**Mad1p, a Phosphoprotein Component of the Spindle Assembly Checkpoint in Budding Yeast**

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**Abstract.** The spindle assembly checkpoint prevents cells from initiating anaphase until the spindle has been fully assembled. We previously isolated mitotic arrest deficient (mad) mutants that inactivate this checkpoint and thus increase the sensitivity of cells to benomyl, a drug that interferes with mitotic spindle assembly by depolymerizing microtubules. We have cloned the MAD1 gene and show that when it is disrupted yeast cells have the same phenotype as the previously isolated mad! mutants: they fail to delay the metaphase to anaphase transition in response to microtubule depolymerization.

MAD1 is predicted to encode a 90-kD coiled-coil protein. Anti-Madlp antibodies give a novel punctate nuclear staining pattern and cell fractionation reveals that the bulk of Madlp is soluble. Madlp becomes hyperphosphorylated when wild-type cells are arrested in mitosis by benomyl treatment, or by placing a cold sensitive tubulin mutant at the restrictive temperature. This modification does not occur in Gl-arrested cells treated with benomyl or in cells arrested in mitosis by defects in the mitotic cyclin proteolysis machinery, suggesting that Madlp hyperphosphorylation is a step in the activation of the spindle assembly checkpoint.

Analysis of Mad1p phosphorylation in other spindle assembly checkpoint mutants reveals that this response to microtubule-disrupting agents is defective in some (mad2, bubl, and bub3) but not all (mad3, bub2) mutant strains. We discuss the possible functions of Mad1p at this cell cycle checkpoint.

**Accurate chromosome replication and segregation require mechanisms that can detect errors in these processes and initiate two responses: repair systems that correct the errors, and delay mechanisms that prevent the cell cycle from proceeding while the repairs are in progress. The combined detection and delay systems are known as cell cycle checkpoints or feedback controls (reviewed in Hartwell and Weinert, 1989; Murray, 1994, 1995).

Genetic analyses in both budding and fission yeast have identified a number of genes that play a role in ensuring that cells complete DNA replication and repair DNA damage before they enter mitosis (Weinert and Hartwell, 1988; Enoch and Nurse, 1990; Al-Khodairy and Carr, 1992; Rowley et al., 1992; Enoch et al., 1992; Weinert et al., 1994; Al-Khodairy et al., 1994, 1995; Ford et al., 1994). For example, the RAD9 gene is required to arrest the cell cycle in response to DNA damage, but is not required for the process of DNA repair (Weinert and Hartwell, 1988). This cell cycle control is not essential for growth in the absence of DNA damage, but irradiated rad9 cells are unable to delay their passage through mitosis in response to the damaged DNA and produce lethally damaged progeny.

The mitotic arrest caused by microtubule polymerization inhibitors suggests the existence of a spindle assembly checkpoint that monitors the status of the spindle and regulates the metaphase to anaphase transition. The ability of single improperly oriented chromosomes (Callan and Jacobs, 1957; Li and Nicklas, 1995; Rieder et al., 1994), partially defective centromeres (Spencer and Hieter, 1992), and excess centromeres (Futcher and Carbon, 1986; Wells, W., and A. W. Murray, unpublished results) to delay anaphase suggests that this checkpoint can detect subtle as well as gross defects in the structure of the spindle. Mutants in the spindle assembly checkpoint have been identified in budding yeast. The bub (budding uninhibited by benzimidazole; Hoyt et al., 1991) and mad (mitotic arrest deficient; Li and Murray, 1991) mutants were isolated from collections of mutants that failed to form colonies in the presence of low doses of benomyl, a microtubule polymerization inhibitor. The mad mutants were distinguished from mutants that affected microtubule assembly by their inability to delay cell division in response to microtubule depolymerization. The divisions that take place in the absence of a fully functional spindle lead to a high frequency of chromosome loss and cell death (Li and Murray, 1991). The spindle assembly checkpoint defined by the mad and...
bub mutants is expected to consist of components that detect defects in spindle structure, and a signal transduction pathway that relays this information and ultimately inhibits a target in the machinery that initiates sister chromatid separation and anaphase. In common with other cell cycle transitions a major regulator of the metaphase to anaphase transition is thought to be the kinase activity of Cdc28p-mitotic cyclin complexes (for reviews see Norbury and Nurse, 1992; Morgan, 1995). Experiments in frog egg extracts and in yeast have shown that the ubiquitin-mediated proteolysis of mitotic cyclins and other proteins is required for sister chromatid separation and entry into the next cell cycle (Holloway et al., 1993; Surana et al., 1993).

Little is known about the biochemical mechanism of the spindle assembly checkpoint. Previous work has shown that BUB1 encodes a protein kinase (Roberts et al., 1994) and that BUB2, BUB3 (Hoyt et al., 1991), and MAD2 (Chen, R-H., and A. W. Murray, unpublished results) encode proteins with unknown biochemical functions. Here we report the cloning of the MAD1 gene and show that it encodes a novel nuclear phosphoprotein that is an essential component of the spindle assembly checkpoint, but is not essential for growth under normal conditions. We find that this protein is hyper-phosphorylated in conditions that activate the spindle assembly checkpoint. The hyper-phosphorylation of Mad1p is only seen in a subset of mad and bub mutants allowing us to provisionally order the function of the known BUB and MAD genes.

Materials and Methods

Yeast Strains and Media

Table 1 lists the strains used in this work. The original mad1 mutants were isolated in the A364A background. All other strains are derivatives of W303, except the bub3A strain MAY 2072 (Hoyt et al., 1991) and the tub2-403 (KH 152) and corresponding TUB2 control (KH146) strain which are S288C derived. Media were prepared and genetic manipulations were performed as described (Sherman et al., 1974). Stock solutions of inhibitors were: benomyl 30 mg/ml in DMSO (added to hot media to maintain solubility), hydroxyurea 200 mg/ml in water, and a-factor 10 mg/ml in DMSO. Microcolony assays were carried out as previously described (Li and Murray, 1991).

Cloning of MAD1

Earlier attempts to clone the MAD1 gene using centromeric and multi-copy libraries were unsuccessful, although we isolated a number of multi-copy suppressors that conferred benomyl resistance on a variety of mad mutants and a mutant (cin1A) with defects in microtubule polymerization (Sears et al., 1990). These suppressors included the transcription factors YAP1 (also named PARI, SNQ2, and PDR4) and CAND1 along with a novel member of the hsp70 family, whose sequence will be reported elsewhere. The two transcription factors had been previously isolated in multi-copy suppressor screens due to their ability to confer multi-drug resistance on yeast cells (Moye-Rowley et al., 1989; Hussain and Lenard, 1991; Schaeil et al., 1992; Wu et al., 1993).

Assuming that the MAD1 gene was not present, or was at best poorly represented, in the available libraries, we constructed a new YCP50-based genomic library. We prepared genomic DNA as previously described (Philippsen et al., 1991) from W303 strain KH 34, carried out a partial SacI digest, isolated 6-10-kb fragments, and ligated them to BamHI cut and phosphatase-treated YCP50 (Rose et al., 1987). This ligation was transformed into Escherichia coli strain XL1-Blue MRF (Strategene, La Jolla, CA) and four pools of ~10,000 colonies each were used to isolate plasmid DNA. This library DNA was then used to transform the mad1-1 strain KH 120. After two to three days of growth on uracil-free plates the Ura+ yeast transformants were scraped off, diluted, and replated onto YPD plates containing 10 µg/ml benomyl. Plasmid DNA was prepared (Ward and Kirschner, 1990) from benomyl-resistant colonies and individual plasmid isolates were re-tested for their ability to confer benomyl resistance to KH 120. One plasmid, pKH130, with a 6.2-kb insert, had this property. A 3.2-kb HindIII-Sal fragment from this plasmid was sufficient to complement mad1 strains. This fragment contained unique Xhol and Xbal sites. When either the Xhol or the XbaI restriction site was filled in, the resulting clone no longer complemented the mad1 mutant, suggesting that these sites lay within, or near, the complementing gene.

To check that the HindIII-Sal fragment contained the MAD1 gene rather than a suppressor we cloned the flanking HindIII fragment into the URA3-containing integration vector pRS306 (Sikorski and Hieter, 1989). This construct was cut with BglIII then transformed into the mad1, ura3 strain, KH 120, to create a mad1 strain in which the genomic locus of the cloned gene was marked with URA3. This strain was then mated with KH 35, a MAD1, ura3 strain, the resulting diploids were sporulated and tetrads were dissected. In 10 tetrads the mad phenotype co-segregated with the Ura+ spores, confirming that the sequences in pKH130 are closely linked to MAD1.
Sequencing, Mapping, and madl Gene Disruptions

We sequenced the whole of the 3,188-bp HindIII-SalI fragment, after cloning various fragments into pBluescript and making EcoHI deletion series (Henikoff, 1984). We found a single large open reading frame that is predicted to encode a protein of 749 amino acids. The sequence of this reading frame was completed and confirmed on the second strand using specifically designed primers.

The Mad1 reading frame was used to make a digoxigenin-labeled probe with a DNA labeling kit (Boehringer-Mannheim Corp., Indianapolis, IN) following the manufacturer’s instructions. This was used to probe a set of filters purchased from the American Type Culture Collection (Rockville, MD) that have DNA from lambda and cosmid clones covering over 96% of the genome of Saccharomyces cerevisiae cross-linked to them. The Mad1 probe specifically recognized a single lambda clone (70269) thereby mapping it to the left arm of chromosome VII between RAD6 and CYP2.

Two madl gene disruptions were made (see Fig. 1): one, madlΔ1, replaces the HindII fragment from nucleotides 994 to 2,186 with a BamHI fragment containing the HIS3 gene (pKH149). The other, madlΔ2, replaces almost the complete Mad1 reading frame from the BgllI site at position 76 to the XmnI site at position 1911 with the URA3 marker (pKH160).

Preparation of Antibodies against Madlp, Immunoblotting, and Immunofluorescence

The BgllI fragment encoding residues 27 to 310 of Madlp was cloned into the BamHI site of pGEX3X (Smith and Johnson, 1988). This glutathione-S-transferase (GST) fusion construct was transformed into E. coli strain HB101 and its expression induced with 0.1 mM IPTG for 3 h at room temperature. Cells were pelleted and resuspended on ice with PBS (140 mM NaCl, 1.8 mM KH₂PO₄, 138 mM NaCl, 2.7 mM KCl, pH 7.2), repelleted, and frozen in liquid nitrogen. The frozen cells were resuspended in five volumes of PBS containing 1 mM EDTA, 1 mM EGTA, 1 mM MgCl₂, 200 mM PMSF, 0.5 μg/ml lysozyme, and 5 μg/ml DNase. To complete their lysis the cells were sonicated briefly. KCl was added to 0.25 M and DTT to 15 mM and the lysate was spun at 35,000 rpm in a rotor (model 50.2 T; Beckman Instruments, Palo Alto, CA) for 1 h. The supernatant was then loaded onto a 10 ml glutathione agarose column (Sigma Chemical Co., St. Louis, MO) which was then washed with PBS (0.25 M KCl and 0.5 mM DTT). The Mad1-GST fusion protein was eluted with 5 mM reduced glutathione in 50 mM Tris, pH 8.1, 0.25 M KCl. The peak fractions were dialyzed extensively into 50 mM Hepes, pH 7.6, 50 mM KCl, 1 mM MgCl₂, 0.1% NaDeoxycholate, 10% glycerol, LPC, 1 mM PMSF, 0.5 mM DTT. The lysate was spun briefly to remove the glass beads and cell debris and then spun for 30 min at 55,000 rpm in a Beckman TL100.2 rotor. 200 μl was loaded onto a Superose 6 10/30 column (Pharmacia Biotech Inc., Piscataway, NJ) running at 0.5 ml/min and 0.5 ml fractions were collected. For sucrose-gradient centrifugation a bead-beaten extract was made in 50 mM Hepes, pH 7.6, 50 mM KCl, 50 mM NaF, 1 mM MgCl₂, 1 mM EGTA, 0.1% NaDeoxycholate, 10% glycerol, LPC, 1 mM PMSF, 0.5 mM DTT, and spun for 30 min at 55,000 rpm in a TL100.2 rotor. A 2 ml sucrose gradient (5–40%) was made using a SG5 gradient mixer (Hoefer Scientific Instruments, San Francisco, CA), 100 μl of extract was loaded on top and spun in a TLS55 swinging-bucket rotor at 55,000 rpm for 14 h. 75 ml fractions were taken from the top of the gradient. The sucrose gradient and the gel filtration column were calibrated with markers from a Pharmacia high molecular weight calibration kit.

For immunofluorescence, spheroplasts were prepared as above then fixed with 3.7% formaldehyde for 5 to 30 min, gently sedimented, washed, and resuspended in 0.7 M sorbitol, 0.1 M KPO4, pH 7.5, before being attached to poly-lysine-coated multi-well microscope slides. The slides were plunged into methanol (−20°C) for 5 min and acetone (−20°C) for 30 s, and then allowed to air dry. After washing with PBS, cells were blocked for 30 min with blotto, and then stained overnight at 4°C with primary antibody diluted (1:5,000 for anti-Madlp, 1:200 for anti-tubulin, 1:1,000 for anti-Raclp, 1:2,000 for mAb 414) in blotto. Cells were washed several times with blotto and then incubated for 1 h at room temperature with a 1:50 dilution of FITC or rhodamine-labeled anti-rabbit, mouse or rat secondary antibodies from Cappel (Organon Teknika Corp., Durham, NC). After washing with blotto and then PBS, the DNA was stained with 1 μg/ml DAPI in PBS and cells were mounted in 90% glycerol, 1 mg/ml phenylendiamine, pH 9.0. Coverslips were sealed with clear nail polish and slides stored at −20°C.

Phosphate Labeling and Phosphatase Digestion

Cells were grown overnight in low-phosphate YPD medium (Rubin, 1975), diluted into fresh medium and grown to a late exponential phase. 1 μg [³²P]orthophosphate (Amersham Corp.) was added to 5 ml cultures which were shaken at room temperature for one hour. Cells were pelleted, washed, and resuspended on ice in RIPA buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton-X 100, 1% NaDeoxycholate, 0.1% SDS, 1 mM PMSF, LPC, 1 mM DTT, 50 mM NaF, 1 mM NaVanadate). An equal volume of glass beads was added and the cells were bead-beaten for 2 min at 4°C. Extracts were clarified, diluted with RIPA buffer and the following: 10 μl of anti-Madlp antibody and 10 μl of protein A-Sepharose 6MB (Pharmacia) beads were added and incubated overnight at 4°C. Immunoprecipitates were washed several times with RIPA buffer, twice with PBS and once again with RIPA before heating the beads in sample buffer and separating the sample by SDS-PAGE. Gels were stained, fixed, dried down, and exposed to film overnight at −80°C.

1. Abbreviations used in this paper: GST, glutathione-S-transferase; LPP, lambda protein phosphatase.
For lambda protein phosphatase (LPP) treatments whole cell extracts were made by bead-beating yeast cells for 2 min in lysis buffer (50 mM Heps, pH 7.6, 75 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, LPC, 50 mM NaF, 1 mM NaVanadate) and DTT was added to 1 mM after clarification. Madlp was immunoprecipitated as described above and the immunoprecipitate was washed three times with lysis buffer, twice with PBS and then with LPP reaction buffer (50 mM Tris, pH 8.0, 2 mM MnCl₂, 1 mM PMSF, LPC, 1 mg/ml acetylated BSA). 20 U of lambda protein phosphatase (New England Biolabs Inc. Beverly, MA) were added to the Madlp immunoprecipitate in 50 µL of LPP reaction buffer and incubated for 30 min at 30°C. Phosphatase inhibitors were 2 mM ZnCl₂, 1 mM NaVanadate and 50 mM NaF. The Sepharose beads were then spun down and heated in sample buffer before separating the sample by SDS-PAGE and analyzing Madlp by immunoblotting.

Results

MAD1 Is Predicted to Encode a Novel Coiled-Coil Protein

We cloned the wild-type MAD1 gene by complementing the benomyl sensitivity of the madi-1 mutant. The complementing plasmid rescued all three madi alleles, did not rescue other benomyl sensitive mutants, and contained a DNA fragment that is closely linked to the MAD1 gene (see Materials and Methods). Sequencing the minimal complementing DNA fragment revealed a 2,250-bp reading frame which spans both an Xhol site and an XbaI site. Filling in either the Xhol site or the XbaI site destroyed the ability of this fragment to complement madi. Two gene disruptions were made, one replacing the 3' half of the MAD1 gene with HIS3 and a second that replaced almost the whole reading frame with the URA3 gene (see Fig. 1 and Materials and Methods). These constructs were used to transform haploid and diploid yeast strains. The diploids, all temperatures tested, are sensitive to anti-microtubule (ORF).

Figure 1. Restriction map of the MAD1 clone and gene disruptions. (a) Restriction map of the MAD1 clone isolated from the YCP50 library showing the position of the open reading frame (ORF). Vector sequences are indicated by the thicker line. (b) Restriction map of the open reading frame (ORF). (c) The bar marks the HinclI fragments that were replaced with the HIS3 gene to create madiΔ1. (d) The bar marks the BglII-XmnI fragment that was replaced with the URA3 gene to create madiΔ2.

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Figure 2. (a) Nucleotide sequence and translation of the MAD1 gene. An unusual stretch of asparagine residues and the putative bi-partite nuclear localization signal are underlined. This sequence is available from Genbank/EMBL/DDBJ under accession number U14632. (b) GCG PEPCOIL prediction for Madlp. This GCG PEPCOIL prediction uses the algorithm of Lupas et al. (1991) with a window of 28 residues.

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indeed have a high probability of forming coiled-coil (see Fig. 2 b) (Lupas et al., 1991). The sequence of Madlp also contains a putative bi-partite nuclear localization sequence (residues 506–527) (Dingwall and Laskey, 1991), many potential phosphorylation sites and an unusual stretch rich in asparagine residues (residues 330–372).

**The MAD1 Gene Encodes a Component of the Spindle Assembly Checkpoint**

We studied madlA strains for defects in the spindle assembly checkpoint. The benomyl sensitivity of the original mad mutants could be rescued with hydroxyurea, a drug that lengthens S phase (Li and Murray, 1991). Since budding yeast can assemble a spindle during S phase, our interpretation of this result is that the enforced delay in S phase allows mad cells enough time to correctly assemble their mitotic spindle before initiating anaphase. Although 1 mg/ml hydroxyurea slightly decreases the benomyl sensitivity of wild-type cells, it does not rescue the benomyl sensitivity of mutants with defects in microtubule polymerization, such as cin1Δ (Fig. 3 a), even at lower concentrations of benomyl (data not shown). Fig. 3 a shows that the mad1Δ.1 deletion mutant behaves like the original mad1 and mad2-1 alleles: at 23°C it is sensitive to benomyl at concentrations of 7.5 μg/ml and above, and this benomyl sensitivity can be rescued by 1 mg/ml hydroxyurea.

We used microcolony assays to monitor the initial rate of cell division of mad1Δ.1 strains on benomyl-containing medium. Individual, unbudded cells were picked with a dissecting needle and their growth on plates containing 12 μg/ml benomyl was analyzed over a period of 8 hours. Fig. 3 b shows that, like the original mad1 alleles, the mad1Δ.1 strain initially divided faster than the wild-type control cells. This behavior of mad1Δ.1 differs radically from strains with defects in microtubule polymerization, which are unable to divide in the presence of benomyl and arrest as large budded cells. This result is consistent with mad1Δ.1 cells being unable to sense or respond to the disruption of their microtubules and thus initiating anaphase before spindle assembly is complete. It has previously been shown that mad mutants fail to maintain the mitotic activity of Cdc28p (monitored as histone H1 kinase activity) when they are treated with benomyl (Li and Murray, 1991). We have confirmed this result with mad1Δ.1 (data not shown).

**Madlp Is a Soluble Nuclear Protein**

To analyze the properties of Madlp we raised polyclonal antibodies, against a GST fusion protein that contained

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*Figure 3. Benomyl sensitivity of the mad1Δ.1 strain (KH 123). (a) Yeast cells (KH 34, KH 123, KH 132, and KH 40) were spotted onto YPD plates containing the indicated concentrations of benomyl and hydroxyurea and were photographed after 3 d of growth at 23°C. (b) Microcolony analysis of the growth of yeast cells (KH 34, KH 123, and KH 40) on YPD plates containing 12 μg/ml benomyl at 23°C. Data was collected from at least 60 cells of each genotype.*

*Figure 4. Immunoblots of yeast extracts with anti-Madlp. (a) Whole cell extracts made from mad1Δ.1 cells (KH 123), wild-type cells (KH 34) or wild-type cells containing the MAD1 gene on a centromeric (pKH130) or two-micron (pKH137) plasmid were immunoblotted with affinity-purified anti-Madlp antibodies. (b) Whole cell extracts made from wild-type (KH 34), the three original mad1 alleles (BEN 24, 27, and 76), or the two mad1 deletion strains constructed (KH 123, KH 131), were immunoblotted with affinity-purified anti-Madlp antibodies. The truncated protein expressed in mad1Δ.1 is indicated (†).*
were stained with affinity-purified anti-Madlp antibodies (b and d) and DAPI (a and c). Patchy nuclear staining is observed in wild-type cells and is absent in the

Figure 5. Immunofluorescent staining of yeast cells with anti-Madlp. Wild-type (a and b) (KH 34) and mad1ΔI (c and d) (KH 123) cells were stained with affinity-purified anti-Madlp antibodies (b and d) and DAPI (a and c). Patchy nuclear staining is observed in wild-type cells and is absent in the mad1ΔI cells. Wild-type diploid cells (KH 36) were stained with DAPI (e and h), anti-Madlp (f and i), and either anti-tubulin (g) or pooled mAbs to the 90-kD component of the SPB (j). Bars, 10 μm.

their rod shape. To assess the hydrodynamic behavior of Madlp we fractionated yeast extracts by gel filtration and sucrose gradient sedimentation. Fig. 6 b shows that Madlp behaves as a very large protein on gel filtration, running ahead of the 670-kD thyroglobulin marker. A similar size for Madlp was obtained by immunoblotting yeast extracts that had been separated in their native state by gradient gel electrophoresis (not shown). However, Madlp sediments relatively slowly through sucrose gradients, peaking in the same fraction as the 4.4 S BSA marker (Fig. 6 c). Neither the sedimentation nor the gel filtration behavior of Madlp was altered by the presence of 1 M NaCl (not shown). Similar fractionation behavior has been reported

Figure 6. Madlp solubility, sizing and sedimentation analyses. (a) The bulk of Madlp behaves as a soluble protein. Yeast cells (KH 34) were spheroplasted and homogenized. Aliquots of this homogenate were then extracted with either 1% Triton X-100, 1 M NaCl or a combination of 1% Triton, 1 M NaCl, 0.25 mg/ml DNase and RNase, before being spun at 100,000 g for 30 min. Pellet and supernatant fractions were immunoblotted with affinity-purified anti-Madlp antibodies. Yeast extracts were made from wild-type (KH 34) cells by bead-beating, spun at 100,000 g for 30 min, and then (b) loaded over a Superose 6 gel filtration column, or (c) fractionated by centrifugation through a 5-40% sucrose gradient. Fractions were immunoblotted with affinity-purified anti-Madlp antibodies. Madlp is the uppermost band in b and c and the molecular masses of marker proteins are indicated.

the amino-terminal half of Madlp. The serum was affinity purified and used to probe Western blots of whole cell extracts from a number of yeast strains. Fig. 4 a shows that a protein of 90 kD that is seen in the wild-type cell extract is absent from the mad1ΔI strain, and present at higher levels in strains containing MADI plasmids. A truncated Mad1 protein is expressed in mad1ΔI where the 3' end of the MADI gene has been removed, and in two of the three previously isolated mad1 alleles (Fig. 4 b). The latter finding provides strong evidence that the reading frame we have identified is indeed the MADI gene and that the antisemur efficiently recognizes both wild-type and a number of mutant Mad1 proteins. The affinity-purified anti-Madlp antisemur still recognizes a number of other proteins in addition to Madlp on immunoblots of whole yeast cell extracts, including a prominent band at 85 kD. However, the cross-reacting proteins are not immunoprecipitated by the antibody (see Fig. 9), nor are they recognized well in formaldehyde-fixed yeast cells (Fig. 5), suggesting that they are unlikely to be closely related to Madlp.

Immunofluorescence with affinity-purified anti–Madlp antibodies was used to localize Madlp within yeast cells. Fig. 5 reveals that the bulk of Madlp appears as discrete patches in the nucleus. The reactivity of Madlp is sensitive to fixation. We found that greater than 5 min of formaldehyde fixation dramatically reduced Madlp staining and further fixation completely abolished it (data not shown). We used a number of antibodies to confirm that the structure of the yeast nucleus was relatively intact after fixation. Fig. 5 (e–j) shows that antibodies to tubulin (Adams and Pringle, 1984) and to the 90-kD component of the spindle–pole body (Rout and Kilmartin, 1990) gave the previously reported patterns, and that there was no consistent co-localization of these proteins with Madlp. In addition, we found no co-localization of Madlp with Raplp (a telomere protein; Klein et al., 1992) or nuclear pore antigens (data not shown). Although we observed coincident localization of some of the Madlp staining and spindle pole bodies in a few cells, we conclude that Madlp has a novel nuclear localization pattern.

Since some coiled-coil proteins form large insoluble oligomeric structures (Fuchs, 1994) we tested the solubility of Madlp. Yeast extracts were made by preparing spheroplasts, douncing them and then extracting the homogenate with buffers containing various salts, detergents and nucleases. The extracts were then spun at 100,000 g to separate soluble from insoluble material. Pellet and supernatant fractions were then run over a Superose 6 gel filtration column, or (c) fractionated by centrifugation through a 5-40% sucrose gradient. Fractions were immunoblotted with affinity-purified anti-Madlp antibodies. Yeast extracts were made from wild-type (KH 34) cells by bead-beating, spun at 100,000 g for 30 min, and then (b) loaded over a Superose 6 gel filtration column, or (c) fractionated by centrifugation through a 5-40% sucrose gradient. Fractions were immunoblotted with affinity-purified anti-Madlp antibodies. Madlp is the uppermost band in b and c and the molecular masses of marker proteins are indicated.

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for other coiled-coil proteins, such as myosin and kinesin (Hackney et al., 1992; Hirano and Mitchison, 1994). We therefore propose that Madlp has a large Stokes’ radius and that it may be rod shaped, consistent with the elongated structure of many coiled-coil proteins.

**Modification of Madlp by Phosphorylation**

Does cell cycle progression or activation of the spindle assembly checkpoint alter the abundance or posttranslational modification of Madlp? To answer these questions we examined cells released from a G1 arrest into normal or benomyl containing medium. Wild-type cells were arrested in G1 with α-factor and then released into YPD or YPD containing 15 μg/ml benomyl. Samples were collected over two cell cycles and analyzed for the abundance of Madlp by immunoblotting. To monitor the timing of the cell cycle, these blots were also probed with antibodies to the mitotic cyclin Clb2p which is degraded at the end of mitosis. Fig. 7 shows that the abundance of Madlp remains constant through the cell cycle and is not affected by benomyl-induced mitotic arrest. During a normal cell cycle there is only a minor variation in the gel mobility of Madlp, however a significant fraction of the protein decreases in gel mobility when cells are arrested in mitosis by benomyl treatment (similar results were obtained with nocodazole; data not shown). The low level of Madlp modification seen in untreated cells may be induced by transient spindle defects that occur in the course of normal spindle assembly. Figs. 9 and 10 clearly demonstrate that the slower migrating bands are forms of Madlp and not cross-reacting proteins that accumulate in mitosis.

The slower migrating form of Madlp appears in mitotically arrested cells whose levels of Clb2p are high, suggesting that modification of Madlp is a specific response to a disrupted spindle. To test this hypothesis we asked whether Madlp was modified when cells arrested at Start with α-factor were treated with benomyl (Fig. 8 a). Half of the α-factor–arrested cells were treated with 30 μg/ml benomyl in the continuous presence of α-factor to prevent their passage through Start. The other half were released from their α-factor arrest into YPD containing benomyl. Fig. 8 a shows that benomyl treatment of cells maintained at the α-factor arrest point causes much less modification of Madlp than releasing cells from α-factor arrest into benomyl. The low level of Madlp modification seen after 2 h of combined treatment with α-factor and benomyl probably represents a fraction of the cells that have broken through the α-factor arrest. This experiment shows that the modification of Madlp is not simply a response to benomyl, but is seen only in cells in the portion of the cell cycle where spindle assembly can occur.

To determine whether other mitotic arrests are accompanied by Madlp modification, we analyzed Madlp in number of other mutants. Fig. 8 b shows that the modification of Madlp is not seen in either cdc23 mutants which prevent the ubiquitin-mediated proteolysis necessary to initiate anaphase and thus arrest early in mitosis with short spindles (Irniger et al., 1995), or in cdc15 mutants which arrest later in mitosis with long spindles (Surana et al., 1993). This result demonstrates that Madlp modification...
requires spindle perturbations in addition to an arrest with high levels of Cdc28-Clb-associated kinase activity.

To test whether other methods of disrupting spindle microtubules lead to the modification of Madlp, we compared synchronous cultures of wild-type and cold-sensitive tub2-403 strains (Huffaker et al., 1988) that had been arrested with α-factor and then released at 15°C. Fig. 8c shows that the tub2 mutant arrests in mitosis with high levels of Clb2p and that a significant fraction of Madlp is modified. In addition, we note that Madlp is transiently modified in the wild-type cells, and that this occurs before the levels of Clb2p peak, when the spindle is presumably being assembled. Such modification can be seen in wild-type cells growing at higher temperatures (see Fig. 7) but is less obvious, consistent with it being a response to temperature-induced spindle perturbations (Dustin, 1984).

Is the reduced gel mobility of Madlp due to phosphorylation? To test this possibility we immunoprecipitated Madlp from logarithmically growing and benomyl-arrested cells and treated the immunoprecipitates with lambda protein phosphatase. Fig. 9a shows that phosphatase treatment eliminates the slower migrating forms of Madlp, consistent with the reduced mobility resulting from phosphorylation. Treatment with lambda protein phosphatase in the presence of phosphatase inhibitors has no effect on the gel mobility of Madlp. As not all protein phosphorylations are accompanied by a gel shift we also analyzed Madlp in cells that were metabolically labeled with [32P]orthophosphate. Extracts were prepared from exponentially growing or cell cycle-arrested cultures, immunoprecipitated with the polyclonal anti-Madlp antiserum, and analyzed by gel electrophoresis and autoradiography. Fig. 9b shows that phosphate incorporation into Madlp occurs to equal extents in exponentially growing, benomyl-arrested, and α-factor–arrested cells, suggesting that Madlp is phosphorylated throughout the cell cycle. Our inability to see more slowly migrating phosphate labeled forms of Madlp in benomyl-treated cells may reflect the relatively low level of labeling, or the poor mitotic arrest produced by benomyl treatment in low phosphate medium, or both.

We also examined the phosphorylation of the N-terminal half of Madlp that is expressed in the mad1Δ/ strain. Although 32P is incorporated into this fragment as efficiently as it is into the wild-type protein (Fig. 9), none of the original mad1 alleles nor this truncated protein (not shown) show the mobility shift that occurs when wild type cells are treated with benomyl (Fig. 10a). These findings indicate that Madlp is the target for two types of phosphorylation: constitutive phosphorylation that appears to occur throughout the cell cycle and on both functional and

Figure 9. Madlp is phosphorylated. (a) Madlp was immunoprecipitated from wild-type extracts made from cells (KH 34) growing in YPD (log) or YPD with 30 μg/ml benomyl. The indicated immunoprecipitates were treated with lambda protein phosphatase, in the presence or absence of phosphatase inhibitors (inh), and then immunoblotted with anti-Madlp. (b) Madlp was immunoprecipitated from [32P]orthophosphate-labeled cells. Wild-type cells (KH 34) were grown in low phosphate YPD medium or the same medium containing either 10 μg/ml α-factor or 30 μg/ml benomyl. The mad1Δ1 (KH 123), mad1Δ2 (KH 131), and bub1Δ (KH 127) cells were all grown in low phosphate YPD medium.

Figure 10. Hyperphosphorylation of Madlp in spindle assembly checkpoint mutants. (a) Wild-type (KH 34) and the three original mad1 alleles (BEN 24, 27, and 76) were grown to log phase and treated with 30 μg/ml benomyl for 3 h. Whole cell extracts were immunoblotted with anti-Madlp. (b) Wild-type (KH 34), mad1Δ1 (KH 123), mad2Δ1 (KH 132), mad3Δ (KH 125), bub1Δ (KH 127), bub2Δ (KH 128), and bub3Δ (MAY 2072) strains were grown to log phase, and then treated with 30 μg/ml benomyl for 3 h. Whole cell extracts were made and immunoblotted with anti-Madlp.
Discussion

We have cloned the MAD1 gene and shown that it encodes a novel nuclear phosphoprotein that is predicted to form coiled-coils. Like the previously isolated mad1 alleles, mad1Δ cells are unable to delay initiation of anaphase in response to microtubule depolymerization and thus suffer a high frequency of chromosome loss and rapid death upon treatment with anti-microtubule drugs. Spindle disruption is accompanied by a Mad1p phosphorylation that reduces the mobility of the protein and this modification requires the Bub1p, Bub3p and Mad2p functions, but not Bub2p and Mad3p, suggesting that this phosphorylation plays an important role in the spindle assembly checkpoint.

Antibodies to Mad1p reveal that the protein has a non-uniform distribution within the nucleus. All cells show a diffuse punctate staining and many also have a small number of brightly staining foci. Neither staining pattern reproducibly colocalizes with nuclear pores, spindle-pole bodies or telomeres, suggesting that the Mad1p may be found at a novel location within the nucleus. Identifying the Mad1p-containing structures will require immunoelectron microscopy or isolating Mad1p homologs from organisms with better cytology. In cell extracts Mad1p is largely soluble, and its hydrodynamic behavior suggests that Mad1p has an elongated shape like many other coiled-coil proteins.

Analyzing the gel mobility and phosphate labeling of Mad1p has revealed both a constitutive level of phosphorylation, seen in all stages of the cell cycle that we analyzed, and a hyper-phosphorylation induced by spindle depolymerization. The former phosphorylation is seen in exponentially growing cells as well as in G1 and mitotically arrested cells, suggesting that it occurs throughout the cell cycle. We do not know the identity of the constitutive phosphorylation site(s), or whether their modification plays any role in the spindle assembly checkpoint. The regulated phosphorylation reduces the gel mobility of Mad1p and is seen only in those parts of the cell cycle in which cells are able to assemble a spindle. The modification is detectable at a low level in normally growing cells, and is much more pronounced in benomyl treated cells suggesting that it may be induced by activation of the spindle assembly checkpoint. The low level of Mad1p hyperphosphorylation in wild type cells could either reflect full activation of the spindle assembly checkpoint in a small fraction of the population with transient spindle defects, or weak activation in all cells during the course of spindle assembly. We find that wild-type cells growing in the cold show higher levels of Mad1p modification, and that the tub2-403 mutant arrests in the cold in mitosis with hyperphosphorylated Mad1p, showing that Mad1p modification is not solely a response to drug-induced spindle perturbations. In addition we find that Mad1p receives little modification in cdc23 and cdc15 mutant arrests, ruling out the possibility that the hyperphosphorylation of Mad1p is simply a byproduct of any cell cycle arrest with active Cdc28-Clb2 complexes. But if cdc23 mutants are first arrested at 37°C and then treated with benomyl without releasing them from the arrest Mad1p does become hyperphosphorylated (data not shown), showing that spindle disassembly at this metaphase-like arrest can be readily detected by the checkpoint.

Examining Mad1p hyper-phosphorylation in mad and bub mutants strengthens the case that this modification plays an important role in the spindle assembly checkpoint. There is no mobility shift in mad1-1, mad1-2, mad1-3, or mad1Δ1. This observation suggests that the proper function of Mad1p is needed for its own modification and is especially significant for mad1-2, which encodes an apparently full length protein. The modification of wild-type Mad1p does not occur in the bub1 and bub3 mutants and is strongly reduced in the mad2 mutant. Although the correlative evidence is strong, proof that Mad1p hyperphosphorylation is necessary for the spindle assembly checkpoint will require the identification and mutation of the regulated Mad1p phosphorylation sites.

We assume that the spindle assembly checkpoint must consist of at least three classes of component: a detection system that monitors the structure of the spindle, a signal transduction pathway that relays this information, and a target in the cell cycle machinery whose regulation can produce a mitotic arrest. Even if the mobility shift of Mad1p is no more than a correlation with the activation of the spindle assembly checkpoint, it represents the first tool for ordering the functions in this pathway. The simplest interpretation of our data is that Mad1p lies in the middle of the checkpoint pathway and that mutants that fail to hyperphosphorylate this protein identify proteins that act upstream of Mad1p, whereas mutants that can modify Mad1p identify proteins that act downstream of Mad1p (Fig. 11). Clearly our results do not allow us to rule out other more complicated models. For example, Bub2p and Mad3p need not be directly downstream of Mad1p but could instead be in a parallel pathway whose action is required for cell cycle arrest but not for the hyperphosphorylation of Mad1p. The isolation of dominant alleles of MAD1, and of the other MAD and BUB genes, that cause a mitotic arrest should enable us to order the function of these genes within the spindle assembly checkpoint more precisely.
Previous work identifies three candidates for the physiological Mad1p kinase. The demonstration that Bublp is a spindle assembly checkpoint. See text for details.

Figure 11. A model suggesting the order of function in the spindle assembly checkpoint. See text for details.
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