Cell Cycle-regulated Trafficking of Chs2 Controls Actomyosin Ring Stability during Cytokinesis[□]

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Submitted December 20, 2004; Revised February 7, 2005; Accepted March 2, 2005

Monitoring Editor: Anthony Bretscher

Cytokinesis requires the coordination of many cellular complexes, particularly those involved in the constriction and reconstruction of the plasma membrane in the cleavage furrow. We have investigated the regulation and function of vesicle transport and fusion during cytokinesis in budding yeast. By using time-lapse confocal microscopy, we show that post-Golgi vesicles, as well as the exocyst, a complex required for the tethering and fusion of these vesicles, localize to the bud neck at a precise time just before spindle disassembly and actomyosin ring contraction. Using mutants affecting cyclin degradation and the mitotic exit network, we found that targeted secretion, in contrast to contractile ring activation, requires cyclin degradation but not the mitotic exit network. Analysis of cells in late anaphase bearing exocyst and myosin V mutations show that both vesicle transport and fusion machineries are required for the completion of cytokinesis, but this is not due to a delay in mitotic exit or assembly of the contractile ring. Further investigation of the dynamics of contractile rings in exocyst mutants shows these cells may be able to initiate contraction but often fail to complete the contraction due to premature disassembly during the contraction phase. This phenotype led us to identify Chs2, a transmembrane protein targeted to the bud neck through the exocytic pathway, as necessary for actomyosin ring stability during contraction. Chs2, as the chitin synthase that produces the primary septum, thus couples the assembly of the extracellular matrix with the dynamics of the contractile ring during cytokinesis.

INTRODUCTION

In animal cells, amoebas, and yeast, cytokinesis occurs centripetally through the formation of a cleavage furrow (reviewed in Guertin *et al.*, 2002). Ingression of the cleavage furrow leads to close juxtaposition of the equatorial plasma membrane and homotypic fusion of this membrane physically divides the cell into two. The force that drives ingression of the cleavage furrow has been mainly attributed to the actomyosin-based contractile ring, which assembles at the onset of cytokinesis along a plane perpendicular to the elongating mitotic spindle. This mechanism contrasts with that for cytokinesis in plant cells, where formation of the dividing cell plate structure occurs centrifugally through local targeting and fusion of vesicles (Field *et al.*, 1999). However, new membrane addition at the cleavage furrow was observed in early studies of cytokinesis in *Xenopus* eggs (Bluemink and de Laat, 1973; Byers and Armstrong, 1986). Recent work in echinoderm, *Drosophila*, and *Caenorhabditis elegans* embryos further confirmed targeted membrane insertion as a general phenomenon in early embryonic cell divisions (Burgess *et al.*, 1997; Jantsch-Plunger and Glotzer, 1999; Skop *et al.*, 2001; Shuster and Burgess, 2002; Xu *et al.*, 2002). Pharmacological or biochemical disruption of the secretory pathway resulted in failed cytokinesis, suggesting that membrane addition at the cleavage furrow comes from targeted vesicular transport and fusion and is required for

cytokinesis, even in cells that use the contractile ring to draw membrane inward (Skop *et al.*, 2001). In conjunction with membrane addition, targeted vesicle fusion in the cleavage furrow may provide timed delivery of factors required for completion of cytokinesis. This was suggested based on results involving disruption of the furrow microtubule arrays that are required for the delivery of vesicles containing specific cargoes to the cleavage furrow (Jesuthasan, 1998). However, there is little data on the function of any proteins and lipid factors delivered by membrane trafficking during cytokinesis or whether membrane addition is similarly important for the much smaller somatic cell to complete cytokinesis.

In this study, we exploit the budding yeast *Saccharomyces cerevisiae* as a model for understanding the function and regulation of targeted exocytosis during cytokinesis. This choice was motivated first by the fruitful studies of vesicular transport carried out using this organism and the availability of a large number of well characterized conditional mutants affecting almost all known aspects of membrane trafficking and recycling (Novick *et al.*, 1980; Schekman and Novick, 2004). Much of the work on vesicular trafficking in budding yeast has been in the context of polarized growth during bud formation. During this process, Golgi-derived vesicles, which contain the Rab GTPase Sec4, are transported via Myo2, a type V myosin, along oriented actin cables into the nascent bud (Pruyne and Bretscher, 2000). Fusion of these vesicles with the plasma membrane requires the conserved exocyst complex (TerBush *et al.*, 1996; Guo *et al.*, 1999). Late in the cell cycle, it has been observed that many of the proteins involved in polarized secretion are retargeted to the bud neck, consistent with a possible role in cytokinesis (Finger *et al.*, 1998).

Cytokinesis in budding yeast, as in animal cells, also involves an actomyosin-based contractile ring, which con-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-12-1090>) on March 16, 2005.

□ The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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stricts during cytokinesis (reviewed in Tolliday *et al.*, 2001). The assembly of the contractile ring occurs in several steps, starting early in the cell cycle with Myo1 localizing to a ring around the bud neck, in a septin-dependent manner (Bi *et al.*, 1998; Lippincott and Li, 1998b). Actin assembles into the contractile ring during mitotic anaphase in a Cyk1- and formin-dependent manner (Lippincott and Li, 1998b; Tolliday *et al.*, 2002). The initiation of actomyosin ring contraction is governed by the mitotic exit network (MEN), a signaling pathway that controls the exit from mitosis through the release of Cdc14, a protein phosphatase critical for the inactivation of mitotic Cdk1, from the nucleolus where Cdc14 is held inactive (reviewed in Bardin and Amon, 2001). Contraction of the ring constricts the membrane and guides the synthesis of a chitin-based primary septum catalyzed by the chitin synthase Chs2, a transmembrane protein. The production and bud neck localization of Chs2 is tightly restricted to late cell cycle stages, in contrast to the other chitin synthases (reviewed in Cabib, 2004). It has been suggested that the formation of the primary septum behind the contractile ring may provide additional support needed for closing the gap between mother and daughter cells (Schmidt *et al.*, 2002).

We first determined the precise time in the cell cycle where the secretory pathway is targeted to the site of cell division and demonstrated that this targeting event is controlled by mitotic exit, but not directly by the MEN. We found that targeted vesicle fusion is important for the completion of cell division. Analysis of contractile ring dynamics suggested that the secretory pathway is required for the localization of Chs2, a membrane-bound factor necessary for the stability of the actomyosin ring during the contraction process.

MATERIALS AND METHODS

Media and Genetic Manipulations

Yeast cell culture and genetic techniques were performed as described in Sherman *et al.* (1974). Media were prepared as follows: yeast extract, peptone, dextrose (YPD) contained 2% glucose, 1% yeast extract, and 2% Bactopeptone (Difco, Detroit, MI). YPR contained 2% raffinose, 1% yeast extract, and 2% Bactopeptone. YPGR contained 2% galactose, 2% raffinose, 1% yeast extract, and 2% Bactopeptone. Synthetic complete (SC) medium was prepared as described in Kaiser *et al.* (1994).

Plasmid and Strain Construction

All strains used in this study are listed in Table 1. pNT138 was generated by digesting the *pGFP-TUB1* insert from pAFS125 (Straight *et al.*, 1997) with *KpnI* and *NotI* for ligation into pRS304 (Sikorski and Hieter, 1989) digested with *KpnI* and *NotI*. To generate a strain expressing Myo1-green fluorescent protein (GFP) and GFP-Tub1, pNT138 was digested with *SnaBI* and transformed into RLY1450 (Tolliday *et al.*, 2003) to produce RLY1994. Labeling of the endogenous copy of *SEC3* was achieved by a PCR-mediated one-step tagging technique (Longtine *et al.*, 1998). Briefly, pFA6a-GFP-HIS3MX6 was used as a template for PCR with primers containing sequences flanking the 3' end of *SEC3*. The resultant PCR product was used to transform RLY261 to produce RLY1995, which was then transformed with pNT138 cut with *SnaBI* to produce RLY1996. To obtain a strain expressing GFP-Tub1 along with Sec4 GFP, RLY261 was transformed first with *SnaBI*-digested pNT138, and then with pRC556 (pEGFP-SEC4) (Schott *et al.*, 2002), to produce RLY1997. The MYO2 (RLY1998) and *myo2-16* (RLY1999) strains were obtained from the Bretscher laboratory (Schott *et al.*, 1999). These strains were transformed with pRC566 (pEGFP-SEC4) to produce RLY2000 and RLY2001. To obtain Sec3-GFP and Sec4-GFP strains expressing the GAL-inducible *clb2* with deletion of the destruction boxes (Surana *et al.*, 1993), RLY1995, RLY1996, and RLY1997 were transformed with pGAL-CLB2ΔDB digested with *NcoI* to produce RLY2066, RLY2002, and RLY2003, respectively. As controls for GAL induction, RLY1995, RLY1996, and RLY1997 were transformed with pRL62 (Lippincott and Li, 1998a) digested with *XcmI* to produce RLY2065, RLY2004, and RLY2005, respectively. To analyze Sec3-GFP localization in the *mob1-77* (Luca and Winey, 1998) background, with over expression of Sic1, RLY656 was mated to RLY1996 and then sporulated to produce *MOB1* and *mob1-77* strains with Sec3-GFP. The resultant strains RLY2039 and RLY2040 were transformed with a 2μ plasmid containing *SIC1* (Luca *et al.*, 2001) to produce RLY2042 and

RLY2041, respectively. The exocyst mutant strains *sec3-2* (RLY1685) and *sec10-2* (RLY1689) were backcrossed three times with RLY387 to put the mutations in the W303 strain background. The resultant crosses produced mutant and congenic wild-type strains RLY1838, RLY1840, RLY2006, and RLY2007, respectively. To obtain a wild-type and *sec3-2* strains expressing Sec4-GFP, RLY1840, and RLY1838 were transformed with pRC566 (Schott *et al.*, 2002) to produce RLY2009 and RLY2008, respectively. The GAL-SEC4 (RLY1900) and dominant negative GAL-SEC4N34 (RLY1901) strains were obtained from Ruth Collins (Cornell University, Ithaca, NY) (Walworth *et al.*, 1989). Cdc14-GFP expression in wild-type and *sec3-2* cells was obtained by transformation of RLY1840 and RLY1838 with pCDC14-5XGFPIT (Yoshida *et al.*, 2002) digested with *StuI*, to produce RLY2013 and RLY2012, respectively. For expression of Myo1-GFP and GFP-Tub1, RLY1838, RLY1840, RLY2006, and RLY2007 were first transformed with pNT119 (Tolliday *et al.*, 2003) digested with *BclI* and then pNT138 digested with *SnaBI* to produce RLY1899, RLY1898, RLY2011, and RLY2010, respectively. MYO2, *myo2-16*, *SEC10*, and *sec10-2* strains expressing Chs2-GFP were created by transforming RLY1998, RLY1999, RLY2007, and RLY2006 with pLP31 (Chs2-GFP) (Tolliday *et al.*, 2003) to create RLY2060, RLY2061, RLY2062, and RLY2063, respectively. RLY2062 and RLY2063 also were expressing Tub1-GFP from transformation with pNT138. The *chs2Δ* strain (RLY1993) was obtained from E. Cabib (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health [NIH], Bethesda, MD) and was transformed with pNT119 to produce RLY2064 (*chs2Δ*, Myo1-GFP).

GFP Localization and Timing by Confocal Fluorescence Microscopy

RLY1994 (Myo1-GFP, GFP-Tub1), RLY1996 (Sec3-GFP, GFP-Tub1), RLY1997 (Sec4-GFP, GFP-Tub1), RLY2062 (Chs2-GFP, GFP-Tub1), RLY1450 (Myo1-GFP), and RLY2064 (*chs2Δ*, Myo1-GFP) and were grown overnight in the appropriate SC + 2% dextrose or YPD + 0.2 mg/ml adenine sulfate to mid-logarithmic phase and placed in a growth chamber for imaging, as adapted from Maddox *et al.* (2000). Briefly, a 25% gelatin (Sigma-Aldrich, St. Louis, MO) solution was prepared in the appropriate SC + 2% dextrose liquid media and heated to mix the gelatin and media. Then, 200 μl of the heated gelatin mix was pipetted onto a glass slide and covered with a second slide perpendicularly, being supported by two slides, with tape for spacers, parallel to the first. After polymerization, the slides were carefully pried apart, and the layer of gelatin on one slide was trimmed to the size of the coverslip. One milliliter of the overnight cultures was pelleted and resuspended in 50 μl of the appropriate SC + 2% dextrose media. Five microliters of the resuspended cells was pipetted onto the gelatin slab and covered with a coverslip. The coverslip was sealed to the slide by using 1:1 Vaseline/lanolin/paraffin (VALAP). Fluorescence images were collected with a PerkinElmer Ultraview spinning disk confocal on a Nikon TE2000 inverted microscope, by using a 100× 1.4 numerical aperture Plan Apo objective lens. The 488-nm wavelength from a krypton-argon laser was selected with a Chroma 488/10-nm bandpass excitation filter. A Chroma single wavelength, 488-nm transmitting dichroic mirror, and HQ550 long-pass emission filter were used. Z-series optical sections were acquired with a Hamamatsu ORCA ER-cooled charge-coupled device camera and with a Prior Proscan focus motor. Image acquisition was initiated every 30 s, and each time point consisted of a stack of 5 z-series images with a distance of 0.5 μm apart, totaling a distance of 2 μm. Exposures of 0.7–0.8 s were used and images were collected by binning 2 × 2 to increase signal over camera noise. MetaMorph imaging software (Universal Imaging, Downingtown, PA) was used for controlling the hardware during imaging, image analysis, and production of two-dimensional (2D) projections of the z-images. The average intensity of Chs2-GFP signal was determined by drawing a line over the Chs2-GFP signal for each time point where the signal was present at the bud neck.

Metaphase Arrest and Release Assays

For strains to be induced by galactose, cells were cultured in YPR. Temperature-sensitive (ts) strains were cultured in YPD. All were grown overnight at room temperature. At ~OD₂₆₀ = 0.200, cells were pelleted and resuspended in either YPGR for GAL induction or YPD for the ts strains with 20 μg/ml nocodazole and grown for ~3 h at room temperature until culture is >90% arrested in metaphase. For GAL induction, cells were pelleted, washed twice in YPGR, and then resuspended in YPGR. For ts strains, after arrest was obtained, cultures were pelleted and resuspended in YPD + 20 μg/ml nocodazole warmed to 37°C. Cells were incubated at 37°C for 30 min, pelleted, washed two times with warmed YPD, and resuspended in 37°C YPD. For analysis of Sec3-GFP and Sec4-GFP localization in cells with an inducible block in mitosis or the MEN, 1-ml aliquots were taken from the culture every 15 min after release from nocodazole arrest, and live cells were quantified for specific GFP-tagged protein localization with a Nikon Eclipse E800 microscope with either a Plan Apo 100×/1.40 or a Plan Apo TIRF 100×/1.45 oil differential-interference contrast objective to assay the number of cells with Chs2-GFP signal at the bud neck. Images were obtained with filtering fluorescent light through a GFP filter set (Chroma Technology, Brattleboro, VT) with a cooled Interline camera (Princeton Scientific Instruments, Monmouth Junction, NJ) by using MetaMorph software. Exposures ranged between 0.2 and 0.7 s. For all the localization experiments, 200 cells were analyzed for each

Table 1. Yeast strains

Name	Genotype	Source
RLY261	<i>MATa ura3-1 his3-11,15 leu2-3,112 trp1 ade2-1 bar1Δ</i>	Elion laboratory
RLY387	<i>MATα ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100λ</i>	Li laboratory
RLY656	<i>MATa ura3 lys2 his3 leu2 mob1-77</i>	Li laboratory
RLY1450	<i>MATa ura3 leu2 his3 trp1 ade2 Δsst1 MYO1-GFP-MYC LEU2::MYO1</i>	Li laboratory
RLY1685	<i>MATa sec3-2 ura3-52 leu2-3,112</i>	C. Kaiser laboratory
RLY1689	<i>MATa ura3-52 sec10-2</i>	C. Kaiser laboratory
RLY1838	<i>MATa ura3 leu2 trp1 his3 sec3-2</i>	This study
RLY1840	<i>MATa ura3 leu2 trp1 his3</i>	This study
RLY1898	<i>MATa ura3 leu2 trp1 his3 MYO1-GFP-MYC-LEU2::MYO1 GFP-TUB1::TRP1</i>	This study
RLY1899	<i>MATa ura3 leu2 trp1 his3 sec3-2 MYO1-GFP-MYC LEU2::MYO1 GFP-TUB1::TRP1</i>	This study
RLY1900	<i>MATα ura3-52 leu2-112::LEU2 PGAL1/10 SEC4</i>	R. Collins laboratory
RLY1901	<i>MATα ura3-52 leu2-112::LEU2 PGAL1/10 SEC4N34</i>	R. Collins laboratory
RLY1993	<i>MATa chs2::TRP1 ade2-101 ura3-52 lys2-801 trpΔ63 his3Δ200 leu2D1</i>	Cabib laboratory
RLY1994	<i>MATa ura3 leu2 his3 trp1 ade2 Δsst1 MYO1-GFP-MYC-LEU2::MYO1 GFP-TUB1::TRP1</i>	This study
RLY1995	<i>MATa ura3 leu2-112 his3 trp1 ade2 Δsst1 SEC3-GFP::HIS3</i>	This study
RLY1996	<i>MATa ura3 leu2-112 his3 trp1 ade2 Δsst1 SEC3-GFP::HIS3 GFP-TUB1::TRP1</i>	This study
RLY1997	<i>MATa ura3 leu2-112 his3 trp1 ade2 Δsst1 GFP-TUB1::TRP1 GFP-SEC4:LEU2</i>	This study
RLY1998	<i>MATα ura3 leu2 lys2 ade2 MYO2::HIS3</i>	Bretscher laboratory
RLY1999	<i>MATα ura3 leu2 lys2 ade2 myo2-16::HIS3</i>	Bretscher laboratory
RLY2000	<i>MATα ura3 leu2 lys2 ade2 MYO2::HIS3 SEC4-GFP:LEU</i>	This study
RLY2001	<i>MATα ura3 leu2 lys2 ade2 myo2-16::HIS3 SEC4-GFP:LEU</i>	This study
RLY2002	<i>MATa ura3 leu2-112 his3 trp1 ade2 Δsst1 SEC3-GFP::HIS3 GFP-TUB1::TRP1 pGAL-clb2ΔDB::URA3</i>	This study
RLY2003	<i>MATa ura3 leu2-112 his3 trp1 ade2 Δsst1 SEC4-GFP:LEU2 GFP-TUB1::TRP1 pGAL-clb2ΔDB::URA3</i>	This study
RLY2004	<i>MATa ura3 leu2-112 his3 trp1 ade2 Δsst1 SEC3-GFP::HIS3 GFP-TUB1::TRP1 pGAL::URA3 (pRL62)</i>	This study
RLY2005	<i>MATa ura3 leu2-112 his3 trp1 ade2 Δsst1 GFP-TUB1::TRP1 SEC4-GFP:LEU2 pGAL::URA3 (pRL62)</i>	This study
RLY2006	<i>MATa leu2 ura3 trp1 his3 ade2-1 sec10-2</i>	This study
RLY2007	<i>MATa leu2 ura3 trp1 his3 ade2-1</i>	This study
RLY2008	<i>MATa ura3 leu2 trp1 his3 sec3-2 GFP-SEC4:LEU2</i>	This study
RLY2009	<i>MATa ura3 leu2 trp1 his3 GFP-SEC4:LEU2</i>	This study
RLY2010	<i>MATa leu2 ura3 trp1 his3 ade2-1 can1-100 GFP-TUB1::TRP1 MYO1-GFP-MYC-LEU2::MYO1</i>	This study
RLY2011	<i>MATa leu2 ura3 trp1 his3 ade2-1 can1-100 sec10-2 GFP-TUB1::TRP1 MYO1-GFP-MYC-LEU2::MYO1</i>	This study
RLY2012	<i>MATa his3 leu2 trp1 ura3 lys2 sec3-2 CDC14-GFP::TRP1</i>	This study
RLY2013	<i>MATa his3 leu2 trp1 ura3 lys2 CDC14-GFP::TRP1</i>	This study
RLY2039	<i>MATa ura3 his3 leu2 trp1 SEC3-GFP::HIS3</i>	This study
RLY2040	<i>MATa ura3 his3 leu2 trp1 mob1-77 SEC3-GFP::HIS3</i>	This study
RLY2041	<i>MATa ura3 lys2 his3 leu2 trp1 mob1-77 SEC3-GFP::HIS3 pYEp13-SIC1:LEU2</i>	This study
RLY2042	<i>MATa ura3 lys2 his3 leu2 trp1 SEC3-GFP::HIS3 pYEp13-SIC1:LEU2</i>	This study
RLY2060	<i>MATa ura3 leu2 lys2 ade2 MYO2::HIS3 CHS2-GFP:LEU2 (pLP31)</i>	This study
RLY2061	<i>MATa ura3 leu2 lys2 ade2 myo2-16::HIS3 CHS2-GFP:LEU2 (pLP31)</i>	This study
RLY2062	<i>MATa leu2 ura3 trp1 his3 ade2-1 GFP-TUB1::TRP1 CHS2-GFP:LEU2 (pLP31)</i>	This study
RLY2063	<i>MATa leu2 ura3 trp1 his3 ade2-1 sec10-2 GFP-TUB1::TRP1 CHS2-GFP:LEU2 (pLP31)</i>	This study
RLY2064	<i>MATa chs2::TRP1 ade2-101 ura3-52 lys2-801 trpΔ63 his3Δ200 leu2D1 MYO1-GFP-MYC LEU2::MYO1</i>	This study
RLY2065	<i>MATa ura3 leu2-112 his3 trp1 ade2 Δsst1 SEC3-GFP::HIS3 pGAL::URA3 (pRL62)</i>	This study
RLY2066	<i>MATa ura3 leu2-112 his3 trp1 ade2 Δsst1 SEC3-GFP::HIS3 pGAL-clb2ΔDB::URA3</i>	This study

time point and each experiment was repeated at least twice. A Nikon labo-
 phot-2 microscope with a Plan 40 0.5 ELWD objective was used to view the
 morphology of the cells to determine the status of the cell cycle. To analyze
 cytokinesis defects in ts strains and the GAL-inducible Sec4 strains, samples
 were taken at time 0 after nocodazole washout and 2 h after. Two milliliter
 samples were fixed in 5% formaldehyde for 1 h at room temperature and
 washed two times with and resuspended in 1× phosphate-buffered saline
 (PBS). Samples were treated with 0.2 mg/ml zymolyase 20T (Seikagaku
 America, Rockville, MD) in sorbitol buffer (1 M sorbitol in 50 mM KPO₄, pH
 7.5) containing 2 mM dithiothreitol for 10 to 20 min at 30°C. After cells lost
 refractile appearance, indicating removal for cell wall, the morphology of the
 cells were analyzed for completion of cytokinesis by comparing the number
 of large-budded cells (undivided) versus unbudded cells or small-budded
 (divided) cells. For assaying actin ring formation, 2-ml samples were collected
 every 15 min after release from arrest and fixed and zymolyase treated as

described above and stained with rhodamine-conjugated phalloidin (Molec-
 ular Probes, Eugene, OR) as described in Pringle *et al.* (1991). Cells expressing
 Cdc14-GFP were treated similarly as described above in relation to arrest and
 release from nocodazole. Two-milliliter samples were taken every 15 min for
 60 min from nocodazole washout, fixed briefly in 1% formaldehyde for 30
 min, washed two times with 1× PBS, adhered to a poly-lysine-coated slide,
 and covered with a mounting solution containing 4,6-diamidino-2-phenylin-
 dole (DAPI) to visualize DNA (Pringle *et al.*, 1991). Two hundred cells were
 counted for release of Cdc14-GFP into the cytoplasm for each time point, with
 this experiment being performed twice. For analysis of Chs2-GFP in wild-type
 (WT), *myo2-16*, and *sec10-2* cells, 2-ml aliquots were taken every 15 min after
 release from nocodazole arrest, lightly fixed in 1% formaldehyde for 30 min,
 and imaged and quantified for Chs2-GFP localization with the fluorescent
 microscope described above. Three-dimensional (3D) images and movies of
 Chs2-GFP in WT and *sec10-2* cells were produced by imaging lightly fixed

cells with the spinning disk confocal microscope described previously, imaging 13 sections in the z-plane, with a spacing of 0.2 μm , with exposures of 0.7 s. Each stack of z-series images was deconvolved and rendered into 3D images and movies by using Autodeblur deconvolution software (AutoQuant Imaging, Troy, NY).

Electron Microscopy

RLY1838 and RLY1840 were cultured overnight in YPD, pelleted, and treated as described above for arrest and release in nocodazole. After washout of nocodazole, cells were collected at 30 min after the release to enrich for cells in cytokinesis. Ten milliliters of cells was pelleted and prepared for electron microscopy (EM) as described in Li (1997).

Analysis of Actomyosin Ring Dynamics in Temperature-sensitive Yeast Strains

RLY1898, RLY1899, RLY2010, and RLY2011 were prepared and treated with nocodazole as described above. At nocodazole washout, cultures were washed in warmed YPD and resuspended in SC-Leu + 2% glucose at 37°C. One milliliter of these cultures was pelleted and resuspended in 50 μl of warmed SC-Leu media. Five microliters of the resuspension of cells was placed on freshly prepared growth slab as described above but prepared with 1.5% agarose instead in SC-Leu + 2% glucose media. Briefly, 250 μl of the heated mixed agarose solution was placed onto a slide. A second slide is placed on top of the agarose and left to polymerize at room temperature for ~10 min. Slides were carefully pried apart, and the remaining agarose slab was trimmed to the size of the cover glass, which is placed on top of the slab after cells are added and sealed with VALAP. The slides were kept at 37°C and immediately set for imaging on a similar spinning disk confocal microscope as described above but with a 37°C incubation chamber (Solvent Scientific, Segensworth, United Kingdom) encompassing the stage of the microscope to allow for imaging at 37°C. Images were collected and analyzed as above. MetaMorph was used to measure the diameter of the Myo1-GFP ring in each frame of the time-lapse series. The kymographs were produced using the MetaMorph add-on for kymographs, by selecting the signal area (Myo1-GFP ring) with a line, to be recorded in the kymograph. Each horizontal line of the kymograph represents fluorescence across the contractile ring at each time point of the time-lapse series.

RESULTS

The Exocytic Machinery Localizes to the Bud Neck 3–4 Minutes before Cytokinesis Onset

To determine the precise timing of localization of the exocytic machinery to the site of cytokinesis (the bud neck), cells expressing GFP-fusion proteins were analyzed using time-lapse confocal microscopy. To obtain a baseline of cytokinetic events under our experimental conditions, cells expressing both Myo1-GFP (myosin II), under the native *MYO1* promoter and Tub1-GFP (α -tubulin), under the native *TUB1* promoter (Straight *et al.*, 1997), were analyzed. The signals of Myo1-GFP at the bud neck and Tub1-GFP along the spindle are distinct and separate, thus allowing for imaging of spindle breakdown, which indicates completion of mitosis, and contraction of the actomyosin ring, which indicates the onset of cytokinesis, in the same cell. Videomicroscopy was performed on cells in anaphase in an asynchronous culture, as indicated by their elongated spindles. Figure 1A (top) shows selected images from a representative time-lapse series (also see Supplemental Movie 1), where the spindle disassembled in less than a minute after the onset of actomyosin ring contraction (time point 4.5). Analysis of 10 time-lapse series showed that the average time separating the two events was 0.4 ± 0.3 min. The entire process of contraction took 4.7 ± 1.3 min (Figure 1B).

The above-mentioned analysis suggests that the onset of contraction and spindle breakdown are nearly concurrent, allowing the use of Tub1-GFP and spindle breakdown as a timing marker for the onset of cytokinesis. To determine the timing of exocytosis relocation to the bud neck relative to the onset of cytokinesis, cells expressing fusion proteins Sec3-GFP (under the native *SEC3* promoter) and Tub1-GFP were analyzed using time-lapse microscopy. Sec3 is a component of the exocyst complex thought to be important for marking the site

to which the rest of the complex localizes. Sec3 localizes to the bud tip during the early parts of the cell cycle then relocates to the bud neck late in mitosis (Finger *et al.*, 1998). To identify the timing of Sec3-GFP localization to the bud neck, cells from an asynchronous culture with elongated spindles but not yet showing bud neck localized Sec3 were chosen for time-lapse imaging. Localization of Sec3-GFP to the bud neck was defined as when the protein accumulated at the bud neck or formed a stable ring-like structure. Figure 1A (middle) and Supplemental Movie 2 show an example of the cells imaged, where Sec3-GFP arrived at the bud neck at time point 0.5, several frames before spindle breakdown at time point 5.5. Analysis of 10 time-lapse series demonstrates that the average time of arrival of Sec3-GFP to the bud neck occurred at 3.9 ± 1.9 min before spindle breakdown (Figure 1B).

Next, we determined the time of vesicle arrival at the bud neck. Sec4 is a Rab GTPase required for exocytosis and was previously used as a marker for detecting Golgi-derived vesicles *in vitro* and *in vivo*. yEGFP-Sec4 was expressed under the *YOP1* promoter carried on the CEN plasmid pRS315 (Schott *et al.*, 2002). As before, cells in anaphase were chosen for imaging where Sec4-GFP had not yet localized to the bud neck and the same criteria used for the localization of Sec3-GFP were applied for Sec4-GFP. As shown in Figure 1A (bottom) and Supplemental Movie 3, Sec4-GFP arrived at the bud neck at time point 0.5, also several frames before spindle breakdown (time point 4). Data from six time-lapse series showed that Sec4-GFP arrived at the bud neck 3.4 ± 1.0 min before spindle breakdown (Figure 1B). These results show that localization of exocytic vesicles as well as the complex required for vesicle fusion at the bud neck occurred 3–4 min before the onset of cytokinesis.

During bud formation, vesicles are targeted to the site of polarized growth through myosin V/actin cable-based transport (Pruyne *et al.*, 1998). To test whether this is also true for trafficking of cytokinesis-associated vesicles, we tested whether Sec4 accumulation at the bud neck was affected by *myo2-16*, a temperature-sensitive mutation in the cargo-binding domain of the major yeast myosin V, which has been shown to depolarize Sec4 from growing bud tips (Schott *et al.*, 1999). To see how the *myo2-16* mutation effects localization of Sec4-GFP specifically at cytokinesis, wild-type and mutant cells were synchronized in metaphase with nocodazole, a microtubule-depolymerizing drug, as described in the *Materials and Methods*. Sec4-GFP localization was analyzed in live cells in late anaphase (around 30–45 min after release from arrest) by fluorescent microscopy. Although bud neck localization of Sec4-GFP was not affected at the nonpermissive temperature of 37°C in wild-type cells, in *myo2-16* cells, Sec4-GFP did not accumulate at the bud neck in anaphase cells but instead occurred in punctuate structures throughout the cytoplasm (Figure 1C), as was seen previously in small-budded cells stained for Sec4 (Schott *et al.*, 1999). Therefore, vesicle trafficking to the cytokinesis site requires the actin cable/myosin V-based transport system.

Mitotic Exit Is Required for Targeting the Exocytic Pathway to the Site of Cell Division

Studies in sea urchin embryos suggested that targeting secretion to the cleavage furrow is dependent on the exit from mitosis, a cell cycle transition that requires degradation of the B-type mitotic cyclin (Shuster and Burgess, 2002). In budding yeast, the known events triggered by mitotic exit are spindle breakdown and onset of actomyosin ring contraction (Tolliday *et al.*, 2001). Because localization of Sec3 and Sec4 to the bud neck occurred several minutes before these events, we tested whether targeted secretion to the

Figure 1. The exocytic machinery arrives at the bud neck before cytokinesis. (A) Representative images of RLY1994 (Myo1-GFP, Tub1-GFP), RLY1996 (Sec3-GFP, Tub1-GFP), and RLY1997 (Sec4-GFP, Tub1-GFP) captured by time-lapse spinning disk confocal microscopy and shown as 2D projections from the collected z-series. Cells were chosen for imaging by the appearance of an elongated spindle (noted by *), indicating the cell is in anaphase (time point 0). To establish the timing of cytokinesis, actomyosin ring contraction was compared with spindle disassembly (top). Myo1-GFP is indicated by an arrow. Localization of Sec3-GFP and Sec4-GFP in the middle and bottom panels (denoted by the arrowheads) occurs before spindle disassembly, which is highlighted by a box drawn around the aligned images. Images were taken at 30-s intervals in all time-lapse series. Bars, 2 μ m. (B) Data compiled from all the time-lapse series show the timing of the localization of the exocytic machinery to the bud neck before contraction of the actomyosin ring and spindle disassembly. (C) RLY2000 and RLY2001 cells imaged 30–45 min after release from nocodazole arrest, at the nonpermissive temperature of 37°C. Bar, 5 μ m.

cytokinesis site is universally dependent on mitotic exit by examining the effect of blocking mitotic exit with a nondegradable mitotic cyclin (Clb2 Δ DB) on the localization of Sec3 and Sec4 to the bud neck. Clb2 Δ DB was previously shown to arrest cells in anaphase (Surana *et al.*, 1993). Cells expressing Sec3-GFP and Clb2 Δ DB under the control of the inducible Gal1 promoter, or the vector (pRL62, no Clb2 Δ DB) control, were cultured in YPR overnight and then shifted into YPGR media containing nocodazole. Under these conditions,

Clb2 Δ DB expression was induced while cells accumulated in metaphase. After 3 h, nocodazole was washed out, and cells were released into YPGR media. Samples were taken every 15 min, and live cells were quantified for localization of Sec3-GFP at the bud neck. As shown in Figure 2A, Sec3-GFP did not localize to the bud neck in cells expressing Clb2 Δ DB and arrested in anaphase as expected, whereas cells without Clb2 Δ DB exhibited Sec3-GFP localization to the bud neck as they underwent cytokinesis. The cells expressing

Figure 2. Mitotic exit is required for targeted exocytosis at the bud neck. RLY2066, RLY2065 (A) and RLY2003, RLY2005 (B) were arrested with nocodazole in YPGR media to induce expression from *GAL-CLBΔDB* or an empty *GAL* plasmid. After release from arrest, samples of the culture were taken every 15 min for viewing of live cells to count for Sec3-GFP or Sec4-GFP localization at the bud neck (■ and ●, left y-axis) and morphology (□ and ○, right y-axis) to determine cell cycle progression. Cells (200) were counted for each time point, and the averages from three experiments are plotted in A and B. (A) Images of RLY2002 (Sec3-GFP, Tub1-GFP) arrested from overexpression of *Clb2ΔDB* are shown to the right of the graph. (C) Images of Sec4-GFP in cells with the empty *GAL* plasmid (RLY2005) and *GAL-CLB2ΔDB* (RLY2003) in YPGR media (a), and WT (RLY2009) and *sec3-2* (RLY2008) at 37°C (b), all between 30 and 45 min after release from nocodazole arrest. Bars, 5 μm. RLY1840 (*wt*) and RLY1838 (*sec3-2*) were analyzed by EM 30 min after release from nocodazole arrest at 37°C (c). The cells are in anaphase as seen by the nuclei (n). Bars, 0.5 μm. (D) RLY2040, RLY2041, and RLY2042 were arrested with nocodazole in YPD and then elevated to 37°C before release from arrest. After release, samples were taken every 15 min to view Sec3-GFP localization at the bud neck. Cells were counted as in A and B.

Clb2ΔDB formed normal actin rings (our unpublished data), as also observed in other cells with mutations in mitotic exit regulators (Lippincott *et al.*, 2001; Luca *et al.*, 2001).

Similar experiments were carried out on cells expressing GFP-Sec4, Tub1-GFP, and *Clb2ΔDB*. After release from the nocodazole arrest, Sec4-GFP localization to the bud neck

was observed in cells with and without Clb2 Δ DB expression (Figure 2B). However, Sec4-GFP localization in the former occurred less frequently, did not seem as intense as in the control cells, and the signal was never seen as a full Sec4-GFP ring (Figure 2C, a). Because blocking mitotic exit prevented Sec3 localization to the bud neck, it was possible that the observed defect in Sec4 accumulation was directly due to a failure of vesicles to fuse with the plasma membrane, followed by vesicle dispersal. To test this possibility, we examined the effect of the *sec3-2* temperature-sensitive mutation on Sec4-GFP localization to the bud neck. *sec3-2* at the nonpermissive temperature blocks secretion and results in accumulation of vesicles in the cytoplasm (Finger and Novick, 1997). In anaphase *sec3-2* cells at the nonpermissive temperature, accumulation of Sec4-GFP at the bud neck was even more prominent than in wild-type cells: Sec4-GFP localized not in a compact region but to a wider area around the bud neck (Figure 2C, b). Electron microscopy of *sec3-2* cells in anaphase also showed that vesicles accumulated in a broad area around the bud neck, in contrast to wild-type cells (Figure 2C, c). This result suggests that the partial defect in Sec4-GFP localization observed in Clb2 Δ DB arrested cells was unlikely to be due to a fusion defect. Taken together, these results suggest that localization of the vesicle fusion machinery and, to a lesser extent, transport of vesicles to the bud neck require inactivation of Cdk1 at exit from mitosis.

The MEN inactivates mitotic Cdk1 by releasing the phosphatase Cdc14 from the nucleolus to activate APC^{Cdh1}, which degrades cyclin, and to promote the transcription and stabilization of the Cdk1 inhibitor Sic1 (Bardin and Amon, 2001). However, it was previously shown that the MEN has a separate control on cytokinesis apart from Cdk1 inactivation (Lippincott *et al.*, 2001; Luca *et al.*, 2001; Menssen *et al.*, 2001). To better understand the regulation of exocytosis in anaphase, we examined whether the localization of Sec3-GFP is directly controlled by the MEN. Here, we bypassed the block in mitotic exit caused by a temperature-sensitive MEN mutant, *mob1-77*, at the nonpermissive temperature by the overexpression of Sic1 from a high copy 2 μ plasmid. *mob1-77* cells at the nonpermissive temperature can form actomyosin contractile rings (Luca *et al.*, 2001). With Sic1 overexpression, cell cycle progression proceeded due to bypass of the mitosis block in *mob1-77* cells, but cytokinesis fails due to lack of contraction of the actomyosin ring, resulting in the formation of chains of cells (Luca *et al.*, 2001). *MOB1* 2 μ + *SIC1*, *mob1-77* + 2 μ *SIC1*, and *mob1-77* cells were arrested with nocodazole in metaphase. During arrest, cells were warmed to the nonpermissive temperature and then released from arrest. Samples were assayed every 15 min for visualization of Sec3-GFP localization at the bud neck. The results in Figure 2D show that the localization of Sec3-GFP to the bud neck occurred normally in *MOB1* + 2 μ *SIC1* and *mob1-77* + 2 μ *SIC1* cells, in contrast to that in *mob1-77* cells, which arrested in anaphase and were unable to localize Sec3-GFP. Hence, targeted exocytosis at the bud neck is dependent on Clb2 degradation but not on the MEN.

Vesicle Transport and Fusion Are Necessary for Successful Cell Division

Having established that vesicle targeting and localization of the exocyst are events that immediately precede cytokinesis, we wanted to test whether these events are required for the completion of cytokinesis. Temperature-sensitive *myo2* (myosin V), *sec3*, *sec10* mutations and a dominant negative *SEC4* (Sec4N34) construct were used. Because these mutations also block bud growth, cells were first synchronized in metaphase by nocodazole treatment at the permissive temperature to allow bud growth and then released from the arrest

Figure 3. Disruption of vesicle transport to or fusion at the plasma membrane blocks completion of cytokinesis, but it does not disrupt the cell cycle. (A) RLY1838, RLY1840, RLY1900, RLY1901, RLY1998, RLY1999, RLY2006, and RLY2007 were analyzed for their effect on cytokinesis. Cells were arrested with nocodazole, switched into nonpermissive conditions, and then released from the arrest. Fixed cells were zymolyase treated and were scored at the time of release from the arrest and 2 h after the release for completion of cytokinesis. (B) Localization of Cdc14-GFP was used to show completion of mitotic exit. RLY2012 and RLY2013 cells were lightly fixed in formaldehyde, stained for DAPI to monitor cell cycle, and Cdc14-GFP release to the cytoplasm from the nucleolus was quantified.

into the nonpermissive temperature (for testing dominant negative Sec4, the cells were arrested with nocodazole and released into media, allowing expression of Sec4N34). The percentage of cells that completed cytokinesis was determined as the reduction of the large-budded population over time. Because we were specifically interested in cytokinesis as opposed to cell separation, the cells were fixed and the cell wall removed by zymolyase treatment before scoring (Lippincott and Li, 1998b). The values presented in Figure 3A represent the percentage of decrease in the large-budded population from 0 to 2 h after release from the nocodazole arrest under nonpermissive conditions. In the wild-type culture, ~60% of the cells divided 2 h after release, compared with <10% in various mutants, suggesting that most of the mutant cells were unable to undergo cytokinesis. Chromosome segregation in these cells, however, occurred normally as seen by staining cells with DAPI after release from the nocodazole arrest (our unpublished data).

It was possible, however, that blocking secretion activates a cell cycle checkpoint response that prevents mitotic exit. To test whether this were true, we examined the release of Cdc14 from the nucleolus, a critical event downstream of the MEN (Bardin and Amon, 2001). Wild-type and mutant cells

Figure 4. Actomyosin rings form in the absence of membrane addition at the bud neck. RLY1838, RLY1840, RLY1998, RLY1999, RLY2006, and RLY2007 were synchronized and subjected to the nonpermissive conditions as described in text. Samples were taken every 15 min after release from arrest and fixed with formaldehyde. Fixed cells were stained with rhodamine-conjugated phalloidin and counted for the number of actin rings seen at the bud neck. Images of RLY1838, RLY1999, and RLY1840 stained with rhodamine phalloidin at the 30-min time point are shown to the right of the graphs. Bar, 5 μ m.

were transformed with a plasmid that expressed GFP-tagged Cdc14 under the native *CDC14* promoter (Cdc14-5 $\times$ GFP) (Yoshida *et al.*, 2002). Previous studies showed that Cdc14 is initially released from the nucleolus at early anaphase through the FEAR network, maintained in the cytosol during late anaphase by the MEN, and then returned to the nucleolus in the next G₁ (Bardin and Amon, 2001). Cdc14-5xGFP cells were released from the nocodazole arrest, and samples were taken every 15 min after release and prepared for visualization of the GFP signal and DNA by DAPI staining. Cdc14-GFP localization was quantified for each time point, and the results are shown in Figure 3B. In *SEC3* cells, Cdc14 resides in the nucleolus in metaphase cells, released into the cytoplasm in anaphase cells at the ~30-min time point, and returned to the nucleolus ~60 min. The same timing of Cdc14 release and resequestration was observed in *sec3-2* cells at the nonpermissive temperature, suggesting that the mutation did not block cytokinesis by preventing mitotic exit. Together, these results suggest that disruption of vesicle transport and fusion prevents the completion of cytokinesis.

Disruption of Vesicle Fusion Affects the Stability of the Actomyosin Ring during Cytokinesis

Another possible explanation for the effect of vesicle transport and fusion mutations on cytokinesis was that the secre-

tory pathway is required for the assembly of the actomyosin ring. In particular, previous work showed that the formation of the actin ring is dependent upon the activity of Rho1, which activates formin family actin nucleators (Tolliday *et al.*, 2002). Rho1 is a membrane-associated GTPase shown to interact with the exocytic machinery (Guo *et al.*, 2001), and it might require membrane-based transport system for its localization and function. To test this possibility, we tested whether the mutations that disrupt vesicle targeting and fusion affect the assembly of the actin ring. Again, cells were released from the nocodazole arrest into the nonpermissive temperature. Samples of cells were taken every 15 min, fixed, and stained with rhodamine-phalloidin. As shown in Figure 4, *sec10-2*, *sec3-2*, and *myo2-16* (myosin V) mutant cells had no defect in actin ring formation, compared with their congenic wild-type strains. Similarly, Myo1 (myosin II) localization to the bud neck also was unaffected in these mutants (Figure 5). Therefore, disruption of vesicle transport and fusion do not affect the formation of the contractile ring.

Next, we asked whether the exocyst mutations affect the contraction of the actomyosin rings. This might be expected if actomyosin ring contraction could not overcome the membrane tension in the absence of vesicle addition. To analyze ring contraction, time-lapse confocal microscopy was carried

Figure 5. Abnormal actomyosin ring contraction in exocyst mutants. RLY1898, RLY1899, RLY2010, and RLY2011 were treated with nocodazole at the nonpermissive temperature (37°C) as discussed in text. At the time of release, cells were prepared for time-lapse imaging, at 37°C. Imaging began as cells reached anaphase, indicated by the appearance of an elongated spindle (time 0). (A) Still images from examples of three time-lapse series produced from reconstruction of z-images of each time point. Initiation of cytokinesis is noted by contraction of the actomyosin ring (arrow) and disassembly of spindle (*). Contraction proceeds until the Myo1-GFP signal is seen as a point in WT cells (top) or as a smaller band in exocyst mutants (middle and bottom), before disappearing altogether. Bars, 2 μ m. (B) Kymographs of the Myo1-GFP signal for WT, *sec3-2*, and *sec10-2* cells. Each horizontal line of the kymograph represents each time point of the image series, derived from a line drawn across the Myo1-GFP signal (shown in drawing).

out on wild-type, *sec3-2*, or *sec10-2* cells expressing Myo1-GFP and Tub1-GFP (α -tubulin). The cells were released from nocodazole arrest at 37°C after a 30-min incubation at 37°C. The cells were then transferred to an imaging platform enclosed in a 37°C chamber. Figure 5A (top) and Supplementary Movie 4 show a typical example of ring contraction in a wild-type cell where contraction continued until Myo1-GFP signal narrowed to a point near the center of the bud neck, which subsequently disappeared. Ring contraction was visibly different in *sec3-2* and *sec10-2* mutant cells. Typ-

ical examples are shown in Figure 5A (middle and bottom) and Supplemental Movies 5 and 6, where contraction started with normal timing, i.e., concurrent with spindle disassembly, however, ring disassembly occurred before the contraction was complete. This difference was better depicted in kymographs where fluorescence profiles across the 3D projections of the Myo1 ring of all frames of the movie were compiled into one image, starting $\sim$ 2 min before the initiation of contraction (Figure 5B). There was no difference between the average size or intensity of the Myo1-GFP ring in the wild-type and mutants at the beginning of each time-lapse series (Table 2). In wild-type cells, the contraction phase of the kymograph was centered and cone-shaped with a pointy apex, whereas those in the mutants had flat, and sometimes off-centered apexes. Table 2 shows that the average width of the apex in the wild-type was $\sim$ 1.5 times smaller than that of *sec3-2* and *sec10-2* cells before complete ring disassembly. *t* tests showed that the differences in apex width between wild-type and *sec3-2* ($t = 0.016$) and between wild-type and *sec10-2* ($t = 0.002$) were significant. Therefore, the mutations that block vesicle fusion led to instability of the contractile ring during cytokinesis.

Table 2. Parameters of actomyosin ring contraction in WT and exocyst mutants

	Length before contraction (μ m)	Length of last-seen Myo1-GFP (μ m)	No. of cells
<i>SEC3</i>	1.85 ± 0.3	0.61 ± 0.2	6
<i>sec3-2</i>	1.88 ± 0.2	0.94 ± 0.3	22
<i>SEC10</i>	1.70 ± 0.2	0.61 ± 0.2	11
<i>sec10-2</i>	1.74 ± 0.4	0.86 ± 0.2	27

Chs2 Is an Exocytic Cargo Required for Contractile Ring Stability

One possible explanation for the instability of the actomyosin ring in the exocyst mutants could be failed delivery of a membrane-bound stabilization factor. Of all proteins known to localize to the bud neck during anaphase, Chs2, a transmembrane protein synthesized and localized to the bud neck late in the cell cycle (Choi *et al.*, 1994; Chuang and Schekman, 1996) is a likely candidate. The activity of Chs2 is required for the production of the primary septum that is laid down behind the contractile ring during cytokinesis (Shaw *et al.*, 1991), and $\Delta chs2$ mutation phenocopies $\Delta myo1$ mutation (Schmidt *et al.*, 2002). To test this possibility, we first examined the precise timing of Chs2 delivery to the bud neck. By time-lapse imaging of cells expressing Chs2-GFP (under the native *CHS2* promoter on the CEN plasmid, pRL73; Tolliday *et al.*, 2003) and Tub1-GFP (α -tubulin), we found that Chs2-GFP arrives at the bud neck 2.4 ± 0.8 min before spindle breakdown, which was slightly later than Sec3 and Sec4 localization (Figure 1). Further analysis of the relative intensity (as percentage of the maximum intensity) of the Chs2-GFP signal showed that in seven of 10 cells recorded, Chs2-GFP at the bud neck peaked after spindle disassembly, indicating that delivery of Chs2-GFP to the plasma membrane continued after contraction initiation (Figure 6A). In the other three cells, Chs2-GFP intensity peaked just before or at the time of spindle disassembly. This result coincides with fluorescence recovery after photobleaching data of Chs2, which showed rapid recovery, consistent with continued delivery to the bud neck (Dobelaere and Barral, 2004).

The delivery of Chs2 to the bud neck was shown not to be dependent on the chitosome, the organelle that targets the other chitin synthases to the plasma membrane (Chuang and Schekman, 1996). To determine whether Chs2 is delivered via Golgi-derived vesicles and the actin cable/myosin V-based transport system, Chs2-GFP localization was analyzed in wild-type and *myo2-16* cells. Cells cultured overnight at room temperature were arrested in media containing nocodazole and then released from the arrest at the nonpermissive temperature of 37°C. Samples were taken every 15 min and quantified for Chs2-GFP localization at the bud neck. In *myo2-16* cells, most Chs2 localized to punctate structures distributed in both the mother and bud (Figure 6B), a pattern similar to the localization of Sec4-GFP in *myo2-16* cells (Figure 1C). This suggests that Chs2 is delivered to the bud neck using the actin cable–myosin V-based transport pathway.

Using the same procedure as described above, we also examined the effects of the *sec10-2* mutation on Chs2 localization at the nonpermissive temperature. Chs2-GFP could localize to the bud neck in *sec10-2* cells, at a slightly lower rate (Figure 6C). However, instead of localizing to a tight band at the bud neck as observed in wild-type cells, Chs2 localizes broadly at the bud neck of the mutant cells (Figure 6, C and D, and Supplemental Movies 7 and 8). Similar changes in localization of other vesicle-associated proteins have been observed in other exocyst mutants (Finger *et al.*, 1998; Figure 2B). To further characterize this abnormal localization, cells from the 45-min time point after nocodazole washout were observed using a spinning disk confocal microscope. A stack of images for each cell were collected along the z-axis and were deconvolved to produce 3D images of Chs2-GFP localization in wild-type and *sec10-2* mutant cells (Figure 6D and Supplemental Movies 7 and 8). Chs2-GFP clearly localized to a ring in the bud neck plasma

membrane in the wild-type, whereas in *sec10-2* cells, Chs2-GFP localizes to the interior of the bud neck in a dispersed pattern, suggesting that Chs2-GFP was not incorporated in the plasma membrane after being delivered to the bud neck. Together, these results demonstrate that Chs2 is deposited at the bud neck before and during actomyosin ring contraction in a myosin V- and exocyst-dependent manner.

We next asked whether Chs2 was acting as a stability factor during the contraction of the actomyosin ring. To analyze the role of Chs2 in actomyosin ring contraction, we used a strain with a deletion of *CHS2* (*chs2* Δ) expressing Myo1-GFP for time-lapse microscopy. *chs2* Δ cells are viable and able to divide but do so inefficiently by the formation of aberrant secondary septa (Shaw *et al.*, 1991). We imaged Myo1 ring contraction in *chs2* Δ cells, along with wild-type and *sec10-2* cells. In these experiments we imaged the Myo1-GFP ring that happened to be in a plane parallel or diagonal to that of the slide. This allowed clear visualization of ring shrinkage during contraction in wild-type cells (Figure 7 and Supplemental Movie 9; Bi *et al.*, 1998). In contrast, in both the exocyst mutant *sec10-2* and the *chs2* Δ cells, the ring abruptly broke at one or two points at the time of cytokinesis and then disassembled in a zipping manner from the broken ends (Figure 7 and Supplemental Movies 10–12). The previous movies (Figure 5 and Supplemental Movies 5 and 6) with the ring in a plane perpendicular to the slides would not have distinguished between this end-directed disassembly from contraction. This result suggests that in $\Delta chs2$ and *sec10* mutant cells, the actomyosin ring does not contract but instead breaks and then rapidly disassembles.

DISCUSSION

Spatially and temporally precise insertion of new membrane at the cleavage furrow has been a well documented phenomenon since the 1970s (Bluemink and de Laat, 1973); however, understanding the molecular basis of this fascinating cellular process has only recently become a focus of the cytokinesis field. Above, we described the characterization of targeted secretion during budding yeast cytokinesis. We show that secretion is targeted to the site of cell division at a precise stage in the cell cycle and is negatively regulated by the mitotic kinase Cdk1. This targeted secretion is mediated through an actin/myosin V-based transport system and the plasma membrane-associated exocyst complex. Inhibition of membrane trafficking during mitosis blocks the completion of cytokinesis, and this effect correlates with a decreased stability of the actomyosin ring during the contraction phase. Further analysis suggested that the membrane protein Chs2, a chitin synthase, is an essential stability factor for the contractile ring. These results establish budding yeast as a model for understanding the regulation and mechanistic roles of membrane trafficking during cytokinesis.

Regulation of Membrane Trafficking during Cytokinesis

Data from studies of embryonic cell divisions clearly indicated that membrane addition to the equatorial region is a late cell cycle event that occurs independently of the contractile ring activity (Bluemink and de Laat, 1973; Drechsel *et al.*, 1997; Shuster and Burgess, 2002). Previous work in yeast also has suggested that the secretory pathway, oriented early in the cell cycle toward the growing bud tip, is targeted to the bud neck region late in the cell cycle (Finger *et al.*, 1998). It was unclear, however, whether this event is associated with cytokinesis or with the process of septum formation and resolution, which follows the closure of the

Figure 6. Chs2 is targeted to the plasma membrane of the bud neck before cytokinesis. (A) Relative intensity profiles of three representative time-lapse series of RLY2062 (Tub1-GFP, Chs2-GFP). Ten cells in total were imaged by acquisition with a spinning disk confocal microscope to provide 2D projection movies, where the intensity of Chs2-GFP was measured for each time point. Time 0 defines spindle disassembly. RLY2060, RLY2061 (B), and RLY2062, RLY2063 (C) expressing Chs2-GFP (pLP31) (Tolliday *et al.*, 2003) were arrested with nocodazole at 25°C. Once arrested, cultures were shifted to 37°C for 30 min and then released from arrest. Samples were taken every 15 min, lightly fixed in 1% formaldehyde for 30 min, and then visualized for Chs2-GFP localization at the bud neck. Examples of the 30-min time points are shown below the graphs. Bars, 5 μ m. (D) Samples from the 45-min time point from C were imaged with a spinning disk confocal microscope, deconvolved, and reconstructed to produce 3D images. Bars, 2 μ m.

plasma membrane. We have observed that localization of the exocyst component Sec3, and the post-Golgi vesicle-associated Rab, Sec4, to the bud neck occurs 3–4 min before the onset of actomyosin ring contraction. The membrane protein Chs2 is deposited at the bud neck through the secretory pathway just before and during the actomyosin ring

contraction. These results suggest that vesicle transport and fusion at the bud neck occur just before and during cytokinesis.

Transport of secretory vesicles toward the site of polarized growth during bud formation is mediated by the actin cable and myosin V-based cytoskeletal system (Pruyne *et al.*,

Figure 7. Chs2 is required for proper actomyosin ring contraction. Time-lapse images of RLY1450, RLY2011, and RLY2064 were captured with a spinning disk confocal microscope at room temperature, with the exception of RLY2011, which was treated and imaged as discussed for Figure 5. Cells were seen in planes that allowed visualization of actomyosin rings from above. Five planes were captured for every 30-s time point, which encompasses the ring. Still images of example cells are excerpts of time-lapse series, including all the time points from before initiation of contraction or disassembly of the actomyosin ring through to completion. The still images were produced from reconstruction of the z-images for each time point. Arrows indicate the direction of the zippering motion for the mutants. Bars, 2 μ m.

1998). Thus, it is not surprising that delivery of secretory vesicles, marked with GFP-Sec4, to the bud neck during cytokinesis also is achieved through this transport system. An interesting question is how this transport system reorients late in mitosis. Here, two events are likely to be important: 1) disassembly of bud tip-oriented actin cables at mitotic entry; and 2) assembly of bud neck-directed cables at mitotic exit. It is interesting to note that the peak for the assembly of bilateral arrays of actin cables around the bud neck occurs rather late during the cell division process, i.e., after disassembly of the contractile ring (Tolliday *et al.*, 2002). However, a few bud neck-oriented cables can be found even before mitotic exit, which could be important for the initial targeting of Sec4-positive vesicles toward the bud neck. Although it is thought that in most metazoan cell types, furrow-bound vesicles are transported mostly along microtubules, there is no definitive evidence ruling out an involvement of actin-based transport mechanisms. Most studies looking at vesicle transport used cytochalasin D (Bluemink and de Laat, 1973; Shuster and Burgess, 2002), a drug that does not always result in loss of actin filaments (Kolega *et al.*, 1991). It is possible that actin plays a role in short-range deposition of secretory vesicles and recycled endocytic vesicles within the cleavage furrow.

It is significant that targeted secretion and actomyosin ring contraction seem to be regulated by different cell cycle pathways (Figure 8A). Previous work showed that the MEN possibly controls contractile ring activation in two ways: 1) inactivation of the mitotic Cdk1 (Bardin and Amon, 2001); and 2) activation of actomyosin ring contraction without involving Cdk1 (Lippincott *et al.*, 2001; Luca *et al.*, 2001; Menssen *et al.*, 2001). The latter was suggested by the observations that the MEN mutants still exhibited a cytokinesis block with a fully assembled contractile ring even when mitotic Cdk1 was inactivated. In fact, mitotic Cdk1 may indirectly inhibit contractile ring activation by inhibiting the MEN. The cell cycle control of cytokinesis-specific secretion, on the other hand, seems to be the opposite: whereas the MEN does not seem to be directly required for localizing the exocyst (Sec3) to the bud neck, nondegradable Clb2 completely blocks the localization of Sec3 and partially prevented targeting of vesicles (Sec4) to the bud neck. This finding is surprisingly consistent with how cytokinetic events are controlled in dividing sea urchin zygotes. It was found that whereas contractile ring activation, as indicated by the ingression of the cleavage furrow, was not strictly dependent on mitotic cyclin degradation, secretion of the extracellular matrix protein hyalin was completely blocked by nondegradable cyclin (Shuster and Burgess, 2002). These findings suggest that, despite the morphological and size differences between budding yeast and sea urchin zygotes, as well as the difference between their transport pathways (microtubules vs. actin-based), the mechanisms by which major cytokinetic events are controlled during the cell cycle seem to be more conserved than one might expect.

The Role of Exocytosis during Cytokinesis

In plant cells, new membrane addition is obviously important for cytokinesis; fusion of vesicles leads to the growth of a cell plate that divides one cell into two (reviewed in Field *et al.*, 1999). When considering why new membranes might be needed for cytokinetic processes that involve actomyosin-driven furrow formation, two possible roles have been discussed (Finger and White, 2002), one mechanical and one biochemical. A mechanical role for membrane insertion could prevent the buildup of membrane tension generated during ingression of the cleavage furrow due to an increase

Figure 8. (A) Cell cycle control of cytokinetic events. Contractile ring activation is controlled directly by the MEN, which promotes CDK1 inactivation but also is inhibited by CDK1. Targeted secretion at cell division site requires CDK1 inactivation but not the MEN. (B) Model for the role of exocytic delivery of Chs2 in cytokinesis. At cytokinesis onset, the septin hourglass, which serves as a scaffold for the actomyosin ring, splits into two separate rings sandwiching the contractile ring. Deposition of Chs2 through vesicles fusion at the bud neck has a necessary stabilizing effect on the actomyosin ring during contraction. CW, cell wall; PM, plasma membrane.

in surface area. A biochemical role could lead to changes in the molecular composition at the cleavage site through delivery of membrane bound factors such as specific lipids and proteins. These factors could be important for force generation by the contractile ring or could facilitate the final membrane fusion that disconnects the two progeny cells. Recent work suggested that membrane trafficking could play a role in the formation of lipid raft domains in the cleavage furrow. Raft components are present in isolated midbodies (Skop *et al.*, 2004) and disruption of these domains impaired cytokinesis (Wachtler *et al.*, 2003).

For a yeast cell, where the bud neck is already very narrow at the onset of cytokinesis (1–2 μm in diameter), cell division does not involve a drastic increase in surface area. It remains possible, however, that the turgor pressure on yeast plasma membrane (Harold, 1990) is too strong to be overcome by the contractile force provided from the actomyosin ring alone. From our results, it seems that membrane insertion definitely performs a biochemical role because the addition of Chs2 by vesicular fusion is required for the stability of the actomyosin ring during the contraction phase. A recent study by Dobbelaere and Barral (2004) found that the rate of Myo1 ring contraction was significantly slowed in *sec3-4* cells compared with the wild-type (Dobbelaere and Barral, 2004). These differences could be

due to a difference in strain background, mutant alleles, or experimental conditions in the two studies. We note, however, that even in the single contraction kymograph shown in that study, the intensity of the Myo1 ring declined faster in the *sec3-4* mutant than in the wild-type (Dobbelaere and Barral, 2004), suggesting that the stability of the Myo1 ring also might be affected. Additionally, without imaging the contractile ring parallel or diagonal to the plane of the slide, it can be difficult to distinguish between contraction and disassembly.

The yeast Myo1 (myosin II) ring forms early in the cell cycle, and its assembly and stabilization require the septin hourglass-like structure that serves as a scaffold for many bud neck-associated components (Bi *et al.*, 1998; Lippincott *et al.*, 2001). At the onset of cytokinesis, the septin hourglass splits into two rings that sandwich the now mature contractile ring (Lippincott *et al.*, 2001). Chs2 could be deposited at this point to assume the septins' role in contractile ring stabilization and also may be important for tethering the contractile ring to the plasma membrane for effective force generation (Figure 8B). Presently, we do not know whether the function of Chs2 in contractile ring stabilization is mediated through a direct interaction of its cytoplasmic domain with the contractile ring or through its enzymatic activity that leads to chitin deposition. The newly deposited chitin

could serve as an extracellular scaffold that recruits a factor that stabilizes the contractile ring; or alternatively, reinforcement of the furrowing plasma membrane by chitin, together with insertion of new membrane, could ease the tension on the actomyosin ring and prevent its breakage. A defect in contractile ring stability could explain the late cytokinesis failure observed in *C. elegans* embryos treated with brefeldin A (Skop *et al.*, 2001). Premature disassembly of the contractile ring could result in regression of the cleavage furrow due to tension in the plasma membrane. Clearly, Chs2 is a yeast specific factor for stabilization of the contractile ring, but proteins with a function linking the contractile ring with the extracellular matrix may exist in animal cells.

ACKNOWLEDGMENTS

We thank C. Kaiser, A. Bretscher, R. Collins, F. Luca, A. Toh-e, E. Cabib, and N. Tolliday for providing strains and plasmids. We are grateful to J. Waters and the Nikon Imaging Center at Harvard Medical School for being very helpful with confocal microscopy, image acquisition, and analysis, and to M. Ericsson for help with electron microscopy. We also thank J. Hastings and A. DeGiacomo for technical assistance, and I. Lister, P. Marina Losada, and C. Field for critical reading of the manuscript and helpful discussions. This work is supported by NIH grant GM-059964 (to R. L.).

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