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

Hongyan Wang,^{*†} Xie Tang,^{*†} Jianhua Liu,^{*} Susanne Trautmann,[‡] David Balasundaram,^{*} Dannel McCollum,[‡] and Mohan K. Balasubramanian^{*§}

^{*}The Institute of Molecular Agrobiolgy, The National University of Singapore, Singapore 117604, Republic of Singapore; and [‡]Department of Molecular Genetics and Microbiology, The University of Massachusetts Medical Center, Worcester, Massachusetts 01605

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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

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[§] Corresponding author. E-mail address: mohan@ima.org.sg.

[†] Hongyan Wang and Xie Tang contributed equally to this work.

to the actomyosin ring (Fankhauser *et al.*, 1995; Balasubramanian *et al.*, 1998). The genes *cdc7*, *cdc11*, *cdc14*, *sid1*, *sid2*, *spg1/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 (Humbel *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 pREP3-1-*sec8* before crosses. Recombinant strains were obtained by tetrad analysis. Yeast transformations were performed by the lithium acetate method (Okazaki *et al.*, 1990). Kanamycin was used at 100 μ g/ml. To eliminate F-actin, yeast cells were treated with latrunculin A (L-12370; Molecular Probes Inc., Eugene, OR) at a concentration of 100 μ M for 3.5 h. To block ER-to-Golgi transport, cells were treated with 100 μ g/ml Brefeldin A (B-7450; Molecular Probes) for 3 h. Cells treated with DMSO and ethanol, respectively, were used as controls. Thiamine was used at a final concentration of 5 μ M to repress transcription from the *nmt1* promoter (Basi *et al.*, 1993).

Microscopy

Fluorescence microscopy was performed essentially as described (Balasubramanian *et al.*, 1997). Cells were viewed using a Leica DMLB microscope with appropriate filters. To visualize DNA, F-actin, and septum material, cells were fixed in 3.7% formaldehyde for 1 min and stained with 4',6-diamidino-2-phenylindole (DAPI), rhodamine-conjugated phalloidin, and Calcofluor (Sigma, St. Louis, MO), respectively, as described (Balasubramanian *et al.*, 1997). For all indirect immunofluorescence, cells were fixed in formaldehyde and probed with anti-GFP antibodies (1:500 dilution; Molecular Probes), anti-Myc antibodies (1:200 dilution; Sigma), anti-Myo2p antibodies (1:400 dilution; Naqvi *et al.*, 1999), anti-Mok1 antibodies

Table 1. *Schizosaccharomyces pombe* strains used in this study

Name	Relevant genotype	Source
MBY797	<i>sec6-GFP-ura4⁺ leu1-32 h⁻</i>	This study
MBY798	<i>sec10-GFP-ura4⁺ leu1-32 h⁻</i>	This study
MBY799	<i>cdc25-22 sec6-GFP-ura4⁺ h⁻</i>	This study
MBY800	<i>cdc25-22 sec10-GFP-ura4⁺ h⁻</i>	This study
MBY803	<i>sec6Δ::ura4⁺/sec6 leu1-32/leu1-32 h⁻/h⁺</i>	This study
MBY804	<i>sec10Δ::ura4⁺/sec10 leu1-32/leu1-32 h⁻/h⁺</i>	This study
MBY810	<i>cdc15-140 sec10-GFP-ura4⁺</i>	This study
MBY813	<i>cdc8-110 sec10-GFP-ura4⁺</i>	This study
MBY814	<i>cdc12-112 sec10-GFP-ura4⁺</i>	This study
MBY816	<i>hht2-GFP-ura4⁺ leu1-32 h⁻</i>	This study
MBY836	<i>sec10-13myc-ura4⁺ leu1-32 h⁻</i>	This study
MBY840	<i>sec6-13myc-ura4⁺ leu1-32 h⁻</i>	This study
MBY843	<i>exo70-13myc-Kan^R sec10-GFP-ura4⁺</i>	This study
MBY850	<i>sec6-13myc-ura4⁺ sec10-GFP-ura4⁺</i>	This study
MBY851	<i>sec10-13myc-ura4⁺ sec6-GFP-ura4⁺</i>	This study
MBY852	<i>exo70-13myc-Kan^R sec6-GFP-ura4⁺</i>	This study
MBY856	<i>sec8-GFP-ura4⁺ leu1-32 h⁻</i>	This study
MBY864	<i>sec8-GFP-ura4⁺ exo70-13myc-Kan^R</i>	This study
MBY866	<i>exo70-13myc-Kan^R leu1-32 h⁻</i>	This study
MBY885	<i>sec8-GFP-ura4⁺ sec6-13myc-ura4⁺</i>	This study
MBY886	<i>sec8-GFP-ura4⁺ sec10-13myc-ura4⁺</i>	This study
MBY887	<i>sec8-1 ura4-D18 leu1-32 h⁺</i>	This study
MBY888	<i>sec8-1</i>	This study
MBY936	<i>cdc25-22 gma12-GFP-ura4⁺</i>	This study
MBY965	<i>sec8Δ::ura4⁺/sec8-13myc-Kan^R-leu1⁺, h⁻/h⁺</i>	This study
MBY972	<i>sec8::81nmt1-sec8-ura4⁺, leu1-32, h⁻</i>	This study

(1:200 dilution; Katayama *et al.*, 1999), or antitubulin antibodies (1:200 dilution; a kind gift from Dr. Keith Gull). Secondary antibodies of anti-rabbit and anti-mouse IgG conjugates (Molecular Probes) were used at 1:200.

Electron microscopy was performed on permanganate-fixed *S. pombe* as described (Armstrong *et al.*, 1993). Briefly, *S. pombe* cells were grown at appropriate temperatures, washed three times in sterile water, and fixed for 1 h in 2% potassium permanganate at room temperature. Fixed cells were harvested by centrifugation and washed three times in sterile water, resuspended in 70% ethanol, and incubated overnight at 4°C. The samples were dehydrated and treated with propylene oxide before infiltration with Spurr's medium, followed by another change of medium and incubation at 65°C for 1 h. Finally, they were embedded in Spurr's resin, and the resin was allowed to polymerize at 60°C overnight. Ultrathin sections were cut on a Jung Reichert microtome (Leica Mikroskopie and Systeme GmbH, Wetzlar, Germany) and examined using a JEM1010 transmission electron microscope (Jeol, Tokyo, Japan) at 100 kV.

Identification and Deletion of Genes for Exocyst Components

sec8⁺ was cloned by complementation of the temperature-sensitive mutant *mut2-1* (*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. O74562), and the only gene on this plasmid encoded a protein homologous to Sec8p in *Saccharomyces cerevisiae*. Three experiments were done to show that *mut2-1* is defective in *sec8*. 1) A genetic cross between *mut2-1* and *sec8-GFP* (marked with *ura4⁺*) showed that *mut2-1* is tightly linked to the *sec8* locus (no recombinants in 20 tetrads). 2)

mut2-1 is fully rescued by recombination and gene conversion upon introduction of PCR fragments of *sec8* that lack promoter and 5' coding sequences. 3) Sequence determination of the *sec8* locus in a *mut2-1* strain revealed that it carried a mutation in the *sec8* gene that changed codon 992 from CAG to TAG, thereby introducing a premature stop codon.

A search through the Sanger Center Fission Yeast Genome Sequencing Project Database for proteins homologous to *S. cerevisiae* Sec6p, Sec10p, Sec15p, and Exo70p identified *S. pombe* Sec6p, Sec10p, Sec15p, and Exo70p. *sec6*⁺, *sec10*⁺, *sec15*⁺, and *exo70*⁺ were found to reside on cosmids SPCC1235.10c (Accession no. O74846), SPAC13F5.06c (Accession no. O13705), SPCC1183.01 (Accession no. O75006), and SPBC582.02 (Accession no. Q10339), respectively.

The entire coding sequences of *sec6*⁺, *sec8*⁺, and *sec10*⁺ were deleted to create the null mutants by replacing the respective coding regions with the *ura4*⁺ gene. The following primer pairs were used to amplify the constructs containing *ura4*⁺ and the flanking 5' and 3' sequences of the respective gene by PCR. MOH595 (CCAGTCCGTAAATATATTAATCAATCTGTCAGTAAATAGAAACGTTTGTAAAGCACTAGGTCTGCTTATAACTTTAAGAAAGCTACAAATCCCAGTGGCTATATGTA), containing 80 base pairs of *sec6*⁺ upstream sequence and 20 base pairs of 5' sequence of *ura4*⁺, and MOH596 (GTAGATCATTAATAATTCAGCAACGACTACTTTGGATCGATATTGACGAAACTTTTGGACATCATTAATCAAAAGGAACATTACTATAGGTAAAGATAAACCCTATC), containing 80 base pairs of downstream sequence of *sec6*⁺ and 20 base pairs of 3' sequence of *ura4*⁺, were used to amplify the construct for knocking out *sec6*. Similarly, TX-1 (TAGTGATTTCTTAGCTCTCCTTTCAAAGATAATACAGTCAATAGACATATCAAGCTAAACTACTGACTATTTGACTTCCGCTACAAATCCCAGTGGCTATATGTA) and TX-2 (TGCACTATGTTTTTATGTTAATAAATTTATTATTATAAAATCATTACTGTGCTATTATAATTAATAATTTCTATATTATACGGAAGAAAGCTACAAATCCCAGTGGCTATATGTA) were used for construction of the *sec8*-null, and MOH597 (CACCTACAACCAAAAGGAAACTTTGATCATTACTTTTCTATTCGAGAATTGTAGATTTAAATTTCTTGTCTATTAAGACGCTACAAATCCCAGTGGCTATATGTA) and MOH598 (TATAATACACTATAAAAGATATTAAGTTTATCTATAGACAAATTCTTACATAATTAAGACATTAACAAAATGAGCGATTGATATTGACGAAACTTTTGTGACATC) were used for construction of the *sec10*-null. The purified fragments were introduced into a wild-type diploid of genotype *leu1-32/leu1-32, ura4-D18/ura4-D18, ade6-210/ade6-216, h⁺/h⁻*. Transformants that had undergone homologous recombination were selected by growth in supplemented minimal medium lacking uracil. Correct integration of the deletion construct was confirmed by PCR assay and nucleotide sequence determination of the genomic DNA from transformants.

Epitope Tagging of *gma12* and *hht2*

Primers *gma12F* (CCTCTGGTACCTAGAACACACGAGTACTTG-GACC) and *gma12R* (CCTCTCCCGGGGATGATGGTTTCAAAA-GATTTTG) were used to amplify the *gma12* ORF (SPCC736.04c, Accession no. Q09174) by PCR, using wild-type genomic DNA as template. Primers MOH768 (CGGCTGGTACCAAGGGGTTTCCGTTGAC) and MOH769 (GGCGGCCCGGTGAGCGTTGCCACGGAG) were used to amplify the *hht2* ORF (SPAC1834.04, 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.

Epitope Tagging and Regulated Expression of the Exocyst 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 MOH584 (GATGGTACCGAACTTTCACAGCAATTATCTG) and MOH585 (CGATCCC-GGGTAAAATTGAACTTCCAGAAAGAG) and cloned into pJK210-GFP. The resulting plasmid pJK210-*sec6*CT-GFP, containing *sec6* fused in frame with GFP sequences, was linearized with *NdeI* and transformed into a wild-type strain of genotype *leu1-32 ura4-D18 ade6-210*. To tag Sec8p with GFP, primers MOH714 (CACCGGTACCAAGCTAATTTCCGTG-TGTACTTT) and MOH715 (CTACCCCGGGGATTTTTCTCGCACCAC-CCACAG) were used to generate a 700-bp *KpnI-SmaI* fragment that was cloned into pJK210-GFP to yield pJK210-*sec8*CT-GFP. This plasmid was linearized with *EcoRI* and transformed into wild-type cells. Similarly, primers MOH586 (GATGGTACCTAGTGA CATTAGGGAATG) and MOH587 (CGATCCCCTGCTCTTTGGGGGCAATAAAG CTTC) were used to generate a 0.9-kb *KpnI-SmaI* fragment of *sec10* that was cloned into pJK210-GFP to generate pJK210-*sec10*CT-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 (Keeney and Boeke, 1994) to generate plasmid pJK210-13myc. A 0.7-kb *NotI/BamHI*-digested fragment containing carboxyl-terminal sequence of *sec6*⁺ was obtained using primers MOH638 (GCTAGCGGC-CGCGGAACCTTTCACAGCAATTATCTG) and MOH639 (GTAG-GATCCGTAATAATTGAACTTCCAGAAAGAG) and was cloned into the *NotI/BamHI* sites of pJK210-13myc. Similarly, a 0.9-kb *NotI/BamHI* fragment of *sec10*⁺ carboxy-terminal sequence was obtained using primers MOH623 (GCGAGCGCGCCCTAGTGGACATTAGGGAATGT-AAG) and MOH624 (GCTAGGATCCGACTGCTCTTTGGGGGCAAT-AAAGC) and cloned into the *NotI/BamHI* sites of pJK210-13myc. These resulting plasmids were linearized with *NdeI* and *SpeI*, respectively, and transformed into wild-type cells.

The tagging of *exo70* with 13myc was done according to the methods described by Bähler *et al.* (1998b). A PCR fragment was generated using plasmid pFA6a-kanMX6 as template and primers *exo70*-5' (CTCATTACGTTAGTATATCAAAATTTACAAAGGCTGATTTAGATTCTTTTATTACAAAGCGCTTTGCTCTCTTCCTACGGATCCCCGGGTTAATTA A; contains 75 base pairs of *exo70* C-terminal sequence and 20 base pairs of 13myc sequence) and *exo70*-3' (TTCAAAGAAAGTGAGAAATGCCAGTACACCCACTTTAGTACTATATTATGGAATTTCAAAGGACCCAAATTCATCGAATTCGAGCTCGTTTAAAC; contains 75 base pairs of *exo70* 3'UTR sequence and 20 base pairs of vector sequence). The PCR fragment was purified and transformed into a wild-type strain. The desired integrations were confirmed by PCR assay and Western blot analysis.

To assess whether the maternal Sec8p was present in *sec8*-null cells, a diploid strain was constructed in which one *sec8* locus was replaced with *ura4*⁺, and the other *sec8* locus was tagged with the Myc epitope marked with *leu1*⁺ and the kanamycin-resistance gene. A 2.4-kb fragment containing Myc and kan^R sequences was obtained from pFA6a-13myc-kanMX6 (Bähler *et al.*, 1998b) by digestion with *SmaI* and *SacI* and then cloned into pJK148 (Keeney and Boeke, 1994) digested with *SmaI* and *SacI*. The resulting plasmid (pJK148-Myc-Kan) was then digested with *KpnI* and *SmaI* and ligated to a 0.7-kb *KpnI-SmaI* fragment containing the carboxy-terminal sequence of *sec8* as described above. This plasmid (pJK148-Myc-Kan-*sec8*CT) was linearized with *XbaI*, transformed into *sec8:ura4*⁺/*sec8*⁺ diploid cells (see above), and selected for kanamycin-resistance. Correct integration was confirmed by PCR assay and immunoblotting.

To construct a *sec8* shut-off strain, we made a tagging cassette containing 5' upstream regulatory sequence of *sec8*, *ura4⁺*, 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 pREP81 was cloned into pSK-*ura4* (Naqvi, N., and Balasubramanian, M. K., unpublished results) to generate pSK-*ura4-81nmt1* (Rajagopalan, S., and Balasubramanian, M. K., unpublished results). A 0.5-kb fragment containing 5' sequence of *sec8* was obtained by PCR using primers MOH724 (CATGGTACCGTATGATCGAGGATACGTACGAGG) and MOH790 (CCATCGATAAGGGTGTGTAAGTAAAGC), and the N-terminal sequence of *sec8* was amplified by PCR using primer MOH746 (CGGGATCCCATATGGATACCAGAGGCTATTCGGAAACG) and MOH727 (CGCTCGAGCTCAGCGGATTGGAGAGCAGTAC), 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-*ura4-81nmt1* 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 ura4-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, ura4-D18, 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 density₅₉₅ < 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 Sec8-1p and then transferred 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 achieved by the addition of 500 μ l of acid-washed glass beads to the cell pellet and subsequent disruption using a mini-bead beater (three cycles of 30-s duration and 2-min cooling intervals). For immunoprecipitation, 500 μ l NP-40 buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 1 mM PMSF, 2 mM Benzamidine) was used to extract soluble proteins. Cell extracts were clarified by centrifugation at 14,000 rpm for 10 min at 4°C. For each immunoprecipitation, 500 μ l of soluble protein was incubated with 2 μ l of anti-GFP antibodies for 1 h at 4°C. Sepharose-Protein G beads were added to the antigen-antibody immunocomplex and incubated for 45 min at 4°C. Beads were washed six times with 1 ml NP-40 buffer, resuspended in gel loading buffer, and heated at 95°C for 3 min.

For detection of Myc- or GFP-tagged proteins from total protein extracts or immunoprecipitates, proteins were separated on 6% SDS-PAGE (Mini-protein II system; Bio-Rad Laboratories, Hercules CA) at 120 V for 1 h and transferred (Trans-Blot system; Bio-Rad Laboratories) at 85 V for 2 h to a nitrocellulose membrane (Pierce Chemical Co., Rockford, IL). The membranes were blocked with 10% nonfat milk in TBS-Tween 20 (50 mM Tris-HCl, 150 mM NaCl, 0.05% 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 anti-mouse 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-aminophthalhydrazide 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 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 μ l of the supernatant was added to 500 μ l 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 μ l 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 Exocyst 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).

Figure 1. Phenotype of *mut2-1* (*sec8-1*) cells. (A) Growth curves of wild-type and *mut2-1* cells. Cell numbers of wild-type and *mut2-1* cells were quantified by counting cells using a hemacytometer after temperature shift from 24 to 36°C. ●, wild-type; ○, *mut2-1*. (B) *mut2-1* is defective in cell separation. *mut2-1* cells were grown at 24°C to log phase and shifted to the restrictive temperature of 36°C. Samples were collected just before the temperature shift (0 h) and 4 h later and stained with 4',6-diamidino-2-phenylindole (DAPI) and rhodamine-conjugated phalloidin to visualize DNA and F-actin (top and middle panels) or with Calcofluor to visualize septa (bottom panels). A constricting actomyosin ring is shown with an arrow. Bar: 10 μ m.

The rescuing DNA encoded a 1088-amino acid polypeptide. Database searches using the predicted protein sequence showed that it was related to *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 *mut2-1* is an allele of *sec8*⁺ (see MATERIALS AND METHODS).

The exocyst is required for polarized cell growth and cell surface expansion in *S. cerevisiae* (TerBush *et al.*, 1996; Roth *et al.*, 1998). In contrast, the *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 *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 μ m and elongate to 12–14 μ m before division

(Figure 3A). After release into rich medium, *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, *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, *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 tetranucleate cells with three septa. On prolonged incubation (12 h) *sec8-1* cells lysed. Cell length and the percentage of septated cells at each time point were also quantified (Table 2), which indicated that *sec8-1* is not defective in cell elongation and is not delayed for septum assembly. These data suggest that *sec8-1* cells are specifically defective in septum cleavage and cell separation.

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 μ m.

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 rota-

tion by 139°). 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.

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

	Time after release from G1				
	0 h	2 h	3 h	4.5 h	4.5 h, Septating cells
WT					
Cell length (μ m)	3.8	5.7	7.8	11.1	12.0
Septated cells (%)	0	0	0	68	
<i>sec8-1</i>					
Cell length (μ m)	3.5	6.1	7.9	11.0	12.1
Septated cells (%)	0	0	0	65	

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

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 in *cdc8-110* (Balasubramanian *et al.*, 1992), *cdc12-112* (Chang *et al.*, 1997), and *cdc15-140* (Fankhauser *et al.*,

Figure 4. Alignment of *S. pombe* Sec6p, Sec10p, Sec15p, and Exo70p with related proteins from *S. cerevisiae*, rat, and human. Amino acid sequences are aligned using ClustalX and Boxshader programs. Identical amino acids are shaded in black, and conservative substitutions are shaded in gray. Rn, *Rattus norvegicus*; Hs, *Homo sapiens*; Sp, *S. pombe*; Sc, *S. cerevisiae*.

[illegible][illegible][illegible][illegible]

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, Sec10-Myc and Sec8-GFP, or Sec10-Myc (B) or cells expressing Sec8-GFP, Exo70-Myc and Sec8-GFP, or Exo70-Myc (C).

1995) mutants. Although Sec10p-GFP was observed as a medial ring at 24°C in all these mutants, at 36°C none of the mutants was able to assemble Sec10p-GFP into ring structures (Figure 6, G and H, and our unpublished data). Thus, the assembly of the exocyst to the medial region appears to depend on the proteins essential for actomyosin ring assembly and actin patch mobilization.

Because the exocyst in *S. pombe* is potentially involved in secretion, we wanted to ascertain whether the localization of the exocyst as a medial ring is dependent on the secretory pathway. Synchronous cells expressing Sec10-GFP were treated with brefeldin A (BFA), a drug blocking membrane trafficking of newly synthesized proteins from endoplasmic reticulum (ER) to Golgi (Turi *et al.*, 1994). Gma12p, a Golgi marker protein that has been reported to relocate from Golgi to ER upon BFA treatment (Brazier *et al.*, 2000), was used to test the efficacy of BFA treatment. The ER in *S. pombe* is distributed primarily around the nuclear membrane region, whereas Golgi is seen as patches throughout the cytoplasm (Brazier *et al.*, 2000). As expected, Gma12p-GFP relocated

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 7, D 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 *81mnt1* 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

Figure 6. Localization of exocyst components in *S. pombe*. (A) Exocyst components localize to the division site and cell tips. *sec8-GFP 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/ μm^2). 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 de-

fective 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 Exo84p-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 exo-

Figure 6 (cont). treated with either LatA in DMSO (bottom panels) or DMSO alone (top panels) upon release into mitosis. Samples were stained with antibodies against GFP and α -tubulin to visualize Sec10-GFP and microtubules. (G and H) Localization of Sec10p to the division site is lost in *cdc12-112* (G) and *cdc15-140* (H) mutants. Cells were grown at 24°C, shifted to 36°C for 4 h, and stained with antibodies against GFP and α -tubulin to visualize Sec10-GFP and microtubules. (I) The localization of Sec10p is insensitive to brefeldin A treatment. *cdc25-22* cells expressing Sec10-GFP or Gma12-GFP (as a marker for the Golgi) were treated with either brefeldin A in ethanol (bottom panels) or ethanol alone (top panels) upon release into mitosis. Samples were stained with DAPI and with antibodies against GFP to visualize Sec10-GFP or Gma12-GFP and DNA. Bars: (A–D) 10 μm ; (E–I) 5 μm .

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. *81nmt1-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 μ m.

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

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 μ m in the two magnified boxes; 1 μ m 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 *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 *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.

The *S. pombe* Exocyst Is Critical for Cell Separation

Mutations in the *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 *et al.*, 1996; Finger and Novick, 1997; Roth *et al.*, 1998). In contrast, *S. pombe* exocyst mutants are capable of polarized growth, cell surface expansion, and division septum assembly. *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 *sec8* temperature-sensitive mutant, a *sec8* shut-off strain as well as the terminal phenotypes of germinated *sec8*-null mutant spores. We have also established that the phenotype of *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 *sec6* and *sec10* also result in a phenotype identical to that observed in the *sec8* mutants. One possibility is that the exocyst is essential only for a subset of secretory events in *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 *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 *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 *et al.*, 1998; Brachet *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-

late in the exocyst mutants should also help unravel the cellular function of the exocyst in *S. pombe*.

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REFERENCES

- Armstrong, J., Craighead, M.W., Watson, R., Ponnambalam, S., and Bowden, S. (1993). *Schizosaccharomyces pombe* ypt5: a homologue of the rab5 endosome fusion regulator. *Mol. Biol. Cell* 4, 583–592.
- Bähler, J., Steever, A.B., Wheatley, S., Wang, Y., Pringle, J.R., Gould, K.L., and McCollum, D. (1998a). Role of polo kinase and Mid1p in determining the site of cell division in fission yeast. *J. Cell Biol.* 143, 1603–1616.
- Bähler, J., Wu, J.-Q., Longtine, M.S., Shah, N.G., McKenzie, A., Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. (1998b). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* 14, 943–951.
- Balasubramanian, M.K., Feoktistova, A., McCollum, D., and Gould, K.L. (1996). Fission yeast Sop2p: a novel and evolutionarily conserved protein that interacts with Arp3p and modulates profilin function. *EBMO J.* 15, 6426–6437.
- Balasubramanian, M.K., McCollum, D., and Gould, K.L. (1997). Cytokinesis in fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* 283, 494–506.
- Balasubramanian, M.K., Helfman, D.M., and Hemmingsen, S.M. (1992). A new tropomyosin essential for cytokinesis in the fission yeast *S. pombe*. *Nature* 360, 84–87.
- Balasubramanian, M.K., Hirani, B.R., Burke, J.D., and Gould, K.L. (1994). The *Schizosaccharomyces pombe* *cdc3⁺* gene encodes a profilin essential for cytokinesis. *J. Cell Biol.* 125, 1289–1301.
- Balasubramanian, M.K., McCollum, D., Chang, L., Wong, K.C., Naqvi, N.I., He, X., Sazer, S., and Gould, K.L. (1998). Isolation and characterization of new fission yeast cytokinesis mutants. *Genetics* 149, 1265–1275.
- Balasundaram, D., Benedik, M.J., Morphew, M., Dang, V.D., and Levin, H.L. (1999). Nup124p is a nuclear pore factor of *Schizosaccharomyces pombe* that is important for nuclear import and activity of retrotransposon Tf1. *Mol. Cell. Biol.* 19, 5768–5784.
- Basi, G., Schmid, E., and Maundrell, K. (1993). TATA box mutations in the *Schizosaccharomyces pombe* *nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. *Gene* 123, 131–136.
- Bowser, R., and Novick, P. (1991). Sec15 protein, an essential component of the exocytotic apparatus, is associated with the plasma membrane and with a soluble 19.5S particle. *J. Cell Biol.* 112, 1117–1131.
- Brachet, V., Pehau-Arnaudet, G., Desaynard, C., Raposo, G., and Amigorena, S. (1999). Early endosomes are required for major histocompatibility

- complex class II transport to peptide-loading compartments. *Mol. Biol. Cell* 10, 2891–2904.
- Brazer, S.C., Williams, H.P., Chappell, T.G., and Cande, W.Z. (2000). A fission yeast kinesin affects Golgi membrane recycling. *Yeast* 16, 149–166.
- Chang, F., Drubin, D., and Nurse, P. (1997). *cdc12p*, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin. *J. Cell Biol.* 137, 169–182.
- Craighead, M.W., Bowden, S., Watson, R., and Armstrong, J. (1993). Function of the *ypt2* gene in the exocytic pathway of *Schizosaccharomyces pombe*. *Mol. Biol. Cell* 4, 1069–1076.
- Eng, K., Naqvi, N.I., Wong, K.C., and Balasubramanian, M.K. (1998). Rng2p, a protein required for cytokinesis in fission yeast, is a component of the actomyosin ring and the spindle pole body. *Curr. Biol.* 8, 611–621.
- Fankhauser, C., Reymond, A., Cerutti, L., Utzig, S., Hofmann, K., and Simanis, V. (1995). The *S. pombe cdc15* gene is a key element in the reorganization of F-actin at mitosis. *Cell* 82, 435–444.
- Fankhauser, C., and Simanis, V. (1994). The *cdc7* protein kinase is a dosage dependent regulator of septum formation in fission yeast. *EMBO J.* 13, 3011–3019.
- Finger, F.P., Hughes, T.E., and Novick, P. (1998). Sec3p is a spatial landmark for polarized secretion in budding yeast. *Cell* 92, 559–571.
- Finger, F.P., and Novick, P. (1997). Sec3p is involved in secretion and morphogenesis in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 8, 647–662.
- Gould, K.L., and Simanis, V. (1997). The control of septum formation in fission yeast. *Genes Dev.* 11, 2939–2951.
- Grindstaff, K.K., Yeaman, C., Anandasabapathy, N., Hsu, S.C., Rodriguez-Boulant, E., Scheller, R.H., and Nelson, W.J. (1998). Sec6/8 complex is recruited to cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells. *Cell* 93, 731–740.
- Guertin, D.A., Chang, L., Irshad, F., Gould, K.L., and McCollum, D. (2000). The role of the *sid1p* kinase and *cdc14p* in regulating the onset of cytokinesis in fission yeast. *EMBO J.* 19, 1803–1815.
- Guo, W., Roth, D., Walch-Solimena, C., and Novick, P. (1999). The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. *EMBO J.* 18, 1071–1080.
- Hsu, S.C., Hazuka, C.D., Foletti, D.L., and Scheller, R.H. (1999). Targeting vesicles to specific sites on the plasma membrane: the role of the sec6/8 complex. *Trends Cell Biol.* 9, 150–153.
- Humbel, B.M., Konomi, M., Takagi, T., Kamasawa, N., Ishijima, S.A., and Osumi, M. (2001). In situ localization of beta-glucans in the cell wall of *Schizosaccharomyces pombe*. *Yeast* 18, 433–444.
- Katayama, S., Hirata, D., Arellano, M., Perez, P., and Toda, T. (1999). Fission yeast α -glucan synthase Mok1 requires the actin cytoskeleton to localize the sites of growth and plays an essential role in cell morphogenesis downstream of protein kinase C function. *J. Cell Biol.* 144, 1173–1186.
- Keeney J.B., and Boeke J.D. (1994). Efficient targeted integration at *leu1-32* and *ura4-294* in *Schizosaccharomyces pombe*. *Genetics* 136, 849–856.
- Kee, Y., Yoo, J.S., Hazuka, C.D., Peterson, K.E., Hsu, S.C., and Scheller, R.H. (1997). Subunit structure of the mammalian exocyst complex. *Proc. Natl. Acad. Sci. USA* 94, 14438–14443.
- Le Goff, X., Woollard, A., and Simanis, V. (1999). Analysis of the *cps1* gene provides evidence for a septation checkpoint in *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* 262, 163–172.
- Leupold, U. (1970). Genetical methods for *Schizosaccharomyces pombe*. *Methods Cell Physiol.* 4, 169–177.
- Lippincott, J., and Li, R. (2000). Involvement of PCH family proteins in cytokinesis and actin distribution. *Microsc. Res. Tech.* 49, 168–172.
- Liu, J., Wang, H., McCollum, D., and Balasubramanian, M.K. (1999). Drc1p/Cps1p, a 1,3-beta-glucan synthase subunit, is essential for division septum assembly in *Schizosaccharomyces pombe*. *Genetics* 153, 1193–1203.
- Luo, W., and Chang, A. (2000). An endosome-to-plasma membrane pathway involved in trafficking of a mutant plasma membrane ATPase in yeast. *Mol. Biol. Cell* 11, 579–592.
- Mallard, F., Antony, C., Tenza, D., Salamero, J., Goud, B., and Johannes, L. (1998). Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. *J. Cell Biol.* 143, 973–990.
- Marks, J., and Hyams, J.S. (1985). Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. *Eur. J. Cell Biol.* 39, 27–32.
- McCollum, D., Balasubramanian, M., and Gould, K. (1999). Identification of cold-sensitive mutations in the *Schizosaccharomyces pombe* actin locus. *FEBS Lett.* 451, 321–326.
- Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* 194, 795–823.
- Naqvi, N.I., Eng, K., Gould, K.L., and Balasubramanian, M.K. (1999). Evidence for F-actin-dependent and -independent mechanisms involved in assembly and stability of the medial actomyosin ring in fission yeast. *EMBO J.* 18, 854–862.
- Naqvi, N.I., Wong, K.C., Tang, X., and Balasubramanian, M.K. (2000). Type II myosin regulatory light chain relieves auto-inhibition of myosin-heavy-chain function. *Nat. Cell Biol.* 2, 855–858.
- Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K., and Okayama, H. (1990). High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* 18, 6485–6489.
- Pelham, R.J., Jr., and Chang, F. (2001). Role of actin polymerization and actin cables in actin-patch movement in *Schizosaccharomyces pombe*. *Nat. Cell Biol.* 3, 235–244.
- Potenza, M., Bowser, R., Muller, H., and Novick, P. (1992). SEC6 encodes an 85 kDa soluble protein required for exocytosis in yeast. *Yeast* 8, 549–558.
- Roth, D., Guo, W., and Novick, P. (1998). Dominant negative alleles of SEC10 reveal distinct domains involved in secretion and morphogenesis in yeast. *Mol. Biol. Cell* 9, 1725–1739.
- Schneppenheim, R., Budde, U., Dahlmann, N., and Rautenberg, P. (1991). Luminography—a new, highly sensitive visualization method for electrophoresis. *Electrophoresis* 12, 367–372.
- Simanis, V. (1995). The control of septum formation and cytokinesis in fission yeast. *Semin. Cell Biol.* 6, 79–87.
- Sparks, C.A., Morpheus, M., and McCollum, D. (1999). Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. *J. Cell Biol.* 146, 777–790.
- Tanaka, K., and Okayama, H. (2000). A Pcl-like cyclin activates the Res2p-Cdc10p cell cycle “start” transcriptional factor complex in fission yeast. *Mol. Biol. Cell* 11, 2845–2862.
- TerBush, D.R., Maurice, T., Roth, D., and Novick, P. (1996). The exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. *EMBO J.* 15, 6483–6494.
- TerBush, D.R., and Novick, P. (1995). Sec6, Sec8, and Sec15 are components of a multisubunit complex which localizes to small bud tips in *Saccharomyces cerevisiae*. *J. Cell Biol.* 130, 299–312.
- Ting, A.E., Hazuka, C.D., Hsu, S.C., Kirk, M.D., Bean, A.J., and Scheller, R.H. (1995). rSec6 and rSec8, mammalian homologs of yeast proteins essential for secretion. *Proc. Natl. Acad. Sci. USA* 92, 9613–9617.
- Turi, T.G., Webster, P., and Rose, J.K. (1994). Brefeldin A sensitivity and resistance in *Schizosaccharomyces pombe*. Isolation of multiple genes conferring resistance. *J. Biol. Chem.* 269, 24229–24236.
- Wong, K.C., Naqvi, N.I., Iino, Y., Yamamoto, M., and Balasubramanian, M.K. (2000). Fission yeast Rng3p: an UCS-domain protein that mediates myosin II assembly during cytokinesis. *J. Cell Sci.* 113, 2421–2432.