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Chs1p and Chs3p, Two Proteins Involved in Chitin Synthesis, Populate a Compartment of the *Saccharomyces cerevisiae* Endocytic Pathway

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In *Saccharomyces cerevisiae*, the synthesis of chitin, a cell-wall polysaccharide, is temporally and spatially regulated with respect to the cell cycle and morphogenesis. Using immunological reagents, we found that steady-state levels of Chs1p and Chs3p, two chitin synthase enzymes, did not fluctuate during the cell cycle, indicating that they are not simply regulated by synthesis and degradation. Previous cell fractionation studies demonstrated that chitin synthase I activity (CSI) exists in a plasma membrane form and in intracellular membrane-bound particles called chitosomes. Chitosomes were proposed to act as a reservoir for regulated transport of chitin synthase enzymes to the division septum. We found that Chs1p and Chs3p resided partly in chitosomes and that this distribution was not cell cycle regulated. Pulse-chase cell fractionation experiments showed that chitosome production was blocked in an endocytosis mutant (*end4-1*), indicating that endocytosis is required for the formation or maintenance of chitosomes. Additionally, Ste2p, internalized by ligand-induced endocytosis, cofractionated with chitosomes, suggesting that these membrane proteins populate the same endosomal compartment. However, in contrast to Ste2p, Chs1p and Chs3p were not rapidly degraded, thus raising the possibility that the temporal and spatial regulation of chitin synthesis is mediated by the mobilization of an endosomal pool of chitin synthase enzymes.

INTRODUCTION

Chitin is a polymer of *N*-acetylglucosamine in the cell wall of *Saccharomyces cerevisiae* that is essential for proper cell morphogenesis and cell division (Shaw *et al.*, 1991). The nomenclature for genes involved in chitin synthesis has recently been revised (Cabib, 1994; Cid *et al.*, 1995). Proteins centrally involved in chitin synthesis include Chs3p, Chs2p, and Chs1p, which are likely to be the catalytic subunits for three biochemically distinct chitin synthase activities designated CSIII, CSII, and CSI, respectively (Bulawa, 1993; Choi and Cabib, 1994). Chitin synthesis in *S. cerevisiae* is temporally and spatially regulated. Late in the G_1 phase of the cell cycle, chitin is deposited in a cell wall structure known as the chitin ring (Cabib and Bowers, 1971; Pringle and Hartwell, 1981). Chs3p, which is essential for CSIII activity, is required for synthesis of the chitin ring (Shaw *et al.*, 1991; Valdivieso *et al.*, 1991; Bulawa, 1992). Chs3p is also required for synthesis of chitin in the lateral cell wall (a minority species) and for synthesis of chitin on the surface of mating projections, thus accounting for most of the cellular chitin (Shaw *et al.*, 1991). After mitosis (early G_1), the chitin in the ring is extended centripetally across the mother-bud junction to form a thin disk of chitin known as the primary septum, a structure required for normal cell separation. Chs2p, which encodes CSII activity, is required for the synthesis of the primary septum (Silverman *et al.*, 1988; Shaw *et al.*, 1991). Because of cell-wall lytic activities involved in cell separation during G_1, repair of the mother-daughter junction is

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required to prevent cell lysis. Although no chitin is detected in the birth scar, Chs1p, which is essential for CSI activity, is required to prevent the lysis of daughter cells at the birth scar in some strain backgrounds, thus, CSI-mediated chitin synthesis aids in repair of the birth scar (Bulawa *et al.*, 1986; Cabib *et al.*, 1989; Cabib *et al.*, 1992). The mechanism that regulates the temporal and spatial deposition of chitin in the cell wall remains unknown.

Chitin synthesis may be regulated at the level of enzyme activity. Each of the chitin synthase enzymes are zymogenic in vitro. CSI and CSII require treatment with trypsin for activation in vitro (Sburlati and Cabib, 1986). Under certain conditions, CSIII exhibits a protease-elicited activity in vitro (Choi *et al.*, 1994b). It is possible that in vivo proteolytic activation of these enzymes at the correct place and time results in the temporal and spatial regulation of chitin synthesis. However, there is no physiological data to support this idea and specific activating proteases are not known (Fernandez *et al.*, 1982; Silverman *et al.*, 1991).

Another hypothesis is that chitin synthases are temporally regulated by a synthesis/degradation mechanism. RNA levels of all three chitin synthases are cell cycle regulated (Pammer *et al.*, 1992). In *S. cerevisiae*, polarized secretion is coordinated with the cell cycle (Schekman and Novick, 1982; Lew and Reed, 1993). As a result, the time of synthesis of plasma membrane proteins that traverse the constitutive secretory pathway may dictate the site of incorporation on the cell surface. Plasma membrane proteins synthesized early in the cell cycle around the time of cell polarization may be incorporated into the plasma membrane at the incipient site of bud emergence. Because of the redirection of polarized secretion to the septal region late in the cell cycle, plasma membrane proteins synthesized during late mitosis may largely be incorporated into the plasma membrane at the region of the mother-bud neck. Rapid internalization and degradation of these proteins may limit their diffusion to other cell surface locations, thus accomplishing spatial regulation. Alternatively, such proteins could be tethered at the neck region before internalization. Some evidence suggests that Chs2p but not Chs1p or Chs3p may be regulated by a temporal synthesis/degradation mechanism (Choi *et al.*, 1994a).

It is possible that Chs3p localization to the vicinity of the incipient chitin ring occurs in G_1 just before START and the polarization of secretion. Therefore, an alternative to the constitutive secretory pathway may be required to localize Chs3p to the vicinity of chitin ring formation. Similarly, Chs1p localization may be required in G_1 just after cytokinesis during a time of isotropic secretion in daughter cells, thus necessitating an alternate mechanism for specific localization to the birth scar. Alternatively, plasma membrane-localized Chs1p and Chs3p could diffuse away from their sites of action and subsequently be relocalized during the next cell cycle.

Chitosomes were originally described as a small, particulate form of chitin synthase from *Mucor rouxii* and hypothesized to play a role in the transport of chitin synthase (Ruiz-Herrera and Bartnicki-Garcia, 1974; Bartnicki-Garcia *et al.*, 1979). In *S. cerevisiae*, CSI activity is associated with the plasma membrane and with a low-buoyant density membrane fraction also termed chitosomes (Schekman and Brawley, 1979; Kang *et al.*, 1985; Flores Martinez and Schwencke, 1988; Leal-Morales *et al.*, 1988). Analysis of membrane markers showed that chitosomes did not appear to be fragments of plasma membrane, vacuole, or mature constitutive secretory vesicles (Kang *et al.*, 1985; Flores Martinez and Schwencke, 1988; Leal-Morales *et al.*, 1988). The origin and role of chitosomes in regulated chitin synthesis has not been addressed. To examine this issue, we produced immunological reagents to probe the origin of chitosomes and the subcellular distribution of chitin synthase enzymes during the cell cycle. We report here that Chs1p- and Chs3p-containing chitosomes may be endosomes that regulate chitin synthesis by mobilization of chitin synthases directly to the site of action.

MATERIALS AND METHODS

Construction of Plasmids and Yeast Strains Expressing Epitope-tagged Proteins

Plasmid pMS-1 is a YEp24 backbone with approximately 8.5 kb of genomic DNA containing *CHS1* (Bulawa *et al.*, 1986). A *Sma*I-*Sal*I fragment containing the entire *CHS1* gene was isolated from pMS-1 and inserted into *Sma*I-*Sal*I-digested pRS316 (Sikorski and Hieter, 1989) to create pRS316(*CHS1*). pJR1265 contains DNA encoding a sixfold repeated Myc epitope in the polylinker of Bluescript II KS^+. A *Cla*I (filled in with Klenow)-*Eco*RI fragment containing part of the *CHS1* coding region was isolated from pRS316(*CHS1*) and inserted into pJR1265 that had been digested with *Nco*I, treated with the Klenow enzyme, and then digested with *Eco*RI to create pJR1265(*MYC-CHS1*). This plasmid contains DNA encoding a fivefold repeated Myc epitope inserted seven amino acids after the N-terminus of Chs1p. A *Sal*I (blunt end)-*Eco*RI fragment was isolated from pJR1265(*MYC-CHS1*) and inserted into a *Cla*I (blunt end)-*Eco*RI backbone fragment of pRS316(*CHS1*) to create pRS316(*MYC-CHS1*), thus recreating the *Sal*I site. An *Xba*I-*Xho*I fragment from pRS316(*MYC-CHS1*) was inserted into *Xba*I-*Xho*I-digested pRS306 (Sikorski and Hieter, 1989) to create pRS306(*MYC-CHS1*). This plasmid was linearized with *Bgl*II and introduced into strain JCY301 (*chs1::HIS3* constructed as per Bulawa and Osmond, 1990) to yield strain Z100 (Table 1).

The gene knockout and adjacent insertion were verified by Southern blot and genetic linkage analysis. Whereas the predicted size of Myc-Chs1p is ~130 kDa, a ~150 kDa Myc-Chs1p species was detected by immunoblot and immunoprecipitation that fractionated as an integral membrane protein in membrane extraction experiments. This species was not observed in immunoblots of untagged control strains and was not immunoprecipitated in the absence of primary antibody. The electrophoretic mobility of this species was not affected by treatment of cells with the *N*-glycosylation-inhibiting drug, tunicamycin, or by incubation of *sec53* mutant cells at the restrictive temperature, suggesting that the anomalous migration in

SDS-PAGE was not due to glycosylation. We were unable to detect Myc-Chs1p in indirect immunofluorescence experiments. A rabbit polyclonal antiserum against Chs3p will be described elsewhere (Chuang and Schekman, 1996).

Pma1p is the major proton ATPase of the yeast plasma membrane (Harris *et al.*, 1994). To detect this protein as a marker of plasma membrane, an integrating plasmid expressing *HA-PMA1* was introduced into yeast. Plasmid pXZ39 contains the entire *HA-PMA1* gene on a *Hin*dIII fragment (Harris *et al.*, 1994). A *Hin*dIII-*Bam*HI fragment containing the N-terminal half of *HA-PMA1* was isolated from this plasmid and inserted into the vector pRS305S/P that had been digested with *Hin*dIII and *Bam*HI, yielding plasmid pRS305(*HA-PMA1*). pRS305S/P was created by digesting pRS305 (Sikorski and Hieter, 1989) with *Sal*I, followed by treatment with the Klenow enzyme and T4 DNA ligase, thus eliminating the *Sal*I site and creating a *Pvu*I site. pRS305(*HA-PMA1*) was linearized with *Sal*I, creating a gapped plasmid that was introduced into strain Z102 (Table 1) to create strain Z106, thus making *HA-PMA1* the only full-length copy of *PMA1* in the cell (Rothstein, 1991).

The gene knockout/adjacent insertion was verified by Southern blot analysis and behaved as a single nuclear gene in genetic crosses. *HA-PMA1* was readily detected by immunoblot and immunoprecipitation as a ~110-kDa protein that behaved as an integral membrane protein in membrane extraction experiments. This protein was not detected in immunoblots from untagged control strains and was not immunoprecipitated in the absence of primary antibody. HA-Pma1p localized to the cell periphery as detected by indirect immunofluorescence.

Assay of CSI Activity

To test for Myc-Chs1p function, CSI activity was measured in digitonin-permeabilized wild-type, *chs1::HIS3*, and *MYC-CHS1* strains (Fernandez *et al.*, 1982). *chs1::HIS3* strains had approximately 10% of the activity of wild-type cells. Residual activity probably was due to CSII and CSIII activities. This defect was restored to approximately 100% of wild type in strains expressing *MYC-CHS1*.

Cell Cycle Synchronization Methods

Strains used for cell cycle synchronization experiments are shown in Table 1. To arrest cells with α-factor mating pheromone, we grew cultures to early log phase (OD_{600} = 0.4) and treated them with 6×10^{-8} M α-factor at 30°C until greater than 90% of the cells were unbudded. Cells were filtered on 0.45-μm cellulose acetate filters and washed once in fresh medium before incubation at 30°C. For *cdc15-2* experiments, we grew cells to early log phase at 25°C and added an equal volume of 50°C YPD medium. Cells were incubated at 37°C until cell cycle arrest occurred (approximately 90% large-budded cells). Cells were released from arrest by addition of an equal volume of 15°C medium and incubated at 25°C. For both synchronization procedures, we assessed cell cycle progression by morphological analysis of lightly sonicated cells and by examining steady-state levels of Clb2p (Grandin and Reed, 1993). Immunoblots were quantified by optical scanning and analysis with Imagequant software (Molecular Dynamics, Sunnyvale, CA).

Subcellular Fractionation and Immunoblot Analysis of Chs1p- and Chs3p-containing Membranes

Approximately 100 OD_{600} units of cells (early log/OD_{600} = 1–4) were treated with 20 mM NaN_3 and 20 mM KF on ice. Cells were centrifuged, washed once in water, and resuspended at 100 OD_{600}/ml in 100 mM Tris-HCl (pH 9.4), 10 mM dithiothreitol, 20 mM NaN_3, and 20 mM KF for 10 min at room temperature. Treated cells were again centrifuged and resuspended at 100 OD_{600}/ml in 10

Table 1. Yeast strains used in this study

Strain	Genotype	Source
YPH499	*MATa ade2-101^{oc} his3-Δ200 leu2-Δ1 lys 2-801^{am} trp1-Δ1 ura3-52*	Hieter lab
YPH500	*MATα ade2-101^{oc} his3-Δ200 leu2-Δ1 lys 2-801^{am} trp1-Δ1 ura3-52*	Hieter lab
JCY301	*MATa chs1::HIS3 ade2-101^{oc} his3-Δ200 leu2-Δ1 lys2-801^{am} trp1-Δ1 ura3-52*	this study
Z100	JCY301 [pRS306(*MYC-CHS1*)]	this study
Z102	*MATa MYC-CHS1 ade2-101^{oc} his3-Δ200 leu2-Δ1 lys 2-801^{am} trp1-Δ1 ura3-52 MATα CHS1 ade2-101^{oc} his3-Δ200 leu2-Δ1 lys 2-801^{am} trp1-Δ1 ura3-52*	this study
Z106	Z102 [pRS305(*HA-PMA1*)]	this study[a]
Z106-2B	*MATα MYC-CHS1 HA-PMA1 ade2-101^{oc} his3-Δ200 leu2-Δ1 lys 2-801^{am} trp1-Δ1 ura3-52*	this study
JTY2053	*MATa end4-1 bar1-1 his4 leu2-ura3*	Thorner lab
Z109-26D	*MATa MYC-CHS1 HA-PMA1 end4-1 bar1-1 ade2-101 his4 leu2 ura3*	this study[b]
Z109-34C	*MATa MYC-CHS1 HA-PMA1 bar1-1 ade2-101^{am} his4 leu2 lys2-101^{oc} ura3*	this study[b]
CSO2B	*MATa cdc12-6 leu2 ura3*	Pringle lab
Z118-6C	*MATa cdc12-6 MYC-CHS1 HA-PMA1 leu2 trp1-Δ1 ura3*	this study[b]
Z120-5A	*MATa bar1-1 MYC-CHS1:URA3 HA-PMA1 ade2-101^{ochre} his4 leu2 lys 2-801^{am} ura3*	this study[b]
13-53	*MATa cdc15-2 ade1 ade2 ura1 his7 lys2 tyr1 gal1*	YGSC[c,d]
DBY2059	*MATα leu2-3,112*	Schekman lab[d]
Z124-5A	*MATa cdc15-2 MYC-CHS1 HA-PMA1 ade2 leu2 trp1-Δ1 gal2*	this study[d]
Z125-8B	*MATa END4 MYC-CHS1 HA-PMA1 ade2-101^{oc} leu2 lys 2-801^{am} ura3*	this study[b]
Z125-8C	*MATa end4-1 MYC-CHS1 HA-PMA1 ade2-101^{oc} leu2 lys 2-801^{am} ura3*	this study[b]
DK102	*MATa ste2::HIS3 sst1-Δ5 ade2-101^{oc} his3-Δ200 leu2-Δ1 lys 2-801^{am} trp1-Δ1*	Stevens lab

[a] Strains containing *HA-PMA1* have *LEU2* integrated at the *PMA1* locus (see MATERIALS AND METHODS).

[b] Strains containing *MYC-CHS1* have *HIS3* and *URA3* integrated at the *CHS1* locus. When crossed to *his3* and/or *ura3* strains, the *HIS3* and/or *URA3* genotype of the segregants was not determined. Myc-Chs1p was verified by immunoblot analysis.

[c] Yeast Genetics Stock Center, University of California, Berkeley (Berkeley, CA).

[d] 13-53 was first crossed to DBY2059 to facilitate genetic analysis. Segregants were crossed and backcrossed to Z106-2B to yield segregant Z124-5A.

mM Tris-HCl (pH 7.5), 1.0 M sorbitol, 20 mM NaN_3, 20 mM KF, and 0.6× YP medium. Recombinant lyticase (Shen *et al.*, 1991) was added to a final concentration of 60 U/OD_{600} and cells were converted to spheroplasts during a 15- to 30-min incubation at 30°C in microcentrifuge tubes. Spheroplasts were centrifuged at 500 × *g* for 4 min in a refrigerated microcentrifuge and resuspended in 50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 20 mM NaN_3, 20 mM KF, 1.0 M sorbitol, and 1 mg/ml concanavalin A (Con A) at a concentration of 100 OD_{600}/ml. Con A coating was carried out in microcentrifuge tubes during a 10-min incubation at room temperature. Con A-coated spheroplasts were centrifuged at 500 × *g* for 4 min in a refrigerated microcentrifuge and resuspended in room temperature lysis buffer composed of 20 mM triethanolamine (pH 7.2), 12.5% (wt/wt) sucrose, and 1× protease inhibitor cocktail (Aris and Blobel, 1991) at a concentration of 100 OD_{600}/ml. Resuspension and homogenization were carried out with a 1-ml Gilson micropipette blue tip and lysis was facilitated by a 10-min incubation at 30°C. Lysed cells were again homogenized with a blue tip before differential centrifugation. Samples were centrifuged at 500 × *g* for 4 min followed by 10,000 × *g* for 5 min. Pellets were resuspended in lysis buffer and each fraction was solubilized in SDS-polyacrylamide gel sample buffer at 50°C for 10 min prior to electrophoresis (Hoeffer Scientific Instruments, San Francisco, CA), semidry transferred to nitrocellulose (Bio-Rad, Hercules, CA), and immunodetected using enhanced chemiluminescence reagents and protocols (Amersham, Arlington Heights, IL). Antibodies were used at the following dilutions: *c-myc* epitope 9E10 ascites (Evan *et al.*, 1985), 1:3000; HA epitope 12CA5 ascites (Wilson *et al.*, 1984), 1:15000; Chs3p serum (Chuang and Schekman, 1996), 1:1000; Vph1p serum (Hill and Stevens, 1994), 1:1000; F1β serum (Maccecchini *et al.*, 1979), 1:5000; Pgk1p serum (Baum *et al.*, 1978), 1:15000; affinity-purified Sec61p antibody (Stirling *et al.*, 1992), 1:10000; Ste2p serum, 1:1600; and Clb2p serum, 1:500 in Blotto containing 0.5 M NaCl. Horseradish peroxidase-conjugated goat anti-rabbit and sheep anti-mouse secondary IgGs were used at 1:10000 (Amersham).

Sucrose gradients were constructed as follows. For the experiment in Figure 2, 1.5 ml of 50% (wt/wt) sucrose in lysis buffer was overlaid with 1.5 ml of 40% and 1.5 ml of 30% sucrose in lysis buffer in a 5-ml ultraclear tube (Beckman, Palo Alto, CA). For the experiment in Figure 5, 45, 40, and 35% (wt/wt) sucrose in lysis buffer was used. The gradient was tilted on its side for 30 to 45 min at room temperature, uprighted gently, and chilled on ice. An aliquot (700 μl) of the 10,000 × *g* supernatant was combined with 300 μl of 60% sucrose in lysis buffer and overlaid on the gradient. Gradients were centrifuged for 20 h at 4°C at 50,000 rpm (~250,000 × *g*) in an SW55 rotor (Beckman). Fractions (25, 0.2 ml) were collected from the top (Isco, Lincoln, NE). Gradient fractions were examined using GDPase assay (Abeijon *et al.*, 1989), immunoblotting (Figure 5), or quantitative immunoblotting (Figure 2) with ^{125}I-labeled protein A or ^{125}I-labeled anti-mouse antibody (ICN Biomedicals, Costa Mesa, CA), followed by PhosphorImager analysis (Molecular Dynamics). Immunoblots in Figure 5 were quantified by optical scanning and analysis with Imagequant software (Molecular Dynamics).

Metabolic Labeling, Subcellular Fractionation, and Immunoprecipitation

Cells were grown in 0.67% yeast nitrogen base, 2% glucose, and appropriate supplements overnight to an OD_{600} of 0.4–0.7. Cells were centrifuged and resuspended at a concentration of ~5 OD_{600}/ml in fresh medium of the same composition and preincubated at the labeling temperature. Promix (Amersham) was added to a concentration of 20–30 μCi/OD_{600}, and labeling was carried out as indicated in the text and figure legends. Chase was initiated by addition of one-tenth volume of 10× chase solution (50 mM methionine, 10 mM cysteine, 4% yeast extract, 20% dextrose) and terminated by addition of NaN_3 and KF to 20 mM followed by incubation on ice. For subcellular fractionation, cells were treated as described above, except approximately 10 OD_{600} U were collected for each sample. Subcellular fractions were denatured with 1% SDS during a 10-min incubation at 50°C and processed for immunoprecipitation as described previously (Wuestehube and Schekman, 1992).

RESULTS

Steady-State Levels of Myc-Chs1p and Chs3p during the Cell Cycle

Cells released from α-factor arrest exhibit cyclical fluctuation in the RNA levels of all three chitin synthases (Pammer *et al.*, 1992). We sought to determine whether the corresponding proteins exhibit a similar fluctuation by examining the steady-state levels of Myc-Chs1p and Chs3p in synchronized cultures and during a time course after release from synchrony.

In agreement with the induction of *CHS1* RNA upon treatment of cells with α-factor mating pheromone (Appeltauer and Achstetter, 1989; Choi *et al.*, 1994a), expression of Myc-Chs1p was induced approximately threefold in response to α-factor treatment (Figure 1). Whereas *CHS3* RNA was not induced by α-factor (Choi *et al.*, 1994a), Chs3p was also induced approximately threefold in response to α-factor treatment (Figure 1). Examination of cultures released from α-factor-induced synchrony indicated that, in contrast to Chs2p (Chuang and Schekman, 1996), the levels of Myc-Chs1p and Chs3p did not significantly fluctuate during the cell cycle (Figure 1B). As a control for the degree of cell cycle synchronization, we examined cellular morphology (Figure 1A) and the steady-state levels of the mitotic cyclin Clb2p (Figure 1B) and found significant fluctuation across the time course, indicating adequate cell cycle synchrony. Chs1p and Chs3p returned to basal level within 135–150 min of removing α-factor. Quantification of these data indicate that the nonspecific band (*), which served as a loading control, fluctuated as much as twofold while Clb2p fluctuated as much as 50-fold. Variation of Chs1p and Chs3p were within twofold.

Because α-factor treatment caused induction of Chs3p and Chs1p that might obscure cell cycle fluctuations in these proteins, alternate synchronization methods were examined. Arrest of *cdc15-2* cultures at the restrictive temperature caused a slight reduction in the levels of Myc-Chs1p (see Figure 3). This reduction may be a result of temperature shift because it was seen in other strains incubated at a high temperature. Examination of cultures released from *cdc15-2*-induced synchrony indicated that the steady-state levels of Myc-Chs1p and Chs3p did not fluctuate significantly during the cell cycle (see Figure 3).

Degradation Rate of Myc-Chs1p

To test the role of synthesis/degradation in the maintenance of Chs1p, we pulsed radiolabeled wild-type cells with [^{35}S]methionine for 10 min followed by a chase of up to 90 min at 30°C. As a control for effective

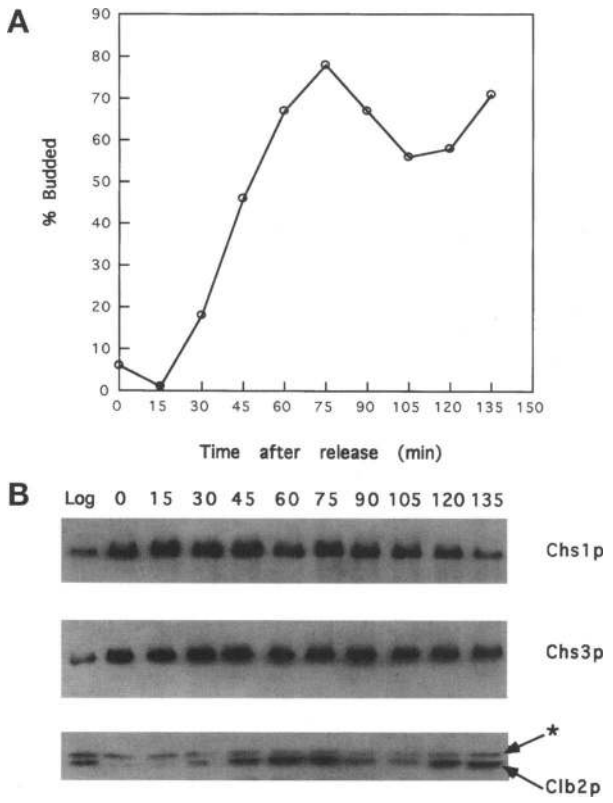

Figure 1. Analysis of the steady-state levels of Myc-Chs1p and Chs3p in cells released from α-factor arrest. (A) Strain Z120-5A *MAT***a bar1-1** was synchronized by arrest with α-factor and percentage of budded cells, and protein levels were followed over a time course (min) after release from arrest (see MATERIALS AND METHODS). The graph shows a morphological analysis of cell cycle synchrony. (B) Protein extracts from cells at each time point were prepared by agitation with glass beads and analyzed by immunoblotting. Clb2p accumulation serves as a marker of mitosis and the * indicates a nonspecific band.

chase, we found that CPY exhibited normal kinetics of maturation. Myc-Chs1p was significantly more stable ($t_{1/2}$ ~90 min) than short-lived plasma membrane proteins such as Ste6p, Ste2p, and Ste3p (Davis *et al.*, 1993; Berkower *et al.*, 1994; Schandel and Jenness, 1994). This result suggests that Chs1p is not rapidly degraded and probably persists for one or more cell cycles. Chs3p was similarly long-lived (Chuang and Schekman, 1996).

Myc-Chs1p- and Chs3p-containing Chitosomes

Subcellular fractionation was used to evaluate the intracellular distribution of Chs1p and Chs3p. In preliminary experiments, two forms of Chs1p were detected. One equilibrated at 42–45% sucrose on density gradients and cosedimented with HA-Pma1p, whereas the other sedimented to 36–38% sucrose, which is slightly higher than the density reported for chitosomes (Leal-Morales *et al.*, 1988). By adapting previously developed techniques, we established a simple differential centrifugation-immunoblotting method for the identification of chitosomes (Duran *et al.*, 1975; Kang *et al.*, 1985; Walworth *et al.*, 1989; see MATERIALS AND METHODS). Con A-coated spheroplasts were lysed and subjected to differential centrifugation which resulted in excellent separation of plasma membrane (HA-Pma1p), endoplasmic reticulum (Sec61p), and mitochondria (F1β) from Myc-Chs1p- and Chs3p-containing chitosomes (Figure 2A), indicating that chitosomes are distinct from these organelles. Pgk1p, a soluble protein, fractionated mainly in the 10,000 × *g* supernatant and served as a control for lysis. A significant amount of vacuole (Vph1p) and Golgi (GDPase) contaminated the chitosome-containing a 10,000 × *g* supernatant fraction. To resolve chitosomes from these organelles, the membranes were sedimented to equilibrium in a 30–50% continuous sucrose gradient. Separation of vacuole (Vph1p) membranes from chitosomes indicated that these are distinct organelles (Figure 2B). Similarly, the GDPase and chitosome peaks were largely resolved, indicating that chitosomes are not identical to Golgi membranes (see Figures 2B and 5).

Distribution of Myc-Chs1p and Chs3p during the Cell Cycle

To test whether chitin synthase enzymes undergo cell cycle-regulated protein transport from the chitosome to the plasma membrane, we used the differential centrifugation-immunoblotting method to analyze cells released from *cdc15-2* synchronization. The block in *cdc15-2* is reversible and arrests cells in late anaphase, allowing relatively synchronous progression through the cell cycle. We looked for a shift of Myc-Chs1p and/or Chs3p from the chitosome containing 10,000 × *g* supernatant to the HA-Pma1p containing 10,000 × *g* pellet. As a control for the degree of cell cycle synchronization, we examined cellular morphology (Figure 3A) and the steady-state levels of Clb2p (Figure 3A, inset) and found significant progression across the time course, indicating adequate cell cycle synchrony. In agreement with Figure 1, there was no significant fluctuation in Chs1p or Chs3p steady-state protein levels during the cell cycle (Figure 3). Additionally, there was no significant change in the chitosome versus plasma membrane distribution of Myc-Chs1p or Chs3p during a time course after release from *cdc15-2* arrest, indicating no cell cycle regulation of the steady-state subcellular distribution of these proteins (see DISCUSSION). The low level of Myc-Chs1p-containing chitosomes at time 0 was not consistently observed. Cells arrested in S phase with hydroxyurea, M phase with nocodazole, or at cytokinesis with *cdc12-6* exhibited no change in the subcellular

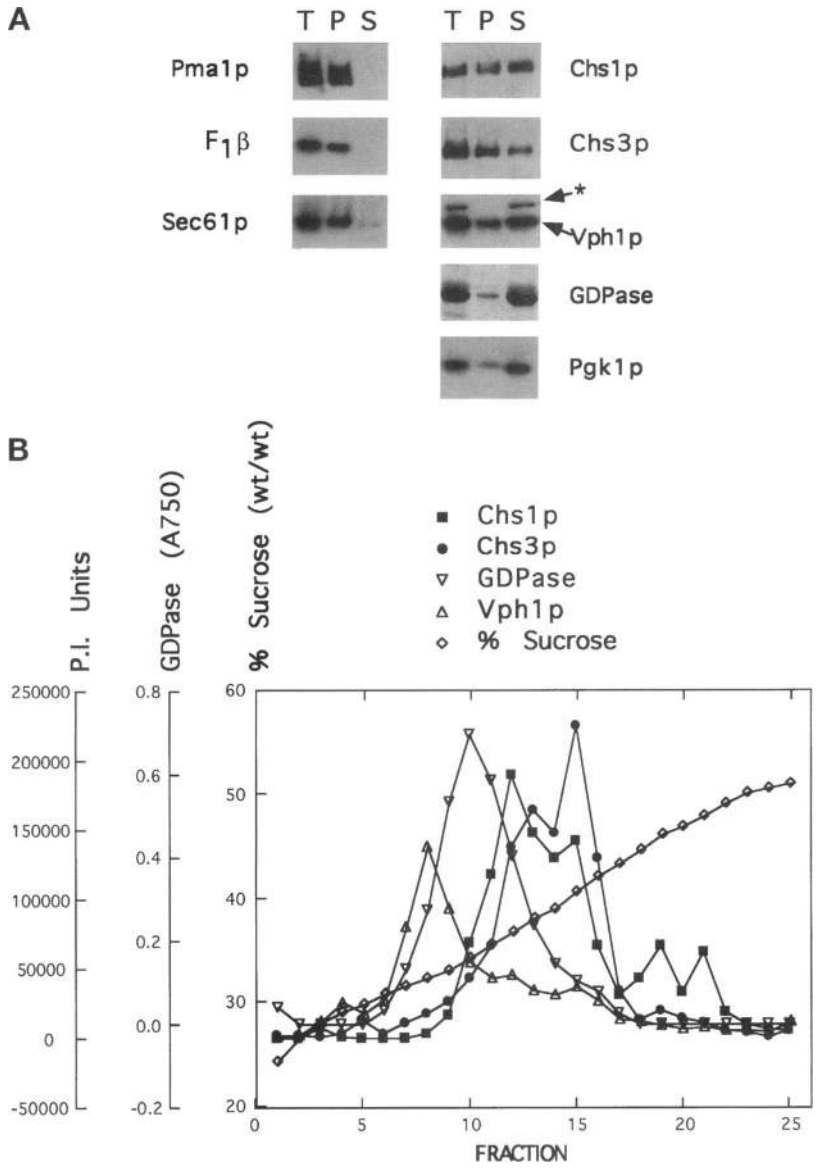

Figure 2. Analysis of the subcellular distribution of Chs1p and Chs3p. (A) Z106-2B cells were converted to spheroplasts, coated with Con A, lysed, and subjected to differential centrifugation-producing fractions that were evaluated with immunoblot. P indicates the 10,000 × *g* pellet and S indicates the 10,000 × *g* supernatant. (B) The 10,000 × *g* supernatant fraction was analyzed by sedimentation to equilibrium in a sucrose gradient. Chs3p, Myc-Chs1p, and Vph1p (* indicates a nonspecific band) were analyzed with quantitative immunoblotting and GDPase activity was measured by biochemical assay. P.I., PhosporImager.

distribution of Myc-Chs1p or Chs3p, supporting the lack of cell cycle regulation of the steady-state distribution of these proteins.

Biogenesis of Chitosomes

Because the plasma membrane contains a major pool of chitin synthase enzymes, we sought to determine whether chitosomal chitin synthases are derived from the plasma membrane. Mutations in *END4* are known to block the internalization step of α-factor receptor endocytosis and block the *SEC6/PEP4*-dependent degradation of Ste6p (Raths *et al.*, 1993; Berkower *et al.*, 1994), making *end4-1* a good tool to evaluate the putative plasma membrane origin of chitosomal chitin synthases. Wild-type and *end4-1* sibling strains were preshifted to 37°C for 30 min, pulse radiolabeled with [^{35}S]methionine for 10 min, chased for 30 min, and processed for separation of chitosomes from plasma membrane. Whereas chitosomes were produced effectively in the wild-type strain, the *end4-1* strain was impaired for the production of chitosomes (Figure 4A). The recovery of Pgk1p, a cytoplasmic protein, in the 10,000 × *g* supernatant indicated adequate cell lysis and the absence of HA-Pma1p in this fraction indicated no plasma membrane fragmentation (Figure 4A). Preshifting *end4-1* cells to the restrictive temperature is required to block chitosome formation. A preshift of 0, 10, and 30 min resulted in an average of

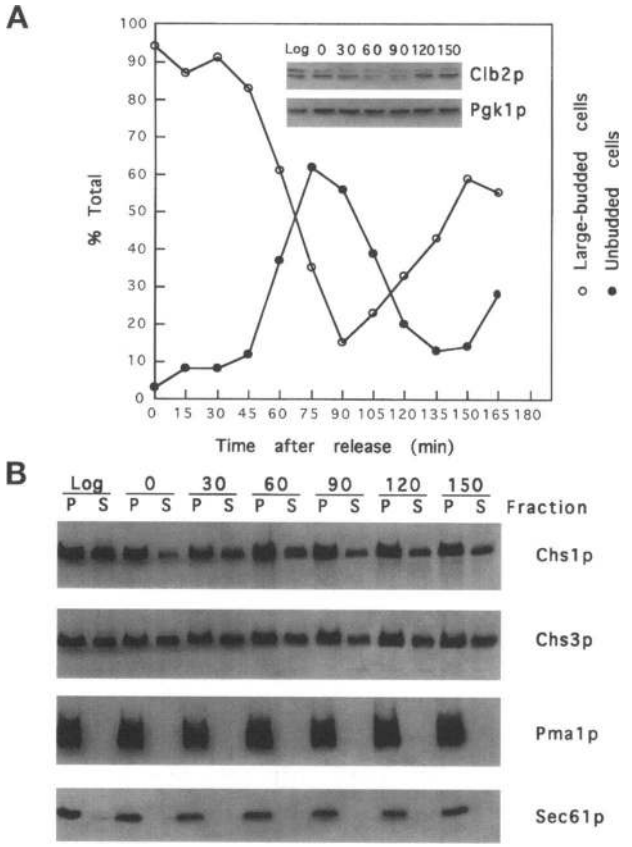

Figure 3. Analysis of the plasma membrane versus chitosome distribution of Chs1p and Chs3p during the cell cycle. (A) Strain Z124-5A *cdc15-2* was arrested at the restrictive temperature and percentage of budded cells, percentage of unbudded cells, and protein levels were followed over a time course after release from arrest. The graph shows a morphological analysis of cell cycle synchrony. Inset, steady-state levels of Clb2p as a control for adequate synchrony. (B) At each time point (min), cells were converted to spheroplasts, coated with Con A, lysed, and subjected to differential centrifugation-producing fractions that were evaluated by immunoblot. P indicates the 10,000 × *g* pellet and S indicates the 10,000 × *g* supernatant. HA-Pma1p and Sec61p served as fractionation controls to show that the 10,000 × *g* supernatant was free of plasma membrane and endoplasmic reticulum.

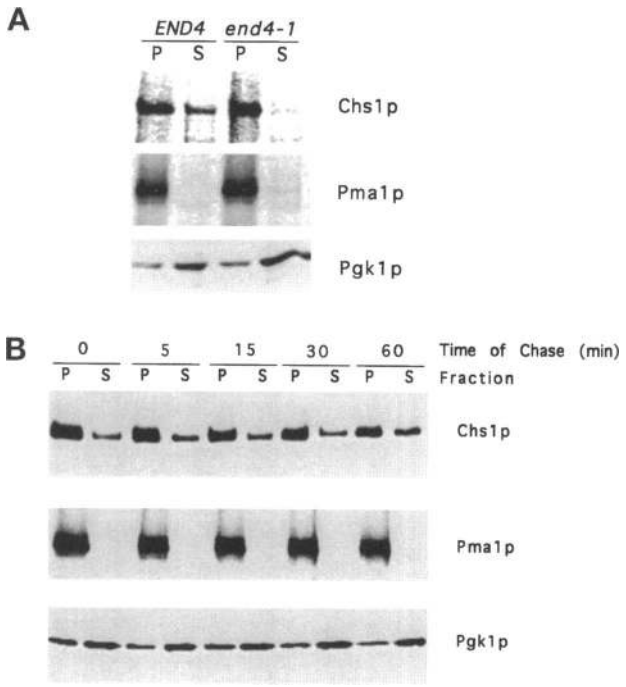

Figure 4. The biogenesis of Myc-Chs1p-containing chitosomes. (A) Sibling strains Z125-8B *END4* and Z125-8C *end4-1* were shifted to 37°C for 30 min, pulse labeled for 10 min, and chased for 30 min. At each time point (min), cells were treated with azide and fluoride, and subcellular fractions, generated as in Figure 3, were immunoprecipitated for Myc-Chs1p, HA-Pma1p, and Pgk1p. Immunoprecipitates were examined by SDS-PAGE and PhosphorImager analysis. P indicates the 10,000 × *g* pellet and S indicates the 10,000 × *g* supernatant. (B) Wild-type strain Z106-2B was pulse labeled at 30°C for 10 min and chased over a time course. Cell fractions were immunoprecipitated for Chs1p, Pma1p, and Pgk1p. Immunoprecipitates were examined by SDS-PAGE and PhosphorImager analysis. P indicates the 10,000 × *g* pellet and S indicates the 10,000 × *g* supernatant.

0-, 2.4-, and 11.5-fold defects, respectively (see DISCUSSION). Of six repetitions of the 30-min preshift experiment, three showed only a 1.5–2.4-fold defect. Three repetitions of the 10-min preshift experiment showed only a 1.5–3.1-fold defect. These results are suspect because plasma membrane contamination of the 10,000 × *g* supernatant fraction was more pronounced, giving the appearance of a diminished defect.

We also sought to determine whether the rate at which chitin synthase enzymes are localized to chitosomes would yield information regarding the biogenesis of this organelle. Wild-type cells were pulse radiolabeled with [^{35}S]methionine at 30°C and chased over a time course. As can be seen by the experiment in Figure 4B, chitosomes were produced rapidly, possibly during labeling, and the percentage of Myc-Chs1p in the chitosome fraction was similar across the time course. These results suggested that there was no delay in transport of Myc-Chs1p from the plasma membrane to the chitosomes and that endocytosis of these proteins was constitutive. Although HA-Pma1p observed in the 10,000 × *g* pellet could represent endoplasmic reticulum or plasma membrane forms, the lack of HA-Pma1p in the 10,000 × *g* supernatant of the 0-, 5-, and 15-min time points suggested that Golgi and vesicular intermediates were not prevalent.

Comparison of Chitosomes and Endosomes

Because transport of Chs1p to chitosomes is dependent on *END4*, we sought to determine whether chitosomes are identical with endosomes. Ste2p, the α-factor receptor, is known to undergo ligand-induced

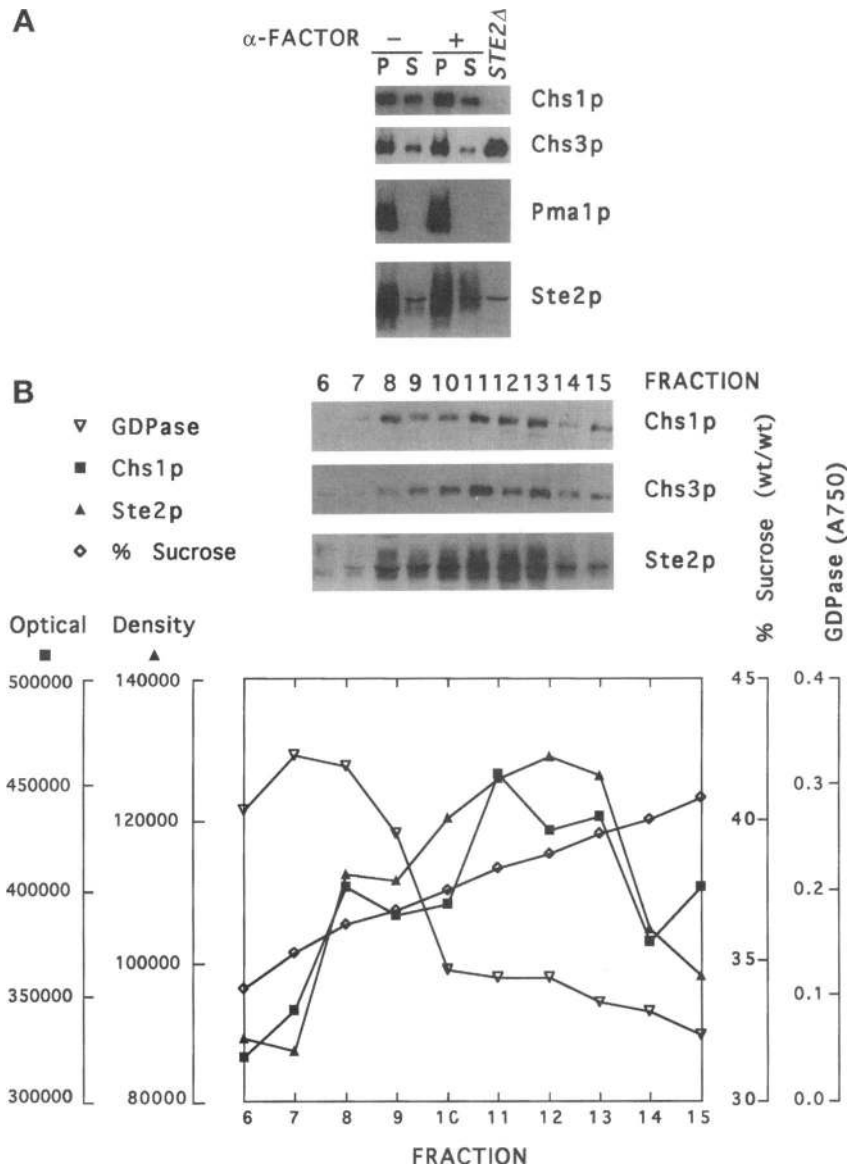

Figure 5. Cofractionation of chitosomal Chs1p and Chs3p with endosomal Ste2p. (A) Z120-5A *MAT***a bar1-1** cells were incubated with 2×10^{-7} M α-factor at 0°C for 50 min. Cells were warmed to 15°C and incubated for 20 min. Cells were treated with azide and fluoride on ice, and cell lysates were fractionated as in Figure 2 and subjected to immunoblot analysis. Ste2p is observed as multiple species. Analysis of total cell lysate from *ste2*Δ strain DK102 indicates a nonspecific band of similar mobility to one of many Ste2p bands. Because DK102 contains no epitope tags, Myc-Chs1p and HA-Pma1p are not observed. (B) The 10,000 × *g* supernatant fraction was sedimented on a linear sucrose gradient. Gradient fractions were subjected to immunoblot analysis and GDPase assay.

endocytosis, followed by transport to the vacuole and degradation (Schandel and Jenness, 1994). We kinetically trapped Ste2p in endosomes by incubating cells at 15°C (Singer-Kruger *et al.*, 1993). Cells treated with α-factor at 0°C and incubated at 15°C exhibited significantly more Ste2p in the 10,000 × *g* supernatant, indicating that ligand-induced endocytosis can be monitored by this method (Figure 5A). The absence of HA-Pma1p in the 10,000 × *g* supernatant controlled for plasma membrane fragmentation. Ste2p from α-factor treated cells exhibited decreased mobility that probably represented ligand-induced hyperphosphorylation (Reneke *et al.*, 1988; Figure 5A). When membranes were examined by sedimentation to equilibrium on sucrose density gradients, a significant cosedimentation of Chs1p, Chs3p, and Ste2p was observed, suggesting that these proteins may reside in the same compartment (Figure 5B). As before, these membranes were resolved from Golgi (GDPase) (Figure 5B) and vacuole (Vph1p) membranes (see Figure 2). Additionally, Chs3p, Chs1p, and Ste2p from these chitosome fractions equilibrated to the same density in Nycodenz gradients.

DISCUSSION

Two observations prompted us to investigate the role of cell cycle-regulated protein trafficking in the control of chitin synthesis. First, chitin appears to be synthesized at three separate times and locations during the

S. cerevisiae cell cycle and separate proteins have been shown to be involved at each time (Shaw *et al.*, 1991). Second, CSI activity has been shown to exist in an undefined internal membrane compartment termed the chitosome (Schekman and Brawley, 1979; Kang *et al.*, 1985; Flores Martinez and Schwencke, 1988; Leal-Morales *et al.*, 1988). We considered the notion that chitosomes serve as an internal reservoir of chitin synthases that await a cell cycle cue for regulated transport to their site of action.

One possibility for regulation is that the chitin synthases are synthesized and degraded in response to cell cycle signals. Data contradictory to this view, with respect to CSI and CSIII, came from an investigation of synthase enzyme stability and cell cycle regulation (Choi *et al.*, 1994a). By examining Chs3p and Chs1p, we have arrived at the same conclusion. First, Chs3p (Chuang and Schekman, 1996) and Chs1p are metabolically stable. Second, in agreement with the lack of cell cycle regulation of CSI (Choi *et al.*, 1994a) and in contrast to the report of cell cycle regulation of *CHS1* RNA and weak cell cycle regulation of *CHS3* RNA (Pammer *et al.*, 1992), we found no regulation of the steady-state levels of Chs3p or Chs1p. In addition, Chs3p is synthesized throughout the cell cycle (Chuang and Schekman, 1996). We conclude that the steady-state levels of Chs3p and Chs1p are not regulated by synthesis and degradation.

Previous work on the intracellular distribution of chitin synthases demonstrated the existence of chitosomes that are distinct from the plasma membrane, the vacuole, and mature secretory vesicles (Flores Martinez and Schwencke, 1988; Leal-Morales *et al.*, 1988). In addition, we have now shown that chitosomes are not fragments of endoplasmic reticulum, mitochondria, or Golgi. It is unlikely that chitosomes are a specialized Pma1p-free domain of plasma membrane. Our procedure to isolate the plasma membrane involved coating spheroplasts with Con A, which recognizes all yeast glycoproteins and thus is unlikely to be excluded from any domains of the cell surface.

Are chitosomes identical to endosomes? The dependence of chitosome formation on *END4* can be interpreted to be direct or indirect. It is possible that the *end4-1* defect blocks the internalization of Chs1p. However, it is also possible that chitosomes are a Golgi-derived, regulated secretory vesicle or compartment and that a block in endocytosis causes such vesicles to enter an alternative pathway such as the Sec1p pathway (Figure 6). Because Chs3p (Chuang and Schekman, 1996) and Chs1p chitosome formation is *END4* dependent and both enzymes cofractionate with internalized Ste2p, it is most likely that chitosomes are identical to Ste2p-containing endosomes. However, metabolically unstable Ste2p is only a transient resident of this compartment on its way to degradation in the vacuole (Schandel and Jenness, 1994),

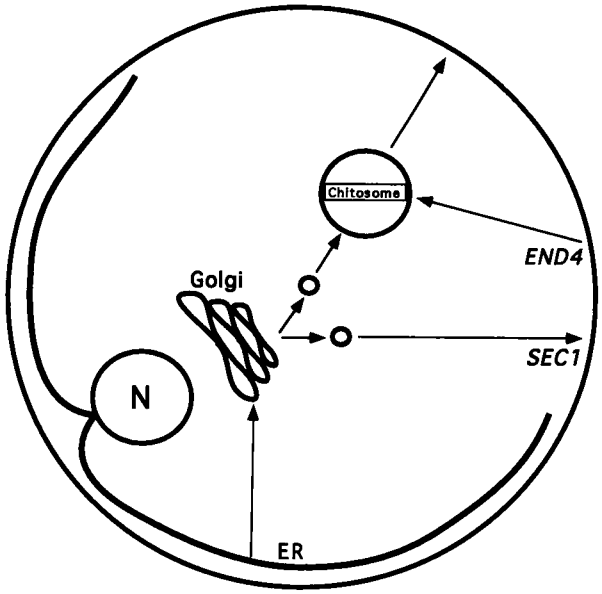

Figure 6. A model for the trafficking of chitin synthase enzymes. Golgi-derived vesicles containing Chs1p and Chs3p may be constitutive secretory vesicles that fuse with the plasma membrane. It is also possible that these vesicles fuse with the chitosome compartment or become chitosomes themselves. An endocytosis defect could directly block the internalization of Chs1p and Chs3p that had been delivered to the plasma membrane via the constitutive (Sec1p-dependent) secretory pathway. Alternatively, a block in endocytosis could indirectly cause newly synthesized Chs1p and Chs3p that are destined for chitosomes to be misdirected into the constitutive secretory pathway and subsequently be trapped at the plasma membrane.

whereas metabolically stable Chs1p and Chs3p are not transported to the vacuole, indicating protein sorting within the endocytic pathway. Further efforts at membrane fractionation will be required to determine whether chitin synthases and endocytic markers cohabit.

If chitosomes represent the sole reservoir of chitin synthases that respond to a cell cycle signal to mobilize to the plasma membrane, some change in the chitosomal pool of these membrane proteins might be expected during the time of the cell cycle that they carry out their function. However, we found no change in the ratio of chitosome to plasma membrane bound Chs3p or Chs1p during the cell cycle. Constitutive synthesis and endocytosis of Chs3p and Chs1p may continually resupply the chitosome, thus obscuring cell cycle-regulated mobilization of these proteins to the plasma membrane. Alternatively, the chitosome supply of Chs3p and Chs1p may exceed the demand for mobilization and small changes in the pools may not be detected.

Chs3p localization to the vicinity of the incipient chitin ring may be required in G_1 just before START and the polarization of secretion, suggesting that it is

impossible to accomplish this localization solely through the constitutive secretory pathway. In a similar manner, Chs1p localization may be required in G_1 just after cytokinesis, during a time of isotropic secretion in daughter cells, thus necessitating an alternate mechanism for specific localization to the birth scar. It is probable that both the constitutive secretory and endocytic pathways are required for transporting Chs3p and Chs1p to endosomes. The function of this localization may be to facilitate cell cycle-regulated recycling of chitin synthase enzymes to their site of action at a time when the constitutive secretory pathway cannot accomplish this function.

Mutations in *CHS6/CSD3* block the majority of chitin synthesis in vivo. However, in contrast to other mutants with this phenotype, *chs6/csd3* has no effect on CSIII activity in vitro (Bulawa, 1992). It is possible that Chs3p in the mutant is unable to interact with its activators in vivo, thus explaining the decrease in cellular chitin. This putative loss of the enzyme-activator complex can be explained if the activator resides at the incipient site of chitin ring formation on the plasma membrane and Chs3p accumulates in the chitosome. The *chs6/csd3* mutant may be defective in cell cycle-regulated recycling of Chs3p and/or Chs1p to its site of action. Cell lysis and preparation of mixed membrane fractions may effectively reconstitute the interaction between Chs3p and its activator, thus explaining CSIII activity in membranes from *chs6/csd3* mutant cells. Our membrane fractionation technique should be useful in addressing the role of *CHS6* and other regulatory genes in the localization of chitin synthases.

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