Ace2p contributes to fission yeast septin ring assembly by regulating mid2+ expression

Claudia S. Petit, Sapna Mehta, Rachel H. Roberts and Kathleen L. Gould*

Howard Hughes Medical Institute, and Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

*Author for correspondence (e-mail: kathy.gould@vanderbilt.edu)

Accepted 12 September 2005
Journal of Cell Science 118, 5731-5742 Published by The Company of Biologists 2005
doi:10.1242/jcs.02687

Summary
The fission yeast Schizosaccharomyces pombe divides through constriction of an actomyosin-based contractile ring followed by formation and degradation of a medial septum. Formation of an organized septin ring is also important for the completion of S. pombe cell division and this event relies on the production of Mid2p. mid2+ mRNA and protein accumulate in mitosis. Recent microarray analyses identified mid2+ as a target of the Ace2p transcription factor, and ace2+ as a target of the Sep1p transcription factor. In this study, we find that Mid2p production is controlled by Ace2p functioning downstream of Sep1p. Consequently, both Sep1p and Ace2p are required for septin ring assembly and genetic analyses indicate that septin rings function in parallel with other Ace2p targets to achieve efficient cell division. Conversely, forced overproduction of Sep1p or Ace2p prevents septin ring disassembly. We find that Ace2p levels peak during anaphase and Ace2p is post-translationally modified by phosphorylation and ubiquitylation. Ace2p localizes symmetrically to dividing nuclei and functions independently of the septation initiation network.

Key words: Schizosaccharomyces pombe, Cytokinesis, Ace2p, Mid2p, Septins

Introduction
Cytokinesis occurs at the end of mitosis and results in a single cell being split into two daughter cells. The genetically tractable yeast Schizosaccharomyces pombe provides a valuable model organism in which to study cell division since it divides by medial fission and its cell cycle is well characterized. In addition, as in animal cells, cytokinesis in S. pombe can only occur through assembly and constriction of an actomyosin ring (Kitayama et al., 1997; Marks et al., 1986). Along with proper actomyosin ring formation and function, successful cytokinesis in S. pombe requires proper selection of a division site (Bahler et al., 1998a; Paoletti and Chang, 2000) and coordinate septation. The septation initiation network (SIN) is a signaling cascade responsible for ensuring the proper timing of actin ring contraction and subsequent septum deposition (Cerutti and Simanis, 2000).

Among the proteins important for successful cytokinesis are septins, a group of conserved GTP-binding proteins present in many organisms (Kinoshita, 2003). Septins are organized into a ring at the site of cell division (An et al., 2004; Faty et al., 2002; Longtine and Bi, 2003) and are important for cytokinesis in a variety of cell types (An et al., 2004; Berlin et al., 2003; Hartwell, 1971; Longtine et al., 1996; Neufeld and Rubin, 1994; Tasto et al., 2003). In S. pombe, septins are not essential but are required for efficient cell separation, as demonstrated by the chained cell phenotype that arises from their loss (An et al., 2004; Berlin et al., 2003; Longtine et al., 1996; Tasto et al., 2003). Like septins, mid2+ is not an essential gene but its loss also causes a chained cell phenotype accompanied by abnormal septin rings (Berlin et al., 2003; Tasto et al., 2003). Fluorescence recovery after photobleaching (FRAP) analysis of Spn1p-green fluorescence protein (GFP) has shown that septin rings are quite dynamic in mid2Δ cells whereas they are very stable in wild-type cells (Berlin et al., 2003). Mid2p production is normally restricted to anaphase but overproduction of a stabilized truncation mutant of Mid2p leads to persistence of septin rings through multiple cell divisions (Tasto et al., 2003). Additionally, normal septin rings can be formed in interphase by mildly overproducing Mid2p even though wild-type cells will not form a stable septin ring until they have passed through mitosis, when Mid2p is normally produced (An et al., 2004). These observations indicate that Mid2p interacts either directly or indirectly with septins to promote their organization into stable ring structures specifically in late mitosis.

mid2+ is periodically expressed, and this expression was initially thought to be under the control of Sep1p (Tasto et al., 2003), a forkhead family transcription factor (Ribar et al., 1997). Microarray data indicate that Sep1p controls the first of four major transcriptional waves in the S. pombe cell cycle (Peng et al., 2005; Rustici et al., 2004). Expression of genes within this cluster peaks during mitosis and generates many factors necessary for mitotic progression. These range from regulators of mitosis (plo1+, ark1+ and rum1+) to genes whose products regulate microtubule dynamics or the actomyosin contractile ring (myp2+, klp5+ and cdc15+). The first cluster also includes ace2+ (Peng et al., 2005; Rustici et al., 2004), a zinc-finger transcription factor (Martin-Cuadrado et al., 2003) that is responsible for a second wave of transcription (Peng et al., 2005; Rustici et al., 2004) whose targets include many genes important for cell separation. Many S. pombe genes expressed during mitosis contain copies of a regulatory motif...
called the pombe cell cycle box (PCB) motif (GNAACG/A) in their promoters (Anderson et al., 2002). A recent study has suggested that this motif participates in periodic expression of genes by recruiting Fkh2p, a forkhead transcription factor, and Mbx1p, a member of the PCB-binding factor (PBF) complex, to promoters (Buck et al., 2004). This study also demonstrated both a decrease in the level and a loss of periodicity of ace2+ expression in the absence of these proteins (Buck et al., 2004). The presence of two PCB motifs at -241 and -389 in the ace2+ promoter is consistent with the hypothesis that Fkh2p and Mbx1p might confer an additional level of regulation on ace2+ expression.

S. pombe cells lacking Ace2p phenocopy sep1Δ cells: they are chained and branched (Martin-Cuadrado et al., 2003). That Ace2p overexpression can largely rescue the sep1Δ phenotype from its single-copy derivative. The nuclear localization of Ace2p was shown to be mediated by a nuclear localization signal (NLS) (Dohrmann et al., 1992; O’Conallain et al., 1999). Although a small amount of Ace2p translocates into the nucleus (Dohrmann et al., 1992; Martin-Cuadrado et al., 2003; Rustici et al., 2004), the majority of the protein accumulates in the daughter nucleus. This indicates that Ace2p localizes symmetrically to daughter nuclei, where it is responsible for activating a set of daughter-specific genes necessary for cell separation (Bidlingmaier et al., 2001; Colman-Lerner et al., 2001; Dohrmann et al., 1992; Weiss et al., 2002). This daughter-specific localization of Ace2p is dependent on both the Cbk1p kinase and its interacting protein, Mob2p (Colman-Lerner et al., 2001; Weiss et al., 2002). The Mob2-Chk1 complex functions as part of a signaling network termed RAM that functions in part to regulate Ace2p activity (Nelson et al., 2003). Once localized to the nucleus, S. cerevisiae Ace2p is at least partially maintained there through the activity of the mitotic exit network (MEN) (Weiss et al., 2002). This signaling cascade, the analog of the S. pombe SIN, plays a crucial role in completion of cell division. Two components of the MEN are Mob1p, which is a protein similar to Mob2p and is needed to activate the Dbf2p kinase and the Cdc14p phosphatase. Cells containing mutant alleles of either MOB1 or CDC14 do not retain Ace2p in the daughter nucleus (Weiss et al., 2002).

Here, we extend the evidence that mid2+ expression is controlled by Ace2p functioning downstream of Sep1p. In the absence of either Sep1p or Ace2p, there is insufficient Mid2p produced to support formation of normal septin rings. As a consequence, cells lacking Sep1p and Ace2p are defective in septin ring function. Conversely, overproduction of either of these transcription factors leads to high levels of Mid2p and delays septin ring disassembly. We provide genetic evidence that septin rings function in parallel with other Ace2p targets to mediate efficient cell separation. Additionally, we report that S. pombe Ace2p levels are regulated by the cell cycle, that Ace2p is post-translationally modified by phosphorylation and ubiquitylation, that Ace2p localizes symmetrically to daughter cell nuclei, and that Ace2p functions independently of the SIN. We have also explored the relative contributions of Sep1p, Fkh2p and Mbx1p to ace2+ regulation.

Materials and Methods

Strains, media and methods

The S. pombe strains used in this study (Table 1) were grown in YE or minimal medium with the appropriate supplements as previously described (Moreno et al., 1991). Strains were constructed by tetrad dissection or random spore analysis. DNA transformations were done by the lithium acetate method (Keeney and Boeke, 1994). Induction of the nmt1 promoter (Basi et al., 1993; Maundrell, 1993) was achieved by washing cells three times in medium lacking thiamine (promoter induced). Centrifugal elutriation was performed using a Beckman J6-E centrifuge.

The ace2+ ORF was tagged at its 3’ end with either the M yc15-K an6, H A3-K an5 or eGFP-K an6 cassette as previously described (Bahir et al., 1998b). Kan+ transformants were screened by whole-cell PCR and then by immunoblotting to confirm the accurate integration and expression of the fusion protein. The nmt1 promoter was introduced at the 5’ end of the sep1Δ open reading frame (ORF) that had been tagged at it 3’ end with the HA3-Kan6 cassette as previously described (Bahir et al., 1998b; Carr et al., 1989). This strain was a generous gift of Jianhua Liu (Genome Institute of Singapore, Singapore).

Molecular biology techniques

PCR amplifications were performed with Taq-Precision Plus (Stratagene) according to the manufacturer’s protocol. Oligonucleotides were synthesized by Integrated DNA Technologies. The ace2+ cDNA was amplified from genomic DNA with oligos
The PCR product was cut with XmaI and BamHI sites on the 5’ and 3’ ends, respectively. The PCR product was cut with XmaI and BamHI and cloned into the thiamine-repressible pREP3X vector to create pKG1341. Automated sequencing was used to determine the fidelity of the amplified product.

### Cytology and microscopy

Strains expressing GFP-tagged proteins were grown in liquid YE medium and visualized live or after ethanol fixation. Microscopy on Strains expressing GFP-tagged proteins was performed on a Carl Zeiss MicroImaging Axiosvert II inverted microscope equipped with an UltraVIEW LCI real-time scanning head confocal (PerkinElmer) and a 488 nm argon laser (for GFP excitation). Images were captured on an Orca-ER charge-coupled device camera (Hamamatsu) using UltraVIEW software and were then processed using Volocity 2.0 software (Improvision). To visualize DNA, cells were stained with DAPI as previously described (Balasubramanian et al., 1997). Septa were aspirated off. 10 mg/ml BSA, divided in half, pulsed down, and the supernatant collected. 100 μl samples were loaded on 4-12% NuPAGE gels (Invitrogen) for electrophoresis. Proteins were resolved by electrophoresis on 4-12% NuPAGE gels performed with anti-Myc (9E10) or anti-HA (12CA5) antibodies. Immunoblots examining ubiquitylation of proteins and effects of the 9E10 or 12CA5 monoclonal antibodies. Immunoprecipitations were performed with monoclonal antibodies specific for Myc (9E10) and HA (12CA5).

### Immunoprecipitations and immunoblotting

Native or denatured cell lysates were prepared in NP-40 buffer as previously described (Gould et al., 1991). Immunoprecipitations were performed with anti-Myc (9E10) or anti-HA (12CA5) antibodies. Proteins were resolved by electrophoresis on 4-12% NuPAGE gels (Novex), 10% Bis-Tris gels or 10% SDS-PAGE gels. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon P; Millipore) for immunoblot analysis performed as previously described (Gould et al., 1991). Immunoprecipitations and immunoblots were performed with anti-Myc (9E10) or anti-HA (12CA5) antibodies. Immunoprecipitation/phosphatase assay

### Immunoprecipitation/phosphatase assay

After immunoprecipitation with anti-Myc antibody, beads were washed twice with 1 ml NP-40 buffer, four times with 1 ml phosphatase buffer (25 mM Heps-NaOH, pH 7.4, 150 mM NaCl, 0.1 mg/ml BSA), divided in half, pulsed down, and the supernatant aspirated off. 10 μl reactions composed of 1X phosphatase buffer, 5733
2 mM MnCl₂ plus 1 µl of λ-phosphatase (New England Biolabs) or 1 µl H₂O were then incubated at 30°C for 45 minutes with gentle mixing every 5 minutes. The beads were washed three times with NP-40 buffer and resuspended in 25 µl 2x lithium dodecyl sulfate sample buffer.

**Northern blotting**

Total RNA was prepared by extraction with hot acid phenol and SDS as detailed previously (Burns et al., 1999). Total RNA (20 µg per sample) was resolved on formaldehyde-agarose gels and capillary blotted to a Duralon-UV membrane. **ace2**⁺, **mid2**⁺ and **his3**⁺ mRNA levels were detected by hybridization of 3²P-labeled DNA probes (PrimeIt-II; Stratagene) generated from fragments of their ORFs. The blots were exposed to Phosphor-Imager screens and visualized with ImageQuant 5.2 on an Amersham Biosciences Typhoon 9200 scanner.

**In vivo ubiquitylation assays**

Ubiquitylation assays were carried out as described previously (Benito et al., 1998). Briefly, strains carrying pREP1-His₆–ubiquitin (pKG1284) were grown at 25°C for 22 hours in the absence of thiamine to induce the expression of His₆–Ub. The cells were then shifted to 36°C for 4 hours. Cell lysates were prepared in the presence of 8 M urea with 100 mM sodium phosphate, pH 8.0, and 5 mM imidazole followed by binding of His₆–Ub-conjugated proteins to a nickel affinity resin (Ni-NTA Superflow; QIAGEN). The beads were washed as previously described (Benito et al., 1998) and ubiquitylated proteins were eluted with 200 mM imidazole. Immunoblot analysis was performed on the eluted proteins as described above.

**Results**

**mid2**⁺ expression is dependent on the transcription factor Ace2p

Recent microarray data indicate that Ace2p rather than Sep1p is responsible for controlling **mid2**⁺ expression (Alonso-Nunez et al., 2005; Peng et al., 2005; Rustici et al., 2004). To confirm this, we examined **mid2**⁺ mRNA expression through the cell cycle in the absence of **ace2**⁺ using a **cdc25-22** block and release protocol. Levels of **mid2**⁺ transcript were significantly throughout the cell cycle of **ace2Δ** cells as compared with wild-type cells, although a small amount of constitutive expression was detectable (Fig. 1A).

The decrease in **mid2**⁺ abundance in **ace2Δ** cells was found to affect Mid2 protein levels dramatically. Mid2p-Myc13 was virtually undetectable in asynchronously growing **ace2Δ** cells in comparison with wild-type cells (Fig. 1B). To ensure that Mid2p expression was not overlooked at a particular cell-cycle stage, we examined Mid2p-Myc13 levels in **ace2Δ** cells that had been synchronized by a **cdc25-22** block and

![Fig. 1.](image-url)
release protocol. Mid2p-Myc13 was detected at very reduced amounts and significantly later in the cell cycle than in wild-type cells (Fig. 1C). These data confirm that Ace2p is required for the transcriptional regulation of mid2+ and Mid2p production.

To confirm that mid2+ is indirectly influenced by Sep1p activity, we first tested whether overexpression of either transcription factor individually could increase Mid2p levels. To regulate expression of sep1+, the nmt1 promoter was integrated into the genome directly upstream of the sep1+ ORF that had been tagged at its 3′ end with sequences encoding three tandem copies of the HA epitope to create the nmt1sep1-HA3 strain (a gift of Jianhua Liu). In the presence of thiamine, these cells displayed a significant cell separation defect (data not shown) and very little Mid2p-Myc13 was produced (Fig. 1D). Increasing the levels of Sep1p-HA3 led to significant overproduction of Mid2p-Myc13 (Fig. 1D). Similarly, overexpression of ace2+ from a plasmid led to a significant elevation of Mid2p-Myc13 abundance (Fig. 1E). Therefore, both transcription factors can increase Mid2p abundance when they are individually overproduced.

To establish that Sep1p affects mid2+ expression indirectly through an effect on ace2+ expression, we studied the effect of overexpressing each transcription factor in strains lacking the other. Whereas overexpression of ace2+ in sep1Δ cells led to abundant Mid2p-HA3 production (Fig. 1F), overexpression of sep1+ in ace2Δ cells did not increase Mid2p-Myc13 abundance (Fig. 1G). These data confirm that Sep1p and Ace2p function in a linear order with respect to the control of mid2+ expression. Similar results have been found with six other Ace2p target genes (Alonso-Nunez et al., 2005).

### Ace2p plays a role in cell separation through Mid2p and the septin ring

We noted that cells overexpressing ace2+ became round and distended (Fig. 2A) and eventually ceased to divide (data not shown). Because Ace2p overproduction caused an accumulation of Mid2p (Fig. 1E), we examined whether Mid2p localized inappropriately under these conditions. Interestingly, Mid2p-GFP localization was detected at the medial region in a ring as in wild-type cells (Fig. 2B, left panels). Mid2p-GFP was also seen in ring-like structures at the ends of cells as if previous Mid2p rings formed in the central region had not completely disassembled following cell separation (Fig. 2B, right panels).

Overproduction of Mid2p causes septin rings to persist at the old cell ends (Tasto et al., 2003). Because of its effect on Mid2p levels, we reasoned that Ace2p overproduction might lead to similar septin ring stabilization. In cells that overproduced Ace2p, Spn3p-GFP was detected in a ring at the medial division site (Fig. 2C). In addition, it was seen in dot-like structures at the ends of cells, corresponding to old division sites (Fig. 2C). This is consistent with the hypothesis that high levels of Mid2p caused by overproduction of Ace2p prevent efficient septin ring disassembly.

In the absence of mid2+, septins are diffusely organized and cover the septum instead of being maintained in a well-defined, cortically restricted stable ring that can be observed to split into two distinct rings at the time of septation (Berlin et al., 2003; Tasto et al., 2003). Since sep1Δ and ace2Δ strains lack significant levels of Mid2p, we reasoned that septin rings are likely to be disorganized in these strains. As expected, compared with wild-type septin rings (Fig. 2D, left panels), Spn3p-GFP formed disorganized rings with no discrete split ring structures in both sep1Δ cells (data not shown) and ace2Δ cells (Fig. 2D, right panel).

![Fig. 2. Ace2p affects cell morphology and septin ring organization.](image-url)
**mid2** is a critical Ace2p target

Six additional targets of Ace2p have been characterized recently and all appear to play some role in cell separation as determined by biochemical and genetic analyses (Alonso-Nunez et al., 2005; Dekker et al., 2004; Martin-Cuadrado et al., 2003). Eng1p and Agn1p encode glucanases (Dekker et al., 2004; Martin-Cuadrado et al., 2003), whereas the functions of Chf4p, Adg1p, Adg2p and Adg3p (Alonso-Nunez et al., 2005) are not yet clear. The phenotypes of strains lacking these proteins are milder than loss of *ace2* or the septins, and show very few defects in cell separation on their own. Nevertheless, we examined whether Ace2p participates in septin ring organization through these targets as well as through Mid2p. In cells lacking *agn1*, *adg1*, *adg2*, *adg3* or *eng1*, Spn3p-GFP localized normally to a tight discrete ring in all cases (Fig. 3A and data not shown). These data suggest that Mid2p functions on its own in septin ring formation whereas other Ace2p targets are more likely to function in other aspects of cell division (Fig. 7). If this were true, it would stand to reason that the chained cell phenotype resulting from *mid2* deletion ([Berlin et al., 2003; Tasto et al., 2003]; Fig. 3B) would be exacerbated by combining *mid2* with deletions of other Ace2p target genes. To begin examining this, we chose *eng1*, *agn1* and *mid2* deletions, which individually and in

![Fig. 3. Mid2p functions in parallel with other Ace2p targets. (A) The spn3-GFP *agn1Δ* (KGY5091), spn3-gfp *adg1Δ* (KGY5093), spn3-GFP *adg2Δ* (KGY5094), and spn3-gfp *adg3Δ* (KGY5095) strains were grown at 32°C and visualized as in (Fig. 2D). (B) *mid2Δ* (KGY3135), *eng1Δ* (KGY5467) and *mid2Δ eng1Δ agn1Δ* (KGY5505) cells were grown at 32°C and stained with DAPI to visualize DNA and Methyl Blue to visualize septa. (C) Quantification of the number of septa in cells from (B) in addition to wild-type (KGY246), *agn1Δ* (KGY5033), and *eng1Δ agn1Δ* (KGY5034) cells. In each case, 200 cells were examined. (D) *spn3-gfp ace2Δ* (KGY4774) containing the pREP41-*mid2* plasmid (pKG2208) was grown at 32°C for 18 hours in media lacking thiamine (–T). Confocal images of live cells were obtained. Z-series optical sections were taken at 0.5 μM spacing. Images were rotated on the Z-axis to visualize the septin rings. Arrows indicate split ring structures that form after septation. Bars, 5 μm.
combination generate the most noticeable phenotypes [(Alonso-Nunez et al., 2005; Dekker et al., 2004); Fig. 3B,C]. Although the double mutants of eng1Δ and either mid2Δ [(Martin-Cuadrado et al., 2005); Fig. 3B,C] or agn1Δ (Alonso-Nunez et al., 2005) were more severe than any single mutant, the mid2Δeng1Δagn1Δ triple mutant accumulated more than three septa in 20% of cells (Fig. 3B,C), a phenotype more severe than any combination of Ace2p-target mutants thus far examined (Alonso-Nunez et al., 2005; Martin-Cuadrado et al., 2005). Finally, we examined the organization of the septin ring in cells lacking Ace2p, but mildly overproducing Mid2p. After 18 hours of induction, those cells with an excess of Mid2p were still chained, indicative of a lack of glucanase activity. However, fluorescence microscopy demonstrated that the septin rings in these cells were organized (Fig. 3D) as they are in wild-type cells (Fig. 2D), forming easily detected split ring structures. This supports the idea that Mid2p, through its role in organizing septin rings, is required separately from Eng1p, Agn1p and the adg+ gene products in cell division.

Ace2p level and localization are regulated by the cell cycle
ace2+ is periodically expressed (Alonso-Nunez et al., 2005) in a Sep1p-dependent manner (Peng et al., 2005; Rustici et al., 2004). The fact that ace2+ mRNA levels were periodic led us to analyze the abundance of Ace2p to see if it mirrored mRNA levels. To examine Ace2p characteristics, sequences encoding 13 copies of the Myc epitope, three copies of the HA epitope, or eGFP were introduced at the 3′ end of the ace2+ ORF by homologous recombination at the endogenous ace2+ locus so that normal expression levels were maintained. The resulting strains were wild type in appearance and growth rate, indicating that the epitope tags had not disturbed Ace2p function. To obtain a synchronous population of cells, a cdc25-

---

**Fig. 4.** Cell-cycle regulation of Ace2p. (A) cdc25-22 ace2-myc13 (KGY4472) cells were shifted to 36°C for 4 hours to block cells in G2. Cell pellets were collected every 15 minutes after shift down to 25°C and were fixed in ethanol or frozen. The septation index (SI) was determined to monitor progression through the cell cycle. The ethanol-fixed samples were stained with DAPI to visualize the nuclei and allow microscopic calculation of the percentage of binucleates (BN). Protein lysates were prepared and resolved by SDS-PAGE. Ace2p-Myc13 levels were determined by immunoblotting with 9E10 antibody. In these and other experiments with multi-septated cells, the fluctuation of Cdc13p abundance (determined as in Fig. 1C) was used to assess mitotic progression and Cdc2p abundance (determined by blotting with anti-PSTAIRE) served as a loading control. (B) Denatured protein lysates prepared from wild-type (KGY246), ace2-myc13 (KGY1124) and ace2-myc13 sep1Δ (KGY1141) strains were resolved on a 4-12% Bis-Tris gel and immunoblotted with antibodies to myc (9E10) (top panel) or to Cdc2p (PSTAIRE) (lower panel). (C) The nmt1sep1-HA ace2-myc13 (KGY4735) strain was grown to mid-log phase in the absence or presence of thiamine (−T/+T) at 32°C for 20 hours. Protein lysates were prepared, resolved on a 4-12% Bis-Tris gel, and immunoblotted as in (B) or with the 12CA5 antibody. (D) The nmt1sep1-HA3 (KGY4221) strain was grown as in (C). Cells were fixed in ethanol, stained with Methyl Blue and DAPI and imaged. (E) The ace2-myc13 mts3-1 (KGY4536) and ace2-HA3, mts3-1 (KGY5314) strains were grown at 25°C and then shifted to 36°C for 4 hours to block proteasome function and arrest cells in mitosis. Ace2p-Myc13 was immunoprecipitated with the 9E10 antibody and Ace2p-HA3 was immunoprecipitated with the 12CA5 antibody, followed by incubation in the presence or absence of λ-phosphatase. (F) The mid2-myc13 mts3-1 (KGY1976; lane 1), mts3-1 (KGY1135; lane 2), ace2-myc13 mts3-1 (KGY5231) strains containing the pREP1-His6-Ubiquitin plasmid (pKG1284; lane 4) and the ace2-myc13 mts3-1 (KGY4536; lane 3) strain without vector were grown at 25°C for 22 hours in the absence of thiamine in order to induce His-Ub production. This was followed by a 4-hour shift to 36°C. Substrates modified with ubiquitin were isolated and detected by immunoblotting with anti-Myc antibodies.
block and release experiment was performed and samples were collected to determine Ace2p-Myc13 levels at each time point. Ace2p-Myc13 was periodically produced through the cell cycle (Fig. 4A). It began to accumulate during anaphase as determined by the coincidence of binucleate formation, and it peaked in abundance concomitantly with the peak of septation.

We next examined how the reduction in ace2 mRNA levels observed in sep1Δ cells (Alonso-Nunez et al., 2005; Peng et al., 2005; Rustici et al., 2004) affected Ace2p protein levels by comparing the overall level of Ace2p-Myc13 in wild-type and sep1Δ cells. As expected, the level of Ace2p-Myc13 was reduced significantly in cells lacking sep1 (Fig. 4B). Conversely, in cells overexpressing sep1-HA3, Ace2p-Myc13 levels were elevated greatly (Fig. 4C), leading to a phenotype very similar to ace2+ overproducing cells (Fig. 4D and data not shown).

Interestingly, Ace2p-Myc13 migrated more slowly on gels as it accumulated and also formed a doublet (Fig. 4E). To determine if this shift corresponded to phosphorylation, immunoprecipitates of Ace2p-Myc13 or Ace2p-HA3 isolated from cells arrested in mitosis were treated with λ-phosphatase. Phosphatase treatment collapsed the multiple Ace2p-Myc13 and Ace2p-HA3 bands into a single band, indicating that Ace2p is indeed phosphorylated in vivo (Fig. 4E).

The fact that Ace2p oscillated in abundance during the cell cycle led us to investigate whether or not Ace2p was ubiquitylated, a modification associated with targeted protein degradation. A plasmid encoding histidine-conjugated ubiquitin was induced in ace2-myc13 mts3-1 cells so that ubiquitin substrates would be modified by this alternative version of the protein. Degradation of these ubiquitylated substrates was prevented by the mts3-1 mutation that inactivates 26S proteasome function and arrests cells primarily in mitosis (Gordon et al., 1996), allowing ubiquitylated proteins to be collected for analysis. Immunoblotting with anti-Myc antibodies revealed that Ace2p-Myc13 is indeed ubiquitylated (Fig. 4F; lane 4). The detection of ubiquitylated Mid2p-Myc13 (Fig. 4F; lane 1), a protein known to be modified in this way (Tasto et al., 2003), served as a positive control. No ubiquitylated proteins were detected from a strain lacking the epitope tag but containing His6-Ub (Fig. 4F, lane 2) and Ace2p-Myc13 was not retained on the Ni-NTA beads in the absence of His6-Ub (Fig. 4F, lane 3). Thus, it is likely that Ace2p is degraded by ubiquitin-mediated proteolysis as cells exit mitosis.

In S. cerevisiae, Ace2p is localized in the cytoplasm through most of the cell cycle. Although it then briefly localizes to the mother cell nucleus, it localizes more significantly and for a longer period of time to the daughter cell nucleus in a MEN-dependent manner at the time of cytokinesis in order to activate daughter-specific gene expression (Colman-Lerner et al., 2001; Weiss et al., 2002). S. pombe Ace2p-GFP was undetectable in the cytoplasm during any stage of the cell cycle in either live (Fig. 5A) or fixed cells (data not shown). Consistent with its periodic abundance determined by immunoblotting, it was detected only in anaphase cells and only in the nucleus (Fig. 5A). To determine more precisely when Ace2p appeared in nuclei, we examined cells expressing both Ace2p-GFP and Sid4p-GFP (Fig. 5B). Sid4p is a constitutive spindle pole body protein (Chang and Gould, 2000) and a useful marker of cell-cycle stage. Ace2p-GFP was not detected in cells during

---

**Fig. 5.** Ace2p localization and regulation. (A) The ace2-GFP (KGY4385) strain was grown at 25°C and confocal images of live cells were obtained. (B) The ace2-GFP sid4-GFP (KGY1311) strain was grown in ethanol and the spindle pole body marker Sid4p was used to detect this cell-cycle stage. 100 cells at each cell-cycle stage were examined to determine the percentage of cells expressing Ace2p-GFP. Representative images of different stages are shown. (C) Ace2p-HA3 was immunoprecipitated from native protein lysates prepared from fields-type (KGY246; lane 1), ace2-HA3 (KGY1123; lane 2), ace2-HA3 fhk2Δ (KGY5388; lane 3) and ace2-HA3 mbx1Δ (KGY5394; lane 4) strains with the 12CA5 antibody. Immunoblotting was performed using antibodies against HA (12CA5; upper panel) or against Cdc2p (PSTAIRE; lower panel) to demonstrate that immunoprecipitation was performed on equal amounts of protein within the lysates. (D) ace2-gfp fhk2Δ (KGY5361) (left panel) or ace2-gfp mbx1Δ (KGY5393) (right panel) cells were grown in at 32°C. Confocal images of live cells were obtained. Bars, 5 μm.
interphase or metaphase. It was detected in 35% of cells at the beginning of anaphase and was present in all cells by the end of anaphase. The Ace2p-GFP signal persisted in the daughter nuclei until cell cleavage (Fig. 5B).

The mitotic regulators Fkh2p and Mbx1p have an effect on Ace2p levels

In addition to Sep1p, two other transcription factors, Fkh2p and Mbx1p, have been reported to impinge on the expression of ace2+ (Buck et al., 2004). To examine their contributions to Ace2p levels, Ace2p-HA3 levels were determined in wild-type, fkh2Δ and mbx1Δ cells. Interestingly, Ace2p-HA3 was present at a slightly increased amount in mbx1Δ or fkh2Δ cells compared with wild-type cells (Fig. 5C, top panel, compare lanes 3 and 4 to lane 2). Consistent with immunoblotting results, Ace2p-GFP was detected easily in the nuclei of cells lacking either fkh2+ (Fig. 5D, left panel) or mbx1+ (Fig. 5D, right panel). These results, in addition to the data showing the effect of Sep1p on Ace2p (Fig. 4B,C), suggest that Fbx1p and Mbx1p might act to repress Ace2p production during interphase.

Timely expression of ace2+ and mid2+ is independent of the SIN

The SIN is a signaling cascade whose function it is to trigger formation of the division septum. Given that it is active only in anaphase when Ace2p also functions, it was possible that the SIN played a role in regulating Ace2p and, consequently, in the production of its targets, such as mid2+. To test this possibility, expression of both ace2+ and mid2+ were examined in a population of cdc7-24 cells synchronized in G2 by centrifugal elutriation and shifted to the non-permissive temperature. Cdc7p is a protein kinase whose activity is required for the SIN (Sohrmann et al., 1998). Inactivation of the kinase upon shift to 36°C prevents septation from occurring although other aspects of the cell cycle such as nuclear division and DNA synthesis continue. Despite the failure of cytokinesis in these cells, mid2+ mRNA level peaked at anaphase as seen by the concomitant increase of number of binucleate cells (Fig. 6A), just as it does in wild type cells (Tasto et al., 2003), and ace2+ expression preceded the mid2+ peak. The independence of eng1+ expression from the SIN has also been observed (Alonso-Nunez et al., 2005). To examine whether precocious activation of the SIN in interphase cells could induce Ace2p production outside of mitosis, expression of both ace2+ and mid2+ were also examined in a population of mid2-GFP cdc16-116 cells synchronized in G2 by centrifugal elutriation and shifted to the non-permissive temperature of 36°C. Cdc16p is a component of the two-component GTPase-activating protein (GAP) for the small GTPase Spg1p, whose activation is required for SIN activity (Furge et al., 1998; Schmidt et al., 1997). When Cdc16p function is blocked, subsequent constitutive activation of Spg1p can drive septation in G2 cells (Minet et al., 1979). Despite precocious septation in synchronized cdc16-116 cells, mid2+ RNA expression remained restricted to anaphase, with the ace2+ RNA peak occurring immediately before it (Fig. 6B). These data indicate that Ace2p production and function are independent of the SIN and suggest, therefore, that S. pombe Ace2p is regulated differently than its S. cerevisiae counterpart.

Discussion

In this study, we analyzed the factors required for Mid2p production and how this regulation contributes to septin ring
organization and cell separation. We find that *mid2* transcript and protein levels are dependent on the zinc-finger transcription factor Ace2p, which itself is regulated by the forkhead transcription factor Sep1p. The regulation of *mid2* by Ace2p contributes specifically to proper septin ring organization. This, in coordination with the activities of other Ace2p targets directed at degrading the primary septum, leads to proper cell separation (Fig. 7).

The cell separation phenotype shared by cells lacking *sep1* (Ribar et al., 1999), *ace2* (Martin-Cuadrado et al., 2003) and *mid2* (Berlin et al., 2003; Tasto et al., 2003), in addition to the fact that Sep1p and Ace2p regulate *mid2* (Alonso-Nunez et al., 2005; Peng et al., 2005; Rustici et al., 2004; Tasto et al., 2003), made it likely that Ace2p was the key factor in the regulation of *mid2*. Genes targeted by *S. cerevisiae* Ace2p share a consensus binding site (Dohrmann et al., 1996) and *S. pombe* Ace2-dependent genes also contain at least one identical site (Alonso-Nunez et al., 2005; Rustici et al., 2004). *mid2* contains two such sites within its promoter located 270 and 290 base pairs upstream of the ATG codon presumed to be the translation start site, but no forkhead consensus binding sites, further suggesting a direct role for Ace2p in *mid2* transcription. Although it was possible that Sep1p and Ace2p acted together to regulate *mid2* expression, our data indicate that Sep1p and Ace2p act in a strictly linear pathway. Six additional Ace2p targets (*eng1*, *agn1*, *adg1*, *adg2*, *adg3*, and *cfh4*) also appear to be regulated in this manner (Alonso-Nunez et al., 2005). Additional microarray studies number as-yet-unknown function. Thirdly, Ace2p controls the expression of four sets of genes. The first group includes genes encoding the glucanases Agn1p and Eng1p; the second group, consisting of *cfh4*, *adg1*, *adg2*, and *adg3*, encodes proteins with as-yet-unknown function. Thirdly, Ace2p controls the expression of *mid2*, whose protein product is responsible for proper septin ring organization, an event necessary for proper cell separation.

**Fig. 7.** Model for transcriptional control of cell separation. In separate waves of transcription, Sep1p and Ace2p promote expression of genes critical for cell separation. Ace2p controls expression of expression of four sets of genes. The first group includes genes encoding the glucanases Agn1p and Eng1p; the second group, consisting of *cfh4*, *adg1*, *adg2*, and *adg3*, encodes proteins with as-yet-unknown function. Thirdly, Ace2p controls the expression of *mid2*, whose protein product is responsible for proper septin ring organization, an event necessary for proper cell separation.

other important roles in cell separation since cells lacking both of the major glucanases in addition to Mid2p have a much more severe cell separation defect than cells lacking any of these genes individually or the two glucanases only ([Alonso-Nunez et al., 2005; Dekker et al., 2004; Martin-Cuadrado et al., 2003; Martin-Cuadrado et al., 2005]; Fig. 3B,C). Additionally, wild-type septin ring organization is re-established through overproduction of Mid2p in *ace2A* cells, further indicating that *mid2* expression is an important function of Ace2p and hence of Sep1p. Restricting formation of functional septin structures by regulating Mid2p abundance would prevent septin ring-binding factors from concentrating at the division site until they were needed. This would be an excellent example of dynamic regulation of protein complex formation. Several proteins that regulate the cell cycle are present constantly throughout the cycle whereas others are regulated in some manner and are only present for a short while. These static and dynamic proteins come together at appropriate moments during the cell cycle in order to form functional complexes that drive the cell cycle forward (de Lichtenberg et al., 2005).

*S. cerevisiae* Ace2p is regulated by the cell cycle both at the RNA and protein levels (Dohrmann et al., 1992). Similarly, in *S. pombe*, *ace2* expression is periodic (Alonso-Nunez et al., 2005; Peng et al., 2005; Rustici et al., 2004). Although protein expression often correlates with mRNA expression, there are instances where protein activity or level does not reflect the amount of its corresponding mRNA (Huang and O’Shea, 2005; Moldoveanu et al., 2000). However, this is not the case with *ace2*; our results show a similar cell-cycle regulation of Ace2p and mRNA, with levels beginning to increase at the onset of anaphase. Protein abundance peaks at late anaphase and then diminishes as mitosis is completed. This timing would be consistent with Ace2p nuclear accumulation requiring activation of the anaphase-promoting complex (APC), which is a multisubunit E3 ubiquitin ligase (Harper et al., 2002; Peters, 2002), as seems to be the case in budding yeast (Ufano et al., 2004). In *S. cerevisiae*, cells with a mutant version of the APC component Swm1p, do not accumulate Ace2p in the daughter nucleus. These mutant cells do not express genes under Ace2p control and exhibit mitotic exit and cell separation defects (Ufano et al., 2004). It remains to be determined, however, whether the APC affects *S. pombe* Ace2p.

*S. pombe* Ace2p-Myc13 and Ace2p-HA3 exhibit a phosphorylation-induced mobility shift during the time that the protein accumulates in cells. Phosphorylation of *S. cerevisiae* Ace2p at three recognition sites for the major cyclin-dependent kinase (CDK) Cdc28p inhibits its nuclear accumulation (O’Conallain et al., 1999). Phosphorylation and retention in the cytoplasm is also associated with a greater Ace2p stability (O’Conallain et al., 1999). *S. pombe* Ace2p contains 11 partial (S/T-P) and 1 exact (S/T-P-X-K/R) CDK consensus sites and therefore Cdc2p, the major *S. pombe* CDK, might have a role in *S. pombe* Ace2p regulation. It is also possible that *S. pombe* Ace2p is a target of the Orb6-Mob2 kinase complex (homologous to *S. cerevisiae* Cbk1p-Mob2) as is Ace2p in *S. cerevisiae* (Colman-Lerner et al., 2001; Nelson et al., 2003; Weiss et al., 2002), although the primary role associated with this event is asymmetric nuclear localization, which is not observed in *S. pombe*. Finally, since phosphorylation of proteins can trigger their degradation, and we observe both...
ubiquitylation and cell-cycle-dependent disappearance of S. pombe Ace2p. Ace2p phosphorylation might affect Ace2p stability. Further experiments will be required to dissect the roles of S. pombe Ace2p phosphorylation in its activation, inhibition and/or degradation.

Consistent with biochemical data, Ace2p-GFP was detected in 35% of cells in early anaphase and, by the end of anaphase, it was visualized in all cell nuclei. Ace2p staining diminished as septation occurred. Whereas the timing of Ace2p accumulation in fission yeast cells is similar to that in S. cerevisiae, the localization differs. S. pombe Ace2p is detected only in nuclei, whereas S. cerevisiae Ace2p first appears in the cytoplasm. Also, S. pombe Ace2p localization is not asymmetric in daughter nuclei as in S. cerevisiae. Thus, the control of Ace2p is likely to differ markedly between the two yeasts, reflecting the specific need of S. cerevisiae to distinguish mother from daughter cell (Colman-Lerner et al., 2001; Laabs et al., 2003; Weiss et al., 2002).

Indeed, whereas the MEN has been implicated in the correct accumulation of S. cerevisiae Ace2p in daughter cell nuclei (Colman-Lerner et al., 2001; Martin-Cuadrado et al., 2003; Weiss et al., 2002), we found no evidence that the analogous pathway in S. pombe, the SIN, is involved in regulating Ace2p. The fact that ace2+, mid2+ (this report) and eng1+ (Alonso-Nunez et al., 2005) are expressed in a timely fashion in cells lacking a functional SIN pathway, and that the effect of the ace2+eng1+ mutation is not observed in cells lacking a functional MEN pathway (Colman-Lerner et al., 2001; Laabs et al., 2003; Weiss et al., 2002), we found no evidence that the analogous pathway in S. pombe, the SIN, is involved in regulating Ace2p.

Despite data indicating that ace2 expression is reduced in cells lacking two other S. pombe cell-cycle transcription factors, Fkh2p and Mbx1p (Buck et al., 2004), we find that Ace2p is still present, indeed at a somewhat increased level, in the absence of either of these two proteins. This result is consistent with the phenotypes of cells lacking fkh2+ (Buck et al., 2004; Bulmer et al., 2004) or mbx1+ (Buck et al., 2004), which are heterogeneous and not notable for significant defects in cell separation. Our biochemical results are also in agreement with microarray analyses of cells lacking Fkh2p, in which the transcript levels of Sep1p targets were found to be elevated, indicating roles of Fkh2p and Mbx1p in the negative regulation of Sep1p or its targets (Rustici et al., 2004). Therefore, with respect to events of late mitosis, a linear model of transcriptional regulation presents itself, with Sep1p regulating ace2 expression among other target genes and Ace2p regulating mid2 expression as well as other genes (Bahler, 2005). Ace2p appears responsible for coordinating cell separation by regulating the production of glucanases and the formation of septin rings that are necessary for both proper glucanase localization and other unknown functions (Fig. 7). The combined activities of Sep1p and Ace2p ensure that mitotic exit, cytokinesis and cell separation are coordinated.

We are grateful to Jianhua Liu, Carlos R. Vazquez de Aldana and Christopher J. McNerney for strains; Joe Tasto for designing oligonucleotides used in this study; and Jennifer Morrell for help with live cell imaging. C.S.P. was supported by NIH training grant 2 T32 CA009582-16. R.H.R. was supported by NSF pre-doctoral training grant DGE-02387141. This work was supported by the Howard Hughes Medical Institute, of which K.L.G. is an Investigator.

References


Dohrmann, P. R., Voth, W. P. and Stillman, D. J. (1996). Role of negative


Moldoveanu, A. I., Shephard, R. J. and Shek, P. N. (2000). Exercise elevates plasma levels but not gene expression of IL-1β, IL-6, and TNF-α in blood mononuclear cells. J. Appl. Physiol. 89, 1499-1504.


