The Septation Apparatus, an Autonomous System in Budding Yeast[□]

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Actomyosin ring contraction and chitin primary septum deposition are interdependent processes in cell division of budding yeast. By fusing Myo1p, as representative of the contractile ring, and Chs2p for the primary septum, to different fluorescent proteins we show herein that the two processes proceed essentially at the same location and simultaneously. Chs2p differs from Myo1p in that it reflects the changes in shape of the plasma membrane to which it is attached and in that it is packed after its action into visible endocytic vesicles for its disposal. To ascertain whether this highly coordinated system could function independently of other cell cycle events, we reexamined the septum-like structures made by the septin mutant *cdc3* at various sites on the cell cortex at the nonpermissive temperature. With the fluorescent fusion proteins mentioned above, we observed that in *cdc3* at 37°C both Myo1p and Chs2p colocalize at different spots of the cell cortex. A contraction of the Myo1p patch could also be detected, as well as that of a Chs2p patch, with subsequent appearance of vesicles. Furthermore, the septin Cdc12p, fused with yellow or cyan fluorescent protein, also colocalized with Myo1p and Chs2p at the aberrant locations. The formation of delocalized septa did not require nuclear division. We conclude that the septation apparatus, composed of septins, contractile ring, and the chitin synthase II system, can function at ectopic locations autonomously and independently of cell division, and that it can recruit the other elements necessary for the formation of secondary septa.

INTRODUCTION

In budding yeast, two processes combine to bring about separation between mother and daughter cell during cell division. One is the contraction of an actomyosin ring (Epp and Chant, 1997; Bi *et al.*, 1998; Lippincott and Li, 1998b), which pulls the plasma membrane centripetally and invaginates it (Figure 1, A and B). The other is the formation of the primary septum, extruded into the invagination through the agency of chitin synthase II (CSII), a plasma membrane-bound zymogenic enzyme (for reviews, see Cabib *et al.*, 1996, 2001). We have shown that the invagination of the membrane caused by contraction of the ring and the formation of the primary septum are interdependent (Schmidt *et al.*, 2002). A defect in either process or in both leads to aberrant cytokinesis with the same characteristics. In this abnormal septation, a fairly wide portion of cell wall at the neck between mother and

daughter cell grows inward, finally closing the channel and generating a thick, amorphous septum. We have interpreted these events as the growth of secondary septa at 90° of the normal direction, because of the absence of a primary septum (Schmidt *et al.*, 2002). Normally, the presence of a primary septum perpendicular to the mother-daughter cell axis determines the direction of deposition of secondary septa, giving rise to a trilaminar septum (Figure 1, C and D).

To study the interrelation between actomyosin ring contraction and primary septum formation, we observed the behavior, as a function of time, of proteins belonging to both systems and carrying fluorescent tags. The close coordination found in this manner between the two systems suggested that they may be parts of a master apparatus for septation. To observe this apparatus independently of the cell division process, we revisited the observation made previously in our laboratory, that temperature-sensitive septin mutants give rise to septum-like structures at locations remote from the mother-daughter cell neck (Slater *et al.*, 1985). These findings were recently confirmed by Cid *et al.* (1998). By using the fluorescence techniques mentioned above, we were able to show that Cdc12p, Myo1p, and

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Figure 1. Scheme of normal septation in *Saccharomyces cerevisiae*. The cell wall is shown in gray and the plasma membrane in brown. The red spots represent CSII. The contractile ring is in blue and the small arrows depict putative connections between the ring and the plasma membrane. The septin ring is not shown for simplicity; however, it would be situated between the contractile ring and the

Chs2p, representing the septin ring, the contractile ring, and the primary septum synthetic system, respectively, congregate in *cdc3* mutants in a manner similar to that of the septation process, but at abnormal sites. These results indicate that the septation machinery can operate autonomously and recruit whatever other elements are needed for its task.

MATERIALS AND METHODS

Culture Conditions and Transformation

Yeast culture conditions and standard genetic procedures were as described by Schmidt *et al.* (2002), except that appropriate synthetic dropout media were used as minimal media. Cells transformed with polymerase chain reaction (PCR) fragments containing a kanamycin-resistance marker were isolated as described by Schmidt *et al.* (2002). *Escherichia coli* DH10B (Invitrogen, Carlsbad, CA) was used for construction and propagation of plasmids.

Yeast Strains Construction

Yeast strains used in this study and their sources are listed in Table 1. Disruption of *CHS2* was performed as described by Crotti *et al.* (2001), to obtain ECY46-3-4A and ECY46-4-15C. For time-lapse microscopy of green fluorescent protein (GFP)-tagged proteins, the diploid strains DHYX100[pMS55], containing Myo1p C-terminally fused to GFP, and DHYX101[pDHR971207], containing Chs2p N-terminally fused to GFP, were used. Because of their larger size and more uniform shape, diploid cells yield a better picture of the localized fluorescent proteins. Because the original cell cycle mutant of *cdc3-1*, 104D7-A, does not have useful markers, it was mated to ECY46-3-4A[pDHR971207] or to YPH499[pMS55]. Segregant uracil prototrophs exhibiting temperature sensitivity were isolated after tetrad dissection (Table 1).

Strains expressing Chs2p C-terminally fused to yellow fluorescent protein (YFP) or Myo1p C-terminally fused to cyan fluorescent protein (CFP) were constructed by the targeted integration of DNA cassettes into strains of YPH499 background as described by Schmidt *et al.* (2002), with the use of plasmids pDH5 (YFP) and pDH3 (CFP) as DNA template. The plasmids were gifts from the Yeast Resource Center, University of Washington (Seattle, WA). The PCR primers and conditions for Chs2p fluorescent tagging were described by Schmidt *et al.* (2002). The primers for *MYO1* were as follows: MYO1GFP 5'-AAATATTGATGATAACAATGCACAGAGTAAATTTTCAGTGGTCGACGGATCCCCGGG-3'; and MYO1GFPB 5'-AAAGGATATAAAGTCTTCCAAATTTTAAAAAAGTTCCGATCGATGAATTCGAGCTGG-3'.

Proper integrations of CFP or YFP fusions were confirmed by PCR. There were no observable cell growth defects associated with the expression of any of the fluorescent proteins, which showed the same localization as GFP-tagged proteins.

Plasmid Construction

The N-terminally tagged GFP-Chs2p plasmid pDHR971024 was constructed by ligating a 714-base pair GFP DNA fragment amplified by PCR from pyEGFP (Cormack *et al.*, 1997) to

plasma membrane in A, whereas in B–D it would have split in two, with one ring above and one below the septation site (Lippincott *et al.*, 2001). (B) Actomyosin ring has started to contract and chitin (green), synthesized through the agency of CSII, is extruded into the invagination thus created. CSII is found all along the invagination of the membrane as cytokinesis progresses (see Figure 3B legend). (C) Formation of the primary septum is complete. (D) Secondary septa (yellow) have been added from both the mother and daughter cell side.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
YPH499	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i>	Sikorski and Hieter, 1989
YPH499-B	<i>MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i>	Crotti <i>et al.</i> , 2001
DHYX100 [pMS55] ^a	<i>MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2Δ1 chs2::TRP1</i>	Cross of YPH499 and YPH499-B
ECY46-3-4A	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs2::TRP1</i>	This study
ECY46-4-15C	<i>MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs2::TRP1</i>	This study
DHYX101 [pDHR971207]	<i>MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/ leu2-Δ1 chs2::TRP1/chs2::TRP1</i>	Cross of ECY46-3-4A and ECY46-4-15C
104D7-A	<i>MATα ura1 lys2 ade1 his7 leu2 tyr1 cdc3-1</i>	Slater <i>et al.</i> , 1985
DHYX102 [pDHR971207]	<i>MATa/MATα ura3-52/URA3 URA1/ura1 lys2-801/lys2 ADE1/ade1 ade2-101/ADE2 trp1-Δ63/TRP1 his3-Δ200/HIS3 HIS7/his7 leu2-Δ1/leu2 TYR1/tyr1 CDC3/cdc3-1 CHS2/chs2::TRP1</i>	Cross of ECY46-3-4A and 104D7-A
DHY102-7D ^b [pDHR971207]	<i>MATα ura3-52 lys2 trp1-Δ63 leu2 chs2::TRP1 cdc3-1</i>	This study
DHY102-9A ^b [pDHR971207]	<i>MATa ura3-52 lys2 trp1-Δ63 leu2 chs2::TRP1 tyr1 cdc3-1</i>	This study
DHYX103 [pMS55]	<i>MATa/MATα ura3-52/URA3 URA1/ura1 lys2-801/lys2 ADE1/ade1 ade2-101/ADE2 trp1-Δ63/TRP1 his3-Δ200/HIS3 HIS7/his7 leu2-Δ1/leu2 TYR1/tyr1 CDC3/cdc3-1</i>	Cross of YPH499 and 104D7-A
DHY103-9B ^b [pMS55]	<i>MATa ura3-52 lys2 his3-Δ200 leu2 tyr1 cdc3-1</i>	This study
DHY104 [pDHR971207]	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs2::TRP1 MYO1::CFP-kanMX6</i>	This study
DHYX105 [pDHR971207]	<i>MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2Δ1 chs2::TRP1/CHS2 MYO1::CFP-kanMX6/MYO1</i>	Cross of DHY104 and YPH499-B
DHY105-8B	<i>MATa ura3-52 lys2-801 ade2-101 leu2-Δ1 trp1-Δ63 his3-Δ200 MYO1::CFP-kanMX6</i>	This study
DHYX106 ^b [pDHR971207]	<i>MATa/MATα ura3-52/ura3-52 lys2-801/lys2 trp1-Δ63/trp1-Δ63 leu2-Δ1/leu2 his3-Δ200/HIS3 CDC3/cdc3-1 chs2::TRP1/chs2::TRP1 MYO1/MYO1::CFP-kanMX6</i>	Cross of DHY104 and DHY102-7D
DHY106-1D ^b [pDHR971207]	<i>MATa ura3-52 lys2 his3-Δ200 trp1-Δ63 leu2 cdc3-1 chs2::TRP1 MYO1::CFP-kanMX6</i>	This study
DHY107	<i>MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CHS2::YFP-HIS3</i>	This study
DHYX108 ^b [pDHR971207]	<i>MATa/MATα ura3-52/ura3-52 lys2/lys2-801 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2/leu2-Δ1 cdc3-1/CDC3 chs2::TRP1/CHS2::YFP-HIS3 MYO1::CFP-kanMX6/MYO1</i>	Cross of DHY106-1D and DHY107
DHY108-11C ^b	<i>MATa ura3-52 lys2 his3-Δ200 trp1-Δ63 leu2 CHS2::YFP-HIS3 MYO1::CFP-kanMX6</i>	This study
DHY108-7B ^b	<i>MATα ura3-52 lys2 his3-Δ200 trp1-Δ63 leu2 cdc3-1 CHS2::YFP-HIS3 MYO1::CFP-kanMX6</i>	This study
DHYX109 ^b [pDHR971207]	<i>MATa/MATα ura3-52/ura3-52 lys2/lys2 his3-Δ200/HIS3 leu2-Δ1/ leu2 trp1-Δ63/trp1-Δ63 CDC3/cdc3-1 CHS2/chs2::TRP1 MYO1::CFP-kanMX6/MYO1</i>	Cross of DHY105-8B and DHY102-7D
DHY109-36AF ^b [pDHR011023]	<i>MATα ura3-52 lys2 his3-Δ200 trp1-Δ63 leu2 cdc3-1 MYO1::CFP-kanMX6</i>	This study
DHY110	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 MYO1::YFP-HIS3</i>	This study
DHYX111 ^b [pDHR971207] [pDHR011021]	<i>MATa/MATα ura3-52/ura3-52 lys2-801/lys2 trp1-Δ63/trp1-Δ63 his3-Δ200/HIS3 leu2-Δ1/leu2 CDC3/cdc3-1 CHS2/chs2::TRP1 MYO1::YFP-HIS3/MYO1</i>	Cross of DHY110 and DHY102-7D
DHY111-4BF ^b [pDHR011021]	<i>MATa ura3-52 lys2 trp1-Δ63 leu2 cdc3-1 his3-Δ200 MYO1::YFP-HIS3</i>	This study
DHY108-1B ^b [pDHR011021]	<i>MATα ura3-52 lys2 trp1-Δ63 his3-Δ200 leu2 cdc3-1 CHS2-YFP-HIS3</i>	This study
DHY113 ^b [pLP17]	<i>MATa ura3-52 lys2 his3-Δ200 leu2 tyr1 cdc3-1</i>	This study
DHY114	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cdc3-1:HIS3</i>	This study
ECY102-1	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cdc3-1:HIS3 myo1::URA3</i>	This study
YMS298-1	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cdc3-1:HIS3 chs2::TRP1</i>	This study

^a Plasmids present in each strain are shown in brackets. ^b Adenine requirement was not checked in this strain.

Figure 2. Contraction of the actomyosin ring, detected with Myo1p-GFP (strain DHYX100[pMS55]). This is shown for comparison with the behavior of Chs2p-GFP. In this and later figures the time is given in minutes and seconds. Note, at time 28:29, the appearance of a new Myo1-GFP ring, due to emergence of a new bud. See also QuickTime movie 1.

YEp352XhoI*CHS2* (Crotti *et al.*, 2001). The oligonucleotide primers (*Xho*I site in bold) used to amplify yEGFP were as follows: GFPXhoIU 5'-CCGGTACTCGAGATGAGTAAAGGAGAAGAACTTTT-CA-3'; and GFPXhoID 5'-CTACGACTCGAGTTTGTATAGTTCATCCATGCCATG-3'.

The PCR conditions were 2 min at 94°C then 25 cycles of 45 s at 94°C, 45 s at 55°C, 2 min at 72°C, followed by 10 min at 72°C. *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used with the manufacturer-supplied buffer. The resulting fragments were cut with *Xho*I and ligated to the vector cut with the same enzyme to yield pDHR971024. To construct a single copy GFP-*CHS2* plasmid, pDHR971024 was digested with *Bam*HI and *Hind*III; the 4.8-kb liberated fragment was isolated and ligated to pRS316 (Sikorski and Hieter, 1989), previously cut with the same enzymes. The resulting plasmid, pDHR971207, complemented the *chs2* null phenotype and Chs2p-GFP localized as reported previously (Chuang and Schekman, 1996).

To obtain C-terminally tagged Cdc12 with either CFP or YFP, an amplified CFP or YFP from pDH3 or pDH5 was exchanged with the GFP-encoding region of pLP17 (Lippincott and Li, 1998a), by cutting both the plasmid and the PCR products with *Bam*HI and *Xba*I, followed by ligation. The oligonucleotides used to amplify CFP and YFP were as follows: FPOUTF 5'-CGGGATCCATGAGTAAAGGAGAAGAAGTTT-3' (*Bam*HI site in bold); and FPOUTB 5'-CGTCTAGATTACTATTGTATAGTTCATCC-3' (*Xba*I site in bold).

The PCR conditions were 3 min at 94°C then 30 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, followed by 10 min at 72°C. Takara Ex *Taq* DNA polymerase (Panvera, Madison, WI) was used with the buffer supplied by the manufacturer.

Cdc12p, tagged with either CFP(pDHR011021) or YFP(pDHR011023), was localized as described previously (Lippincott *et al.*, 2001) under permissive conditions. In general no difference was noted in the behavior of the fluorescent proteins, whether their genes were integrated in the genome or present on a single copy plasmid.

For the targeted integration of the *cdc3-1* mutation into the genome of strain YPH499, plasmid pDHR010607 was constructed as follows. The *cdc3-1* allele with ~200 base pair up- and downstream sequence was amplified from genomic DNA of strain 104D7 by PCR with primers 5'-CGGAATTCTCGTTATGGCCACATTATG-3' and 5'-CGGAATTCTTCTGTTATGGCCACATTATG-3' (*Eco*RI sites in bold). PCR conditions were 5 min at 94°C, followed by 25 cycles of

94°C for 1 min, 55°C for 1 min, and 72°C for 4 min. The resulting fragment was cut with *Eco*RI and cloned into the *Eco*RI site of pBluescript II KS+. The plasmid thus generated was cut with *Mlu*I; a *HIS3* gene was cut from pJJ217 with *Bam*HI. Both fragments were blunt-ended with T4 DNA polymerase and ligated. The resulting plasmid, pDHR010607, thus contains the *cdc3-1* allele with a *HIS3* gene integrated 73 base pairs downstream from the reading frame, followed by 151 base pairs of downstream chromosomal sequence. This construct was amplified by PCR with the primers described above. Yeast strain YPH499 was transformed with the PCR fragment and among the histidine-prototrophic transformants, *cdc3-1* mutants were identified by their phenotype. Correct integration of the fragment was confirmed by PCR. The resulting strain is DHY114.

Microscopy and Image Processing

For still pictures, GFP fluorescence was observed with an Axioskop microscope (Carl Zeiss, Thornwood, NY) equipped with a 41018 filter set (exciter, 470 nm; dichroic, 495 nm; and emitter 500 nm; Chroma Technology, Brattleboro, VT). For time-lapse pictures, cells were observed with an Axiovert 35 microscope (Carl Zeiss) and one of the following filter combinations: for observation of GFP, 71012 (exciter, 470 nm; dichroic, 500 nm; and emitter 530/550 nm); for CFP, 71009 (exciter, 414/430 nm; dichroic, 505 nm; and emitter 530/550 nm); and for YFP, 71009 (exciter, 487 nm; dichroic, 505 nm; and emitter, 530/550 nm) (all from Chroma Technology). For observation of 4,6-diamidino-2-phenylindole (DAPI) fluorescence, the D 360/40× excitation filter was used in combination with the GFP dichroic and emission filters (Chroma Technology). Images were recorded with a Photometrics Cool Snap HQ charge-coupled device camera (Roper Scientific, Trenton, NJ) operated by IPLab software (Scanalytics, Billerica, MA). An objective heater (Bioptechs, Butler, PA) was used to maintain the temperature of slides at 37°C.

Electron Microscopy

Electron microscopy was performed as described previously (Schmidt *et al.*, 2002).

Figure 3. Changes in Chs2p during septation. (A) Appearance and disappearance of a Chs2p-GFP ring in two pairs of cells undergoing septation in strain DHYX101[pDHR971207]. At time 37:01 for the first pair and 47:01 for the second, fluorescent patches, presumably representing endocytic vesicles, appear in both mother and daughter cells. See also QuickTime movie 2. (B) Detailed view of Chs2p-GFP changes during septation in strain DHYX101[pDHR97127]. The ring seen at time 0, assumes later an X shape (see time 5:17 or 6:10) as the fluorescent protein is found spread over both mother and daughter cell portions of the plasma membrane. As in A, fluorescent patches are seen leaving the neck region at the end of septation. See also QuickTime movie 3.

Figure 4. Double labeling with Myo1pCFP and Chs2pYFP, to determine the colocalization and relative timing of the contractile ring and of the CSII system (strain DHY108-11C). Chs2p seems to linger a little longer than Myo1p after cytokinesis.

RESULTS

Contraction of Actomyosin Ring and Deposition of Primary Septum Are Coordinated in Space and Time

As previously reported, Myo1p, a component of the contractile ring, is present in a ring structure at the mother cell-bud neck from the time of bud emergence (Epp and Chant, 1997; Bi *et al.*, 1998; Lippincott and Li, 1998b). At cytokinesis, the ring contracts rapidly and disappears (Figure 2). The behavior of Chs2p, the catalytic subunit of CSII, the chitin synthase responsible for primary septum formation, is quite different. The protein only appears a short time before septum formation and is immediately degraded after cell division (Choi *et al.*, 1994; Chuang and Schekman, 1996). The degradation seems to take place in the vacuole, because it requires a vacuolar protease, Pep4p (proteinase A; Chuang and Schekman, 1996), and transport to the vacuole seems to occur by endocytosis, because it depends on End4p (Chuang and Schekman, 1996). As a consequence of the transient appearance of Chs2p, in asynchronous cultures many more cells show a Myo1p-GFP fluorescent ring at the neck than a Chs2p-GFP ring (Schmidt *et al.*, 2002). However, time-lapse photography of growing cells showed that the time interval required for contraction of the Myo1p ring, ~7 min, was similar to that between appearance and disappearance of Chs2p (compare Figures 2 and 3A). Although the Myo1p-GFP fluorescence vanished completely after contraction, that of Chs2p-GFP was found in patches that traveled rapidly through both mother and daughter cell before disappearing (Figure 3A). In the light of the evidence mentioned above about degradation of Chs2p in the vacuole after septation, it seems evident that the patches represent endosomal vesicles transporting Chs2p to its final destination. Aside from the late endocytic phase, the behavior of Chs2p differs from that of Myo1p in another aspect. During contraction, the Myo1p-GFP ring does not change in shape, only in diameter (Figure 2). Chs2p-GFP, however, shows initially a ring-like localization, but later the fluorescence is distributed over two lines

Figure 5. Formation of delocalized septa in a *cdc3* mutant (strain DHY108-7B), after incubation for 3 h at 37°C. (A) Cell is shown with three delocalized septa (arrows), whereas the cell in B has only one. (C and D) High-magnification images of delocalized septa, one with both secondary septa (C) and the other with only one (D). The arrowheads show the translucent chitin layer. Note also the cytoplasm trapped by the septa.

Figure 6. Double mutants *cdc3 myo1* or *cdc3 chs2* show elongated cells at 37°C but no delocalized septa. (A–D) A *cdc3 myo1* mutant (strain EY102-1). (E–H) A *cdc3 chs2* mutant (strain YMS298-1). (A and E) Cells grown at 26°C. (B, C, D, F, G, and H) Cells shifted to 37°C for 4 h. (B and F) Arrows point to newly grown elongated buds.

of opposing curvature that resemble an X (Figure 3B). This difference in appearance correlates with the distinct localization of Myo1p and Chs2p in the cell. Myo1p is a part of the contractile ring, which is in the cytoplasm, inside the plasma membrane (Figure 1A). On the other hand, Chs2p is bound to the plasma membrane (Sburlati and Cabib, 1986), which becomes invaginated during cytokinesis (Figure 1, A and B). Our results clearly indicate that Chs2p does not only remain at the vertex of the invagination but also is spread through all the invaginated portion of the plasma membrane (Figure 1B). This may occur either by diffusion of the protein or by addition of more Chs2p during cytokinesis.

Whereas the above-mentioned results show that the movements of Myo1p and Chs2p are closely correlated, it

was desirable to observe both proteins in the same cell and at the same time. For this purpose, we constructed fusions of Myo1p with CFP and Chs2p with YFP and obtained a strain containing both fusions. The difference in excitation wavelength of CFP and YFP is sufficient for independent observation of both labels. CFP localization was somewhat difficult to observe because of a diffuse fluorescence in the cell and a more fuzzy appearance of the areas of localized fluorescence. Nevertheless, it could be easily seen that Myo1p and Chs2p are both localized at the neck and that their changes occur simultaneously, although Chs2p seems to linger a few more minutes after Myo1p is no longer visible (Figure 4). However, from Figures 2 and 3 it seems that the contraction of the actomyosin ring and the presence of

Chs2p at the neck have the same duration. Therefore, the delay may be an artifact, due to the weaker signal of CFP compared with YFP. The latter, in turn, has a weaker signal than GFP and this explains why the Chs2p-containing vesicles are not seen well in Figure 4.

Septation-like Process at Abnormal Locations in Septin Mutants

The determination of the behavior of Myo1p and Chs2p during septation confirmed the previous indications that contractile ring function and formation of the primary septum are tightly interlocked aspects of the same process. Would it be possible to observe this process independently from the other cell division events? To answer this question, we revisited a finding reported in 1985 by our laboratory, i.e., that temperature-sensitive septin mutants (designated at that time as cytokinesis mutants) exhibit septum-like structures at different positions in the cell cortex when exposed to a nonpermissive temperature (Slater *et al.*, 1985). The availability of methods that allow the localization of proteins in the cell suggested that the time may be ripe to gather more information about the aberrant septa. We concentrated our effort in the *cdc3* mutation, which was the most extensively investigated in the previous study. It was necessary, however, to cross the original strain, to endow it with convenient markers, thereby changing somewhat the genetic background. The strains finally used herein showed a similar phenotype to that of strain 104D7 (Slater *et al.*, 1985) at the nonpermissive temperature, with the formation of elongated and distorted buds and the appearance of aberrant septa (Figure 5). The latter are rather variable in shape, in consonance with their abnormal nature and the different locations at which they arise. With an appropriate plane of section, an electron-translucent line is seen (Figure 5, C and D, arrowheads), similar to the line of the chitin primary septum present in the middle of a trilaminar septum (Shaw *et al.*, 1991; Schmidt *et al.*, 2002). Trapped between the septa and the cell wall is a portion of cytoplasm, surrounded by a membrane (Figure 5, C and D). Previous observations with colloidal gold-wheat germ agglutinin labeling showed the chitinous nature of the electron-lucent line (Slater *et al.*, 1985).

If the aberrant septa arise by a similar process to that of normal ones, they should be defective or absent in strains that cannot construct normal septa, such as *myo1* or *chs2* mutants (Schmidt *et al.*, 2002). To examine this possibility, we carried out a deletion of either *MYO1* or *CHS2* in a *cdc3-1* strain. The double mutants grew rather slowly at 26°C (generation time, 4.5–5 h in synthetic complete medium), forming large clumps (Figure 6, A and E), as previously observed in the *myo1* or *chs2* single null mutants (Shaw *et al.*, 1991; Schmidt *et al.*, 2002). When shifted to 37°C, they gave rise to elongated buds, characteristic of the *cdc3* mutation (Figure 6, B and F). The optical density of the culture doubled at the higher temperature, before the cells eventually died. Examination of the double mutants by electron microscopy after 4 h at 37°C showed the thick septa typical of *myo1* and *chs2* mutants (Shaw *et al.*, 1991; Schmidt *et al.*, 2002), but no delocalized septa in *cdc3-1 chs2* and *cdc3-1 myo1* (Figure 6, C and D, and G and H, respectively) and an occasional localized thickening of the cell wall in *cdc3-1 myo1* that may be similar in nature to the aberrant septa found in the *myo1*

single mutant (our unpublished data). In either case, however, no well-formed delocalized septa were detected.

With the assurance that the mutant phenotype was as expected and the delocalized septa behaved like the normal ones, we proceeded to observe the localization of Myo1p and Chs2p, each fused with an appropriate fluorescent protein. At the permissive temperature, Myo1p-GFP was found only at the neck between mother and daughter cell (Figure 7 and Table 2) in the same percentage of cells previously reported for wild type (Schmidt *et al.*, 2002). After incubation for 3–6 h at 37°C, however, the protein localized to various abnormal positions in the cell cortex, the most common of which was the tip of an elongated bud (Figure 7 and Table 2).

Figure 7. Localization of Myo1p-GFP in the *cdc3* mutant (strain DHY103-9B[pMS55]) at permissive (26°C) and nonpermissive (37°C) temperatures. (A and B) At 26°C, the protein is only present at the neck between mother and daughter cell. (C–F) At 37°C, Myo1p-GFP is still concentrated in small areas, but these are now at different positions in the cell, with a preference for the tip of elongated buds. (C) No buds were visible in the two bottom cells at left in a phase-contrast photograph (our unpublished data), despite the presence of the fluorescent spots. The lines in A and B represent 5 μ m. The line in A refers only to that panel, that in B refers to panels B–F.

Table 2. Localization of GFP fusions with Myo1p, Chs2p, and Cdc12p after incubation of *cdc3-1* mutants at 37°C^a

Time, h	Myo1GFP ^b		GFPChs2 ^c		Cdc12GFP ^d	
	Localized	Mislocalized	Localized	Mislocalized	Localized	Mislocalized
		%		%		%
0	24	0	1.8	0	43	1.7
2	0	10	0	0.7	0.6	7.4
3	0	6			0	17
4 ^e			0	1.9		

^a For Myo1GFP and Cdc12GFP, 200 cells were counted. For GFPChs2p, that is found less frequently, 1500 cells were counted for $t = 0$ and 700 for the other two time points.

^b Strain DHY103-9B[pMS55].

^c Strain DHY102-9A[pDHR971207].

^d Strain DHY113[pLP17].

^e Counting was done at 4 h rather than 3 h because in this strains elongated buds appeared later than in the other two.

Chs2p, although much more rarely found, because of its short presence at the septation site (Schmidt *et al.* 2002), showed a similar change in localization when the cells were shifted from 26 to 37°C (Table 2). We also observed that a septin (Cdc12p) was mislocalized at 37°C (Table 2).

Time-lapse photography of cells embedded in solid growth medium and incubated for 2–4 h at 37°C showed in several cells disappearance of the Myo1p-GFP patches (Figure 8). The process differed to some extent from that observed in wild-type cells. The fluorescent patches showed some displacement while shrinking and the shrinkage was often not uniform. The movement may be due to the localization of the patch to a growing part of the cell, as opposed to the neck, which does not change appreciably during growth. As for the irregular shrinkage, it is perhaps not unexpected, because of the abnormal position of the actomyosin ring that would be here almost parallel, rather than perpendicular, to the plasma membrane and the cell wall.

We were also able to observe disappearance of Chs2p-GFP patches, with concomitant appearance of putative vesicles (Figure 9). This process seemed to be slower than in normal septation, which is not surprising in consideration of the aberrant topology.

We asked next whether Chs2p and Myo1p would colocalize at the abnormal positions. Again, Myo1p was fused to CFP and Chs2p to YFP. As expected, the presence of Chs2p-

YFP was much rarer than that of Myo1p-CFP. However, in most cases in which Chs2p-YFP was visible, Myo1p-CFP was found in the same location (Figure 10). Those instances in which Myo1p-CFP was not found with Chs2p-YFP may be due to the earlier disappearance of Myo1p during septation (Figure 4).

Finally, we wanted to find out whether septins other than Cdc3p would colocalize with Myo1p and Chs2p. It has been proposed that septins act as scaffolds for proteins involved in cytokinesis and septation (Hales *et al.*, 1999). If the aberrant septa are similar to the normal ones and the septins are necessary for their formation, they should be present at the same sites. As mentioned above, we had already observed that the septin Cdc12p was mislocalized at 37°C in the *cdc3* mutant. In further experiments, either Myo1p-YFP and Cdc12p-CFP or Myo1-CFP and Cdc12p-YFP were present in the same cell. With both combinations, colocalization of the two proteins was detected (Figure 11). We were also able to observe colocalization of Cdc12-CFP and Chs2p-YFP (our unpublished data). Thus, all three components of the septation apparatus, septins, contractile ring, and the chitin synthase II system, are found together at abnormal locations in the *cdc3* mutant. Meaningful statistics of double-labeled cells could not be obtained, mainly because one of the two proteins under observation was always labeled with CFP, which made counting difficult: CFP-fused proteins lost the

Figure 8. Shrinking and disappearance of a Myo1p-GFP patch in the *cdc3-1* mutant (strain DHY103-9B[pMS55]) at 37°C, presumably representing contraction of an improperly localized ring. Note that the patch seems to change somewhat its localization, possibly due to growth of the bud. See also QuickTime movie 4, in which shrinkage of an additional Myo1p-GFP patch can be observed.

Figure 9. Generation and later disappearance of patches from a Chs2p-GFP spot in the *cdc3-1* mutant at 37°C (strain DHY102-9A[pDHR971207]). Arrows show the position of the initial patch at different times, until its disappearance.

fluorescence at 37°C and only partially regained it at lower temperatures; they also bleached very rapidly and in their presence there was a high background of fluorescence in the cells (Figures 4, 10, and 11). Thus, double labeling could be seen in favorable cases, but accurate counting could not be done.

Mitosis Is Not Required for the Formation of Delocalized Septa

In Figure 5A, three delocalized septa are shown in a single cell pair, in agreement with previous observations (Slater *et al.*, 1985). If the formation of each septum required a mitotic event, as in the normal cell cycle, such a pair of cells should contain six nuclei. We determined by DAPI staining the number of nuclei of *cdc3-1* cells (strain DHY103-9B[pMS55]) exposed to 37°C and bearing an elongated bud. After 3- and 6-h incubation, 15 and 17%, respectively, of the cells (41 and 47 cells counted) had two nuclei and all of the others had only one. Furthermore, by two-channel, time-lapse photography of Myo1p-GFP and DAPI (vital stain) we observed in several cells shrinkage and disappearance of a Myo1p-GFP patch in a cell pair, without nuclear duplication (Figure 12). Taken together, these results show that formation of septa at ectopic locations takes place independently of mitosis.

DISCUSSION

The interdependence between actomyosin ring contraction and chitin primary septum deposition that we found previously (Schmidt *et al.*, 2002) indicated that these two processes are functionally related. The observations by fluorescence microscopy reported herein show the close correlation between the two events (Figures 2–4). They also point to the physical separation of the two systems: the contractile ring is in the cytoplasm, whereas Chs2p is attached to the plasma membrane. Accordingly, the actomyosin ring shows a simple contraction, whereas Chs2p follows the morphological changes of the membrane during cytokinesis (Figures 2 and 3). In agreement with this physical separation, we were unable to detect an interaction between Chs2p and Myo1p by the two-hybrid method (Schmidt *et al.*, 2002). One striking

difference between Myo1p and Chs2p, as observed in their fluorescent form, is seen at the time of their disappearance (Figures 2–4). Whereas Myo-GFP simply vanishes, the fluorescence of Chs2-GFP is transferred to patches, probably representing endocytic vesicles, which move rapidly through both mother and daughter cell before disappearing. This behavior is in excellent agreement with the previous finding that Chs2p is immediately degraded in the vacuole after its participation in septum formation in a process that requires endocytosis (Chuang and Schekman, 1996). We are not aware of a similar direct visualization of such an endocytic process related to a cell cycle event.

The strict coordination and interdependence between actomyosin ring contraction and primary septum formation suggested that they may act together as a largely autonomous system. This idea finds support in our previous observation that structures similar to septa, but totally misplaced relative to the normal septation site, are found next to the cell wall of septin mutants incubated at a nonpermissive temperature. Importantly, the structures showed a trilaminar appearance, with an electron-lucent layer in the middle, similar to the chitin primary septum layer in wild-type cells and staining as chitin with colloidal gold-wheat germ agglutinin (Slater *et al.*, 1985). Moreover, we have shown now that the delocalized septa are absent when mutations that lead to aberrant septation in normal cells are introduced in the *cdc3* strain. The availability of fluorescence methods to observe protein localization encouraged us to apply these techniques to the study of the abnormal septa. Our findings in *cdc3* cells incubated at 37°C may be summarized as follows: 1) Myo1p shows localization at abnormal positions, compatible with those of the septum-like structures; 2) the Myo1p patches contract, albeit in a somewhat irregular manner; 3) Chs2p also mislocalizes and Chs2p spots were seen to disappear while patches, presumably representing endocytic vesicles, moved away from the site; 4) Chs2p colocalizes with Myo1p; and 5) the septin Cdc12p also colocalizes with Myo1p and Chs2p. Taken together, these observations strongly suggest that the proteins detected by fluorescence were in the process of constructing the abnormal septa seen by electron microscopy. We speculate that the *cdc3* mutation results in an abnormal septin ring or septin complex. As a

Figure 10. Colocalization of Myo1p-CFP and Chs2p-YFP in the *cdc3-1* mutant (strain DHY108-7B) at 37°C. On the left side, arrows point to the Myo1p-CFP fluorescent areas, which are less clearly visible than those of Chs2p-YFP. The diffuse fluorescence seen in the left side panels was always observed when using proteins fused to CFP.

result, the septins assemble in an unusual location next to the plasma membrane. Thereafter, an essentially normal septation would occur at the chosen site (Figure 13). Thus, Myo1p and other components of the contractile ring and later Chs2p and accessory subunits (such as an activator of the zymogen form of the enzyme) would be recruited to the site (Figure 13). Our scheme conserves the idea that the septins serve as a scaffold for septation. This notion is supported by the finding that a defect in a septin leads to delocalization of the septum, coupled to the fact that at least one other septin (Cdc12p) is at the alternative site. However,

Figure 11. Colocalization of Myo1p and Cdc12p in the *cdc3-1* mutant at 37°C. Left, two examples in which Myo1p was fused to CFP and Cdc12p to YFP (strain DHY109-36AF[pDHR011023]). Right, two examples where Myo1p was fused to YFP and Cdc12p to CFP (strain DHY111-4BF[pDHR011021]).

a recent genome-wide screen for two-hybrid interactions involving proteins that participate in cell polarization failed to detect interactions between septins and known components of the contractile ring or Chs2p (Drees *et al.*, 2001). Thus, the interactions may be indirect, as is the case for Chs3p (DeMarini *et al.*, 1997).

It has not been possible to demonstrate directly that the sites at which we find the three proteins in the *cdc3* mutant are the same as where the septum-like structures are observed by electron microscopy. The problem cannot be approached by immunoelectronmicroscopy, because by the time the structures are completed the proteins involved in their formation are no longer there. In one aspect, the localization of Myo1p, Chs2p and Cdc12p differs somewhat from that of the septa seen by electron microscopy. The proteins are found more often at the tip of elongated buds. It should be kept in mind, however, that the cells are growing, therefore many septa that were made at the tip of buds may be found later at a different position in elongated cells.

The septation machinery seems to be able to recruit whatever other elements are necessary to build at least one secondary septum, because cell wall material is observed between the chitin layer and the plasma membrane on the luminal side of the cell (Figure 5). On the side of the trapped portion of cytoplasm, a very thin secondary septum can be seen in some (Figure 5C) but not all cells. This is not surprising because little material can be gathered from the small portion of cytoplasm closed off by the primary septum.

From all the above-mentioned information, it may be concluded that actomyosin ring contraction and primary septum formation must be strictly coordinated, as shown by the similar aberrant phenotype caused by a defect in either component (Schmidt *et al.*, 2002). We do not know how this coordination is achieved, but our results show that it is independent from mitosis. Multiple septa can occur in cells that at most underwent a single mitotic event and shrinking of Myo1p patches can be observed in cells with a single nucleus (Figure 12).

Thus, septins, contractile ring, and the chitin synthase II system constitute a relatively autonomous entity for the performance of septation. The septation apparatus, like the

Figure 12. Disappearance of a Myo1p-GFP patch in a *cdc3-1* cell without nuclear division (strain DHY103-9B[pMS55]). After incubation for 3 h at 37°C, the cells were stained with DAPI (1 μ g/ml final concentration; Schenkman *et al.*, 2002) in vivo and enrobed in agar. Myo1p-GFP and nuclei were visualized at different excitation wavelengths and recorded by time-lapse photography. Five representative frames are shown for Myo1p-GFP (A–E) and for DAPI (F–J).

mitotic apparatus, may be considered as a piece of machinery designed to execute a specific task. Both septation and mitotic apparatus are normally subject to regulation by cell cycle and other controls but also have their internal organization. This concept underlines the importance of studying the interactions that so precisely coordinate the functions of the septation apparatus components.

Figure 13. Proposed mechanism for the formation of the abnormal septa in the *cdc3* mutant at 37°C. The septin and the contractile ring are shown by marks at both ends to avoid crowding the figure. See text for explanations.

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