

# End3p-Mediated Endocytosis Is Required for Spore Wall Formation in *Saccharomyces cerevisiae*

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## ABSTRACT

During sporulation in *Saccharomyces cerevisiae*, vesicles transported to the vicinity of spindle pole bodies are fused to each other to generate bilayered prospore membranes (PSMs). PSMs encapsulate the haploid nuclei that arise from the meiotic divisions and serve as platforms for spore wall deposition. Membrane trafficking plays an important role in supplying vesicles for these processes. The endocytosis-deficient mutant, *end3Δ*, sporulated poorly and the spores produced lost resistance to ether vapor, suggesting that *END3*-mediated endocytosis is important for sporulation. End3p-GFP localized to cell and spore peripheries in vegetative and sporulating cells and colocalized with actin structures. Correspondingly, the actin cytoskeleton appeared aberrant during sporulation in *end3Δ*. Analysis of meiosis in *end3Δ* mutants revealed that the meiotic divisions occurred with wild-type kinetics. Furthermore, PSMs were assembled normally. However, the levels of proteins required for spore wall synthesis and components of the spore wall layers at spores were reduced, indicating that *end3Δ* mutants are defective in spore wall synthesis. Thus, *END3*-mediated endocytosis is important for spore wall formation. Additionally, cytological analyses suggest that trafficking between the plasma membrane and PSMs is important earlier during sporulation.

**I**N *Saccharomyces cerevisiae*, the initiation of the sporulation program in *MATa/MATα* diploid cells is triggered by deprivation of nitrogen in the presence of a nonfermentable carbon source (KUPIEC *et al.* 1997). During sporulation, two major processes, meiosis, which reduces the ploidy of the cell, and spore formation are tightly coordinated to ensure the production of viable progeny (KUPIEC *et al.* 1997). During the second meiotic division, novel bilayer membranous structures termed prospore membranes (PSMs) are formed by the fusion of vesicles at the cytoplasmic face of spindle pole bodies (SPBs). PSMs then grow and engulf each of the four haploid nuclei (NEIMAN 1998; MORENO-BORCHART and KNOP 2003). Once formed, PSMs serve as a platform for the deposition of the multi-layered spore wall that is laid down between the inner and outer lipid bilayer (LYNN and MAGEE 1970; SMITS *et al.* 2001; MORENO-BORCHART and KNOP 2003). The molecular mechanisms underlying the formation and coordination of PSMs and spore walls are not well understood. However, a combination of classical genetics and genomic approaches have identified a number of molecules essential for these processes (ENGBRECHT *et al.* 1998; RABITSCH *et al.* 2001; BRIZA *et al.* 2002; ENYENIHI and SAUNDERS 2003; COLUCCIO *et al.* 2004)

*SPO14* encodes the single yeast phosphatidylcholine

(PC)-specific phospholipase D (PLD), which hydrolyzes PC, producing phosphatidic acid (PA) and choline (ROSE *et al.* 1995; RUDGE and ENGBRECHT 1999). Spo14p localizes to, and is essential for, PSM formation (RUDGE *et al.* 1998, 2004). Furthermore, the enzymatic activity of Spo14p is required for PSM formation at the SPBs (RUDGE *et al.* 1998). Another gene required for PSM formation is *SSO1*, a syntaxin, which functions in secretory vesicle fusion at the plasma membrane (AALTO *et al.* 1993). *Sso1p* is also essential for sporulation (JÄNTTI *et al.* 2002; ENYENIHI and SAUNDERS 2003; ÖYEN *et al.* 2004). Recently, cytological and biochemical analyses revealed that deletion of *SPO14* or *SSO1* results in the accumulation of vesicles in the vicinity of SPBs in sporulating cells, indicating that these proteins function specifically in vesicle fusion for PSM formation (H. NAKANISHI, personal communication; M. MORISHITA, unpublished data).

During spore formation, vesicles are continuously transported to PSMs to bring and deposit spore wall materials (LYNN and MAGEE 1970; SMITS *et al.* 2001). The spore wall consists of four discrete layers: mannan, glucan, chitosan, and dityrosine. The first two layers are similar to vegetative cell walls, while the outer layers are unique to spores and ensure their resistance to environmental stresses and harmful chemicals such as ethyl ether (BRIZA *et al.* 1994; SMITS *et al.* 2001). The spore wall layers are laid down in a specific order in which the mannan, glucan, chitosan, and dityrosine layers overlay outward in a step-wise manner (TACHIKAWA *et al.* 2001; COLUCCIO *et al.* 2004). Many proteins and enzymes re-

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**TABLE 1**  
**Strain list**

Strain	Genotype	Source
AN120	<i>MATa/MAT<math>\alpha</math> ura3/ura3 his3/his3 leu2/leu2 trp1/trp1 arg4/ARG4 rme::LEU2/RME</i>	NEIMAN <i>et al.</i> (2000)
Y4733	AN120, plus <i>end3::KanMX/end3::KanMX</i>	This laboratory
Y4734	AN120, plus <i>end3::KanMX/end3::KanMX</i>	This laboratory
Y592	<i>MATa/MAT<math>\alpha</math> ho::LYS2/ho::LYS2 trp1-hisG/trp1-hisG ura3/ura3 leu2-k/leu2::hisG sps1::TRP1/sps1::TRP1</i>	IWAMOTO <i>et al.</i> (2005)
Y4931	AN120, plus <i>GSC2::GFP(TRP1)/GSC2::GFP(TRP1)</i>	This laboratory
Y4962	<i>MATa/MAT<math>\alpha</math> ura3/ura3 his3/his3 leu2/leu2 trp1/trp1 rme::LEU2/rme::LEU2 end3::KanMX/end3::KanMX GSC2::GFP(TRP1)/GSC2::GFP(TRP1)</i>	This laboratory
Y4535	AN120, plus <i>CHS3::GFP(TRP1)/CHS3::GFP(TRP1)</i>	This laboratory
Y4823	Y4733, plus <i>CHS3::GFP(TRP1)/CHS3::GFP(TRP1)</i>	This laboratory
Y5095	AN120, plus <i>END3::GFP(HIS5)/end3::KanMX</i>	This laboratory

quired for spore wall formation are transported to PSMs through vesicle trafficking. Dtr1p, a transporter for di-tyrosine (FELDER *et al.* 2002), is transported from the Golgi to PSMs (M. MORISHITA, unpublished data), indicating that vesicle trafficking between the Golgi and PSMs is essential for spore formation (NEIMAN 1998; MORENO-BORCHART and KNOP 2003). Since Fks2p/Gsc2p, hereafter referred to as Gsc2p, a glucanase required for glucan synthesis (MAZUR *et al.* 1995), and Chs3p, a chitin synthase required for the synthesis of the chitosan layer (PAMMER *et al.* 1992), are present at the plasma membrane in vegetative cells, recycling of these proteins from the plasma membrane of the mother cell to PSMs may be necessary for spore wall formation. Consistent with this idea, Gsc2p and Chs3p mislocalize in mutants defective for the sporulation-specific *SPS1* gene, which encodes a putative protein kinase (FRIESEN *et al.* 1994; IWAMOTO *et al.* 2005). Sps1p has been proposed to regulate the movement of these enzymes to build the spore wall (IWAMOTO *et al.* 2005). However, the pathway of this protein movement has not been elucidated.

Endocytosis, the uptake of membrane proteins and lipids, extracellular ligands, and soluble molecules from the cell surface, is important for membrane trafficking between the plasma membrane and internal compartments. Endocytosis is essential for the maintenance of plasma membrane functions such as cell growth regulation, cell polarity establishment, nutrient uptake, and ion homeostasis by control of the protein and lipid composition of the plasma membrane, regulation of signaling pathways, and uptake of nutrients and pathogens (GELI and RIEZMAN 1998). In *S. cerevisiae*, a number of genes have been identified in screens for mutants defective in the internalization of pheromone receptor or the fluid-phase endocytic marker, lucifer yellow (RATHS *et al.* 1993; MUNN *et al.* 1995). Two of these mutants, *end3* and *end4*, show clear defects in internalization, temperature-sensitive growth defects, and defects in organization of the actin cytoskeleton (RATHS *et al.* 1993; BÉNÉDETTI *et al.* 1994). End4p/Sla2p is a phosphatidyli-

nositide 4,5 bisphosphate binding protein and has been proposed to recruit Sla1p, which is required for assembly of the endocytic complex at the plasma membrane, and to regulate actin-dependent endocytic internalization (WESP *et al.* 1997; GOURLAY *et al.* 2003; SUN *et al.* 2005). *END3* encodes a component of the endocytic complex, which is essential for the internalization step of endocytosis and regulation of actin organization (TANG *et al.* 2000). Additionally, the actin cytoskeleton is essential for endocytosis (QUALMANN *et al.* 2000; ENQVIST-GOLDSTEIN and DRUBIN 2003; KAKSONEN *et al.* 2003; MERRIFIELD 2004). Interestingly, analysis of a large number of actin mutations revealed a strong correlation between defects in endocytosis and sporulation, suggesting that endocytosis is important for sporulation (WHITACRE *et al.* 2001). However, the precise defect in sporulation in these mutants was not examined.

To investigate the role of endocytosis in sporulation, we characterized the *end3 $\Delta$*  mutant. Here we show that the *end3 $\Delta$*  mutant shows defects in both endocytosis and actin organization during sporulation. The *end3 $\Delta$*  mutant exhibits normal progression through meiosis and PSM assembly; however, the formation of the spore wall is aberrant. Thus, the *END3*-mediated endocytosis pathway is important in spore wall formation. Additionally, cytological analyses suggest that trafficking between the plasma membrane and PSMs is important for PSM assembly.

## MATERIALS AND METHODS

**Strains, media, and plasmids:** Routine growth and manipulation of *S. cerevisiae* strains were performed as described (ROSE *et al.* 1990). Strains used in this study are all derived from the sporulation-proficient SK-1 strain background and are listed in Table 1. DNA-mediated transformation of yeast cells was performed using the lithium acetate procedure (ITO *et al.* 1983). Gene replacements were performed by the one-step method (ROTHSTEIN 1983). The *end3::KanMX* deletion mutant, which replaces the entire open reading frame with the marker *kanMX4* (WACH *et al.* 1994), was made by PCR amplifi-

cation of regions ~300 bp upstream and downstream of the disrupted open reading frame from previously constructed knockout strains purchased from Research Genetics (Huntsville, AL). GFP chromosomal fusions were constructed using reagents *FKS2-GFP* and *CHS3-GFP* (LONGTINE *et al.* 1998) and the reagent *END3-GFP* (SHEFF and THORN 2004). All integrants were confirmed using the PCR and appropriate synthetic oligonucleotide primers. *yEP-GFP-SPO14* is described in detail elsewhere (SCIORRA *et al.* 2002). *SPR28-GFP* was expressed from a low-copy-number (CEN) *URA3* plasmid (pSB19) (DE VIRGLIO *et al.* 1996).

**Analysis of sporulation:** Cells were grown on yeast extract peptone (YP)-glycerol medium overnight, transferred to YPAD (YPD + 10 mM adenine) medium, and incubated overnight. Cells were replica plated onto SPO (2% potassium acetate, 0.1% dextrose, 0.125% yeast extract, 0.05% amino acid drop-out powder) medium and incubated at 24°, 30°, or 33° for 2 days; sporulation was monitored by differential interference contrast (DIC) microscopy. For liquid cultures, cells were grown on YPAD medium for 2 days after overnight incubation on YP-glycerol medium and inoculated into 2 ml YPAD liquid medium for 24 hr, and then 135  $\mu$ l of saturated culture was inoculated into 10 ml YP-acetate medium. Cells were grown in YP-acetate medium for 16 hr, collected by centrifugation, and resuspended in an equal volume of SPM (1% potassium acetate, 0.02% raffinose, 0.1% amino acid drop-out powder) to induce sporulation. For analysis of GFP fusion proteins, live cells were examined by fluorescein-5-isothiocyanate on a Zeiss Axioskop 2 fluorescence microscope at various times after induction of sporulation. To monitor progression in sporulation and to ensure that cells were at the appropriate stage, parallel cultures were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI).

**Ether sensitivity assay:** Cells were sporulated on SPO medium at 30° for 2 days, replica plated to YPAD medium, immediately exposed to 0.6 ml ether vapor for 15 min, and then exposed to an additional 2 ml of ether for 15 min. The plates were incubated at 30° overnight.

**Vacuolar marker staining:** Cells were induced to sporulate in liquid as described above. FM4-64, *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl)hexatrienyl pyridinium dibromide (Molecular Probes, Eugene, OR) (VIDA and EMR 1995) was added at a final concentration of 2  $\mu$ g/ml (1 mg/ml stock solution in DMSO) and incubated on ice for 5 or 10 min. The cells were collected by centrifugation, inoculated into fresh SPM, and incubated with shaking at 30°. At various times, 15 mM NaN<sub>3</sub> was added and cells were observed by fluorescence microscopy.

**Actin staining with rhodamin-phalloidin:** Sporulating or mitotically dividing cells were fixed in PM buffer (1 M potassium phosphate pH 6.8, 1 M MgSO<sub>4</sub>) with 3% formaldehyde at 30° for 1 hr. After washing with PM buffer three times, fixed cells were resuspended in 1 ml PM containing 1% Triton X-100 and incubated at room temperature for 3 min for cell permeabilization. Permeabilized cells were washed with PM buffer, resuspended in 20  $\mu$ l rhodamine-phalloidin (Molecular Probes)/PM buffer, and incubated with shaking at 30° for 1 hr. Cells were mounted on a glass slide and 8  $\mu$ l of a 300- $\mu$ M DAPI solution was added to visualize DNA.

**In vivo BODIPY-PC analysis:** Cells were sporulated in liquid as described above and 2-decanoyl-1-[*O*-(11-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino)undecyl]-sn-glycerol-3-phosphocholine (BODIPY-PC) (4  $\mu$ M final concentration) was added directly to the culture after a 4-hr incubation at 30°. Cells were harvested at 1.5 hr after the addition of BODIPY-PC. Lipids were extracted and analyzed by thin-layer chromatography as described (RUDGE *et al.* 2001). All assays were performed in triplicate. The percentage of

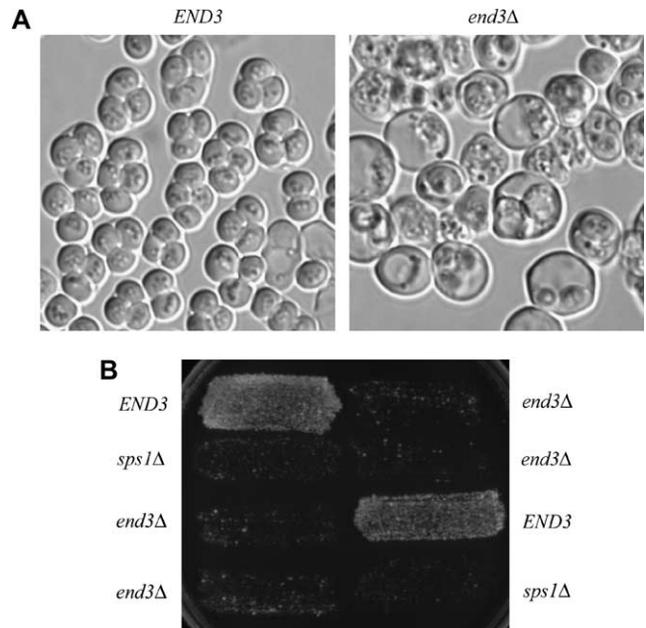


FIGURE 1.—*end3Δ* is defective in spore formation. (A) Sporulation in wild-type (AN120) and *end3Δ* (Y4733) cells. DIC microscopy of cultures induced in sporulation showed *end3Δ*-produced aberrant spores. (B) Ether vapor sensitivity test. Wild-type (AN120), *end3Δ* (Y4733 and Y4734), and *sps1Δ* (Y592) cells were incubated on sporulation medium for 2 days, replica plated onto rich medium, and then immediately exposed to ether vapor. The photo of the plate was taken after overnight incubation at 30°.

conversion of BODIPY-PC to -PA was determined from the pixel intensities obtained from the image using AlphaEase FC4.0 imager soft (Alpha Innotech).

**Calcofluor White staining:** Cells were sporulated in liquid as described above. One milliliter of culture was harvested, washed once with water, and resuspended in 1 mg/ml Calcofluor White (CW) solution. Cells were incubated at room temperature for ~5 min, washed three times with water, and resuspended in water for fluorescence microscopy using DAPI optics.

## RESULTS

**The *end3Δ* mutant is defective in sporulation:** *END3* encodes a component of the endocytic machinery required for the internalization step of endocytosis (BÉNÉDETTI *et al.* 1994). To investigate the role of endocytosis in sporulation, we constructed an *end3* deletion (*end3::KanMX*) in the SK-1 strain background, which sporulates rapidly and efficiently (FAST 1973). Contrary to a previous report of temperature-sensitive growth of the *end3* deletion mutant, in which the N-terminal three-fourths was deleted (BÉNÉDETTI *et al.* 1994), the *end3Δ* SK-1 strain grew similarly to wild type at all temperatures examined (data not shown). The *end3Δ* mutation, however, conferred a sporulation-deficient phenotype (Figure 1; Table 2). DIC microscopy revealed that *end3Δ* sporulated poorly and that the spores produced appeared aberrant (Figure 1A). A large percentage of the

**TABLE 2**  
Sporulation efficiency

Strain	Relevant genotype	Temperature	No. of spores/cell				
			4	3 <sup>a</sup>	2	1	0
AN120	<i>END3/END3</i>	24°	68.7 (9.3)	20.3 (4.2)	5.4 (2.7)	0.3 (0.2)	5.2 (3.2)
		30°	65.6 (2.7)	19.2 (3.9)	1.2 (0.5)	0.2 (0.2)	13.7 (4.9)
		33°	70.6 (3.7)	26.6 (3.8)	0.0 (0.0)	0.2 (0.4)	2.7 (0.6)
Y4733	<i>end3Δ/end3Δ</i>	24°	25.8 (2.8)	28.1 (2.4)	16.8 (2.9)	9.8 (3.3)	19.6 (8.3)
		30°	3.5 (0.5)	11.8 (3.5)	11.5 (4.1)	17.3 (3.1)	55.9 (10.0)
		33°	3.3 (1.2)	8.0 (1.8)	14.3 (2.3)	26.0 (3.5)	48.3 (6.1)

Frequencies of spore formation are shown as percentages.

<sup>a</sup> Values may be overrepresented since tetrads and three-spored asci are indistinguishable in wild-type cells, while three-spored asci in *end3Δ* mutant cells are clearly distinguished from tetrads. Standard deviation for each value is shown in parentheses. *n* = 300.

sporulating *end3Δ* cells contained one-, two-, and three-spored asci, which were rarely seen in wild type, although a small population in *end3Δ* contained complete tetrads at 30° (3.5% *end3Δ* vs. 65.6% *END3*) and at 33° (3.3% *end3Δ* vs. 70.6% *END3*). This phenotype was less severe at 24° (25.8% *end3Δ* vs. 68.7% *END3*) (Table 2). We used 30° for all subsequent experiments.

The outermost layer of the spore wall consists of dityrosine, which fluoresces when exposed to ultraviolet (UV) light. *end3Δ* spores emitted only very weak fluorescence under UV light (data not shown), suggesting that dityrosine molecules are not efficiently incorporated into *end3Δ* spores. Consistent with this result, *end3Δ* spores were sensitive to ether vapor, similar to the *sps1Δ* mutant, which is defective in the deposition of spore wall components (FRIESEN *et al.* 1994; IWAMOTO *et al.* 2005), at a concentration that had no effect on wild-type spores (Figure 1B). Taken together, these results indicate that the *end3Δ* mutant fails to produce mature spores.

#### Endocytosis is impaired in *end3Δ* during sporulation:

To examine endocytosis, we monitored uptake of the vacuole membrane lipophilic dye, FM4-64, in wild type and in the *end3Δ* mutant during vegetative growth and sporulation (Figure 2, Tables 3 and 4). Wild-type and *end3Δ* mutant cells were incubated in rich or sporulation medium for 5 hr and labeled with FM4-64 (as described in MATERIALS AND METHODS). During vegetative growth, wild-type cells rapidly internalized FM4-64 and displayed strong vacuolar staining by 20 min after removal of dye (Figure 2A; Table 3). In *end3Δ* mutants, the majority of the cells displayed very weak or no signal; strong vacuolar staining was observed in only a small number of cells after 60 min (Figure 2A; Table 3), indicating that *end3Δ* is defective in uptake of this dye during vegetative growth.

In wild-type sporulating cells, FM4-64 was observed at the plasma membrane, punctate structures, and vacuoles (Figure 2B; Table 4). Greater than 80% of the cells no longer showed plasma membrane staining 10 min

after removal from the dye and vacuolar staining increased steadily throughout the time course. In addition, some PSMs were stained by FM4-64. In contrast, *end3Δ* mutants had persistent plasma membrane staining; the majority of cells lost plasma membrane staining only after 60 min after removal from dye. In addition, significant vacuolar staining was observed only after 60 min, although not to the extent of wild type (Figure 2B; Table 4). Similar to wild type, some cells contained PSMs stained with FM4-64. Thus, endocytosis in the *end3Δ* mutant is also impaired during sporulation.

***end3Δ* exhibits defects in the actin cytoskeleton during sporulation:** Endocytosis is tightly coordinated with the organization and movement of actin (ENGQVIST-GOLDSTEIN and DRUBIN 2003). Endocytosis-deficient mutants show defects in the actin cytoskeleton and, vice versa, mutants defective in actin or actin-related proteins have defects in endocytosis (KÜBLER and RIEZMAN 1993; RATHS *et al.* 1993; MUNN *et al.* 1995). It has been previously demonstrated that actin mutants are defective in sporulation (WHITACRE *et al.* 2001) and that actin patches are seen in spores (DOYLE and BOTSTEIN 1996). To examine the actin cytoskeleton during sporulation, fixed cells were labeled with rhodamine-conjugated phalloidin to visualize F-actin at various times throughout sporulation (Figure 3, A and B). In wild type, actin was observed as an extensive array of patches and cables in mitotically dividing cells (Figure 3A, vegetative cells). Cells arrested in G<sub>1</sub> for entry into meiosis almost exclusively exhibited actin patches (Figure 3A, 0 hr). Interestingly, in addition to patches, network-like actin filaments appeared in cells undergoing the first and second meiotic divisions (Figure 3A, 2–5 hr). The actin filaments were maintained until meiosis was completed and then actin patches were observed around and/or inside the individual spores (Figure 3A, 6 and 7 hr). In *end3Δ* cells, large actin clumps were observed in the cytoplasm of sporulating cells (Figure 3B, 0, 2, and 4 hr) and huge actin aggregates were seen around spores (Figure 3B,

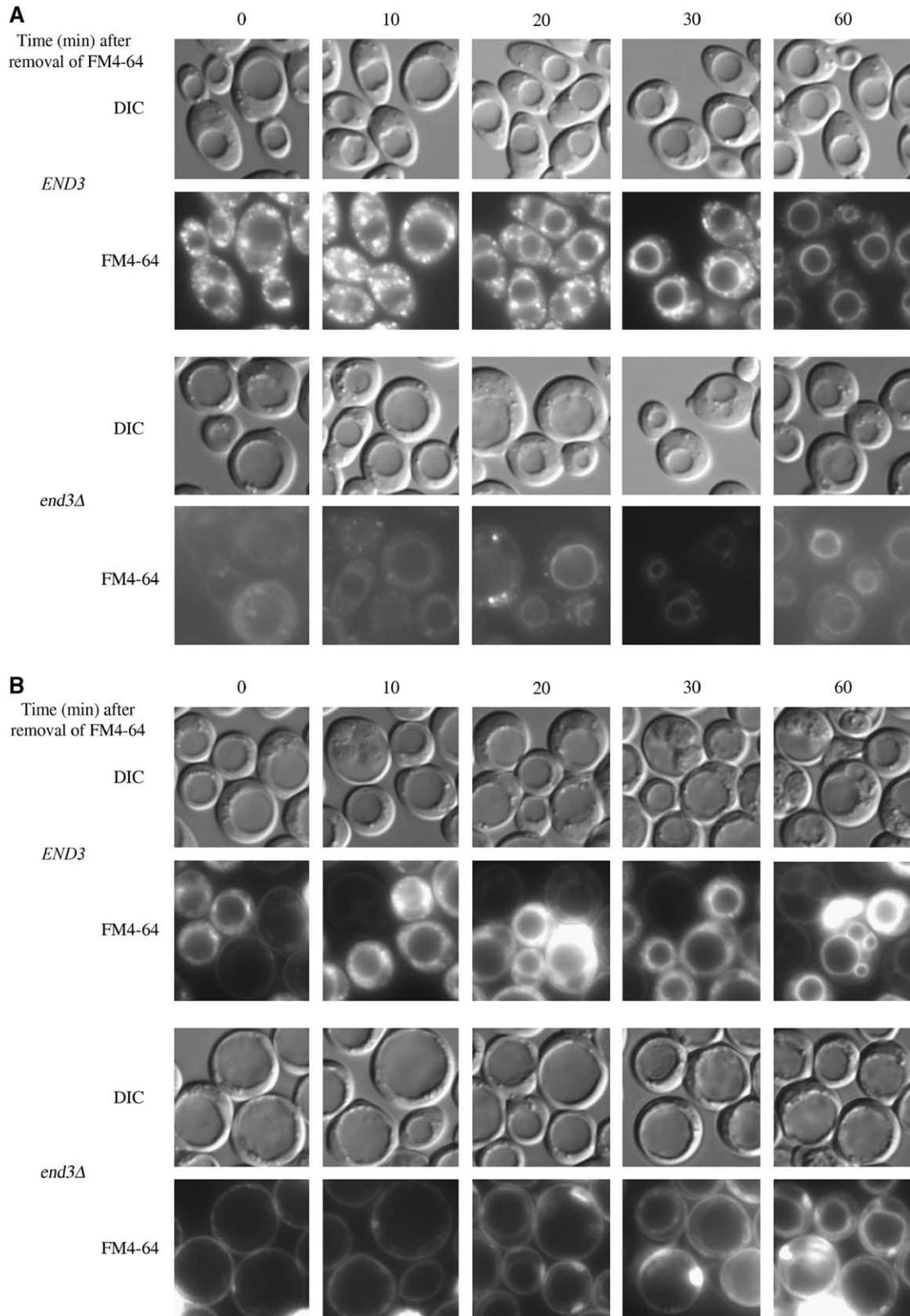


FIGURE 2.—*end3Δ* is defective in the uptake of FM4-64. (A) Vegetative and (B) sporulating wild-type (AN120) and *end3Δ* mutant (Y4733) cells were stained with the lipophilic styryl marker FM4-64, as described in MATERIALS AND METHODS. Fluorescence (bottom) and DIC (top) images of the same cells are shown. In wild-type cells, FM4-64 was observed as punctate signals in the cytoplasm and eventually at vacuoles by 60 min after removal of the dye during vegetative growth. In addition to vacuole staining of the mother cells, FM4-64 was detected at spores during sporulation. In *end3Δ*, only weak or no signals for FM4-64 were detected at vacuoles during vegetative growth, and plasma membrane staining persisted for 60 min after removal of dye during sporulation.

**TABLE 3**  
**Quantification of FM4-64 uptake: vegetative cells**

Time (min) after removal of FM4-64	Vacuoles		Faint cytoplasm or no signal	
	Punctate	Strong Weak		
<i>END3</i> : % of cells labeled with FM4-64				
0	98.7	0.0 0.0	1.3	
10	85.3	0.7 6.7	7.3	
20	22.1	60.3 11.4	6.2	
30	10.0	65.3 23.0	1.7	
60	0.0	62.3 36.0	1.7	
<i>end3Δ</i> : % of cells labeled with FM4-64				
0	0.0	0.0 1.0	99.0	
10	17.7	0.7 3.7	77.9	
20	2.3	2.0 25.0	70.7	
30	0.7	6.0 53.3	40.0	
60	0.7	35.3 56.7	7.3	

Five hundred cells were counted for vegetative wild-type and *end3Δ* strains.

6 hr; marked by arrow), which were not seen in wild-type cells. Additionally, actin filaments were observed in some *end3Δ* cells (Figure 3B, 2 and 4 hr), although not to the extent of wild type. Thus, the actin cytoskeleton is aberrant in the *end3Δ* mutant.

**Meiosis and PSM assembly are normal in the *end3Δ* mutant:** To determine at what stage of sporulation End3p-mediated endocytosis functions, we examined several molecular and cytological markers of sporulation in the *end3Δ* mutant. The expression of sporulation-specific genes (*i.e.*, *HOP1*, HOLLINGSWORTH and BYERS 1989; *NDT80*, XU *et al.* 1995; and *DIT1*, BRIZA *et al.* 1994) was not affected in the *end3Δ* mutant (data not shown). Analysis of progression through the meiotic

divisions was analyzed by DAPI staining in a time course of sporulation (Figure 4). The meiotic divisions occurred with normal kinetics; however, the efficiency of the second meiotic division was slightly reduced in *end3Δ* mutants ( $71.8\% \pm 4.2$  *end3Δ* vs.  $91.8\% \pm 0.6$  *END3*; Figure 4). Thus, *end3Δ* mutants induce the sporulation program and progress through meiosis similarly to wild type.

We next examined whether PSM assembly, an early event in spore formation, is perturbed in the *end3Δ* mutant. To that end, we examined GFP-Spo14p, the major yeast PLD, which is essential for and marks PSMs (RUDGE *et al.* 1998, 2004) in live cells induced to sporulate. The *end3Δ* mutant showed clear GFP-Spo14p signals at PSMs analogous to the pattern observed in wild-type cells (Figure 5A). To determine whether inactivation of End3p had any effect on Spo14p function, we monitored Spo14p PLD activity by measuring conversion of internalized BODIPY-PC to BODIPY-PA. As previously reported (RUDGE *et al.* 2001), hydrolysis of BODIPY-PC occurs almost exclusively by the action of Spo14p, as *spo14Δ* strains did not generate any appreciable BODIPY-PA (Table 5). BODIPY-PC can be internalized by endocytosis and flip-flop action of lipids at the plasma membrane (RUDGE *et al.* 2001). BODIPY-PC was efficiently internalized in *end3Δ* mutants under the conditions used. Furthermore, the conversion rate of BODIPY-PA/BODIPY-PC revealed that *end3Δ* retained the equivalent efficiency of Spo14p PLD activity (Table 5). Therefore, we conclude that PSM assembly and PA production by Spo14p PLD activity are normal in the *end3Δ* mutant.

After PSM closure, spore wall components are deposited in the lumen between the double membranes (LYNN and MAGEE 1970). Septins are organized at the leading edge of the PSM and are believed to facilitate spore wall deposition (FARES *et al.* 1996). Septin localization is

**TABLE 4**  
**Quantification of FM4-64 uptake: sporulating cells**

Time (min) after removal of FM4-64	Plasma membrane	Punctate	Vacuoles	Prospore membranes	Faint cytoplasm or no signal
<i>END3</i> : % of cells labeled with FM4-64					
0	30.6	46.6	21.4	0.6	0.8
10	17.4	51.0	26.2	3.6	1.8
20	6.6	63.2	24.2	4.8	1.2
30	7.2	31.9	45.3	12.2	3.4
60	10.0	2.2	66.6 <sup>a</sup>	18.6	2.6
<i>end3Δ</i> : % of cells labeled with FM4-64					
0	90.8	1.2	0.0	3.4	4.6
10	66.8	16.8	0.2	2.8	13.4
20	58.8	25.1	9.8	2.2	4.1
30	29.9	31.3	21.0	11.4	6.4
60	16.4	5.8	56.0 <sup>a</sup>	14.8	7.0

Five hundred cells were counted for sporulating wild-type and *end3Δ* strains.

<sup>a</sup> After 60 min 85% of wild-type and 51% of *end3Δ* cells displayed strong vacuolar staining.

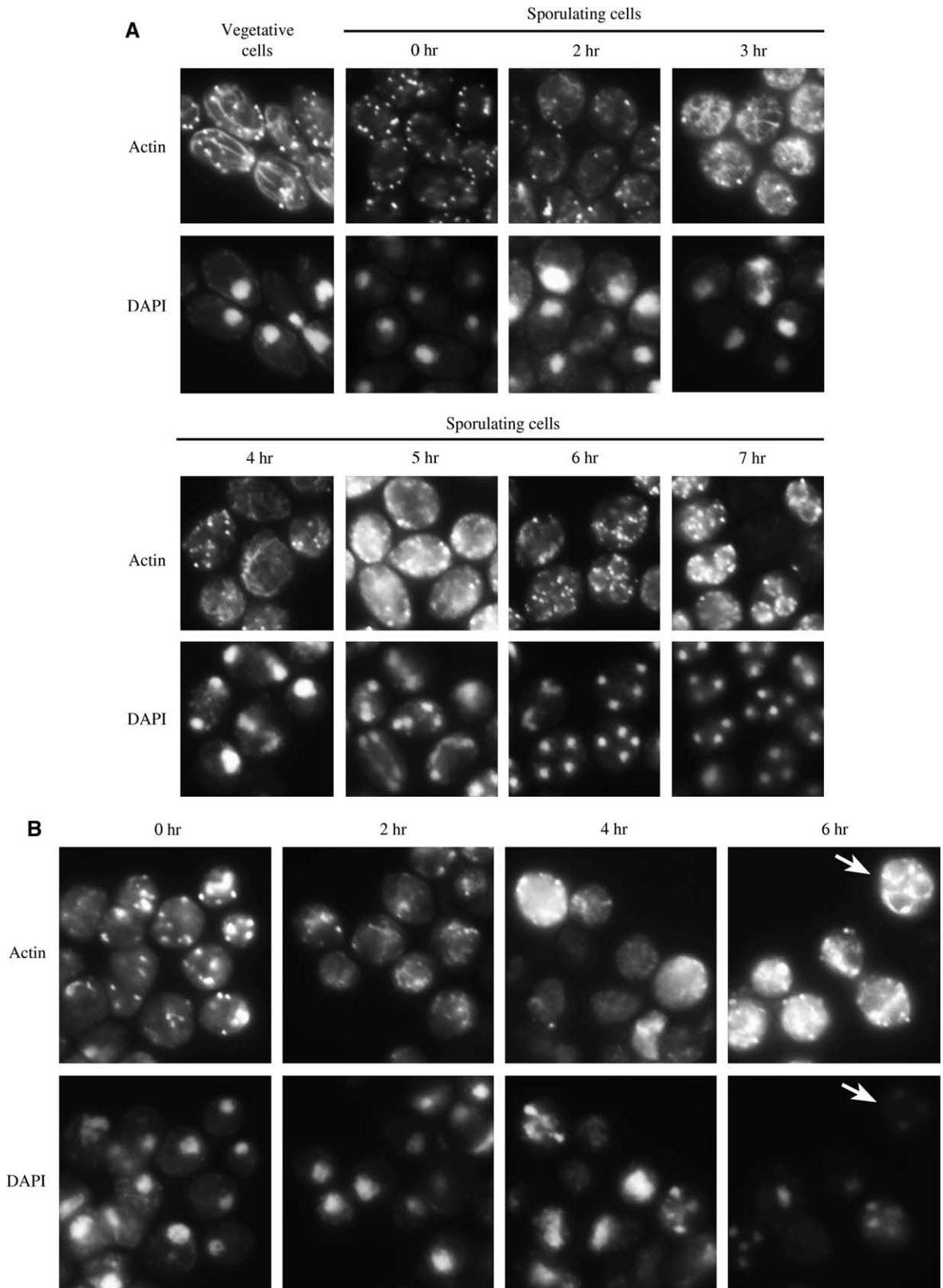


FIGURE 3.—Actin organization in wild type and *end3Δ* during sporulation. (A) Wild-type (AN120) and (B) *end3Δ* (Y4733) cells cultured in sporulation medium were harvested at indicated times, fixed, and then stained with rhodamine-phalloidin and DAPI as described in MATERIALS AND METHODS. (A) In wild type, vegetative cells showed actin patches and cables throughout the cells (vegetative cells), while arresting cells contained primarily actin patches (0 hr). During meiosis, network-like actin filaments appeared (2–4 hr) and as sporulation proceeded actin patches were observed around the maturing spore peripheries (6 and 7 hr). (B) *end3Δ* cells showed accumulation of large actin clumps in the cytoplasm and around the spore peripheries. Arrows show cell that completed meiosis and displayed actin clumps around spores.

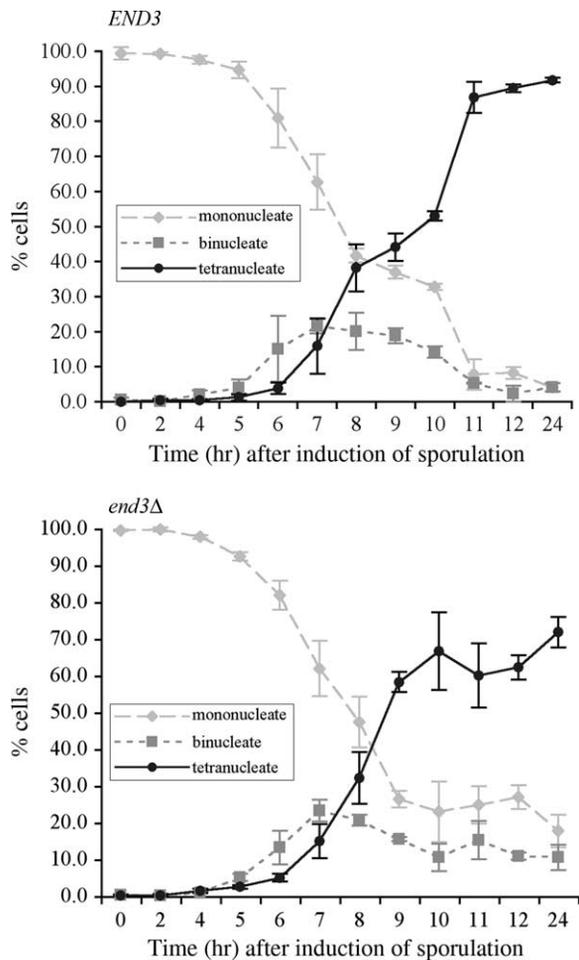


FIGURE 4.—Meiotic kinetics in *end3Δ* is similar to that in wild type. At indicated times after induction of sporulation, cells were stained with DAPI. Mono-, bi-, and tetranucleate cells were counted ( $n = 300$ ). *end3Δ* underwent meiosis with a kinetics similar to that of wild type, although the efficiency of completion of the second meiotic division was slightly lower in *end3Δ*.

altered in mutants where PSM closure is not sensed or has failed (TACHIKAWA *et al.* 2001). To determine if PSM closure occurs in *end3Δ*, Spr28p-GFP, a sporulation-specific septin (DE VIRGILIO *et al.* 1996), was examined in cells induced in sporulation. Septins form parallel bar structures and then eventually surround the spores. Analogous localization patterns were observed in both wild-type and *end3Δ* cells (Figure 5B), suggesting that septins organize properly in the absence of End3p. Thus, PSMs are assembled and undergo closure in *end3Δ* mutants.

***end3Δ* is defective in spore wall synthesis:** Next we examined spore walls in the *end3Δ* mutant. The spore wall contains four distinct layers: mannan, glucan, chitosan, and dityrosine from the inner to the outer layer, which are formed outwardly step-by-step. Proteins required for the formation of the spore wall layers are deposited at PSMs; Gsc2p and Chs3p are responsible for synthesis of the glucan and chitosan layer, respec-

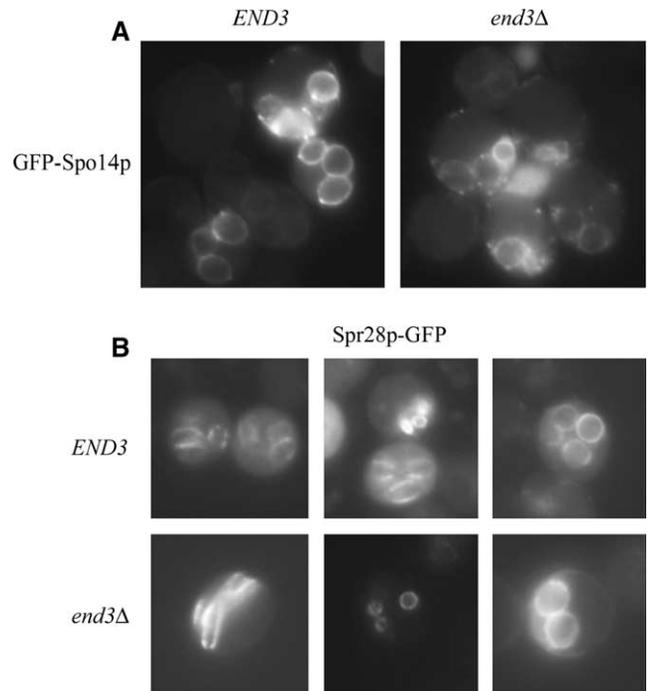


FIGURE 5.—PSM formation and closure are normal in *end3Δ*. Localization of (A) GFP-Spo14 and (B) Spr28p-GFP in sporulating wild type (AN120) and *end3Δ* (Y4733). Both wild-type and *end3Δ* cells showed a PSM staining pattern of GFP fluorescence for GFP-Spo14p and an analogous pattern of Spr28-GFP signals, bar structures, and PSMs.

tively, and both localize to PSMs (IWAMOTO *et al.* 2005). We constructed *GSC2-GFP* or *CHS3-GFP* in wild type and the *end3Δ* mutant and observed their localization during sporulation (Figure 6). Weak but detectable Gsc2p-GFP staining was observed at spores and in the cytoplasm in *end3Δ* cells (Figure 6A). However, unlike wild type, Chs3p-GFP was detected primarily in the cytoplasm, although a limited number of cells showed faint fluorescence at spores (Figure 6B). These results indicate that both Gsc2p and Chs3p are not efficiently localized in *end3Δ* mutants.

We also stained sporulating cells with CW, which binds to chitin at bud scars and chitosan in the spore wall. CW, however, cannot access the chitosan layer once the outermost dityrosine layer is formed. In wild type, the majority of the cells contained mature spores and thus were not stained by CW, although some immature

TABLE 5

PLD-catalyzed hydrolysis of internalized BODIPY-PC

	<i>END3</i>	<i>spo14Δ</i>	<i>end3Δ</i>
% BODIPY-PA <sup>a</sup>	0.82 ± 0.11	0.02 ± 0.02	0.85 ± 0.20

<sup>a</sup> The percentage conversion of intracellular BODIPY-PC to BODIPY-PA was determined as described in MATERIALS AND METHODS. The mean values ±SD from three independent experiments are shown.

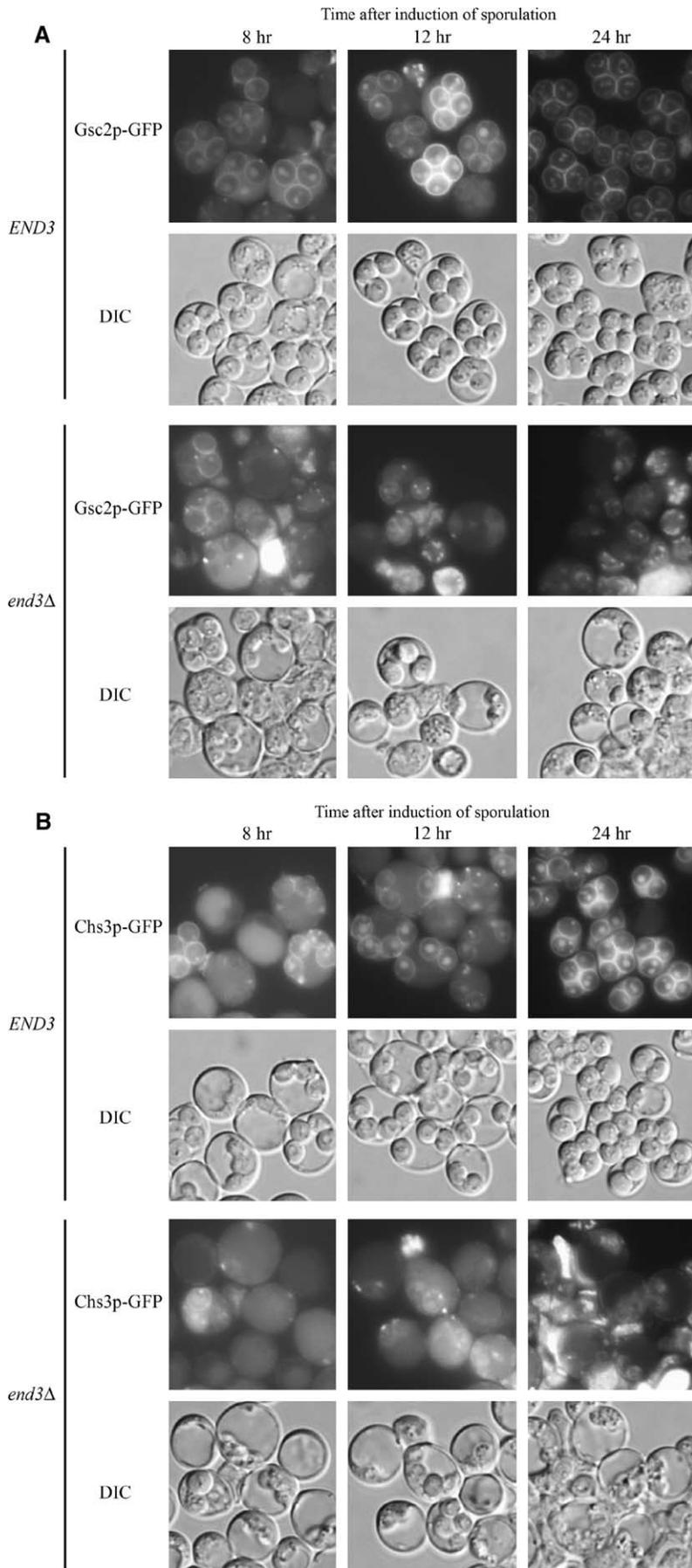


FIGURE 6.—Spore wall maturation in *end3Δ*. Localization of (A) Gsc2p-GFP and (B) Chs3p-GFP in *end3Δ* cells. Gsc2p-GFP and Chs3p-GFP localize to PSMs in wild-type cells (top). *end3Δ* cells displayed Gsc2p-GFP and Chs3p-GFP fluorescence in the cytoplasm and only very weak fluorescence at PSMs. Strains used here were *GSC2::GFP END3*, Y4931; *GSC2::GFP end3Δ*, Y4962; *CHS3::GFP END3*, Y4535; and *CHS3::GFP end3Δ*, Y4823. (C) CW staining in *end3Δ* cells. Wild-type AN120) and *end3Δ* (Y4733) cells were incubated at 30° for 24 hr and stained with CW, which binds to chitin at bud scars and the chitosan layer of spores. In wild type (left), the majority of the spores were mature and not stained, although some immature spores showed bright CW fluorescence. While in *end3Δ* (right), many spores were stained with a lower intensity of CW fluorescence compared with that in wild type, suggesting that *end3Δ* has defects in the formation of the outer layers of spores, chitosan, and ditryosine.

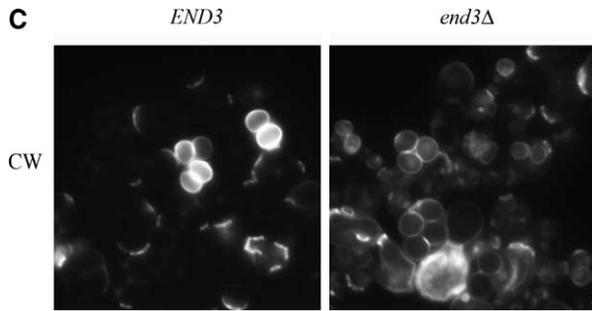


FIGURE 6.—Continued.

spores were present as observed by intense CW staining (Figure 6C). Consistent with the aberrant Chs3p-GFP localization, the intensity of CW staining in *end3Δ* spores was much lower than that in the immature wild-type spores (Figure 6C). Furthermore, a much larger proportion of sporulating *end3Δ* cells stained with CW (Figure 6C), suggesting that dityrosine is not incorporated into the *end3Δ* spores. This is consistent with the weak dityrosine fluorescence observed under UV light. Taken together, these results indicate that the *end3Δ* mutation is impaired in spore wall synthesis.

**End3p-GFP localizes to actin patches:** In the endocytic complex, End3p interacts with Pan1p, which has been reported to associate with actin patches (TANG *et al.* 2000). To examine whether End3p also associates with actin patches and how End3p behaves during sporulation, the *END3* gene was fused with *GFP* and End3p-GFP fluorescence was monitored in sporulating cells. The End3p-GFP fusion was functional as assessed by sporulation efficiency (data not shown). End3p-GFP localized to the peripheries of vegetative cells (data not shown) and in sporulating cells (Figure 7). End3p-GFP patches showed complete colocalization with actin stained with rhodamine-conjugated phalloidin at the surface and/or inside of the spores in addition to the periphery of the mother cell (Figure 7). Thus, End3p associates with the actin cytoskeleton at cell peripheries in both vegetative and sporulating cells.

**FM4-64 is transported to PSMs during sporulation:** The *end3Δ* mutant is defective in sporulation, resulting from defects in the formation of the spore wall (Figure 6). However, FM4-64 and BODIPY-PC are internalized in *end3Δ* mutants, although not as efficiently as in wild type. Thus, it appears that these molecules can be internalized independently of End3p. To examine if this End3p-independent pathway of internalization is important at earlier stages of sporulation, we conducted FM4-64 staining in sporulating cells expressing the PSM marker GFP-Spo14p and examined the cells after 1 hr of incubation, at a time when there is significant FM4-64 staining in both wild-type and *end3Δ* cells (Figure 2B; Table 4). Figure 8 shows different stages of PSM formation, such as initiation, development, engulfment, and shaping of PSMs in cells labeled with FM4-64 and

GFP-Spo14p. The FM4-64 signal was detected at vacuoles and at PSMs at all stages of PSM formation (Figure 8, *END3*). FM4-64 also labeled PSMs in the *end3Δ* mutant (Figure 8, *end3Δ*). Therefore, some FM4-64 molecules at the plasma membrane are internalized and delivered to PSMs during sporulation even in *end3Δ* mutant cells. Thus, it is likely that multiple pathways allow for communication between the plasma membrane and PSMs and that trafficking between these compartments is important throughout sporulation.

## DISCUSSION

Spore morphogenesis requires the *de novo* synthesis of a complicated, stratified structure within the mother cell. Previous work has shown that vesicles derived from the Golgi carry lipids and proteins important for spore formation. However, the contributions of other membrane compartments, such as the plasma membrane, vacuoles, and endoplasmic reticulum, have not been investigated. The plasma membrane syntaxin Sso1p is required for sporulation (JÄNTTI *et al.* 2002; ÖYEN *et al.* 2004), in particular, in the process of vesicle fusion at SPBs (H. NAKANISHI, personal communication). Thus, the endocytic pathway may play a role in recycling plasma membrane proteins and lipids to PSMs for spore morphogenesis. Furthermore, the fact that the spore wall contains mannoproteins and  $\beta$ -1,3-glucan, which are also components of the vegetative cell wall, suggests that there is communication between these different organelles.

To determine whether trafficking between the plasma membrane and the PSM is important for sporulation, we constructed and characterized mutants harboring a deletion of the endocytic complex gene, *END3*, during sporulation. This was possible because, unlike the previously reported deletion mutant (BÉNÉDETTI *et al.* 1994), the *end3Δ* mutant in SK-1 exhibited no growth defect; presumably this discrepancy is due to differences in strain background. The *end3Δ* mutant showed significant defects in internalization of the dye FM4-64, during both vegetative growth and sporulation; however, measurable uptake did occur. This is consistent with previous studies in which FM4-64 is transported to the vacuole even in mutants with strong endocytic defects (VIDA and EMR 1995). Thus, it is likely that there are alternative pathways independent of End3p-mediated endocytosis for uptake of FM4-64 and other molecules. The *end3Δ* mutant did produce aberrant spores consistent with either a partial block in endocytosis or the existence of additional pathways. The aberrant *end3Δ* spores appear immature, containing little or no dityrosine, indicating that the End3p-mediated endocytosis pathway plays an important role in the process of spore formation.

The *end3Δ* mutants underwent meiosis with wild-type kinetics, suggesting that endocytosis is not required for

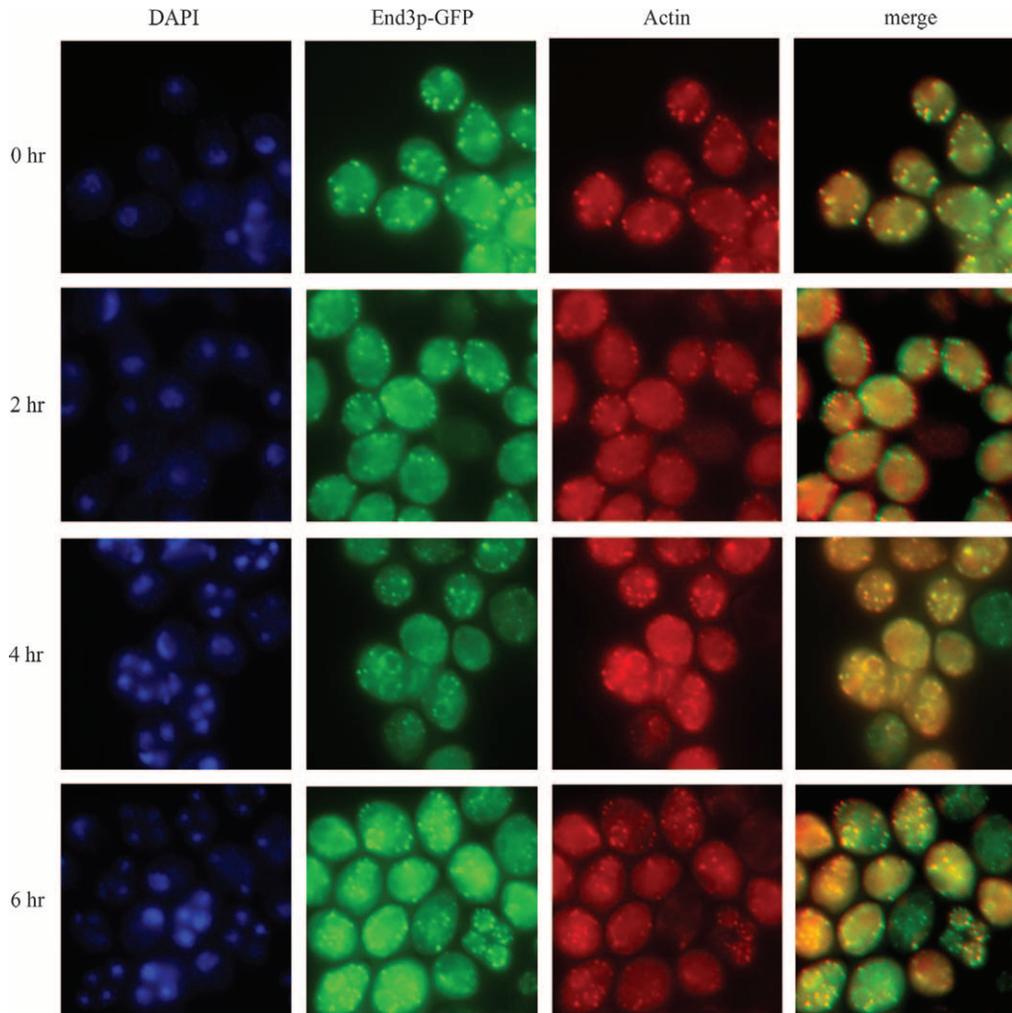


FIGURE 7.—End3p-GFP associates with the actin cytoskeleton during sporulation. *END3-GFP* cells (Y5095) incubated in sporulation medium were harvested at indicated times, fixed, and then stained with rhodamine-phalloidin and DAPI. Photos show DAPI, End3p-GFP rhodamine-phalloidin, and merged images of End3p-GFP and rhodamine-phalloidin (merge). End3p-GFP was detected as bright patches at the cell periphery (0 and 2 hr) and around spore walls (4 and 6 hr). End3p-GFP colocalized to actin patches at all the stages of sporulation (merge).

the early stages of sporulation. However, there was a small reduction in the number of cells that completed the second meiotic division. This may be due to the inability to observe discrete DAPI-staining bodies in a mutant that is aberrant in producing mature spores. Alternatively, endocytosis may play a subtle role during meiosis.

Spo14p PLD activity is essential for PSM formation (RUDGE *et al.* 1998). Fluorescence microscopy analysis revealed that GFP-Spo14p localized properly to PSMs in *end3Δ* cells and an *in vivo* biochemical assay showed that *end3Δ* retained wild-type Spo14p PLD enzymatic activity. These results indicate that deletion of *END3* does not affect the localization and function of Spo14p at PSMs. Consistent with this result, *end3Δ* shows identical kinetics of PSM formation, including vesicle fusion and growth of PSMs, to those of wild type. Furthermore, spetins are properly organized in the *end3Δ* mutant, indicating that closure occurs. Thus, End3p-mediated endocytosis is not essential for PSM assembly. However, the finding that FM4-64 is internalized and present at PSMs even in the absence of End3p, suggests that trafficking between the plasma membrane and PSMs is important for

PSM formation. Whether FM4-64 is transported directly to PSMs or via a plasma membrane-vacuole-PSM route is not clear from these experiments and remains an interesting question for future study.

Once PSM closure occurs, proteins required for spore wall synthesis, including Gsc2p and Chs3p, are deposited at spores. Neither Gsc2p-GFP nor Chs3p localized normally to spores in *end3Δ* mutants. In vegetative *end3Δ* cells, chitin on the cell surface is delocalized, suggesting that Chs3p fails to undergo endocytosis and thus is not properly targeted to the bud neck (BÉNÉDETTI *et al.* 1994). Similarly, during sporulation Chs3p and Gsc2p are endocytosed from the plasma membrane of the mother cell and redirected to PSMs (IWAMOTO *et al.* 2005); in the absence of *END3*, plasma membrane Chs3p and Gcs2p are inefficiently internalized and redirected to PSMs. Since Dtr1p-GFP is delivered to the PSMs from the Golgi (M. MORISHITA, unpublished data) and late-acting *SEC* genes are essential for PSM formation (NEIMAN 1998), Golgi-to-PSM trafficking clearly plays a critical role in the early stages of spore formation.

End3p is a component of the endocytic complex essential for the internalization step of endocytosis, which

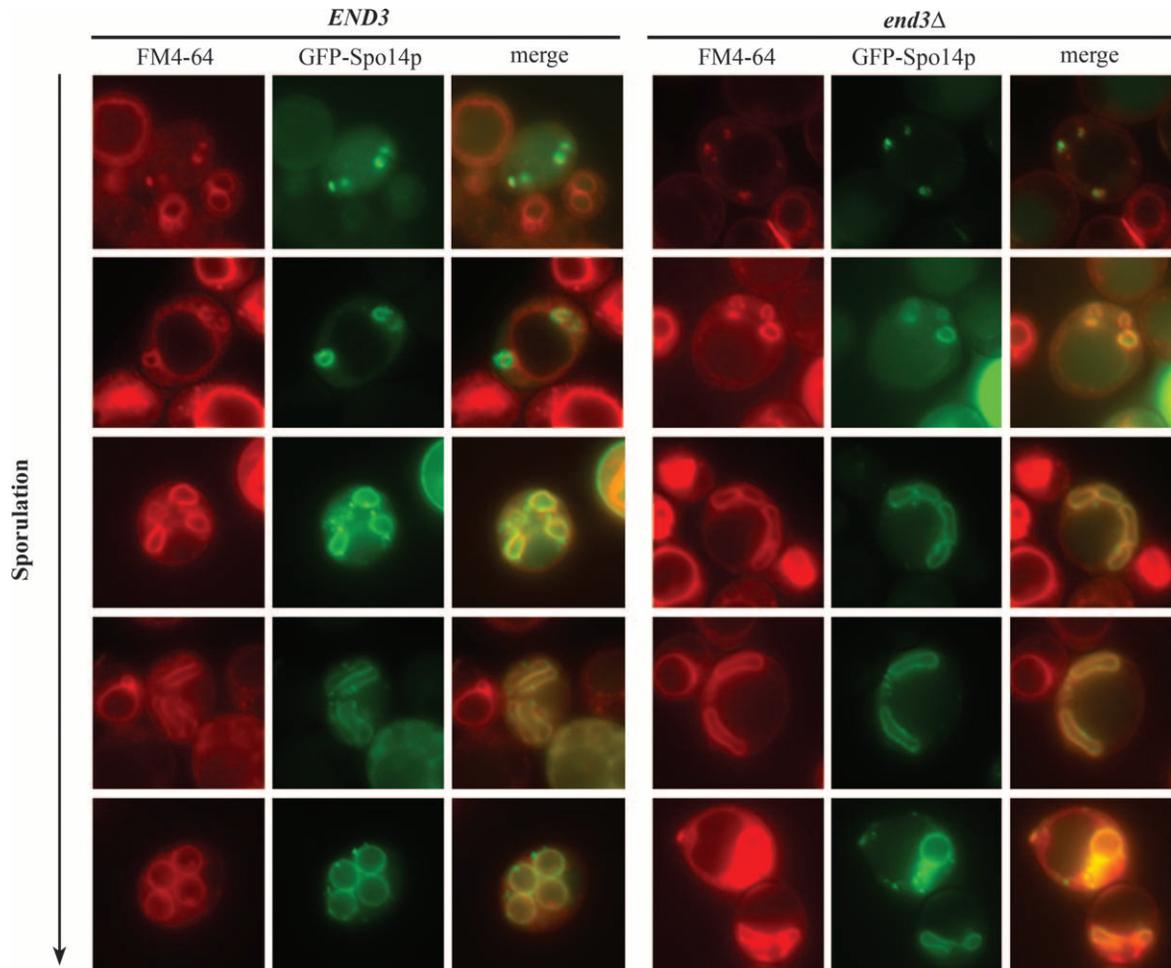


FIGURE 8.—FM4-64 is internalized and transported to PSMs in wild type and *end3Δ* during sporulation. Wild-type (AN120) and *end3Δ* (Y4733) cells expressing GFP-Spo14p incubated for 5 hr after the induction of sporulation were stained with FM4-64. Photos of cells at different stages of sporulation were taken. FM4-64 was internalized and transported to vacuoles, and PSMs, marked by GFP-Spo14p, in *end3Δ* at all stages of sporulation were examined, indicating that there may be alternative pathway(s) independent of End3p-mediated endocytosis to transport FM4-64 during sporulation.

includes, Pan1p, Ent1/2p, and Sla1p (TANG *et al.* 2000). During endocytosis, internalization, vesicle formation, vesicle scission, and vesicle transport require a functional actin cytoskeleton. Many mutants defective in endocytosis have mutations in actin or actin-related genes (*i.e.*, *ACT1*, MUNN *et al.* 1995; *SLA2*, WESP *et al.* 1997; *SAC6*, KÜBLER and RIEZMAN 1993; *ARP2/3*, MOREAU *et al.* 1997; *PRK1/ARK1*, SMYTHE and AYSOUGH 2003; and *MYO5*, JONSDOTTIR and LI 2004). Interestingly, several alleles of the essential *ACT1* gene have been reported to confer a sporulation-deficient phenotype (WHITACRE *et al.* 2001); the severity of the sporulation defect correlates well with the severity of the endocytic defects observed, suggesting that endocytosis is important for sporulation. In addition, actin patches have previously been observed in spores (DOYLE and BOTSTEIN 1996). Taken together with this study, it appears that the actin cytoskeleton plays an important role during yeast sporulation in large part due to the role it plays in endocytosis. Detailed analysis of actin reveals

that the actin cytoskeleton undergoes changes throughout sporulation, forming patches and network-like filaments. It is noteworthy that this is the first observation of filamentous actin in sporulating cells. It is possible that this filamentous actin is important for the cell cycle changes that occur during meiosis. At late stages of sporulation, actin patches are observed around the peripheries of each of the four spores, raising the possibility that endocytosis plays an important role even after the spore is constructed (KONO *et al.* 2005). Consistent with what has been observed in vegetative cells, *end3Δ* mutant cells exhibit defects in actin organization during sporulation (Figure 3B). Furthermore, End3p localizes to actin patches (Figure 7), indicating that, similar to vegetative cells, endocytosis and actin organization are tightly coordinated for the process of sporulation.

In conclusion, our results provide direct evidence that endocytosis is important for sporulation and contributes to vesicle trafficking of plasma membrane proteins required for spore formation. Future work will elucidate

the specific route that internalized proteins and lipids follow and determine the contribution of End3p-independent pathways for trafficking from the plasma membrane of the mother cell to the new plasma membrane of the developing spore.

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