Chs5/6 Complex: A Multiprotein Complex That Interacts with and Conveys Chitin Synthase III from the *Trans*-Golgi Network to the Cell Surface

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In *Saccharomyces cerevisiae*, the polysaccharide chitin is deposited at the mother bud junction by an integral membrane enzyme, chitin synthase 3 (Chs3p). The traffic of Chs3p to the cell surface from the *trans*-Golgi network (TGN) depends on two proteins, Chs5p and Chs6p, which sort selected cargo proteins into secretory vesicles. We have found that Chs5p forms a large higher-order complex of around 1 MDa with Chs6p and three Chs6 paralogs: Bch1p, Bud7p, and Bch2p. The Chs5/6 complex transiently interacts with its cargo, Chs3p, and the presence of Chs3p in the complex is dependent on every subunit. Chs5p and Chs6p have unique and crucial roles in Chs3p transport because either a *chs5Δ* or *chs6Δ* mutant drastically reduces the level of Chs3p bound to the remaining subunits of the complex. Bch1p and Bud7p appear to have a redundant function in Chs3p transport because deletion of both is necessary to displace Chs3p from the complex. The role of Bch2p in Chs3p binding is the least important. Chs5p is essential for structural integrity of the Chs5/6 complex and may act as a scaffold through which the other subunits assemble. Our results suggest a model of protein sorting at the TGN that involves a peripheral, possibly coat, complex that includes multiple related copies of a specificity determining subunit.

INTRODUCTION

Protein sorting in the secretory pathway involves coat proteins and possibly segregated lipid phases of the membrane bilayer. Although a great deal can be said about the sorting of proteins that traffic from the ER or are recycled from the *cis*-Golgi or intermediate compartment, much less is understood about sorting at the *trans*-Golgi network (TGN) en route to the cell surface (Lee *et al.*, 2004). It is clear that lipids, specifically PI4P, play a role in this process, as does the GTP-binding protein Arf and its GEF and GAP partners (Walch-Solimena and Novick, 1999; Hama *et al.*, 1999; Audhya *et al.*, 2000; Poon *et al.*, 2001; Wong *et al.*, 2005). However, no coat protein complex has yet been identified in a sorting role at the TGN, at least for those proteins that traffic directly to the cell surface. We have previously found that some secretory proteins employ clathrin for traffic to an endosome en route to the cell surface, but others including the yeast plasma membrane ATPase appear to flow directly from the TGN (Harsay and Schekman, 2002). For such proteins the sorting mechanism is unknown.

We have focused on the traffic of a cell surface enzyme responsible for the deposition of chitin at the mother-bud junction in dividing yeast cells. One particular enzyme, chitin synthase 3 (Chs3p), builds a chitin ring at the G1 phase of the yeast cell cycle. Unlike most plasma membrane proteins whose transport to the cell surface is tightly controlled

at the level of gene expression and protein synthesis, the control of Chs3p transport to the plasma membrane at the mother-bud junction is achieved by regulation of protein localization (Chuang and Schekman, 1996). Several proteins have been identified as essential for regulation of Chs3p transport to the cell surface. Chs7p functions as a chaperone that controls the export of Chs3p from the endoplasmic reticulum (Trilla *et al.*, 1999). Chs4p is required to activate and link Chs3p to the septins at the mother-bud neck (Bulawa, 1993; DeMarini *et al.*, 1997). Chs5p and Chs6p are essential for the transport of Chs3p from the Golgi apparatus to the plasma membrane (Santos and Snyder, 1997; Santos *et al.*, 1997; Ziman *et al.*, 1998).

In steady state, Chs3p localizes to both the plasma membrane and internal punctate structures, colocalized with TGN and early endosome markers (Chuang and Schekman, 1996; Santos and Snyder, 1997; Ziman *et al.*, 1996; Valdivia *et al.*, 2002). These internal organelles, termed chitosomes, constitute a reservoir of Chs3p for mobilization to the plasma membrane in a stress- and possibly cell cycle-regulated manner (Chuang and Schekman, 1996; Ziman *et al.*, 1996). Heat shock redistributes Chs3p to the plasma membrane in a Rho1p/Pkc1p-dependent manner (Valdivia and Schekman, 2003). At the G1 phase of the cell cycle, Chs3p is found at the incipient bud site, whereas in the other cell cycle stages Chs3p is found in chitosomes (Chuang and Schekman, 1996). Thus, the itinerary of Chs3p presents an opportunity to investigate the mechanism and regulation of traffic of a plasma membrane protein with distinctive and known genetic requirements.

In *chs5Δ* or *chs6Δ* cells, the localization of Chs3p to the incipient bud site at the onset of bud emergence and the redistribution to lateral cell wall upon heat stress are impaired (Ziman *et al.*, 1998; Santos and Snyder, 1997; Santos *et al.*, 1997; Valdivia and Schekman, 2003). When exocytosis

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Abbreviations used: TGN, *trans*-Golgi network; CHS, chitin synthase.

Table 1. Strains used in this study

Name	Genotype	Reference
DDY1810	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451</i>	Drubin/Barnes lab
YSS27	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX</i>	This study
YSS28	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS6-Stag-TEV-ZZ::KanMX</i>	This study
YSS77	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX chs3::LEU2</i>	This study
YSS78	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX chs6::LEU2</i>	This study
YSS96	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX bch1::LEU2</i>	This study
YSS112	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX bud7::TRP1</i>	This study
YSS95	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX bch2::LEU2</i>	This study
YSS109	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX chs6::URA3 bch1::LEU2</i>	This study
YSS108	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX chs6::LEU2 bud7::TRP1</i>	This study
YSS126	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX chs6::LEU2 bch2::NatMX</i>	This study
YSS104	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX bch1::LEU2 bud7::TRP1</i>	This study
YSS127	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX bch1::LEU2 bch2::NatMX</i>	This study
YSS110	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX bch2::LEU2 bud7::TRP1</i>	This study
YSS111	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX chs6::URA3 bch1::LEU2 bud7::TRP1</i>	This study
YSS125	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX chs6::URA3 bch1::LEU2 bch2::NatMX</i>	This study
YSS114	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX chs6::LEU2 bch2::NatMX bud7::TRP1</i>	This study
YSS128	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX bch1::LEU2 bud7::TRP1 bch2::NatMX</i>	This study
YSS115	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS5-Stag-TEV-ZZ::KanMX chs6::URA3 bch1::LEU2 bud7::TRP1 bch2::NatMX</i>	This study
YSS99	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS6-Stag-TEV-ZZ::KanMX chs5::LEU2</i>	This study
YSS116	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS6-Stag-TEV-ZZ::KanMX BUD7-3xHA::TRP1</i>	This study
YSS117	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS6-Stag-TEV-ZZ::KanMX BCH2-3xHA::TRP1</i>	This study
YSS133	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS6-Stag-TEV-ZZ::KanMX chs5::LEU2 BUD7-3xHA::TRP1</i>	This study
YSS134	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS6-Stag-TEV-ZZ::KanMX bch1::LEU2 BUD7-3xHA::TRP1</i>	This study
YSS140	<i>MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 pre1-451 CHS6-Stag-TEV-ZZ::KanMX chs5::LEU2 BCH2-3xHA::TRP1</i>	This study
JTY3605	<i>MATa leu2-1 ura3-52 trp1-63 his3-200 lys2-801a bar1::KanMX</i>	Thorner Lab
YRB48.5	<i>MATa leu2-1 ura3-52 trp1-63 his3-200 lys2-801a bar1::KanMX chs6::HIS3</i>	This laboratory
YRB56.1	<i>MATa leu2-1 ura3-52 trp1-63 his3-200 lys2-801a bar1::KanMX bch1::HIS3 bch2::LEU2</i>	This laboratory
YRB86.6	<i>MATa leu2-1 ura3-52 trp1-63 his3-200 lys2-801a bar1::HIS3 bch1::LEU2 bud7::KanMX</i>	This laboratory

is blocked in a *sec6-4* temperature-sensitive mutant, newly synthesized Chs3p accumulates in post-Golgi transport vesicles; however, in *chs5Δ* or *chs6Δ* the incorporation of newly synthesized Chs3p into the transport vesicles is blocked, suggesting a role for Chs5p and Chs6p in protein sorting at the TGN (Valdivia *et al.*, 2002). In vivo evidence revealed that both Chs5p and Chs6p are required for chitin synthase III (CSIII) activity; nevertheless, in vitro experiments demonstrated that a membrane extract from *chs5Δ* lacked CSIII activity, whereas the extract from *chs6Δ* retained CSIII activity (Bulawa, 1992). In addition to its role in Chs3p traffic, Chs5p is also essential for the cell surface transport of at least two other cell wall component proteins: Fus1p and Crh2p. During mating, Chs5p is required for polarized transport of Fus1p to the shmoo tip to promote cell fusion (Santos and Snyder, 2003). Crh2p, a GPI-anchored cell wall protein, is mis-localized from the polarized growth site in *chs5Δ* (Rodriguez-Peña *et al.*, 2002). Here, we show that Chs5p forms a higher-order macrocomplex with Chs6p and its three paralogs: Bch1p,

Bud7p, and Bch2p. This Chs5/6 complex transiently binds to Chs3p and is important for the anterograde transport of Chs3p from the TGN to the plasma membrane. We propose a model invoking multiple determinants of protein sorting within a multisubunit coat-like complex.

MATERIALS AND METHODS

Strains, Growth Conditions, and Reagents

Yeast strains used in this study are listed in Table 1. Yeast cells were grown in rich medium (Sherman, 1991) or in complete synthetic medium (CSM) dropout mixes (Q-biogene, Carlsbad, CA). Geneticin (Invitrogen, Carlsbad, CA) and clonNAT (Hans-Knöll Institute für Naturstoff-Forschung, Jena, Germany) were used at 200 and 100 μ g/ml, respectively. Sensitivity to Calcofluor was assessed by growth on YPD plates supplemented with 20 μ g/ml Fluorescent Brightener 28 (Sigma, St. Louis, MO) in the dark at 24°C for 3 d. PCR was carried out using either Taq polymerase (Promega, Madison, WI) or Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN). Yeast transformation was performed using standard procedures (Ausubel *et al.*, 1987–1995).

TAP-fusion strains, YSS27 and YSS28, were constructed by in-frame integration into DDY1810 of PCR products amplified from pKW804 (a gift from K. Weis,

University of California, Berkeley, CA) encoding Stag-TEV-ZZ::KanMX at the 3' end of genes (Cheeseman *et al.*, 2001). *BUD7-3xHA* and *BCH2-3xHA* in YSS116, YSS117, YSS133, YSS134, and YSS140 were created using the same strategy as previously described (Longtine *et al.*, 1998). All fusion constructs were confirmed by immunoblotting for expression of fusion proteins. Deletion mutants were constructed by integration of disruption cassettes into YSS27 or YSS28. All deletion constructs were confirmed by PCR. Disruption cassettes for complete deletion of *CHS3*, *CHS5*, or members of the *CHS6* family with *LEU2*, *TRP1*, or *NatMX* were PCR amplified from pFA6a-LEU (kindly provided by P. Stromhaug, University of Missouri, Columbia, MO), pFA6a-TRP1 (Longtine *et al.*, 1998), and pDD1080 (a gift from D. Drubin, University of California, Berkeley), respectively. A *chs6::URA3* disruption cassette was created by PCR amplification from total DNA isolated from YRB143 (R. Barfield, this laboratory).

Affinity-purified polyclonal antibodies against Chs3p, Clc1p (this laboratory) and Apl2p (G. Payne, University of California, Los Angeles); rabbit antisera against Tlg1p, Gas1p, and Bgl2p (H. Pelham, MRC Laboratory of Molecular Biology, Cambridge) and Arf1p, Chs5p, and Chs6p (this laboratory); and a monoclonal antibody (mAb) against Chc1p (Lemmon *et al.*, 1988) were used in this study. Anti-HA mAb HA.11 was purchased from Covance (Denver, PA). HRP-conjugated secondary antibodies were from Amersham Biosciences (Piscataway, NJ).

Purification of the Chs5/6 Complex

Tandem affinity purification of the Chs5/6 complex was performed with IgG Sepharose affinity purification followed by S-protein agarose affinity purification (Cheeseman *et al.*, 2001). TAP-fusion strains were grown overnight in YPD to OD₆₀₀ = 0.7–1.0, and cells were harvested by centrifugation (4500 × g for 20 min) and converted to spheroplasts. Methods for converting cells to spheroplasts were previously described (Chuang and Schekman, 1996; Ziman *et al.*, 1998). Cells were resuspended at ~40 OD₆₀₀ cell equivalents per ml in pretreatment buffer (0.1 M Tris or 0.1 M HEPES, pH 9.4, and 40 mM β-mercaptoethanol) at room temperature for 10 min and converted to spheroplasts using 60 U lyticase per OD₆₀₀ cell equivalent in spheroplast buffer (20 mM HEPES, pH 7.4, 0.7 M sorbitol, 0.75 × YPD, and 4 mM β-mercaptoethanol) at 30°C for 25 min. Spheroplasts were centrifuged at low speed (500 × g for 10 min), resuspended at ~100 OD₆₀₀ cell equivalents per ml in lysis buffer (50 mM HEPES, pH 7.4, 0.4 M NaCl, 1% TX-100, and 1 mM PMSF), and lysed by homogenizing 15 times with a Dounce Tissue Homogenizer (Bellco Biotechnology, Vineland, NJ) followed by incubation on ice for 30 min. The following steps were carried out at 4°C. The cell lysate was centrifuged in a SS-34 rotor at 2000 rpm for 5 min and 10,500 rpm for 20 min. Supernatant fractions were precleared by incubating with water- and lysis buffer-washed CL-6B Sepharose (Sigma) for 30 min and then incubated with prewashed IgG Sepharose (Amersham Biosciences; 500 μl slurry per 1000 OD₆₀₀ cell equivalents) for 3 h. IgG Sepharose-bound protein fractions were washed with lysis buffer and incubated overnight in a small volume of lysis buffer plus 1 U AcTEV protease (Invitrogen) per 100 OD₆₀₀ cell equivalents. The TEV eluate was collected and incubated with prewashed S-protein agarose (Novagen, Madison, WI; 60 μl slurry per 1000 OD₆₀₀ cell equivalents) for 3 h. S-protein agarose was washed with lysis buffer and resuspended in 20 μl 2× SDS protein sample buffer to elute the purified complex. Proteins were resolved by SDS-PAGE, visualized with Sypro Red Protein Gel Stain (Invitrogen) and then scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For protein detection by immunoblotting, blots were developed using ECL Plus kits (Amersham Biosciences). Quantification was performed with Image Quant Software (Molecular Dynamics).

To capture the transient interaction between the Chs5/6 complex and Chs3p, we used in vivo cross-linking with dithiobis(succinimidylpropionate) (DSP; Pierce, Rockford, IL). After cells were converted to spheroplasts and centrifuged at 500 × g, the pellet was washed once and then resuspended at ~50 OD₆₀₀ cell equivalents per ml in cross-link buffer (20 mM HEPES, pH 7.4, 0.7 M sorbitol, and 100 mM KOAC). DSP from a freshly made 100 mM stock was added to a final concentration of 5 mM, incubated at room temperature for 30 min, and stopped with 100 mM Tris, pH 7.5, for 15 min. The spheroplasts were then lysed as described above. For analysis of the purified complex by blue native PAGE, we applied the TEV eluate on a 4–8% gradient gels as previously described (Schagger and von Jagow, 1991; Schagger *et al.*, 1994) and visualized Chs5p by immunoblotting.

Subcellular Fractionations and Sucrose Gradient Sedimentations

The analysis of subcellular fractionation of Chs3p on step sucrose gradient was adapted from Valdivia and Schekman (2003). Briefly, 10 OD₆₀₀ of cells (OD₆₀₀ ~ 0.5–0.8) were harvested, washed with water, resuspended in 0.3 ml of lysis buffer (5% sucrose in 20 mM triethanolamine, pH 7.2, 1 mM EDTA, and 1 mM PMSF), and lysed by agitation with glass beads. Unlysed cells were removed by centrifugation at 500 × g for 4 min. A 0.2-ml aliquot of the total cell lysate was overlaid on a step sucrose/EDTA gradient (0.4 ml 55%/1 ml 45%/0.7 ml 35% sucrose [wt/wt] in 20 mM triethanolamine, pH 7.2, and 5 mM EDTA) and centrifuged at 55,000 rpm in a TLS55 rotor at 4°C for 2.5 h.

Fractions (0.2 ml) were manually collected from the top, solubilized in SDS protein sample buffer, and analyzed by SDS-PAGE and immunoblotting.

Gel Filtration

For determination of the size of the Chs5/6 complex by gel filtration, we fractionated 0.2 ml of TEV eluate on a 24-ml Superose 6 column (Amersham Biosciences) at a constant flow rate of 0.2 ml/min at 4°C. Fractions of 1 ml were collected, affinity-purified with 40 μl slurry of prewashed S-protein agarose as described above, and analyzed by SDS-PAGE and Sypro Red Protein Gel Stain. Standards used in gel filtration include Blue Dextran, 2000 kDa; thyroglobulin, 669 kDa; catalase, 232 kDa; BSA, 67 kDa; and cytochrome C, 12.4 kDa.

RESULTS

Chs5p Forms Stable Interactions with Chs6p and Its Three Paralogs: Bch1p, Bud7p, and Bch2p

In preliminary experiments, we found that Chs5p and Chs6p were extracted from a membrane fraction by a high salt wash and that in detergent-solubilized samples the two proteins cofractionated with Chs3p during velocity sedimentation on a sucrose gradient. In addition, Chs5-RFP overlapped with Chs6-GFP and Chs3-GFP in living cells. Finally, by coimmunoprecipitation, Chs5p and Chs6p were found in a complex. To further identify other interaction partners of Chs5p and Chs6p, we pursued these preliminary results with greater precision using a modified version of the tandem affinity purification (TAP) tag (Rigaut *et al.*, 1999) integrated at the C-terminal coding sequence of the *CHS5* locus (*CHS5-TAP*). This construct was functional as judged by Calcofluor sensitivity (see Figure 6A). Figure 1A shows that four other protein species were specifically copurified with Chs5-TAP (N. Krogan, personal communication, University of California, San Francisco, CA). Using deletion mutations, we found that these proteins corresponded to Chs6p, Bch1p, Bud7p, and Bch2p, all of which are paralogs in *Saccharomyces cerevisiae* (Figure 1B). The four Chs6-family members fall into two subfamilies (Table 2): Chs6p and Bch2p are more closely related to each other and represent one subfamily; Bch1p and Bud7p, which are more distant from Chs6p and Bch2p, belong to the other subfamily. Bud7p functions in the generation of the bipolar budding pattern in diploid cells (Zahner *et al.*, 1996), whereas the functions of Bch1p and Bch2p are unknown. We calculated a molar ratio of the copurified subunits to be ~5:4:1:1:1 for Chs5p, Bch1p, Bud7p, Chs6p, and Bch2p (Table 3) and named this the Chs5/6 complex. The different stoichiometries of the copurified Chs6-family proteins seem to reflect their different expression levels in steady state with Bch1p being the most abundant and Chs6p, Bud7p, and Bch2p of similar low abundance (unpublished data). The interactions between subunits in this Chs5/6 complex appear to be stable because no cross-linking reagent was required to recruit the copurified subunits. Furthermore, this complex remained intact in high salt and high detergent (0.4 M NaCl and 1% TX-100) during the purification.

The Chs5/6 Complex Is a Higher-Order Multisubunit Complex

Because Chs5p functions in the transport of at least several cell surface proteins, we considered the possibility that the components copurified with Chs5-TAP represented more than one type of complex. One hypothesis is that Chs5p forms distinct heterodimers with each member of the Chs6p family to transport specialized cargos. To identify a protein complex directly involved in Chs3p transport, we used a *CHS6-TAP* strain instead of *CHS5-TAP* because in *chs6Δ* there is no defect in the transport of Fus1p to the cell surface

Figure 1. Purification of a Chs5/6 complex. (A) Purification of the Chs5/6 complex using Chs5-TAP reveals four additional copurified polypeptides. Different fractions of TAP purification from a *CHS5-TAP* strain and an untagged control strain were analyzed on SDS-PAGE and stained with Sypro Red. (B) Analysis of deletion mutants identified those four polypeptides as members of the Chs6-family proteins: Chs6p, Bch2p, Bch1p, and Bud7p.

(Santos and Snyder, 2003) or in the bipolar budding pattern of yeast diploid cells (A. McKenzie III, K. Nakashima, and J. Pringle, personal communication, University of North Carolina, Chapel Hill, NC; Trautwein *et al.*, 2006). A functional

Table 2. Members of the Chs6 protein family

Protein	Length (aa) ^b	Function	% identity to ^a			
			Chs6p	Bch2p	Bud7p	Bch1p
Chs6p	746	Chs3p transport	100	43	25	23
Bch2p	765	Unknown	43	100	28	24
Bud7p	746	Bud-site selection	25	28	100	53
Bch1p	724	Unknown	23	24	53	100

^a BLASTP search against *Saccharomyces cerevisiae* genome database (NCBI).

^b SGD, *Saccharomyces* Genome Database.

Table 3. Subunit stoichiometry of the Chs5/6 complex

Protein	Predicted molecular weight (kDa) ^a	Molar ratio ^b
Chs5-Stag	75.3	1.00
Bch1p	82	0.83
Bud7p	84.8	0.19
Chs6p	86	0.20
Bch2p	88	0.17

^a SGD, *Saccharomyces* Genome Database.

^b To calculate the molar ratio of each protein, the intensity of Sypro Red-stained protein is quantified, divided by the predicted molecular weight, and normalized against the value of Chs5-Stag.

CHS6-TAP construct was integrated at the *CHS6* locus. Chs6-TAP specifically copurified Chs5p, Bch1p, Bud7p, and Bch2p (Figure 2A). The presence of Bch1p in the copurified complex was detected by Sypro Red total protein staining. We confirmed the copurification of Bud7p and Bch2p by immunoblotting because Chs6-TAP migrated at about the same position as Bud7p and the protein expression levels of Bud7p and Bch2p were lower than the expression level of Bch1p. We noted that the stoichiometry of the subunits copurified by Chs6-TAP appeared similar to that of the subunits copurified by Chs5-TAP. The amounts of copurified Chs5p and Bch1p were similar, whereas the levels of copurified Bud7p and Bch2p were much lower. The results of Chs5-TAP and Chs6-TAP purifications are inconsistent with the notion that Chs5p forms individual heterodimeric complexes with each of the Chs6 family members. Instead, the presence of copurified Bch1p, Bud7p, and Bch2p by Chs6-TAP suggests the possibilities of a five-subunit multiprotein complex or subcomplexes of Chs5p, Chs6p, and at least one of the Chs6-family members.

To further characterize the nature of the Chs5/6 complex, we next determined the native molecular weight using gel filtration chromatography. After the Chs5-TAP complex had been purified with IgG Sepharose, the TEV eluate was applied to a Superose 6 gel filtration chromatography column, and individual chromatographic fractions were affinity-purified with S-protein agarose. In a wild-type background, total protein analysis of the purified gel filtration fractions revealed that all subunits of the Chs5/6 complex cofractionated in a broad profile with a single peak of around 1 MDa (Figure 2B). In the *bch1Δ bud7Δ* double mutant, Chs5, Chs6p, and Bch2p comigrated in a similarly large macrocomplex (Figure 2B). Thus, deletion of two of the Chs6-family members did not disrupt or reduce the size of the complex. The observed native size of the Chs5/6 complex is much larger than the combined molecular weight of Chs5p and Chs6-family proteins (~420 kDa), indicating that the nature of this complex is of higher order; all five subunits appear to oligomerize in a large multisubunit structure. In addition, in the course of developing methods to monitor the Chs5/6 complex, we observed several large forms of Chs5p that migrated slowly on a native gel containing Coomassie blue (Figure 2C). Surprisingly, these apparently large species persisted even when all four *CHS6*-related genes were deleted (Figure 2C). The similarity of these large forms in wild-type and quadruple mutant backgrounds suggested that these large forms contained only Chs5p and that the Chs5/6 complex had disintegrated during electrophoresis. We also did not detect Chs6p comigrating in these forms (unpublished data). Thus, Chs5p may self-assemble and form a scaffold for the four Chs6-family subunits.

Figure 2. Subunits of the Chs5/6 complex associate together in a higher-order multiprotein complex. (A) TAP purification using Chs6-TAP reveals the presence of Chs5p, Bch1p, Bud7p, and Bch2p in the purified complex. The presence of Bch1p was detected by Sypro Red staining of SDS-PAGE gel. Because of low protein expression levels, the presence of Bud7p and Bch2p was detected using epitope-tagged (HA) versions of Bud7p and Bch2p, whose chromosomal loci were replaced with *BUD7-3xHA* and *BCH2-3xHA*, respectively. Nonspecific protein bands recognized by anti-HA antibody are denoted with asterisks. (B) Size determination of the Chs5/6 complex by gel filtration chromatography. TEV eluates from *CHS5-TAP* and *CHS5-TAP bch1Δ bud7Δ* were fractionated on Superose 6 gel filtration column and subsequently affinity purified with S-protein agarose. Purified fractions were analyzed by SDS-PAGE and Sypro Red staining to monitor the comigration of all subunits of the Chs5/6 complex. The approximate native molecular weight was determined from a plot of elution volumes of standards versus natural log of their molecular weights. Vo is void volume as determined by Blue Dextran, 2000 kDa; 1, thyroglobulin, 669 kDa; 2, catalase, 232 kDa; 3, BSA, 67 kDa; and 4, cytochrome C, 12.4 kDa. (C) Self-assembly of Chs5p in large structures. Purified Chs5/6 complexes by Chs5-TAP from the TEV eluate of wild type and *chs6Δ bch1Δ bud7Δ bch2Δ* quadruple mutant were analyzed on 4–8% gradient blue native PAGE. Several large forms of Chs5p self-oligomerization were monitored by immunoblotting with antiserum against Chs5p and denoted with asterisks.

The Chs5/6 Complex Transiently Interacts with Chs3p

We considered the possibility that the Chs5/6 complex interacts with a cargo molecule, a feature shared by several vesicle coat protein complexes (reviewed in Bonifacino and Lippincott-Schwartz, 2003; Kirchhausen, 2000). All coats include subunits that recognize specific sorting signals on cargo proteins that are substrates for packaging into transport vesicles. Given the transient nature of a cargo-coat interaction, we developed an *in vivo* cross-linking approach coupled with TAP purification to detect a brief contact between the Chs5/6 complex and one likely cargo, Chs3p. In the presence of a reversible chemical cross-linker, DSP, Chs3p copurified with Chs5-TAP and Chs6-TAP (Figures 3, A and B, and 5A). Other cell surface–destined cargos such as Gas1p and Bgl2p, which also populate the TGN compartment, were not detected in the copurified fraction. To identify other proteins involved in the transport of Chs3p, we also probed for proteins known to function in other export routes from the TGN. Arf1p, a small GTPase involved in intra-Golgi COPI vesicle transport and TGN export of the clathrin-coated vesicle (CCV), copurified with Chs5-TAP (Trautwein *et al.*, 2006). Apl2p, a subunit of the AP-1 complex was also detected in the copurified fraction. However,

little if any clathrin light and heavy chain were enriched by cross-linking to Chs5-TAP or Chs6-TAP. Most of the clathrin was found in the S-protein agarose flowthrough fraction. Furthermore, the signal intensity of clathrin light and heavy chain did not increase at higher levels of DSP, as was observed with the putative cargo protein. Thus, the low level of clathrin in the eluted fraction may constitute background. In contrast, Tlg1p, a t-SNARE that is essential for localization of Chs3p to the bud neck (Holthuis *et al.*, 1998), was enriched at higher DSP concentrations in the Chs5-TAP copurified fraction.

Members of the Chs6 Family Are Distinguishably Important for the Binding of Chs3p to the Chs5/6 Complex

In an effort to evaluate the physiological relevance of the interaction between Chs3p and the Chs5/6 complex, we repeated the experiment in a *chs6Δ* strain. In a *chs6Δ* background the level of Chs3p coisolated with Chs5-TAP was significantly reduced (Figure 4). However, the levels of coisolation of Apl2p and Tlg1p were not reduced in *chs6Δ*, perhaps because the other Chs6p paralogs may function to interact with other effectors or cargo molecules.

Figure 3. The Chs5/6 complex transiently interacts with Chs3p and other proteins. (A) Transient interaction between the Chs5/6 complex and its cargo, Chs3p, as well as other candidate molecules were captured by in vivo cross-linking with DSP. TAP purification using Chs5-TAP was performed with DSP concentrations at 0, 1, 2, and 5 mM. Purified complexes were incubated with DTT to reverse the cross-linking and then analyzed by SDS-PAGE and immunoblotting. (B) Quantification of data in A. The levels of proteins in the purified fractions were normalized with the levels of input loaded on the gel (1/8000 of total input). Concentrations of DSP are indicated as follows: open bars, 0 mM; light gray-shaded bars, 1 mM; medium gray-shaded bars, 2 mM; and black bars, 5 mM.

Because the three Chs6p paralogs, Bch1p, Bud7p, and Bch2p, copurified in a Chs5/6 complex, we next examined their role in the binding of Chs3p to the Chs5/6 complex. We constructed all possible combinations of single, double, triple, and quadruple deletion mutants of the Chs6-family members and examined the level of Chs3p copurified by Chs5-TAP (Table 4). In the single mutants, only *chs6Δ* showed a drastic reduction in the amount of Chs3p bound to the remaining subunits of the mutant Chs5/6 complex, suggesting that Chs6p has an important and unique function in binding Chs3p to the Chs5/6 complex. In the double mutants, the same effect was pronounced in combinations including *chs6Δ*. Surprisingly, the combination of *bch1Δ bud7Δ* showed a strong reduction in the level of cross-linked Chs3p, whereas the other double combinations *bch1Δ bch2Δ* or *bch2Δ bud7Δ* did not impair the binding of Chs3p to the mutant Chs5/6 complex. These results indicated that Bch1p and Bud7p have a redundant role because the deletion of both subunits was required to displace Chs3p from the Chs5/6 complex. In the triple mutants, all the combinations

Figure 4. Comparison between levels of copurified proteins from wild-type and *chs6Δ* backgrounds. TAP purification using Chs5-TAP was performed with 5 mM DSP in vivo cross-linking. Levels of copurified proteins were analyzed by quantitative immunoblotting and presented as a percentage of the protein levels in the loaded input (1/8000 of total input). Copurified proteins from wild-type and *chs6Δ* backgrounds are indicated by gray-shaded bars and black bars, respectively.

Table 4. Effects of different Chs6-family subunit disruptions on the binding ability of Chs3p to the remaining subunits of the Chs5/6 complex, Calcofluor resistance, and Chs3p transport to cell surface

Mutant Background	% copurified Chs3p ^a	Calcofluor resistance ^b	Chs3p transport to PM ^c
Single mutants			
<i>chs6Δ</i>	18 ± 6.8	+	No
<i>bch1Δ</i>	109 ± 5.7	–	Yes
<i>bud7Δ</i>	98	–	Yes
<i>bch2Δ</i>	90 ± 23.9	–	Yes
Double mutants			
<i>chs6Δ bch1Δ</i>	33 ± 13.5	+	No
<i>chs6Δ bud7Δ</i>	41 ± 4.3	+	No
<i>chs6Δ bch2Δ</i>	24 ± 8.3	+	No
<i>bch1Δ bud7Δ</i>	17 ± 4.5	+	No
<i>bch1Δ bch2Δ</i>	121 ± 21.2	–	Yes
<i>bud7Δ bch2Δ</i>	130 ± 27.1	–	Yes
Triple mutants			
<i>chs6Δ bch1Δ bud7Δ</i>	7 ± 0.1	+	No
<i>chs6Δ bch1Δ bch2Δ</i>	6	+	No
<i>chs6Δ bch2Δ bud7Δ</i>	19	+	No
<i>bch1Δ bud7Δ bch2Δ</i>	7	+	No
Quadruple mutant			
<i>chs6Δ bch1Δ bud7Δ bch2Δ</i>	3 ± 0.2	+	No

^a Percentage of Chs3p copurified with Chs5-TAP in various mutant backgrounds compared to the copurified level of Chs3p from wild-type strain.

^b Calcofluor resistance was determined on YPD agar supplemented with 20 μg/ml Calcofluor (see Figure 6A).

^c Chs3p transport to the cell surface was determined by Calcofluor resistance and subcellular distribution of Chs3p on step sucrose gradients (see Figure 6, A and B). PM, plasma membrane.

Figure 5. Chs5p is essential for Chs3p binding and structural stability of the Chs5/6 complex. (A) Purification of the Chs5/6 complexes from wild-type and *chs5Δ* backgrounds using Chs6-TAP was performed with 5 mM DSP in vivo cross-linking. The levels of copurified Chs3p were analyzed by quantitative immunoblotting and shown as percentage of the Chs3p level in the loaded input (1/8000 of total input). Copurified Chs3p from wild-type and *chs5Δ* backgrounds are indicated by gray-shaded bar and black bar, respectively. (B and C) The presence of Chs5p is critical for association of the other subunits in the Chs5/6 complex. The copurified levels of Bch1p from wild-type and *chs5Δ* backgrounds are shown in B. TAP purification using Chs6-TAP was performed and analyzed by SDS-PAGE and Sypro Red staining. In C, the copurified levels of Bud7-3xHA by Chs6-TAP from wild-type, *chs5Δ*, and *bch1Δ* backgrounds and of Bch2-3xHA from wild-type and *chs5Δ* backgrounds were analyzed by immunoblotting and compared. All purifications started with equivalent cell numbers; the expression levels of Bud7-3xHA and Bch2-3xHA were slightly decreased in *chs5Δ*, and Bud7-3xHA was up-regulated in *bch1Δ*. (D) The Chs6-family proteins are not essential for complex association of the other subunits. Purified complex by Chs5-TAP from wild-type and various CHS6-family mutant backgrounds were analyzed by SDS-PAGE and Sypro Red staining.

strongly reduced the amount of bound Chs3p. In the quadruple mutant, the amount of copurified Chs3p was almost undetectable. The fact that the quadruple mutant produced a slightly more pronounced effect than those seen in the single, double, or triple mutants suggested that Bch2p may also have a role in Chs3p binding, though less importantly, perhaps in maintaining structural stability of the Chs5/6 complex.

Chs5p Is Essential for Chs3p Binding and for Maintaining the Integrity of the Chs5/6 Complex

We next assessed the importance of Chs5p for the interaction between the Chs5/6 complex and Chs3p. Using a Chs6-TAP in a *chs5Δ* background, we found that the recovery of copurified Chs3p was strongly dependent on Chs5p (Figure 5A). During

the analysis of the Chs6-TAP purification results from wild-type and *chs5Δ* strains, we noticed that the coisolation of the Bch1p protein in the complex was also dependent on Chs5p (Figure 5B). Next we monitored the levels of Bud7-3xHA copurified by Chs6-TAP in wild-type, *chs5Δ*, and *bch1Δ* backgrounds (Figure 5C). In a wild-type background, the level of Bud7-3xHA in the copurified fraction was enriched compared with the input control lane (Figure 5C). Deletion of Bch1p appeared to increase the steady state expression level of Bud7-3xHA. The enrichment of copurified Bud7-3xHA was still observed in *bch1Δ* mutant (Figure 5C), suggesting that Bch1p is not necessary for the coisolation of Bud7-3xHA with Chs6-TAP. In the *chs5Δ* mutant, the steady-state levels of Bud7-3xHA and Bch2-3xHA were slightly decreased. However, in

Figure 6. Interaction between the Chs5/6 complex and Chs3p is required for the anterograde transport of Chs3p from the TGN to the cell surface. (A) The transport of Chs3p to the cell surface in wild type and mutants lacking different subunits of the Chs5/6 complex was assessed. Level of chitin synthesis by CSIII activity at the cell wall was monitored by growth on a Calcofluor plate. Mutant backgrounds are abbreviated as follows: 6, *chs6Δ*; 1, *bch1Δ*; 7, *bud7Δ*; and 2, *bch2Δ*. (B) Subcellular distribution of Chs3p was analyzed by step sucrose gradient. Total cell lysates from wild type, *chs6Δ*, *bch1Δ bud7Δ*, and *bch1Δ bch2Δ* were overlaid on step sucrose gradient, centrifuged at 55,000 rpm for 2.5 h. Fractions were collected manually from the top and analyzed by SDS-PAGE and quantitative immunoblotting. PM marker: Gas1p_m, mature form; Gas1p_i, immature form (ER); Golgi marker: Tlg1p.

the absence of Chs5p Bud7-3xHA disappeared in the copurified fraction (Figure 5C). Similarly, the level of copurified Bch2-3xHA was undetectable in the *chs5Δ* mutant (Figure 5C). All three Chs6 paralogs required Chs5p for efficient copurification with Chs6-TAP; Chs5p appears to be the centerpiece of the complex through which all the other subunits interact. The results of Chs5-TAP purification in various deletion backgrounds of the *CHS6*-like genes further indicated that the Chs6-family members are important but not essential for the structural integrity of the complex. Deletion of one or more Chs6-family members did not affect the ability of the remaining subunits to form a complex and copurify with Chs5-TAP (Figures 5D and 1B).

The Binding of Chs3p to the Chs5/6 Complex Is Critical for the Anterograde Transport of Chs3p from the TGN to the Plasma Membrane

To understand the biological significance of the interaction between Chs3p and Chs5/6 complex, we monitored the

transport of Chs3p to the cell surface in the deletion backgrounds that showed impairment of Chs3p binding to the Chs5/6 complex. One method to indirectly assess the transport of Chs3p to the cell surface is to measure the levels of chitin on the yeast cell wall. Wild-type levels of chitin render cells sensitive to the chitin-binding dye Calcofluor (Roncero and Duran, 1985). In contrast, cells with a defect in Chs3p transport such as *chs3Δ*, *chs5Δ*, and *chs6Δ* are resistant to Calcofluor (Bulawa, 1993). We examined the phenotype of deletions of the *CHS6*-family genes. Cells were plated on control and YPD plates supplemented with 20 μ g/ml Calcofluor at 24°C and growth was assessed after 3 days. *chs6Δ*, *bch1Δ bud7Δ*, and other mutant backgrounds that impaired the binding of Chs3p to the Chs5/6 complex were Calcofluor resistant, indicating that the transport of Chs3p was blocked (Figure 6A; Table 4; Trautwein *et al.*, 2006).

We confirmed the defect of Chs3p transport by subcellular fractionation of yeast membranes using step sucrose gradients (Figure 6B). In wild-type cells Chs3p fractionated in two

peaks: one coincided with intracellular organelle markers, Tlg1p and an immature form of Gas1p, and the other with plasma membrane marker, a mature form of Gas1p. In *chs6Δ* and *bch1Δ bud7Δ*, the distribution of Chs3p was restricted to the intracellular fractions, confirming a block in Chs3p transport to the cell surface. As a control, the *bch1Δ bch2Δ* double mutant, which did not affect Chs3p binding to the Chs5/6 complex, produced a membrane distribution of Chs3p similar to that of wild type. Thus, by functional criteria, we confirmed the conclusion that multiple Chs6-family subunits influence the sorting and traffic of Chs3p to the cell surface. The effects of different subunits on Chs3p sorting and transport to the cell surface is summarized in Table 4.

DISCUSSION

The mechanism of cargo protein selection for traffic from the TGN to the cell surface has not been illuminated in as much detail as the corresponding events early in the secretory pathway. Although many cellular proteins required in vesicular transport (e.g., Rabs, SNAREs, exocyst complex, and elements of the cytoskeleton) have been described, the actual sorting of proteins from *trans*-Golgi residents and the morphogenesis of a mature transport vesicle are not understood in any depth. One extreme view is that no sorting is required; rather all the proteins that remain after clathrin-mediated sorting to the endosome and COPI-mediated retrieval to an emerging *trans*-Golgi cisterna are transported by default to the cell surface. An investigation of the genetic and biochemical requirements for sorting in the anterograde direction late in the secretory pathway could distinguish between the models of selective transport or secretion by default.

We have investigated the genetic and biochemical requirements for the transport of Chs3p from the TGN to the plasma membrane at the mother-bud junction in yeast. Specifically, our attention was drawn to two proteins, Chs5p and Chs6p, required for the TGN to the cell surface traffic of Chs3p. *chs5Δ* and *chs6Δ* mutants accumulate Chs3p in intracellular deposits corresponding to the TGN and early endosome, and Chs5p has been localized to the TGN in normal cells (Santos and Snyder, 1997; Valdivia *et al.*, 2002). Interrupted transport in the *chs5Δ* and *chs6Δ* mutants is restored when any subunit of the clathrin AP-1 complex is deleted (Valdivia *et al.*, 2002). We suggested that clathrin and AP-1 mediate the cycling of Chs3p between the TGN and the early endosome and that this pathway serves to restrain an underlying Chs5p- and Chs6p-independent route of Chs3p traffic to the cell surface. The normal purpose of this diversion of Chs3p between the TGN and the early endosome may be to maintain an intracellular reservoir of Chs3p that is called upon when cells are stressed or possibly in the G1 phase of the cell cycle when the synthesis of chitin at the mother-bud junction is maximum.

Unlike other cellular functions that are essential for traffic from the Golgi complex to the cell surface, e.g., many Sec proteins, Chs5p is required for only a subset of trafficked proteins in addition to Chs3p (e.g., Fus1p, Crh2p, and possibly a protein necessary for establishing the bipolar budding pattern of yeast diploid cells; Rodriguez-Peña *et al.*, 2002; Santos and Snyder, 2003). Surprisingly, Chs6p is not required for the transport of Fus1p or the organization of the diploid-cell bipolar budding pattern (Santos and Snyder, 2003; Trautwein *et al.*, 2006; A. McKenzie III, K. Nakashima, and J. Pringle, personal communication); however, one or more of the three paralogs of Chs6p may substitute in these processes.

Given the phenotype of *chs5Δ* and *chs6Δ* mutants, the intracellular location of Chs5p and the limited repertoire of cargo proteins whose traffic depends on Chs5p and Chs6p, we consider these proteins attractive candidates for subunits of a sorting complex, possibly a novel coat, involved in selective anterograde transport late in the secretory pathway. For this reason we evaluated the molecular characteristics of Chs5p and Chs6p and developed an approach to detecting their intracellular target proteins.

Native TAP-tagged forms of Chs5p and Chs6p revealed a complex that includes one or another member of the *CHS6* family of gene products. Even complexes defined by the Chs6-TAP included Chs6 paralogs. This is in contrast to the composition of the sorting determinant of the COPII coat, Sec23/24, which in yeast includes one copy of Sec23p and one copy of Sec24p or one of its two paralogs, Iss1p and Lst1p (Kurihara *et al.*, 2000; Peng *et al.*, 2000; Shimoni *et al.*, 2000). In mammalian cells the COPII situation is a bit more complex, with two isoforms of the assembly GTPase, Sar1p, two paralogs of Sec23p, and four of Sec24p (Kuge *et al.*, 1994; Paccaud *et al.*, 1996; Tang *et al.*, 1999; Shoulders *et al.*, 2004). Nonetheless, the active species in this case is a heterodimer, whereas the Chs5/6 complex must be a higher-order oligomer. In spite of this structural difference, the two complexes may share the feature of one structural subunit (Sec23p for COPII and Chs5p for the Chs5/6 complex) and one subunit that specifies cargo sorting (Sec24p and its paralogs for COPII and Chs6p and its paralogs for the Chs5/6 complex).

The TAP-tagged Chs5p and Chs6p proteins could be cross-linked *in vivo* to the cargo protein Chs3p and to one or more other proteins that may travel together in a common carrier vesicle. However, other cargo proteins or clathrin subunits were undetected perhaps because they were much less efficiently cross-linked to Chs5p or Chs6p. Most importantly, the specificity of Chs3p cargo selection was established with the demonstration that deletion of *CHS5* or *CHS6* strongly reduced the coisolation of Chs3p with the Chs6-TAP or Chs5-TAP, respectively. These data make it hard to establish the subunit most responsible for direct contact between Chs3p and the sorting subunit of the Chs5/6 complex.

The paralogs of Chs6p could serve to extend the range of cargo molecules sorted into vesicles en route from the TGN to the cell surface. However, among the paralogs it appears that Chs6p is not uniquely responsible for capture of Chs3p. The pair Bch1p and Bud7p serves a parallel role in Chs3p transport such that their deletion results in a Chs3p traffic and cell wall chitin defect. It may be that the Chs6 paralogs serve both in membrane protein sorting and in structural stability of the Chs5/6 complex. In this respect, the Chs5p subunit appears to play a critical role. Deletion of *CHS5* results in a defect in the assembly of Chs6 paralogs with each other. Resolution of the precise roles of these subunits requires an evaluation of the purified complex in the context of a biochemical reaction that reflects the traffic function of the Chs5/6 complex.

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