Targeting of Chitin Synthase 3 to Polarized Growth Sites in Yeast Requires Chs5p and Myo2p

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Abstract. Chitin is an essential structural component of the yeast cell wall whose deposition is regulated throughout the yeast life cycle. The temporal and spatial regulation of chitin synthesis was investigated during vegetative growth and mating of Saccharomyces cerevisiae by localization of the putative catalytic subunit of chitin synthase III, Chs3p, and its regulator, Chs5p. Immunolocalization of epitope-tagged Chs3p revealed a novel localization pattern that is cell cycle-dependent. Chs3p is polarized as a diffuse ring at the incipient bud site and at the neck between the mother and bud in small-budded cells; it is not found at the neck in large-budded cells containing a single nucleus. In large-budded cells undergoing cytokinesis, it reappears as a ring at the neck. In cells responding to mating pheromone, Chs3p is found throughout the projection. The appearance of Chs3p at cortical sites correlates with times that chitin synthesis is expected to occur. In addition to its localization at the incipient bud site and neck, Chs3p is also found in cytoplasmic patches in cells at different stages of the cell cycle. Epitope-tagged Chs5p also localizes to cytoplasmic patches; these patches contain Kex2p, a late Golgi-associated enzyme. Unlike Chs3p, Chs5p does not accumulate at the incipient bud site or neck. Nearly all Chs3p patches contain Chs5p, whereas some Chs5p patches lack detectable Chs3p. In the absence of Chs5p, Chs3p localizes in cytoplasmic patches, but it is no longer found at the neck or the incipient bud site, indicating that Chs5p is required for the polarization of Chs3p. Furthermore, Chs5p localization is not affected either by temperature shift or by the myo2-66 mutation, however, Chs3p polarization is affected by temperature shift and myo2-66. We suggest a model in which Chs3p polarization to cortical sites in yeast is dependent on both Chs5p and the actin cytoskeleton/Myo2p.
CHS4/CAL2/CSD4 (Roncero et al., 1988; Bulawa, 1993), and CHS5/AL3 (Roncero et al., 1988; Santos, B., M.H. Valdèvieso, and A. Durà, unpublished results). Chs3p has significant homology with other chitin synthases and is thought to be the catalytic component of this activity. Overexpression of CHS4 increases CSIII activity three- to tenfold indicating that Chs4p might be an activator of Chs3p (Bulawa, 1993). Recently, the CHS5 gene has been cloned and found to encode a novel protein containing internal repeats and a lysine-rich carboxy terminus (accession number YLR330w; Santos, B., M.H. Valdèvieso, and A. Durà, unpublished results). In addition to its role in chitin synthesis, Chs5p is also required for cell fusion during mating (Santos, B., M.H. Valdèvieso, and A. Durà, unpublished results). The precise function of Chs5p and how it participates in chitin synthesis is not known.

Little is known about the regulation of chitin synthesis, but the existence of different chitin synthases with separate roles during the yeast life cycle suggests the need for specific temporal and spatial regulation of each one. Increased mRNA levels for each chitin synthase gene are detected at the particular stage of the cell cycle in which the encoded enzyme is thought to function, suggestive of transcriptional regulation (Pammer et al., 1992; Choi et al., 1994a). In addition, each of the chitin synthases is present in the cell as a zymogen that can be activated in vitro by proteolysis indicating that posttranslational regulation also occurs (Choi et al., 1994a, b). Spatial regulation requires either a mechanism for proper targeting of the active synthase and/or a strictly localized activation of a randomly dispersed enzyme at the site where chitin synthesis is required. Studies of the subcellular localization of CSI and CSII by sucrose gradient sedimentation have shown these enzymes are present in two different membrane populations, chitosomes and plasma membrane (Leal-Morales et al., 1994). Several chitin synthases (for example Chs3p) lack a recognizable signal peptide for secretion, and the delivery of these enzymes to the plasma membrane, the location of chitin synthesis, has been suggested to require a specific transport system. Chitosomes have been speculated to be part of such a transport system that controls the spatial regulation of chitin synthesis (Bartnicki-García, 1990).

Several cytoskeletal elements have been implicated in the regulation of CSIII and/or activating factors. Spatial control of chitin synthesis may depend in part upon a ring of 10-nm filaments lying underneath the plasma membrane in the mother-bud neck (Byers and Goetsch, 1976). This ring is believed to be formed by a family of septin proteins, the products of the CDC3, CDC10, CDC11, and CDC12 genes; mutations in any of these genes result in diffused deposition of chitin rather than the tight ring in the neck (Roberts et al., 1983; Longtime et al., 1996). The actin cytoskeleton and polarity establishment proteins also participate in the organization of chitin in the cell wall. Mutants defective in actin (Novick and Botstein, 1985), Myo2p, a type V myosin (Johnston et al., 1991), or other proteins affecting actin function (Amatruda et al., 1990; Haarer et al., 1990; Rodriguez and Paterson, 1990; Amatruda et al., 1992; Liu and Bretsch, 1992; Donnelly et al., 1993; Haarer et al., 1994) display an altered pattern of chitin deposition in which chitin is present over the whole cell surface and is no longer restricted to the neck. There is also a correlation between actin delocalization and altered chitin deposition in polarity and bud emergence mutants including cdc24 (Sloat et al., 1981), cdc42 (Adams et al., 1990), cdc43 (Adams et al., 1990), bem2 (Kim et al., 1994), rho3, and rho4 (Matsui and Toh-e, 1992). How the actin cytoskeleton affects chitin distribution is not known. Actin may affect either the distribution of chitin synthases, their activators, or cell wall structures which interact with chitin.

To understand more about how chitin synthesis occurs at specific cellular locations, we have used indirect immunofluorescence to determine the subcellular localization of Chs3p and its regulator Chs5p in vegetatively growing and pheromone-treated cells. We find that Chs3p is located at sites of polarized cell growth and in cytoplasmic patches; Chs5p is found only in patches. Furthermore, we demonstrate that localization of Chs3p to polarized cortical sites requires both Chs5p and Myo2p. Our data suggest a pathway by which Chs3p and the actin cytoskeleton target Chs3p to polarized growth sites in yeast.

**Materials and Methods**

**Yeast Strains, Media, and Microbiological Techniques**

Yeast strains used in this study are listed in Table I. Genetic methods and growth media were as described in Guthrie and Fink (1991). Yeast transformants were by the lithium acetate method of Ito et al. (1983).

**Construction of Epitope-tagged Chs3p and Chs3p Strains**

A strain containing the CHS3::3XHA allele (Y1306) was constructed using the PCR epitope-tagging method of Schneider et al. (1995). Primers 5′-GAATTTGAAAGGAGAATATTCTCTAATGCGAGAGAGAGAGAGAGAGAAGAGACTGGG-3′ and 5′-CACACACCATATATCATATGTCAGTACAGGAAAATTATTTCATATGTCATATATAGGAATATTGAATATTGGAGTGG-3′ were used to amplify a region of pMPY-3xHA, the resulting 1.5-kb PCR product contains the complete URA3 gene flanked by direct repeats encoding the three copies of the hemagglutinin HA epitope (Schneider et al., 1995), and is flanked by 57 bp of CHS3 sequence. This DNA fragment was used to transform Y604. For two strains, correct integration occurred immediately upstream of the CHS3 stop codon as determined by PCR analysis. These strains were streaked onto 5-fluoro-orotic acid (5-FOA) plates to select cells that have lost the URA3 gene through a recombination event between the two repeated epitope coding regions; this event leaves a single in-frame 3XHA-encoding epitope sequence at the 3′ end of the CHS3 gene. Proper formation of the HA-tagged allele was confirmed by PCR and immunoblot analyses.

Strain Y1304 containing the CHS5::3XHA allele was constructed by PCR similar to the method described above except that primers 5′-GAGTAGCGAATAATGTCTAGGGAACAAAAGCTGG-3′ and 5′-ACTGACTGTAGGGCGAATTGG-3′ were used. 3′-CATGTTACGTTTCCGTTTTAGAACCTGGTC-9 and 5′-CACACCATATATCATATGTCAGTACAGGAAAATTATTTCATATGTCATATATAGGAATATTGGAGTGG-3′ were used. 3′-CACACCATATATCATATGTCAGTACAGGAAAATTATTTCATATGTCATATATAGGAATATTGGAGTGG-3′ were used.

To construct the 3XHA::CHS5 (Y1305) strains, primers 5′-CATGTTACGTTTCCGTTTTAGAACCTGGTC-9 and 5′-CACACCATATATCATATGTCAGTACAGGAAAATTATTTCATATGTCATATATAGGAATATTGGAGTGG-3′ were used to amplify a region of pMPY-3xHA or pMPY-3xMYC, respectively (Schneider et al., 1995). After integration and recombination, the strains contained a single in-frame triple epitope (HA or MYC) after the second codon at the 5′ end of the CHS5 coding sequence, which was confirmed by PCR and immunoblot analyses.

**Yeast Immunoblot Analysis**

Cells were grown in 25 ml of YPDA (rich medium supplemented with ad-
and analyzed by immunoblot analysis as described above. Monoclonal anti-HA or anti-myc antibodies were used for detection of epitope-tagged Chs3p and Chs5p, respectively. Rabbit polyclonal antibodies against Pma1p, Anp1p, Kre2p, and carboxypeptidase Y (kindly provided by Dr. C. Slayman, S. Munro, H. Busey, and P. Novick, respectively) were used as markers of different cellular compartments. Chs3p, Chs5p, Pma1p, and Anp1p levels were examined in all fractions; Kre2p and Cpy1p levels were examined in the odd numbered fractions. Using these conditions the plasma membrane migrates primarily at the bottom of the gradient (Leal-Morales et al., 1994; Fig. 5); the smaller amounts of Pma1p in the lighter fractions may represent material in intermediate compartments. Blots were scanned using a model 1650 transmittance/reflectance scanning/densitometer (BioRad Labs, Hercules, CA).

**Indirect Immunofluorescence**

Indirect immunofluorescence was performed as outlined by Gehring and Snyder (1990) and Pringle et al. (1991). Cells were fixed in 3.7% formaldehyde for 60 min and washed three times with 1.2 M sorbitol, 50 mM potassium phosphate buffer, pH 7.4 (solution A), and resuspended in solution A. Spheroplasts were prepared incubating cells in solution A containing 5 µg/ml Zymolyase 100T, 0.03% glusulase, and 0.2% 6-mercaptopentanol at 37°C for 45 min. Spheroplasts were washed and allowed to settle onto poly-l-lysine–coated slides. They were then washed once with 0.15 M NaCl, 0.05 M sodium phosphate, pH 7.4, 0.1% BSA (PBS/BSA), twice with PBS/BSA containing 0.1% NP-40, and once with PBS/BSA. For detection of HA-tagged proteins, primary antibody incubations were performed overnight at 4°C using the mouse 12CA5 antibody, and secondary antibody incubations were performed for 2 h at room temperature using CY3-conjugated goat anti-mouse antibodies (Jackson Immuno-research, West Grove, PA). Before use, both primary and secondary antibodies were preabsorbed to whole yeast wild-type cells lacking the epitope tags and spheroplasts to remove nonspecific antibodies (Burns et al., 1994). Washes before and after the secondary antibody incubations were similar to those described above. Samples were mounted in 70% glycerol, 2% n-propyl gallate, and 0.25 µg/ml HOECHST 33258.

For actin and Chs5p double labeling, immunofluorescence was performed as described above, but an additional methanol/acetone fixation was carried out after cells had settled onto the poly-l-lysine–coated slides. Monoclonal anti-actin antibodies (mAb C4) and rabbit anti-HA poly-

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**Table I. Strain List**

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<th>Strain</th>
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<th>Source</th>
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Wild-type MY02 (Y1313) or myo2-66 mutant (Y1314) strains containing the CHS3::3XHA allele were grown at the permissive temperature (24°C) and then transferred at the restrictive temperature (37°C) as described in Materials and Methods. Samples were taken at different times, fixed, and prepared for immunofluorescence; the percentage of cells at different stages of the cell cycle and the localization of epitope-tagged Chs3p in those cells were determined. N, number of cells scored.
clonal antibodies (BABC0) were used. For detection of primary antibodies, CY3-conjugated sheep anti-rabbit antibodies (Sigma) and FITC-conjugated goat anti-mouse antibodies (Cappel Laboratories, Malvern, PA) were used.

For colocalization experiments of 3Xmyc::Chs5p and Chs3p:3XHA, cells (Y1310) were stained with a mouse anti-my c antibody (9E10) and a rabbit anti-HA polyclonal antibody. For double immunofluorescence experiments with Golgi-associated proteins, myc-epitope-tagged Chs5p was detected as described above and rabbit anti-Ann1p or rabbit anti-Kex2p antibodies were used. For the double immunofluorescence experiment with Chs5p and Kex2p, strain Y1303 containing HA-epitope-tagged Chs5p was transformed with plasmid pBM-KX22 which contains the KEX2 gene under the control of the GAL1 promoter (Redding et al., 1991). Cells were grown in medium containing 2% galactose and immunofluorescence was performed as described using monoclonal anti-HA and affinity-purified rabbit anti-Kex2p antibodies (kindly provided by Dr. R. Fuller, Stanford University, Stanford, CA). For detection of primary antibodies, CY3-conjugated goat anti-mouse antibodies and FITC-conjugated goat anti-rabbit antibodies (Cappel Laboratories) were used.

For immunofluorescence experiments in pheromone-treated cells, cells were grown in YPDA to early log phase, α-factor (Sigma) was added to a final concentration of 5 μg/ml, and cells were incubated with shaking at 30°C for 45 min. Cultures were supplemented with a second addition of α-factor and incubated with shaking at 30°C for another 45 min. Microscopic examination of the cultures revealed that after 90 min most of the cells (~90%) were unbudded and had formed shmooes. Cells were fixed with formaldehyde and processed for indirect immunofluorescence as described above.

Analysis of temperature-sensitive myo2, sec, and end4 Δ mutant strains was as follows: myo2-66 mutants were grown in YPDA at room temperature until mid-log phase. To shift cells to the restrictive temperature, an aliquot of the culture was diluted fivefold into prewarmed YPDA at 37°C. Samples were taken at 0, 20, 60, and 120 min and fixed with formaldehyde as described in Lilley and Brown (1994). Control cultures of wild-type cells were treated in a similar fashion. In parallel with these experiments, myo2-66 cells were also diluted and incubated at room temperature. For immunolocalization of Chs5p in sec and end4 Δ mutants, strains Y1930, Y1931, Y1932, Y1933, and Y1934 were grown at room temperature until mid-log phase and then diluted twofold in prewarmed YPDA at 37°C. Samples were taken at 0, 30, 60, 90, and 120 min and fixed with formaldehyde. Control experiments in which cells were incubated at the permissive temperature (~24°C) were also performed. Indirect immunofluorescence was performed as described above.

Results

Epitope Tagging of Chs5p

To gain insight into how Chs5p might participate in generating functional CSIII activity, we determined its subcellular distribution. The Chs5 gene was tagged at its genomic locus with three copies of the hemagglutinin (HA) epitope coding sequence or three copies of the c-myc monoclonal antibody and total cell extracts detects a 170–180-kD protein and a less abundant protein of ~65 kD in each tagged strain (Fig. 1A; data not shown for the myc-tagged strain). These proteins are not detected in strains lacking the epitope tag (Fig. 1A). The same two species are also detected when antibodies against the amino terminus of Chs5p are used (data not shown). Chs5p is expected by its predicted primary amino acid sequence to be a 73-kD polypeptide. However, epitope-tagged Chs5p migrates substantially slower than expected. Chs5 is predicted to encode an acidic protein with seven potential N-glycosylation sites (Santos, B., M.H. Valdivieso, and A. Duran, unpublished results). To determine whether the difference in predicted and observed mobility is due to N-linked glycosylation, cells containing the HA-tagged Chs5 protein were incubated in the presence of tunicamycin, a potent inhibitor of N-linked glycosylation (Orlean et al., 1991). Samples were taken after treatment with tunicamycin for different lengths of time and analyzed by immunoblot analysis. As shown in Fig. 1C, the mobility of Chs5p:3XHA does not change upon incubation of cells in the presence of tunicamycin. Control experiments analyzing Chs5p (Fig. 1C, see below), or similar experiments analyzing Ax1Δ2p, an integral membrane glycoprotein (Roemer et al., 1996), result in detection of a lower molecular weight protein of the predicted size after tunicamycin treatment in each case. Thus, Chs5p is not N-glycosylated. The mobility of Chs5p does not change by pretreatment of protein extracts with other disrupting agents including 8 M urea, high salt or Na2CO3 (pH 11.0) before separation in the SDS-polyacrylamide gels (data not shown). The predicted Chs5p sequence is rich in serine and threonine residues (16%); perhaps phosphorylation, O-linked glycosylation, or other posttranslational modifications account for the substantial deviation from the expected mobility.

Chs5p Localizes in Cytoplasmic Patches

To determine the subcellular localization of epitope-tagged Chs5p, indirect immunofluorescence was performed on the 3XHA::CHS5, CHS5::3XHA, and 3Xmyc::CHS5 strains using anti-HA or anti-myc monoclonal antibodies. As shown in Fig. 2 for 3XHA::CHS5 cells, staining of haploid cells containing the HA-tagged Chs5 protein revealed a punctate localization pattern. (Staining of 3Xmyc::CHS5 cells is shown below in Figs. 3 and 7.) Chs5p is localized in cytoplasmic patches in cells at different stages of the cell cycle. The number of patches ranges from 3 to 15, and their size, shape, and intensity of staining appears heterogeneous. Chs5p patches are distributed throughout the cell, and they are observed in both mother and daughter cells. The same localization pattern is observed in diploid cells (data not shown). The Chs5p localization does not overlap with nuclei or mitochondria as visualized by DNA staining with Hoechst. It also does not overlap with actin patches as determined by indirect immunofluorescence using anti-actin antibodies (data not shown). The localiza-

1. Abbreviation used in this paper: HA, hemagglutinin.
Figure 1. Immunoblot analyses. (A) Proteins from total yeast cell extracts of strains Y1303 or Y1308 (chs3Δ) containing Chs3p tagged with the triple HA epitope at the amino terminus (+) were separated in 8% polyacrylamide gels containing SDS, and an immunoblot was prepared and probed with a monoclonal anti-HA antibody. Control yeast cell extracts of a strain lacking the epitope, the 170–180-kD and 65-kD species cannot be detected even upon long exposures. An identical blot with the CHS5 gene expressed from the GAL1 promoter indicates that Chs3p and Kex2p usually colocalize (Fig. 3B); 76% of Chs3p patches (203 patches counted) contain Kex2p, and 88% of Kex2p patches contain Chs3p (175 patches scored). Thus, these immunofluorescence studies suggest that Chs3p resides, at least in part, in a late Golgi/secretory compartment.

To attempt to characterize the Chs3p compartment further, the localization of Chs3p:3XHA was examined in end4Δ mutants which are blocked in endocytosis (Raths et al., 1993), and in temperature-sensitive sec16-4, sec7-1, or sec6-4 cells incubated at the restrictive temperature (37°C) (Novick et al., 1980, 1981). These latter three mutants are defective in transport from the endoplasmic reticulum to the Golgi, from the Golgi to the secretory vesicle compartment, and from secretory vesicles to the plasma membrane, respectively. The punctate staining pattern of Chs3p was not altered in end4Δ mutants, in sec7-1 or sec6-4 cells incubated at 37°C for 0.5, 1.0, 1.5, or 2.0 h (Fig. 4), or in any of the sec mutants incubated at the permissive temperature. In contrast, punctate Chs3p staining was not detected in sec16-2 cells incubated at 37°C for any of the times indicated above (0.5–2 h); a slight increase in uniform cytoplasmic staining was observed. Accumulation in an endoplasmic reticulum-like pattern (i.e., nuclear
periphery) was not detected. Staining with anti-Anp1p antibodies yielded similar results (not shown). These data are consistent with the hypothesis that Chs5p lies in a late Golgi compartment (see Discussion).

### Chs5p Is in a Vesicle Compartment

The punctate staining pattern of Chs5p and its colocalization with Kex2p patches, suggest that Chs5p may be part of a vesicular compartment. To independently test this possibility, subcellular fractionations were performed using conditions that optimize separation of chitosomes (Leal-Morales et al., 1994). Lysates of 3Xmyc::CHS5 CHS3::3XHA yeast cells were prepared and subjected to sucrose density gradient centrifugation. Fractions were collected and analyzed by immunoblot analysis using antibodies that recognize HA, c-myc, Anp1p, Kre2p, Pma1p (a plasma membrane marker) (Chang and Slayman, 1991; Chang and Fink, 1995), and carboxypeptidase Y (Cpy1p; a protein whose three different isoforms are located in the vacuole, endoplasmic reticulum, or Golgi apparatus; Stevens et al., 1982). As shown in Fig. 5, A and B, all of the detectable Chs5p partitions in a vesicular fraction that is similar in density to the Golgi fraction as monitored by Anp1p, Kre2p, and the carboxypeptidase Golgi isoform (data not shown for the latter two proteins, but the results are similar to the Anp1p data). This compartment is distinct from the plasma membrane (which primarily migrates at the bottom of the gradient [Fractions 1–3] under these conditions; see Materials and Methods), endoplasmic reticulum, and vacuole. Thus, consistent with the immunofluorescence results, Chs5p is present in a membrane vesicle compartment.

### Chs3p, the Catalytic Component of CSIII, Is a Glycoprotein

Little is known about the regulation or localization of Chs3p, the catalytic subunit of CSIII (Valdivieso et al., 1991). To analyze the subcellular distribution of Chs3p and to test the hypothesis that Chs5p may affect the localization of Chs3p, the latter protein was tagged and localized. Three copies of the HA epitope coding sequence

![Figure 2. Localization of epitope-tagged Chs5p. Haploid yeast cells containing Chs5p tagged at the COOH terminus with triple HA (Y1303) or Chs5p without an epitope tag (Y604) were stained by indirect immunofluorescence with an anti-HA monoclonal antibody (left panels). Hoechst 33258 DNA staining of the same cells is shown in the right panels.](image-url)
gel containing SDS, transferred to a filter, and probed with "CHS3::3XHA"

that the Chs3::3XHA protein is functional.
cero et al., 1988; Valdivieso et al., 1991); in contrast, the
with mutations in "CHS3" after the last codon of
were integrated into the "CHS3" gene after the last codon of the open reading frame at the genomic locus (Schneider et al., 1995) to generate the "CHS3::3XHA" allele. Strains with mutations in "CHS3" are resistant to Calcofluor (Roncero et al., 1988; Valdivieso et al., 1991); in contrast, the "CHS3::3XHA" strain is sensitive to Calcofluor indicating that the "CHS3::3XHA" protein is functional.

Protein extracts prepared from strains containing the "CHS3::3XHA" allele were separated in a polyacrylamide gel containing SDS, transferred to a filter, and probed with anti-HA monoclonal antibodies. Four reactive bands were detected: one very high molecular mass protein at the top of the gel, two other proteins of ~150 and 180 kD and a smaller protein of 70 kD that may be a degradation product (Fig. 1 B). None of these bands were evident in protein samples prepared from strains lacking the HA construct. In the absence of protein modification, Chs3p is predicted to be a 131-kD polypeptide. The observation that most Chs3p isoforms migrate slower than expected suggests the presence of posttranslational modifications. The predicted Chs3p sequence contains seven potential transmembrane domains and three potential N-glycosylation sites (Valdivieso et al., 1991). We therefore examined whether this protein is N-glycosylated by incubating cells containing epitope-tagged Chs3p in the presence of tunicamycin. As shown in Fig. 1 C, a new 130-kD form of Chs3p is detected after tunicamycin treatment, close to the size predicted by sequence. The amounts of the high molecular mass and the 150-kD proteins are reduced; interestingly, the level of the 180-kD species does not appear to change. This experiment indicates that Chs3p is modified by N-glycosylation; other types of modification may occur as well.

The Chs3::3XHA Protein Localizes to the Incipient Bud Site, the Neck, and Cytoplasmic Patches

The subcellular distribution of the epitope-tagged Chs3p was determined by indirect immunofluorescence using anti-HA monoclonal antibodies. As shown in Fig. 6, Chs3p is localized at the cell periphery and in cytoplasmic patches. Both haploids and diploids exhibit similar localization patterns (Fig. 6). No staining is detected in the isogenic untagged parental strain (e.g., Fig. 2).

The distribution of Chs3p changes during the cell cycle (Fig. 6). In unbudded cells Chs3p is detected as weakly or moderately staining cytoplasmic patches (Fig. 6 B-a), or as a strongly staining large cortical patch at the presumptive bud site (Fig. 6 B-b). In favorable views from the end of the cell, the cortical patch usually appears as a diffuse ring. In some unbudded diploid cells (~3%; n = 204), Chs3p diffuse rings are located at both poles of the cell (Fig. 6 B-g), suggesting that these cells contain Chs3p material that remains after cytokinesis (see below) and a new Chs3p ring that appears at the incipient bud site. In small-budded cells Chs3p is maintained as a ring at the neck and some cytoplasmic patches are still visible (Fig. 6 D-c and d). Chs3p staining is usually more intense on the mother side of the neck. In cells that have a bud greater than or equal to half the size of the mother cell and a single nucleus, Chs3p is located in strongly staining cytoplasmic patches (Fig. 6 B-e). Mitotic cells also have Chs3p patches. In cells undergoing cytokinesis (large-budded cells with two completely separated nuclei), Chs3p is usually found at the neck (Fig. 6 A and B-f). In these latter cells, staining at the neck is sometimes more intense on the mother side of the neck than on the daughter side, but in other cells the signal is distributed equally (Fig. 6 A). In cells in which Chs3p is polarized at the incipient bud site or at the neck, cytoplasmic patches are also detected but they are fewer and stain more faintly. In summary, Chs3p is often polarized at the incipient bud site and the neck in yeast. The presence of high levels of chitin at these sites is presumably due to the polarized targeting of Chs3p.
Colocalization of Epitope-tagged Chs3p and Chs5p in Cytoplasmic Patches

To test whether Chs3p and Chs5p are present in the same cytoplasmic compartment, indirect immunofluorescence was performed on a strain expressing Chs3p and Chs5p epitope-tagged with HA and myc, respectively. A strain containing 3Xmyc::CHS5 and CHS3::3XHA was constructed and stained using anti-myc monoclonal and rabbit polyclonal anti-HA antibodies. As shown in Fig. 7, the staining of Chs3p at polarized sites is unique for Chs3p, however, the staining pattern of the Chs5p and Chs3p patches are very similar. All patches containing Chs3p also contain Chs5p and most, but not all (~75%), of Chs5p patches localize to Chs3p patches (72 cells and 451 Chs5p spots counted). The patches that are unique to Chs5p generally stain more weakly. We do not know whether the patches in which only Chs5p is found also contain low levels of Chs3p that are not detected in these studies or if they are truly unique for Chs5p. The colocalization of Chs3p and Chs5p is not due to crossreaction of secondary antibodies for two reasons: (1) staining of single epitope-tagged strains yields a signal only with the appropriate secondary antibody and (2) there are regions that are uniquely stained for Chs3p (polarized growth sites) and Chs5p (some of the cytoplasmic patches). In summary, these data indicate that Chs3p and Chs5p colocalize in many cytoplasmic patches. This result raises the possibility that Chs5p may be involved in processing or transporting Chs3p to the membrane.

Localization of Chs3p Requires Chs5p

To test whether Chs5p participates in the regulation of the subcellular distribution of Chs3p, localization of Chs3p in a chs5Δ background was examined. The entire coding region of the CHS3 gene except for the last segment encoding the 55 carboxy-terminal amino acids was replaced by the ADE2 gene, as described (Santos, B., M.H. Valdivieso, and A. Durán, unpublished results). chs5Δ strains are viable, but they have less chitin (~25% of wild-type levels) and lack detectable CSIII activity in vitro (Bulawa, 1993; Choi et al., 1994b). Importantly, in the absence of Chs5p, Chs3p::3XHA localizes to cytoplasmic patches, but it is no longer found at the incipient bud site or the neck (Fig. 8). Immunoblot analysis shows that both the number and amount of the three high molecular weight Chs3p isoforms is similar in the presence or absence of Chs5p, indicating that disruption of CHS5 does not affect either protein levels or modification of Chs3p (Fig. 1B). Localization and protein levels of Chs5p in a chs3Δ background were also examined. In the absence of Chs3p, the Chs5p punctate localization pattern (data not shown) and protein levels (Fig. 1A) are not affected. In summary, Chs5p is required for the polarization of Chs3p to the cortex.

The localization of Chs3p in chs5Δ cells was also examined using sucrose density gradient centrifugation experiments. As shown in Fig. 5C, in wild-type cells, a large proportion of Chs3p::3XHA in our cell lysate preparations migrates in the vesicular fractions in a pattern similar to that of Chs5p; some Chs3p::3XHA appears in a slightly heavier fraction that could represent a different vesicular compartment. A small amount of protein is also detected in the high density region close to the plasma membrane fraction at the bottom of the gradient. In chs5Δ cells, no Chs3p is detected in the high density fraction, and much of Chs3p is shifted to the lighter density fractions; most Chs3p::3XHA...
Localization of Proteins Involved In Chitin Synthesis

Thus, the density profile of Chs3p is altered in chs5Δ cells. These data, together with the immunolocalization results indicate that Chs5p is required for proper membrane targeting of Chs3p.

Localization of Chs3p and Chs5p in Pheromone-treated Cells

In addition to vegetative growth, chitin is also synthesized during mating; chitin deposition occurs at the subapical portion of the shmoo tip (Lipke et al., 1976; Schekman and Brawley, 1979). CSIII is the activity responsible for the synthesis of chitin during mating (Roncero et al., 1988; Valdivieso et al., 1991). chs3 mutants are unable to synthesize chitin in response to pheromone but mate at a moderate efficiency (Roncero et al., 1988; Valdivieso et al., 1991). By contrast, chs5 mutants are able to induce some chitin synthesis in response to pheromone, but the mating efficiency is severely decreased, suggesting two roles for Chs5p, one in chitin synthesis and the other in mating (Santos, B., M.H. Valdivieso, and A. Durán, unpublished results).

To understand the role of CSIII in chitin synthesis during mating, the localization of epitope-tagged Chs3p and Chs5p was examined in MATα cells treated with α-factor. Chs5p is localized in cytoplasmic patches that preferentially accumulate at the tip of the shmoo in ~30% of the cells (n = 118) (Fig. 9). The distribution of Chs3p in pheromone-treated cells is slightly different from its localization pattern in vegetative cells; in cells incubated with mating pheromone Chs3p is primarily at the cell periphery of the mating projection with very few cytoplasmic patches. By contrast, in chs5Δ strains Chs3p is primarily found in cytoplasmic patches and it is no longer polarized to the periphery, similar to the defect in cortical localization of Chs3p observed in chs5Δ strains during vegetative growth (Fig. 9). Localization of Chs5p in shmoo is not affected by the absence of Chs3p (data not shown). These results indicate that Chs5p is required for the polarization of Chs3p to the shmoo projection in addition to the requirement for Chs5p for polarization of Chs3p in vegetative cells.

Localization of Epitope-tagged Chs3p and Chs5p in myo2-66 Mutants

The MYO2 gene encodes an unconventional essential form of myosin implicated in polarized growth and secretion (Johnston et al., 1991). Temperature-sensitive myo2-66 mutants form enlarged unbudded cells at the restrictive temperature indicating that bud initiation is inhibited (Prendergast et al., 1990; Johnston et al., 1991). myo2-66 mutant cells lose actin and chitin polarization, and accumulate cytoplasmic vesicles. However, many types of secretion continue in these cells (Govindan et al., 1995).

To determine whether the abnormal deposition of chitin in myo2-66 cells is due to mislocalization of Chs5p or Chs3p, we examined the localization of these two proteins in wild-type (MYO2) and myo2-66 mutant strains grown at the permissive temperature (24°C) and after shifting to 37°C for 20 min, 1 or 2 h. Previous studies have shown that shifting wild-type cells from room temperature to 36°C causes a dramatic, although transient, rearrangement of

Figure 5. Sucrose density gradient centrifugation analysis. (A and B) Cells lysates were prepared from yeast strain (Y1310) containing 3Xmyc::CHS5 and CHS3::3XHA and fractionated in 10–65% sucrose density gradients by centrifugation for 20 h. Fractions (abscissa in A) were collected from the bottom of the gradient and analyzed by immunoblot analysis using antibody probes to HA, c-myc, Pma1p, Anp1p, and Cpy1p. Each fraction was analyzed for all of the samples except Cpy1; only odd-numbered fractions were analyzed for that protein. Several sample blots are shown in B (Only the bottom ~150-kD Chs3p band, which has most of the Chs3p material is shown.). For each protein, two separate immunoblots were analyzed; the amount of immunoreactive material was quantified for both blots (the results were similar for each blot) and the average is shown in A as a percentage of the total protein. (C) Fractionation of epitope-tagged Chs3p in a wild-type and chs5Δ mutant. Samples were prepared from strain Y1309 and analyzed in parallel with the wild-type samples described in A. For comparison, the Chs3p and Chs5p peaks from the wild-type strain are shown.
the actin cytoskeleton (Palmer et al., 1992; Lew and Reed, 1993; Lillie and Brown, 1994). Actin polarity is initially lost and then reforms after prolonged incubation (1–2 h). In contrast, myo2-66 mutants do not repolarize actin after prolonged incubation at 37°C. Myo2p localization is also perturbed by temperature shift but reappears with a time course similar to that of actin spot depolarization and repolarization (Lillie and Brown, 1994).

Analysis of Chs3p::3XHA localization reveals that it is clearly affected by both the shift to 37°C and by the myo2-66 mutation. In wild-type cells, after 20 min at 37°C, Chs3p staining is reduced in intensity and the protein accumulates in cytoplasmic patches; it is not polarized at the membrane or at the neck (Fig. 10, Table II). However, after 2 h at 37°C, Chs3p begins to repolarize (Fig. 10, Table II). The time course of disappearance/reappearance is similar to that of actin and Myo2p described previously (Lillie and Brown, 1994). In the myo2-66 mutant Chs3p is often less polarized even at room temperature. After shifting to the restrictive temperature Chs3p polarization disappeared, as expected in response to the temperature shift, but unlike the wild-type strain, it did not recover after 2 h (Fig. 10, Table II). In contrast to that observed for Chs3p, the localization of Chs5p in either wild-type or myo2-66 cells was...
not perturbed by incubation at 37°C suggesting that its localization is not dependent on actin and/or Myo2p. In summary, these different data indicate that Chs3p requires not only Chs5p, but also actin and/or Myo2p for proper localization to the cortex.

Discussion

Chs3p Localizes to Polarized Growth Sites

During vegetative growth, CSIII is involved in the synthesis of the chitin ring at the base of the emerging bud and the chitin in the lateral wall (Cabib et al., 1974; Shaw et al., 1991). We found that Chs3p is present at the cell periphery and in cytoplasmic patches and undergoes a dynamic localization during the cell cycle. Chs3p localizes to the incipient bud site in unbudded cells, and the neck in small budded cells and in large budded cells undergoing cytokinesis. The polarization of Chs3p at the incipient bud site and at the neck in small budded cells is consistent with the appearance of chitin during this time (Hayashibe and Katohda, 1973; Shaw et al., 1991) and its expected role in bud formation. The reappearance of Chs3p at the neck is consistent with a role for CSIII activity during cytokinesis, which has not been noted previously. The localization pattern of Chs3p supports the premise that this protein is the catalytic component of CSIII activity (Valdivieso et al., 1991), and that chitin synthesis occurs at the site of chitin accumulation.

The localization of Chs3p to the incipient bud site and/or neck region is unique, but has similarities to other yeast proteins. The polarity proteins Spa2p, Cdc42p, Myo2p, Smy1p, and Axl2p all localize at the incipient bud site and, like Chs3p, many of these proteins also appear at the neck in cells undergoing cytokinesis (Snyder, 1989; Snyder et al., 1991; Ziman et al., 1993; Lillie and Brown, 1994; Roemer et al., 1996). However, these proteins form a patch at the incipient bud site (instead of a diffuse ring like Chs3p) and the proteins remain associated with the bud tip of small budded cells rather than at the neck. (The one exception is Axl2p which localizes both to the tip of small budded cells and to the neck of medium and large budded cells.) Chs3p localization is most similar to that of the septins which localize as a ring at the incipient bud site and remain at the neck until after cytokinesis (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim et al., 1991). However, in contrast to the septins, Chs3p disappears from the neck region and forms cytoplasmic patches once buds reach a medium-large size; later it reappears at the neck. In addition, in cells undergoing cytokinesis, only one broad ring is observed rather than two as seen for the septins. Finally, unlike any of the morphogenic proteins mentioned above, Chs3p is found in brightly staining cytoplasmic patches in addition to cortical sites.

The changes of Chs3p localization in the cell cycle suggest a complex regulation pattern of this protein. Several aspects of Chs3p polarization are probably temporally regulated similar to the other polarity proteins, in that Chs3p accumulates its destination after the G1/S transition and after mitosis. As found for actin (Lew and Reed, 1993), activation of the G1 Cdc28-Cln kinase may be necessary for Chs3p polarization to the incipient bud site, and inactivation of the G2 Cdc28-Cib kinase may be necessary for redistribution to the neck region during cytokinesis. In addition, Chs3p delocalizes when cells reach a medium-sized bud, an event that may require endocytosis since the protein is probably an integral membrane protein (it has seven potential transmembrane domains; Valdivieso et al., 1991) and is membrane-bound (Fig. 5). This event probably requires inactivation of the G1 Cdc28-Cln kinase and activation of the G2 Cdc28-Cib kinase. Finally, it is also likely that Chs3p mRNA and protein levels are regulated during the cell cycle. CHS3 contains an MCB box upstream of its open reading frame, similar to many other polarity genes, including AXL2 and SPA2 (Roemer et al., 1996). MCB elements have been shown to mediate preferential G1/S expression (Andrews and Herskowitz, 1989). We therefore expect that the level of Chs3p is most prevalent after the G1/S transition, consistent with the observation that Chs3p staining is most intense in unbudded cells with polarized distributions of the protein and in budded cells (i.e., cells which have traversed the G1/S transition).

Chs5p and Chs3p Localize to Cytoplasmic Patches

Chs5p is involved in chitin synthesis and is specifically required for CSIII activity (Choi et al., 1994b), but before this study the mechanism by which Chs5p participates in chitin synthesis was not known. Immunolocalization experiments show that epitope-tagged Chs5p is localized in cytoplasmic patches. A subset of these patches also con-
tain Chs3p. Since Chs3p is glycosylated, it must move through parts of the secretory pathway, and it is likely that the patches represent a secretory compartment(s). Consistent with this interpretation, Chs5p fractionates with vesicular compartments (Fig. 5), and usually colocalizes with Kex2p, a late Golgi protein (Redding et al., 1991). Kex2p is overexpressed in our localization experiments, and Redding et al. (1991) present evidence to suggest that the foci in the Kex2p overexpressing cells are late Golgi compartments (Redding et al., 1991). However, it probably cannot be rigorously excluded that some of these patches are late, post-Golgi, secretory compartments. Two additional lines of evidence indicate that the patches represent a late secretory compartment: First, Chs5p localization is not disrupted by two mutants blocked late in the secretory pathway, sec7-1 and sec6-4, or by the end4Δ mutant; however, localization is disrupted in sec16-2 cells which are blocked early in the secretory pathway. Second, in a chs5Δ mutant Chs3p is present in patches; the Chs3 protein in these cells is glycosylated, as indicated by the presence of the same high molecular weight isoforms observed in wild-type cells. Since N-glycosylation occurs in the endoplasmic reticulum and Golgi apparatus, the patches must be either late- or post-Golgi compartments.

Immunolocalization of chitin synthases or their regulators has not been reported previously. However, by cell fractionation experiments chitin synthases in different fungi and CSI and CSII in S. cerevisiae have been shown to be present in two different subcellular locations: an internal chitosome compartment and the plasma membrane (Durán et al., 1975, 1979; Leal-Morales et al., 1988, 1994). Our experiments indicate that the Chs3p patches are a subset of the Kex2p/late secretory compartment and raise the possibility that the chitosome compartment is a late secretory compartment. Chs5p is required for secretion of Chs3p to the plasma membrane, but not other proteins whose transport is dependent upon the general secretory machinery. For example, secretion of Exg1p, an extracellular exo-1,3β-glucanase (Vázquez de Aldana et al., 1991) is not affected in chs5 mutants (Santos, B., M.H. Valdivieso, and A. Durán, unpublished results). Therefore, these Chs5p patches may represent a specific and unique subset of the late secretory pathway, and the presence of Chs3p in these patches raises the possibility that the patches may represent a storage compartment. Alternatively, and not mutually exclusive with the former possibility, Chs5p may be involved in interacting with the late Golgi and generating a unique secretory vesicle pathway, perhaps one that targets a unique class of secretory vesicles to particular sites in the cell (e.g., polarized growth sites).

Figure 8. Immunolocalization of Chs3p in a chs5Δ strain. Haploid chs5Δ cells (Y1309) containing Chs3p tagged with triple HA were stained by indirect immunofluorescence with anti-HA monoclonal antibodies. Hoechst 33258 DNA staining of the same cells is also shown.
Chs5p Is Required for Chs3p Localization

The observation that Chs3p and Chs5p reside in the same internal cytoplasmic compartment raises the possibility that Chs5p is important for targeting Chs3p to the membrane. In a chs5Δ mutant, Chs3p is no longer polarized at the cell cortex and instead accumulates in cytoplasmic patches, indicating that Chs5p is required for localization of Chs3p to the cell periphery. Immunoblot analysis indicates that disruption of the CHS5 gene does not affect the levels of Chs3 protein; furthermore, the same three Chs3p isoforms that are observed in wild-type cells are present in chs5Δ cells, indicating that Chs5p does not affect protein processing. These results indicate that chs5 mutants do not have wild-type levels of chitin because the Chs3p does not reach its cortical destination.

Vegetative chs5 cells have reduced levels of chitin (25% that of wild-type cells), but they still have more chitin than chs3Δ cells (10% that of wild-type cells; Roncero et al., 1988; Valdivieso et al., 1991). In the absence of Chs5p, either a low level of Chs3p might reach polarized sites independently of Chs5p, or Chs3p patches might anchor throughout the cell surface (rather than at incipient bud sites and the neck) and deposit chitin over the entire surface of the cell. Staining of chs5 cells with wheat germ agglutinin, which binds chitin, indicates that the lateral cell wall chitin is probably increased compared to chs3 mutants, suggesting the latter possibility (Roncero et al., 1988). The overall low level of CSIII activity in chs5Δ cells may be due to its failure to interact with a specific CSIII activator at the cell cortex (e.g., Chs4p; see below).

How Chs5p is involved in targeting Chs3p to its correct location is not known. Chs5p lacks a signal sequence, but contains small blocks of homology with Sec16p, a coat vesicle protein that also migrates with an unexplained slow mobility in SDS-polyacrylamide gels (Espenshade et al., 1995). These features raise the possibility that Chs5p resides on the outside of vesicles. Consistent with this possibility, Chs5p does not accumulate in the endoplasmic reticulum in sec16-2 strains incubated at the restrictive temperature; such a result would be expected if Chs5p associated within late secretory organelles. Association of Chs5p with the outside of vesicles would ideally position it to interact with either the transport machinery or components at the target site (the incipient bud site and neck region) and thereby help target Chs3p to those sites. Alternatively (and not exclusive with the former possibility), Chs5p might also be involved in generating or segregating the specific Chs3p secretory vesicles from the late Golgi apparatus.

Subcellular Localization in Pheromone-treated Cells

During mating chitin is normally increased threefold and is deposited at the subapical portion of the shmoo tip (Lipke et al., 1976; Schekman and Brawley, 1979). We found that in pheromone-treated wild-type cells, Chs3p is almost entirely at the periphery of the mating projection and very few cytoplasmic patches are observed. Chs5p is in cytoplasmic patches that sometimes accumulate at the shmoo tip. As in vegetative cells, Chs3p polarization is severely compromised in chs5Δ mutants and found in patches, indicating that Chs5p may be involved in targeting Chs3p to the plasma membrane in mating cells.

Chs5p is required for chitin synthesis and also for cell fusion in mating (Santos, B., M.H. Valdivieso, and A. Durán, unpublished results). In contrast, Chs3p is primarily important for chitin synthesis, and chs3Δ cells only have a mild mating defect. Therefore, Chs5p has functions other than the targeting of Chs3p. Consistent with this interpretation, in vegetative cells Chs5p patches are observed that lack detectable Chs3p. Interestingly, two other proteins, Fus1p and Fus2p, that are required for cell fusion during mating, localize with a similar pattern to that of

Figure 9. Localization of epitope-tagged Chs3p and Chs5p in α-factor treated cells. MATα strains Y1303 (3XHA::CHS5), Y1306 (CHS3::3XHA), and Y1309 (CHS3::3XHA chs5Δ) were incubated with α-factor mating pheromone. After 90 min, the cells were fixed and stained with anti-HA monoclonal antibodies. (Left two panels) WT, wild-type cells. (Right panel) chs5Δ cells. The epitope-tagged protein in the cells is indicated.
Chs3p and Chs5p, respectively. Fus1p is localized to the plasma membrane at the projection tips of pheromone-induced cells (Trueheart et al., 1987). Fus2p is present in punctate spots that accumulate within the projection or near the projection tip (Elion et al., 1995). Both Fus2p and Chs5p have weak homology with cytoskeletal proteins. FUS1 and FUS2 genes are not expressed during vegetative growth and are induced upon pheromone treatment (Trueheart et al., 1987; Elion et al., 1995). The fact that Chs5p has additional functions in cell fusion during mating other than chitin synthesis, suggests that the Chs5p patches could transport other proteins, for example, Fus1p and Fus2p. Consistent with this possibility, chs5Δ mutants are defective in cell fusion (Santos, B., M.H. Valdivieso, and A. Durán, unpublished results).

### Myo2p Is Required for Chs3p Polarization

Previous studies have indicated that the actin cytoskeleton is important for both polarized growth and chitin deposition (Novick and Botstein, 1985). Temperature-sensitive actin mutants have delocalized chitin (Novick and Botstein, 1985) and an aberrant cell wall (Gabriel and Kopecka, 1995). Other mutants affecting the actin cytoskeleton also exhibit delocalized chitin, including cdc42 (Adams et al., 1990), cdc24 (Sloat et al., 1981), ben2 (Kim et al., 1994), cdc43 (Adams et al., 1990), tpm1 (Liu and Bretscher, 1995).

**Table II. Localization of Chs3p in Wild-Type and myo2-66 Mutant**

<table>
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<tr>
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<th>Unbudded</th>
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<td>rt, 0 hr</td>
<td>17.4</td>
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<td>0.5</td>
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Wild-type MYO2 (Y1313) or myo2-66 mutant (Y1314) strains containing the CHS3::3XHA allele were grown at the permissive temperature (24°C) and then transferred at the restrictive temperature (37°C) as described in Materials and Methods. Samples were taken at different times, fixed, and prepared for immunofluorescence; the percentage of cells at different stages of the cell cycle and the localization of epitope-tagged Chs3p in those cells were determined. N, number of cells scored.
1992), myo2 (Johnston et al., 1991), and pfy1 (Haarer et al., 1990). Since Chs3p fails to localize properly in myo2-66 cells shifted to the restrictive temperature, our results suggest that in addition to Chs5p, Chs3p requires Myo2p and/or the actin cytoskeleton for proper polarization. In addition, the delocalized chitin observed in a myo2-66 mutant (Johnston et al., 1991) is most likely due to a lack of polarization of Chs3p.

Myo2p has been suggested to be involved in transporting a class of secretory vesicles from the mother cell along actin cables into the bud (Johnston et al., 1991; Lilie and Brown, 1994); myo2Δ cells accumulate vesicles at the restrictive temperature, although the identity of these vesicles is unknown (Harsay and Bretscher, 1996). It has been suggested that the cargo of these vesicles are components necessary for cell wall assembly, such as chitin synthases, chitinase, endoglucaanases, or even proteins needed for mating (Govindan et al., 1995). Because Chs3p patches accumulate in an myo2-66 mutant and the protein does not localize to the incipient bud site or the neck, it is likely that the vesicles contain Chs3p and may be the cargo of a Myo2p motor.

An alternative hypothesis for how Myo2p affects chitin synthesis is that Myo2p may help organize the bud site. myo2-66 mutants arrest as unbudded cells, similar to many bud emergence mutants, and fail to localize many components that accumulate at the incipient bud site (Johnston et al., 1991). Components at this site may be important for targeting CSHII to that site. Septins are deposited at the incipient site and Chs3p has a localization pattern similar to these proteins. Recently, an activator of CSHII, Chs4p, has been shown to directly or indirectly associate with neck filaments (Longtime et al., 1996). Thus, we speculate that interaction of Chs3p with neck filament-associated components at the incipient bud site (perhaps using Chs5p and Chs4p) is important for localization of the activity at that site.

In summary, these results demonstrate that Chs3p is polarized to bud formation and cytokinesis sites in yeast, and that Chs3p and Myo2p are required for this targeting. Further elucidation of the mechanism by which Chs3p helps target Chs3p to cortical locations will require additional studies.

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