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.

Chitin is an important structural component of many organisms, and in many fungi it helps form the cell walls and septa. In *Saccharomyces cerevisiae*, chitin is present at very low levels; however, it is essential for cell growth (Shaw et al., 1991). Chitin deposition is spatially and temporally regulated throughout the yeast cell cycle and life cycle (for review see Bulawa, 1993; Cid et al., 1995). During vegetative growth, chitin is primarily present in the neck of the bud or in bud scars; smaller amounts of chitin are also detected in the lateral wall. Chitin appears to be deposited at the bud neck in at least two stages. During bud formation, a ring of chitin is laid down in the cell wall at the incipient bud site and at the base of an emerging bud. Later, following nuclear separation and cytokinesis, a thin disk of chitin is synthesized within the chitin ring to form the primary septum between the mother cell and bud. This structure is reinforced by the addition of secondary septa composed of glucan and mannan. The cells separate with the help of a chitinase that presumably digests some of the primary septum, leaving most of the chitin in the mother cell as a bud scar. In addition to vegetative growth, chitin synthesis also occurs during mating and sporulation (for review see Bulawa, 1993; Cid et al., 1995). Before mating, cells exposed to mating pheromones form projections; chitin is deposited at the subapical portion of the projection tips. During sporulation, four spore wall layers are formed; one layer is made of chitosan, a deacetylated derivative of chitin.

Three chitin synthase activities (CSI, CSII, and CSIII), have been described so far; each one has a different function. CSI, encoded by the *CHS1* gene, acts as a repair enzyme by replenishing chitin hydrolyzed by the excessive action of chitinase during cell separation (Cabib et al., 1989, 1992). CSII is encoded by the *CHS2* gene and is responsible for chitin formation in the central disk of the primary septum (Silverman et al., 1988; Shaw et al., 1991). CSIII activity is required for the formation of the chitin ring at the base of the bud and lateral wall during vegetative growth, and chitin synthesis during mating and sporulation (Shaw et al., 1991; Valdivieso et al., 1991). At least three genes are necessary for CSIII activity: *CHS3/CAL1/CSD2/DIT101* (Roncero et al., 1988; Valdivieso et al., 1991; Bulawa, 1992; Pammer et al., 1992; Cabib, 1994),

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CHS4/CAL2/CSD4 (Roncero et al., 1988; Bulawa, 1993), and *CHS5/CAL3* (Roncero et al., 1988; Santos, B., M.H. Valdivieso, and A. Durán, 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. Valdivieso, and A. Durán, unpublished results). In addition to its role in chitin synthesis, Chs5p is also required for cell fusion during mating (Santos, B., M.H. Valdivieso, and A. Durán, 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., 1994*a*). 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., 1994*a*,*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 Bretscher, 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 Chs5p 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 transformations were by the lithium acetate method of Ito et al. (1983).

Construction of Epitope-tagged Chs5p 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′-GAATTTGAAAGGGAAGATATTCTCAATCGGAAGGAGGAAAGTGACTCCTTCGTTGCAAGGGAACAAAAGCTGG-3′ and 5′-CACACAACCATATATCAACTTGTAAGTATCACAGTAAAAATATTTTCATACTGTCTACTATAGGGCGAATTGG-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 occured 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′-GGCAACAGCAACAGTAATAAGAAGAAGAATAAGAAGAATAAGAAGAAAGGGAAAAAGAAAAGGGAACAAAAGCTGG-3′ and 5′-CGAAAAAATAAACGTGCGTCGTGGAACTCATTGAAGGCATCCATTAATCACTATAGGGCGAATTGG-3′ were used.

To construct the *3XHA::CHS5* (Y1303) and *3Xmyc::CHS5* (Y1305) strains, primers 5′-CATGTTACGTTTCCGTTTTAGAACCTGGTCGAGTAGCGAATAATGTCTAGGGAACAAAAGCTGG-3′ and 5′-CGCCAATGAGGCATCCAACTTACCTACTGTTAACAGTACATCAACTGACTGTAGGGCGAATTGG-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-

Table I. Strain List

Strain	Genotype	Source
Y604	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200*	This laboratory
Y1303	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 3XHA::CHS5*	This study
Y1304	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 CHS5::3XHA*	″
Y1305	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 3Xmyc::CHS5*	″
Y1306	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 CHS3::3XHA*	″
Y1307	*MATα ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 CHS3::3XHA*	″
Y1308	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 CHS5::3XHA chs3::HIS3*	″
Y1309	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 CHS3::3XHA chs5::ADE2*	″
Y1310	*MATα ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 CHS3::3XHA 3Xmyc::CHS5*	Spore progeny from Y1305 × Y1307
Y951	*MATα ura3-52 leu2-3 myo2-66*	P. Novick
Y1311	*MATα ura3-52 leu2-3 3XHA::CHS5*	Spore progeny from Y1303 × Y951
Y1312	*MAT***a** *ura3-52 lys2-801 trp1-901 his3-Δ200 myo2-66 3XHA::CHS5*	″
Y1313	*MATα ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 CHS3::3XHA*	Spore progeny from Y1306 × Y951
Y1314	*MAT***a** *ura3-52 trp1-901 myo2-66 CHS3::3XHA*	″
NY415	*MAT***a** *ura3-52 sec16-2*	P. Novick
NY759	*MAT***a** *ura3-52 his4-619 sec7-1*	″
NY17	*MAT***a** *ura3-52 sec6-4*	″
NY1180	*MAT***a** *ura3 lys2 his4 leu2 his4 bar1-1 end4::LEU2*	″
Y1929	*MATα ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 3XHA::CHS5*	This study
Y1930	*MATα ura3-52 lys2-801 ade2-101 trp1-901 3XHA::CHS5*	Spore progeny from NY415 × Y1929
Y1931	*MATα ura3-52 trp1-901 sec16-2 3XHA::CHS5*	″
Y1932	*MATα ura3-52 lys2-801 trp1-901 sec7-1 3XHA::CHS5*	Spore progeny from NY759 × Y1929
Y1933	*MATα ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 sec6-4 3XHA::CHS5*	Spore progeny from NY17 × Y1929
Y1934	*MAT***a** *ura3 lys2 trp1-901 end4::LEU2 3XHA::CHS5*	Spore progeny from NY1180 × Y1929

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.

enine) to early-log phase (OD_{600} = 0.5; 5 × 10^6 cells/ml), washed, and then lysed using glass beads in 500 μl of lysing buffer (1 mM DTT, 0.1% NP-40, 250 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) containing the protease inhibitors phenyl-methanesulfonyl fluoride (1 mM), leupeptin (1 μg/ml), pepstatin (1 μg/ml), SBTI (10 μg/ml), and TPCK (10 μg/ml). Lysates were centrifugated 10 min at 14,000 *g* to remove unlysed cells. Total cell lysates were mixed with an equal volume of twofold concentrated Laemmli sample buffer (Sambrook et al., 1989) and boiled 5 min before loading onto 8% polyacrylamide minigels containing SDS. After electrophoresis, proteins were blotted onto Immobilon-P (Millipore, Bedford, MA), probed with anti-HA antibodies (mAb 12CA5, purchased from BABCO, Richmond, CA), anti–c-*myc* monoclonal antibodies (9E10; provided by Dr. P. Novick, Yale University, New Haven, CT) or anti-actin antibodies (mAb C4, purchased from ICN Biochemicals, Inc., Aurora, OH), and the reactive bands were detected using anti–mouse alkaline phosphatase-conjugated antibodies (Amersham Corp., Arlington Heights, IL) and CDP-Star (Boehringer Mannheim Corp., Indianapolis, IN) detection reagent.

Tunicamycin treatment was performed on 40-ml cultures of strains Y1303 and Y1306 grown in YPDA to mid-log phase. Cells were concentrated fourfold in fresh YPDA medium, grown for an additional 30 min, and then treated with a final concentration of 10 μg/ml of tunicamycin (Sigma Chem. Co., St. Louis, MO) at 30°C, and 2.5-ml samples were taken at 0, 30, 60 and 90 min. These samples were processed for immunoblot analysis as described above. For Chs3p, alkaline phosphatase (NBT/BCIP) color detection system was used.

Sucrose Density Gradient Centrifugation

Cell lysates were prepared from strains Y1309 and Y1310 and analyzed as described by Leal-Morales et al. (1994), which optimizes the separation of chitosomes from other intracellular organelles. Briefly, cells were grown in YPDA to mid-log phase (2 × 10^7 cells/ml), and 3 g of cells (wet weight) were resuspended in 6 ml of 17% sucrose (wt/vol) in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA containing the protease inhibitors described above and 6 ml of glass beads. Cells were broken by vortexing and the crude extract was centrifuged at 1,500 *g* for 10 min. The supernatant was layered on top of 33 ml of linear sucrose gradient (10–65%, wt/vol) in 50 mM Tris-HCl, 1 mM EDTA, pH 7.5. The tubes were centrifuged in an SW28 rotor at 25,000 rpm for 20 h at 4°C (Beckman, Instrs., Fullerton, CA). 1.25-ml fractions were collected from the bottom of the tube using a peristaltic pump 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. Bussey, 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 Gehrung 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 6.8 (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% β-mercaptoethanol 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-

clonal antibodies (BABCO) 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-myc 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-Anp1p or rabbit anti-Kre2p 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 shmoos. Cells were fixed with formaldehyde and processed for indirect immunofluorescence as described above.

Analysis of temperature-sensitive *myo2*, *sec*, and *end4Δ* mutant strains was as follows: *myo2-66* mutants were grown in YPDA at room temperature with shaking 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 Lillie 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 *end4Δ* 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°) 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)[1] epitope coding sequence or three copies of the c-*myc* epitope coding sequence using a PCR approach (Schneider et al., 1995; see Materials and Methods). Three strains were prepared. The HA epitope coding segment was integrated either at the NH_2-terminal coding region (after the second codon) or at the COOH terminus (after the last codon of the open reading frame) to generate *3XHA::CHS5* and *CHS5::3XHA* strains, respectively. The c-*myc* epitope coding region was integrated after the second codon to generate *3Xmyc::CHS5.* In each case the resulting epitope-tagged protein is functional. Mutations in the *CHS5* gene cause reduced chitin levels in the cell wall and resistance to Calcofluor, a compound that interferes with chitin synthesis (Roncero et al., 1988); the *3XHA::CHS5*, *CHS5::3XHA*, and *3Xmyc::CHS5* strains are each sensitive to Calcofluor similar to wild-type strains indicating that they have normal levels of chitin in the cell wall (data not shown).

Immunoblot analysis with an anti-HA or anti-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. 1 *A*; data not shown for the myc-tagged strain). These proteins are not detected in strains lacking the epitope tag (Fig. 1 *A*). 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. Durán, 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. 1 *C*, the mobility of Chs5p::3XHA does not change upon incubation of cells in the presence of tunicamycin. Control experiments analyzing Chs3p (Fig. 1 *C*; see below), or similar experiments analyzing Axl2p, 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 Na_2CO_3 (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.

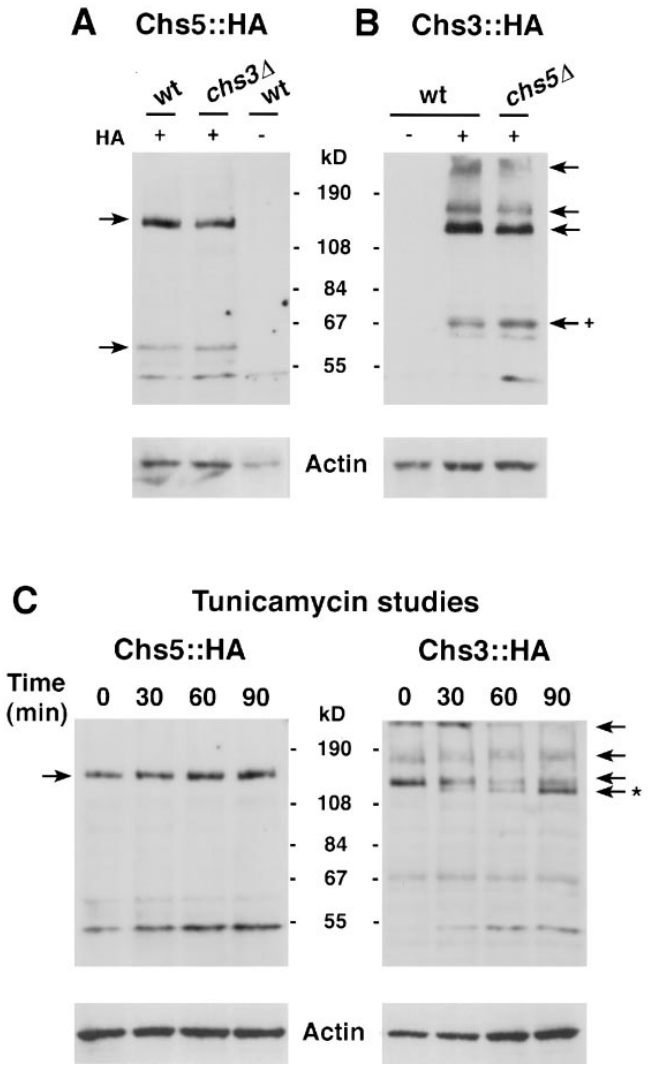

Figure 1. Immunoblot analyses. (*A*) Proteins from total yeast cell extracts of strains Y1303 or Y1308 (*chs3Δ*) containing Chs5p tagged with the triple HA epitope at the amino terminus (+) were separated in 8% polyacrylamide gels containing SDS, and an immunoblot was prepared and probed with a monoclonal anti-HA antibody. Control yeast cell extracts of a strain lacking the epitope (Y604) were included (−). Specific Chs5p::3XHA reactive bands are indicated by arrows. A ~50-kD endogenous polypeptide is recognized by the anti-HA monoclonal antibody and is present in all of the lanes. In the wild-type *CHS5* samples lacking the epitope, the 170–180-kD and 65-kD species cannot be detected even upon long exposures. An identical blot with the same samples was probed with monoclonal C4 anti-actin antibodies. (*B*) Proteins from total cell extracts of strains Y1306 and Y1309 (*chs5Δ*) containing epitope-tagged Chs3p were separated by SDS-PAGE, and immunoblots were prepared and probed as in *A*. Arrows indicate different detected Chs3p isoforms; + indicates a possible degradation product. (*C*) Yeast strains Y1303 and Y1306 containing Chs5p::3XHA or Chs3p::3XHA were treated with 10 μg/ml tunicamycin and samples were taken at different times (0, 30, 60, and 90 min). Protein extracts were prepared, immunoblotted, and probed as described above. The new Chs3p isoform is indicated with an asterisk.

tion pattern of Chs5p is identical for all three strains (*3XHA::CHS5*, *CHS5::3XHA*, and *3Xmyc::CHS5*), indicating that the position and type of epitope does not affect the localization pattern. Staining was not detected in an isogenic parental strain that lacks the HA-tagged protein (Fig. 2).

Chs5p Colocalizes with Kex2p

The punctate Chs5p staining pattern is similar to that described previously for Golgi-associated proteins (Franzusoff et al., 1991; Redding et al., 1991; Antebi and Fink, 1992; Cooper and Bussey, 1992). To determine whether Chs5p colocalizes with known Golgi proteins, its localization was compared with that of Anp1p, an early-Golgi compartment protein (Chapman and Munro, 1994; Munro, S., personal communications), α-1,2-mannosyltransferase Kre2p/Mnt1p, a medial-Golgi compartment protein (Lussier et al., 1995), and Kex2p, a late Golgi protease (Franzusoff et al., 1991; Redding et al., 1991), by double-label indirect immunofluorescence. Staining of haploid *3Xmyc::CHS5* cells with rabbit polyclonal anti-Anp1p antibodies and anti-myc monoclonal antibodies revealed that Anp1p and epitope-tagged Chs5p reside in distinct patches (~82% do not overlap; 134 spots counted for Chs5p and 150 counted for Anp1p); these patches are usually in distinct parts of the cell (data not shown). Double indirect immunofluorescence of *3Xmyc::CHS5* cells containing the *KRE2* gene on a 2-μm multicopy plasmid with polyclonal anti-Kre2p antibodies and anti-myc antibodies revealed that the two proteins are often in the same general region of the cell; however, as shown in Fig. 3 *A*, the localization of Chs5p and Kre2p also does not overlap (at least 75% of the patches are distinct; 134 spots counted for Chs5p and 164 counted for Kre2p) and within individual cells, the number of Chs5p and Kre2p patches usually differs. Interestingly, double staining of *3XHA::CHS5* cells containing the *KEX2* gene expressed from the *GAL1* promoter indicates that Chs5p and Kex2p usually colocalize (Fig. 3 *B*); 76% of Chs5p patches (203 patches counted) contain Kex2p, and 88% of Kex2p patches contain Chs5p (175 patches scored). Thus, these immunofluorescence studies suggest that Chs5p resides, at least in part, in a late Golgi/secretory compartment.

To attempt to characterize the Chs5p compartment further, the localization of Chs5p::3XHA was examined in *end4Δ* mutants which are blocked in endocytosis (Raths et al., 1993), and in temperature-sensitive *sec16-2*, *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 Chs5p 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 Chs5p 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

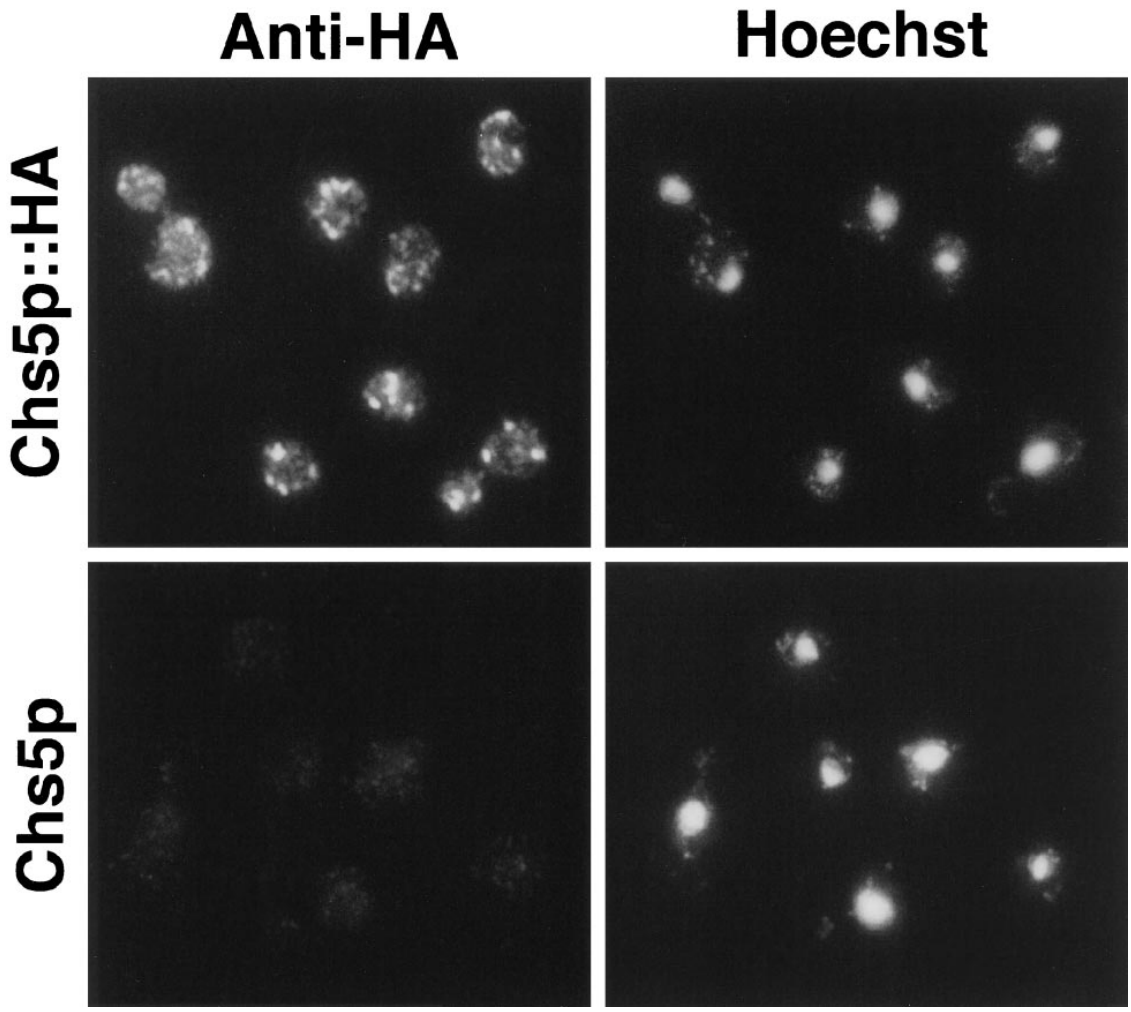

Figure 2. Localization of epitope-tagged Chs5p. Haploid yeast cells containing Chs5p tagged at the COOH terminus with triple HA (Y1303) or Chs5p without an epitope tag (Y604) were stained by indirect immunofluorescence with an anti-HA monoclonal antibody (*left panels*). Hoechst 33258 DNA staining of the same cells is shown in the right panels.

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

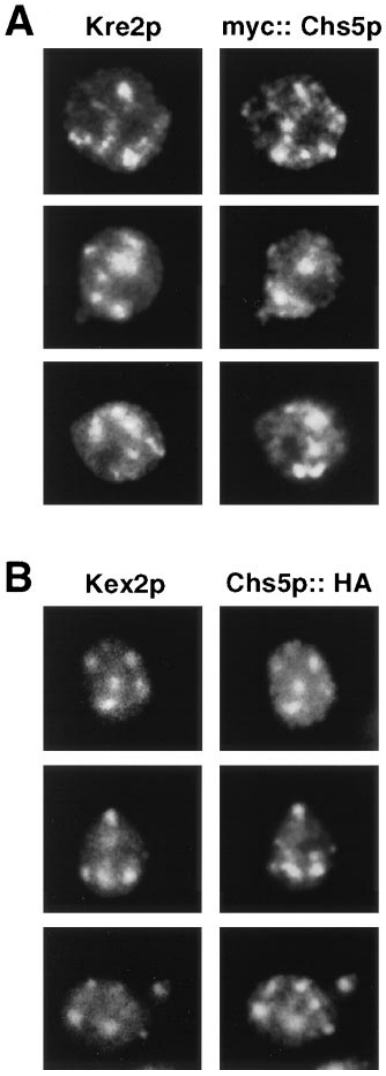

Figure 3. Double indirect immunofluorescence of epitope-tagged Chs5p and the Golgi proteins, Kre2p and Kex2p. (*A*) Haploid yeast cells containing *3Xmyc::CHS5* (Y1305) and the *KRE2* gene on a multicopy plasmid were stained with affinity-purified anti-Kre2p antibodies (Lussier et al., 1995) and anti-myc antibodies. Most of the patches are distinct (*top and bottom cell*), but occasionally some cells are found where a small subset of patches overlap (e.g., middle cell). (*B*). Haploid *3XHA::CHS5* cells (Y1303) overexpressing *KEX2* were stained with affinity-purified anti-Kex2p antibodies (Redding et al., 1991) and anti-HA antibodies. Most of the patches overlap, but a small fraction of patches appear specific to one protein (e.g., see bottom cell).

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.

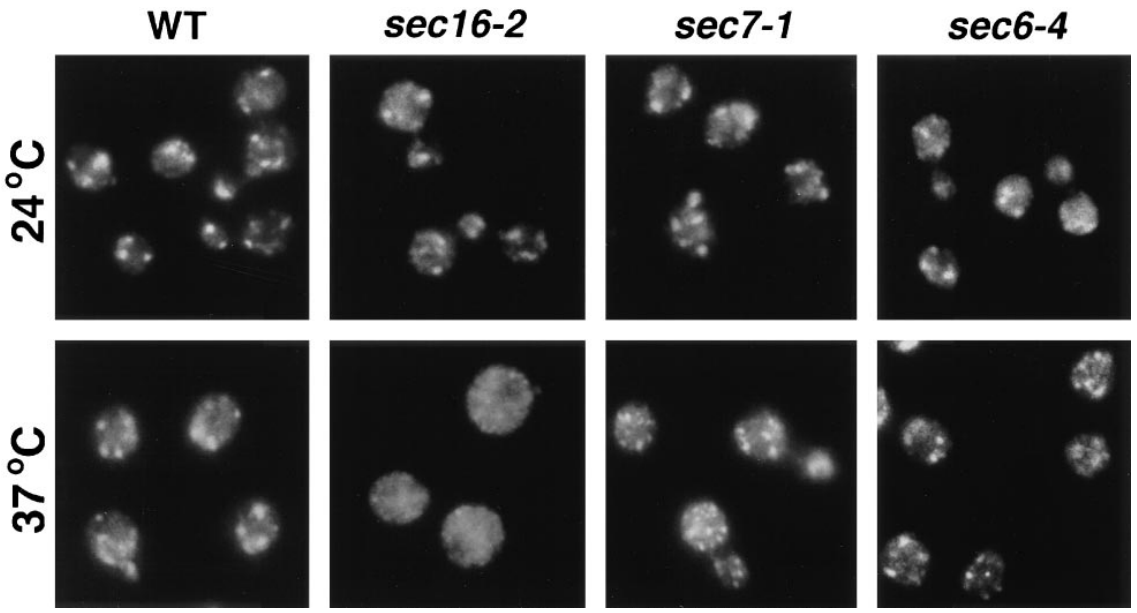

Figure 4. Localization of Chs5p in *sec* mutants. Wild-type (*WT*) and *sec* mutant yeast strains containing the *3XHA::CHS5* construct were incubated either at 24°C (the permissive temperature for the mutant strains; *top panels*) or for 2 h at 37°C (the restrictive temperature; *bottom panels*). At the restrictive temperature, Chs5p localized to patches in *sec6-4* and *sec7-1* cells, which are blocked in Golgi to secretory vesicle and in vesicle to plasma membrane transport, respectively; this pattern is indistinguishable from wild-type cells. In *sec16-2* cells, which are blocked in ER to Golgi transport, patches above background were not evident, but a slight increase in staining throughout the cytoplasm is observed. Similar results were observed for cells incubated at 0.5, 1 h, and 1.5 h at 37°C.

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 *CHS5* 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., 1994*b*). 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. 1 *B*). 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. 1 *A*) 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. 5 *C*, 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

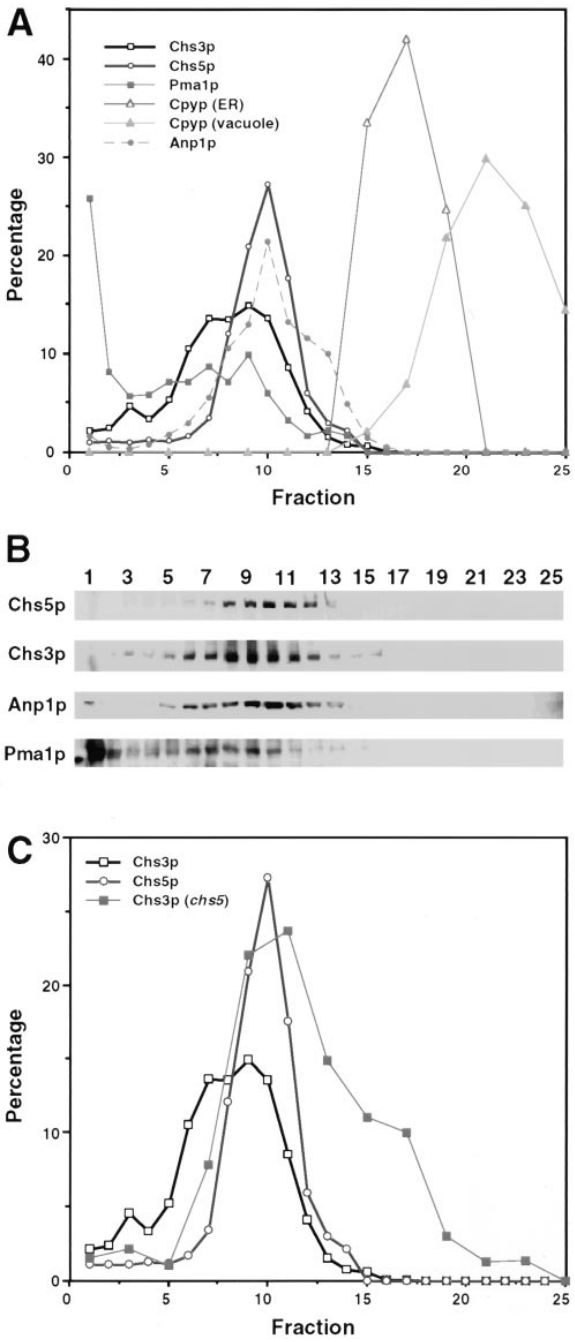

Figure 5. Sucrose density gradient centrifugation analysis. (*A* and *B*) Cells lysates were prepared from yeast strain (Y1310) containing *3Xmyc::CHS5* and *CHS3::3XHA* and fractionated in 10–65% sucrose density gradients by centrifugation for 20 h. Fractions (abscissa in *A*) were collected from the 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.

migrates in the region containing Chs5p. 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 *MAT***a** 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 shmoos 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

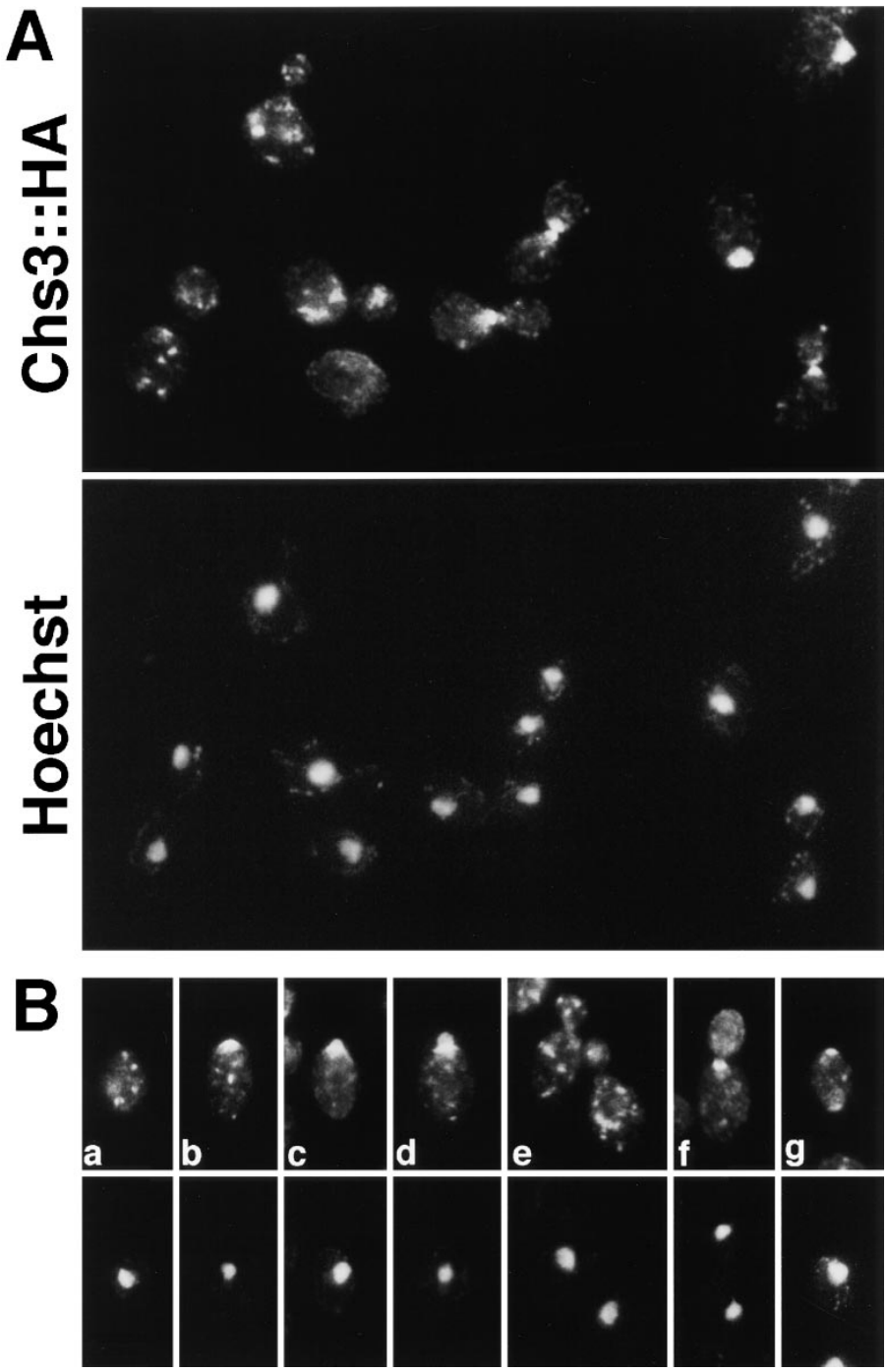

Figure 6. Localization of epitope-tagged Chs3p. (*A*) Haploid yeast cells containing Chs3p tagged at the COOH terminus with the triple HA epitope at its genomic locus (Y1306) were stained by indirect immunofluorescence with anti-HA monoclonal antibody (*upper panel*). Hoechst 33258 DNA staining of the same cells is shown in the lower panel. (*B*) Distribution of epitope-tagged Chs3p throughout the cell cycle. Diploids cells containing two copies of the *CHS3::3XHA* allele (Y1306 × Y1307) were stained as described above. Representative cells are depicted. (*a*) An unbudded cell with Chs3p in cytoplasmic patches. (*b*) An unbudded cell with Chs3p polarized at the incipient bud site (This cell contains a diffuse ring which is not evident in this plane of view.). Some cytoplasmic patches are still visible. (*c* and *d*) Two cells with an emerging or a small bud, respectively. Chs3p is localized at the neck and very faint cytoplasmic patches are evident. (*e*) Medium-sized budded cell with one nucleus containing Chs3p in cytoplasmic patches. Polarization of Chs3p is not observed at this stage of the cell cycle or in mitotic cells. (*f*) Large budded cell with two completely separated nuclei, Chs3p is localized to the neck as a ring. Sometimes the staining is brighter in the mother side of the cell (see *A*). (*g*) Unbudded cell with Chs3p staining at both poles. In cells with staining at the incipient bud site and neck, staining is usually evident as a ring when viewed from the end of the cell or different focal planes.

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

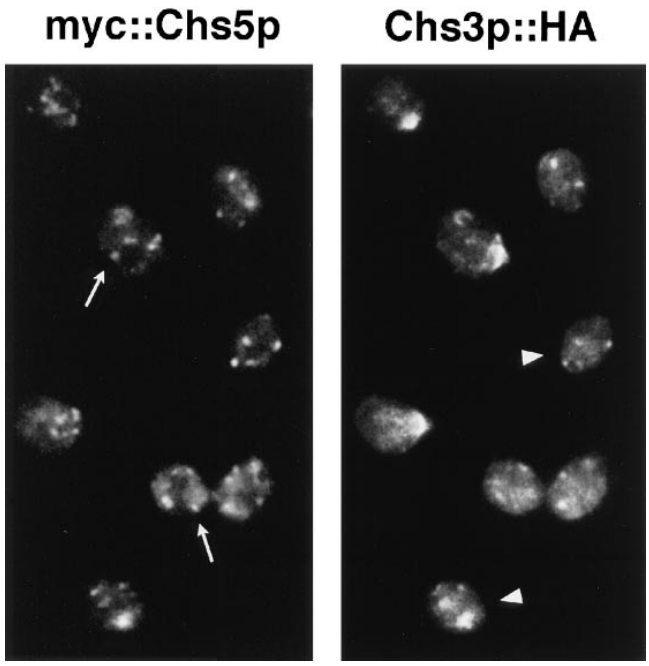

Figure 7. Double-label indirect immunofluorescence of epitope-tagged Chs3p and Chs5p. Haploid yeast cells (Y1310) containing Chs5p tagged with the myc epitope and Chs3p tagged with the triple HA epitope were stained with anti-myc antibodies and polyclonal anti-HA antibodies. The Chs3p cytoplasmic patches colocalize with Chs5p patches (for example see cells with arrowheads), but the polarized Chs3p does not. Some Chs5p patches do not appear to colocalize with Chs3p (e.g., *small arrows*).

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-Clb 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-Clb 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., 1994*b*), 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-

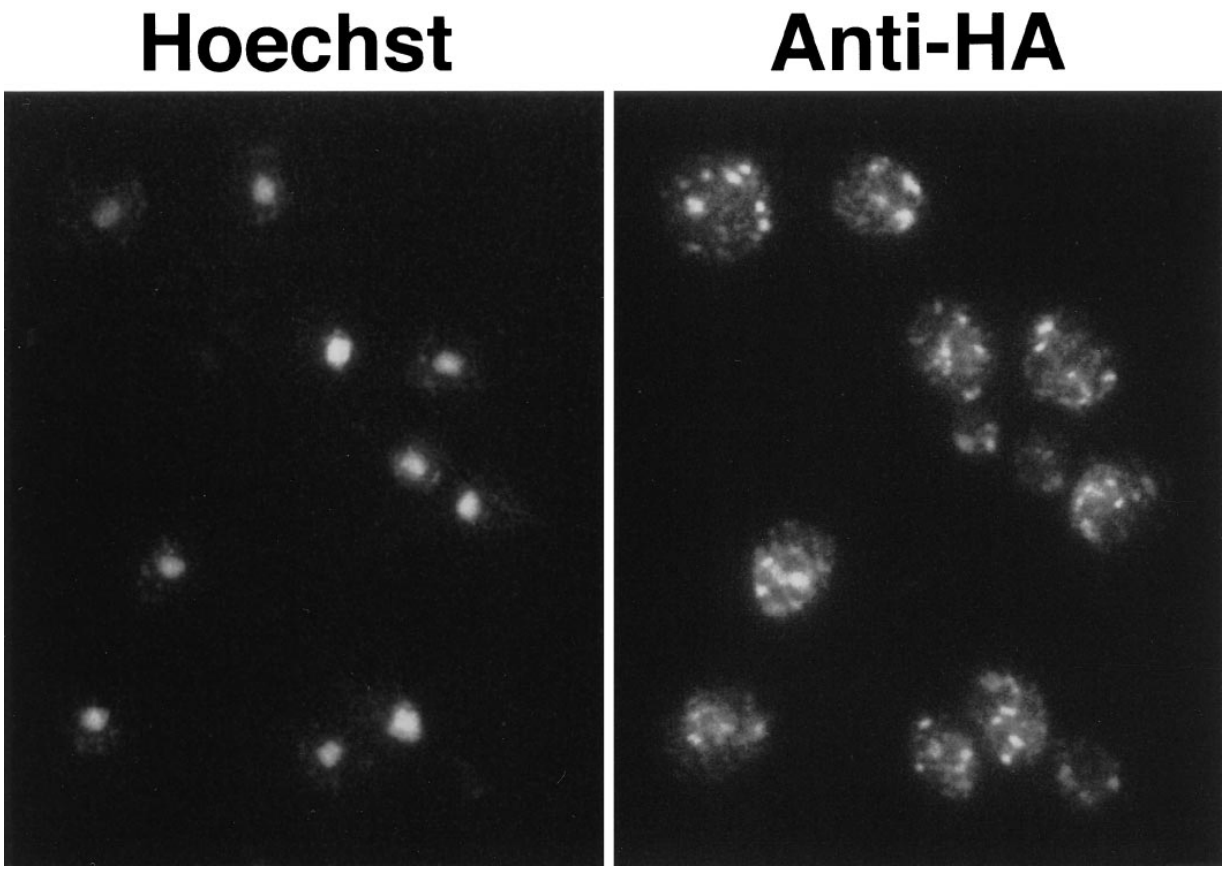

Figure 8. Immunolocalization of Chs3p in a *chs5Δ* strain. Haploid *chs5Δ* cells (Y1309) containing Chs3p tagged with triple HA were stained by indirect immunofluorescence with anti-HA monoclonal antibodies. Hoechst 33258 DNA staining of the same cells is also shown.

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-1,3 β-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).

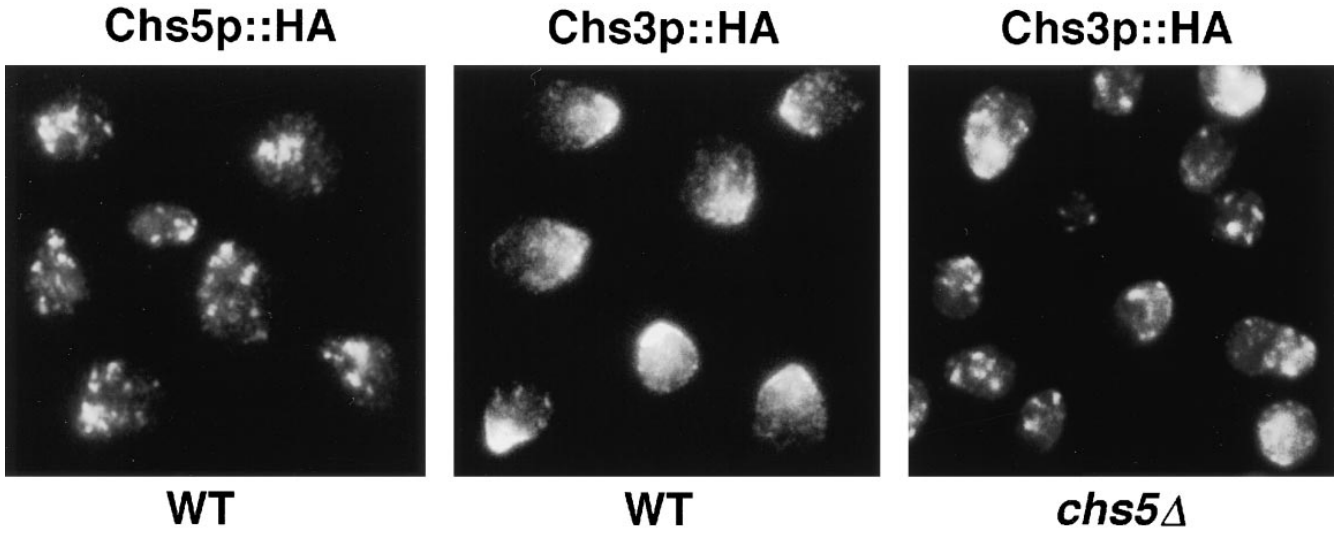

Figure 9. Localization of epitope-tagged Chs3p and Chs5p in α-factor treated cells. *MAT**a*** strains Y1303 (*3XHA::CHS5*), Y1306 (*CHS3::3XHA*), and Y1309 (*CHS3::3XHA chs5Δ*) were incubated with α-factor mating pheromone. After 90 min, the cells were fixed and stained with anti-HA monoclonal antibodies. (*Left two panels*) *WT*, wild-type cells. (*Right panel*) *chs5Δ* cells. The epitope-tagged protein in the cells is indicated.

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

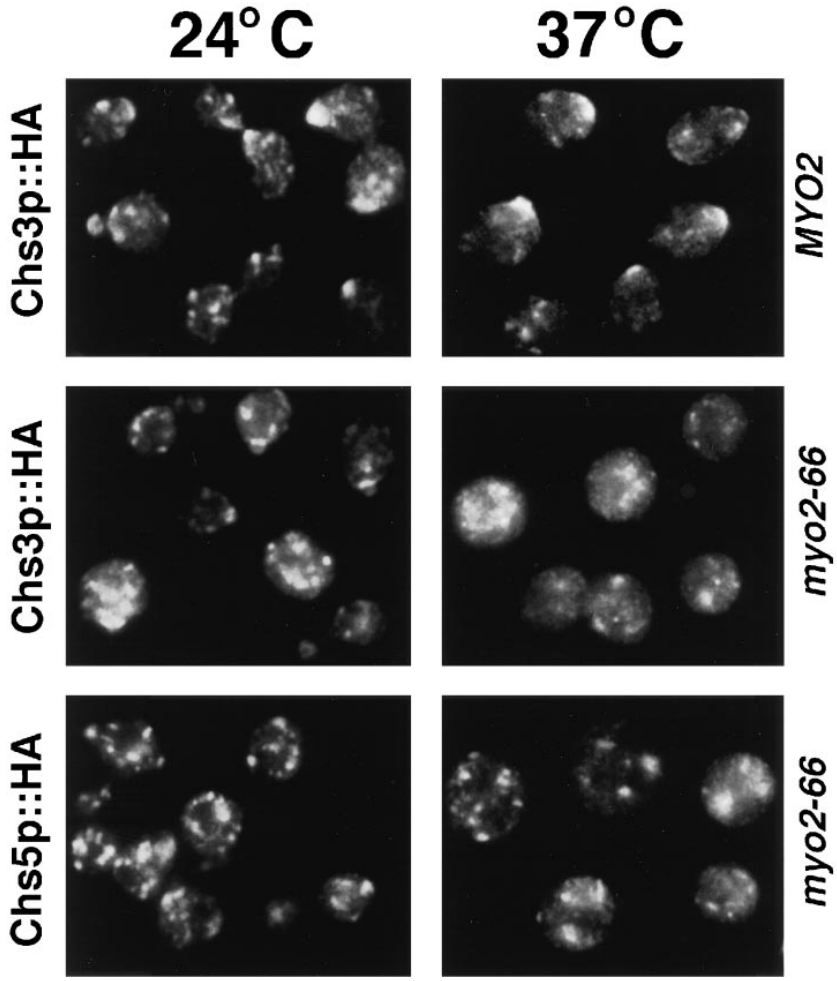

Figure 10. Immunolocalization of epitope-tagged Chs3p and Chs5p in *myo2-66* mutants. Strains Y1313 (*MYO2*) and Y1314 (*myo2-66*) containing the *CHS3::3XHA* allele and strain Y1312 (*myo2-66*) containing the *3XHA::CHS5* allele were incubated at the permissive temperature (24°C) and then transferred to the restrictive temperature (37°C) for 2 h. Cells were fixed and stained with anti-HA monoclonal antibody by indirect immunofluorescence as described in Materials and Methods.

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

	Unbudded		Small Budded		Large Budded 1 Nucleus		Large Budded 2 Nuclei		N
MYO2									
rt, 0 hr	17.4	45.3	12.5	1.2	0.0	17.0	2.8	3.6	247
37°, 20′	6.2	52.8	0.5	6.2	0.0	28.2	0.9	5.2	210
37°, 2 hr	11.7	66.1	16.7	0.4	0.0	2.5	2.1	0.4	239
rt, 2 hr	13.8	48.1	13.3	0.5	0.0	20.0	1.4	2.8	210
myo2-66									
rt, 0 hr	12.2	57.3	5.4	4.9	0.0	12.2	2.4	5.4	204
37°, 20′	0.5	72.2	0.0	2.8	0.0	12.5	0.4	11.5	216
37°, 2 hr	0.0	93.8	0.0	0.8	0.0	1.5	0.0	3.7	261
rt, 2 hr	10.9	46.3	13.4	2.4	0.0	20.3	2.4	4.6	246

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.

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), *bem2* (Kim et al., 1994), *cdc43* (Adams et al., 1990), *tpm1* (Liu and Bretscher,

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; Lillie and Brown, 1994); $myo2^{ts}$ 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, endoglucanases, 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 CSIII to that site. Septins are deposited at the incipient site and Chs3p has a localization pattern similar to these proteins. Recently, an activator of CSIII, 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 Chs5p and Myo2p are required for this targeting. Further elucidation of the mechanism by which Chs5p helps target Chs3p to cortical locations will require additional studies.

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