The Multiprotein Exocyst Complex Is Essential for Cell Separation in *Schizosaccharomyces pombe*

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*Schizosaccharomyces pombe* cells divide by medial fission through the use of an actomyosin-based contractile ring. A multilayered division septum is assembled in concert with ring constriction. Finally, cleavage of the inner layer of the division septum results in the liberation of daughter cells. Although numerous studies have focused on actomyosin ring and division septum assembly, little information is available on the mechanism of cell separation. Here we describe a mutant, sec8-1, that is defective in cell separation but not in other aspects of cytokinesis. *sec8-1* mutants accumulate ~100-nm vesicles and have reduced secretion of acid phosphatase, suggesting that they are defective in exocytosis. Sec8p is a component of the exocyst complex. Using biochemical methods, we show that Sec8p physically interacts with other members of the exocyst complex, including Sec6p, Sec10p, and Exo70p. These exocyst proteins localize to regions of active exocytosis—at the growing ends of interphase cells and in the medial region of cells undergoing cytokinesis—in an F-actin–dependent and exocytosis-independent manner. Analysis of a number of mutations in various exocyst components has established that these components are essential for cell viability. Interestingly, all exocyst mutants analyzed appear to be able to elongate and to assemble division septa but are defective for cell separation. We therefore propose that the fission yeast exocyst is involved in targeting of enzymes responsible for septum cleavage. We further propose that cell elongation and division septum assembly can continue with minimal levels of exocyst function.

**INTRODUCTION**

Cytokinesis is the stage in the cell division cycle during which the boundaries between the two daughter cells are assembled and individual daughter cells are liberated. Given the complex nature of this event, spatial and temporal regulations are key issues underlying this process. Cytokinesis in a variety of eukaryotes is achieved through the use of an actomyosin-based contractile ring. The constriction of the actomyosin ring generates the force necessary for cell cleavage. Newly synthesized membrane is inserted at the site of division concomitant with the constriction of the actomyosin ring. Although this process has been studied at a descriptive level for decades, it is only recently that we are beginning to gain a molecular framework for understanding the mechanism and regulation of cytokinesis.

The fission yeast *Schizosaccharomyces pombe* is an attractive model organism for the study of cytokinesis. Like animal cells, *S. pombe* cells divide through the use of an actomyosin ring. This ring is assembled at the onset of mitosis. At the end of mitosis, the actomyosin ring constricts concomitant with the formation of the division septum. Genetic studies in *S. pombe* have identified many genes important for various steps in cytokinesis (Simanis, 1995). The genes mid1, plo1, and pom1 are required to position the actomyosin ring, and the division septum. Mid1p and Plo1p act possibly in a signaling pathway that integrates nuclear positioning with the position of the actomyosin ring (Bähler et al., 1998a). The genes cdc3, cdc4, cdc8, cdc12, rng2, rng3, rng4, rng5/myo2, rlc1, and act1 are required for the assembly of the actomyosin ring. The identity of their gene products as actin cytoskeleton elements is consistent with the idea that they interact to effect actomyosin ring assembly (Balasubramanian et al., 1992, 1994; Chang et al., 1997; Eng et al., 1998; McCollum et al., 1999; Naqvi et al., 1999, 2000; Wong et al., 2000). After actomyosin ring assembly, the function of the ring component Cdc15p, a SH3 domain-containing protein, is required for the assembly of the F-actin patches adjacent
to the actomyosin ring (Fankhauser et al., 1995; Balasubramanian et al., 1998). The genes cdc7, cdc11, cdc14, sid1, sid2, spo1/sid3, and sid4, which encode signaling molecules (collectively referred to as the Septation Initiation Network [SIN]), regulate division septum assembly during actomyosin ring constriction (Fankhauser and Simanis, 1994; Gould and Simanis, 1997; Balasubramanian et al., 1998; Sparks et al., 1999; Guertin et al., 2000). Genetic studies indicate that the activation of the SIN pathway might regulate Cps1p, a 1,3-β-glucan synthase essential for the assembly of the division septum (Le Goff et al., 1999; Liu et al., 1999). After assembly of the primary septum (composed primarily of unbranched 1,3-β-glucan and 1,3-α-glucan) and the secondary septa (composed of branched 1,3-β-glucan, α-galactomannan, and 1,3-α-glucan), the primary septum is cleaved to liberate two daughter cells (Humel et al., 2001). Although the mechanisms of actomyosin ring assembly, constriction, and division septum assembly have received considerable attention, very little is known about how cleavage of the primary septum is achieved to effect the liberation of the two daughter cells.

In this study, we describe the characterization of sec8-1, a mutant defective in cell separation. Sec8p is a component of the exocyst complex that plays a key role in delivery of secretory vesicles in a number of organisms (Ting et al., 1995; TerBush et al., 1996; Grindstaff et al., 1998). The exocyst localizes to regions of active secretion in fission yeast. We come to the interesting conclusion, based on analysis of a series of mutations in members of the S. pombe exocyst complex, that the exocyst complex is rate-limiting for cell separation but that only low levels of exocyst function are required for cell elongation and division septum assembly.

MATERIALS AND METHODS

Media, Reagents, and Genetics

S. pombe strains used in this study are listed in Table 1. Yeast cells were grown on YES medium or minimal media with appropriate supplements (Moreno et al., 1991). Crosses were performed by mixing appropriate strains directly on YPD plates (Moreno et al., 1991), except that sec8-1 was transformed with plasmid pREP3–sec8 before crosses. Recombinant strains were obtained by tetrad analysis. Yeast transformations were performed by the lithium acetate protocol (Okazaki et al., 1990). Kanamycin was used at 100 μg/ml. To eliminate F-actin, yeast cells were treated with latrunculin A (composed of branched 1,3-glucan, 2000). Genetic studies indicate that the actomyosin ring of S. pombe mutant sec8-1 (sec8-1). An S. pombe genomic library (Balasundaram et al., 1999) was introduced into mut2-1 mutant cells, and transformants were selected at 36°C. One plasmid was found to be able to reverse the temperature sensitivity. Nucleotide sequence determination and BLAST searches suggested that the rescuing DNA was located on cosmid SPCC970 (SPCC970.09, Accession no. U74562), and the only gene on this plasmid encoded a protein homologous to Sec8p in Saccharomyces cerevisiae. Three experiments were done to show that sec8-1 is defective in sec8. 1) A genetic cross between mut2-1 and sec8-GFP (marked with ura4+) showed that sec8-1 is tightly linked to the sec8 locus (no recombinants in 20 tetrads). 2)
Epitope Tagging and Regulated Expression of the Exo70 Gene Products

Chromosomal copies of sec6−, sec8−, sec10−, and exo70− were tagged by the carboxy-terminal addition of GFP and/or the Myc epitope. To tag Sec6p with GFP, a 0.8-kb KpnI/SmaI fragment of the sec6− C-terminal sequence was obtained by PCR using the primers MOH854 (GATGCTGAGGAAAATGTAGGAGGCAGAAGGGAC) and MOH855 (CGATGGCGGCTCTTCTCCGGCGAC) and cloned into pJK210-GFP. The resulting plasmid pJK210-sec6-C-T-GFP, containing sec6 fused in frame with GFP sequences, was linearized with NotI and transformed into a wild-type strain of genotype leu1-32 ura4-16 Adh1-20. To tag Sec8p with GFP, primers MOH714 (CACGGCTACAGTATTTTGGATGCTATTC) and MOH715 (CTACGCGGAGATTTTCTCGGACCACCCAG) were used to generate a 700-bp KpnI-SalI fragment that was cloned into pJK210-GFP to yield pJK210-sec8-C-T-GFP. This plasmid was linearized with EcoRI and transformed into wild-type cells. Similarly, primers MOH856 (GATGTTCTACTGAGGGATTTTG) were used to amplify the sec8− ORF (SPCC736.04c, Accession no. P09988), which was cloned into pJK210-GFP to yield pJK210-sec8-C-T-GFP. This plasmid was linearized with SpeI and introduced into wild-type cells. In each case, transformants were selected on supplemented minimal medium lacking uracil, and putative integrants were subjected to PCR and Western blot analyses to confirm the desired integration.

A similar strategy was used for Myc tagging. A 1.2-kb BamHI/BglII digested fragment containing the 13myc sequence and terminator from pFA6a-13myc (Bähler et al., 1998b) was cloned into the BamHI site of pJK210 (Keener and Boeke, 1994) to generate plasmid pJK210-13myc. A 0.7-kb NotI/BamHI-digested fragment containing carboxy-terminal sequence of sec6− was obtained using primers MOH638 (GCTAGCGGGCGCCGGCGACTGCTCTTTTGCGGAATACCGCTTC) and MOH639 (GATCCGCTCGGCGACTGCTCTTTTGCGGAATACCGCTTC), and cloned into pJK210-GFP. This plasmid was linearized with SpeI and introduced into wild-type cells. In each case, transformants were selected on supplemented minimal medium lacking uracil, and putative integrants were subjected to PCR and Western blot analyses to confirm the desired integration.

Epitope Tagging of gma12 and hht2

Primers gma12F (CCCTCCTGTAACCTCAAGACACACACGATCTGGCACC) and gma12R (CTTCCTCCTGAGGAGTAGTACGGTCTCAAAGGATTTC) were used to amplify the gma12 ORF (SPCC736.04c, Accession no. Q09174) by PCR, using wild-type genomic DNA as template. Primers MOH768 (CGCCGTTGACAAAGCCATTTTGCTCGTGACTGAC) and MOH769 (CGCCGTTGACAAAGCCATTTTGCTCGTGACTGAC) were used to amplify the hht2 ORF (SPAC135.04, Accession no. P09988), using wild-type genomic DNA as template. These fragments were cloned into pJK210-GFP (Naqvi, N. and Balasubramanian, M. K., unpublished results) as KpnI/SmaI fragments to generate pJK210-gma12-GFP and pJK210-hht2-GFP. These plasmids were linearized and transformed into a wild-type strain, and colonies were selected for growth on medium lacking uracil. Correct integrations were confirmed by PCR assay.

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To construct a sec8 shut-off strain, we made a tagging cassette containing 5’ upstream regulatory sequences of sec8, urad-1, the 81nmt1 promoter, and the coding sequences of sec8 sequentially to replace the sec8 gene with the 81nmt1 promoter-controlled sec8. The 81nmt1 promoter region from pEP18 was cloned into pSK-urad-1 (Naqvi, N., and Balasubramanian, M. K., unpublished results) to generate pSK-urad-81nmt1. A 0.5-kb fragment containing 5’ sequence of sec8 was obtained by PCR using primers MOH724 (CAT-GTGACCGTATGATCGAGGATACGTACGAGG) and MOH790 (CCATCCGATAAGGGTTGTGAATCAAGC), and the N-terminal sequence of sec8 was amplified by PCR using primer MOH746 (CGGGATCCCATATGGATACCAGAGGCTATTCGGAAACG) and MOH790 (CCCTCGAGCGACGGATCCGGTTGGAAGCAGCAGA), respectively. The first fragment was digested with KpnI and Clal, and the latter fragment was digested with NdeI and SacI. These two fragments were cloned into pSK-urad-81nmt1 sequentially to yield a plasmid that was then linearized with SacI and XhoI. The linearized DNA was used to transform wild-type cells of genotype leu1-32 urad-D18 ade6-210. Transformants were selected on medium lacking uracil, and correct integration was confirmed by PCR assay. The sec8 shut-off strain was maintained in minimal medium lacking thiamine. To shut off Sec8p expression, cells were grown in minimal medium containing thiamine at 30°C for 14 h.

Synchronization by Nitrogen Starvation

MBY888 (sec8-1) was generated by crossing MBY887 (sec8-1, urad-1D18, leu1-32, h+) to wild-type strain 972 (Leupold, 1970). MBY888 and wild-type cells were grown in minimal medium overnight at 24°C to early log phase (optical density 0.4). Cells were washed three times with minimal medium lacking nitrogen, resuspended in the same medium, and grown for 18 h at 24°C to arrest in the G1 phase. Cells were shifted to 36°C for 1 h to inactivate Sec8p and then transfered into YES (rich medium) to release cells from G1 and allow mitotic cell cycle progression at 36°C. Cell samples were taken just before the release (0) and every hour after the release (1–8).

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were performed essentially as described (Naqvi et al., 1999). Cells were grown to exponential phase in YES medium at 24°C and harvested. Cell lysis was carried out by acid-washed glass beads to the cell pellet and subsequent disruption using a mini-bead beater. Cell extracts were clarified by centrifugation and the resulting supernatant was used to extract soluble proteins. PMSF, 2 mM Benzamidine) was used to extract soluble proteins. For each immunoprecipitation, 500 µl of soluble protein extracts or immunoprecipitates, proteins were separated on 6% gel and transferred to nitrocellulose membranes. The membranes were incubated with 0.1% Tween 20, pH 7.6) for 1 h at room temperature. Primary anti-GFP and anti-Myc antibodies were used at 1:700 and 1:1000 dilutions, respectively. Peroxidase-conjugated anti-rabbit and antimouse IgG (Sigma) were used at 1:4000 dilutions, and the enhanced chemiluminescent signal was detected using a 1:1 mixture of ECL1 (2.5 mM 3-aminoethylidrazide dissolved in DMSO, 0.4 mM p-coumaric acid, 100 mM Tris-HCl, pH 8.5) and ECL2 (0.02% H2O2, 100 mM Tris-HCl, pH 8.5; Schneppenheim et al., 1991).

Measurement of Acid Phosphatase Secretion

Acid phosphatase secretion was assayed as follows (modified from Craighead et al., 1993; Tanaka and Okayama, 2000). Because up to 40% of acid phosphatase is secreted into the medium in fission yeast, enzyme activity was assayed in the culture supernatant. Cells were grown to log phase in minimal medium (MM) at 24°C, pelleted, washed twice with MM, and resuspended in fresh MM at 24°C or 36°C. Samples were taken at 0 h (time of resuspension) and at hourly intervals thereafter. For each sample, 1 ml of culture was centrifuged, and 500 µl of the supernatant was added to 500 µl of substrate solution (2 mM p-nitrophenyl phosphate, 0.1 M sodium acetate, pH 4.0; prewarmed to 30°C) and incubated at 30°C for 5 min. Reactions were stopped by the addition of 500 µl of 1 M sodium hydroxide. The absorbance at 405 nm was measured, using the 0-h sample as a blank control.

RESULTS

mut2-1 Identifies a Gene Product Important for Cell Separation and Is Defective in the Exocytosis Component Sec8p

We performed a screen to identify mutants defective in cytokinesis in S. pombe. To visualize nuclei easily, a strain was constructed in which the coding region of the histone H3 gene (hht2) was fused to green fluorescent protein sequences (hht2-GFP). As expected, Hht2-GFP localized to the nucleus throughout the cell cycle (our unpublished results). This starting strain (MBY816) was mutagenized by UV irradiation, and the resulting ts mutants were subjected to microscopic analysis to detect mutants that accumulated multiple nuclei (Tang, X., and Balasubramanian, M.K., unpublished results). The characterization of one such mutant, mut2-1, is described in this study. Mut2-1 cells grew and formed colonies at 24°C (permissive temperature) but were unable to do so at 36°C (restrictive temperature). Although wild-type cells continued to grow and divide upon temperature shift from 24 to 36°C, the cell number of a mut2-1 strain did not increase after an identical temperature shift (Figure 1A), whereas the number of attached cell bodies increased, indicating failed cell separation. To better characterize the phenotype of mut2-1, we monitored changes in the subcellular distribution of F-actin and cell wall material after a shift from 24 to 36°C (Figure 1B). Under permissive conditions, F-actin rings and septa in the majority of mut2-1 cells resembled those found in wild-type cells (Figure 1B, 0 h). After 4 h at 36°C, >50% of mut2-1 cells contained four nuclei, indicative of the successful completion of two rounds of mitosis despite the aberrant cytokinesis (Figure 1B, 4 h). Interestingly, under these conditions, assembly and constriction of the actomyosin ring were not impaired in mut2-1 cells (Figure 1B, arrow). In addition, mut2-1 cells were also capable of assembling medial division septa (Figure 1B). However, the septa apparently could not be disassembled in mut2-1 cells, leading to the accumulation of elongated cells with one or three septa. Thus, mut2-1 identifies a protein important for cell separation after assembly of the division septum.

To identify the gene responsible for the mut2-1 phenotype, a plasmid rescuing the temperature-sensitive lethality of mut2-1 was identified (see MATERIALS AND METHODS).
The rescuing DNA encoded a 1088-amino acid polypeptide. Database searches using the predicted protein sequence showed that it was related to \textit{S. cerevisiae} Sec8p (16\% amino acid identity), a component of the exocyst, as well as to Sec8p-like proteins from humans (13\% identity, Figure 2). Several lines of evidence established that \textit{mut2-1} is an allele of \textit{sec8}/H11001 (see MATERIALS AND METHODS).

The exocyst is required for polarized cell growth and cell surface expansion in \textit{S. cerevisiae} (TerBush \textit{et al.}, 1996; Roth \textit{et al.}, 1998). In contrast, the \textit{sec8-1} mutant described in this study appeared to be defective only in septum disassembly and cell separation. To test the role of Sec8p in cell elongation, wild-type and \textit{sec8-1} cells were synchronized by nitrogen starvation and monitored for the ability to undergo polarized cell growth and septum assembly. This protocol provides a convenient means to assess the function of a protein in cell elongation, because wild-type cells start at the length of 4 \(\mu\)m and elongate to 12–14 \(\mu\)m before division (Figure 3A). After release into rich medium, \textit{sec8-1} cells were able to elongate, enter mitosis, and assemble division septa with kinetics similar to that of wild-type cells (Figure 3B). However, unlike wild-type cells, \textit{sec8-1} cells failed to disassemble the division septa, leading to the accumulation of binucleate cells with a medial division septum. Even although septum cleavage and cell separation failed, \textit{sec8-1} cells reinitiated polarized growth and underwent a second round of mitosis and division septum assembly 7 h after release into the rich medium. Septum cleavage again failed in these cells resulting in the accumulation of tetraneucleate cells with three septa. On prolonged incubation (12 h) \textit{sec8-1} cells lysed. Cell length and the percentage of septated cells at each time point were also quantified (Table 2), which indicated that \textit{sec8-1} is not defective in cell elongation and is not delayed for septum assembly. These data suggest that \textit{sec8-1} cells are specifically defective in septum cleavage and cell separation.
Identification of sec6^+, sec10^+, sec15^+, and exo70^+ Sequences from the S. pombe Genome Database

The exocyst in S. cerevisiae is a multprotein complex comprised of Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (TerBush et al., 1996). We have identified Sec8p in S. pombe as a homologue of one component of the exocyst. We therefore searched the S. pombe databases to determine whether other exocyst components were also present in fission yeast. Interestingly, homologues of S. cerevisiae Sec6p, Sec10p, Sec15p, and Exo70p were also present in S. pombe (see MATERIALS AND METHODS). We were unable to identify proteins related to Sec3p, Sec5p, or Exo84p. The alignments of the S. pombe exocyst proteins (named like their S. cerevisiae homologues) with their counterparts in other organisms are shown in Figure 4. These four exocyst proteins in S. pombe share ~20% identities in sequences and align through their entire lengths with their homologues. Thus, several components of the exocyst complex are conserved in S. pombe.

The Exocyst Components Interact In Vivo

Immunoprecipitation experiments were performed in order to determine whether S. pombe Sec6p, Sec8p, Sec10p, and Exo70p form a complex in vivo, as has been demonstrated with their counterparts in other organisms. A number of strains expressing either c-Myc- or GFP-tagged versions of Sec6p, Sec8p, Sec10p, and Exo70p were constructed. To test the interaction between Sec8p and Sec6p, protein extracts from strains expressing sec8-GFP alone, sec8-GFP and sec6-Myc, or sec6-Myc alone were immunoprecipitated using anti-GFP antibodies and analyzed using a Myc mAb. Sec6p-Myc was only detected in the immunoprecipitates from the sec8-GFP sec6-Myc strain (Figure 5A), suggesting that these two proteins associate in vivo. To test the interaction of Sec8p with Sec10p, similar immunoprecipitations were performed using extracts of strains expressing sec8-GFP alone, sec8-GFP and sec10-Myc, or sec10-Myc alone. Sec10-Myc was detected only in the immunoprecipitates from sec8-GFP sec10-Myc cells (Figure 5B). Thus, Sec8p also interacts with Sec10p. Finally, the interaction of Sec8p with Exo70p was demonstrated in similar experiments (Figure 5C). In addition, we observed interactions in the other pairwise combinations (Sec6p-Sec10p, Sec6p-Exo70p, and Sec10p-Exo70p; unpublished results). Thus, the exocyst components Sec6p, Sec8p, Sec10p, and Exo70p physically interact with each other in S. pombe.

Sec6p, Sec8p, Sec10p, and Exo70p Localize to the Division Site

The subcellular localization of Sec8p was determined by tagging the chromosomal copy of sec8^+ with GFP sequences. In this strain, the expression of Sec8p-GFP was under the control of the sec8^+ promoter. The sec8-GFP cells resembled wild-type cells in morphology and growth rates, establishing that the addition of GFP did not compromise the function of Sec8p. However, the Sec8p-GFP signal was prone to rapid photobleaching. Therefore, indirect immunofluorescence was performed to visualize Sec8p-GFP. In interphase cells, identified as uninucleate cells with uncondensed chromosomes, tip localization was observed in 55% (Figure 6A, marked with arrowheads). In early mitotic cells, tip localization was absent and Sec8-GFP was seen as a ring in the...
medial region of the cell that resembled the actomyosin ring (Figure 6A, cells marked with 1 and 4). However, in late mitotic cells, unlike the actomyosin ring, which undergoes constriction, medial staining of Sec8p-GFP was detected as double rings (Figure 6A, cells marked with 2 and 3). To examine whether these structures were real ring structures, confocal microscopy and 3D-projection software were used to determine the localization of Sec8-GFP. When Sec8-GFP double ring images were rotated, they appeared clearly as rings (Figure 6B; arrow marks the entire ring visualized upon rotation by $139^\circ$). Essentially identical localization patterns were observed for Sec6p-GFP, Sec10p-GFP, Sec6p-Myc, Sec10p-Myc, and Exo70p-Myc (Figure 6, A, C, and D). Thus, consistent with their coimmunoprecipitation, components of the exocyst also colocalized in S. pombe cells, supporting the hypothesis that the exocyst components interact in vivo.

To investigate the localization of exocyst components in relation to the actomyosin ring, we examined the localization of Sec10p-GFP and Myo2p (an actomyosin ring component) in the same cells. Both proteins assembled into ring structures at early mitosis and approximately colocalized (Figure 6E, top panel). However, in cells undergoing actomyosin ring constriction (Figure 6E, bottom panel), constriction of the Sec10-GFP rings was not observed. Instead, the Sec10-GFP rings split into a pair of rings on either side of the constricting actomyosin ring.

The Medial Localization of the Exocyst Is Dependent on the Actomyosin Ring but not on Exocytosis

Given that the S. pombe exocyst components assembled as a medial ring that colocalized approximately with the actomyosin ring at early mitosis, we addressed the roles of the F-actin cytoskeleton and of proteins important for actomyosin ring formation in the assembly of the exocyst complex at the division site. First, we monitored the localization of

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**Table 2.** Cell elongation and septum assembly in wild-type and Sec8-1 cells.

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<tr>
<th>Time after release from G1</th>
<th>WT</th>
<th>sec8-1</th>
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<td></td>
<td>0 h</td>
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<tr>
<td>Cell length (μm)</td>
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<td>5.7</td>
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<td>Septated cells (%)</td>
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Figure 3. sec8-1 cells are not defective in polarized cell growth. Wild-type cells (A) and sec8-1 cells (B) were synchronized in G1 by growth in nitrogen-free medium for 18 h at 24°C, and shifted to 36°C for 1 h to inactivate the Sec8-1p protein. Cells were then resuspended in rich medium to allow cell cycle progression at 36°C. Samples were collected just before resuspending in rich medium (0 h) and at intervals thereafter and stained with Calcofluor to visualize septa. Bar: 10 μm.
Sec10-GFP after treatment of G2-synchronized cells with latrunculin A (LatA), a drug that prevents actin polymerization. Although DMSO alone did not affect assembly of medial Sec10-GFP rings, cells treated with LatA in DMSO were unable to assemble medial Sec10p-GFP rings (Figure 6F). Thus, the proper assembly of Sec10p and, by inference, the other exocyst components as a medial ring at the division site is F-actin dependent. We then examined Sec10p-GFP localization incdc8-110 (Balasubramanian et al., 1992), cdc12-112 (Chang et al., 1997), andcdc15-140 (Fankhauser et al., 1999).
from Golgi to ER upon treatment with BFA (Figure 6I). In contrast, the localization of Sec10p was not affected by BFA (Figure 6I), indicating that the exocyst localization is independent of exocytosis. Thus, the exocyst complex in *S. pombe* could serve as a landmark for the targeting of the exocytic machinery.

**Phenotype of Exocyst Null Mutants**

Although exocyst mutants in *S. cerevisiae* are defective in polarized growth (Hsu et al., 1999), *sec8-1* mutants in *S. pombe* appear to be unaffected with respect to polarized growth. Given this dramatic difference in phenotype, it seemed possible that *sec8-1* was not defective in a polarized growth function of Sec8p. In this case, a *sec8* null mutant would be expected to show a stronger phenotypic defect with respect to cell growth. To test this, we replaced *sec8*+ with *ura4*+ in a diploid strain. By analysis of meiotic products from the heterozygous strain, we found that spores bearing the *sec8*-null mutation were incapable of forming colonies. Thus, Sec8p is essential for cell viability. To characterize the terminal phenotype, the mutant spores were germinated and stained to visualize F-actin, DNA, and septa (Figure 7A). The mutant spores were capable of germination, cell elongation, mitosis, actomyosin ring assembly, and septum assembly. However, the septa assembled in the germinating mutant cells were not cleaved. Cell growth, cell elongation, and mitosis continued in the unseparated mutant cells, leading eventually to the accumulation of tetranucleate cells with septa placed between each pair of nuclei. Similar results were obtained with *sec6* and *sec10* null mutants (Figure 7D and E).

To ensure that the phenotype was not due to inherited maternal exocyst proteins, we tested whether the maternal Sec8p was present in *sec8*-null cells using a diploid strain in which one *sec8* locus was replaced with *ura4*+ and the other was tagged with Myc and *leu1*+. We examined whether the maternal Sec8-Myc protein was present in the germinated null mutant cells. Spores were germinated in medium selective for *ura4*+ or *leu1*+ and stained with antibodies against Myc and Mok1p to visualize Sec8-Myc and the α-glucan synthase Mok1p (Katayama et al., 1999). Although Sec8-Myc localization was clearly observed in *sec8*-Myc cells (our unpublished results), it was not observed in the *sec8* null cells (Figure 7B), suggesting that there was no significant carry-over of maternal Sec8p in these cells. Mok1p, used as a control, was observed in both cases as expected.

To analyze the *sec8* loss-of-function phenotype using a different approach, we constructed a *sec8* shut-off strain in which *sec8* transcription was under the control of the low-strength and thiamine-repressible 81*nm1t1* promoter. On growth under repressing conditions, *sec8* shut-off cells again appeared defective only in the disassembly of division septa but not in polarized cell growth (Figure 7C). We conclude that the exocyst is essential for septum disassembly and cell separation, whereas cell elongation and division septum assembly might require reduced levels of exocyst function or might be independent of it.

**sec8-1 Mutant Cells Are Defective in Exocytosis**

The exocyst in *S. cerevisiae* and mammals is involved in membrane trafficking from the Golgi apparatus to the

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**Figure 5.** Sec6p, Sec8p, Sec10p, and Exo70p associate in vivo. (A) Protein extracts were prepared from cells expressing Sec8-GFP (lanes 1), Sec8-GFP and Sec6-Myc (lanes 2), or Sec6-Myc alone (lanes 3). Total lysates (right panel) and immunoprecipitates prepared using anti-GFP antibodies (left panel) were blotted using anti-Myc (upper panels) and anti-GFP antibodies (lower panels). (B and C) Similar experiments were conducted using cells expressing Sec8-GFP, Exo70-Myc and Sec8-GFP, or Sec8-GFP, Sec6-Myc and Sec8-GFP, or Sec8-GFP, Sec6-Myc alone (lanes 3).
Figure 6. Localization of exocyst components in *S. pombe*. (A) Exocyst components localize to the division site and cell tips. *sec8-GFP* and *sec6-Myc* cells were stained with antibodies against GFP and Myc. In the merged micrograph, Sec8-GFP is in red, Sec6-Myc in green, and DNA in blue. Arrowheads: tip localization; 1, 4: a single ring structure; 2, 3: double ring structures. (B) Sec8-GFP appeared as two rings in late mitotic cells using confocal microscopy and 3D viewing. Top panel, cell with 0° rotation; lower panel, cell with 139° rotation. (C and D) Merged micrographs of Sec8-GFP with Sec10-Myc (C) and Exo70-Myc (D). Sec8-GFP is in red, Sec10-Myc or Exo70-Myc in green, and DNA in blue. (E) The localization of Sec10p in relation to Myo2p, an actomyosin ring marker. *cdc25-22* cells expressing Sec10p-GFP were synchronized in G2 by incubation at 36°C for 4 h and allowed to enter mitosis by shifting to 24°C. Cells were stained with antibodies against GFP and Myo2p. (F) The localization of Sec10p is dependent on intact F-actin structures. *cdc25-22* cells expressing Sec10p-GFP were...
plasma membrane (Hsu et al., 1999). To test whether the exocyst in S. pombe has a role in exocytosis, we used electron microscopy to ask if the targeting and fusion of secretory vesicles with the plasma membrane could occur normally in sec8 mutant cells. Presumed secretory vesicles (100 nm in diameter) were observed only rarely in wild-type cells (Figure 8A). In contrast, 60–100 such vesicles were detected in every section in the sec8-1 mutant (Figure 8B; average three vesicles/μm²). These vesicles were stained intensely after permanganate fixation and most likely represent post-Golgi secretory vesicles (Armstrong et al., 1993). In mutant cells undergoing septum assembly, most of the vesicles were clustered approximately in the vicinity of the septa. These observations suggested that targeting of secretory vesicles to the correct location occurs in sec8-1 cells but that the subsequent docking and/or fusion with the plasma membrane failed. During interphase, sec8-1 cells were also found to accumulate ~100 nm vesicles, indicating that Sec8p might also participate in exocytic events during interphase. Similarly, sec8 shut-off cells accumulated a large number of ~100 nm vesicles under repressing conditions (Figure 8D), whereas cells under nonrepressing conditions resembled wild-type cells (Figure 8C).

To test whether sec8 mutants are defective in exocytosis using a different approach, we monitored the transport of the enzyme acid phosphatase through the S. pombe secretory pathway in sec8-1 cells (Figure 8E). The activity of secreted acid phosphatase was assayed using the culture supernatant (see MATERIALS AND METHODS). Wild-type cells at 36°C secreted acid phosphatase about twice as fast as at 24°C. sec8-1 cells secreted much less acid phosphatase than wild-type cells at both temperatures. After 4 h, they secreted 67% of the level of activity of wild-type cells at 24°C and 42% of the activity at 36°C. Thus, sec8-1 is indeed defective in exocytosis.

**DISCUSSION**

**An Exocyst Complex in the Fission Yeast S. pombe**

Previous studies of cytokinesis in the fission yeast S. pombe have focused on actomyosin ring assembly, actin patch movement, signaling events that control septum delivery, and on the study of enzymes responsible for septum assembly (Simanis, 1995). However, little information was available on the regulation of cell separation. In this study, we have described the isolation of sec8-1, a mutant that is defective in cell separation after assembly of the division septum. Molecular cloning established that Sec8p is a component of the exocyst protein complex with homologues in several other organisms including the prototypic Sec8p from the budding yeast S. cerevisiae. The exocyst is a multiprotein complex that has been identified in a number of organisms (Hsu et al., 1999). In budding yeast the exocyst consists of seven core subunits: Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (Potenza et al., 1992; TerBush and Novick, 1995; TerBush et al., 1996; Guo et al., 1999). In addition, the budding yeast exocyst complex interacts with its targeting factor Sec3p and the rab-related GTPase Sec4p (Finger et al., 1998; Guo et al., 1999). Although homologues of Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo70 have been identified in other organisms including in mammalian cells, Sec3p and Exo84-related proteins have been identified only in S. cerevisiae (Kee et al., 1997). The exocyst proteins appear to be important for transport between the Golgi apparatus and the plasma membrane and have been implicated in targeting and fusion of Golgi derived vesicles with the plasma membrane (Bowser and Novick, 1991; Potenza et al., 1992; Roth et al., 1998; Hsu et al., 1999).

Using sequences of the budding yeast exocyst proteins, we have identified proteins related to Sec6p, Sec10p, Sec15p, and Exo70 in S. pombe. These four proteins are ~20% identical in protein sequence with the budding yeast, plant, and rat counterparts. Using biochemical methods we have shown that Sec6p, Sec8p, Sec10p, and Exo70p interact physically. We therefore conclude that an exocyst-like complex is present in S. pombe. However, the S. pombe exocyst complex appears to lack proteins related to the budding yeast Sec5p and Exo84p. It will be interesting to test if proteins structurally related to the budding yeast Sec5p and Exo84p associate with the S. pombe exocyst complex. The fact that sec8-1 mutants accumulate ~100 nm vesicles at the restrictive temperature indicates that the exocyst complex in S. pombe, as in budding yeast and in mammalian cells, is important for exocytic events. The accumulation of ~100-nm vesicles in interphase as well as mitotic cells suggests that the exocyst might participate in exocytic events in all phases of the cell cycle. Independent experiments on secretion of acid phosphatase in wild-type and sec8-1 cells also conforms a role for the S. pombe exocyst in exocytosis. Whether the exocyst is required for all exocytosis events remains to be established.

**The S. pombe Exocyst Localizes to Regions of Active Secretion**

We show that the fission yeast exocyst proteins localize to both cell tips as well as the site of cell division. In early mitosis, the exocyst colocalizes with the actomyosin ring and later splits into two rings upon constriction of the actomyosin ring. We have shown that the localization of the exocyst complex to the division site is dependent on an intact F-actin cytoskeleton and also on the molecules that are important for actomyosin ring assembly. Thus, the actomyosin ring might serve as a spatial landmark for targeting of the exocyst complex. It is also possible that the exocyst complex might be transported to the division site along the F-actin cables (Marks and Hyams, 1985; Balasubramanian et al., 1996; Pelham and Chang, 2001) that are attached to the actomyosin ring. The function of Cdc15p, an SH3 domain containing protein is also essential for assembly of the exocyst.
cyst at the division site (Fankhauser et al., 1995). It is interesting to note that Cdc15p is also related to proteins of the PACSIN family, which are important for membrane transport events (Lippincott and Li, 2000). It is likely that Cdc15p might participate in membrane transport events pertaining to cytokinesis and might allow the targeting of proteins that specify exocytic events or allow the localization of proteins that themselves utilize the exocytic pathway during cytokinesis.

The localization of the exocyst appears to be independent of secretion because disruption of the Golgi apparatus by treatment with BFA does not impair the ability of the exocyst complex to localize to the actomyosin ring. The secretion-independent localization of the S. pombe exocyst is different from the situation in S. cerevisiae, where it has been shown that the localization of all members of the exocyst complex (with the exception of the targeting subunit, Sec3p) depends on the secretory pathway (Finger et al., 1998). We

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**Figure 7.** (A, D, and E) Null mutants of sec8 (A), sec6 (D), and sec10 (E) are defective in cleavage of the septum. Mutant spores were germinated in appropriate selective media and stained with DAPI, phalloidin, and Calcofluor to visualize DNA, F-actin, and septa, respectively. (B) Germinated sec8-null mutant cells do not contain detectable maternal Sec8p (see text for details). (C) sec8 shut-off cells are defective in cell separation. §1nmt1-sec8 cells were grown in thiamine-containing medium for 14 h at 30°C and stained with DAPI, phalloidin, and Calcofluor to visualize DNA, F-actin, and septa, respectively. Bar: 10 µm.
Figure 8. sec8 mutants are defective in exocytosis. (A-D) sec8-1 mutant and sec8 shut-off cells accumulate large numbers of secretory vesicles. (A and B) Wild-type (A1, A2) and sec8-1 (B1–B3) mutant cells were grown at 24°C and shifted to 36°C for 4 h, fixed, and processed for electron microscopic analysis. Higher magnifications of the framed regions in A1 and B1 are also shown (A1' and B1'). Arrows point at presumed secretory vesicles; arrowheads point at division septa. (C and D) sec8 shut-off cells were grown in medium lacking (C) or containing (D) thiamine at 30°C, fixed, and processed for electron microscopic analysis. Note that in the mutant cell forming a septum (D), vesicles were accumulated in the vicinity of the septum. The average number of vesicles in wild-type (5 cells were quantified) and sec8-1 (6 cells were quantified) were 1 and 53, respectively. Bars: 0.5 μm in the two magnified boxes; 1 μm in all other cells. (E) sec8-1 secretes less activity of acid phosphatase. Wild-type (squares) and sec8-1 (triangles) cells were assayed for secreted acid phosphatase activity at 24°C (open) and 36°C (filled).
have been unable to find a Sec3p-like protein in \textit{S. pombe}. Thus, in the absence of a Sec3p-like protein the other components might have evolved additional secretion-independent mechanisms to achieve their intracellular localizations in \textit{S. pombe}. Thus, the exocyst complex might localize to the division site in a secretion-independent and F-actin-dependent manner to direct exocytic events.

\textbf{The \textit{S. pombe} Exocyst Is Critical for Cell Separation}

Mutations in the \textit{S. cerevisiae} exocyst members appear to block fusion of all post-Golgi vesicles with the plasma membrane. As a result, these mutants are unable to expand the cell surface and perish because of failure of all exocytic events (TerBush and Novick, 1995; TerBush \textit{et al.}, 1996; Finger and Novick, 1997; Roth \textit{et al.}, 1998). In contrast, \textit{S. pombe} exocyst mutants are capable of polarized growth, cell surface expansion, and division septum assembly. \textit{S. pombe} exocyst mutants appear to be specifically defective in cleavage of the division septum and cell separation. Given the differences in the phenotypes of exocyst mutants in the two yeasts, we have established the terminal phenotypes of the exocyst mutants using several approaches. We have investigated the terminal phenotypes of a \textit{sec8} temperature-sensitive mutant, a \textit{sec8} shut-off strain as well as the terminal phenotypes of germinated \textit{sec8}-null mutant spores. We have also established that the phenotype of \textit{sec8}-null mutant spores is not likely to be influenced by maternal carry-over of Sec8p from the heterozygous diploid. In addition, we have also shown that null mutations in \textit{sec6} and \textit{sec10} also result in a phenotype identical to that observed in the \textit{sec8} mutants. One possibility is that the exocyst is essential only for a subset of secretory events in \textit{S. pombe}. This conclusion is similar to that obtained from studies in MDCK cells where it has been shown that the exocyst is only important for delivery of proteins to the basolateral membranes but not to the apical membranes (Grindstaff \textit{et al.}, 1998). Thus, the exocyst might be essential for delivery of proteins important for septum cleavage, but not for proteins involved in cell elongation and division septum assembly. It is possible that the lethality of the exocyst null mutants results from inappropriate cleavage of cell wall rather than the septum after prolonged incubation at the restrictive conditions. Alternatively, the exocyst might participate in all secretory events in wild-type \textit{S. pombe} cells. In its absence, however, other pathways might substitute for the exocyst in some exocytic events. Previous studies have shown that additional mechanisms exist in budding yeast and mammalian cells for the delivery of proteins from the Golgi apparatus to the plasma membrane via early and recycling endosomes (Mallard \textit{et al.}, 1998; Brachet \textit{et al.}, 1999; Luo and Chang, 2000). Currently, it is unclear if transport from Golgi apparatus to the plasma membrane via endosomes requires exocyst function. In this model, the exocyst is rate limiting for the delivery of proteins important for septum cleavage and is redundant with other mechanisms important for targeting proteins required for polarized growth and division septum assembly. A third possibility is that in all the mutants that we have analyzed in this study, a low level of exocyst activity might persist that might be sufficient for cell elongation and division septum assembly but not for cell separation. A further investigation of these possibilities will require the isolation and characterization of a bank of temperature-sensitive mutant alleles of the various exocyst components, followed by detailed cell biological and biochemical characterization of these mutants using secretion assays. The identification of the contents of the 100-nm vesicles that accumu-

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