

Role of Cell Cycle-regulated Expression in the Localized Incorporation of Cell Wall Proteins in Yeast[□]

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The yeast cell wall is an essential organelle that protects the cell from mechanical damage and antimicrobial peptides, participates in cell recognition and adhesion, and is important for the generation and maintenance of normal cell shape. We studied the localization of three covalently bound cell wall proteins in *Saccharomyces cerevisiae*. Tip1p was found only in mother cells, whereas Cwp2p was incorporated in small-to-medium-sized buds. When the promoter regions of *TIP1* and *CWP2* (responsible for transcription in early G₁ and S/G₂ phases, respectively) were exchanged, the localization patterns of Tip1p and Cwp2p were reversed, indicating that the localization of cell wall proteins can be completely determined by the timing of transcription during the cell cycle. The third protein, Cwp1p, was incorporated into the birth scar, where it remained for several generations. However, we could not detect any role of Cwp1p in strengthening the birth scar wall or any functional interaction with the proteins that mark the birth scar pole as a potential future budding site. Promoter-exchange experiments showed that expression in S/G₂ phase is necessary but not sufficient for the normal localization of Cwp1p. Studies of mutants in which septum formation is perturbed indicate that the normal asymmetric localization of Cwp1p also depends on the normal timing of septum formation, composition of the septum, or both.

INTRODUCTION

The establishment and maintenance of asymmetry are of vital importance for the growth, differentiation, and morphogenesis of cells and organisms. For example, the yeast *Saccharomyces cerevisiae* grows asymmetrically, producing a bud that becomes the daughter cell (reviewed by Pringle *et al.*, 1995; Lew and Reed, 1995; Pruyne and Bretscher 2000a,b). In the G₁ phase of the cell cycle, a bud site is selected in a mating type-dependent manner. After the cell cycle-commitment point START, proteins required for bud initiation are recruited to this site, and a bud is formed in a process that depends largely on the delivery of vesicles containing new cell surface material by the polarized actin cytoskeleton. Growth occurs exclusively in the bud, first preferentially at the tip and later isotropically, until the time of cytokinesis. The actin cytoskeleton and cell surface growth are then redirected toward the mother-bud neck, where a chitinous primary septum is formed, followed by the deposition of secondary septa on both sides of the pri-

mary septum. Cells then separate by partial digestion of the primary septum, and a new cell cycle is initiated.

The cell wall of yeast is essential for growth and is involved in the establishment and maintenance of asymmetry, as illustrated by the loss of both buds and polarized localization of cytoskeletal proteins when the cell wall is removed (Reck-Peterson *et al.*, 1999). The cell wall is comprised of glucans, mannoproteins, and a small amount of chitin (reviewed by Orlean, 1997; Smits *et al.*, 2001; Klis *et al.*, 2006). The mannoproteins can be divided into four classes: 1) noncovalently linked proteins, such as Bgl2p (Klebl and Tanner, 1989; Cappellaro *et al.*, 1998); 2) proteins that are linked covalently to the cell wall through disulfide bridges (Orlean *et al.*, 1986; Moukadiri *et al.*, 1999); 3) PIR-proteins, which are linked via an alkali-sensitive bond to β -1,3-glucan (Kapteyn *et al.*, 1999b; Castillo *et al.*, 2003; Ecker *et al.*, 2006); and 4) a set of \sim 40 glycosylphosphatidylinositol (GPI)-dependent cell wall proteins (Caro *et al.*, 1997; Hamada *et al.*, 1998; Yin *et al.*, 2005), which are covalently linked to β -1,3-glucan through β -1,6-glucan (Kapteyn *et al.*, 1996; Kollár *et al.*, 1997) and may or may not also be linked directly to β -1,3-glucan through an alkali-sensitive bond (Kapteyn *et al.*, 2001). In general, the cell wall proteins are thought to protect the cell from the environment, and deletions of some cell wall protein genes with homologies to glycoside hydrolases produce the expected kinds of phenotypes (Yin *et al.*, 2005). However, deletions of other genes encoding individual cell wall proteins, even those that are highly expressed, often have surprisingly little effect (van der Vaart *et al.*, 1995; Hagen *et al.*, 2004).

Cell wall proteins are processed through the secretory pathway, where they are O- and often also N-glycosylated and may receive a GPI anchor at their carboxy terminus. When GPI-

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Abbreviations used: CFW, calcofluor white; DOC, sodium deoxycholate; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; ORF, open reading frame; TCA, trichloroacetic acid.

Table 1. Strains used in this study

| Strain | Relevant genotype | Source |
|-----------|---|--------------------------------|
| FY833 | <i>MATa his3-Δ200 leu2-Δ1 lys2Δ202 trp1-Δ63 ura3-52</i> | Winston <i>et al.</i> (1995) |
| JV96 | As FY833 except <i>cwp1::LEU2</i> | Vossen <i>et al.</i> (1997) |
| YEF473 | <i>MATa/α his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52</i> | Bi and Pringle (1996) |
| YEF473A | <i>MATa his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52</i> | Segregant from YEF473 |
| M-272 | As YEF473 except <i>gin4-Δ9/gin4-Δ9</i> | Longtine <i>et al.</i> (1998b) |
| M-717 | As YEF473 except <i>bni1Δ::HIS3/bni1Δ::HIS3</i> | M. Longtine ^a |
| ICY018 | As YEF473 except <i>she3Δ::His3MX6/she3Δ::His3MX6</i> | See text |
| ICY028 | As YEF473 except <i>chs2Δ::His3MX6/chs2Δ::His3MX6</i> | See text |
| KBY1012 | <i>MATa his3 leu2 lys2 ura3-52 myo4::ura3</i> | Beach and Bloom (2001) |
| DDY172-2A | As YEF473A except <i>chs4-Δ1</i> | DeMarini <i>et al.</i> (1997) |
| DDY181 | As YEF473 except <i>CHS3/chs3-Δ1</i> | DeMarini <i>et al.</i> (1997) |
| DDY181-2D | As YEF473A except <i>chs3-Δ1</i> | Segregant from DDY181 |
| KNY1048 | As YEF473 except <i>chs6Δ::TRP1/chs6Δ::TRP1 bud7Δ::TRP1/bud7Δ::TRP1 ymr237wΔ::kanMX6/ymr237wΔ::kanMX6 ykr027wΔ::His3MX6/ykr027wΔ::His3MX6</i> | K. Nakashima ^b |
| AM273 | As YEF473 except <i>axl2Δ::His3MX6</i> | A. McKenzie ^b |
| AM476 | As YEF473 except <i>rax2Δ::His3MX6/rax2Δ::His3MX6</i> | A. McKenzie ^b |
| AM503 | As YEF473 except <i>rax1Δ::kanMX6/rax1Δ::kanMX6</i> | A. McKenzie ^b |
| AM775 | As YEF473 except <i>chs5Δ::TRP1/chs5Δ::TRP1</i> | A. McKenzie ^b |
| LSY252 | As YEF473A except <i>cwp1Δ::His3MX6</i> | See text |
| LSY265 | As YEF473 except <i>cwp1Δ::His3MX6/cwp1Δ::His3MX6</i> | See text |
| YHH415 | As YEF473 except <i>bud8-Δ1/bud8-Δ1</i> | Harkins <i>et al.</i> (2001) |
| YHH615 | As YEF473 except <i>bud9-Δ1/bud9-Δ1</i> | Harkins <i>et al.</i> (2001) |

^a Constructed by mating strains YJZ426 and YJZ427 (Harkins *et al.*, 2001).

^b Nakashima, McKenzie, and Pringle, unpublished data. The strains have complete deletions of the indicated ORFs.

dependent cell wall proteins arrive at the plasma membrane, the GPI anchor is thought to be processed, resulting in its cleavage and linkage of the protein to the β -1,6-glucan through a GPI remnant (Kollár *et al.*, 1997; Lipke and Ovalle, 1998; Kapteyn *et al.*, 1999a; Terashima *et al.*, 2003).

Interestingly, more than half of the genes encoding cell wall proteins in *S. cerevisiae* are transcribed in a cell cycle-dependent manner (Caro *et al.*, 1998; Spellman *et al.*, 1998), compared with only 13% for the genome as a whole. This suggests that many cell wall proteins may be produced only when needed during the different stages of bud formation and cell division. Because of the rearrangements of the actin cytoskeleton during the cell cycle (see above), cell cycle-specific synthesis of a cell surface protein can also lead to its localized incorporation at discrete regions of the cell surface, as shown by the integral plasma membrane proteins Axl2p, Bud8p, and Bud9p (Lord *et al.*, 2000; Schenkman *et al.*, 2002). Consistent with this model, the GPI-dependent cell wall protein Crh1p seems to be synthesized in late G₁ and then incorporated specifically at the presumptive bud site and in the surface of the emerging small bud (Spellman *et al.*, 1998; Rodríguez-Peña *et al.*, 2000). However, cell cycle-specific synthesis does not seem to be the only mechanism by which cells can achieve the localized incorporation of cell wall proteins. For example, the GPI-dependent cell wall protein Crh2p seems to be synthesized throughout the cell cycle and yet is localized primarily to a ring at the base of the bud by a mechanism that depends on a number of factors, including the septin ring at that location (Spellman *et al.*, 1998; Rodríguez-Peña *et al.*, 2000, 2002).

To investigate further the patterns and mechanisms of incorporation of GPI-dependent proteins into the cell wall, we tagged three different proteins with green fluorescent protein (GFP). All three proteins seemed to be incorporated into specific regions of the wall, and further investigation suggested that timed transcription during the cell cycle is

involved in this localized incorporation. In addition, our studies suggest that incorporation of one of these proteins, Cwp1p, specifically into the birth scar also depends on the formation of a normal septum at the proper time.

MATERIALS AND METHODS

Strains, Growth Conditions, and Genetic Procedures

S. cerevisiae strains used in this study are listed in Table 1, and PCR primers are listed in Supplemental Table 1. Yeast cells were grown in YPD or appropriate selective media (Adams *et al.*, 1996) at 28°C unless otherwise specified. Growth was routinely monitored by optical density (OD)₆₀₀ measurements using spectrophotometers in their linear ranges (OD₆₀₀ < 0.4; cultures were diluted before reading when necessary) and calibration curves relating OD₆₀₀ to cell number. For mating, sporulation, tetrad dissection, and transformation, standard procedures were used (Adams *et al.*, 1996). Molecular genetic procedures were also standard (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995) except where noted. Strains containing complete deletions of the *CWP1*, *CHS2*, and *SHE3* open reading frames (ORFs) in the YEF473 background were constructed by the PCR method (Baudin *et al.*, 1993); plasmid pFA6a-His3MX6 (Longtine *et al.*, 1998a) was used as template with primers LS137 and LS138 (*CWP1*), ML176 and ML177 (*CHS2*), or IC101 and IC102 (*SHE3*). In all cases, transformants were checked for the desired chromosomal insertions using PCR with other primer pairs including LS139 and LS144 (*CWP1*), ML176 and ML178 (*CHS2*), or IC103 and 5'TTEFCHK (*SHE3*).

Plasmids

Multiplicity plasmids expressing GFP-Cwp1p from the *CWP1* promoter (pAR213) or GFP-Cwp2p from the *CWP2* promoter (pAR205) were described previously (Ram *et al.*, 1998a). Both constructs were subcloned into YCplac33 (Gietz and Sugino, 1988) using HindIII and BamHI for pAR213 and HindIII and EcoRI for pAR205, creating the low-copy plasmids pGS-GFP-CWP1-low and pGS-GFP-CWP2-lowR, respectively. The orientation of *GFP-CWP2* relative to the vector sequences was then reversed (necessary for the promoter-exchange experiments described below) in two steps. First, the PstI-EcoRI fragment of pGS-GFP-CWP2-lowR was subcloned into the corresponding sites of pBluescript II KS. Second, the HindIII-PstI fragment of the resulting plasmid was subcloned into the corresponding sites of YCplac33, yielding pGS-GFP-CWP2-low.

To create a plasmid expressing GFP-tagged Tip1p, *TIP1* was first cloned by gap repair. A linear gap-repair plasmid was created by PCR using primers GS2 and GS3 and plasmid YEplac181 (Gietz and Sugino, 1988) as template.

The resulting fragment was transformed into strain FY833, selecting for Leu⁺. Plasmids were recovered into *Escherichia coli*, and the desired plasmid (pGS-TIP1) was identified by restriction analysis and PCR using primers GS22 and GS23. XhoI and KpnI restriction sites were then generated directly downstream of the *TIP1* signal sequence in a two-step PCR reaction using *Pfu* polymerase (Fermentas, St.-Leon Rot, Germany) and plasmid pGS-TIP1 as template. Primer pairs GS6/GS7 and GS8/GS9 were used in the first reactions, and the combined purified products were used together with primers GS6 and GS8 in the second reaction. The final PCR product and plasmid pGS-TIP1 were digested with RsrII and XbaI and ligated, and the product was checked by restriction analysis. A XhoI-KpnI fragment containing the GFP (S65T) sequences from plasmid pREP4-GFP(S65T) (a derivative of plasmid pREP4; Maundrell, 1993; kindly supplied by Dr. F. Hochstenbach, Department of Medical Biochemistry, AMC, University of Amsterdam, Amsterdam, The Netherlands) was subcloned into the XhoI-KpnI sites to create pGS-GFP-TIP1-high. The HindIII-EcoRI fragment of pGS-GFP-TIP1-high was then subcloned into YCplac33 to create pGS-GFP-TIP1-low.

For promoter-exchange experiments, EagI (for *CWP1* and *TIP1*) or NotI (for *CWP2*) sites were generated just upstream of the start codons using two-step *Pfu*-polymerase PCR reactions (see above) with plasmids pGS-GFP-CWP1-low, pGS-GFP-CWP2-low, and pGS-GFP-TIP1-low as templates. pGS-EagI-CWP1 was created using primer pairs GS24/GS25 and GS26/GS27 in the first reactions and primers GS24 and GS26 in the second reaction. The final product was digested with PstI and Sall and then cloned into PstI/Sall-digested pGS-GFP-CWP1-low. pGS-NotI-CWP2 was created using primer pairs GS56/GS29 and GS57/GS31 in the first reactions and primers GS56 and GS57 in the second reaction. The final product was digested with HindIII and KpnI and then cloned into HindIII/KpnI-digested pGS-GFP-CWP2-low. The resulting plasmid was again digested with KpnI, and the 1309-base pair KpnI fragment from pGS-GFP-CWP2-low was cloned into this site. The orientation of this last insertion was checked by restriction analysis. pGS-EagI-TIP1 was created using primer pairs GS32/GS33 and GS34/GS35 in the first reactions and primers GS32 and GS34 in the second reaction. The final product was digested with RsrII and KpnI and then cloned into RsrII/KpnI-digested pGS-GFP-TIP1-low. In all three cases, insertion of the restriction sites did not affect the localization patterns of the proteins (our unpublished data). Promoters were then exchanged by digesting the plasmids with either HindIII and EagI or HindIII and NotI and then ligating promoter and residual plasmid fragments from different origins. Products were checked by restriction analysis. In this way, six new plasmids were created: pCWP1-GFP-CWP2, pCWP1-GFP-TIP1, pCWP2-GFP-CWP1, pCWP2-GFP-TIP1, pTIP1-GFP-CWP1, and pTIP1-GFP-CWP2.

The MS2 tag was cloned into plasmid pAR213 (see above) using primers GS52 and GS53 and plasmid pIII/MS2-2 (Beach *et al.*, 1999) as template. The PCR product was digested with BamHI and cloned into the unique BclI site just downstream of the *CWP1* ORF, creating pGS-CWP1-MS2.

Cell Cycle Synchronization

Some cultures were synchronized by adding hydroxyurea (Sigma-Aldrich, St. Louis, MO) to an exponential phase culture ($\sim 3 \times 10^6$ cells/ml) at 30°C to a final concentration of 200 μ M. After a 3-h incubation, the culture was released by washing three times in medium prewarmed to 30°C and incubating further at that temperature. In addition, synchronization with α -factor was performed by washing cells from an exponential phase culture ($\sim 1.5 \times 10^6$ cells/ml) at 30°C and resuspending them at 1.5×10^5 cells/ml in medium at 30°C containing 2 μ g/ml α -factor (Sigma-Aldrich). After 3 h, >90% of the cells were unbudded, and the culture was released by washing the cells with water and resuspending them at 1.5×10^6 cells/ml in medium at 25°C.

Microscopy

For observations of GFP-tagged proteins by fluorescence microscopy, cells were harvested, washed once in phosphate-buffered saline (PBS), and kept on ice for at least 15 min. For chitin staining, calcofluor white (CFW) (Sigma-Aldrich) was added to a final concentration of 20 μ g/ml. In most experiments, cells were observed and imaged using an Olympus BH-2 microscope equipped with a Hamamatsu (Ammersee, Germany) C5985 charge-coupled device camera and Object Image (<http://simon.bio.uva.nl/object-image.html>) 1.62n2 software. In some experiments, cells were observed and photographed on a Nikon Microphot SA microscope using an Apo 60X/1.40 numerical aperture oil immersion objective and Kodak T-Max 400 film.

Cell Fractionation, Cell Wall Digestion, and Western Blotting

Cells were harvested from an exponential phase culture ($\sim 3 \times 10^6$ cells/ml). Proteins from the culture supernatant were precipitated using the sodium deoxycholate (DOC), trichloroacetic acid (TCA) method (Ozols, 1990). First, DOC was added to a final concentration of 200 μ g/ml. After 10 min at 4°C, TCA was added to a final concentration of 6% (wt/vol), and the mixture was left overnight at 4°C. The precipitate was then pelleted at $10,000 \times g$ and washed extensively with 80% (vol/vol) acetone. Meanwhile, the cells were washed and homogenized as described previously (Montijn *et al.*, 1994;

Kapteyn *et al.*, 1995), and cell walls were spun down at $3000 \times g$. The resulting supernatant was precipitated with DOC and TCA as described above ("cytosolic fraction"). Meanwhile, the pellet from the $3000 \times g$ spin was washed extensively with 1 M NaCl and then boiled twice for 10 min in 2% SDS, 100 mM EDTA, 40 mM β -mercaptoethanol, and 50 mM Tris-HCl, pH 7.8, to solubilize noncovalently linked cell wall proteins and proteins from the plasma membrane and other membranous compartments (Klis *et al.*, 1998). The combined supernatants constitute the "SDS-soluble fraction." The SDS-extracted cell walls were washed with 10 mM Tris-HCl, pH 7.8, and then treated overnight at 37°C with 0.8 U of recombinant *Trichoderma harzianum* endo- β -1,6-glucanase (Bom *et al.*, 1998) per gram (wet weight) of cell walls to release the GPI-dependent cell wall proteins (Kapteyn *et al.*, 1996).

Proteins were separated on 3–20% gradient polyacrylamide gels containing 0.1% SDS (Laemmli, 1970) and transferred electrophoretically onto Immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA) (Montijn *et al.*, 1994). Glycoproteins were visualized by staining the membranes with peroxidase-labeled 1 μ g/ml concanavalin A (Sigma-Aldrich) in PBS containing 3% (wt/vol) bovine serum albumin, 2.5 mM CaCl₂, and 2.5 mM MnCl₂ (Klis *et al.*, 1998). Cwp1p was also visualized using polyclonal anti-Cwp1p antiserum (Shimoi *et al.*, 1995), as described previously (Kapteyn *et al.*, 1996). The blots were visualized using ECL Western blotting detection reagents (Amersham Europe, Braunschweig, Germany) according to the manufacturer's instructions.

RESULTS

Incorporation of *Tip1p* and *Cwp2p* into Specific Regions of the Cell Wall and Its Dependence on Their Times of Expression

Of genes encoding the ~ 40 GPI-dependent cell wall proteins in *S. cerevisiae*, more than half are transcribed in a cell cycle-dependent manner (see *Introduction*). To begin examining the consequences of such expression patterns for protein localization and function, we generated GFP-fusion genes on plasmids for three such proteins: *Tip1p*, *Cwp2p*, and *Cwp1p*. The GFP sequences were inserted directly downstream of the signal peptide sequences so that the first amino acids of each mature fusion protein are from GFP. The resulting fusion proteins were extractable by β -1,6-glucanase but not by SDS, indicating that they were incorporated normally into the cell wall, and they were recognized by the lectin concanavalin A after separation by SDS-PAGE, indicating that they were glycosylated (our unpublished data). The genes encoding these proteins are expressed in different phases of the cell cycle: *TIP1* is transcribed in late M and/or early G₁ phase, well before bud emergence, whereas *CWP1* and *CWP2* are transcribed in S/G₂ phase (Caro *et al.*, 1998; Spellman *et al.*, 1998).

GFP-*Tip1p* showed little, if any, incorporation into buds: unbudded cells fluoresced at the cell surface, as did the mother portions of budded cells, but little fluorescence was seen in buds of any size (Figure 1A and Table 2). These observations suggest that *Tip1p* is incorporated into the cell wall predominantly or exclusively in unbudded, G₁-phase cells, soon after *TIP1* is transcribed.

Similarly, GFP-*Cwp2p* fluorescence was readily detectable in unbudded cells and in the mother portions of budded cells but was weak or undetectable in very small buds (Figure 1B). However, in contrast to GFP-*Tip1p*, GFP-*Cwp2p* signal was clearly present in most medium-sized and large buds and indeed was usually as strong or stronger there than in the corresponding mother cells (Figure 1B and Table 2). In a culture synchronized with α -factor, GFP-*Cwp2p* fluorescence was also clearly present in the growing buds observed after release and was almost always stronger there than in the mother cells (our unpublished observations). These observations suggest that *Cwp2p* is incorporated into the cell wall predominantly or exclusively during S/G₂ phase, soon after *CWP2* is transcribed. Further bud growth after the bulk of GFP-*Cwp2p* has been incorporated could explain why there is a decrease in the percentage of buds that fluoresce very strongly in large-budded cells compared with small-budded cells (Table 2).

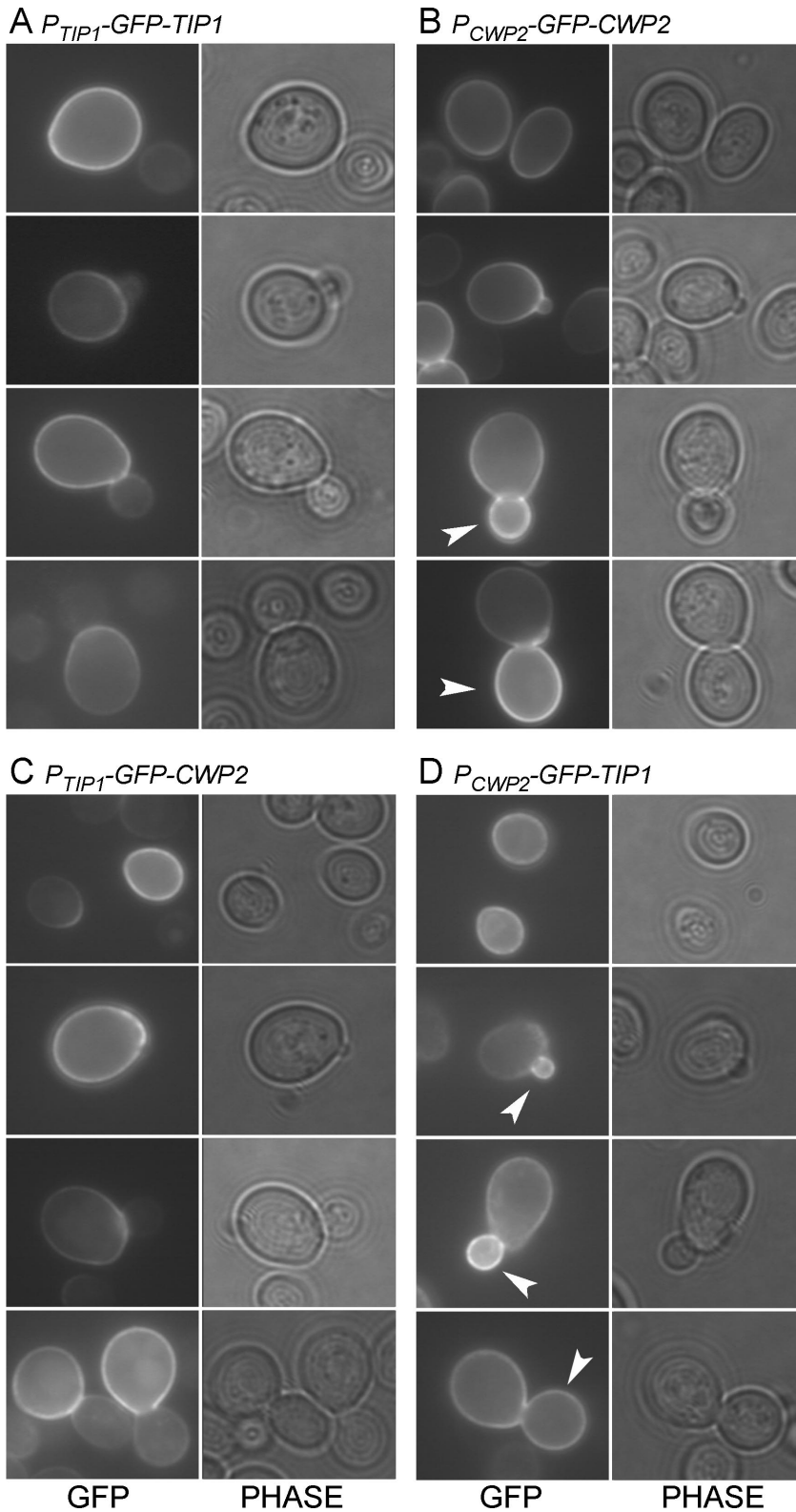


Figure 1. Localization of GFP-Tip1p and GFP-Cwp2p. Fusion proteins were expressed from low-copy plasmids in strain FY833. For each construct, >200 cells showing wall fluorescence were observed, and representative cells from four successive phases of the cell cycle are shown. (A) GFP-Tip1p expressed from its own promoter (plasmid pGS-GFP-TIP1-low). (B) GFP-Cwp2p expressed from its own promoter (plasmid pGS-GFP-CWP2-low). (C) GFP-Cwp2p expressed from the *TIP1* promoter (plasmid pTIP1-GFP-CWP2). (D) GFP-Tip1p expressed from the *CWP2* promoter (plasmid pCWP2-GFP-TIP1). Phase, phase-contrast images; arrowheads, buds with strong fluorescence.

The actin cytoskeleton is responsible for directing secretory vesicles to the sites of cell surface growth, and the apparent timing of Tip1p and Cwp2p incorporation correlates in each case with the polarization status of the actin cytoskeleton (see *Introduction*) at the time of gene expression.

We therefore hypothesized that the timing of transcription might determine the observed patterns of incorporation. To test this, we exchanged the promoters of the two genes. Strikingly, this exchange essentially reversed the incorporation patterns of the two GFP-fusion proteins. When ex-

Table 2. Distribution of GFP fluorescence

| Promoter | GFP-fusion | Small-budded cells ^a | | | Large-budded cells ^a | | |
|----------|------------|---------------------------------|-----------|-----------|---------------------------------|----------------|------------------|
| | | b < m (%) | b = m (%) | b > m (%) | b < m (%) | b = m (%) | b > m (%) |
| TIP1 | Tip1p | 100 | 0 | 0 | 98.5 | 1 ^b | 0.5 ^b |
| | Cwp2p | 100 | 0 | 0 | 98 | 2 ^b | 0 |
| | Cwp1p | 100 | 0 | 0 | 99 | 1 ^b | 0 |
| CWP2 | Cwp2p | 19 | 31 | 50 | 14 | 71 | 15 |
| | Tip1p | 40 | 25 | 35 | 21 | 67 | 12 |
| CWP1 | Cwp2p | 30 | 21 | 49 | 8 | 67 | 25 |
| | Tip1p | 32 | 16 | 52 | 11 | 57 | 32 |

^a From cultures of cells expressing each construct (see Figures 1 and 2), we counted ≥ 100 budded cells with bud diameter $< 50\%$ that of the mother cell (small-budded cells) and ≥ 100 budded cells with bud diameter $> 50\%$ that of the mother (large-budded cells). Bud wall fluorescence intensity was scored as lower than (b < m), equal to (b = m), or greater than (b > m) that of the mother.

^b Examination of the CFW staining suggested that each of these cells had a septum and thus that each was actually a pair of unbudded cells.

pressed from the *TIP1* promoter, GFP-Cwp2p was no longer incorporated into growing buds but was instead found almost exclusively in unbudded cells and in the mother portions of budded cells (Figure 1C and Table 2). Conversely, GFP-Tip1p expressed from the *CWP2* promoter was preferentially incorporated into medium-sized buds, which typically fluoresced more brightly than their mothers (Figure 1D and Table 2). Thus, the localizations of Tip1p and Cwp2p to specific regions of the wall seem to be determined largely or entirely by their times of expression during the cell cycle, as controlled by the 5'-upstream sequences.

Localization of Cwp1p to Birth Scars

CWP1 is expressed concurrently with *CWP2* (Caro *et al.*, 1998; Spellman *et al.*, 1998). However, the incorporation patterns of GFP-Cwp2p and GFP-Cwp1p were very different. When expressed under its own promoter from a low-copy vector, GFP-Cwp1p was incorporated mainly in the region of the birth scar (which is marked by a relatively low intensity of CFW staining and by the somewhat protuberant shape of the cell; Chant and Pringle, 1995) at one pole of the cell (Figure 2A). In many cases, some GFP fluorescence was also seen in the lateral walls of the cells, but the signal at the birth scar was much more intense, and lateral wall signal was never observed in the absence of birth scar signal. In addition, some intracellular GFP signal was often observed (Figure 2A). This signal probably corresponded to the vacuole (Kunze *et al.*, 1999); its strength varied greatly between experiments (cf. Figure 2B). The signal at the birth scar was stable: cells with more than four bud scars were observed that still showed a clear GFP-Cwp1p signal at the birth scar (Figure 3).

When the same GFP-Cwp1p construct was expressed from a high-copy vector, there was strong GFP fluorescence in medium-sized and large buds as well as strong signal at the birth scars (Figure 2B; Ram *et al.*, 1998a). The bud fluorescence resembled that seen with GFP-Cwp2p (Figure 1B), suggesting that the mechanism for directing Cwp1p specifically to the birth scar is saturable and that excess protein is incorporated into the bud wall by the "default" pathway for S/G₂-synthesized cell wall proteins (as seen normally with GFP-Cwp2p).

Dependence of Cwp1p Localization on Both Promoter and Other Sequences

To begin investigating the mechanisms of Cwp1p localization, we performed additional promoter-exchange experi-

ments. When expressed from the *CWP2* promoter, GFP-Cwp1p localized both to birth scars and to medium-sized and large buds (Figure 2C), much as when it was expressed from its own promoter on a high-copy plasmid (Figure 2B). These results seem consistent with the data indicating that the *CWP1* and *CWP2* promoters are expressed concurrently (see above) but with substantially stronger expression from the *CWP2* promoter in the rich medium used here (Wodicka *et al.*, 1997). Thus, upon expression from the *CWP2* promoter, the mechanism for targeting Cwp1p to the birth scar would be saturated, and the excess protein would follow the default pathway to the surface of the bud.

In contrast, when GFP-Cwp1p was expressed from the *TIP1* promoter, its localization resembled that of GFP-Tip1p (Figure 1A and Table 2) or of GFP-Cwp2p expressed from the *TIP1* promoter (Figure 1C and Table 2); that is, fluorescence was seen almost exclusively in unbudded cells and in the mother portions of budded cells (Figure 2D and Table 2). Although this fluorescence sometimes seemed stronger around the birth and bud scars than elsewhere on the cell surface, it was much less localized than when GFP-CWP1 was expressed at its normal time in the cell cycle. These results suggest two conclusions. First, incorporation into the unbudded cell wall (and hence into the mother cell wall) seems to be a default pathway for cell wall proteins expressed in late M/early G₁ phase, because all three proteins expressed from the *TIP1* promoter followed this pattern (Table 2). Second, normal timing of expression (based on an appropriate promoter) seems to be critical for proper Cwp1p localization.

However, expression from the *CWP1* promoter is not sufficient to target a protein to the birth scar: when either GFP-Tip1p or GFP-Cwp2p was expressed from this promoter, it was incorporated generally into growing buds (Figure 2, E and F, and Table 2), essentially as seen when either protein was expressed from the *CWP2* promoter (see Figure 1, B and D). Thus, sequences downstream of the promoter, either in the coding region or in the 3'-untranslated region, also seem to be critical for normal Cwp1p localization, and proteins without such sequences seem to follow the default pathway for G₂-synthesized cell wall proteins.

The Lag between CWP1 Transcription and Cwp1p Incorporation

Although *CWP1* is transcribed in late S to early G₂ phase when the nucleus is in the mother cell, Cwp1p seems to be incorporated into the cell wall much later and specifically in

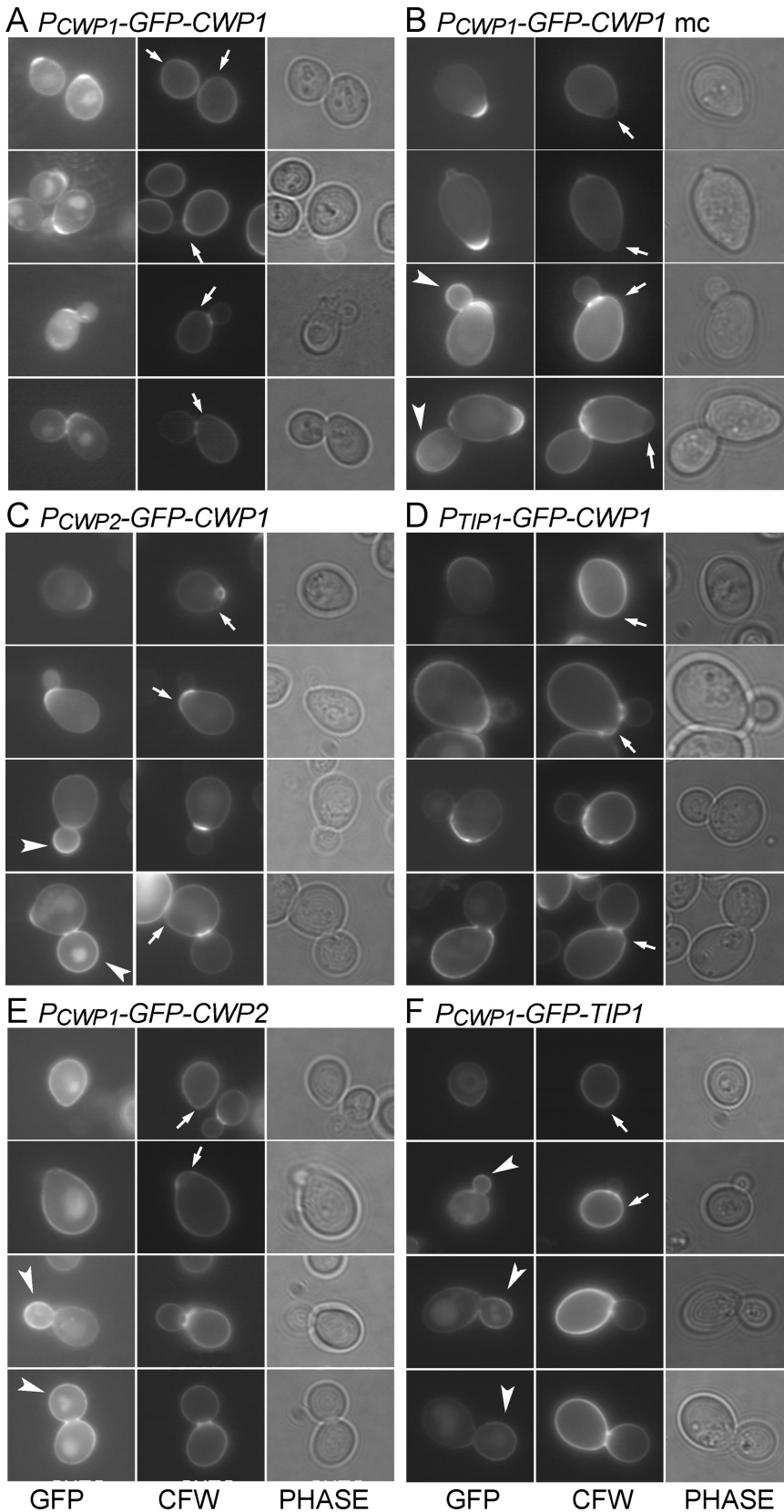


Figure 2. Localization of GFP-Cwp1p to the birth scar and its dependence on both promoter sequences and sequences within or downstream of the coding region. GFP-tagged proteins were expressed from low-copy (A and C–F) or high-copy (B) plasmids in strain FY833. For each construct, >200 cells showing wall fluorescence were observed, and representative cells from four successive phases of the cell cycle are shown. (A and B) GFP-Cwp1p expressed from its own promoter (plasmids pGS-GFP-CWP1-low and pAR213). (C) GFP-Cwp1p expressed from the *CWP2* promoter (plasmid pCWP2-GFP-CWP1). (D) GFP-Cwp1p expressed from the *TIP1* promoter (plasmid pTIP1-GFP-CWP1). (E) GFP-Tip1p expressed from the *CWP1* promoter (plasmid pCWP1-GFP-TIP1). (F) GFP-Cwp2p expressed from the *CWP1* promoter (plasmid pCWP1-GFP-CWP2). Phase, phase-contrast images; arrows, birth scars; arrowheads, buds with strong fluorescence.

the daughter cell, because the birth scar region of the wall is synthesized after cytokinesis as the secondary septum on the daughter side of the neck. This hypothesis was supported by

observations on cells that had been synchronized at the DNA replication checkpoint using hydroxyurea. Even 90 min after release, when the cells were about to begin sepa-

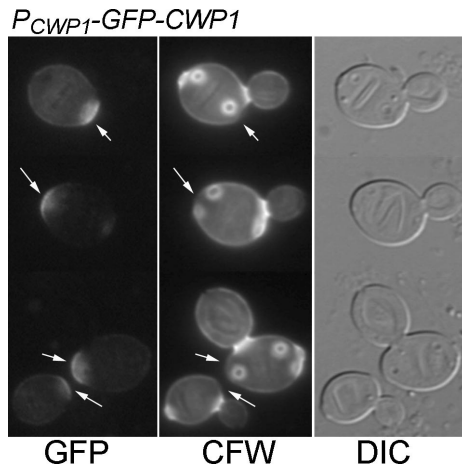


Figure 3. Stability of Cwp1p birth scar localization. Cells of strain LSY265 containing plasmid pGS-GFP-CWP1-low were grown at 30°C and stained with CFW, and cells were observed by DIC and fluorescence microscopy. Arrows, birth scars.

rating (Figure 4A), most cells did not show GFP-Cwp1p fluorescence at the neck (our unpublished data). The cells that did had already formed septa, as seen from the differential interference contrast (DIC) microscopy images and CFW staining (Figure 4B). Similarly, in an unsynchronized population of the same strain, 43 of 43 large-budded cells with GFP-Cwp1p fluorescence at the neck had completed septum formation as judged by DIC. In later samples of the synchronized population, most cells had separated, and most daughter cells did show fluorescence at the birth scar (our unpublished data). Thus, it seems that GFP-Cwp1p is indeed incorporated primarily soon after septum formation, just before cell separation in some cells, and probably during or after cell separation in others.

These observations imply that there is a delay either in the translation of *CWP1* mRNA or in the incorporation of Cwp1p into the wall. If the mRNA were translated immediately after transcription, Cwp1p should be detectable in an intracellular compartment where it would reside (in amounts similar to those finally incorporated) until incorporation into the wall. However, immunoblot analysis of asynchronously growing cultures showed that the Cwp1p in the cytosolic and SDS-soluble fractions together amounted to considerably <10% of the Cwp1p extractable from the cell wall using β -1,6-glucanase (Figure 5). It is therefore unlikely that the protein resides intracellularly, or even in the plasma membrane, for any significant period.

Although *CWP1* is transcribed in the mother-cell nucleus before nuclear division, its product is found specifically in the daughter cell. A similar situation is known for the transcription factor Ash1p (Bobola *et al.*, 1996; Sil and Herskowitz, 1996). In this case, the *ASH1* mRNA is delivered specifically into the bud, and translation is inhibited while the mRNA is transported (Long *et al.*, 1997; Takizawa *et al.*, 1997). Several genes involved in this process have been identified, including *BN11*, *SHE3*, and *MYO4* (Jansen *et al.*, 1996; Takizawa *et al.*, 1997; Takizawa and Vale, 2000; Beach and Bloom, 2001), and it has been proposed that stem-loop structures present in the 3'-untranslated region of the mRNA are important for its localization (Chartrand *et al.*, 1999, 2002; Gonzalez *et al.*, 1999). Although the *CWP1* mRNA also contains a large predicted stem-loop structure in its 3'-untranslated region (according to the Mfold 2.3 and 3.0

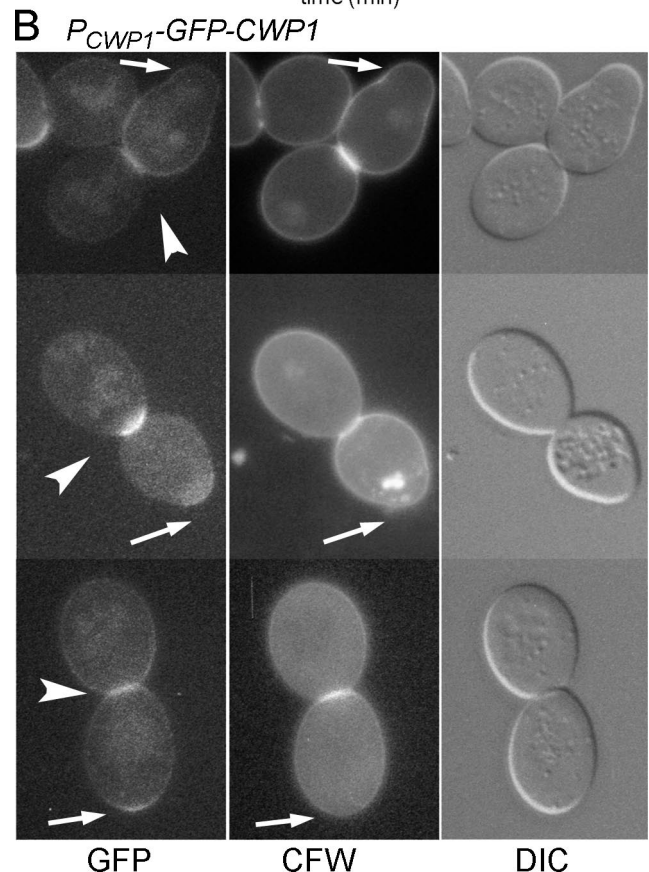
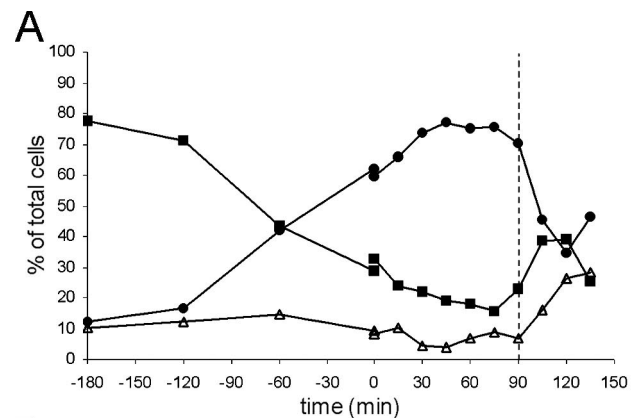


Figure 4. Incorporation of GFP-Cwp1p into the secondary septum of the daughter cell after cytokinesis. Cells of strain LSY265 containing plasmid pGS-GFP-CWP1-low were synchronized by adding hydroxyurea at $t = -180$ min and washing it out at $t = 0$ (see *Materials and Methods*). (A) Synchronization shown as percentages of un-budded (■), small-budded (△), and large-budded (●) cells. (B) Fluorescence and DIC images of large-budded cells from the 90-min sample (A, dashed line). Arrows, birth scars of the mother cells; arrowheads, GFP-Cwp1p on the daughter side of the necks.

algorithms [Zuker *et al.*, 1999; Mathews *et al.*, 1999]), two types of experiments suggested that mRNA localization is not involved in the asymmetric distribution of Cwp1p. First, deletion of *BN11*, *SHE3*, or *MYO4* did not prevent the asymmetric localization of GFP-Cwp1p to the birth scar in many cells (Figure 6). Second, direct examination of *CWP1* mRNA localization using the MS2 CP-GFP system (Beach *et al.*,

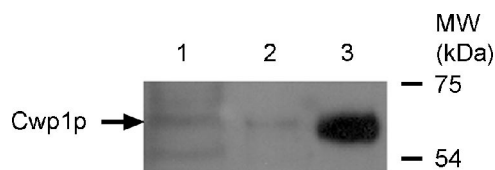


Figure 5. Abundance of Cwp1p in different cellular fractions. Proteins from strain FY833 were fractionated as described in *Materials and Methods* and analyzed by Western blotting using anti-Cwp1p antibodies. Lane 1, cytosolic fraction; lane 2, SDS-soluble fraction; lane 3, proteins released by β -1,6-glucanase digestion. Equal cell equivalents were loaded in each lane. The culture supernatant was also examined (see *Materials and Methods*) to determine whether Cwp1p was released from the cells (Ram *et al.*, 1998b), but no Cwp1p was detected.

1999) in strain JV96 did not detect any specific localization to the bud (our unpublished observations).

Dependence of Cwp1p Localization on Normal Septum Formation

The incorporation of Cwp1p into the daughter cell wall soon after septum formation suggested that the two processes might be linked. To explore this possibility, we examined the localization of GFP-Cwp1p in several mutants in which septum formation is perturbed. Although mutants lacking the formin Bni1p are viable, *bni1Δ/bni1Δ* diploid cells often display defects such as the formation of incomplete, asymmetric, and/or misplaced septa and a delay in cell separation (Bi *et al.*, 2000; Vallen *et al.*, 2000). In a wild-type strain, unseparated cells that already had detectable GFP-Cwp1p signal at the daughter side of the septum were uncommon (see above), but such cells were frequently observed in a *bni1Δ/bni1Δ* strain (Figure 6A, short arrow), even when the septum was misplaced or otherwise abnormal, suggesting that Cwp1p incorporation followed closely upon septum formation even when cell separation was delayed. Moreover, in some cells in which the septum was incomplete and only partially traversed the neck, GFP-Cwp1p was present on both sides of the septum (Figure 6A, arrowhead), sup-

porting the hypothesis that Cwp1p incorporation is linked both spatially and temporally to septum formation.

This hypothesis received further support from observations on mutant cells lacking Gin4p, a protein kinase that is involved in the organization of the ring of septin proteins at the neck, which in turn influences the pattern of septum formation (Longtine *et al.*, 1996, 1998b, 2000; Gladfelter *et al.*, 2001; Caviston *et al.*, 2003). In a *gin4Δ/gin4Δ* diploid strain, many cells showed seemingly normal GFP-Cwp1p localization to the birth scar and to the daughter side of the neck in unseparated cells with relatively normal-looking septa (Figure 7A, long and short arrows). However, in many other cells, the neck region seemed elongated and relatively disorganized, and GFP-Cwp1p was observed in bars or patches that seemed to coincide with patches of increased CFW staining (Figure 7A, arrowheads). These observations suggested that Cwp1p incorporation might be linked specifically to the deposition of septal chitin.

To investigate this possibility further, we first examined mutants defective in Chs3p, the catalytic subunit of chitin synthase III, or in one of its accessory proteins. Chitin synthase III is primarily responsible for the synthesis of the chitin ring at the incipient bud site and the chitin of the lateral cell wall (Shaw *et al.*, 1991; Kollár *et al.*, 1995; Cabib and Durán, 2005) as well as for increased chitin synthesis in response to various stresses (Valdivieso *et al.*, 2000; Valdivia and Schekman, 2003), but it can also contribute to the formation of a remedial septum in the absence of *CHS2* (Cabib and Schmidt, 2003). However, GFP-Cwp1p seemed to localize normally in a *chs3Δ* mutant (Figure 7B); in a mutant (DDY172-2A) with a deletion of *CHS4*, which encodes a protein necessary for the normal localization and activation of Chs3p (DeMarini *et al.*, 1997; Trilla *et al.*, 1997; Ono *et al.*, 2000) (our unpublished data); and in a mutant with a deletion of *CHS6*, which encodes a protein involved in the trafficking of Chs3p through the secretory system (Ziman *et al.*, 1998; Valdivia *et al.*, 2002; Valdivia and Schekman, 2003) (our unpublished data).

Chs6p is one of a family of four proteins in *S. cerevisiae*. One of its homologues, Bud7p, is implicated in the vesicular transport of the bud site selection marker proteins Bud8p

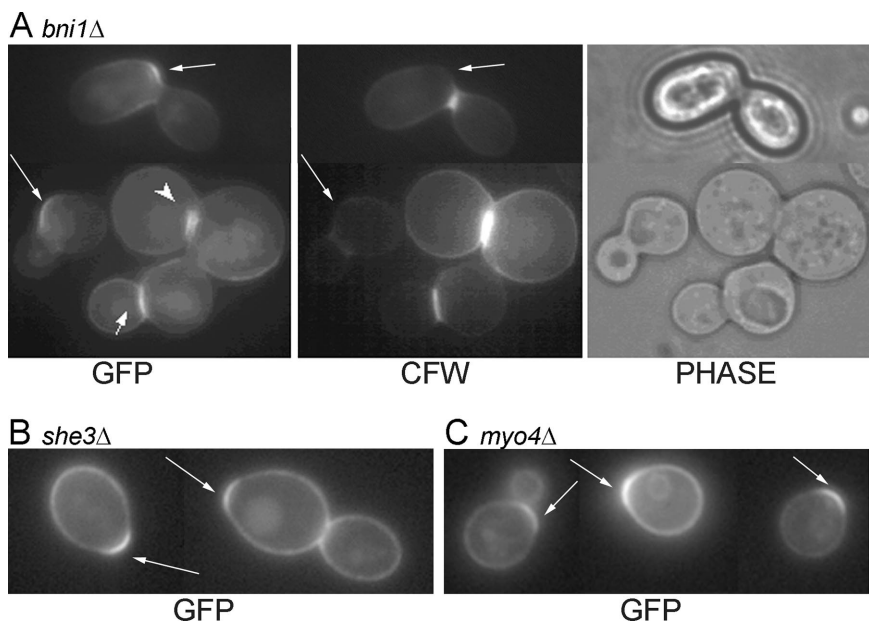


Figure 6. Asymmetric localization of Cwp1p in mutants defective in mRNA localization. Strains (A) M-717 (*bni1Δ/bni1Δ*), (B) ICY018 (*she3Δ/she3Δ*), and (C) KBY1012 (*myo4Δ/myo4Δ*) were transformed with plasmid pGS-GFP-CWP1-low and examined for the localization of GFP-Cwp1p. Phase, phase-contrast images; long arrows, birth scars; short arrow, a septum with GFP-Cwp1p on the daughter side; arrowhead, GFP-Cwp1p localized on both sides of an incomplete septum.

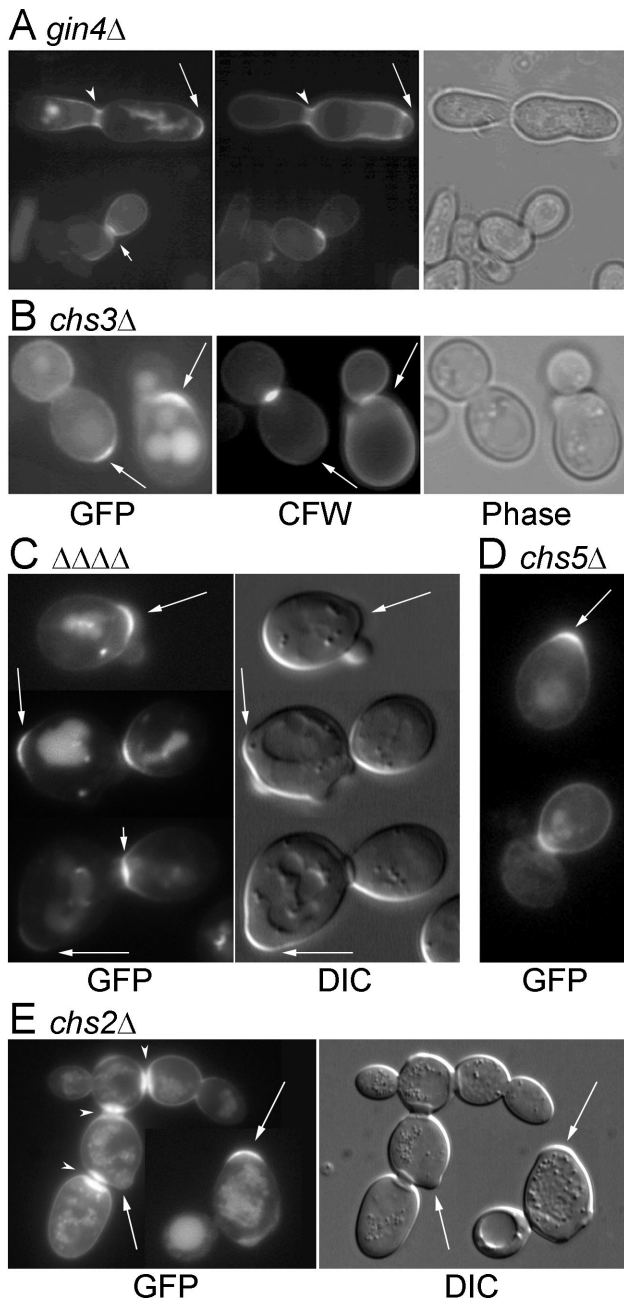


Figure 7. Dependence of Cwp1p localization on normal septum formation. Strains (A) M-272 (*gin4Δ/gin4Δ*), (B) DDY181-2D (*chs3Δ*), (C) KNY1048 (*chs6Δ/chs6Δ bud7Δ/bud7Δ ymr237wΔ/ymr237wΔ ykr027wΔ/ykr027wΔ*), (D) AM775 (*chs5Δ/chs5Δ*), and (E) ICY028 (*chs2Δ/chs2Δ*) were transformed with plasmid pGS-GFP-CWP1-low and examined for the localization of GFP-Cwp1p. KNY1048 and ICY028 were grown at 30°C. Long arrows, birth scars; short arrows, fluorescent daughter sides of septa; arrowheads, GFP-Cwp1p spread through an elongated neck (A) or on both sides of an aberrant septum (E).

and Bud9p (Zahner *et al.*, 1996; our unpublished data), and it seems likely that the other homologues, Ykr027p and Ymr237p, play similar roles in the transport of other specialized cargoes to specific sites at the cell surface. Thus, we also examined GFP-Cwp1p localization in a strain with deletions of these four genes as well as in a mutant with a deletion of

CHS5, which encodes a protein that seems to function together with all four members of the Chs6p/Bud7p family (Valdivia *et al.*, 2002; Sanchatjate and Schekman, personal communication; Trautwein and Spang, personal communication; Nakashima, McKenzie, and Pringle, unpublished data). However, in both cases, GFP-Cwp1p still seemed to localize normally to birth scars (Figure 7, C and D).

In contrast to the results with mutants lacking Chs3p or its accessory proteins, Cwp1p localization was strongly affected by an absence of chitin synthase II (Chs2p), the enzyme primarily responsible for synthesis of the primary septum (Shaw *et al.*, 1991; Schmidt *et al.*, 2002; Lesage *et al.*, 2005). In a *chs2* deletion strain, GFP-Cwp1p localization was rather variable from cell to cell. Nonetheless, many cells did show a well defined concentration of GFP-Cwp1p at the birth scar (Figure 7E). In addition, in the many necks that persist in the clumps of such a strain (reflecting its inefficient septum formation and cell separation; Shaw *et al.*, 1991; Schmidt *et al.*, 2002), GFP-Cwp1p was often highly concentrated. However, the fluorescence signal was typically bright on both sides of the neck (Figure 7E), instead of showing the normal restriction to the daughter side. These data suggest that the asymmetric localization of Cwp1p to the nascent birth scar requires the normal timing of septum formation, normal septum structure, or both.

Possible Functions of Cwp1p

Previous studies have reported that deletion of *CWP1* is nonlethal and indeed produces no obvious phenotype other than a slightly increased sensitivity to cell surface-interactive chemicals (Shimoi *et al.*, 1995; van der Vaart *et al.*, 1995; Dielbandhoesing *et al.*, 1998). We also found that haploid and diploid *cwp1Δ* cells did not differ significantly from wild type in growth rate, overall cell morphology, or appearance after staining chitin with CFW (our unpublished data). The localization of Cwp1p to the birth scar suggested that it might have a role in cell division and/or in bud site selection (which involves cortical marker proteins that are localized to the cell poles; Lord *et al.*, 2000; Harkins *et al.*, 2001; Kang *et al.*, 2004). However, because the percentage of budded cells with septa visible by DIC microscopy did not differ between *cwp1Δ* and wild-type strains (31 and 32% for exponentially growing populations of strains LSY265 and YEF473, respectively), there seemed to be no delay in cell separation. Moreover, using several approaches, we were unable to obtain any evidence for a role of Cwp1p in bud-site selection or for interaction of Cwp1p with the bud site selection marker proteins. Haploid and diploid *cwp1Δ* cells displayed normal axial and bipolar budding; localization of the marker protein Bud9p to the birth scar was unaffected in *cwp1Δ* cells; and Cwp1p localization to the birth scar seemed to be independent of Bud9p and of the other marker proteins Bud8p, Rax2p, and Axl2p as well as of Bud7p and Rax1p, which seem to be involved in the trafficking of Bud8p, Bud9p, and Rax2p (Supplemental Figure 1).

DISCUSSION

Localized Incorporation of Yeast Cell Surface Proteins

Although studies of individual proteins and several large-scale surveys (Kumar *et al.*, 2002; Huh *et al.*, 2003) have described the localizations of many yeast proteins, cell surface proteins have mostly escaped attention because of the technical constraints imposed by their specific requirements for incorporation into the plasma membrane or cell wall. Thus, surprisingly little is known about the specific localiza-

tions of integral and GPI-anchored plasma membrane proteins, PIR proteins, and GPI-dependent cell wall proteins. Among the exceptions are the integral membrane proteins involved in bud site selection, which localize to the division sites and cell poles as noted above, and the enzymes that synthesize cell wall chitin and β -1,3-glucan, which localize dynamically to the bud neck or bud tip at appropriate times in the cell cycle (Chuang and Schekman, 1996; Drgonová *et al.*, 1996; DeMarini *et al.*, 1997; Utsugi *et al.*, 2002). In addition, specific localizations have been reported for several GPI-dependent cell wall proteins, including the α -agglutinin Sag1p/Ag α 1p, which was found at the projection (shmoo) tip of Mata cells after stimulation with Mata mating pheromone (Wojciechowicz and Lipke, 1989), and the proteins Crh1p and Crh2p, which were found in small buds and the neck region (Crh1p) and all over the wall but preferentially in the neck region (Crh2p) (Rodríguez-Peña *et al.*, 2000). At least in pheromone-treated cells, such localizations may reflect the distribution of lipid rafts enriched in the GPI-proteins (Bagnat and Simons, 2002).

In this study, we have demonstrated specific patterns of incorporation for three additional GPI-dependent cell wall proteins. We found Tip1p to be incorporated specifically in mother cells before bud emergence, Cwp2p to be incorporated specifically in small to medium-sized buds, and Cwp1p to be incorporated stably into the birth scar region. In the cases of Cwp1p and Cwp2p, these observations differ somewhat from those reported previously (Ram *et al.*, 1998a), probably because the earlier study used high-copy plasmids that resulted in overexpression of the GFP-tagged proteins, whereas we have now expressed the proteins from their normal promoters on low-copy plasmids.

Mechanisms for Localized Incorporation of Cell Surface Proteins

In *S. cerevisiae*, the actin cytoskeleton is responsible for delivering secretory vesicles to specific areas of the cell surface; as actin organization changes during the cell cycle, the patterns of vesicle delivery show corresponding changes (Lew and Reed, 1995; Pringle *et al.*, 1995; Pruyne and Bretscher, 2000b). Because plasma membrane and cell wall proteins travel to the cell surface in secretory vesicles, and because the traffic of such vesicles to the cell surface is normally rapid (Novick *et al.*, 1981; Pastor *et al.*, 1982; Novick and Schekman, 1983), it seems that in the absence of additional mechanisms, the synthesis of such a protein at a specific time in the cell cycle would automatically determine its incorporation into the membrane or wall in a specific pattern. Thus, the normal pulse of *AXL2* expression in late G_1 seems to allow the incorporation of Axl2p specifically at the presumptive bud site (at which it remains as the bud emerges); artificial expression of *AXL2* in S/G2 led to the incorporation of Axl2p throughout the bud membrane (Lord *et al.*, 2000). Similarly, the induction of α -agglutinin expression by Mata pheromone (Lipke *et al.*, 1989), coinciding with the polarization of the actin cytoskeleton toward the pheromone source (Hašek *et al.*, 1987), seems to be sufficient to explain its localized incorporation into the shmoo tip (Wojciechowicz and Lipke, 1989). This simple model also seems to explain the behavior of the cell wall proteins Tip1p and Cwp2p: when either of these proteins is expressed from the *TIP1* promoter in late M/early G_1 phase, it is incorporated specifically into the wall of the unbudded G_1 cell and not into growing buds, but when either protein is expressed from the *CWP2* or *CWP1* promoter in S/early G_2 phase, it is incorporated specifically into the wall of the growing bud. In each case, the site of incorporation coincides with the direc-

tionality of actin-based secretion at that phase of the cell cycle.

In contrast, the behavior of Cwp1p presents a more complicated picture. The normal expression of *CWP1* in late S/early G_2 seems to be critical for the normal pattern of Cwp1p incorporation, because expression from the *TIP1* promoter in late M/early G_1 causes Cwp1p to be incorporated specifically, and without apparent delay, into the wall of the unbudded cell, much as are Tip1p and Cwp2p when expressed at this time (although the Cwp1p pattern does show more heterogeneity than is evident with either of the other two proteins). However, when Cwp1p is expressed at normal levels from its own promoter in late S/early G_2 , it is incorporated with high specificity, and after a long delay, into the birth scar of the daughter cell. This behavior is strikingly different from that of Tip1p and Cwp2p when expressed at the same time, indicating that the *CWP1*-encoded mRNA and/or protein must contain (a) signal(s) that diverts Cwp1p from the general flow of secretory material produced around the time at which *CWP1* is transcribed. Interestingly, the mechanism that produces this diversion seems to be saturable, because when *CWP1* is overexpressed in late S/early G_2 (by being expressed either from its own promoter on a high-copy plasmid or from the stronger *CWP2* promoter), it is incorporated into the surface of the growing bud as well as at the birth scar.

An explanation for the behavior of Cwp1p must account for at least two unusual features, namely, the long delay between gene transcription and protein incorporation into the wall and the incorporation specifically into the daughter side of the septum. Both features might have a common explanation if the *CWP1* mRNA, like the mRNAs of *ASH1* (Chartrand *et al.*, 2002; Gu *et al.*, 2004) and certain other genes (Shepard *et al.*, 2003; Gerber *et al.*, 2004), were delayed in translation while being transported specifically into the bud, where it might be translated only at the time of cytokinesis, when the actin cytoskeleton would deliver it to the forming septum. This mechanism seemed the more likely because a large stem-loop structure, such as is thought to be critical for the transport of *ASH1* mRNA (Chartrand *et al.*, 1999, 2002; Gonzalez *et al.*, 1999), is also predicted to be present in the 3'-untranslated region of the *CWP1* mRNA. However, we could obtain no evidence to support this hypothesis. In particular, we could detect no enrichment of *CWP1* mRNA in the bud using methods that have worked for other mRNAs, and we found that normal Cwp1p localization did not depend on any of several proteins that are essential for *ASH1* mRNA transport. *CWP1* mRNA was also not detected in other studies of the mRNAs that interact with known components of the mRNA-localization machinery (Shepard *et al.*, 2003; Gerber *et al.*, 2004). The localization of Cwp1p to the bud surface when *CWP1* is transcribed at the normal time in the cell cycle but at higher-than-normal levels showed that *CWP1* mRNA does have the potential to be translated at that time. However, because we did not detect any substantial pool of Cwp1p other than that covalently bound to the cell wall, we conclude that a delay in mRNA translation must account, at least in part, for the delay in Cwp1p incorporation into the wall. The mechanism of this translation delay seems to be novel and should be interesting to investigate further.

Another interesting feature of Cwp1p incorporation is its apparent dependence on the normal timing of septum formation and/or the normal structure of the septum. Most conspicuously in the *chs2* mutant, but also in some cells of the *bni1* and *gin4* mutants, Cwp1p was found localized to both sides of the septal region rather than asymmetrically to

the daughter side. This phenotype may simply reflect the inefficient septum formation in these mutants; thus, if Cwp1p is delivered to the neck before the septum is complete, it may become incorporated symmetrically along with other septum components rather than being confined to the daughter side. Moreover, the remedial septum in a *chs2* mutant is formed differently from the normal primary and secondary septa in wild-type cells, which may also disturb the generation of asymmetry. Alternatively, the altered distribution of Cwp1p may reflect a specific association with other septal components whose distribution is altered in these mutants.

Functional Significance of Localized Incorporation of Cell Wall Proteins

What purposes are served by the localized incorporation of cell wall proteins? The cell cycle-regulated incorporation of the bulk of the cell wall mannoproteins into the wall (presumably of the bud) in G₂/M phase has been shown to correlate with a decreased cell wall permeability at that stage (de Nobel *et al.*, 1991), which may result from an increased cross-linking of the glucan network by some of the proteins. However, it is difficult to see the significance of the localized incorporation of Tip1p (in unbudded cells) and Cwp2p (in buds). Because every unbudded cell was once a bud and every bud soon becomes an unbudded cell, the initial asymmetry generated by this localized incorporation is only transient, and lasts less than one cell cycle. Because the phenotypes of *tip1Δ* and *cwp2Δ* mutants are very mild (van der Vaart *et al.*, 1995; Dielbandhoesing *et al.*, 1998), they also provide little guidance, and we are presently without a clear hypothesis as to the functions of these proteins or of their cell cycle-regulated and initially asymmetric incorporation.

In the case of Cwp1p, its localization primarily to the birth scar and persistence at that site suggest strongly that it should have a role in some specific function of that region of the cell wall. One well established function of the birth scar pole is that it houses both the transient marker used in the axial budding pattern of haploid cells and a persistent marker used in the bipolar budding pattern of diploid cells (see above). However, despite performing a seemingly full suite of tests, we were unable to detect any role of Cwp1p in either axial or bipolar budding or any functional interaction of Cwp1p with the proteins that serve as the bud site selection markers.

During the separation of mother and daughter cells, digestion of the chitinous primary septum proceeds from the daughter side (Colman-Lerner *et al.*, 2001; Ríos Muñoz *et al.*, 2003), with the result that the division site on the mother cell (the bud scar) retains both a thick chitin ring and a layer of chitin across the entire scar, whereas the division site on the daughter cell (the birth scar) contains a thinner or nonexistent chitin ring and little or no chitin in the wall within the ring (Streiblová and Beran, 1963; Beran *et al.*, 1972; Molano *et al.*, 1980; Roberts *et al.*, 1983; Shaw *et al.*, 1991; Chant and Pringle, 1995; Powell *et al.*, 2003). Perhaps for this reason, the bud scar is a rigid structure that expands little or not at all as the cell grows during subsequent generations, whereas the birth scar is a more plastic structure that expands both during division and during the subsequent growth of the cell (Bartholomew and Mittwer, 1953; Streiblová and Beran, 1963; Bacon *et al.*, 1966; Talens *et al.*, 1973; Molano *et al.*, 1980; Chant and Pringle, 1995; Powell *et al.*, 2003). Thus, it seems plausible that the birth scar might be an intrinsically weaker structure than the bud scar and the rest of the cell wall, and thus in need of reinforcement from special birth scar-local-

ized proteins such as Cwp1p, whose expression is known to be induced by cell wall stress (Ram *et al.*, 1998b; Jung and Levin, 1999; Terashima *et al.*, 2000), and that can cross-link the glucan network because of its multiple possible linkages (Kapteyn *et al.*, 2001). However, *cwp1Δ* cells form septa at normal rates, have bud and birth scars of normal shape, and are hardly, if at all, more susceptible to environmental stress (van der Vaart *et al.*, 1995; Dielbandhoesing *et al.*, 1998; our unpublished data; see *Results*). Thus, we are presently without a good clue to the function of Cwp1p or of its specific localization to the birth scar.

It remains possible that the roles of Tip1p, Cwp2p, and Cwp1p are obscured because these proteins are functionally redundant with other proteins that have not yet been identified or tested for such possible redundancy. If further studies bring deeper insight into the specific roles of these proteins in the cell wall, this may also clarify the functional significance, if any, of their specifically timed, and thus localized, patterns of incorporation.

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