In budding yeast, contraction of the actomyosin ring and formation of the primary septum at cytokinesis depend on each other

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Summary

Saccharomyces cerevisiae chs2 **mutants are unable to synthesize primary septum chitin, and** ***myo1*** **mutants cannot construct a functional contractile ring. The morphology of the two mutants, as observed by electron microscopy, is very similar. In both cases, neither an invagination of the plasma membrane, which normally results from contraction of the actomyosin ring, nor generation of a chitin disc, the primary septum, is observed. Rather, both mutants are able to complete cytokinesis by an abnormal process in which lateral walls thicken gradually and finally meet over an extended region, giving rise to a thick septum lacking the normal trilaminar structure and often enclosing lacunae. Defects in** ***chs2*** **or** ***myo1*** **strains were not aggravated in a double mutant, an indication that the corresponding proteins participate in a common process. In contrast, in a** ***chs3*** **background the** ***chs2*** **mutation is lethal and the** ***myo1*** **defect is greatly worsened, suggesting that the synthesis of chitin catalyzed by chitin synthase III is necessary for the functionality of the remedial septa. Both** ***chs2*** **and** ***myo1*** **mutants show abnormalities in budding pattern and a decrease in the level of certain proteins associated with budding, such as Bud3p, Bud4p and Spa2p. The possible reasons for these phenotypes and for the interdependence between actomyosin ring contraction and primary septum formation are discussed.**

Key words: Yeast, *Saccharomyces cerevisiae*, Cytokinesis, Primary septum, Contractile ring, Budding pattern

Introduction

In budding yeast, cytokinesis starts with the invagination of the plasma membrane at the neck between the mother and daughter cell. Concomitantly, the polysaccharide chitin is extruded into the invagination as the latter advances toward the center of the mother-daughter channel (Fig. 1B) (Cabib et al., 1996). Eventually, this process results in pinching off of the membranes and separation of the two cells by a chitin disk, the primary septum (Fig. 1C). Subsequently, secondary septa are added from both mother and daughter cells, so that the complete septum has a trilaminar structure, with the chitin disk in the middle (Fig. 1D). The formation of primary septum chitin is catalyzed by one of the three yeast chitin synthases, ChsII. Its putative catalytic subunit, Chs2p, is encoded by the *CHS2* gene (Silverman et al., 1988). Null mutants of *CHS2* have abnormal and very thick septa but are able to survive (Bulawa and Osmond, 1990; Shaw et al., 1991).

Recently, it has been found that the invagination of the plasma membrane at cytokinesis is brought about by the contraction of an actomyosin ring (Epp and Chant, 1997; Lippincott and Li, 1998; Bi et al., 1998), as is the case for division of mammalian cells. Mutants in *MYO1*, which encodes the type II myosin required for function of the contractile ring, show abnormal septation (Rodríguez and Paterson, 1990) but are still able to complete cytokinesis (Bi et al., 1998).

Several aspects of the septation process remain obscure: for instance, can synthesis of the primary septum take place in the absence of contractile ring closure and vice versa? Is at least one of these two processes required for viability? What is the nature of the remedial septation that takes place in *chs2* and *myo1* mutants?

We have reinvestigated the events that occur during cytokinesis in different mutants and have come to the conclusion that contractile-ring closure and formation of the chitin primary septum are interdependent phenomena. The cell can survive even when both mechanisms are not functional. The remedial septa formed under those conditions are probably a modification of the secondary septa laid down at the end of normal cell division. Another chitin synthase, ChsIII, is required for the successful execution of the repair mechanism.

Materials and Methods

Strains and growth conditions

Yeast strains are listed in (Table 1). Culture media for *Escherichia coli* were described in Ausubel et al. (Ausubel et al., 1994). *E. coli* DH10B (Gibco-BRL) was used as plasmid host. Yeast cells were grown either in YEPD (2% glucose, 2% peptone, 1% yeast extract) or in SD (2% glucose, 0.7% Difco Yeast Nitrogen Base). Generation time was determined by monitoring the OD_{660}.

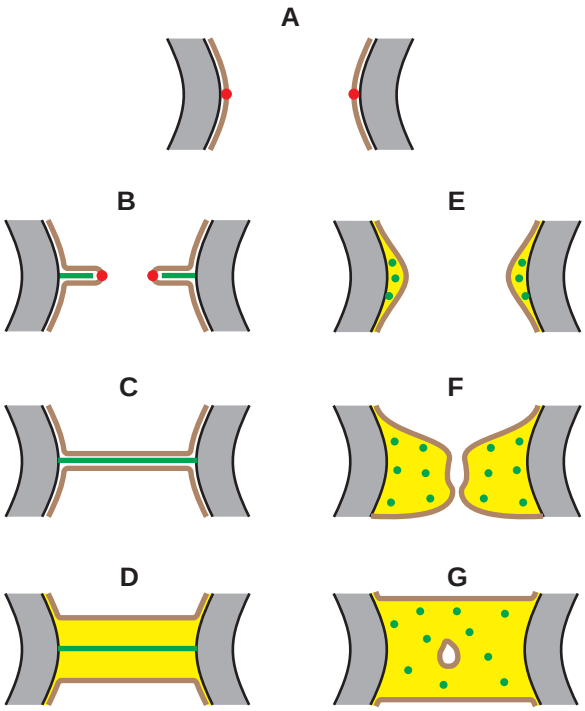

Fig. 1. Schematic view of septation in wild-type (A-D) and in *chs2* or *myo1* mutants (A,E-G). The neck region between the mother and daughter cell is represented, with the cell walls as the grey area and the plasma membrane as the brown line. The red spots indicate the location of Chs2p. Chitin is shown in green. In (B), the membrane invaginates and chitin is laid down in the invagination. Continuation of this process leads to generation of the primary septum disk and to pinching off of the membrane (C). Next, secondary septa (yellow) are built up from both the mother and daughter cell sides. A trilaminar septum results (D). In *chs2* and *myo1* mutants, invagination of the plasma membrane in a small area and growth of a primary septum do not take place. Instead, inward growth of cell wall material over a large portion of the plasma membrane pushes the membrane toward the center of the channel (E,F), finally closing the latter and generating a thick and uniform septum (G). In our hypothesis, this represents growth of secondary septa, which would occur at 90° to the normal direction. Thus, in the cell cycle, stage E would start between the normal times for (C) and (D). The green spots in (E-G) designate chitin formed by the action of chitin synthase III that is required for the remedial septa but not for the normal secondary septa. In (G), a lacuna resulting from the uneven fusion of the advancing secondary septum is also shown.

Yeast transformation

Yeast transformation was performed according to Gietz et al. (Gietz et al., 1992). For kanamycin selection, the standard transformation protocol (Ausubel et al., 1994) was followed. After heat shock, the cells were suspended in 5 ml of YEPD medium and incubated overnight at room temperature before being spread on YEPD plates containing 250 mg/L G418 (Gibco-BRL). After a three-day incubation at 30°C, the colonies were replicated onto fresh G418 plates and incubated for another two days at 30°C before resistant colonies were picked.

Plasmid construction

General methods of DNA manipulation were used as described in Ausubel et al. (Ausubel et al., 1994) except when following procedures recommended by the manufacturers of the enzymes and DNA isolation kits used (New England Biolabs; Qiagen). To obtain *URA3*-containing vectors with genes *SPA2*-3x*HA*, *MYO1-c-myc* and *MYO1-GFP*, a 2.9 kb *Pvu*I fragment from pRS316 was ligated to the large fragment of the *Pvu*I-digested plasmids pBU4 (M. Snyder), pLP7 and pLP8 (Lippincott and Li, 1998), respectively. This exchanged the *LEU2* markers of the original plasmids with the *URA3* gene and yielded plasmids pMS53, pMS54 and pMS55 (Table 2).

To construct pEC28 for the detection of synthetic lethality with *chs3* mutants, plasmid pHV7 (Valdivieso et al., 1991) containing the *CHS3* gene was cut with *Cla*I and *Pvu*II. The digest was treated with Klenow fragments to remove the overhang; the 5.2-kb fragment containing *CHS3* was isolated and ligated to the *ADE2*-harboring vector pRS412 (Brachmann et al., 1998) that had been cut with *Sma*I. For construction of pMS52, used in the detection of synthetic lethality with *chs2* mutants, pMS11, containing the *CHS2* gene in the *Bam*HI/*Hind*III sites of pRS316 ,was cut with *Kpn*I/*Bam*HI. The 3.9-kb fragment was purified and ligated to *Kpn*I/*Bam*HI-digested pRS412.

Gene disruption

Disruption of *CHS2* and *CHS3* was performed according to Crotti et al. (Crotti et al., 2001). For disruption of *MYO1*, a 6.2-kb fragment containing the *MYO1* ORF and 200 bp each of 5′ and 3′ flanking sequences was amplified by PCR from chromosomal DNA with oligonucleotides 5′-CCCTCCTTGGAATTTGG-3′ and 5′-GACTT-TATTTCCGCTGC-3′. The fragment was then treated with Klenow fragments and T4 polynucleotide kinase and ligated to *Eco*RV-linearized pBluescript II KS+ vector (Stratagene). From the resulting

Table 1. Strains used in this study

Strain	Genotype	Source
ECY46-4-1B	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs3::LEU2*	Crotti et al., 2001
YMS8	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs3::LEU2 chs2::TRP1* pEC28	This study
YMS11	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs2::TRP1*	Crotti et al., 2001
YMS57	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 myo1::TRP1*	This study
YMS75	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs3::LEU2 myo1::URA3*	This study
YMS135	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs2::TRP1 myo1::URA3*	This study
YMS159	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 BUD3::GFP-kanMX6*	This study
YMS160	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 BUD4::GFP-kanMX6*	This study
YMS161	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs2::TRP1 BUD3::GFP-kanMX6*	This study
YMS163	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 myo1::TRP1 BUD3::GFP-kanMX6*	This study
YMS164	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs2::TRP1 BUD4::GFP-kanMX6*	This study
YMS166	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 myo1::TRP1 BUD4::GFP-kanMX6*	This study
YMS167	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CHS2::GFP-kanMX6*	This study
YMS171	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 myo1::TRP1 CHS2::GFP-kanMX6*	This study
YPH499	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	Sikorski and Hieter, 1989

Table 2. Plasmids used in this study

Plasmid	Description	Source
pBU4	pRS315-*SPA2-HA*	M. Snyder
pEC28	pRS412-*CHS3*	This study
pHV7	YCp50-*CHS3*	Valdivieso et al., 1991
pLP7	pRS315-*MYO1-c-myc*	Lippincott and Li, 1998
pLP8	pRS315-*MYO1-GFP*	Lippincott and Li, 1998
pMS11	pRS316-*CHS2*	This study
pMS48	pBluescript II KS+*myo1::TRP1*	This study
pMS50	pBluescript II KS+*myo1::URA3*	This study
pMS52	pRS412-*CHS2*	This study
pMS53	pRS316-*SPA2-HA*	This study
pMS54	pRS316-*MYO1-c-myc*	This study
pMS55	pRS316-*MYO1-GFP*	This study
pRS316	*CEN URA3*	Sikorski and Hieter, 1989
pRS412	*CEN ADE2*	Brachmann et al., 1998

plasmid a 3.3-kb *Mfe*I fragment was cut out and replaced with a blunt-ended 1.0-kb *TRP1* or 1.2-kb *URA3* fragment, both generated by PCR with subsequent Klenow filling-in and phosphorylation. This yielded plasmids pMS48 and pMS50, respectively. Plasmids were cut with *Eco*RI/*Cla*I (pMS48) or *Eco*RI (pMS50) before transformation of yeast strains.

GFP-tagging of Bud3p, Bud4p and Chs2p

PCR-based C-terminal tagging of Bud3p, Bud4p and Chs2p was performed as described by Longtine et al. (Longtine et al., 1998). Primers consisted of a 40-bp sequence homologous to the target gene (followed, in the case of *CHS2*, by a linker encoding a stretch of 8 glycines) and a 20-bp sequence homologous to the pFA6a-GFP(S65T)-kanMX6 plasmid. The oligonucleotide sequences were as follows:

BUD3UP: 5′-GAACTGCGCTGTTGGTGGCCCAGAGAAACT-GAAATTTTATCGGATCCCCGGGTTAATTAA-3′

BUD3DOWN: 5′-TTGCATTAAATTAAAAAGAAAAAAAAA-ATCAATAAAACACGAATTCGAGCTCGTTTAAAC-3′

BUD4UP: 5′-GACTCGAACAACCGGTCATAATTTGAAGCAA-GATTTTAATCGGATCCCCGGGTTAATTAA-3′

BUD4DOWN: 5′-TCATCAGATTATATGCTGTTTTCATTCCA-TTAATCACCTTGAATTCGAGCTCGTTTAAAC-3′

CHS2UP: 5′-CTCTAAATTAGACTTACCAAATGTTTTCCACA-AAAAGGGCGGTGGAGGTGGAGGTGGAGGTGGACGGATCCC-CGGCTTAATTAA-3′

CHS2DOWN: 5′-AAAGAGGGAATGACGAGAAATTAGCTG-AAAAATACTGGCAGAATTCGAGCTCGTTTAAAC-3′

PCR conditions were: one minute at 94°C, then 30 cycles of one minute at 94°C, one minute at 55°C, two minutes at 72°C, followed by 15 minutes at 72°C with Takara ExTaq polymerase (Takara) and the supplied buffers. After transforming yeast with the resulting fragments, G418-resistant transformants were selected (see above).

Electron microscopy

Cells were fixed and embedded essentially as described by van Tuinen and Riezman (van Tuinen and Riezman, 1987). Aliquots of cells were washed in 0.1 M sodium phosphate buffer, pH 7.2 (PB) and fixed by suspension in 3% formaldehyde with 0.5% glutaraldehyde for two hours at room temperature. The cells were subsequently washed twice in PB, treated for 30 minutes with aqueous 1% metaperiodate, rinsed in PB and quenched for 30 minutes in 50 mM NH_4Cl in PB. After rinsing in PB, the cells were enrobed in low melting point agarose, cut into small blocks and dehydrated in cold ethanol. The cells were embedded in LR White resin (Electron Microscopy Science). Thin sections were stained in aqueous 1% uranyl acetate, followed by lead citrate and carbon-coated before examination in the electron microscope.

Fluorescence microscopy

GFP fluorescence was observed with a Zeiss Axioskop microscope equipped with a Chroma 41018 filter set (exciter 470 nm, dichroic 495 nm, emitter 500 nm). To enhance fluorescence intensity, cultures were saturated with oxygen for 20 minutes at room temperature before observation.

Synthetic lethality

For assessment of synthetic lethality, segregation of an *ADE2*-containing plasmid from strains carrying an *ade2-101* mutation was monitored by the appearance of red sectors. The screen for mutants synthetically lethal with *chs3::LEU2* was performed in strain ECY46-4-1B complemented by a plasmid harboring the *CHS3* gene in addition to the *ADE2* marker (pEC28). Correspondingly, for detection of synthetic lethality with *chs2::TRP1*, YMS11 was complemented by a plasmid containing *ADE2* and *CHS2* (pMS52). After disruption of the genes under investigation, synthetic lethality with either *chs2* or *chs3* mutations leads to an inability of the strain to segregate the *ADE2*-containing plasmid, hence yielding white colonies devoid of red sectors when grown on medium containing 8 mg/L of adenine.

Determination of budding pattern

For the determination of budding pattern, strains were grown overnight in YEPD medium at 30°C to 10^7 cells/ml, then spread on YEPD plates at room temperature. Cell pairs were then separated with the use of a micromanipulator, and the position of emerging new buds was recorded at 30-minute intervals.

Protein sample preparation

0.1-1.0 L cultures of yeast were grown to 10^7 cells/ml, centrifuged at 3,000 ***g*** and washed with ice-cold water. Cell pellets were frozen at –80°C. 50 μl of wet cells were mixed with 100 μl of water and 150 μl of 2× sample buffer (63 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS [sodium dodecyl sulfate], 0.0025% bromphenol blue) with 1mM phenylmethylsulfonyl fluoride and 1% 2-mercaptoethanol. After heating the cell suspension at 95°C for five minutes, 200 μl glass beads were added and the cells were broken by vortexing for three 30-second periods. The liquid was aspirated with a Pasteur pipette and centrifuged at 13,000 ***g*** for two minutes. Samples of the supernatant were loaded on the polyacrylamide gel. In the case of Pma1p, both the addition of 2-mercaptoethanol and the heating at 95°C were omitted.

Electrophoresis and western blot

40 μg of protein were loaded on Tris-glycine gels (thickness 1 mm, 4-12% or 4-20% polyacrylamide, Invitrogen). The electrophoresis was run for two hours in 25 mM Tris, 192 mM glycine and 0.1% SDS at 125 V. Proteins were transferred onto PVDF membranes (Invitrogen) at 30 V for two hours in 12 mM Tris, 96 mM glycine, 0.1% SDS and 20% methanol. After transfer, membranes were blocked for one hour in 5% nonfat dry milk in TTBS/0.9% NaCl (0.1 M Tris chloride, pH 7.5, 0.9% NaCl, 0.1% Tween 20) and incubated overnight at 4°C in 20 ml of the same buffer, containing 10 μl of one of the following antibodies: a) anti-c-myc 9E10 (Santa Cruz Biotechnology) for detection of Myo1-c-mycp; b) anti-HA (Roche) for detection of Spa2-HAp; c) anti-Hsp104p (Stressgen); d) anti-Rho1p (Drgonová et al., 1999); e) anti-Cdc42 y-191 (Santa Cruz Biotechnology); f) anti-Bud3p (S. Sanders); g) anti-Bud4p (S. Sanders); or h) 2 μl of anti-Pma1p (C. Slayman).

The blots were washed three times for 15 minutes with TTBS/0.5M NaCl (0.1 M Tris-chloride, pH 7.5, 0.5 M NaCl, 0.1% Tween 20) and incubated for two hours at room temperature with these secondary antibodies: 10 μl/20 ml of anti-mouse IgG-POD/Fab fragments-HRP

(Roche) for a) and b) or 2 μl/20 ml of anti-rabbit-HRP (Pierce) for c)-h) in TTBS/0.9% NaCl. The blots were then washed again three times for 15 minutes each time with TTBS/0.5 M NaCl, rinsed with water and developed with the ECL detection kit (Pierce) and Kodak BioMax ML film.

Results

A mutual requirement between contractile ring and primary septum

As mentioned above, cytokinesis in budding yeast starts with invagination of the plasma membrane and simultaneous deposition of chitin in the invagination (Fig. 2A). Continuation of this process results in the pinching off of the membrane, while a thin chitin disk, the primary septum, separates the mother and daughter cell (Fig. 2B). Subsequent deposition of secondary septa, both from the mother and daughter side, generates the complete, trilaminar septum (Fig. 2C) [for electron microscope images showing in detail the steps in septum formation see Cabib et al. (Cabib et al., 1974)]. In *chs2* mutants, which are deficient in chitin synthase II (the enzyme responsible for the formation of primary septum chitin), cytokinesis does occur but in an aberrant manner. No localized invagination of the membrane can be seen. Rather, a wide area of the cell wall at the neck between the mother and daughter cell thickens gradually, squeezing the membrane (Fig. 2D) and finally causing its pinching off. In this case, the closure of the channel is an irregular process, as shown by the lacunae that often remain in the thick septa formed in the *chs2* strains (Fig. 2E) (Shaw et al., 1991). Note that although the cell wall pushes the plasma membrane in, the wall growth occurs by secretion and extrusion of material from the interior of the cell through the membrane itself.

Cells defective in Myo1p, an essential component of the contractile ring (Lippincott and Li, 1998), are similarly able to complete cytokinesis (Bi et al., 1998) despite abnormalities in septation (Rodríguez and Paterson, 1990). In fact, we have examined *myo1*Δ and *chs2*Δ mutants by light and electron microscopy and found their cytokinesis defect to be indistinguishable. Both mutants are characterized by the

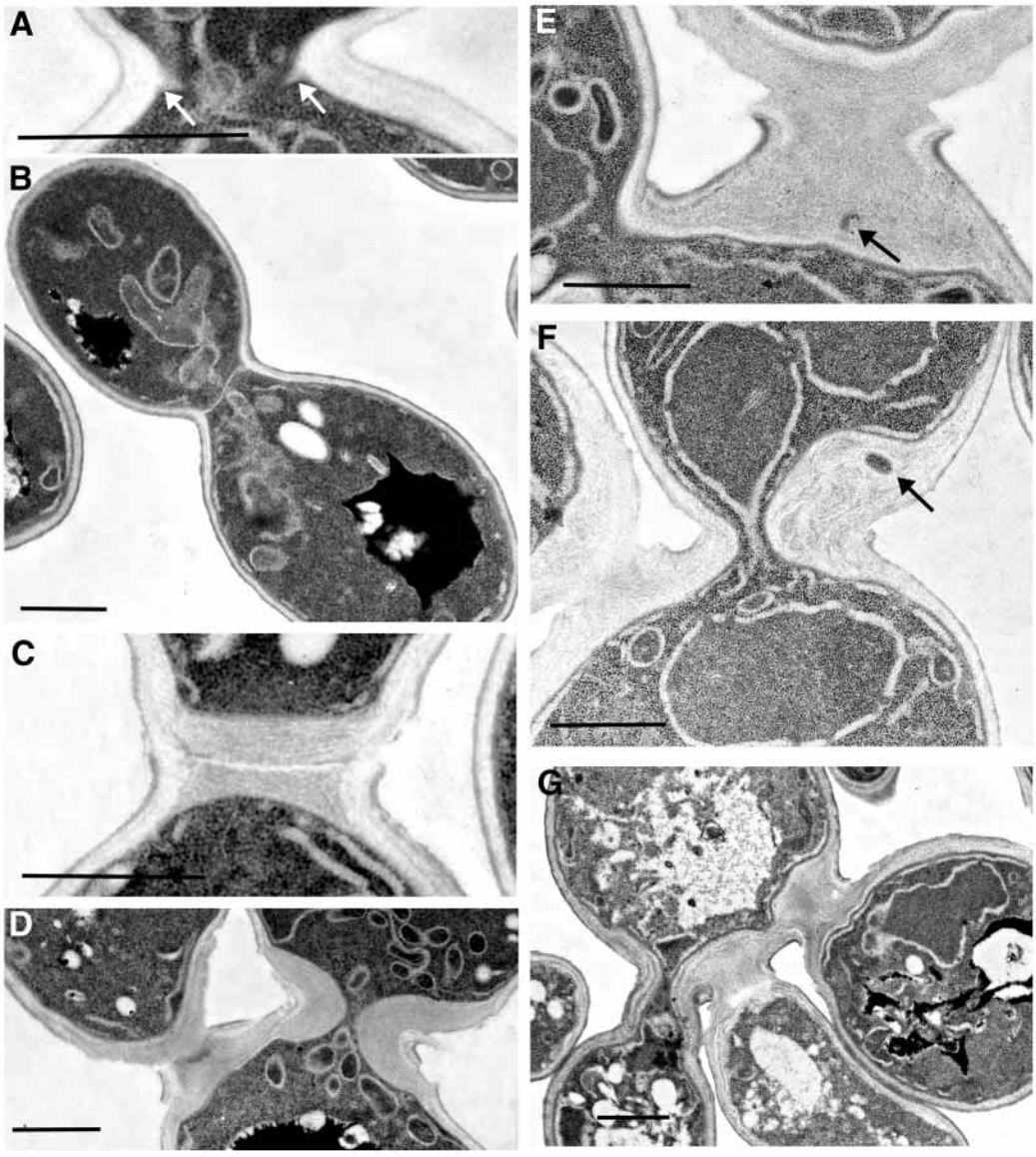

Fig. 2. Electron microscopy of septa in wild-type (YPH499), *chs2*Δ (YMS11) and *myo1*Δ (YMS57) strains. (A) early invagination in cytokinesis of wild-type; (B) completed primary septum in wild-type; (C) completed trilaminar septum in wild-type; (D) an advancing abnormal septum (right) and a completed septum (left) in the *chs2*Δ mutant; (E) a completed septum in the *chs2*Δ mutant, showing a lacuna; (F), an advancing abnormal septum in the *myo1*Δ strain; (G), an advancing septum (left) and two completed septa in the *myo1*Δ mutant. White arrows indicate an advancing primary septum; black arrows point to lacunae. Bars represent 1 μm.

formation of large clumps (Fig. 3), inward growth of cell walls at the neck (Fig. 2D,F,G) and thick septa, occasionally enclosing lacunae (Fig. 2D,E,G).

These observations suggest that ring contraction and primary septum formation are processes that depend on one another. Thus, interference with one function would also block the other. A prediction of this hypothesis is that *myo1 chs2* double mutants should have the same phenotype as the single mutants. That turned out to be the case. By using a variation of the white-red selection devised by Bender and Pringle (Bender and Pringle, 1991) we found that null mutations of *CHS2* and *MYO1* are not synthetically lethal. In fact, the double mutant hardly differs from the single mutants, both in morphology and in generation time (Fig. 3C).

Both Chs2p (Chuang and Schekman, 1996) and Myo1p (Lippincott and Li, 1998; Bi et al., 1998) localize at the neck between mother cell and bud, although they reach that area at different points in the cell cycle. Because of the close relationship between their function, we asked whether either protein is localized correctly in the absence of the other. Chs2p-GFP and Myo1p-GFP fusion proteins were used in these experiments. Morphological observations showed that Myo1p-GFP totally complemented a *MYO1* defect, whereas a strain carrying an integrated copy of *CHS2-GFP* was largely normal but still exhibited some clumping. Myo1p rings were easily visible both in the wild-type and in the *chs2*Δ mutant (Fig. 4A,B,C,D). In the wild-type, 26% of the cells showed rings (536 cells counted, mother cell and bud counted as two cells); in a *chs2*Δ strain, 21% of the cells had rings (404 cells counted). Chs2p-GFP could be seen in the wild-type in only 1.7% of the cells (700 cells counted, Fig. 4E,F). This is not surprising because Chs2p is apparently synthesized just before septum formation and is degraded immediately afterwards (Choi et al., 1994; Chuang and Schekman, 1996). If one assumes that Chs2p will be at the neck only during contraction of the actomyosin ring, its presence there would last only

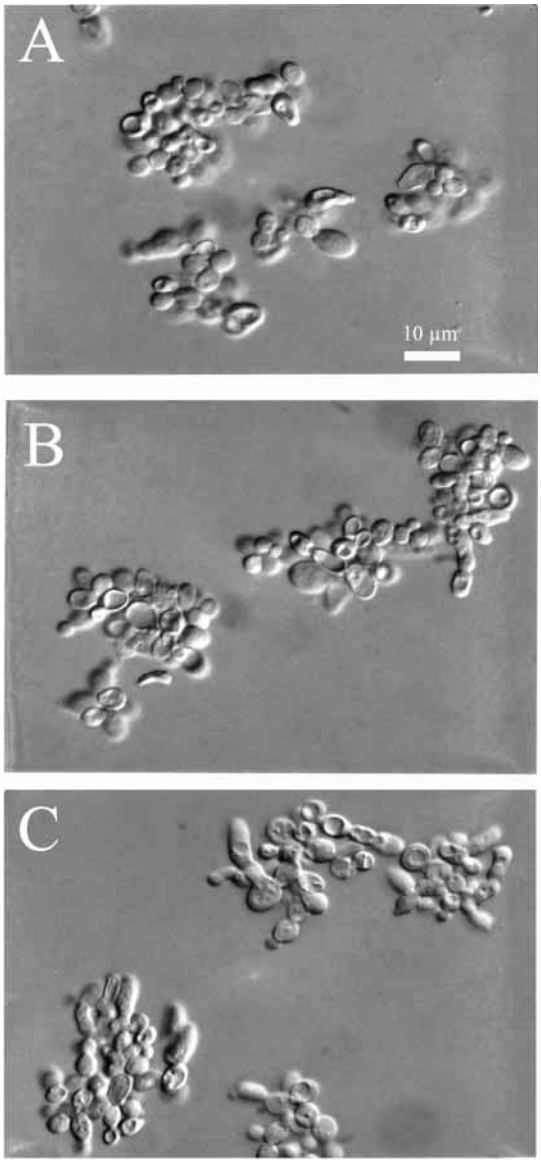

Fig. 3. Morphology of single and double mutants in *MYO1* and *CHS2* as seen by differential interference contrast. The strains used are YMS11 (*chs2*Δ) (A), YMS57 (*myo1*Δ) (B) and YMS135 (*chs2*Δ *myo1*Δ) (C). The generation time in YEPD at 30°C was 145 minutes for YMS11, 226 minutes for YMS57 and 230 minutes for YMS135.

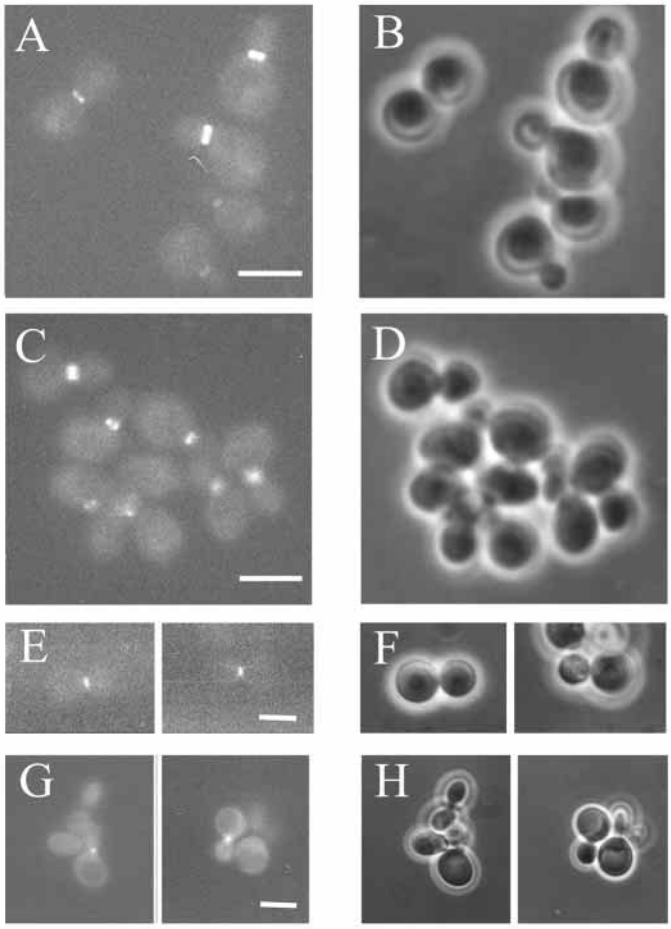

Fig. 4. Localization of Chs2p or Myo1p in strains lacking the other protein. (A) Myo1p-GFP rings in wild-type (YPH499[pMS55]). (C) Myo1p-GFP rings in a *chs2*Δ strain (YMS11[pMS55]). (E,G) Chs2p-GFP rings in wild-type (YMS167) and in a *myo1*Δ strain (YMS171), respectively. The pictures on the right (B,D,F,H) are the corresponding frames, as seen under phase contrast. Bars represent 5 μm.

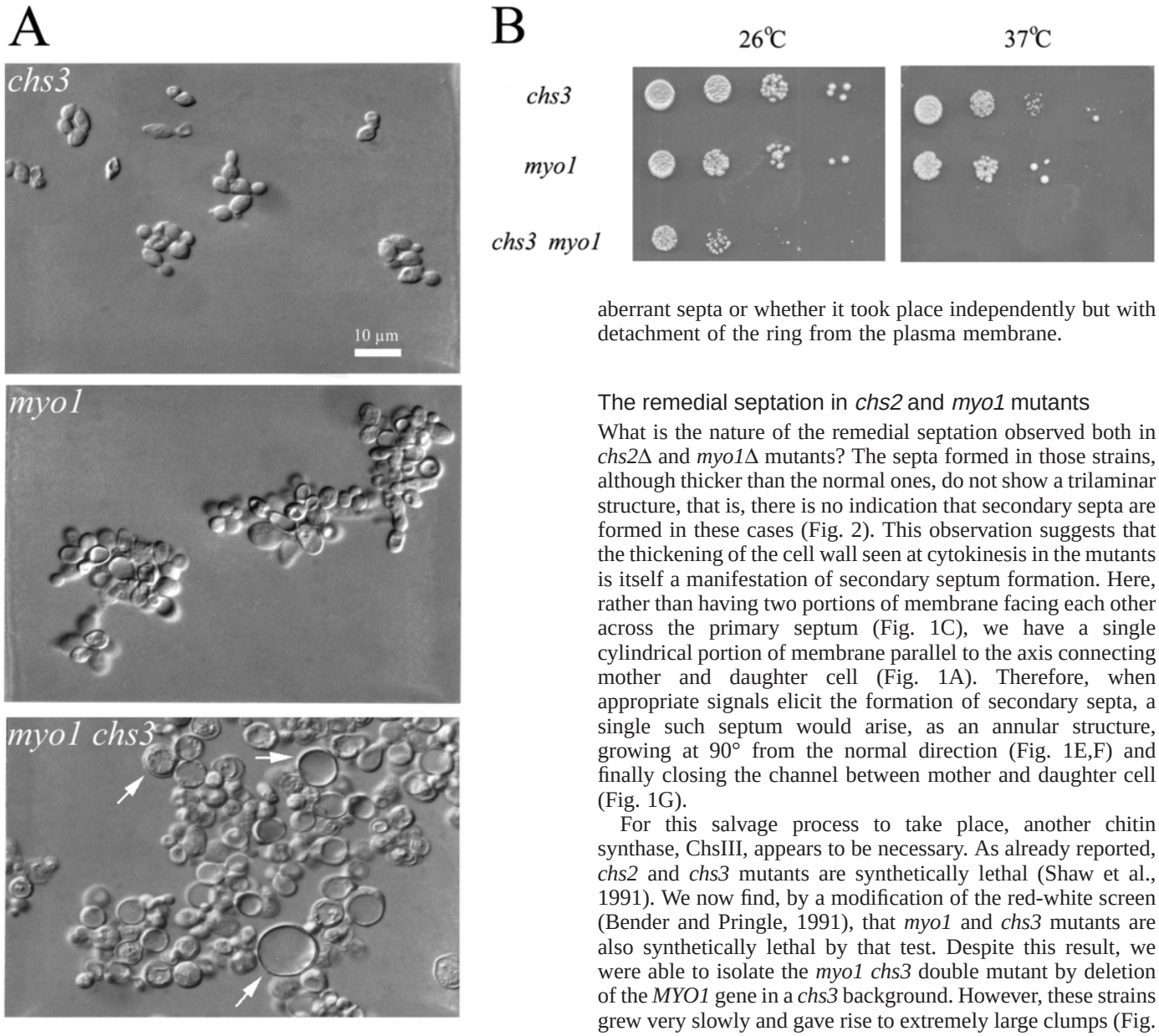

Fig. 5. Morphology by differential interference contrast (A) and temperature sensitivity (B) of single and double mutants in *MYO1* and *CHS3*. In (A), arrows point to cells that are enlarged and lysed. In (B), successive tenfold dilutions are shown. The strains used are ECY46-4-1B (*chs3*Δ), YMS57 (*myo1*Δ) and YMS75 (*myo1*Δ *chs3*Δ). The generation times of the strains in YEPD at 30°C were 160 minutes for ECY46-4-1B, 226 minutes for YMS57 and 800 minutes for YMS75.

approximately five minutes, about 5% of the cell cycle (Bi et al., 1998). In *myo1*Δ strains, Chs2p-GFP could also be detected (Fig. 4G,H) in about the same proportion as in wild-type (1.5%; 700 cells counted). This result indicates that Chs2p does not need a functional contractile ring to attain its localization.

By using time-lapse fluorescent microscopy, we were able to show that at least some of the myosin rings in a *chs2* mutant (as in Fig. 4C) do contract (results not shown). However, this result is not easily interpretable, because it is not possible to ascertain whether the contraction was caused by closure of the aberrant septa or whether it took place independently but with detachment of the ring from the plasma membrane.

The remedial septation in *chs2* and *myo1* mutants

What is the nature of the remedial septation observed both in *chs2*Δ and *myo1*Δ mutants? The septa formed in those strains, although thicker than the normal ones, do not show a trilaminar structure, that is, there is no indication that secondary septa are formed in these cases (Fig. 2). This observation suggests that the thickening of the cell wall seen at cytokinesis in the mutants is itself a manifestation of secondary septum formation. Here, rather than having two portions of membrane facing each other across the primary septum (Fig. 1C), we have a single cylindrical portion of membrane parallel to the axis connecting mother and daughter cell (Fig. 1A). Therefore, when appropriate signals elicit the formation of secondary septa, a single such septum would arise, as an annular structure, growing at 90° from the normal direction (Fig. 1E,F) and finally closing the channel between mother and daughter cell (Fig. 1G).

For this salvage process to take place, another chitin synthase, ChsIII, appears to be necessary. As already reported, *chs2* and *chs3* mutants are synthetically lethal (Shaw et al., 1991). We now find, by a modification of the red-white screen (Bender and Pringle, 1991), that *myo1* and *chs3* mutants are also synthetically lethal by that test. Despite this result, we were able to isolate the *myo1 chs3* double mutant by deletion of the *MYO1* gene in a *chs3* background. However, these strains grew very slowly and gave rise to extremely large clumps (Fig. 5A). Staining with methylene blue showed that many cells in the clump were dead (results not shown). Frequently, cells were enlarged, lysed and practically empty (Fig. 5A, arrows). Furthermore, the double mutant could not grow at all at 37°C (Fig. 5B).

An abnormal budding pattern in *chs2* and *myo1* mutants

Observation of some of the mutants used in this study suggested an abnormal budding pattern. However, accurate determinations of the budding pattern by the usual methods were practically impossible, owing to the clumping manifested by those strains (Fig. 3). Therefore, an alternative procedure was used. Cells with a single bud, rare but still present in the mutants, were micromanipulated with a spore-dissecting apparatus to established positions on a plate. The plate was incubated at room temperature and observation of the cells was carried out every 20-30 minutes. Thus, the budding pattern (Chant and Herskowitz, 1991) for two generations could be

Table 3. Non-axial budding in Δ*chs2* and Δ*myo1* strains

Strain	Relevant genotype	Non-axial budding, %	Total observations
YPH499	Wild-type	0	43
YMS57	*myo1::TRP1*	23	117
YMS11	*chs2::TRP1*	53	115
YMS164	*chs2::TRP1 BUD4::GFP-kanMX6*	42	99
YMS135	*chs2::TRP1 myo1::URA3*	51	124

determined. However, a polar pattern could not be easily distinguished from a random one with this method; therefore the patterns are recorded in (Table 3) as axial or non-axial, a distinction that could easily be made. Both *chs2*Δ and *myo1*Δ mutants exhibited an abnormal, non-axial budding pattern, with a more pronounced defect in *chs2*Δ mutants. A *chs2*Δ *myo1*Δ double mutant was not more defective than a *chs2*Δ mutant (Table 3). Some abnormality in budding pattern of *myo1* mutants was reported by Rodríguez and Paterson (Rodríguez and Paterson, 1990), although the defect was less noticeable in their strains, which contained partial deletions of *MYO1*.

We also examined the levels of Bud3p and Bud4p, two proteins required for the axial budding pattern (Chant and Herskowitz, 1991), in *chs2* and *myo1* mutants. Both proteins were severely reduced in the mutants, especially in the *myo1* strains (Fig. 6A). Another protein showing a low level in the mutant strains is Spa2p (Fig. 6), which localizes at bud tips and is involved in cell polarization (Sheu et al., 1998). In contrast, other proteins usually localized at the cell cortex, such as Rho1p, Cdc42p and the plasma membrane ATPase Pma1p, were not diminished in the mutants (Fig. 6A). This result prompted us to find out whether the localization of Bud3p and Bud4p in double rings at the junction between mother cell and bud (Chant et al., 1995; Sanders and Herskowitz, 1996) was maintained in *chs2* and *myo1* mutants. GFP-fusions of Bud3p and Bud4p were constructed for this purpose. Surprisingly, fluorescence microscopy showed that the rings were indeed present both in wild-type and mutants (Fig. 7). An interesting observation was that the double rings showed much greater separation in the mutants than in wild-type (Fig. 7), perhaps as a consequence of the thicker septa present in the mutant strains. In contrast with the results obtained with native Bud3p and Bud4p (Fig. 6A), the level of the GFP fusion proteins was not much diminished in the mutants (Fig. 6B). However, the budding pattern of the mutant harboring the Bud4p-GFP fusion in a *chs2* background was as different from wild-type as that of the *chs2* strain containing the unmodified Bud4p (Table 3). Therefore, the low level of the Bud proteins in *chs2* and *myo1* mutants does not appear to be the cause of the abnormal budding pattern. Rather, both the aberrant budding and Bud-protein depletion seem to result somehow from the abnormal septation of the *chs2* and *myo1* mutants. We will return to this point in the Discussion.

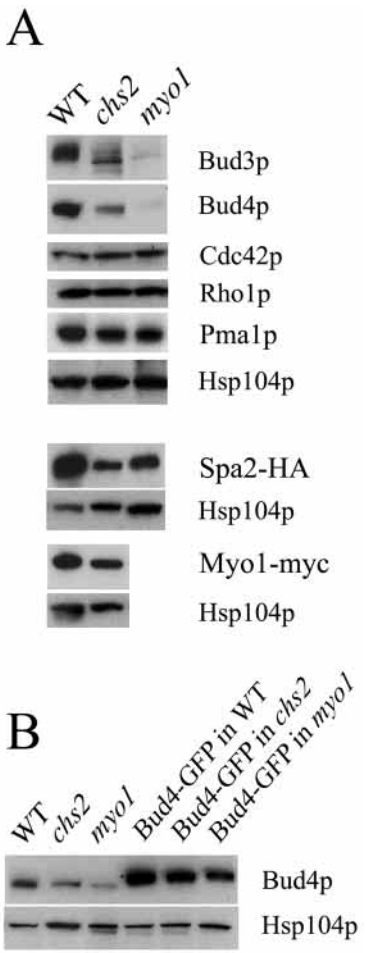

Fig. 6. There are low levels of some proteins (Bud3p, Bud4p and Spa2p) in *chs2* and *myo1* mutants (A). In (B), the decrease in level of Bud4p, when fused to GFP, is much less than with the native protein. The strains used are YPH499 (wild-type), YMS11 (*chs2*Δ), YMS57 (*myo1*Δ), YMS160 (Bud4p-GFP), YMS164 (*chs2*Δ, Bud4p-GFP) and YMS166 (*myo1*Δ, Bud4p-GFP). For the detection of Spa2p-HA and Myo1p-c-myc, YPH499 containing pMS53 or pMS54, respectively, was used. Hsp104p was detected as a loading control. In this figure, relevant genotypes are listed above the blot and detected proteins on the right side of it. For the western blotting technique used see Materials and Methods.

Discussion

The recent discovery that cytokinesis in *S. cerevisiae* includes the contraction of an actomyosin ring prompted us to re-examine the process of septation in this organism. Mutants unable to construct a chitin primary septum (as *chs2* mutants) or a contractile ring (as *myo1* mutants) exhibit a remarkable similarity in their aberrant mechanism of cytokinesis and septation. As judged from observations by electron microscopy, in both cases invagination of the plasma membrane and the pinching off of the membrane in a restricted area does not take place. Instead, the lateral cell wall at the neck between mother cell and bud grows inward over a wide region, gradually closing the gap between the two cells and giving rise to a thick septum lacking the normal three layers. In this process, a wide portion of plasma membrane becomes involved in the closing of the septum, rather than the very small area pinching off in normal cytokinesis. The thick aberrant septum, as it grows, may pinch off the membrane at more than one point, thus giving rise to the cytoplasm-enclosing lacunae that are frequently observed (Fig. 1G; Fig. 2E,F). As

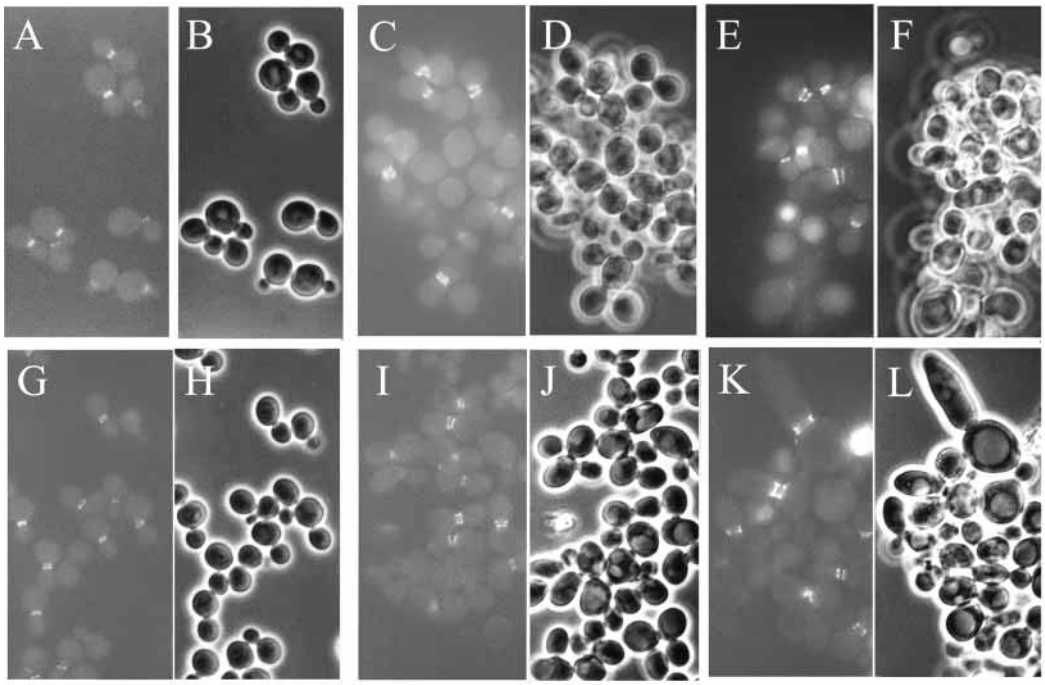

Fig. 7. Fluorescent rings of Bud3p-GFP (A,C,E) and Bud4p-GFP (G,I,K). (B,D,F,H,J,L) are phase-contrast pictures of the same cells. For Bud3p,(A) and (B) show wild-type (YMS159), C and D *chs2*Δ (YMS161), E and F *myo1*Δ (YMS163). For Bud4p, G and H represent wild-type (YMS160), I and J *chs2*Δ (YMS164), K and L *myo1*Δ (YMS166). Double rings in *myo1* and *chs2* mutants are more separated from each other (about 1.5 times) than those in wild-type.

mentioned in the Results, we interpret the salvage mechanism of septation found in *chs2* and *myo1* mutants as the growth of secondary septa at 90° of the normal direction. This is because of lack of the orientation, which is usually provided by the primary septum (Fig. 1E-G).

Thus, it appears that primary-septum formation and actomyosin ring contraction are parts of the same process and are interdependent. This concept is consistent with the finding that *chs2* and *myo1* mutants are not synthetically lethal. In fact, the double mutant does not differ significantly from the single ones, either in morphology or growth rate. In contrast, *chs2* and *chs3* mutations are synthetically lethal (Shaw et al., 1991), and a *myo1 chs3* double mutant grows extremely slowly, forming very large clumps, which contain many lysed cells (Fig. 5). Thus, *CHS3* seems to be necessary for the salvage route that leads to the abnormal but ultimately successful cytokinesis found in *myo1* or *chs2* mutants. In this connection, it is interesting that we observed a high concentration of chitin, as judged from wheat germ agglutinin-gold staining in the thick septa of *chs2* mutants (Shaw et al., 1991). Those strains also showed a higher level of chitin than wild-type in their cell wall (Shaw et al., 1991) and an enhanced activity of chitin synthase III (Crotti et al., 2001). An increase in chitin content (Rodríguez-Medina et al., 1998) and in the activity of chitin synthases (Cruz et al., 2000) has also been reported in *myo1* mutants. The extra chitin may be needed to reinforce the growing aberrant septa by cross-linking to β-glucans (Kollár et al., 1995; Kollár et al., 1997). This reinforcement would not be needed for normal secondary septa, whose function is not to effect cytokinesis but to fill in bud and birth scars. These fillings protect the cells after a chitinase hydrolyzes part of the primary septum chitin to effect cell separation (Kuranda and Robbins, 1991). Indeed, *chs3* mutants assemble trilaminar septa, which lacks only the external chitin ring laid down at early budding (Shaw et al., 1991).

It is interesting to compare the process of septation in *S. cerevisiae* with that of the fission yeast, *Schizosaccharomyces pombe*. *S. pombe* also form a contractile ring (Gould and Simanis, 1997), as well as primary and secondary septa, except that in this case the primary septum appears to consist of β(1→3)glucan rather than chitin. It has been recently reported that ring contraction and formation of the primary septum are both required for cytokinesis, as we found in *S. cerevisiae* (Le Goff et al., 1999; Liu et al., 1999). The difference with budding yeast is that in *S. pombe*, a defect in either structure is lethal, because there is no alternate route for cytokinesis. This lethality may be caused by the lack of chitin in the wall of vegetative cells (Arellano et al., 2000) or by the larger distance that the salvage septa would have to cover to close the gap in the cigar-shaped cells.

In addition to their septation defects, *chs2* and *myo1* mutants show abnormalities in their budding pattern (Table 3). Concomitantly, they are depleted in some proteins necessary for axial budding, such as Bud3p and Bud4p, and in a protein localizing at the tip of a new bud, Spa2p (Fig. 6A). It would be tempting to assume that the decrease in the Bud proteins is the cause of the abnormal pattern. However, Bud3p and Bud4p double rings were normally formed in *myo1* and *chs2* mutants (Fig. 7), albeit with more separation between them than in the wild-type. Furthermore, the strains in which the rings were observed harbored Bud3p-GFP or Bud4p-GFP fusion proteins, which were maintained at much higher levels than the original Bud3p and Bud4p (Fig. 6B). Nevertheless, those strains had the same axial budding defect as the others. Therefore, it is not the depletion of the proteins in itself that causes the aberrant budding pattern. Rather, the reason for this behavior may reside in the abnormal septation of *chs2* and *myo2* strains. As shown in the scheme of Fig. 1B, the wide area along which repair septa make contact may result in the loss of a fairly large portion of plasma membrane, some of it remaining entrapped in lacunae. This part of the plasma membrane may contain binding sites where certain proteins, such as Bud3p, Bud4p or

Spa2p, would attach in the next budding event. Elimination of those sites may lead to the abnormal budding pattern and to enhanced degradation of those proteins with a resulting decrease in their cellular level.

Finally, why are formation of the chitin primary septum and contraction of the actomyosin ring interdependent processes? First of all, this is not due to delocalization of the proteins of interest in the mutants used in this study. Chs2p localized correctly in *myo1* mutants and so did Myo1p in *chs2* mutants (Fig. 4). Second, Chs2p and Myo1p do not seem to interact directly. This notion is supported by the lack of effect of one protein on the localization of the other. Furthermore, we were unable to detect two-hybrid interaction between the two proteins (results not shown). Finally, Chs2p is located in the plasma membrane and in secretory vesicles, whereas Myo1p is in the cytoplasm. Fractionation of cell extracts by centrifugation in density gradients showed that the two proteins are in fractions of very different density (results not shown). It is not difficult to understand why formation of the chitin septum requires contraction of the ring. Chitin synthase II, the enzyme responsible for the formation of primary septum chitin, is localized in the plasma membrane (Sburlati and Cabib, 1986) at the neck between the mother cell and the bud (Fig. 4). Invagination of the plasma membrane coupled to continuing extrusion of chitin from the center of the invagination will automatically generate an inwardly growing 'doughnut' of the polysaccharide that will ultimately evolve into a disk. If there is no invagination, some chitin may build up at the neck but no disk will result.

The reciprocal need for chitin septum formation in order that the ring may contract is less easily understood. We propose an explanation on the basis of the high turgor pressure of fungal cells. Because of the elevated intracellular osmolarity, relative to that of the medium, water tends to enter the cell and the resulting turgor pressure pushes the plasma membrane against the cell wall. In fact, even a small hole or weak area in the cell wall results in cell lysis and in the blowing out of cell membrane through the opening (Cabib et al., 1989). Thus, in order to have contraction, the force developed by the actomyosin ring must exceed that generated by the turgor pressure. Here is where the extrusion of chitin may help, both by pushing the membrane inward and, through a sort of ratcheting action, by preventing it from moving back towards the cell wall. Therefore, in our conception, primary septum formation and actomyosin ring contraction are integral parts of the same cytokinetic process. Note that mammalian cells complete cytokinesis without the need of a septum. This is consistent with our hypothesis, because mammalian cells are devoid of turgor pressure.

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