Swm1p subunit of the APC/cyclosome is required for activation of the daughter-specific gene expression program mediated by Ace2p during growth at high temperature in *Saccharomyces cerevisiae*

Sandra Ufano[1,2], M. Evangelina Pablo[1], Arturo Calzada[1,3], Francisco del Rey[1] and Carlos R. Vázquez de Aldana[1,*]

[1]Instituto de Microbiología-Bioquímica, Departamento de Microbiología y Genética, CSIC / Universidad de Salamanca, Campus Miguel de Unamuno, 37007, Salamanca, Spain
[2]Centro Nacional de Biotecnología, CSIC/UAM, Cantoblanco, 28049, Madrid, Spain
[3]Centro de Investigación del Cáncer, CSIC / Universidad de Salamanca, Campus Miguel de Unamuno, 37007, Salamanca, Spain
*Author for correspondence (e-mail: cvazquez@usal.es)

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Summary

***SWM1* was originally identified for its role in the late steps of the sporulation process, being required for spore wall assembly. This protein, recently identified as one of the core subunits of the anaphase-promoting complex (APC) is also required to complete cell separation in vegetative cells during growth at high temperature. Mutants lacking *SWM1* show a thermosensitive growth defect that is suppressed by osmotic support in the culture medium. At the restrictive temperature, *swm1* mutants are unable to complete separation, forming chains of cells that remain associated and, with prolonged incubation times, the stability of the cell wall is compromised, resulting in cell lysis. This separation defect is due to a reduction in expression of *CTS1* (the gene encoding chitinase) and a group of genes involved in cell separation (such as *ENG1*, *SCW11*, *DSE1* and *DSE2*). Interestingly, these genes are specifically regulated by the transcription factor Ace2p, suggesting that Swm1p is required for normal expression of Ace2p-dependent genes during growth at high temperatures. Although no defect in Ace2p localization can be observed at 28°C, this transcription factor is unable to enter the nucleus of the daughter cell during growth at 38°C. Under these growth conditions, *swm1* cells undergo a delay in exit from mitosis, as determined by analysis of Clb2p degradation and Cdc28p-Clb2p kinase assays, and this could be the reason for the cytoplasmic localization of Ace2p.**

Key words: Cytokinesis, APC, Cell separation, Chitinase, Ace2p

Introduction

Cell growth and morphogenesis is a complex process that is controlled by intricate signalling pathways and is tightly linked to the cell cycle. It has been extensively studied in *Saccharomyces cerevisiae* (for a review, see Madden and Snyder, 1998) and involves several different processes that need to be perfectly coordinated in time and space to be successfully completed, such as specific programs of gene transcription (Cho et al., 1998; Spellman et al., 1998), selection of the new bud site (Chant, 1999), transport of new material to growth sites, or cell wall synthesis (Smits et al., 1999; Smits et al., 2001). Many of these processes involve cytoskeleton reorganization (Pruyne and Bretscher, 2000). To ensure that each daughter cell receives only one copy of each chromosome, exit from mitosis and cytokinesis must not occur before chromosome segregation has been completed. Thus, the onset of cytokinesis (which involves actomyosin ring contraction and delivery of new plasma membrane to an ingressing division furrow) only occurs after the completion of previous events in the cell cycle, owing to the presence of control mechanisms.

In the budding yeast *S. cerevisiae*, a signalling cascade known as the mitotic exit network (MEN) controls exit from mitosis and ensures that this transition occurs only after sister-chromatid separation has been initiated and the genetic material has been segregated between the mother and the daughter cell. Cytokinesis in budding yeast is analogous to that of animal cells and involves actomyosin ring contraction and synthesis of the division septum at the mother-daughter junction as an extension of the vegetative cell wall, which ultimately needs to be cleaved to allow the two cells to become two independent entities. The MEN is composed of highly conserved proteins that include the protein kinases Cdc5p, Cdc15p, Dbf2p and Dbf20p, the phosphatase Cdc14p, the GTPase Tem1p and its GEF Lte1p, and Mob1p, a protein that binds Dbf2p and Dbf20p (for a review, see Bardin and Amon, 2001; McCollum and Gould, 2001). This regulatory network is required for inactivation of mitotic cyclins by ubiquitin-dependent proteolysis. A ubiquitin ligase, the anaphase-promoting complex (APC) or cyclosome, together with a specificity factor, ubiquitinates mitotic cyclins and targets them for degradation by the 26S proteasome (reviewed in Harper et

al., 2002; Peters, 2002; Zachariae and Nasmyth, 1999). The dual-specificity phosphatase Cdc14p is essential for the inactivation of mitotic cyclins and acts through two different mechanisms. First, it dephosphorylates the APC specificity factor Cdh1p/Hct1p, promoting its association with the APC and the consequent activation of this complex. Second, it dephosphorylates the Sic1p inhibitor, which also contributes to inactivation of mitotic cyclin-dependent kinases (CDKs) (Jaspersen et al., 1999; Visintin et al., 1998). In addition, Cdc14p upregulates transcription of *SIC1* by dephosphorylating the transcription factor Swi5p, which promotes its translocation to the nucleus (Knapp et al., 1996; Moll et al., 1991; Visintin et al., 1998). The MEN also controls the onset of cytokinesis, because actomyosin ring contraction and septum deposition do not occur when MEN function is abrogated, even when progress from mitosis to G_1 is artificially allowed by suppression of mitotic CDK activity (Lippincott et al., 2001; Luca et al., 2001).

Another two proteins also play an important role in the completion of cell separation: the protein Mob2p, which is closely related to Mob1p, and the protein kinase Cbk1p, which is similar to Dbf2p. Cbk1p is a protein kinase of the Cot-1/Orb6/Ndr/Warts family, whose members are important regulators of cell morphogenesis and proliferation. *S. cerevisiae cbk1*Δ cells fail to degrade the septum separating the mother and daughter cells, which results in the formation of chains of connected cells (Bidlingmaier et al., 2001; Racki et al., 2000). These cells also display other defects in cell morphogenesis, such as round cells, random budding patterns and abnormal mating projections. The cell separation defect of *cbk1*Δ cells is probably due to reduced activity of the transcription factor Ace2p, which is essential for the transcription of a group of genes expressed at the M/G_1 transition and whose products are required for septum degradation and cell separation (Bidlingmaier et al., 2001; Dohrmann et al., 1992; Doolin et al., 2001; Racki et al., 2000). Ace2p exhibits Mob2- and Cbk1p-dependent localization to the daughter cell nucleus at the end of mitosis, and controls daughter cell-specific transcription of genes that are necessary for septum degradation (Colman-Lerner et al., 2001; Weiss et al., 2002). Furthermore, the nuclear accumulation of Ace2p is cell-cycle regulated, because this protein associates with the Cdc28 kinase, which presumably phosphorylates three Ser/Thr residues, preventing its nuclear entry until late mitosis and early G_1 (O'Conallain et al., 1999). In addition, MEN function is also required for Ace2p nuclear accumulation, because the protein remains in the cytoplasm in *cdc14-1* or *mob1-77* mutants (Weiss et al., 2002).

For a daughter cell to become separated from its mother, specific enzymatic processes leading to partial degradation of the cell wall components at the mother-daughter junction are activated, such as chitinase (encoded by *CTS1*), an endo-1,3-β-glucanase (encoded by *ENG1*) or the product of the *SCW11* gene (reviewed by Cabib et al., 2001). Chitinase partially removes chitin, which is deposited in the cell wall between the mother and daughter cells, forming the primary septum, whereas Eng1p and Scw11p might be required for dissolution of the β-glucan that composes the secondary septum (Baladrón et al., 2002; Kuranda and Robbins, 1991). Transcriptional regulation of these genes is important in order to avoid untimely gene expression, which will presumably result in cell lysis. Their transcription is cell-cycle regulated and depends on the protein encoded by *ACE2*. This transcription factor is very similar to Swi5p but, in spite of their similar DNA binding sequences, each of them regulates the expression of specific target genes. For example, Swi5p controls the expression of the genes encoding HO endonuclease (Dohrmann et al., 1992), cyclins Pcl2p and Pcl9p (Aerne et al., 1998) and the cell wall protein Pir1p (Doolin et al., 2001), whereas Ace2p regulates the expression of *CTS1*, *ENG1*, *SCW11*, *DSE1* and *DSE2* (Baladrón et al., 2002; Dohrmann et al., 1992; Doolin et al., 2001). There is also a third group of genes, whose expression is dependent on both transcription factors, including *EGT2*, *SIC1*, *ASH1* and *RME1* (Doolin et al., 2001).

In this report, we present evidence that Swm1p, recently identified as one of the subunits of the APC/cyclosome complex (Hall et al., 2003; Passmore et al., 2003; Yoon et al., 2002), is important for cell separation and for maintaining cell integrity during growth at high temperature (38°C). In addition, we show that *swm1* cells display a delay in Clb2p degradation and in the inactivation of CDK activity at the end of mitosis during growth at 38°C. The high CDK activity levels could account for the inability of the cells to accumulate Ace2p in the nucleus of the daughter cell at the end of mitosis. Consequently, the cells fail to activate the daughter-specific gene expression program regulated by Ace2p that is required for cell separation.

Materials and Methods

Yeast strains and growth conditions

Table 1 lists the yeast strains used in this study. Yeast cells were grown vegetatively in YEPD (1% yeast extract, 2% peptone, 2% glucose). Transformants carrying the *kanMX4* gene were selected on YEPD plates containing geneticin (200 μg ml^{-1}) and transformants containing the *hph* selection marker on YEPD plates containing hygromycin B (300 μg ml^{-1}). Strains YPA24 (wt) and YPA207 (*swm1::hisG*/*swm1::hisG*) have been described previously (Ufano et al., 1999). Strain SEP3 was derived from strain W9317 ($CLB2^{HA}$) by replacing the *SWM1* gene with the *swm1::kanMX4* cassette contained in plasmid pSU29. To construct the isogenic diploid strain LS61 containing a deletion of *ACE2*, strains W303-1A and α131-20 were transformed with a linear fragment containing the *ace2::hph* deletion cassette, and transformants were selected on YEPD plates containing hygromycin B. After verifying the deletions by PCR, the haploid strains were mated to generate the homozygous diploid strain LS61 (*ace2::hph/ace2::hph*). A similar approach was used to construct the double mutant diploid strains LS70 (*swm1::hisG*/*swm1::hisG ace2::hph/ace2::hph*) but using strains YPA202 and YPA203 as recipients for transformation.

The *ace2::hph* deletion cassette was constructed using the technique described by Goldstein and McCusker (Goldstein and McCusker, 1999). Specific oligonucleotide primers were used to amplify two fragments: one containing the 5′ flanking region (–372 to –5) and the other the 3′ flanking region (401 bp downstream from the stop codon). These fragments were fused by recombinant PCR to the *hph* cassette (Goldstein and McCusker, 1999) and the amplified fragment was used directly for gene replacement. Plasmid pSU50, containing the *CTS1* coding region under the control of the strong constitutive promoter TPI, was constructed by PCR amplification of the *CTS1* coding region with specific oligonucleotides (5′-CCATGGCACTCCTTTACATC-3′ and 5′-GTCGACTTAAAAGT-AATTGCTTTCC-3′), which generated *Nco*I and *Sal*I sites at the ends, and cloning the resulting fragment between the same sites of plasmid pYX112 (R&D Systems; http://www.rndsystems.com), which

Table 1. Yeast strains used in this study

Strain	Genotype	Source
W303-1A	*MAT***a** *ura3 ade2 leu2 his3 trp1 can1*	(Ufano et al., 1999)
α131-20	*MAT*α *ura3 ade2 leu1 can1cyh2*	(Ufano et al., 1999)
YPA202	α131-20 *swm1::hisG*	(Ufano et al., 1999)
YPA203	W303-1A *swm1::hisG*	(Ufano et al., 1999)
W9317	W303-1A $CLB2^{HA}$	(Schwab et al., 1997)
SEP3	W303-1A $CLB2^{HA}$ *swm1::kanMX4*	This work
YPA24	*MAT***a**/*MAT*α *ura3/ura3 ade2/ade2 leu2/+ his3/+ trp1/+ +/leu1 can1/can1 +/cyh2*	(Ufano et al., 1999)
YPA207	YPA24 *swm1::hisG/swm1::hisG*	(Ufano et al., 1999)
TD28	*MAT***a** *ura3-*Δ*52 ino1* can^r	F. del Rey
LS40	TD28 *swm1::kanMX4*	This work

contains the constitutive TPI promoter. The dominant allele *ACE2-1* cloned in a yeast-shuttle vector has been described previously (Racki et al., 2000), and was kindly provided by C. Herbert (CNRS, Gif-Sur-Yvette, France), whereas plasmids pACE2-YFP and pACE2-G128E-YFP (Colman-Lerner et al., 2001) were kindly provided by A. Colman-Lerner (The Molecular Sciences Institute, Berkeley, CA).

Gene expression analysis

Cells (1.3×10^9) were collected at different times after transfer to the restrictive temperature (38°C) and total RNA was prepared using the method described by Percival-Smith and Segall (Percival-Smith and Segall, 1984). For northern blot analysis, 5 μg of RNA was denatured and transferred to Hybond membranes (Amersham Biosciences; http://www.apbiotech.com) using the manufacturer's instructions. The DNA probes used to detect the different transcripts were internal fragments of the coding region of each gene amplified by PCR using specific oligonucleotide pairs, except for *ACT1* (the 1.7-kb *Bam*HI-*Hin*dIII fragment of plasmid pYactI) (Ng and Abelson, 1980). PCR probes were as follows: *SWM1*, a 360 bp fragment from +38 to +398; *CTS1*, a 734 bp fragment from +41 to +774; *DSE1*, a 914 bp fragment from +505 to +1418; *DSE2*, a 610 bp fragment from +50 to +659; *SCW11*, a 646 bp fragment from +18 to +663; *EGT2*, a 1010 bp fragment from +39 to +1048; *ACE2*, a 462 bp fragment from +19 to +480.

Whole-genome transcriptional analyses were carried out using Yeast GeneFilters Microarrays (Research Genetics; http://mp.invitrogen.com), with the conditions described by the manufacturer. Briefly, $polyA^+$ mRNA was prepared from wild-type or *swm1* mutant cells grown for 4 hours at 38°C using an mRNA purification kit (Amersham Biosciences) as specified by the manufacturer. ^{33}P-Labeled cDNA probes were prepared and hybridized to DNA filters. Membranes were scanned using a Molecular Imager FX System (BioRad; http://www.bio-rad.com) using a resolution of 50 μm and the resulting images were processed using Pathways software (Research Genetics).

Microscopy techniques

For light microscopy, cells were fixed in ethanol and stained with DAPI (4′,6-diamino-2-phenylindole) or Calcofluor, as previously described (Pringle, 1991). Samples were viewed and photographed as wet mounts using a Leica DMXRA microscope equipped for Nomarski optics and epifluorescence. Pictures were taken with a Photometrics Sensys CCD camera.

Samples were prepared for electron microscopy according to the protocol described by Wright and Rine (Wright and Rine, 1989). In brief, cells from strains YPA24 and YPA207, which had been incubated for 8 hours at 38°C in YEPD or YEPD supplemented with 1 M sorbitol, were fixed directly by the addition of 0.1 volumes of a 10× fixation solution (10% glutaraldehyde, 2% methanol, 0.4 M potassium phosphate, pH 7) to the culture medium, pelleted and then incubated in fixation solution on ice for 30 minutes. Samples were washed and treated with 1% sodium metaperiodate for 15 minutes, washed again and resuspended in 50 mM ammonium phosphate for another 15 minutes. Fixed cells were embedded in agar, dehydrated through a graded series of ethanol and then embedded in LR White Resin (London Resin Company, London, UK). Thin sections were stained with uranyl acetate and Reynold's lead citrate and examined under a Zeiss EM900 electron microscope.

Nocodazole-induced cell cycle arrest

Cultures were incubated with shaking in the presence of nocodazole (15 mg ml^{-1}) for 2.5 hours, transferred to 38°C for 1.5 hours and finally released from the nocodazole arrest at the restrictive temperature. Samples were collected for fluorescence-activated cell sorting (FACS), western analysis or kinase assays at different time intervals after the release.

Protein extract preparation

Soluble protein extracts were prepared as described previously (Moreno et al., 1991). Cells were collected, washed and broken in 30 ml of histone buffer using glass beads. The HB buffer contained 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM 4-morpholinepropanesulfonic acid (pH 7.2), 15 mM $MgCl_2$, 15 mM EGTA, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 20 μg ml^{-1} each leupeptin and aprotinin. The glass beads were washed with 500 ml of histone buffer and the supernatant was recovered. Protein concentrations were measured using the BCA assay kit (Pierce; http://www.piercenet.com). Protein extracts and immunoprecipitates were electrophoresed using 10% or 8-16% gradient SDS-polyacrylamide gels.

Western-blot analysis

For Western blots, 40 μg total protein extracts from each sample was blotted onto nitrocellulose and proteins were detected using anti-haemagglutinin (HA) antibody (12CA5, 1:500; Roche; http://www.roche.com) or anti-Pgk1 antibody (mouse monoclonal; Molecular Probes; http://www.probes.com). Horseradish peroxidase-conjugated anti-mouse antibodies and the ECL kit (Amersham Biosciences) were used.

Kinase activity assays

Total $p34^{CDC28}$ protein kinase activity was assayed after immunoprecipitation with 12CA5 antibody, using histone H1 (Roche) as substrate (Moreno et al., 1991). $p34^{CDC28}$ was immunoprecipitated from 0.8 mg soluble protein extracts with 0.5 μl of anti-HA antibodies. Immunoprecipitates were incubated in a 0.1 mM ATP, 0.5 mg ml^{-1} histone H1 and 20 μCi ml^{-1} [γ-^{32}P]ATP reaction mix at 30°C for 30 minutes. Reactions were stopped with 1× final Laemmli-SDS sample buffer and denatured for 5 minutes at 100°C. Samples were run on a 12% SDS-polyacrylamide gel. Phosphorylated histone H1

was detected by autoradiography and quantified using a Fujifilm BAS1200 PhosphorImager.

Chitinase assays

Chitinase activity was assayed on culture supernatants and whole cells using the protocol described previously (Kuranda and Robbins, 1991). In brief, culture supernatants or whole cells were incubated for 1 hour at 30°C with 200 μM 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside (Sigma; http://www.sigma-aldrich.com) in 0.1 M sodium citrate buffer, pH 3.0, in a final volume of 100 μl. The reaction was stopped by the addition of 2.9 ml of 0.5 M glycine, NaOH buffer (pH 10.4) and the 4-methylumbelliferone released was measured with a fluorescence spectrophotometer (excitation at 350 nm, emission at 400 nm). One unit of activity was defined as nanomoles of 4-methylumbelliferone released per hour.

Results

swm1 mutants have a thermosensitive growth defect

We have previously reported that *SWM1* expression is induced during sporulation and that its product is required for spore cell wall construction (Ufano et al., 1999). The fact that a basal level of expression was also detected during vegetative growth prompted us to analyse the phenotypes arising from the absence of Swm1p during this part of the yeast life cycle. Because the main defect of *swm1* spores is in the maturation of the spore cell wall, the possible contribution of Swm1p to the construction of the vegetative cell wall was monitored by growing the cells under conditions described to challenge the integrity of this structure. These include growth at high temperature (38°C), growth in the presence of the cell-wall-disturbing agents Calcofluor white or Congo Red, or growth on plates containing low levels of caffeine, which has been shown to inhibit the growth of cells containing mutations in the Pkc1p/Slt2p mitogen-activated-protein kinase pathway (Costigan et al., 1992; Posas et al., 1993). Growth of *swm1* mutants in YEPD medium containing caffeine or Congo Red was indistinguishable from the isogenic wild type, but deletion of *SWM1* resulted in a weak sensitivity to Calcofluor white. Interestingly, *swm1* mutants were unable to form colonies at 38°C, a defect that was suppressed by the addition of 1 M sorbitol to the culture medium (Fig. 1). Similar results were found when the strains were cultured in liquid medium at 38°C. *swm1* cells ceased proliferation after 6-8 hours at the restrictive temperature and, after prolonged incubation (12-16 hours or more), the cells appeared non-refractile under the light microscope, which suggests cell lysis (data not shown). The addition of 1 M sorbitol to the liquid cultures rescued the lysis defect of the mutant strain. These results suggest that, in addition to the previously described function in sporulation, *SWM1* also plays a role during vegetative growth, sharing some phenotypes with mutants defective in cell wall construction.

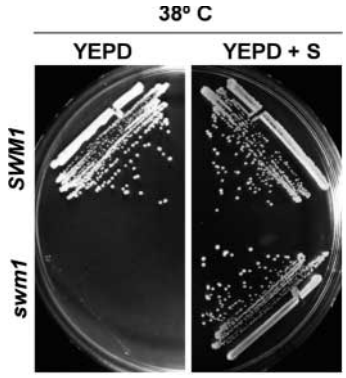

Fig. 1. Growth of wild-type and *swm1* mutants. Growth of wild-type (YPA24) and mutant (YPA207) cells at 38°C in solid medium. Cells were streaked out onto YEPD or YEPD plates supplemented with 1 M sorbitol (YEPD +S) and incubated for 48 hours at 38°C.

Morphological defects and altered chitin distribution at the restrictive temperature

To further characterize the effects of Swm1p depletion during vegetative growth, the morphology of mutant cells at different times after transfer to the restrictive temperature was examined. Microscopic inspection of cells grown at 28°C revealed no obvious differences between mutant and wild-type cells. However, after 8 hours of incubation at the restrictive temperature, chains of connected cells were observed in diploid *swm1* cells that were absent in the isogenic wild-type strain grown under the same conditions (Fig. 2). The cells in the chains and branched chains were tightly bound (they could

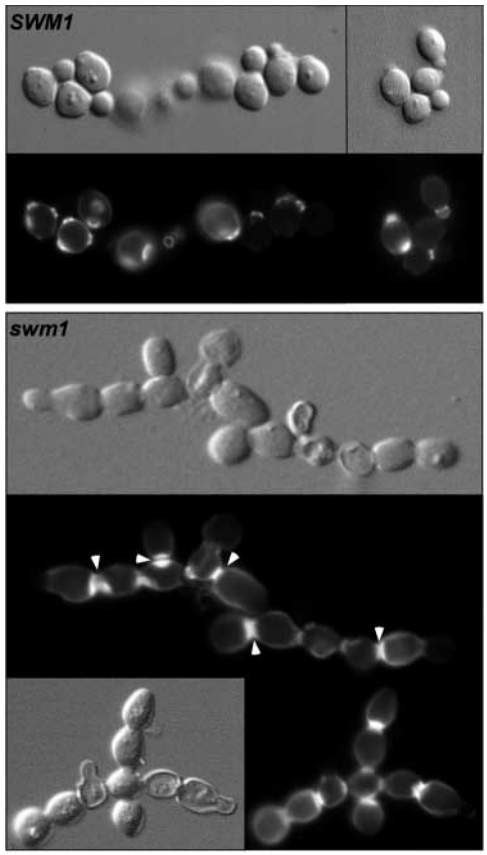

Fig. 2. Microscopic appearance of wild-type and *swm1* mutant cells during growth at 38°C. Diploid wild-type (YPA24) or *swm1* mutant (YPA207) cells were grown for 8 hours at the restrictive temperature on YEPD medium, washed and stained with Calcofluor. Photographs of differential interference contrast microscopy or Calcofluor-stained cells are shown. Arrowheads indicate the position of thickened septa.

not be separated by sonication). DNA staining with DAPI revealed that most of the cells contained a single nucleus, indicating that there was no defect in nuclear division or segregation in the mutant cells (data not shown). This phenotype suggests a defect in the last stages of the cell cycle, either in cytokinesis or in completion of cell separation.

To distinguish between these two possibilities, cells were stained with Calcofluor white, a specific chitin-binding dye in *S. cerevisiae*. In wild-type cells, chitin is primarily deposited at the neck between the mother and the daughter cells and, after cell separation, remains on the mother cell as circular rings (the 'bud scars'). A low level of chitin also accumulates throughout the cell wall, which results in a dim Calcofluor-white staining of the cell wall of the mother cell, whereas the daughter cells stain more weakly (Fig. 2). Calcofluor-white staining revealed that the chains present in *swm1* cells after 8 hours of incubation at the restrictive temperature showed an intense staining at the neck region between mother and daughter cell, indicating that cytokinesis had been completed. In addition, the cells had an aberrant chitin deposition, because this polymer was also spread right across the surface of the cells. It has been described that mutants with severe cell wall defects (e.g. *fks1* or *gas1* mutants) or mutants with defects in polarized growth (e.g. *act1*, *cdc24* or *myo2*) show a significant increase in chitin synthesis and in some cell wall proteins as part of a cellular response to ensure cell viability (Madden et al., 1992; Popolo et al., 1997; Ram et al., 1998; Smits et al., 1999). Therefore, these results suggest that the depletion of Swm1p produces defects in mother-daughter cell separation and also in cell wall assembly during growth at high temperature.

Abnormal ultrastructure of the septum in *swm1* mutants cells

To analyse the nature of the defects produced in the cell wall by growth at 38°C in greater detail and to confirm that cytokinesis had been completed, wild-type and mutant cells were grown in sorbitol-containing medium for 8 hours and then prepared for electron microscopy (Fig. 3). In wild-type cells, a normal structure of the cell wall was observed, in which an inner layer (which often appeared as an electron-transparent layer) was surrounded by an external, osmiophilic layer, mainly corresponding to mannoproteins (Fig. 3A). Electron-microscopic examination of *swm1* cells revealed that the cell wall had a more-or-less-normal morphology, although this structure was thicker than in wild-type cells (Fig. 3B). In addition, although a septum was present in *swm1* cells, confirming that cytokinesis had been completed, the main differences between mutant and wild-type cells were found in this region, which separates mother and daughter cells. Wild-type cells contain a normal three-layer structure in which the primary septum (composed of chitin) is sandwiched between two secondary septa composed mainly of glucans and mannans (reviewed by Cabib et al., 2001). In these cells, the chitin ring was also observed as two electron-translucent regions located at both ends of the neck (Fig. 3C). Mutant cells lacking *SWM1* showed an extremely thick septum (Fig. 3D-F), although the three-layer structure was still apparent, because the chitin ring and the primary septum could be observed, but the region corresponding to the secondary septum was thickened. This morphology is reminiscent of the defects described for *chs2* mutants, in which an extremely thick septum with an amorphous aspect is formed, although no primary septum is present in *chs2* mutants (Shaw et al., 1991).

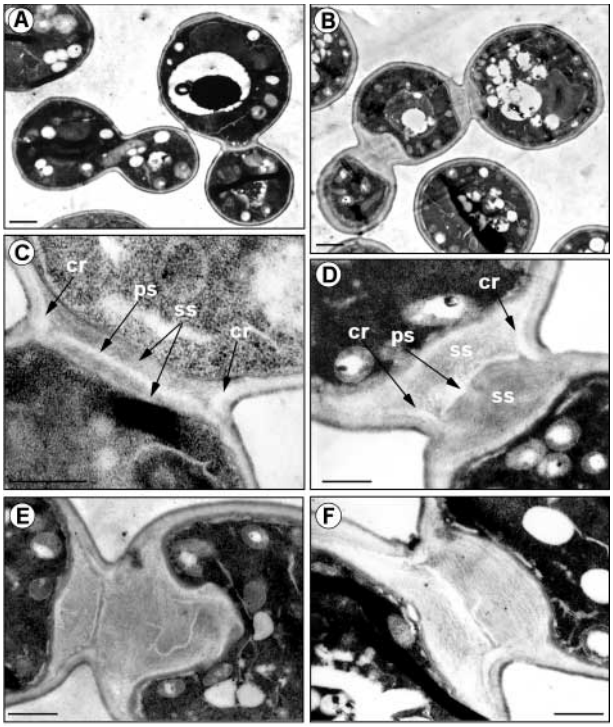

Fig. 3. Electron microscopic appearance of wild-type and *swm1* mutant cells incubated for 12 hours at 38°C. (A,C) Wild-type strain YPA24; (B,D-F) *swm1* strain YPA207. Cells grown in YEPD supplemented with 1 M sorbitol are shown at low magnification in (A,B), whereas higher magnifications of the septal region are shown in (C-F). The structure of the septum is clearly visible in wild-type cells, in which the chitin-rich primary septum (ps) is surrounded by the secondary septum (ss), composed mainly of glucans and mannoproteins. The chitin ring (cr) synthesized by Chs3p is visible at both sides of the primary septum. In mutant cells (D-F), the same structure is visible but the region corresponding to secondary septum (ss) is thickened. Scale bars: 1 μm (A,B), 0.5 μm (C-F).

Cell separation defect caused by lack of chitinase

Cell separation is a complex process that requires the controlled action of hydrolytic enzymes to degrade the components of the cell wall partially in the septum region (reviewed by Cabib et al., 1997). In *S. cerevisiae*, the major role in cell separation is performed by the chitinase encoded by *CTS1* (Kuranda and Robbins, 1991), which specifically hydrolyses the chitin present in the primary septum. Its activity can be readily assayed in culture supernatants (in which the activity accumulates) using a fluorogenic substrate. For this reason, chitinase activity was measured in culture supernatants from wild-type and mutant cells at different times after transfer of the fresh cultures to 38°C. As can be seen in Fig. 4A, in wild-type cells, chitinase activity accumulated slowly as the incubation progressed (from 0 U to over 130 U after 10 hours), whereas no such accumulation was detected in *swm1* cells. This result suggests that the defect in cell separation observed in cells lacking the *SWM1* gene at the restrictive temperature

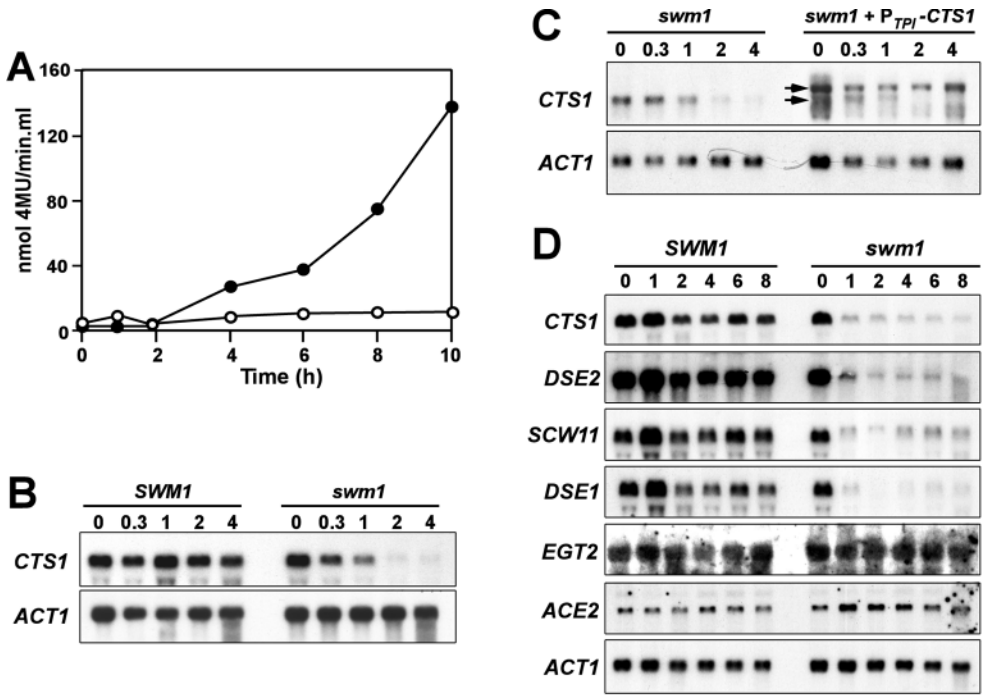

Fig. 4. (A) Chitinase activity in culture supernatants. Wild-type (YPA24, black circles) and mutant cells (YPA207, white circles) cells were grown overnight in YEPD medium at 28°C and then diluted into fresh YEPD medium that was transferred to 38°C. At the indicated times, aliquots were taken, cells were removed by centrifugation and chitinase activity was measured using the fluorogenic substrate 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside. Activity is expressed in nmoles 4-methylumbelliferone (4-MU) released per minute and per millilitre of culture. (B) Expression of *CTS1* in wild-type and mutant cells. RNA was purified from wild-type (strain TD28) and *swm1* (strain LS40) cells at the indicated times (hours) after transfer to 38°C. RNA blots were hybridized sequentially with radioactively labelled probes for *CTS1* and *ACT1*. (C) Analysis of *CTS1* mRNA stability. RNA was purified from *swm1* cells (strain LS40) transformed with vector (left, *swm1*) or pSU50 (right, *swm1*+P*TPI*-*CTS1*) containing the *CTS1* ORF under the control of the *TPI* promoter (P*TPI*-*CTS1* allele) at the indicated times (hours) after transfer to 38°C. RNA blots were hybridized with radioactively labelled probes for *CTS1* and *ACT1*. Arrows indicate the position of the transcript corresponding to the heterologous promoter (upper) or the chromosomal locus (lower). (D) Expression of genes involved in cell separation in *swm1* cells. RNA was purified from wild-type (strain TD28) and *swm1* cells (strain LS40) at the indicated times (hours) after transfer to 38°C. RNA blots were hybridized sequentially with radioactively labelled probes for *CTS1*, *DSE1*, *DSE2*, *SCW11*, *EGT2* and *ACE2*. The *ACT1* gene was used to test for equal RNA loading in all lanes.

might be due, among other reasons, to a reduction in chitinase activity.

In order to assess whether the defect in chitinase activity was due to a transcriptional defect or to some post-transcriptional modification, *CTS1* expression was analysed by northern blot after the cells had been transferred to 38°C. The results (Fig. 4B) indicated that, in contrast to control cells, in which no significant variation was detected, *CTS1* expression in *swm1* cells slowly decreased and almost disappeared after 2 hours of incubation at the restrictive temperature. To rule out the possibility that the reduction in *CTS1* expression was due to a defect in mRNA stability, the *CTS1* promoter was replaced with the constitutive *TPI* promoter, generating the P*TPI*-*CTS1* allele. The resulting construct was introduced into mutant cells and northern-blot analysis was performed after transfer of the cells to the restrictive temperature. The results showed that the mRNA corresponding to the chromosomal *CTS1* gene disappeared after 2 hours of incubation at 38°C (Fig. 4C, lower band), whereas the mRNA corresponding to the P*TPI*-*CTS1* construct (Fig. 4C, upper band) remained more or less constant during the experiment. The morphology of the cells carrying the P*TPI*-*CTS1* construct was also analysed by microscopic inspection, because the *CTS1* transcript levels produced from this construct were considerably higher than from the native gene and did not decrease when the cells were transferred to elevated temperatures. Overexpression of *CTS1* under these conditions did not complement the cell separation defect observed in *swm1* cells. Taken together, these results indicate that Swm1p is required for the correct expression of the *CTS1* gene during growth at 38°C and, consequently, for normal chitinase activity but that the separation defect is not only due to a reduction in this enzymatic activity.

Deleting *SWM1* affects the expression of genes involved in cell separation

Mother-daughter separation following cytokinesis is a complex process that requires the activity of the *CTS1* gene product, although other genes are also involved in this process. Apart from chitinase, other enzymatic activities must be present at the mother-daughter junction to dissolve additional cell wall components, and several known (Eng1p) or putative glucanases (Sun4p/Scw3p and Scw11p) are also required for the process to be successfully completed (Baladrón et al., 2002; Cappellaro et al., 1998; Mouassite et al., 2000). To examine the nature of the separation defect of *swm1* mutants in more detail and to

attempt to identify other genes whose expression might also be affected during growth at the restrictive temperature, the transcriptional profiles of wild-type (TD28) and *swm1* mutant cells (LS40) were analysed. Cells were grown asynchronously in YEPD medium at 38°C for 4 hours, and RNA was purified and used to compare the transcriptional patterns using microarray membranes (Tables 2, 3). Similar to the results of the northern-blot analysis, *CTS1* expression was markedly reduced in *swm1* cells (3.5- to 4.5-fold reduction), *CTS1* being one of the genes whose expression was most compromised in mutant cells during growth at the restrictive temperature. Additionally, the expression of several other genes encoding cell-wall-related proteins was reduced in *swm1* cells, such as *DSE2* (a gene that could be involved in mother-daughter separation), *ENG1*/*DSE4* (which encodes an endo-1,3-β-glucanase involved in cell separation), *RHK1* (a mannosyltransferase-encoding gene) or *FIT2* and *FIT3* (which encodes two glycosylphosphatidylinositol cell-wall proteins

Table 2. Genes with a higher expression in wild-type cells (more than twofold)

Gene or ORF[a]	WT/mut ratio[b]	Function[c]
CTS1	3.9	Endochitinase; cell separation.
DSE2	3.8	Protein involved in cell separation.
FIT2	3.6	GPI-CWP; facilitator of iron transport.
FIT3	3.0	GPI-CWP; facilitator of iron transport.
RNR2	3.0	Ribonucleotide reductase.
TIM54	2.8	Mitochondrial inner membrane protein.
HTA2	2.7	Histone H2A.
HTB2	2.6	Histone H2B.
SML1	2.5	Protein that negatively affects dNTP pools.
RNR4	2.4	Ribonucleotide reductase, small subunit.
CUP1	2.4	Metallothionein.
YMR009w	2.4	None known.
YOL014w	2.4	None known.
CPR6	2.3	Cyclophilin.
ENG1/DSE4	2.1	Endo-1,3-β-glucanase; involved in cell separation.
IMD4	2.1	Protein with similarity to inosine-5′-monophosphate dehydrogenase.
SCC3	2.1	Cyclophilin of the endoplasmic reticulum membrane.
SSA1	2.1	Cytoplasmic chaperone and heat shock protein of the HSP70 family.
SSA4	2.1	Protein chaperone of the HSP70 family.
SSA2	2.0	Cytoplasmic protein chaperone of the HSP70 family.
RHK1	2.0	Mannosyltransferase involved in *N*-glycosylation.
AHA1	2.0	Activator of heat-shock protein 90 ATPase.

[a]ORF, open reading frame.
[b]This is the mean of the normalized ratios of expression in the wild type and mutant strains (three independent experiments).
[c]According to *Saccharomyces* Genome Database.

Table 3. Genes with a higher expression in *swm1* cells (more than twofold)

Gene or ORF[a]	mut/WT ratio[b]	Function[c]
PIR3	5.3	Cell wall protein of the PIR family.
YLR042c	5.3	Putative glycosylphosphatidylinositol-anchored protein of unknown function.
SED1	4.0	Cell wall glycoprotein important for cell wall integrity and stress resistance.
GIC2	3.4	Effector of Cdc42p, important for bud emergence.
BNA4	3.1	Kynurenine 3-hydroxylase. Biosynthesis of nicotinic acid.
HXT4	3.0	Moderate- to low-affinity hexose transporter.
PRY2	2.8	Protein expressed under starvation conditions.
SVS1	2.7	Serine- and threonine-rich protein required for vanadate resistance.
COS8	2.5	Member of the COS family of subtelomere-encoded proteins.
ASH1	2.4	GATA-type transcription factor required for pseudohyphal growth.
AFR1	2.4	Protein involved in morphogenesis of the mating projection.
MAL32	2.3	Maltase.
COS3	2.2	Member of the COS family of subtelomere-encoded proteins.
PST1	2.2	Glycosylphosphatidylinositol protein of the Sps2p-Ecm33p-Ycl048p family.
PIR1	2.1	Cell wall protein of the PIR family.
HXT3	2.1	Low-affinity hexose transporter.
TOS6	2.1	Probable cell wall protein; has weak similarity to Mid2p.
YGR146c	2.1	Protein of unknown function.
YRO2	2.1	Protein paralog of Mrh1p; has similarity to heat-shock protein Hsp30.
SUL1	2.1	Sulphate permease.
YMR057c	2.1	None known.
CLN3	2.0	G1/S-phase-specific cyclin.
CCW14	2.0	Mannoprotein from the cell wall.

[a]ORF, open reading frame.
[b]This is the mean of the normalized ratios of expression in the mutant and wild type strains (three independent experiments).
[c]According to SGD.

involved (GPI-CWPs) in iron transport) (Baladrón et al., 2002; Colman-Lerner et al., 2001; Protchenko et al., 2001).

Conversely, the expression of a group of genes encoding glycosylphosphatidylinositol or protein with internal repeats (PIR) cell wall proteins (*SED1*, *PIR1*, *PIR3*, *PST1*, *CCW14*, *TOS6* or *YLR042c*) was significantly elevated in *swm1* cells, as was the expression of *SVS1* or *PRY2* (whose products are located in the extracellular region). The increase in expression of this group of cell-wall-related genes could be part of the response of *swm1* cells to cell wall damage when they are transferred to 38°C, similar to what has been described for the *fks1* mutant (Terashima et al., 2000). Additionally, the expression of *GIC2* (a putative effector of Cdc42p), *ASH1* (encoding a GATA-type transcription factor required for pseudohyphal growth) and *CLN3* was also increased in *swm1* cells.

Expression of Ace2p-regulated genes is decreased at high temperature

The previous results suggested that the expression of several genes whose products are involved in cell separation (e.g. *CTS1*, *DSE2* and *ENG1*) is reduced in *swm1* cells during growth at 38°C. Interestingly, transcription of these three genes is cell-cycle regulated, peaking during G_1 phase, and their expression depends on Ace2p (Baladrón et al., 2002; Colman-Lerner et al., 2001; Doolin et al., 2001). In addition, it has been shown that expression of two other genes (*SCW11* and *DSE1*) also requires the transcription factor Ace2p, because no transcripts can be detected in *ace2* mutant strains (Doolin et al., 2001). Because the data for *SCW11* and *DSE1* were not clear in our membrane filters, northern blot analysis was used to compare the expression of these genes between wild-type and mutant cells after transfer of the cells to the restrictive temperature. In wild-type cells, no significant change was detected in any of the genes (Fig. 4D). However, in *swm1* cells, the expression pattern of three genes (*DSE1*, *DSE2* and *SCW11*) was almost identical to that found for *CTS1*: the transcripts disappeared after 1-2 hours of incubation at 38°C. This suggests that the expression of the group of genes controlled by Ace2p is affected in a similar fashion in *swm1* cells. The expression of *EGT2*, another gene whose deletion results in the formation of chains of cells but that is regulated by Swi5p and Ace2p (Doolin et al., 2001; Kovacech et al., 1996), was also assessed by northern blot analysis, but no change was detected in the mutant cells (Fig. 4D). The normal transcription of *EGT2* in *swm1* cells, which is temporarily co-regulated (in late M early G_1 phase) with *CTS1*, indicated that the effect of *SWM1* deletion was not general for all late M and early G_1 genes, and that it could be specific for the set of genes that are regulated by Ace2p (e.g. *CTS1*, *DSE1*, *DSE2*, *SCW11* and *ENG1*).

The expression of *ACE2* was also analysed in wild-type and mutant cells after transfer to the restrictive temperature in order to test whether the phenotype of *swm1* cells might be due to a defect in the expression of this transcription factor during growth at high temperature, thus indirectly affecting the expression of *CTS1* and its co-regulated genes. *ACE2* expression was similar in wild-type and mutant cells after transfer to the restrictive temperature (Fig. 4D), ruling out the possibility of an indirect effect on *CTS1* expression. The effect of overexpression of *ACE2* was also analysed during growth at 38°C in order to test whether it could compensate some of the defects observed in *swm1* cells. We found that increasing the copy number of this transcription factor was not sufficient to restore the ability of mother and daughter cells to separate, nor to compensate for the cell lysis observed in mutant cells. Taken together, these results indicated that Swm1p is required for the correct expression, during growth at high temperature, of the group of genes that are regulated by the transcription factor Ace2p, and that this effect is not due to a transcriptional defect of *ACE2*.

Swm1p is required for entrance of Ace2p to the nucleus of the daughter cell

Ace2p is a zinc-finger protein synthesized during G_2 that has been shown to shuttle between the nucleus and cytoplasm in mother and daughter cells (Jensen et al., 2000; O'Conallain et al., 1999). During the M-to-G_1 transition, Ace2p accumulates specifically in the daughter nucleus, where it activates a daughter-specific expression program that is required for cell separation, and this accumulation requires the function of the Cbk1p-Mob2p kinase complex (Colman-Lerner et al., 2001; Weiss et al., 2002). To investigate the nature of the separation defect that *swm1* mutants show during growth at the restrictive temperature, the localization of Ace2p was analysed in wild-type and mutant cells using a fusion protein between Ace2 and yellow fluorescent protein (YFP) that has been described to localize to the daughter nucleus during growth at 28°C (Colman-Lerner et al., 2001). A similar localization pattern was found during growth at 38°C (Fig. 5A). Ace2-YFP accumulated in the nucleus of the daughter cell of large budded cells in both wild-type and *swm1* mutant cells during growth at 28°C (Fig. 5B), consistent with the absence of defect at this temperature. However, after the shift to the restrictive temperature, differences were observed in *swm1* cells. Thus, after 2 hours of incubation at 38°C, Ace2-YFP was still present in the daughter nucleus of some of the chains, although a significant proportion of cells showed diffuse fluorescence in the cytoplasm. After 8 hours of incubation, no fluorescence in the nucleus of mutant cells could be observed and, in some cases, a faint fluorescence accumulated at the bud necks and sites of polarized growth, where Cbk1p and Mob2p have been reported to localize (Colman-Lerner et al., 2001; Weiss et al., 2002). A similar diffuse cytoplasmic localization for Ace-GFP has been reported previously for mutants impaired in MEN function, such as *mob1-77* or *cdc14-1* (Weiss et al., 2002). Therefore, these results suggest that the cell separation phenotype observed in *swm1* cells during growth at the restrictive temperature could be due to a defect in accumulation of Ace2p in the nucleus of the daughter cell.

A similar result was obtained when the localization of the Ace2-G128E mutant protein was analysed. This mutant version of Ace2p was isolated as a dominant suppressor of the defects associated with the loss of the *CBK1* gene (Racki et al., 2000) and it has been shown to accumulate in mother and daughter nuclei (Fig. 5A) (Colman-Lerner et al., 2001). Localization of the mutant Ace2-G128-YFP protein was similar in wild-type and *swm1* cells during growth at 28°C (Fig. 5C), accumulating in both the mother and daughter

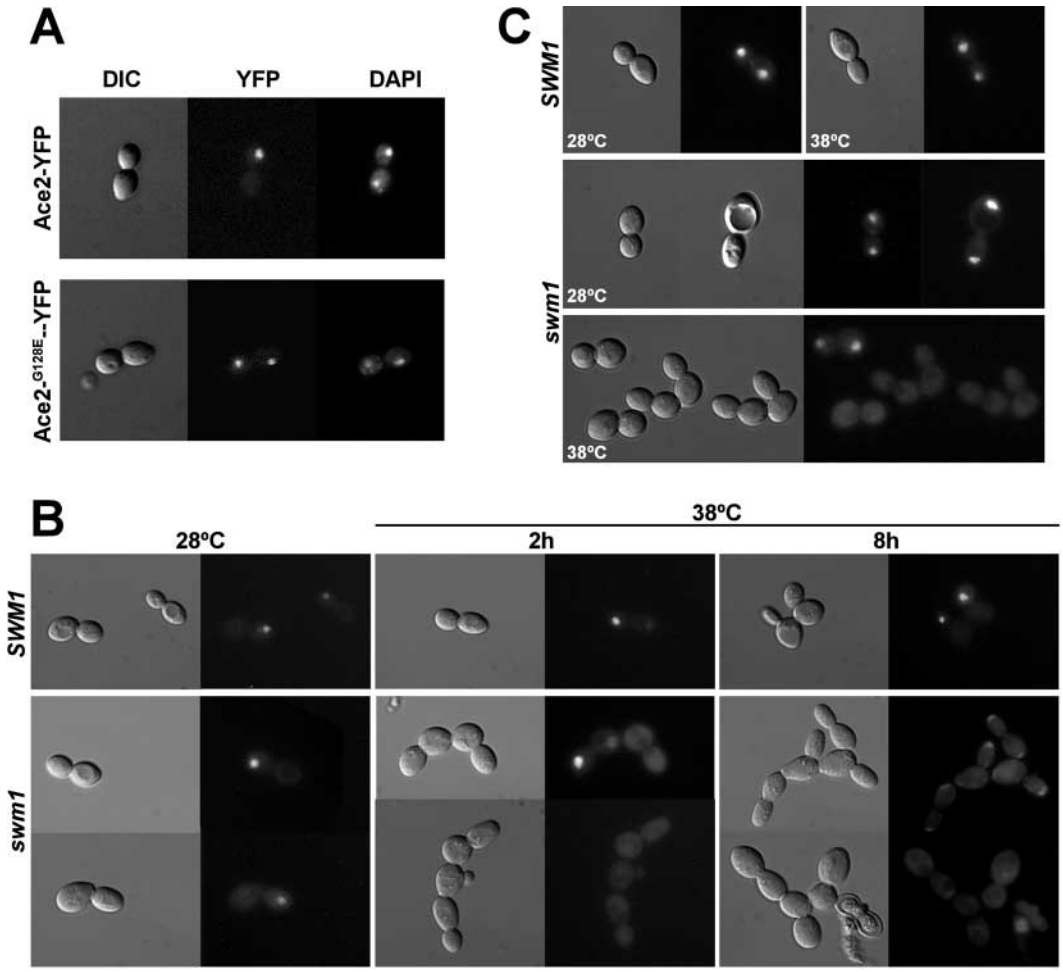

Fig. 5. Localization of Ace2p in wild-type and *swm1* mutants. (A) Diploid wild-type (YPA24) cells carrying the Ace2-YFP or Ace2-G128E-YFP fusion proteins were grown at 38°C for 4 hours and stained with DAPI. (Left) Differential interference contrast microscopy (DIC); (middle) YFP fluorescence; (right) DAPI fluorescence. (B) Diploid wild-type (YPA24) or *swm1* (YPA207) cells were transformed with plasmid pACE2-YFP and the localization of the protein was determined by fluorescence microscopy during growth at 28°C or after 2 hours or 8 hours of incubation at 38°C. (Left) Differential interference contrast microscopy; (right) YFP fluorescence. (C) Localization of Ace2-G128Ep in wild-type and *swm1* mutants. Diploid wild-type (YPA24) or *swm1* (YPA207) cells were transformed with plasmid pACE2-G128E-YFP and the localization of the protein was determined by fluorescence microscopy during growth at 28°C or after 4 hours of incubation at 38°C. (Left) Differential interference contrast microscopy; (right) YFP fluorescence.

nuclei. However, during growth at 38°C, the fluorescence in *swm1* was diffuse and cytoplasmic, indicating a defect in nuclear accumulation. Furthermore, expression of the *ACE2*-G128E allele from a yeast shuttle plasmid that suppresses a *cbk1* deletion (Racki et al., 2000) does not complement the separation defect of *swm1* cells (results not shown). Thus, these result suggest that Swm1p function is required for proper localization of Ace2p at the end of mitosis in order to allow mother and daughter cells to separate.

Cells with the *swm1* mutation have a defect in exit from mitosis at the restrictive temperature

Because Swm1p has recently been identified as one of the subunits of the APC (Hall et al., 2003; Passmore et al., 2003; Yoon et al., 2002), a complex that is essential for degradation of the mitotic cyclin Clb2 (Lim et al., 1998; Wasch and Cross, 2002), and because Cdc28p phosphorylation prevents nuclear accumulation of Ace2p (O'Conallain et al., 1999), the defect observed in *swm1* cells might be due to a defect in regulation of CDK/Clb2 activity during growth at the restrictive temperature. To study whether *swm1* cells show defects in the downregulation of Cdc28/Clb2 activity, we used nocodazole block-and-release experiments to analyse the events from mitosis until entry into the G_1 phase in wild-type and *swm1* mutants cells carrying an epitope-tagged version of the *CLB2* gene (the $CLB2^{HA}$ allele) (Schwab et al., 1997). Exponentially growing cultures were arrested with nocodazole (150 minutes), incubated at the restrictive temperature for 1.5 hours and then released from the block at the same temperature. Samples were taken at 10 minute intervals and processed for FACS analysis and protein detection. Flow cytometry analysis demonstrated that wild-type cells resumed growth 40 minutes after the release, whereas cell cycle progression was blocked in *swm1* cells (Fig. 6A). Microscopic inspection of the cells stained with propidium iodide confirmed that, 90 minutes after release,

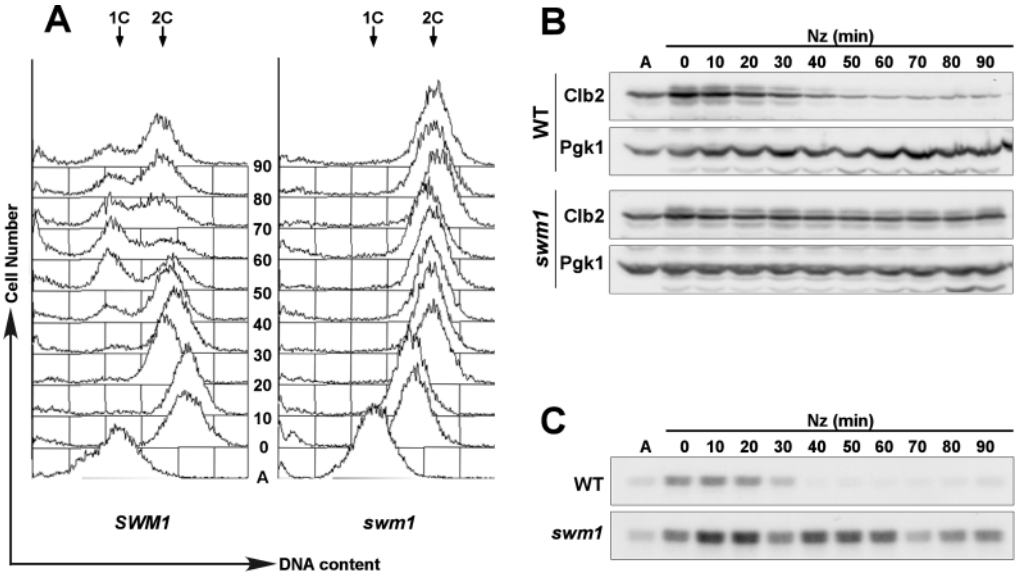

Fig. 6. Cells with the *swm1* mutation have a defect in exit from mitosis. Cultures of isogenic wild-type (W9317) or *swm1* mutant (SEP3) cells containing an epitope-tagged version of the *CLB2* gene (*CLB2*^{HA}) were arrested in mitosis with nocodazole (2.5 hours) before transfer to the restrictive temperature for the *swm1* mutation (38°C, 1.5 hours). Cultures were then released from nocodazole arrest and incubated at 38°C. Samples were taken at the indicated intervals after the release (minutes) and processed for FACS analysis (A), Clb2 and Pgk1 western analysis (B) or Clb2-associated H1 kinase activity assays (C). In each panel asynchronous cultures (A) are also shown for reference.

swm1 mutant cells were still arrested in telophase, with large buds and well-separated nuclei (data not shown).

Degradation of the mitotic cyclin Clb2p and the kinase activity associated with the Cdc28-Clb2 complex were monitored at the same time intervals in wild-type and mutants cells. Western-blot analysis using anti-HA antibodies revealed that, in wild-type cells, Clb2p protein levels began to decrease at the same time as the cells entered a new cell cycle (30-40 minutes after the release; Fig. 6B). However, degradation of Clb2p in *swm1* cells was much slower and did not completely disappear during the time analysed in this experiment. The activity of the Cdc28-Clb2p complex at each time point was also analysed in vitro after immunoprecipitation of the complex with anti-HA antibodies, using histone H1 as substrate (Fig. 6C). Similar to the results found for Clb2 protein levels, the kinase activity of the complex almost disappeared 40 minutes after the release in wild-type cells, whereas inactivation of kinase activity in *swm1* cells was delayed. Accordingly, these results indicate that, during growth at 38°C, *swm1* mutants show a delay in mitotic exit, as previously observed for other components of the APC complex (Irniger and Nasmyth, 1997; Irniger et al., 1995) and, consequently, they accumulate high CDK kinase activity levels.

Discussion

In this study, we describe and characterize the phenotypes associated with the loss of the *SWM1* gene during vegetative growth. Although *SWM1* was originally identified as a gene induced during the sporulation process and required for proper maturation of the spore cell wall (Ufano et al., 1999), the fact that a basal level of transcription was detected during vegetative growth prompted us to analyse the role of this gene during this part of the cell life cycle. While this work was in preparation, three independent groups reported the identification of Swm1p as one of the core subunits of the APC (Hall et al., 2003; Passmore et al., 2003; Yoon et al., 2002). The APC is a multiple-subunit E3 ubiquitin ligase responsible for initiating the metaphase-to-anaphase transition once the chromosomes have become attached and aligned at the metaphase plate, and for promoting mitotic exit once chromosome segregation is complete (reviewed in Harper et al., 2002; Peters, 2002; Zachariae and Nasmyth, 1999). Chromosome separation during the cell-cycle transition from metaphase to anaphase requires the proteolytic destruction of anaphase inhibitors, such as Pds1p and Cut2p, mediated by the APC (Zachariae and Nasmyth, 1999). APC-dependent proteolysis is also required at the end of mitosis to promote mitotic exit and cytokinesis. In particular, the degradation of B-type cyclins seems to be crucial for CDK inactivation, leading to spindle disassembly, cytokinesis and entry into a new round of DNA replication (Peters, 2002; Zachariae and Nasmyth, 1999). Most subunits of this complex are essential for cell viability (e.g. Apc1p, Apc2p, Cdc23p or Cdc27p), although some of them (Apc9p, Apc10p, Cdc26p and Swm1p) are dispensable for vegetative growth.

Our results indicate that *swm1* mutants show a clear delay in exit from mitosis, resulting in the accumulation of high Clb2 levels and the associated kinase activity during growth at high temperature. This indicates that Swm1p, like other subunits of the APC (Irniger and Nasmyth, 1997; Irniger et al., 1995), is required at the end of mitosis under these growth conditions. However, this delay in CDK inactivation does not lead to a complete block of the cell cycle, because the cells were able to divide and form chains of connected cells. This was not unexpected, because it has been shown that deletion of *CDH1/HCT1*, which leads to constant Clb cyclin levels throughout the cell cycle, is not lethal and does not prevent exit from mitosis (Schwab et al., 1997; Visintin et al., 1997). Additional mechanisms to reduce the activity of the Clb-CDK kinases at the end of mitosis are also present in the cells, such as direct binding of the Sic1p inhibitor (Mendenhall, 1993; Schwob et al., 1994; Schwab et al., 1997). The slight reduction in CDK activity observed in *swm1* cells at longer times after release could be brought about by such a mechanism.

Characterization of the defects of *swm1* mutant cells revealed that two main defects are associated with the loss of this gene in our background. First, in agreement with a role at the end of mitosis, in the mutant cells incubated at the

restrictive temperature cytokinesis and septum formation were aberrant and, in addition, the cells were unable to complete mother-daughter separation, forming chains that remained associated. Second, a defect in cell wall construction that caused the lysis of the cells when the incubation at elevated temperature was prolonged in the absence of osmotic support, a phenotype traditionally associated with mutants having defects in cell wall construction. The fact that these defects in *swm1* cells were only apparent at 38°C could be due to a defect in APC stability, which could be more compromised during growth at high temperature. This might be similar to the possible role of the Cdc26p subunit, which has been suggested to be required for maintaining complex stability during growth at high temperature, because it is essential for growth at 38°C but seems to be unnecessary for normal growth at 28°C (Araki et al., 1992). Interestingly, no such phenotypes have been previously described for other subunits of the APC, which raises the question of whether they are specific for *swm1* mutants or are also shared by mutants in other nonessential subunits of the complex. Experiments are under way in our laboratory to answer this question.

Swm1p is required for the expression of Ace2p-dependent genes at high temperature

Budding yeast cytokinesis occurs as cells exit mitosis, actomyosin ring contraction and septum formation accomplishing the fission of the mother and daughter cell cytoplasms (Bi et al., 1998; Schmidt et al., 2002). After cytokinesis is complete, the septum is degraded, allowing the separation of mother and daughter cells. From electron micrographs of *swm1* cells, it was apparent: (i) that the mother-daughter pair did not separate after the daughter cells had completed the septum; and (ii) that the structure of this region was extremely thick, in part resembling the defects described for *chs2* mutants. Cells deficient in Chs2p show a clumpy phenotype, with thick septa in which the primary septum is completely absent (Shaw et al., 1991). The aberrant morphology of the septum in *swm1* cells must have a different origin than in *chs2* mutants because, in *swm1* cells, the primary septum was clearly visible and presented a normal aspect. Furthermore, *CHS2* expression in wild-type and *swm1* mutants was similar during growth at the restrictive temperature (data not shown). One interesting possibility is that the synthesis of the secondary septum in wild-type cells would be tightly regulated in time and perfectly coordinated with other events of the cell cycle, and that this regulation (especially the signal to stop synthesis) is disturbed in *swm1* cells during growth at high temperature, resulting in cells with thick secondary septa.

Related to the defect in the synthesis of the septum, *swm1* cells also showed defects in mother-daughter cell separation. This process requires localized degradation of the components of the cell wall. Two main enzymatic activities required for partial degradation of the cell wall components have been described. Chitinase, encoded by the *CTS1* gene, is the enzyme that partially removes chitin from the primary septum (Kuranda and Robbins, 1991), whereas the Eng1p endo-1,3-β-glucanase might degrade the β-1,3-glucans present in the secondary septum (Baladrón et al., 2002). Here, we show that one of the reasons for the separation defect observed in *swm1* cells is a reduction in extracellular chitinase activity, a consequence of a rapid decrease in *CTS1* transcription after transfer of the cells to the restrictive temperature. In addition to *CTS1*, a genome-wide transcriptional analysis allowed the identification of other genes whose expression was reduced in *swm1* during growth at restrictive temperature. The products of some of these genes are also involved in cell separation, such as Eng1p, Scw11p, Dse1p and Dse2p (Baladrón et al., 2002; Cappellaro et al., 1998; Doolin et al., 2001). Interestingly, all these genes are dependent on the transcription factor Ace2p (Dohrmann et al., 1992; Doolin et al., 2001).

Nuclear localization of Ace2p at the end of mitosis requires Swm1p

Cell separation and septum degradation occur only after mitotic exit and completion of cytokinesis, indicating the existence of a tight temporal coordination of the events that occur during the mitosis-to-G_1 transition. CDK activity plays a negative role in the regulation of Ace2p localization, preventing its entry into the nucleus until late mitosis and early G_1 (O'Conallain et al., 1999). It has been proposed that cell-cycle regulated entry of Ace2p into the nucleus would be associated with dephosphorylation of one threonine and two serine residues located at the C-terminus of the protein, which are potential Cdc28p phosphorylation sites. Specific accumulation of Ace2p in the daughter nucleus during late mitosis also requires the participation of Mob2p and Cbk1p (Colman-Lerner et al., 2001; Weiss et al., 2002). Mob2p-Cbk1p form a complex with Ace2p that is co-transported into the nucleus, and it has been suggested that Mob2p-Cbk1p-mediated phosphorylation of Ace2p inhibits its export from the nucleus, thus allowing its accumulation in the daughter nucleus (Weiss et al., 2002). In addition, MEN signalling is also required for correct localization of Mob2p and Ace2p, because they do not localize properly to the nucleus in *cdc14-1* or *mob1-77* mutants (Weiss et al., 2002). Interestingly, in budding yeast, the protein phosphatase Cdc14p is required for dephosphorylation of Cdh1p, Sic1p and its transcription factor Swi5p at the end of mitosis (Visintin et al., 1998), suggesting that this phosphatase might also be involved in dephosphorylation of Ace2p.

Our results indicate that *swm1* mutants have a defect in the nuclear accumulation of Ace2p when incubated at high temperature, because the Ace2-YFP fluorescence was mainly found in the cytoplasm under these conditions. Interestingly, the Ace2-G128E-YFP protein, which shows nuclear localization independent of Cbk1p, was also found in the cytoplasm at the restrictive temperature, suggesting that the nature of the defect in *swm1* cells would be different from that of *cbk1* cells. This is supported by the fact that the *ACE2-G128E* allele complements the separation defect of *cbk1* mutants (Racki et al., 2000) but fails to rescue that of *swm1* cells. These results therefore suggest that Swm1p acts independently of Cbk1-Mob2p to regulate the nuclear import of Ace2p. The cytoplasmic localization of both Ace2-YFP and Ace2-G128E-YFP in *swm1* cells during growth at the restrictive temperature is most probably due to the defect in Clb2p degradation and the consequent delay in CDK inactivation observed in *swm1* mutants grown under restrictive conditions, because CDK activity acts as a negative signal for Ace2p nuclear entry (O'Conallain et al., 1999). Although we

favour this idea, it is also possible that the function of Swm1p is specifically required for the polyubiquitination of a substrate or set of substrates whose degradation is essential for nuclear accumulation of Ace2p during growth at high temperature. It is interesting that no defect in the transcription of Swi5-regulated genes was observed in our whole-genome analysis, because nuclear localization of Swi5p is also negatively regulated by Cdc28p phosphorylation (Moll et al., 1991; Nasmyth et al., 1990). Thus, these results might simply indicate different sensitivities to the phosphorylation level between Ace2p and Swi5p or different affinities for the phosphatase that catalyses the dephosphorylation reaction. Alternatively, because additional proteins are required for the nuclear accumulation of Ace2p, it is also possible that some of them could be targets of the cell-cycle control as a redundant mechanism to ensure that activation of the daughter-specific gene expression program that leads to cell separation will only be activated after mitosis is complete.

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