Fission yeast mfr1 activates APC and coordinates meiotic nuclear division with sporulation

Miguel A. Blanco*, Laetitia Pelloquin* and Sergio Moreno‡

Instituto de Microbiología Bioquímica, CSIC/Universidad de Salamanca, Edificio Departamental, Campus Miguel de Unamuno, 37007 Salamanca, Spain

*These two authors contributed equally to this work
‡Author for correspondence (e-mail: smo@gugu.usal.es)

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SUMMARY

Meiosis is the developmental program by which sexually reproducing diploid organisms generate haploid gametes. In yeast, meiosis is followed by spore morphogenesis. These two events are normally coordinated in such a way that spore formation is dependent upon completion of the meiotic nuclear divisions. Here we describe a meiosis-specific protein, mfr1, that is involved in this coordination. mfr1 is an activator of the anaphase-promoting complex (APC), which is necessary for the rapid degradation of the cdc13 cyclin at the end of meiosis II, prior to the formation of spores. An *mfr1* null mutant completes meiosis II but remains with high levels of cdc13 and cdc2 kinase activity and has considerably delayed spore formation. By analogy with the mitotic cell cycle, where proteolysis and inactivation of cdc2 kinase are necessary to trigger mitotic exit and cytokinesis, we propose that at the end of meiosis rapid and timely proteolysis of cyclins is required to switch on the differentiation program that eventually leads to the formation of haploid gametes.

Key words: Cell cycle, Meiosis, APC, Proteolysis, Fizzy, mfr1, Yeast

INTRODUCTION

Progression through mitosis is controlled by cyclin-dependent kinases (cdk), which drive cells into metaphase, and by the anaphase-promoting complex (APC), a ubiquitin ligase that triggers sister chromatid separation, mitotic exit and cytokinesis (Cerutti and Simanis, 2000; Zachariae and Nasmyth, 1999). In a normal cell cycle, destruction of securins (cut2 in *S. pombe*, Pds1 in *S. cerevisiae*) is required for sister chromatid separation and chromosome segregation, while destruction of mitotic cyclins and the consequent loss of cdk activity drives exit from mitosis and cytokinesis. Degradation of mitotic cyclins is also required in G_1 for the assembly of pre-replicative complexes in the DNA and, in fission yeast, for G_1 arrest in response to mating pheromones or nutrient starvation (Blanco et al., 2000; Kitamura et al., 1998; Stern and Nurse, 1998; Yamaguchi et al., 1997; Yamaguchi et al., 2000).

Two APC activators have been described in all organisms analyzed so far. These are proteins containing seven WD repeats that associate to and activate APC. In fission yeast, slp1 (known as Cdc20 in budding yeast, *Fizzy* in *Drosophila* and $p55^{cdc}$ in animal cells) initiates APC-dependent degradation of securin and mitotic cyclins during the metaphase-to-anaphase transition. A second APC activator, ste9/srw1 (or Hct1/Cdh1 in *S. cerevisiae*, *Fizzy-related* in *Drosophila* or Hct1/Cdh1 in animal cells), binds to APC and continues with the degradation of cyclins up to the end of G_1. ste9/srw1 (or Hct1/Cdh1) binding to APC is negatively regulated by cdk phosphorylation (Blanco et al., 2000; Jaspersen et al., 1999; Kramer et al., 2000; Yamaguchi et al., 2000; Zachariae et al., 1998). In S-phase and G_2, when cdc2 is active, ste9/srw1 is phosphorylated and does not associate with APC. At the end of mitosis, ste9/srw1 is dephosphorylated, binds to APC, and promotes the degradation of mitotic cyclins in G_1. This ordered activation of APC by slp1 in metaphase/anaphase and by ste9/srw1 in anaphase/G_1 is essential for mitotic exit, cytokinesis, and for G_1 arrest (Blanco et al., 2000; Kim et al., 1998; Kitamura et al., 1998; Yamaguchi et al., 1997; Yamaguchi et al., 2000).

Little is known about the regulation of APC in meiosis. Meiosis is a specialized cell division with two major differences relative to the mitotic cell cycle. First, following premeiotic DNA synthesis, recombination takes place between homologous chromosomes in meiotic prophase. Second, after recombination two consecutive nuclear divisions occur without an intervening S-phase. In the first division (reductional division or meiosis I), homologous chromosomes are separated. In the second (equational division or meiosis II), sister chromatids segregate (Roeder, 1997). Once the two nuclear divisions have been completed, a differentiation program is induced to generate haploid germ cells. In yeast, the four haploid nuclei formed are packaged into spores.

Here, we describe a third activator of APC (mfr1 for meiotic fizzy-related 1), which is specific for meiosis in fission yeast. APC^{mfr1} is involved in the rapid and timely degradation of the cdc13 M-phase cyclin at the end of meiosis II, which is necessary to inactivate the cdc2 kinase and thereby to bring about sporulation.

MATERIALS AND METHODS

Fission yeast strains and methods

The fission yeast strains used in this study are listed in Table 1. Growth

Table 1. *Schizosaccharomyces pombe* strains

Strain	Genotype	Source
Sp448	*h⁻ pat1-114 leu1-32*	P. Nurse
Sp785	*h⁺/h⁻ ade6-M210/ade6-M216 leu1-32/leu1-32*	This study
Sp813	*h⁺ lid1::lid1-myc:kanMX*	K. Gould
Sp963	*h⁺/ h⁻ mfr1Δ::ura4⁺/mfr1Δ::ura4⁺ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-d18/ura4-d18*	This study
Sp964	*h⁻/h⁻ pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32*	This study
Sp965	*h⁻/h⁻ pat1-114/pat1-114 mrf1Δ::ura4⁺/mfr1Δ::ura4⁺ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-d18/ura4-d18*	This study
Sp966	*h⁻/h⁻ pat1-114/pat1-114 mfr1Δ::ura4⁺/mfr1Δ::ura4⁺ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-d18/ura4-d18 pJK148mfr1-HA/pJK148mfr1-HA*	This study
Sp967	*h⁻/h⁻ pat1-114/pat1-114 mfr1Δ::ura4⁺/mfr1Δ::ura4⁺ ade6-M210/ade6-M216leu1-32/leu1-32 ura4-d18/ura4-d18 pJK148mfr1-HA/pJK148 mfr1-HA lid1::lid1-myc:kanMX/ lid1::lid1-myc:kanMX*	This study
Sp969	*h⁻/h⁻ pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32 pJK148cdc13⁺*	This study
Sp970	*h⁻/h⁻ pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32 pJK148cdc13-des2*	This study

conditions and strain manipulations were as described previously (Moreno et al., 1991). Diploids strains were generated by mating in MEA plates or by protoplast fusion (Sipiczki and Ferenczy, 1977), and the identity of these strains was confirmed by Southern blotting. Yeast transformation was carried out using the lithium acetate transformation protocol (Norbury and Moreno, 1997). Experiments in liquid culture were carried out in minimal medium (EMM) containing the required supplements, starting with a cell density of 2-4×10^6 cells/ml, corresponding to mid-exponential phase growth. For sporulation in liquid culture, cells were incubated in EMM + supplements, and shifted to EMM-NH_4Cl + supplements, a nitrogen-free version of minimal medium.

Synchronous meiosis in *pat1-114/pat1-114* temperature-sensitive mutants was performed as follows. *h⁻/h⁻ pat1-114/pat1-114* diploid cells were cultured in rich YES medium at 25°C for one day and transferred to EMM + supplements (100 μg/ml) for another day. These cells were then washed and transferred to EMM-NH_4Cl + supplements (50 μg/ml) at a density of 2-3×10^6 cells/ml. After 14 hours at 25°C, most cells were arrested in G_1. The culture was then shifted to 34°C, in the presence of 0.5 g/l NH_4Cl and 50 μg/ml supplements, to induce meiosis.

Cloning of *mfr1⁺*

The *mfr1⁺* sequence was identified by searching the fission yeast database deposited at the Sanger Centre (UK) (http://www.sanger.ac.uk/Projects/S_pombe/) for homologues of ste9 using the BLAST 2.0 (Basic Local Alignment Search Tool) algorithm. To isolate a DNA fragment containing the *mfr1⁺* gene, an *S. pombe* genomic library containing partially digested Sau3A DNA fragments constructed in pUR18 plasmid was screened by colony hybridization using a *mfr1⁺* ORF probe generated by PCR. A 3.8 kb genomic fragment containing the 1.3 kb *mfr1⁺* open reading frame flanked by 1.9 kb in the 5′ untranslated region and 0.6 kb in the 3′ untranslated region was cloned into pTZ18R (pTZ18R *mfr1⁺*-genomic) and sequenced.

Disruption of *mfr1⁺*

The *mfr1* null allele was generated by a PCR-based approach as described previously (Bahler et al., 1998). The *S. pombe ura4⁺* gene was amplified by PCR using the KS-ura4⁺ plasmid as template and the primers 5′-ATTTATTCACGAAATAGGAATCTCAACATTTCC-CTTCCATCCCGACTAACTTCAACTTATAAAGAATTG-GTTGACACGCCAGGGTTTTCCCAGTCACGAC-3′ and 5′-ATC-ACCTGATAATCCTGGAACGAATGCTGAAGAGGATGAAGATG ATGATGGGGTTGAGATGATTGATGTTGTTTGAAGCGGATA-A-CAATTTCACACAGGA-3′ (5′-ends are targeting sequences corresponding to 76 nucleotides immediately upstream and downstream from the *mfr1⁺* ORF and 3′-ends are 24 underlined nucleotides corresponding to sequences on either side of pBluescript multiple cloning site). *S. pombe ura4-d18* diploid cells were transformed with this 2.2 kb PCR-amplified fragment, and the *ura4⁺* transformants were checked for the correct integration of the DNA fragment in the *mfr1⁺* locus by Southern blot analysis. A heterozygous diploid was sporulated and the spores germinated in rich medium. After tetrad dissection the four spores were able to grow. Haploid *ura4⁺* cells deleted for *mfr1* were viable.

HA-tagging of *mfr1⁺*

The plasmid pTZ18R *mfr1⁺* containing the 3.8 kb genomic fragment was used to introduce a *Not*I restriction site just before the *mfr1⁺* stop codon by site-directed mutagenesis using the Muta-gene phagemid in vitro mutagenesis kit (BioRad) and the oligonucleotide 5′-AGTAC-ATTAATTCGCGGCCGCTAATCAAACAACATC-3′ (the *Not*I site is underlined). A 110 pb fragment containing three HA-epitope repeats was then cloned in frame in the *Not*I site. The construction was confirmed by DNA sequencing. A genomic fragment containing *mfr1-3xHA* was then subcloned into the integrative pJK148 plasmid containing the *leu1⁺* marker and used to transform the haploid strains *h⁻ pat1-114 mfr1::ura4⁺ ade6-M210 leu1-32* and *h⁻ pat1-114 mfr1::ura4⁺ ade6-M216 leu1-32.* Stable single-copy integrants were obtained and checked by Southern blot and a diploid strain was prepared by protoplast fusion. This diploid strain essentially behaved like a wild-type *h⁻/h⁻ pat1-114/pat1-114 mfr1⁺/ mfr1⁺* (Fig. 2).

Protein extracts, western blots and kinase assays

Total protein extracts were prepared as described previously (Blanco et al., 2000). For western blot analysis, 50-75 μg of total protein extract was run on a 14% SDS-PAGE gel (30:0.15 acrylamide:bisacrylamide ratio), transferred to nitrocellulose, and probed with SP4 anti-cdc13 (1:400), anti-cig1 (1:250) affinity-purified polyclonal antibodies, or with anti-myc 9E10 (1:1000) or anti-HA 12CA5 (0.15 μg/ml) monoclonal antibodies. Goat anti-rabbit or goat anti-mouse antibodies conjugated to horseradish peroxidase (Amersham) (1:3,500) were used as secondary antibodies. As loading controls, rabbit affinity-purified anti-cdc2 C2 antibodies (1:250) were used. Immunoblots were developed using the ECL kit (Amersham) or Super Signal (Pierce). Immunoprecipitations with cdc2 antibodies and cdc2 protein kinase assays were performed as described previously (Benito et al., 1998).

Coimmunoprecipitation analysis of mfr1 and lid-myc

Total protein extracts were prepared from 3×10^8 cells using HB buffer (Moreno et al., 1989; Moreno et al., 1991). Cell extracts were spun at 4°C in a microfuge for 15 minutes, and protein concentrations were determined using the BCA protein assay kit (Pierce). Approximately 3.5 mg of total protein extracts were subjected to immunoprecipitation by consecutive incubation with the monoclonal anti-HA 12CA5 (1 μg) or anti-myc 9E10 (1 μg) antibody for 1 hour on ice, and protein A-Sepharose or protein G-Sepharose (Pharmacia-Biotech) for 30 minutes at 4°C with agitation. Immunoprecipitates were washed six times with 1 ml of HB buffer. Lysates and immunoprecipitates were resolved on 12% SDS-polyacrylamide gels, followed by western blot analysis as above.

Flow cytometry and microscopy

About 10^7 cells were spun down, washed once with water, fixed in 70% ethanol and processed for flow cytometry or DAPI staining, as described previously (Moreno et al., 1991; Sazer and Sherwood, 1990). A Becton-Dickinson FACScan was used for flow cytometry. To estimate the proportion of cells in meiosis I, meiosis II or in sporulation, we determined the percentage of cells with one, two or four nuclei after DAPI staining and the percentage of asci with mature spores under phase contrast microscopy.

For mfr1 subcellular localization, indirect immunofluorescence was performed as previously described (Sohrmann et al., 1996) except that the cells were fixed in 4% *p*-formaldehyde (Sigma) in PEM and digested for 5 minutes in 1 mg/ml zymolyase 20T. Monoclonal anti-HA (1:200) or polyclonal anti-sad1 (1:25) antibodies and anti-mouse Cy3-conjugated (Jackson) or anti-rabbit FITC-conjugated (Kappel) secondary antibodies (1:500) were used to detect mfr1-HA and sad1, respectively. Lid1(APC4)-myc localization was done using rabbit polyclonal antibodies (Upstate Biotechnology) against the myc epitope (1:50).

RNA preparation and northern blots

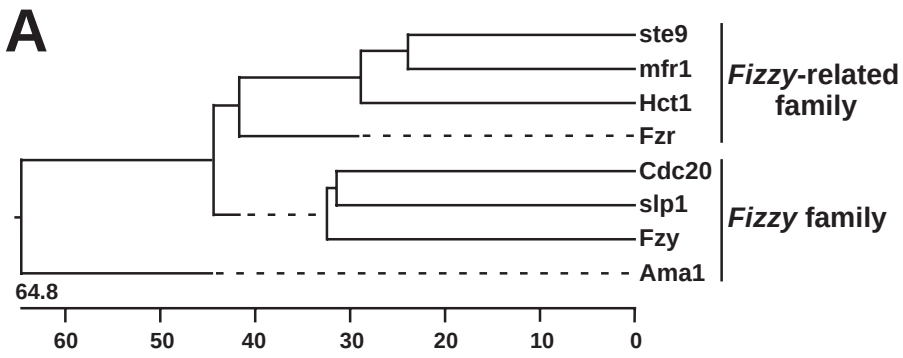
RNA from cells was prepared by lysis with glass beads in the presence of phenol (Moreno et al., 1991). RNA gels were run in the presence of formaldehyde, transferred to GeneScreen Plus (NEN, Dupont) and probed with the *mfr1*$^+$ open reading frame according to the manufacturer's instructions.

RESULTS

mfr1 is a meiosis-specific protein

In order to identify additional proteins related to the APC activator ste9/srw1 we searched the *S. pombe* genome sequence database at the Sanger Centre (UK) using the BLAST 2.0 algorithm. A new open reading frame (SPBC660.02) was identified encoding a protein that showed 40% identity to ste9/srw1. This protein was more similar to the *Fizzy*-related family than to the *Fizzy*-family of APC activators (Fig. 1).

This gene was not expressed during the mitotic cell cycle and was dramatically induced during meiosis (Fig. 2A). We therefore named this gene *mfr1*$^+$ (for meiotic fizzy-related 1). To study the expression of *mfr1*$^+$, we constructed the diploid strain (Sp966) *h*$^-$/*h*$^-$ *pat1-114/pat1-114 mfr1-3xHA/mfr1-3xHA*. These cells are temperature sensitive for the *pat1*$^+$ gene and contain a functional version of mfr1 tagged at the C terminus with three copies of the influenza virus hemagglutinin (HA) epitope. Exponentially growing cells (Fig. 2, Exp) were pre-synchronized in G_1 by nitrogen starvation at 25°C for 14 hours (Fig. 2, 0 hours). Nitrogen was added back and the



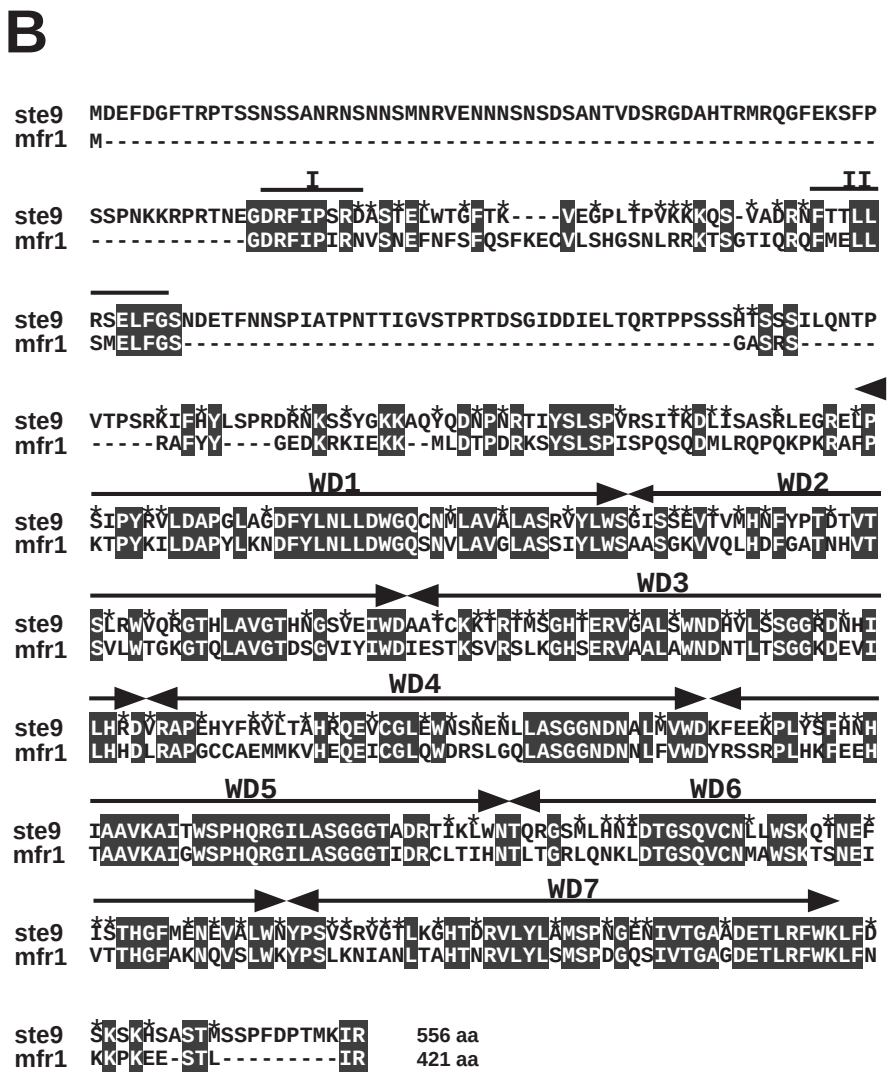


Fig. 1. Fission yeast mfr1 is related to ste9/srw1. (A) mfr1 belongs to the Fizzy-related family of APC activators. Phylogenetic tree using the clustal method with PAM250 residue weight table. (B) Protein sequence comparison between ste9 and mfr1. Black boxes indicate identity; asterisks indicate related amino acids. The homology between ste9 and mfr1 extends beyond the seven WD repeats, including domains I and II previously described (Cebolla et al., 1999).

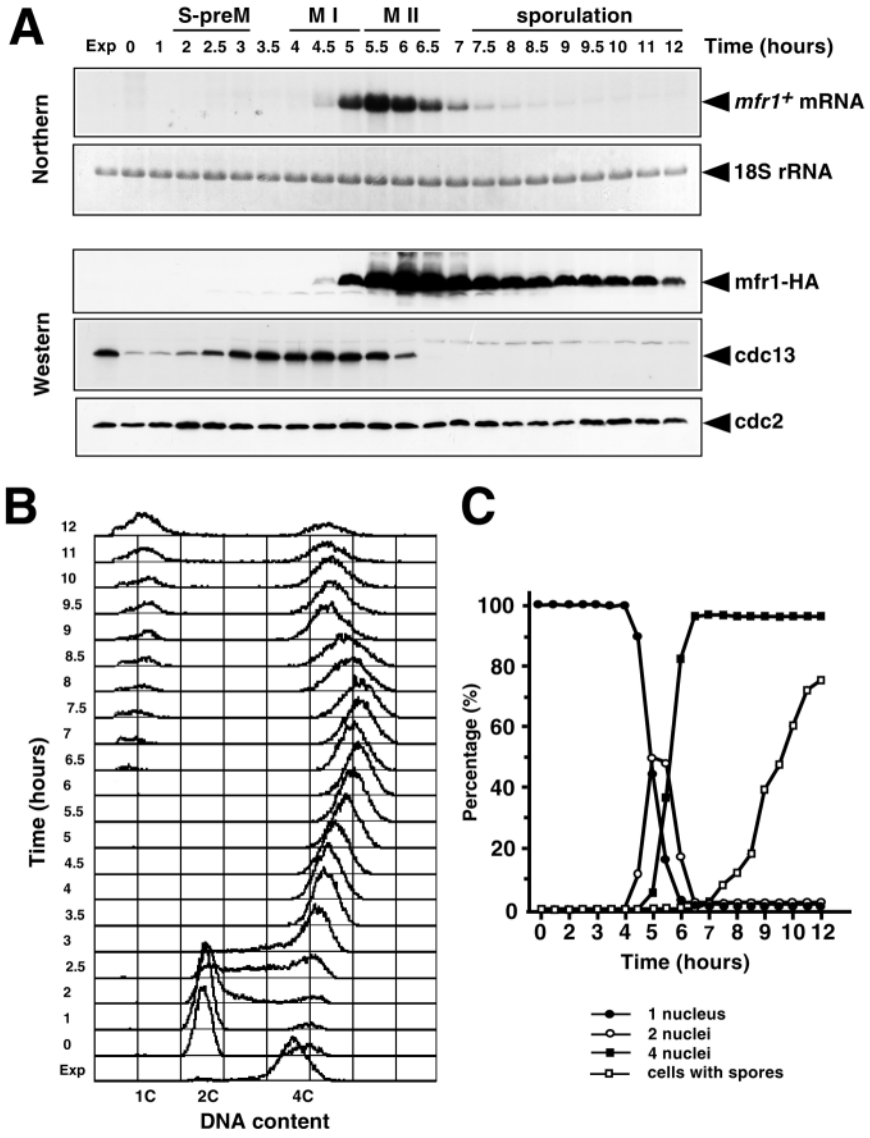


Fig. 2. *mfr1*$^+$ is expressed exclusively during meiosis. Cells of the diploid strain Sp966 were presynchronized in G_1 by nitrogen starvation for 14 hours at 25°C. Nitrogen was added and the culture was incubated at 34°C to inactivate the pat1-114 temperature-sensitive protein kinase. (A) Northern and western blots showing *mfr1*$^+$ mRNA and protein levels. Cdc13 and cdc2 protein levels are shown. Exp, mitotic exponentially growing cells before nitrogen starvation. (B) FACS analysis. Pre-meiotic S-phase occurred between 2 and 3 hours. Cells with 1C DNA content observed after 6.5 hours correspond to haploid spores that are released when cells are sonicated before flow cytometry. (C) Percentage of cells with 1, 2 and 4 nuclei and percentage of asci. Meiosis I occurred between 4 and 5 hours, meiosis II between 5 and 6 hours and sporulation after 7.5 hours.

cultures were incubated at 34°C to inactivate the pat1 kinase (Bahler et al., 1991). Under these conditions, the cells underwent synchronous meiosis (Fig. 2B,C); *mfr1*$^+$ mRNA was expressed during meiosis I, peaking at meiosis II (Fig. 2A). mfr1 protein levels followed those of the mRNA and remained high throughout the second meiotic nuclear division and sporulation (Fig. 2A). The *mfr1*$^+$ gene contains an intron with a stop codon. This intron seems to be spliced immediately after transcription since we failed to observe any significant delay between the expression of the gene and the synthesis of the protein (Fig. 2A). This experiment indicates that *mfr1*$^+$ mRNA and protein are meiosis-specific.

The *mfr1* mutant is defective in spore formation

To investigate the function of mfr1, a deletion of *mfr1*$^+$ was constructed by one-step gene replacement. The complete open reading frame of *mfr1*$^+$ was replaced with the *ura4*$^+$ marker and transformed into a diploid *ura4-d18* diploid strain. Stable *ura4*$^+$ transformants were selected and successful disruptants were identified by Southern blot analysis. Diploid cells, in which one copy of *mfr1*$^+$ was deleted, were sporulated and tetrads were dissected. All tetrads gave rise to four colonies, of which two were *ura4*$^+$ and two were *ura4-d18*. Haploid *ura4*$^+$ cells, deleted for the *mfr1*$^+$ gene, showed no apparent growth defects and were able to mate with the same efficiency as wild-type cells (data not shown). We conclude that mfr1 has no obvious function in the mitotic cell cycle, which is consistent with the fact that *mfr1*$^+$ is expressed only during meiosis.

In order to test whether mfr1 has a function in meiosis, a homozygous *h*$^+$*/h*$^-$ *mfr1Δ/mfr1Δ* diploid strain was constructed. These cells were able to complete both meiotic nuclear divisions but showed severe defects in spore formation (Fig. 3A). Sporulation was delayed considerably with respect to wild-type cells. After 24 hours in sporulation medium, no asci with spores in the *mfr1Δ/mfr1Δ* mutant were observed, while in the wild-type isogenic strain a high percentage of the asci contained four spores. After 48 hours, 80% of the *mfr1Δ/mfr1Δ* mutant cells contained spores, of which only 3% were four-spore asci (Fig. 3B). In order to test whether the spores formed were viable or not, 200 randomly chosen spores from the wild type and 200 spores from the *mfr1Δ/mfr1Δ* strain were micromanipulated in rich medium (YES) and spore viability was examined after 4 days at 32°C. Spore viability was similar in the wild type and the *mfr1* mutant (68% versus 71%, respectively). All spores from the *mfr1Δ/mfr1Δ* strain gave raise to haploid colonies, confirming that chromosome segregation during meiosis is normal.

For close examination of this phenotype, an *h*$^-$*/h*$^-$ *pat1-114/pat1-114 mfr1Δ/mfr1Δ* diploid strain was constructed and compared with *h*$^-$*/h*$^-$ *pat1-114/pat1-114* control cells in a synchronous meiosis experiment (Fig. 4). In both cases, cells underwent premeiotic DNA replication between 2 and 3 hours, meiosis I between 4 and 5 hours and meiosis II between 5 and 6 hours (Fig. 4A,B). Whereas after 8 hours, 80% of the control cells had undergone sporulation, not even one ascus was observed after 10 hours in the *mfr1Δ/mfr1Δ* mutant, confirming

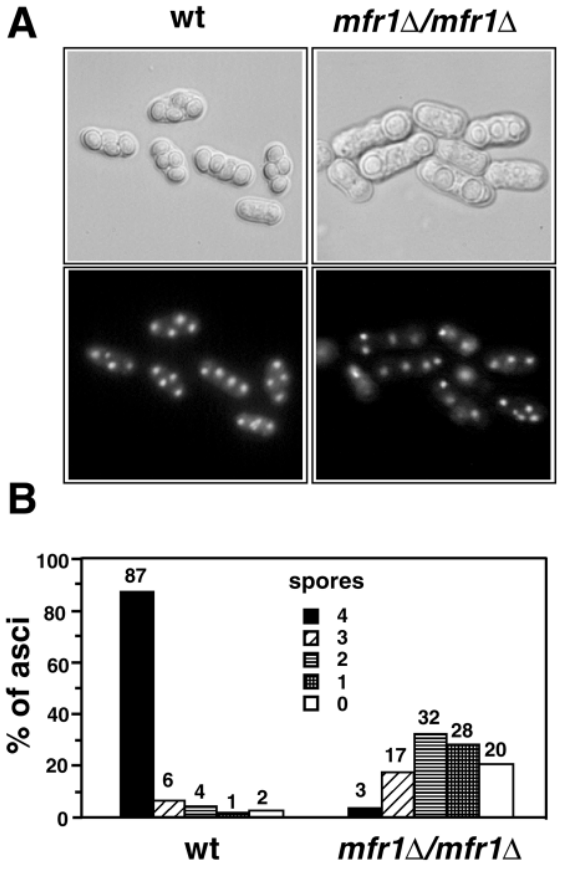


Fig. 3. *mfr1*$^{+}$ is required for sporulation. A wild-type diploid (Sp785) and a *mfr1Δ/mfr1Δ* mutant (Sp963) were sporulated on agar plates containing malt extract (sporulation medium). (A) Photographs of cells after 48 hours using interference microscopy (top) and DAPI staining (bottom). In the *mfr1Δ/mfr1Δ* mutant most cells contained four nuclei, as in the wild type. These nuclei were difficult to photograph because they were on different focal planes. (B) Percentage of asci after 48 hours with 4, 3, 2, 1 and 0 spores.

that sporulation is severely impaired (Fig. 4A,B). These experiments clearly show that mfr1 is required at the end of the meiotic nuclear divisions for spore formation. Tubulin staining with the TAT-1 antibodies of the *mfr1Δ/mfr1Δ* mutant cells after 5, 6 and 7 hours revealed that the meiotic spindles disassemble (data not shown), indicating that these cells complete meiosis II.

In meiosis II, at the metaphase to anaphase transition, the cytoplasmic face of the spindle-pole bodies (SPB) differentiate into multilayered plaques (Hirata and Shimoda, 1994; Tanaka and Hirata, 1982). During anaphase, the inner side of the SPB forms the meiotic spindle while the outer side of the plaques serves as platform for the assembly of the forespore membrane (Hirata and Shimoda, 1994; Tanaka and Hirata, 1982). The forespore membrane grows by vesicle fusion and eventually encapsulates each of the four haploid nuclei. Finally, spore walls are synthesized by accumulating wall materials between the inner and outer membranes of the forespore (Hirata and Shimoda, 1994; Tanaka and Hirata, 1982). This morphological alteration of the SPB

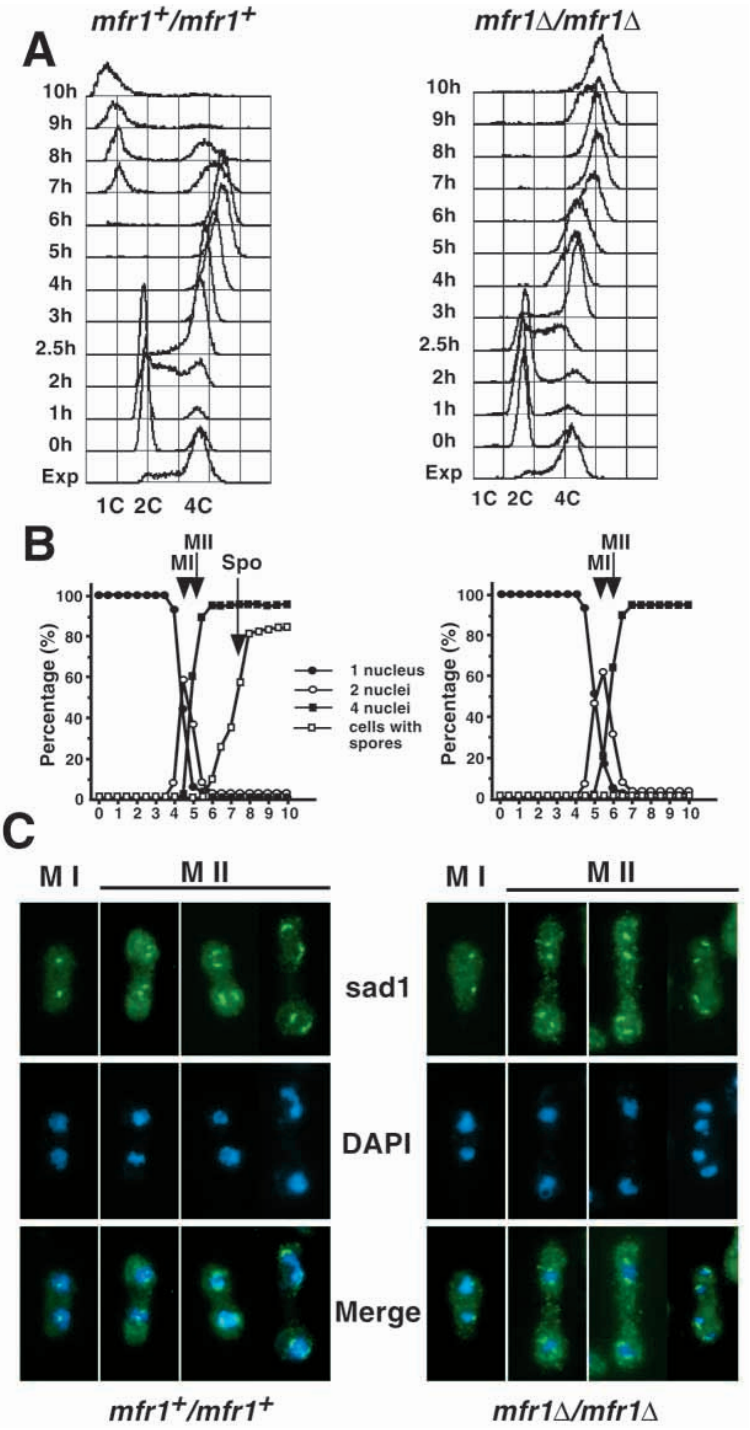


Fig. 4. The *mfr1Δ/mfr1Δ* mutant completes meiosis II but fails to undergo sporulation. The strains *h*$^{-}$*/h*$^{-}$ *pat1-114/pat1-114* (Sp964) and *h*$^{-}$*/h*$^{-}$ *pat1-114/pat1-114 mfr1Δ/mfr1Δ* (Sp965) were synchronized through meiosis as indicated in Fig. 2. (A) FACS analysis. Pre-meiotic S-phase took place between 2 and 3 hours in both cases. Haploid spores (as cells with 1C DNA content) were observed in the control cells after 7 hours but not in the *mfr1Δ/mfr1Δ* mutant. Exp, exponentially growing cells before nitrogen starvation. (B) Percentage of cells with 1, 2 and 4 nuclei and of asci with spores during the experiment. Spores were not observed in the *mfr1Δ/mfr1Δ* strain during this experiment. (C) Spindle-pole body (SPB) staining with anti-sad1 antibodies and DAPI staining in cells undergoing meiosis I (M I) and meiosis II (M II). The two cells shown in the right panels in each case have modified SPBs.

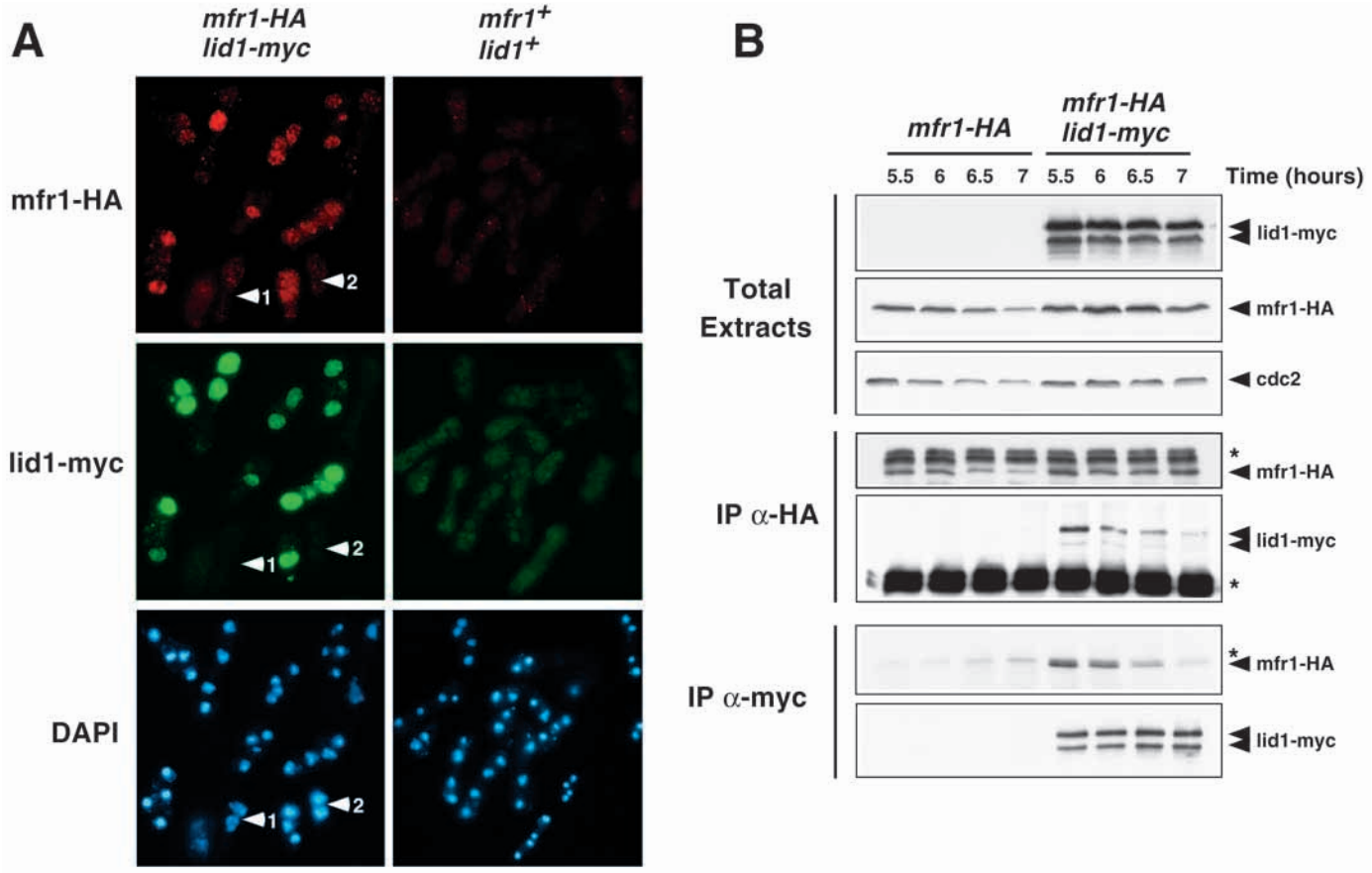


Fig. 5. mfr1 colocalizes and interacts with APC during meiosis. (A) Cells of the diploid strain Sp967, containing mfr1 tagged with three copies of the HA epitope and lid1(APC4) tagged with 9 copies of the myc epitope, were induced to undergo synchronous meiosis as indicated in Fig. 2. After 6 hours, cells were fixed and stained with anti-HA monoclonal antibodies, anti-myc polyclonal antibodies, and with DAPI. Nuclei 1 and 2 are in meiosis I. Cells in the right panels are control cells (Sp964) lacking the HA and the myc tags stained with anti-HA and anti-myc antibodies. (B) The diploid strains Sp967 and the control Sp966 with identical genotype except that it is wild type for *lid1*$^+$ were synchronized in meiosis. Samples were taken at 5.5, 6, 6.5 and 7 hours for immunoprecipitation with anti-HA or anti-myc antibodies. Immunoprecipitates were run on SDS-PAGE gels and probed with anti-HA or anti-myc antibodies. Western blots of total extracts were probed with anti-myc and anti-HA antibodies to check the levels of the tagged proteins. Western blot with anti-cdc2 is shown as loading control. * IgG heavy chain.

produces a transient change in shape from a dot into a crescent when stained with anti-sad1 antibodies (Hagan and Yanagida, 1995). We carried out immunofluorescence in wild type and *mfr1Δ/mfr1Δ* mutant cells using anti-sad1 antibodies, observing that in the *mfr1Δ/mfr1Δ* mutant the SPB undergoes the normal transition from a dot to a crescent (Fig. 4C). This observation indicates that the defect in sporulation in the *mfr1* mutant is not due to a failure in SPB differentiation, as is the case in some fission yeast sporulation mutants (Ikemoto et al., 2000).

mfr1 colocalizes and interacts with APC in meiosis

We next studied the localization of mfr1 in cells undergoing meiosis. We used the diploid strain Sp967 (see Table 1) where both copies of *mfr1*$^+$ have been deleted and two copies of *mfr1-3xHA* were integrated in the *leu1* loci. In this strain, *mfr1-3xHA/mfr1-3xHA* fully complements the sporulation defect of the *mfr1Δ/mfr1Δ* mutant and essentially behaved like the wild type (Fig. 2). mfr1 was not detected in cells during meiosis I (Fig. 5A, cells 1 and 2) but became detectable in anaphase II, accumulating in the area around the nucleus where the forespore membrane was being formed (Fig. 5A). In most cells, mfr1 was located around one, two, three and, very seldom, around the four nuclei simultaneously (Fig. 5A). Since mfr1 could be a regulator of APC, we tested whether mfr1 colocalizes with this complex. The strain Sp967 contains both chromosomal copies of the *lid1*$^+$ gene, encoding subunit 4 of APC (APC4), modified by addition of 9 copies of the myc epitope (Berry et al., 1999). Staining of APC4 with anti-myc antibodies showed a pattern similar to that of mfr1 (Fig. 5A). APC4 colocalized with mfr1 in 90% of the nuclei (Fig. 5A), suggesting that the majority of mfr1 is associated with APC.

To confirm that mfr1 interacts with APC in meiosis, we examined whether mfr1 coprecipitates with APC. A *pat1-114* diploid strain containing mfr1-3xHA and lid1(APC4)-9xmyc (Sp967) and a control strain with identical genotype but with the wild-type *lid1*$^+$ gene (Sp966) were induced to undergo synchronous meiosis as described before. Samples were taken at different times (5.5, 6, 6.5 and 7 hours) for immunoprecipitation with anti-HA or anti-myc antibodies. As shown in Fig. 5B, mfr1 was present in immunoprecipitates of lid1(APC4) and vice versa, indicating that mfr1 not only

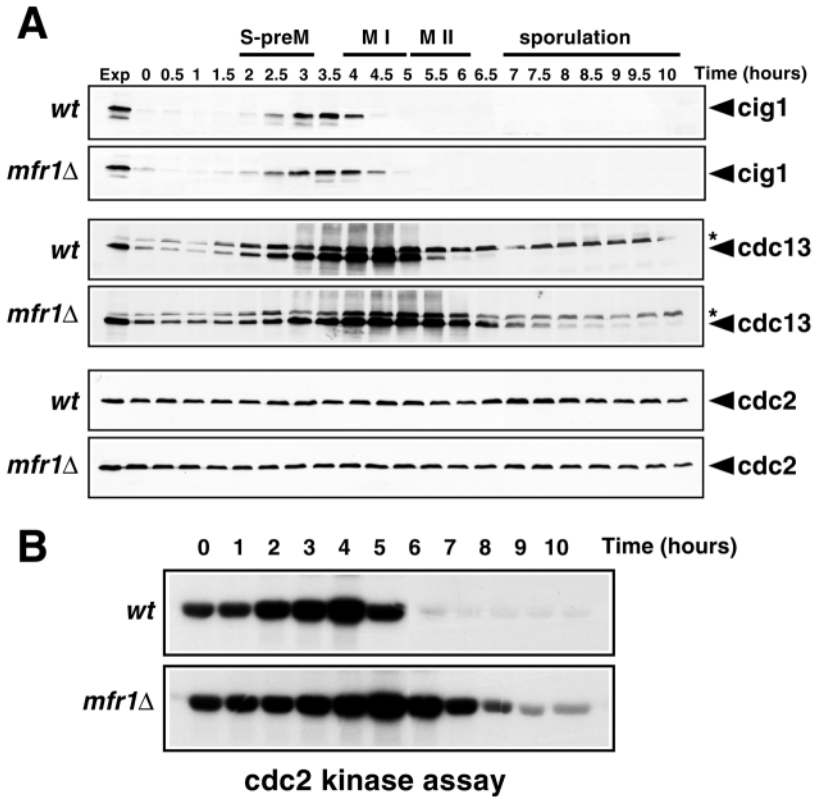


Fig. 6. Cdc13 cyclin is stabilized in the *mfr1Δ/mfr1Δ* mutant. (A) Strains *h⁻/h⁻ pat1-114/pat1-114* (Sp964) and *h⁻/h⁻pat1-114/pat1-114 mfr1Δ/mfr1Δ* (Sp965) were synchronized through meiosis as indicated in Fig. 2. Samples were taken every 30 minutes and protein extracts were analyzed by western blot with antibodies to cig1, cdc13 and cdc2. Exp, exponentially growing cells before nitrogen starvation. (B) Cdc2 protein kinase assays after immunoprecipitation with cdc2 antibodies. Samples were taken every hour.

colocalizes with APC but also interacts with APC in vivo during meiosis.

mfr1 is necessary for the rapid and timely degradation of cdc13 cyclin

During mitosis in fission yeast, active cdc2/cyclin kinase prevents cytokinesis before anaphase by inhibiting actomyosin ring constriction and septation. This control mechanism prevents the constricting ring and the septum from cutting the nuclei in half before sister chromatid segregation (Balasubramanian et al., 2000; Le Goff et al., 1999). At the end of anaphase, after sister chromatid segregation and spindle disassembly, the actomyosin ring constricts, the division septum is formed, and two daughter cells are generated. Therefore, downregulation of cdc2/cyclin in anaphase is essential for cytokinesis (Cerutti and Simanis, 1999; He et al., 1997; Kim et al., 1998).

By analogy with this control mechanism that prevents cytokinesis until cdc2/cyclin complexes have been inactivated, we reasoned that in meiosis the cdc2/cyclin kinase might prevent sporulation before the completion of meiosis II. If mfr1 functions as a meiosis-specific activator of APC required for the degradation of cyclins, failure to destroy cyclins after meiosis II will keep the cdc2/cyclin complexes active and could inhibit sporulation. In order to test this hypothesis, we analyzed the protein levels of cig1 and cdc13 M-phase cyclins in wild-type and *mfr1Δ/mfr1Δ* cells (Fig. 6A). cig1 and cdc13 are B-type cyclins known to be destroyed during mitosis (Blanco et al., 2000; Moreno et al., 1989). In both wild-type and *mfr1Δ/mfr1Δ* cells, cig1 protein levels rose during premeiotic DNA replication and dissapeared as cells were undergoing meiosis I (Fig. 6A). Cdc13 was rapidly destroyed in wild-type cells during meiosis II (Fig. 6A, see also Fig. 2A). In contrast, cdc13 was significantly stabilized in the *mfr1Δ/mfr1Δ* mutant as compared to the wild type (Fig. 6A), suggesting that this cyclin may be one target of APC^{mfr1}. Consistent with this, cdc2 protein kinase activity and the mitotic spindle persisted for longer in the *mfr1Δ/mfr1Δ* mutant cells than in the wild type (Fig. 6B; data not shown). Thus, high levels of cdc13 at the end of anaphase II maintain cdc2/cyclin kinase active, presumably by inhibiting sporulation in the *mfr1Δ/mfr1Δ* mutant.

cdc13 stabilization inhibits sporulation

Finally, we tested whether expression of the non-degradable cdc13-des2 destruction box mutant (Yamano et al., 1996) could inhibit sporulation. Fission yeast cells expressing stable *cdc13-des2*, in single copy under its own promoter, are viable (J. M. de Prada, M. A. Blanco and S. Moreno, manuscript in preparation). We integrated a genomic copy of *cdc13-des2* at the *leu1* locus in the *h⁻/h⁻ pat1-114/pat1-114* strain. Single-copy integrants were obtained and induced to undergo a synchronous meiosis (it should be noted that these cells are diploid and therefore contain two wild-type copies of $cdc13^+$ and one mutant copy of *cdc13-des2*). As a control, we used an *h⁻/h⁻ pat1-114/pat1-114* strain in which one additional copy of wild-type $cdc13^+$ had been integrated at the *leu1* locus. Cells expressing *cdc13-des2* were able to complete the meiotic nuclear divisions (data not shown) but were severely impaired for sporulation (Fig. 7A,B). After 9 hours at 34°C, only 13% of the cells expressing *cdc13-des2* were able to form four-spore asci, as compared to 43% for the control cells. Moreover, 34% of these cells did not contain a single spore versus 4% in the control (Fig. 7B). This confirms that stabilization of cdc13 at the end of meiosis is inhibitory for sporulation and mimics the phenotype of the *mfr1Δ/mfr1Δ* mutant.

DISCUSSION

Here we describe mfr1, a fission yeast protein closely related to ste9/srw1, which is a member of a highly conserved family of proteins containing seven WD repeats, similar to Hct1/Cdh1 of budding yeast and *Fizzy-related* (*Fzr*) of higher eukaryotes (Blanco et al., 2000; Kitamura et al., 1998; Kramer et al., 2000; Kramer et al., 1998; Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997; Yamaguchi et al., 1997; Yamaguchi et al., 2000). These proteins function as activators of APC to promote poly-ubiquitination and degradation of mitotic cyclins

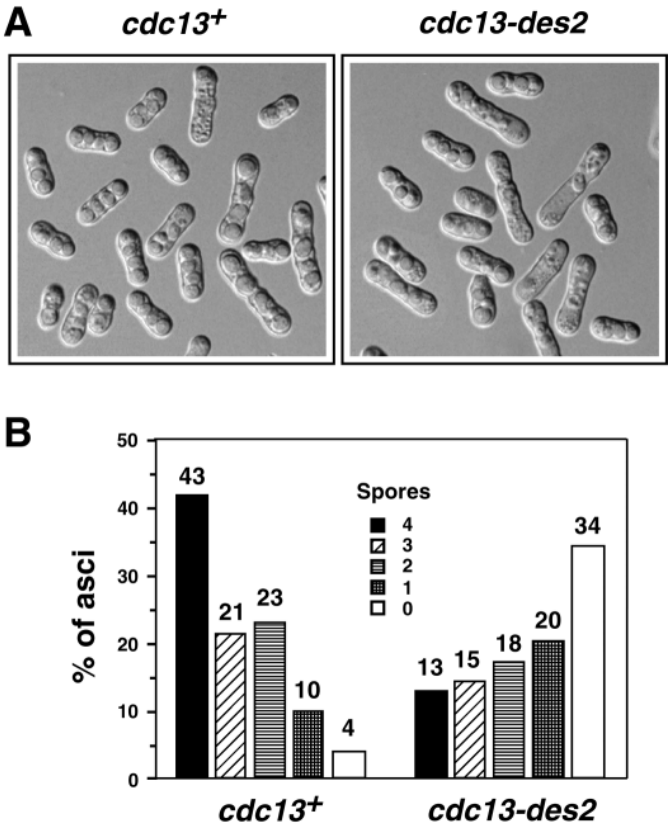


Fig. 7. Expression of non-degradable *cdc13-des2* destruction box mutant inhibits sporulation. The diploid strain *h⁻/h⁻ pat1-114/pat1-114* (Sp964) was transformed with pJK148-*cdc13*$^+$ or pJK148-*cdc13-des2* to generate the strains Sp969 and Sp970, respectively. Single-copy integrants at the *leu1* locus were isolated and induced to undergo a synchronous meiosis. (A) Photographs of cells after 9 hours at 34°C. (B) Percentage of asci with 4, 3, 2, 1 and 0 spores.

at the end of mitosis and in G_1. We present several lines of evidence indicating that mfr1 is a meiosis-specific activator of APC involved in the degradation of the cdc13 cyclin at the end of meiosis II. (1) The *mfr1*$^+$ gene is expressed exclusively during meiotic nuclear divisions; (2) mfr1 colocalizes and interacts with APC; (3) mfr1 is necessary for the rapid and timely degradation of the M-phase cyclin cdc13 but not for cig1; (4) the *mfr1Δ* mutant completes meiosis II but fails to undergo sporulation; (5) stabilization of the M-phase cyclin cdc13 prevents the formation of spores at the end of meiosis II.

APC^{mfr1} functions specifically at the end of meiosis

ste9 and mfr1 are closely related APC activators. ste9 functions in G_1 to promote the degradation of the mitotic cyclins cdc13 and cig1 (Blanco et al., 2000; Yamaguchi et al., 2000). *ste9Δ* mutants are unable to arrest the cell cycle in G_1 and are therefore sterile. We observed that the *mfr1Δ* mutant has no defect in the mitotic cell cycle. Haploid cells lacking mfr1 arrest normally in G_1 upon nitrogen starvation and mate like the wild type. On the other hand, we found that ste9 is not required for sporulation since a diploid *h⁻/h⁻ pat1-114/pat1-114 ste9Δ/ste9Δ* mutant underwent meiosis and sporulation at 34°C with similar kinetics to a control *h⁻/h⁻ pat1-114/pat1-114 ste9*$^+$*/ste9*$^+$ (data not shown). Thus, APC^{ste9} and APC^{mfr1} must act at different stages of the fission yeast life cycle: APC^{ste9} in the G_1 phase of the mitotic cell cycle and APC^{mfr1} in the 'G_1 phase' that follows the end of meiosis. They are both necessary to inactivate cdc2/cyclin complexes in order to permit the onset of differentiation programs: mating in the case of APC^{ste9}, and sporulation in the case of APC^{mfr1}.

In the fission yeast mitotic cell cycle, at the end of anaphase, the actomyosin ring constricts and the division septum is synthesized. There is evidence that cdc13 proteolysis and inactivation of cdc2 kinase are necessary for actomyosin ring constriction (He et al., 1997). Here we show that in the *mfr1Δ/mfr1Δ* mutant the cdc13 cyclin is stabilized and cdc2 kinase activity remains high. Expression of a stable allele of cdc13 inhibits sporulation, suggesting that inactivation of cdc2/cdc13 marks the successful completion of chromosome segregation in both mitosis and meiosis and permits the formation of a septum or a spore wall, respectively. Currently we are unable to rule out the possibility that other as yet unidentified targets of mfr1 might need to be degraded for sporulation.

Mfr1 orthologues in other organisms

In budding yeast, a protein named Spo70 related to Cdc20 and Hct1, which is expressed only in meiosis, has been described in the genomic analysis of genes expressed during meiosis (Chu et al., 1998). While our manuscript was in a late stage of preparation, a regulator of APC in *S. cerevisiae*, Ama1, that is identical to Spo70 was reported (Cooper et al., 2000). Like mfr1, Ama1 is a meiosis-specific activator of APC that triggers the degradation of Clb1 cyclin. There are, however, some differences between Ama1 and mfr1. Ama1 is more similar to the Fizzy family of APC activators while mfr1 is closer to the Fizzy-related family (Fig. 1A). This is consistent with the fact that Ama1 is required earlier in meiosis than mfr1. *ama1* mutant cells arrest with a single nucleus, suggesting a role for Ama1 in meiosis I, while mfr1 is needed once cells complete the two meiotic nuclear divisions. It is therefore possible that there could be several APC complexes acting in meiosis: APC^{Ama1} with a role in meiosis I and APC^{mfr1} acting at the end of meiosis.

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