Exomer: a coat complex for transport of select membrane proteins from the trans-Golgi network to the plasma membrane in yeast

Chao-Wen Wang,1,2 Susan Hamamoto,1,2 Lelio Orci,3 and Randy Schekman1,2

1Department of Molecular Cell Biology and 2Howard Hughes Medical Institute, University of California, Berkeley, Berkeley, CA 94720
3Department of Cell Physiology and Metabolism, University Medical Center, 1121 Geneva 4, Switzerland

A yeast plasma membrane protein, Chs3p, transits to the mother–bud neck from a reservoir comprising the trans-Golgi network (TGN) and endosomal system. Two TGN/endosomal peripheral proteins, Chs5p and Chs6p, and three Chs6p paralogues form a complex that is required for the TGN to cell surface transport of Chs3p. The role of these peripheral proteins has not been clear, and we now provide evidence that they create a coat complex required for the capture of membrane proteins en route to the cell surface. Sec7p, a Golgi protein required for general membrane trafficking and functioning as a nucleotide exchange factor for the guanosine triphosphate (GTP)-binding protein Arf1p, is required to recruit Chs5p to the TGN surface in vivo. Recombinant forms of Chs5p, Chs6p, and the Chs6p paralogues expressed in baculovirus form a complex of approximately 1 MD that binds synthetic liposomes in a reaction requiring acidic phospholipids, Arf1p, and the nonhydrolyzable GTPγS. The complex remains bound to liposomes centrifuged on a sucrose density gradient. Thin section electron microscopy reveals a spiky coat structure on liposomes incubated with the full complex, Arf1p, and GTPγS. We termed the novel coat exomer for its role in exocytosis from the TGN to the cell surface. Unlike other coats (e.g., coat protein complex I, II, and clathrin/adaptor protein complex), the exomer does not form buds or vesicles on liposomes.

Introduction

Vesicular trafficking provides a continuous exchange of proteins and lipids between membranes in a eukaryotic cell (for review see Rothman and Wieland, 1996; Schekman and Orci, 1996). Coat proteins are believed to confer much of the specificity associated with protein sorting into transport vesicles (Le Borgne and Hoflack, 1998a,b; Springer and Schekman, 1998). To date, three classes of coated vesicles have been identified: clathrin/adaptor-coated vesicles mainly involved in transport between the TGN and the endosomes (Robinson, 1994); coat protein complex I (COPI), which is responsible for both retrograde transport from the Golgi back to the ER and intra-Golgi transport (Orci et al., 1997; Spang and Schekman, 1998); and COPII, which mediates anterograde transport from the ER to the Golgi apparatus (Bednarek et al., 1996; Schekman and Orci, 1996).

Coat assembly is initiated by activation of the ADP ribosylation factor (ARF) family of small G proteins (Arf1p and the closely related Sar1p) by which membrane-selective nucleotide exchange catalysts activate Arf1p or Sar1p for membrane attachment (Serafini et al., 1991; Donaldson et al., 1992; Pulmer et al., 1993; Barlowe et al., 1994). Arf1p regulates the recruitment of COPI and most clathrin-containing coats, leading to membrane deformation into coated buds and vesicles. Likewise, COPII vesicles form when Sar1p-GTP recruits the inner coat complex (the Sec23/24p heterodimer) and the outer coat (the Sec13/31p heterotetramer; Matsuoka et al., 1998; Antonny et al., 2003; Lee et al., 2004).

Although coat proteins account for much of the vesicular traffic in a cell, no such involvement of coat proteins has been documented in the formation of vesicles or tubules that convey membrane and secretory proteins directly from the TGN to the cell surface. As an example of this limb of the secretory pathway, we have studied the transport of a cell wall biosynthetic enzyme, Chs3p (chitin synthase III), from the TGN/endosome membranes to the plasma membrane of the mother–bud junction in yeast. Chs3p is a multispanning transmembrane protein...
that is required for chitin ring formation during the G1 phase of the cell cycle and, subsequently, in lateral cell wall chitin synthesis (Shaw et al., 1991). Unlike other Golgi surface proteins, Chs3p export is regulated in response to cell cycle and stress signals (Shaw et al., 1991; Valdivia and Schekman, 2003). However, throughout the cell cycle, it is maintained in an intracellular reservoir by being recycled between the TGN and the early endosomes. This recycling is mediated by clathrin and an adaptor protein complex (AP-1). Chs5p and Chs6p are peripheral proteins that are required to transport Chs3p from the reservoir to the cell surface (Santos et al., 1997; Ziman et al., 1998). \( chs5 \Delta \) and \( chs6 \Delta \) mutants accumulate Chs3p in the TGN/endosome membranes, and the deletion of clathrin or subunits of AP-1 restores Chs3p traffic to the cell surface by some unknown bypass pathway (Valdivia et al., 2002). Thus, at least two mechanisms of traffic from the TGN/endosome membrane to the cell surface are possible for Chs3p. Each pathway involves an unexplored protein-sorting event that packages Chs3p into secretory vesicles that are delivered to the bud plasma membrane by the standard secretory pathway (Valdivia et al., 2002).

The Chs5 and Chs6 proteins are restricted to yeast and fungi, which may imply an organism-specific role such as the biosynthesis of yeast cell wall chitin. Yeast cells have three additional Chs6-like proteins (Bch1p \([\text{YMR237W}]\), Bud7p, and Bch2p \([\text{YKR027W}]\)), and their roles relative to Chs6p and the traffic of Chs3p are not yet understood (Satchatjate and Schekman, 2006; Trautwein et al., 2006). To pursue the role of Chs5p and Chs6p in the sorting and packaging of Chs3p, we have cloned and characterized the gene products, including the three paralogues of Chs6p. We have demonstrated that these Chs5 and Chs6 proteins are associated with each other in a complex that may make direct contact with Chs3p (Sanchatjate and Schekman, 2006). In this study, we identified Sec7p, a TGN-localized Arf1p nucleotide exchange factor that is required for the membrane association of Chs5p and regulating the interaction of Chs5p and Arf1p. We elucidated the biochemical requirements for membrane recruitment of a complex of Chs5p, Chs6p, and the Chs6p paralogues and describe a novel coat structure termed exomer, which forms when the complex is recruited to synthetic membranes in the presence of Arf1p and GTP\(\gamma\)S.

Results

Chs5p membrane association is mediated by Sec7p

In a previous study, Santos and Snyder (2000) found Chs5p localized to puncta in cells marked by the TGN/endosome resident protein Kex2p. We confirmed the late Golgi localization of Chs5p-RFP by comparing its localization in vivo with Sec7p-GFP (late Golgi marker) and Anp1p-GFP (early Golgi marker) using double staining live cell imaging (Fig. S1, A–C; available at http://www.jcb.org/cgi/content/full/jcb.200605106/DC1). To determine whether this localization is perturbed when Golgi traffic is disrupted, we used a functional, integrated Chs5p-GFP to examine its localization in wild type and in cells defective in secretory protein traffic from the Golgi complex. In wild-type cells, Chs5p-GFP localized to spots dispersed within the cytoplasm, as was determined previously (Santos and Snyder, 2000), and this distribution was similar at 26 and 37°C.

We also expressed Chs5p-GFP in two temperature-sensitive mutants in which the secretory function of the Golgi apparatus can be altered. A temperature-sensitive allele of the phosphatidylinositol 4-kinase (PIK1) gene blocks protein secretion and accumulates exaggerated Golgi structures (Flanagan et al., 1993;
Sec7p in regulating this association, we next evaluated the recovery of bound Chs5p in incubations containing the ARF GEF inhibitor brefeldin A (BFA). We observed a reduction in the retention of Chs5p in an incubation containing GTPγS and 2 μg/ml BFA (Fig. 1 C). A similar effect of BFA in vivo has been reported by Trautwein et al. (2006). These experiments suggest that activated Arf1p-GTP, presumably by contact with Sec7p, recruits Chs5p to the Golgi membrane to initiate its role in the traffic of Chs3p.

**Lipid-binding and Chs6 interaction domains of Chs5p**

In addition to binding to activated Arf1p, Chs5p may interact by additional contact with the Golgi membranes. To determine whether Chs5p interacts with lipids, we expressed GST hybrid forms of Chs5p and Chs6p in *Escherichia coli*, and the purified GST hybrid proteins were probed for lipid interaction using an overlay assay on PIP strips. GST-Chs5p but not GST or GST-Chs6p showed significant interaction with most anionic lipids (unpublished data). A similar spectrum was seen with 6× His-tagged Chs5p expressed in yeast (unpublished data).

Figure 2.

**Functional mapping of Chs5p.** (A) Mapping Chs5p lipid interaction and Chs6p interaction regions. Full-length Chs5p (aa 1–671) contains two motifs: FN3 (aa 79–160) and BRCT (aa 160–260). The fragments positive for lipid interaction are shown by a plus sign. (B) Wild-type (WT; SEY6210) cells harboring GST, GST-Chs5 (aa 1–79), and GST-Chs5 (aa 401–671) cloned into pRS424 were streaked on SD-Trp plates in addition to 50 (CF 50) or 100 μg/ml (CF 100) calcofluor. For each construct, two colonies were restreaked and examined on the plates.
domain—Chs6p (Fig. 2 A). These two domains (the lipid-binding domain within the C terminus of Chs5p and the Chs6 interaction domain within the N terminus of Chs5p) were compared by a competition assay evaluating their functional importance in chitin synthesis. The overexpression of GST hybrids containing the N-terminal domain of Chs5p interfered with Chs3p traffic as judged by the calcofluor growth test, whereas a GST hybrid containing the C-terminal domain of Chs5p did not impair chitin synthesis (Fig. 2 B). Thus, the interaction of Chs5p and Chs6p may be crucial for the transport of Chs3p.

A purified recombinant complex of Chs5p, Chs6p, and Chs6 paralogues

To further investigate the role of activated Arf1 in Chs5p membrane recruitment (Fig. 1), we sought to isolate the Chs5p- and Chs6p-containing complex (Fig. 2) for functional tests. Efforts to express the proteins in stable oligomeric forms in E. coli and yeast resulted in poor yields. However, expression in baculovirus proved more reliable. We created baculovirus vectors containing N-terminally 6× His-tagged Chs5p and untagged versions of one or more copies of Chs6p and its paralogues (Bch1p, Bud7p, and Bch2p; Fig. 3 A). Recent evidence has suggested that Chs5p interacts with Chs6p and each of the Chs6 paralogues and that complexes include more than one copy of Chs6p and its paralogues (Sanchatjate and Schekman, 2006; Trautwein et. al., 2006). The baculovirus system allowed us to evaluate complex formation by coexpressing multiple combinations of these recombinant Chs5p and Chs6p proteins. Consistent with previous observations (Sanchatjate and Schekman, 2006; Trautwein et. al., 2006), we found that Chs5p copurified with each Chs6p or its parologue when the two were coexpressed and that multiple paralogues of Chs6p copurified with Chs5p from cells expressing two, three, or all Chs6p and Chs6p paralogues (Fig. 3 A and not depicted).

In most cases, the apparent abundance of Chs5p, based on Sypro red staining intensity, approximated the abundance of the sum of the Chs6 and Chs6p paralogues. Bch2p was a notable exception, perhaps because of a lower virus titer. Infection with a larger Bch2 baculovirus stock increased the abundance of this species in the isolated Chs5p complex (unpublished data). The ratios of the Chs6p species in the complex differed from those detected in the complex isolated from wild-type yeast cells.
(Sanchatjate and Schekman, 2006), probably reflecting the different level of CHS gene expression in these circumstances. The recombinant expression of Chs6p and its paralogues without Chs5p also resulted in complexes including Chs6p and one or more Chs6p paralogues (unpublished data).

Affinity-purified complexes were evaluated by gel filtration on a Superose 6 fast protein liquid chromatography (FPLC) column to determine the size and composition of the complex. Complex isolated from cells expressing Chs5p and all four Chs6p and its paralogues (Chs5–Chs6(all)) fractionated at a position consistent with a size slightly $>1$ MD with coincident chromatography of the most abundant Chs6p species (Fig. 3 B). Similar patterns of filtration were seen with complexes containing only one Chs6p paralogue, although a complex of Chs5p and Chs6p fractionated somewhat heterogeneously. An independent method of separation, velocity sedimentation on a sucrose density gradient, confirmed that Chs5p, Chs6p, and Chs6p paralogues (Chs5–Chs6(all)) were present in a large complex (Fig. 3 C). This pattern of cosedimentation was not altered in the Chs5–Chs6(all) samples treated with 3 M urea, 1% Triton X-100, or 1 M KCl (unpublished data). Overall, recombinant Chs5p, Chs6p, and Chs6p paralogues, like those isolated from wild-type yeast cells, appear to be self-organized into a large and stable complex.

Myristoylated Arf1p-GTPγS recruits the Chs5–Chs6(all) complex to liposomes

Because genetic evidence suggests that more than just one of the Chs6p and its paralogues is required for traffic of Chs3p to the cell surface (Sanchatjate and Schekman, 2006; Trautwein et al., 2006), we used the complex including Chs5p, Chs6p, and three Chs6 paralogues (Chs5–Chs6[all]) to evaluate the role of activated Arf1p in membrane recruitment. Recombinant myristoylated Arf1p (mArf1p: Q71L; GTPase deficient) was purified from E. coli and mixed with GTPγS and liposomes formulated with synthetic phospholipids with various levels of selected acidic phospholipids (Fig. 2 A, Spang et al., 1998). EDTA was included to stimulate spontaneous GTP/GDP exchange on Arf1p (Antony et al., 1997). After 1 h at 30°C, MgCl2 was added to stabilize Arf1-GTPγS, and the samples were supplemented with Chs5–Chs6[all] and incubated for a further 10 min at 22°C. For each liposome formulation, three samples were prepared: complete, without Arf1p, and without GTPγS. Liposomes and bound proteins were separated from unbound materials by flotation sedimentation on a sucrose density shelf. Fig. 4 A documents the recovery of proteins bound to liposomes, and Fig. 4 B displays a quantitative representation of proteins recovered in the floated fractions. In most samples, Arf1p bound to liposomes in the presence or absence of GTPγS. However, recruitment of the Chs5–Chs6[all] complex was optimum in incubations that contained Arf1p and GTPγS.

Certain liposome formulations (e.g., phosphatidylcholine/phosphatidylethanolamine [PE]/phosphatidylserine/phosphatidic acid [PA] with a high concentration of PI(4)P or phosphatidylinositol-4,5-bisphosphate) recruited Chs5–Chs6[all] in the absence of Arf1p or GTPγS (Fig. 4, A and B). Other formulations (e.g., major-minor mix optimized for COPII assembly; Matsuoka et al., 1998) displayed a substantial GTPγS requirement for recruitment of the Chs5–Chs6[all] complex. From these results, we conclude that Arf1p binds directly to the Chs5–Chs6[all] complex and facilitates the association of the complex with liposomes, particularly with certain formulations containing one or more of several acidic phospholipids.

The major-minor mix formulation, which was optimized for the recruitment of activated Sar1p and COPII to liposomes (Matsuoka et al., 1998) and which also works well for the recruitment of activated Arf1p and coatomer (COPI; Spang, et al., 1998), was reexamined for wild-type Arf1p and Chs5–Chs6[all] assembly in the presence of various nucleotides. Fig. 4 C shows a substantial dependence on GTPγS (or GMP-PNP), with much less of the complex recruited in the presence of GDP or GTP. GTP hydrolysis during recruitment and liposome sedimentation may explain the failure to retain comparable amounts of the Chs5–Chs6[all] complex on liposomes incubated with Arf1p-GTP. Likewise, a Golgi-enriched membrane fraction incubated with the Chs5–Chs6[all] complex confirmed that the binding of Chs5–Chs6[all] in a reaction was stimulated by GTPγS (unpublished data).

We next compared recruitment of the Chs5–Chs6[all] complex to the other subcomplexes formed with Chs5p, Chs6p, or fewer paralogues of Chs6p (Fig. 4 D). Chs5p alone was very inefficiently recruited to major-minor liposomes. Combinations including one paralogue were recruited in a manner largely independent of GTPγS. However, two paralogues known to be important in Chs3p traffic (Chs6p and Bch1p) were recruited to membranes in an Arf1p-GTPγS–dependent manner comparable with Chs5–Chs6[all] (Fig. 4 D).

Stoichiometric recruitment of the Chs5–Chs6(all) complex to liposomes

To provide evidence that the recruitment is under control by a specific, regulated process, we varied the concentration of Arf1-GTPγS and the Chs5–Chs6[all] complex and the time of incubation to discover the optimum conditions of assembly on major-minor mix liposomes. Two-stage recruitment assays were performed. Liposomes were incubated with $\sim 1 \mu$M mArf1p and GTPγS in a first-stage binding reaction as in Fig. 4 and were mixed with the Chs5–Chs6[all] complex for 15 min at 22°C. Membrane-bound protein complexes were collected by flotation on a step sucrose cushion, and the protein content was measured by Sypro red staining of gels (Fig. 5 A). Chs5–Chs6[all] complex binding to liposomes was saturated at $\sim 0.5 \mu$M in this experiment. Conversely, at a fixed Chs5–Chs6[all] concentration of $\sim 0.8 \mu$M, Arf1p binding increased nonsaturably (Fig. 5 B, ii), whereas binding of the Chs5–Chs6[all] complex appeared to approach saturation at around 1.5 $\mu$M Arf1p (Fig. 5 B, i). Excess bound Arf1p may not be functional or accessible to the Chs5–Chs6[all] complex. Titration at the low range of Arf1p, where the linear membrane association of Arf1p and Chs5p was shown (Fig. 5 B, iii), indicated an $\sim 7:5:1$ molar stoichiometry of the Arf1/Chs5–Chs6[all] complex, reflecting either a substantial fraction of bound Arf1p that is not accessible to the Chs5–Chs6[all] complex or incomplete density gradient recovery of Chs5–Chs6[all] compared with activated Arf1p bound to liposomes.
The relationship of Arf1p exchange and Chs5–Chs6[all] recruitment in a reaction was evaluated in a time course experiment. Protein binding to liposomes was conducted with Arf1p at approximately the maximum Chs5–Chs6[all] ratio achieved in the titration experiment in Fig. 5 A. Incubations included EDTA to promote Arf1p nucleotide exchange. Samples were collected at the indicated times at 30°C, mixed with MgCl₂, and chilled on ice for the duration. The kinetics of binding paralleled the rate of EDTA-stimulated Arf1p nucleotide exchange as measured by the change in tryptophan fluorescence of activated Arf1p (Fig. 5 C and not depicted; Antonny et al., 1997). These results support our conclusion that GTPγS-activated Arf1p recruits the Chs5–Chs6[all] complex to the membrane.

Chs5–Chs6[all] complex forms a coated surface on liposomes

Given the similarity between the Chs5–Chs6[all] complex and the COPs (coatamer and COPII) in regard to Arf1p-GTPγS (or Sar1p–GMP-PNP)–dependent recruitment to liposomes, we examined the influence of this assembly on the buoyant density of liposomes. COPII proteins that assemble on liposomes in the presence of Sar1p–GMP-PNP cause membranes to shift to a higher buoyant density, reflecting the formation of protein-coated surfaces and synthetic COPII vesicles (Matsuoka et al., 1998). We formulated major-minor mix liposomes with Texas red–PE and conducted assembly incubations for the complete reaction containing Arf1-GTPγS and Chs5–Chs6[all] as described above. Samples were applied to a 10–50% linear sucrose gradient and centrifuged to equilibrium for 16 h at 4°C. In control incubations, in samples of liposomes incubated with the Chs5–Chs6[all] complex without Arf1p or GTPγS or liposomes incubated with Arf1p and GTPγS alone (unpublished data), fluorescent lipid peaked at the top of the gradient (fraction 1). In a complete reaction, most of the fluorescent lipids and Arf1p sedimented into the gradient but remained in the low density liposome fractions (Fig. 6, C and D; fractions 2–8), whereas most of the unbound Chs5–Chs6[all] complex sedimented to a high density with no apparent lipid cofractionation (Fig. 6, B and C). Thus, at least some liposome-bound Chs proteins may coat membranes sufficiently to influence membrane buoyant density.
The unambiguous assignment of a membrane coat requires inspection by thin section electron microscopy. Samples were prepared from a complete incubation (Fig. 7 A), an incubation of liposomes and Arf1p-GTPγS alone (Fig. 7 C), and an incubation with Arf1p, Chs5–Chs6[all] complex, and GDP (Fig. 7 B). A spiky coat appeared uniformly distributed along the surface of liposomes incubated under conditions in which the Chs5–Chs6[all] complex binds to the membrane but not in the control conditions. Although some membrane profiles appeared elongated, no coated buds or small coated vesicles were evident. Thus, the Chs5–Chs6[all] complex, which we now call exomer, appears to form coats on the membrane. However, unlike the COPs, exomer by itself does not deform membranes to induce the formation of buds and transport vesicles.

**Discussion**

Vesicular traffic in several limbs of the secretory pathway is initiated by the GTP-binding protein-dependent recruitment of coat proteins that sequester cargo molecules in a bud and pinch the membrane to form a transport vesicle. Clathrin, COPI, and COPII are well-known examples of this seemingly general feature (for review see Rothman and Wieland, 1996; Schekman and Orci, 1996). However, one clear gap in our knowledge concerns the mechanism of sorting and transport of membrane and secretory proteins from the TGN to the cell surface. Although some proteins use clathrin to traverse the endosome en route to the cell surface (Ang et al., 2004), others do not, and, until now, the general view has been that the direct path out of the TGN may involve tubular carriers formed without the intervention of coat proteins. Some regulatory proteins control the formation of transport carriers at the TGN by modulating lipid composition (for example, activation of phospholipase D by protein kinase C; Simon et al., 1996). Other proteins, such as FAPPs (four-phosphate adaptor proteins), are phosphoinositide-binding proteins that are found associated with TGN carriers and also interact with Arf1p (Godi et al., 2004). Protein kinase D is recruited to the TGN through interaction with diacylglycerol and...
is subsequently activated by phosphorylation to promote carrier fission (Liljedahl et al., 2001).

We have investigated traffic of the plasma membrane enzyme that makes the chitin ring at the mother–bud junction in growing yeast cells. This protein, Chs3p, has an interesting itinerary and set of genetic requirements for its traffic that are somewhat distinct from the requirements of other cell surface proteins. Specifically, the action of two peripheral membrane proteins, Chs5p and Chs6p, suggest a special machinery to convey Chs3p from the TGN to the cell surface (Valdivia et al., 2002). Chs5p and Chs6p form subunits of a complex, including one or more additional Chs6p paralogues that also facilitate the traffic of Chs3p (Sanchatjate and Schekman, 2006; Trautwein et al., 2006). Chs5p is required for the transport of at least two other proteins in addition to Chs3p, one of which, Fus1p, a cell surface protein required for yeast cell fusion, does not depend on Chs6p (Santos and Snyder, 2003). By analogy to the paralogues of Sec24p that form dimers with Sec23p in the COPII coat (Shimoni et al., 2000), we propose that Chs5p, Chs6p, and its paralogues may recognize distinct sets of membrane cargo proteins at the TGN for packaging into mature secretory vesicles.

Our strategy has been to identify conditions that promote the recruitment of Chs5p and Chs6p to the TGN/endosomal membrane and then to reconstruct this interaction with synthetic membranes. Two lines of evidence support a role for activated Arf1p in Chs5p–Chs6p assembly at the TGN. Trautwein et al. (2006) found that Arf1p-GTPγS binds Chs5p and Chs6p in a crude lysate of yeast. In vivo, nucleotide exchange on Arf1p is promoted by several proteins sharing a GEF domain that was first identified in a peripheral Golgi protein, Sec7p, which is required for secretory traffic within the Golgi complex. Mutations within the Sec7 GEF domain block traffic of all or most secretory proteins and disperse Chs5p from its normal punctate TGN/endosomal localization (Fig. 1A). BFA blocks GEF activity and reduces the recruitment of Chs5p to immobilized
Arf1p-GTPγS (Fig. 1 C). Arf1p-GTP may tether Chs5p to membrane much as it does for coatomer in COP I vesicle formation and adaptor proteins (AP-1 and -2) for clathrin-coated vesicles and as Sar1p-GTP does for Sec23p–Sec24p in COP II coat assembly.

Coat recruitment and assembly on artificial membranes is stimulated by acidic phospholipids. Recombinant Chs5p interacts with Chs6p and with a variety of acidic phospholipids (Figs. 2 and 4). Recombinant complex expressed in baculovirus-infected Sf-9 cells as combinations of Chs5p, Chs6p, and one or more of the Chs6p paralogues fractionates as an ~1-MD complex that binds to artificial membranes in an Arf1-GTPγS (or GMP-PNP)–dependent manner (Fig. 4). Optimal interaction occurs on liposomes that are similar in composition to those formulated for the recruitment and assembly of the COP II and I coats (Matsuoka et al., 1998; Spang et al., 1998). Complexes formed with binary combinations of Chs5p and Chs6p or one other Chs6p parologue are somewhat less strictly dependent on Arf1p-GTPγS for membrane recruitment. Thus, we suggest that the native mixed complex (i.e., Chs5–Chs6[all]) is the likely form recruited to TGN membranes in vivo.

The Chs5–Chs6[all] complex binds to and perturbs the density of liposome membranes (Fig. 6). Thin section microscopy of fixed, membrane-bound complex reveals an electron-dense coat whose morphology is quite distinct from that seen for other coating complexes associated with cargo traffic (Fig. 7). Unlike COP II and I, which assemble onto and vesiculate artificial liposomes, the Chs5–Chs6[all] coat forms a spiky structure but does not pinch the membrane into buds and small vesicles. We propose to call the Chs coat the exocomer to reflect its role in the exocytosis of Chs3p and select additional proteins. Although this coat also traffics a subset of other cargo proteins, the subunits are dispensable for normal cell viability, and the CHS5 and CHS6 genes are largely restricted to fungi that make chitin. It seems likely that other coats of similarly restricted roles will be uncovered in yeast and in other organisms, but the null phenotype of such coat subunits may not be nearly as dramatic as one normally associates with a general block in secretion.

The absence of coated buds and small vesicles in the preparation of exocomer-coated liposomes suggests that other factors cooperate with the exocomer to convey cargo proteins out of the TGN donor compartment. Chs3p interacts with Chs5p–Chs6p (Sanchatjate and Schekman, 2006; Trautwein et al., 2006), and it is possible that cargo-coat contact may promote the shape change that accompanies vesicle morphogenesis. Alternatively or in addition, the exocomer may engage elements of the cytoskeleton, perhaps actin directly, to draw cargo molecules into tubules much as has been shown for cooperative interaction of clathrin, actin, and the Arp2/3 complex in cell surface invagination and endocytosis in yeast (Kaksonen et al., 2003, 2005). Indeed, genetic studies link Chs5p and Chs6p to elements of the cytoskeleton (Tong et al., 2004; Lesage et al., 2005). A biochemical reconstitution approach with exomer, liposomes, and cytoskeletal proteins may now be used in an effort to recapitulate the formation of more fully developed TGN to cell surface traffic intermediates.

### Materials and methods

**Yeast strains and materials**

GFP was integrated at the C-terminal codon of the CHS5 locus by a PCR-based one-step transformation procedure (Longtine et al., 1998). Primers for C-terminal GFP integration were 5′-AAGAGAAATAGAAGATGGAATTTGAAAGGAAAAAGAAAGCCTCCGCGGTGAATAAA and 3′-ATAGAAAGATGATATATTCTGGTACGATGCTCCGCGGTGAATTTAA and 3′-ATAGAAAGATGATATATTCTGGTACGATGCTCCGCGGTGAATTTAA and 3′-ATAGAAAGATGATATATTCTGGTACGATGCTCCGCGGTGAATTTAA and 3′-ATAAGAAGATGATATATTCTGGTACGATGCTCCGCGGTGAATTTAA and 3′-ATAAGAAGATGATATATTCTGGTACGATGCTCCGCGGTGAATTTAA. Deletion primers to generate chs5Δ were 5′-GTTTGATGGTTAAGTTTGTTTAACTTACCTGGACGCCGGTGAATTTAAG and 3′-CTTATGTTTCTATTTATTATACATACGGTTATGACC GAATTCCAGCTCGTATTAAA. Deletion primers to generate chs6Δ were 5′-GTTTGATGGTTAAGTTTGTTTAACTTACCTGGACGCCGGTGAATTTAAG and 3′-CTTATGTTTCTATTTATTATACATACGGTTATGACC GAATTCCAGCTCGTATTAAA.

Yeast strains used in this study include the following: SEY6210 (MATα, leu2-3, ura3-52, his3-Δ200, lys2-801, trp1-901, suc2-39, CWS512), Chs5-GFP::HIS3 (SEY6210, CWY559 (MATα pkl1-b3::TRP1 ade2-101och his3-200 leu2-1 lys2-801 trp1-1 ura3-52), Chs5::HIS3, pks4/S12, CWY612 (MATα ura3-52 leu2-311 trp1-1 his3-111, 15 sec4-7, Chs5-5p::kan, CWY506 (Arf1-pA::HIS3 SEY6210, CWY624 (chs5Δ::LEU2 SEY6210), and P69-4A (MATα, trp1-101, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, lys2::GAL1-HIS3, GAL2-ADE2, me2::GAL7-lacZ). All additional reagents were purchased from Sigma-Aldrich unless specified. Lipids were obtained from Avanti Polar Lipids, Inc., and PIP strips were purchased from Echenicher. Sproyo red protein staining dye was purchased from Invitrogen. Complete protease inhibitor cocktail was obtained from Roche Molecular Biochemicals. E. coli BL21 (DE3) coexpressing yeast N-myristoyltransferase and Arf1 (wild type and Q71L) were provided by R. Kuhn (Emory University, Atlanta, GA). Anti-Chs5 antiserum was described previously (Sanchatjate and Schekman, 2006). Anti-GST antibody was purchased from Santa Cruz Biotechnology, Inc. DH10Bac competent cells and the Bacto-bac baculovirus expression system were purchased from Invitrogen. Glutathione-Sepharose fast flow, pGEX vector, and the Superose 6 gel filtration column were obtained from GE Healthcare. Dynabead M-500 subcellular was purchased from Dynal.

**Protein purification**

GST, GST-Chs5, GST-Chs6, and other GST-Chs5 fragments shown in Fig. 1 B were constructed in the pGEX vector (GE Healthcare), and proteins were purified from E. coli BL21 (DE3) coexpressing yeast N-myristoyltransferase and Arf1 (wild type and Q71L) (Randozio et al., 1992), expressed in yeast N-myristoyltransferase and either wild-type or dominant-activated (Q71L) Arf1. The purification procedures have been published previously (Randozio et al., 1992). Chs5-GFP::KAN was grown to an OD600 = 0.5–1.0 followed by 200 μM IPTG induction for 3 h at 22°C. Cells were resuspended in 20 ml PBS buffer, lysed by sonication, and centrifuged at 12,000 rpm for 10 min. The clear cell lysate was incubated with 5 ml glutathione-Sepharose (prewashed by PBS) at 0°C for 3 h. Beads and adsorbed proteins were poured into a column, and 3 x 30 ml PBS aliquots were applied to the column to remove nonspecific material. Bound proteins were eluted with 12 ml 100 mM of reduced glutathione. For the lipid-protein overlay assay, we adjusted purified proteins to 150 μg/ml and followed the procedures recommended by the manufacturer [Echelon]. mArf1p was purified from an E. coli BL21 (DE3) strain that coexpressed yeast N-myristoyltransferase and either wild-type or dominant-activated (Q71L) Arf1. The purification procedures have been published previously (Randozio et al., 1992), except a Sephacryl S-100 column was used for the gel filtration step. Based on the mobility shift by electrophoresis and in agreement with the literature, we confirmed that >75% of the E. coli–purified mArf1p, either wild-type or the dominant-activated mutant, was N-myristoylated. The purification resulted in ~80% pure mArf1p.

The Chs5p–Chs6p complex was purified using the Bac-to-Bac baculovirus expression system [Invitrogen]. To make the recombinant viruses for the baculovirus expression system, we used pFastBac (Invitrogen) to clone all Chs5p-like proteins, and pFastBac-HT was used to clone Chs5p, resulting in an Nterminal 6× His tag. Genes were cloned independently into constructs followed by transformation into DH10Bac competent cells for the production of recombinant bacmid vectors. Bacmid DNA was then transfected into Sf-9 insect cells, and viruses were amplified separately and kept frozen at ~8°C for later use; the same virus stocks were thawed from a small aliquot, and the virus titer remained constant. 500 ml of insect cultures (Sf-9) were infected with the indicated virus stock; for example, 500 μl of insect virus stock of His-Chs5, Chs6, Bch1, Bch7, and Bch2 was added, respectively, into the same culture to assemble the Chs5–Chs6[all] complex. Infected cultures were harvested after 4 d of growth at room temperature.
Cells were lysed in 20 ml of lysis buffer I (50 mM Hepes, pH 7.4, 450 mM KOAc, 0.1 mM EGTA, 20 mM imidazole, and 10% glycerol) supplemented with 1× protease inhibitor cocktail and 1 mM PMSF. The lysate was centrifuged at 12,000 rpm for 10 min (5534 rotor; Sorvall), and the resulting supernatant was incubated with 5 ml Ni-nitrotriacetic acid (NTA) agarose at 4°C for 3–4 h. Beads were washed first with 20 ml of lysis buffer I followed by washing twice with wash buffer II (50 mM MES, pH 6.5, 450 mM KOAc, 0.1 mM EGTA, 40 mM imidazole, 1% glycerol, and 1 mM PMSF), three additional washes with wash buffer III (50 mM Hepes, pH 7.4, 450 mM KOAc, 250 mM sorbitol, 0.1 mM EGTA, 40 mM imidazole, 10% glycerol, and 1 mM PMSF), and one wash with HKG (50 mM Hepes, pH 7.4, 50 mM KOAc, and 1% glycerol). Bound proteins were eluted with HKGI buffer (50 mM Hepes, pH 7.4, 50 mM KOAc, 10% glycerol, and 0.5 M imidazole), and the eluate was dialyzed against HKG with three changes of buffer. Precipitation occurred during dialysis, and the protein aggregates were removed by centrifugation at 14,000 rpm at 4°C for 5 min. The protocol results in a >90% pure protein complex at a protein concentration of ~0.5 mg/ml. If necessary, the complex was concentrated by loading a 1-ml aliquot of the Ni-NTA fraction on a 400-nm Nuclepore polycarbonate membrane.

Cytochalasin D (100 nM) was added to the membranes and allowed to bind for 5 min. The membranes were washed three times with ice-cold 20 mM Hepes, pH 7.4, 1.2 mM MgCl2, 2.5 mM MgAc, 300 mM NaCl, and 0.1 mM CaCl2. Membranes were incubated with 1 ml of a 1 mg/ml solution of purified complexes and with 1 ml of lysis buffer B88, 1× protease inhibitor cocktail, and 1 mM PMSF, for 1 h on ice. Samples were centrifuged using a TLA100.3 rotor (Beckman Coulter) at 55,000 rpm for 30 min before examination. Microscopy was performed using a fluorescence microscope (Eclipse E600; Nikon). Images were captured by a CCD camera (C4742-95; Hamamatsu) using Image-Pro software (Media Cybernetics). For thin section microscopy, we fixed a standard reaction as described in the Liposome recruitment assay section with 2% glutaraldehyde and 1% osmium tetroxide in cacodylate buffer for 1 h on ice. Samples were centrifuged using a TLA100.3 rotor (Beckman Coulter) at 55,000 rpm for 30 min. Membrane pellet fractions were processed for thin section electron microscopy as described previously (Orci et al., 1993).

Online supplemental material

Fig. S1 shows that Chs5p colocalized with Sec7p to the late Golgi compartment. Although the Chs5p-GFP/RFP signal was diffusely distributed at the restrictive temperature in the sec74 strain, other Golgi marker signals were focused and more exaggerated, indicating that the Golgi membrane did not disperse in the sec74 strain. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200605106/DC1.

We thank Drs. Eugene Futai, Jinoh Kim, and other members of the Schekman laboratory for discussion and encouragement and Bob Lesch and Crystal Chan for technical assistance. We thank Pierre Cosson for the suggestion of the name exomer as well as for improving the manuscript. We thank Drs. David Drubin and Georjana Barnes for use of their FPLC system and microscope, Jasper Rine for access to his fluorescence microscope (Eclipse E600), and images were captured by a CCD camera (C4742-95; Hamamatsu) using Image-Pro software (Media Cybernetics). For thin section microscopy, we fixed a standard reaction as described in the Liposome recruitment assay section with 2% glutaraldehyde and 1% osmium tetroxide in cacodylate buffer for 1 h on ice. Samples were centrifuged using a TLA100.3 rotor (Beckman Coulter) at 55,000 rpm for 30 min. Membrane pellet fractions were processed for thin section electron microscopy as described previously (Orci et al., 1993).

Submitted: 16 May 2006
Accepted: 18 August 2006

References

Ang, A.L., T. Taguchi, S. Francis, H. Folsch, L.J. Murrells, M. Pypaert, G. Warren, and I. Mellman. 2004. Recycling endosomes can serve as intermediates during transport from the Golgi to the plasma membrane of MDCK cells. J. Cell Biol. 167:531–543.

Antony, B., S. Berard-Foufour, P. Chardin, and M. Chabre. 1997. N-terminal hydrophobic residues of the G-protein ADP-ribosylation factor-1 insert into membrane phospholipids upon GDP to GTP exchange. Biochemistry. 36:4655–4664.

Antony, B., P. Gounon, R. Schekman, and L. Orci. 2003. Self-assembly of minimal COPII cages. EMBO Rep. 4:419–424.
A novel coat for traffic from the trans-Golgi network • Wang et al.