Faithful anaphase is ensured by Mis4, a sister chromatid cohesion molecule required in S phase and not destroyed in G₁ phase

Kanji Furuya, Kohta Takahashi, and Mitsuhiro Yanagida¹

CREST Research Project, Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa-oiwake-cho, Sakyo-ku, Kyoto 606, Japan

The loss of sister chromatid cohesion triggers anaphase spindle movement. The budding yeast Mcd1/Scc1 protein, called cohesin, is required for associating chromatids, and proteins homologous to it exist in a variety of eukaryotes. Mcd1/Scc1 is removed from chromosomes in anaphase and degrades in G₁. We show that the fission yeast protein, Mis4, which is required for equal sister chromatid separation in anaphase is a different chromatid cohesion molecule that behaves independent of cohesin and is conserved from yeast to human. Its inactivation in G₁ results in cell lethality in S phase and subsequent premature sister chromatid separation. Inactivation in G₂ leads to cell death in subsequent metaphase–anaphase progression but missegregation occurs only in the next round of mitosis. Mis4 is not essential for condensation, nor does it degrade in G₁. Rather, it associates with chromosomes in a punctate fashion throughout the cell cycle. *mis4* mutants are hypersensitive to hydroxyurea (HU) and UV irradiation but retain the ability to restrain cell cycle progression when damaged or sustaining a block to replication. The *mis4* mutation results in synthetic lethality with a DNA ligase mutant. Mis4 may form a stable link between chromatids in S phase that is split rather than removed in anaphase.

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In eukaryotic cells, replication of chromosomal DNA occurs once during S phase followed by separation of duplicated DNAs into two daughter nuclei during M-phase. DNA replication is initiated at the onset of S phase in particular chromosomal regions, the origins, which are unwound and stabilized by single-strand-binding protein. The replication machinery is large and complex, containing a number of enzyme subunits including DNA polymerases and helicase. However, other factors are necessary for initiating replication such as the origin recognition complex, which is bound to origin sequences throughout the cell cycle. Cdc6 and the MCM complex are also needed and are loaded onto the origins to form the prereplicative complex (e.g., see Rowles and Blow 1997). Moreover, an essential protein kinase Cdc7/Dbf4 must act prior to replication, so that the replication machinery can interact with the prereplicative complex and thereby ensure continuing replication. The proteins described above are still part of the gene products required for the quality control of DNA replication. When chromosomal DNAs are damaged or replication is blocked,

entry into mitosis is restrained until the damage is recognized and repaired (e.g., see Kitazono and Matsumoto 1998). A block of mitotic entry upon damage or inhibition of replication can be accomplished by inactivation of cyclin-dependent protein kinases (CDKs). This checkpoint system is important for maintaining the genome with high fidelity, otherwise damaged chromosomal information will be transmitted to the nuclei of progeny cells.

To maintain the genome at high fidelity, there is an enigmatic aspect in chromosomal structure, that is, the mechanism by which sister chromatids are linked after duplication of chromosomal DNA. This sister chromatid link, alternatively called sister chromatid cohesion, must be dissociated before chromosomes are segregated into daughter cells (Holloway et al. 1993). Sister cohesion is thought to oppose the splitting forces exerted by kinetochore microtubules. If such linkage is absent, precocious sister chromatid separation will take place. The loss of sister cohesion in normal anaphase may thus trigger spindle movement. Until quite recently, very little or nothing has been known about the proteins required for sister chromatid cohesion. The first such protein to be identified was the *Drosophila* MEI-S332 protein required

¹Corresponding author.

E-MAIL yanagida@kozo.biophys.kyoto-u.ac.jp; FAX 81 75 753 4208.

for the maintenance of sister chromatid cohesion in male and female meiosis (Kerrebrock et al. 1995). This protein localizes to meiotic and mitotic centromeres (although it is not essential for mitosis) until sister chromatids separate and are dissociated from chromosomes at anaphase (Moore et al. 1998), suggesting that the destruction or the release of this protein might be required for sister chromatid separation. In fission yeast, meiotic sister chromatid cohesion is deficient in the recombination-defective *rec8-110* mutant (Molnar et al. 1995). Aberrant segregation is caused by precocious segregation of sister chromatids at anaphase I. In the same organism, mitotic centromeres are separated in metaphase-arrested *mis6* mutants defective in equal segregation of sister chromatids in mitosis (Saitoh et al. 1997). *Mis6* is essential for the formation of a centromere-specific chromatin in the G₁/S phase and maintains the connection of sister centromeres until anaphase.

In budding yeast, Mcd1p/Scclp, a sister-chromatid cohesion protein called cohesin, was identified by genetic screening and found to form the complex with Smc1p, an SMC protein, known to be involved in the structural maintenance of chromosomes (Guacci et al. 1997; Michaelis et al. 1997). Mcd1p/Scclp is similar to fission yeast Rad21 (Birkenbihl and Subramani 1992) and Rec8, and proteins homologous to Mcd1p/Scclp are present in higher eukaryotes. The *mcd1* mutant is defective in sister chromatid cohesion and chromosome condensation. Scclp associates with chromosomes in an Smc1p-dependent manner, dissociates from chromosomes at the metaphase-to-anaphase transition, and is degraded in G₁ by the anaphase promoting complex (APC)/cyclosome. Thus Rad21/Mcd1p/Scclp-like proteins may form a common cohesion apparatus that forms a complex with two SMC proteins and is regulated by association with and removal from chromosomes in the cell cycle. The other protein, Scc2p, was discovered to be required for sister chromatid cohesion in budding yeast (Michaelis et al. 1997). Its behavior in the cell cycle and relationship to the cohesin complex has not been reported.

In this report we show that the *Mis4* protein, initially identified by its requirement for stable maintenance of minichromosomes in fission yeast (Takahashi et al. 1994), is required for sister chromatid cohesion, and its function is quite distinct from cohesin. Fission yeast *mis* mutants are temperature-sensitive lethal at the restrictive temperature (36°C) and can grow at the permissive temperature (26°C), but show high loss rates of minichromosomes at 26°C or semi-permissive temperatures. Twelve *mis* genes have been identified. The *mis5*⁺ gene encodes one of the MCM subunits essential for replication (Takahashi et al. 1994; Adachi et al. 1997) whereas *mis6*⁺ codes for a centromere protein (Saitoh et al. 1997). The *mis1*⁺ and *mis10*⁺ genes encode the subunits of DNA polymerase δ (H. Tatebe, H. Kondoh, M. Yanagida, unpubl.). The phenotype of *mis4* is similar to that of *mis6*, as not only minichromosomes but also regular chromosomes were missegregated at 36°C (Takahashi et al. 1994). In addition to the *mis* phenotype, the *mis4* mutant displays a low frequency of the *cut* phenotype

(the occurrence of septation or cytokinesis in the absence of normal nuclear division; Hirano et al. 1986), and hypersensitivity to hydroxyurea (HU) and UV irradiation at permissive and semi-permissive temperatures (Takahashi et al. 1994). *Mis4* thus appears to play an important role in maintaining the quality of the genome against cell cycle aberration, damage, and replication inhibition.

Results

Missegregation in mis4-242 is preceded by a prior lethal event

To determine which stage is defective during the cell cycle, cultures of *mis4-242* were synchronized. Small, early G₂ cells were collected by use of an elutriator rotor from an exponentially grown culture at 26°C, the permissive temperature. Then the culture was transferred to 36°C, the restrictive temperature (0 min, Fig. 1A). Viability of the mutant cells remained high until 60 min but then decreased and reached 50% at about 100 min while the cells proceeded across the first mitosis at around 80–100 min. To distinguish the timing of metaphase and anaphase, the frequencies of cells showing short or elongated spindles were measured; cells containing short spindles (<2.5 μ m in length), which corresponded to prophase through metaphase, reached a maximum at 80 min, whereas those displaying elongated anaphase spindles (>2.5 μ m and up to 14 μ m) peaked at 100 min. Thus *mis4-242* cells synchronized culture in early G₂ at 36°C appeared to lose viability as they progress from metaphase through anaphase. Further decrease of viability occurred in the succeeding S phase and cell separation (around 120–140 min). The G₁ phase is very short in fission yeast.

In sharp contrast to the above observations, chromosome segregation and spindle movements were apparently normal during the first mitosis (micrographs in Fig. 1B taken at 100 min: left, DNA stain; right, anti-tubulin antibody stain). Missegregation defects were quite frequently produced only later during the second round of mitosis at 200–220 min (Fig. 1C). Different types of missegregation are shown in Figure 1D (taken at 220 min). Unequal segregation of sister chromatids was most common. Chromatids remaining along the anaphase spindle (indicated by arrowheads) were also seen. The kinetochore microtubules might fail to pull the chromatids toward the poles.

mis4 mutants lose viability during S phase

We examined whether the missegregation defect could be produced in the first mitosis, if mutant cells previously arrested in G₁ by nutrient starvation were transferred to the growing medium at 36°C. Viability of the mutant cells sharply decreased between 3 and 4 hr (Fig. 2A), while the cells traversed S phase (Fig. 2C, right panel). Abnormal missegregation phenotypes were frequently produced during the following first mitosis (7 hr,

Figure 1. A lethal event in the first cell cycle precedes the abnormal second mitosis in a G_2 -synchronized culture of *mis4-242* cells. Mutant cells were grown at 26°C, and small early G_2 cells were collected by an elutriator rotor and then cultured at 36°C for 5 hr in synthetic EMM2 medium. (A) Cell viability (%) (▲) was measured by plating of cells at 26°C. Spindle indexes (%) were obtained by estimation of the frequencies of cells that contained the spindle stained by anti-tubulin antibody. The length of the spindle was classified into short (<2.5 μ m; □) or long (>2.5 μ m; ■). Short spindles peaked at 80 and 200 min, whereas long spindles peaked at 100 and 220 min. The decrease of cell viability occurred when the two spindle indexes were high in the first mitosis. Abnormal mitosis occurred only in the second mitosis. (B) Mitotic cells in the first mitosis at 36°C showing normal anaphase. Micrographs taken at 100 min. (Left) Chromatin DNA staining by DAPI; (right) spindle staining by anti-tubulin antibody. (C) Frequencies of cells showing equal sister chromatid separation in normal anaphase (○; examples shown in B) and unequal abnormal anaphase (●; examples in D). (D) Mitotic cells in the second mitosis at 36°C showing unequal sister chromatid separation. Micrographs were taken at 220 min. (Arrowheads) Chromatids remaining along the spindle. Bar, 10 μ m.

Fig. 2B,D). These results showed that *mis4-242* cells cultured from G_1 at 36°C became inviable during the S phase, and chromosome missegregation occurred in the succeeding mitosis.

The 160-kD product of *mis4*⁺ is an evolutionarily conserved protein

The *mis4*⁺ gene was isolated by a search of plasmids that fully suppressed the temperature-sensitive phenotype of *mis4-242* by use of a genomic library of *Schizosaccharomyces pombe*. Thirty-nine independent clones were obtained, and they were all found to overlap, indicating that they were derived from a single genetic locus. The cloned DNA was integrated into the chromosome by homologous recombination with the *ura4*⁺ gene marker (see Materials and Methods), followed by tetrad analysis with the *mis4-242* mutant, and verified to be derived from the *mis4*⁺ locus. The plasmid pKT221 was further subcloned, and the resulting pKF201 contained a minimal functional sequence (5.1 kb). The nucleotide sequence was determined, and one coding region representing the *mis4*⁺ gene was present. The predicted polypeptide contained 1583 amino acids interrupted with seven presumed introns (Fig. 3A). This coding region was also present in the *S. pombe* genome database.

A database search revealed that the fungus *Coprinus cinereus* Rad9 (2157 amino acids; Seitz et al. 1996) and the budding yeast *Scc2* (1493 amino acids; Michaelis et al. 1997) showed a significant similarity from the central to the carboxy-terminal region of the polypeptides as illustrated in Figure 3B. Their overall identity was not high, but three regions showed significant sequence identity (22%–38%). It was also noted that mouse and human cDNA sequences (HUMHBC4244

and AA062272) similar to the carboxy-terminal regions of these proteins exist in the databases, indicating that Mis4-like proteins are present in mammalian organisms.

The hemagglutinin antigen (HA) sequence was fused to the carboxyl terminus of Mis4. The resulting fusion protein was expressed in *S. pombe* cells either after integration into the chromosome or introduced as a multicopy plasmid. As shown in Figure 3C, HA-tagged Mis4 was detected at the expected molecular mass of 160 kD (lane 2, integrated single copy; lane 3, overproducing plasmid). Nonintegrated cells carrying the vector plasmid did not show the band (lane 1). Comparison of the immunoblotting intensity of Cut2 also tagged with the same HA construct indicated that the amount of Mis4 in cells was approximately four-fold higher than Cut2, that is, ~30,000 molecules per cell.

In block and release experiments carried out with a *cdc25* mutant, cells were arrested in late G_2 and then released to the permissive temperature. The amount of Mis4 protein did not greatly fluctuate during the cell cycle; it showed an increase (1.5-fold) in late mitosis, but otherwise the level remained roughly constant. The control mitotic cyclin (Cdc13) decreased in exiting of mitosis (Fig. 3D). Mis4 did not degrade in the G_1 -arrested *cdc10* mutant cells, and the level did not change in the cyclosome subunit mutants (data not shown). Mis4 is thus present throughout the cell cycle, whereas budding yeast Mcd1p/Scclp is destroyed in G_1 .

The *mis4*⁺ gene is essential for viability

Gene disruption of *mis4*⁺ was achieved by a single-step replacement (see Materials and Methods). The coding region was replaced with the *S. pombe ura4*⁺ gene. The

Figure 2. A lethal event occurs in S phase when *mis4-242* mutant cells are released from G₁ arrest. Mutant cells were nitrogen source-starved at 26°C to arrest then in G₁. Cells were then released in complete medium at 36°C. (A) Cell viability (●; decreasing from 3 to 4 hr after the shift) and cell number (○; increasing around 8–9 hr). (B) Frequency of cells showing unequal chromosome segregation (peaking at 7 hr). (C) DNA content measured by FACS analysis showed that DNA replication in *mis4-242* cells took place around 3–4 hr, coinciding with the occurrence of cell inviability. (wt) Wild-type control. (D) Examples of mutant cells showing abnormal anaphase. Cells stained by DAPI. Bar, 10 μm.

presence of the disrupted gene in the genomes of heterozygous diploids was verified by genomic Southern hybridization (data not shown). Tetrad analysis of the sporulated diploids indicated that only two spores were viable and that all viable spores were nondisrupted Ura⁺. The *mis4⁺* gene was therefore absolutely required for viability. The *mis4*-disrupted cells were observed after DAPI staining, chromosome condensation was observed in mitosis, and missegregation phenotypes resembling *mis4-242* were seen (data not shown).

HU-sensitive *mis4-242* maintains replication checkpoint control

mis4-242 was found to be hypersensitive to HU; like *cdc2-3w* it did not form colonies in 5 mM HU at 26°C (Fig. 4A). Mutant cells cultured at 26°C in liquid medium containing 15 mM HU were greatly elongated (Fig.

4C), although they contained a 1C DNA content determined by FACS analysis (Fig. 4B), showing that HU-induced replication checkpoint control, which restrained entry into mitosis, was maintained in the mutant. Note that cell viability did not decrease under these conditions for up to 8 hr (Fig. 4D).

The HU-sensitive phenotype of the mutant was further examined at 36°C. Mutant cells initially arrested in G₁ at 26°C were released in rich medium at 36°C either in the presence or the absence of HU. These cells contained 1C DNA even after 7 hr in the presence of HU whereas cells without HU completed S-phase at 4 hr (Fig. 4E). Cells were highly elongated in the presence of HU for 7 hr at 36°C (Fig. 4F), indicating that replication checkpoint control remained in the mutant even at that temperature. Cell viability in the presence of HU remained high until 4 hr but then decreased and reached 10% after 6 hr (Fig. 4G). This timing of inviability was 1–2 hr slower than that of mutant cells that lost viability during the progression through S phase in the absence of HU. Notably, however, most mutant cell nuclei after 6 hr in the presence of HU contained only a 1C DNA content. The prolonged S-phase block by HU in *mis4-242* thus caused severe cell lethality.

mis4-242 cells are sensitive to UV irradiation

In addition to HU-sensitivity, *mis4-242* cells were UV-irradiation sensitive at 26°C (Fig. 5A). To determine whether the DNA damage checkpoint control was retained after irradiation, the frequency of septum formation after damage was measured. Wild-type cells treated with UV irradiation showed a rapid decrease in the septum index as a result of the great delay for mitotic entry (Fig. 5B, left). Cells were elongated with a single nucleus (data not shown). The septation index was also reduced in *mis4-242* mutants after irradiation (right panel). The recovery of this index, however, was delayed (the mutant took 2–3 hr, rather than 1.5 hr as shown by the wild type). These results show that *mis4-242* mutants retain damage-sensing checkpoint control, but appear to be impaired in the process of repair or recovery.

mis4 is synthetic lethal with a DNA ligase mutation at 26°C

We looked for mutations that interacted with *mis4-242*. For this purpose, a number of genetic crossings were done between *mis4-242* and various *cut* or *cdc* mutations, and the phenotypes were tested. A strong interaction with the *cdc17* mutation, which is defective in DNA ligase, was found. The *mis4-cdc17* double mutant did not form colonies at 26°C, but formed small colonies at 22°C (Fig. 5C). Weak interactions were found for other double mutants with *mis5*, *cdc20*, or *cdc22*, all defective in replication; the double mutants failed to form colonies below the restrictive temperature of single mutants.

Figure 3. The gene product of *mis4*⁺ is similar to that of *C. cinereus* Rad9 and proteins present in yeast and mammals. (A) Isolation of plasmid carrying the *mis4*⁺ gene. The temperature-sensitive phenotype of *mis4-242* was rescued (indicated by +) by pKT221 and some of its subclones. (Arrow) Coding region; (vertical white lines) introns. pKF201 is the minimal functional clone that contains the whole coding region. (B) Schematic representation of *S. pombe* Mis4, *S. cerevisiae* Scc2, and *C. cinereus* Rad9. Partial sequences derived from human and mouse cDNAs are also shown. The conserved regions (22% to 38% identity) are hatched. Mouse AA062272 and human HMHBC4244 sequences are similar to the carboxyl termini of Mis4/Scc2/Rad9. The DDBJ accession number for the nucleotide sequences of Mis4 is AB016866. (C) Immunoblot of *S. pombe* extracts containing Mis4-HA integrated into the chromosome (lane 2) or a plasmid containing Mis4-HA (lane 3). (lane 1) Control extracts of cells without integration and carrying the vector. (D) The immunoblot patterns of *cdc25* mutant block and release experiments, showing the levels of Mis4, Cdc2 (PSTAIR), and mitotic cyclin (Cdc13). Cyclin destruction occurred 30 min after the shift to the permissive temperature (26°C). The Mis4 protein band slightly increased its mobility after 50 min (when Cdc13 levels dropped), but this was not reproducible.

Hypercondensation of chromosome occurs, but whole sister chromatids are separated in metaphase-arrested mis4-242 cells

Incorrect chromosome segregation in *mis4* mutants strongly suggested that Mis4 may act to hold sister chromatids together until anaphase. Therefore, we examined the phenotypes of mitotically arrested *mis4* mutants by introducing a second mutation that blocked cells in metaphase. Mis4 sister chromatids were condensed and held together in metaphase-arrested *cut9* mutant cells; Cut9 is an essential component of the 20S APC/cyclo-some complex required for anaphase-promoting proteolysis (Yamada et al. 1997). The double mutant *cut9-mis4* was constructed and examined. Single *cut9* and double *cut9-mis4* mutants were arrested in G₁ by nitrogen starvation and then released in rich medium at 36°C. In both mutant strains, Cdc2 protein kinase activity was measured and found to be high for 5–8 hr (Fig. 6A), and neither Cut2 nor Cdc13 was degraded (Fig. 6B). In single *cut9* mutants at 36°C, chromosomes were highly condensed (Samejima and Yanagida 1994) whereas in the double *cut9-mis4* mutants, they were also highly condensed but scattered (Fig. 6C). *mis4-242* thus differs from budding yeast *mcd1-1*, which was shown to be defective in chromosome condensation (Guacci et al. 1997).

To show that sister chromatids were separated in double mutant cells, FISH analysis (Uzawa and Yanagida 1992; Funabiki et al. 1993) was performed with three kinds of probes, namely, sequences of rDNA located on chromosome III, unique sequences derived from the centromere II (cen II), and the telomere of the short arm of chromosome II (tel II). As seen in a typical example of cells in Figure 6D–F, the regions of rDNA, centromeres,

and telomeres were all separated in double mutant cells, but never in single *cut9* mutants. These results definitively demonstrate that Mis4 is needed to associate all the regions of sister chromatids in metaphase-arrested cells.

Localization of Mis4 to the nuclear chromatin region

The results described above suggest that Mis4 is a nuclear chromosomal protein. Mis4 is indeed localized to the nucleus. The single copy gene of *mis4*⁺ was tagged with jellyfish green fluorescent protein (GFP) and integrated into the chromosome (see Materials and Methods). The carboxyl terminus of Mis4 was tagged with GFP and the promoter was kept native. This construct was functional, as cells substituting the temperature-sensitive mutant gene with the GFP construct could grow normally. As shown in Figure 6G, the nuclear chromatin in cells expressing GFP-Mis4 was fluorescent with intense nuclear punctate signals. Note that fluorescence signals are enriched in rDNA (Uzawa and Yanagida 1992) as well as other restricted chromatin regions. No decrease of the signals was detected during mitosis. Punctate GFP signal was also observed in anaphase nuclear chromatin. In *nda3* mutant cells expressing Mis4-GFP, the signals were localized to condensed chromosomes (Fig. 6H). Some of the chromosomal punctate signals were also seen. Mis4, at least its fraction, was associated with chromosomes throughout the cell cycle.

Premature sister chromatid separation occurs in *mis4-242* cells during the post-replicative interphase

A question was raised whether sister chromatids were

Figure 4. *Mis4* is sensitive to HU and becomes lethal in the S-phase block induced by HU. (A) Like *Cdc2-3w* cells *mis4-242* cells failed to form colonies in the presence of 5 mM HU at 26°C. (B) FACS analysis indicating that replication was blocked (DNA content is 1C) for 6 hr in *mis4-242* in the presence of 15 mM HU at 26°C. (C) Mutant cells elongated with the interphase single nucleus. Bar, 10 μ m. (D) Viability of mutant cells blocked by 15 mM HU remained high for 6 hr at 26°C. (E–G) Mutant cells were nitrogen starved to cause arrest in G_1 at 26°C and then released in complete medium at 36°C in the presence or the absence of 15 mM HU. (E) The DNA content of mutant cells remained 1C for 7 hr in the presence of HU but increased to 2C after 4 hr in the absence of HU. (F) Mutant cells were elongated at 36°C in the presence of HU. (G) Viability of mutant cells decreased to 50% and 20% after 3 and 5 hr, respectively, in the absence of HU ($\circ$), whereas viability decreased to 50% and 20%, respectively, after 5 and 7 hr in the presence of HU ($\bullet$). Mutant cells became inviable while the DNA content was still 1C.

prematurely separated in *mis4-242* interphase cells after replication. The mutant cells were initially arrested in G_1 by nitrogen starvation and then released in complete medium at 36°C. DNA replication occurred about 4 hr after the shift (Fig. 7A). Mitosis took place around 7 hr (see Fig. 2). Cells were collected at 2, 4, and 6 hr, and to visualize behavior of the centromere DNA of chromosome I (*cen1*) in living cells (see Materials and Methods; Straight et al. 1996; Nabeshima et al. 1998) the *cen1*-GFP technique was applied to score the frequencies of cells revealing the two separated signals of the unique *cen1* DNA. A large fraction of interphase cells showed the two split *cen1* signals after 4–6 hr (~30% and 40% after 4 and 6 hr, respectively). Examples of cells showing the split *cen1*-GFP dots in interphase are shown in Figure 7C. Hence, we concluded that the sister chromatids were prematurely separated after the S phase and continued to be split until the following mitosis in *mis4*

mutant cells. Note that these interphase cells revealing the two split signals did not contain the spindle at all, so that separation was not attributable to the spindle force. The decrease of viability coincided with the increase of split signals, suggesting that the cause for the loss of viability was the failure to form the sister chromatid cohesion.

Viability of metaphase-arrested mis4 mutant cells is quickly lost

As S phase in growing fission yeast cells was initiated immediately after mitotic anaphase, we wanted to assure that *mis4* cells truly lost viability during the first mitosis when cells were shifted from early G_2 (see Fig. 1). For this purpose, viability of the double mutants *cut9-mis4* and *cut7-mis4* was measured and compared with that of single mutants (*cut7* mutant was arrested at prophase; Hagan and Yanagida 1990). Viability of the double mutants, which were cultured from G_2 and arrested at metaphase at 36°C, decreased more rapidly (reduced to 10%–15% after 2 hr) than single *cut9* and *cut7* mutants (60%–70%), suggesting that *Mis4* was needed to maintain viability in mitotically arrested cells. The viability of double mutant *cdc25-mis4* cells (similarly cultured as described above but arrested in late G_2), on the other hand, decreased much more slowly (60% after 2 hr) than single *mis4* mutants (30%), indicating that viability is lost in *mis4* mutant much faster in metaphase- rather than in G_2 -arrested cells.

Discussion

This study describes results demonstrating that fission yeast *Mis4* is required for sister chromatid cohesion, its loss leading to premature sister chromatid separation. Properties of *Mis4* differ from those of the previously identified *Rad21/Mcd1p/Scc1p* cohesin. In *Xenopus*, *XRAD21* forms a complex with the two SMC subunits (*XSMC1* and *XSMC3*) and two other unknown subunits, *p155* and *p95* (Losada et al. 1998). This five-member *Xenopus* cohesin complex is required for sister chromatid cohesion: Immunodepletion of the complex causes defects in sister chromatid cohesion in subsequent mitosis. In *S. pombe*, besides *Rad21* (Birkenbihl and Subramani 1992), proteins highly similar to *Smc1* and *Smc3* are present, and their complex products were obtained (T. Sutani, J. Morishita, T. Tomonaga, M. Yanagida, unpubl.). *Mis4* does not seem to be one of the unknown subunits in the cohesin complex. Immunoprecipitation with antibodies against *Smc3* or *Rad21* did not coprecipitate *Mis4* (K. Furuya, T. Sutani and M. Yanagida, unpubl.). In other experiments with anti-Cut3 antibodies, *Mis4* was not coprecipitated with *Cut3*, indicating that *Mis4* is the subunit of neither cohesin nor condensin. *Mis4* may exist in an oligomeric complex, as it sediments at ~10S in sucrose gradient centrifugation (K. Furuya, K. Takahashi, and M. Yanagida, unpubl.), distinct from the 14S peak of the five-member cohesin.

Figure 5. UV sensitivity and instability of chromosomes in *mis4-242*. (A) *mis4-242* cells are (●) sensitive to UV irradiation at 26°C. (○) Wild-type control. (B) The septation indices of wild-type and *mis4-242* cells were equally reduced by 100 joules of UV irradiation at 26°C (●), but the recovery of the septation index to the level of normal growing cells was significantly delayed in mutant cells (○) 0 joules of UV irradiation. (C) The double mutant *mis4-242-cdc17-K42* did not form colonies even at 26°C.

Properties of Mis4 suggest that the manner in which Mis4 is involved in sister chromatid cohesion is distinct from Rad21/Sccl/Mcd1, the subunit of cohesin. First, Mis4 is not directly related to condensation whereas Mcd1 is shown to be required for chromosome condensation (Guacci et al. 1997). However, the requirement of cohesin (Mcd1p) for chromosome condensation has recently been challenged in a *Xenopus* system by Losada et al. (1998). Second, Mis4 is not the target of the APC/cyclosome whereas Mcd1/Sccl requires the APC/cyclosome for its destruction (Michaelis et al. 1997). Mis4 is present in anaphase- and G₁-arrested cells; Mis4-GFP signals were clearly visualized in anaphase chromosomes. Third, Mis4 is required to maintain viability during the S phase, though replication per se is not blocked in *mis4* mutants. Synthetic lethality with a DNA ligase mutant suggests that gaps or nicks, causing chromosome instability or recombination, might be produced in *mis4* mutant cells. UV and HU hypersensitivity also suggest the presence of chromosomal defects generated by *mis4* mutation. During ongoing replication and G₂, correction of damage on one sister is normally facilitated by the close proximity of the undamaged sister, but loss of cohesion interferes with such interactions. Recombinational repair might also be defective in *mis4* mutant as the sisters are farther away. But these possibilities remain to be determined. Taken together, we conclude that Mis4 belongs to a distinct class of proteins required for sister chromatid cohesion. It functions differently from Rad21/Sccl/Mcd1, and is unrelated to the amino acid sequences of SMC proteins. Mis4, together with *C. cinereus* Rad9, budding yeast Sccl, mouse AA062272, and human HUMHBC4244 may constitute a novel family of sister chromatid adhesion molecules. We propose naming Rad9/Sccl/Mis4 adherin to distinguish it from

the original SMC-interacting cohesin. We discuss possible roles of Mis4 for sister chromatid cohesion, and propose a simple model.

Mis4 is an essential component of sister chromatid cohesion

Punctate nuclear signals of Mis4-GFP suggest that Mis4 may preferentially associate with distinct chromosomal sites. In mitotically arrested cells, the dotted images were also seen on hypercondensed chromosomes. The punctate signals might colocalize with repetitive regions such as rDNA. As the rDNA repeats are located at the ends of chromosome III, Mis4 may be enriched in other repetitive chromosomal ends. Mis4 did not appear to be enriched in the centromeric regions, however. Mis4 may recognize DNA sequence motifs or protein(s) enriched in such particular chromosomal regions. Further investigation is needed to precisely identify Mis4 localization, however. The conserved carboxy-terminal regions in Rad9/Sccl/Mis4 adherin molecules form no nucleotide binding sequence nor known DNA-binding motif, and their molecular function is unknown. Three Cdc2 kinase phosphorylation sites are present in the amino termini of Mis4 and Rad9, but not in Sccl. The amino-terminal region may have a regulatory function, as that of Mis4 also contains the nuclear localization signal (Y. Toyoda, K. Furuya, and M. Yanagida, unpubl.).

We show that whole chromatids are separated in metaphase-arrested *mis4* mutant cells using three different hybridization probes for the FISH analysis. Premature sister chromatid separation in *mis4* mutants is also demonstrated in interphase G₂ cells by inactivation of mutant Mis4 protein in G₁. Separation is not caused by the spindle force, as G₂ cells in fission yeast are completely

A cohesion protein required for S phase and M phase

Figure 6. Sister chromatid separation occurs in metaphase-arrested *mis4-242* cells. (A) Single *cut9* and double *cut9-mis4* mutant cells initially arrested in G₁ by nitrogen starvation and then released in complete medium and cultured at 36°C for 8 hr. The H1 kinase activity was high in both single *cut9* and double *cut9-mis4* mutant cells. (B) Cut2 and Cdc13 proteolysis was absent. (C) DAPI-stained single *cut9* and double *cut9-mis4* mutant cells. Both mutant strains were arrested at metaphase with condensed chromosomes. (D–F) FISH analysis applied to arrested single *cut9* and double *cut9-mis4* cells with probes from rDNA (D) the unique centromere region cenII (E), and the unique telomere region telII (F). Counter staining by anti-SPB antibody or DAPI is also shown with the merged image. Bar, 10 μ m. (G) Localization of Mis4–GFP in the nucleus. The *mis4⁺* gene was tagged with GFP and integrated into the chromosome as a single-copy gene with the native promoter. In living cells, the GFP signal was seen in nuclear chromatin. Signals were seen in the nucleus throughout the cell cycle. Punctate nuclear signals were also seen, which appeared to localize to rDNA and telomeres. (H) Mis4–GFP signals were seen on condensed chromosomes produced in the mitotically arrested β -tubulin mutant *nda3-311* at 20°C.

devoid of spindles. Great reduction in the level of Mis4 protein in mutant cells probably causes the loss of connection between sister chromatids and results in the premature separation phenotype. Mis4 may thus provide a direct link between chromatids or may alter chromatids so that they have a structure capable of interacting with cohesin. No genetic interaction, however, has been found between *mis4* and *rad21* mutations so far. It is of considerable interest to determine whether these two chromatid cohesion molecules are functionally interrelated.

Requirement for Mis4 in S phase and M phase

When *mis4* mutants had been inactivated in G₁ or G₂, a cell cycle defect was produced in the subsequent S or M phase, respectively, suggesting that Mis4 was required for both these phases. Viability was kept to be relatively high in the gap stages between S phase and M phase. The

failure to form sister chromatid cohesion is probably the cause of mutant cell death during S phase; this defect could not be restored when cells had passed this phase. The requirement for Mis4 in M phase is intriguing, as the progression of the first mitosis is an unrecoverable lethal event but cytologically indistinguishable from normal mitosis. One explanation of this phenotype is that mutant cells could not establish, during the first mitosis, a particular chromosome conformation that is specifically required for the reforming of sister chromatid cohesion in the subsequent S phase. The major chromosome reorganization reported to take place at the onset of mitosis in vertebrate cells (Losada et al. 1988) may also take place in *S. pombe*. The lack of Mis4 in the reorganization step may not affect sister chromatid separation immediately thereafter but may lead to the loss of the ability to reform cohesion in the subsequent S phase. If sister cohesion is not made in S phase, premature sister chromatid separation inevitably occurs in G₂ and gives

Figure 7. Sister centromeres are prematurely separated in the interphase state of *mis4-242* after replication. The *cen1* DNA was visualized by use of the LacI system; (Straight et al. 1996). Lac repressor was tagged with GFP and an NLS and expressed in a *S. pombe* strain containing LacO repeats integrated near *cen1* (Nabeshima et al. 1998) *mis4-242* cells (and a wild-type control) expressing the LacI-NLS-GFP and integrated with the LacO repeat were arrested in G₁ by nitrogen starvation at 26°C, shifted to complete medium, and cultured at 36°C for 8 hr. (A) FACS analysis indicating that replication occurred in *mis4-242* cells after 4 hr and was completed before 6 hr as in the wild type (not shown). (B) *cen1*-GFP signals were visualized, and the frequencies of cells revealing two signals (○) graphed with the frequencies of viable cells (●). Because mitotic cells started to increase after 7 hr, data for the two split signals were not taken after that time. (C) Example of *mis4-242* cells showing the two split *cen1* signals. The wild-type control is also shown. Bar, 10 μm.

rise to nearly random chromosome segregation in the following mitosis. Bi-orientation of sister centromeres will not be achieved in metaphase if sister chromatid cohesion is entirely abolished. A similar loss of cohesion, in the restricted region, was reported for the mutant of Mis6, a centromere-specific connector protein essential for association of sister centromeres that acts during the G₁/S phase (Saitoh et al. 1997).

Mis4 and the onset of anaphase

The removal of cohesin from chromosomes upon entry into anaphase requires Esp1, which is essential for sister chromatid separation (Ciosk et al. 1998). Anaphase-promoting proteolysis triggers the destruction of Pds1, an inhibitor of Esp1, resulting in the activation of Esp1, which may subsequently promote disruption of the linkage between chromatids by removing Scc1. However, we have not yet found any genetic and biochemical relations between Mis4 and Cut1 (a homolog of Esp1) or between Mis4 and Cut2 (a homolog of Pds1; Kumada et al. 1998). Therefore, we consider a different mechanism for the implication of Mis4 in the onset of anaphase.

We propose a simple model in which Mis4 associates with chromosomes throughout the cell cycle and forms the connection between sister chromatids directly or indirectly through another linking protein. Disruption of the link might occur by splitting of the oligomeric Mis4 complex into halves or removal of the linking protein from chromosomes in anaphase while Mis4 remains associated with chromosomes. Post-translational modification such as protein dephosphorylation (Ohkura et al. 1989) may be important in disconnecting the linkage. Many other models may be possible, but the one described here is among the simplest. During S phase, Mis4 should be added to a newly replicated DNA. If Mis4 is

inactive at the time of replication, the connection between chromatids will not be made, resulting in precocious separation of sister chromatids after replication. Once formed, sister cohesion would be maintained even if Mis4 is later inactivated, but would result in normal-looking mitosis with a cryptic irreversible defect. Identification of actual chromosome defects at the molecular level will be an important step toward understanding the regulation of sister chromatid cohesion. Both cohesin and adherin are essential for sister chromatid cohesion, and the loss of either one results in premature chromatid separation. Determination of their hierarchical relationship, if any exists, will also be a subject for future investigation.

Materials and methods

Strains and media

S. pombe haploid strains were used. Handling of temperature-sensitive strains, *mis4*, *cut9*, *cdc25*, and *cdc17* and media used were described previously (Takahashi et al. 1994; Yamada et al. 1997). The strain used for integration of the cloned *mis4*⁺ gene onto the chromosome was h⁻ *mis4-242* *ura4*.

Plasmids

Procedures for gene cloning in *S. pombe* by transformation were described previously (Takahashi et al. 1994). An *S. pombe* genomic library was used for isolation of the *mis4*⁺ gene. The selection marker was the *S. cerevisiae* *LEU2* gene. Integration plasmid pYC6 containing the *ura4*⁺ gene was used for integration of a 3-kB *Bam*HI-*Bgl*III fragment into the chromosome by homologous recombination. The carboxyl terminus of *mis4*⁺ was tagged in frame with the 3× HA antigen. A plasmid carrying the tagged *mis4*⁺ was able to suppress the temperature-sensitive phenotype of *mis4-242*. The vector plasmid used was pSK248.

Molecular biological methods

The nucleotide sequence of a 7-kb long *Bam*HI-*Bgl*III fragment in pKT221 was determined. Three of the presumed introns in the carboxyl terminus were confirmed by PCR amplification of cDNA. 12CA5, an antibody against HA, was used for immunoblotting to detect Mis4-HA. Cells were irradiated at 36°C in a warm room with temperature control. The FACScan analysis was performed according to the procedures described by Costello et al. (1986). Cells collected were washed in 70% ethanol. DNA contents were measured by a Becton-Dickinson FACScan apparatus after propidium iodide staining.

Gene disruption

The procedures described by Rothstein (1983) were followed. The *Bgl*III-*Pst*I fragment in pKT221 was subcloned, and an internal *Hind*III fragment was removed and substituted with the *S. pombe ura4⁺* gene. The resulting plasmid, pKF230, was digested with *Sna*BI and *Spe*BI and used for transformation of a diploid (5A/1D). Stable transformants for the *Ura⁺* marker were isolated, and gene disruption was verified by genomic Southern hybridization. The resulting heterozygous diploid $\Delta 10$ was sporulated and tetrads dissected.

Synchronous culture

The procedures described by Moreno et al. (1989) and Kinoshita et al. (1990) were followed. A Beckman elutriator rotor was used for collecting small G₂ cells. Procedures for nitrogen starvation were employed to concentrate cells in G₁ followed by release into complete medium (Saka and Yanagida 1993). EMM2-N lacking NH₄Cl was used for starvation (2×10^7 /ml for 24 hr at 26°C). A cell concentration of 1×10^6 /ml was employed for the shift into complete medium at 36°C. Block and release experiments with the *cdc25* mutant were done as described (Funabiki et al. 1996; Yamada et al. 1997).

GFP constructs

The use of GFP for tagging the carboxyl terminus of Mis4 and integrating of the construct into the chromosome was done as described (Nabeshima et al. 1995). The integration vector pYC11 was used, and the resulting plasmid pMIS4-GFP was used for transformation of the strain *h⁻ leu1 mis4-242* after linearization. The resulting *Leu⁺ Ts⁺* integrants were used for microscopy. A fission yeast strain was constructed to visualize cenDNA (Nabeshima et al. 1998). A haploid *S. pombe* strain *h⁻ lys1 his7* was transformed simultaneously with the two plasmids pMK24A and pMK2A. pMK24A carried the GFP-LacI-NLS (Straight et al. 1996) driven by the promoter region of *dis1⁺* (Nabeshima et al. 1995) and the *his7⁺* gene; NLS is the nuclear location signal. The plasmid pMK2A contained the LacO repeat and the *lys1⁺* gene, which is tightly linked to the *cen1* locus (~30 kb; Takahashi et al. 1992). The resulting *Lys⁺ His⁺* transformants were isolated. Transformant MKY5A contained the integrated GFP-LacI-NLS gene at the *his7* locus and also the LacO array at the *lys1* locus.

Microscopy

FISH analyses and immunofluorescence microscopy with anti-tubulin antibody were done as described by Funabiki et al. (1993). Staining of DNA in living cells was done as described

previously (Chikashige et al. 1994). Detailed procedures for the culture of cells for microscopy were described by Nabeshima et al. (1997). A MRC-1024 confocal microscope was used with excitation at 488 nm and the filter 522DF32.

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Faithful anaphase is ensured by Mis4, a sister chromatid cohesion molecule required in S phase and not destroyed in G₁ phase

Kanji Furuya, Kohta Takahashi and Mitsuhiro Yanagida

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