Proper metaphase spindle length is determined by centromere proteins Mis12 and Mis6 required for faithful chromosome segregation

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High-fidelity chromosome transmission is fundamental in controlling the quality of the cell division cycle. The spindle pole-to-pole distance remains constant from metaphase to anaphase A. We show that fission yeast sister centromere-connecting proteins, Mis6 and Mis12, are required for correct spindle morphogenesis, determining metaphase spindle length. Thirty-five to sixty percent extension of metaphase spindle length takes place in mis6 and mis12 mutants. This may be due to incorrect spindle morphogenesis containing impaired sister centromeres or force unbalance between pulling by the linked sister kinetochores and kinetochore-independent pushing. The mutant spindle fully extends in anaphase, although it is accompanied by drastic missegregation by aberrant sister centromere separation. Hence, metaphase spindle length may be crucial for segregation fidelity. Suppressors of mis12 partly restore normal metaphase spindle length. In mis4 that is defective in sister chromatid cohesion, metaphase spindle length is also long, but anaphase spindle extension is blocked, probably due to the activated spindle checkpoint. Extensive missegregation is caused in mis12 only when Mis12 is inactivated from the previous M through to the following M, an effective way to avoid missegregation in the cell cycle. Mis12 has conserved homologs in budding yeast and filamentous fungi.

[Key Words: Cell cycle control; checkpoint; fission yeast; kinetochore; chromosome segregation; sister chromatid cohesion]

Received March 22, 1999; revised version accepted May 14, 1999.
For polyubiquitination, the anaphase-promoting complex (APC)/cyclosome, which is essential for polyubiquitination of mitotic cyclin and Cut2, must be activated [King et al. 1995; Sudakin et al. 1995; Yamano et al. 1996; Yamashita et al. 1996; Funabiki et al. 1997; Yamada et al. 1997, Kim et al. 1998]. In fission yeast mutants defective in APC/cyclosome subunits [Hirano et al. 1988, Yanagida 1998] or in proteasome [Gordon et al. 1993], cells are arrested at a metaphase-like stage with the short spindle and highly condensed chromosomes. Cyclin and Cut2 remain without anaphase proteolysis in these mutants. The onset of anaphase is thus regulated by ubiquitin-mediated proteolysis and this is thought to ensure harmonious chromosome segregation.

At least three evolutionarily conserved groups of proteins are responsible for sister chromatid cohesion. Rad21/Mcd1p/Scc1p [Guacci et al. 1997; Michaelis et al. 1997, Losada et al. 1998; Uhlmann and Nasmyth 1998] forms the complex called cohesin with two other proteins [Scc1p and Scc3p] of the SMC family. The second type of chromatid cohesion molecule is Mis4/Scc2p, called adherin [Furuya et al. 1998]. These become essential during the S phase and appear to link two sister chromatids together until anaphase. The third is Ctf7p/Eco1p, which is required for the establishment of cohesion during DNA replication [Skibbens et al. 1999; Toth et al. 1999]. The loss of these cohesion molecules apparently leads to the failure to hold sister chromatids at replication, and therefore sister chromatids are prematurely separated. Esp1p was shown to be essential for the removal of Scc1p from sister chromatids in anaphase [Ci osk et al. 1998], but similar events have not been found in fission yeast (T. Nakamura, K. Kumada, and M. Yanagida, unpubl.). Cut1 protein appeared to be needed for activating the anaphase spindle on Cut2 proteolysis [Kumada et al. 1998], loading of Cut1 onto the anaphase spindle required the carboxy-terminal-conserved amino acids.

More restricted sister chromatid linking has been reported for fission yeast Mis6, which is required to connect the sister centromeres in metaphase-arrested cells [Saitoh et al. 1997]. Whereas sister chromatid cohesion molecules such as Rad21 and Mis4 locate along the entire length of chromatids, Mis6 tagged with GFP is visualized only at centromeres. Fission yeast centromeres are large and complex (see e.g., Takahashi et al. 1992), and Mis6 is shown to associate with the inner regions essential for precise segregation. In temperature-sensitive mis6-302 cultured at the restrictive temperature (36°C), sister chromatids are separated but radically missegregated [Takahashi et al. 1994]. Mis6 is necessary to construct specialized chromatin present in the inner centromeres; smeared nucleosome ladders obtained by the inner centromere DNA probes were abolished in mis6 mutant [Saitoh et al. 1997]. Drastic missegregation phenotype in anaphase is produced only after cells proceed across from G1/S to M at 36°C. Mis6 normally acts during G1 or at the onset of S phase but may be functionally restored at a later stage. If mis6 mutant cells are cultured at 36°C from G1 and then arrested at metaphase, sister centromeres are already separated, but other parts of chromatids are associated, suggesting that Mis6 is involved in linking sister centromeres. If, however, the sister cohesion protein Mis4 is inactivated, the entire length of chromatids including centromeres are prematurely separated in the presence of Mis6 [Furuya et al. 1998]. Mis4 is independently needed for sister centromere connection.

In fission yeast, behavior of centromeric DNA during mitosis has been visualized in living cells with GFP–LacI–NLS, which can bind to the LacO repeats integrated near the cen1 [Nabeshima et al. 1998]. Combined with the images obtained by GFP–Sad1, a GFP-tagged spindle pole body (SPB) protein, spindle, and centromere dynamics in mitosis in the wild-type cells are now fairly well understood. Spindle dynamics in S. pombe consists of three phases: Phase 1 is the duration of spindle formation, whereas in phase 2, the spindle has a constant length. Sister chromatid separation occurs at the end of phase 2. Phase 2 consisting of the stages similar to metaphase and anaphase A in higher eukaryotes is crucial for understanding the onset of anaphase. Spindle elongation [similar to anaphase B] occurs in phase 3. The durations of phases 1, 2, and 3 are, respectively, 2.5, 7.0, and 15 min at 26°C and 1.4, 4.0, and 6.2 min at 36°C.

Among 12 mis (mini-chromosome instablity) genetic loci identified [Takahashi et al. 1994], only 3, mis4, mis6, and mis12, gave rise to the missegregation phenotype of regular chromosomes at 36°C. We were interested in how regular chromosomes in mis12-537 mutant cells were missegregated and undertook to characterize the phenotypes of mis12-537 and identify the gene product. Mis12 plays a unique role in regulating the functional centromeres during the cell cycle.

Results

Expansion of the metaphase spindle in mis12-537

To determine whether spindle formation and elongation was normal in the mis12-537 mutant, the gene for Sad1–GFP, the SPB protein [Hagan and Yanagida 1995] tagged with GFP [Nabeshima et al. 1998], was expressed by multicopy plasmid in wild-type and mis12-537 at 36°C. Time-lapse images of living cells in the culture medium at 36°C (images taken after 3.5–8 hr because the phenotype was produced in the second mitosis, see below) were taken at 30-sec intervals by a confocal microscope [Nabeshima et al. 1997]. The elevated dosage of Sad1–GFP results in extra localization at the nuclear envelope, a convenient marker for the shape of the nucleus. More than 20 living mis12 cells and 6 wild-type cells were observed. Two living mis12 cells and one wild-type control cell are shown, respectively, in Figure 1, A and B (the number indicates minutes). The spindle is fully extended in the mutant, although daughter nuclei produced are unequal in size (see nuclear images at 16.5 and 10.5 min).

Another striking anomaly was discovered in mutant cells with regard to spindle length of phase 2, the period corresponding to metaphase to anaphase A in higher eu-
karyotes. Phase 2 is crucially important for understanding mitosis as the duration and length of the phase-2 spindle appears to be precisely regulated (Nabeshima et al. 1998). Centromeres move quickly back and forth along the spindle in phase 2. Time course of the pole-to-pole distance (defining spindle length) for three mis12 sample cells (#1–3, Fig. 1C) demonstrates that the three phases of spindle dynamics clearly exist in mis12, but the spindle in phase 2 is much longer than that of wild type. In Figure 1D, the data for all mis12 cells examined are shown: the average pole-to-pole distance [3.7 ± 0.7 µm] is 60% longer in mis12 (solid bars) and wild-type cells (open bars) are plotted. Frequencies are expressed as the number of living cells for which mitosis was analyzed. (E) Plot of duration of time for phase 2 measured for the same number of mis12 (shaded bars) and wild-type cells (open bars). (F) Two mis12 mutant cells cultured at 36°C for 5 hr (left) and 8 hr (right) and expressing the cen1–GFP. The number indicates minutes.

Aberrant centromere separation in mis12

To gain more information about the phenotype of mis12, it was observed how the sister centromeres (cen) behaved during mitosis. For this procedure, the cen1–GFP probe used previously (Nabeshima et al. 1998) was applied to visualize cen DNA. Briefly, LacI–GFP was expressed in S. pombe cells integrated with the tandem LacO sequences near the cen1. LacI–GFP bound to the LacO site was an appropriate marker for the cen1 DNA. As the cen1–GFP signal was very faint at 36°C compared with Sad1–GFP, simultaneous observation of cen1–GFP and Sad1–GFP at 36°C has not been successful (it was possible at 26–30°C; Nabeshima et al. 1998). In 8 of 13 cells undergoing the second mitosis, the cen1–GFP signal was rapidly split in mis12 apparently as in wild type (Fig. 1F left, 0–1 min). In other cases (5/13), the centromere signal movement was abnormal. Four cells showed the movement of cen1–GFP to one direction without splitting, followed by separation (right, 6 min), leading to two cen1–GFP signals in one divided nucleus (indicated by the arrowheads). As S. pombe cells contain three haploid chromosomes, aberrant centromere movement, if it occurred for each chromosome at the same frequency, could explain the lethality of mis12 cells by their production of aneuploidy cells (see Discussion).
Aberrant metaphase spindle size in the mis6 mutant

A similar set of experiments was done with mis6-302. Fifteen mutant cells analyzed all revealed the three distinct spindle phases as in mis12. Time-lapse images of Sad1–GFP in one mis6 cell at 36°C are shown in Figure 2A. Aberrant pear-shaped nuclear formation (seen at 9 min in A) followed by unequal nuclear division (at 13.5 min) is also seen in the mutant cells. Two mutant cells after 6.0 and 4.2 hr at 36°C (#1 and #2, respectively) in Figure 2B (left) showed expansion of the phase-2 spindle. Average length of the phase-2 spindle in mis6 [3.1 ± 0.5 µm; Fig. 2B, right, solid columns] was 35% longer than that of wild type [open column, 2.3 ± 0.2 µm]. Mis6 is thus also required for correct spindle morphogenesis at metaphase. The duration of phase 2 (4.9 ± 1.7 min) in mis6 was more variable than the wild type. To further confirm that metaphase spindle length was extended in the mis6 mutant, spindle length of metaphase-arrested cut9 mis6 and cut9 cells was measured [cut9 is defective in polyubiquitination of mitotic cyclin and Cut2; Funabiki et al. 1996a]. Single and double mutants were first arrested in G1 by nitrogen starvation and then released to the complete medium at 36°C. After 8 hr, metaphase cells were accumulated because of the inactivation of Cut9 mutant protein. In metaphase-arrested cut9 mis6 double mutant, sister centromeres were prematurely disassociated because of the inactivation of the Mis6 mutant protein, but not in the single cut9 [Saitoh et al. 1997]. At that time, the metaphase spindle length of cut9 mis6 was 25% longer than that of single cut9 mutant [data not shown].

Spindle size and centromere proteins

Spindle extension blocked in a sister chromatid cohesion mutant, mis4

Spindle length in phase 2 was normal in top2 and cut14 mutants at 36°C [Nabeshima et al. 1998], respectively, and both were defective in chromosome condensation and separation. In nuc2 and cut9 mutants blocked at metaphase because of the defects in anaphase promoting proteolysis [Yamada et al. 1997], spindle length was similar to that of wild-type metaphase cells. In contrast, spindle length in phase 2 was aberrant in mis4-242. The phase-2 spindle formed in mis4 was twice [4.6 ± 0.1 µm] the length of wild type, but it did not actually elongate further for a long period [Fig. 3A,B], whereas sister chromatid separation had already occurred (this was confirmed by FISH with the centromere probe, data not shown). The mutant spindle increased without interruption for a long period [Fig. 3A,B], whereas sister chromatid separation had already occurred (this was confirmed by FISH with the centromere probe, data not shown). The mutant spindle increased without interruption for a long period [Fig. 3A,B], whereas sister chromatid separation had already occurred (this was confirmed by FISH with the centromere probe, data not shown).
mature sister chromatid dissociation, because the mitotic spindle was further elongated in mis4 when the spindle checkpoint protein mad2 was deleted (K. Furuya and M. Yanagida, unpubl.).

In metaphase-arrested cut9 mis4 at 36°C [cells were initially nitrogen starved and then shifted to the complete medium at 36°C], spindle length was 75% longer (3.7 ± 0.8 µm in fixed and anti-Sad1 antibody-stained cells) than in single cut9 [2.1 ± 0.5 µm] at 36°C (data not shown).

Mis12, a 30-kD protein locating at centromeres throughout the cell cycle

The mis12+ gene was isolated by chromosome and cosmid mapping (Materials and Methods). The isolated gene rescued the temperature-sensitive phenotype of mis12-537. The mis12+ gene encodes a novel protein containing 259 amino acids. Database search showed that S. cerevisiae, Aspergillus nidulans, and Magnaporthe grisea coding sequences [YAL034W-A, ENAC000133, and mgae0004dc05l, respectively] significantly resemble the predicted sequence of Mis12 [Fig. 4A]. The S. cerevisiae gene product was designated Mtw1p [Mis twelve-like protein]. Sequence similarity was confined in the 88 amino-terminal amino acids. Protein structure prediction with the program COILS indicates that the central regions of Mis12 (100–150) and Mtw1p (101–150) might be coiled–coil.

Gene disruption was performed by one-step replacement. Most of mis12+ was replaced by the S. pombe ura4+ gene. Ura+ heterozygous diploid cells obtained by homologous integration were dissected. Only two spores were viable, and both were Ura+, suggesting that Mis12 was essential for cell viability. mis12 null cells divided two to four times after germination. They were observed after DAPI stain (Fig. 4B): The daughter nuclei with unequal sizes were highly frequent, showing that the temperature-sensitive and null phenotypes were similar.

To confirm that the Mis12 protein had the expected molecular weight, immunoblotting was performed. The HA [hemagglutinin antigen]-tagged Mis12 protein band with the expected molecular weight was detected in cell extracts carrying the integrated Mis12–HA gene by anti-HA antibody [Fig. 4C, lane 2]. The integrated HA-tagged Mis12 sedimented around 4-12S in sucrose gradient centrifugation, whereas Mis6 was broadly distributed, forming a peak at 15S (data not shown); this suggested that Mis12 and Mis6 belonged to different molecular complexes although their localizations were similar (see below).

Intracellular localization of Mis12 was visualized by GFP tagging (the mis12–GFP gene integrated onto the chromosome with the native promoter for A and D or expressed in plasmid for B and C). The GFP signals were clustered into one dot at the SPBs in interphase (the top cell in Fig. 5A, the bottom cell in early mitosis), whereas two or three signals were seen along the spindle in mitosis [Fig. 5B], visualized by anti-tubulin staining [TUB]. In mitotically-arrested nda3-311 (β-tubulin) mutant, the single Mis12–GFP signal was seen on each of the hyper-condensed chromosomes [Fig. 5C]. These results showed that Mis12 was localized at centromeres throughout the cell cycle.

The Mis12–GFP signals seen in living cells were consistent with those of fixed cells. Localization of Mis12–GFP strongly resembled that of Mis6–GFP (Saitoh et al. 1997). The Mis12–GFP signal was clustered until late G2 (0 min in Fig. 5D), and became multiple in M (1 min), often seen as two or three dots along the short rod (2–6 min) during the putative metaphase, and then rapidly separated (6.5–7 min), followed by further separation in anaphase B. Mis12 appeared to be bound to the centromere of all the chromosomes.

Figure 4. Mis12 is an essential conserved protein. (A) Sequences of S. cerevisiae, A. nidulans, and M. grisea are similar to the amino terminus of Mis12. (Top) Identical amino acids are boxed, similar ones are shaded. (Bottom) Alignment of Mis12 and Mtw1p. The predicted coiled–coil exists in the middle [amino acids 100–150], while the conserved regions are in the amino terminus [amino acids 1–88]. (B) Gene disruption of mis12+ led to the missegregation phenotype with the large and small daughter nuclei. Gene-disrupted cells were stained by DAPI. Bar, 10 µm. (C) Identification of Mis12 protein by immunoblotting. The carboxyl terminus of the mis12+ gene was tagged with HA and integrated onto the chromosome of mis12-537 by homologous recombination [the promoter was native]. The temperature-sensitive phenotype was rescued in the resulting integrant, which grew normally. Mis12 was detected at the expected molecular mass [lane 2]. The band intensity increased in cells overproducing Mis12–HA by multicopy plasmid [lane 1], but the band was not detected in extracts carrying the vector [lane 3].
Requirement of Mis12 for maintaining inner centromere structure

The chromatin immunoprecipitation method was used to identify the centromere DNA that interacts with Mis12. Four primers, imr1, cnt1, otr2 (dhII), and lys1 were used (Fig. 6A; Takahashi et al. 1992). Combination of Mis12–HA and anti-HA antibody produced the PCR products (lane 1), when the inner centromere primers cnt1 and imr1 were used, but not the outer region primers otr2 or lys1. Beads alone without antibody (lane 2) did not produce any PCR DNA. These results indicated that Mis12 interacted with the inner centromere DNAs.

As Mis6 was also shown to be an inner-centromere-interacting protein (Saitoh et al. 1997), we examined whether Mis12 formed the complex with Mis6. To this end, immunoprecipitation was done with HA–Mis12 and Myc–Mis6, both of which were expressed by the genes integrated onto the chromosome. Most of the Mis12–HA was precipitated (Ppt) by anti-HA antibodies, but no Mis6–Myc was detected in the precipitates (Fig. 6B). There was no cross-precipitation between Mis12–HA and Mis6–Myc, showing that the Mis6 and Mis12 did not form the stable complex.

To answer the question whether the Mis6 mutant protein could interact with the inner centromere, the mutant mis6-302 gene was tagged with HA and integrated onto the chromosome. Mis6-302–HA was immunoprecipitated after formaldehyde fixation. The levels of the PCR products were reduced at 26°C and negligible at 36°C, indicating that the primary defect in mis6-302 was due to its deficient interaction with the centromeres (data not shown).

To determine whether association of Mis6 to the inner centromere region required active Mis12, a mis12-537 strain expressing the integrated Mis6–HA was made, cultured at 36°C for 6 hr, and extracts were prepared and immunoprecipitated by anti-HA antibody (Fig. 6C). Nearly identical amounts of cen–DNA were precipitated from the wild-type (lane 1) and mis12 mutant (lane 2). Lanes 4, 5, and 6 are loading control. Hence, mis6–HA could interact with the centromere in the absence of functional Mis12. Conversely, the mis6-302 strain integrated with the Mis12–HA gene was used. Immunoprecipitated Mis12–HA in mis6-302 was bound to the inner centromere regions (lane 8). Mis6 and Mis12 appeared to interact independently with the centromeres.

Loss of centromere-specific chromatin digestion pattern in the mis12 mutant

The inner centromere DNAs of S. pombe has a specialized chromatin (Polizzi and Clarke 1991; Takahashi et al. 1992). In the mis6-302 mutant, the smeared chromatin pattern is abolished at 36°C (Saitoh et al. 1997). The same result was obtained in mis12-537 at 36°C (Fig. 6D): The inner centromere probes for hybridization, cnt1 and imr1, failed to show the smeared pattern in mis12 mutant cultured at 36°C for 8 hr (the ethidium bromide staining pattern shown at the right end). The patterns for the wild-type control are also shown. Mis12 is thus required for maintaining the inner centromere structure.

Missegregation occurs after the passage of mis12 in the previous mitosis

To understand the role of Mis12, it is important to determine how its action is implicated in the cell cycle. First, chromosome missegregation (unequal nuclear division seen by DAPI stain was verified to be missegregation by FISH; Fig. 7A) took place in mis12-537 cells after one cycle of cell division at 36°C (Fig. 7B). The phenotype did not appear for the first division [1–3 hr]. The majority of cells of fission yeast in the asynchronous culture were in G2. Eighty percent of the binucleate cells
after the second division (4–8 hr) contained missegregation (unequal-sized nuclei). Coincident with the second mitosis (2nd M), a sharp decrease of cell viability only after missegregation was observed. Control G1-arrested cultures were directly released to YPD at 36°C, and vice versa. (D) Nuclear chromatin was prepared from wild-type and the mis12 mutant cultured at 36°C for 8 hr, and digested with micrococcal nuclease for 1, 2, 4, and 8 min, followed by agarose gel electrophoresis and Southern hybridization with the three DNA probes, otr1, imr1, and cnt1 (Saitoh et al. 1997). The ethidium-bromide staining patterns are shown at right with the size markers. The smeared nucleosome pattern in the inner centromere was abolished in mis12 mutant.

The cut4 mis12 double-mutant strain carrying the integrated Mis6–GFP was constructed to examine whether sister centromeres could be prematurely separated in metaphase (cut4 is defective in polyubiquitination of mitotic cyclin and Cut2; Yamashita et al. 1996). The double mis12-537 cells were nitrogen starved to arrest in G1 at 26°C, then cultured at 36°C under the same G1 condition for 4 hr and released to the rich YPD medium at 36°C (Fig. 7D). The first mitosis took place 9–10 hr after cells were exposed to 36°C, but abnormal chromosome segregation was negligible (~4%). Only in the second mitosis (12 hr), was missegregation observed. Control G1-arrested culture was directly released to YPD at 36°C, and ~60% of the cells produced aberrant missegregation in the second mitosis that took place 9–10 hr after the shift to 36°C. This experimental result clearly showed that the delayed phenotype is not due to the rate of protein inactivation, and passing the first mitosis at 36°C is crucial to produce the phenotype. Mutant Mis12 protein was thus not temperature sensitive for folding but not for function. Taken together, missegregation in mis12 occurred only after cells had passed through the previous mitosis to the subsequent mitosis at 36°C without proper Mis12 function.

One possibility is that Mis12 is temperature sensitive for folding but not for function. To examine this possibility, we performed the following experiment: mis12-
A mutant was first cultured in the synthetic EMM2 at 36°C for 3.5 hr and then at 36°C in the complete YPD for 3 hr. The signal of Mis6–GFP was then observed in methanol-fixed cells. Under this culture condition, the second mitosis could take place as cut4 was able to go through mitosis in the synthetic EMM2 at 36°C (Yamashita et al. 1996). The Mis6–GFP signals were frequently resolved in four to six dots in the second mitosis of cut4 mis12 (Fig. 7E, right), suggesting that sister centromeres were separated in the metaphase-arrested cells. However, less than three signals were observed in mitotically arrested single cut4 mutant cells (left). The Mis6–GFP signal in the first mitosis of cut4 mis12 (middle) was similar to that of single cut4.

mis12 suppresses dis1, whereas cold-sensitive mutations leading to normal spindle length suppress mis12

To identify gene products that might interact with Mis12, we made a number of crosses between mis12-537 and known mitotic mutations. We found that mis12-537 (but not mis6) could suppress the cold-sensitive phenotype of dis1 mutation [Fig. 8A]. Dis1 plays a role in linking the kinetochore to the mitotic SPBs (Nabeshima et al. 1998). In the dis1 mutant, the prophase spindle continued to elongate without sister chromatid separation. Suppression did not work in the opposite direction, however: The temperature-sensitive phenotype of mis12 was not suppressed by the dis1 null. Instead, at 33°C, the double-mutant mis12 dis1 null was synthetically lethal. These results suggested that Mis12 is functionally related to Dis1.

We screened for mutations that could suppress the temperature-sensitive phenotype of mis12-537 and isolated 500 spontaneous revertants that produced colonies at 36°C. Twenty four of them were cold sensitive; they were unable to form colonies at 22°C. Although genetic analyses of the mutants have not been completed, all of these mutants curiously display deviation from the rod shape. Identification of the genes responsible for the cold-sensitive mutations is under investigation. Two
strains, 163 and 165, were examined to learn whether suppression of the temperature-sensitive phenotype of mis12 led to the decrease in spindle length. Mutant cells were stained by anti-sad1 antibodies, and their pole-to-pole distance in mitotic cells was measured at 36°C (Fig. 8B). For both strains, the spindle length in a large fraction of cells was identical to that of the wild type (open arrowheads), showing that the mis12 phenotype of long metaphase spindle was greatly diminished when suppressor mutations were combined. These results are consistent with the hypothesis that metaphase spindle expansion and drastic missegregation in mis12 are closely related.

Discussion

This paper reports a novel centromere protein, Mis12, in fission yeast. It is essential for viability, and its loss leads to a dramatic missegregation of chromosomes. It is not required for sister chromatid separation, but is absolutely required for correct segregation of chromosomes. Budding yeast Mtw1p shares similarity with Mis12 in the amino-terminal sequence and the central putative coiled-coil motif. Gene disruption indicates that MTW1 is essential for viability with the cell cycle arrest phenotype showing a large bud with the nucleus often located in the neck between two daughter cells (G. Goshima and M. Yanagida, unpubl.). Mtw1p is not included in the known centromere-interacting proteins. Intracellular localization of Mtw1p remains to be determined.

Certain properties of Mis12 are similar to Mis6 (Saitoh et al. 1997). Both colocalize at the centromeres, and co-precipitate with the inner centromere DNAs rich in A or T clusters. The inner centromere-specific chromatin structure is abolished in mis12 as in mis6 cells. Therefore, Mis12 and Mis6 proteins are essential for creating the architecture of the inner centromere. Moreover, both Mis6 and Mis12 were discovered to affect spindle length during the metaphase–anaphase transition period. We assume that this property is important for faithful chromosome segregation [see below]. A budding yeast kinetochore protein Ctf19p, which provides a link between the mitotic spindle and the kinetochore (Hyland et al. 1999), might have similar function. Mis6 and Mis12 differ considerably in other aspects. They neither cosediment nor are coimmunoprecipitated, indicating that they do not function together in the same complex. Mis12 [but not Mis6] interacts with Dis1 [Nabeshima et al. 1995; Naka- seko et al. 1996], possible mediator between kinetochores and the SPBs. Bipolar spindle assembly in budding yeast is achieved by motors that antagonize each other by exerting forces in opposite directions (Hoyt et al. 1997). Considering the continuous elongation of the prophase spindle in dis1 because of the lack of pulling force [Nabeshima et al. 1998] and abnormal metaphase spindle length in mis12 perhaps due to the loss of sister-centromere cohesion, their double-mutant phenotype might be obtained by the change in the force balance in the spindle.

Mis12 seems to function in the previous M phase for proper chromosome segregation. This surprising conclusion is reached on the basis of several results. If Mis12 is
inactive from \(G_1\) (or from \(G_2\)), the immediate mitosis is completely normal, but in the second round of mitosis, viability sharply decreases with extensive chromosome missegregation. Preheating in \(G_1\) for 4 hr at 36°C confirmed that Mis12 had to be inactive in the first mitosis for producing missegregation in the second mitosis. Sister centromeres were prematurely separated at metaphase in the second mitosis. An interpretation of these results is that sister centromeres are connected by the action of functional Mis12. A simple model is that Mis12 may establish a centromere structure during the first mitosis that can be built on to ensure correct segregation in the second mitosis. Mis6 association with centromeres is not dependent on Mis12, thus, at least two independent functions must exist. One implication is that drastic missegregation occurs only when the mutant Mis12 protein is inactivated from the first M through to the second M phase. This would be a highly effective way to maintain segregation fidelity of chromosomes, as the centromere defect due to mis12 mutation could be repaired over a broad time span even in late \(G_2\) if active Mis12 were supplied. Missegregation occurs in the mis6 mutant if Mis6 is continuously inactive from \(G_1\) to M (Saitoh et al. 1997). The defect could be fixed if active Mis6 were supplied anytime from \(G_1/S\) to M. These late remedy systems explain a part of the high fidelity in chromosome segregation. We found recently that a class of centromere chromatin protein was localized at the centromere in a Mis6 (but not Mis12)-dependent manner, supporting the theory that Mis6 and Mis12 are functionally distinct [K. Takahashi and M. Yanagida, in prep.].

Two other conclusions emerging from the present study may have some general implications. One is that centromere-connecting proteins actively participate in correct morphogenesis of the mitotic spindle. Mutations in the centromere proteins affect overall spindle size. Kinetochore, however, are apparently not required for spindle formation per se. Heald et al. (1996, 1997) demonstrated using frog extracts that microtubules and DNA beads self-assemble into the bipolar spindle structure in the absence of centrosomes and kinetochores. Functional kinetochores were not required for the assembly of a bipolar spindle in budding yeast (Sorger et al. 1995). The present study showed that the kinetochore proteins are a major determinant for correct metaphase spindle length in fission yeast. Expansion of the metaphase spindle is not due to extension of the centromere DNAs, neither cen1–GFP [this study] nor GFP–Mis6 [data not shown] revealed any stretching of the centromere signals in mutant cells.

The expanded metaphase spindle might be explained by defects in the steps leading to correct spindle morphogenesis. Alternatively, it might be explained by the unbalanced force in the spindle. The metaphase spindle length can be assumed to be set by the point at which the force that the linked sister kinetochores exert to pull the poles toward each other is balanced by other, kinetochore-independent forces that tend to push the poles apart from each other. In mis12 and mis6 mutants, defects in kinetochore function and sister kinetochore linkage diminish the force that pulls the poles toward each other, resulting in the force balance causing the longer metaphase spindle. The metaphase spindle length became normal and the frequency of missegregation was greatly reduced at 36°C when cold-sensitive suppressor mutations were combined with mis12-537. In these suppressor mutants, the kinetochore function might be strengthened, inducing the stronger pulling force of the poles, which in turn reduces the length of the metaphase spindle. We recently found that the metaphase spindle in one cold-sensitive mutant is shorter than normal at the permissive temperature (G. Goshima et al., unpubl.). We consider that both models, defective in the force balance or the spindle-size control, are equally possible in causing chromosome missegregation. These two models, however, are not necessarily mutually exclusive, because the longer the spindle is, the more difficult it will be for a kinetochore that is close to one pole to be captured by microtubules that emanate from the opposite pole. The expanded metaphase spindle thus may have a crucial relation to the missegregation phenotype.

The other conclusion is that all three phases of spindle dynamics exist in mis6 and mis12, although the metaphase spindle is exceedingly long. The decrease in the fidelity of sister-chromatid separation is not due to the loss of the spindle pulling force, as the unequal-sized daughter nuclei were fully separated. Full extension of the spindle in mis6 and mis12 suggests that defects in these mutants do not activate spindle checkpoint. Mis6 and Mis12 may be involved in recruiting the checkpoint components onto the spindle, so that the spindle made in mis6 and mis12 mutants is deficient in spindle checkpoint. This possibility can be tested, but other explanations also are possible.

How, then, are sister chromatids missegregated in mis6 and mis12 mutants in spite of the presence of apparently normal spindle extension? We have no firm answer to this important question, other than the longer metaphase spindle discussed above. One clue is that we have not observed lagging chromosomes in mis12 (or mis6) mutants. Lagging chromosomes located along the elongated anaphase spindle are found in many mutants (Ohkura et al. 1988; Ekwall et al. 1995). In mis6 and mis12, however, chromosomes are always brought to the poles. Behavior of the cen1–GFP signal visualized in mis12 is another clue to understanding missegregation. In >50% of mis12 cells, the cen1 signals are rapidly separated toward the poles as in wild type, but in the remaining cell populations, the cen1–GFP signals behave abnormally. These results suggest that there is a high probability that the “entry gate” into sister centromere separation, rather than the force-generating machinery for this separation, is defective in mutant cells. Aberrant sister centromeres separate only after they move along on the elongating anaphase B spindle, remaining in one side of the cell. One of the sister kinetochores may not function properly, or the link between one kinetochore and one pole may be broken immediately before anaphase. Biorientation of the sister chromatids in the
spindle may be impaired as hypothesized previously (Saitoh et al. 1997), so that one sister kinetochore frequently becomes defective in interacting with the kinetochore microtubules. Alternatively, biorientation is established, but the link between the kinetochore and the pole is fragile because of the elongated metaphase spindle, so that the trigger of anaphase spindle movement may break one of the two pole-to-kinetochore connections. In another case, microtubules from the same direction might capture the sister centromeres of one chromosome that have lost biorientation so that the sister chromatids would be brought to the same pole, whereas other chromosomes are correctly separated by spindle extension. These cases are thought to be possible causes for aneuploidy. Molecular understanding of the centromere defects would be crucial for solving the high-fidelity segregation mechanism supported by Mis6 and Mis12.

Materials and methods

Strains, media, and culture media

The complete YPD (1% yeast extract, 2% polypeptone, 2% glucose) and the minimal EMM2 medium (Mitchison 1970) were used for the culture of S. pombe. EMM2-N was used for nitrogen starvation. LB medium [0.5% yeast extract, 1% polypeptone, 1% NaCl at pH 7.5] was used to grow Escherichia coli MM294. S. pombe mutant strains mis12-537, mis6-302, mis4-242, cut9-665, and nda3-KM311 were described previously (Hiraoka et al. 1984; Takahashi et al. 1994; Saitoh et al. 1997; Yamada et al. 1997; Furuya et al. 1998). S. pombe strain used for visualization of cen1–GFP was described previously (Nabeshima et al. 1998). The strain MKY7B-8 containing plasmid with GFP–LacI–NLS under the nmt1 promoter was a modification of the MKY7A-4 strain described. This strain was grown in the presence of 2 μM nmt1-KM311 and nda3-KM311.

Isolation of the miss2+ gene

The miss2+ gene was isolated by chromosome mapping and cosmid walking. By extensive crossing, miss2-537 was mapped near cut3 (10 cm) and sds23 (21 cm) in chromosome II. Cosmids in this region (146, 337, 1734, 1709, 409; Mizukami et al. 1993) were introduced into mis2-537 by transformation with a helper plasmid pYC11 (Chikashige et al. 1994). Cosmid c409 could suppress the temperature-sensitive phenotype of the miss2+ gene; a 700-bp long 5’-upstream sequence was included. The 8x Myc tag was added to the carboxyl terminus of the miss2+ gene by PCR amplification of the Myc epitope. Mis12–GFP, Mis12–HA, and Mis6–Myc were integrated onto the genome by an integration vector pYC11 as described (Nabeshima et al. 1997), although concanavalin A was not used.

Isolation of cold-sensitive suppressors

Haploid h+ leu1 mis12-537 cells grown at 26°C were plated on YPD at 36°C for 4 days. Five hundred Ts+ revertants were obtained from 5 × 10⁶ cells, of which 24 strains did not produce colonies at 22°C. These Ts+ Cs- strains were cultured at 36°C and stained by anti-Sad1 antibodies.

Nitrogen starvation

The procedures for nitrogen source starvation experiments with cut9 miss6 and cut9 miss4 were described previously (Saitoh et al. 1997; Furuya et al. 1998). Single mis12 mutant cells [5 × 10⁶/ml grown in EMM2 at 26°C] were washed in EMM2-N and the concentrated cell suspension (2 × 10⁶/ml) was cultured at 26°C for 22 hr. Cells were then released into the rich YPD medium and cultured at 36°C. The procedures of FACScan analysis were described previously (Costello et al. 1986).

Chromatin immunoprecipitation, micrococcal nuclease digestion, and immunological methods

The Chip method adapted to S. pombe was described in Saitoh et al. (1997). For miss6 and mis12 mutant strains, cells cultured at 36°C for 6 hr were used for formaldehyde fixation. The procedures for micrococcal nuclease digestion were described by...
Takahashi et al. [1992] with a modification. Cells were cultured at 36°C for 8 hr, and treated with micrococcal nuclease (250 U/ml, Worthington Biochem). Plasmids pKT110, pKT108, and pYC148 were used as hybridization probes. For immunoblotting and precipitation, cell extracts were prepared with the HB buffer (Moreno et al. 1989). Immunoprecipitation was done by anti-HA antibody [12CA5, BABCO] conjugated with protein A-Sepharose. Anti-Myc antibody 9E10 (Calbiochem) was used.

Acknowledgments

The present study was supported by CREST of Japan Science and Technology Corporation, and a grant from the Human Frontier Science Project Organization. S.S. acknowledges the Japan Society for Promotion of Science, which awarded him a postdoctoral research fellowship.

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Note added in proof

The nucleotide sequence data reported in this paper will appear in DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers: AB027472 for the S. pombe mis12+ gene and the p19SKP1-like gene; AB027473 for the S. cerevisiae MTW1 gene.

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*Genes Dev.* 1999, 13:

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