The endo-β-1,3-glucanase eng1p is required for dissolution of the primary septum during cell separation in *Schizosaccharomyces pombe*

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Summary

Schizosaccharomyces pombe **cells divide by medial fission throughout contraction of an actomyosin ring and deposition of a multilayered division septum that must be cleaved to release the two daughter cells. Although many studies have focused on the actomoysin ring and septum assembly, little information is available concerning the mechanism of cell separation. Here we describe the characterization of *eng1*$^+$, a new gene that encodes a protein with detectable endo-β-1,3-glucanase activity and whose deletion is not lethal to the cells but does interfere in their separation. Electron microscopic observation of mutant cells indicated that this defect is mainly due to the failure of the cells to degrade the primary septum, a structure rich in β-1,3-glucans, that separates the two sisters cells. Expression of *eng1*$^+$ varies during the cell cycle, maximum expression being observed before septation, and the protein localizes to a ring-like structure that surrounds the septum region during cell separation. This suggests that it could also be involved in the cleavage of the cylinder of the cell wall that covers the division septum. The expression of *eng1*$^+$ during vegetative growth is regulated by a C2H2 zinc-finger protein (encoded by the SPAC6G10.12c ORF), which shows significant sequence similarity to the *Saccharomyces cerevisiae* ScAce2p, especially in the zinc-finger region. Mutants lacking this transcriptional regulator (which we have named *ace2*$^+$) show a severe cell separation defect, hyphal growth being observed. Thus, ace2p may regulate the expression of the *eng1*$^+$ gene together with that of other genes whose products are also involved in cell separation.**

Movies available online

Key words: Cell separation, β-1,3-glucanase, Ace2p, Cytokinesis, Primary septum hydrolysis

Introduction

Cytokinesis is the final stage of the cell cycle during which the daughter cells physically separate and become two independent entities. In a variety of organisms, this is achieved by the use of an actomyosin-based contractile ring, which provides the force necessary for cell cleavage. Newly synthesized membrane is inserted at the division site, concomitant with constriction of the actomyosin ring (reviewed by Guertin et al., 2002; Hales et al., 1999; Robinson and Spudich, 2000). In yeast and fungi, cytokinesis is coupled with the synthesis of a separation septum: an extension of the cell wall that completely surrounds these cells.

Schizosaccharomyces pombe cells are cylindrical, growing by elongation of their ends and dividing by medial septation followed by cleavage of the septum, a process known as binary fission (for a review, see Johnson et al., 1982). Cytokinesis and cell division are brought about by the action of the actomyosin ring, whose constriction is perfectly coordinated with the synthesis of the primary septum. Genetic studies have identified many genes that are important in the different steps of cytokinesis, such as for the positioning and assembly of the actomyosin ring, the localization of actin patches at the site of cell division, and for the physical assembly of the division septum (Balasubramanian et al., 1998; Balasubramanian et al., 2000; Chang et al., 1996; Gould and Simanis, 1997; Le Goff et al., 1999a; McCollum and Gould, 2001; Simanis, 1995). The *mid1*$^+$, *plo1*$^+$ and *pom1*$^+$ genes are required for the division plane to be established and for correct positioning of the actomyosin ring (Bähler and Pringle, 1998; Bähler et al., 1998a). Once the division plane has been established, the medial ring is formed, and this process is dependent on many genes, including *cdc3*$^+$, *cdc4*$^+$, *cdc8*$^+$, *rng2*$^+$, *rng3*$^+$ and *myo2*$^+$ (reviewed by Balasubramanian et al., 2000; Guertin et al., 2002; Le Goff et al., 1999a). F-actin patches are subsequently recruited to the medial ring, where they form the actomyosin-contractile ring. Coordination of ring contraction and the nuclear cycle requires a network of regulatory proteins that are collectively referred to as the septation initiation network (SIN). These proteins also control the formation of the primary septum during constriction of the actomyosin ring. Genetic studies have indicated that activation of the SIN pathway might regulate cps1p, a β-1,3-glucan synthase subunit essential for

the assembly of the division septum (Le Goff et al., 1999b; Liu et al., 2000). This septum has a three-layer structure, with a central primary septum (mainly composed of linear β-1,3-glucan) surrounded on both sides by two secondary septa (composed of β-1,6-branched β-1,3-glucan and β-1,6-glucan) (Humbel et al., 2001).

Cell separation requires dissolution of the primary septum for the daughter cells to become two independent entities. Upon completion of mitosis, the primary septum undergoes rapid autolytic degradation, accompanied by local erosion of the adjacent regions of the cell wall. Although the mechanism of actomyosin ring assembly, constriction and formation of the division septum have received considerable attention, very little is known about how the cleavage of the cell wall and primary septum is achieved. To address this question, mutants showing complete or partial defects in cell separation, resulting in the formation of chains of cells, have been previously isolated and classified in 16 different groups, named *sep1*$^+$ to *sep16*$^+$ (Grallert et al., 1999; Sipiczki et al., 1993). *sep1*$^+$ encodes a transcription factor highly homologous to the HNF-3/forkhead family present in higher eukaryotic cells and also in other microorganisms (Ribár et al., 1997). Interestingly, another two members of this family of transcription factors (the *Saccharomyces cerevisiae* Fkh1p and Fkh2p proteins) have also been implicated in cell separation (Hollenhorst et al., 2000). *sep15*$^+$ has recently been cloned and characterized, and found to encode an essential protein that shows a high degree of similarity to Med8p, one of the subunits of the mediator complex of *S. cerevisiae* RNA polymerase II (Zilahi et al., 2000). In addition, recent work has pointed to the importance of the exocyst complex in cell separation (Wang et al., 2002). The exocyst is an octameric protein complex present in many organisms and is involved in tethering vesicles to specific sites on the plasma membrane. Based on the fact that mutants in different subunits show a defect in cell separation, it has been proposed that this complex might be involved in the delivery of hydrolytic proteins that are important for cell cleavage.

Here we demonstrate that cell separation in *S. pombe* is an enzymatic process that requires the hydrolysis of certain components of the cell wall. The characterization of *eng1*$^+$, a gene encoding a protein with endo-β-1,3-glucanase activity that transiently localizes to the septum region in a ring-like structure, indicated that this protein is involved in cell separation. According to observations obtained from transmission electron microscopy, the eng1p glucanase seems to be required for dissolution of the β-1,3-glucan material that composes the primary septum.

Materials and Methods

Strains, growth conditions and genetic manipulations

The *S. pombe* strains used in this study are listed in Table 1. Yeast cells were grown on YES medium or minimal media (EMM) with appropriate supplements (Moreno et al., 1991). Yeast transformations were performed with the lithium acetate method (Ito et al., 1983). For overexpression experiments using the *nmt1*$^+$ promoter, cells were grown in EMM containing 15 μM thiamine up to the logarithmic phase. Then, the cells were harvested, washed three times with EMM, and inoculated in fresh medium (without thiamine) at an OD_{595} of 0.01.

Synchronization of strains carrying the thermosensitive *cdc25-22* mutation was achieved by growing the cells at the permissive temperature (25°C) to early log phase (OD_{595}=0.5) and then shifting the cultures to 37°C for 4 hours. Cells were released from arrest by transfer to 25°C, and samples were taken every 20 minutes.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
h20	*h*$^-$ *leu1-32*	S. Moreno*
h123	*h*$^-$ *ura4-d18*	S. Moreno*
PPG148	*h*$^-$ *ura4-d18 cdc25-22*	S. Moreno*
YAB14	*h*$^-$ *ura4-d18 eng1::kanMX4*	This study
YAB18	*h*$^-$ *leu1-32 eng1-GFP*	This study
YAB60	*h*$^-$ *ura4-d18 cdc25-22 eng1::kanMX4*	This study
LE25	*h*$^-$ *ura4-d18 ace2::kanMX4 ura4*$^+$	This study
LE26	*h*$^-$ *ura4-d18 spaC4G8.13c::ura4*$^+$	This study

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Plasmid and DNA manipulations

The oligonucleotides used for different DNA manipulations are shown in Table 2. Construction of plasmid pAB10, carrying the *eng1*$^+$ coding sequence under the control of the *nmt1*$^+$ promoter, was achieved by PCR amplification of the coding sequence of the endo-β-1,3-glucanase using oligonucleotides 423 and 302 (which introduced *Xho*I and *Bgl*II sites at the ends) and cloning of the resulting fragment between the *Xho*I and *Bam*HI sites of plasmid pREP3X.

Construction of null mutants and GFP-tagged strains

The entire coding sequences of *eng1*$^+$ (SPAC821.09), *ace2*$^+$ (SPAC6G10.12c), and SPAC4G8.13c were deleted to create the null mutants by replacing the coding sequences by the *ura4*$^+$ or *kanMX4* cassette. The deletion cassettes were constructed using the recombinant PCR approach described by Wach (Wach, 1996). For this purpose, DNA fragments of 300-500 bp corresponding to the 5′ and 3′ flanking regions of each gene were PCR amplified using specific oligonucleotide pairs. The resulting fragments were then fused, by recombinant PCR, to the *kanMX4* cassette (which confers resistance to the G418 antibiotic) or to the *ura4*$^+$ gene. For *eng1::kanMX4*, the oligonucleotide pairs 755-359 and 360-756 were used to amplify, respectively, specific regions of the 5′ and 3′ ends, which were subsequently fused to the *KanMX4* cassette obtained from plasmid pFA-*KanMX4* (Wach et al., 1994). A similar approach was implemented to construct the *ace2::kanMX4* cassette (using oligonucleotides 675, 676, 677 and 678) or the SPAC4G8.13c::*ura4*$^+$ module (with oligonucleotides 679, 680, 681 and 682).

The C-terminally GFP-tagged strain was constructed by direct chromosome integration of PCR fragments generated using the pFA6a-GFP-kanMX6 plasmid as a template and oligos 434 and 435 (Bähler et al., 1998b). The amplified fragment contained the GFP coding region fused in frame to the last codon of the *eng1*$^+$ gene and the *kanMX6* cassette that was used to select for transformants. Correct integration of the DNA fragment was verified by PCR.

RNA isolation and northern blot analysis

Cells (1.3×10^9) were collected at different time intervals after release from the restrictive temperature (37°C) or from different mutant strains, and total RNA was prepared using the method described previously (Percival-Smith and Segall, 1984). For northern blot analysis, 5 μg of RNA was used. The DNA probes used to detect the different transcripts were: *eng1*$^+$, a 534 bp internal fragment (from +1901 to +2435) obtained by PCR; *ura4*$^+$, a 1.7 kb *Bam*HI-*Hin*dIII fragment obtained from plasmid pSK-*ura4*$^+$; and

Table 2. Oligonucleotides used for gene deletion and epitope tagging

Name	Sequence (5′-3′)
***eng1::kanMX4* cassette**	
755	TAACTCGAGCTTAAAAAGGGCTGGCTGTTAAG
359	GGACGAGGCAAGCTAAACAGAAGTTCCTAACAATAAAGTAAGTGA
360	GAGCTCGAATTCATCGATGATGCATGACCAAAGTCCGGAATTGTA
756	TAAAGATCTATACCATCGTTTCATCTTTGTTG
P_{nmt1}-*eng1*$^+$ fusion	
423	TAACTCGAGGGAACTATGAGTTCCTATTTA
302	TAAAGATCTTTTGTTTTGCATCGTTGAGTTCG
***eng1*$^+$-*GFP* fusion**	
434	GCTTGTGGTAATGCGTGCTATGACTCCTCTATATACGGTTGCTCCAATGGTGCACTTGTTGCTGCTCGGATCCCCGGGTTAATTAA
435	TATCCAAAAAGGGTTTCAAGTTGAGAGTAGTTCACGTTCCAGACGTGTATTATGAACAAAATGTAGGAATTCGAGCTCGTTTAAAC
***ace2::kanMX4* cassette**	
675	CTGGGTCTGTGTTTACACTTG
676	GGGGATCCGTCGACCTGCAGCGTACTGTCTTGTTTACTTAGGATAGCGAG
677	AACGAGCTCGAATTCATCGATGATATAATGATGACAGCTGTACAACTTG
678	GGTGCGAGTGAAAGCAAACAC
SPAC4G8.13c*::ura4*$^+$ cassette	
679	TCTCATTTGTCGCACTCTCTC
680	GCAAGCTTATCGATACCGTCGCCACTGACCTTTGACGCTCCATCGATG
681	TAAGCTGGCATGCCTGCAGCCCGGAAGACAGCAGTGGCCTCGACAAGC
682	TAAACAACTCTTGGTCGACGG

act1$^+$, a 1.1 kb fragment containing the whole coding region obtained by PCR.

Microscopy techniques

For light microscopy, cells were fixed in 3.7% formaldehyde and stained with DAPI (4′,6-diamino-2-phenylindole) or Calcofluor White as previously described (Balasubramanian et al., 1997). Samples were viewed using a Leica DMRXA microscope equipped for Nomarski optics and epifluorescence and photographed with a Photometrics Sensys CCD camera. For time-lapse photography, cells were mounted on medium containing 0.5% agar. Confocal microscopy was performed on a Zeiss Axiovert microscope equipped with a LSM510 laser scanning system, and the images were analysed with LSM510 software.

For scanning electron microscopy (SEM), cells were harvested, washed in 0.1 M sodium phosphate buffer, pH 7.4, prefixed with glutaraldehyde (5% glutaraldehyde in phosphate buffer) for 1 hour, washed twice in buffer, and placed in 1% osmium tetraoxide for 1 hour at 4°C. The material was subsequently washed in distilled water and dehydrated in a graded acetone series. The dehydrated cells were mounted on specimen holders, air-dried, coated with gold, and examined under a Zeiss DSM 940 scanning electron microscope. For transmission electron microscopy (TEM), the cells were stained with potassium permanganate according to the protocol described previously (Johnson et al., 1973). Electron photomicrographs were taken with a Jeol Jam-1010 electron microscope.

Assay for β-glucanase activity

β-1,3-glucanase activity was assayed in cell extracts or in culture supernatants as previously described (Baladrón et al., 2002). Determination of the reducing sugars released in the reactions was performed by the methods of Somogyi (Somogyi, 1952) and Nelson (Nelson, 1957). One unit of activity was defined as the amount of enzyme that catalysed the release of reducing sugar groups equivalent to 1 μmol of glucose per hour, and specific activity was expressed as units per milligram of protein or per milligram of dry cell weight. For activity against PNPG, the amount of *p*-nitrophenol released was determined spectrophotometrically by measuring optical density at 410 nm. One unit of enzyme catalyzed the release of 1 μmol of *p*-nitrophenol per hour under the reaction conditions used.

Results

eng1p is an endo-β-1,3-glucanase

A search of the *S. pombe* database (Sanger Centre) for proteins with sequence similarity to the *S. cerevisiae* endo-β-1,3-glucanase ScEng1p allowed the identification of the eng1p protein (the product of the SPAC821.09 ORF). This protein shares common features with ScEng1p, such as the presence of a predicted sequence signal at the N-terminal end, a region with a high concentration of Ser/Thr, and a domain of around 700 amino acids with strong sequence similarity to members of the glycosyl hydrolase family 81 (GHF81) (Henrissat and Bairoch, 1996; Baladrón et al., 2002; Mouyna et al., 2002). However, the structure of the *S. pombe* protein is slightly different from that of *S. cerevisiae*, because the Thr-rich region is located at the C-terminal end of the proteins instead of at the N-terminal region (Fig. 1A).

To analyse whether the protein identified on the basis of sequence similarity also showed endoglucanase activity, the coding region was PCR-amplified using specific oligonucleotides and was cloned under the control of the strong inducible promoter *nmt1*$^+$, which is regulated by thiamine (Forsburg, 1993). The resulting plasmid (pAB10) was introduced into strain h20 and β-glucanase activity was assayed on cell extracts using laminarin (a β-1,3-glucan polymer) as substrate. The results showed that in presence of thiamine (*nmt1*$^+$ promoter repressed) the level of β-glucanase activity was similar to that found in cells carrying the vector alone (pREP3X). However, in cells that had been growing for 16 hours in the absence of thiamine (*nmt1*$^+$ promoter induced), a four- to fivefold increase in β-glucanase activity was observed (Fig. 1B). This result indicates that the *S. pombe* eng1p has β-glucanase activity.

Substrate specificity was tested under the same conditions described above, using two other substrates: pustulan (a linear β-1,6-glucan) and p-nitrophenyl-β-D-glucopyranoside (PNPG), a synthetic compound that is only cleaved by glucanases with an exo-hydrolytic mode of action. As shown in Fig. 1C, no activity was detected against these two

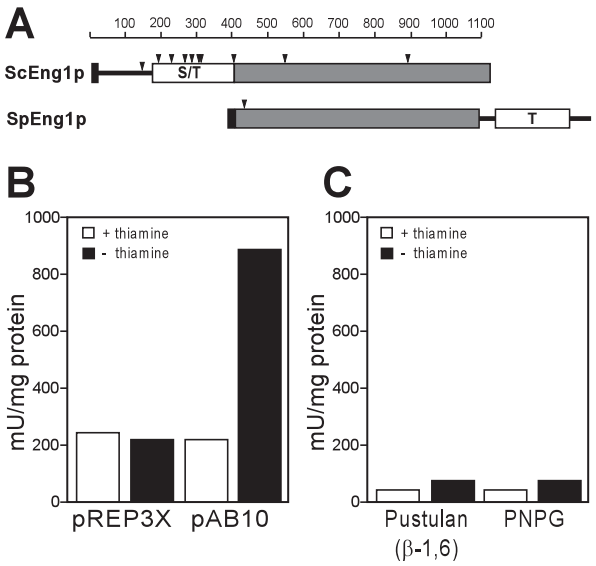

Fig. 1. eng1p is an endo-β-1,3-glucanase. (A) Schematic representation of the *S. cerevisiae* (ScEng1p) and *S. pombe* endo-β-1,3-glucanases (Speng1p). The structure of each protein is shown at the same scale (indicated at the top as the number of amino acids), with a gray rectangle indicating the conserved region between the proteins. A black box in the N-terminal region indicates the predicted secretory signal sequence, while triangles mark the position of putative N-glycosylation sites. White boxes represent Ser/Thr-rich regions (indicated by S/T) or the Thr-rich domain (marked with a T). (B) β-glucanase activity against laminarin (β-1,3-glucan) in cells (strain h20) transformed with plasmid pAB10 (carrying P_{nmt1}-*eng1*) or vector alone (pREP3X). Cells were grown for 16 hours in the presence (white bars) or absence (black bars) of thiamine to induce the expression of the *eng1*$^+$ gene. (C) Enzymatic activity of cells overexpressing *eng1*$^+$ against pustulan (β-1,6-glucan) or *p*-nitrophenyl-β-D-glucoside (PNPG).

compounds, confirming that eng1p, as previously described for other GFH81 proteins (Baladrón et al., 2002), is specific for β-1,3-glucans and that it acts by cleavage of the internal bonds of the polymer chains.

eng1p is involved in cell separation in *S. pombe*

To investigate the function of eng1p endo-β-1,3-glucanase during the cell cycle of fission yeast, *eng1*Δ cells were generated using a PCR-based system (Wach, 1996). *eng1*Δ mutants were viable at all temperatures and showed no apparent growth defect in either rich or minimal medium. When the morphology of mutant cells was analysed by microscopic observation, a defect in cell separation was apparent: most of the mutant cells in the culture were clustered in groups of four cells (95% of cells; *n*=210 cells) compared with the wild-type strain (3% of cells; *n*=234 cells). Staining with Calcofluor, a dye that in *S. pombe* shows greater affinity for the β-1,3-glucans present in the primary septum that separates the two sisters cells, revealed that a septum had been assembled between the cells (Fig. 2). In addition, DAPI staining of DNA indicated that each cell contained a single nucleus (not shown). These results therefore indicate that *eng1*Δ cells are able to complete nuclear segregation and

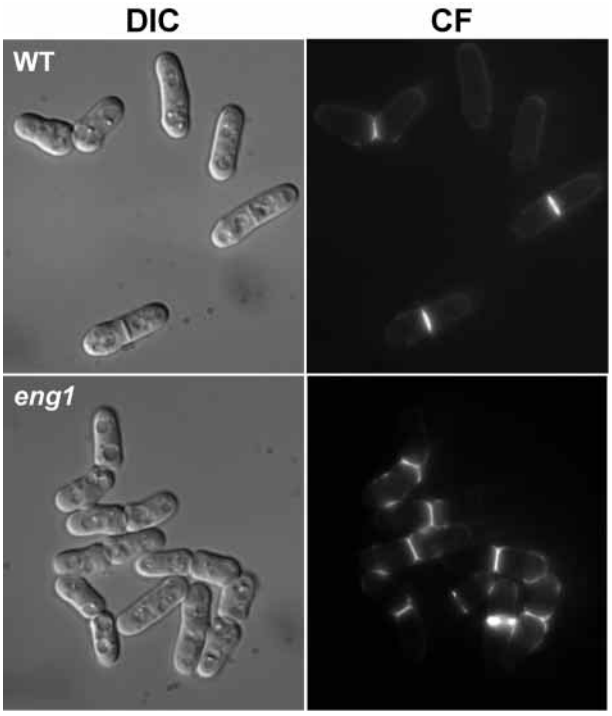

Fig. 2. Microscopic appearance of wild-type and *eng1*Δ mutant cells. Wild-type (h20) or the isogenic *eng1*Δ mutants cells (YAB14) were grown in rich medium (YES), washed, and stained with Calcofluor. Photographs of differential interference contrast microscopy (DIC) or Calcofluor-stained cells (CF) are shown.

cytokinesis normally but that they have a defect in cell separation, resulting in the formation of groups of cells.

To analyse the separation defect in greater detail, cell growth was monitored using time-lapse differential interference contrast (DIC) microscopy. In wild-type cells, cell division produced two equivalent cells that separated immediately after cell division (Fig. 3A). In contrast, *eng1*Δ cells failed to complete septum dissolution and cell separation (Fig. 3B, black arrows). However, even under these conditions, they were able to reinitiate polarized growth and undergo a new round of mitosis and cell division, constructing new septa (white arrows) that resulted in the formation of groups of four cells (see supplementary movies: http://jcs.biologists.org/supplemental). A similar morphology, four connected cells, was observed when cells were prepared for scanning electron microscopy (Fig. 3C). All of these results therefore suggest that the protein encoded by *eng1*$^+$ is involved in cell separation.

eng1p is required for degradation of the primary septum

To further assess the nature of the separation defect of *eng1*Δ mutants, transmission electron microscopy was used to compare the morphology of the septum region between the wild-type and the mutant strain. In wild-type cells, the three-layer structure of the septum was apparent, with a clear primary septum surrounded by two darker layers corresponding to the secondary septum (Fig. 4A). In these cells, it was observed that the primary septum was being degraded centripetally, from the

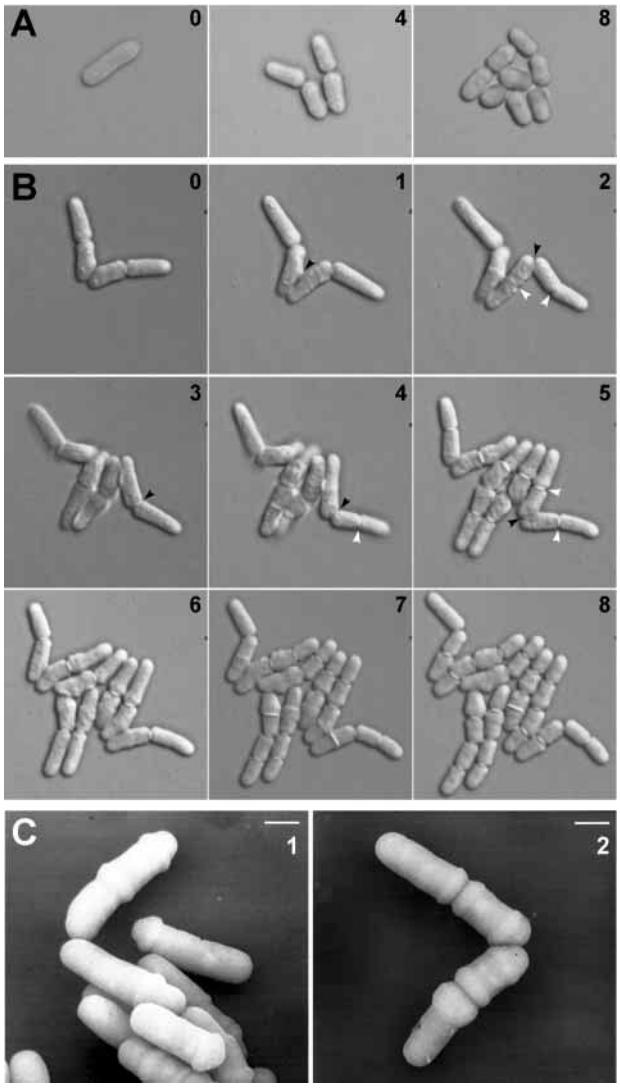

Fig. 3. Time-lapse DIC images of wild-type (A) and *eng1* mutant cells (B). Black arrowheads mark the position of the previous septum while white arrowheads indicate the new septum formed before dissolution of the previous one. Numbers indicate the hours elapsed. (C) Scanning electron microscopy of wild-type (panels 1) and *eng1*Δ cells (panel 2). Bars, 2 μm.

cortex to the midpoint of the septum, and no remnants of this structure were seen in the region from which the two cells had already detached themselves. Inspection of the septal region in mutants lacking the eng1p endo-β-1,3-glucanase also revealed the typical three-layer structure, indicating that the septum had been normally assembled. However, in this case it was evident that cell separation had not proceeded in the usual way, because abundant cell wall material that had not been correctly degraded was present between the two sister cells (indicated by arrows in Fig. 4B,C). Interestingly, most of the extra material clearly corresponded to the β-1,3-glucan-rich primary septum. These observations therefore indicate that cell separation in *S. pombe* requires enzymatic degradation of the primary septum, and that the endo-β-1,3-glucanase eng1p is required for this process.

The endo-β-1,3-glucanase eng1p localizes to the septal region

To determine the subcellular localization of eng1p, the coding sequence of the green fluorescent protein (GFP) was fused in-frame before the *eng1*$^+$ stop codon using a PCR based approach (Bähler et al., 1998b). The C-terminus was chosen because a putative signal sequence is present at the N-terminal end of the protein, which could be important for entry into the secretory pathway and for the proper localization of this β-glucanase. All the resulting strains contained the fusion under the control of the native *eng1*$^+$ promoter and the fusion protein was functional.

Since eng1p may exert its function in a cell-cycle-regulated manner during cytokinesis, the localization of the eng1p-GFP protein was first analysed in a synchronous population of *S. pombe* cells. To this end, a strain containing a *cdc25-22* thermosensitive allele, which arrests at the G_2/M boundary, and the eng1p-GFP fusion was constructed. Cells were arrested by incubation at the restrictive temperature for 4 hours and then transferred to the permissive temperature. The fluorescence corresponding to the eng1p-GFP appeared 90 minutes after release, coinciding with the first peak of synchronous septation (Fig. 5). Interestingly, in all the cells observed, eng1p-GFP was localized to the region of the cell where polarized growth occurs at this time point (i.e. the septum). Thus, eng1p appears to be synthesized periodically during the cell cycle and seems to accumulate in the septum region, in agreement with the proposed role in cell separation.

The localization of the eng1p-GFP fusion was further analysed in cells that had also been stained with Calcofluor to assess septum formation. The localization of eng1p-GFP was found to be almost coincident with the position of the primary septum, as shown when the fluorescence from Calcofluor and GFP were overlain (Fig. 6A). Interestingly, the green fluorescence observed in cells carrying the eng1p-GFP fusion reporter was more intense in a circumference surrounding the primary septum (indicated by the Calcofluor fluorescence) rather than in the septum itself, suggesting that the protein could be localized in a ring structure. To confirm this observation, confocal microscopy was used. The results of the 3D reconstruction of the green fluorescence found in cells carrying the eng1p-GFP indicated that in the cell wall this β-glucanase is localized to a ring-like structure that completely surrounds the primary septum (Fig. 6B; see supplementary movies online).

eng1$^+$ expression and β-1,3-glucanase activity peak during the septation process

The above results suggested that the endoglucanase-encoding gene *eng1*$^+$ is expressed periodically during the cell cycle. To confirm this point, the expression of this gene was monitored by northern blot analysis in *cdc25-22* mutant cells that had been synchronized by arrest-release. When the level of *eng1*$^+$ mRNA was examined, a periodic cell cycle variation was found, maximum accumulation being observed 20-40 minutes before the peak of septation (Fig. 7A), in good agreement with the results obtained from the protein localization experiments. As a control for RNA loading in all the lanes, a gene that displayed no cyclic variation such as *ura4*$^+$ was used.

β-glucanase activity against laminarin was also assayed in

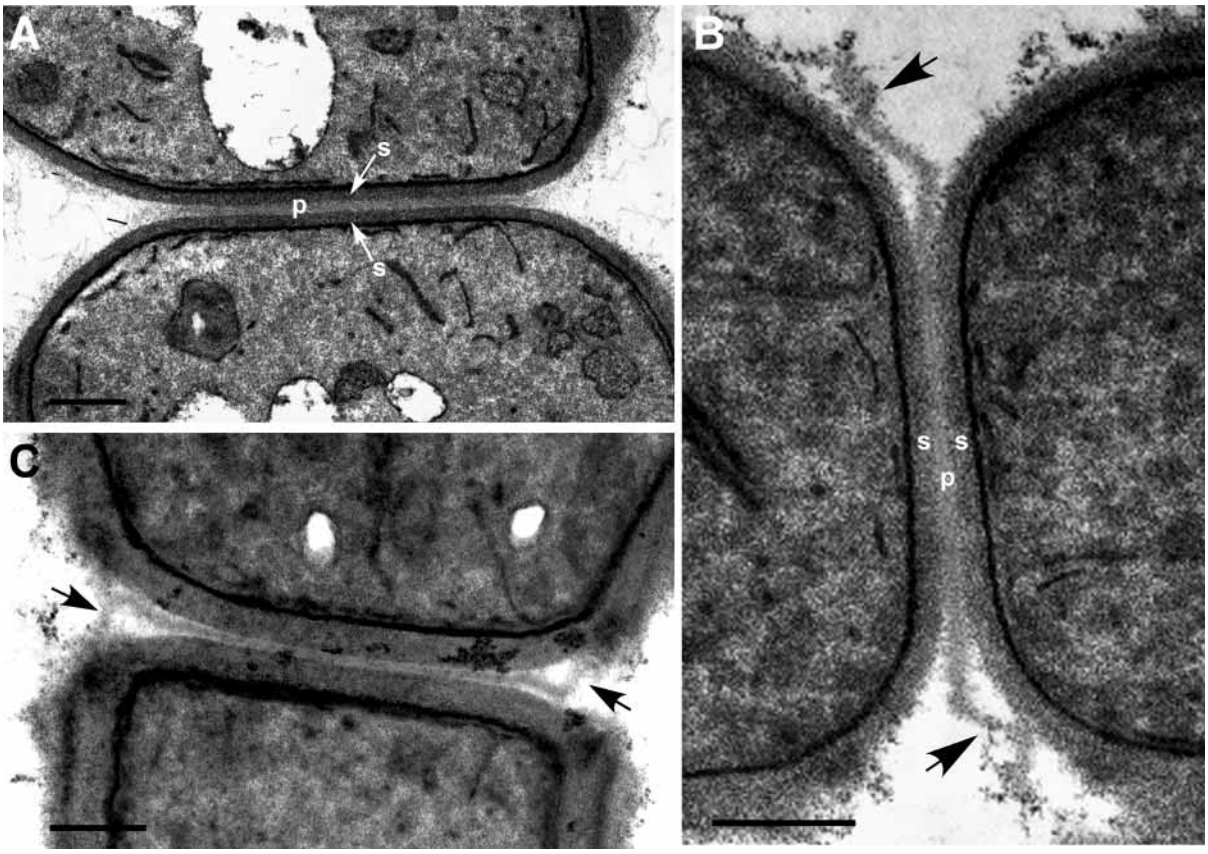

Fig. 4. Electron microscopy ultrastructure of wild-type (A) and *eng1*Δ mutant septa (B,C) during the separation process. p, primary septum; s, secondary septum. Arrows mark the remnants of the primary septum that has not been dissolved in mutant cells. Bars, 0.5 μm.

cdc25-22 synchronised cells at the same time intervals as those used for northern analysis. The results, shown in Fig. 7B, indicated the existence of a periodic increase in β-glucanase activity, that slowly rose to reach a maximum coincident with the septation peak. The maximum activity was detected 20-40 minutes after the time of maximum mRNA accumulation (compare the septation index of both experiments), suggesting that the protein accumulates until the separation process is completed. To check that the increase in activity was indeed due to the product of *eng1*$^+$, this gene was deleted in the *cdc25-22* mutant strain. When β-glucanase activity was measured in the double *cdc25-22 eng1*Δ mutant, only a basal level of activity (mainly due to the protein encoded by the *eng2*$^+$ gene, data not shown) was detected in all samples. Thus, β-1,3-glucanase activity in *S. pombe* periodically oscillates during the cell cycle, maximum accumulation coinciding with the septation and cell separation processes, in good agreement with the expression pattern of the *eng1*$^+$ gene.

eng1$^+$ expression is regulated by the *ace2*$^+$ transcription factor

In budding yeast, ScAce2p regulates the expression of a group of genes involved in cell separation, such as chitinase (*CTS1*), endo-β-1,3-glucanase (*ENG1*), and *YHR143w* (Dohrmann et al., 1992; Doolin et al., 2001; Baladrón et al., 2002), and for this reason *ace2* mutants display a cell separation defect, forming large aggregates of cells. BLAST searches of the *S. pombe* genome revealed the presence of two proteins with significant similarity to ScAce2p: namely, the products of the SPAC6G10.12c (e value=–21) and SPAC4G8.13c (e value=–10) ORFs. However, this latter protein is more similar to the *S. cerevisiae* Crz1p transcription factor (e value=–23), suggesting that the former could be the functional homolog of ScAce2p. To test this possibility, both genes were independently deleted in a wild-type background by replacing the coding region with the *kanMX4* (for the SPAC6G10.12c ORF) or *ura4*$^+$ (for SPAC4G8.13c) marker genes. The resulting strains, LE25 (lacking SPAC6G10.12c, which will be referred to as *ace2*$^+$ based on the homologies to ScAce2p, see below) and LE26 (lacking SPAC4G8.13c), were used to analyse *eng1*$^+$ expression and to check the morphological appearance of the cells.

Northern analysis was performed in the two mutant strains and in the isogenic wild-type strain to test whether either of the two putative transcriptional regulators were controlling the expression of *eng1*$^+$ in *S. pombe*. As can be seen in Fig. 8A, the accumulation of *eng1*$^+$ mRNA is slightly reduced in the LE26 strain (lacking SPAC4G8.13c) but was almost absent in the *ace2*Δ mutant. This observation therefore clearly indicates that *eng1*$^+$ expression requires ace2p, which is similar to what has been described for the *S. cerevisiae ENG1* gene (Baladrón et al., 2002), although a minor contribution of the SPAC4G8.13c protein to its regulation cannot be ruled out.

The morphology of *ace2Δ* cells was also analysed by

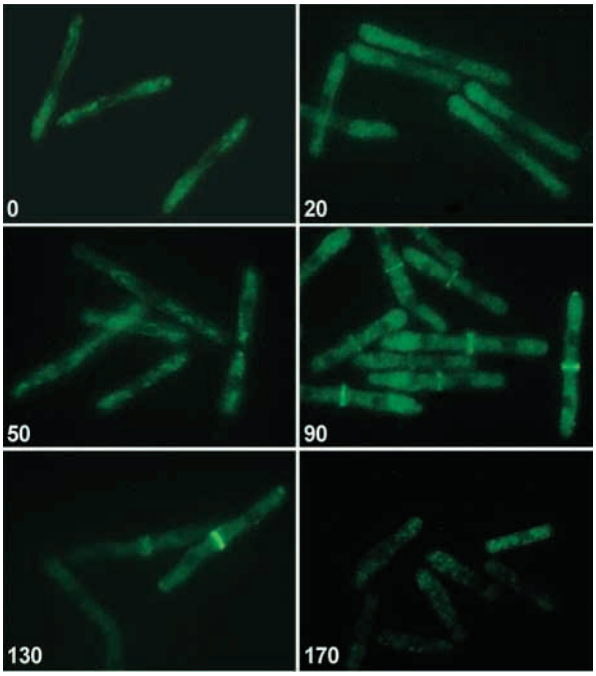

Fig. 5. eng1p protein is synthesized periodically during the cell cycle. *cdc25-22* mutant cells carrying the *eng1-GFP* fusion allele integrated in the chromosome were arrested by incubation at the restrictive temperature (37°C) for 4 hours and then transferred to the permissive temperature (25°C) to monitor the localization of the fusion protein. Numbers indicate minutes after transfer to the permissive temperature.

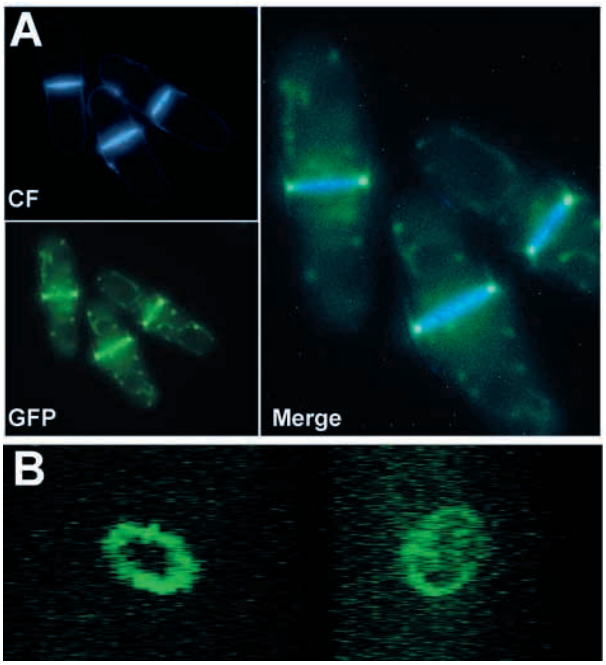

Fig. 6. eng1p localizes to the septum. (A) Wild-type cells containing the *eng1-GFP* fusion allele were grown to early-log phase and stained with Calcofluor before microscopic observation. Calcofluor fluorescence (CF), GFP fluorescence and the overlay of both images (Merge) are shown. (B) Three-dimensional reconstruction of eng1p localization. Cells containing the *eng1-GFP* fusion allele were observed under a confocal microscope and z-sections of 0.3 μm were taken. The image was reconstructed using LSM510 software.

microscopic inspection (Fig. 8B). In contrast to wild-type cells (panel 1) or mutants lacking SPAC4G8.13c (panel 2), *ace2*Δ-null mutants displayed a severe cell separation defect (panel 3), resulting in the formation of mycelial cells with a branched morphology, as has been reported for other genes such as *sep1*$^+$ and *spl1*$^+$ (Sipiczki et al., 1993). Thus, the product of the SPAC6G10.12c ORF appears to be a functional homolog of ScAce2p, regulating the expression of a group of genes required to complete cell separation in *S. pombe* (one of which is the endo-β-1,3-glucanase encoded by *eng1*$^+$).

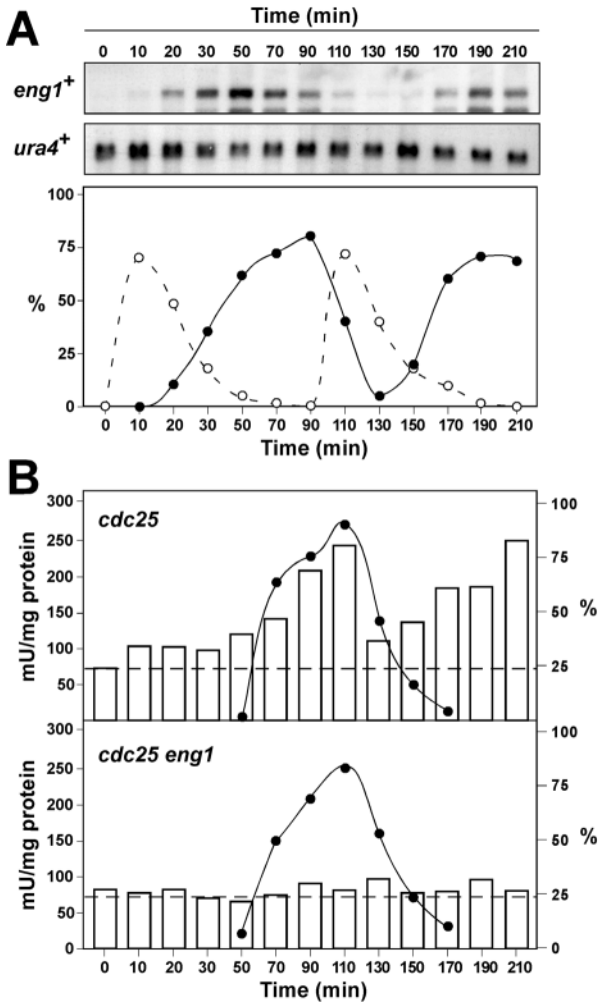

Fig. 7. *eng1*$^+$ expression and β-1,3-glucanase activity vary periodically during the cell cycle. Synchrony was induced by arrest-release of *cdc25-22* mutants and samples were taken at the indicated time points (minutes) for RNA extraction (A) or β-glucanase activity determinations (B). (A) RNA was probed with specific probes for *eng1*$^+$ or *ura4*$^+$. The graph represents the anaphase index (○) or septation index (●) at each time point. In this experiment, the peak of septum formation occurred at 70-90 minutes. (B) β-glucanase activity was assayed using laminarin as substrate in samples from a *cdc25-22* (upper panel) or *cdc25-22 eng1*Δ (lower panel) mutant strains. The septation index for each strain is indicated (●). The anaphase index was determined by counting the percentage of anaphase cells (cells with two nuclei and without a septum) after DAPI staining. The septation index was determined by counting the percentage of cells with septum after calcofluor staining.

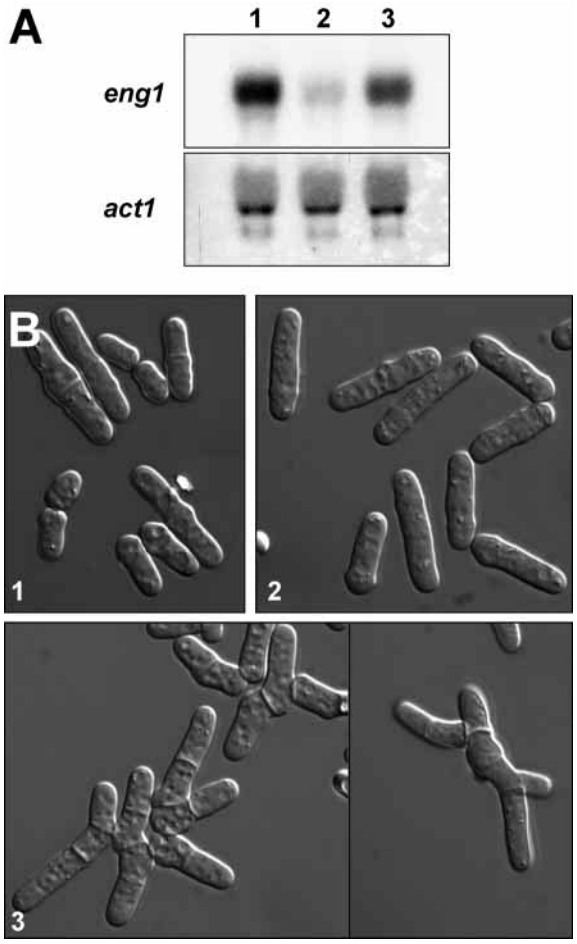

Fig. 8. *ace2*$^+$ is required for cell separation. (A) Expression of *eng1*$^+$ and *act1*$^+$ genes in wild-type (lane 1), *ace2*-null mutants (cells lacking the SPAC6G10.12C ORF, lane 2), or cells containing a deletion of SPAC4G8.13c (lane 3). (B) Morphology of the mutant cells grown in rich medium to early-log phase. Wild-type (1) and mutants lacking SPAC4G8.13c (2) show a normal morphology, while *ace2* mutants (3) have a clear mycelial and branched phenotype, indicating severe cell separation defects.

Discussion

In this work we describe the identification of a new *S. pombe* gene, *eng1*$^+$, that codes for a protein with significant sequence similarity to other glycosyl hydrolases grouped in family 81 (GHF81) (Henrissat and Bairoch, 1996). Like other members of this family of proteins, eng1p shows detectable β-1,3-glucanase activity in vitro and is active against linear β-1,3-glucans (laminarin) with an endo-hydrolytic mode of action. Several observations suggest that this enzymatic activity would be involved in cell separation in *S. pombe*. First, the endo-glucanase eng1p localizes to the septum region at the time of cell separation. Second, mutants lacking this gene form short chains of cells that do not separate completely. Finally, microscopic observation of mutant septa reveals the presence of remnants from the uncleaved primary septum. Although previous reports have postulated the participation of hydrolases in the dissolution of the primary septum or the cell wall that surrounds it, to our knowledge this is the first description of a protein for which enzymatic activity (β-glucanase) has been demonstrated to be directly involved in cell separation in *S. pombe*.

Cell separation in *S. pombe* requires enzymatic hydrolysis of the primary septum

The goal of cytokinesis is common in all organisms: to physically separate a mother cell into two daughter cells. This is achieved by a common set of mechanisms and involves the use of an actomyosin contractile ring that provides the mechanical force required for the separation process. However, fungi and yeast cells synthesize a division septum behind the ring as it constricts, generating new cell wall material between the daughter cells. Thus, cell separation in these organisms requires dissolution of both the primary septum and the cylinder of cell wall that surrounds it (Robinow and Hyams, 1989).

In budding yeast, the chitin-rich primary septum is synthesized by the action of chitin synthase II [the ScChs2p protein (Schmidt et al., 2002; Shaw et al., 1991)], after which a secondary septum is laid down at both sides. Cell separation in *S. cerevisiae* requires partial hydrolysis of the primary septum between the mother cell and the new daughter cell, a process that is mediated by the action of the endo-chitinase encoded by *CTS1* (for a review, see Cabib et al., 2001). In addition, other cell wall components, such as glucans, must be hydrolysed for cell separation to be completed, and the endo-β-1,3-glucanase ScEng1p has recently been shown to participate in this process (Baladrón et al., 2002). Interestingly, both these proteins localize to the mother-daughter neck region during the time of cell separation, but in an asymmetrical manner, according to the division pattern of budding yeast. They are only present at the daughter side of the septum because the genes that encode them are expressed only in newborn cells (Baladrón et al., 2002; Colman-Lerner et al., 2001).

In fission yeast, the primary septum, which is rich in linear β-1,3-glucan (Horisberger and Rouver-Vauthey, 1985; Humbel et al., 2001), is the first part to be laid down in a centripetal fashion until it completely closes and compartmentalizes the two daughter cells. Cps1p, an integral membrane protein known as the putative catalytic subunit of β-1,3-glucan synthase, is essential for division septum assembly and it localizes to the division site (Ishiguro et al., 1997; Le Goff et al., 1999b; Liu et al., 1999; Liu et al., 2002; Cortés et al., 2002) Following this, each daughter contributes cell wall material to its own side of the primary septum, building a secondary septum mainly composed of β-1,6-branched β-1,3-glucan and β-1,6-glucan (Horisberger and Rouver-Vauthey, 1985; Humbel et al., 2001). Separation of the sister cells requires two degradative processes: erosion of the surrounding cylinder of cell wall at its junction with the septum and dissolution of the primary septum (Robinow and Hyams, 1989). It has been proposed that degradation of the primary septum might be a mechanical process triggered by rupture of the cell wall of the mother cell (Sipiczki and Bozsik, 2000). Here we have shown that cell separation requires enzymatic hydrolysis of the primary septum in a process that is mediated by the endo-β-1,3-glucanase encoded by *eng1*$^+$. The fact that this protein is present in a ring-like structure surrounding the septum before cell separation has started suggests that eng1p could perhaps

be involved in the dissolution of the cell wall material that surrounds the separation septum, a process that may trigger cell separation. Once the cell wall has been dissolved, the phenotype of *eng1*Δ mutants clearly supports the idea that the endo-β-1,3-glucanase eng1p would be involved in the degradation of the fibrillar β-1,3-glucans that are so abundant in the primary septum of fission yeast. However, we were unable to detect the constriction of the fluorescent ring concomitant with the disappearance of the primary septum, perhaps due to the fact that the eng1p-GFP fluorescence is very faint and rapidly disappears or, alternatively, because they do not occur in a synchronic manner. In the absence of eng1p, cell separation is delayed in comparison with wild-type cells, and may be achieved either through participation of other hydrolytic enzymes or through mechanical rupture of the cell wall in a process in which the primary septum is not completely hydrolysed. The observation that the phenotype of *eng1*Δ cells is much less severe than that of other previously identified mutants (such as *sep1*Δ or *spl1*Δ) or that brought about by the *ace2* deletion clearly suggests that additional proteins (perhaps other hydrolytic activities) would also be involved in cell separation. In this context, and similar to the case of *S. cerevisiae*, other proteins with sequence similarity to glucanases are present in the *S. pombe* genome. Thus, a protein related to eng1p has recently been reported in *S. pombe* [the product of the SPAC23D3.10c ORF (Baladrón et al., 2002)]. This protein, named eng2p, also exhibits endo-β-1,3-glucanase activity, but *eng2*Δ mutants do not have a cell separation defect (A.B.M.-C. and F.d.R., unpublished), which suggests that eng2p is not involved in cell separation. Additionally, three genes that show similarity to yeast exo-β-1,3-glucanases and two proteins related to fungal α-glucanases are also present in the *S. pombe* genome, although no data about their putative involvement in morphogenetic processes are yet available.

The spatial regulation of hydrolases during cell separation is also important for the process to be achieved successfully. Unlike cell separation in *S. cerevisiae*, which is an asymmetric process resulting in the formation of two cells of different size, *S. pombe* cells divide by medial septation, to generate two equivalent cells. Asymmetries in *S. pombe* cells have been found only in spindle-pole bodies (Sohrmann et al., 1998), mating-type switching (Klar, 1990), and in *for3* mutants, which lack a formin required for symmetry to be maintained during cell growth (Feierbach and Chang, 2001). This difference is also reflected in the localization of the proteins involved in septum degradation: while in *S. cerevisiae CTS1* and *ENG1* expression occurs only in the daughter cell and the proteins are located at the daughter side of the septum (Baladrón et al., 2002; Colman-Lerner et al., 2001), in fission yeast the protein appears to be localized in a symmetrical fashion in a ring surrounding the primary septum. Thus, whereas in budding yeast it is the daughter cell that separates from the mother, in fission yeast both sister cells seems to contribute equally to the cell separation process.

It has recently been proposed that the exocyst, a multiprotein complex involved in the late steps of the exocytic pathway, is essential in *S. pombe* for the delivery of proteins that are important for cell cleavage, including putative hydrolytic enzymes (Wang et al., 2002). Mutants deficient in any of the subunits of this complex accumulate 100 nm vesicles, although the content of these vesicles has not yet been characterized. Since eng1p localizes to the septum region at the time of cell cleavage, it is therefore possible that it could be one of the components being transported in such vesicles.

Temporal regulation of *eng1*$^+$ expression requires the ace2p protein

In addition to the tight spatial regulation observed for the eng1p protein, we also observed a strict temporal regulation of β-1,3-glucanase activity during the cell cycle, the maximum being reached at the time of cell separation. Furthermore, the variation in activity is in good agreement with the expression pattern of *eng1*$^+$, indicating that this activity is regulated at transcriptional level and not by any other post-transcriptional mechanism. The expression of *eng1*$^+$ requires the product of the SPAC6G10.12c gene, a protein that shows strong sequence similarity to the ScAce2p especially in the DNA-binding region, although the product of the SPAC4G8.13c ORF could have a minor role in its regulation. Similarly to what has been described for ScAce2p (Dohrmann et al., 1992), deletion of the *S. pombe* protein results in a severe cell separation defect because null mutants are unable to separate and show a hyphal and branched pattern of growth similar to that of *sep1*Δ or *spl1*Δ mutants (Grallert et al., 1999; Ribár et al., 1999; Sipiczki et al., 1993). Based on these functional and sequence similarities, we have named the SPAC6G10.12c ORF *ace2*$^+$. The phenotype of *ace2*Δ mutants is much more severe than that observed in *eng1*Δ mutants, which indicates that ace2p might regulate the expression of other genes also involved in the cell separation process, including additional hydrolytic enzymes required for the dissolution of other cell wall components.

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