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Functional Characterization of the Mad1p, a Spindle Checkpoint Protein in Fission Yeast

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Abstract Defects in the mitotic spindle or in the attachment of chromosomes to the spindle are believed to release an activated form of spindle checkpoint complex that inhibits APC-dependent ubiquitination and subsequently arrests the cell cycle at metaphase. When the spindle assembly is disrupted, the fission yeast mitotic arrest deficient (*mad*) mutants fail to arrest and rapidly lose viability. To enhance our understanding of the molecular mechanisms for the pathway of checkpoint function, the functional characterizations of Mad1p from *Schizosaccharomyces pombe* involved in this process have been carried out. Yeast two-hybrid and various deletion analyses of *S. pombe* Mad1p reveal that the C terminus of Mad1p is critical for the binding of Mad2p and maintenance of Mad1p-Mad2p interaction. In addition, it was found that the Mad1p region (residues 206–356) is essential for Mad1p-other checkpoint components. Mad1p truncating this region is sufficient to bind Mad2p but abolishes the checkpoint function, indicating that the checkpoint function is necessary for interaction of Mad1p-other checkpoint components. The possible functions of *S. pombe* Mad1p at the cell cycle checkpoint are discussed.

Key words: *Schizosaccharomyces pombe*, Mad1p, spindle checkpoint, cell cycle

The accuracy of chromosome segregation is dependent upon the correct and timely attachment of sister chromatid kinetochores to the microtubules of the mitotic spindle [25]. This attachment process must be completed before sister chromatid separation at anaphase can take place. Errors in this process can lead to inaccurate separation of sister chromatids, which generate genomic instability that in turn provokes developmental defects, cancer, or death [3, 24]. The kinetochore-microtubule interaction is monitored by the spindle checkpoint system, which delays anaphase entry until both kinetochores of each duplicated chromosome pair acquire stable attachment to spindle microtubules [28]. Precisely, how spindle checkpoint signals are transmitted and received is not yet fully understood. However, it seems clear that unattached kinetochores produce a signal that blocks anaphase onset by inhibiting Cdc20/Fizzy, a protein required for exit from mitosis, which associates with and activates the APC/C (Anaphase Promoting Complex/ Cyclosome) [9, 21]. It is the APC/C, which catalyzes the ubiquitination of key regulatory proteins such securins and cyclins, whose subsequent destruction is required for sister chromatid separation and mitosis exit, respectively [7, 14].

Components of the spindle checkpoint were first identified in the budding yeast *Saccharomyces cerevisiae*. *MAD* (mitotic arrest deficient) [19], *BUB* (budding uninhibited by benzimidazole) [15], and *MPS1* [12] genes are required for the spindle checkpoint to prevent anaphase by inhibiting the ubiquitin-mediated degradation of anaphase inhibitor Pds/Cut2 [10, 31]. Although *MAD* and *BUB* genes are not essential for cell viability, mutations in these genes increase the chromosome loss rate, suggesting that they regulate the metaphase-to-anaphase transition during normal cell cycles [15, 19]. In budding yeast, Mad1p is a nuclear protein whose phosphorylation increases greatly upon spindle depolymerization, and is phosphorylated by Mps1 whose function is required for spindle pole body duplication and spindle checkpoint function [11, 30]. Phosphorylation of Mad1 depends on Mad2, Bub1, and Bub3, but not on Bub2 and Mad3 [11]. *MAD2* is a novel gene and its gene product forms a complex with Mad1 and becomes phosphorylated when the spindle checkpoint is activated [4]. A combination of genetic and biochemical evidence suggests that Mps1, Bub1, and Bub3 are believed to function upstream of Mad1 and Mad2, while Bub2 and Mad3 might act downstream [8, 11, 29].

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Homologues of spindle checkpoint components have been identified in fission yeast Mad2 [13] and Bub2 [27], in the frog *Xenopus laevis* Mad2 [6] and Mad1[5], and human Mad2 [20] and Mad1 [17]. The conservation of the spindle checkpoint proteins in eukaryotes indicates that the checkpoint is essential for the cell cycle and its mechanism has been conserved throughout evolution.

Although these genes have been identified and their functions have provided a more detailed understanding of the mechanisms of the spindle assembly checkpoint, little is known about the molecular genetic analysis of the Mad1p in fission yeast. To gain further insight into these questions, the characterization of the *S. pombe mad1*$^+$ gene and the relationship between Mad1p and Mad2p are reported here.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

All strains used in this study are listed in Table 1. Standard *S. pombe* growth conditions and genetic manipulations were performed as previously described [26]. Cells were grown in yeast extract (YE) or EMM minimal medium with appropriate supplements [1]. Thiabendazole (TBZ; 10 mg/ml stock in dimethyl sulfoxide [DMSO]) was added to plates to a final concentration indicated. Yeast transformation was carried out by the lithium acetate method [18] and yeast two-hybrid analysis was performed by the manufacturer's protocol (Clonetech, Palo Alto, CA, U.S.A.).

Construction of *mad1*$^+$ Gene Truncations

To map the critical functional region of Mad1p, the N- and C-terminal Mad1p truncation constructs were made by PCR amplified from *S. pombe* total genomic DNA using *pfu* DNA polymerase according to the manufacturer's instructions (Stratagene, La Jolla, CA, U.S.A.) and primers: 5'-ggCCC-gTCgACATgAgCTCC AAgCTgAC-3' (full-length Mad1p), 5'-ggCCCgTCgACCATTgCAgAgTTgAAA-3' (residues 145–689), 5'-ggCCCgTCgACCCAgCAATTACAAgAg-3' (residues 206–689), 5'-ggCCCgTCgACCgCAAATgA AAAA gAC-3' (residues 357–689), and 5'-ggCCgTCgACTCAA-TCATTTTTgTC-3'; 5'-ggCCCgTCgACATgAgCTCCAA-gCTgAC-3' and 5'-ggCCCgTCgACCTCAgCATTCAgCATC-3' (residues 1–544) (restriction enzyme site underlined). The amplified PCR products were purified and subcloned into the *Sal*I site of *S. pombe* expression plasmid pREP 1 [23]. To analyze the Mad2p-binding region in Mad1p, PCR amplification was performed using primers: 5'-ggCC-CCCgggATgAgCTCCAAgCTgAC-3' and 5'-ggCCCCCggg-TC AAgAATTTAATTCAg T-3' (residues 1–308); 5'- ggAA-CCCggggAAgAgTTTAATTACT-3' (residues 308–689), 5'-ggAACCCggggACggAgCTATATCgT-3' (residues 471–689), 5'-ggAACCCgggggAgA ggATTAAAgCT-3' (residues 535–689), and 5'-ggCCCCCgggTCAATCATTTTTgTC-3'; 5'-ggCCCCCgggTCATTCTCTC TCggCTAC-3' (residues 471–589), 5'-ggCCCCCgggTCAAAATT CggggCCTgA-3' (residues 471–660), and 5'-ggAACCCggggACggAgCT-ATATCgT-3'; 5'-ggAACCCggggACggAgCTATATCgT-3' and 5'-ggAACCCgggCTCAgCATTCAgCATC-3' (residues 471–544) from *S. pombe* genomic DNA and amplified PCR products were subcloned into the *Sma*I site of the pGBT 9 plasmid (Clonetech, Palo Alto, CA, U.S.A.).

mad1$^+$ and *mad2*$^+$ Gene Disruption

For the disruption of *mad1*$^+$, a 3.2-kb *Eco*RV-*Xba*I genomic fragment was subcloned into pBluescript SK(+) (Stratagene, La Jolla, CA, U.S.A.), and a 0.83-kb *Hin*dIII fragment containing the coding region of the *mad1*$^+$ gene was replaced with a 1.8-kb *Hin*dIII fragment of the *S. pombe ura4*$^+$ gene. Digestion of this plasmid (pDM1) with *Eco*RI produced a 2.4-kb fragment containing the *mad1*$^+$ gene interrupted by the *ura4*$^+$ gene and this linear fragment was transformed into haploid and diploid strains (ED 665 and SP 286). A *mad2*$^+$*::kanMX4* strain was generated by inserting a 1.44-kb fragment encoding the *kanMX4* gene, a G418 resistance marker, into the *Spe*I-*Pvu*II sites of the *mad2*$^+$ gene in plasmid pCM2. A 2.1-kb fragment containing the disruption of the *mad2*$^+$ gene was isolated from this plamid and the linear fragment was transformed into ED 668 haploid strain. Cells were plated onto medium containing G418 and grown at 30°C. To generate the *mad1*$^+$ and *mad2*$^+$ double disrupted mutant, *mad2*$^+$*:: kanMX4* strain was transformed with DNA fragment containing the *mad1*$^+$ gene interrupted by the *ura4*$^+$ gene. Correctly targeted gene disruptions were identified by genomic Southern blot analysis.

Table 1. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype	Characteristics	Source
ED 665	h$^-$	*ade6-210 leu1-32 ura4-D18*	Lab stock
ED 668	h$^+$	*ade6-210 leu1-32 ura4-D18*	Lab stock
SP 286	h$^+$/h$^+$	*ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18*	Lab stock
KDM1	h$^-$	*mad1::ura4*$^+$ *ade6-210 leu-32 ura4-D18*	This study
KDM2	h$^+$	*mad2::kanMX4 ade6-210 leu-32 ura4-D18*	This study
KDM3	h$^+$	*mad1::ura4*$^+$ *mad2::kanMX4 ade6-210 leu-32 ura4-D18*	This study

Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis was performed using a *S. pombe mad2*$^+$ gene subcloned into the *GAL4* activation domain (AD) of the plasmid pGAD containing the *LEU2* gene of *Saccharomyces cerevisiae* (Clonetech, Palo Alto, CA, U.S.A.). The truncated *mad1*$^+$ genes were subcloned using the *Sma*I site downstream of the *GAL4* DNA binding domain in the plasmid pGBT9, which carries the *TRP1* gene. Strain HF7c (MATa, *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3, 112 gal4-542 gal80-538 LYS2::GAL-HIS3 URA3:(GAL4 17mers)3-CYC1-lacZ*) was transformed with pGBT9 containing the truncated *mad1*$^+$ gene series, and transformants were selected on medium lacking tryptophan. The cells harboring pGBT9/truncated *mad1*$^+$ genes were transformed with the *mad2*$^+$ gene, and colonies able to grow on medium lacking leucine, tryptophan, and histidine were screened. Yeast transformations and 3-AT (3-amino-1,2,4-triazole) assays were carried out as described in the Clonetech Matchmaker protocol.

RESULTS

Characterization of *S. pombe mad1*$^+$ Gene

The spindle checkpoint *mad1*$^+$ gene in the fission yeast was identified as the homologue of budding yeast *S. cerevisiae MAD1* and human HsMAD1 [11, 16, 17]. However, the *S. pombe mad1*$^+$ gene was isolated as a suppressor from the cytokinesis mutant *sun1*, which was defective in actin ring placement (unpublished data). BLAST searching of *S. pombe* genomic project data reported that the *S. pombe mad1*$^+$ gene was derived from a region on chromosome II (GenBank/EMBL/DDBJ accession number Z95620) and a 2,120 bp open reading frame (ORF) containing one intron encoding a protein of 689 amino acids.

When treated with a microtubule polymerization inhibitor, such as TBZ, fission yeast cells arrest in mitosis with unseparated sister chromatids. The mitotic arrest by a microtubule polymerization inhibitor suggests the spindle checkpoint monitors the status of the spindle and regulates the metaphase-to-anaphase transition. Deletions in the *mad* genes, *mad1Δ* and *mad2Δ* mutants, result in failure of cell cycle arrest in mitosis and allow cells to enter anaphase in the absence of a functional spindle [25]. To determine the correlation of *mad1Δ* and *mad2Δ* mutant, a *mad1Δ mad2Δ* double mutant has been made by replacing the putative *mad1*$^+$ ORF with the *ura4*$^+$ gene and *mad2*$^+$ ORF with the *kanMX4* gene. The double disrupted haploid strains grew as well as wild-type, *mad1Δ*, and *mad2Δ* cells at all temperatures tested. Spotting serial dilutions of *mad* mutant cells onto YEA plates containing the microtubule depolymerizing agent TBZ showed that they are unable to form colonies on medium 10 μg of TBZ per ml (Fig. 1a).

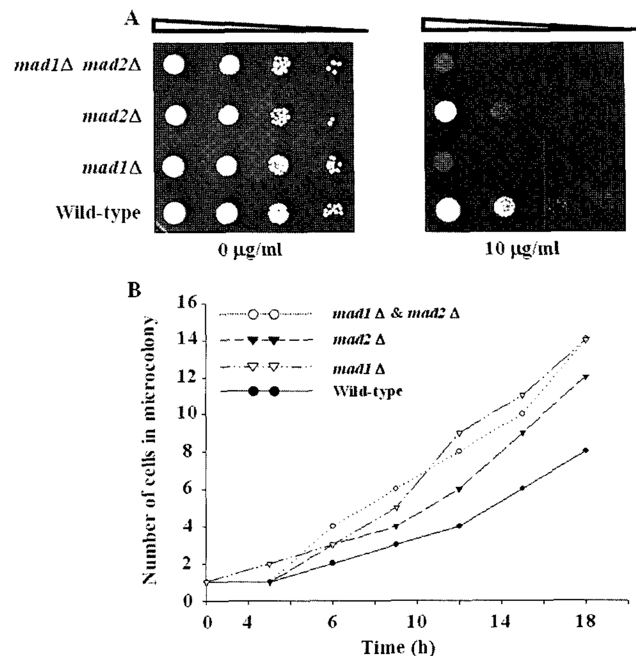

Fig. 1. TBZ sensitivity of *mad1Δ*, *mad2Δ*, and *mad1Δ mad2Δ* strains.

(A) Serial dilutions (1/10) of cells (wild-type, *mad1Δ*, *mad2Δ*, and *mad1Δ mad2Δ*) were spotted onto either a complete medium plate or a complete medium plate containing indicated concentrations of TBZ, and were incubated at 30°C for 4 days. (B) Microcolony analysis of the growth of yeast strains (wild-type, *mad1Δ*, *mad2Δ*, and *mad1Δ mad2Δ*) on complete medium plates containing 10 μg/ml TBZ at 30°C. Data were collected from at least 50 cells of each genotype.

The *mad1Δ* cells were more sensitive than *mad2Δ* cells and *mad1Δ mad2Δ* double mutant cells were observed to be the same as *mad1Δ* cells. In addition, microcolony assays were used to monitor the initial rate of cell division of *mad1Δ*, *mad2Δ*, and *mad1Δ mad2Δ* cells on YEA medium containing TBZ (Fig. 1b). Undivided cells were picked with a dissecting needle and their growth on plates containing 10 μg/ml TBZ was observed over a period of 18 h. The *mad* mutants were unable to sense or respond to the disruption of their microtubules and thus entered anaphase before the spindle checkpoint was completed. The *mad1Δ*, *mad2Δ*, and *mad1Δ mad2Δ* mutant cells divided faster than the wild-type control cells and the behavior of *mad1Δ mad2Δ* double mutant cells resembled those of *mad1Δ* cells. The *mad1Δ mad2Δ* double mutant cells did not show more sensitivity than *mad1Δ* and *mad2Δ* mutant cells. In addition, overexpression of the *mad1*$^+$ gene in *mad* deletion mutants (*mad1Δ*, *mad2Δ*, and *mad1Δ mad2Δ*) showed that only *mad1Δ* strain was rescued from TBZ sensitivity (data not shown). These results were consistent with Mad1p function upstream of Mad2p and suggested that formation of the Mad1p-Mad2p complex was important for checkpoint function.

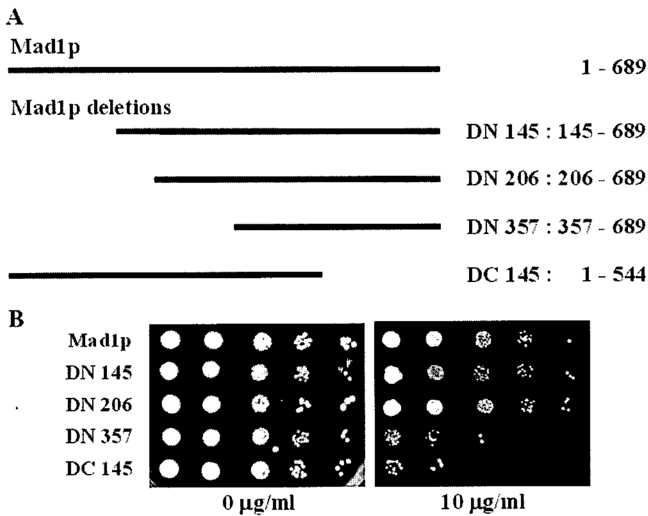

Fig. 2. The checkpoint region in Mad1p.
(A) Summary of the different truncated Mad1p mutations. Constructed Mad1p deletion genes were expressed in the *mad1Δ* strain. (B) Various Mad1p deletions were assayed for their ability to complement the TBZ sensitivity of the *mad1Δ* strain. Transformatns were spotted onto minimal medium plates containing TBZ or not, and were grown at 30°C for 4 days. TBZ was used at 10 μg/ml.

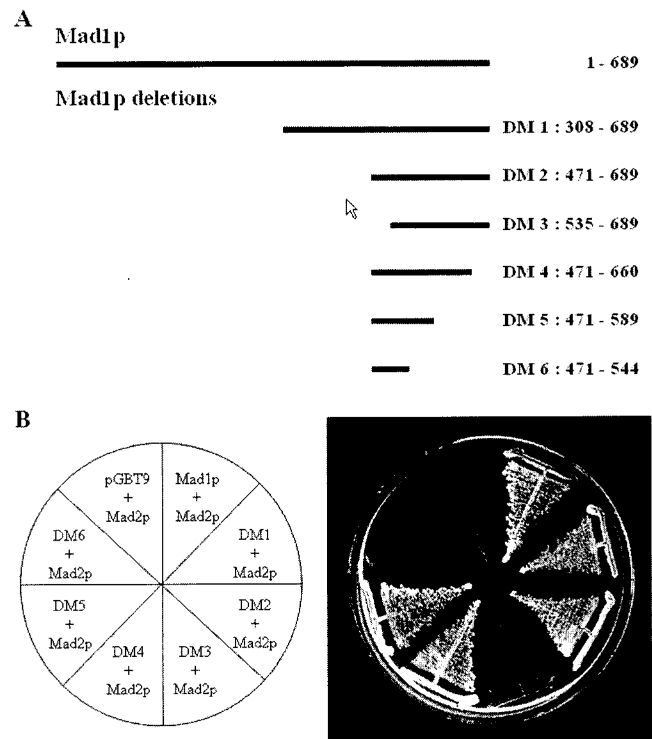

Fig. 3. The Mad2p binding domain in Mad1p.
(A) Construction of Mad1p deletion mutations for assay of the Mad2p binding region. Deleted Mad1p genes were fused to the GAL4 DNA binding domain in pGBT9. (B) Mad1p deletion mutations were tested for their inter interaction with Mad2p, the endogeneous Mad2p in *S. cerevisiae* by yeast two-hybrid assay. Yeast strains containing Mad2p and various Mad1p deletion mutations were streaked on minimal medium (-Leu/-Trp/-His) and incubated at 30°C for 3 days.

Analysis of the Checkpoint Region in Mad1p

To map the functional region for spindle checkpoint in Mad1p, various deletion mutations were generated in Mad1p and these truncated Mad1p proteins were expressed in the *mad1Δ* strain to determine their ability to rescue the TBZ sensitivity (Fig. 2a). Analyzing their function to rescue the TBZ sensitivity of the *mad1Δ* strain indicated that the C-terminus of Mad1p is critical for its function. Mad1p missing the C-terminal 144 amino acids could not complement a *mad1* mutant, whereas deletion of the N-terminal 205 amino acids still rescued the TBZ sensitivity of the *mad1* mutant (Fig. 2b). A Mad1p region lacking 483 amino acids from the C-terminus included a highly glutamic acid-rich region (75–205), which was less well conserved in Mad1p homologues in other organisms. In addition, removal of 144 and 205 amino acids from the N-terminus of Mad1p could not abolish their ability to complement a *mad1* mutant. A Mad1p protein starting at 357 (alanine) was nonfunctional, but additional residues, 206–356, did rescue the TBZ sensitivity of the *mad1* mutant. These results suggest that the region of Mad1p between amino acids 206 and 356 is structurally or functionally important for checkpoint function.

Identification of the Mad2p Binding Region in Mad1p

In species such as budding yeast, frog, and humans [4, 5, 17], Mad1p was proposed to complex with Mad2p and recruited Mad2p to unattached kinetochores. Having established an interaction of Mad1p and Mad2p, the Mad2p binding region in Mad1p was investigated for a spindle checkpoint.

To further map the Mad2p binding region in Mad1p, various truncated Mad1p proteins were constructed and their ability to bind Mad2p was tested by yeast two-hybrid assays (Fig. 3a). Various deletions of Mad1p were constructed and fused to the *GAL4* DNA binding domain (in pGBT9), and these proteins were tested for their interaction with the endogenous Mad2p in a *S. cerevisiae* strain by the *HIS* reporter gene (Fig. 3b). In this experiment, the region spanning amino acids 471 to 589 in Mad1p was detected as a minimal segment for binding to Mad2p. Mad1p lacking the N-terminal 534 amino acids or C-terminal 145 amino acids failed to bind Mad2p. In studies of the *S. cerevisiae* Mad1p, it was found that residues 529–649 failed to bind Mad2p efficiently and the extreme C-terminus was clearly critical for its function [4]. However, a similar region of the *S. pombe* Mad1p (residues 471–589) was sufficient for the interaction of the Mad1p and Mad2p in a two-hybrid assay. In addition, the human homologue of Mad1p showed that the region containing amino acids 456 to 584, an analogues region of *S. pombe* Mad1p (residues 471–589), was defined as a minimal segment for binding human Mad2p [17]. Although the

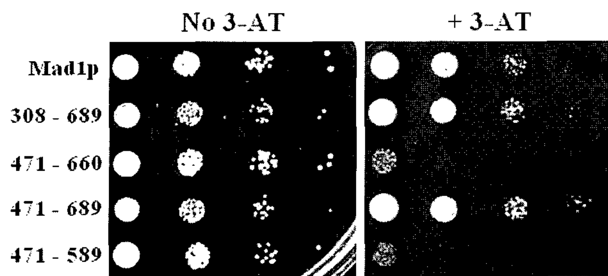

Fig. 4. 3-AT sensitivity of Mad1p deletion mutants.
Cells containing various Mad1p deletion mutations were spotted onto either a minimal medium plate or minimal medium plate containing 10 mM 3-AT. Cells were diluted 10-fold from the corresponding spot on the left. Used Mad1p deletion mutations are indicated on the left.

extreme C-terminus of *S. pombe* Mad1p is not critical for its Mad2p interaction, it cannot be ruled out that this region may affect the stability of the Mad1p-Mad2p complex. In order to confirm this possibility, the response of various truncated Mad1p to 3-AT (3-amino-1,2,4-triazole) that normally suppress growth of transformants containing noninteracting hybrid proteins or unstable interaction by inhibition of the *HIS3* gene product was tested. If the extreme C-terminal region of Mad1p is functionally important for stability of Mad1p-Mad2p interaction, cells containing C-terminal truncated Mad1p would be expected to be hypersensitive to 3-AT. Transformants containing various Mad1p missing the C-terminal regions were unable to form colonies on medium containing 10 mM 3-AT, whereas the cells containing N-terminal Mad1p truncation still did (Fig. 4). These results support that the region of C-terminal Mad1p is necessary for stability of Mad1p-Mad2p interaction.

DISCUSSION

The fission yeast Mad1p was identified, which is an essential component of the spindle checkpoint. Ever since the *mad1* mutant was characterized in 1991 [19], its homologues have been studied from a wide variety of organisms, including humans [17], mouse [29], *Xenopus* [5], and budding yeast [11]. Structurally, all three proteins maintain very similar coiled-coil motifs, and loss of proteins function results in a premature escape from a mitotic block. Antibodies to *S. pombe* Mad1p and sequence analysis reveal that it encodes a 79-kDa protein with homology to other organisms and is predicted to form coiled-coils (data not shown). Like the previously identified budding yeast *mad1*$^+$ gene, the *S. pombe mad1*$^+$ gene is not essential for cell division, and functions as a spindle checkpoint protein, because *S. pombe mad1Δ* cells are viable but 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. Analysis of TBZ sensitivity, microcolony assays, and overexpression of Mad1p in *S. pombe mad* mutants support the idea that Mad1p functions upstream of Mad2p and Mad1p-Mad2p complex are important for the spindle checkpoint. The frog homologue of Mad1p, Xmad1, has been reported to recruit Xmad2 to unattached kinetochores, and budding yeast Mad1p was observed to have a nuclear localization pattern [5, 11]. It was attempted to localize Mad1p in yeast cells; however, it was not possible to detect Mad1p with the monoclonal anti-Mad1p antibody. When overexpressed, the GFP-Mad1p fusion protein in pREP 41 plasmid, Bulk of Mad1p, appeared as discrete patches in nuclear envelopment (data not shown).

Mad1p is less conserved than Mad2p in their homologues in higher eukaryotes. The sequence of Mad1p is predicted to be coiled-coil with a C-terminal globular domain. The level of conservation is higher toward the C-terminus, and it has been shown through the yeast two-hybrid experiment that the C-terminal region (residues 471–589) of Mad1p is critical for its Mad2p interaction. In studies on the human homologue of Mad1p [17], it was found that a similar region (residues 465–584) was sufficient for the interaction of the human Mad1p and Mad2p in a two-hybrid assay. However, a similar region of *S. cerevisiae* Mad1p (residues 529–649) failed to bind efficiently to Mad2p [4]. Although genetic and biochemical differences exist in functional domains between the yeast and human proteins, these results suggest that the fission yeast checkpoint system is evolutionarily conserved with higher eukaryotes. The extreme C-terminus of *S. pombe* Mad1p was clearly necessary for maintenance of Mad1p-Mad2p interaction. Cells containing C-terminal truncated Mad1p, which was enough to interact with Mad2p, were dramatically hypersensitive to 3-AT in the yeast two-hybrid experiment, whereas cells containing Mad1p lacking the N-terminus were barely detectable at the 3-AT sensitivity.

It has recently been reported that *S. pombe* cells lacking Mad1p failed to localize Mad2p to the nuclear periphery or the chromatin domain and to regulate its entry into the nucleus [16]. In addition, Xmad1, the frog homologue of Mad1p, recruited Mad2p to unattach kinetochores during mitosis [5]. This suggests that formation of the Mad1p-Mad2p complex is important for checkpoint function. However, it was found that N-terminal truncated Mad1p (residues 357–689), which was sufficient to bind Mad2p, was unable to rescue the TBZ sensitivity of the *mad1Δ* strain, suggesting that the checkpoint function was required for interaction of Mad1p-other checkpoint components. Genetic studies in budding yeast demonstrated that Mad1p became hyperphosphorylated and formed a complex with Bub1p/Bub3p, and the formation of this complex occurred in a Mad2p- and Mps1p-dependent manner and was found at significantly higher levels once the spindle checkpoint

was activated [2]. However, in *S. pombe*, the molecular events that occur once they are in the complex remain largely unknown, and little is known about the conserved Mad1p domain for other protein interaction. Mad1p missing the N-terminal region (residues 206–689) could still rescue a *mad1* mutant, whereas deletion of the N-terminal 354 amino acids abolished the checkpoint function. This suggests that the Mad1p region (residues 206–356) is important for Mad1p-other components complex. Further studies will be necessary to determine whether other regions of the Mad1p are required for other checkpoint proteins to bind.

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