Dynamin and clathrin are required for the biogenesis of a distinct class of secretory vesicles in yeast

Sangiliyandi Gurunathan, Doris David and Jeffrey E. Gerst¹

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

¹Corresponding author
e-mail: jeffrey.gerst@weizmann.ac.il

Yeast produce two classes of secretory vesicles (SVs) that differ in both density and cargo protein content. In late-acting secretory mutants (e.g. *snc1^{ala43}* and *sec6-4*), both low- (LDSV) and high-density (HDSV) classes of vesicles accumulate at restrictive temperatures. Here, we have found that disruptions in the genes encoding a dynamin-related protein (*VPS1*) or clathrin heavy chain (*CHC1*) abolish HDSV production, yielding LDSVs that contain all secreted cargos. Interestingly, disruption of the *PEP12* gene, which encodes the t-SNARE that mediates all Golgi to pre-vacuolar compartment (PVC) transport, also abolishes HDSV production. In contrast, deletions in genes that selectively confer vacuolar hydrolase sorting to the PVC or protein transport to the vacuole (i.e. *VPS34* and *VAM3*, respectively) have no effect. Thus, one branch of the secretory pathway in yeast involves an intermediate sorting compartment and has a specific requirement for clathrin and a dynamin-related protein in SV biogenesis.

Keywords: clathrin/dynamin/secretory vesicles/yeast

Introduction

The biogenesis of carrier vesicles is an important aspect of membrane transport along the secretory pathway. At the core of the process is the delivery of cargo to sites of newly forming vesicles, the recruitment of coat proteins via activation of GTP-binding proteins, the ordered assembly of the coat, and the budding off of the vesicle (reviewed in Robinson, 1997; Springer *et al.*, 1999; Wieland and Harter, 1999). Carrier vesicles mediate the trafficking of both secreted and lysosomal/vacuolar cargo proteins early in the pathway [endoplasmic reticulum (ER) to Golgi], following which, proteins destined to reach the lysosome/vacuole are sorted separately in the *trans*-Golgi (reviewed in Rothman and Wieland, 1996; Schekman and Orci, 1996). Many lysosomal/vacuolar proteins contain specific sorting signals that deliver them via clathrin-coated vesicles to the late endosome/pre-vacuolar compartment (PVC) and from there to the lysosome/vacuole (reviewed in Traub and Kornfeld, 1997; Kirchhausen, 1999; Rohn *et al.*, 2000). In contrast, secretory vesicles (SVs), which are thought to be derived from the *trans*-Golgi, deliver mature secreted proteins to the plasma membrane (PM) (reviewed in Traub and Kornfeld, 1997; Gu *et al.*, 2001).

In the case of SVs, however, no proteinaceous coat has been shown to be required for their biogenesis (Gu *et al.*, 2001). Thus, neither clathrin nor the various adaptor complexes (e.g. AP1, AP2, AP3 and Gga) have been shown to play a direct role in the formation of vesicles involved in constitutive Golgi to PM transport (Seeger and Payne, 1992; Cowles *et al.*, 1997; Chen and Graham, 1998; Yeung *et al.*, 1999; Black and Pelham, 2000; Costaguta *et al.*, 2001).

Though less well studied than secretory granules, dense core vesicles and synaptic vesicles (reviewed in Hannah *et al.*, 1999; Cremona and De Camilli, 2001; Tooze *et al.*, 2001), which are required for the regulated delivery of specific cargo to the cell surface, SVs perform the bulk of constitutive Golgi to PM transport. In addition, they delineate multiple independent transport routes to the PM. For example, protein cargo may be sorted to different surfaces (i.e. apical versus basolateral) in polarized epithelial cells, as well as to the same surface in hepatocytes, using independent vesicle populations (Saucan and Palade, 1994; Yoshimori *et al.*, 1996; reviewed in Ikonen and Simons, 1998; Mostov *et al.*, 2000). Thus, there must exist unique sorting mechanisms and, perhaps, distinct molecular requirements for the biogenesis of SVs.

Biochemical and genetic studies using yeast have elucidated many of the genes and molecular mechanisms that underlie protein trafficking and secretion. Genetic screens for temperature-sensitive yeast mutants, defective at different steps along the secretory (e.g. *sec*), endosomal (e.g. *end*) and vacuolar transport pathways (e.g. *vps*, *vam* and *pep*), have defined a large number of gene products that function in vesicle biogenesis, transport, docking and fusion (Novick *et al.*, 1980; Munn and Riezmann, 1994; Wuestehube *et al.*, 1996; Bryant and Stevens, 1998). To isolate the original *sec* mutants, alterations in the buoyant density of yeast were used to identify cells deficient in exocytosis and which accumulated secreted cargo (e.g. invertase) when shifted to restrictive conditions. Initial cell fractionation studies demonstrated that one type of vesicle of 100–120 nm could be purified by column chromatography from lysates derived from late-acting *sec* mutants (Walworth *et al.*, 1987). However, it was shown later that exocytic cargo is, in fact, transported by at least two routes (Harsay and Bretscher, 1995). Late-acting *sec* and *snc* mutants, which are deficient in Golgi to PM transport, accumulate two distinct types of SVs of similar size. One type is of higher density (HDSVs) and contains the soluble secreted enzymes invertase and acid phosphatase, as well as the bulk of exoglucanase activity, while the other is of lower density (LDSVs) and contains a plasma membrane H⁺-ATPase activity among its cargo (Harsay and Bretscher, 1995; David *et al.*, 1998). Integral membrane proteins involved in vesicle targeting and fusion, including

the Snc1,2 v-SNAREs and Sso1,2 t-SNAREs, are present in both vesicle types (David *et al.*, 1998; Lustgarten and Gerst, 1999). Thus, the secretory pathway in yeast is bifurcated, but uses shared trafficking components. Moreover, the endocytic pathway is not involved directly in SV biogenesis, as an *end4* mutation that abolishes receptor-mediated endocytosis does not block LDSV or HDSV formation (Harsay and Bretscher, 1995).

The membrane of origin for the SVs that accumulate in late-acting *sec* and *snc* mutants is thought to be the trans-Golgi, as secreted proteins undergo processing in the Golgi and mutations that block vacuolar protein sorting (VPS) do not block exocytosis. However, several works have suggested that a post-Golgi intermediate compartment could play a role in the trafficking of secreted proteins in yeast. For example, the Chs3 chitin synthase is distributed between the plasma membrane and its own membrane-bound compartment, called the chitosome (Chuang and Schekman, 1996; Ziman *et al.*, 1998). Also, the loading of copper onto Fet3, a ceruloplasmin-like protein that is involved in iron transport from the cell surface, was shown to require endosomal transport (Yuan *et al.*, 1997). Finally, a mutant form of the Pma1 H⁺-ATPase is trafficked directly to the cell surface in yeast bearing a mutation in *VPS1*, which abolishes all Golgi to endosome transport (Luo and Chang, 2000). In contrast, Pma1 is trafficked via a PVC-like compartment in cells bearing mutations in other genes (e.g. *VPS8*, *VPS36*, etc.) that are involved in endosome to Golgi retrograde trafficking. Thus, Pma1 delivery to the PM may involve SVs derived from an intermediate compartment.

To determine whether endosomal sorting pathways are involved in the biogenesis of SVs, we created yeast with mutations in both late-acting secretory genes and genes involved in vacuolar protein sorting. Here, we show that a deletion in the *VPS1* gene, which encodes the dynamin-related GTPase, abolishes formation of the HDSV class of vesicles in *snc1^{ala43}* and *sec6* cells at restrictive temperatures. A marked reduction in the formation of SVs was observed and the enzymatic activities used to identify the HDSV class of vesicles were shown to sediment now at the density corresponding to the LDSV class. Co-precipitation experiments also revealed that both HDSV and LDSV cargo are transported together in the same vesicles. Thus, biogenesis of the HDSV class is blocked in these mutants. Identical results were also obtained with cells lacking the clathrin heavy chain and the Pep12 Golgi to endosome t-SNARE. In contrast, mutations in genes required for the specific sorting of hydrolases to the vacuole did not affect HDSV biogenesis. Thus, one branch of the exocytic pathway in yeast utilizes a dynamin-related protein and clathrin, as well as an intact Golgi to endosome sorting pathway.

Results

Disruption of *VPS1* in *snc* null cells leads to synthetic lethality

Yeast late secretory mutants (e.g. *sec6*, *sec9* and *sncΔ*) accumulate two distinct populations of exocytic vesicles, LDSVs and HDSVs (Harsay and Bretscher, 1995; David *et al.*, 1998; Lustgarten and Gerst, 1999). To determine whether the vacuolar protein sorting and secretory path-

ways overlap, we disrupted representative *VPS* genes involved in protein (i.e. CPY) sorting to the vacuole in these secretion mutants, and examined them for synthetic defects. We chose two of three genes that have been implicated in the delivery of vacuolar hydrolase-containing carrier vesicles from the Golgi to the PVC, *VPS1* and *VPS34*. *VPS1* encodes a Golgi-localized dynamin-related protein that is required for the biogenesis of both clathrin-coated vesicles destined to reach the PVC and non-clathrin-coated vesicles involved in the delivery of alkaline phosphatase (ALP) to the vacuole (Nothwehr *et al.*, 1995; Bryant and Stevens, 1998). Mutations in *VPS1* result in the secretion of vacuolar hydrolases and ALP, but do not result in defects in endocytosis. *VPS34* encodes a phosphatidylinositol 3-kinase (PI 3-kinase) that is found in a hetero-oligomeric complex with the *VPS15* gene product, which is required for the delivery of vacuolar hydrolases to the PVC (Stack and Emr, 1994). Mutations in *VPS34* result in the secretion of hydrolases, but also result in defects in autophagy and endocytosis (Bryant and Stevens, 1998; Kihara *et al.*, 2001). In addition, we also examined *VAM3*, which encodes a vacuole-localized multifunctional t-SNARE involved in the docking and fusion of both CPY-containing and ALP-containing vesicles (Darsow *et al.*, 1997). Mutations in *VAM3* block both Vps-dependent transport of hydrolases from the PVC to the vacuole, as well as the ALP transport route that bypasses the PVC and delivers proteins directly to the vacuole (reviewed in Bryant and Stevens, 1998).

Disruptions of *VPS1*, *VPS34* and *VAM3* were made in yeast lacking the *SNC* genes. This strain has been well characterized and has been used to show that Snc1 and -2 are the v-SNAREs that confer both exocytosis and endocytosis (Protopopov *et al.*, 1993; Gurunathan *et al.*, 2000). Cells lacking the *SNC* genes grow poorly, are defective in secretion and accumulate both LDSVs and HDSVs constitutively. These phenotypes are suppressed either by employing a galactose-inducible *SNC1* gene (and growing cells on galactose-containing medium) or by introducing a temperature-sensitive *SNC1* gene (*snc1^{ala43}*) into the strain (Protopopov *et al.*, 1993; Gurunathan *et al.*, 2000).

snc vps1Δ, *snc vps34Δ* and *snc vam3Δ* cells were created by transformation and examined for growth and the secretion of CPY under conditions in which *SNC1* was expressed (on galactose-containing medium) (Figure 1). We found that *snc vps1Δ*, *snc vps34Δ* and *snc vam3Δ* cells all secreted CPY onto nitrocellulose filters, while *snc* cells expressing *SNC1* did not (Figure 1A). Thus, mutations in *VPS/VAM* genes result in the missorting of CPY, as expected. In the absence of *SNC1* induction, *snc* null cells fail to grow at 37°C and on amino acid-rich medium (YPD), but are viable at ≤30°C (Protopopov *et al.*, 1993). We next examined the growth of *snc vps1Δ*, *snc vps34Δ* and *snc vam3Δ* cells under different conditions (Figure 1B). All cells were found to be temperature sensitive at 37°C in the presence of *SNC1* expression (on galactose), due to the presence of the *vps* mutations. However, only *snc vps1Δ* cells were unable to grow at all in the absence of *SNC1* expression (i.e. on glucose-containing medium) at any temperature. This observed synthetic lethality suggests that the *SNC* and *VPS1*

Fig. 1. Disruption of certain *VPS* genes leads to synthetic lethality in *snc* cells. (A) *vps* mutations lead to the secretion of CPY in *snc* cells. *snc* cells (JG8) carrying a control plasmid, a plasmid expressing *SNC1*, or having a deletion in a *VPS* gene (e.g. *VPS1*, *VPS34* and *VAM3*; strains JG9-VPS1, JG9-VPS34 and JG9-VAM3) were patched onto selective medium containing galactose. Following growth, cells were replica plated onto nitrocellulose filters and grown on the same medium for 36 h, prior to western blotting with anti-CPY antibody. (B) Deletion of *VPS1* results in synthetic lethality in *snc* cells. The growth of *snc* null cells (JG8) carrying a control plasmid or bearing disruptions in *VPS1*, *VPS34* or *VAM3* genes (strains JG9-VPS1, JG9-VPS34 and JG9-VAM3) was compared. All strains carry a galactose-inducible *SNC1* expression plasmid and were grown on galactose-containing medium (Gal) and then replica plated onto glucose medium (Glu) for 24 h (to deplete *Snc1*). Next, yeast were replica plated onto either galactose- or glucose-containing media at different temperatures, or onto amino acid-rich medium (YPD), and were allowed to grow for 48 h. (C) Expression of *snc1^{ala43}* suppresses the synthetic lethality seen in *snc vps1Δ* mutants. *snc* and *snc vpsΔ* mutants were transformed with a centromeric plasmid expressing *snc1^{ala43}* and tested for temperature sensitivity. Cells were grown for 36 h after replica plating. (D) Deletion of *CHC1* or *PEP12* results in synthetic lethality in *snc* cells. The growth of *snc* null cells (JG8) carrying a control plasmid or bearing disruptions in the *CHC1* or *PEP12* genes (JG9-CHC1 and JG9-PEP12) was compared, as described above (B). *snc* null cells carrying a constitutive *SNC1* expression plasmid (pADH-SNC1) were also used as controls.

products function together to bring about an essential process.

We next tested whether expression of the temperature-sensitive *snc1^{ala43}* allele can suppress the synthetic lethality seen between *snc* and *vps1Δ* mutations (Figure 1C). We found that *snc1^{ala43} vps1Δ* cells remained viable up to 35°C, as shown previously for *snc1^{ala43}* cells (Gurunathan *et al.*, 2000). Thus, *snc1^{ala43} vps1Δ* cells can be used to examine the effects of the *vps1Δ* mutation upon vesicle biogenesis.

Fewer SVs are produced in *snc vps1* cells

To determine whether any of the *vps* mutations alter the accumulation of SVs in *snc* cells, we examined the morphology of *snc vps1Δ*, *snc vps34Δ* and *snc vam3Δ* cells by thin-section electron microscopy after shifting the cells to glucose-containing medium for 16 h to deplete *Snc1* (half-life ~10 h) (Figure 2). *snc* cells typically accumulate 100 nm vesicles of the order of 15 vesicles/μm² in the

Fig. 2. Thin-section electron microscopy of *snc vps* mutants. *snc*, *snc vps1Δ*, *snc vps34Δ* and *snc vam3Δ* mutants (JG8, JG9-VPS1, JG9-VPS34 and JG9-VAM3) carrying a galactose-inducible *SNC1* gene were grown to log phase on galactose-containing media and then shifted to glucose-containing media for 16 h. Cells were harvested, fixed and subjected to thin-sectioning and electron microscopy. Control = *snc* cells. Arrows indicate areas of SV accumulation. Bars = 1 μm. Insets in the upper left-hand corners show enlarged regions from cells shown in the figure.

absence of *SNC1* expression (Lustgarten and Gerst, 1999; Marash and Gerst, 2001), and have fragmented, though functional, vacuoles (Protopopov *et al.*, 1993; David *et al.*, 1998). We found that all *snc vps* mutants accumulated aberrant membranous structures, including fragmented vacuolar-like bodies (Figure 2). In addition, all mutants accumulated vesicles that are of the order of SVs in size, though they differed in the number of vesicles present per unit area. We found that *snc vps1Δ* cells had only 7.2 ± 0.6 vesicles/μm², while *snc*, *snc vam3Δ* and *snc vps34Δ* cells had 14.1 ± 0.7 , 11.8 ± 1 and 12.3 ± 0.7 vesicles/μm², respectively. Thus, the *vps1Δ* mutation strongly influences the number of SVs formed.

Only LDSVs accumulate in *snc1^{ala43} vps1* mutants

As introduction of the *vps1Δ*, but not *vps34Δ*, mutation in the late-acting *snc* secretory mutant results in synthetic lethality and decreases the formation of SVs, we reasoned that this dynamin-related protein might be required for SV biogenesis while *Vps34* is not. To prove this, we purified SVs from *snc1^{ala43}*, *snc1^{ala43} vps1Δ*, *snc1^{ala43} vps34Δ* and *snc1^{ala43} vam3Δ* cells by cell fractionation and density gradient centrifugation. Log phase cultures were moved to low phosphate- and low glucose-containing medium to induce the production of acid phosphatase and invertase, which serve as markers for the HDSV class of vesicles. To induce vesicle formation, pre-warmed (37°C) medium was used, while control cells were maintained at 26°C.

We found that both LDSVs and HDSVs accumulate in temperature-shifted *snc1^{ala43}* cells (Figure 3A), as shown

Fig. 3. The HDSV peak is absent in SV preparations from *snc1^{ala43}vps1* cells. SVs were purified from temperature-shifted (37°C) and non-shifted (26°C) *snc1^{ala43}* (A; JG8-SNC1A43T) and *snc1^{ala43} vps1Δ* (B; JG9-VPS1A43K) cells by Nycodenz density gradient centrifugation (see Materials and methods). Density, protein concentration and the activities of various enzymatic markers were assayed and plotted. Acid phosphatase and exoglucanase activities are expressed in arbitrary units based upon the absorbance measured at 415 nm, ATPase activity in arbitrary units based upon the absorbance measured at 820 nm and invertase activity in arbitrary units based upon the absorbance measured at 540 nm.

previously for *snc*, *sec6* and *sec9* cells (Harsay and Bretscher, 1995; David *et al.*, 1998; Lustgarten and Gerst, 1999). The densities at which the LDSVs (as detected by H⁺-ATPase activity) and HDSVs (as detected by invertase, acid phosphatase and exoglucanase activities) sediment were 1.148 and 1.164 g/ml Nycodenz, which were similar to those reported previously (Harsay and Bretscher, 1995; David *et al.*, 1998; Lustgarten and Gerst, 1999). For easy

comparison, we note that the LDSVs typically elute between fractions 8 and 10, while HDSVs elute in fractions 16–18. Identical results were obtained with temperature-shifted *snc1^{ala43} vps34Δ* and *snc1^{ala43} vam3Δ* cells, which also accumulated LDSVs at 1.147 and 1.147 g/ml and HDSVs at 1.168 and 1.165 g/ml, respectively (Figure 4). Thus, neither *vps34Δ* nor *vam3Δ* mutations affect SV biogenesis in yeast. Surprisingly, we found

Fig. 4. The HDSV peak is present in SV preparations from *snc1^{ala43}vps34Δ* and *snc1^{ala43}vam3Δ* cells. SVs were purified from (A) temperature-shifted (37°C) and non-shifted (26°C) *snc1^{ala43}vps34Δ* (JG9-VPS34A43K) and (B) *snc1^{ala43}vam3Δ* (JG9-VAM3A43K) cells by Nycodenz density gradient centrifugation. Density, protein concentration and enzymatic activities are represented as in Figure 3.

no peak of invertase, acid phosphatase or exoglucanase activities present in fractions that correspond to the HDSV class of vesicles in *snc1^{ala43}vps1Δ* cells (Figure 3B). In contrast, the peak activities of these markers were found to elute at 1.148 g/ml Nycodenz, which corresponds to the LDSV class. Thus, it would appear that either the enzymes of the HDSV class are sorted to the LDSVs or the biogenesis of the HDSVs is blocked entirely in *vps1* mutants.

To determine whether production of the HDSV class is blocked specifically in *snc1^{ala43}vps1Δ* cells, we examined

the membranes present in the different fractions by uranyl acetate staining and electron microscopy (Figure 5). In *snc1^{ala43}*, *snc1^{ala43}vps34Δ* and *snc1^{ala43}vam3Δ* cells, we found that many (thousands) 100 nm vesicles were apparent in the fractions that correspond to either the LDSV or HDSV classes (Figure 5). Both the LDSVs and HDSVs are similar in size and appearance to one another and, thus, cannot be distinguished except by density gradient centrifugation (Harsay and Bretscher, 1995; David *et al.*, 1998). In contrast, few, if any, vesicles were ever observed on grids containing fractions from

Fig. 5. Absence of SVs in the high-density fractions obtained from *snc1^{ala43}vps1Δ* cell preparations. Aliquots of fractions corresponding to the peaks of enzymatic activity were processed for negative staining, as described in Materials and methods. Representative samples of the LDSV and HDSV fractions from *snc1^{ala43}*, *snc1^{ala43} vps1Δ*, *snc1^{ala43} vps34Δ* and *snc1^{ala43} vam3Δ* cells (JG8-SNC1A43T, JG9-VPS1A43K, JG9-VPS34A43K and JG9-VAM3A43K) are shown. Bars = 100 nm.

snc1^{ala43} vps1Δ cells that correspond to the HDSV class (Figure 5). Thus, production of the HDSV class is blocked entirely in the absence of Vps1.

LDSV and HDSV markers are present in the same vesicle in *vps1* cells

As no HDSV class of vesicle is produced in *snc1^{ala43} vps1Δ* cells, it was important to determine whether biogenesis of the HDSVs was completely abolished or simply that their buoyant density became altered. In the case of the former, it would be expected that the HDSV enzymatic markers would be present in the same vesicle as the LDSV markers. To examine this, a hemagglutinin (HA)-tagged form of Pma1 (a membranal LDSV marker) was introduced into a *sec6 vps1Δ* strain created for this experiment. Disruption of *VPS1* in the *sec6* background gave results identical to *snc1^{ala43} vps1Δ* cells with respect to the accumulation of only the LDSV population at restrictive temperatures (Figure 6B). In contrast, both populations of vesicles were present in temperature-shifted *sec6* cells (Figure 6A).

Purified LDSVs from *sec6 vps1Δ* cells were subjected to immunoprecipitation (IP) with anti-HA antibodies and resolved by SDS-PAGE and western blotting. The resulting blots were then detected for both Pma1 and invertase (a luminal HDSV marker) (Figure 7). We

found that invertase co-precipitated with the Pma1 marker, suggesting that both proteins are carried in the same vesicle population. About 70% of available invertase co-precipitated with Pma1, after normalization for Pma1 recovery. This co-precipitation was essentially abolished upon the addition of Triton X-100 to the IP buffer. Likewise, invertase did not co-precipitate with Pma1 from vesicle preparations made from *sec6 vps1Δ* cells that did not express HA-tagged Pma1, nor from mixed vesicle preparations derived from *sec6* cells expressing the tagged protein. Together with the data showing reduced SV production in *snc vps1Δ* yeast (Figure 2), it appears that HDSV biogenesis is blocked in these cells and that only one population of exocytic vesicles is formed.

Biogenesis of HDSVs is blocked in *chc1Δ* and *pep12Δ* mutants

As mutations in *VPS1* block HDSV biogenesis, resulting in the production of a single class of vesicle, we examined the involvement of other key genes that act upon endosomal sorting. First, since Vps1/dynamin is required for both the clathrin-dependent and -independent sorting of proteins to the vacuole, we examined whether mutations in the clathrin heavy chain gene (*CHC1*) affect HDSV biogenesis. Disruption of *CHC1* in yeast is not lethal, but strongly affects vacuolar protein sorting (Seeger and Payne, 1992). We found that a null mutation in *CHC1* also led to synthetic lethality in *snc* null cells (Figure 1D), as seen with the *vps1Δ* mutation. Thus, clathrin may also be involved in SV biogenesis. To test this further, we created a *sec6 chc1Δ* strain, which is viable at 26°C (data not shown), and purified SVs from temperature-shifted cells (Figure 8A). We found that like *sec6 vps1Δ* cells, *sec6 chc1Δ* yeast accumulated only LDSVs at the restrictive temperature (peak invertase, acid phosphatase and exoglucanase enzymatic activities were present at ~1.149 g/ml Nycodenz). Thus, clathrin heavy chain is required for HDSV production.

Since mutations in *VPS1* and *CHC1* both block HDSV production, it suggests that Vps1/dynamin and clathrin are required for the biogenesis of this vesicle population in yeast. However, it is unclear whether these vesicles are derived directly from the Golgi or from an intermediate compartment. As the *vps34Δ* mutation has no effect upon HDSV biogenesis, it suggests that such a compartment might not be involved. To test this directly, however, we deleted *PEP12*, which encodes an endosome-localized t-SNARE involved in the fusion of hydrolase-containing vesicles with the PVC (Becherer *et al.*, 1996; Bryant and Stevens, 1998), in *snc* and *sec6* cells. We presumed that mutations in *PEP12* would have an effect if HDSV cargo is normally targeted to the PVC, prior to sorting and packaging into the HDSVs. We found that *snc pep12Δ* cells were inviable on glucose-containing medium at all temperatures (Figure 1D) and that vesicles purified from *sec6 pep12Δ* cells sedimented at the density corresponding to LDSVs (peak invertase, acid phosphatase and exoglucanase enzymatic activities were present at 1.151 g/ml Nycodenz) (Figure 8B). Thus, Pep12, a t-SNARE involved in Golgi to PVC transport, is essential for the formation of HDSVs, implying that both vacuolar

Fig. 6. The HDSV peak is absent in SV preparations from *sec6 vps1Δ* cells. SVs were purified from (A) temperature-shifted (37°C) *sec6* cells (NY778) and (B) both shifted and non-shifted (26°C) *sec6 vps1Δ* (SG1) cells by Nycodenz density gradient centrifugation. Density, protein concentration and enzymatic activities are represented as in Figure 3.

and HDSV cargo proteins are sorted to the PVC, prior to segregation.

Biogenesis of HDSVs is blocked in a *chc1* temperature-sensitive strain

Although deletions in the *VPS34* and *VAM3* genes do not affect HDSV biogenesis, we could not entirely rule out the possibility that pleiotropic effects arising from the *VPS1*, *CHC1* or *PEP12* gene disruptions somehow lead to this block. To eliminate this possibility, we created a *sec6* strain bearing a temperature-sensitive mutation in *CHC1*

by integration of the *chc1-521* allele into the *CHC1* locus. Unlike *sec6* cells, *sec6 chc1-521* cells secrete CPY onto filters at restrictive temperatures (data not shown). We next made vesicle preparations from temperature-shifted and non-shifted cells, and examined the distribution of enzymatic markers therein by density gradient centrifugation and biochemical analysis (Figure 9). Like that found for *sec6 chc1Δ* mutants (Figure 8A), vesicles purified from temperature-shifted *sec6 chc1-521* cells also sedimented at the density corresponding to LDSVs (e.g. peak invertase, acid phosphatase and exoglucanase activities were found

Fig. 7. LDSV and HDSV markers co-precipitate in vesicles derived from *sec6 vps1Δ* cells. (A) Aliquots of vesicles from the LDSV peak derived from *sec6 vps1Δ* cells (SG1) expressing HA-tagged Pma1 (*sec6 vps1Δ PMA1HA*) were subjected to immunoprecipitation (IP) using anti-HA antibodies. IPs were performed either in the presence (+) or absence (–) of added HA peptide (75 μg), as a competitive blocker. Following western blotting, blots were probed with either anti-HA antibodies (to detect Pma1) or anti-invertase antibodies, as shown. Equal amounts of vesicles were electrophoresed and detected in parallel (Input). (B) As control, IPs of vesicles from *sec6 vps1Δ* cells expressing HA-tagged Pma1 (*sec6 vps1Δ PMA1HA*) were also performed in the presence (+) or absence (–) of Triton X-100 (1%). (C) As an additional control, IPs were performed on vesicles derived from *sec6 vps1Δ* cells not expressing HA-tagged Pma1 (*sec6 vps1Δ*). (D) As a final control, IPs of vesicles derived from *sec6* cells (NY778) expressing HA-tagged Pma1 (*sec6 PMA1HA*) were also performed in the presence (+) or absence (–) of HA peptide. Samples from both LDSV and HDSV populations were mixed together prior to IP.

at 1.144 g/ml Nycodenz). Thus, a block in HDSV biogenesis can be demonstrated even in a temporal fashion.

CPY is secreted by the LDSV population

As *vps* mutants mis-sort and secrete CPY, this hydrolase should be present in SVs derived from these cells. To determine in which population CPY is secreted, we used western analysis to probe samples of the density gradients prepared using temperature-shifted *snc1^{ala43} vps1Δ* and *sec6 vps1Δ* cells, which accumulate only LDSVs, as well as *snc1^{ala43} vps34Δ* cells, which accumulate both populations.

In *snc1^{ala43} vps1Δ* cells, we found that both the p2 form of CPY and its receptor, Vps10, are present in vesicles and elute in those fractions corresponding to the LDSVs (Figure 10). Other secreted cargo molecules, such as the GPI-anchored protein, Gas1, the Snc v-SNAREs, and the Sso t-SNAREs also elute in the LDSV fractions. Finally, both Pma1 and invertase, which we have shown to co-precipitate (Figure 8), were found to be present in LDSV fractions derived from *sec6 vps1Δ* cells (Figure 10). Thus, as expected, all mis-sorted and secreted cargos are present in the LDSVs when HDSV biogenesis is abolished.

In *snc1^{ala43} vps34* cells, wherein both SV populations accumulate upon temperature shifting, we found that both p2 CPY and Vps10 remain in those fractions corresponding to the LDSVs. In contrast, Sso, Snc and Gas1 are spread throughout the LDSV and HDSV peaks, and invertase is enriched in the late fractions (Figure 10), as expected. Thus, CPY and Vps10 are secreted via the LDSV population of vesicles even when HDSV biogenesis is intact.

Discussion

Because the secretory and endosomal sorting pathways overlap in yeast, we examined whether *vps* mutations affect the production of SVs in cells bearing late-acting *sec* or *snc* mutations. Since the molecular requirements for SV biogenesis are unknown, this would seem to be an ideal way of determining what these requirements might be. Here we have shown that the *VPS1* gene, which encodes an 80 kDa yeast dynamin-like GTPase, is required for the production of a single class of SVs in yeast. Disruption of *VPS1* in a strain bearing a late-acting secretion defect (*snc* null cells) led to synthetic lethality and a large reduction in the number of vesicles observed by electron microscopy (Figures 1B and 2). Moreover, the disruption of *VPS1* in cells bearing a temperature-sensitive *SNC1* allele resulted in the accumulation of only one class of SV in density gradients, after cells were shifted to the restrictive temperature (Figure 3). Finally, both LDSV and HDSV cargo proteins were found to co-precipitate in vesicle preparations derived from *sec6 vps1Δ* cells (Figure 7). Thus, Vps1 is required for the biogenesis of the HDSV class of vesicles. In contrast, no requirement for Vps34, a PI 3-kinase necessary for the transport of hydrolases to the PVC/late endosome, could be shown for SV biogenesis. Therefore, we must consider a role for dynamin in the derivation of transport vesicles that deliver soluble secreted enzymes to the cell surface.

As a null mutation in *VPS34* does not compromise LDSV and HDSV biogenesis and the amount of vesicles produced (Figures 2 and 4), it is likely that this lipid kinase plays no role in SV formation. Mutations in *VPS34* clearly affect the trafficking of hydrolases to the vacuole, as well as endocytosis and autophagy, but a normal vacuolar structure is maintained in the absence of the protein (Stack and Emr, 1994; Bryant and Stevens, 1998; Kihara *et al.*, 2001). Therefore, the role of Vps34 (and PI-3P) could be in the sorting of hydrolases to vesicles destined to reach the PVC or in the formation of specific vesicles that transport the hydrolases. Neither possibility rules out the likelihood that an intermediate compartment is involved in the trafficking of secreted proteins to the cell surface, of which there is some evidence (Chuang and Schekman, 1996; Yuan *et al.*, 1997; Ziman *et al.*, 1998; Luo and Chang, 2000). On the contrary, we found that a mutation in *PEP12*, which encodes the t-SNARE required for all Golgi to PVC transport, completely blocks HDSV production (Figure 8B), while a mutation in the *VAM3* vacuolar t-SNARE gene does not (Figure 4). Thus, it would seem likely that HDSV-destined cargo is sorted to the PVC prior to being packaged into HDSVs. That Vps34 is not involved in this process is, therefore, suggestive of the

Fig. 8. The HDSV peak is absent in SV preparations from *sec6 chc1Δ* and *sec6 pep12Δ* cells. SVs were purified from (A) temperature-shifted (37°C) *sec6 chc1Δ* cells (SG3) and (B) *sec6 pep12Δ* (SG2) cells by density gradient centrifugation. Density, protein concentration and enzymatic activities are represented as in Figure 3.

idea that it functions only in cargo (e.g. hydrolase) selection and not in vesicle biogenesis *per se*.

Vps1/dynamin is required for all biosynthetic transport to the vacuole, including the clathrin-independent, AP3-mediated, ALP route as well as the clathrin-dependent, AP1-mediated, CPY (hydrolase) route (reviewed in Bryant and Stevens, 1998). In addition, we now demonstrate that Vps1 plays an important role in HDSV biogenesis, along with clathrin. Thus, this work demonstrates, perhaps for the first time, the involvement of both dynamin and clathrin in the constitutive exocytic pathway and lends credence to the idea that coat proteins may be necessary

for the biogenesis of some SV types (see model, Figure 11). Yet, is their role in SV biogenesis direct? For example, it may be that Vps1 and clathrin are required only for the transport of secreted (and vacuolar) cargo from the Golgi to the PVC. HDSVs containing secreted cargo molecules may then be derived from the PVC in both a clathrin- and Vps1-independent fashion. Since biogenesis of the LDSV class appears to occur independently of clathrin and Vps1, it is possible that HDSVs are derived in the same way.

In contrast to the HDSV route, our work suggests that the second (LDSV) route to the cell surface is dynamin and clathrin independent, and is the default path when the

Fig. 9. The HDSV peak is absent in SV preparations from temperature-shifted *sec6 chc1-521* cells. SV preparations were obtained from temperature-shifted (2 h, 37°C) or non-shifted (26°C) *sec6 chc1-521* cells (SG4) by Nycodenz density gradient centrifugation. Density, protein concentration and enzymatic activities are represented as in Figure 3.

Fig. 10. CPY is secreted by the LDSV population of vesicles. Aliquots of fractions obtained through density gradient centrifugation were subjected to SDS-PAGE. Following western blotting, blots containing samples from various preparations (e.g. *sec6 vps1Δ*, *snc1^{ala43} vps1Δ* and *snc1^{ala43} vps34Δ*) were probed with antibodies against various proteins. Concentrations included: anti-HA (1:1000, for Pma1); anti-invertase (1:1000); anti-Sso (1:3000); anti-Vps10 (1:5000); anti-Snc (1:5000); anti-Gas1 (1:5000); and anti-CPY (1:1000). Detection was performed by chemiluminescence. Samples of total cell lysates (TCL) from temperature-shifted *sec18* cells and *pep4Δ* cells were used to show the different forms of CPY [i.e. p2 and mature (m)].

Golgi to PVC route is blocked (i.e. in *vps1*, *pep12* and *chc1* mutants). The fact that no significant block in exocytosis has been observed in these mutants probably stems from the fact that the LDSV route is unaffected. Thus, *vps1*,

Fig. 11. A model for secretory vesicle biogenesis in yeast. The exocytic pathway in yeast is bifurcated. Proteins destined to be secreted from the cell can be trafficked from the Golgi to the cell surface either by LDSVs, low density secretory vesicles (typified by the Pma1 H⁺-ATPase), or by HDSVs, high density secretory vesicles (typified by invertase or acid phosphatase). Proteins normally secreted via the HDSV route require an intact Golgi to endosome (PVC) sorting pathway. Mutations that inhibit either the biogenesis (e.g. *chc1* or *vps1*) or docking and fusion (e.g. *pep12*) of carrier vesicles that confer Golgi to endosome trafficking abolish not only hydrolase (i.e. CPY) sorting, but also the HDSV sorting route. Under such circumstances, both vacuolar and secreted cargo proteins are exported from the cell via the LDSV route. Mutations that block hydrolase sorting to carrier vesicles (e.g. *vps34*) or endosome to vacuole sorting (e.g. *vam3*) have no effect upon HDSV biogenesis. None of the mutations tested affected biogenesis of the LDSV vesicles, indicating that this default route is independent of known Golgi to endosome sorting requirements.

pep12 and *chc1* were never identified as *sec* mutants in earlier screens. Interestingly, when the trafficking of LDSVs is inhibited, cells grow more slowly. For example, overexpression of *VSM1*, which encodes a protein that binds directly to the Snc v-SNAREs and induces the accumulation of LDSVs in *sec9-4* t-SNARE mutants, strongly inhibits cell growth (Lustgarten and Gerst, 1999). It would appear, however, that as long as one branch of the

Table I. Strains used in this study

Name	Genotype	Source
SP1	<i>MATa can1 his3 leu2 trp1 ura3 ade8</i>	M.Wigler
JG8	<i>MATa his3 his4 leu2 trp1 snc1::URA3 snc2::ADE8</i> pTGal-SNC1 or pLGal-SNC1	J.Gerst
JG8-SNC1A43L	<i>MATa his3 his4 leu2 trp1 snc1::URA3 snc2::ADE8</i> pLADH-SNC1 ^{ala43}	J.Gerst
JG8-SNC1A43T	<i>MATa his3 his4 leu2 trp1 snc1::URA3 snc2::ADE8</i> pTADH-SNC1 ^{ala43}	J.Gerst
JG9-VPS1	<i>MATa his3 his4 trp1 snc1::URA3 snc2::ADE8 vps1::LEU2</i> pTGal-SNC1	this study
JG9-VPS1A43K	<i>MATa his3 his4 trp1 snc1::URA3 snc2::ADE8 vps1::LEU2</i> pKADH-SNC1 ^{ala43}	this study
JG9-VPS34	<i>MATa his3 his4 trp1 snc1::URA3 snc2::ADE8 vps34::LEU2</i> pTGal-SNC1	this study
JG9-VPS34A43K	<i>MATa his3 his4 trp1 snc1::URA3 snc2::ADE8 vps34::LEU2</i> pKADH-SNC1 ^{ala43}	this study
JG9-VAM3	<i>MATa his3 leu2 trp1 snc1::URA3 snc2::ADE8 vam3::LEU2</i> pTGal-SNC1	this study
JG9-VAM3A43K	<i>MATa his3 his4 trp1 snc1::URA3 snc2::ADE8 vam3::LEU2</i> pKADH-SNC1 ^{ala43}	this study
JG9-PEP12	<i>MATa his3 his4 trp1 snc1::URA3 snc2::ADE8 pep12::LEU2</i> pTGal-SNC1	this study
JG9-CHC1	<i>MATa his3 his4 trp1 snc1::URA3 snc2::ADE8 chc1::LEU2</i> pTGal-SNC1	this study
BJ5462	<i>MATa ura3 trp1 pep4::HIS3 prb1Δ can1 GAL</i>	G.Galili
NY1217	<i>MATa ura3 leu2 sec18-1</i>	P.Novick
NY778	<i>MATa ura3 leu2 sec6-4</i>	P.Novick
SG1	<i>MATa ura3 sec6-4 vps1::LEU2</i>	this study
SG2	<i>MATa ura3 sec6-4 pep12::LEU2</i>	this study
SG3	<i>MATa ura3 sec6-4 chc1::LEU2</i>	this study
SG4	<i>MATa leu2 sec6-4 chc1::chc1-521::URA3</i>	this study

secretory pathway is intact, yeast remain viable. On the other hand, late-acting *sec*-type mutants die at restrictive temperatures because both branches of the exocytic pathway are inactivated due to the loss of a shared component.

Many questions remain unresolved. For example, we do not know the compartment of origin for the LDSV class of vesicles. They may either be Golgi derived or may originate from a second endosomal compartment, but in a Vps1- and clathrin-independent fashion. Support for an endosomal source of the LDSVs comes from the work of Luo and Chang (2000), who showed that Pma1 (an LDSV marker) is trafficked through an intermediate compartment. Perhaps, then, both exocytic routes in yeast involve the sorting of secreted cargo molecules through different types of endosomes. If so, there should be distinct molecular requirements (i.e. targeting signals) for the sorting of proteins to the HDSV class of vesicle, vis à vis the LDSVs. Another question that arises is why is there a need for two types of SVs originating from two different paths? Since some secreted proteins require proteolytic processing prior to secretion (e.g. α -mating factor), the existence of multiple endocytic compartments might allow for the controlled segregation of those proteins that are activated by processing and those that might otherwise be inactivated. This idea is supported by the fact that Kex2, a proteolytic enzyme involved in α -mating factor maturation, is sorted along with Vps10 in clathrin-coated vesicles to the PVC (Deloche *et al.*, 2001). Finally, it is clear that mutations in genes encoding other proteins directly involved in Golgi to PVC vesicle biogenesis (i.e. components of the AP1 or Gga coats) and transport (i.e. Vps21, Vps45, etc.) should abolish production of the HDSV class of vesicles. This will be the subject of further investigations.

Overall, the existence of intermediate compartments on exocytic routes appears to be conserved in evolution. For example, an endosome to PM trafficking pathway in mammalian cells has already been established. In particu-

lar, newly synthesized transferrin receptors (Futter *et al.*, 1995), asialoglycoprotein receptor H1 (Leitinger *et al.*, 1995) and MHC class II presentation molecules (Peters *et al.*, 1991) all transit an endosomal intermediate before reaching the surface (reviewed in Ikonen and Simons, 1998; Mostov *et al.*, 2000). Likewise, the biogenesis of synaptic vesicles (and recycling of synaptic proteins) also occurs via recycling endosomes (Calakos and Scheller, 1996; Hannah *et al.*, 1999). Thus, it is likely that branching of the exocytic pathway evolved early in evolution, the importance of which is only now being appreciated.

Materials and methods

Media and genetic manipulations

Yeast were grown in media containing 2% glucose or 3.5% galactose. Amino acid-rich medium (YPD: yeast extract/bactopeptone/dextrose), synthetic minimal medium (SC) and SC drop-out medium, lacking an essential amino acid or nucleotide base, were used. Media were prepared similarly to that described by Rose *et al.* (1990). Phosphate-depleted synthetic medium was prepared as described in Guthrie and Fink (1991). Standard methods were used for the introduction of DNA into yeast and the preparation of genomic DNA (Rose *et al.*, 1990).

Plasmids

Previously described plasmids included: pADH-SNC1 (Gerst *et al.*, 1992), pTGal-SNC1 (Protopopov *et al.*, 1993) and pLADH-SNC1^{ala43} (Gurunathan *et al.*, 2000).

Plasmid pCKR2 (*vps1::LEU2* in pUC12HP, a gift from T.H.Stevens, University of Oregon), was digested with *SacI* and *XbaI*, and was used for the disruption of *VPS1*. For the disruption of *VPS34*, a *vps34::LEU2* construct, pVPS34L, was generated by replacing *HIS3* with a *LEU2* marker in the original *vps34::HIS3* knockout plasmid (a gift from S.Emr, University of California, San Diego, CA). *LEU2* was inserted into the *BglII* site of *HIS3* via blunt-end ligation. Disruption of *VPS34* was performed by transforming cells with the *SpeI* and *XbaI* *vps34::LEU2* fragment derived from pVPS34L. Plasmid pCB95 (*pep12::LEU2* in pRS414, a gift of S.Emr) was digested with *StuI* and *ScaI*; the appropriate fragment was purified and used for the disruption of *PEP12*. A *vam3::LEU2* disruption in Bluescript KS (a gift of S.Emr) was used to generate a linear DNA fragment containing the deletion by PCR, using primers complementary to the *VAM3* coding sequence. Plasmid pCHC- Δ 10 (*chc1::LEU2* in pUC9, a gift of G.Payne, University of California, Los Angeles, CA) was digested with *HindIII* and used for the disruption

of *CHC1*. Plasmid YIpchc512-ΔCla (a gift of G.Payne) was digested with *Xba*I and used to integrate the clathrin ts allele, *chc1-521*, at the *CHC1* locus.

A *kan^r* plasmid expressing *SNC1^{ala43}* was constructed by subcloning a blunt-ended *EcoRV* and *Sall* fragment encoding the *kan^r* gene into the *Hind*III-digested and subsequently blunt-ended pLADH-*SNC1^{ala43}* to yield pKADH-*SNC1^{ala43}*. A centromeric plasmid expressing HA-tagged Pma1, pX28, was a gift of J.Haber, Brandeis University.

Yeast strains

Yeast strains used are listed in Table I. *vpsΔ* gene disruptions in *snc* or *sec6-4* cells were created by transformation of the JG8 or NY778 strains, respectively, with the appropriate disruption constructs (see Plasmids). Disruptions were verified by PCR and by the analysis of CPY secretion using a filter assay.

CPY secretion assay

To measure CPY secretion on filters, *snc* and *snc vpsΔ* cells were patched onto selective synthetic media containing galactose and grown for 2–3 days. *sec6* and *sec6 vpsΔ* cells were grown on glucose-containing medium. Plates were replica plated onto 82-mm-diameter nitrocellulose filters (Schleicher and Schuell; BA-S 85), which were then placed yeast side up onto fresh plates. Cells were grown on the filters for another 2 days. Filters were then washed with phosphate-buffered saline (PBS), dried and incubated in blocking buffer (5% non-fat dry milk, 0.1% Tween-20 in PBS) for 1 h. Next, filters were incubated for 2 h with anti-CPY antibody (1:1000) (gift of S.Emr) in 1% non-fat dry milk, 0.1% Tween-20 in PBS. Filters were washed, incubated with anti-rabbit secondary antibody (1:10 000), and CPY was detected by enhanced chemiluminescence (Amersham).

Electron microscopy

Fixation, thin-sectioning and electron microscopy of yeast were performed using standard procedures. Uranyl acetate staining of membranes from density gradients was performed by first diluting 2–3 fractions of a given peak of enzymatic activity in the gradient buffer (Harsay and Bretscher, 1995) lacking Nycodenz, followed by the addition of glutaraldehyde to a final concentration of 3%. After 2–4 h with shaking, the membranes were pelleted at 100 000 g and resuspended in 20 μl of buffer. Next, grids bearing collodion support film were incubated with the membranes for 1 min, followed by staining with 1% uranyl acetate (1 min). Membranes were visualized by electron microscopy.

The number of vesicles per square micrometer was determined by counting the number of SVs in photographs of cross-sectioned cells (*n* = 30). Next, the combined total area was determined by weighing cut-outs of cells and dividing them by the weight of a similarly enlarged 1 μm² cut-out. Dividing the former by the latter yields the number of SVs per square micrometer.

Cell fractionation and density gradient centrifugation

Cell fractionation and density gradient centrifugation were performed as described by Harsay and Bretscher (1995) with modifications by David *et al.* (1998). All strains were grown to early exponential phase (0.5–0.8 OD₆₀₀) in 2–4 l of synthetic minimal medium and then resuspended in phosphate-depleted low glucose medium to induce acid phosphatase and invertase, respectively. Cells were grown for 2 h in pre-warmed medium (37°C) to induce vesicle formation, while control cells were maintained at 26°C.

After density gradient centrifugation using Nycodenz (Sigma), the density of the fractions obtained was determined by measuring the refractive index. These values were converted to g/ml of Nycodenz, based on a standard curve generated using standards (15, 20, 25 and 30% Nycodenz having densities of 1.1201, 1.1468, 1.1743 and 1.1949 g/ml, respectively). Samples were taken to determine the protein concentration, using the Bradford assay (Bio-Rad). Enzymatic assays for acid phosphatase, exoglucanase, invertase and ATPase activities were performed as described previously (Harsay and Bretscher, 1995).

Immunoprecipitation

Immunoprecipitation (IP) of vesicles from the peak fractions of enzymatic activity was performed. Briefly, 2 μl of anti-HA antibody (gift of M.Wigler, Cold Spring Harbor Laboratory) were added to 50 μl aliquots of the gradient fractions, in a final volume of 500 μl of gradient buffer (Harsay and Bretscher, 1995) lacking Nycodenz, and incubated for 12 h at 4°C. As controls, either 75 μg of HA peptide or Triton X-100 (1% final concentration) were added to separate reactions. Next, washed

protein A-agarose (15 μl bed volume) was added to the mixture and incubated for an additional 2 h at 4°C. The beads were then pelleted, washed with 1 ml aliquots of ice-cold gradient buffer and the proteins extracted upon the addition of 15 μl of SDS-PAGE sample buffer. After SDS-PAGE, IP samples were blotted onto nitrocellulose membranes and detected with either anti-HA or anti-invertase (gift of Eitan Bibi, Weizmann Institute) antibodies.

Acknowledgements

The authors thank E.Bibi, S.Emr, G.Galili, J.Haber, G.Payne, T.Stevens and M.Wigler for the generous gifts of reagents or strains; special thanks to Vera Shindler for electron microscopy, and to Scott Emr and Greg Payne for helpful advice. This work was supported by a grant from the Minerva Foundation, Germany. S.G. was supported by a post-doctoral fellowship from the Feinberg Graduate School. J.E.G. holds the Henry Kaplan Chair in Cancer Research.

References

- Becherer,K.A., Rieder,S.E., Emr,S.D. and Jones,E.W. (1996) Novel syntaxin homologue, Pep12p, required for the sorting of luminal hydrolases to the lysosome-like vacuole in yeast. *Mol. Biol. Cell*, **7**, 579–594.
- Black,M.W. and Pelham,H.R.B. (2000) A selective transport route from Golgi to late endosomes that requires the yeast Gga proteins. *J. Cell Biol.*, **151**, 587–600.
- Bryant,N.J. and Stevens,T.H. (1998) Vacuole biogenesis in *Saccharomyces cerevisiae*: protein transport pathways to the yeast vacuole. *Microbiol. Mol. Biol. Rev.*, **62**, 230–247.
- Calakos,N. and Scheller,R.H. (1996) Synaptic vesicle biogenesis, docking and fusion: a molecular description. *Physiol. Rev.*, **76**, 1–29.
- Chen,C.Y. and Graham,T.R. (1998) An *arfΔ* synthetic lethal screen identifies a new clathrin heavy chain conditional allele that perturbs vacuolar transport in *Saccharomyces cerevisiae*. *Genetics*, **150**, 577–589.
- Chuang,J.S. and Schekman,R. (1996) Differential trafficking and timed localization of two chitin synthase proteins, Chs2p and Chs3p. *J. Cell Biol.*, **135**, 597–610.
- Costaguta,G., Stefan,C.J., Bensen,E.S., Emr,S.D. and Payne,G.S. (2001) Yeast Gga coat proteins function with clathrin in Golgi to endosome transport. *Mol. Biol. Cell*, **12**, 1885–1896.
- Cowles,C.R., Odorizzi,G., Payne,G.S. and Emr,S.D. (1997) The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell*, **91**, 109–118.
- Cremona,O. and De Camilli,P. (2001) Phosphoinositides in membrane traffic at the synapse. *J. Cell Sci.*, **114**, 1041–1052.
- Darsow,T., Rieder,S.E. and Emr,S.D. (1997) A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. *J. Cell Biol.*, **138**, 517–529.
- David,D., Sundarababu,S. and Gerst,J.E. (1998) Involvement of long chain fatty acid elongation in the trafficking of secretory vesicles in yeast. *J. Cell Biol.*, **143**, 1167–1182.
- Deloche,O., Yeung,B.G., Payne,G.S. and Schekman,R. (2001) Vps10p transport from the *trans*-Golgi network to the endosome is mediated by clathrin-coated vesicles. *Mol. Biol. Cell*, **12**, 475–485.
- Futter,C., Connolly,C.N., Cutler,D.F. and Hopkins,C.R. (1995) Newly synthesized transferrin receptors can be detected in the endosome before they appear on the cell surface. *J. Biol. Chem.*, **270**, 10999–11003.
- Gerst,J.E., Rodgers,L., Riggs,M. and Wigler,M. (1992) *SNC1*, a yeast homolog of the synaptic vesicle-associated membrane protein/synaptobrevin gene family: genetic interactions with the RAS and CAP genes. *Proc. Natl Acad. Sci. USA*, **89**, 4338–4342.
- Gu,F., Crump,C.M. and Thomas,G. (2001) *Trans*-Golgi network sorting. *Cell Mol. Life Sci.*, **58**, 1067–1084.
- Gurunathan,S., Chapman-Shimshoni,D., Trajkovic,S. and Gerst,J.E. (2000) Yeast exocytic v-SNAREs confer endocytosis. *Mol. Biol. Cell*, **11**, 3629–3643.
- Guthrie,C. and Fink,G.R. (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press, New York, NY.
- Hannah,M.J., Schmidt,A. and Huttner,W.B. (1999) Synaptic vesicle biogenesis. *Annu. Rev. Cell Dev. Biol.*, **15**, 733–798.
- Harsay,E. and Bretscher,A. (1995) Parallel secretory pathways to the cell surface in yeast. *J. Cell Biol.*, **131**, 297–310.

- Ikonen,E. and Simons,K. (1998) Protein and lipid sorting from the *trans*-Golgi network to the plasma membrane in polarized cells. *Semin. Cell Dev. Biol.*, **9**, 503–509.
- Kihara,A., Noda T, Ishihara N and Ohsumi Y. (2001) Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J. Cell Biol.*, **152**, 519–530.
- Kirchhausen,T. (1999) Adaptors for clathrin-mediated traffic. *Annu. Rev. Cell Dev. Biol.*, **15**, 705–732.
- Leitinger,B., Hille-Rehfeld,A. and Spiess,M. (1995) Biosynthetic transport of the asialoglycoprotein receptor H1 to the cell surface occurs via endosomes. *Proc. Natl Acad. Sci. USA*, **92**, 10109–10113.
- Lustgarten,V. and Gerst,J.E. (1999) Yeast *VSM1* encodes a v-SNARE binding protein that may act as a negative regulator of constitutive exocytosis. *Mol. Cell Biol.*, **19**, 4480–4494.
- Luo,W.-J. and Chang,A. (2000) An endosome-to-plasma membrane pathway involved in trafficking of a mutant plasma membrane ATPase in yeast. *Mol. Biol. Cell*, **11**, 579–592.
- Marash,M. and Gerst,J.E. (2001) t-SNARE dephosphorylation promotes SNARE assembly and exocytosis in yeast. *EMBO J.*, **20**, 411–421.
- Mostov,K.E., Verges,M. and Altschuler,Y. (2000) Membrane traffic in polarized epithelial cells. *Curr. Opin. Cell Biol.*, **12**, 483–490.
- Munn,A.L. and Riezman,H. (1994) Endocytosis is required for the growth of vacuolar H⁺-ATPase-defective yeast: identification of six new *END* genes. *J. Cell Biol.*, **127**, 373–386.
- Nothwehr,S.F., Conibear,E. and Stevens,T.H. (1995) Golgi and vacuolar membrane proteins reach the vacuole in *vps1* mutant yeast cells via the plasma membrane. *J. Cell Biol.*, **129**, 35–46.
- Novick,P., Field,C. and Schekman,R. (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell*, **21**, 205–215.
- Peters,P.J., Neefjes,J.J., Oorschot,V., Ploegh,H.L. and Geuze,H.J. (1991) Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature*, **349**, 669–676.
- Protopopov,V., Govindan,B., Novick,P. and Gerst,J.E. (1993) Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S.cerevisiae*. *Cell*, **74**, 855–861.
- Robinson,M.S. (1997) Coats and vesicle budding. *Trends Cell Biol.*, **7**, 99–102.
- Rohn,W.M., Rouille,Y., Waguri,S. and Hoflack,B. (2000) Bi-directional trafficking between the *trans*-Golgi network and the endosomal/lysosomal system. *J. Cell Sci.*, **113**, 2093–2101.
- Rose,M.D., Winston,F. and Hieter,P. (1990) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Rothman,J.E. and Wieland,F.T. (1996) Protein sorting by transport vesicles. *Science*, **272**, 227–234.
- Saucan,L. and Palade,G.E. (1994) Membrane and secretory proteins are transported from the Golgi complex to the sinusoidal plasmalemma of hepatocytes by distinct vesicular carriers. *J. Cell Biol.*, **125**, 733–741.
- Schekman,R. and Orci,L. (1996) Coat proteins and vesicle budding. *Science*, **271**, 1526–1533.
- Seeger,M. and Payne,G.S. (1992) A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast. *EMBO J.*, **11**, 2811–2818.
- Springer,S., Spang,A. and Schekman,R. (1999) A primer on vesicle budding. *Cell*, **97**, 145–148.
- Stack,J.H. and Emr,S.D. (1994) Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific PI 3-kinase activities. *J. Biol. Chem.*, **269**, 31552–31562.
- Tooze,S.A., Martens,G.J.M. and Huttner,W.B. (2001) Secretory granule biogenesis: rafting to the SNARE. *Trends Cell Biol.*, **11**, 116–122.
- Traub,L.M. and Kornfeld,S. (1997) The *trans*-Golgi network: a late secretory sorting station. *Curr. Opin. Cell Biol.*, **9**, 527–533.
- Walworth,N.C. and Novick,P.J. (1987) Purification and characterization of constitutive secretory vesicles from yeast. *J. Cell Biol.*, **105**, 163–174.
- Wieland,F. and Harter,C. (1999) Mechanisms of vesicle formation: insights from the COP system. *Curr. Opin. Cell Biol.*, **11**, 440–446.
- Wuestehube,L.J., Duden,R., Eun,A., Hamamoto,S., Korn,P., Ram,R. and Schekman,R. (1996) New mutants of *Saccharomyces cerevisiae* affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex. *Genetics*, **142**, 393–406.
- Yeung,B.G., Pahn,H.L. and Payne,G.S. (1999) Adaptor complex-independent clathrin function in yeast. *Mol. Biol. Cell*, **10**, 3643–3659.
- Yoshimori,T., Keller,P., Roth,M.G. and Simons,K. (1996) Different biosynthetic transport routes to the plasma membrane in BHK and CHO cells. *J. Cell Biol.*, **133**, 247–256.
- Yuan,D.S., Dancis,A. and Klausner,R.D. (1997) Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. *J. Biol. Chem.*, **272**, 25787–25793.
- Ziman,M., Chuang,J.S., Tsung,M., Hamamoto,S. and Schekman,R. (1998) Chs6p-dependent anterograde transport of Chs3p from the chitosome to the plasma membrane in *Saccharomyces cerevisiae*. *Mol. Biol. Cell*, **9**, 1565–1576.

Received September 13, 2001; revised November 27, 2001;
accepted December 13, 2001