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Novel WD-Repeat Protein Mip1p Facilitates Function of the Meiotic Regulator Mei2p in Fission Yeast

SATOKO SHINOZAKI-YABANA, YOSHINORI WATANABE,* AND MASAYUKI YAMAMOTO

Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo, Tokyo 113-0033, Japan

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In fission yeast, the onset of meiosis is triggered by activation of the RNA-binding protein Mei2p. We screened for a high-copy-number suppressor of the ectopic meiosis induced by expression of an active form of Mei2p. Consequently we isolated a truncated form of a novel gene, named *mip1*, from a fission yeast genomic library. The *mip1* gene encoded a protein of 1,313 amino acids which carried a WD-repeat motif in the C-terminal region and was apparently conserved among eukaryotes. Mip1p was cytoplasmic, and two-hybrid and immunoprecipitation analyses demonstrated that Mip1p was bound to Mei2p in vivo. Genetic evidence indicated that wild-type Mip1p was required for the function of Mei2p to induce meiosis and that the truncated form of it (Mip1-15p) dominantly interfered with Mei2p. Mip1p appeared to be involved also in conjugation, associating with Ste11p, which is a key transcription factor for sexual development. Furthermore, Mip1p was essential for cell growth, to which neither Mei2p nor Ste11p is relevant. These results suggest that Mip1p assists functional expression of a number of proteins required for proliferation and sexual development in fission yeast.

Sexual differentiation in the fission yeast *Schizosaccharomyces pombe* proceeds under nutrient starvation (4, 27, 28). Ste11p, which is a high-mobility-group protein, serves as a key transcription factor in this process. Upon starvation, Ste11p activates a number of genes required for mating and/or meiosis, including the mating-type genes (*mat1-P* and *mat1-M*) and *mei2* (21). The *mei2* gene encodes a pivotal regulator of meiosis (3, 20, 25). Pat1p (Ran1p) kinase (6, 11, 17), which is active during the mitotic cell cycle, phosphorylates Mei2p on two amino acid residues, Ser438 and Thr527, and thereby blocks the function of Mei2p (24). In diploid cells, the gene products of *mat1-P* and *mat1-M* cooperate to turn on expression of *mei3*, which encodes an inhibitor of Pat1p kinase and hence induces derepression of Mei2p (12, 13, 24, 26). Thus, *mei2* is activated both transcriptionally and posttranscriptionally in diploid cells under starved conditions. If a mutant Mei2p carrying alanine residues in place of Ser438 and Thr527 (Mei2-SATA) is expressed in proliferating cells, the cells cease growth immediately and enter meiosis, indicating that dephosphorylated Mei2p has the ability to switch the mitotic cell cycle to the meiotic one (24).

Mei2p is an RNA-binding protein with three RNA recognition motifs that is required for both induction of premeiotic DNA synthesis and promotion of the first meiotic division (meiosis I). Cytoplasmic Mei2p performs the former function, and the RNA-binding ability of Mei2p is essential for it. To perform the latter function, Mei2p must move into the nucleus, where it forms a distinct dot structure. A specific RNA species, named meiRNA, has been shown to promote meiosis I as a cofactor that assists nuclear transport of Mei2p (25, 29). However, the molecular function of Mei2p in the cytoplasm as well as that in the nucleus remains unknown.

To identify novel regulators or downstream targets of Mei2p, we designed a genetic screen that exploited the fact that expression of Mei2-SATAp causes ectopic meiotic differentiation. We screened for high-expression suppressors of this phenotype and identified a novel gene, *mip1*. The *mip1* gene encodes a WD-repeat protein which appears to be widely conserved among eukaryotes but whose function is unknown. The original isolate of *mip1* was a truncated form and inhibited Mei2p function strongly if overexpressed. However, subsequent genetic and biochemical studies suggested that the product of the wild-type *mip1* gene is involved in the activation or functioning of Mei2p, interacting physically with it. Mip1p also appeared to bind to Ste11p, possibly assisting its role in conjugation. In addition, Mip1p plays an essential role in cell growth. We propose that the conserved WD-repeat protein Mip1p is likely to play a general role in the cytoplasm in contributing to functional expression of a certain class of proteins.

* Corresponding author. Mailing address: Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo, Tokyo 113-0033, Japan. Phone: 81-3-5841-4387. Fax: 81-3-5802-2042. E-mail: ywatanab@ims.u-tokyo.ac.jp.

MATERIALS AND METHODS

Strains and media. The *S. pombe* strains used in this study are listed in Table 1. Yeast media YE, SD, SSA, and MEA were used for routine culture of *S. pombe* strains (14). Liquid minimal medium MM (14) and its nitrogen-free derivative MM-N were used for growth and starvation experiments. SD, MM, and MM-N used in this study contained only 1% glucose.

Mating and sporulation assay. Mating and sporulation frequencies were calculated according to equations previously described (8), by counting unmated or unsporulated cells, zygotes, asci, and free spores in a sample.

Cloning and nucleotide sequence determination of *mip1*. A heterothallic strain expressing *mei2-SATA* from the *nmt1* promoter (JX383) was transformed with an *S. pombe* genomic library that carried *Sau*3AI digests of the chromosomal DNA at the *Bam*HI site of the vector pART1. Transformants were plated on thiamine-free SSA and incubated at 30°C for 4 to 6 days. Colonies formed were isolated, and plasmids recovered from them were transfected into *Escherichia coli*. The suppression activity of the recovered DNA clones was reexamined by repeating transfection into the host strain. The *mip1-15* clone was isolated in this screen. A full-length *mip1* clone was isolated by colony hybridization from an *S. pombe* genomic library that carried on average 5-kb-long *Bgl*II fragments at the *Bam*HI site of pBluescript. Nucleotide sequence analysis of each clone was performed by the dideoxy-chain termination method. Subclones for the sequencing were produced by progressive deletion with exonuclease III and S1 nuclease. The open reading frame (ORF) region of *mip1* has been sequenced in both directions at least once.

TABLE 1. Fission yeast strains used in this study

Strain	Genotype[a]
JY362	*h*$^+$/*h*$^-$ *ade6-M216/ade6-M210 leu1/leu1*
JY741	*h*$^-$ *ade6-M216 leu1 ura4-D18*
JY765	*h*$^+$/*h*$^-$ *ade6-M216/ade6-M210 leu1/leu1 ura4-D18/ura4-D18*
JZ409	*h*$^-$ *ade6-M216 leu1 pat1-114*
JZ725	*h*$^-$ *ade6-704 leu1 ura4-D18*
JX383	*h*$^-$ *ade6-M210 leu1::nmt1-mei2-SATA<<ura4*$^+$ *ura4-D18*
JX564	*h*$^+$/*h*$^-$ *ade6-M216/ade6-M210 leu1/leu1 ura4-D18/ura4-D18 mip1*$^+$*/mip1::ura4*$^+$
JX1017	*h*$^+$/*h*$^-$ *ade6-M216/ade6-M210 leu1/leu1 ura4*$^+$*/ura4-D18*
JW191	*h*$^+$ *ade6-704 leu1::nmt1-mei2-SATA<<ura4*$^+$ *ura4-D18 sup3-5*
JW192	*h*$^-$ *ade6-704 leu1::nmt1-mei2-SATA<<ura4*$^+$ *ura4-D18 adh1-mip1-15-FLAG<<sup3-5*
JW193	*h*$^-$ *ade6-704 leu1::nmt1-mei2-SATA<<ura4*$^+$ *ura4-D18 adh1-mip1-15-FLAG<<sup3-5 mip1::ura4*$^+$
JW195	*h*^{90} *ade6-704 leu1 adh1-mip1-15-FLAG<<sup3-5*
JW196	*h*^{90} *ade6-704 leu1 adh1-mip1-15-FLAG<<sup3-5 ura4-D18 mip1::ura4*$^+$
JW197	*h*$^-$ *ade6-M216 leu1 ura4-D18 mip1::mip1-310<<ura4*$^+$

[a] *leu1::nmt1-mei2-SATA<<ura4*$^+$ indicates that *leu1*$^+$ is disrupted by the *nmt1-mei2-SATA* construct and that *ura4*$^+$ locates in the vicinity of it. *adh1-mip1-15-FLAG<<sup3-5* indicates that *sup3-5* is in the vicinity of *adh1-mip1-15-FLAG*. The exact location of this array on the chromosome has not been determined.

Gene disruption. One-step gene disruption (19) of *mip1* was carried out as follows. A 2.1-kb *Bgl*II-*Eco*RV fragment was replaced by a *ura4*$^+$ cassette. Cells of a wild-type homothallic diploid strain, JY765 (*ura4-D18/ura4-D18*), were transformed with a *mip1::ura4*$^+$ DNA fragment and then spread on SD plates without uracil. Stable Ura$^+$ transformants were selected, and Southern blot analysis confirmed that one of the *mip1* alleles was properly disrupted in these Ura$^+$ cells.

Construction of a *mip1* temperature-sensitive allele. To generate a temperature-sensitive allele of *mip1*, we used the method described by Francesconi et al. (5), with minor modifications. Briefly, a 2.8-kb *Bgl*II-*Xho*I fragment, carrying most of the *mip1* ORF including the C terminus and the WD repeat but lacking the N terminus and the promoter region, was cloned into pUC119. The *Xho*I site was artificially created at the C terminus of the *mip1* ORF. The resultant plasmid was used as a template for PCR to introduce replication errors. A pair of oligonucleotide primers for pUC119, M13F (5′-CACGACGTTGTAAAACGAC-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′), were used for PCR amplification. Amplified fragments were digested with *Sph*I and *Kpn*I and cloned between the *Sph*I and *Kpn*I sites of pUC119 carrying the *ura4*$^+$ cassette. The mutagenized library thus obtained was linearized at the *Hin*cII site within the *mip1* ORF and transformed into haploid strain JY741. Integration of an entire plasmid at the chromosomal *mip1* locus by homologous recombination was expected to result in uracil prototrophy. Ura$^+$ transformants were selected at 25°C on an SD plate. Approximately 3,000 Ura$^+$ transformants were replicated to YE plates and examined for the ability to grow at 36°C. Two strains were found to be temperature sensitive. PCR analysis of the genomic DNA isolated from these Ura$^+$ clones confirmed the integration of the mutagenized plasmid at the proper locus. A plasmid pREP81-*mip1* was able to complement the thermosensitive phenotype of these mutant strains at the restrictive temperature. The temperature-sensitive allele of *mip1* carried by one of them (JW197) was named *mip1-310* and analyzed further.

Construction of FLAG-tagged *mip1* clones and *adh1-mip1-15-FLAG* integrants. To construct pREP2/42-*mip1-FLAG* and pREP2/42-*mip1-15-FLAG*, two synthetic oligonucleotides (5′-TCGACCGACTACAAGGACGACGATGACAAGTGACCC-3′ and 5′-GGGTCACTTGTCATCGTCGTCCTTGTAGTCGG-3′) were annealed and inserted between a synthetic *Sal*I site at the C terminus of the *mip1* ORF and the *Sma*I site on pREP2/42 (10). To obtain a *mip1-15* integrant, JZ725 (*h*$^-$ *ade6-704 leu1 ura4-D18*) was transformed with plasmid pANS1-*mip1-15-FLAG*. pANS1 contains the *adh1* promoter, the *sup3-5* marker, and the *nmt1* terminator. Transformants that generated white colonies, indicating stable integration of the plasmid, were selected. A resultant *h*$^-$ *ade6-704 leu1 ura4-D18 adh1-mip1-15-FLAG<<sup3-5* strain was used in crosses to generate JW192, JW193, JW195, and JW196.

Yeast two-hybrid and three-hybrid assays. The yeast two-hybrid assay was performed as previously described (1). A DNA fragment encoding the C-terminal region of Mei2p (amino acids [aa] 429 to 733) was cloned into pAS2 to express Mei2p fused with the Gal4 DNA-binding domain (GDB). DNA fragments encoding either full-length Mip1p or its truncated version Mip1-15p were cloned into pACT2 to express Mip1p and Mip1-15p fused with the Gal4 transcriptional activator domain (GAD). The latter plasmids were named pACT2-*mip1* and pACT2-*mip1-15*, respectively. We used a pair of plasmids, pSE1111 (pACT2-*SNF4*) and pSE1112 (pAS1-*SNF1*), as a control that exhibits positive interaction in the two-hybrid system (1). Positive results were judged by the appearance of blue color generated by degradation of the substrate by induced β-galactosidase activity.

The yeast three-hybrid assay was performed with the commercially available pBridge vector system (Clontech), with modifications. We replaced the GAD sequence in pBridge with the LexA sequence, producing pLB. The nucleotide sequence for the C-terminal region of either Mei2p or Mei2-SATAp was cloned into pLB and fused to the LexA sequence in frame. This generated pLB-*mei2* and pLB-*mei2-SATA*. The nucleotide sequence for the Pat1p ORF was amplified by PCR and subcloned into pLB-*mei2* and pLB-*mei2-SATA* so that it could be expressed from a promoter. A kinase-negative version of Pat1p ORF, in which the ATP-binding site was disrupted by replacing Lys47 with Arg (12), was constructed by in vitro mutagenesis and cloned similarly. The four versions of pLB-*mei2/pat1* plasmids thus constructed were introduced into the tester strain L40 together with pACT2-*mip1* to assay the interaction between Mei2p and Mip1p.

Construction of HA-tagged *mei2* and *ste11* genes. To construct tagged *mei2*$^+$, a three-hemagglutinin epitope (3HA) fragment was cloned between a *Not*I site created at the C terminus of the *mei2* ORF and the *Sac*I site on pREP41-*mei2*, thus generating pREP41-*mei2-3HA*. pREP41-*3HA-ste11* was constructed by inserting a 3HA fragment into the synthetic *Nde*I site at the initiation codon on pREP41-*ste11*.

Immunoprecipitation. For Mip1p-Mei2p immunoprecipitation experiments, we transformed JY741 doubly with pREP41-*mei2-3HA* and either pREP42-*mip1-FLAG*, pREP42-*mip1-15-FLAG*, or the vector pREP42. For Mip1p-Ste11p immunoprecipitation experiments, we transformed JY741 doubly with pREP41-*3HA-ste11* and either pREP42-*mip1-FLAG*, pREP42-*mip1-15-FLAG*, or the vector pREP42. Cells were harvested and washed once with STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3 [pH 8.0]) and kept at −70°C. HBIP buffer (25 mM morpholinepropane sulfonic acid [MOPS], 5 mM EGTA, 15 mM $MgCl_2$, 50 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 0.8% NP-40, 10% glycerol, 150 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 40 μg of aprotinin per ml, 20 μg of leupeptin per ml, 2 protease inhibitor cocktail tablets [Complete Mini; Boehringer Mannheim] per ml) was then added to the cells. They were disrupted by glass beads and centrifuged at 15,000 rpm for 5 min at −5°C. Immunoprecipitation was performed by incubating the supernatant on ice for 40 min with mouse anti-FLAG monoclonal antibody M2 (Sigma) and 25 μl of 50% (vol/vol) protein G-Sepharose (4FF; Pharmacia Biotech).

Fluorescence microscopy of Mei2p-green fluorescent protein (GFP). Diploid cells transformed doubly with pGFT81-*mei2* (24) and either pREP2-*mip1-FLAG* or pREP2-*mip1-15-FLAG* were cultured in MM at 30°C to mid-log phase and then shifted to MM-N and incubated further for 4 h at 29°C. Cells were fixed with methanol at −20°C overnight. After washed once with PEMS buffer, they were stained with Hoechst 33342 to visualize nuclei and observed with a chilled charge-coupled device camera (Hamamatsu) attached to an Axiophot fluorescence microscope (Carl Zeiss).

Immunofluorescence microscopy. Cells were fixed with 3% paraformaldehyde at 30°C for 40 min. For double staining of Mei2p-GFP and Mip1-15p-FLAG, rabbit anti-GFP polyclonal antibodies (Clontech) and mouse anti-FLAG monoclonal antibody M2 (Sigma) were used. They were further detected by BODIPY-FL-conjugated anti-rabbit immunoglobulin G and Cy3-conjugated anti-mouse immunoglobulin G, respectively.

Nucleotide sequence accession number. The sequence data for *mip1* are available from EMBL/GenBank/DDBJ under accession no. AB032552.

RESULTS

Screening for high-copy-number suppressors of the activated *mei2* allele. Haploid strain JX383 carried an integrated *mei2-SATA* allele driven by the *nmt1* promoter. In the absence of thiamine, the *nmt1* promoter was derepressed and *mei2-SATA* was expressed. JX383 then underwent ectopic meiotic development and failed to form colonies on a thiamine-depleted plate (24). To isolate novel factors relevant to *mei2* function, we screened for high-copy-number suppressors of JX383 which could recover growth of the strain on a thiamine-free SSA plate. We obtained five efficient high-copy-number suppressors, but three of them were found to suppress the activity of the *nmt1* promoter and were discarded. One of the

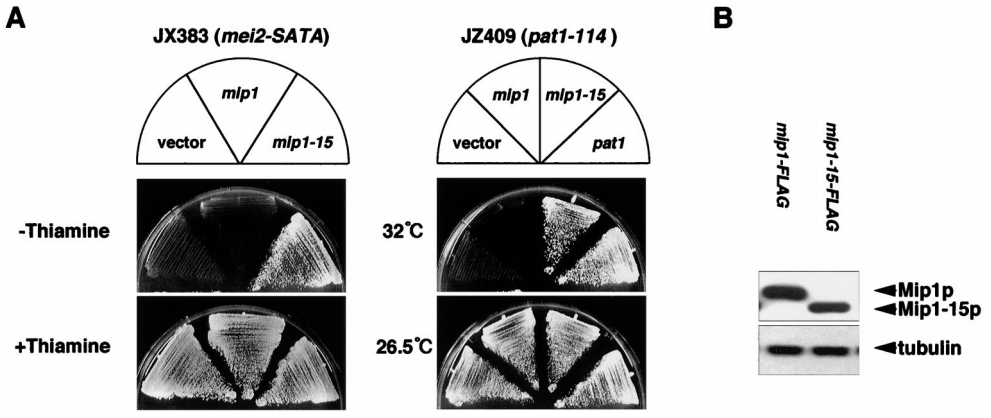

FIG. 1. Suppression of ectopic meiosis of the *nmt1-mei2-SATA* and *pat1-114* strains by overexpression of *mip1-15*. (A) JX383 (h^- *nmt1-mei2-SATA*) was transformed with either pREP41-*mip1*, pREP41-*mip1-15*, or the vector pREP41. JZ409 (h^- *pat1-114*) was also transformed with either pREP41-*mip1*, pREP41-*mip1-15*, pREP41-*pat1*, or the vector pREP41. Each transformant of JX383 was grown on a plate containing thiamine, restreaked on an SSA plates with or without 2 μM thiamine, and incubated at 32°C. Each transformant of JZ409 was grown at 26.5°C, spread on an SSA plate, and incubated at either 32 or 26.5°C. (B) To examine protein stability, the wild-type strain JY741 was transformed with either pREP41-*mip1-FLAG* or pREP41-*mip1-15-FLAG*, and total extracts were prepared from the transformants grown in thiamine-free MM for 16 h. Proteins extracted from 10^6 cells were separated by SDS-PAGE and subjected to Western blot analysis using an anti-FLAG antibody (upper panel) or the antitubulin antibody Tat1 (lower panel).

remaining two was *rcd1*, which encodes a regulator of transcription of *ste11* (18). The functional relationship between *rcd1* and *mei2* is under investigation. Here we report the analysis of the last clone, *mip1*.

By sequencing the 5-kb-long insert of the clone, we found a large ORF which apparently lacked the initiation codon. Then we isolated the 5′-terminal region of the gene by colony hybridization and determined the complete ORF sequence. We named this gene *mip1* (Mei2p-interacting protein). It turned out to correspond to SPAC57A7.11 defined by the genome sequence project. The *mip1* gene encoded 1,313 amino acid residues, and the original suppressor clone lacked the N-terminal 173 residues. We named this truncated *mip1* allele *mip1-15*.

To confirm that *mip1-15* was responsible for the suppression, we subcloned *mip1-15* ORF to pREP41, which carried the moderate *nmt1* promoter. This plasmid efficiently suppressed *mei2-SATA*. Surprisingly, however, the full-length *mip1* ORF connected to the same promoter could not suppress *mei2-SATA* (Fig. 1A). This difference was not due to the stability of the protein products (separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) (Fig. 1B), indicating that the property of Mip1p in relation to *mei2-SATA* was changed by the deletion of the N-terminal residues. Overexpression of *mip1-15*, but not *mip1*, also suppressed the *pat1*-driven meiosis (Fig. 1A). The $pat1^{ts}$ mutant enters meiosis at the restrictive temperature (2, 7) because inactivation of Pat1p kinase leads to loss of inhibitory phosphorylation of Mei2p (24). Thus, these results suggest that Mip1-15p may interfere with the function of Mei2p itself or its downstream factor.

***mip1* encodes a novel protein carrying a WD-40 repeat.** Using the BLAST homology search algorithm, we found that Mip1p was highly similar to the products of putative genes assigned in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* by the respective genome projects. These proteins all carried a WD repeat (15) in the C terminus and another highly conserved domain in the N terminus (Fig. 2). A short nucleotide sequence found in the human cancer genome anatomy project also appeared to encode the N-terminal region of these proteins (Fig. 2). Thus, this protein family is likely to be widespread among eukaryotes. The Mip1p homologues of other organisms have been identified only in the sequencing project, and their functions remain unknown.

***mip1* is essential for cell proliferation.** Using the $ura4^+$ cassette, we disrupted one copy of the *mip1* gene in diploid strain JY765. The resultant $mip1^+/mip1::ura4^+$ diploid JX564 was subjected to sporulation and tetrad analysis. Each ascus generated only two viable spores, both of which were Ura^-. These results strongly suggest that disruption of *mip1* is lethal. Microscopic observation revealed that ~40% of *mip1*Δ spores germinated and underwent one to three rounds of cell division before halting growth. The length of the arrested cells was smaller than that of the wild type (data not shown; see below).

We constructed haploid *mip1*Δ strains carrying either pREP81-*mip1* or pREP81-*mip1-15*. Both strains could grow in the absence of thiamine, although cells with pREP81-*mip1-15* grew more slowly (doubling time of 6.0 h in MM) than cells with pREP81-*mip1* (3.4 h). To investigate the terminal phenotype of *mip1*Δ, we shifted them to thiamine-containing medium to shut off the *nmt1* promoter. The pREP81-*mip1* cells, how-

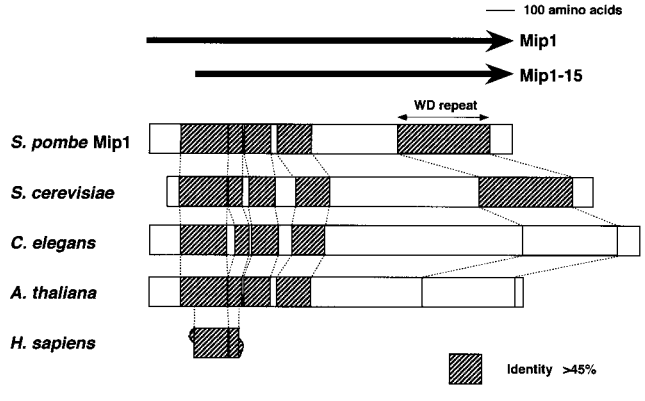

FIG. 2. Schematic illustration of the structure of *S. pombe* Mip1 protein (SPAC57A7.11) and its *S. cerevisiae* (SCYHR186C), *C. elegans* (C10C5.6), and *A. thaliana* (T16O11.22) homologues. A fragmentary sequence derived from the human cancer genome anatomy project is also shown (ng92c11.s1). The regions highly conserved (>45% identity) between Mip1p and the homologues are hatched.

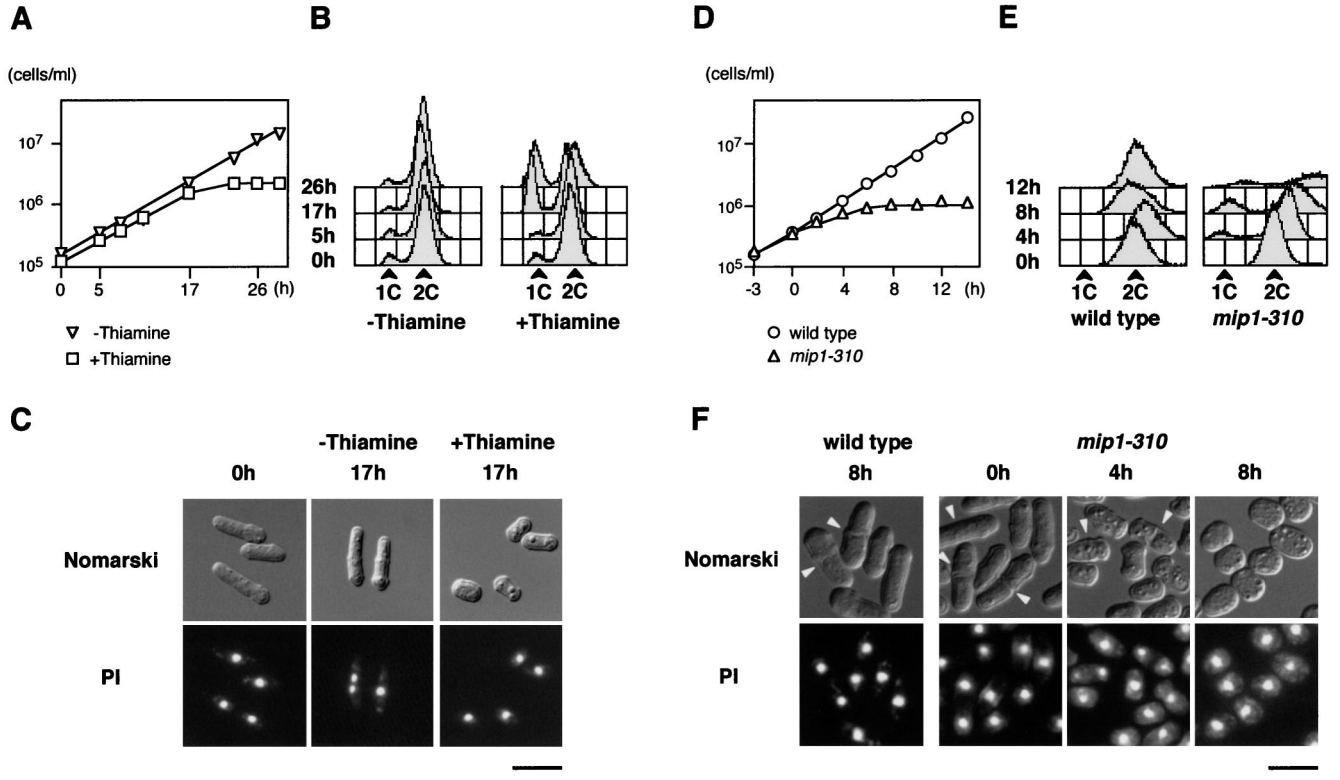

FIG. 3. *mip1*$^+$ is essential for cell growth. (A) Growth of *mip1*Δ cells transformed with pREP81-*mip1-15*. Cells were grown in MM to a concentration of about 10^5/ml; cell number was measured chronologically after the addition of 2 μM thiamine to the culture (□). Results for a control culture with no thiamine addition are also shown (▽). (B) Flow cytometry of the cells at 0, 5, 17, and 26 h after the addition of thiamine. (C) Nomarski micrographs of *mip1*Δ cells carrying the *mip1-15* plasmid cultured with or without thiamine for the indicated time. Fluorescence micrographs of the same cell population stained with the DNA-binding dye propidium iodide (PI) are also shown. Bar, 10 μm. (D) Growth of *mip1-310* cells. Cells were grown in YE medium to a concentration of about 10^5/ml at 26.5°C. They were shifted up to 36°C (0 h), and cell number was measured chronologically (△). Results for a control wild-type strain are also shown (○). (E) Flow cytometry of the cells at 0, 4, 8, and 12 h after the shift. (F) Morphology of *mip1-310* and control wild-type cells cultured at 36°C for the indicated time. Arrowheads indicate dividing cells. Fluorescence micrographs of the same cells stained with propidium iodide are also shown. Bar, 10 μm.

ever, continued proliferation, indicating that the repressed level of *mip1* expression from this plasmid was still enough to sustain cell growth. In contrast, pREP81-*mip1-15* cells could not grow on the thiamine-containing medium. Upon addition of thiamine to the pREP81-*mip1-15* liquid culture, most cells started to arrest growth after approximately three rounds of cell division (Fig. 3A). The arrested cells were short, and flow cytometry indicated that nearly 40% of the cells arrested with 1C DNA content (Fig. 3B and C).

To further explore the mitotic role of *mip1*, we aimed to construct a temperature-sensitive *mip1* allele and succeeded in isolating *mip1-310* as such (see Materials and Methods). At 26.5°C, *mip1-310* cells were indistinguishable from wild-type cells in either growth rate or morphology. Upon a shift to 36°C, however, *mip1-310* cells began to grow at a lower rate and continued cell division with reduced cell size for some while (Fig. 3D and F at 4 h). They finally terminated cell division, exhibiting a very short cell length (Fig. 3D and F at 8 h). The viability of these cells was less than 10% at 4 h after the shift. The *mip1-310* mutant could proliferate only below 34°C.

During the shutoff or thermal inactivation of Mip1p, a significant number of cells with 1C DNA content appeared (Fig. 3B and E). This may be explained by supposing that cell division with reduced cell size indirectly led to the accumulation of cell population with 1C DNA content, as was originally noted in the *wee1* cell cycle mutant (16). Unlike the *wee1* mutant, however, the *mip1* mutant was deficient in cell growth and inviable. Thus, Mip1p is likely to be required for facilitating cell cycle control in addition to supporting cell growth.

***mip1* plays a positive role in meiosis.** To see whether *mip1* has some role relevant to meiosis, we examined sporulation of an h^+/h^- *mip1*Δ/*mip1*$^+$ diploid strain JX564. This strain proliferated normally but sporulated at only 37% efficiency, while the efficiency of the wild-type JX1017 was 65% (Fig. 4A). Most of the unsporulated JX564 cells were arrested with a single nucleus, suggesting that the entry to meiosis was impaired in them. Despite their clear difference in meiotic proficiency, JX564 and JX1017 proliferated at nearly the same rate. This suggests that Mip1p plays a positive role in promoting the entry to meiosis but that its amount is not much in excess of the level needed to meet this end in wild-type diploid cells.

To further demonstrate the participation of Mip1p in meiosis, we compared meiotic development induced by *mei2-SATA* in *mip1*$^+$ and *mip1*Δ backgrounds. Two haploid strains were constructed for this purpose. One was a *mip1*Δ strain in which both *nmt1-mei2-SATA* and *adh1-mip1-15* were integrated (JW192), and the other was an isogenic *mip1*$^+$ strain (JW193). The *adh1-mip1-15* construct was integrated in order to support mitotic growth of the *mip1*Δ strain. They were shifted to thiamine-depleted medium to express *mei2-SATA*. Strikingly, even when *mei2-SATA* was maximally expressed, *mip1*Δ cells did not initiate meiosis and continued proliferation, whereas *mip1*$^+$ entered meiosis efficiently (Fig. 4B and C). These results support the idea that the wild-type *mip1*

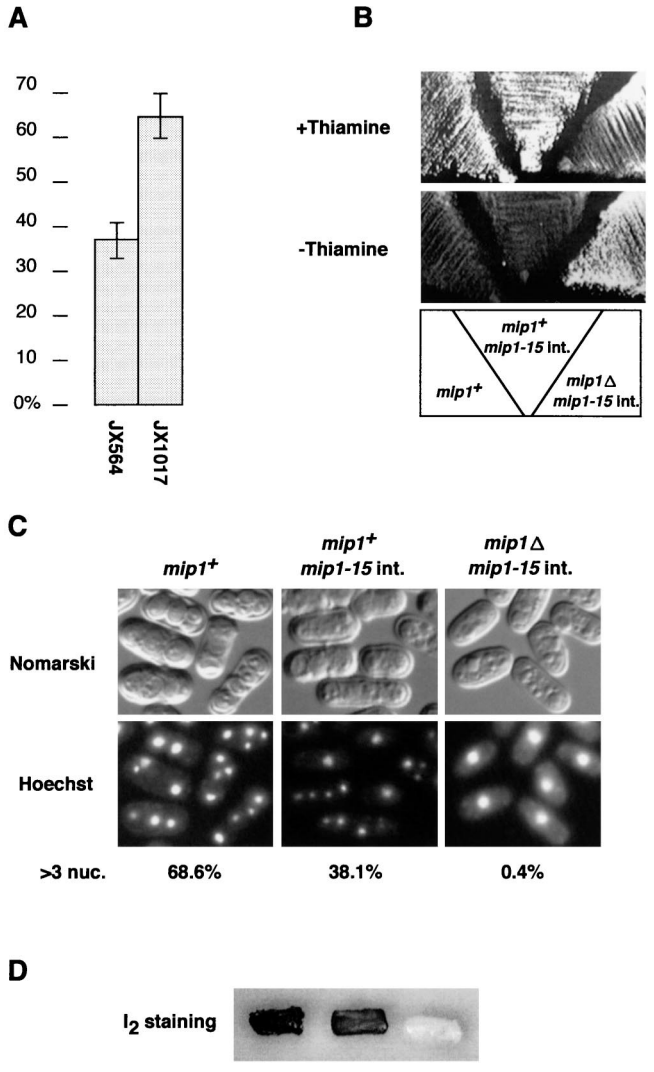

FIG. 4. Mip1p is required for the entry to meiosis. (A) Lowered sporulation efficiency in diploid cells heterozygous for *mip1*. The wild-type strain JX1017 and the *mip1*$^+$/*mip1*Δ strain JX564 were streaked on sporulation medium MEA and incubated at 30°C for 3 days. The sporulation frequency of each strain was scored under the microscope. The error bar for each data represents the standard deviation of three independent measurements. (B to D) Requirement of *mip1*$^+$ for haploid meiosis induced by *mei2-SATA*. JW192 (*nmt1-mei2-SATA adh1-mip1-15*) and the isogenic *mip1*Δ strain JW193 were streaked on an SSA plate with or without thiamine and incubated at 32°C for 3 days (+Thiamine) or 5 days (−Thiamine) (B). The same strains were cultured in liquid MM at 30°C for 48 h. Cells were fixed with ethanol, stained with Hoechst 33342, and photographed (C). They were also spread in patches on an SSA plate, incubated at 30°C for 4 days, and stained with I_2 vapor (D). Nuc., nuclei.

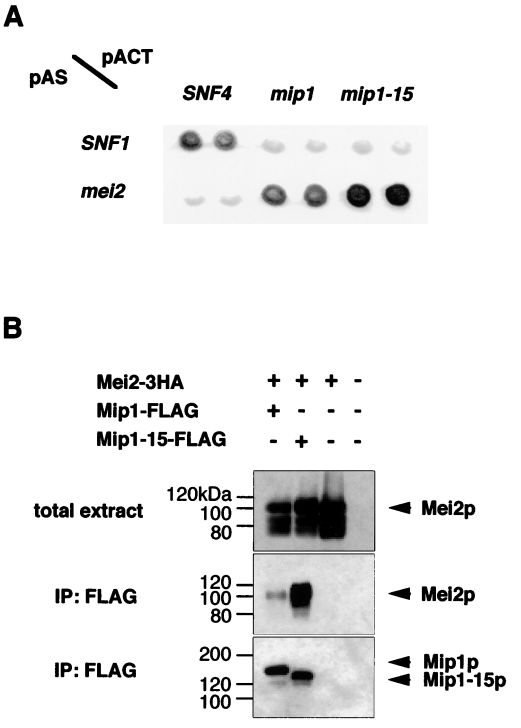

FIG. 5. Mip1p and Mei2p form a complex in vivo. (A) Yeast two-hybrid analysis. The interaction between Mip1p and Mei2p and that between Mip1-15p and Mei2p are positive, as judged by the blue coloring of the host cells. The intensity of the blue color is reproduced in black and white here. The pair *SNF1* and *SNF4* represents a standard positive control. (B) Coimmunoprecipitation of Mip1p and Mei2p. Crude extracts were prepared from vegetative cells expressing tagged proteins as indicated and subjected to immunoprecipitation (IP) with an anti-FLAG antibody. Each precipitate was separated by SDS-PAGE and examined for the presence of Mei2 protein by Western blotting using an anti-HA antibody. The immunoblotting patterns of total crude extracts are also shown.

allele performs a positive function in *mei2*-driven meiosis. The *mip1-15* allele is apparently defective in this function.

Mip1p interacts physically with Mei2p. A number of genes are involved in the genetic cascade controlling meiotic entry in fission yeast. Among them, *mei2* is apparently the most downstream factor whose activation is sufficient for the induction of meiosis. As shown above, Mip1p is required for the *mei2*-driven meiosis, whereas its truncated version Mip1-15p interferes with it. These results suggested the possibility that Mip1p might directly interact with Mei2p. To examine this, we performed a yeast two-hybrid assay (1). The C-terminal region of Mei2p (aa 429 to 733), which could perform the essential function of Mei2p (24), was fused to GDB. Full-length Mip1p and its truncated version Mip1-15p were fused to GAD and tested for interaction with the Mei2p bait by assaying β-galactosidase activity. Both versions of Mip1p were found to interact with Mei2p specifically. Apparently Mip1-15p interacted with Mei2p more strongly than full-length Mip1p (Fig. 5A).

To confirm the association of Mip1p and Mei2p in vivo, we expressed FLAG-tagged Mip1p and 3HA-tagged Mei2p in wild-type cells. Mip1p was immunoprecipitated using an anti-FLAG antibody, and coprecipitation of Mei2p was investigated. As shown in Fig. 5B, Mei2p was coimmunoprecipitated efficiently with Mip1-15p and less efficiently with full-length Mip1p. Therefore, we conclude that Mip1p interacts with Mei2p in vivo and that Mip1-15p has acquired a strengthened affinity to Mei2p.

Mip1p is located in the cytoplasm. To examine subcellular localization of Mip1p, we fused GFP to the C terminus of Mip1p. The Mip1p-GFP fusion protein was expressed from the *nmt1* promoter on pREP41. This fusion protein was functional because it could complement *mip1*Δ. Fluorescence microscopy revealed that Mip1p-GFP was distributed uniformly in the cytoplasm but was scarce in the nucleus (Fig. 6). This localization of Mip1p was similar to that of Mei2p in the premeiotic stage, suggesting that Mip1p is likely to interact with Mei2p at this stage (24, 29).

In contrast to wild-type Mip1p, overexpression of Mip1-15p counteracted Mei2p and blocked the initiation of meiosis (Fig. 1). To gain a deeper insight into this observation, we compared

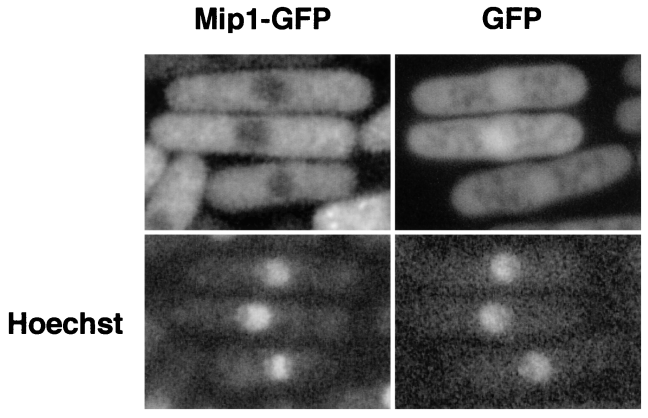

FIG. 6. Subcellular localization of Mip1p tagged with GFP. Wild-type strain JY362 transformed with either pREP41-*mip1-GFP* or pREP41-*GFP* was grown in liquid MM and examined for GFP fluorescence without fixation. A fluorescence micrograph of the same cells stained with the DNA-binding dye Hoechst 33342 is also shown for each transformant.

the effect of overexpression of the two versions of Mip1p on Mei2p localization. We coexpressed Mei2p-GFP together with either Mip1p-FLAG or Mip1-15p-FLAG in diploid strain JY765. Each tagged protein was indistinguishable from its untagged prototype in function. The diploid transformants were shifted to the nitrogen-depleted medium and monitored for progression of meiosis and localization of Mei2p-GFP (Fig. 7). Control cells carrying only the vector exhibited horse-tail nuclei during meiotic prophase, as previously reported (18a). Mei2p formed a dot in the nucleus at this phase, being located also in the cytoplasm homogeneously. Cells overproducing Mip1p-FLAG behaved like the control cells (Fig. 7A and B). In contrast, most cells overproducing Mip1-15p-FLAG failed to enter meiosis and arrested at G_1 (Fig. 7A). Mei2p-GFP was mislocated in the latter cells, forming patches mostly in the cytoplasm (Fig. 7B). These patches were apparently colocated with Mip1-15p-FLAG (Fig. 7C), consistent with the observed coimmunoprecipitation of the two proteins. The inhibition of meiotic entry by Mip1-15p overproduction was alleviated by elevated expression of Mei2p (data not shown). Thus, we conclude that Mip1-15p associates Mei2p tightly and induces abnormal aggregation of the proteins, thereby blocking Mei2p function.

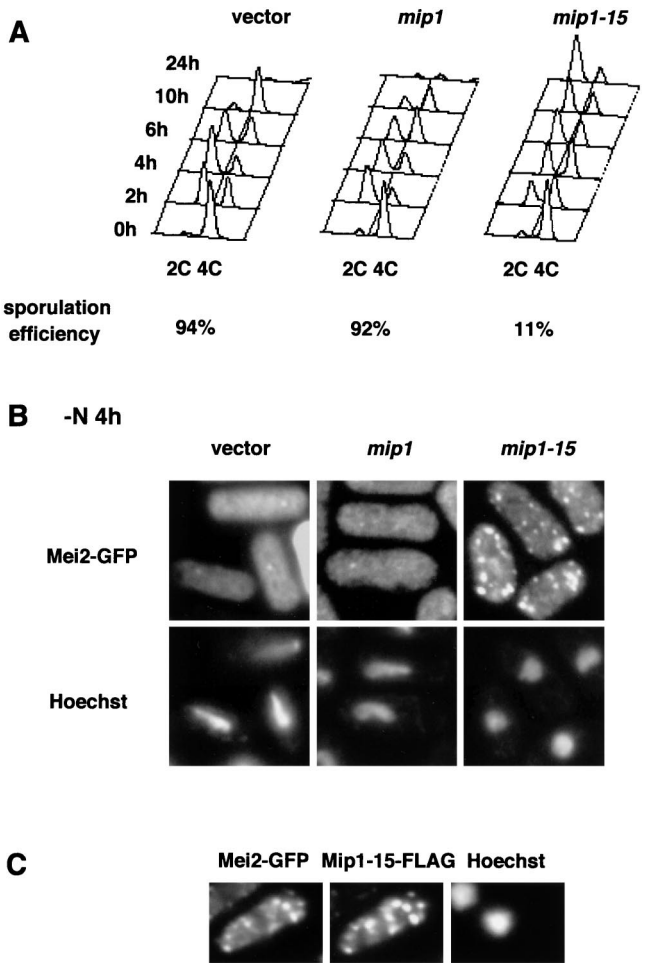

FIG. 7. Aggregation of Mei2p induced by Mip1-15p overproduction. (A) Flow cytometry of the wild-type diploid strain JY765 carrying pGFT81-*mei2* and either pREP2-*mip1-FLAG*, pREP2-*mip1-15-FLAG*, or the vector pREP2. Each strain was grown to 4×10^6 cells/ml in MM and shifted to MM-N to induce meiosis. Cells were harvested at intervals after the shift and subjected to flow cytometry. Sporulation efficiency of each strain was determined 24 h after the shift. (B) The same strains as in panel A were fixed by methanol at 4 h after the shift and observed for fluorescence of Mei2p-GFP. Fluorescence micrographs of cells stained with the DNA-binding dye Hoechst 33342 are also shown. (C) Cells producing Mei2p-GFP and Mip1-15p-FLAG were fixed by formaldehyde at 4 h after the shift. They were doubly immunostained with anti-GFP antibodies and a monoclonal anti-FLAG antibody and subjected to fluorescence microscopy.

Mip1p interacts with Ste11p, which is required for conjugation. We observed that overexpression of *mip1-15* reduced efficiency of conjugation in wild-type haploid cells (data not shown). Furthermore, haploid *mip1*Δ cells carrying the integrated *adh1-mip1-15* allele hardly underwent mating, while *mip1*$^+$ cells carrying the same allele could mate considerably (Fig. 8A). These results suggested that, as was true with meiosis, Mip1p might have a positive role in conjugation that is inhibited by Mip1-15p. Overexpression of Mip1-15p could inhibit conjugation in *mei2*Δ cells, suggesting that this effect is not mediated by Mei2p (data not shown). It has been known that *ste11* and some of its target genes are essential to initiate conjugation in *S. pombe* (21). Therefore, we examined transcription of *ste11* itself and its target genes *mat1-Pc* and *mat1-Pi* in the diploid Mip1-15p overproducer. Expression of *ste11* was not much affected in this strain, but expression of *mat1-Pc* and *mat1-Pi* was impaired (Fig. 8B). Consistently, expression of *mei3*, which depends on the function of *mat1-Pi* (22, 26), was not detectable in this strain (Fig. 8B). The conjugation defect caused by Mip1-15p overproduction was alleviated by elevated expression of Ste11p (data not shown). These results suggested that the function of Ste11p was impaired by Mip1-15p. We examined whether Mip1-15p and Ste11p could be coimmunoprecipitated. As shown in Fig. 8C, Ste11p was precipitated efficiently with Mip1-15p and less efficiently with Mip1p. These results support the conclusion that Mip1p plays a positive role in conjugation, possibly by activating or assisting Ste11p directly.

We investigated the behavior of the temperature-sensitive *mip1-310* mutant in mating and meiosis. At every tested temperature up to 33°C, which was close to the limit for mitotic proliferation of the mutant, *mip1-310* cells performed mating and meiosis almost as efficiently as wild-type cells. This analysis hence turned out to be uninformative.

Mip1p interacts only with unphosphorylated Mei2p. As shown above, Mip1p interacted with two different proteins, Mei2p and Ste11p. It is noteworthy that these proteins are both

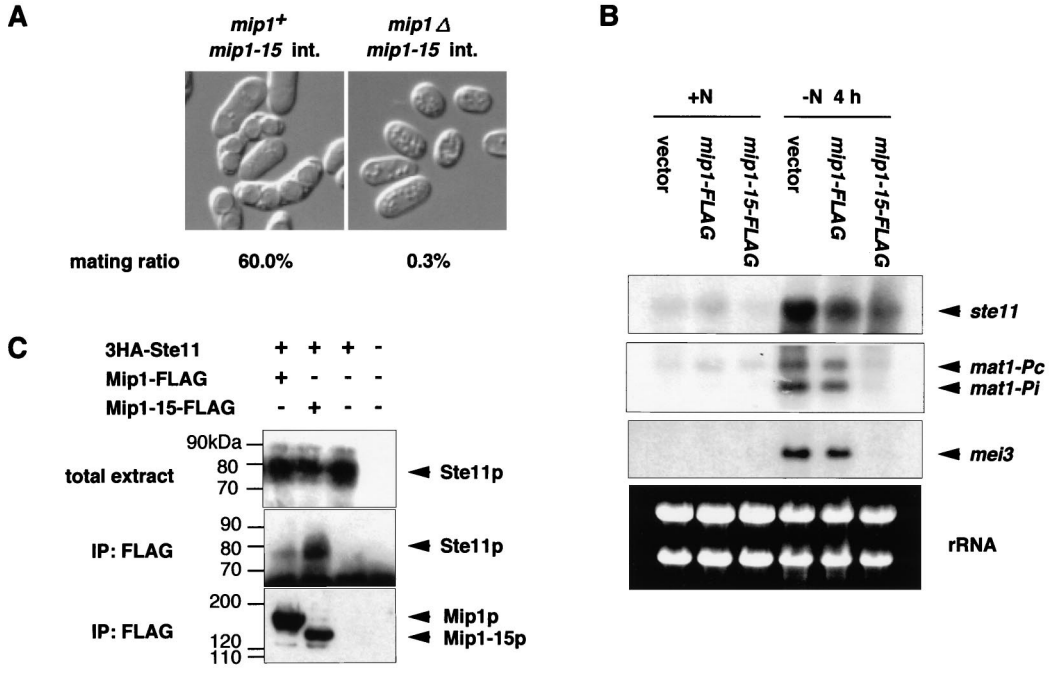

FIG. 8. *mip1* is required for the mating process. (A) h^{90} *mip1-15* integrant (int.) strains with or without the authentic $mip1^+$ gene (JW195 or JW196) were streaked on sporulation medium SSA and incubated at 30°C for 4 days. The mating frequency of each strain was scored under the microscope. (B) Northern blot analysis of *mip1-15*-overexpressing cells. Total RNA was extracted from the same strains as analyzed in Fig. 7, either growing exponentially (0 h) or starved of nitrogen for 4 h. RNA (10 μg) was loaded in each lane after denaturation by formamide, electrophoresed, transferred to a nylon membrane, and hybridized with the indicated probes. rRNA in each sample, stained with ethidium bromide, are shown in the lower panel to verify nearly equal loading of RNA in each lane. (C) Coimmunoprecipitation of Mip1p and Ste11p. Crude extracts were prepared from cells expressing tagged proteins as indicated and growing mitotically. They were subjected to immunoprecipitation (IP) with an anti-FLAG antibody. The precipitates were subjected to SDS-PAGE and examined for the presence of Ste11 protein by Western blotting using an anti-HA antibody. Immunoblotting patterns of the crude extracts are also shown.

phosphorylated by Pat1p kinase (9, 24), implying that Mip1p may recognize the phosphorylation sites in these proteins. Therefore, we examined whether interaction of Mei2p with Mip1p could be affected by the phosphorylation by Pat1p. To do so, we used a yeast three-hybrid assay. As demonstrated in Fig. 9, Mei2p lost the ability to interact with Mip1p when Pat1p kinase was expressed in the host cell. Expression of kinase-negative Pat1p (Pat1-Dp) had no effect. Unphosphorylatable Mei2-SATAp interacted with Mip1p efficiently even in the presence of Pat1p kinase. These results strongly suggest that Mip1p interacts with Mei2p when it is not phosphorylated by Pat1p.

Consistent with the above idea, a shortened form of the Mei2p C-terminal region (aa 532 to 750) which lacked the two phosphorylation sites (Ser438 and Thr527) failed to interact with Mip1p in two-hybrid assay (data not shown), whereas the Mei2p C-terminal region carrying aa 429 to 733 could do so (Fig. 5A). This observation may suggest further that these phosphorylation sites, in their unphosphorylated form, serve as the binding sites for Mip1p.

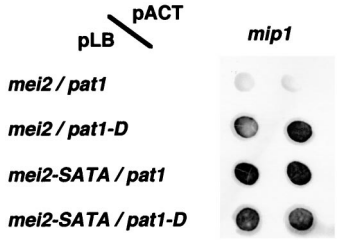

FIG. 9. Interaction of Mip1p with unphosphorylated Mei2p, examined by yeast three-hybrid analysis. Either LexA-Mei2p or LexA-Mei2-SATAp was expressed together with either active Pat1p or kinase-negative Pat1-Dp from a single plasmid. GAD-Mip1p was expressed from another plasmid. For each combination, two independent transformants were assayed for β-galactosidase activity. The intensity of the blue color is reproduced in black and white here.

DISCUSSION

We have identified a novel WD-repeat protein, Mip1p, which interacts with the meiotic regulator Mei2p in fission yeast. In the screening for high-copy-number suppressors of ectopic meiosis induced by active Mei2p, we isolated a truncated form of *mip1* missing the N-terminal region of the ORF. This *mip1-15* allele inhibited meiotic differentiation if overexpressed. However, we concluded that wild-type Mip1p plays a positive role in meiotic differentiation for the following reasons: (i) unlike *mip1-15*, overexpression of wild-type *mip1* does not interfere with meiosis; (ii) reduction of the *mip1* gene dosage by half in a diploid cell lowered the frequency of meiotic entry; and (iii) the negative effect of *mip1-15* on *mei2*-driven meiosis can be alleviated by overproduction of Mei2-SATAp in the presence of wild-type *mip1* but not in its absence.

Two-hybrid and immunoprecipitation experiments demonstrated that Mip1p interacts physically with Mei2p in vivo. Thus, Mip1p may bind and assist Mei2p in the process of its activation or function, thereby facilitating meiotic entry. The N-terminal truncation in Mip1-15p likely converted this protein into a dominant negative form inhibiting Mei2p function. This model is consistent with the observation that Mip1-15p but not Mip1p causes abnormal aggregation of Mei2p in the

cell. In addition, Mip1-15p was bound to Mei2p more tightly than Mip1p, and the meiotic defect caused by Mip1-15p was alleviated by elevated expression of Mei2p from a plasmid.

We presently have no firm picture about the molecular function of Mip1p, but the following discussion may be noteworthy. Although interaction of wild-type Mip1p with Mei2p was demonstrated, this interaction appeared to be relatively weak, suggesting that Mip1p may interact with Mei2p transiently rather than permanently. Results of the three-hybrid analysis indicate that Mip1p interacts with unphosphorylated Mei2p but not with Mei2p phosphorylated by Pat1p. This may mean that Mip1p interacts with Mei2p when the protein is produced de novo in the cytoplasm, prior to its phosphorylation by Pat1p. If this is the case, Mip1p may assist protein folding of nascent Mei2p and/or a subsequent crucial step for Mei2p activation. The period of their interaction will be very short, and once Mei2p is phosphorylated by Pat1p, Mip1p may dissociate from it. This scenario is consistent with the idea that Mip1p is a kind of molecular chaperon, acting to assist protein folding and subsequent processing required for the proper function of the target protein.

In addition to Mei2p, a crucial transcription factor for sexual development, Ste11p, is associated with Mip1p in vivo. Coimmunoprecipitation demonstrated that Ste11p interacts weakly with Mip1p but more strongly with Mip1-15p. Mip1-15p, but not Mip1p, inhibited conjugation if overexpressed. Furthermore, cells expressing *mip1-15* did not conjugate in the absence of wild-type *mip1* allele but became proficient in mating if *mip1*$^+$ was present, indicating that *mip1*$^+$ plays a positive role in conjugation. Thus, the relationship of Mip1p with Ste11p appears very similar to the relationship of Mip1p with Mei2p, suggesting that Mip1p participates in conjugation through the interaction with Ste11p. It is interesting that Mip1p can recognize and regulate two totally different proteins, Mei2p and Ste11p, the former an RNA-binding protein and the latter a high-mobility-group transcription factor. A common feature of these two proteins is their possession of conserved phosphorylation motifs by Pat1p kinase, which are relevant to their nuclear localization (9, 24, 29). We have demonstrated that the interaction of Mip1p with Mei2p depends on the phosphorylation state of the motifs. Thus, these motifs themselves may be the sites of interaction with Mip1p.

As discussed above, Mip1p plays a crucial role for sexual development by interacting with Mei2p and Ste11p, which are key factors for meiosis and conjugation. However, they are not the only targets of Mip1p in the cell. Gene disruption of *mip1* indicated that the function of *mip1* is essential for cell proliferation. Neither Mei2p nor Ste11p is the target molecule interacting with Mip1p during mitotic proliferation, because these proteins are dispensable for asexual growth (21, 23). Therefore, there must be another target(s) of Mip1p required for mitotic cell growth. A notable feature of the truncated *mip1-15* allele is that it dominantly inhibits meiosis and conjugation if overexpressed but does not interfere with, and even to some extent supports, mitotic growth. It is hence presumable that Mip1p functions in somewhat different modes in mitotic growth and in sexual development.

It is particularly intriguing that Mip1p is evolutionarily conserved among eukaryotes. Several Mip1p homologues have been identified in other organisms by genome sequencing projects, but none of them have been analyzed for function. The present work with fission yeast is the first example of a functional analysis of this family. Given that Mip1p is required for cell growth as well as sexual development and therefore is likely to interact with several proteins in fission yeast, its molecular function is probably ubiquitous and fundamental. We propose that fission yeast Mip1p plays a critical role in the cytoplasm in assisting a certain class of proteins to express their function. Although more extensive analysis is required to elucidate the precise molecular function of Mip1p, this report provides an important basis for further studies of this conserved WD-repeat protein family.

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