Mechanisms for targeting of the *Saccharomyces cerevisiae* GPI-anchored cell wall protein Crh2p to polarised growth sites

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Summary

The cell wall is an essential structure that preserves the osmotic integrity of fungal cells and determines cellular morphology during developmental programs. The high number of different wall components demands a variety of processes to deliver precursors and synthetic proteins to the proper location at the right time for wall development and modification. Here, the specificity of the mechanisms that regulate the temporal and spatial localisation of cell wall proteins to sites of polarised growth in *Saccharomyces cerevisiae* is investigated. For this purpose, the localisation of Crh2p, a cell wall glycosylphosphatidylinositol (GPI)-anchored mannoprotein that we have recently described as involved in cell wall construction and localised to polarised growth sites, was followed using a Crh2p-GFP fusion protein. Crh2p distribution was studied in several genetic backgrounds affected in different steps of the cell polarity establishment machinery or/and bud morphogenesis. Crh2p is localised at the mother-bud neck in *bud1* cells following the random budding pattern characteristic of this mutant. The Crh2p distribution was greatly altered in a *cdc42-1* mutant, indicating complete dependence on an organised actin cytoskeleton for polarised Crh2p distribution. The usual deposition of Crh2p in a ring at the base of growing buds was lacking in *cdc10-11* cells growing under restrictive temperature conditions, whereas Crh2p deposition at the septum region was absent in both *cdc10-11* and *cdc15-lyt1* cells. These results point to the dependence of Crh2p localisation at the bud-neck on both septins and septum integrity. Furthermore, in the absence of Bni4p, a scaffold protein involved in the targeting of the chitin synthase III complex to the bud neck, Crh2p was not longer found at the neck in large-budded cells undergoing cytokinesis. Finally, Crh2p was not properly localised in cells deleted in *CHS5*, which encodes a protein involved in the transport of Chs3p, and was completely mislocalised in *sbe2/sbe22* mutants, suggesting that the transport systems for Chs3p and Crh2p are to a certain extent coincident. The transport of other GPI-cell wall proteins, such as Cwp1p, however, does not depend on these systems as the localisation of the latter protein was not affected in either of these mutants.

Key words: *Saccharomyces cerevisiae*, Glycosidase, Cell wall, Crh2p, Polarity, GFP

Introduction

In fungal cells, the cell wall is an essential structure that maintains cell morphology, regulates the uptake of substances and protects the cell from the external environment by preserving its osmotic integrity. The *Saccharomyces cerevisiae* cell wall is basically composed of mannosylated proteins, β-1,3 glucan, β-1,6 glucan and chitin. This apparently rigid structure is, however, very dynamic. As cells increase in size during growth, the cell wall needs to incorporate new materials to the sites of growth, presumably through the secretory pathway (Santos and Snyder, 2000). The cell wall also needs to be remodelled during morphogenetic processes involving changes in cell morphology, such as mating, sporulation or pseudohyphal growth, and must be weakened for bud emergence (Cabib et al., 2001; Molina et al., 2000; Cid et al., 1995; Orlean, 1997; Smits et al., 2001). In all these situations growth occurs at defined positions on the cell surface and involves asymmetric growth from one region of the cell to form particular cell structures or shapes.

During vegetative growth, the initiation of bud development comprises diverse molecular steps: recognition of the appropriate site for budding, polarisation of the actin cytoskeleton, secretion and cell wall synthesis. The site for bud formation is determined in a cell-type-dependent fashion. In haploid strains, mother cells select a bud site immediately adjacent to their previous daughter, and the daughter cells bud next to the birth site (axial budding pattern). In diploids, mother cells select bud sites adjacent to their daughter cells or on the opposite end of the cell (bipolar budding pattern) (reviewed by Madden and Snyder, 1998). Ras1-like GTPase Bud1p/Rsr1p and its regulators Bud2p and Bud5p function in all cell types as the general bud-site selection machinery that recruits the proteins required for bud formation to the cell-type-specific landmarks (for reviews, see Cabib et al., 1998; Madden and Snyder, 1998). In addition, it is currently well known that the polarity in *S. cerevisiae* is under the coordinated control of Rho GTPases and cyclin-dependent protein kinases. One of the major functions of the essential Rho GTPase Cdc42p is to

regulate the polarisation of the actin cytoskeleton in yeast, which in turn targets the secretory vesicles and other factors that support growth (Johnson, 1999; Pruyne and Bretscher, 2000a; Pruyne and Bretscher, 2000b).

The biosynthesis and assembly of cell wall components needs to be tightly controlled both temporally, in order to be perfectly coordinated with other cellular events such as cell cycle or developmental programs, and spatially, to be coupled with cytoskeleton dynamics for polarised growth. To date, most of the work related to the temporal and spatial regulation of cell wall components has focused on the biosynthesis and deposition of chitin, a polysaccharide that is synthesised at the plasma membrane. Spatial control of chitin deposition depends on the actin cytoskeleton and polarity establishment proteins. Mutants defective in actin (Novick and Botstein, 1985), Myo2 (a type V myosin) (Johnston et al., 1991) or proteins related to actin function (Donnelly et al., 1993; Haarer et al., 1994; Liu and Bretscher, 1992) or mutants altered in polarity and bud emergence (Adams et al., 1990; Kim et al., 1991; Sloat et al., 1981) display altered chitin deposition patterns. Proper chitin location also depends on the structure formed by the family of septin proteins (Cdc3p, Cdc10p, Cdc11p and Cdc12p). This family of proteins assembles as a ring at the mother-bud neck, thereby providing a scaffold for the organisation of proteins at this specific region of the cell surface (Field and Kellogg, 1999; Ford and Pringle, 1991; Longtine et al., 1996).

The localisation of Chs3p [which is responsible for the bulk of chitin at the ring between mother and daughter cells and in the lateral cell wall (Shaw et al., 1991; Valdivieso et al., 1991)], both in time and space, is essential for chitin synthesis during vegetative growth. Chs3p localises to sites of polarised growth in a cell-cycle-dependent manner, displaying a similar pattern to that of chitin deposition (Santos and Snyder, 1997). Moreover, the proper targeting of CSIII (Chitin Synthase III) activity depends on several other genes, such as those encoding the septins and *CHS4*, *CHS5*, *CHS6*, *CHS7*, *MYO2* and *BNI4*. In the absence of any of the proteins encoded by these genes, Chs3p is no longer present in polarised cortical sites (DeMarini et al., 1997; Santos et al., 1997; Trilla et al., 1999; Ziman et al., 1998). A model has emerged in which Chs4p links Chs3p to Bni4p and, hence, to the septins (DeMarini et al., 1997). Targeting of Chs3p to polarised growth sites requires the protein Chs5p, a protein from the trans-Golgi network (Santos et al., 1997; Santos and Snyder, 1997). It has been suggested that Chs5p-containing vesicles target Chs3p to the neck with the help of the actin cytoskeleton (Madden and Snyder, 1998). The role of two other proteins, namely Chs6p and Chs7p, in the transport of Chs3p has also been characterised (Trilla et al., 1999; Ziman et al., 1998). In addition, two Golgi proteins (Sbe2p and Sbe22p) have recently been identified and associated with the transport of Chs3p as this protein is mislocalised in *sbe2 sbe22* mutants. It has been suggested that these proteins are also involved in the transport of mannoproteins to the cell surface (Santos and Snyder, 2000).

In contrast to the knowledge about the mechanisms that control chitin synthesis at the cell membrane, little is known about the mechanisms that control the spatial and temporal distribution of mannoproteins at the cell wall. The yeast genome encodes approximately 40 different GPI and 4 Pir cell wall mannoproteins (Smits et al., 1999). Many of these proteins are expressed in a cell-cycle-dependent manner (Spellman et al., 1998), with most of their encoding genes being transcribed in the M/G1 phase. Mannoproteins, which are located in an outer layer of the cell wall, play an important role in determining cell wall porosity, acting either as structural proteins or as enzymes involved in cell wall construction and remodelling (Kapteyn et al., 1999; Orlean, 1997).

We have recently described a family of cell wall proteins (Crh1p, Crh2p and Crr1p); these proteins are involved in cell wall assembly and are differently expressed during the yeast life cycle (Rodriguez-Peña et al., 2000). Crh1p and Crh2p, which are both GPI-anchored cell wall proteins (Hamada et al., 1998), although differently regulated through the cell cycle (*CRH2* transcript levels are stable throughout the mitotic cycle but *CRH1* has two transcription peaks at G1 and M/G1), localise to polarised growth sites. The localisation of these proteins is reminiscent of the distribution of chitin at the cell wall (Rodriguez-Peña et al., 2000), suggesting a probable role for these proteins in the integration of this polymer within the cell wall matrix. Bearing in mind this characteristic localisation pattern, in this study we were prompted to characterise the mechanisms controlling the localisation of the Crh2p cell wall protein, studying the dependence of this localisation on proteins involved in polarity selection, transport systems and bud morphogenesis.

Materials and Methods

Strains and growth media

The *S. cerevisiae* strains used in this work are indicated in Table 1. For routine cultures, *S. cerevisiae* was grown on YED (2% yeast extract and 2% glucose) or YEPD (YED + 2% peptone). The *Escherichia coli* strain used as plasmid host was DH5α [*supE44; Δlac*U*169* (*ϕ*80*lacZ*ΔM15); *hsdR17; recA1; endA1; gyrA96; thi1; relA1*]. For selective growth, bacteria were grown on LB medium containing 100 mg/l ampicillin.

Yeast manipulations

Yeast transformation was carried out by the lithium acetate protocol (Gietz and Woods, 1994). Auxotrophies of the transformants were verified on SD plates (20 g/l glucose, 1.67 g/l yeast nitrogen base without amino acids, 5 g/l ammonium sulphate and the appropriate amount of amino acids) lacking a particular amino acid or nitrogen base. The sensitivity/resistance of the segregants to geneticin (encoded by the *KanMX4* module) was tested in 200 mg/l geneticin-YEPD plates.

Cell polarity and morphogenetic defects were evaluated under fluorescence microscopy after staining cell wall chitin with Calcofluor White (Sigma), as described elsewhere (Pringle, 1991).

For studying the dependency of the polarised localisation of Crh2p on the actin cytoskeleton, cells from the asynchronous wild-type strain 1783 transformed with the plasmid pJV40G were grown to early log phase in YEPD medium at 24°C, and α-factor (Sigma) was added to 10 μg/ml. After 3 hours, more than 80% of cells were observed to have shmoos. At this time cells were harvested by gentle centrifugation, washed once with fresh medium and finally resuspended in fresh medium containing Latrunculin-B (LAT-B, Calbiochem) from a 10 mM DMSO stock to a final concentration of 200 μM or DMSO as a control. The cells were incubated at 24°C for 1 hour. Samples corresponding to different steps of the treatment were harvested by gentle centrifugation, washed twice with PBS buffer, resuspended in PBS and finally observed under a Fluorescence Microscope Eclipse TE 2000-U (Nikon, Tokyo).

Table 1. *S. cerevisiae* strains used in this work

Strain	Genotype	Source or reference
JC223	*MATα rsr1::URA3 leu2-3,112 ura3-52*	J. Chant (Harvard University, Cambridge, MA)
FY1679	*MATa ura3-52 his3D200 leu2Δ1 trp1D63*	EUROFAN collection (Frankfurt, Germany)
DJTD2-16D	*MATα cdc42-1^{ts} ura3 his4 leu2 trp1 gal2*	Johnson and Pringle, 1990
VCY1	*MATα cdc10-11^{ts} ura3-52 leu2-3,112 trp1-1 his4 can^{R}*	Cid et al., 1998
1784	*MATα ura3-52 leu2-3,112 trp1-1 his4 can^{R}*	D. Levin (John Hopkins University, Baltimore, MD)
L2C24d	*MATα cdc15-lyt1^{ts} ura3-52*	Jiménez et al., 1998
10510A	*MATa bni4::Kan^{R} ura3-52 his3D200 trp1D63*	EUROFAN collection (Frankfurt, Germany)
15Daub	*MATa bar1Δ ura3 leu2 his2 ade1 trp1*	A. Durán (Salamanca University, Salamanca, Spain)
HVY260	15Daub *chs5::ADE1*	A. Durán
JM95	15Daub *crh2::Kan^{R}*	This work
JM96	HVY260 *crh2::Kan^{R}*	This work
237	15Daub *chs4::URA3*	C. Roncero (Salamanca University, Salamanca, Spain)
Y603	*MATα ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200*	Santos and Snyder, 2000
Y1949	*MATa ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 sbe2::HIS3 sbe22::TRP1*	Santos and Snyder, 2000

Molecular biology techniques

Standard molecular biology techniques for DNA manipulations and bacterial transformations were performed as described previously (Sambrook et al., 1989). Restriction enzymes were provided by Boehringer-Mannheim. In the construction of *crh2Δ* strains, the complete ORF was deleted, except for the start and stop codons. Disruptions were performed by the SFH (Short Flanking Homology) PCR technique (Wach et al., 1997), which allows the replacement of the target ORF by a selection marker. The strategy for *CRH2* deletion using the *KanMX4* marker from the plasmid pFA6a-KanMX4 (Wach et al., 1994) was similar to that previously described (Rodriguez-Peña et al., 2000) except that the *HIS3* cassette was replaced by *Kan^{r}*.

The YCp(*CDC42Sc*) plasmid containing a wild-type *CDC42* gene in a *LEU2* centromeric plasmid has been previously described (Ziman et al., 1991).

GFP constructions

To determine Crh2p localisation, we used the previously described (Rodriguez-Peña et al., 2000) internal in-frame fusion with the green fluorescent protein from *Aequorea victoria* (GFP) to Crh2p. This cassette was cloned in centromeric pRS416 *URA3* (pJV40U), YCplac111 *LEU2* (pJV40L) or episomic YEp352 *URA3* (pJV40G) and YEplac181 *LEU2* (pJV40F) plasmids to fit genetic background requirements. The pJV40G construction has been described previously (Rodriguez-Peña et al., 2000). pJV40U was obtained by cloning the 3.1 kb *Bam*HI/*Hin*dIII insert from pJV40G into the *Bam*HI/*Hin*dIII-cleaved pRS416 vector (Sikorski and Hieter, 1989), pJV40L by cloning the same insert from pJV40G into the *Bam*HI/*Hin*dIII-cleaved YCplac111 vector (Gietz and Sugino, 1988) and finally, pJV40F was obtained by cloning the 3.1 kb *Bam*HI/*Hin*dIII insert from pJV40G into the *Bam*HI/*Hin*dIII-cleaved YEplac181 vector (Gietz and Sugino, 1988).

The Cwp1-GFP fusion protein used in this work was obtained from the episomic plasmid pAR213 generously donated by Glaxo-Wellcome (Ram et al., 1998). The 2.6 kb *Xba*I/*Hin*dIII insert from pAR213 was cloned into the pRS416 vector digested with the same enzymes, giving rise to the pAR214 plasmid.

When necessary, sequence verification of the clones was carried out on an automated DNA sequencer (ABI 377, Applied Biosystems).

Confocal microscopy techniques

Cells were grown overnight in YEPD at 24°C or 28°C and then transferred to fresh medium. After 3 hours of incubation they were harvested by gentle centrifugation, washed twice with PBS buffer and finally resuspended in PBS. In the case of thermosensitive mutants, sample preparation was identical, except that cells were grown overnight in YED or YEPD at 24°C and then transferred to fresh medium at the same temperature or at 37°C when the expression of the mutant phenotype was required (3 hours of incubation for *cdc10-11* and 5 hours for *cdc42-1* and *lyt1* mutants). Samples were observed under an Eclipse TE-300 (Nikon, Tokyo) microscope attached to a Bio-Rad MRC1024 confocal system (BioRad, Hampstead, UK).

Propidium iodide staining for the detection of lysed cells was performed as described previously (De la Fuente et al., 1992).

Results

Crh2p localisation follows the signals for bud site selection and requires a polarised actin cytoskeleton

Bud1p, Bud2p and Bud5p form the GTPase signalling module that is required for selection of the budding site in both axial and bipolar yeast budding patterns. Mutants defective in any of these genes exhibit the same phenotype: a random budding pattern. To analyse whether Crh2p is recruited to the site of bud emergence and to the mother-daughter neck in a Bud1p-dependent way, we followed the localisation of Crh2p-GFP by means of confocal microscopy (see Materials and Methods) in a haploid *bud1/rsr1* mutant (strain JC223; see Table 1) bearing the Crh2-GFP construction in a centromeric plasmid (pJV40L). As previously described (Rodriguez-Peña et al., 2000), Crh2p, although present at the lateral cell wall, was mainly localised to incipient budding sites in the early stages of budding, as a ring at the bud neck as the bud grows and accumulated in the septum at the time of cytokinesis in the wild-type strain (Fig. 1A). Crh2p also localized to the bud scars in a ring-like fashion, marking the previous division site (Fig. 1A). Consistent with the *bud1* phenotype, Crh2p was also localised in the haploid *bud1* background at the incipient bud site or in the birth scar (Fig. 1B), following a random budding pattern instead of the axial budding pattern of the wild-type haploid cells (Fig. 1A). Therefore, the localisation of the cell wall protein Crh2p does not depend on the mechanisms implied in the bud-site selection pattern. Once the cell selects a position for budding, the cellular machinery necessary for bud development is recruited at this point, including proteins located in the extracellular matrix and involved in cell wall assembly, such as Crh2p.

Additionally, we investigated the distribution of Crh2p in a *cdc42-1*^{ts} mutant (DJTD2-16D strain; see Table 1). Cdc42p is an essential GTPase necessary for organisation of the actin

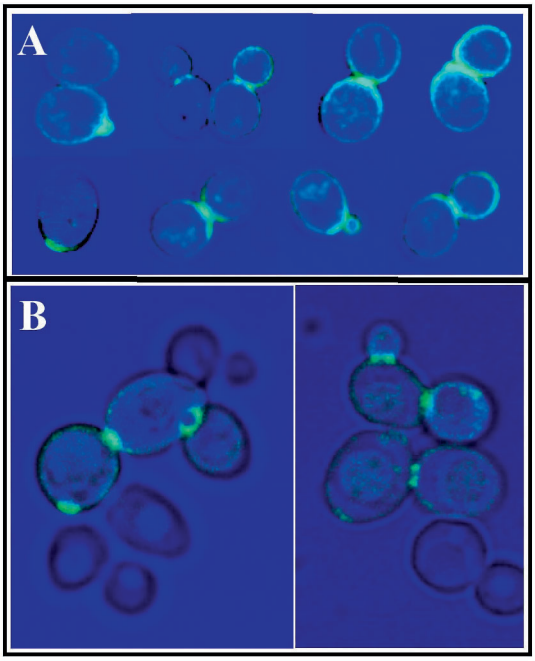

Fig. 1. Localisation of Crh2p-GFP in wild-type and *bud1* cells. Crh2p-GFP was examined by confocal microscopy in exponentially growing wild-type cells transformed with the pJV40U plasmid (FY1679, see Table 1) (A) and *bud1* (JC223) cells bearing the plasmid pJV40L (B).

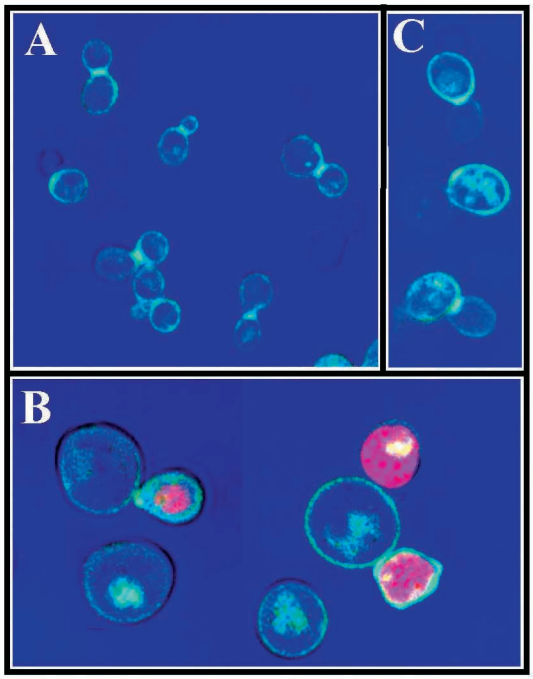

Fig. 2. Crh2p-GFP distribution in a *cdc42-1*^{ts} strain. *cdc42-1*^{ts} cells containing the pJV40G construct were incubated either at permissive growth conditions (24°C) (A) or under restrictive growth conditions (37°C for 5 hours) (B). Polarised distribution of Crh2p was lacking at the restrictive temperature. *cdc42-1*^{ts} cells transformed with the pJV40G (Crh2-GFP) and YCp(*CDC42*Sc) showed a normal polarised localisation pattern at the restrictive temperature (C). Lysed cells were stained with propidium iodide.

cytoskeleton and for cell polarisation (Park et al., 1997). At the permissive temperature the mutant grows and buds normally, but at restrictive temperatures the nuclear cycle continues but bud formation is blocked. The cytoplasmic actin network appears disorganised, and depolarised growth leads to round unbudded cells (Adams et al., 1990). The *cdc42-1* strain was transformed with the pJV40G plasmid. A multicopy plasmid was used in this case because there was a decrease in the GFP signal when cells were incubated at 37°C. No changes were observed in the distribution of the Crh2p-GFP as a function of the plasmid used. When cells were grown at the permissive temperature (24°C), we did not observe variations with respect to the wild-type pattern, the protein being present at the lateral cell wall, incipient budding sites and at the region of the septum (Fig. 2A). However, when cells were shifted to the restrictive temperature (37°C for 5 hours), the mutant phenotype was clearly expressed, with a high percentage of larger, rounded and unbudded cells. Under these conditions the distribution of Crh2p was significantly altered, being mainly localised to the lateral cell wall (Fig. 2B). As expected, the polarised distribution completely disappeared, suggesting that the organisation of the actin cytoskeleton is necessary for targeting Crh2p to the cell wall at sites of polarised growth. To test this possibility, Crh2p localisation was followed in synchronised cells treated with the actin inhibitor Latrunculin B (see Materials and Methods). No polarised distribution of Crh2p was observed in cells treated with this drug (data not shown). The altered distribution of Crh2p in a *cdc42-1* mutant at 37°C was totally corrected when these cells were co-transformed with a plasmid bearing a wild-type *CDC42* gene [YCp(*CDC42Sc*)], which confirmed the results described above (Fig. 2C).

Bud neck localisation of Crh2p depends on the septin ring and septum integrity during cytokinesis

Previous work from different groups have demonstrated the importance of the septins in the correct positioning of various proteins that need to be located at the mother-bud neck, such as Chs3p (DeMarini et al., 1997). In view of the localisation pattern of Crh2p in chitin-rich areas of the cell surface, we investigated the role of septins in the spatial deposition of the GPI-cell wall protein Crh2p following its localisation in the temperature-sensitive *cdc10-11* septin mutant (VCY1). The septin ring in this mutant disassembles after a shift from the permissive (24°C) to restrictive (37°C) temperature. In cells grown at 24°C, Crh2p-GFP (expressed from pJV40G plasmid) localised to the mother-bud neck and the lateral cell wall of the *cdc10-11* mutant strain, following its usual localisation pattern (Fig. 3A). A similar result was also observed in the isogenic wild-type strain grown at 37°C (Fig. 3B). When *cdc10-11* mutant cells were shifted to 37°C for 3 hours, they developed elongated buds that were unable to complete cytokinesis (Cid et al., 1998). Under these conditions, Crh2p is mainly present in the lateral wall of mother cells (Fig. 3C-E) but, in accordance with the loss of bud neck landmarks in this mutant, Crh2p completely disappeared from the base of the elongated buds (Fig. 3C-E), in contrast to the normal strong accumulation of this protein in the septum of wild-type cells, especially during cytokinesis (Fig. 3A-B). Interestingly, in the elongated buds formed under the restrictive temperature, Crh2p diffusely marked both sides of the lateral cell wall at the mother-bud

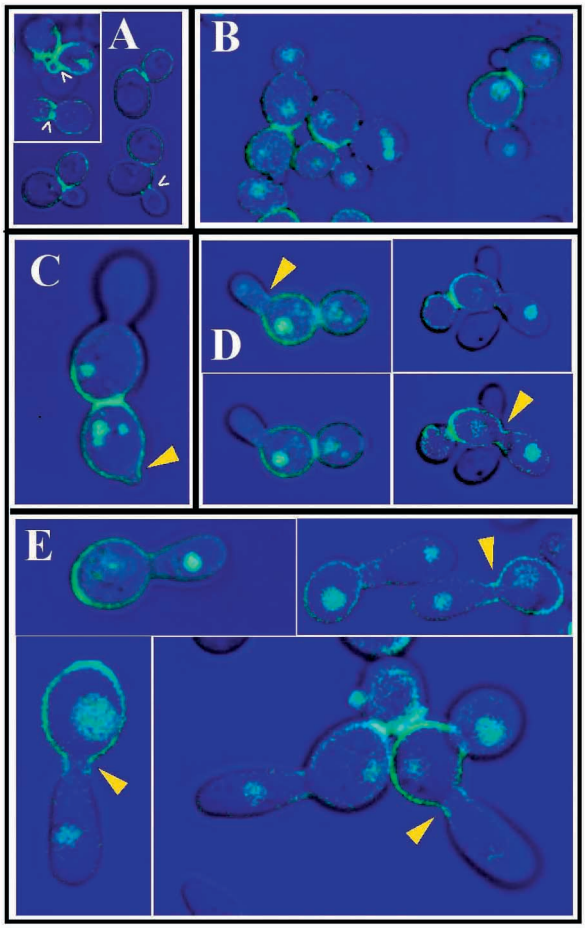

Fig. 3. Localisation of Crh2p-GFP at the mother-bud neck depends on septin integrity. Crh2p-GFP was followed by confocal microscopy in *cdc10-11* cells (VCY1) growing at permissive conditions (24°C) (A). White arrowheads indicate the typical ring-like distribution of the protein in small and medium budded cells. Cellular localisation of Crh2p-GFP in the *cdc10-11* (C-E) and the isogenic wild-type cells (strain 1784) (B) growing at the restrictive temperature (37°C). Yellow arrowheads indicate the signal of fluorescence detected in the enlarged mother-bud neck region in different stages of the bud development. Two different slides of the same cells are shown in D.

neck. By contrast, no conspicuous Crh2p rings were formed under these conditions at the base of new buds, including small (Fig. 3C), medium (Fig. 3D) and large (Fig. 3E) sized buds, suggesting that accurate Crh2p deposition at the mother-bud neck clearly depends on septin ring integrity. This is consistent with the distribution pattern of chitin in septin mutants (DeMarini et al., 1997) or mutants with *GIN4* deleted (Longtine et al., 1998). Gin4p is a protein kinase involved in septin assembly, in which the chitin detected by calcofluor staining shows a diffuse band on both sides of the neck whose intensity decreases with the distance from the mother-bud neck instead of its usual staining, which is restricted to the mother-cell side of the neck (Longtine et al., 1998). The signal corresponding to the GFP-fusion protein could be occasionally detected in the neck region of cells that were in the late stages of the cell cycle. Since the mutation is not 'leaky', we interpret that those cells had already started budding at the time of the

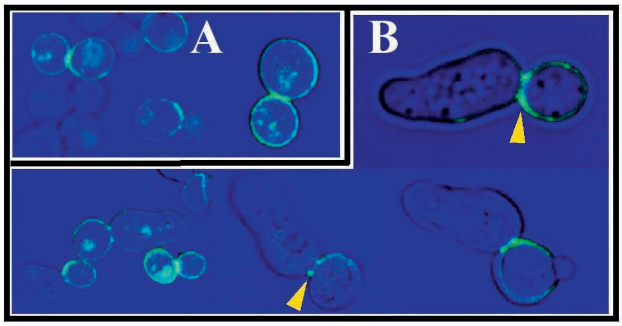

Fig. 4. Localisation of Crh2p-GFP in a *cdc15-lyt1* strain. Crh2p-GFP was examined by confocal microscopy in *cdc15-lyt1* cells (L2C24d strain) growing at the permissive temperature (24°C) (A) or 5 hours after shifting to the restrictive temperature (37°C) (B). Yellow arrowheads indicate the presence of Crh2p-GFP in a conspicuous ring at the mother-bud neck of cells expressing the mutant phenotype. In those cells that do not express the mutant phenotype the fusion protein is correctly localised at the septum region (B, bottom-left).

temperature shift and thus maintain its correct localisation (Fig. 3C-E).

The enhanced signal of Crh2p-GFP at cytokinesis in wild-type cells could be caused by the association of Crh2p with the developing septum, as the septin mutant, which fails to form septa, clearly lacked Crh2p enrichment at this region. We next characterised the localisation of Crh2p in yeast cells bearing the *cdc15-lyt1* thermosensitive mutation (mutant L2C24d) (Jimenez et al., 1998). The *CDC15* gene encodes a protein kinase essential for exit from the M phase in the *S. cerevisiae* cell cycle (Surana et al., 1993). Cells carrying the *lyt1* mutation are unable to septate at 37°C, but the septins remain at the mother-daughter neck (Jimenez et al., 1998). When *lyt1* cells transformed with the Crh2p-GPF construction (pJV40G plasmid) were grown at the permissive temperature (24°C), the distribution of the protein was identical to that of the wild-type (Fig. 4A). As in the *cdc10-11* mutant, Crh2p was not accumulated at the septal region between the mother and elongated buds at the restrictive temperature (5 hours at 37°C). However, in contrast to *cdc10-11*, in most of these cells Crh2p localised to the mother-bud neck in a conspicuous ring-like structure (Fig. 4B). All these data suggest that the deposition of Crh2p at the neck between mother and daughter cells during cytokinesis depends both on septins, for proper localisation of a Crh2p ring structure, and on septum integrity, for the deposition of Crh2p at the septum structure itself.

Bud neck localisation of Crh2p depends on Bni4p but not on Chs4p

A model has been suggested for the spatial localisation of CSIII activity, in which the septin complex localises Bni4p through the interaction of this protein with Cdc10p and, at the same time, Bni4p localises the chitin synthase III complex (including Chs3p and Chs4p) through its interaction with Chs4p. In order to test the requirement of Bni4p for Crh2p distribution, a *bni4*Δ strain (10510A) was transformed with the pJV40U plasmid, and Crh2p-GFP was monitored by confocal microscopy. As previously described (DeMarini et al., 1997),

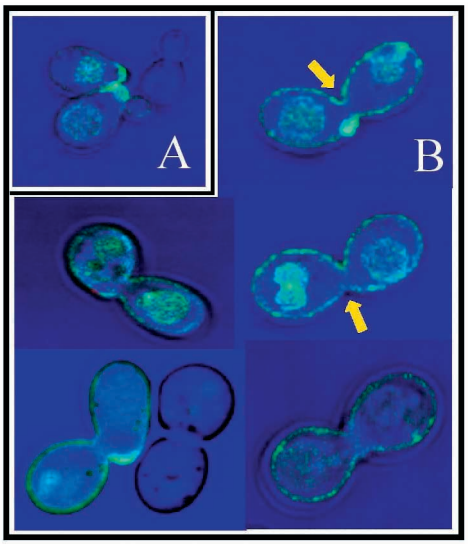

Fig. 5. Bni4p is required for the proper Crh2p localisation during late stages of the cell cycle. *bni4* cells (10510A strain) were transformed with the plasmid pJV40U and grown at 28°C. Crh2p-GFP localise to the site of bud emergence during early stages of the cell cycle (A), but no Crh2p was present at the septum during cytokinesis (B), as indicated by yellow arrows.

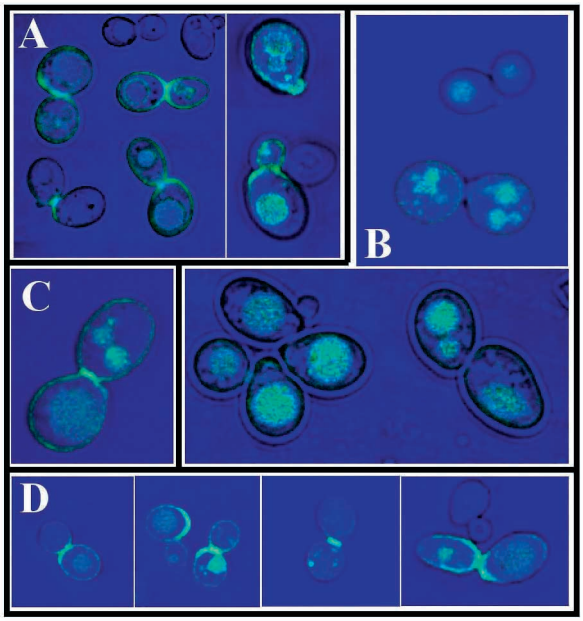

Fig. 6. Localisation of Crh2p-GFP and Cwp1-GFP in a *chs5* strain. Wild-type cells (JM95 strain) transformed with the plasmid pJV40U (Crh2p-GFP) (A) and *chs5* cells (JM96 strain) transformed with the same plasmid (B-C) or the plasmid pAR214 (Cwp1-GFP) (D) were grown at 28°C and analysed by confocal microscopy.

the majority of *bni4* mutant cells showed enlarged bud necks and some protuberances at previous division sites (Fig. 5B). As shown in Fig. 5A, Crh2p localised at incipient budding sites independently of Bni4p. During cytokinesis, it localised to both mother and daughter cells at the lateral cell wall and also in the protuberances at previous division sites. However, no Crh2p was found at the bud-neck region at the time of cytokinesis (Fig. 5B), suggesting that the deposition of Crh2p in this area, late in the cell cycle, depends on the presence of Bni4p. This result prompted us to test whether, like Chs3p, which depends both on Bni4p and Chs4p, Chs4p was also necessary for the proper localisation of Crh2p. To address this, Crh2-GFP was followed in a *chs4*Δ mutant (237 strain) transformed with the pJV40F plasmid. However, in contrast to Chs3p, Crh2p distribution at the bud neck does not depend on Chs4p at any stage, early or late, in the cell cycle (data not shown).

Crh2p localisation is dependent on Chs5p and Sbe2p/Sbe22p transport systems

Among the different steps required to achieve correct bud growth, the secretory machinery plays an essential role, as it is required to specifically direct new plasma membrane and cell wall material to the growth site (Igual et al., 1996; Madden and Snyder, 1998). However, the molecular mechanisms that control this process are poorly understood.

Recently two pathways have been described for the transport of cell wall components to the cell surface. One of them is dependent on Chs5p, and it is required for the transport of Chs3p to the bud neck. The second pathway involves the Sbe2p/Sbe22p redundant proteins, which could be important for cell wall mannoprotein transport.

Previous results (Rodriguez-Peña et al., 2000), as well as evidence in this work, indicated a probable functional relationship between Crh2p and chitin deposition. Therefore, we were prompted to evaluate the dependence of Crh2p localisation on the aforementioned pathways. To this end, we first analysed the localisation of the Crh2p-GFP fusion protein in wild-type and *chs5* isogenic strains. A *chs5* mutant strain (HVY260) and its isogenic wild-type (15Daub) were transformed with the plasmid pJV40U. However, Crh2p localisation was difficult to follow in this background, probably because of the problem of the competition of Crh2p-GFP with the native Crh2p. In an attempt to solve this problem, the *CRH2* gene was deleted in both strains by means of the SFH-PCR technique (see Materials and Methods). The double mutant *crh2 chs5* (JM96) was viable and did not exhibit any variation in growth with respect to the strain bearing the single mutation *chs5*. We then compared Crh2p-GFP localisation in both isogenic *crh2* (JM95) and *crh2 chs5* strains. In the JM95 strain, Crh2p was mainly observed (85%, (n=50) of the cells with detectable fluorescence signal) at correct positions and sometimes in vacuoles (this background is extensively vacuolated) (Fig. 6A). By contrast, the localisation pattern of Crh2p changed in the JM96 strain (*chs5* mutant), where most of the Crh2p protein accumulated in internal vesicles (90% of cells, n=50) (Fig. 6B), and only occasionally did a residual signal of the protein reached the expected sites (Fig. 6C). These results pointed out that Crh2p localisation is dependent on Chs5p and suggest that the transport systems for Chs3p and Crh2p are to, a certain extent, coincident. Interestingly, the transport of another GPI cell wall protein, Cwp1p, does not depend on this transport system as the Cwp1p-GFP localisation pattern was similar to one previously described (Ram et al., 1998) and did not vary between *chs5* (Fig. 6D) and its isogenic wild-type (data not shown).

Two proteins (Sbe2p and Sbe22p) from the Golgi apparatus

have recently been implicated in the transport of Chs3p. Although the secretion of invertase and exoglucanase was not affected in the *sbe2 sbe22* mutant, the participation of Sbe2p and Sbe22p in the transport of other mannoproteins has been suggested, as deduced from the reduced mannoprotein layer of the *sbe2 sbe22* strain (Santos and Snyder, 2000). To investigate the possible role of the Sbe2p and Sbe22p proteins in the transport of Crh2p to the cell surface, we followed Crh2-GFP localisation in an *sbe2 sbe22* background (Y1949) and compared this with its distribution in the isogenic wild-type strain (Y603), both of which were transformed with the pJV40U plasmid. Crh2p-GFP was correctly localised in the wild-type strain grown at 24°C, marking the lateral cell wall, although mainly accumulating in the mother-bud neck and septum during cytokinesis as well as in bud scars (Fig. 7A). However, in the absence of Sbe2p and Sbe22p, Crh2p was completely delocalised. Instead of its usual localisation pattern, the whole cargo of the protein was retained, and it accumulated in an internal compartment of the cell, being unable to reach the cell surface either at the lateral cell wall or at polarised growth sites (Fig. 7B). Interestingly, and in accordance with the above described non-dependence of Cwp1p transport on Chs5p, the transport and correct localisation of the GPI cell wall protein Cwp1p does not depend on Sbe2p and Sbe22p, as judged from the similar pattern of fluorescence observed in both the *sbe2 sbe22* mutant (Fig. 7C) and its isogenic wild-type strain (data not shown). Taken together, these results confirm previous observations that indicated that Chs5p and Sbe2p/Sbe22p play a selective role in transport of proteins to the cell wall. Our observations clearly show that these two transport systems are not specific for chitin synthesis (Chs3p) but are also required for other aspects of the biogenesis or modification of the cell wall.

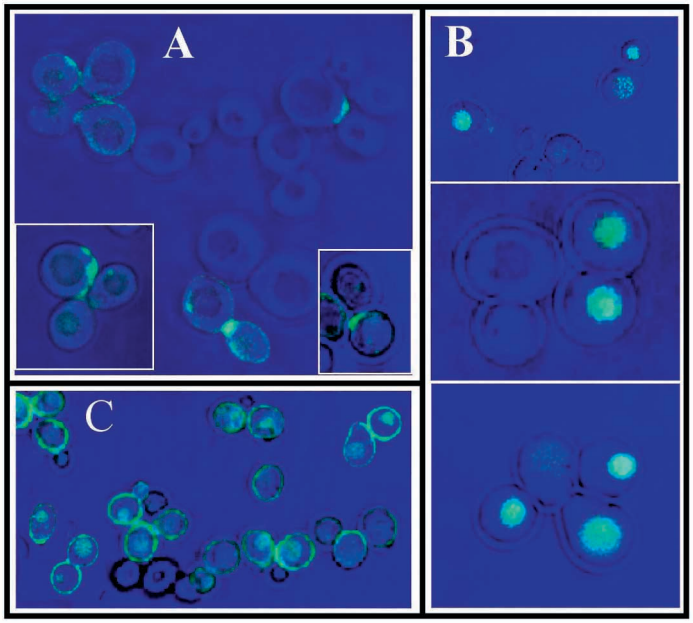

Fig. 7. Crh2p-GFP localisation depends on the presence of Sbe2p and Sbe22p. Crh2-GFP was followed in wild-type (Y603 strain) (A) and *sbe2 sbe22* cells (Y1949 strain) (B) growing at 24°C. Crh2p was properly distributed in wild-type cells marking septa and bud scars. However the fusion protein completely mislocalised in *sbe2 sbe22* cells. C shows the localisation of Cwp1-GFP (plasmid pAR214) in *sbe2 sbe22* cells growing at 24°C.

Discussion

The temporal and spatial control of assembly of cell wall components in the fungal cell is a critical process, but it is poorly understood, with the exception of the control of chitin deposition, which has been substantially analysed.

Our results represent new insights into the mechanisms that control the spatial localisation of proteins associated with the cell wall matrix to polarised growth sites. We have specifically studied the cell wall mannoprotein Crh2p, a protein involved in cell wall assembly and covalently attached to the cell wall glucan (Hamada et al., 1998) (J.M.R.-P., C.R., A.A. et al., unpublished). Correct localisation of Crh2p at the mother-bud neck is first controlled by the cellular machinery responsible for the selection of the new budding site. In the absence of Bud1p, the cell polarity establishment protein complex is recruited by the cell to the mother-bud neck, but it is not recruited to the correct place. Crh2p is deposited following the random budding pattern of *bud1* cells. Therefore, Crh2p recruitment relies on other cell polarity cues rather than on the recognition of bud site selection elements. In fact, correct Crh2p localisation to polarised growth sites is completely dependent on Cdc42p. Both actin cytoskeleton polarisation and organisation of the septin ring are disrupted in a *cdc42*^{ts} mutant under the restrictive temperature. The lack of Crh2p polarisation in Latrunculin-B-treated cells demonstrates that the actin cytoskeleton is necessary for targeting Crh2p to the cell wall at sites of polarised growth. This polarisation is also dependent (as explained below) on the presence of a functional septin ring. To our knowledge, this is the first cell wall protein whose localisation depends on the polarity signalling pathway. The importance of the actin cytoskeleton in chitin deposition has also been reported previously. Chitin is delocalised in temperature-sensitive actin mutants (Novick and Botstein, 1985), and *cdc42* mutants also show defects in correct chitin deposition (Adams et al., 1990). In accordance with the important role for the actin cytoskeleton in the correct organisation and construction of the cell wall, an aberrant cell wall is formed over the surface of the isodiametrically growing *act1* mutant cells (Gabriel and Kopecka, 1995). In this context, our data indicate that proteins localised extracytoplasmically and involved in cell wall construction, such as Crh2p, are unable to reach their correct destination at the cell surface in the absence of an organised actin cytoskeleton.

The data reported here clearly demonstrate that Crh2p, a GPI cell wall mannoprotein localised in the extracellular matrix, depends on the septin ring for proper deposition at the mother-bud neck. Many other proteins that function at the mother-bud neck, such as Chs3p, Bni4p, Chs4p (DeMarini et al., 1997), Bud3p (Chant and Pringle, 1995) or Bud4p (Sanders and Herskowitz, 1996), depend on septins for their localisation. Septins provide a scaffold for the organisation of these proteins (and probably other proteins still unidentified) needed at this specific region of the cell surface (Field and Kellogg, 1999), some of them being required for chitin deposition. However, to date no other proteins directly involved in cell wall construction itself other than those involved in chitin

deposition (such proteins are associated with the plasma membrane and not with the cell wall structure) have been shown to be organised by the septin scaffold. Our results clearly extend the previous role of septins in chitin localisation to other proteins involved in cell wall construction and suggest that septins could play an important role in the organisation of the whole cell wall assembly process at the mother-bud neck. Moreover, proper localisation of Crh2p at this region during cytokinesis depends on septum integrity. In wild-type cells, Crh2p accumulates at the septum during the cytokinesis stage, probably because the cell needs the protein there at this time for the formation of the secondary septum. However, in *cdc10-11* and *cdc15-lyt1* mutants, which are unable to septate, Crh2p is not accumulated at this region. It is likely that in the absence of specific unknown septum landmarks, proteins involved in septum formation, including Crh2p, are not recruited to this site.

In the absence of Bni4p, Crh2p is not able to localise to the mother-bud neck, in particular to the septum in cells undergoing cytokinesis. DeMarini et al. have offered evidence that Bni4p provides a link between septins and Chs3p through its interaction with Chs4p, at least in the periods immediately before and during bud emergence (DeMarini et al., 1997). More recently it has been proposed that Bni4p is required for bud-neck localisation of Chs4p at bud emergence but not at cytokinesis, suggesting the existence of other chitin targeting mechanisms late in the cell cycle (Kozubowsky et al., 2001). The data reported here suggest that Bni4p might be not only related to the Chs3p distribution but also involved in the interaction between septins and other proteins involved in cell wall construction, such as Crh2p. In contrast to Chs3p, proper localisation of Crh2p does not depend on Chs4p either in bud emergence or during cytokinesis. Bni4p could therefore be part of the mechanisms involved in the localisation and recruitment of the cell wall protein Crh2p to bud neck late in the cell cycle, at the time of cytokinesis, when this protein needs to be accumulated at this region for proper septum formation. Supporting this idea, Bni4p has been localised as a ring on both sides of the neck at cytokinesis (Kozubowsky et al., 2001). Probably Bni4p has a more general function as a protein scaffold for proteins required for polarised growth at the bud-neck region, early at bud emergence and later during cytokinesis.

A critical step in proper localisation of proteins involved in polarised growth, including those necessary for cell wall construction, is the transport of these proteins to their respective sites of action. The secretory pathway must deliver all the proteins required for these events to discrete growth sites at the cell surface. Myo2p, a yeast class V myosin, has been implicated in the transport of a class of secretory vesicles, from the mother to the bud, that facilitate the transport of a specific set of proteins (Govindan et al., 1995). These authors proposed that the cargo of the vesicles that are rapidly accumulated in a *myo2-66* mutant could be the components necessary for cell wall assembly, such as chitin synthases, chitinase or endoglucanases. Which specific proteins are transported in these vesicles is now beginning to be understood. Work by Santos and Snyder (1997) demonstrated that Chs3p is one of these proteins (Santos and Snyder, 1997). The localisation of Chs3p requires Myo2p and the actin cytoskeleton in addition to Chs5p. Chs5p, a protein from the trans-Golgi network, is necessary for the proper secretion of Chs3p, and the possibility that this protein might be associated with the outside of secretory vesicles, facilitating their interaction with either the transport machinery or components at the bud site and bud-neck region, has been suggested (Santos and Snyder, 1997). We wondered whether Chs5p might be involved in the delivery of other proteins that contribute to cell wall construction. The work reported here demonstrates the dependence on Chs5p for the proper localisation of Crh2p to polarised growth sites and particularly to the mother-neck region. Therefore, Chs5p-containing vesicles may not represent, as previously thought, a specific and unique subset of vesicles involved in the transport of Chs3p but may also be involved in the localisation of other proteins that participate in the process of cell wall assembly, such as Crh2p. Interestingly, the transport of other secreted proteins such as Exg1p, - an extracellular exo-β 1,3 glucanase - is not affected in *chs5* mutants (Santos et al., 1997). Likewise, the localisation of the GPI cell wall Cwp1p does not depend on Chs5p, as reported here.

Another pathway for the transport of cell wall components to the cell surface has recently been proposed by Santos and Snyder (Santos and Snyder, 2000). This pathway, involving two Golgi proteins - Sbe2p and Sbe22p - must be interconnected with the one based on Chs5p, as Chs3p is also mislocalised in the *sbe2/sbe22* mutant. A substantially diminished outer layer of mannoproteins has been observed in this mutant. However, no specific mannoproteins have been associated so far with this pathway. Neither the secretion of invertase nor that of exoglucanase is defective in this mutant (Santos and Snyder, 2000). Moreover, preliminary results from this work suggested that transport of other two cell wall proteins (Cwp1p and Pir2p) do not depend on this pathway. Here we demonstrate that the transport and localisation of Cwp1 does not depend on this pathway. However, the transport of Crh2p to the cell wall at the sites of polarised growth and even to the lateral cell wall is completely impaired in a *sbe2 sbe22* mutant, meaning that transport of this mannoprotein is dependent on the Sbe2p/Sbe22p pathway. Our data point to the notion that Crh2p and the machinery for chitin synthesis, in particular Chs3p, are clearly associated. The localisation of Crh2p is not only reminiscent of and resembles the distribution of chitin in the cell wall, but also depends on the mechanisms already described for the transport of Chs3p; at least partially for Chs5p and completely for Sbe2p and Sbe22p. However, the localisation of Crh2p does not requires chitin deposition, since its localisation is maintained in both *chs2* and *chs3* mutants (Rodriguez-Peña et al., 2000). Interestingly, however, and in accordance with the association of Crh2p deposition with sites of chitin localisation, Crh2p is strongly accumulated in the aberrantly thickened (and very chitin-rich) septa seen in a *chs2* mutant (Rodriguez-Peña et al., 2000).

From many studies it is now becoming clear that the secretion of proteins through the secretory pathway involves different vesicles that transport specific sets of cargo proteins (Chuang and Schekman, 1996; Govindan et al., 1995; Madden and Snyder, 1998). The data presented here support this hypothesis and offer new insight into the cargo of these vesicles. Why is the transport of Crh2p dependent on the Chs5p and Sbe2p/Sbe22p pathways, whereas other cell wall proteins such as Cwp1p, Exg1 and Pir2p, are not? Cwp1p and Crh2p are both GPI cell wall proteins (containing a

glycosylphosphatidylinositol-derived structure) covalently linked to β-1,6 glucan. In contrast, Pir2p, which does not contain a GPI anchor site, is covalently linked to β-1,3 glucan, and Exg1p is mainly secreted to the culture supernatant. Therefore, the differences in the dependence on the mechanisms of transport described above between the cell wall proteins Crh2p, Cwp1p, Pir2p and Exg1p cannot be explained in terms of their association with different structural components of the cell wall. An attractive hypothesis to account for these results is the existence of specific transport systems that could be involved in the localisation of functionally related cell wall proteins required for cell wall construction at specific sites of the cell surface and/or in a particular stage of cell growth.

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