Chitin synthase III requires Chs4p-dependent translocation of Chs3p into the plasma membrane

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Accepted 24 April 2007

Journal of Cell Science 120, 1998-2009 Published by The Company of Biologists 2007

doi:10.1242/jcs.005124

Summary

In *Saccharomyces cerevisiae*, Chs4p is required for chitin synthase III (CSIII) activity and hence for chitin synthesis. This protein is transported in vesicles in a polarized fashion independently of the other Chs proteins. Its association with membranes depends not only on prenylation, but also on its interaction with other proteins, mainly Chs3p, which is the catalytic subunit of CSIII and is able to properly direct Chs4p to the bud neck in the absence of prenylation. Chs4p is present in functionally limiting amounts and its overexpression increases Chs3p accumulation at the plasma membrane with a concomitant increase in chitin synthesis. In the absence of Chs4p, Chs3p is delivered to the plasma membrane but fails to accumulate there because it is rapidly endocytosed and accumulates in intracellular vesicles. A blockade of endocytosis stops

Chs3p internalization, triggering a significant increase in chitin synthesis. This blockade is independent of Chs4p function, allowing the accumulation of Chs3p at the plasma membrane even in the *chs4Δ* mutant. However, the absence of Chs4p renders CSIII functionally inactive, independently of Chs3p accumulation at the plasma membrane. Chs4p thus promotes Chs3p translocation into the plasma membrane in a stable and active form. Proper CSIII turnover is maintained through the endocytic internalization of Chs3p.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/120/12/1998/DC1>

Key words: Chitin, Endocytic turnover, *S. cerevisiae*

Introduction

In *S. cerevisiae*, chitin synthesis occurs in a highly organized fashion. It begins during the initial steps of budding, forming a growing ring that will remain at the mother side after cell division. This ring constitutes a kind of structural scaffold for the formation of the cell wall at the neck (Schmidt et al., 2003). The chitin ring contains most of the cellular chitin and is synthesized by CSIII (Cabib et al., 1996). Cell separation occurs through the formation of a chitinous disk, which accounts for ~10% of the total chitin and is made by CSII. In addition, yeast cells display a small (<10%) amount of chitin along their lateral cell walls, also made by CSIII during the later stages of bud formation (Cabib et al., 1996).

The isolation of mutants with reduced levels of chitin has allowed the identification of several proteins required for CSIII activity (Roncero, 2002). Chs3p encodes the catalytic subunit of CSIII, whereas the other Chs proteins participate in the intracellular sorting of Chs3p to the plasma membrane, where it synthesizes chitin. Exit of Chs3p from the ER depends on Chs7p (Trilla et al., 1999), which mediates the incorporation of Chs3p into COPII vesicles and its exit to the Golgi (Kota and Ljungdahl, 2005). Chs5p and Chs6p function in the Golgi, and both are required for the exit of Chs3p from the Golgi to the plasma membrane through their participation in the formation of specialized Chs3p-containing vesicles in the trans-Golgi network (TGN), which are later delivered to the plasma membrane in a polarized fashion. Chs6p belongs to a family of recently described proteins named ChAPs, which mediate the cargo into the TGN-derived vesicles (Trautwein et

al., 2006), whereas Chs5p is unique and plays a more general role in vesicle formation at the TGN (Trautwein et al., 2006). Not surprisingly, *chs5Δ* mutants accumulate additional phenotypes not related to chitin synthesis (Santos et al., 1997). Recently, it has been shown that Chs5p, together with Chs6p and its three paralogues, form a vesicular coat complex at the TGN that is required for the capture of membrane proteins in route to the cell surface (Wang et al., 2006). Previous work has described alternative routes in the sorting of Chs3p that bypass the requirement for Chs5p and Chs6p (Ortiz and Novick, 2006; Valdivia et al., 2002).

During its transit through the ER and Golgi, Chs3p is assumed to undergo posttranslational modifications that render it active at the plasma membrane (Bulik et al., 2003). Later on, Chs3p is endocytosed from the plasma membrane (Holthuis et al., 1998), probably in an inactive form, to populate a pool of stable vesicles in the early endosomal compartment (Valdivia et al., 2002; Ziman et al., 1996). Such vesicles, together with those derived from anterograde transport, serve as a TGN reservoir of Chs3p ready to be transported to the membrane after its sorting in this compartment (Valdivia et al., 2002). However, the role of endocytosis turnover in the regulation of chitin synthesis has not been explored experimentally.

Chs4p was originally identified from mutants deficient in chitin synthesis. In *S. cerevisiae*, Chs4p plays two different roles in CSIII regulation: it is required for eliciting CSIII activity (Trilla et al., 1997), and hence for chitin synthesis, but also for the interaction of Chs3p with Bni4p and, through this, with septins (DeMarini et al., 1997). This allows proper CSIII

localization at the neck and correct formation of the chitin ring (Sanz et al., 2004). However, this latter function is independent of CSIII activation since *bni4Δ* cells contain normal amounts of chitin (Sanz et al., 2004). Accordingly, Chs4p colocalizes with Chs3p at the neck, a localization that is altered in the absence of Bni4p (DeMarini et al., 1997; Sanz et al., 2004). Chs3p and Chs4p interact physically (DeMarini et al., 1997; Ono et al., 2000) and this interaction is required for promoting chitin synthesis (Ono et al., 2000) and for the proper localization of chitin (DeMarini et al., 1997).

Although the role of Chs4p in the anchoring of CSIII to the septin ring appears straightforward, the molecular mechanism involved in CSIII activation remains obscure. In the *chs4Δ* mutants CSIII is inactive and its activity can only be elicited in vitro with protease treatment. In addition, *CHS4* overexpression reduces CSIII zymogenicity in the wild type (Trilla et al., 1997). However, Chs4p does not show similarity to any known protease, it lacks protease activity in vitro and Chs3p has never been shown to be processed proteolytically in vivo (Cos et al., 1998; Santos and Snyder, 1997). Therefore, the zymogenic nature of CSIII in the *chs4Δ* mutant is probably a biochemical artifact. Recently it has been shown that Chs4p is prenylated, pointing to Chs4p as being a catalytic part of CSIII (Grabinska et al., 2007). Yeasts also contain a functional homologue of Chs4p, Shc1p, which promotes chitin synthesis during sporulation but cannot direct chitin ring assembly (Sanz et al., 2002).

In the past few years it has become evident that chitin levels are related to the amount of Chs3p in the plasma membrane. Chitin synthesis increases in cell-wall-deficient strains, such as the *fkslΔ* or the *gas1Δ* mutants, linked to a higher delivery of Chs3p to the plasma membrane according to fluorescence microscopy (Carotti et al., 2002; Garcia-Rodriguez et al., 2000). It was later shown at the biochemical level that activation of the PKC response that occurs in these mutants increases the amount of Chs3p in the plasma membrane

(Valdivia and Schekman, 2003), providing a convincing explanation for these results. More recent findings have indicated that the localization of Chs3p in the plasma membrane is altered in *sac1Δ* (Schorr et al., 2001) and *ypt32Δ/ypt31Δ* (Ortiz and Novick, 2006; Sciorra et al., 2005) mutants, suggesting that the lipid composition and activity of Rab proteins influence Chs3p delivery to the plasma membrane. This evidence points to the translocation of Chs3p into the plasma membrane as a major step in the regulation of chitin synthesis in yeast. Here we analyze the role of Chs4p in CSIII activation, showing that Chs4p is required for correct Chs3p translocation to the plasma membrane, a process that is linked to the activation of CSIII.

Results

Chs4p is located along the plasma membrane independently of other Chs proteins

Chs3p and Chs4p appear to colocalize at the neck region of the budding yeast (DeMarini et al., 1997), which together with the role of Chs4p in CSIII activation leads to the assumption that the interaction of Chs3p and Chs4p at the neck triggers CSIII activation (Roncero, 2002). Here, closer scrutiny revealed that in addition to neck localization Chs4p-GFP was also uniformly distributed along the plasma membrane of both mother and daughter cells, where Chs3p-GFP was apparently not present (Fig. 1A, compare upper and lower figures). Similar results were obtained with confocal microscopy, since Chs4p appeared uniformly distributed along the yeast plasma membrane, with a more marked accumulation at the neck, whereas Chs3p was localized only at the neck region and in some intracellular vesicles (Fig. 1B). Some Chs3p remained irregularly associated with the plasma membrane because of the artificial accumulation of Chs3p-GFP at the ER. These distributions were confirmed by subcellular fractionation in continuous sucrose gradients (Fig. 1C). Most Chs3p was localized in a pool of intracellular vesicles partially coincident

Fig. 1. Intracellular distribution of Chs4p and Chs3p. (A,B) Chs4p-GFP and Chs3p-GFP were localized by fluorescence (A) and confocal microscopy (B) in CRM233 and CRM103 strains, respectively. (C) Subcellular fractionation in continuous sucrose gradients of cell extracts obtained from the strain JAY29 containing plasmid pRS314-*CHS4-GFP*. Protein amounts were determined in even fractions by western blot using specific antibodies. The graph shows the quantitative analysis of these western blots data.

with Pep12p, and only minor amounts of Chs3p were localized in the plasma membrane, as previously reported (Santos and Snyder, 1997). Chs4p distribution was clearly bimodal, a significant part of the protein colocalizing with Pma1p, and hence along the plasma membrane. The remaining Chs4p appeared to be located in vesicles with a similar density to that of vesicles containing Chs3p. We were unable to detect Chs4p-containing vesicles microscopically, either using living fluorescence (Fig. 1) or immunofluorescence techniques (not shown). Neither Chs4p nor Chs3p migrated in the lower density fractions, supporting the association of both proteins with membranes. These results show that a significant part of Chs4p localizes along the plasma membrane independently of Chs3p and suggest that the amount of Chs4p interacting with Chs3p at the neck is rather limited.

In light of this distribution it is uncertain how Chs4p might arrive at the plasma membrane. Fig. 2A shows that Chs4p-GFP distribution was not significantly disturbed in any of the *chsΔ* mutants. Thus, Chs4p intracellular traffic must be independent of Chs3p and all CSIII associated machinery, including Chs5p, a protein with broad functions in protein sorting in the TGN. In order to explore this transport in more detail we controlled Chs4p expression from the *GALI* promoter. After transfer of the cells from raffinose to galactose, we determined Chs4p-GFP localization (Fig. 2B). As early as 30 minutes after the transfer, Chs4p was faintly detected at the neck; longer inductions increased the amount of Chs4p at the neck and virtually no signal was detected along the plasma membrane after 80 minutes. A faint signal appeared in some buds 2 hours after incubation (Fig. 2B, arrowheads) and a significant

accumulation along the plasma membrane was only observed 3 hours after induction. Identical results were observed when induction was carried out in a $\Delta chs3$ strain (Fig. 2B). A general blockade of secretion in the *sec6*, *sec7* or *sec16* thermosensitive mutants strongly reduced Chs4p accumulation at the neck (not shown). These results indicate that Chs4p transport was mostly polarized, depending on the general secretion machinery, although this polarization is independent of its interaction with Chs3p.

The C-terminal region of Chs4p is required for its association with plasma membrane but not for biological function

The above results strongly point to Chs4p as a membrane-associated protein, even though it lacks transmembrane domains and its prenylation domain appears dispensable for function (DeMarini et al., 1997; Ono et al., 2000; Trilla et al., 1997). To study this association, we generated different Chs4p mutant proteins, including several C-terminal truncations and elimination of the prenylation site (Chs4p^{C693S}). A preliminary analysis indicated that all mutations created were functional and behaved similarly, and hence we concentrated on the characterization of two of them: the truncated Chs4p Δ 590, which lacks the last 106 amino acids of the protein, and the non-prenylated Chs4p^{C693S}. Both proteins accumulated at normal levels in the cell, interacted correctly with Bni4p and Chs3p, and restored calcofluor sensitivity in the *chs4Δ* mutant (data not shown). The functionality of these proteins was assessed quantitatively by measuring chitin levels. The *CHS4Δ*590 and *CHS4*^{C693S} strains contained 272.0±10.7 and 269.9±25 nmoles

Fig. 2. Intracellular distribution of Chs4p-GFP. (A) Localization of Chs4p-GFP in the indicated strains. Note the accumulation at the septum but also the defined staining of the cell periphery in mother and daughter cells. (B) Intracellular accumulation of Chs4p-GFP in the indicated strains after transfer of the cells to galactose. Arrowheads indicate Chs4p deposition along bud membrane. Numbers indicate the time (in minutes) after induction. Wt and *chs3Δ* were transformed with the pRS314-*GALI-CHS4-GFP* plasmid. All the strains used in the figure are W303 derivatives.

of N-acetyl-glucosamine/100 mg cells, respectively, accounting for ~92% of that in the wild type (292.0 ± 21.6 nmoles of N-Acetyl-glucosamine/100 mg cells). This was a clear indication that both proteins were biologically active. They also promoted chitin synthesis at the neck, in a distribution identical to the full protein (not shown). The *CHS4* Δ 590 and *CHS4*^{C693S} strains showed a modest increase in calcofluor resistance compared with the wild type, but showed an identical pattern of sensitivity to SDS, caffeine and zymolyase, arguing against additional defects in cell wall construction. Similar conclusions were obtained in the BY4742 genetic background (not shown). These results somehow disagree with a report published during the revision of this manuscript (Grabinska et al., 2007). Thus, we also determined quantitatively the chitin levels in the *CHS4*^{C693S} mutant in the BY4742 background. This strain contained 82% of the chitin levels determined for the corresponding wild type, in closer agreement with the recently reported data. The differences observed are probably a result of technical differences (see Discussion).

In addition, we found Chs4p Δ 590-GFP and Chs4p^{C693S}-GFP localized at the neck, similarly to the control (Fig. 3A and not shown) in either genetic background. However, neither protein was detected along the plasma membrane (Fig. 3A, and not shown) in thousands of cells examined. In agreement with this result, sucrose subcellular fractionations revealed that Chs4p Δ 590-3xHA and Chs4p^{C693S}-3xHA localized mostly in the vesicle fractions but were absent from the plasma membrane fractions (not shown). However, both Chs4p Δ 590 and Chs4p^{C693S} retained their physical interaction with Bni4p and Chs3p, prompting us to test their localization in strains lacking these proteins. The absence of Bni4p or Chs3p only modestly reduced Chs4p-GFP localization (Sanz et al., 2004) (Fig. 3B). Chs4p Δ 590-GFP localization was similar to that of the wild-type Chs4p in the Δ *bni4* mutant (Fig. 3B). However, the absence of Chs3p or the simultaneous absence of Bni4p and Chs3p reduced

Chs4p Δ 590-GFP accumulation at the neck. In comparison with wild-type Chs4p, only one-third of the cells showed neck localization, and in all cases, the staining intensity was much lower than in the control (Fig. 3B, see arrowheads). In the double *bni4* Δ *chs3* Δ mutant, some Chs4p Δ 590-GFP accumulated in the vacuolar compartment (Fig. 3B) and the total amount of the protein detected by western blotting was approximately one-quarter of that found in the control (not shown). These results highlight the direct role of Chs3p in the correct positioning of the non-prenylated Chs4p as well as the relevance of Chs4p association with membranes.

We confirmed these results biochemically by comparing the association of Chs4p-3xHA and Chs4p Δ 590-3xHA with membranes (Fig. 3C). Chs4p appeared mostly associated with the particulate fraction (Po), although a significant part of the protein was released from the sediment fraction after urea treatment. This behavior was independent of the presence of Bni4p and Chs3p. By contrast, a significant part of Chs4p Δ 590 appeared in the supernatant fraction of the wild-type strain, although this amount did not increase in the *chs3* Δ *bni4* Δ double mutant. In the wild type, urea treatment released Chs4p Δ 590 similarly to the control, but nearly all the protein was solubilized in the *chs3* Δ *bni4* Δ double mutant.

Taken together, these results indicate that the C-terminal region of the protein mediates Chs4p association with the plasma membrane and is directly involved in the lateral diffusion of this protein along the plasma membrane. However, the interaction of Chs4p with other proteins such as Bni4p and Chs3p also contributes to the association of Chs4p with membranes, affording the accumulation of Chs4p Δ 590 at the neck, together with the biological functionality of this protein. Although all our tests indicate that Chs4p Δ 590 and Chs4p^{C693S} behaved similarly we cannot exclude the fact that the C-terminal region of Chs4p serves an additional purpose other than prenylation.

Fig. 3. Localization of Chs4p Δ 590-GFP. (A,B) Chs4p-GFP and Chs4p Δ 590-GFP were expressed in the indicated strains, all of them W303 derivatives. Cells of the wild type (A) are shown at higher magnification to show details. Note the absence of plasma membrane staining in all Chs4p Δ 590-GFP-containing cells. Arrowhead indicates neck localization in the double mutant. Note the high levels of fluorescence in the vacuole. Numbers in each picture indicate the percentage ($n > 300$) of cells showing defined staining at the neck or at the bud. (C) Subcellular fractionations comparing Chs4p and Chs4p Δ 590 association to particulate (Po) and soluble (So) fractions with and without 4% urea treatment. The figure represents a western blot analysis using the 3xHA tagged version of each protein. Numbers below bands indicate the percentage of the band in each fraction after quantitative densitometry.

Chs3p accumulation at the plasma membrane depends on Chs4p

The unexpected finding that Chs4p was self-associated with the plasma membrane opened new alternatives regarding the function of this protein. One possibility is that Chs4p decorates the plasma membrane and later mediates Chs3p insertion into the plasma membrane, promoting CSIII activity. To test this hypothesis, we assessed Chs3p-GFP localization in several strains.

Chs3p-GFP localized at the neck in the wt strain (Fig. 4A), also showing a discrete dotted distribution, in agreement with previous data (Santos and Snyder, 1997; Trautwein et al., 2006). In the *chs4Δ* strain, dots were more prominent and the number of cells showing neck staining was reduced to approximately one-fifth of that of the wild type ($n=500$). Fluorescence at the neck was also significantly reduced in comparison with the controls (Fig. 4A, see arrowheads).

This result somehow contradicts those of other authors reporting a diffuse localization of Chs3p in a *chs4Δ* strain after immunofluorescent localization (DeMarini et al., 1997). Accordingly, we localized Chs3p-3xHA by immunofluorescence in wild-type and *chs4Δ* strains (Fig. 4B). In both cases ~15% of the cells ($n=230/240$) showed a polarized staining (Fig. 4B). In the wild type, most Chs3p was localized as a well-defined line at the neck region of budded cells. By contrast, Chs3p localization appeared more diffuse in the *chs4Δ* mutant, as described elsewhere (DeMarini et al., 1997). Quantitative analysis of the data (Fig. 4B, lower graph) indicated that most wild-type cells ($80.5\pm0.5\%$) showed a discrete localization of Chs3p, whereas only $20.1\pm3.4\%$ of the *chs4Δ* mutant did so. Accordingly, the diffuse staining pattern is more common in the *chs4Δ* mutant, and most cells show diffuse fluorescence polarized at the budding site (Fig. 4B), including a significant number of cells showing bud staining ($11.5\pm0.3\%$). This pattern is very rarely observed in the wild

type ($<1.1\%$). As previously reported (Santos and Snyder, 1997), Chs3p was retained in Golgi vesicles in the *chs5Δ* mutant, which showed a very prominent dotted stain (Fig. 4B). Apparently, the localization of Chs3p is altered in the *chs4Δ* and *chs5Δ* mutants, but polarization of Chs3p only occurs in the *chs4Δ*. Interestingly, this diffuse staining was dotted, contrary to that observed in the wild type. Both approaches thus suggested that in the absence of Chs4p, Chs3p would not associate with the plasma membrane and would tend to accumulate in polarized intracellular vesicles. Chs3p-containing vesicles obtained from the wild type or *chs4Δ* mutant had similar equilibrium densities (not shown), suggesting that Chs3p finally accumulates in the TGN in both cases.

To confirm these results, we analyzed Chs3p distribution by a completely different approach using subcellular fractionation. In this case, we used discontinuous sucrose gradients, which would allow us to detect Chs3p accumulation in the plasma membrane (Fig. 5). According to previous data (Valdivia and Schekman, 2003), Chs3p-3xHA expressed from plasmid pHV7-C-HA was distributed in two peaks in the wild-type strain. A significant part of this protein ($25.5\pm0.6\%$) was found in fractions 5-7, together with most of the plasma membrane marker Pma1p. The remaining Chs3p appeared in vesicular fractions 1-4. In the *chs4Δ* strain, the situation was rather different because only a marginal amount ($6.9\pm2.1\%$) of Chs3p localized with Pma1p (fractions 5-7), almost all of the protein being in vesicular fractions 1-4. Similar results were obtained using an integrated Chs3p-3xHA construct (see below), confirming that Chs4p promotes Chs3p accumulation at the plasma membrane.

Chs4p promotes the activation of CSIII activity along Chs3p translocation into the plasma membrane
Previous results indicated that the levels of Chs4p may be

Fig. 4. Intracellular distribution of Chs3p in the *chs4Δ* mutant. (A) Localization of Chs3p-GFP in the indicated strains. Arrowheads indicate Chs3p-GFP localization at the neck in the *chs4Δ* strain. Note the reduced intensity of the label compared with the wild type. (B) immunofluorescence localization of Chs3p-3xHA in wild-type, *chs4Δ* and *chs5Δ* strains (Y1306 background). Note the high definition of staining in the wild type in contrast to the diffuse and dotted pattern in the *chs4Δ*. Chs3p was retained prominently in Golgi vesicles in the *chs5Δ*. Data from wild type and *chs4Δ* are also expressed in graph format as percentages ($n=230/240$) of cells with the indicated distribution pattern, referred to the number of cells with defined staining. Discrete distribution refers to cells presenting staining sharply defined at the plasma membrane; Diffuse, polarized staining not sharply defined; Bud, cells showing diffuse staining at bud periphery.

Fig. 5. Immunodetection of Chs3p-HA and markers Pma1p and Pep12p in discontinuous sucrose gradients using cell extracts from CRM872 (*chs3Δ*) and CRM278 (*chs3Δ chs4Δ*) strains transformed with the pHV7-C-HA. Western blots and their quantitative analyses are shown. Data are from a single representative experiment, but average values for several experiments are indicated in the text.

related to the degree of CSIII functionality (Trilla et al., 1997). Therefore, we tested our hypothesis by measuring Chs3p distribution and CSIII activity in a strain overexpressing *CHS4*. In this case we used a strain with the Chs3p-3xHA construction integrated in the chromosome (Santos and Snyder, 1997). Fig. 6A compares the amount of Chs3p reaching the plasma membrane fraction in the *chs4Δ*, pRS314-*CHS4* and pRS424-*CHS4* strains and the calcofluor resistance and chitin levels measured in each strain. These strains respectively represent the absence, wt and overexpression levels of Chs4p. As reported above, only a marginal amount of Chs3p localized in the plasma membrane fractions in the *chs4Δ* mutant compared to wt, which was correlated to the reduced chitin levels and

calcofluor resistance. The strain overexpressing *CHS4* showed a significant increase in the amount of Chs3p localized in the membrane; this matches the higher levels of chitin in this strain and the hypersensitivity to calcofluor. Interestingly, Chs4p overexpression produced a delocalization of the chitin synthesis from the neck.

Fig. 6B shows the correlation between Chs3p distribution and the CSIII activity measured in the same samples. As expected, CSIII was very low in all the fractions obtained from the *chs4Δ* mutant regardless of the amount of Chs3p in the sample, a clear indication for CSIII being inactive in this mutant. On the other hand, plasma membrane samples (6-7) were very enriched in CSIII activity in the overexpressing

Fig. 6. Plasma membrane levels of Chs3p and biological activity. (A) Quantification of Chs3p-3xHA levels in the plasma membrane fraction after discontinuous sucrose gradients and western analysis as in Fig. 2C. Protein extracts were obtained from the JAY29 (*chs4Δ*) strain transformed with the indicated plasmids. Data represent the amount of Chs3p-3xHA localized in fractions 5-7 corresponding to the plasma membrane and are the average of three experiments. They are compared with resistance to 0.025 mg/ml Calcofluor and chitin levels (nmol/100 mg cells), measured as indicated in the Materials and Methods. Calcofluor staining of these strains is shown in the lower images. (B) Distribution of Chs3p-3xHA in discontinuous sucrose gradients using extracts obtained from the *chs4Δ* and pRS424-*CHS4* strains as in A. Lower panel represents specific CSIII activity (mU) in each fraction.

strain, whereas CSIII activity was low in the vesicle fractions (1-4), despite the higher amounts of Chs3p in these samples. These results point to a direct relationship between the amount of Chs3p in the plasma membrane and CSIII activity, and indicate that the Chs3p accumulated in vesicles is essentially inactive. Remarkably, proteolytic treatment of the vesicle fractions did not elicit CSIII activity in either strain (data not shown).

These results clearly assign a role for Chs4p in the intracellular traffic of Chs3p. The sorting of Chs3p at the TGN has been shown to depend on Chs5p and Chs6p, a requirement that can be bypassed by the upregulation of Ypt32p, which restores chitin synthesis in the corresponding *chs5Δ* and *chs6Δ* mutants (Ortiz and Novick, 2006). It was therefore tempting to know whether *YPT32* overexpression might also suppress the requirement for Chs4p. Fig. 7 shows that the overexpression of Ypt32p under the *GALI* promoter restored chitin synthesis (Fig. 7A) and calcofluor sensitivity (not shown) in the *chs6Δ* mutant, but not in *chs4Δ*. Accordingly, *YPT32* upregulation did not promote Chs3p accumulation at the plasma membrane in the *chs4Δ* mutant since only 1% of the cells ($n=294$) showed Chs3p at the neck (Fig. 7B). Apparently, the absence of Chs4p cannot be bypassed by higher amounts of Ypt32p in the cell, suggesting that Chs4p functions after the Golgi sorting.

Chs4p is involved in the proper endocytic turnover of Chs3p

The results discussed above revealed Chs4p as a new protein involved in Chs3p intracellular trafficking. However the experiments described did not address whether Chs4p is required for the arrival of Chs3p at the plasma membrane or for its stabilization at that site. To answer this question we analyzed the behavior of Chs3p after endocytosis blockade.

In the *end4Δ* mutant Chs3p-GFP was retained along the plasma membrane and its accumulation at the neck was visually reduced. Accordingly, vesicle staining was significantly reduced compared with that of the wild type (Fig.

8A). Confocal microscopic analysis showed that Chs3p-GFP distribution was fairly uniform throughout the plasma membrane (not shown). Similar results have been reported using the *tlg1Δ* mutant (Holthuis et al., 1998). Interestingly, this mutant, as well as *tlg1Δ* (not shown) showed discrete patches of chitin synthesis along its surface (Fig. 8A) after calcofluor vital staining. This increase corresponds to a ~threefold increase in chitin and CSIII levels compared with wild-type levels (not shown). Clearly, CSIII regulation depends on Chs3p internalization. The *end4Δchs4Δ* double mutant also showed a uniform distribution of Chs3p-GFP along the plasma membrane, a clear indication that Chs3p arrives at the plasma membrane even in the absence of Chs4p. However, calcofluor staining of this strain revealed very little fluorescence (Fig. 8A), indicating low chitin levels because of an inactive CSIII. In conclusion, these data strongly suggest that Chs4p mediates Chs3p stabilization at the plasma membrane, such stabilization being required, but not sufficient for CSIII activation. This type of behavior is independent of the intrinsic Chs3p stability, which remains constant under all conditions tested regardless of the presence of Chs4p (data not shown) (Chuang and Schekman, 1996).

Although the results presented here are conclusive, the double *end4Δchs4Δ* mutant grew significantly slower than the single *end4Δ* mutant and some of the phenotypes observed could be pleiotropic. We therefore used Latrunculin A treatment to block endocytosis. Wild type and *chs4Δ* mutants containing the Chs3p-GFP construct were treated with different concentrations of latrunculin A and Chs3p-GFP localization was assessed. In both the wild type and *chs4Δ* strains Chs3p-GFP was uniformly distributed along the plasma membrane, and its distribution was nearly identical, showing a discrete but clear accumulation at the neck region (Fig. 8B). Under the same treatment, the Chs3p-GFP in the *chs6Δ* mutant remained in the TGN (Fig. 8B, lower row). Lower doses and shorter times of treatment with latrunculin provided additional information. After minutes of treatment (see figure legend for

Fig. 7. Effect of *YPT32* overexpression on chitin synthesis. (A) Calcofluor staining of the indicated strains. Upper row shows the strains transformed with the pRS314 plasmid and the lower row those transformed with pRS314-*GALI-YPT32*. Cells were grown in galactose and stained as indicated. (B) Chs3p-GFP localization after *YPT32* overexpression. All the indicated strains contained the YpLac111-*CHS3-GFP* plasmid and the additional indicated plasmids. Note that such overexpression does not modified Chs3p-GFP accumulation in the neck in either wt or *chs4Δ* strains. Cells were grown in galactose and observed microscopically as described.

Fig. 8. Chitin synthesis and Chs3p distribution after endocytosis blockade. (A) Calcofluor staining and Chs3p-GFP distribution visualized microscopically as described in independent cultures. The indicated strains (BY4742 background) were transformed with the YpLac111-*CHS3*-GFP plasmid and thus contained two functional copies of Chs3p. Note the uniform distribution of Chs3p-GFP along the plasma membrane in the *end4Δ* mutants regardless of the presence of Chs4p. (B) Chs3p-GFP distribution after Latrunculin A treatment. CRM103 (*chs3Δ*), CRM892 (*chs3Δ chs4Δ*) and CRM1077 (*chs3Δ chs6Δ*) strains transformed with the YpLac111-*CHS3*-GFP plasmid were grown to early log phase and treated with 50 μ M latrunculin for one hour. (C) Same as in B but with treatment with 15 μ M latrunculin for 15 minutes. Note that the fluorescence intensity is not uniform along the cell surface.

exact conditions), many wild-type cells showed a clear gradient of Chs3p-GFP distribution from the neck region (Fig. 8C), whereas none of the cells ($n=64$) showed increased staining at the bud. The aspect of *chs4Δ* cells under these conditions was somewhat different and many buds ($21.7 \pm 5.9\%$, $n=94$) showed an increased accumulation of Chs3p along their surface (Fig. 8C). This kind of distribution in the *chs4Δ* mutant is not unexpected owing to the failure of Chs3p to anchor to Bni4p in the absence of Chs4p. During this treatment, neither Bni4p-YFP nor the septin Cdc3p-GFP underwent significant changes in their localization (not shown). Longer incubations with latrunculin eventually rendered Chs3p distribution that was uniform in both strains (Fig. 8B).

Chs4p-GFP distribution was not significantly altered in the *end4Δ* mutant, suggesting that Chs4p was not undergoing endocytic turnover (Fig. 9). The distribution of Chs4p along the plasma membrane precludes any knowledge about its role in the lateral activation of chitin synthesis in the *end4Δ* mutant. We did observe, however, that the Chs4p Δ 590-GFP protein was absent along the plasma membrane providing us with an opportunity to test this role. Chs4p Δ 590-GFP distribution in the *end4Δ* mutant was almost identical to that of the wild type, being only located at the neck and absent along the rest of the plasma membrane (compare Fig. 9 with Fig. 3A). Interestingly, calcofluor staining showed that the *end4Δ* Chs4p Δ 590 cells contained a similar amount and distribution of chitin along their cell walls to cells containing the wild-type Chs4p (Fig. 9). Therefore CSIII must remain active even in the apparent absence of Chs4p.

Taken together, these results convincingly show that Chs3p is translocated to the plasma membrane in the absence of Chs4p, but is then rapidly endocytosed. A blockade of endocytosis redistributes either active or inactive Chs3p along the plasma membrane from the original site of translocation.

Discussion

Chs4p localization depends on multiple signals

Previous studies suggested that the physical interaction between Chs4p and Chs3p at the neck would be responsible for triggering CSIII activation (DeMarini et al., 1997; Trilla et al., 1997). However, it is unclear how this occurs, because this activation is independent of the CSIII anchoring to the septins through Bni4p (Sanz et al., 2004). Part of our ignorance stems from the fact that very little is known about the Chs4p protein.

Our work indicates that Chs4p is a very stable membrane-associated protein that is transported in vesicles in a polarized

Fig. 9. Chs4p distribution in the absence of endocytosis. Calcofluor staining and Chs4p distribution in the *end4Δ chs4Δ* mutant transformed with pRS315 plasmids containing either the *CHS4*-GFP or *CHS4Δ*590-GFP constructs. Chs4p-GFP distribution did not change as a result of the *end4Δ* mutation, independently of the C-terminal region of the protein (compare with Fig. 3A). Yeast cells were grown at 25°C.

fashion. In this form, Chs4p arrives at the neck and is later redistributed along the plasma membrane. This transport is independent of Chs3p and the rest of the chitin synthesis machinery, suggesting that Chs3p and Chs4p would be transported independently. Chs4p-transporting vesicles showed similar sedimentation properties to those of Chs3p, making the separation of both types of vesicles technically difficult. The arrival of Chs4p at the plasma membrane is also independent of Bni4p (Sanz et al., 2004), raising the question of how Chs4p eventually becomes associated with membranes in the absence of any transmembrane domain or putative signal peptide. Our biochemical and microscopic data clearly indicate that the prenylation of Chs4p is a major determinant in its association with membranes and this prenylation appears to be responsible for the lateral redistribution of Chs4p from the neck. Nevertheless, the situation is more complex since the nonprenylated Chs4p still interacts with Bni4p and Chs3p, and this interaction is sufficient to promote the accumulation of non-prenylated Chs4p at the neck. Thus, the non-prenylated Chs4p maintains most of its biological functionality in the formation of the chitin ring. Although both Bni4p and Chs3p participate in the process, the interaction of Chs4p with Chs3p must clearly be more important in view of the stronger defect in the localization of the non-prenylated Chs4p observed in the *chs3Δ* or *chs3Δ bni4Δ* mutants. The association of Chs4p with membranes is crucial for its correct intracellular turnover and when this is compromised the protein is degraded in the vacuolar compartment. However, in all our experiments, we observed a residual amount of non-prenylated protein still showing the correct behavior, indicating a more complex set of interactions of Chs4p, probably involved in its association to transport vesicles.

Taken together, these results suggest that Chs4p prenylation is responsible for some of the biochemical properties of the protein, in agreement with the reduced functionality of the non-prenylated protein as stated in this and some other recent studies (Grabinska et al., 2007). The quantitative differences observed between these two studies is probably due to technical reasons because of the use of different genetics backgrounds combined with the use of centromeric plasmids in our work instead of chromosomal mutations. At this stage it remains unclear what the biological function of Chs4p is along the plasma membrane. A tentative hypothesis associated it with the synthesis of chitin in lateral walls, which accounts for 10% of the total (Molano et al., 1980), in accordance with the modest reduction in chitin synthesis observed in the Chs4pΔ590 and *CHS4*^{C693S} strains. However, cells containing Chs4pΔ590 or *CHS4*^{C693S} stained normally with WGA-FITC (not shown) and showed a completely normal pattern of sensitivity to compounds affecting the cell wall regardless of the genetic background used, suggesting that synthesis of the lateral chitin was normal. However, it is possible that none of the tests used provided sufficient sensitivity to detect small changes in cell wall composition. Therefore, this possibility should be kept in mind. In the same way, it is possible that the Chs4pΔ590 or *CHS4*^{C693S} strains are not identical albeit they behaved similarly in all our tests. An alternative possibility to explain prenylation function would be that Chs4p might have broader functions in the cell (see below).

The Chs4p/Chs3p interaction promotes Chs3p accumulation at the plasma membrane and activation of the CSIII

Study of the interaction of Chs4p with Chs3p has provided very little information about how Chs4p might elicit CSIII activity. Since the proteolytic activation of CSIII by Chs4p is unlikely to occur in vivo (see above), we looked for other explanations. Microscopic analysis with Chs3p-GFP indicated that Chs3p localizes poorly in the *chs4Δ* mutant and tends to accumulate in vesicles. Immunofluorescence revealed that Chs3p-3xHA localization in *chs4Δ* was diffuse (Fig. 2B) (DeMarini et al., 1997), partially accumulating in polarized vesicles but not in the plasma membrane. Although this microscopic analysis is not conclusive, it does offer a new working hypothesis: Chs3p would not accumulate at the plasma membrane in the absence of Chs4p. These observations were fully confirmed with subcellular fractionations on discontinuous sucrose gradients: Chs3p was not present in the plasma membrane fractions in the absence of Chs4p and the overexpression of *CHS4* increased the amount of Chs3p in the plasma membrane. The fact that Chs3p distribution was still clearly polarized in the *chs4Δ* mutant, but not in the *chs5Δ* mutant (Fig. 2B), suggests that Chs4p acts after Chs3p sorting in the TGN. It was therefore not surprising that the overexpression of *YPT32* (Fig. 7) and the activation of the PKC response (Carotti et al., 2002) are unable to suppress the requirement of Chs4p for chitin synthesis or Chs3p accumulation at the plasma membrane, because both of these are known to suppress the absence of Chs5p and Chs6p in the TGN (Ortiz and Novick, 2006; Valdivia and Schekman, 2003).

If Chs4p does act as an activator of the CSIII, as proposed, the Chs4p-dependent accumulation of Chs3p at the plasma membrane should be linked to CSIII activation. This indeed proved to be the case, because only the Chs3p at the plasma membrane was enzymatically active (Fig. 6B). In clear agreement with our data, *CHS4* overexpression has been shown to increase CSIII almost twofold (Trilla et al., 1997), which corresponds very well to the increased amount of Chs3p delivered to the plasma membrane. In addition, the higher levels of Chs4p can be directly related to the higher amounts of chitin and to hypersensitivity to calcofluor. In these cases, the increase was much more modest, probably owing to metabolic constraints (Lagorce et al., 2002). It is interesting to note that overexpression of Chs4p produced a partial delocalization of chitin from the neck (Fig. 6A, lower row), although the amount of polymer increased only very modestly.

It may be unambiguously concluded that Chs4p promotes Chs3p accumulation at the plasma membrane concomitantly to the activation of CSIII, confirming the proposed role of Chs4p as a direct activator of CSIII.

Endocytic turnover is a critical step in CSIII regulation

Considering that Chs5p, Chs6p and Chs7p participate in the anterograde transport of Chs3p, all the data presented so far pointed to Chs4p as a new step in this process. However, our data did not exclude the possibility that Chs4p function could be related to the endocytic turnover of Chs3p (Holthuis et al., 1998; Ziman et al., 1996). It has been assumed that inactivation of CSIII depends on endocytosis of Chs3p, although this has not been proved experimentally. Our data confirm that endocytosis is a general requirement for the downregulation of

CSIII, because the *end4Δ* mutant contained significantly higher levels of chitin and CSIII activity than the wild type. Calcofluor vital staining indicated that chitin synthesis occurred in patches, contrary to Chs3p-GFP, which appeared uniformly distributed along the plasma membrane (Fig. 8); this suggests that only part of the CSIII remains active after the endocytosis blockade. Similar results were observed after Latrunculin A treatment and calcofluor staining (not shown). Based on these data, it is probable that at least part of the increased accumulation of Chs3p at the plasma membrane after a temperature upshift (Valdivia and Schekman, 2003) would be related to the reduction in endocytosis turnover caused by the heat shock effect on actin polymerization (Delley and Hall, 1999).

The double *end4Δchs4Δ* mutant did not show appreciable calcofluor staining, although Chs3p was evenly distributed along the plasma membrane in this mutant (Fig. 8A). These results were later fully confirmed by endocytosis blockade with latrunculin, which also produced a uniform accumulation of Chs3p at the plasma membrane in the *chs4Δ* mutant (Fig. 8B) but without triggering chitin synthesis (not shown). Apparently, Chs3p is able to reach the plasma membrane normally in the absence of Chs4p, but in an inactive form. Analysis soon after endocytosis blockade fully confirmed this hypothesis: Chs3p diffused from the neck in the wild type, but from the bud in *chs4Δ*, where it was partially transported in the absence of the Chs4p-dependent mechanisms that anchor CSIII to the neck (DeMarini et al., 1997; Sanz et al., 2004). This is in good agreement with the polarized distribution of Chs3p observed with immunofluorescence in the *chs4Δ* mutants: such vesicles will only originate where Chs3p has previously been delivered through its polarized transport. These vesicles are probably only visible with immunofluorescence because glutaraldehyde fixation prevents rapid vesicle turnover and the eventual accumulation of Chs3p at the TGN. One of the most puzzling observations was the calcofluor staining of the *end4Δ* Chs4p-Δ590 strain (Fig. 9). This strain showed normal chitin synthesis compared with the Chs4p strain, even though Chs4p-Δ590 failed to relocalize along the plasma membrane. These results indicate that CSIII remains active independently of the presence of Chs4p once it has become activated at the neck through the interaction between Ch4p and Chs3p. Thus, Chs4p does not appear to behave as a catalytic part of CSIII.

What is the exact role of Chs4p in CSIII function? Our analysis indicates that Chs4p and Chs3p are independently delivered to the plasma membrane in a polarized fashion. This polarization favors physical contact between both proteins promoting the stabilization of Chs3p at the plasma membrane and the concomitant activation of the CSIII. In wild-type cells, the combination of endocytic turnover with a low lateral diffusion (Valdez-Taubas and Pelham, 2003) owing to the indirect anchoring of Chs3p to septins allowed the polarized deposition of Chs3p and a restricted synthesis of chitin at the neck. Thus, disruption of endocytosis led to an immediate lateral diffusion of Chs3p (Fig. 8B, upper row) despite the maintenance of Bni4p and the septum machinery (not shown). Based on this model it may be interpreted that the role of Chs4p in Chs3p stabilization at the plasma membrane could be linked to its neck-anchoring function. This, however, cannot be the case since the *bni4Δ* mutant showed normal levels of CSIII activity despite Chs3p delocalization; indeed, *bni4Δ* mutants

contain higher levels of chitin (Sanz et al., 2004), which could be related to a reduction in Chs3p-endocytic turnover because of its altered localization. Higher amounts of Chs4p increased Chs3p stabilization and the consequent increase in CSIII and chitin synthesis; this increase saturates the septum machinery, favoring the lateral diffusion of active CSIII and the physical expansion of chitin synthesis (Fig. 6A, lower row).

What is the direct effect of Chs4p on Chs3p? There is no clear answer to this, but our data argue against Chs4p being a catalytic part of CSIII. Therefore, the most plausible scenario is that Chs4p mediates a posttranslational modification in Chs3p that is responsible for stabilization and activation. In the absence of Chs4p, Chs3p can be stabilized in the plasma membrane by blocking endocytosis, but no active CSIII can be obtained, probably because of an inappropriate conformational state. The non-prenylated Chs4p retains most of its biological properties, thus it would be still able to promote some type of conformational change in Chs3p, which would be compatible with the data recently reported (Grabinska et al., 2007). The nature of this Chs4p-induced conformational change is unknown, and it is unclear to date whether it would be mediated directly or indirectly by Chs4p.

The modest reduction in biological functionality observed for the C-terminal-truncated Chs4p proteins, together with the absence of any cell-wall-associated phenotype, raises doubts concerning the role of Chs4p distribution along the plasma membrane, and hence about the biological relevance of Chs4p prenylation. Nevertheless, the proposed model for Chs4p function is compatible with a more general role for Chs4p in promoting the translocation of other proteins into the plasma membrane. In favor of this possibility, *chs4Δ* shows synthetic growth defects in the absence of several proteins (Lesage et al., 2005), some of which, such as Lst4p or Ast1p, are not involved in chitin synthesis but in the later steps of protein sorting and delivery (Bagnat and Simons, 2001; Rubio-Teixeira and Kaiser, 2006), thus linking them functionally to Chs4p. This could also provide an evolutionary explanation for the presence of relatively well-conserved Chs4p homologues from bacteria to humans (Roncero, 2002). Such a possibility remains to be tested experimentally.

Materials and Methods

Strains, growth conditions and genetic methods

The yeast strains used throughout this work are described in supplementary material Table S1. All strains were obtained by the single-step method (Rothstein, 1983). *S. cerevisiae* strains were grown in YEPD (1% Bacto yeast extract, 2% peptone, 2% glucose) or SD medium (2% glucose, 0.7% Difco yeast nitrogen base without amino acids). Growth supplements were added to the SD medium when required. Yeast cells were typically grown at 28°C, but all *end4Δ* strains were routinely grown at 25°C. Bacterial and yeast strains were manipulated by standard techniques. Calcofluor resistance was tested by a plate assay on SD medium buffered with 50 mM potassium biphthalate, pH 6.2, as described (Trilla et al., 1999).

Construction of plasmids

DNA manipulations were accomplished following standard techniques. Truncated Chs4p proteins were obtained by site-directed mutagenesis, using pRS314-*CHS4* (Trilla et al., 1997) as template. Oligonucleotides contained two stop codons at the appropriate sites in order to avoid alterations in the mRNA. The *CHS4*^{C693S} mutant lacking the prenylation site was generated by site-directed mutagenesis. All mutations were confirmed by direct sequencing. The tagged versions of the truncated proteins were constructed by replacing their N-terminal regions by the corresponding regions from plasmids pRS315-*CHS4-GFP* (Sanz et al., 2004) or pRS315-*CHS4-3xHA* (Trilla et al., 1997) using an internal *NheI* site present in the middle of the *CHS4* ORF. In all cases, the tags were inserted immediately after the initial methionine codon. Multicopy *CHS4*-containing plasmids were generated by cloning the complete *CHS4* gene into pRS424. *CHS4-GFP* was also placed under

the control of the *GALI* promoter by inserting the *CHS4-GFP* gene using a *Bst*NI site (49 bp upstream from the starting codon) in the *Sma*I site of the pRS314-*GALI* plasmid (Mumberg et al., 1994).

The *YPT32* gene was amplified by PCR and fully sequenced. It was cloned into a pRS314 plasmid under the control of the *GALI* promoter (Mumberg et al., 1994). The *Chs3-GFP* construct was kindly provided by J. Rodriguez-Medina (University of Puerto Rico). It contains the GFP tag at the C-terminal region of *Chs3p* in the centromeric YpLac111 plasmid. The protein is expressed from its own promoter and is biologically functional (not shown).

Unless otherwise indicated, all the different plasmids containing *CHS3* or *CHS4* constructs were assayed in the corresponding deletant strains, *chs3Δ* or *chs4Δ*. Therefore, these strains contained a single copy of each gene in a centromeric plasmid. This system guarantees similar levels of expression as the genomic alleles while maintaining the versatility of having different constructs in plasmids to test them easy test in multiple deletion strains.

Subcellular fractionation

Subcellular fractionations were carried out using mid-logarithmic phase (OD_{600} 0.5–1) cells grown in SD medium. Cells were placed on ice, collected by centrifugation, and washed with ice-cold 10 mM $NaNO_3$, 10 mM NaF , 50 mM Tris-HCl, pH 7.5, and then with 5 mM EDTA, 50 mM Tris-HCl, pH 7.5.

For discontinuous gradients (Valdivia et al., 2002), 30 OD units of cells were resuspended in 0.4 ml lysis buffer containing a 1× protease inhibitor cocktail (PMSF 1 mM, aprotinin 1 μ g ml⁻¹, leupeptin 1 μ g ml⁻¹, pepstatin 1 μ g ml⁻¹) and broken by agitation with glass beads. Cells debris was removed by centrifugation (500 g for 5 minutes) and clarified lysates (0.2 ml) were loaded onto a 30–55% (w/w) sucrose step gradient (0.3 ml 55%, 0.75 ml 45%, 0.5 ml 41%, 0.3 ml 37% and 0.25 ml 30% sucrose in 10 mM Tris-HCl pH 7.5, 5 mM EDTA). Gradients were centrifuged at 200,000 g in an SW65 rotor (Beckman, Fullerton, CA) for 3.5 hours at 4°C. Seven 0.3-ml fractions were collected manually from the top, and the sucrose concentration was determined according to the refractive index.

For continuous sucrose gradients, 350 OD units of cells were finally resuspended in 1.5 ml lysis buffer [10% sucrose (w/w) in 10 mM HEPES, pH 7.5, 1 mM EDTA] containing 1× protease inhibitor cocktail. Cells were broken as above and the clarified supernatant was overlaid on 10 ml of a linear sucrose gradient (15–60%, w/w) in 10 mM HEPES pH 7.5, plus 1 mM EDTA. The tubes were centrifuged in an SW40 rotor at 170,000 g for 18 hours at 4°C. 0.4 ml fractions were collected from the bottom of the tube using a peristaltic pump. The fractions in both gradients were analyzed by immunoblotting.

For simple subcellular fractionations, 15–20 OD units of cells were collected and the total cellular extracts were obtained as above, split in two, and treated with 4% urea or water as a control. Extracts were incubated for 30 minutes in ice and centrifuged at 120,000 g for 30 minutes. Supernatant (So) and pellet fractions were recovered and the pellets were resuspended in the same volume as the supernatant to give the Po fractions. Samples were analyzed by immunoblotting.

Immunoblot analysis.

Proteins were analyzed by western blotting after SDS-PAGE and immunoblotting (Trilla et al., 1999), using commercial mouse monoclonal antibodies against the HA epitope (Boehringer) or GFP (Clontech). Rabbit polyclonal antibodies against Pma1p (Serrano et al., 1986) and mouse monoclonal antibodies against Pep12p (Molecular Probes) were used as markers of different cellular compartments. Western blots were developed using the ECL kit (Amersham) and when required they were quantified using a scanning densitometer (BioRad GS800) and Quantity One software (BioRad). Quantitative data are typically represented as the relative amounts of a protein in each fraction. All western blots reported were repeated at least three times and a single representative experiment is shown. Where appropriate, the data from several Western blots were quantified and the average values are presented.

Microscopic techniques

Calcofluor vital staining was observed in cells grown in the presence of 50 mg ml⁻¹ calcofluor for 2 hours at 28°C. This treatment highlights the sites of active chitin synthesis in the cell wall (Sanz et al., 2004). For GFP visualization, yeast cells containing the corresponding centromeric plasmid were grown to early logarithmic phase in SD medium supplemented with 0.2% adenine. *Chs3p*-3xHA was localized in Y1306 strains by indirect immunofluorescence (IF) as described (Trilla et al., 1999), using an Alexa Fluor 594 goat anti-mouse (Molecular Probes) as secondary antibody. Latrunculin A treatment to block endocytosis was carried out on logarithmically growing cells, using 15–100 μ M final concentrations of the drug. Latrunculin was dissolved in DMSO (10 mM) and the same amount of the solvent was added to control samples. Microscopic observations were initiated after 15 minutes of treatment.

All microscopic observations were performed using a Leica RX150 epifluorescence microscope with a 100 W Hg lamp, using the appropriate filters: UV for calcofluor; GFPblue for GFP observation, and N2.1 for IF. Images were obtained with a Sensys digital camera and were processed with Adobe Photoshop software. Where indicated, images were obtained with a Leica spectral TCS-SL

confocal microscope, using the recommended laser excitation for the fluorochrome. Pictures within the same composition were processed identically for comparative purposes. Statistical analysis of protein distribution was performed after counting several pictures from each experiment carried out in duplicate, and standard deviations are included for reference. The total number of cells counted in each experiment is indicated.

Analytical determinations

The determination of CSIII activity was performed as described (Choi and Cabib, 1994) in the absence of proteolytic activation. The addition of Ni²⁺ and Co²⁺ to the reaction mixture allowed the specific measurement of CSIII activity in the presence of CSI and CSII activities. Chitin was determined enzymatically as described previously (Trilla et al., 1999), using chitinase and colorimetric determination of N-acetylglucosamine.

We thank P. Perez and Y. Sanchez for critical comments about the manuscript, and N. Skinner for language revision. Thanks are also due to J. Rodriguez-Medina and R. Serrano for materials and to R. Valle for technical assistance. A.R. is a predoctoral fellow of the Junta de Castilla y Leon. This research was supported by the CICYT BIO2004-00280, the JCyL CS102C05 and the EU LSHB-CT-2004-511952 Grants.

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