Control of localization of a spindle checkpoint protein, Mad2, in fission yeast

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Summary

To ensure accurate chromosome segregation, the spindle checkpoint delays the onset of sister chromatid separation when the spindle is not attached to a kinetochore. Mad2, a component of the checkpoint, targets fission yeast Slp1/budding yeast Cdc20/human p55CDC and prevents it from promoting proteolysis, which is a prerequisite to sister chromatid separation. The protein is localized to unattached kinetochores in higher eukaryotes, and it is thought to be required for activation of the checkpoint as well. In this study, Mad2 and its target Slp1 were visualized in a tractable organism, fission yeast *Schizosaccharomyces pombe*. When cells were arrested at a prometaphase-like stage, the Mad2-Slp1 complex was stable and the two proteins were colocalized to unattached kinetochores. When the spindle attachment was completed, the complex was no longer detectable and only Mad2 was found associated to the spindle. These results would suggest that unattached kinetochores provide sites for assembly of the Mad2-Slp1 complex. During interphase, Mad2 was localized to the nuclear periphery as well as to the chromatin domain. This localization was abolished in a yeast strain lacking Mad1, a protein that physically interacts with Mad2. Mad1 may anchor Mad2 to the nuclear membrane and regulate its entry into the nucleus.

Key words: Spindle checkpoint, Mad2, Slp1

Introduction

Duplicated chromosomes are connected to each other and form sister chromatids. During mitosis, the spindle radiates from opposite poles and is attached to kinetochores, a specialized chromosome structure composed of centromeric DNA and proteins. At anaphase, sister chromatids separate and are guided to daughter cells by the attached spindle. Accurate chromosome segregation requires that (1) kinetochores of all sister chromatids bind to the bipolar spindle that extends from opposite poles and (2) sister chromatids separate only after the completion of proper spindle attachment to kinetochores. The spindle checkpoint is a surveillance system that regulates cell cycle progression in compliance with the above requirements. It delays the onset of sister chromatid separation until all sister chromatids bind to the bipolar spindle (Amon, 1999; Shah and Cleveland, 2000). Genetic studies in budding yeast originally identified a number of components required for the function of the spindle checkpoint. At present, the proteins identified are Mad1, 2 and 3, Bub1 and 3 and Mps1 (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). Homologous genes have been identified in other eukaryotes, including human (Jin et al., 1998; Li and Benezra, 1996), mouse (Taylor and McKeon, 1997), fly (Basu et al., 1998), frog (Chen et al., 1996) and fission yeast (Bernard et al., 1998; He et al., 1997), indicating that accuracy of chromosome segregation is insured by a similar strategy in many types of eukaryotic cells.

The cohesin protein complex (Guacci et al., 1997; Michaelis et al., 1997) maintains the link between the sister chromatids. At anaphase, cohesin is cleaved by a specific protease separase (budding yeast Esp1/fission yeast Cut1), which triggers sister chromatid separation (Uhlmann et al., 2000). Throughout most of the cell cycle, separase forms a complex with securin (budding yeast Pds1/fission yeast Cut2) and is kept as an inactive form (Nasmyth et al., 2000; Yanagida, 2000). Separase is released from the complex and activated after securin is removed and degraded by ubiquitin-dependent proteolysis (Ciosk et al., 1998; Kumada et al., 1998). Therefore, the destruction of securin leads to the activation of separase and to the subsequent sister chromatid separation. Securin contains a short amino-acid motif termed the 'destruction box', a signature sequence for proteins to be selectively destroyed at or after the onset of anaphase. Proteins that contain a destruction box are ubiquitinated by the action of the APC/Cyclosome protein complex (King et al., 1995; Sudakin et al., 1995). Following ubiquitination, proteins with the destruction box are degraded by the 26S proteasome. The ubiquitination of securin also requires another protein, budding yeast Cdc20 (Schwab et al., 1997; Visintin et al., 1997)/fission yeast Slp1 (Matsumoto, 1997)/human p55CDC (Weinstein et al., 1994), which serves as a substrate-specific activator of APC. When Cdc20 is overexpressed, securin can be degraded during interphase, whereas other destruction box proteins will remain stable. In cells lacking Cdc20, securin remains stable, and sister chromatids will not separate (Visintin et al., 1997).

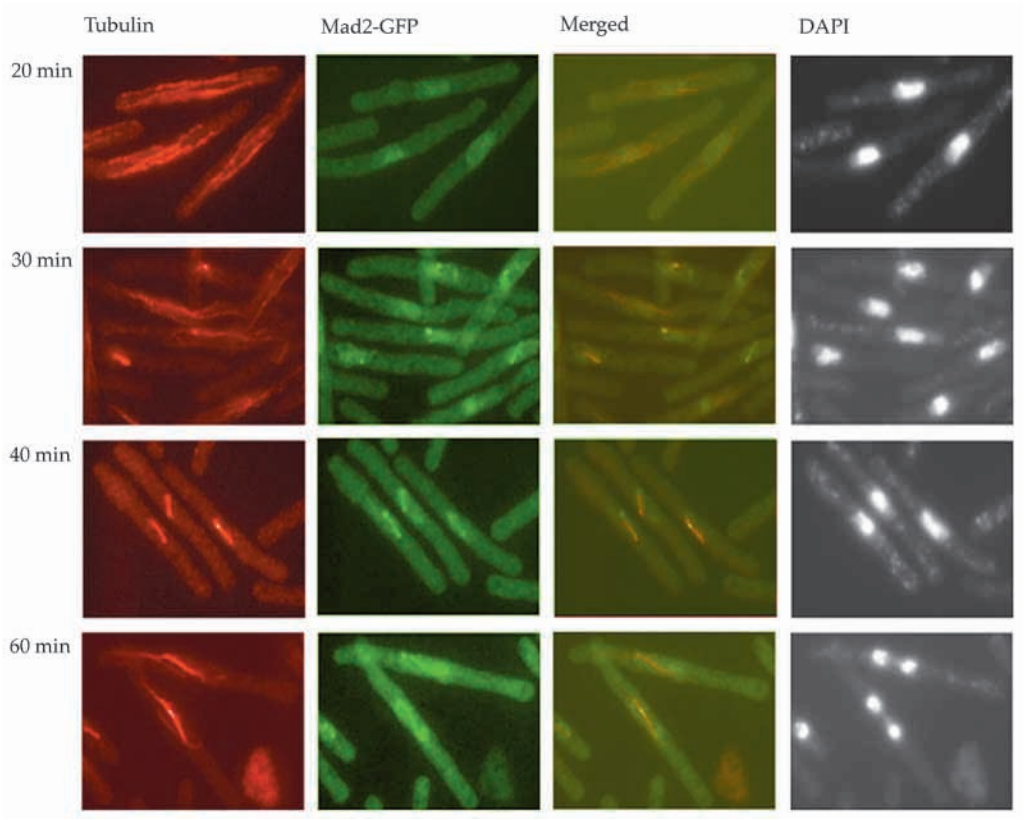


Fig. 1. Mad2 localization in mitosis. Samples were collected 20, 30, 40 or 60 minutes after the release from the cdc25-block. The cells were stained with anti-tubulin antibody (red) and DAPI. Mad2-GFP (green) was visualized in the same cells.

Mad2 mediates the cross-talk between the spindle checkpoint and the mechanism that regulates securin proteolysis as it physically interacts with Slp1/Cdc20/p55CDC. In yeast, expression of a mutant allele of *slp1*$^{+}$*/CDC20*, which is defective in binding to Mad2 abolishes the spindle checkpoint in a dominant manner (Hwang et al., 1998; Kim et al., 1998). In humans, Mad2 and p55CDC associate with each other in early mitosis (Fang et al., 1998). The association is transient, and the Mad2-p55CDC complex dissociates in late mitosis (Wassmann and Benezra, 1998). Biochemical experiments have demonstrated that Mad2 inhibits APC-dependent ubiquitination (Fang et al., 1998; Li et al., 1997). These results indicate that Mad2 targets Slp1/Cdc20/p55CDC and prevents them from promoting the securin proteolysis at such time when the spindle attachment to kinetochores is incomplete.

In higher eukaryotes Mad2 changes its localization in a cell-cycle-dependent manner (Chen et al., 1996; Howell et al., 2000; Li and Benezra, 1996). It is preferentially found on the nuclear periphery, although it is also distributed throughout the cell (Kallio et al., 1998). At or near the onset of mitosis, Mad2 translocates into the nucleus. From prophase to prometaphase if the spindle attachment is incomplete, it localizes to kinetochores. Similarly, if spindle formation is inhibited by a poison such as nocodazole, Mad2 can be found associated with unattached kinetochores. The localization of Mad2 on unattached kinetochores would suggest that unattached kinetochores play an important role in activating the spindle checkpoint so as to delay the onset of sister chromatid separation.

Bub1, another component of the spindle checkpoint, has also been shown to translocate in the cell cycle. From prophase to prometaphase, mouse Bub1 is localized to kinetochores (Taylor and McKeon, 1997). Once kinetochores are aligned on the metaphase plate, it diffuses throughout the nucleus. Fission yeast Bub1 behaves similarly. It, however, remains at kinetochores in later mitosis (Bernard et al., 1998) as well as meiosis (Bernard et al., 2001). It has been proposed that fission yeast Bub1, besides its role in the spindle checkpoint, plays an additional role in maintenance of sister kinetochore cohesion in meiosis I.

Mad2 forms protein complexes with other components of the spindle checkpoint. The Mad1-Mad2 complex, which exists throughout the cell cycle (Chen et al., 1999), is necessary for localization of Mad2 on unattached kinetochores (Chen et al., 1998). Mad2 also forms a complex with Mad3 in budding yeast (Hardwick et al., 2000). Because the Mad2-Mad3 complex also contains Cdc20, this complex may function at the endpoint of the checkpoint signaling cascade.

In this study we have used a simple tractable organism, fission yeast, to investigate localization of Mad2 in conjunction with its target, Slp1. When the spindle attachment is not complete, these two proteins form a complex and are colocalized on unattached kinetochores. During later stages of mitosis, the complex dissociates and the majority of Mad2, but not Slp1, is found on the spindle. Additionally we have also shown that Mad1 is required for the localization of Mad2 to the nuclear periphery during interphase.

Materials and Methods

Yeast strains and media

YEA media was used to grow *S. pombe* strains (Beach et al., 1985). *nda3-KM311* mutant strains were grown in YPD medium containing 1% yeast extract, 2% glucose and 2% polypeptone (Waco Co., Japan).

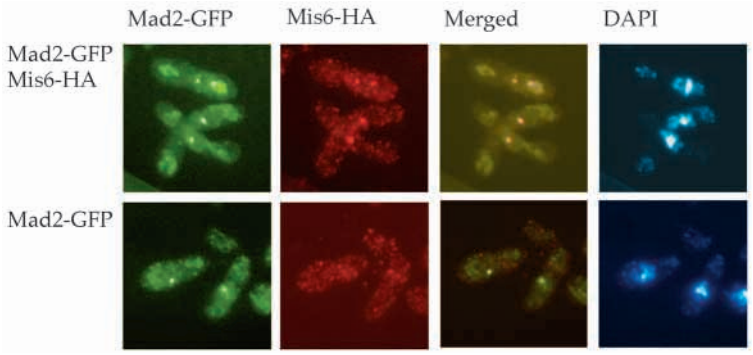
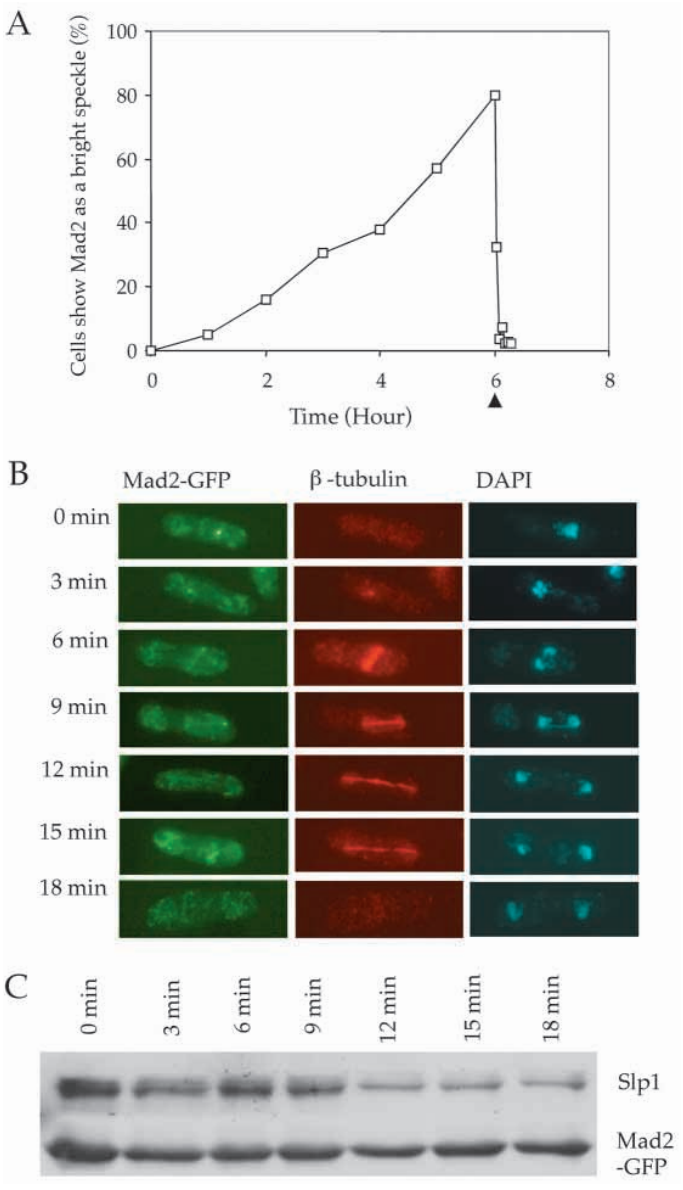


Fig. 2. Mad2 on unattached kinetochores. Mad2 was localized in the *nda3-KM311* mutant cells that were expressing Mis6-HA (top panels). Mis6-HA was visualized by indirect immunofluorescent staining (red), DNA by DAPI (blue) and Mad2 by GFP (green). As a negative control, indirect immunofluorescent staining for HA epitope was performed with cells not expressing Mis6-HA (lower panels).

For synchronization, *nda3-KM311* mutant cells were first cultured at the permissive temperature (33°C) before being downshifted to the restrictive temperature (20°C) for 6 hours. The *cdc25-22* mutant strain was cultured at the permissive temperature (26°C), followed by incubation at the restrictive temperature (36°C) for 4 hours (Alfa et. al., 1993). The block and release experiment using the *nda3-KM311* mutant was preformed as described (Hiraoka et al., 1984) except for modifications. The cell cycle block was released by shifting the temperature up to 33°C, and cells were collected every 3 minutes by filtration. Cells were fixed either by methanol or formaldehyde for direct or indirect immunofluorescence, respectively, as described in the following section.



Cloning of *mad1⁺* gene, its disruption and tagging

With the full-length *mad2⁺* gene as bait, a part of the *mad1⁺* gene was identified through the yeast two-hybrid screen. Nucleotide sequence analysis indicated that a cosmid clone, c1055, contains the full-length *mad1⁺*. A *Sal*I-*Sph*I fragment of the cosmid, which contained the *mad1⁺* gene, was subcloned into pUC119 to construct 119gMad1. The *ura4⁺* was inserted between *Nco*I and *Nru*I sites of the *Sal*I-*Sph*I fragment on 119gMad1. This insertion would allow expression of the first 34 amino acids, at most, of the Mad1 protein. The linear DNA fragment, which contained the *ura4⁺* gene in place of a part of the *mad1⁺* gene, was used for transformation. Stable Ura⁺ diploids were examined by southern blot as well as tetrad analysis. The examination indicated that deletion of the *mad1⁺* gene does not affect cell growth under normal conditions.

The gene encoding the HA epitope, flanked by *Not*I sites at both ends, was inserted into a *Not*I site generated right before the termination codon of the *mad1⁺* gene on 119gMad1. The linear DNA fragment resulting from digest with *Sal*I and *Sph*I was used for replacement of the disrupted allele of *mad1* with the HA-tagged allele by double homologous recombination.

Protein analysis

Cells were washed and suspended in modified HB buffer as described by Yamada et al. (Yamada et al., 2000). Yeast extract was prepared by beating cells in the presence of glass beads for 30 seconds, five times. The cell extracts were centrifuged at 10,000 ***g*** for 5 minutes,

Fig. 3. (A) Mad2-GFP as a speckle in *nda3-KM311* mutant. The cells were collected every hour during the synchronization at the restrictive temperature. After incubation for 6 hours (indicated by the arrowhead), the cells were released into the permissive temperature. Note that the samples were collected every 3 minutes after the point indicated by the arrowhead. Mad2-GFP was visualized under the microscope, and the percentage of the cells showing Mad2 as speckles versus total cell number was calculated. Over 100 cells were examined at each time point. (B) Mad2 is released from kinetochores after metaphase. A block and release experiment using the *nda3-KM311* mutant was performed to stain tubulin by indirect immunofluorescence (red) and DNA by DAPI (blue). Mad2-GFP (green) was visualized in the same cells. (C) Slp1 and Mad2 protein expression after metaphase arrest. Slp1 and Mad2 protein expression in the *nda3-KM311* mutant cells was analyzed 0, 3, 6, 9, 12 and 15 minutes after the release. The proteins were blotted using anti-Slp1 or anti-Mad2 antibody.

with aliquots of the supernatant either used for western blot or immunoprecipitation. For SDS-PAGE, protein extracts were loaded at 150 to 200 μg/lane, whereas 1.5 to 2.0 mg of protein extract was used for immunoprecipitation.

Cytological techniques

PEM buffer (100 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9) was added to the fixed cells at 30% (v/v) of the final methanol concentration. Cells were then washed with PBS three times and stained with 1 μg/ml DAPI. For tubulin staining, the methanol fixation method was employed. Cells were fixed with methanol as described above and were treated with 0.1 mg/ml zymolase 100T in PEMS buffer (PEM, 1.2 M sorbitol) at 37°C for 10 minutes. The cells were then washed with PEMS containing 0.1% TritonX-100 for 2 minutes and washed with PEM three times. For blocking, PEMBAL buffer (PEM, 0.1% bovine serum albumin) was used. After 1 hour of blocking, cells were stained with the primary antibody (tat1) and incubated over night. Cells were washed with PEMBAL for 30 minutes three times, followed by incubation with a CY3 conjugated secondary antibody for 4 hours. Finally cells were washed with PEMBAL three times and stained with 1 μg/ml of DAPI. All steps were performed at room temperature. For Slp1 staining, cells were first fixed by formaldehyde at the final concentration of 3.7% for 30 minutes. After performing two wash steps with PEM, spheroplasts were obtained by incubation at 37°C for 7 minutes using 0.5 mg/ml zymolase and 0.3 mg/ml novozyme. The following steps are identical to those for tubulin staining, except that the Slp1 antibody was used as a primary antibody. The Mad2-GFP signal was observed under the microscope using the same cells as was used for tubulin or Slp1 immunostaining.

Results

Localization of Mad2 in normal mitosis

In order to visualize Mad2 we tagged GFP (green fluorescent protein) to the C-terminus of Mad2 at its native locus. Strains expressing Mad2-GFP grew normally and efficiently arrested in response to a defect in the formation of the spindle (see below), similar to the wild-type strain, suggesting that GFP-tagging did not affect the biological activity of Mad2. Temperature-sensitive *cdc25-22* mutant cells expressing Mad2-GFP were first arrested at the boundary of G2/mitosis at the restrictive temperature (36°C), followed by their release upon shifting to the permissive temperature (26°C). The shift to the permissive temperature allowed cells to proceed through the cell cycle in a highly synchronous fashion (Alfa et al., 1993). For the first 20 minutes after the shift, most of the cells were in interphase (as determined by indirect immunofluoresence staining of cytoskeltal microtubules). Mad2-GFP was localized to the nuclear periphery, as well as in the chromatin domain (Fig. 1). After the cells entered into mitosis (30 minutes after the shift), the cytoskeletal microtubules were disassembled and a very short spindle structure became visible. At this early stage of mitosis, the majority of Mad2-GFP was seen concentrated into a single bright speckle, which localized to the end of the mitotic spindle. In fission yeast, three centromeres are clustered at the spindle pole body (SPB) during interphase (Funabiki et al., 1993). The bright speckle of Mad2-GFP may indicate that Mad2 is colocalized at the SPB together with the three centromeres at early mitosis. As mitosis proceeded, Mad2-GFP was observed to shift to the mitotic spindle (40 to 60 minutes after the release; Fig. 1).

Mad2 in a β-tubulin mutant, *nda3-KM311*

In higher eukaryotes, Mad2 will localize to unattached kinetochores if the spindle-kinetochore attachment is incomplete (Chen et al., 1996; Waters et al., 1998). Using the synchronous culture of the *cdc25* mutant, mitosis proceeded normally, but we were unable to anticipate at what time point spindle-kinetochore attachment failed. In order to capture Mad2-GFP localization to unattached kinetochore, we employed a cold-sensitive *nda3-KM311* mutant, which is unable to form a spindle because of a mutation in the β-tubulin gene (Hiraoka et al., 1984). At the restrictive temperature (20°C), the mutant will arrest during mitosis with condensed chromosomes but with no spindle (Hiraoka et al., 1984). Importantly, the arrest induced by the *nda3* mutation requires a functional spindle checkpoint (He et al., 1997). As shown in Fig. 2, Mad2-GFP was concentrated as one or two speckles in the mutant after 6 hours of incubation at the restrictive temperature. When the mutants were incubated longer at the restrictive temperature, some of them exhibited three speckles, which might represent the three chromosomes of fission yeast (not shown). In the same cells, Mis6 a kinetochore-specific protein (Saitoh et al., 1997) was colocalized with Mad2-GFP, indicating that the fission yeast Mad2 is localized to unattached kinetochores.

The cell cycle arrest caused by the *nda3* mutation is reversible. If the arrested cells are shifted back to the permissive temperature (33°C), cell cycle progression is re-initiated and the cells will proceed synchronously (Hiraoka et al., 1984). We examined localization of Mad2-GFP in the cells that were released from the *nda3*-induced arrest. The progression of mitosis was monitored both by visualizing chromosomes using DAPI staining and by indirect immunofluorescent staining of the spindle. We also determined the relative abundance of Slp1, which drops in late mitosis, as an additional means of monitoring cell cycle progression (Yamada et al., 2000). After the *nda3* mutant was arrested at 20°C for 6 hours, approximately 80% of the cells exhibited the bright speckles of Mad2-GFP (Figs 3A,B). Three minutes after the release, in response to a shift to the permissive temperature, most of the bright speckles became less intense. At this time point, short spindles became visible in those cells (Fig. 3B), although separation of sister chromatids was not apparent. The reduction in the intensity of the Mad2-GFP signal during the 3 minutes immediately following release from metaphase arrest was highly reproducible. We analyzed the digital images in a quantitative manner and categorized each speckle into either of two groups. In one group, speckles remained as intense as they had at time 0, whereas the second group was scored for a reduced intensity. The analysis indicated that in 30% of the cells, Mad2-GFP speckle intensity did not change (Fig. 3A). Although we could not demonstrate the biological significance of the change in the speckle intensity, it might be possible that the bright speckles represented Mad2-GFP localized to unattached kinetochores. The sudden reduction in the intensity of the Mad2-GFP is similar to that of Bub1 (Bernard et al., 2001). Six minutes after the release, anaphase was initiated and Mad2-GFP was localized to the spindle. During the following 9 minutes, the GFP-Mad2 remained with the spindle. When the spindle disappeared 18 minutes after the release, Mad2-GFP was not observed localized to any distinctive structure (Fig. 3B). Analysis of the levels of Mad2 and Slp1 indicated that although

Mad2 was constant throughout mitosis, Slp1 abundance dropped after anaphase was initiated (Fig. 3C).

Mad2 in other mitotic mutants

We also examined the localization of Mad2-GFP in other mutants that arrest at specific stages of mitosis. In the temperature-sensitive *cut7* mutant, spindle formation is blocked shortly after duplication of the SPB. The mitotic spindle radiating from the opposite poles cannot interdigitate and thus remains as a V shape (Hagan and Yanagida, 1992). The *cut7*-specific defect generates a condition whereby the bipolar attachment of the spindle to kinetochores is not achieved. Previously, we demonstrated that this defect causes an arrest in a *mad2*$^+$-dependent manner (Kim et al., 1998). At the restrictive temperature the majority of Mad2-GFP was found on condensed chromosomes as one or more speckles (Fig. 4). Although we did not examine the colocalization with Mis6, these speckles of Mad2-GFP would represent the position of unattached kinetochores. The *dis1*$^+$ gene encodes an M-phase-specific kinetochore protein with a presumed role in establishing a connection between kinetochores and microtubules (Nakaseko et al., 2001). Its defect would result in an anomaly in the spindle-kinetochore interaction. Indeed, sister chromatids do not separate in the *dis1* cold-sensitive mutant, although the spindle will elongate (Ohkura et al., 1988). In the *dis1* mutant, Mad2-GFP was localized as speckles in the chromatin domain (Fig. 4). Mad2-GFP was localized similarly in the three mutants *nda3*, *cut7* and *dis1*, in which the spindle-kinetohore attachment are defective. Other mutants, such as the *cut4* and *nuc2*, display an arrest of the cell cycle in response to a lack of APC activity (Yamada et al., 1997; Yamashita et al., 1996). In these mutants the interaction between the spindle and kinetochores is presumably normal, and we found that the majority of the Mad2-GFP was localized to the spindle (Fig. 4).

Interaction between Slp1 and Mad2

Slp1 is a target of the spindle checkpoint (Kim et al., 1998). When spindle formation is incomplete, Mad2 is thought to bind to Slp1 and prevent proteolysis by Slp1; proteolysis is necessary for the onset of anaphase. In order to determine at which mitotic stages Mad2 is interacting with Slp1, we performed immunoprecipitation using cell extracts prepared from various cell cycle mutants. In late G2 extracts prepared from the *cdc25* mutant, Slp1 was not detectable. Extracts, immunoprecipitated with the antibody to Slp1, did not reveal the presence of either Slp1 or Mad2, although the extracts did contain Mad2 (Fig. 5A). These results demonstrated that the antibody to Slp1 did not directly interact with Mad2 and thus could serve as a negative control. In contrast, precipitates from the *nda3* mutant contained both Slp1 and Mad2, indicating that the two proteins formed a complex when spindle formation was not completed (Fig. 5A). In extracts prepared from the *nuc2* mutant, which causes an arrest owing to defective APC subunit, the levels of Mad2 and Slp1 were similar to those found in the *nda3* mutant. However the immunoprecipitates using the antibody to Slp1 contained Slp1 but very little Mad2. Similar results were obtained using extracts prepared from another APC mutant (*cut9*) and the *mts3* mutant defective in a subunit of the 26 S proteasome (Fig. 5A). These results would suggest that the Mad2-Slp1 complex, which is stable when the spindle formation is incomplete, is disassembled prior to the onset of anaphase.

We next examined localization of Slp1 using indirect immunofluorescent staining. First, in the G2-phase-arrested *cdc25* mutant, detection of the antibody to Slp1 revealed speckle-like signals (Fig. 5B). Considering that in the *cdc25* mutant, Slp1 was not detectable by immunoblotting (Fig. 5A), the staining may represent a non-specific recognition by the antibody. Second, in the *nda3* mutant, Slp1 was observed as speckles in the chromatin domain. These speckles were colocalized with Mad2-GFP, indicating that in the *nda3* mutant, Slp1 is also localized to unattached kinetochores (Fig. 5B). In other mitotic mutants such as *nuc2* and *mts3*, we did not observe Slp1 localized to any distinctive cellular location, although the Slp1 protein was detectable by western blot (Figs 5A,B).

Interaction between Mad1 and Mad2

In species such as budding yeast, frog and human (Chen et al., 1999; Chen et al., 1998; Jin et al., 1998), Mad2 also interacts with Mad1, which is another component of the spindle checkpoint. We isolated a part of the fission yeast *mad1*$^+$ gene by the yeast two-hybrid screen using Mad2 as bait. The full-length *mad1*$^+$ gene was identified in a cosmid clone 1055 (Mizukami et al., 1993). In order to confirm the physical interaction between Mad1 and Mad2, we tagged the C-terminus of Mad1 with an HA epitope. Immunoprecipitates with the antibody to Mad2 contained Mad2 as well as Mad1-HA (Fig. 6A). If a similar experiment was performed with extracts prepared from cells that did not express Mad2, the precipitates did not contain Mad1-HA. Thus, the immunoprecipitation demonstrated a specific interaction between Mad1 and Mad2.

To determine the role of Mad1 in localization of Mad2, we examined localization of Mad2-GFP in cells lacking Mad1 (*Δmad1*). As shown in Fig. 6B, although Mad2-GFP was localized to the nuclear periphery and chromatin domain in the wild-type cells during interphase, it was not found in the corresponding locations in Δmad1. To confirm that the abundance of Mad2-GFP was similar in the two cell lines, we examined its levels by western blot. We found that both the wild-type strain and Δmad1 expressed Mad2-GFP at similar levels (Fig. 6C). These results indicate that Mad1 is required for localization of Mad2.

Discussion

Localization of Mad2 in fission yeast

We have determined the localization of Mad2 by visualization of Mad2-GFP in fission yeast released from the *cdc25* block or released from the *nda3* block. In addition, we have arrested the cell cycle at specific stages using existing mutants and then examined the resulting localization of Mad2. Our results have consistently indicated that (1) during interphase, the majority of Mad2 is localized to the nuclear periphery and chromatin domain, (2) in the prometaphase-like stage, during which the spindle does not attach to kinetochores, Mad2 concentrates to unattached kinetochores and (3) once the spindle attachment is complete, Mad2 localizes to the spindle. These results are similar, but not identical, to those obtained in higher eukaryotes (Chen et al., 1996; Li and Benezra, 1996; Waters et al., 1998).

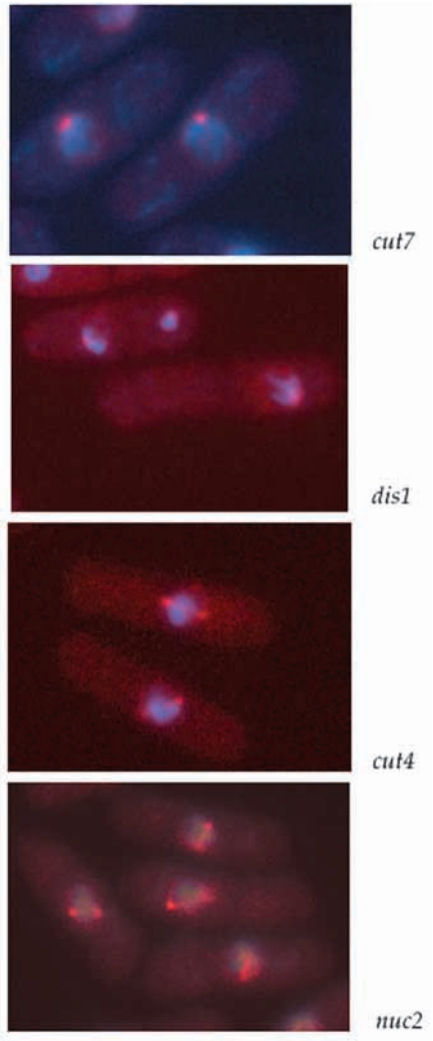


Fig. 4. Mad2-GFP in mitotic mutants. Mad2-GFP was visualized in *cut7*, *dis1*, *cut4* and *nuc2* mutants at their restrictive temperature. DAPI (blue) was used to stain DNA, and the GFP-fluorescence was converted to red.

Upon release from the *nda3* block, the kinetochore-associated Mad2-GFP signal rapidly decreased. Three minutes after the release, the spindle can be recognized as a very short fiber. At this early time point, the intensity of Mad2-GFP has already decreased. We would speculate that the attachment of the spindle to the kinetochore can be completed within three minutes of the release and that this decrease in intensity of Mad2-GFP reflects the movement of Mad2 away from kinetochores upon completion of the spindle attachment. Unlike in higher eukaryotes, all three kinetochores of fission yeast are clustered near the SPB, a structure equivalent to the centrosome (Funabiki et al., 1993). This configuration may be beneficial, as it allows the spindle to be attached in such a short period of time.

In order to demonstrate Mad2 localization to kinetochores, we have used Mis6 as a kinetochore-specific marker (Saitoh et al., 1997). CHIP (chromatin immunoprecipitation) is another method by which localization can more precisely be shown. If successful, one could determine in which region of the

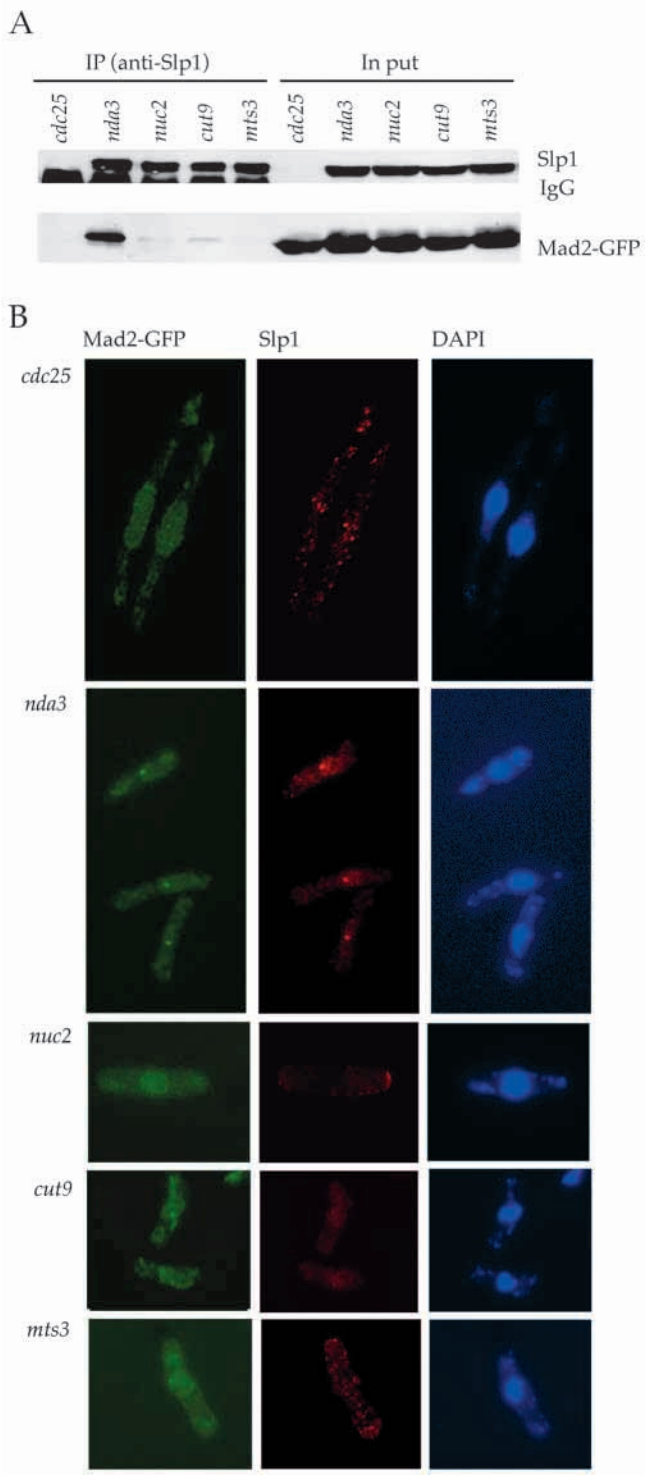


Fig. 5. (A) The Slp1-Mad2 complex formed in *nda3-KM311* mutant cells. The protein binding was observed by a coimmunoprecipitation experiment using the anti-Slp1 antibody in *cdc25-22*, *nda3-KM311*, *nuc2*, *cut9* or *mts3* mutants (left). Mad2-GFP and Slp1 were blotted using an anti-GFP (lower panel) or anti-Slp1 antibody (upper panel), respectively. The total amount of protein in the cells was analyzed by straight western blotting (right). (B) Visualization of Mad2 and Slp1 in *nda3-KM311*, *cdc25-22*, *nuc2*, *cut9* or *mts3* mutants. The cells were stained using an anti-Slp1 antibody (red) or DAPI (blue). Mad2-GFP was observed in the same cells as those used for Slp1 staining and DAPI (green).

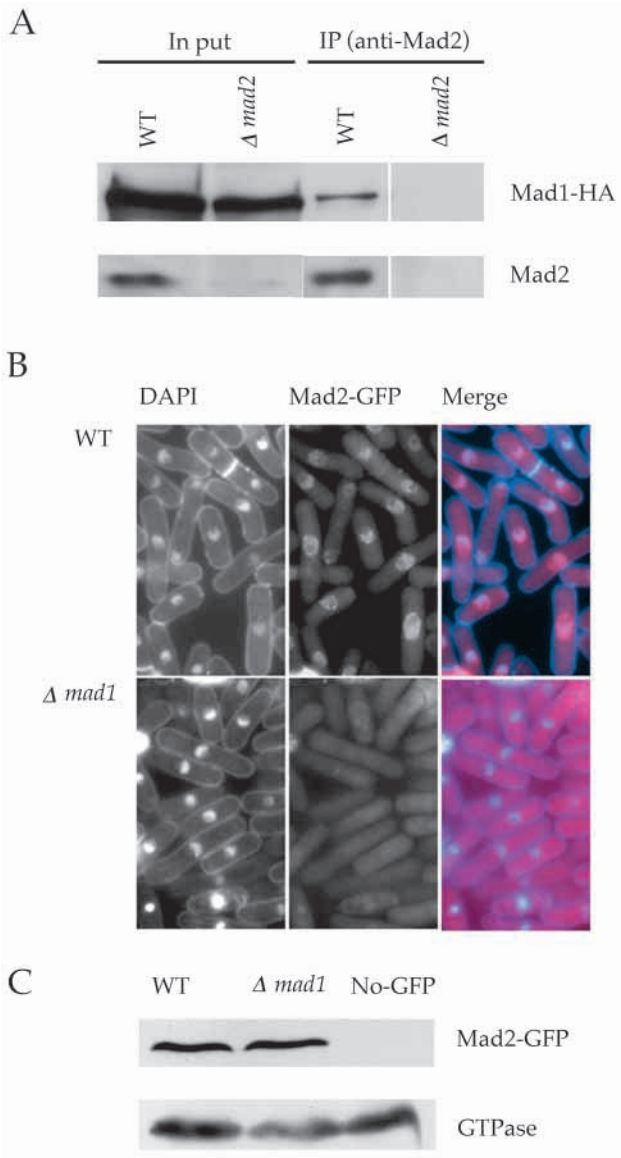


Fig. 6. (A) The Mad1-Mad2 complex in the wild-type strain. A coimmunoprecipitation experiment using an anti-Mad2 antibody was performed in order to visualize the Mad1-Mad2 protein interaction (right). The total amount of proteins was analyzed by straight western blotting (left). Mad1 and Mad2 precipitates were analyzed using an anti-Mad2 antibody (lower panels), whereas Mad1-HA was blotted using an anti-HA antibody (top panels). Mad2 knockout strain (*Δmad2)* was used as a negative control. (B) Localization of Mad2-GFP in the *Δmad1* strain. Mad2-GFP (middle) was observed in the wild-type strain (upper panel) or *Δmad1* strain (lower panel). DNA was stained with DAPI in the same cells (left). (C) Mad2-GFP expresses at the same level in both the wild-type and *Δmad1* strain. Mad2-GFP protein level was analyzed by western blot using an anti-GFP antibody (upper panel). GTPase was blotted using anti-Spi1 antibody as a loading control (lower panel). A non-tagged Mad2 strain was used as a negative control for Mad2.

kinetochore a protein is localized to. However, this method proved unsuccessful in examining Mad2 localization. As recently shown in PtK1 cells, Mad2 is a transient component of the kinetochore with a half-life of ~24 to 28 seconds (Howell et al., 2000). A CHIP assay may require a more stable interaction between the protein and its target site.

The role of unattached kinetochores and catalytic model

In this study we have also examined the localization of Slp1, a target of Mad2, and found that Slp1 and Mad2 colocalize to unattached kinetochores when formation of the spindle is inhibited by the *nda3* mutation. The colocalization coincides with the formation of the Mad2-Slp1 complex. Although the complex is readily detectable in extracts prepared from cells arrested by the *nda3* mutation, it is not detectable in extracts prepared from cells arrested by other mutations such as the *nuc2*, *cut9* or *mts3*. These mutations affect ubiquitin-dependent proteolysis, causing arrest of a cell cycle progression at a later stage of mitosis. These results strongly support the catalytic model (Kallio et al., 1998), in which unattached kinetochores are believed to catalyze the formation of the Mad2-Slp1 complex.

Mad2 is localized to the spindle after the transition from metaphase to anaphase. The role of Mad2 at the spindle is still to be revealed. Because the Mad2-Slp1 complex is not detectable after the transition, we would speculate that Mad2 on the spindle plays a role unrelated to the regulation of Slp1.

Silencing the spindle checkpoint

Although the levels of Mad2 and Slp1 are similar among cell extracts prepared from the *nda3*, *nuc2*, *cut9* and *mts3* mutants, the two proteins are only found assembled into the complex when extracts are prepared from the *nda3* mutant. One would speculate that the complex, which is assembled when attachment of the spindle is incomplete, is disassembled upon the completion of the attachment. The disassembly would be necessary for Slp1 to promote ubiquitin-dependent proteolysis. We therefore postulate that an active mechanism is responsible for silencing the spindle checkpoint and disassembly of the Mad2-Slp1 complex. Closer examination of Mad2, as well as Slp1, would reveal the molecular mechanism behind the silencing.

The role of Mad1

In cells lacking Mad1, Mad2 fails to localize to the nuclear periphery or the chromatin domain. It has recently been demonstrated that Mad1 recruits Mad2 to unattached kinetochores in frogs (Chen et al., 1998). Our result indicates that Mad1 plays an additional role – that of anchoring Mad2 to the nuclear periphery and probably regulating its entry into the nucleus. Every time a cell enters mitosis, kinetochores are unattached for a certain period of time. Thus, the spindle checkpoint is activated as part of the normal cell cycle. The Mad1-Mad2 complex, which is stable throughout the cell cycle (Chen et al., 1999), would have to be regulated in such a way, so it would be able to enter into the nucleus at or around the onset of mitosis.

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