Secretion is required for late events in the cell-fusion pathway of mating yeast

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Summary

Secretory vesicles accumulate adjacent to the contact site between the two cells of a yeast mating pair before they fuse, but there is no direct evidence that secretion is required to complete fusion. In this study, temperature-sensitive secretion (*sec*^{ts}) mutants were used to investigate the role of secretion in yeast cell fusion. Cell fusion arrested less than 5 minutes after inhibiting secretion. This rapid fusion arrest was not an indirect consequence of reduced mating pheromone signaling, mating-pair assembly or actin polarity. Furthermore, secretion was required to complete cell fusion when it was transiently inhibited by addition and removal of the lipophilic styryl dye, FM4-64. These results indicate that ongoing secretion is required for late events in the cell-fusion pathway, which include plasma-membrane fusion and the completion of cell-wall remodeling, and they demonstrate a just-in-time delivery mechanism for the cell-fusion machinery.

Key words: Secretion, Cell fusion, Membrane fusion

Introduction

Cell fusion is a fundamental biological process required for fertilization and for the development of multinucleated cells such as skeletal muscle fibers. Cell fusion has also been implicated in the transdifferentiation of stem cells and in the generation of aneuploidy leading to cancer. The defining feature of cell fusion is a membrane fusion event that joins the plasma membranes of two cells. In contrast to the membrane fusions associated with intracellular membrane transport and viral infection, the mechanism of membrane fusion between cells is poorly understood, and relatively little is known about the processes that lead to and regulate plasma-membrane fusion (Chen et al., 2007; Oren-Suissa and Podbilewicz, 2007).

Cell-fusion events in diverse biological systems are preceded by specialized secretory processes. Before a mouse sperm can fuse with an egg, it releases an acrosomal vesicle containing hydrolytic enzymes required to penetrate the zona pellucida surrounding the egg. Izumo, a sperm protein required for membrane fusion with the egg, appears on the cell surface after acrosomal exocytosis (Inoue et al., 2005). In *Neurospora*, there is a burst of endocytic and exocytotic traffic immediately before fusion between hyphal cells (Hickey et al., 2002). In *Drosophila* myoblasts, pairs of electron-dense vesicles assemble on either side of the contact site and are thought to release their contents into the intercellular space before cell fusion (Doberstein et al., 1997). These prefusion vesicles accumulate in the *sing* and *sltr/D-WIP* mutants, which have myoblast fusion defects (Estrada et al., 2007; Kim et al., 2007).

Mating in *S. cerevisiae* provides a genetically accessible model system to investigate the mechanism of cell fusion (Chen et al., 2007; Ydenberg and Rose, 2008). Many mutations are known to inhibit mating, and these mutations have been used to help define the stages of the cell-fusion pathway. Yeast has two haploid mating types, *MAT**a*** and *MAT*α, which fuse to form diploids. Mating begins with an exchange of mating pheromones between haploid cells of opposite mating types. Activation of the pheromone-response signaling pathway results in cell-cycle arrest, the induction of mating-specific genes and growth of a mating projection in the direction a potential mating partner. When the tips of two mating projections come into contact, the cells bind to each other and then remodel their cell walls to first form a unified wall surrounding the mating pair and then degrade the cell walls at the contact point. The two plasma membranes fuse shortly after they come into contact. The fusion pore connecting the membranes opens abruptly and then gradually expands to allow the two haploid nuclei to merge. After mating is complete, diploid daughter cells bud from the neck connecting the two parent cells of the fused zygote.

As in *Drosophila* myoblasts, secretory vesicles are often found adjacent to the contact site in yeast mating pairs (Baba et al., 1989; Gammie et al., 1998). These vesicles accumulate in the *fus2* and *prm1* mutants, which arrest with pairs of cells that are bound to each other, but not fused, suggesting that cell fusion is mediated in part by localized exocytosis of secretory vesicles containing components essential for fusion (Gammie et al., 1998; Heiman and Walter, 2000; Jin et al., 2008).

In this study, temperature-sensitive secretory (*sec*^{ts}) mutants were used to investigate the role of secretion in the fusion of yeast mating pairs. By initiating mating interactions at a permissive temperature and then increasing the temperature to block secretion, it was possible to determine whether secretion is required for late mating events without inhibiting early events such as the polarized growth of a mating projection. The results revealed that cell fusion arrests almost immediately after inhibiting secretion. Although secretion is required for pheromone signaling, polarized morphogenesis, mating-pair assembly, and the maintenance of actin polarity, the rapid cell-fusion arrest after inhibiting secretion cannot be explained by defects in any of these processes. The results therefore suggest that ongoing secretion is essential for just-in-time delivery of proteins needed for plasma-membrane fusion, and possibly also to complete the cell-wall remodeling process that immediately precedes plasma-membrane fusion.

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Results

Ongoing secretion is required to complete cell fusion

Cell fusion can be detected by measuring cytoplasmic mixing with a β-galactosidase complementation assay. The principle underlying this assay is that the Δμ and Δω fragments of β-galactosidase are not functional in isolation, but can bind to each other to produce an active enzyme (Ullmann et al., 1965). Previous studies with myoblasts established that Δμ and Δω will bind to each other after cell fusion, but not after cells are lysed and diluted into assay buffer (Mohler and Blau, 1996). To measure cell fusion in yeast, β-galactosidase activity was measured after mating *MAT**a** lacZ-Δμ* cells to *MATα lacZ-Δω* cells. Test matings between some of the cell fusion mutants described in Table 1 were used to validate the assay (Fig. 1A). As expected from previous studies (Heiman and Walter, 2000; Trueheart et al., 1987), fusion was reduced by approximately 50% in the *fus1* and *prm1* mutants and by >98% in the *fus1 fus2* double mutant. Furthermore, absolutely no β-galactosidase activity could be detected after a mock mating between two *MAT**a*** strains.

To investigate whether secretion is required for cell fusion, β-galactosidase complementation assays were performed using *sec*^{ts} mutant strains. Cell fusion was tested in six different *sec*^{ts} mutants to ensure that the results reflect a general requirement for secretion rather than the specific phenotype of any single mutant (Table 2). The *sec*^{ts} mutants were originally shown to grow and secrete normally at 25°C, but not at 37°C (Novick et al., 1980). In the cell-fusion assays, the temperature was increased to 34.5°C instead of 37°C because wild-type yeast cannot form mating pairs at 37°C, but fuse normally at 34.5°C. The *sec*^{ts} mutants chosen for this study failed to grow at 34.5°C (data not shown).

A two-stage mating assay was used to investigate whether secretion is required to complete cell fusion (Fig. 1B). In the first stage, *MAT**a** sec*^{ts} and *MATα sec*^{ts} mutants expressing the Δμ and Δω constructs were mixed and incubated for 80 minutes at 25°C to initiate mating. In the second stage, aliquots were incubated for 60 additional minutes at either 34.5°C to inhibit secretion or at 25°C to measure the amount of cell fusion that could occur with normal secretion. A sample was collected at the end of the first incubation to measure the amount of fusion that occurred before the temperature shift. During the second incubation, all of the *sec*^{ts} mutants continued to fuse at 25°C, but not at 34.5°C. By contrast, raising the temperature to 34.5°C did not inhibit cell fusion in wild-type mating pairs or in the *cdc6*^{ts} mutant, which cannot initiate DNA synthesis at 34.5°C. In conclusion, ongoing secretion is required to complete cell fusion.

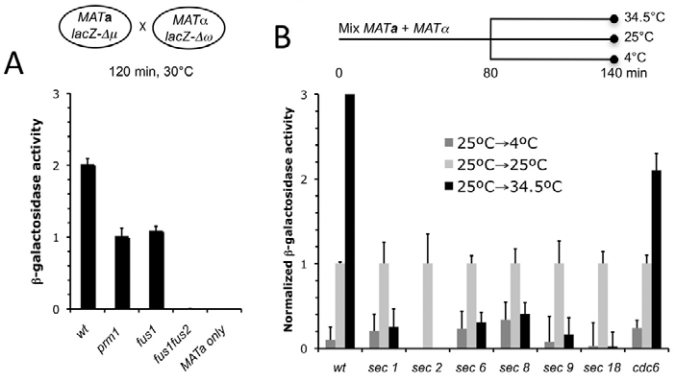

Fig. 1. Secretion is required to complete cell fusion. (**A**) Validation of the β-galactosidase complementation assay for cell fusion. *MAT**a*** strains expressing *lacZ-Δμ* were mated to *MATα* strains expressing *lacZ-Δω* for 120 minutes at 30°C. (**B**) *sec*^{ts} mutants cannot fuse after a temperature shift to 34.5°C. *MAT**a*** strains expressing *lacZ-Δμ* were mated to *MATα* strains expressing *lacZ-Δω* for a total of 140 minutes. Three sets of mating were done for each mutant. After 80 minutes at 25°C, one set was shifted to 34.5°C to test for fusion after inhibiting secretion, the second set was allowed to continue mating at 25°C, and the third set was placed on ice to arrest mating. The mean galactosidase activity for each strain after 140 minutes at 25°C was set to 1 to compensate for differences in growth rates and β-galactosidase fragment expression. Error bars represent s.d.

Secretion is required for cell fusion after washing out a reversible inhibitor of plasma-membrane fusion

Cell fusion intermediates can be observed by mating *MAT**a*** cells expressing cytoplasmic GFP to non-fluorescent *MATα* cells (Grote, 2008). Before fusion, prezygotes (unfused mating pairs) have GFP restricted to one side. After fusion, GFP diffuses across the fusion pore and fills the cytoplasm of the zygote. Mutants such as *fus1* with delayed cell-wall remodeling accumulate prezygotes with a wide neck and a flat interface between cells (Gammie et al., 1998). By contrast, mutants such as *prm1,* with a defect in plasma-membrane fusion, accumulate prezygotes with a finger of

Table 1. Cell-fusion mutants

Mutant	Cell-fusion phenotype	Reference
fus1Δ	Cell-wall remodeling, fusion pore expansion	(Nolan et al., 2006; Trueheart et al., 1987)
fus2Δ	Cell-wall remodeling	(Trueheart et al., 1987)
prm1Δ	Plasma-membrane fusion	(Heiman and Walter, 2000; Jin et al., 2004)
erg6Δ	Plasma-membrane fusion, pheromone signaling	(Jin et al., 2008)
fig1Δ	Plasma-membrane fusion, cell-wall remodeling	(Aguilar et al., 2006; Erdman et al., 1998)
sst2Δ	Supersensitivity to mating pheromones	(Chan et al., 1983)

Table 2. *sec*^{ts} mutants

sec^{ts} mutant	Secretory function	Reference
sec1-1	SNARE-complex regulator	(Carr et al., 1999)
sec2-59	GTP-exchange protein for Sec4 Rab	(Walch-Solimena et al., 1997)
sec6-4	Exocyst-complex component	(TerBush et al., 1996)
sec8-9	Exocyst-complex component	(TerBush et al., 1996)
sec9-4	t-SNARE on plasma membrane	(Brennwald et al., 1994)
sec18-1	NSF-ATPase for SNARE-complex disassembly	(Grote et al., 2000)

membrane-bound cytoplasm that projects from one cell into its mating partner (Heiman and Walter, 2000). The cytoplasmic finger is caused in part by differences between the cytoplasmic osmolarity of the two cells, and can only form if the cell wall between the two plasma membranes has been degraded (Heiman and Walter, 2000; Jin et al., 2004). Neither *FUS1* nor *PRM1* is absolutely essential for cell fusion, so a significant percentage of *fus1* and *prm1* mating pairs are able to fuse, with the amount of fusion dependent upon environmental conditions such as the temperature and Ca^{2+} concentration (Aguilar et al., 2006).

Surprisingly, fusion of *prm1* mating pairs was almost completely blocked by FM4-64, a styryl dye often used to trace the yeast endocytic pathway (Vida and Emr, 1995) (Fig. 2A). FM4-64 reversibly partitions into membranes, cannot flip-flop across a membrane, and its fluorescent yield increases by more than 300-fold within the hydrophobic interior of the membrane (Brumback et al., 2004). Although the overall number of *prm1* mutant prezygotes increased on FM4-64 medium, the percentage of prezygotes with fingers was the same as observed in the absence of FM4-64 (FM4-64, 30.7±1.9%, n=3; control, 33.0±6.4%, n=3). Inhibition was specific for *prm1* because matings with *erg6* and *fig1* mutants, which also have defects at the plasma-membrane-fusion stage of mating (Aguilar et al., 2006; Jin et al., 2008), were not inhibited by FM4-64 (Fig. 2B). Thus, FM4-64 enhances the plasma-membrane fusion defect of *prm1* mating pairs.

The inhibitory effects of FM4-64 on cell fusion can be reversed by washing the dye out of the plasma membrane. In particular, less than 1% of *prm1* mating pairs fused during a 2.5-hour incubation on FM4-64 medium. When this mating mix was washed and then incubated for an additional hour on fresh medium, 30% of the mating pairs were fused. At least 70% of these fusions occurred between cells that had assembled into mating pairs during the 2.5-hour incubation on FM4-64 medium.

To determine whether secretion is required for mating pairs to proceed to fusion after releasing the FM4-64 block, *prm1* mutant and *sec6-4 prm1* double-mutant mating pairs were assembled in the presence of FM4-64, washed, and then chased at either 25°C or 34.5°C. The *prm1* single-mutant mating pairs could fuse at both temperatures, but the *sec6-4 prm1* double-mutant mating pairs could only fuse at 25°C (Fig. 2C). This result confirms that secretion is essential for a late event in the cell-fusion pathway.

Cell fusion arrests within minutes after inhibiting secretion

To determine how quickly cell fusion arrests after inhibiting secretion, the first step was to identify two *sec*^{ts} alleles, *sec6-4* and *sec18-1*, that are rapidly and almost completely inactivated by a shift to 34.5°C. The *sec6-4* mutant has a more specific phenotype than *sec18-1* because Sec6 is only required for exocytosis, whereas Sec18 participates in all membrane fusions in the secretory and endocytic pathways. Then again, the *sec18-1* mutant has a tighter phenotype and a lower threshold for growth inhibition (30°C for *sec18-1* vs 33°C for *sec6-4*). The rapid onset of the secretion defects in these two mutants was characterized in a previous study by measuring the secretion of newly synthesized [^{35}S]p120 (Grote et al., 2000). The extent of the secretion defects at 34.5°C and 37°C was quantified by measuring invertase secretion (Table 3). At 34.5°C, the *sec18-1* mutant had less invertase secretion than *sec6-4* (3.4% vs 11%). Interestingly, the *sec1-1* and *sec2-41* mutations inhibited cell fusion at 34.5°C despite the fact that they inhibited secretion by only 63%, indicating that a partial inhibition of secretion is sufficient to block cell fusion.

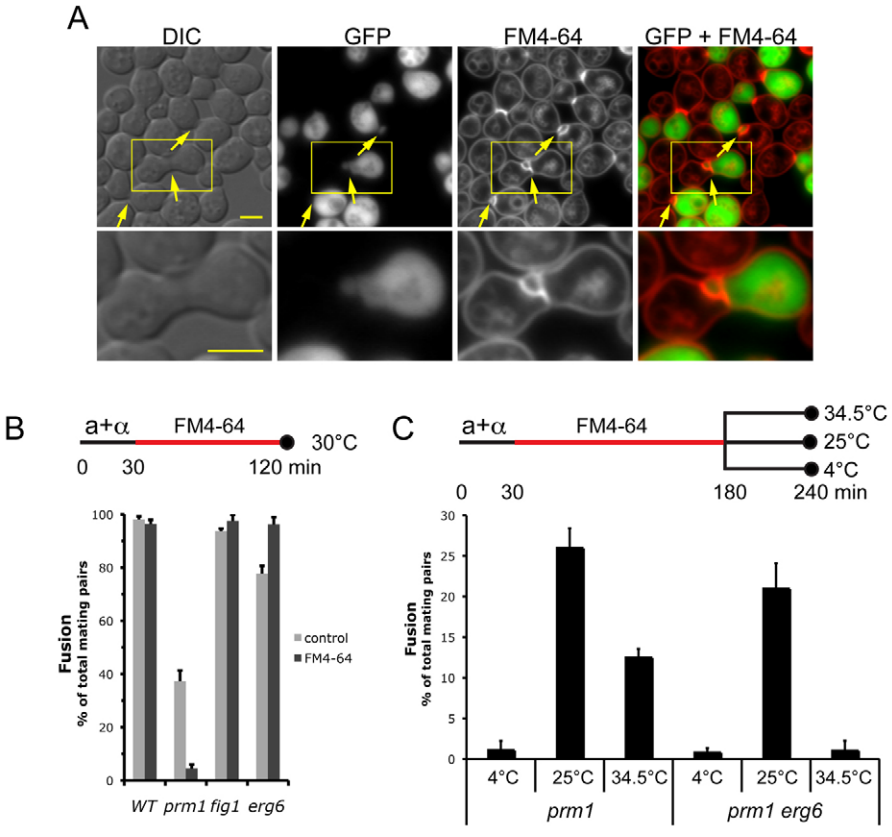

Fig. 2. Secretion is required for cell fusion after reversible inhibition by FM4-64. (**A**) FM4-64 inhibits plasma-membrane fusion in *prm1* mating pairs. *MAT**a** GFP* and *MAT*α cells were mated for 30 minutes on standard medium, transferred to an agarose pad with FM4-64, and then incubated for 90 additional minutes at 30°C. Some FM4-64-treated *prm1* mating pairs arrested with a cytoplasmic finger (arrows), indicating that FM4-64 inhibits plasma-membrane fusion. Scale bars: 5 μm. (**B**) FM4-64 inhibition is specific for the *prm1* mutant. The frequency of cell fusion was monitored by cytoplasmic GFP transfer. (**C**) Secretion is required for fusion after washing out FM4-64. To accumulate FM4-64 arrested prezygotes, pairs of *prm1* or *prm1 sec6* double-mutant strains were mated at 25°C for 30 minutes on standard SC medium followed by 150 minutes on FM4-64 medium. After washing FM4-64 out of the plasma membrane, the mating pairs were incubated for 60 minutes at the indicated temperatures. Error bars represent s.d.

Table 3. Invertase secretion from *sec*^{ts} mutants[a]

SEC allele	25°C	34.5°C	37°C
SEC	70.6 (100)	77.9 (100)	85.7 (100)
sec1-1	74.7 (106)	29.1 (37)	–3.1 (–4)
sec2-59	58.1 (82)	29.8 (38)	0.0 (0)
sec6-4	83.3 (118)	11.0 (14)	3.7 (4)
sec18-1	72.7 (103)	3.4 (4)	–0.2 (0)

[a]Invertase expression was induced for 45 minutes at the indicated temperatures. Values indicate the percentage of invertase secreted and, in parentheses, the percentage secretion compared with the wild-type (*SEC*) control.

Time-lapse microscopy of *MAT**a*** *GFP* cells mating to nonfluorescent *MAT*α cells was used to determine how quickly cell fusion arrests after inhibiting secretion. In these experiments, the time of fusion was defined by the first image with a measurable amount of cytoplasmic GFP in the *MAT*α cell. In a microscopic field of wild-type cells mated at 25°C, the first fusion event occurred after approximately 1 hour, and fusions then continued at a steady pace for more than 1 hour (Fig. 3A). The *sec6-4* and *sec18-1* mutants also fused at a steady pace at 25°C. By contrast, fusion arrested shortly after shifting the temperature of *sec6-4* and *sec18-1* matings from 25°C to 34.5°C, but the temperature shift had no effect on the rate of fusion between wild-type cells (Fig. 3B; supplementary material Fig. S1). The final *sec6-4* fusion occurred 5.4 minutes after initiating the temperature shift, and the final *sec18-1* fusion occurred at 4.3 minutes (Table 4). These times represents an upper limit for the amount of time that cell fusion can continue in the absence of secretion because it took 3 minutes to increase the slide temperature to 34°C (supplementary material Fig. S2). Cell fusion arrested 8.1 minutes after inhibiting secretion in only one cell of a wild-type × *sec6-4* mating pair, indicating that both cells in a mating pair must have an active secretory pathway to complete cell fusion. The rapid arrest of cell fusion after inhibiting secretion indicates that secretion is required for a very late event in the cell-fusion pathway.

Reduced pheromone signaling cannot account for the cell-fusion block

In addition to the well-known role of pheromones in the initiation of mating, pheromone signaling is also required at later stages in the cell-fusion process (Brizzio et al., 1996). Pheromones and their receptors are transported via the secretory pathway, therefore inhibiting secretion could block cell fusion indirectly via reduced pheromone signaling. Pheromone signaling is bidirectional. *MAT**a*** cells release **a**-factor, which binds to the **a**-factor receptor expressed on *MAT*α cells. Conversely, *MAT*α cells secrete α-factor, which

Table 4. Time of the final fusion after inhibiting secretion[a]

Mating	Final temperature (°C)	Final fusion time (mean minutes ± s.d.)	*n*
sec6-4 × *sec6-4*	34.5	5.4±2.0	5
sec18-1 × *sec18-1*	34.5	4.3±1.5	3
sec18-1 × *sec18-1*	31.0	6.2±0.9	4
WT × *sec6-4*	34.5	8.1±2.0	4
sst2Δ × *sec6-4*	34.5	7.6±0.4	3

[a]The temperature was shifted from 25°C to the indicated temperature 70-90 minutes after the initiation of mating for all samples except for *sst2Δ* × *sec6-4* mating, which was shifted at 150 minutes.

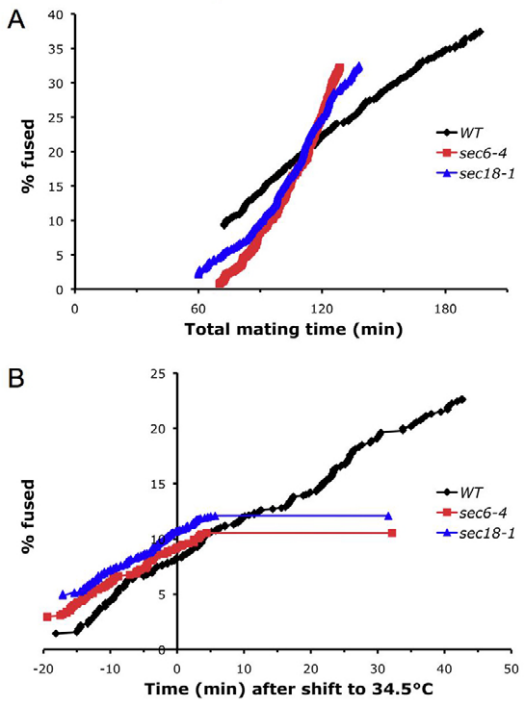

Fig. 3. Rapid arrest of cell fusion after inhibiting secretion. (**A**) Fusion kinetics at 25°C. Each point on the curves represents the opening of one or more fusion pores. (**B**) Fusion kinetics before and after increasing the temperature from 25°C to 34.5°C. Cells were mated for 70-90 minutes before the temperature shift. In the *sec6-4 and sec18-1* matings, the final data point represents the end of the recording.

binds to the α-factor receptor expressed on *MAT**a*** cells. To further investigate the dependence of pheromone signaling on secretion, a *FUS1-lacZ* reporter was used to measure the transcriptional response to mating pheromones in the context of genuine mating pairs formed between combinations of wild-type and *sec6-4* cells (Fig. 4). At 34.5°C, *sec6-4* cells were unable to extend mating projections or assemble into mating pairs (data not shown). Nevertheless, they were able to both send and respond to pheromone signals, as determined by *lacZ* expression. No significant difference in signaling was found if secretion was inhibited in the pheromone-responsive cell. However, **a**-factor and α-factor signaling were both reduced if the cell releasing the pheromone was a *sec6-4* mutant. After controlling for the effect of temperature on signaling in wild-type mating pairs (α-factor signaling was reduced by 28%), shifting *sec6-4* cells to 34.5°C reduced pheromone signaling by 30-40%.

Previous work suggested that a 30-40% reduction in pheromone signaling should not inhibit cell fusion (Brizzio et al., 1996; Jin et al., 2008). An *sst2Δ* mutation was used to directly test whether reduced pheromone release contributes to the rapid cell-fusion block after inhibiting secretion. The *sst2Δ* mutation renders cells supersensitive to mating pheromones, and thereby allows them to fuse to mating partners with reduced pheromone synthesis (Brizzio et al., 1996; Chan et al., 1983; Dohlman et al., 1996). Nevertheless, fusion between *sec6-4* and *sst2Δ* cells could not continue for any longer than fusion between *sec6-4* and wild-type cells after shifting the temperature to 34.5°C (Table 4), indicating that the rapid arrest of cell fusion is not a consequence of reduced α-factor secretion.

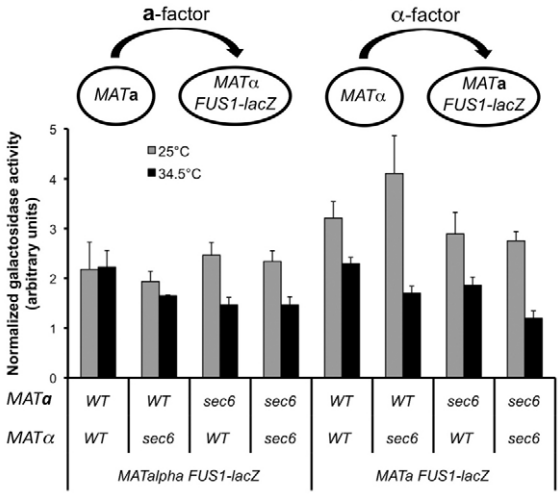

Fig. 4. Secretion and pheromone signaling. Pheromone signaling was measured with a *FUS1-lacZ* reporter in mating pairs assembled from combinations of wild-type and *sec6-4* mutant strains. The reporter was expressed in *MAT*α cells to measure **a**-factor signaling and in *MAT**a*** cells to measure α-factor signaling. Error bars represent s.d.

Secretion and cell-wall remodeling

Cell fusion in mating yeast involves an elaborate cell-wall remodeling process. Mating pairs first assemble through binding interactions between cell-wall-associated agglutinins followed by the synthesis of a unified cell wall surrounding the mating pair. Then, the cell walls at the contact site are selectively degraded to allow the two plasma membranes to come into contact and fuse. The proteins that mediate these processes are delivered to the cell surface via the secretory pathway (Cappellaro et al., 1998; Lesage and Bussey, 2006). Thus, inhibiting secretion could block cell fusion by inhibiting mating-pair assembly or cell-wall degradation.

The assembly of *MAT**a** sec6-4* and *MAT*α *sec6-4* cells into sonication-resistant mating pairs was measured to investigate the role of secretion in the early stages of cell-wall remodeling. Secretion was required for mating-pair assembly, as expected. However, if mating was initiated during an 80-minute incubation at 25°C, mating-pair assembly continued for 20 minutes after raising the temperature to 34.5°C (supplementary material Fig. S3). In conclusion, mating pair assembly is not blocked quickly enough after inhibiting secretion to contribute to the rapid cell-fusion arrest.

Inhibition of cell-wall degradation could cause a rapid cell fusion arrest because the two plasma membranes are thought to fuse immediately after they come into contact. In support of this concept, cell-fusion intermediates that have completed cell-wall remodeling but not plasma-membrane fusion are rarely observed in wild-type matings (Jin et al., 2004). A specific role for secretion in cell-wall degradation was suggested by an accumulation of small vesicles adjacent to the contact site in several cell-fusion mutants with defects in cell-wall remodeling (Brizzio et al., 1996). Thus, inhibiting secretion could block cell-wall degradation, and this block could account for the rapid arrest of cell fusion.

The role of secretion in cell-wall remodeling was thus further investigated by assaying for defects in mating pairs assembled from *sec*^{ts} mutants. In these experiments, *MAT**a** GFP* cells were mated to *MAT*α *RFP* (mCherry or DsRed) cells to more precisely define the junction between cells. The mating mixtures were first incubated for 30 minutes at 25°C to initiate mating and then incubated for 90 minutes at 34.5°C to inhibit secretion (Fig. 5A). In *sec*^{ts} × *sec*^{ts} mating assays, the interface between prezygotes

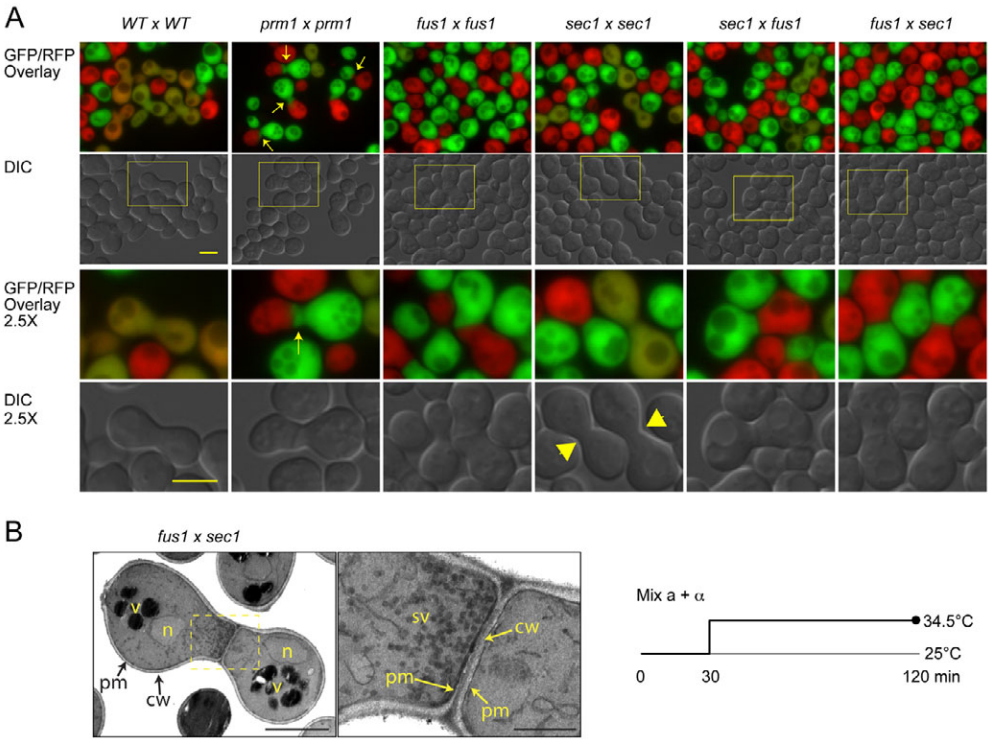

Fig. 5. Secretion is required for cell-wall remodeling. *MAT**a** GFP* and *MAT*α *RFP* cells with the indicated mutations were mated for 30 minutes at 25°C followed by 90 minutes at 34.5°C. The *MAT**a** GFP* strain is listed first in all labels. (**A**) WT mating pairs fuse, producing a yellow zygote filled with both GFP and RFP. *prm1* mating pairs arrest as prezygotes with cytoplasmic fingers (arrows) indicating membrane contact. *sec1-1* mating pairs have a narrow neck (arrowheads). *fus1* × *fus1*, *sec1-1* × *fus1* and, *fus1* × *sec1-1* mating pairs arrest as prezygotes with a broad, flat interface indicating that the two cells are separated by cell walls. Scale bars: 5 μm. (**B**) Electron micrograph of a permanganate-fixed *fus1* × *sec1* mating pair with a cell wall (cw) separating the two plasma membranes (pm). Note that secretory vesicles (sv) accumulate in the cell on the left. Each cell has one nucleus (n) and multiple vacuoles (v). Scale bars: 5 μm, left; 1 μm, right.

Table 5. Prezygote accumulation in combinatorial matings[a]

		*MAT**a** GFP*			
		WT	*fus1Δ*	*prm1Δ*	*sec1-1*
MATα RFP	WT	0.0	15.6	5.7	3.5
	fus1Δ	12.3	93.4	21.1	50.0
	prm1Δ	2.1	18.5	80.6	9.2
	sec1-1	5.0	66.1	10.8	28.8

[a]The indicated strains were mixed and then incubated first for 30 minutes at 25°C, and then for 90 minutes at 34.5°C. The percentage of mating pairs that arrested as prezygotes was scored by microscopy. At least 100 live mating pairs were scored for each mating.

was not wide enough to easily determine whether the cell walls at the contact site had been degraded. The junction between cells normally expands over time, but this cannot occur in *sec^{ts}* mating pairs because secretion is required for cell-surface growth. As an alternative, *sec^{ts}* mutants were mated to *fus1* partners. The cell walls separating the two cells of a mating pair can be degraded from either side, so most wild-type × *fus1* mating pairs are able to fuse (Trueheart et al., 1987). By contrast, *sec1-1* × *fus1* mating pairs arrested with a broad flat interface indicating a defect in cell-wall remodeling. Secretion in general, rather than a specific function of Sec1, is required for cell-wall remodeling, because prezygotes also accumulated in *sec6-4* × *fus1* and *sec18-1* × *fus1* matings (supplementary material Fig. S4). Furthermore, reduced pheromone release is not the sole cause of the cell-wall-remodeling defect in *sec^{ts}* mutants because an increased sensitivity to pheromone did not promote fusion in *sec6-4* × *fus1 sst2* matings.

sec1-1 × *fus1* prezygotes were examined by electron microscopy to provide a higher-resolution view of the interface between mating partners (Fig. 5B). These images confirmed that the two cells are separated by cell walls, and also revealed significant secretory vesicle accumulation adjacent to the contact site, which was limited to one cell (presumably the *sec1-1* mutant) of the prezygote.

In a quantitative experiment, *sec1-1*, *fus1*, *prm1* and wild-type strains were mated in all possible combinations (Table 5). As expected, the strongest mating defects were found in bilateral *fus1* and *prm1* matings. Significant prezygote accumulation was also found in *fus1* × *sec1* matings, but not in *prm1* × *sec1* matings or in *fus1* × *prm1* matings. In conclusion, secretion appears to be specifically required to degrade cell walls at the contact site. However, cell-wall-remodeling defects were observed after inhibiting cell fusion for 90 minutes, but cell fusion arrested in less than 5 minutes after inhibiting secretion. Thus, these results do not rule out the possibility that secretion is also required for plasma-membrane fusion, which occurs after cell-wall remodeling.

Inhibiting secretion does not arrest cell fusion indirectly via effects on the actin cytoskeleton

Cell polarity in yeast is reinforced by positive feedback between the secretory pathway and the actin cytoskeleton, both of which are polarized towards specific sites on the plasma membrane. Secretory vesicles are transported along actin filaments to exocytic sites, and they deliver polarity factors such as Cdc42 to promote actin polymerization (Wedlich-Soldner et al., 2003). One report showed that actin was almost completely depolarized 5 minutes after shifting the *sec15-1* mutant to 37°C (Aronov and Gerst, 2004). This finding suggests that the cell-fusion block found after inhibiting secretion could be a secondary consequence of the loss of polarized actin. However, actin in wild-type cells is also rapidly depolarized after raising the temperature (Lillie and Brown, 1994), and the previous study did not control for the effects of a heat shock on actin polarity.

The *sec18-1* mutant was used to distinguish between the consequences to actin of excess heat and a secretion block. In this mutant, secretion is almost completely blocked at 32°C (10% invertase secretion), a temperature that does not induce a heat shock response. Wild-type and *sec18-1* mutant cells were grown at 25°C and then shifted to either 32°C or 38°C. Actin polarity was then analyzed by Rhodamine-phalloidin staining (Fig. 6A). In mitotic cells, phalloidin stains actin patches, which are associated with endocytosis, and weakly stains actin cables, which are used as tracks for long-range transport. In small-budded wild-type cells at 25°C, actin patches were concentrated in the bud and actin cables were polarized towards the bud. A similar pattern was found in the *sec18-1* mutant at 25°C, but actin cables were less abundant. When the temperature was raised to 38°C, actin polarity was lost within 10 minutes in both wild-type and *sec18-1* mutant cells, confirming that a heat shock depolarizes actin. After 2 hours at 32°C, actin was almost completely depolarized in the *sec18-1* mutant, but not in the wild type, consistent with a requirement for secretion in the maintenance of actin polarization. In contrast to this long-term effect, actin polarity was only modestly reduced during the first 20 minutes after shifting the *sec18-1* mutant to 32°C, and there was also a slight reduction in wild-type cells. Therefore, actin does not appear to be rapidly depolarized after inhibiting secretion.

Lifeact-GFP was used to label actin filaments in live cells to investigate the relationship between actin polarity and secretion in mating pairs (Riedl et al., 2008). Lifeact is a 17-residue peptide from the N-terminus of Abp140. Similarly to Abp140-GFP, Lifeact-GFP binds preferentially to actin cables, but also interacts with actin patches (Riedl et al., 2008; Yang and Pon, 2002). However, Lifeact-GFP expressed from the *TEF1* promoter had much brighter fluorescence than Abp140-GFP, and there was no need to grow cells on lactate medium, as required for Abp140-GFP (Yang and Pon, 2002). Another advantage of Lifeact-GFP is that it binds actin filaments with low affinity and is therefore less toxic than GFP-actin. Indeed, Lifeact-GFP had no measurable effect on mating efficiency when expressed in wild-type cells, whereas cell fusion was completely inhibited by GFP-actin.

Wild-type and *sec18-1* mutant *MAT**a*** cells expressing Lifeact-GFP were mated to *MATα* cells expressing mCherry as a marker for cytoplasmic mixing. Images were collected before and 6-8 minutes after shifting from 25°C to 32°C (Fig. 6B). Actin cables were found in both wild-type and *sec18-1* mating pairs, and the percentage of mating pairs with at least one polarized actin cable was unaffected by the short incubation at 32°C. Most mating pairs had several actin cables, some of which were not polarized towards the contact site. Nevertheless, the temperature shift did not appear to affect the number or distribution of actin cables in either wild-type or *sec18-1* mutant mating pairs. In summary, actin cables and patches are not depolarized by a short (6-8 minute) shift of *sec18-1* cells or mating pairs to 32°C. By contrast, cell fusion arrested 6.2 minutes after shifting *sec18-1* mating pairs to 31°C (Table 4), which should affect actin polarity even less than a shift to 32°C. Therefore, the cell fusion block is unlikely to be an indirect consequence of actin depolarization.

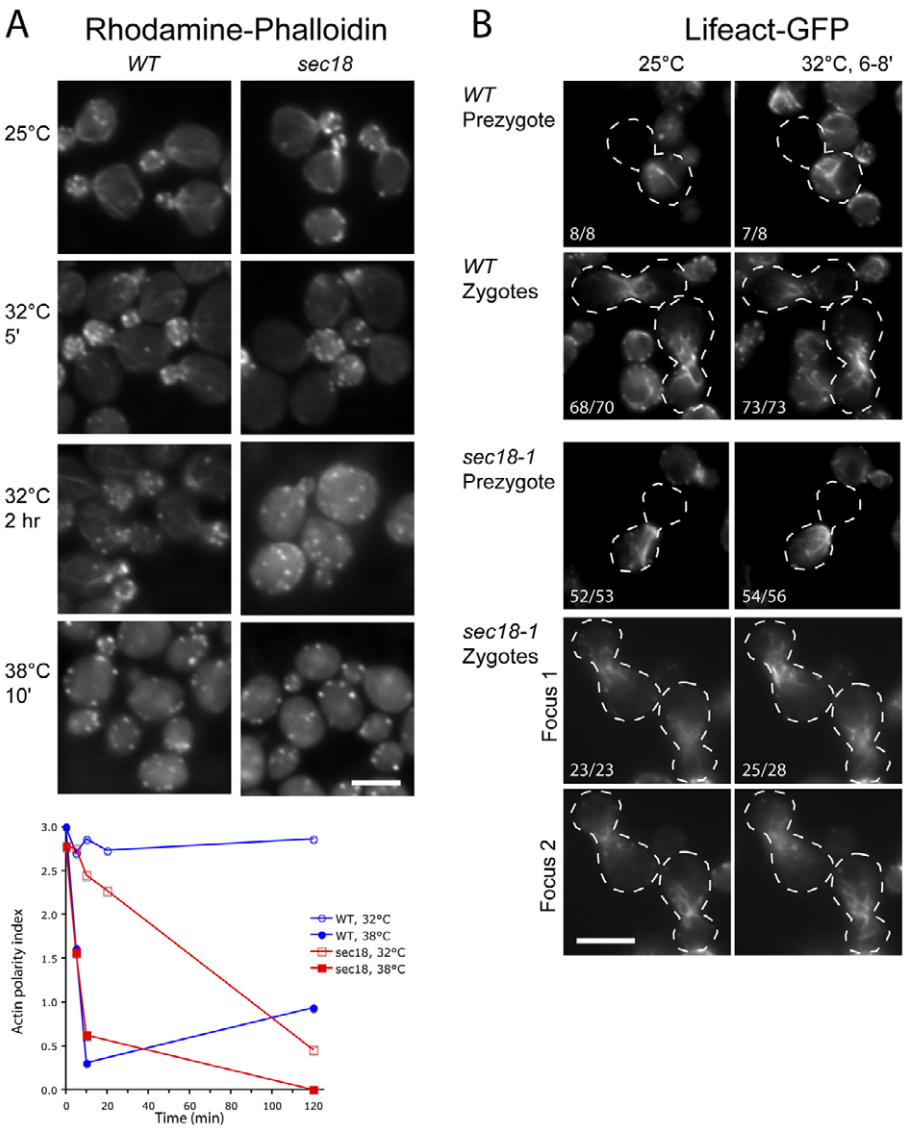

Fig. 6. Actin polarity is maintained during the first minutes after blocking secretion. (**A**) Heat shock and secretory inhibition have distinct effects on actin localization. Mitotic cells were shifted from 25°C to either 32°C or 38°C for the indicated times. Actin was stained with Rhodamine-phalloidin, and actin polarity was scored as described in the methods. (**B**) Actin-cable localization in mating pairs. Wild-type or *sec18-1* mutant *MAT***a** cells expressing Lifeact-GFP were mated to *MAT*α *RFP ce*lls with the same *SEC18* allele. After 60-100 minutes of mating, the temperature was shifted from 25°C to 32°C. Images were collected before and 6-8 minutes after the temperature shift. Mating pairs are enclosed with dotted lines. Two focal planes separated by 2 µm are shown for the *sec18-1* zygotes (Focus 1 and Focus 2). The number of mating pairs with polarized actin cables and the total number of mating pairs of each type are indicated. RFP fluorescence was used to help distinguish prezygotes from zygotes, but is not shown so that Lifeact-GFP can be seen more clearly. Scale bars: 5 µm.

Discussion

Secretion is essential for growth and remodeling of the cell surface and is therefore required at several steps in the mating pathway. The availability of conditional secretory mutants in which secretion is rapidly inhibited after raising the temperature made it possible to investigate the contribution of secretion to late mating events. When plasma-membrane fusion was reversibly blocked by FM6-64 in *sec*^{ts} *prm1* mating pairs, fusion could only resume after washing out the inhibitor if the conditions were permissive for secretion. Furthermore, cell fusion arrested within minutes after shifting the temperature to inhibit secretion in *sec*^{ts} mating pairs. These results demonstrate a just-in-time delivery mechanism for at least one essential component of the cell fusion machinery in yeast.

Secretion, pheromone signaling and cell fusion

Yeast mating initiates with an exchange of pheromone signals that activate transcription of mating-specific genes, arrest the cell cycle and promote polarized growth towards a potential mating partner. Secretion is essential for this process because α-factor is secreted and the **a**-factor exporter and both pheromone receptors must be continually delivered to polarized sites on the cell surface to replace proteins internalized by endocytosis (Chen and Davis, 2000; Jenness and Spatrick, 1986; Kolling and Hollenberg, 1994). Mating-pair assembly increases the efficiency of pheromone signaling by concentrating pheromone release sites and pheromone receptors on opposite sides of the contact site to create a structure analogous to a neuronal synapse. Enhanced pheromone signaling is thought to be important because mutants affecting **a**-factor biosynthesis have cell-wall-remodeling defects (Brizzio et al., 1996). It was therefore surprising to find that raising the temperature of a *sec6-4* mutant to 34.5°C had no effect on the transcriptional response to mating pheromones if the mutation was in the pheromone receiving cell, and only a modest (30-40%) reduction in signaling if the mutation was in the pheromone-releasing cell. For comparison, invertase secretion from the *sec6-4* mutant was inhibited by 86% at 34.5°C. Thus, wild-type cells must have significantly more pheromone receptors on their cell surface than are required for maximum signaling, and are also likely to release more pheromones than required.

Previous studies established that a 30-40% reduction in pheromone signaling should not affect later events in the mating

pathway. In a *ste5*^{ts} mutant, signaling was reduced by 60% at 25°C with no effect on cell-wall remodeling or plasma-membrane fusion. Raising the temperature to 30°C caused an 80% reduction in pheromone signaling with only a 10% reduction in cell-wall remodeling and no effect on plasma-membrane fusion (Jin et al., 2008). Similarly, a 50% reduction in pheromone signaling resulting from attenuated *MFA1* expression did not affect cell fusion (Brizzio et al., 1996). Consistent with these results, amplification of the pheromone response in the mating partner of a *sec*^{ts} mutant did not promote cell-wall remodeling or permit plasma-membrane fusion to continue for more than 8 minutes after a temperature shift. Thus, reduced pheromone signaling is not the underlying cause of the cell-wall remodeling and plasma-membrane fusion phenotypes.

Actin, secretion and cell fusion

Cell fusion is inhibited by mutations in several proteins with actin-related functions (Chen et al., 2007; Ydenberg and Rose, 2008). For example, selective hydrolysis of the cell wall at the contact site requires the formin Bni1, which nucleates growth of polarized actin cables, and the tropomyosins Tpm1 and Tpm2, which stabilize actin cables (Evangelista et al., 1997; Liu and Bretscher, 1992; Matheos et al., 2004; Qi and Elion, 2005). Actin cables are required for myosin-dependent transport of secretory vesicles to the contact site. Thus, disrupting actin cables would block secretion, and thereby inhibit cell fusion. However, actin cables are also required to transport Fus2 to contact sites (Sheltzer and Rose, 2009). Since Fus2 is required for normal cell-wall remodeling in mating pairs, cell fusion would be inhibited by the loss of actin cables even if vesicle fusion with the plasma membrane was unaffected.

The intimate connections between actin and secretion raised the possibility that the cell fusion block is actually an indirect consequence of a failure to deliver proteins required to establish and maintain actin polarity. Compelling evidence against this model is provided by the fact that cell fusion in temperature-shifted *sec*^{ts} mating pairs arrested before there was any notable loss of actin polarity. The persistence of actin polarity after inhibiting secretion runs counter to current dogma, but is fully consistent with previous work showing that actin cables depolarized gradually during a 1 hour shift of the *sec4*^{ts}, *sec6*^{ts}, *sec10*^{ts} and *sec15*^{ts} mutants to 35°C (Pruyne et al., 2004). In conclusion, the secretory vesicle cargo proteins most acutely required for cell fusion are distinct from the cargo proteins that maintain actin polarity.

FM4-64 inhibits plasma-membrane fusion

FM4-64 reversibly inhibited plasma-membrane fusion in *prm1* mating pairs. Prm1 is thought to coordinate the activity of the core fusion proteins, thereby preventing the fusion process from degenerating into lysis and increasing the free energy available to fuse membranes of altered lipid composition (Jin et al., 2004; Jin et al., 2008). In line with this hypothesis, the hobbled fusion proteins of *prm1* mating pairs might provide insufficient free energy to fuse membranes containing FM4-64. Interestingly, FM4-64 actually stimulated fusion of *erg6* mating pairs, which have defects in ergosterol synthesis that lead to a reduction in plasma-membrane fusion (Jin et al., 2008). Thus, although FM4-64 and ergosterol depletion both inhibit fusion in *prm1* mating pairs, they appear to have opposing rather than synergistic effects on the biophysical properties of the membrane.

How does FM4-64 inhibit fusion? FM4-64 partitions into the external leaflet of the plasma membrane, which is the proximal membrane leaflet when two plasma membranes fuse. Positive curvature in the proximal leaflet has been shown to inhibit membrane fusion in a variety of protein-free, cell-free and in vivo settings (Chernomordik and Kozlov, 2008). However, FM4-64 does not have a notably cone-shaped structure that might induce positive curvature, and oleoyl lysophosphatidylcholine (LPC 18:1), a standard reagent used to generate positive membrane curvature, inhibited the fusion of *prm1* mating pairs by less than 50% when present at a saturating concentration (200 μM) that completely inhibited growth (data not shown). An alternative model based upon the bilayer-couple hypothesis (Sheetz and Singer, 1974) is that FM4-64 inhibits membrane deformations required for fusion by increasing the surface area of the outer leaflet of the plasma membrane. A third possibility is that FM4-64 interacts with a membrane protein involved in fusion, as previously described for the related styryl dye FM1-43 (Gale et al., 2001; Mazzone et al., 2006). Any such interaction would have to be rapidly reversible and susceptible to modulation by the sterol composition of the membrane. Although further study is clearly required to elucidate the mechanism of inhibition, FM4-64 provides a useful tool for the analysis of plasma-membrane fusion in yeast mating pairs.

The ability to wash FM4-64 out of the plasma membrane made it possible to demonstrate that secretion is required to recover from the FM4-64-arrested state and resume fusion. The most straightforward interpretation of this result is that secretion is required for plasma-membrane fusion. However, previous observations that mating pairs with persistent cytoplasmic fingers rarely proceed to fusion (Jin et al., 2004) and that a new cell wall can grow between the two plasma membranes of an arrested mating pair (Jin et al., 2008) allow for the possibility that the relatively small number (20%) of mating pairs that fused had not yet achieved plasma-membrane contact at the time when the FM4-64 was removed. Thus, the resumption of cell fusion after FM4-64 washout could depend upon secretion to deliver either glucanases for cell-wall remodeling and/or fusion proteins for plasma-membrane merger.

Secretion is required for very late events in cell fusion

Cell fusion arrested 4-5 minutes after raising the temperature to inhibit secretion in the *sec6-4* and *sec18-1* mutants, indicating that ongoing secretion is required at a late stage in the fusion process. The final events before cytoplasmic mixing are cell-wall remodeling and plasma-membrane fusion. Secretion is certainly required for the overall process of cell-wall remodeling because glucanases and other cell-wall-remodeling enzymes are delivered via the secretory pathway (Cappellaro et al., 1998; Lesage and Bussey, 2006). This dependency was confirmed by the observation that *sec*^{ts} × *fus1* mating pairs arrested as prezygotes with intervening cell walls after a 90 minute shift to 34.5°C.

Although secretion is required for cell-wall remodeling, cell-wall remodeling is unlikely to arrest immediately after blocking secretion. The enzymes responsible for cell-wall remodeling are covalently linked to the cell wall via disulfide or GPI remnant bonds (Cappellaro et al., 1998; Lesage and Bussey, 2006). Immediately after secretion is inhibited, there will be a cohort of enzymes on the plasma membrane that have not yet been transferred to the cell wall. Once linked to the cell wall, these enzymes cannot be rapidly internalized by endocytosis and degraded. Thus, enzymes that are already at the cell surface should be able to continue degrading the cell wall for more than 5 minutes. In support of this interpretation, mating-pair assembly,

which is also mediated by cell-wall proteins (Dranginis et al., 2007; Lu et al., 1994; Lu et al., 1995), can continue for 20 minutes after inhibiting secretion.

Another significant observation indicating that cell-wall remnants are not responsible for the rapid cell-fusion arrest is that cell fusion arrested within minutes after shifting *WT* × *sec6-4* mating pairs to 34.5°C. Mutants with cell-wall-remodeling defects must be deleted from both cells in a mating pair to strongly inhibit fusion (Trueheart et al., 1987) suggesting that the cell wall can be degraded by enzymes released from either cell in a mating pair. Thus, *WT* × *sec6-4* mating pairs should not have a cell-wall-remodeling defect. In conclusion, the rapid arrest in cell fusion after blocking secretion is probably not caused by a failure to complete cell-wall remodeling.

A closer examination of the kinetics of the cell fusion arrest in *sec*^{ts} mating pairs is consistent with the possibility that inhibition of secretion blocked cell fusion in some mating pairs that had already completed cell-wall remodeling. There was a 4.3 minute delay after initiating the 25°C to 34.5°C temperature shift in *sec18-1* mating pairs before cell fusion was blocked, but secretion was not instantaneously inhibited at the beginning of the 4.3 minute interval because it took 3 minutes to increase the temperature to 34°C and because SNARE proteins primed by Sec18-1 before the temperature shift must be depleted before secretion is completely inhibited (Banerjee et al., 1996). In addition, the plasma membranes in some mating pairs could remain in contact for a few minutes before they fuse to allow time for a complex of fusion proteins to assemble. If this reasoning is correct, secretion must be required for plasma-membrane fusion.

Models

There are many potential explanations for the dependence of cell fusion upon just-in-time delivery through the secretory pathway. One possibility is that fusion proteins must be continually delivered to the cell surface to replace proteins removed by endocytosis. A related model is that ongoing polarized secretion is required to maintain a concentrated patch of fusion proteins at the contact site, thereby ensuring that fusion proteins on the two cells are able to interact once the two membranes come into contact. A third possibility is that a mating event associated with plasma membrane contact provides a signal to target fusion proteins to the plasma membrane. All of these models predict that proteins essential for cell fusion will be concentrated in the secretory vesicles of mating yeast.

Materials and Methods

Strains

Yeast strains (supplementary material Table S1) were constructed by standard techniques including lithium acetate transformations and isogenic genetic crosses. The *sec*^{ts} mutant alleles were from Peter Novick (UCSD, San Diego, CA). The *cdc6*^{ts} allele was from Marc Solomon (Yale University, New Haven, CT). KanMX-knockout strains were from Michael Snyder (Yale University). *SST2* was disrupted by homologous recombination. Cells were grown overnight to early log phase for all experiments using either rich YPD medium or selective synthetic complete (SC) dropout medium.

Plasmids

URA3 marked integrating plasmids for GFP (pEG311), mCherry (pEG640) and DsRed (pEG223) expression have been previously described (Nolan et al., 2006). GFP (pEG745) and mCherry (pEG743) expression plasmids with a hygromycin-resistance (*Hph-NTI*) marker were constructed by inserting a *Sac*I-*Sma*I fragment from pAG32 (Goldstein and McCusker, 1999) into vectors digested with *Sac*I and *Eco*RV. The Lifeact-GFP expression plasmid pEG844 was constructed by inserting complementary oligonucleotides encoding 17 amino acids from the N-terminus of Abp140 into the *Bam*HI site at the 5′ end of the *GFP* gene in pEG745. The *lacZ-Δμ* and *lacZ-Δω* open reading frames were subcloned from MFG retroviral vectors (Mohler and Blau, 1996) into pEG311, replacing the GFP insert. The *prm1Δ* cassette from pEG676 (Jin et al., 2008) was subcloned as an *Xba*I-*Pvu*II fragment into pAG32 digested with *Spe*I and *Hpa*I (Goldstein and McCusker, 1999) to create the *prm1::Hph-NTI* plasmid pEG767. The P_{FUS1}-*FUS1(1-254)-lacZ* reporter plasmid pSB234 (Trueheart et al., 1987) was from Susan Michaelis (Johns Hopkins, Baltimore, MD). Plasmid p1307 containing an *sst2::URA3* deletion cassette was from Charlie Boone (University of Toronto, Toronto, Canada).

β-galactosidase ω-complementation assay

Equal amounts (0.15 ODU) of *MAT**a*** *lacZ-Δμ* and *MATα lacZ-Δω* cells were mixed and collected on a 2.5-cm nitrocellulose filter. The filters were incubated on YPD plates as indicated. Mating pairs were then washed off the filters into ice-cold TAF buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaN_3, 20 mM NaF). 0.3 OD units of cells from each sample were pelleted, resuspended in 100 μl buffer 1 (100 mM HEPES, pH 7.3, 150 mM NaCl, 5 mM L-aspartate, 1% BSA, 0.05% Tween 20), and then lysed by three cycles of freezing in liquid nitrogen and thawing in a 37°C water bath. An aliquot of the lysate was incubated with 2.23 mM chlorophenol Red, β-D-galactopyranoside (CPRG) in buffer 1 at 30°C. The reaction was stopped by adding $NaCO_3$ to a final concentration of 300 mM, and product was quantified by reading the absorbance at 578 nm. The OD_{578} readings were corrected for protein in the lysate, which was measured using a Bradford assay.

Invertase secretion

Invertase secretion was assayed as previously described (Novick and Schekman, 1979). Invertase expression was induced by shifting cells from high (2%) to low (0.1%) glucose. The cells were then incubated at the indicated temperature for 45 minutes. The percentage of invertase secreted was calculated from measurements of external and internal invertase activity. These measurements were corrected by subtracting a background corresponding to the invertase activity of uninduced cells.

FUS1-lacZ expression

*MAT**a*** and *MATα* cells expressing the *FUS1-lacZ* reporter as indicated were grown separately, washed to remove soluble pheromones, mixed, collected on filters, and then incubated over SC agar plates at the indicated temperature. After incubation for 80 minutes, the cells were collected in ice-cold TAF buffer. β-galactosidase activity was assayed using the colormetric substrate o-nitrophenyl-β-D-galactopyranoside (ONPG), as previously described (Jin et al., 2008).

Light microscopy

For endpoint assays, *MAT**a*** *GFP* and *MATα RFP* cells were mated on filters incubated over SC plates as indicated. Mating pairs were imaged with an Axioplan2 imaging microscope (Zeiss) and an Orca ER digital camera (Hamamatsu), and scored for fusion as previously described (Jin et al., 2004). Time-lapse images of yeast mating were collected as previously described (Nolan et al., 2006). The temperature was controlled using stage and objective heaters (PeCon, Erbach, Germany) and monitored using the temperature probe of an Omega 871A digital thermometer embedded in the agarose pad of a slide. The focus was manually adjusted during the first 5 minutes after the temperature shift to compensate for thermal expansion.

Actin polarization

Cells were cultured overnight in YPD medium at 25°C to an OD_{600} of 0.6 and then diluted tenfold into YPD medium prewarmed to the indicated temperature. At the indicated times, prewarmed paraformaldehyde was added to a final concentration of 3.7%. After 20-40 minutes of fixation in YPD medium, the cells were pelleted and resuspended in PBS with 3.7% formaldehyde. Actin was stained with Rhodamine-phalloidin, as previously described (Baggett et al., 2003). Actin polarity was scored in small-budded cells as follows: cells with full polarization of actin cables and patches received 3 points; cells with partial cable polarization and fully polarized patches received 2 points; cells with depolarized cables and partially polarized patches received 1 point; and cells with complete depolarization received 0 points. The mean score from 50 cells was calculated to yield the actin polarity index.

For the *Lifeact-GFP* matings, a series of DIC, GFP and mCherry images were collected from five adjacent microscopic fields before and 6-8 minutes after shifting the mating pairs to 32°C. To increase the likelihood of detecting cortical actin cables, six GFP images at focal planes separated by 1 μm were collected from each field.

Mating with FM4-64

Standard SC growth medium has a low pH and a high concentration of divalent cations, both of which inhibit the partitioning of FM4-64 into membranes. Custom SC medium was prepared using yeast nitrogen base lacking divalent cations (Sunrise Science Products, San Diego, CA). The medium was supplemented with 0.2 mM $MgCl_2$ and 20 mM MES, pH 6. Equal amounts (0.1 ODU) of *MAT**a*** and *MATα* cells were collected on a filter and incubated over a standard SC plate for 30 minutes. The cells were washed off the filter into 1 ml of custom SC medium, concentrated to 10 μl by centrifugation at 500 ***g*** for 1 minute, transferred to agarose pads prepared from custom SC medium supplemented with 8 μM FM4-64, overlayed with a 22 mm^2 coverslip to spread out the cells, and then incubated as indicated. To release the FM4-64 block, mating pairs were washed off the FM4-64 pads into 1 ml of custom

medium pre-warmed to 34.5°C, washed twice with custom medium, transferred to an agarose pad prepared from custom medium supplemented with 7 mM Ca^{2+} to promote fusion (Aguilar et al., 2006), and then incubated for 1 hour at 25°C or 34.5°C before imaging.

Electron microscopy

MATa fus1Δ × *MATα sec1-1* cells (4 OD units each) were mixed, collected on an 82 mm nitrocellulose filter, and incubated over a YPD plate for 30 minutes at 25°C followed by 90 minutes at 34.5°C. The mating pairs were then processed for electron microscopy, as previously described (Jin et al., 2008).

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