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Role of Septins and the Exocyst Complex in the Function of Hydrolytic Enzymes Responsible for Fission Yeast Cell Separation[V]

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Cell separation in *Schizosaccharomyces pombe* is achieved by the concerted action of the Eng1 endo-β-1,3-glucanase and the Agn1 endo-α-1,3-glucanase, which are transported to the septum and localize to a ringlike structure that surrounds the septum. The requirements for the correct localization of both hydrolases as a ring were analyzed using green fluorescent protein fusion proteins. Targeting to the septum required a functional exocyst, because both proteins failed to localize correctly in *sec8-1* or *exo70*Δ mutants, suggesting that Agn1 and Eng1 might be two of the cargo proteins present in the vesicles that accumulate in exocyst mutants. Septins and Mid2 were also required for correct formation of a ring. In their absence, Eng1 and Agn1 were found in a disklike structure that spanned the septum, rather than in a ring. Even though septin and *mid2*Δ mutants have a cell separation defect, the septum and the distribution of linear β-1,3-glucans were normal in these cells, suggesting that mislocalization of Eng1 and Agn1 might be the reason underlying the failure to separate efficiently. Thus, one of the functions of the septin ring would be to act as a positional marker for the localization of hydrolytic proteins to the medial region.

INTRODUCTION

Cytokinesis is the final stage of the cell cycle during which the daughter cells separate physically and become two independent entities. In a variety of organisms, the force necessary for cell cleavage is provided by a contractile actomyosin ring (CAR) accompanied by the synthesis of new membrane, which is inserted at the division site. In the fission yeast *Schizosaccharomyces pombe*, the CAR—consisting of actin, myosin, and other proteins—is assembled in early mitosis and persists through anaphase. At the end of anaphase, septation is triggered by the septation initiation network pathway of cell cycle regulators (McCollum and Gould, 2001) and the CAR begins to close, guiding the closure of the plasma membrane behind it; at the same time, the cell wall of the septum is synthesized outside the plasma membrane (for a review, see Feierbach and Chang, 2001). On completion of the cell wall, and after the cell membranes have closed, cell-cell separation occurs by digestion of the primary septum and the surrounding cell wall, mediated by the Eng1 and Agn1 endo-glucanases (Martín-Cuadrado *et al*., 2003; Dekker *et al*., 2004). Over the past few years, a variety of mutants defective in cell separation have been described, including mutations in components of the exocyst complex (*sec6*$^+$, *sec8*$^+$, *sec10*$^+$, and *exo70*$^+$); in an anillin homologue (*mid2*$^+$); septins, or the endo-glucanases *agn1*$^+$ and *eng1*$^+$ (Wang *et al*., 2002; Berlin *et al*., 2003; Martín-Cuadrado *et al*., 2003; Tasto *et al*., 2003; An *et al*., 2004; Dekker *et al*., 2004).

The exocyst is a multiprotein complex that has been identified in a number of organisms (Hsu *et al*., 1999). Exocyst proteins seem to be important for the targeting and fusion of Golgi-derived vesicles and the plasma membrane. This complex is thought to be essential for directing vesicles to their precise sites of fusion, and its activity is controlled by several members of the Ras superfamily of small GTP-binding proteins (for reviews, see Finger and Novick, 1998; Hsu *et al*., 1999; Lipschutz and Mostov, 2002; Novick and Guo, 2002; Hsu *et al*., 2004). In *S. pombe*, the exocyst is composed of five subunits (Sec6, Sec8, Sec10, Sec15, and Exo70), and mutants in the subunits of this complex are capable of polarized growth, cell surface expansion, and division septum assembly, although they are specifically defective in cleavage of the division septum and cell separation (Wang *et al*., 2002).

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Abbreviations used: CAR, contractile actomyosin ring.

Based on the fact that mutants in different subunits show a defect in cell separation, it was proposed that this complex might be involved in the delivery of the hydrolytic proteins that are important for cell cleavage to the septum. Two such enzymes could be the Eng1 and Agn1 endo-glucanases, because these are responsible for the main activities required for cell separation (Martín-Cuadrado *et al*., 2003; Dekker *et al*., 2004; Alonso-Nuñez *et al*., 2005).

Septins are a family of GTP-binding proteins conserved from yeast to mammalian cells that function during cytokinesis (Longtine *et al*., 1996; Field and Kellog, 1999; Kartmann and Roth, 2001; Faty *et al*., 2002; Longtine and Bi, 2003). Its first members were identified in budding yeast as the proteins altered in mutants defective in cytokinesis (Hartwell, 1971), and similar roles have been shown in other organisms (Neufeld and Rubin, 1994; Kinoshita *et al*., 1997). Increasing evidence indicates that septins also function in actin recruitment, secretion, and membrane deposition (Adam *et al*., 2000; Dent *et al*., 2002; Kinoshita *et al*., 2002). The budding yeast *S. cerevisiae* contains five septins during mitosis: Cdc3, Cdc10, Cdc11, Cdc12, and Shs1/Sep7, which assemble into a patch at the incipient bud site during G_1 (Gladfelter *et al*., 2001). As the cell cycle progresses, septins reorganize at the mother-bud neck into an hourglass-shaped collar of cortical filaments (Frazier *et al*., 1998). The collar remains at the neck throughout the rest of the cell cycle, and at cytokinesis splits into two rings, which then disassemble. In this organism, septins are required for cytokinesis and are thought to perform multiple functions at the mother-daughter bud neck. These functions include regulating the cell cycle and cell shape, providing a boundary that restricts certain determinants to particular cortical domains, and serving as a scaffold for the localization of many signaling proteins, bud site selection proteins, and chitin synthases (reviewed by Longtine *et al*., 1996; Field and Kellog, 1999; Gladfelter *et al*., 2001; Faty *et al*., 2002). With the completion of the *S. pombe* genome sequence (Wood *et al*., 2002), homologues of the *S. cerevisiae* septins Cdc3, Cdc10, Cdc11, and Cdc12 were identified and named Spn1, Spn2, Spn3 and Spn4, respectively. *S. pombe* septin (*spn*) mutants are viable, but they have a defect in cell separation and accumulate in chains of cells (Berlin *et al*., 2003; Tasto *et al*., 2003; An *et al*., 2004). Consistent with this phenotype, septins occur at the division site only after the contractile ring has been fully assembled. Septins organize at the site of cell division late in mitosis, where they form a ring that splits as the septum forms, although this ring does not constrict with actomyosin ring invagination; instead, it dissipates after cell cleavage (Berlin *et al*., 2003; Tasto *et al*., 2003). Thus, in contrast to septins in budding yeast, fission yeast septins are not essential for cell viability and may function primarily in the late stages of cytokinesis (An *et al*., 2004).

Formation of the septin ring requires Mid2, a protein related to *S. cerevisiae* Bud4, *C. albicans* Int1, and anillins present in multicellular eukaryotes (Field and Alberts, 1995; Sanders and Herskowitz, 1996; Oegema *et al*., 2000; Gale *et al*., 2001). Like septins, *mid2*$^+$ is not an essential gene, but the protein is required for the proper organization of septin rings at the site of cell division, and its loss causes a chained cell phenotype (Berlin *et al*., 2003; Tasto *et al*., 2003). Mid2 production is normally restricted to anaphase and is solely responsible, directly or indirectly, for septin ring coalescence (An *et al*., 2004) and for the stability of this structure, because septin rings are dynamic in *mid2*Δ cells, whereas they are very stable in wild-type cells (Berlin *et al*., 2003). Mild overproduction of Mid2 leads to the persistence of septin rings and the inhibition of mitotic progression (Tasto *et al*., 2003; An *et al*., 2004). These observations suggest that Mid2 interacts either directly or indirectly with septins to promote their organization into stable ring structures in late mitosis.

In this work, we investigate the mechanisms involved in targeting the endo-β-glucanases Agn1 and Eng1 to the septum. Correct localization of the two hydrolytic enzymes in a ring that surrounds the septum requires a functional exocyst, the septins, and Mid2. In mutants lacking any of these components, Eng1 and Agn1 are found as a disk instead of as the normal ring. We propose that septins and Mid2 would act as positional markers to direct the secretion of Eng1 and Agn1 to the septum edging to form a ring in a process mediated by the exocyst. Electron microscopy analysis revealed that the *spn*Δ and *mid2*Δ mutants are defective in septum digestion but not in septum organization. We also suggest that the separation defect of *spn*Δ and *mid2*Δ mutants arises from abnormal localization of the hydrolytic enzymes.

MATERIALS AND METHODS

Strains, Growth Conditions, and Plasmids

The *S. pombe* strains used in this study are listed in Table 1. Yeast cells were grown on YES medium or minimal medium (EMM) with appropriate supplements (Moreno *et al*., 1991). For overexpression experiments using the *nmt1*$^+$ promoter, cells were grown in EMM containing 15 μM thiamine up to the logarithmic phase. The cells were then harvested, washed three times with EMM, and inoculated in fresh medium (without thiamine) at an $OD_{595} = 0.01$. 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 h. Cells were released from arrest by transfer to 25°C. Plasmid pAB10, carrying the *eng1*$^+$-coding sequence under the control of the *nmt1*$^+$ promoter has been described previously (Martín-Cuadrado *et al*., 2003). To overexpress *agn1*$^+$, cells were transformed with plasmid pND02, which contains the *agn1*$^+$ open reading frame under the control of the P3X-*nmt1* promoter (Dekker *et al*., 2004). The Eng1-green fluorescent protein (GFP), Agn1-GFP, Mid2-GFP, Spn3-GFP, and Sec8-GFP constructions used have also been described previously (Wang *et al*., 2002; Martín-Cuadrado *et al*., 2003; Tasto *et al*., 2003; Alonso-Nuñez *et al*., 2005). The *eng1*$^+$ gene was tagged at the 3′ end with the sequence encoding the monomeric red fluorescent protein (mRFP; Campbell *et al*., 2002) by a PCR-mediated strategy as described previously (Bähler *et al*., 1998). Construction of strains carrying the Eng1-GFP, Eng1-RFP, or Agn1-GFP reporters and mutations in different genes was performed using standard genetic methods.

Fluorescence Microscopy and Photobleaching

For light microscopy, cells were fixed in 3.7% formaldehyde and stained with 4′,6-diamino-2-phenylindole or Calcofluor White as described previously (Balasubramanian *et al*., 1997). Samples were viewed using a Leica DMRXA microscope equipped for Nomarski optics and epifluorescence and photographed with a Photometrics Sensys charge-coupled device camera. Confocal microscopy was performed on a Leica TCS SL spectral confocal microscope and the images were analyzed with the Leica Confocal Software. For three-dimensional confocal images, cells were grown in log-phase cultures, placed on glass slides, and imaged on 25–30 image planes 0.2 μm apart. Images were reconstructed in three dimensions using the three-dimensional module of the program. Fluorescence recovery after photobleaching (FRAP) was performed using a Zeiss confocal microscope LSM system. The region of the cell to be bleached was demarcated and subjected to the laser beam, which scanned the area 100 times in less than a second. Fluorescent recovery images were captured at different time intervals.

Transmission Electron Microscopy

To observe the septum, the specimens were prefixed with 2% glutaraldehyde (GA) in 0.1 M sodium phosphate buffer (phosphate-buffered saline [PBS], pH 7.2) at 4°C for 1 h, postfixed with 1.2% potassium permanganate at 4°C overnight, block-stained with 1% uranyl acetate at 4°C for 1 h, and embedded in Quetol 653 as described previously (Konomi *et al*., 2003). The ultrathin sections were stained in 4% uranyl acetate and 0.4% lead citrate and were viewed with a TEM H-800 transmission electron microscope (Hitachi, Tokyo, Japan) operating at 125 kV.

Immunoelectron Microscopy

To observe the primary septum, linear β-1,3-glucan was detected by immunoelectron microscopy (IEM) as described previously (Humbel *et al*., 2001). The specimens for IEM were fixed with a mixture of 0.5% GA and 3%

Table 1. Yeast strains used in this study

Strain	Genotype	Source
HC125	*ppb1::ura4 leu1-32 ura4-D18 h*$^+$	Y. Sánchez laboratory
KGY972	*h*$^-$	K. Gould laboratory
KGY1285	*exo70::ura4 agn1-GFP::Kan*^{R} *leu1-32 ura4-D18 ade6-210 h*$^-$	This work
KGY1286	*exo70::ura4 eng1-GFP::Kan*^{R} *leu1-32 ura4-D18 ade6-210 h*$^-$	This work
KGY1330	*spn4::ura4 agn1-GFP::Kan*^{R} *leu1-32 ura4-D18 ade6-210 h*$^+$	This work
KGY3135	*mid2::ura4 leu1-32 ura4-D18 ade6-M210 h*$^-$	K. Gould laboratory
KGY3682	*sec8-GFP::ura4 leu1-32 h*$^+$	K. Gould laboratory
KGY3693	*sec8-GFP::ura4 mid2::ura4 ade6-M210 leu1-32 ura4-D18 h?*	K. Gould laboratory
KGY3986	*spn4::ura4 leu1-32 ura4-D18 ade6-M210 h*$^+$	K. Gould laboratory
KGY5376	*spn3-GFP::kan*^{R} *sec8-1 leu1-32 ura4-D18 h?*	K. Gould laboratory
KGY5377	*sec8-GFP::ura4 spn4::ura4 leu1-32 h?*	K. Gould laboratory
MBY192	*ura4-D18 leu1-32 h*$^-$	Balasubramanian laboratory
MBY888	*sec8-1 h*$^-$	Balasubramanian laboratory
MBY919	*exo70::ura4 leu1-32 ura4-D18 h*$^-$	Balasubramanian laboratory
MBY1047	*spn1::kanMX4 ade6-210 leu1-32 h*$^+$	Balasubramanian laboratory
PPG3119	*mid2::ura4 ade6-210 leu1-32 ura4-D18 h*$^+$	P. Pérez laboratory
spn4D	*spn4::ura4 ade6-210 leu1-32 ura4-D18 h*$^-$	J. Bähler laboratory
YAB50	*eng1::kanMX4 ura4-D18 leu1-32 h*$^-$	Laboratory stock
YAB136	*eng1-GFP::Kan*^{R} *leu1-32 ura4-D18 h*$^-$	Laboratory stock
YSAB59	*sec8-1 eng1::kanMX4 agn1::ura4 leu1-32 ura4-D18 h*$^+$	This work
YSAB61	*sec8-1 agn1::ura4 ura4-D18 h*$^-$	This work
YSAB66	*sec8-1 eng1::kanMX4 h*$^-$	This work
YSAB69	*sec8-1 eng1-GFP::Kan*^{R} *h*$^-$	This work
YSAB130	*exo70::ura 4 eng1::kan MX4 leu1-32 ura4-D18 h*$^-$	This work
YSAB135	*mid2::ura4 eng1::kanMX4 ade6-210 leu1-32 ura4-D18 h*$^-$	This work
YSAB141	*spn4::ura4 eng1-GFP::Kan*^{R} *ade6-210 leu1-32 ura4-D18 h*$^+$	This work
YSAB149	*spn4::ura4 eng1::kanMX4 ade6-210 leu1-32 ura4-D18 h*$^-$	This work
YSAB153	*spn1::kanMX4 agn1-GFP::Kan*^{R} *ade6-210 leu1-32 ura4-D18 h*$^-$	This work
YSAB155	*mid2::ura4 agn1-GFP::Kan*^{R} *ade6-210 leu1-32 ura4-D18 h*$^-$	This work
YSAB157	*mid2::ura4 eng1-GFP::Kan*^{R} *ade6-210 leu1-32 ura4-D18 h*$^-$	This work
YSAB160	*spn1::kanMX4 eng1::kanMX4 ade6-210 leu1-32 h*$^-$	This work
YSAB162	*ppb1::ura4 agn1-GFP::Kan*^{R} *leu1-32 ura4-D18 h*$^-$	This work
YSAB163	*spn1::kanMX4 eng1-GFP::Kan*^{R} *ade6-210 leu1-32 h*$^-$	This work
YSAB164	*sec8-1 agn1-GFP::Kan*^{R} *leu1-32 ura4-D18 h*$^-$	This work
YSAB168	*ppb1::ura4 eng1-GFP::Kan*^{R} *leu1-32 ura4-D18 h*$^-$	This work
YSAB192	*eng1-RFP mid2-GFP leu1-32 h*$^-$	This work
YSAB194	*eng1-RFP spn3-GFP mid2::KanMX4 leu1-32 h*$^+$	This work
YSAB199	*eng1-RFP agn1-GFP ade6-210 leu1-32 h*$^-$	This work

paraformaldehyde in 0.1 M PBS, pH 7.2, at 4°C for 2 h. After washing with PBS, cells were treated with 1% sodium metaperiodate for 15 min and 50 mM ammonium chloride for 30 min, and embedded in LR White resin (medium grade; London Resin, Berkshire, England) followed by polymerization for 24 h at 50°C. Immunostaining with mouse monoclonal linear β-1,3-glucan antibody (mouse IgG, diluted 1:2000; Biosupplies, Parkville, Victoria, Australia) was performed on ultrathin sections collected on nickel grids after blocking with normal goat IgG (chromatographically purified; Zymed Laboratories, South San Francisco, CA) diluted 1:3 in 50 mM Tris-buffered saline containing 0.1% bovine serum albumin for 15 min, as described previously (Kamasawa *et al.*, 1992). An ultrasmall colloidal gold-conjugated goat anti-mouse IgG (diluted 1:100; Aurion, Wageningen, The Netherlands) was used as secondary antibody. After immunostaining, the sections were fixed with 1% GA in 0.1 M PBS, and the colloidal gold particles were enhanced by silver deposition, using high-efficiency silver enhancement reagents (R-GENT SE-EM; Aurion). The grids were stained in 4% uranyl acetate and viewed with a TEM H-800 device (Hitachi) at 125 kV.

Immunoblotting and Protein Methods

Total cell extracts of *S. pombe* were prepared by breaking the cells with glass beads in lysis buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 137 mM NaCl, 10% glycerol, and 0.5% NP-40). For immunoblotting, 50 μg of protein extracts was resolved by SDS-PAGE on 8% gels. Protein transfer, blotting, and enhanced chemiluminescence detection were performed using standard procedures. Anti-GFP (BD Living Colors monoclonal antibody JL-8; BD Biosciences, San Jose, CA) or anti-tubulin antibodies (monoclonal anti-α-tubulin antibody, clone B-5-1-2; Sigma-Aldrich, St. Louis, MO) were used.

β-1,3-Glucanase activity was assayed in cell extracts as described previously (Baladrón *et al.*, 2002). For whole-cell activity measurement, 10^7 cells were resuspended in 50 mM acetic buffer, pH 5.5, containing 20 mM sodium azide before the assay. One unit of activity was the amount of enzyme that catalyzed 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.

FACS Analysis

For fluorescence-activated cell sorting (FACS) analysis, cells were prepared using the method of Hutter and Eipel (1979), staining them with propidium iodide. DNA contents and cell size were measured using a BD Biosciences FACScan apparatus.

RESULTS

Eng1 Is Secreted to the Septum Region

In a previous study, we reported that Eng1 is an endo-1,3-β-glucanase involved in dissolution of the primary septum in *S. pombe* that localizes to the septum region late in the cell cycle as a ringlike structure (Martín-Cuadrado *et al.*, 2003). Here, we analyzed the behavior of a functional Eng1-GFP fusion in different mutants to investigate the requirements for such localization.

FRAP experiments were carried out to study Eng1 dynamics in the septum region. In the first experiment, the bleached region corresponded to half of the Eng1 ring region (Figure 1A). Eng1-GFP was found to recover slowly, with a faint fluorescence that occurred 6 min after photobleaching and then increased progressively ($t_{1/2}$ = 6 ± 1 min; n = 7).

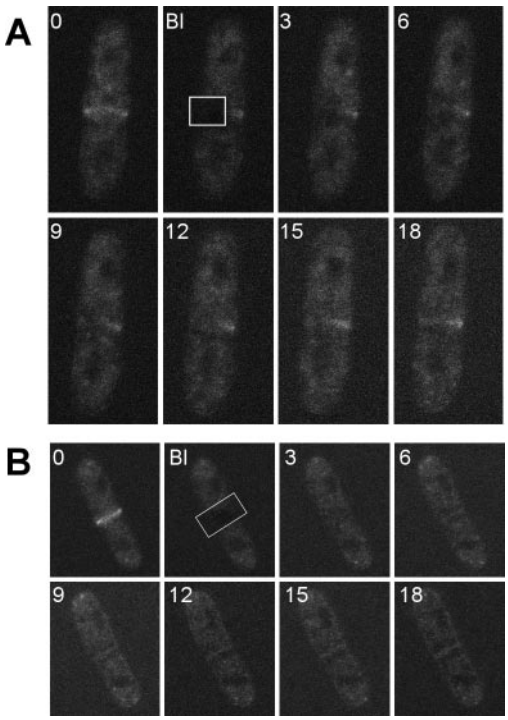

Figure 1. Eng1 is continuously transported to the septum region. GFP fluorescence in cells carrying an Eng1-GFP fusion was photobleached in the area shown. Images were taken before (0), immediately after (Bl), and at 3-min intervals after photobleaching. Cells in which partial (A) or total (B) bleaching of the septum was achieved are shown.

To rule out the possibility that diffusion of the protein might occur in the septum region, the same experiment was repeated, bleaching all the fluorescence from the septum. The results were similar to those obtained previously; the fluorescence began to show up around 6 min after bleaching and increased thereafter ($t_{1/2} = 6 \pm 1$ min; n = 5) (Figure 1B). We conclude that the Eng1 ring is not a static structure and that, normally, continuous transport of Eng1 to the septum region occurs.

The Exocyst Is Required for Eng1 Ring Formation

The exocyst is multiprotein complex involved in the late steps of the exocytic pathway, and it has been proposed that this complex is essential in *S. pombe* for the delivery of proteins important for cell cleavage, including putative hydrolytic enzymes (Wang *et al*., 2002). To investigate whether this complex is involved in targeting Eng1 to the septum, the localization of Eng1-GFP was analyzed in mutants in different subunits of this complex. We first chose the *exo70*$^+$ gene, because it is the only nonessential subunit of the complex, even though *exo70*Δ mutants are conditionally lethal. Eng1-GFP fluorescence was normally seen in the septum region in *exo70*Δ cells grown at 25°C, but the fluorescence was markedly reduced when the mutants were incubated at 37°C (Figure 2A, left). Under these conditions, only a faint signal was observed in some of the septa, accompanied by an increase in intracellular fluorescence. Eng1-GFP localization was also analyzed in the thermosensitive *sec8-1* mutant and similar results were obtained. At the permissive temperature (25°C), the *sec8-1* mutant had some separation defects, even though Eng1-GFP was normally seen in the septum region (Figure 2A, right). During growth at the restrictive temperature (37°C), the fluorescence disappeared from the septum, and it accumulated intracellularly. In wild-type cells, Eng1 localized normally at 37°C, suggesting that the defect in localization was not due to the increased temperature (our unpublished data). These results indicate that exocyst function is required for the targeting of Eng1 to the septum.

We also observed that even though Eng1 was targeted to the septum region in *exo70*Δ and *sec8-1* mutants during growth at the permissive temperature, the fluorescence pattern was different to that seen in wild-type cells because it was more intense at the center of the septum than at the ends. To analyze Eng1-GFP localization in further detail, confocal microscopy was used (Figure 2B). In wild-type cells Eng1 localized to a defined ring in the septum region, whereas in *exo70*Δ cells the ring was present, but the fluorescence was more diffuse and could also be seen in the internal region of the ring. This defect was more severe in *sec8-1* mutants, in which in most septa Eng1-GFP localized as a disk rather than as a ring. Eng1 recovery dynamics was also analyzed by FRAP experiments in the *sec8-1* mutants grown at 25°C. FRAP experiments indicated that recovery of fluorescence was clearly slower in this mutant compared with the wild-type strain, because no recovery was observed after 18 min of the bleaching ($t_{1/2} = > 18$ min; n = 5). Thus, the exocyst is required for normal Eng1 ring formation.

To check that the absence of fluorescence in the septum region during growth at the restrictive temperature was not due to a reduction in the amount of Eng1-GFP, protein levels were measured by Western analysis. The results indicated that Eng1 levels did not vary substantially during the first 8 h of growth at the restrictive temperature in wild-type, *exo70*Δ, or *sec8-1* mutants (Figure 3A). Intracellular β-1,3-glucanase activity was also measured in wild-type, *exo70*Δ, and *sec8-1* mutants grown at the permissive temperature and 7 h after transfer to the restrictive temperature. β-1,3-glucanase activity was higher in the two mutants compared with wild-type cells (Figure 3B), confirming the notion that protein stability is not compromised in these mutants. As a control for the specificity of the activity measured, double *exo70*Δ *eng1*Δ and *sec8-1 eng1*Δ mutants were used; β-glucanase activity levels in these strains were similar to those found in the *eng1*Δ mutants. These results therefore indicate that Sec8 and Exo70 (and consequently exocyst function) are required for correct targeting of Eng1 to the septum.

Cell Death in sec8-1 Mutants Is Not Due to Abnormal Glucanase Activity

Because *sec8-1* mutants had higher levels of β-1,3-glucanase activity than those found in the wild-type strain, it is possible that the lysis phenotype of *sec8-1* mutants at the restrictive temperature might be due either to the abnormal localization of this hydrolytic activity or to its intracellular accumulation. To test these hypotheses, the growth of *sec8-1 eng1*Δ mutants was analyzed at the restrictive temperature. Because the *agn1*$^+$ gene codes for an endo-1,3-α-glucanase that is also involved in cell separation (Dekker *et al*., 2004; Alonso-Nuñez *et al*., 2005), we also constructed the *sec8-1 agn1*Δ and the triple *sec8-1 eng1*Δ *agn1*Δ mutants. No growth defect was seen in any of the mutants at the permissive temperature (Figure 3C). When the strains were incubated in solid medium at the restrictive temperature, the three mutants (*sec8-1 eng1*Δ, *sec8-1 agn1*Δ, and *sec8-1 eng1*Δ *agn1*Δ) were unable to grow, like the *sec8-1* strain. Addition of an osmotic stabilizer (such as sorbitol) to the medium completely complemented the growth defect of all the mutants at the restrictive temperature (Figure 3C). These results

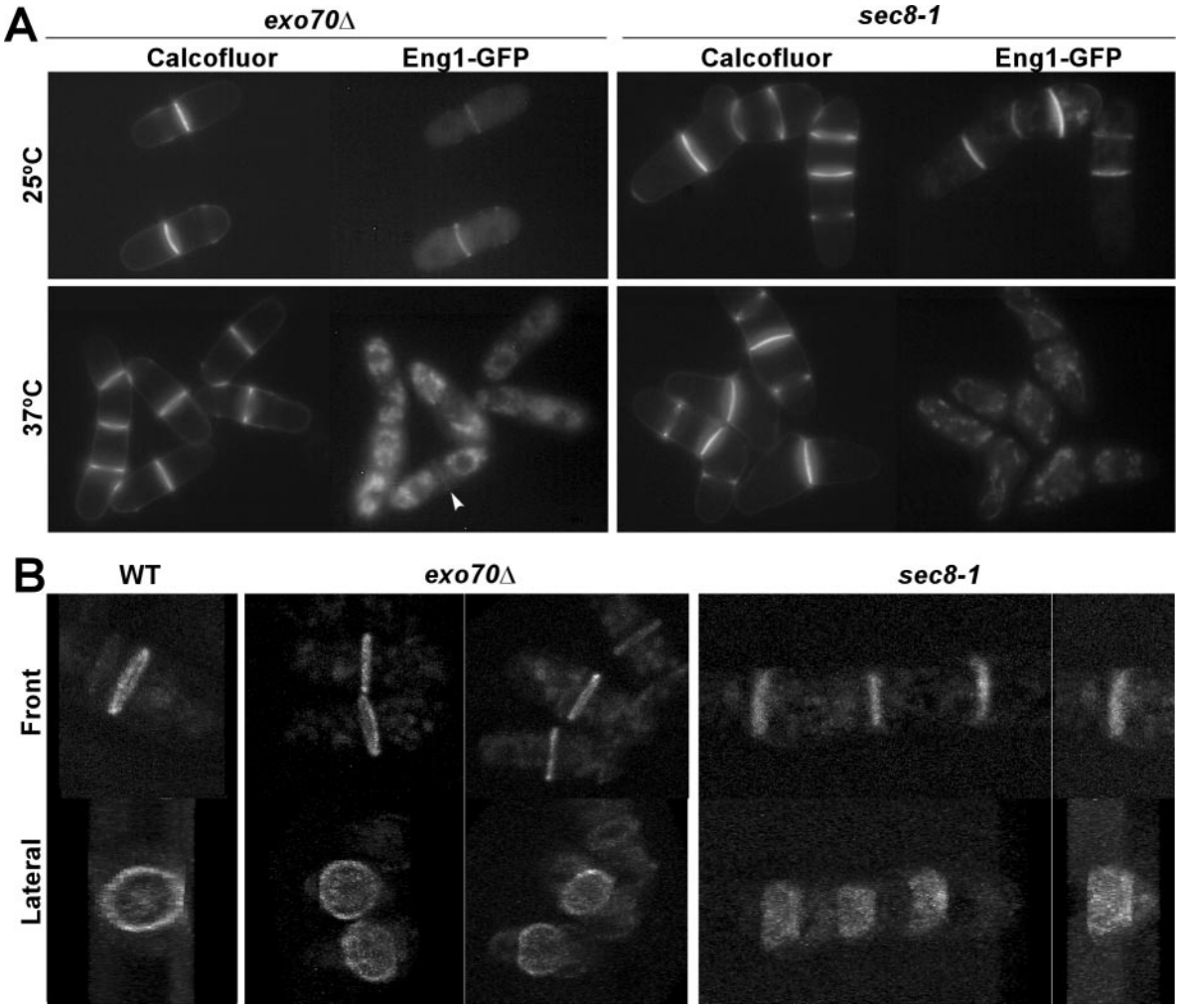

Figure 2. Eng1 localization in exocyst mutants. (A) *exo70*Δ (left) and *sec8-1* (right) mutants expressing Eng1-GFP (YSAB137 and YSAB69, respectively) were incubated at 25°C or 37°C for 4–6 h, and the cells were stained with Calcofluor White to visualize the septum and the cell wall. Images of the Calcofluor-stained cells or Eng1-GFP are shown. (B) Wild-type (YSAB136), *exo70*Δ (YSAB137), and *sec8-1* (YSAB69) mutants expressing Eng1-GFP grown at 25°C were imaged for GFP fluorescence using confocal three-dimensional microscopy. Eng1-GFP structures were rendered in three dimensions. Frontal and lateral views of the Eng1-GFP septum structures are shown for each mutant (see Supplemental Movies).

therefore indicate that the inability of *sec8-1* mutants to grow at restrictive temperature does not arise from the accumulation or abnormal localization of the main hydrolytic enzymes involved in cell separation.

Septins and Mid2 Are Required for Eng1 Ring Formation but Not for Eng1 Glucanase Activity

In *S. pombe*, septins and Mid2 are required for normal cell separation because deletion of the septin genes or *mid2*$^+$ produces a chained cell phenotype (Berlin *et al*., 2003; Tasto *et al*., 2003; An *et al*., 2004). Because the phenotype of septin and *mid2*Δ mutants is similar to that of *eng1*Δ cells, the localization of Eng1-GFP was analyzed in mutants lacking those genes. We found that Eng1-GFP was present in the septum region in the *mid2*Δ and *spn1*Δ mutants but that the pattern of fluorescence was different to that seen in wild-type cells because it was more intense at the center of the septum than at the sides, similar to what was observed in *sec8-1* mutants. This observation suggested that Eng1 could be present in the septum region as a disk rather than as a ring. To analyze Eng1 localization in greater detail, we used confocal microscopy. Three-dimensional reconstruction of the Eng1-GFP signal in wild-type cells clearly revealed the presence of a ring (Figure 4A). However, in *mid2*Δ and *spn1*Δ mutants it was evident that Eng1-GFP was targeted to the septum in a structure that spread all over the septum, resembling a disk. Similar results were observed in *spn4*Δ mutants (our unpublished data). In addition, Eng1 recovery was at least threefold slower in *spn1*Δ and *mid2*Δ than in wild-type cells, as measured by FRAP experiments ($t_{1/2} =>$ 18 min; n = 5). This indicated that Mid2 and septins are not only required to restrict Eng1 localization to a ring at the septum edging but also for the normal turnover of the protein.

Even though Eng1 was present in the septum in *spn*Δ and *mid*2Δ cells, these mutants showed a cell separation defect that resulted in the formation of groups of four connected cells. Anti-GFP antibodies were used to determine the amount of Eng1 in *mid2*Δ, *spn1*Δ, and *spn4*Δ strains carrying Eng1-GFP (Figure 4B). Eng1-GFP protein levels were similar

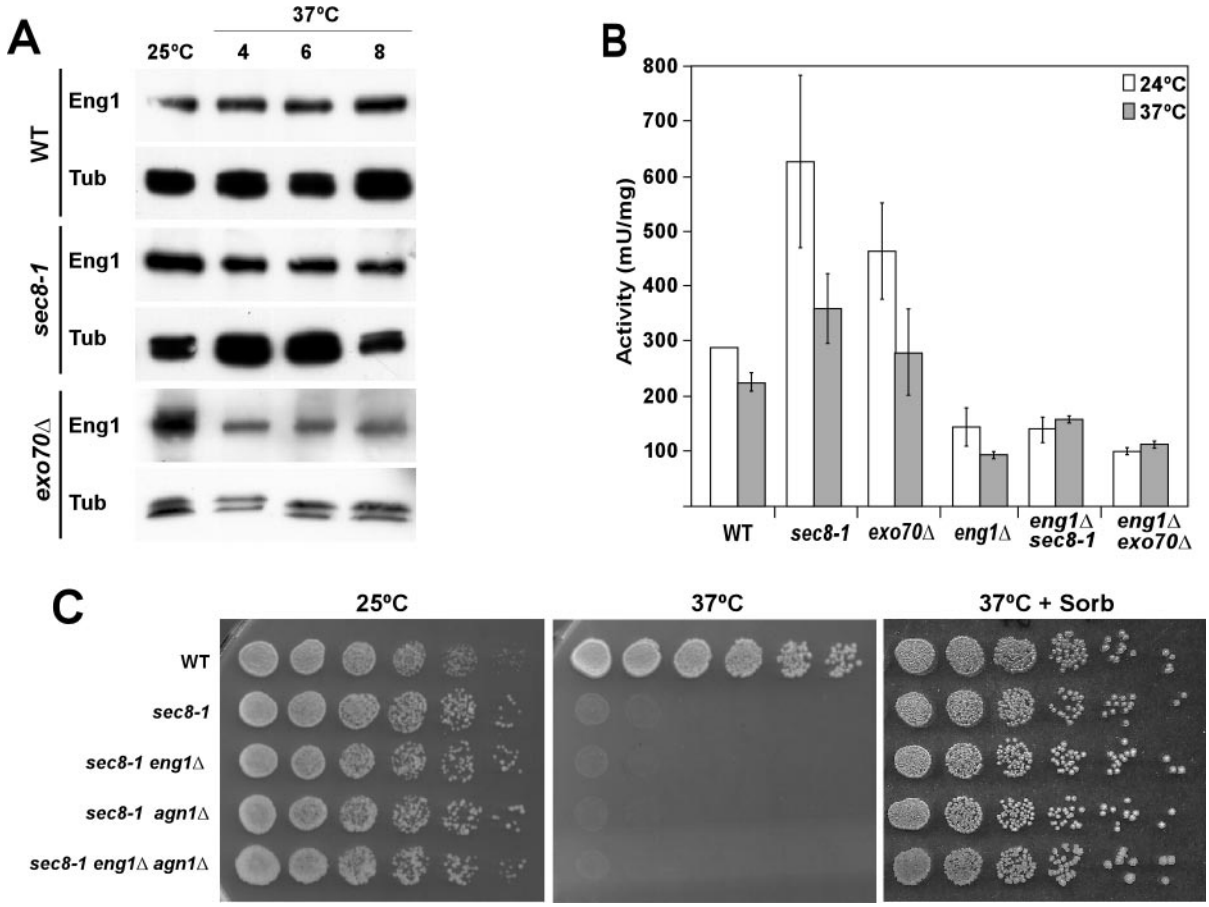

Figure 3. Exocyst mutants have normal levels of Eng1. (A) Cell extracts were prepared from wild-type (YSAB136) and *sec8-1* (YSAB69) or *exo70*Δ (YSAB137) mutants containing Eng1-GFP during growth at the permissive temperature (25°C) or at the indicated times (hours) after transfer to 37°C. Samples were immunoblotted with anti-GFP or anti-tubulin antibodies. (B) β-1,3-Glucanase activity was measured in cell extracts from wild-type (MBY192) and *sec8-1* (MBY888), *exo70*Δ (MBY919), *eng1*Δ (YAB50), *sec8-1 eng1*Δ (YSAB66), and *exo70*Δ *eng1*Δ (YSAB130) mutant cells during growth at 25°C (white rectangles) or 7 h after transfer to the restrictive temperature (gray rectangles). Activity is shown as milliunits per milligram of protein and is the mean of two independent experiments. Error bars indicate the SD. (C) Lysis of *sec8-1* mutants is not due to the increase in hydrolytic activity. Serial dilutions of wild-type (MBY192), *sec8-1* (MBY888), *sec8-1 eng1*Δ (YSAB66), *sec8-1 agn1*Δ (YSAB61), or *sec8-1 eng1*Δ *agn1*Δ (YSAB59) cells were plated onto YES medium and incubated at 25°C or 37°C for 2 d or onto YES medium supplemented with 1 M sorbitol and incubated at 37°C.

or even slightly higher in the mutant strains than in wild-type cells, ruling out the possibility that the separation defect might arise from a reduction in Eng1 levels. One interesting possibility to explain the separation defect of *mid2*Δ and *spn*Δ mutants is that the Eng1 protein secreted in those mutants might be inactive and might be unable to cleave the β-1,3-glucans of the septum. To test this idea, β-1,3-glucanase activity was measured in whole cells to determine whether the extracellular secreted protein was active in those mutants (Figure 4C). Interestingly, we found that β-1,3-glucanase activity was around twofold higher in *spn1*Δ, *spn4*Δ, and *mid2*Δ cells than in wild-type cells, in good agreement with the increase in protein levels detected by Western analysis. This indicated that the secreted Eng1 was fully active in the mutants analyzed. Similar results were obtained when β-1,3-glucanase was measured in whole cells extracts (our unpublished data), suggesting that neither septins nor Mid2 regulate Eng1 activity. The double *spn1*Δ *eng1*Δ, *spn4*Δ *eng1*Δ, and *mid2*Δ *eng1*Δ mutants were used to test the specificity of the activity measured, and the values obtained were similar to those found for *eng1*Δ mutants (our unpublished data). Together, the above-mentioned results indicate that it is very likely that abnormal Eng1 localization, rather than decreased hydrolytic activity, contributes to the cell separation defect of *spn*Δ and *mid2*Δ mutants.

Localization of the α-Glucanase Agn1 Is Also Dependent on the Exocyst, Septins, and Mid2

To check whether the localization defect was specific for Eng1 or was more general, we analyzed the localization of the Agn1 endo-α-1,3-glucanase, which is also involved in cell separation and forms a ring in the septum region (Dekker *et al*., 2004; Alonso-Nuñez *et al*., 2005). Similar to the results described for Eng1-GFP, we found that Agn1-GFP was readily detected in *sec8-1* mutants during growth at the permissive temperature but that it failed to localize to the septum at the restrictive temperature (Figure 5A). This defect was not due to a reduction in Agn1 protein levels during incubation at the restrictive temperature, as assessed by Western analysis (Figure 5B); these protein levels remained stable for at least 8 h of incubation at 37°C in both wild-type and *sec8-1* mutants. Confocal microscopy was used to test

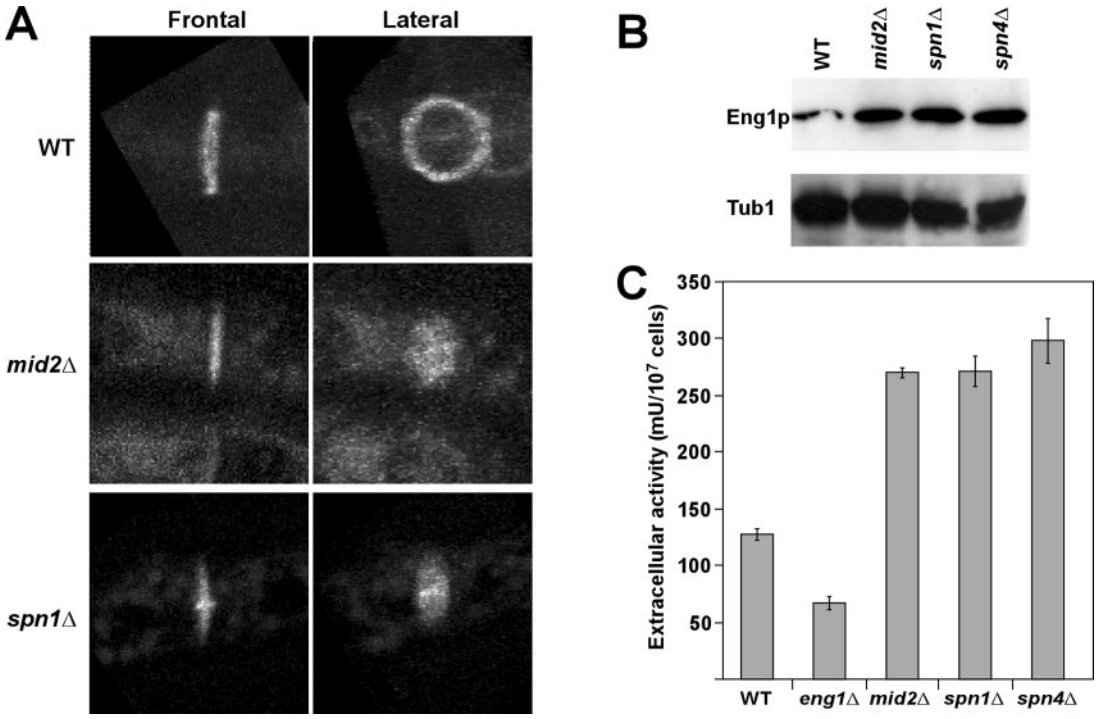

Figure 4. Septins and Mid2 are required for Eng1 ring formation but not for activity. (A) Wild-type (MBY192), *mid2*Δ (YSAB157), and *spn1*Δ (YSAB163) mutants expressing Eng1-GFP were imaged for GFP fluorescence using confocal three-dimensional microscopy. Eng1-GFP structures were rendered in three dimensions. Frontal and lateral views of the Eng1-GFP septum structures are shown for each mutant (see Supplemental Movies). (B) Eng1 protein levels. Cell extracts were prepared from wild-type (MBY192), *mid2*Δ (YSAB157), *spn1*Δ (YSAB163), and *spn4*Δ (YSAB141) mutants containing Eng1-GFP during growth at 25°C. Samples were immunoblotted with anti-GFP antibodies or anti-tubulin as a loading control. (C) Extracellular β-1,3-glucanase activity was measured in whole cells from wild-type (MBY192), *eng1*Δ (YAB50), *mid2*Δ (PPG3119), *spn1*Δ (MBY1047), and *spn4*Δ (spn4Δ) mutants during growth at 25°C. Activity is shown as mU/10^7 cells.

for the formation of the Agn1-GFP ring in wild-type and several different mutants, including *sec8–1* (during growth at 25°C), *mid2*Δ, *spn1*Δ, and *spn4*Δ. Three-dimensional reconstruction of the Agn1-GFP signal in wild-type cells clearly revealed the presence of a ring, but in all the mutant strains tested the fluorescent signal was found in the form of a disk, similar to the abnormal distribution found for Eng1-GFP (Figure 5C). Thus, the exocyst, Mid2 and septins are also required for the correct localization of the Agn1 endo-α-1,3-glucanase in a ring that surrounds the septum.

To determine whether the abnormal localization of Agn1-GFP and Eng1-GFP in *mid2*Δ and septin mutants was a direct consequence of the loss of the gene products or whether it was a byproduct of the cell separation defect, we analyzed the localization of Agn1-GFP and Eng1-GFP in another mutant that also displays multiple uncleaved septa. Mutants lacking the calcineurin gene *ppb1*$^+$ display this phenotype and form groups of four cells (Yoshida *et al.*, 1994). We observed that Eng1-GFP assembled normal rings in a large percentage of the septa in *ppb1*Δ mutants, as did Agn1-GFP (Figure 5D). Thus, Eng1 and Agn1 are not necessarily mislocalized in all cell separation mutants but are specifically affected by the absence of Mid2 and the septins.

Eng1 Localizes between the Septin Rings and Colocalizes with Agn1

To analyze in more detail the relationship between the septins and Eng1, we studied the localization of both proteins in the same cell using a strain that simultaneously expressed Eng1-RFP and Mid2-GFP. In wild-type cells, Eng1-RFP localized as a discrete region sandwiched between the two septin rings, although the Eng1-RFP fluorescence also extended to the exterior of the cell, consistent with a localization in the cell wall that surrounds the septum (Figure 6A). Interestingly, Eng1-RFP was never found in the septum region in cells with a single septin ring (our unpublished data). Only when the septin ring split, Eng1-RFP started to accumulate, indicating that Eng1 secretion occurs after septum assembly has been initiated. The effect of disturbing the septin ring in Eng1/septin colocalization was tested using a *mid2*Δ strain expressing Eng1-RFP and Spn3-GFP. As described previously (Berlin *et al.*, 2003; Tasto *et al.*, 2003), in this strain the septin ring was unstable and quickly formed a disk. As expected, the Eng1-RFP disk was always found in the region between the two abnormal septin rings (Figure 6B). Thus, Eng1 localizes in a discrete region of the cell wall that is delimited by the two septin rings.

We next tested whether Eng1 and Agn1 rings have the same localization in the septum region. To this end, we used a wild-type strain containing Eng1-RFP and Agn1-GFP. As expected from our previous experiments, we found that Eng1 and Agn1 show a perfect colocalization in the septum region (Figure 6C). Thus, these results indicate that Eng1 and Agn1 form a ring in the cell wall that surrounds the septum, between the two septin rings.

Relationship between the Septin Ring and the Exocyst Complex

Given that proper Eng1 and Agn1 localization depend upon both functional septin rings and the exocyst complex, we

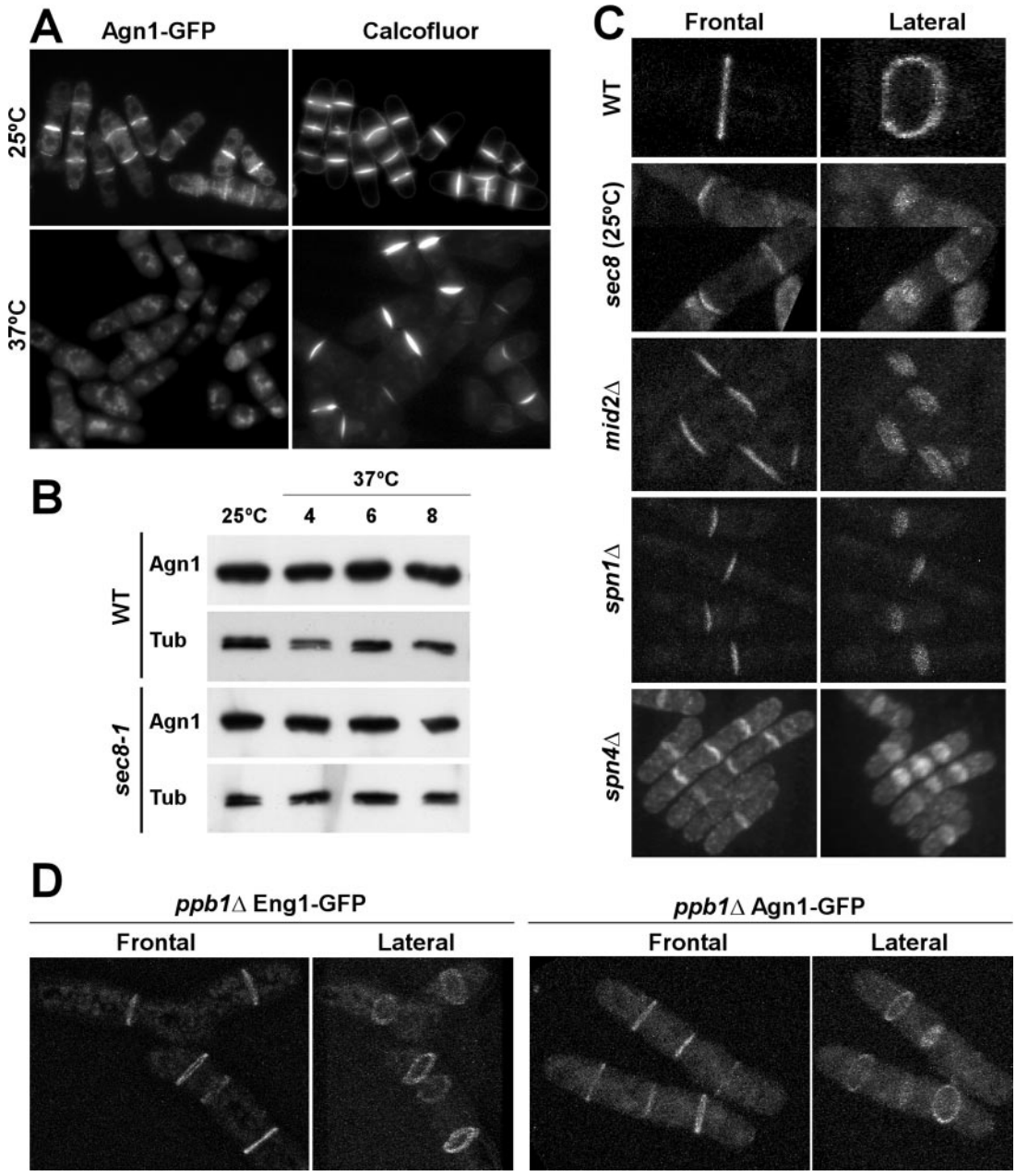

Figure 5. The exocyst, septins, and Mid2 are required for Agn1 ring formation. (A) *sec8-1* mutants expressing Agn1-GFP (YSAB164) were incubated at 25°C or 37°C for 4 h, and the cells were stained with Calcofluor White to visualize the septum and the cell wall. Images of the Calcofluor-stained cells or Agn1-GFP are shown. (B) Agn1 protein levels in *sec8-1* cells. Cell extracts were prepared from wild-type (MBY192) or *sec8-1* (YSAB164) mutants containing Agn1-GFP during growth at the permissive temperature (25°C) or at the indicated times (hours) after transfer to 37°C. Samples were immunoblotted with anti-GFP antibodies or anti-tubulin as a loading control. (C) Wild-type (MBY192), *sec8-1* (YSAB164), *mid2*Δ (YSAB155), *spn1*Δ (YSAB153), and *spn4*Δ (KGY1330) mutants expressing Agn1-GFP were imaged for GFP fluorescence using confocal three-dimensional microscopy. The structures were rendered in three dimensions and frontal and lateral views are shown for each mutant. *sec8-1* Agn1-GFP cells were grown at 25°C (see Supplemental Movies). (D) Three-dimensional reconstruction of the Eng1-GFP (left) and Agn1-GFP (right) fluorescence in *ppb1*Δ mutants. Live *ppb1*Δ mutants carrying Eng1-GFP (YSAB168) or Agn1-GFP (YSAB162) were imaged for GFP fluorescence using confocal three-dimensional microscopy. Eng1-GFP and Agn1-GFP structures were rendered in three dimensions and frontal and lateral views of the structures are shown for each mutant (see Supplementary Movies).

next tested whether septins and exocyst components depended upon one another for correct localization. First, Sec8-GFP localization was examined in wild-type cells and in the absence of *mid2* or *spn4*. We did not detect appreciable differences between these strains (Figure 7, A–C). In all cases, Sec8 formed a ring that was more diffuse than the rings formed by septins, Mid2, Eng1, or Agn1. In the reciprocal experiment, septins localized normally in the *sec8-1* mutation at restrictive temperature (Figure 7, D and E). These results are consistent with the idea that Eng1 and Agn1 require at least two independent targeting mechanisms to localize and function correctly.

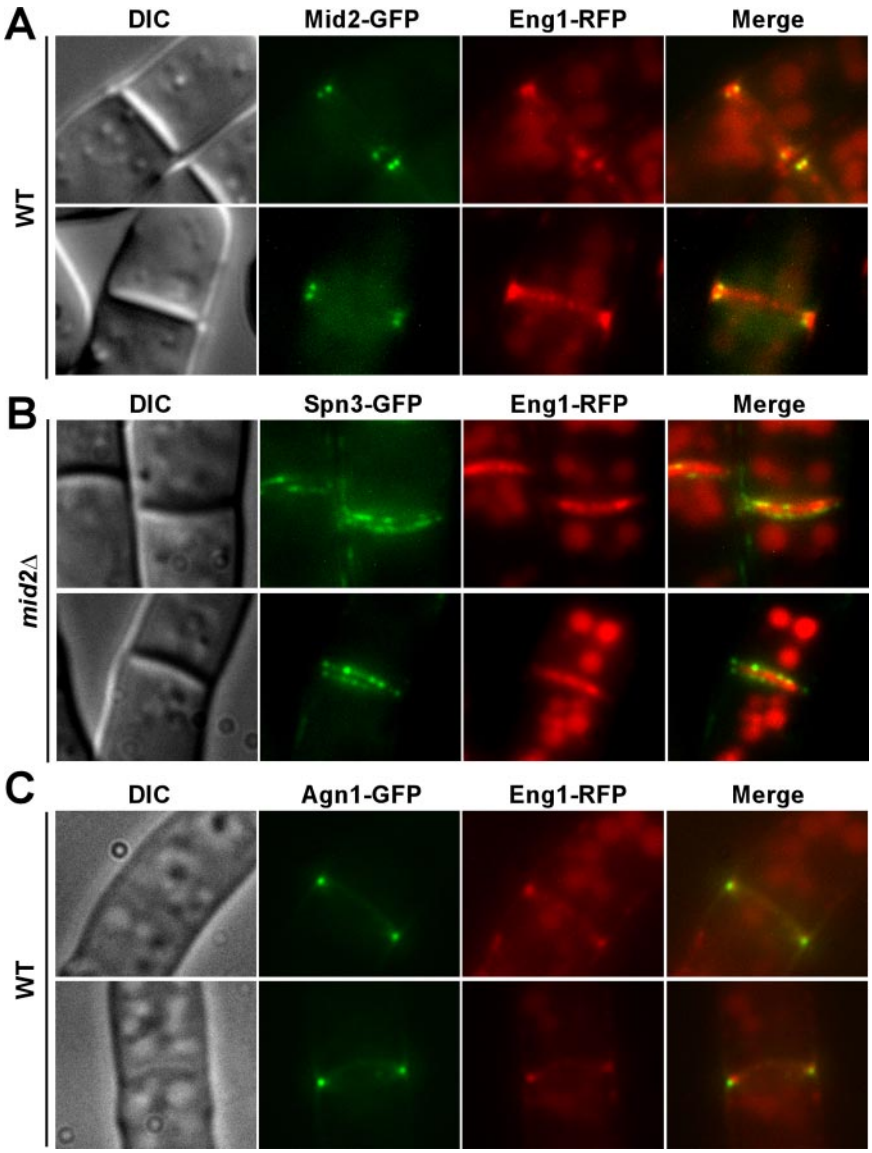

Figure 6. Eng1 localizes between the septin rings and colocalizes with Agn1. (A) Exponentially growing wild-type cells expressing Eng1-RFP and Mid2-GFP (YSAB192) were imaged for differential interference contrast (DIC), RFP, and GFP fluorescence. Overlay of the red and green channels is also shown in the merge images. (B) *mid2*Δ mutants expressing Eng1-RFP and Spn3-GFP (YSAB194) were imaged for DIC, RFP, and GFP fluorescence. Overlay of the red and green channels is also shown in the merge images. (C) Exponentially growing wild-type cells expressing Eng1-RFP and Agn1-GFP (YSAB199) were imaged for DIC, RFP and GFP fluorescence. Overlay of the red and green channels is also shown in the merge images.

Altering Eng1 Protein Levels Affects Cell Separation in mid2Δ and spnΔ Mutants

To investigate whether the cell separation defect of *mid2*Δ and *spn*Δ mutants was due to the abnormal localization of the hydrolytic enzymes involved in the cleavage of the septum and septum edging, we analyzed the phenotype of *spn1*Δ and *mid2*Δ mutants in which the levels of Eng1 and Agn1 were increased. We tested whether the overexpression of *eng1*$^+$ and/or *agn1*$^+$ from the *nmt1*$^+$ promoter could complement the separation defect. Because the phenotype of *spn1*Δ and *mid2*Δ mutants is difficult to quantitate by microscopic inspection of the cells, we used FACS analysis to analyze a population of cells from each mutant. The profile obtained for *spn1*Δ mutants revealed the presence of different peaks: one corresponding to cells with a 2c DNA content (marked in black in Figure 8A), and several additional peaks that corresponded to groups of unseparated cells of increasing size. Whereas *eng1*$^+$ or *agn1*$^+$ overexpression did not fully complement the cell separation defect, we found that the amount of separated cells (cells with a 2c DNA content) was increased in a similar proportion in *spn1*Δ mutants overexpressing *eng1*$^+$ or *agn1*$^+$: the percentage of separated cells rose from 6 to 25% for cells carrying P*nmt1-eng1*$^+$ and from 5 to 29% in cell overexpressing *agn1*$^+$ (Figure 8A). Simultaneous overexpression of *eng1*$^+$ and *agn1*$^+$ resulted in severe cell lysis in *spn1*Δ mutants, cells breaking in the septum region (Figure 8B), that was not complemented by the addition of sorbitol to the medium. However, a substantial increase in the number of separated cells could be detected at short incubation times in the absence of thiamine before cell lysis occurred (from 7 to 55%; Figure 8A). Similar results were found for *mid2*Δ mutants (our unpublished data).

The effect of deleting *eng1*$^+$ in *mid2*Δ and *spn4*Δ strains was also analyzed in a similar manner. The microscopic appearance of double *mid2*Δ *eng1*Δ or *spn4*Δ *eng1*Δ mutants was similar to the single *mid2*Δ or *spn4*Δ mutants, because in both cases chains containing more than four cells were rarely observed (Figure 8C). However, FACS analysis showed that the number of chained cells increased in the two double mutants as compared with the single deletion strains (Figure 8D). The more severe separation defect ob-

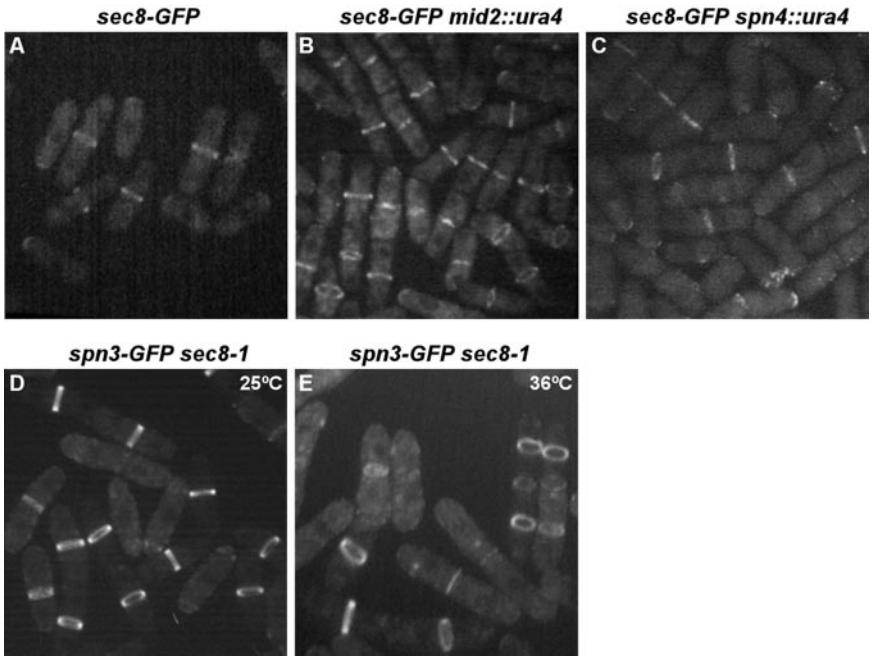

Figure 7. Relationship between exocyst and septins. (A–C) Wild-type (A) (KGY3682), *mid2::ura4* (B) (KGY3693), and *spn4::ura4* (C) (KGY5377) cells expressing *sec8-GFP* were grown at 25°C and imaged for GFP fluorescence using confocal three-dimensional microscopy. (D and E) *spn3-GFP* was visualized in live *sec8-1* cells (KGY5376) grown at 25°C (D) or shifted to 36°C for 4 h (E).

served in the double mutants (*mid2*Δ *eng1*Δ or *spn4*Δ *eng1*Δ) suggests that Eng1, although abnormally located in the septum of *mid2*Δ and *spn4*Δ mutants, is still able to degrade the primary septum, even though more inefficiently that in wild-type cells. Absence of the β-glucanase activity, by deletion of *eng1*$^+$, exacerbates the phenotype in cell separation in these strains. Together, our results indicate that Eng1 and Agn1 are limiting activities in *mid2* and septin mutants and that a failure to efficiently degrade the primary septum is a major cause of the mid2 and septin phenotypes.

Septum Organization and Linear β-1,3-Glucan Distribution Are Normal in mid2Δ and spn4Δ Cells

The results pointing to inefficient septum degradation as a major contributor to the mid2 and septin phenotypes prompted us to examine the ultrastructure of the septum in these mutants. We predicted that septum organization and the distribution of glucans would be normal in *mid2*Δ and *spn4*Δ cells if the cell separation defect was indeed due solely to delayed septum degradation. To study this, *mid2*Δ and *spn4*Δ cells were fixed and observed using transmission electron microscopy (TEM). Previous TEM analyses of septum morphology in fission yeast had revealed a three-layered structure composed of a central, electron-transparent layer of primary septum, and two electron-dense layers of secondary septa (Osumi and Sando, 1969; Johnson *et al*., 1973). In both *mid2*Δ and *spn4*Δ cells, the septa displayed the standard three-layered ultrastructure, and no defects in septum morphology or organization were observed (Figure 9, A and B).

We next examined the distribution of linear β-1,3-glucan in *mid2*Δ and *spn4*Δ cells using immunoelectron microscopy. A recent study has described the differential localization of linear β-1,3-glucan and β-1,6-glucan in the primary and secondary septa, respectively (Humbel *et al*., 2001). Interestingly, linear β-1,3-glucan localized exclusively to the primary septum and persisted until just before cell separation, suggesting that linear β-1,3-glucan may be targeted for degradation during this process (Humbel *et al*., 2001). Like the distribution in wild-type cells (Figure 9C), we found that linear β-1,3-glucan concentrated to the primary septum of *mid2*Δ and *spn4*Δ cells (Figure 9, D and E). Together, these data indicate that the formation and organization of septa in *mid2*Δ and *spn4*Δ cells are normal and suggest that a defect in septum degradation may be the reason why these mutants fail to separate efficiently.

We also examined *mid2*Δ and *spn4*Δ cells in the process of dividing. In wild-type cells, digestion of the primary septum was probably rapid because only a few cells from an asynchronous culture were captured in the process. Moreover, very little primary septum material was observed between the daughter cells once division had begun (Figure 10A). As digestion of the septum approached completion, the new cell ends began rounding, in agreement with published reports (Osumi and Sando, 1969) (Figure 10B). In *mid2*Δ and *spn4*Δ cells, however, primary septum material was retained between the dividing cells, suggesting a defect in septum digestion (Figure 10, C and E). In addition, we observed *mid2*Δ and *spn4*Δ cells with rounded new ends that were still in the process of dividing (Figure 10, D and F). This observation suggests that cells continue growing, even though division is incomplete, and is consistent with the long delay in cell separation associated with these mutants.

DISCUSSION

In this work, we investigated the mechanisms required for targeting the two main enzymatic activities necessary for cell separation in *S. pombe*, the Eng1 endo-β-1,3-glucanase and the Agn1 endo-α-1,3-glucanase to the septum. These enzymes are required to achieve efficient cell separation in the sense that Eng1 is required for controlled dissolution of the primary septum (Martín-Cuadrado *et al*., 2003) and Agn1 is involved in degradation of the septum edging (Dekker *et al*., 2004).

In fission yeast, a separation septum is synthesized as an extension of the cell wall during cytokinesis, and it has a three-layered structure. The internal layer, called the pri-

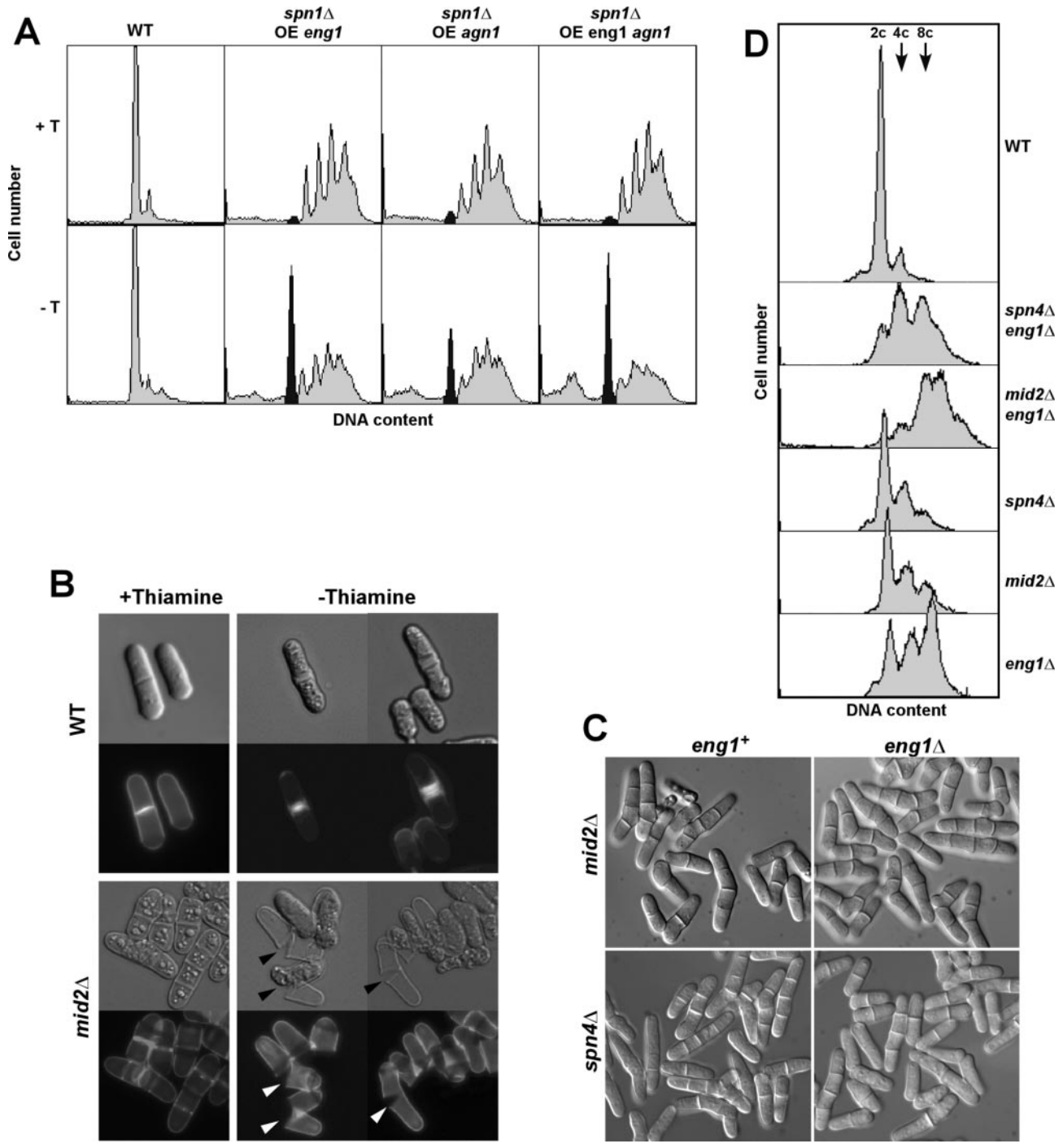

Figure 8. Effects of altering Eng1 and Agn1 levels in *mid2*Δ and *spn*Δ mutants. (A) FACS analysis of *spn1*Δ cells overexpressing *eng1*$^+$, *agn1*$^+$ or both genes at the same time from the *nmt1*$^+$ promoter. Cells were grown in the presence of thiamine to early log phase, washed three times, and transferred to medium with (+T) or without (−T) thiamine. After 15 h of incubation, samples were prepared for FACS analysis. A representative plot for each strain is shown. (B) Microscopic appearance of wild-type (MBY192) and *mid2*Δ (PPG3119) cells overexpressing *eng1*$^+$ and *agn1*$^+$. Cells were grown in the presence or absence of thiamine for 20 h and stained with aniline blue before pictures were taken. (C) Microscopic appearance of *mid2*Δ (PPG3119), *spn4*Δ (spn4Δ), *mid2*Δ *eng1*Δ (YSAB135), and *spn4*Δ *eng1*Δ (YSAB149) mutant strains. (D) FACS analysis of wild-type (MBY192) and *eng1*Δ (YAB50), *mid2*Δ (PPG3119), *spn4*Δ (spn4Δ), *mid2*Δ *eng1*Δ (YSAB135), and *spn4*Δ *eng1*Δ (YSAB149) mutant strains. Cells were grown to early log phase before samples were prepared for FACS analysis. A representative plot for each strain is shown.

mary septum, is rich in linear β-1,3-glucan (Horisberger and Rouver-Vauthey, 1985; Humbel *et al*., 2001), and its synthesis requires the Cps1/Bgs1 protein, which encodes a subunit of the β-1,3-glucan synthase complex (Ishiguro *et al*., 1997; Le Goff *et al*., 1999; Liu *et al*., 1999; Liu *et al*., 2000). The outer layers, forming the secondary septa, are mainly composed of

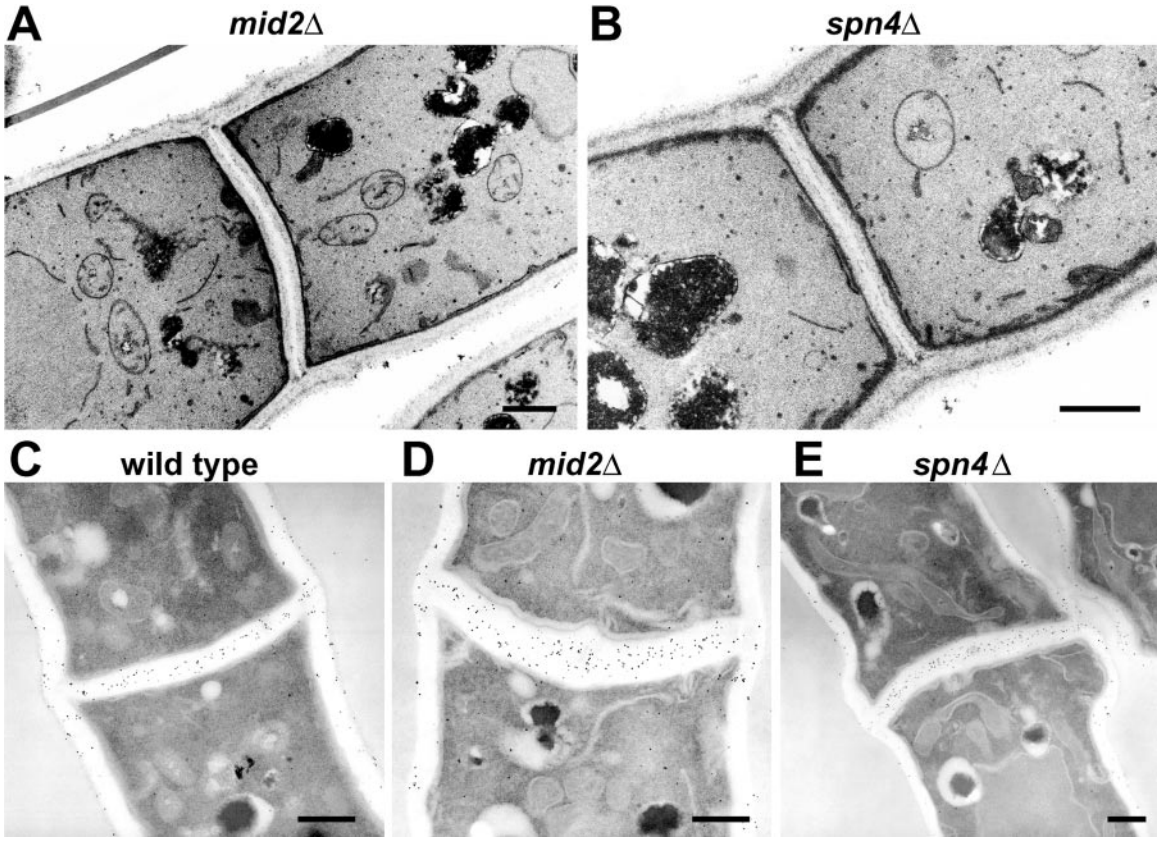

Figure 9. Septum organization and linear β-1,3-glucan distribution are normal in *mid2*Δ and *spn4*Δ cells. (A) *mid2*Δ (KGY3135) and (B) *spn4*Δ (KGY3986) cells were grown at 30°C, fixed, and processed for electron microscopic analysis. Ultrathin sections were stained in 4% uranyl acetate and 0.4% lead citrate to emphasize the layered structure of the cell wall and septum. The electron-transparent middle layer represents the primary septum, which is bordered by two electron-dense layers of secondary septum. Wild-type (KGY972) (C) and *mid2*Δ (KGY3135) (D) and *spn4*Δ (KGY3986) (E) mutants were grown at 30°C, fixed, and processed for immunoelectron microscopy. Sections were labeled for linear β-1,3-glucan and detected using silver-enhanced ultrasmall gold particles. Linear β-1,3-glucans are concentrated in the primary septum. Bar (A–E), 500 nm.

β-1,6-branched β-1,3-glucan and β-1,6-glucan (Horisberger and Rouver-Vauthey, 1985; Humbel *et al*., 2001). Once the septum has been assembled, cell separation requires dissolution of the cylinder of the cell wall at its junction with the septum and controlled degradation of the primary septum (Johnson *et al*., 1982). To achieve efficient cell separation and avoid cell lysis, a perfect temporal and spatial regulation of hydrolytic activities is required, and these processes must also be perfectly coordinated with other events occurring in the cell cycle. At least two different levels of regulation are involved in this process, namely, cell cycle-regulated expression of the genes and targeted secretion to the septum. The transcriptional regulation of *eng1*$^+$, *agn1*$^+$, and other genes involved in cell separation has recently been addressed in several studies (Dekker *et al*., 2004; Rustici *et al*., 2004; Alonso-Nuñez *et al*., 2005). Here, we focused on analysis of the requirements for the targeting of these enzymes to the septum of *S. pombe* cells.

The two main hydrolases required for cell separation, Eng1 and Agn1, are present in a ringlike structure at the septum region (Martín-Cuadrado *et al*., 2003; Alonso-Nuñez *et al*., 2005). Here, we have shown that both proteins not only colocalize in the same region of the cell, the cell wall that surrounds the septum before cell separation has started, but also they have similar requirements for targeting to this region of the cell, the exocyst complex, and the septin ring. To reach their final destination, the hydrolases involved in cell separation are transported to the septum region in secretory vesicles. The exocyst is a multiprotein complex involved in the late steps of the exocytic pathway and is important for cell separation in *S. pombe*, and it has been proposed that it would be required for the delivery of cell separation proteins to the septum (Wang *et al*., 2002). Our results support this idea, because Eng1 and Agn1 never reached their final destination, but instead accumulated intracellularly, when *sec8-1* mutants were incubated at the restrictive temperature, and, in addition, the amount of Eng1 present in the septum in *exo70*Δ cells was strongly reduced at 37°C. We confirmed that this defect was not due to a reduction in Eng1 or Agn1 protein levels by Western analysis and observed that the intracellular β-1,3-glucanase activity measured in vitro was similar, or even higher, in the mutants than in wild-type cells. These results therefore clearly indicate that a functional exocyst complex is required for the targeting of Eng1 and Agn1 to the septum. Although definitive confirmation will require purification of the vesicles that accumulate in *sec8-1* mutants and biochemical characterization of the cargo proteins, our results strongly support the idea that both endo-glucanases are transported to the septum in such secretory vesicles. Interestingly, when Eng1 and Agn1 reached the septum (in *exo70*Δ or *sec8]-1* at the permissive temperature), they were found in a structure

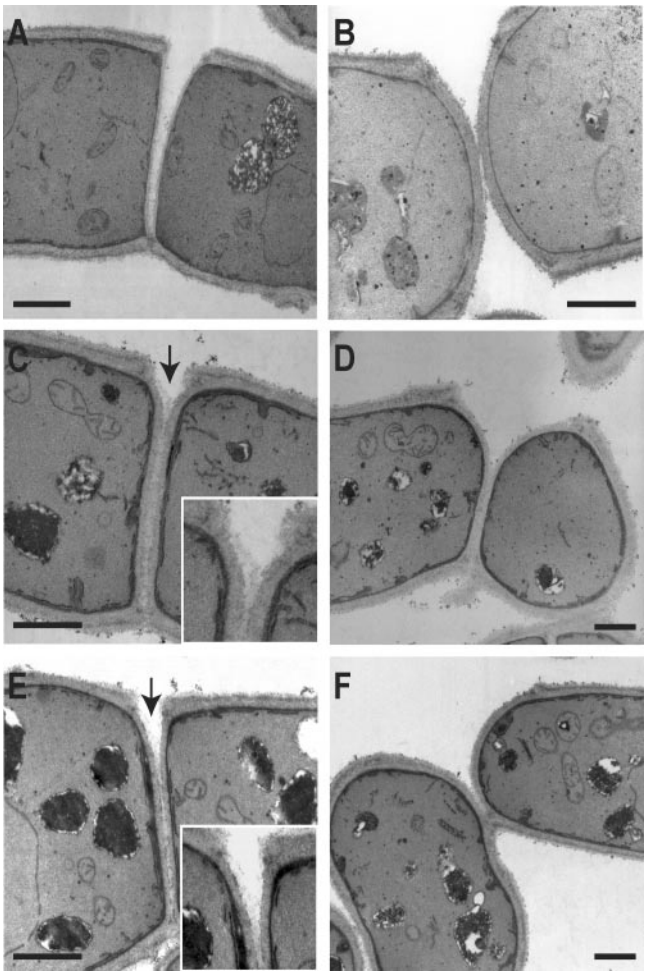

Figure 10. Digestion of the primary septum is defective in *mid2*Δ and *spn4*Δ cells. Wild-type (KGY972) (A and B) and *mid2*Δ (KGY3135) (C and D) and *spn4*Δ (KGY3986) (E and F) mutant cells were grown at 30°C, fixed, and processed for electron microscopic analysis as described under *Materials and Methods*. Left (A, C, and E), typical cells in the early stages of septum digestion. Black arrows point to undigested primary septum material (C and E). Right (B, D, and F), cells at later stages of separation. Bar (A–F), 1 μm.

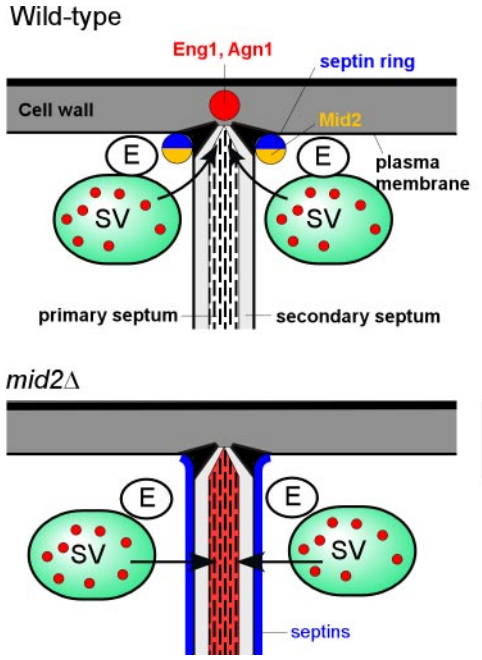

Figure 11. Model representing the targeting of Eng1 and Agn1 to the septum region. In wild-type cells (top), the septins would act as positional markers to guide the secretory vesicles (SVs) in a process mediated by the exocyst (E) to allow the localization of Eng1 and Agn1 as a ring. In *mid2*Δ mutants (bottom), abnormal distribution of septins in a disk structure results in the mislocalization of Eng1 and Agn1 and the formation of an abnormal structure resembling a disk (see text for details).

that resembled a disk rather than the normal ring. This defect was similar to that observed in *spn*Δ mutants, but it was not due to abnormal septin positioning, because septin rings were normally assembled in *sec8-1* mutants at both temperatures. We have no clear explanation for this defect, but it is possible that when exocyst function is reduced, then the secretory vesicles are not properly directed to their correct destination.

Septins and Mid2 are also required for Eng1 and Agn1 localization to the septum. In fission yeast, septin mutants have a mild phenotype, and the only apparent defect is a delay in cell separation lasting about a single generation time, resulting in the formation of groups of four cells (Longtine *et al*., 1996). Mid2 is required to maintain the stability of the septin ring, and *mid2*Δ mutants show a phenotype very similar to that of *spn*Δ mutants (Berlin *et al*., 2003; Tasto *et al*., 2003). Interestingly, *mid2*$^+$ is induced by Ace2 at the end of mitosis, suggesting that stabilization of the septin ring would be important during this moment of the cell cycle. In fact, it has recently been shown that Mid2 is the main factor required for septin ring coalescence and organization of the septin ring (An *et al*., 2004). We have shown here that Eng1/Agn1 form a ring in the septum region that is localized between the two septin rings. Our results suggest that the organization of the septin ring is a prerequisite for the assembly of the Eng1/Agn1 ring at the septum edging so that efficient cell separation can occur and that when the septin ring assembly fails, so does that of the separation ring. In *mid2*Δ and *spn*Δ mutants, failure in septin ring assembly results in abnormal localization of the hydrolytic enzymes, but not in a decrease in their enzymatic activity (at least that of Eng1p). This suggest that one of the functions of the septin ring in *S. pombe* could be to act as positional cues for the targeting of the hydrolytic enzymes required for cell separation. This idea is strengthened because the formation of a disk is specific to *mid2*Δ and *spn*Δ mutants and not a general consequence of the cell separation defect, because *ppb1*Δ mutants showed normal rings. Interestingly, Spn3 and Mid2 also localize correctly in *ppb1*Δ mutants (Tasto *et al*., 2003). An alternative possibility is that in fission yeast septins act as barriers to compartmentalize the cortex around the cleavage site, similar to the function that has been recently described for *S. cerevisiae* septins (Dobbelaere and Barral, 2004). Although this has been demonstrated for membrane and cytoplasmic proteins, it is more difficult to envision how septins, located at the cytoplasmic side of the plasma membrane, can interact with extracellular proteins located in the cell wall. Perhaps the establishment of a specialized region in the cell wall required for cell separation (in which the Eng1 and Agn1 hydrolases are concentrated) is just the consequence of targeted secretion mediated by the exocyst and the septins. The apparent in-

dependence of the exocyst and septin ring for localization to the medial region might reflect the primary role of the actin cytoskeleton in directing and maintaining both structures at the cell division site (Tasto *et al*., 2003). However, subtle alteration in exocyst localization in *mid2* and *spn* mutants that were undetectable by light microscopy cannot be ruled out.

A model accounting for the formation of the Eng1/Agn1 ring is shown in Figure 11. In wild-type cells, the septin ring (stabilized by Mid2) acts as a positional marker to direct the targeting of exocyst-mediated secretory vesicles to the region of the cell where the membrane and the septum contact each other. This would allow the release of the cargo proteins (including the hydrolytic enzymes Eng1 and Agn1) into the septum edging as a ring that completely surrounds the septum. Once this ring has been assembled, cell separation would proceed in a centripetal manner, from the septum edging toward its center. Although the main defect of *eng1*Δ mutants is their inability to cleave the primary septum, we cannot rule out that this protein might also play some role in the controlled dissolution of the septum edging, because β-1,3-glucans are also present in this region.

In *mid2*Δ mutants, the stability of the septin ring is compromised, and the ring rapidly disassembles and completely covers the cleavage furrow, forming a structure that is also similar to a disk (Berlin *et al*., 2003; Tasto *et al*., 2003). In these mutants, disorganization of the septin ring will result in abnormal Eng1/Agn1 localization, which are found as a disk rather than a ring. This defect would probably be caused by the fusion of secretory vesicles all over the cleavage furrow rather than at the ends, releasing their content to the septum region instead of to the septum edging. In septin mutants, the situation could be similar to that described for *mid2*Δ cells, because it has been shown that Mid2 does not localize properly in *spn4*Δ mutants (Berlin *et al*., 2003; Tasto *et al*., 2003). In these mutants, cell separation would proceed from the inside to the outside, a process that could be much more inefficient than in wild-type cells. This would explain, at least in part, the delay in cell separation observed in *mid2*Δ and *spn*Δ mutants.

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