly like the *cdc* mutant alone: the cell cycle arrests at the nonpermissive temperature, and the single and double mutants lose viability at the same rate when incubated at the nonpermissive temperature. In contrast, if the arrest of the *cdc* mutant depends on the spindle checkpoint, inactivation of the checkpoint will allow the *mad cdc* double mutants to pass through mitosis at the restrictive temperature even though the spindle is defective. In a haploid strain, initiating anaphase in cells that have not aligned their chromosomes properly on a bipolar mitotic spindle will lead to chromosome loss and generate dead cells. Thus, combining such *cdc* mutations with *mad* mutations will produce double mutants that fail to arrest in mitosis and lose viability faster than the *cdc* mutant alone when the strains are incubated at the nonpermissive temperature (Figure 2). A second test for whether the spindle checkpoint detects a defect is to examine the cell cycle progress. If the cell cycle arrest of a *cdc* mutant is suppressed in the *mad cdc* double mutant, the spindle checkpoint must be required for the arrest. This analysis is only meaningful if most of the cells in the starting population are viable and the degree of synchrony in the population is high. Unfortunately, for several of the mutations that do show genetic interactions with the *mad* mutations, the double mutants are difficult to synchronize, and populations of these strains contain many dead cells, even at the permissive temperature.

A number of components of the spindle, such as individual microtubule motors, are not essential for cell viability (reviewed in Hoyt and Geiser 1996). Although the loss of such components may compromise spindle function, cells can survive without them if the spindle checkpoint delays anaphase until alternative mecha-

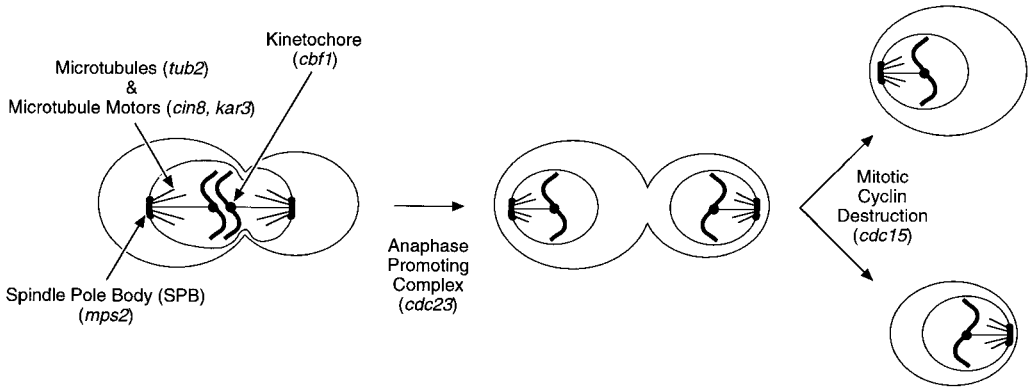

Figure 1.—Mitosis in budding yeast. Cells are shown in metaphase, anaphase, and G1. The mutants used in this article that compromise various structures and reactions are indicated.

nisms have correctly aligned the chromosomes on a bipolar spindle. This model predicts that the combined loss of a nonessential spindle component and the spindle checkpoint would be lethal, since the double mutant cells would no longer be able to delay anaphase to allow backup mechanisms to assemble a functional spindle (Figure 2). This prediction has been confirmed by the isolation of several checkpoint mutations (*bub2*, *bub3*, *mps1*, and a dominant *CDC20* allele, *CDC20-50*) in a screen for mutations that kill cells lacking Cin8, a kinesin with a role in spindle assembly (Geiser *et al.* 1997).

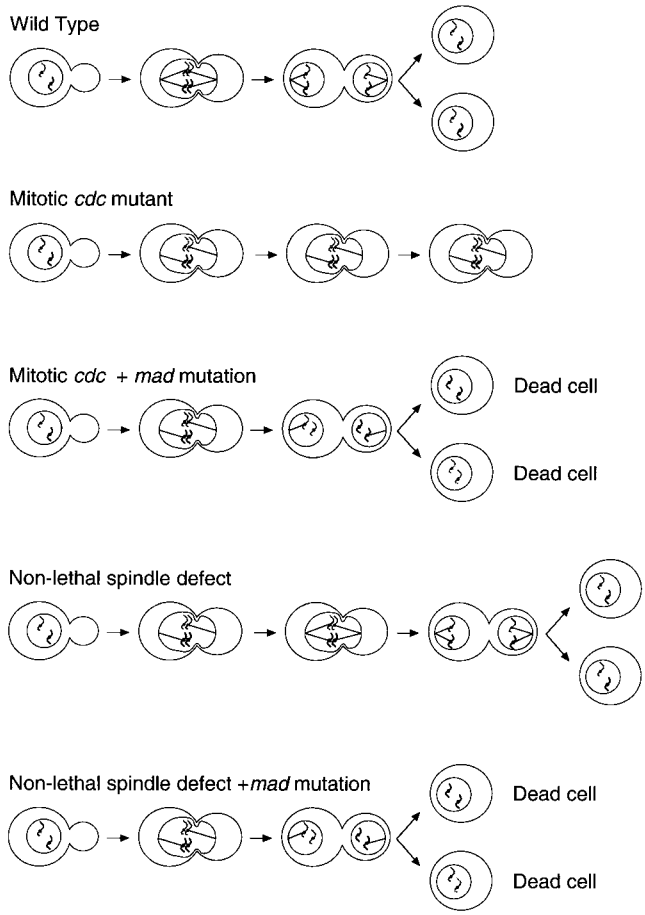

Figure 2.—Interactions between *mad* mutations and mutations that cause spindle defects. The effect of inactivating the spindle checkpoint on cell division in wild-type cells, *cdc* mutants that affect spindle structure, and cells with nonlethal spindle defects. See text for further details.

Tubulin and spindle pole body mutants activate the spindle checkpoint: The *mad* mutants were originally isolated as mutants that had increased sensitivity to benomyl, an inhibitor of microtubule polymerization. The mutants failed to arrest in mitosis and died rapidly when treated with benomyl (Li and Murray 1991; Hardwick and Murray 1995), suggesting that the defects in microtubule polymerization activated the spindle checkpoint. To confirm that this phenotype resulted from the effect of benomyl on microtubule polymerization, we tested the interaction of the *mad* mutations with a mutation in *TUB2*, the yeast β-tubulin gene (Huffaker *et al.* 1988). When cells of a strain carrying the cold-sensitive *tub2-403* mutation are incubated in rich medium at 14°, they arrest in mitosis as large-budded cells that lack polymerized microtubules and remain viable for >6 hr. In contrast, *tub2-403 mad1*, *tub2-403 mad2*, and *tub2-403 mad3* double mutants lose viability during incubation at 14°, with <25% of the cells remaining viable at 6 hr (Figure 3A). In this experiment, cells were shifted to the restrictive temperature from a block at the G1/S phase boundary created by hydroxyurea treatment because release from a G1 arrest at 14° is very slow. These experiments show that the combination of the *mad* mutations with the *tub2-403* mutation increases the rate at which cells that cannot polymerize microtubules die in the cold. These observations demonstrate that the *MAD*-dependent spindle checkpoint is required to arrest cells that cannot assemble microtubules.

Mutations that prevent spindle pole body duplication have no effect on microtubule polymerization, but they cause cells to assemble monopolar rather than the normal bipolar spindles. Two mutants, *mps2* and *cdc31*, that have this phenotype arrest in mitosis, whereas a third, *mps1*, fails to arrest in mitosis despite the absence of a bipolar spindle (Baum *et al.* 1986; Weiss and Winey 1996). The observation that *mps1 mps2* double mutants

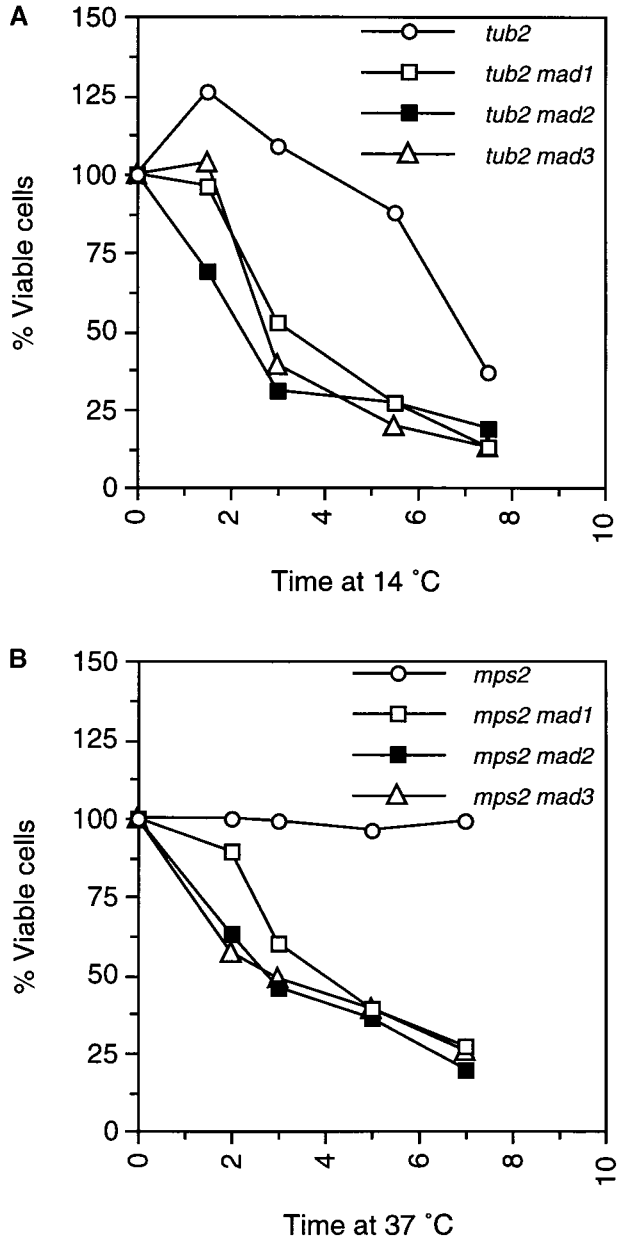

Figure 3.—*mad* mutations increase the rate at which tubulin and spindle pole body mutants die. The indicated strains were arrested in G1 by nutrient starvation at 23° and released into rich medium at the nonpermissive temperature for (A) *tub2-403* (14°) or (B) *mps2-1* (37°). Samples were taken at the indicated time points, diluted, and plated for viability on rich medium at 23°. Values are expressed relative to the number of viable cells at time zero.

fail to arrest in mitosis led to the suggestion that *mps1* mutants lacked a checkpoint for spindle pole body duplication. Subsequent analysis showed that *mps1* mutants, like the *mad* mutants, could not respond to spindle depolymerization, and demonstrated that Mps1 functions in the Mad-dependent spindle checkpoint (Hardwick *et al.* 1996; Weiss and Winey 1996). The inability of *cdc31-2 mps1-1* double mutants to arrest in mitosis suggested that the spindle checkpoint was required to arrest cells with monopolar spindles in mitosis (Weiss and Winey 1996), as did the observation that the *cdc31-2* and *mps2-1* mutations both led to the hyperphosphorylation of Mad1, a biochemical marker for activation of the checkpoint (Hardwick *et al.* 1996).

We tested whether Mad proteins were needed to detect monopolar spindle mutations by combining the temperature-sensitive *mps2-1* mutation with the *mad1*, *mad2*, or *mad3* mutations and monitoring the rate of death at 37° as a measure of exit from mitosis. The single *mps2-1* mutant arrests in mitosis and remains fully viable for at least 7 hr, when G1 stationary phase cells are inoculated into rich medium at 37°. All the *mps2-1 mad* double mutants, however, die rapidly, with 50% of the cells becoming inviable within 3–4 hr of incubation at 37° and 25% of the cells remaining viable at 7 hr (Figure 3B). This observation demonstrates that all the Mad proteins are required for cells to detect a monopolar spindle and arrest in mitosis as viable cells.

Microtubule motor defects activate the spindle checkpoint: Microtubule motors move along the surface of microtubules, and genetic and cell biological experiments have implicated motors in many aspects of mitosis and meiosis in a wide variety of organisms. These include formation of a bipolar spindle (Enos and Morris 1990; Hagan and Yanagida 1990, 1992), the regulation of spindle length (Hoyt *et al.* 1992; Roof *et al.* 1992), the movement of chromosomes along microtubules (Hyman and Mitchison 1991; Lombillo *et al.* 1995), the regulation of microtubule stability (Walczak *et al.* 1996), and distributive chromosome segregation in meiotic cells (Ashfar *et al.* 1995). Despite these many roles, no single microtubule motor in yeast is essential for viability at 30° (Hoyt and Geiser 1996). This observation can be explained in two ways: either there is full overlap between the function of different motors so that loss of one motor has no effect on the robustness or fidelity of mitosis, or there is partial overlap, and the spindle checkpoint delays the onset of anaphase until cells with motor defects have aligned their chromosomes correctly. A variety of observations support the latter view. Detailed analysis of the dynamics of mitosis reveals that defects in different motors affect different phases of mitosis (Straight *et al.* 1998); cells that lack the minus-end-directed motor, Kar3, delay in mitosis (Meluh and Rose 1990), and cells that lack the plus-end-directed motor, Cin8, show elevated frequencies of chromosome loss (Hoyt *et al.* 1992). In addition, the inability to recover *mad2-1 kar3* double mutants (Roof *et al.* 1991) suggests that the proliferation of cells lacking Kar3 depends on a functional spindle checkpoint, and the recovery of *mad* and *bub* mutations as synthetic lethal mutations with *cin8* suggests that the lack of the Cin8 motor causes defects that are lethal in the absence of the spindle checkpoint (Geiser *et al.* 1997).

We tested the interaction of microtubule motors and the spindle checkpoint by combining the *kar3* and *cin8*

TABLE 2

Viability of double mutants with defects in the spindle checkpoint and microtubule motors

	Spore genotypes			
	MAD KAR3	*MAD kar3*	*mad KAR3*	*mad kar3*
mad1	15	12	17	2*,**
mad2	18	21	26	7*,**
mad3	27	16	23	11**
	MAD CIN8	*MAD cin8*	*mad CIN8*	*mad cin8*
mad1	22	13	16	0*,**
mad2	31	20	21	6*,**
mad3	19	15	21	3*,**

* Null hypothesis that frequency of double mutants is the product of the frequency of single mutants is rejected at $P < 0.005$.

** Null hypothesis that all four genotypes are equally viable is rejected at $P < 0.05$.

At least 20 tetrads were dissected for each cross, and the genotypes of the products were deduced from the auxotrophic markers that marked gene disruption mutations. Neither *KAR3* nor CIN8 are linked to any of the *MAD* genes. Data were analyzed by the χ^2 test. By applying this test to the pooled data from all the crosses, the viabilities of *mad1*, *mad2*, *mad3*, *kar3*, and *cin8* single mutants were not significantly different from that of wild-type cells.

TABLE 3

Viability of double mutants with defects in the spindle checkpoint and the kinetochore component Cbf1

	Spore genotypes			
	MAD CBF1	*MAD cbf1*	*mad CBF1*	*mad cbf1*
mad1	24	36	37	4*,**
mad2	27	34	33	4*,**
mad3	20	34	34	20

At least 20 tetrads were dissected for each cross, and the genotypes of the products were deduced from the auxotrophic markers that marked gene disruption mutations. *CBF1* is not linked to any of the *MAD* genes. Data were analyzed by the χ^2 test. By applying this test to the pooled data from all the crosses, the viabilities of *mad1*, *mad2*, *mad3*, and *cbf1* single mutants were not significantly different from that of wild-type cells.

* Null hypothesis that frequency of double mutants is the product of the frequency of single mutants is rejected at $P < 0.005$.

** Null hypothesis that all four genotypes are equally viable is rejected at $P < 0.05$.

mutations with the *mad1*, *mad2*, and *mad3* mutations. Diploids that were heterozygous for a *mad* mutation and a defect in a microtubule motor were sporulated and subjected to tetrad dissection. Because both mutations were marked by the insertion of standard genetic markers, the genotype of every spore could be unambiguously deduced from its pattern of growth on selective media. The results of this analysis are presented in Table 2. In all but one of the crosses (that between *mad3* and *cin8*), fewer double-mutant spores were recovered than any other genotype. We tested the statistical significance of these findings in two ways. First, we tested whether the results we obtained were significantly different from those expected if all four genotypes were equally likely to survive. Second, we tested whether the viability of the double-mutant spores was less than the viability of the single *mad* mutant spores' viability multiplied by the viability of the spores lacking only the microtubule motor. Both tests indicated that the viability of the *kar3 mad1*, *kar3 mad2*, *cin8 mad1*, *cin8 mad2*, and *cin8 mad3* double mutants was significantly less than expected, showing that viability of mutants lacking Kar3 or Cin8 is dependent on the integrity of the spindle checkpoint. For the *kar3 mad3* double mutant, the low spore viability of the *kar3 MAD3* spores makes it impossible to determine whether the viability of *kar3* cells depends on Mad3.

The absence of a kinetochore component activates the spindle checkpoint: Many observations suggest that interactions between the kinetochore and the spindle microtubules play an important part in sensing the structure of the mitotic and meiotic spindles (reviewed in Rudner and Murray 1996; Wells 1996). These suggest that the spindle checkpoint monitors spindle integrity by detecting kinetochores that have not attached to microtubules or by measuring the tension exerted by microtubules on the kinetochore.

We assayed the interaction between kinetochore mutations and *mad* mutations. In our hands, none of the mutations in the essential components of the kinetochore (Ndc10, Ctf13, and Cbf3/Cep3; reviewed in Hyman and Sorger 1995) gave a robust cell cycle arrest in the W303 strain background, making it impossible to assess whether the phenotype associated with any of these mutations was altered by loss of the spindle checkpoint. Instead, we assayed the interaction between the *mad* mutations and *cbf1*, which eliminates a nonessential protein that binds to the CDEI element of the centromeric DNA (Cai and Davis 1990). We constructed diploids that were heterozygous for genetically marked *cbf1* and *mad* deletions, sporulated the diploids, and subjected them to tetrad dissection. Very few *cbf1 mad1* or *cbf1 mad2* spores were recovered from these crosses, demonstrating that the viability of *cbf1* cells depends on the integrity of the spindle checkpoint (Table 3). Like *kar3*, the *cbf1* mutation was viable when combined with the *mad3* mutation. This observation suggests that the *mad3* mutant is, in some sense, less defective in the spindle checkpoint than *mad1* or *mad2* mutants. Others have shown spindle checkpoint-dependent arrests in response to *ctf13* mutations and mutations in the kinetochore DNA in the S288C strain background (Wang and Burke 1995; Pangilinan and Spencer 1996).

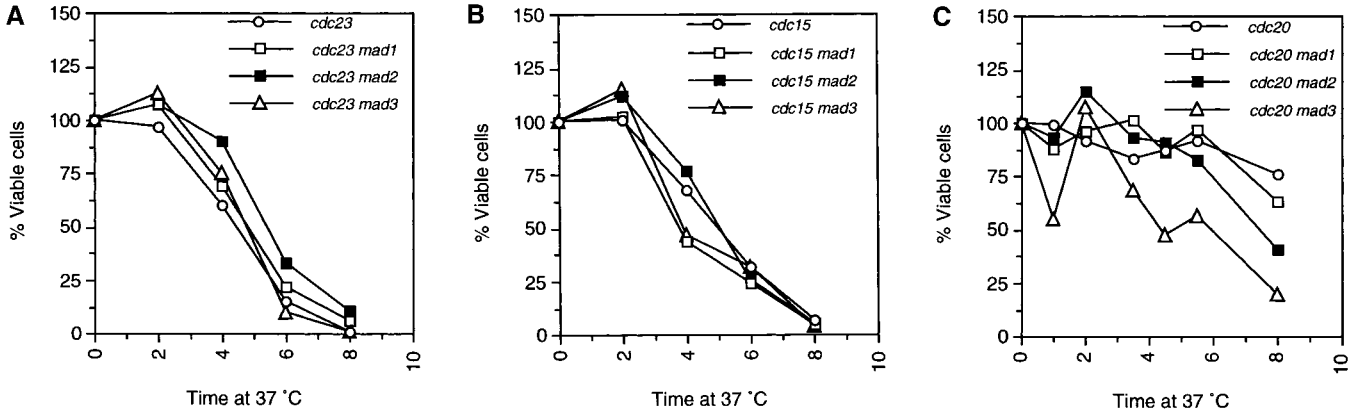

Figure 4.—*mad* mutations do not increase the rate of cell death in mutations that impair mitotic protein degradation. The indicated strains were arrested in G1 by nutrient starvation at 23° and released into rich medium at 37° for (A) *cdc23-1*, (B) *cdc15-2*, or (C) *cdc20-1* strains. Samples were taken at the indicated time points, diluted, and plated for viability on rich medium at 23°. Values are expressed relative to the number of viable cells at time zero. All experiments were repeated three times. Although the results of individual experiments with *cdc20-1* show some variability, there is no consistent difference between the rate at which the *cdc20-1* and *cdc20-1 mad* double mutants die.

The arrest of proteolysis-defective mutants is checkpoint independent: Experiments in budding yeast and frog egg extracts support the idea that the spindle checkpoint prevents anaphase by inhibiting the activation of the cyclin proteolysis machinery (Hoyt *et al.* 1991; Li and Murray 1991; Minshull *et al.* 1994; Hardwick and Murray 1995). If the critical event for inducing anaphase is the activation of this machinery, mutations that block the proteolysis of mitotic cyclins should arrest the cell cycle in mitosis, even in cells that lack the spindle checkpoint. We tested this hypothesis by examining the effect of *mad* mutations on the arrest phenotype of *cdc15* and *cdc23*, two mutations with defects in the proteolysis of mitotic cyclins. The *cdc23-1* mutation affects a component of the anaphase-promoting complex, the multiprotein complex that catalyzes the ubiquitination of cyclin B and Pds1, a protein that regulates the cohesion of sister chromatids (Irniger *et al.* 1995). In contrast, Cdc15 is a protein kinase whose inactivation arrests cells in anaphase, apparently because they are unable to completely degrade cyclin B (Schweitzer and Philippsen 1991; Zachariae and Nasmyth 1996; Jaspersen *et al.* 1998).

We combined *cdc23-1* and *cdc15* with *mad* mutations and examined the phenotypes of the double mutants at the nonpermissive temperature (Figure 4, A and B). In all cases, the results were the same: the arrest phenotype of the double mutants was indistinguishable from that of the single proteolysis mutants.

We also examined the interaction of the *mad* mutations with *cdc20-1*, which results in arrest in mitosis with increased numbers of microtubules. Cdc20 is a member of a conserved family of proteins that binds to the APC and plays an essential but poorly defined role in APC-mediated proteolysis (Fang *et al.* 1998b; Kallio *et al.* 1998). Evidence in budding yeast suggests that Cdc20 preferentially targets Pds1 rather than cyclin B for destruction, whereas Hct1/Cdh1, another member of this family, shows a preference for ubiquitination of cyclin B and is responsible for the destruction of cyclin B in G1-arrested cells (Schwab *et al.* 1997; Visintin *et al.* 1997). Drosophila mutations in *fizzy*, the homolog of *CDC20*, arrest the embryonic cell cycle in metaphase and block the destruction of both cyclins A and B (Dawson *et al.* 1995; Sigrist *et al.* 1995). We compared the phenotypes of *cdc20-1 mad* double mutants with *cdc20-1* mutants (Figure 4C). Like *cdc20-1* mutants, the *cdc20-1 mad* double mutants arrested in mitosis as large-budded cells (data not shown) and remained viable, demonstrating that this arrest is independent of the spindle checkpoint.

A specific checkpoint arrests the cell cycles of cells whose DNA has been damaged (Weinert and Hartwell 1988). The *cdc13-1* mutation leads to the accumulation of single-stranded DNA at telomeres, and it arrests cells with a G2 DNA content and a short spindle (Garvik *et al.* 1995). This arrest is completely suppressed by mutations in the DNA damage checkpoint (Lydall and Weinert 1995). To test whether DNA damage could activate the spindle checkpoint, we examined the interaction between *cdc13* and *mad* mutations (Figure 5). By this test, the *cdc13-1* arrest was independent of the spindle checkpoint, since *cdc13-1 mad* double mutants, like the *cdc13-1* single mutant, remained arrested as large-budded cells when shifted to 37° (data not shown), and the single and double mutants die at similar rates during prolonged incubation at 37°. These findings show that the components of the spindle checkpoint are not needed for the DNA damage checkpoint. Weiss and Winey (1996) reached similar conclusions to ours by showing that the spindle checkpoint mutation *mps1-1* failed to overcome the cell cycle arrest caused by DNA

damage in the *cdc9-1* and *cdc13-1* mutations or the arrest caused by defects in APC-mediated proteolysis in *cdc16-1*, *cdc20-1*, and *cdc23-1* mutations.

DISCUSSION

We have analyzed the defects that the spindle checkpoint detects. Cells with defects in microtubule polymerization or spindle pole body duplication die rapidly at the nonpermissive temperature if they lack the spindle checkpoint. Spores with defects in the checkpoint and microtubule motors or a kinetochore component survive at a greatly reduced frequency, suggesting that the survival of cells with these defects depends on the integrity of the spindle checkpoint. In contrast, mutations that inhibit the anaphase-promoting complex or activate the DNA damage checkpoint arrest the cell cycle independently of the spindle checkpoint. These observations confirm the original conclusion that the spindle checkpoint is restricted to monitoring the function of the mitotic spindle (Hoyt *et al.* 1991; Li and Murray 1991).

Benomyl and nocodazole treatments and the *tub2-403* mutation all reduce the stability of yeast microtubules in both the spindle and the cytoplasm (Huffaker *et al.* 1988; Jacobs *et al.* 1988; Hoyt *et al.* 1990; Stearns *et al.* 1990). These perturbations all induce a cell cycle arrest or delay that is abolished by *mad* (Li and Murray 1991) and *bub* mutations (Hoyt *et al.* 1991). Tubulin mutants with defects that are restricted to cytoplasmic microtubules pass through mitosis and produce two

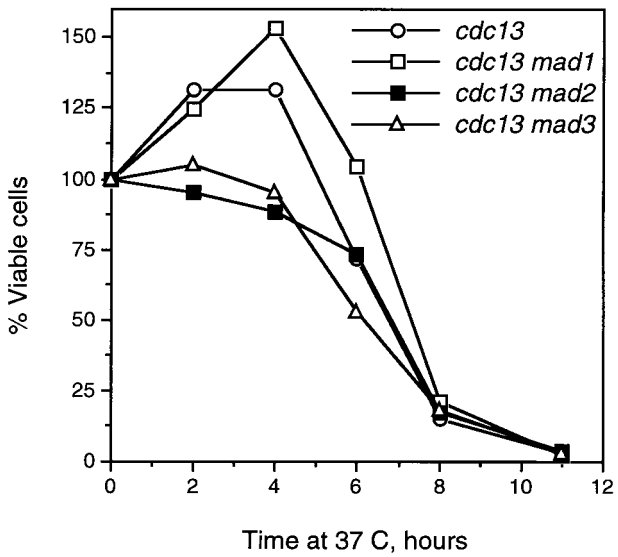

Figure 5.—*mad* mutations do not affect the behavior of a mutation that activates the DNA damage checkpoint. The indicated *cdc13* strains were arrested in G1 by nutrient starvation at 23° and released into rich medium at 37°. Samples were taken at the indicated time points, diluted, and plated for viability on rich medium at 23°. Values are expressed relative to the number of viable cells at time zero.

daughter nuclei (Sullivan and Huffaker 1992). Although these cells initiate anaphase normally, they are defective in the positioning of the nucleus within the cell, and as a result, both daughter nuclei are often found in one of the two progeny cells (Sullivan and Huffaker 1992). This observation suggests that the spindle checkpoint only monitors the intranuclear spindle and does not respond to defects in nuclear positioning during mitosis. Examination of mutations that affect spindle positioning suggests that there is an independent checkpoint that delays the completion of nuclear division and cytokinesis in cells that have undergone anaphase but have not succeeded in placing one set of chromosomes in the bud (Yeh *et al.* 1995; Li *et al.* 1998).

In contrast to the *tub2-403* mutant or nocodazole-treated cells, the number of spindle microtubules in the *cdc20-1* mutant is greater than that in wild-type cells (O'Toole *et al.* 1997). The mitotic arrest in *cdc20-1* cells is not eliminated in *mad* mutants, raising the possibility that the Mad-dependent checkpoint is not involved in detecting spindle lesions caused by excess microtubules. We favor two alternative possibilities, either that *cdc20* mutations have direct effects on the anaphase-promoting complex and indirect effects on microtubule distribution, or that the *cdc20-1* allele is closely linked to another mutation that affects the number of spindle microtubules. The latter idea is supported by the observations that mutations in *fizzy*, the Drosophila homolog of Cdc20, do not have obvious effects on spindle morphology, but arrest cells in mitosis and prevent the proteolysis of both cyclins A and B (Dawson *et al.* 1995; Sigrist *et al.* 1995). Since cyclin A is not protected from proteolysis by the spindle checkpoint in frog (Minshull *et al.* 1994) and clam (Hunt *et al.* 1992) eggs, the phenotype of the fly mutant suggests that the arrest of *cdc20/fizzy* mutants reflects a defect in some step required for cyclin proteolysis rather than a defect in activation of the spindle checkpoint. This suggestion is strengthened by the observations that Cdc20 and its relative, Hct1, appear to act as specificity factors for APC-mediated protein degradation (Schwab *et al.* 1997; Visintin *et al.* 1997), and that Cdc20 and its homolog in fission yeast are targets that the spindle checkpoint inhibits to produce a mitotic arrest (Hwang *et al.* 1998; Kim *et al.* 1998). Finally, independently isolated, temperature-sensitive *cdc20* alleles or extensively backcrossed *cdc20-1* strains show a mitotic arrest without increased antimicrotubule staining compared to other metaphase-arrested mutants (A. Amon, personal communication; L. Hwang and A. W. Murray, unpublished data).

Mutations that affect spindle pole body function can also activate the *MAD*-dependent checkpoint. *mps2-1*, a monopolar spindle mutant, arrests in mitosis, and *mad mps2-1* double mutants die faster than *mps1* mutants. Our attempts to test the interaction of the checkpoint with other spindle pole body mutations, such as *cdc31* and *ndc1*, have been frustrated by the rapid diploidiza-

tion of these mutant strains, which hampers genetic analysis. Mutations in Mps1, a protein kinase whose activity is required for spindle pole body duplication, also inactivate the spindle checkpoint (Hardwick *et al.* 1996). *mps1-1* mutants fail to arrest in response to the spindle pole body defects or microtubule depolymerization (Hardwick *et al.* 1996; Weiss and Winey 1996). This analysis suggests that like the *mad* mutants, *mps1* mutants fail to respond to several different defects in the spindle. Although the role of Mps1 in spindle pole body duplication makes it tempting to speculate that the spindle pole is involved in the checkpoint, there is no evidence that Mps1 is localized to the spindle pole body and that spindle pole body duplication and checkpoint functions of Mps1 clearly occur at different points in the cell cycle (Winey *et al.* 1991; Hardwick *et al.* 1996; Weiss and Winey 1996).

Preventing microtubule polymerization or spindle pole body duplication leads to gross defects in spindle structure that clearly make normal chromosome segregation impossible. As a result, it has been possible to isolate conditional lethal alleles in genes involved in spindle pole body duplication and microtubule polymerization. In contrast, mutants that lack single microtubule motors are viable, suggesting that there is considerable overlap between the functions of different motors involved in spindle and chromosome segregation (Hoyt *et al.* 1992; Roof *et al.* 1992). Such overlap may reflect the formidable task of assembling an enormous macromolecular assembly that will capture, align, and then accurately segregate the chromosomes. The spindle checkpoint may help spindles function correctly in the presence of environmental perturbations, stochastic defects in chromosome alignment, and mutant alleles of genes involved in spindle function. The interaction of motor mutants with the spindle checkpoint supports this idea. All double-mutant combinations of *mad* mutations with *cin8* or *kar3*, except *mad3 kar3*, show greatly reduced spore viability, indicating that the survival of strains with nonlethal defects in the spindle depends on the action of the spindle checkpoint.

The final spindle component we examined was the kinetochore, the specialized structure that attaches chromosomes to microtubules. Evidence in insect spermatoctyes (Li and Nicklas 1995), mammalian tissue culture cells (Rieder *et al.* 1995), and budding yeast (Wells and Murray 1996) indicates that normal kinetochores that are not attached to or improperly aligned on the spindle can activate the spindle checkpoint. In addition, a subset of mutations in the centromeric DNA that impair kinetochore function (Spencer and Hieter 1992), as well as a temperature-sensitive mutation in *CTF13*, an essential kinetochore function, both induce a mitotic delay that depends on the spindle checkpoint (Wang and Burke 1995; Pangilinan and Spencer 1996). We tested whether elimination of a nonessential kinetochore component engaged the checkpoint. The Cbf1 protein binds to the CDEI element of centromeric DNA, and although this interaction is not essential for viability, disrupting it increases the frequency of chromosome loss (Cai and Davis 1990). The *mad1 cbf1* and *mad2 cbf1* double mutants show greatly reduced spore viability, suggesting that the survival of *cbf1* mutants depends on the ability of the spindle checkpoint to delay anaphase until chromosomes with compromised kinetochore function have aligned correctly on the spindle.

How many different aspects of the spindle does the spindle checkpoint monitor? If there were multiple detection systems that each monitored a single feature of the spindle, different checkpoint mutants could inactivate different detection systems, with the result that the different mutants would fail to respond to different subsets of spindle defects. For example, a mutation that inactivated surveillance of the spindle pole body but had no effect on kinetochore monitoring would not arrest the cell cycle in cells with unduplicated spindle pole bodies, but it would still arrest in response to defects at the kinetochore. We have not observed this type of qualitative specificity. The *mad1*, *mad2*, and *mad3* mutations show similar interactions with a wide range of spindle defects. The only exception to this conclusion is the observation that *mad3* mutations allow the survival of *kar3* or *cbf1* mutants, whereas the *mad1* and *mad2* mutations do not. This result is not easy to interpret. In a quantitative assay, interactions of different strengths can easily be compared with each other. In a qualitative assay, however, such as the assessment of whether two mutations are synthetically lethal with each other, a continuous variable (the strength of the interaction between two mutations) is converted into an all-or-none output. Thus, there are two possible interpretations of the observation that *mad3* is not synthetically lethal with *kar3* or *cbf1*: (i) compared to wild-type cells, *mad3* reduces the delay that these mutations cause, but the reduced delay is still sufficient to allow a colony to grow; or (ii) the delay these mutations cause is the same as that in wild-type cells. Distinguishing between these possibilities is likely to require conditional alleles of checkpoint genes.

Although we cannot rule out the future discovery of mutants that fail to respond to a specific spindle defect, we favor the idea that the spindle checkpoint monitors only one aspect of the spindle, the interaction between kinetochores and microtubules. Three arguments support this hypothesis. The first is that defects in microtubule polymerization, formation of a bipolar spindle, microtubule motors, and the kinetochore will all impair the ability of the kinetochore to capture and move along microtubules. The second is that the *ndc10-1* mutation, which disrupts kinetochore function, abolishes the ability of nocodazole to arrest the budding yeast cell cycle (Tavormina *et al.* 1997). The third is that many components of the spindle checkpoint have been found at the kinetochore and respond to changes in kinetochore-

microtubule interactions. One is an unidentified phosphoepitope, detected with the 3F3/2 antibody, which is present only on those kinetochores that are not both attached to microtubules and under tension (Gorbsky and Ricketts 1993; Nicklas *et al.* 1995). The others are the vertebrate homologs of the budding yeast Mad1 (Chen *et al.* 1998; Jin *et al.* 1998), Mad2 (Chen *et al.* 1996; Li and Benezra 1996), Bub1 (Taylor and McKeon 1997; Chan *et al.* 1998; Jablonski *et al.* 1998), and Bub3 (Basu *et al.* 1998; Taylor *et al.* 1998) checkpoint proteins. All these proteins are preferentially recruited to those kinetochores that have not attached to microtubules, suggesting that their recruitment plays an important role in monitoring the interaction between kinetochores and microtubules. Future studies should provide molecular details of how cells monitor the interaction between kinetochores and microtubules and then use this information to regulate the progress of the cell cycle.

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