[SVI-6]

Mad1p, a Component of the Spindle Assembly Checkpoint in Fission Yeast, Suppresses a Novel Septation-defective Mutant, *sun1*, in a Cell Division Cycle

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Abstract

Schizosaccharomyces pombe is suited for the study of cytokinesis as it divides by forming a septum in the middle of the cell at the end of mitosis. To enhance our understanding of the cytokinesis, we have carried out a genetic screen for temperature-sensitive *S. pombe* mutants that show defects in septum formation and cell division. Here we present the isolation and characterization of a new temperature-sensitive mutant, *sun1*(septum uncontrolled), which undergoes uncontrolled septation during cell division cycle at restrictive temperature (37℃). In *sun1* mutant, actin ring and septum are positioned at random locations and angles, and nuclear division cycle continues. These observations suggest that the *sun1* gene product is required for the proper placement of the actin ring as well as precise septation. The *sun1* mutant is monogenic recessive mutation unlinked to previously known various *cdc* genes of *S. pombe*. In a screen for *sun1*$^+$ gene to complement the *sun1* mutant, we have cloned a gene, *sus1*$^+$(*su*ppressor of *s*un1 mutant), that encodes a protein of 689 amino acids. The predicted amino acid sequence of *sus1*$^+$ gene is similar to the human hMad1p and *Saccharomyces cerevisiae* Mad1p, a component of the spindle checkpoint in eukaryotic cells. The null mutant of *sus1*$^+$ gene grows normally at various temperatures and has the increased sensitivity to anti-microtubule drug while *sun1* mutant shows no sensitivity to microtubule destabilizing drugs. The putative *S. pombe* Sus1p directly interacts with *S. pombe* Mad2p in yeast two-hybrid assays. These data suggest that the newly isolated *sus1*$^+$ gene encodes *S. pombe* Mad1p and suppresses *sun1* mutant defective in controlled septation in a cell division cycle.

Keywords: *Schizosaccharomyces pombe*; cell division cycle; cytokinesis; spindle checkpoint; *mad1*

Introduction

An important question in cell division cycle is how cells divide into two daughter cells. Cells must assure correct spatial and temporal coordination of mitosis with cytokinesis for each daughter cell to receive a full set of chromosomes together with a proper complement of cytoplasm and organelles following cell division. Most eukaryotic cells undergo a cytoplasmic division late in the M phase of the cell division cycle. In higher eukaryotic cells, a contractile process brought about by sliding of actin and myosin filament forms a furrow where constriction and cleavage occurs. A number of studies reveal that the position of the cleavage plane is determined during mitosis, and many proteins such as actin, myosin, septin, profilin, and calmodulin are involved in this process (Strome, 1993). Though considerable research has been conducted toward understanding the mechanisms that control the onset of cytokinesis, there is little information on the signals and regulatory processes.

The fission yeast *Schizosaccharomyces pombe* provides a simple eukaryotic model for the study of cell division cycle. In *S. pombe*, cells grow mainly by extension at their tips and divide by binary fission after forming middle placed septum (Minet, *et al.*, 1979). F-actin is found as multiple cortical patches at the growing end of cells, and it relocates to form an equatorial ring whose position anticipates the site of septum formation after the onset of mitosis (Mark and Hyams, 1985). Structural studies of cortical actin have demonstrated that it is composed of spiral actin filaments that are associated with an invagination of the plasma membrane (Mulholland, *et al.*, 1994).

Two functions of actin ring have been known in cell cycle: One, it may mark the position of septum formation, and, two, it may play a contractile faculty in actively closing plasma membrane. The conservation of actin ring components suggested that *S. pombe* actin ring might be similar at a molecular level to the contractile ring in higher eukaryotes. The actin ring and the subsequent cell division site are marked by a signal from the premitotic nucleus (Chang and Nurse, 1996). In contrast with animal cells, the mitotic spindle, itself, is not required for formation and placement of the medial ring, but the position of the nucleus may determine the site of ring formation in *S. pombe* (Chang and Nurse, 1996; Chang, *et al.*, 1996).

Previous studies of *S. pombe* have identified several defective mutants in mitosis and cytokinesis (Bahler and Pringle, 1998; Chang, *et al.*, 1997; Chang and Nurse, 1996; Edamatsu and Toyoshima, 1996; Kitayama, *et al.*, 1997; May, *et al.*, 1997; Sohrmann, *et al.*, 1996). These mutants were further organized into three groups by their phenotypes, the actin ring contraction and/ or septum deposition mutants (*cdc7, cdc11, cdc14,* and *spg1*); the controlling actin ring assembly mutants (*cdc3, cdc4, cdc8, cdc12, cdc15, myo2,* and *rng2*); and the ring placement mutant (*mid1/dmf1*). Although these genes have been identified and their functions have provided a more detailed understanding of mechanisms of cytokinesis and septation, how cytokinesis and septaiton are coordinated to the rest of cell cycle is unclear.

To gain further insight into these questions, here we have reported an isolation and characterization of a novel temperature-sensitive *S. pombe sun1* mutant that exhibits a frequent mislocalization and/or misorientation of division septa at restrictive temperature(37℃). We have also cloned a suppressor $sus1^+$ gene that rescues the *sun1* mutant. Evidences supporting $sus1^+$ as *S. pombe* $mad1^+$ include the sequence conservation

of Sus1p with human hMad1p and *Saccharomyces cerevisiae* Mad1p, the hypersinsitivity of its null mutation to the low doses of microtubule depolymerizing drugs, and its direct interaction with *S. pombe* Mad2p.

Results

Isolation of *sun1* mutant defective in septum formation and cytokinesis

To isolate *S. pombe* mutants defective in cytokinesis, we have performed a genetic screen that enriched for mutants having phenotypes such as rereplication of chromosomal DNA, abnormal segregation of chromosomal DNA, improper septum positioning, and elongated size, after a shift to restrictive temperature (37℃). In this procedure, we isolated one interesting mutant by single mutation that was specifically defective in normal septum formation. At restrictive temperature (37℃), this mutant exhibited a variety of defects in septum positioning and structure; hence it was named *sun1* (septum uncontrolled). After 6 hours at restrictive temperature, *sun1* cells accumulated abnormal or misplaced septum and exhibited continuous nuclear division without cytokinesis (Figure 1A, B). *sun1* mutant cells did not arrest the cell cycle homogeneously but exhibited delayed cell cycle compared to wild type cells at restrictive temperature (Figure 2A). Septum was usually formed obliquely or longitudinally (Figure 1A; d, e) and was often misplaced (Figure 1A; b, c). Occasionally, cells with two septa were observed (Figure 1A; f). Septation patterns of *sun1* cells showed that septa have a random distribution and/or a frequent misorientation (Figure 2C). The abnormal septum localization in *sun1* mutant cells resembled that of *mid1* mutant cells (Chang, *et al.*, 1996; Sohrmann, *et al.*, 1996), but complementation and linkage analysis revealed that *sun1* was unlinked to *mid1* as well as various other mutations affecting cytokinesis.

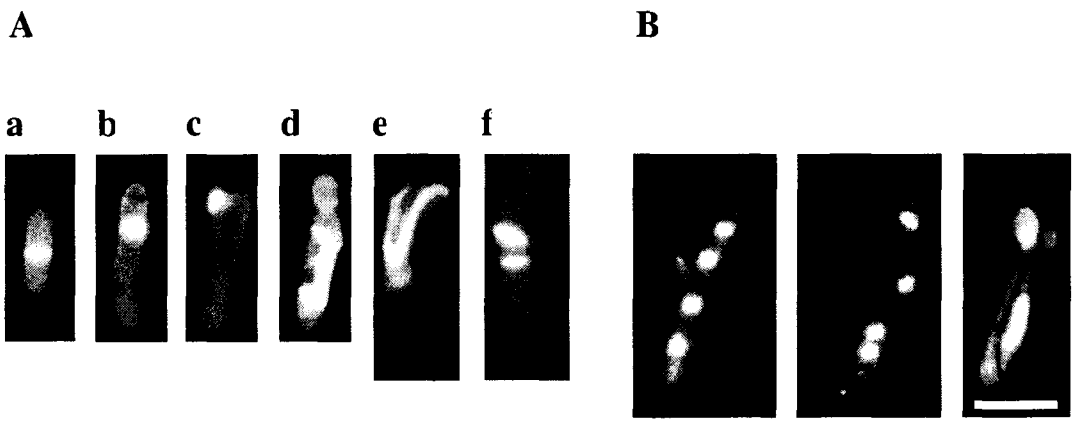

Figure 1. Septation patterns and DAPI staining of *sun1* mutant. (A) Septum localization. Cells were cultured at 23℃ to exponential growth phase, then shifted and grown for 6 hr at 37℃. Cells were fixed with formaldehyde, and stained by Calcofluor. (a) wild type cell and (b, c, d, e, and f) *sun1* cells. (B) DAPI staining of the *sun1* cells. *sun1* cells were grown at 23℃ to exponential growth phase, then shifted and cultured at 37℃. Cells were taken at 6 hours after temperature shift, fixed with formaldehyde, and stained with DAPI to visualize nuclear. Bar, 10μm.

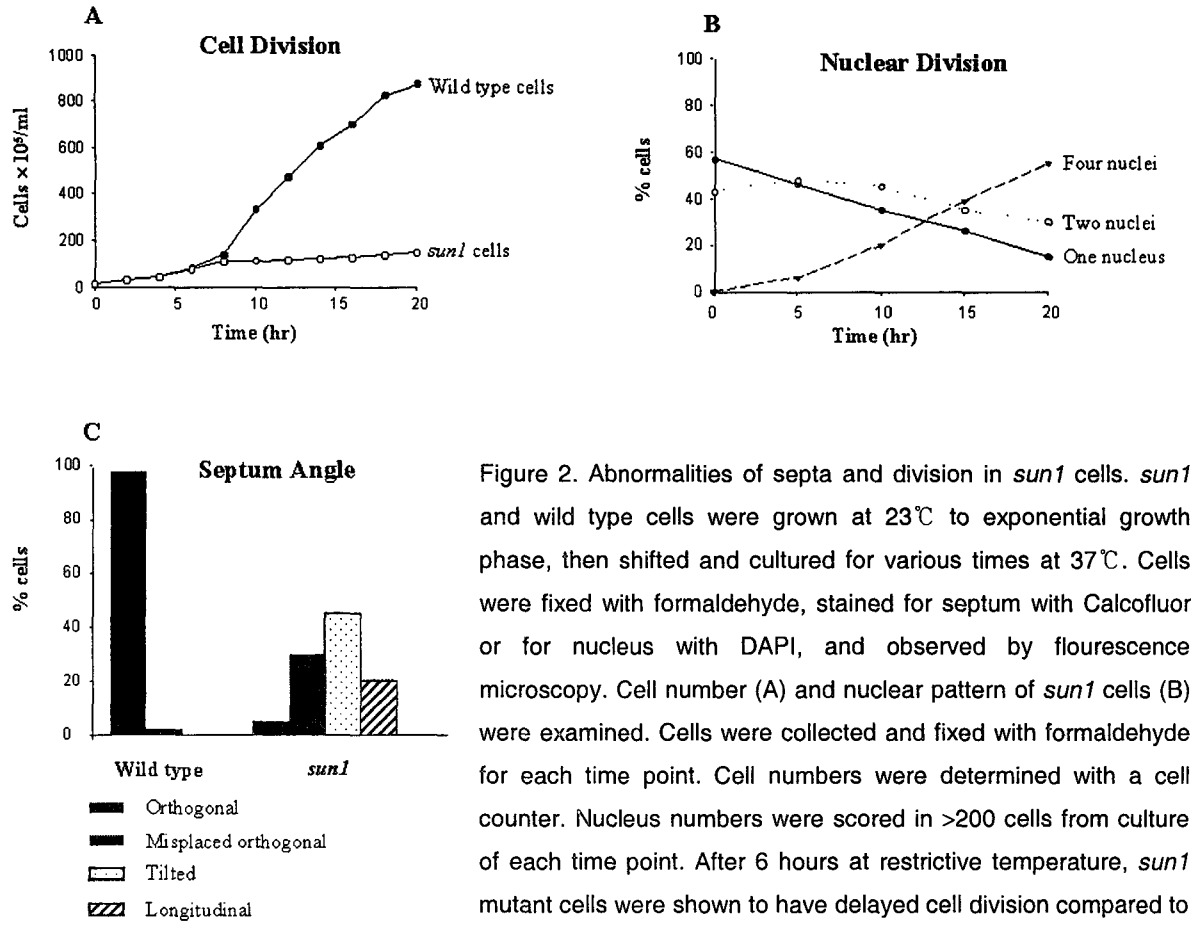

Figure 2. Abnormalities of septa and division in *sun1* cells. *sun1* and wild type cells were grown at 23℃ to exponential growth phase, then shifted and cultured for various times at 37℃. Cells were fixed with formaldehyde, stained for septum with Calcofluor or for nucleus with DAPI, and observed by flourescence microscopy. Cell number (A) and nuclear pattern of *sun1* cells (B) were examined. Cells were collected and fixed with formaldehyde for each time point. Cell numbers were determined with a cell counter. Nucleus numbers were scored in >200 cells from culture of each time point. After 6 hours at restrictive temperature, *sun1* mutant cells were shown to have delayed cell division compared to wild type (A), but nuclear division cycle continued (B). Septum angle was analyzed on >200 wild type, and *sun1* scored relative to the long axis of the cell after growing 6 hours at 37℃ (C). Septa with angle of 80°-90° were designated as orthogonal septa, which have an orthogonal form, and displaced septa from the middle of the cell were determined as misplaced. Septa that placed longitudinally in the cell were designated as longitudinal. The remainders of the septa were scored as tilted.

Misplacement of actin ring in *sun1* mutant

The actin ring is organized primarily as cortical actin patches at the cell ends during interphase and forms the medial contractile ring during mitosis in wild type *S. pombe* (Marks, *et al.*, 1986). Also, the actin ring serves to guide and position the septum in *S. pombe* (Marks and Hyams 1985). Therefore, mutants defective in actin ring assembly can be isolated on the basis of the accumulation of disorganized septal material in the medial region of the cell (Balasubramanian, *et al.*, 1994; Chang, *et al.*, 1996; McCollum, *et al.*, 1995). To verify that *sun1* cells have defects in actin ring assembly, exponential phase growing cells were shifted to 37℃, taken after 2 and 6 hours, and we observed the pattern of F-actin and DNA by staining with rhodamine-conjugated phallodin and DAPI. In contrast to the actin ring of wild type cells (Figure 3A), *sun1* cells displayed misplaced and disorganized medial rings at restrictive temperature and the actin rings were thinner as observed in wild type cells (Figure 3B, C, D, and E). After 6 hours at restrictive temperature, *sun1* cells containing four nuclei and showing developed abnormal actin distributions ceased cell division, although nuclear cycles

proceeded without cytokinesis (Figure 3E). These observations suggest that *sun1* mutant defect is in medial ring placement. Previous analysis has shown that mitotic spindle is not involved in actin ring formation or placement (Chang, *et al.*, 1996). In order to explore if the *sun1* mutation also causes defects in spindle formation, exponential phase growing cells were shifted to 37℃, taken after 2 and 6 hours, and we observed the pattern of spindle and DNA by staining with anti-tubulin and DAPI. At restrictive temperature (37℃), *sun1* cells exhibited microtubules network similar to those observed in wild type cells (Figure 4). These data suggest that the microtubule networks were not affected in *sun1* mutant.

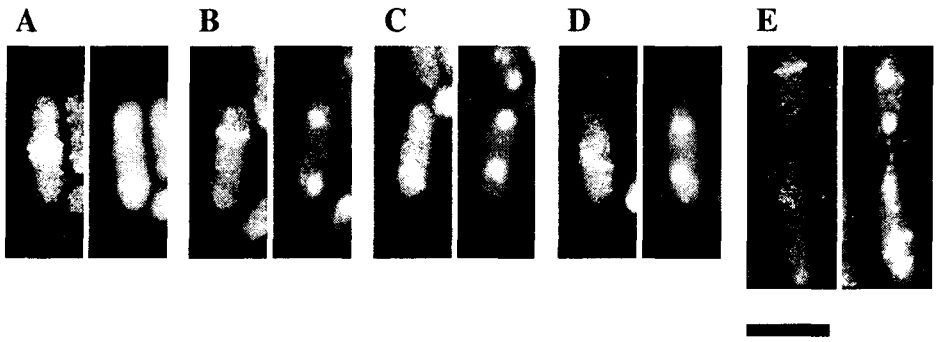

Figure 3. Actin ring formation in *sun1* cells. *sun1* cells were grown at 23℃ to exponential growth phase, then shifted and cultured at 37℃. (A) wild type cell, *sun1* cells were taken at 2 hours (B, C, and D) and 6 hours (E) after temperature shift, fixed with formaldehyde, and stained with rhodamine-conjugated phallodin to visualize actin (left panels) and with DAPI to visualize DNA (right panels). Bar, 10μm.

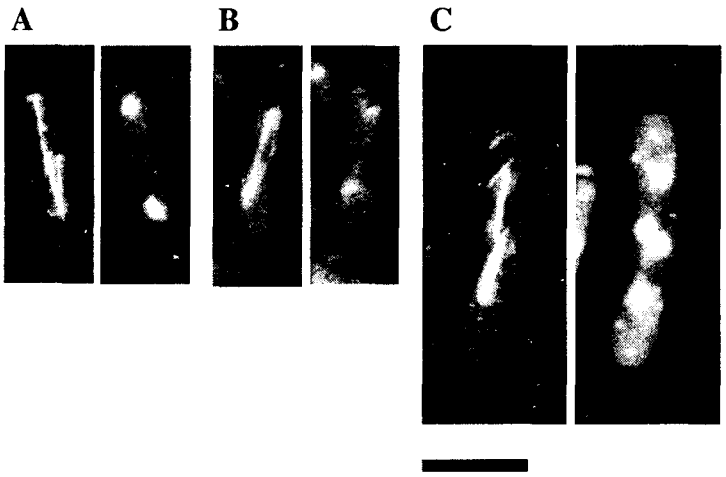

Figure 4. Microtubules in *sun1* cells. *sun1* cells were grown at 23℃ to exponential growth phase, then shifted and cultured at 37℃. (A) wild type cell, *sun1* cells were taken at 2 hours (B) and 6 hours (C) after temperature shift, fixed with formaldehyde, and processed for immunofluorescence with anti-tubulin antibody (left panels) and DAPI to visualize DNA (right panels). Bar, 10μm.

Isolation of a gene that rescue the *sun1* mutant

In order to identify genes involved in cytokinesis, we have screened for genes that could restore the defective cytokinesis in *sun1* mutant by transforming *S. pombe* genomic library constructed in the plasmid vector pWH5 (Wright, *et al.*, 1986). In this process, we obtained a genomic DNA clone that rescued the temperature-sensitive phenotypes of *sun1* mutant, and this plasmid was recovered from the colony rescued *sun1* mutant phenotype. The smallest insert, which was sufficient to rescue *sun1* mutant, was found in 4.2 kb fragment (Figure 5A a). Nucleotide sequencing of the 4.2 kb fragment revealed that this insert was derived from a region on chromosome II that had been sequenced as part of the *S. pombe* genomic project (Genbank/EMBL/DDBJ accession number Z95620). Analysis of this fragment indicated that a 2120 bp open reading frame(ORF) containing one intron encoded a protein of 689 amino acids with a predicted molecular weight of 80024 Daltons. We thought that we identified a multicopy suppressor of the *sun1* mutant, which we have named as *sus1*$^+$ (*su*ppressor of *s*un1 mutant). In order to characterize whether a gene rescue the *sun1* mutant is a suppressor or *sun1*$^+$ itself, we made a deletion mutant of *sus1*$^+$. A *sus1::ura4*$^+$ deletion construct was made in which 836 bp(the majority of the coding region) of the *sus1*$^+$ coding region was replaced with *ura4*$^+$ (Figure 5A; c: see Materials and methods). Linear fragment containing the *sus1*$^+$ knockout construct was used to transform haploid and diploid yeast cells. Ura$^+$ transformants were selected, and homologous integration was confirmed by southern blotting (Figure 6). Haploid colonies in which the wild type copy of the *sus1*$^+$ had been deleted by *ura4*$^+$ grew as well as wild type cells at restrictive temperature. Crossing of *sun1* cell and the null mutant of the *sus1*$^+$ showed that two genes are unlinked, indicating that the isolated plasmid did not contain the *sun1* gene, but a multicopy suppressor. Because a multicopy suppressor could encode a potential substrate or a regulator of the *sun1* encoded protein, we studied it further. A database searches using the BLAST program found that the predicted peptide was similar to a *Saccharomyces cerevisiae* and human spindle checkpoint component MAD1p (Figure 5B). Alignment of the predicted peptide with *S.cerevisiae* and human MAD1p showed that Sus1p shared 24.5% and 22% identity respectively. Using the COILS algorithm (Lupas, *et al.*, 1991), a number of previously characterized sequence motifs were identified. The sequence of Sus1p contains a putative bipartite nuclear localization signal sequence (residues 575 - 592) (Dingwall and Laskey, 1991), potential phosphorylation sites, and coiled-coil domains (data not shown). These results were similar to previously described domain motifs of *S. cerevisiae* Mad1p (Hardwick and Murray, 1995).

Characterization and genetic interaction of *sus1*$^+$ gene in *S. pombe*

In an attempt to elucidate whether *sus1*$^+$ is a function homologue of Mad1, we tested the response of the deletion mutant of *sus1*$^+$ to agents that normally interfere with mitotic spindle assembly by depolymerizing microtubule. If *sus1*$^+$ is identified as a *S. pombe mad1*$^+$, cells lacking Sus1p would be expected to be hypersensitive to agents, such as TBZ, because loss of function of *mad1*$^+$ can not arrest cell cycle progression in the presence of a defective spindle (Hoyt, *et al.*, 1991). A haploid strain containing a

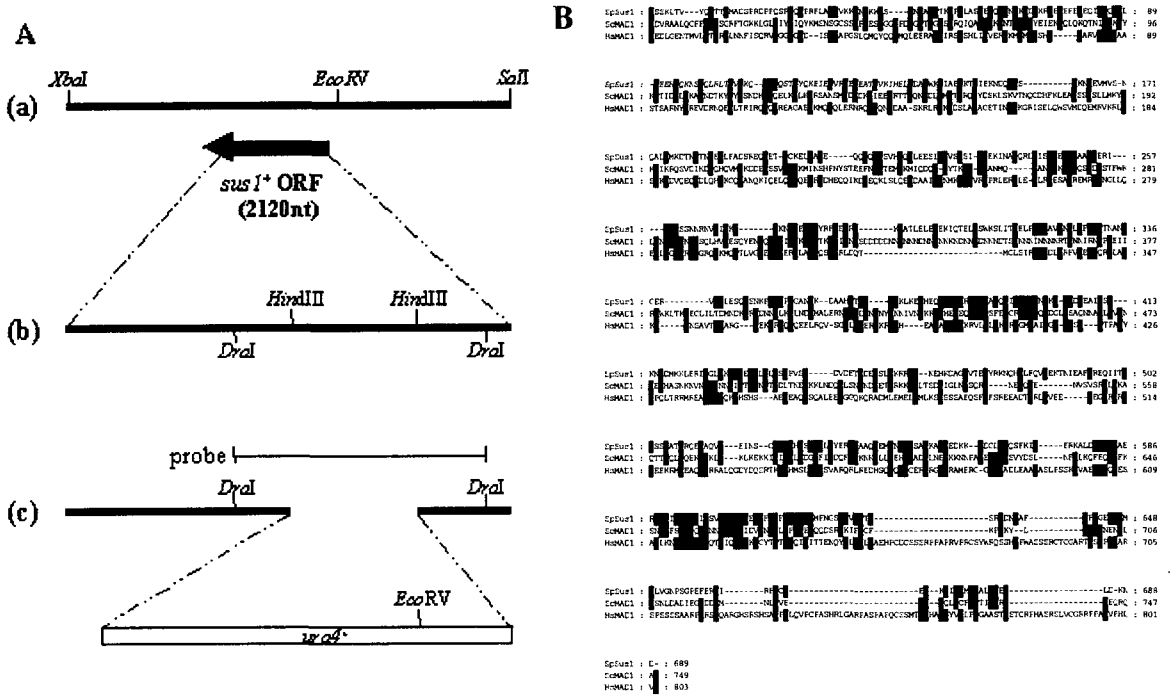

Figure 5. () Restriction map of the *sus1*$^+$ gene and disruption. (a) Restriction map of the *sus1*$^+$ gene isolated from the pWH5 containing *S.pombe* genomic library. The 4.2 kb genomic DNA included insert that rescues *sun1* mutant cells. The arrow indicated coding region and orientation of the single open reading frame (ORF). (b) Restriction map of the *sus1*$^+$ open reading frame. (c) Construction of *sus1*$^+$ gene disruption. *Hind*III fragment of *sus1*$^+$ gene was replaced with a 1.8 kb *ura4* insert. (B) Alignment of the amino acid sequence from the *S.pombe* Sus1p (SpSus1) with that of Mad1p from Human (HsMAD1) and from *S.cerevisiae* (ScMAD1). Boexs indicated identities among all three proteins. Alignment was performed with DNASTAR " MegAlign" program.

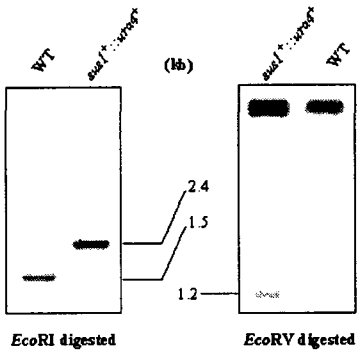

Figure 6. Southern blot analysis of the *sus1*$^+$ gene disruption. Genomic DNA isolated from wild-type cells and *sus1*$^+$*::ura4*$^+$ cells was digested with *Eco*RI or *Eco*RV. Digested fragments were resolved on a 0.8% agarose gel and blotted to Hybond-N nylon membrane (amersham pharmacia). Blots were probed with *Dra*I fragment as indicated in Figure 4A. Southern hybridization of genomic DNA digested with *Eco*RI gave rise to a signal at 2.4 kb in *suk1*$^+$*::ura4*$^+$ cells, indicating that this band originated from the *sus1*$^+$ locus including the *ura4*$^+$ gene. In genomic DNA digested with *Eco*RV, a signal at 1.2 kb was also detected in *suk1*$^+$*::ura4*$^+$ cells, but not in the wild-type cells. This band was expected from the restriction map of *sus1*$^+$ gene containing the *ura4*$^+$ gene.

sus1$^+$ deletion was unable to form colonies on medium containing 10μg/ml TBZ, whereas the wild type cells still does (Figure 7). At the permissive temperature, the *sun1* mutant showed the same pattern of TBZ sensitivity as the wild type cells (Figure 7B). To identify interactions between Sus1p and other proteins, we searched *S. pombe* cDNA library by yeast two-hybrid system. Approximately 5 × 10^6 independent transformants of the *S. pombe* library were screened. Several transformants that showed growth on medium lacking leucine, tryptophan, and histidine containing 3-aminotriazole and production of β-galactosidase in filter assay were identified. One of the positive clones containing an open reading frames was identical to the product of the *S. pombe mad2*$^+$ (Figure 8) (He, *et al.*, 1997). Mad1p is a nuclear protein whose phosphorylation increases greatly upon spindle depolymerization and rises transiently during normal mitosis (Hardwick and Murray, 1995). In budding yeast, genetic and biochemical evidence indicates that Mad1p is phosphorylated by Msp1, a protein kinase required for the spindle checkpoint. Phosphorylation of Mad1p appears to be important for spindle checkpoint because its phosphorylation requires the function of Mad2p (Hardwick, *et al.*, 1996). Conservation of the Mad1p-Mad2p interaction in budding yeast, frog, and human indicates the importance of this complex (Chen *etal.*, 1998; Jin *et al.*, 1998). Taken together these results demonstrate that *sus1*$^+$ encodes the Mad1p in S. *pombe*, and Mad1p suppresses the *sun1* mutant.

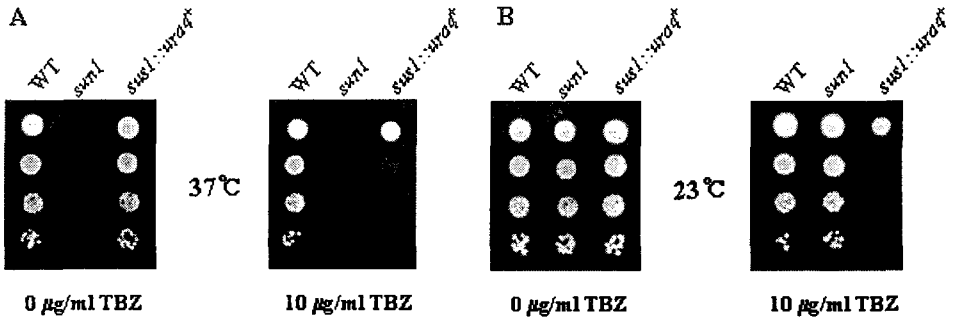

Figure 7. Hypersensitivity of the *sus1Δ* cell to thiabendazole (TBZ). Serial dilutions (1/10) of cells (wild-type, *sun1* and *sus1Δ*) were spotted onto complete medium plates containing indicated concentrations of TBZ, and were incubated at 37℃ (A) and 23℃ (B) until colonies appeared. *sus1Δ* cell was unable to form colonies on medium containing 10μg/ml TBZ. However, wild type and *sun1* cells did not show any sensitivity to TBZ.

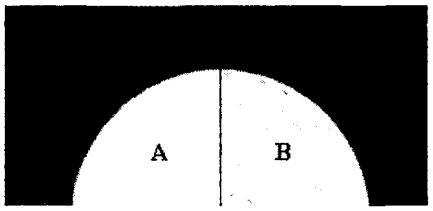

Figure 8. Two-hybrid interaction between Sus1p and Mad2p. Interaction of Sus1p and Mad2p was tested by β-galactosidase filter assay. Transformants grown on minimal selection plates were transferred on a piece of filter paper, immersed in liquid nitrogen for 30 sec, and thawed on another piece of filter paper containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The paper was incubated at 30℃ until a blue color was detected. Yeast cells containing pGBT9/*sus1*$^+$ without pGAD/*mad2*$^+$ (A) or with pGAD/*mad2*$^+$ (B) were used. Only yeast cells containing pGBT9/*sus1*$^+$ with pGAD/*mad2*$^+$ were detected the colour development.

Discussion

Cells assure correct spatial and temporal coordination of mitosis with cytokinesis so that each daughter cell will receive a full set of chromosomes together with a proper complement of cytoplasmic materials and organelles. Proper placement and orientation of the cleavage plane are also important during development, since they determine overall organization of cell shape, size, and segregation of localized determinants in development (Rhyu and Knoblich, 1995). We have described, in this report, the isolation and characterization of the fission yeast *sun1* mutant defective in normal septum formation caused by single mutation. Its phenotype is recessive and temperature sensitive, and on shift to restrictive temperature (37℃), the mutant shows abnormal septation as described. Shifting the *sun1* mutant to restrictive temperature results in a high percentage of arrested cells with an abnormal or a misplaced septum. Also, *sun1* cells exhibit delayed cell division compared to wild type; accumulate as four nucleate cells with elongated and aberrant phenotypes; and do not produce anucleate cells. These data indicate that *sun1* cells with abnormal or misplaced septum are unable to execute cytokinesis and septation. Rhodamine-conjugated phallodine staining shows that *sun1* cells exhibit actin rings at variable locations and angles, suggesting that the misplaced actin rings mark the abnormal positions of septum formation. In animal cells, the mitotic spindle is thought to induce and position the cleavage furrow (Oegema and Mitchison 1997). However, *sun1* cells has a defect in medial ring positioning, yet it has virtually no spindle defect. The *sun1* phenotypes are analogous to those of *mid1*mutant, which is required for the placement of medial ring in *S. pombe*. *mid1* mutant cells exhibit random placement of actin rings and septa, but the mitotic spindle is positioned normally (Chang, *et al.*, 1996; Sohrmann, *et al.*, 1996). Therefore the phenotypic analysis of *sun1* suggests that *sun1* gene product may be involved in the correct placement of the medial ring. Many *sun1* cells with a misplaced septum do not complete cell division, exhibit cell cycle delay, and do not product anucleate cells at restrictive temperature. The analysis presented above indicated that Sun1p also may be involved in cell cycle checkpoint control that coordinates nuclear and cell division.

In addition to the isolation and characterization of *sun1* mutant, we have also characterized the *sus1*$^+$ gene, which was obtained as a suppressor of *sun1* mutant. Database searches using the predicted 689 amino acid polypeptide sequence as a query indicated that Sus1p was most closely related to the budding yeast Mad1p, a protein known to be required for activation of spindle assembly checkpoint (Hardwick and Murray, 1995). Structurally, all three proteins (*S. cerevisiae* MAD1p, human MAD1p, and Sus1p) maintain very similar coiled-coil motifs. Deletion of *sus1*$^+$ gene induces sensitivity to low doses of microtubule depolymerizing drug, such as TBZ, indicating that a loss of *sus1*$^+$ gene function results in a premature escape from a mitotic block and Sus1p was a member of the spindle checkpoint family. From the yeast two-hybrid system, Sus1p directly interacts with the Mad2p of *S. pombe*. In *S. cerevisiae*, Mad2p tightly binds Mad1p, and their association between Mad1p and Mad2p is essential for the function of the spindle checkpoint (Chen, *et al.*, 1999). Based on these results, we propose Sus1p as a *S. pombe* Mad1p. When treated with microtubule polymerization inhibitor, budding yeast cells arrest in mitosis with unseparated sister chromatids. The mitotic arrest by microtubule polymerization inhibitor suggests spindle checkpoint monitors the status of the spindle and

regulates the metaphase to anaphase transition. Mutation of the *MAD 1* gene results in failure of cell cycle arrest in mitosis, and allows cells to enter anaphase in the absence of a functional spindle in budding yeast (Li and Murray, 1991). Mad1p is a nuclear protein whose phosphorylation increases greatly upon spindle depolymerization, and accumulates transiently during normal mitosis (Hardwick and Murray, 1995). However, we did not establish how Ma1p suppressed the misplaced actin ring resulting from loss of Sun1p function. Genetic and molecular studies suggest that early septation genes and inhibitory genes closely interact to regulate the timing of septaion (Marks *et al.,* 1992). The *S. pombe dma1* gene, a component of the spindle assembly checkpoint, suppresses the *S. pombe cdc16* mutation (Murone and Simanis, 1996). The *S. pombe cdc16* gene is required for control of septum formation. Also, *S. pombe zfs1* gene, which is required to prevent septum formation and to exit from mitosis if the mitotic spindle is not assembled, has previously been isolated as multicopy suppressor of *cdc16* mutant (Beltraminelli, *et al.,* 1999). These observations suggest that Mad1p may be involved in negative regulation of Sun1p. Identification of the Sun1p and elucidation of its mechanism with Mad1p will be the subject of future studies.

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