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Yeast Exocytic v-SNAREs Confer Endocytosis

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In yeast, homologues of the synaptobrevin/VAMP family of v-SNAREs (Snc1 and Snc2) confer the docking and fusion of secretory vesicles at the cell surface. As no v-SNARE has been shown to confer endocytosis, we examined whether yeast lacking the *SNC* genes, or possessing a temperature-sensitive allele of *SNC1* (*SNC1*^{ala43}), are deficient in the endocytic uptake of components from the cell surface. We found that both *SNC* and temperature-shifted *SNC1*^{ala43} yeast are deficient in their ability to deliver the soluble dye FM4–64 to the vacuole. Under conditions in which vesicles accumulate, FM4–64 stained primarily the cytoplasm as well as fragmented vacuoles. In addition, α-factor–stimulated endocytosis of the α-factor receptor, Ste2, was fully blocked, as evidenced using a Ste2-green fluorescent protein fusion protein as well as metabolic labeling studies. This suggests a direct role for Snc v-SNAREs in the retrieval of membrane proteins from the cell surface. Moreover, this idea is supported by genetic and physical data that demonstrate functional interactions with t-SNAREs that confer endosomal transport (e.g., Tlg1,2). Notably, Snc1^{ala43} was found to be nonfunctional in cells lacking Tlg1 or Tlg2. Thus, we propose that synaptobrevin/VAMP family members are engaged in anterograde and retrograde protein sorting steps between the Golgi and the plasma membrane.

INTRODUCTION

Protein and lipid transport along the secretory pathway in eukaryotes involves the selective packaging and delivery of cargo from one compartment to the next. This is accomplished by carrier vesicles and tubulovesicular structures that bud from a given donor compartment and fuse with a specific target compartment. The mechanism by which carrier membranes identify their correct target and undergo docking and fusion, requires conserved families of membrane-associated proteins, termed SNAREs (reviewed in Rothman and Warren, 1994; Pfeffer, 1996; Rothman, 1996; Hay and Scheller, 1997; Gerst, 1999). On encroachment to the target compartment, SNAREs from the donor or vesicular membranes (v-SNAREs) form complexes in *trans* with cognate SNARE partners from the opposing target membrane (t-SNAREs). According to x-ray crystallographic data, the synaptic fusion complex consists of four α-helices arrayed in parallel to form a four-helix bundle, with three helices donated by the t-SNAREs and one by the v-SNARE (Sutton *et al.*, 1998). Originally categorized as three distinct and structurally conserved families (e.g., syntaxin, SNAP-25, and synaptobrevin/VAMP families), SNAREs also have been typed according to the charged amino acid (i.e., glutamine [Q] or arginine [R]) present in the ionic layer within the bundle (Fasshauer *et al.*, 1998). According to this type of classification, the neuronal four-helix bundle consists of two Q-SNAREs and one R-SNARE.

SNAREs play an essential role in both the docking and fusion of membranes, and may be required for both forward (anterograde) and reverse (retrograde) membrane-trafficking events in the early secretory pathway (Pelham, 1997; Gotte and Fischer von Mollard, 1998; Nichols and Pelham, 1998). Therefore, one might expect that similar mechanisms are operant on both protein export and protein retrieval pathways, to and from the plasma membrane (PM) (exocytosis and endocytosis, respectively). At the level of ER-Golgi transport, the v- and t-SNAREs that confer anterograde and retrograde transport already have been identified. For example, the ER-localized t-SNARE, Ufe1 (Q-type), and its v-SNARE partners, Sec22 (R-type) and Bet1 (nonR, nonQ-type), are involved in retrograde Golgi–ER transport of ER-retained proteins (Lewis and Pelham, 1996; Lewis *et al.*, 1997; Stone *et al.*, 1997; Spang and Schekman, 1998). Other SNARE-like proteins, Sec20 and Tip20, also may contribute to this trafficking event (Lewis *et al.*, 1997). In contrast, ER–Golgi anterograde transport is conferred by the Golgi-localized t-SNARE, Sed5 (Q-type), and the Bos1 (Q-type) and Sec22 (R-type) v-SNAREs (Hardwick and Pelham, 1992; Lian and Ferro-Novick, 1993; Banfield *et al.*, 1994; Lian *et al.*, 1994). A fourth helix may be contributed by Bet1, which is present on ER-derived transport vesicles and is found in complexes with Bos1 and Sed5 (Lian and Ferro-Novick, 1993; Søgaard *et al.*, 1994; Stone *et al.*, 1997; Spang and

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Table 1. Strains used in this study

Name	Genotype	Source
W303-ab	*MAT***a**/α *can1 his3 leu2 lys2 trp1 ura3 ade2*	J. Hirsch
W303-1a	*MAT***a** *can1 his3 leu2 lys2 trp1 ura3 ade2*	J. Hirsch
JG8	*MAT***a** *can1 his3 leu2 trp1 snc1::URA3 snc2::ADE8 pTGAL-SNC1 or pLGAL-SNC1*	J. Gerst
JG9-TLG2	*MAT***a** *can1 his3 trp1 snc1::URA3 snc2::ADE8 tlg2::LEU2*	This study
JG9-TLG2, SNC1A43	*MAT***a** *can1 his3 trp1 snc1::URA3 snc2::ADE8 tlg2::LEU2* pTADH-SNC1^{ala43}	This study
JG9-TLG1	*MAT***a** *can1 his3 leu2 snc1::URA3 snc2::ADE8 tlg1::TRP1*	This study
JG9-TLG1, SNC1A43	*MAT***a** *can1 his3 leu2 snc1::URA3 suc2::ADE8 tlg1::TRP1* pLADH SNC1^{ala43}	This study
JG10	*MAT***a** *can1 his3 lys2 trp1 ade2 snc1::URA3 snc2::LEU2* pTGAL-SNC1	This study
SP1	*MAT***a** *can1 his3 leu2 trp1 ura3 ade8*	M. Wigler
NY778	*MATα ura3-52 leu2-3,112 sec6-4*	P. Novick

Schekman, 1998). Thus, Sec22 and Bet1 are v-SNAREs that are shared between anterograde and retrograde trafficking events. Another SNARE shared between two compartments is Vti1 (Q-type). Vti1 confers Golgi-to-prevacuolar compartment (PVC) anterograde transport, by partnering with the Pep12 t-SNARE, as well as PVC-to-Golgi retrograde transport, by pairing with Sed5 (Fischer von Mollard *et al.*, 1997; Lupashin *et al.*, 1997; Fischer von Mollard and Stevens, 1999).

We are studying yeast members of the synaptobrevin/VAMP family of R-type v-SNAREs, which confer secretory vesicle docking and fusion. The yeast proteins, Snc1 and Snc2 (Gerst *et al.*, 1992; Protopopov *et al.*, 1993), are archetypal exocytic v-SNAREs (Gerst, 1997) that traffic secretory vesicles, and deliver proteins and lipids to the cell surface (Protopopov *et al.*, 1993; David *et al.*, 1998). Their functions are largely redundant, as either isoform recouples the docking and fusion of low-density secretory vesicles (LDSVs) and high-density secretory vesicles (HDSVs) that accumulate in *snc* cells and late-acting *sec* mutants (David *et al.*, 1998). However, these v-SNAREs differ somewhat in their ability to interact genetically and physically with other proteins of the secretory apparatus (Gerst, 1997; Lustgarten and Gerst, 1999). Interestingly, the requirement of these v-SNAREs in secretion can be bypassed by mutations in genes (e.g., *VBM1/ELO3* and *VBM2/ELO2*) involved in ceramide and sphingolipid synthesis (David *et al.*, 1998). Subsequently, we have determined that a ceramide-activated protein phosphatase regulates vesicle docking and fusion in these cells by dephosphorylating the t-SNAREs involved in exocytosis and promoting formation of a t-t-SNARE exocytic complex (Marash and Gerst, unpublished observations).

In addition to their ability to form stable ternary SNARE complexes with Q-type exocytic t-SNAREs, Sso1,2 and Sec9 (Brennwald *et al.*, 1994; Rossi *et al.*, 1997), Snc proteins coimmunoprecipitate with the Tlg1 and Tlg2 t-SNAREs (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a). These t-SNAREs act at the level of the late-Golgi/endosomes to confer retrograde protein transport from the cell surface (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a; Seron *et al.*, 1998; Coe *et al.*, 1999) and are required for Snc1 retrieval to the Golgi (Lewis *et al.*, 2000). In particular, Tlg2 was shown to localize to early endosomes and to be involved in endosome biogenesis. This suggests a prominent role for Tlg2 in endocytosis (Abeliovich *et al.*, 1998; Seron *et al.*, 1998) as well as in the cytoplasm-to-vacuole transport route (Abeliovich *et al.*, 1999). As no yeast v-SNARE has been shown to localize to endosomes or to confer endocytosis per se, we hypothesized that the Snc v-SNAREs might be strong candidates for fulfilling such a function.

Here, we show that endocytic events (i.e., delivery of the vital dye FM4–64 or the Ste2 mating factor receptor to the vacuole) are severely impaired in *snc* cells, as well as in cells possessing a novel temperature-sensitive allele of *SNC1* (*SNC1*^{ala43}) after being shifted to restrictive temperatures. Genetic experiments further reveal that inactivating mutations in either *TLG1* or *TLG2* decrease substantially the growth rate of *snc* or *SNC1*^{ala43} cells. These results of synthetic enhancement, along with those of previous coimmunoprecipitation studies, imply that a functional relationship exists between these endosomal/TGN t-SNAREs and the Snc exocytic v-SNAREs. Coupled with earlier findings (Abeliovich *et al.*, 1998; Seron *et al.*, 1998), these results demonstrate that the Snc exocytic v-SNAREs are involved in endocytic events and, perhaps, the biogenesis of endocytic vesicles as well. Thus, we propose that members of the synaptobrevin/VAMP family of v-SNAREs are involved in a dynamic cycle of anterograde and retrograde trafficking events in vivo to deliver proteins and lipids both to and from the PM.

MATERIALS AND METHODS

Media, DNA, and Genetic Manipulations

Molecular cloning techniques were performed as described (Sambrook *et al.*, 1989). Polymerase chain reaction (PCR) and the subcloning of PCR products were carried out as described previously (Gerst *et al.*, 1991).

Yeast were grown in standard growth media containing either 2% glucose or 3.5% galactose as a carbon source. Synthetic complete and drop-out media were prepared in a manner similar to that described elsewhere (Rose *et al.*, 1990). Standard methods were used for the introduction of DNA into yeast and for the preparation of genomic DNA (Rose *et al.*, 1990).

Yeast Strains

Yeast strains used in this study are listed in Table 1. *TLG2* gene disruption in the *snc* background was accomplished by transforming a 2.4-kb fragment from plasmid pTLG2T, which bears the *tlg2::TRP1* disruption, into JG8 cells. *TLG1* gene disruption in the *snc* background was accomplished by transforming a 3-kb fragment from plasmid pTLG1L, which bears the *tlg1::LEU2* disruption, into JG8 cells. Transformants were selected on galactose-containing medium and then were examined for growth at different temperatures

on both glucose and galactose-containing medium, as well as for labeling of the vacuole using FM4–64 (1 μg/ml). Disruptions of the *TLG* loci were verified by PCR or Southern analysis. A *snc* null strain in the W303 background (JG10) was created by the sequential disruption of *SNC1* and *SNC2*, using the *snc1::URA3* and *snc2::LEU2* constructs described previously (Gerst *et al.*, 1992; Protopopov *et al.*, 1993). Viability was maintained on galactose-containing medium, using either the pTGAL-SNC1 or pAHGAL-SNC2 plasmids that express *SNC1* or *SNC2*, respectively (Protopopov *et al.*, 1993; David *et al.*, 1998).

Plasmids

Vectors included the 2-μm plasmids YEp13 M4 and pAD4Δ, which bear the *LEU2* marker, while the latter has an *ADH1* constitutive promoter upstream to the polycloning site. Centromeric vectors included pRS315 and YCplacIII, which bear a *LEU2* marker, and pSE358, which bears a *TRP1* marker.

Previously described plasmids included: pADH-SNC1 (Gerst *et al.*, 1992); pADH-HASNC1 and pTGAL-SNC1 (Protopopov *et al.*, 1993); pALT-SNC1 (Couve *et al.*, 1995); and pAHGAL-SNC2 (David *et al.*, 1998). Plasmids pRS314STE2-green fluorescent protein (GFP) and pRS314ste2Δtail-GFP, which express the *STE2-GFP* or *STE2Δ-GFP* gene fusions (Stefan and Blumer, 1999), respectively, were generously provided by Dr. Kendall Blumer (Washington University School of Medicine, St. Louis, MO). A plasmid, pMD53, that expresses a *STE2HA* gene was kindly provided by Dr. Jamie Konopka (State University of New York at Stony Brook, NY).

The plasmid created for this study, pALT-SNC1^{ala43}, which bears a *SNC1*^{ala43} allele, was derived by the mutagenesis of *SNC1* in pALT-SNC1 (Couve *et al.*, 1995). Site-directed mutagenesis to alter codon 43 (from methionine to alanine) was performed using a mutagenic oligonucleotide (SNCM-A; 5′GCTACTTTATTTATGTTATCTCTCGCGATTCCCACGGTATCATCAATTTCC-3′), containing an *Nru*I restriction endonuclease site, according to the ALTER protocol recommended by the supplier (Promega, Madison, WI). *SNC1*^{ala43} expression constructs included: pADH-SNC1^{ala43}, which was created by subcloning a *Sal*I–*Sac*I fragment containing *SNC1*^{ala43} from pALT-SNC1^{ala43} into the *Sal*I–*Sac*I sites of pAD4Δ; pTADH-SNC1^{ala43}, which was created by subcloning a *Bam*HI fragment containing *ADH1-SNC1*^{ala43} from pADH-SNC1^{ala43} into the *Bam*HI site of pSE358; and pLADH-SNC1^{ala43}, which was created by subcloning this fragment into the *Bam*HI site of pRS315.

pTLG2, which contains a 1.2-kb genomic fragment of *TLG2*, was created by subcloning a fragment generated by PCR with oligonucleotides TLG2F (5′-AGTCGTTACGTCGACGTTTAGAGATAG-3′) and TLG2R (5′-ACCGTGAAAGAGCTCTCATCAAAGTAG-3′) into pGEM (Promega). A *TLG2* disruption construct, pTLG2T, was created by subcloning a blunt-ended *TRP1* selectable marker into the *Bgl*II site of *TLG2* in pTLG2, which could be released by digestion with *Sac*I and *Nde*I. pTLG1, which contains a 1.7-kb genomic fragment of *TLG1*, was created by subcloning a fragment generated by PCR with oligonucleotides TLG1F (5′-GCTATGAGCCTCGAGCCCGCTAATAAATGC-3′) and TLG1R (5′-CTTCACCTCCGCGGCCGCAAATGGAATTCC-3′) into pGEM. A *TLG1* disruption construct, pTLG1L, was created by subcloning the *LEU2* selectable marker into the *Xba*I site of *TLG1* in pTLG1, resulting in a fragment that could be released by digestion with *Xho*I and *Not*I.

Snc1-GFP and Snc1^{ala43}-GFP expression plasmids, pADH-SNC1GFP and pADH-SNC1^{ala43}GFP, were created by splice-overlap extension (Horton *et al.*, 1989). In the first PCR reaction, *SNC1* and *SNC1*^{ala43} were amplified separately with oligonucleotides JG90 (Gerst *et al.*, 1992) and JG600 (5′-GTTCTTCTTTACTTCGACTAAAAGTGAACAGCA-3′), resulting in *SNC1* gene fusions that lack their stop codons but encode the first 10 amino acids of GFP. In the second reaction, GFP was amplified from plasmid pRS314-STE2GFP using oligonucleotides JG601 (5′-TGCTGTTCACTTTAGTCGAAGTAAAGGAGAAGAA-3′) and JG602 (5′-TCGAAGCTGAGCTCCTATTTGTATAGTTCATCCAT-3′), resulting in a gene that encodes the last 10 amino acids of Snc1 fused to full-length GFP. The products of the first and second reactions then were combined in a third PCR reaction, but in the absence of the oligonucleotide. This led to the extension of the annealed products to yield the appropriate 1.2-kb *SNC1-GFP* fusions, as verified by subcloning into pGEM and sequencing. The pGEM constructs, pSNC1GFP and pSNC1^{ala43}GFP, then were digested with *Sal*I and *Sac*I to release the gene fusion fragments, which were subcloned into pAD4Δ to give plasmids pADH-SNC1GFP and pADH-SNC1^{ala43}GFP.

A *CEN* plasmid that expresses *STE2HA*, pLADH-STE2HA, was created by subcloning a *Sac*I and blunt-ended *Sph*I fragment from pMD53 into the *Sac*I and *Sma*I sites in YCplacIII.

FM4–64 Labeling and Fluorescence Microscopy

Cells were grown to log-phase on glucose- or galactose-containing medium before harvesting. For each sample to be visualized, 1 OD_{600} unit of cells (*snc* or *SNC1*^{ala43}) was incubated for 0.5 h with FM4–64 (Molecular Probes, Eugene, OR) (1 μg/ml) with shaking at 26°C. Cells then were resuspended in fresh medium lacking the dye and were allowed to grow for 1 h at 26°C. For measuring uptake at restrictive temperatures, *SNC1*^{ala43} cells were incubated with FM4–64 30 min after first shifting to the restrictive temperature (37°C). After 30 min of labeling, cells were resuspended in fresh prewarmed medium lacking the dye and were allowed to grow for 0.5–1 h at 37°C. After the chase, cells were resuspended in a volume of 100 μl of fresh medium and were mixed with an equal volume of a solution of 2.6% low–melting point agarose cooled to 50°C. Cells then were transferred to a glass slide, covered with a coverslip, and visualized by light and fluorescence microscopy using the rhodamine channel. GFP fluorescence was visualized via the fluorescein isothiocyanate (FITC) channel in strains expressing GFP fusion proteins.

Thin-sectioning and Electron Microscopy of Yeast

Thin-sectioning and electron microscopy of yeast were carried out essentially as described (Zelicof *et al.*, 1996)

Enzymatic Assays and Metabolic Labeling

Invertase activity was assayed by the method of Goldstein and Lampen (Goldstein and Lampen, 1975) and is expressed in units based on absorption at 540 nm, where 1 unit = 1 μmol glucose released/min/100 mg of dry cells. Pulse-chase studies for Ste2 uptake, using [^{35}S]-methionine (Amersham, Arlington Heights, IL), were performed essentially as described (Hicke and Riezman, 1996). *snc* cells grown on galactose-containing medium at 26°C were shifted to glucose-containing medium for 30 h before pulse-labeling (30 min) with [^{35}S]-methionine (0.1 mCi/OD_{600} unit). After a chase (30 min) in medium containing 5 mM methionine and cysteine, cells were treated with α-factor (1 μM) for up to 120 min. Cell extracts were prepared and Ste2HA was immunoprecipitated using anti-HA antibodies. Immunoprecipitated Ste2HA was solubilized in SDS-containing sample buffer, was heated for 15 min at 37°C, and was resolved on 10% SDS-PAGE. For *snc* cells expressing *SNC1*^{ala43}, yeast were either maintained continually at 26°C during pulse-chase labeling and treatment with α-factor or were shifted to the restrictive temperature (37°C) after labeling and coincident to the chase.

RESULTS

Mutation of Methionine-43 to Alanine in Snc1 Yields a Temperature-sensitive Exocytic v-SNARE

An earlier study reported that substitutions in the conserved α-helical domain of VAMP2 resulted in proteins that are unable to be retrieved from the PM via endocytosis (Grote *et al.*, 1995; Grote and Kelly, 1996). In particular, mutations in

a methionine residue (met-46) of the heptad repeat motif, that is, positioned −3 relative to the designated ionic layer, resulted in a loss in retrieval to vesicles (Grote *et al.*, 1995; Grote and Kelly, 1996). In contrast, this mutation did not block the ability of the protein to form a stable SDS-resistant SNARE complex with its partner t-SNAREs.

When expressed in yeast, VAMP1,2 proteins are unable to substitute for Snc proteins in conferring exocytosis and do not undergo retrieval from the PM, as demonstrated by immunogold labeling and electron microscopy (Gerst, 1997). However, the fusion of amino acids 1–49 of Snc1 (which includes the layers −7 to −1 upstream to the designated ionic layer) to the downstream cytoplasmic and transmembrane regions of VAMP2 conferred retrieval of the Snc–VAMP chimera from the cell membrane to secretory vesicles (Gerst, 1997). Since the Snc and VAMP proteins are structural and functional homologues, we proposed that the targeting signal for Snc retrieval from the PM to vesicles (via the endocytic pathway) might be identical to that shown for VAMP. To test this possibility, we created an analogous mutation (e.g., met-43 to ala-43) in the Snc1 v-SNARE by site-directed mutagenesis.

We first examined whether this allele (*SNC1*^{ala43}) could confer normal exocytic function to *snc* yeast. We tested this allele for function in two *snc* strains, one (JG8) having a conditional lethal phenotype (Protopopov *et al.*, 1993) and the other (JG10) having a lethal phenotype (this study). The differences between these strain backgrounds (SP1 and W303, respectively) are unclear, but similar results have been noted for mutations in other yeast genes (i.e., *CHC1*, which encodes the clathrin heavy chain) (Munn *et al.*, 1991). Both *snc* strains were maintained using a galactose-inducible *SNC1* gene on galactose-containing medium. On glucose-containing medium, JG8 *snc* cells are unable to grow at temperatures higher than 30°C, are deficient in secretion, and accumulate secretory vesicles (Protopopov *et al.*, 1993). In contrast, constitutive expression of *SNC1*^{ala43} from a single-copy plasmid in this strain conferred viability up to 35°C, although the growth rate of these cells was slower than that conferred by native Snc1 (Figure 1A, left and right panels). Moreover, *SNC1*^{ala43}expression in JG10 *snc* cells (which are inviable on glucose-containing medium) was found to confer viability at temperatures up to 26°C (Figure 1A, left panel). Thus, the *SNC1*^{ala43}allele is at least partially active at permissive temperatures and might be used as a tool to induce the *snc* phenotype more rapidly than switching *snc* cells from galactose- to glucose-containing medium.

We next tested whether exocytosis is blocked at restrictive temperatures in *snc* cells (JG8) expressing *SNC1*^{ala43}. We examined the secretion of invertase, a soluble secreted enzyme that is present in HDSVs and is used as a marker for secretion competence. We found that on depression in low glucose-containing medium, invertase synthesis and secretion in *SNC1*^{ala43}cells at 26°C was similar to that conferred by native *SNC1* (Figure 1B). Moreover, invertase was secreted at levels over 3-fold higher than *snc* cells, which served as control cells (Figure 1B). However, after shifts (i.e., at 0.5 h, 1 h, or 2 h) to restrictive temperatures (37°C), invertase secretion from cells expressing *SNC1*^{ala43} declined by 2- to 3-fold relative to that observed at permissive temperatures (values from a 2-h shift are shown). A similar decline was observed in control *snc* cells. However, cells expressing native *SNC1* synthesized and secreted even more invertase at the higher temperature. Thus, the *SNC1*^{ala43} allele encodes a protein capable of conferring near-normal growth and secretion at the permissive temperature, but not at the restrictive temperature.

We then examined the accumulation of secretory vesicles in JG8 cells expressing *SNC1*^{ala43} at permissive and restrictive temperatures (Figure 1C). As expected, few vesicles were found to accumulate in cells grown at 26°C (Figure 1C, lower left panel) but were clearly evident after a 2-h shift to restrictive conditions (Figure 1C, lower right panel). This contrasts both with *snc* cells, which accumulate vesicles constitutively at 26°C (Figure 1C, upper left panel), and *snc* cells that express *SNC1* (Figure 1C, upper right panel), which do not accumulate vesicles under any conditions (Protopopov *et al.*, 1993; David *et al.*, 1998). We noted that the number of vesicles per square micrometer was very similar between *snc* cells and *SNC1*^{ala43} cells shifted to 37°C (our unpublished results). Thus, *SNC1*^{ala43} appears to be a temperature-sensitive allele that can induce a secretion block similar to late-acting *sec* mutants.

Snc1^{ala43} Is Unable To Recycle Efficiently from the PM at Restrictive Temperatures

In earlier studies, a mutant VAMP protein, VAMP2^{ala46}, was found to be unable to undergo retrieval from the PM of PC12 cells (Grote *et al.*, 1995; Grote and Kelly, 1996). While this protein was presumed to function normally, due to its ability to interact with partner t-SNAREs (Grote *et al.*, 1995), its contribution to exocytic function was unknown. Here, we have shown that an analogous mutation in *SNC1* results in a protein that is greatly lessened in its ability to confer exocytosis from yeast lacking the native *SNC* genes. Nevertheless, we presume that Snc1^{ala43} must interact productively with the *SSO* and *SEC9* gene products at temperatures ≤ 35°C for vesicle docking and fusion to occur in JG8 cells under these conditions.

To determine whether the mutant Snc1 protein is retained on the membrane, like VAMP2^{ala46}, we examined its intracellular localization in a late-acting *sec* mutant under permissive conditions and conditions in which secretory vesicles accumulate. We created *GFP* gene fusions with *SNC1* and *SNC1*^{ala43}and expressed them in *sec6* cells (Figure 1D). We have used these cells previously to demonstrate the localization of Snc protein primarily to the PM at permissive conditions and to secretory vesicles at restrictive temperatures (Protopopov *et al.*, 1993; Gerst, 1997). We found that at 26°C, both Snc1-GFP and Snc1^{ala43}-GFP labeled primarily the PM but also could be detected in the vacuole. However, at 37°C Snc1-GFP was not found on the PM, but it did intensely label the cytoplasm and punctate areas therein. This is probably indicative of its localization to secretory vesicles, as previously shown (Protopopov *et al.*, 1993; Gerst, 1997), as well as, perhaps, to endosomes. In contrast, the localization of Snc1^{ala43}-GFP was more restricted to areas corresponding to the PM at 37°C, although labeling of the vacuole was still apparent. The latter observation is probably due to overexpression of the fusion protein. However, continual labeling of the PM at 37°C suggests that Snc1^{ala43} is unable to redistribute in the cell as efficiently as native Snc1, as was shown previously for VAMP2^{ala46} in mammalian cells. Moreover, a recent study utilizing the same me-

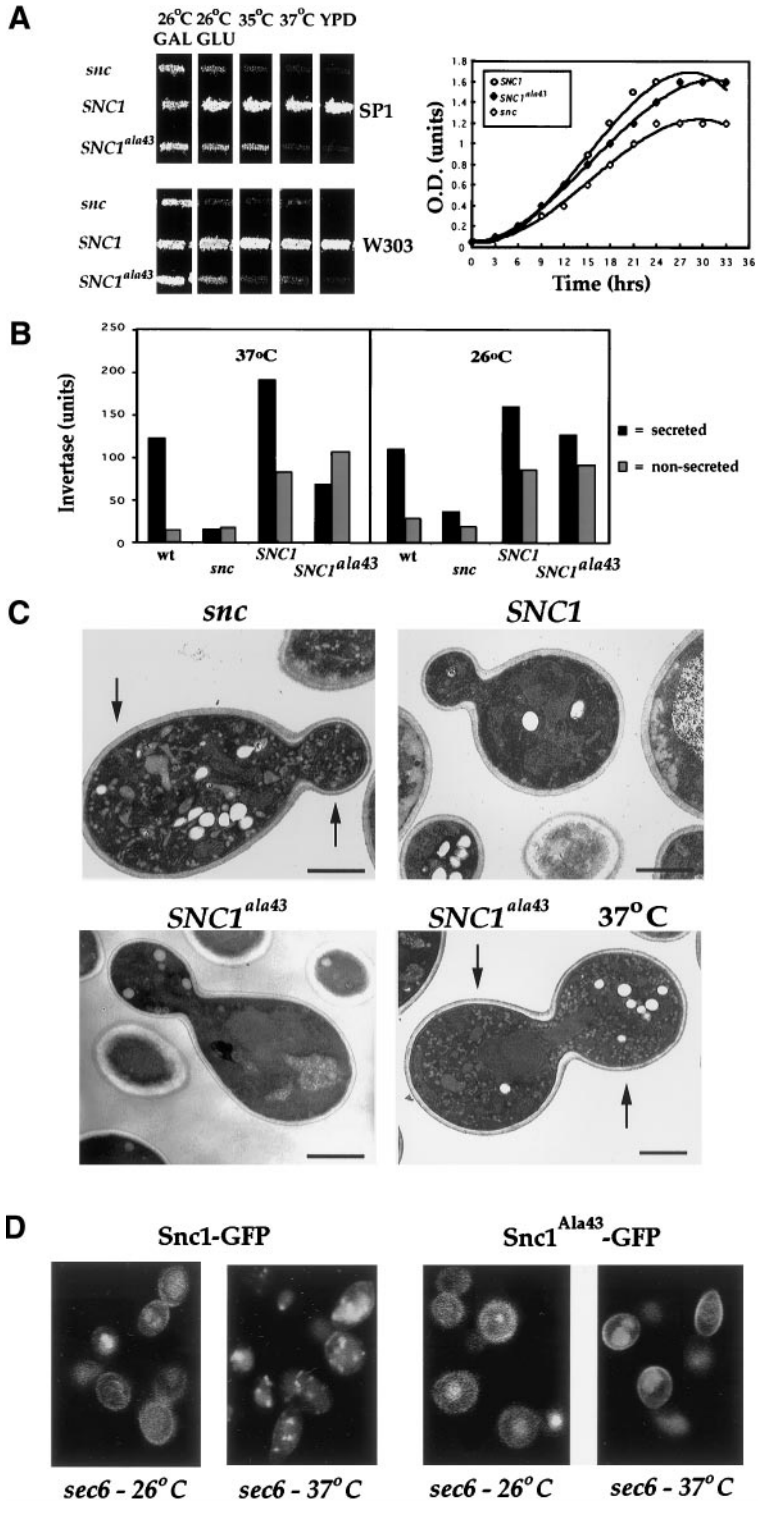

Figure 1. Mutation of methionine-43 to alanine in Snc1 yields a thermosensitive exocytic v-SNARE. (A) *SNC1*^{ala43} encodes a thermosensitive v-SNARE. Left panel: *snc* cells (SP1 background–JG8 and W303 background–JG10) transformed with a control vector (*snc*) or plasmids constitutively expressing *SNC1* or *SNC1*^{ala43} were patched onto synthetic complete plates containing galactose. Cells were grown for 3 d at 26°C, before replica plating onto medium containing glucose to deplete *SNC1*. After 24 h, patches were replica plated onto the following: galactose- (GAL) and glucose-containing (GLU) plates at 26°C; prewarmed glucose-containing plates at 35°C and 37°C; and amino acid-rich medium (YPD). Plates were incubated for 30 h. Right panel: *snc* cells (JG8) transformed with a control vector (*snc*), or plasmids expressing *SNC1* or *SNC1*^{ala43}, were grown to log phase on galactose-containing medium and then were shifted to glucose-containing medium for 24 h to deplete *SNC1*. Cells were seeded in fresh glucose-containing medium at 26°C, and optical density (measured at 600 nm) was monitored as a function of time. (B) Invertase secretion is blocked in *SNC1*^{ala43} cells after a shift to restrictive temperatures. Wild-type (SP1), *snc* (JG8), and *snc* cells (JG8) transformed with plasmids expressing *SNC1* or *SNC1*^{ala43} were grown to log phase on galactose-containing medium, shifted to glucose medium for 24 h at 26°C and then derepressed for 2 h on low glucose (0.05%) medium at either 26°C or 37°C. Both secreted (black) and nonsecreted (gray) invertase activities were measured. (C) *SNC1*^{ala43} cells accumulate secretory vesicles at restrictive temperatures. *snc* cells (JG8) expressing a control vector (*snc*), *SNC1*, or *SNC1*^{ala43} were grown to log phase on glucose-containing medium. Cells were maintained at 26°C, or were shifted for 2 h to 37°C (indicated as 37°C), and were processed for electron microscopy. Bar indicates 1 μm. (D) Localization of Snc1-GFP and Snc1^{ala43}-GFP in *sec6* cells. *sec6–4* cells transformed with plasmids expressing Snc1-GFP or Snc1^{ala43}-GFP were maintained at 26°C or were shifted for 2 h to 37°C, and then were processed for confocal microscopy.

thionine-to-alanine mutation in Snc1 also demonstrated preferential labeling of the PM (Lewis *et al.*, 2000).

Delivery of the Soluble Dye, FM4–64, to the Vacuole Is Impaired in snc Cells and SNC1^{ala43} Cells Shifted to Restrictive Temperatures

Snc proteins are structural and functional homologues of the synaptobrevin/VAMP family of v-SNAREs and are involved in the docking and fusion of at least two classes of secretory vesicles in yeast (Protopopov *et al.*, 1993; Gerst, 1997; David *et al.*, 1998; Gerst, 1999). Ironically, no v-SNARE has been identified as acting on the endocytic pathway. Moreover, presumably all SNARE-like molecules in yeast have been identified by homology and have been shown to act earlier in the secretory pathway (Gotte and Fischer von Mollard, 1998; Nichols and Pelham, 1998). Thus, it seemed likely to us that the Snc v-SNAREs also participate in endocytosis. Supporting evidence for this comes from studies showing physical interactions between the Snc proteins and two t-SNAREs of the TGN/endosomal sorting pathway, Tlg1,2 (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a).

We reasoned that if Snc proteins are required for endosomal protein sorting (i.e., the fusion of endocytic vesicles with early endosomes, endosome-derived vesicles with late endosomes/PVC, or both), then the update of vital dyes to the vacuole should be impaired in *snc* cells. We examined whether the uptake of FM4–64, a dye that labels membranes of the endocytic pathway and is delivered to the vacuole (Vida and Emr, 1995), is normal in *snc* cells. *snc* yeast (JG8) were shifted from galactose- to glucose-containing medium, the cells then were incubated with FM4–64 and were examined in vivo using fluorescence microscopy (Figure 2A). We found that cells maintained continually on galactose showed normal vacuolar morphology (e.g., bright staining of the circular vacuolar compartment). In contrast, cells shifted to glucose-containing medium (to induce the *snc* phenotype) showed a retardation in the ability of the stain to reach the vacuole. Since the half-life of Snc proteins is approximately 8 h (Couve *et al.*, 1995), defects in dye sorting become evident only after this time. At 12 h after the shift, multiple vacuolar bodies labeled by FM4–64 were evident. In addition, disperse labeling of the cytoplasm (where carrier vesicles accumulate) also was observed. By 30 h, FM4–64 labeling was dispersed primarily throughout the cytoplasm, despite the fact that some vacuoles can be seen by light microscopy (Nomarski) and electron microscopy (Figures 2A and 3B) (Protopopov *et al.*, 1993). This suggests that FM4–64 delivery is deficient in the absence of Snc protein and that the dye may stain vesicles that accumulate under these conditions (Protopopov *et al.*, 1993). Moreover, the results imply that vacuolar maintenance is deficient in *snc* yeast, resulting in the appearance of fragmented vacuoles. Yet, as the processing and delivery of a vacuolar hydrolase, carboxypeptidase Y (CPY), to the vacuole occurs in *snc* cells (David *et al.*, 1998), we believe that the vacuoles present therein are functionally intact.

Next, we examined FM4–64 uptake and delivery in *SNC1*^{ala43} cells at permissive and restrictive temperatures (Figure 2B). *snc* cells (JG8) expressing *SNC1*^{ala43}, *SNC1*, or a control vector were grown on glucose-containing medium for 24 h. After a 1- to 1.5-h shift to restrictive conditions (37°C), we found that FM4–64 was unable to reach the vacuole and stained primarily the cytoplasm, as well as fragmented vacuolar compartments, in cells expressing *SNC1*^{ala43}. Similar labeling results were obtained with *snc* control cells at either 26°C or 37°C. In contrast, *SNC1*^{ala43} cells maintained at permissive conditions were indistinguishable from cells expressing native *SNC1*. Together these experiments (Figure 2, A and B) indicate that FM4–64 reaches the vacuole in a manner dependent on Snc protein function. Similar results were obtained for the uptake of another dye, lucifer yellow, using both *snc* and temperature-shifted *SNC1*^{ala43} cells (our unpublished results).

Ligand-mediated Delivery of the Ste2 Mating Factor Receptor to the Vacuole Is Abolished in snc Cells and SNC1^{ala43} Cells Shifted to Restrictive Temperatures

Since dye delivery to the vacuole is deficient in *snc* and temperature-shifted *SNC1*^{ala43} cells, we hypothesized that endocytosis and endosomal protein sorting may be blocked. To verify this using a protein known to be retrieved from the PM and delivered to the vacuole, we utilized a fusion protein composed of the Ste2 α-factor receptor and GFP, Ste2-GFP (Stefan and Blumer, 1999). This protein has been shown previously to undergo ligand-dependent endocytosis with kinetics similar to those of native Ste2 (Stefan and Blumer, 1999). In addition, we utilized a deletion construct that removes 131 residues of the carboxyl terminus of Ste2 (Ste2Δ-GFP) and is unable to undergo retrieval from the PM (Stefan and Blumer, 1999).

Both *snc* cells and *SNC1*^{ala43} cells expressing either Ste2-GFP or Ste2Δ-GFP were examined for their ability to deliver the fusion protein to the vacuole by confocal fluorescence microscopy (Figure 3). Before treatment with α-factor (zero time), we found that Ste2-GFP and Ste2Δ-GFP fluorescence were restricted primarily to the PM in both cell types (Figure 3, A–C). This pattern was similar to that seen previously with these constructs (Stefan and Blumer, 1999). Little to no overlap with the vacuolar marker, FM4–64, was observed at the zero time (Figure 3, A–C).

α-factor treatment of *snc* cells (JG8) maintained on galactose (which express *SNC1*) resulted in a time-dependent recruitment of the Ste2-GFP fusion protein, but not Ste2Δ-GFP, to the vacuole as detected by a shift in the FITC fluorescence staining of the cell (Figure 3A). By 5 min, nearly all Ste2-GFP fluorescence had disappeared from the PM into the vacuolar region. Moreover, nearly all FITC fluorescence was eliminated from the cell by 11 min, presumably due to protein degradation that occurs in cells having their full complement of vacuolar hydrolases. In contrast, cells expressing the Ste2Δ-GFP protein did not show a significant loss of FITC labeling from the PM and did not show colabeling in the vacuolar region, as assayed for up to 45 min (Figure 3A; our unpublished results). Thus, Ste2 regulation in the presence of Snc1 alone is similar to what has been described in earlier studies.

In *snc* cells (JG8) shifted to glucose-containing medium for 24 h, we found that both Ste2-GFP and Ste2Δ-GFP were present primarily on the PM in untreated cells (Figure 3B). In addition, FM4–64 labeling of the vacuole was deficient and stained the cytoplasm, as described in Figure 2 (note the

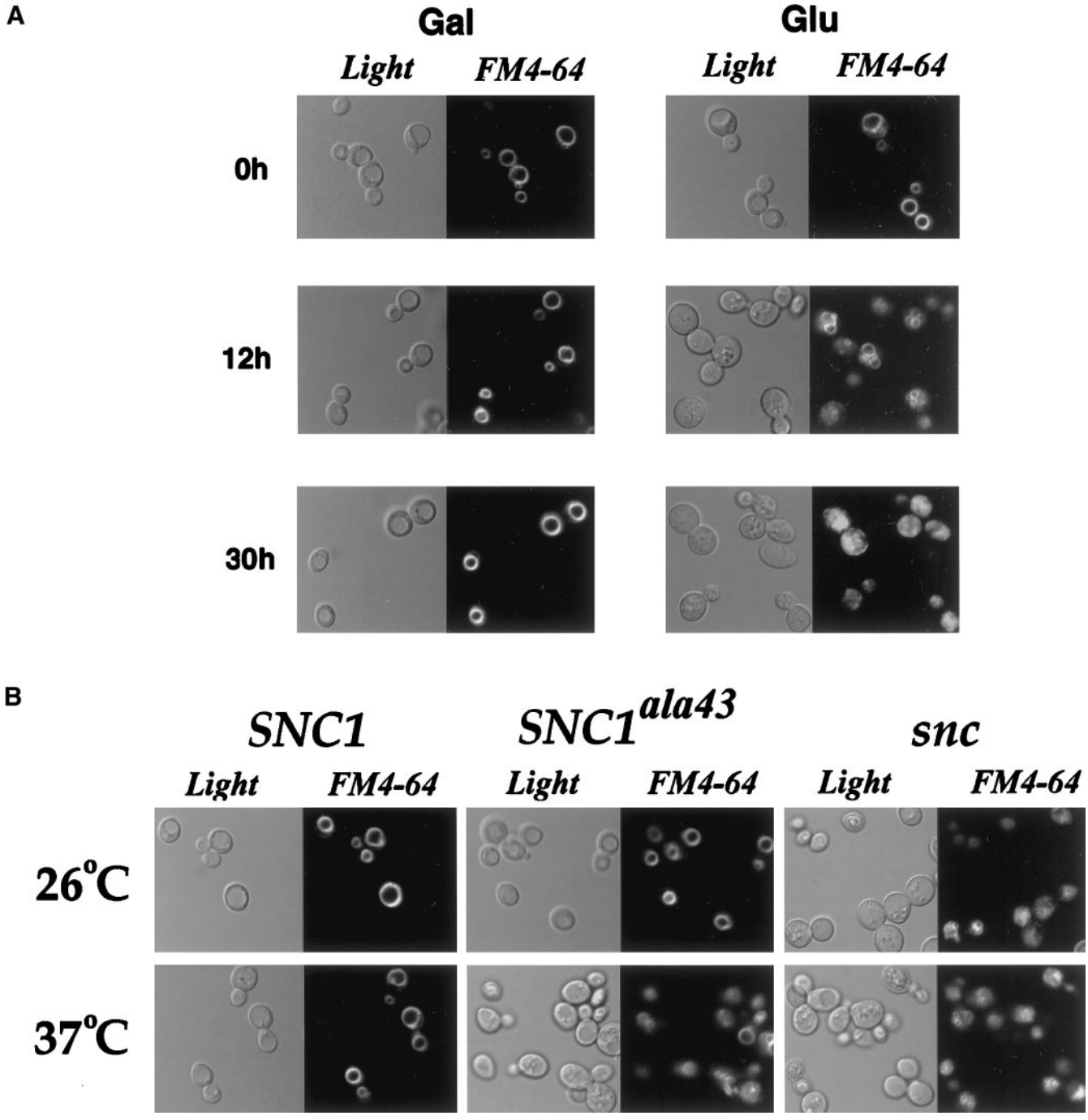

Figure 2. Delivery of FM4–64 to the vacuole is impaired in *snc* cells and *SNC1*^{ala43} cells shifted to restrictive temperatures**.** (A) Delivery of FM4–64 to the vacuole is impaired in *snc* cells. *snc* cells (JG8) maintained on galactose-containing medium (GAL), or shifted to glucose-containing medium (GLU) for various times at 26°C, were incubated with FM4–64 (see MATERIALS AND METHODS) and were processed for microscopy. Cells were visualized using light (Nomarski optics) and fluorescence (rhodamine channel) microscopy. (B) Delivery of FM4–64 to the vacuole is impaired in *SNC1*^{ala43}cells shifted to restrictive temperatures. *snc* cells (JG8) expressing a control vector (*snc*), *SNC1*, or *SNC1*^{ala43} were incubated with FM4–64 at 26°C or were shifted for 30 min to 37°C before labeling with FM4–64 at the restrictive temperature (see MATERIALS AND METHODS).

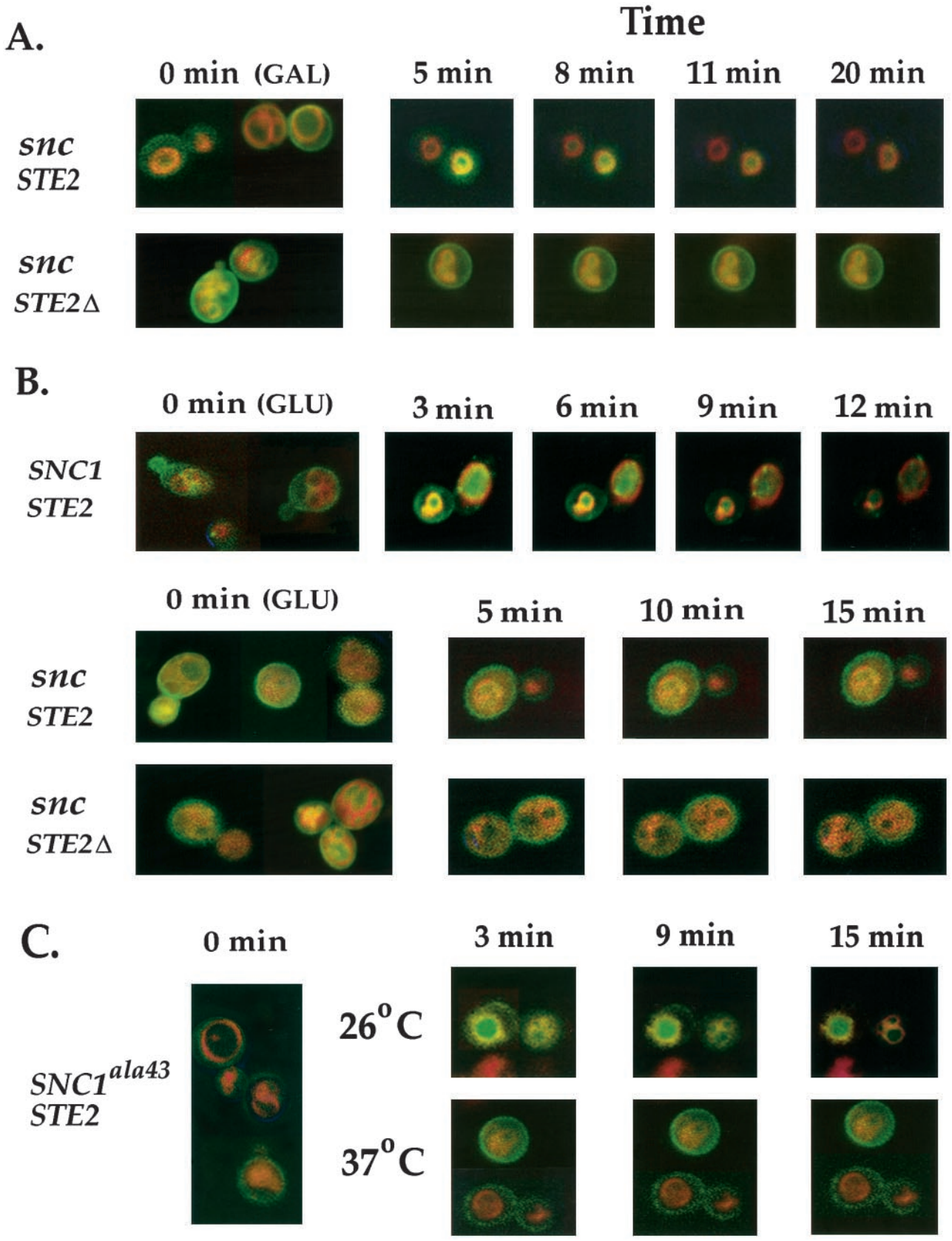
A.
Time
0 min (GAL)
5 min
8 min
11 min
20 min
snc
STE2
snc
STE2Δ
B.
0 min (GLU)
3 min
6 min
9 min
12 min
SNC1
STE2
0 min (GLU)
5 min
10 min
15 min
snc
STE2
snc
STE2Δ
C.
0 min
3 min
9 min
15 min
SNC1ala43
STE2
26°C
37°C

Figure 3.

dye exclusion from the vacuolar bodies in Figure 3B). In contrast to the results obtained on galactose-containing medium (Figure 3A), no translocation of either the full-length or truncated Ste2-GFP fusion proteins to the cytoplasm or vacuole was observed, even after long-term treatment (up to 45 min) with α-factor (Figure 3B; our unpublished results). Thus, ligand-mediated retrieval of Ste2 to the vacuole appears fully blocked in *snc* cells. As a control, *snc* cells expressing *SNC1* constitutively showed a rapid translocation of Ste2-GFP to the vacuole on α-factor treatment. The Ste2-GFP protein was degraded more quickly (i.e., near-complete degradation by 12 min) than on galactose-containing medium, probably due to the enhanced metabolic rate of the cells.

A similar experiment was performed using *snc* cells (JG8) expressing *SNC1*^{ala43} (Figure 3C). We found that at 26°C, Ste2 underwent internalization and delivery to the vacuole on treatment with α-factor, although we noted that the rate of internalization was slower than that seen in *snc* cells expressing native *SNC1* (Figure 3B). In contrast, no translocation of Ste2-GFP was observed whatsoever in cells shifted to 37°C for 1.5 h, indicating that ligand-mediated endocytosis was fully blocked.

Vacuolar Degradation of Ste2 Is Inhibited in snc Cells

To verify that Ste2 is not degraded in the vacuole in *snc* cells, as suggested by the GFP fluorescence data shown above, we examined ligand-mediated degradation of an epitope-tagged form of Ste2 by pulse-chase analysis. *snc* cells (JG8) expressing HA-tagged Ste2 were shifted from galactose-containing medium to glucose-containing medium for 30 h before pulse-chase with [^{35}S]-methionine and subsequent treatment with α-factor. At different times after α-factor treatment, samples were removed and Ste2HA was immunoprecipitated and exposed for autoradiography (Figure 4A). In control WT cells expressing Ste2HA, a broad band of approximately 47 kDa was initially detected before treatment (0 min). After 2 min of treatment with α-factor a higher molecular weight (54 kDa), possibly ubiquitinated (Hicke and Riezman, 1996) and/or hyperphosphorylated, form of Ste2 was evident. Afterward, both bands disappeared as a function of time. By 20 min, these bands were almost completely absent, which is suggestive of targeted degradation of the protein. In WT or *snc* cells lacking the Ste2HA expression vector, we noted that no bands were present within this molecular weight range (our unpublished results). In other experiments performed using *end4* mutant cells expressing Ste2HA, we noted that both Ste2HA bands were apparent for up to 90 min (our unpublished results).

When examined in *snc* cells expressing Ste2HA, two bands of approximately 47 kDa were detected before treatment with α-factor (0 min), whereas by 2 min the higher molecular weight form (54 kDa) seen in WT cells also was observed. We believe that the lower molecular weight form may represent incompletely processed Ste2 protein, as it appeared to mature as a function of time. More work is necessary to determine the exact nature of the different bands observed in our labeling experiments. In contrast to WT cells, Ste2HA did not disappear from α-factor–treated *snc* cells with the same apparent kinetics (Figure 4A). Interestingly, the two major molecular weight forms of Ste2HA were readily apparent, even up to 120 min after treatment with α-factor.

Next, when examined in *snc* cells expressing *SNC1*^{ala43}, we found that the Ste2HA protein was completely stable in temperature-shifted (37°C) cells for at least 60 min after treatment with α-factor (Figure 4B). In contrast, α-factor treatment of *SNC1*^{ala43} cells maintained at 26°C resulted in the total disappearance of Ste2HA by 60 min (Figure 4B). We note that the same two major bands (i.e., 47 and 54 kDa) were observed in *SNC1*^{ala43} cells, although other minor bands could be detected.

These results suggest that Ste2 degradation is severely delayed or, perhaps, completely blocked in the absence of the Snc v-SNARE or in temperature-shifted *SNC1*^{ala43} cells. Along with the data presented in Figure 3, we propose that ligand-mediated uptake and delivery of Ste2 to the vacuole is negligible in the absence of a functional Snc v-SNARE. Since hydrolase (i.e., CPY) delivery from the Golgi to the vacuole does occur in the absence of these v-SNAREs (David *et al.*, 1998), it suggests that the endosomal trafficking defects seen in *snc* or temperature-shifted *SNC1*^{ala43} cells are probably specific to components being retrieved from the cell surface.

Genetic Interactions Between the TLG and SNC Genes in Yeast

The Tlg2 and Tlg1 t-SNAREs are proposed to mediate retrograde trafficking of proteins from the PM to endosomes and from endosomes to the Golgi, respectively (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a; Seron *et al.*, 1998; Coe *et al.*,

Figure 3 (facing page). Ligand-mediated delivery of Ste2 to the vacuole is abolished in *snc* cells and *SNC1*^{ala43} cells shifted to restrictive temperatures. (A) Delivery of Ste2-GFP, but not Ste2Δ-GFP, to the vacuole occurs in *snc* cells grown on galactose. *snc* cells (JG8) expressing either Ste2-GFP (*snc STE2*) or a C-terminal truncated form of Ste2-GFP (*snc STE2*Δ), which does not undergo endocytosis, were grown to log phase at 26°C before labeling with FM4–64 (see MATERIALS AND METHODS). Cultures were split, whereby half were mounted in soft agar containing no additions, while the other half were mounted in agar containing α-factor (1 μM) and were processed immediately for microscopy. A composite image of untreated cells is representative of the zero time (0 min), while specific α-factor–treated cells were monitored as a function of time. (B) Delivery of Ste2-GFP to the vacuole is abolished in *snc* cells grown on glucose. *snc* cells (JG8) expressing *SNC1* constitutively or bearing a control plasmid were transformed with plasmids expressing Ste2-GFP to give *SNC1 STE2* and *snc STE2* strains, respectively. In addition, *snc* cells expressing the truncated form of Ste2-GFP (*snc STE2*Δ) also were used. Cells were grown to log phase at 26°C, before labeling with FM4–64. Next, samples of cells were mounted in agar containing no additions, while an equal amount was mounted in agar containing α-factor (1 μM) before visualization, as described above. (C) Delivery of Ste2-GFP to the vacuole is abolished in *SNC1*^{ala43} cells shifted to the restrictive temperature. *snc* cells (JG8) expressing *SNC1*^{ala43} were grown to log phase and either incubated with FM4–64 at 26°C or shifted for 30 min to 37°C before labeling with FM4–64 at the restrictive temperature. Cells that were treated with α-factor (1 μM) were monitored over time by microscopy.

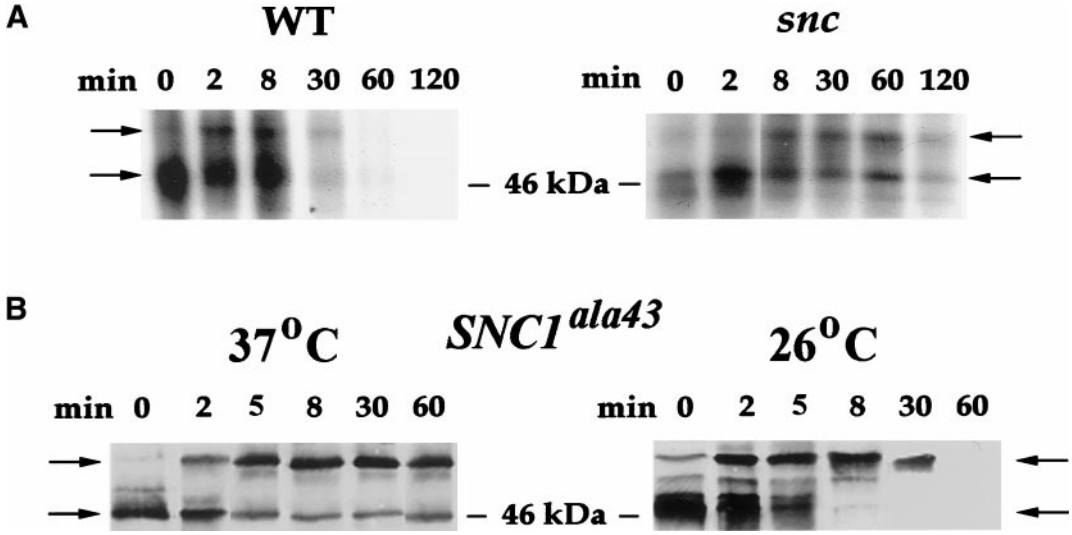

Figure 4. Vacuolar degradation of Ste2 is severely delayed or blocked in *snc* cells and temperature-shifted *SNC1*^{ala43} cells. (A) Vacuolar degradation of Ste2 is delayed in *snc* cells. Wild-type (WT) and *snc* cells grown on glucose-containing medium at 26°C for 30 h were pulse-labeled with [^{35}S]-methionine and chased for 30 min in medium containing 5 mM methionine/cysteine, before treatment with α-factor (1 μM). At various times after α-factor treatment, cell extracts were prepared and Ste2HA was immunoprecipitated using anti-HA antibodies. Ste2HA was resolved by SDS-PAGE and was detected by autoradiography. The lower and upper arrows indicate the respective 46–47-kDa and 54-kDa forms of Ste2HA observed before and after treatment with α-factor. (B) Vacuolar degradation of Ste2 is blocked in temperature-shifted *SNC1*^{ala43} cells. *snc* cells expressing *SNC1*^{ala43} were grown on glucose-containing medium at 26°C for 30 h before pulse-chase labeling and exposure to α-factor (1 μM) for different amounts of time. Cells either were maintained at the permissive temperature (26°C) during labeling and α-factor treatment or were shifted to the restrictive temperature (37°C) during the chase and thereafter. Ste2HA was resolved on gels and detected as described above.

1999). In addition, it is possible that their functions overlap to some degree at the endosomal level (Lewis *et al.*, 2000). We reasoned that if the Snc and Tlg proteins confer retrograde trafficking events from the cell surface, then combined deletions in both the *SNC* and *TLG* genes could result in an enhancement of growth defects. As Tlg2 was shown to coimmunoprecipitate with Snc2, and Tlg1 with Snc1 (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a), it was important to determine whether these physical interactions had any functional significance in vivo.

Cells lacking the individual *TLG* genes tend to grow normally, while those lacking the *SNC* genes in the SP1 background are conditional lethal (Protopopov *et al.*, 1993; Holthuis *et al.*, 1998a). Thus, even if the Snc v-SNAREs are directly involved in Tlg-mediated trafficking steps, we still expect these t-SNAREs to confer some retrograde functions in the absence of the v-SNAREs. These are likely to come about either by utilizing another v-SNARE or, more likely, by forming functional t-t-SNARE fusion complexes (Marash and Gerst, unpublished observations). To test this, we disrupted either *TLG1* or *TLG2* in *snc* cells by gene-targeted disruption (see Experimental Procedures) and monitored them for growth and trafficking phenotypes. We found that in the presence of *SNC1* expression (i.e., on galactose-containing medium), *snc tlg2* cells grew normally (Figure 5,A and B) but were deficient in their ability to deliver FM4–64 to the vacuole (Figure 5D), as shown previously (Abeliovich *et al.*, 1998; Seron *et al.*, 1998). In contrast, in the absence of *SNC* gene expression *snc tlg2* cells grew poorly at normally permissive temperatures (Figure 5, A and B) and had a cell division time approximately 10% longer than *snc* cells (Figure 5A, right panel).

Similar results were observed between combined mutations in *TLG1* and the *SNC* genes (Figure 5, A and B). *snc tlg1* cells grew normally on galactose-containing medium (Figure 5, A and B), although FM4–64 delivery to the vacuole was severely altered (Figure 5D). In the absence of *SNC1* gene expression, *snc tlg1* cells grew very poorly and appeared even more inhibited for growth than *snc tlg2* cells (Figure 5, A and B). *snc tlg1* cells were unable to grow at either 15°C or 30°C (Figure 5A), unlike *snc tlg2* cells (Figure 5A; our unpublished results). Thus, combined *snc* and *tlg* mutations result in severe growth deficiencies, indicating that functional Snc–Tlg1 and Snc–Tlg2 interactions are likely to occur in vivo and contribute to shared membrane-trafficking events.

To provide additional evidence for a functional interaction between the *SNC* and *TLG* genes, we expressed *SNC1*^{ala43} in *snc tlg* strains and examined cell growth, the uptake of FM4–64, and invertase secretion. We reasoned that since the Snc1^{ala43} mutant is already deficient in its ability to be endocytosed, as shown in Figure 1D, then its expression in *tlg* cells might not enhance endocytic uptake, secretion, or cell growth, as it does in *snc* yeast (Figure 1). Indeed, we found that *snc tlg1* or *snc tlg2* cells expressing *SNC1*^{ala43} grew identically to *snc tlg1* or *snc tlg2* cells on glucose-containing medium (Figure 5C, right panel). In addition, no amelioration in the uptake of FM4–64 was observed (Figure 5D). In contrast, *SNC1*^{ala43} tlg cells grown on galactose-containing medium (to induce the expression of

native *SNC1*) grew identically to wild-type or *tlg* mutant cells, as expected (Figure 5C, left panel). Next, we examined the secretion of invertase from *snc tlg1* or *snc tlg2* cells expressing *SNC1*^{ala43}. We found that these cells behaved identically to *snc* cells (see Figure 1) in their ability both to synthesize and secrete only small amounts of enzyme at 26°C (our unpublished results). Thus, while the *SNC1*^{ala43} allele is able to ameliorate exocytosis in *snc* cells (Figure 1), it is unable to do so in the absence of either *TLG1* or *TLG2*. This further implicates the *SNC* and *TLG* genes as interacting in a functional manner.

DISCUSSION

In yeast, the Bet1, Sec22 (ER-Golgi), and Vti1 (Golgi-PVC) v-SNAREs have been shown to participate in both anterograde and retrograde trafficking events by virtue of their ability to interact with t-SNARE partners that are present on both donor and acceptor compartments (Gotte and Fischer von Mollard, 1998; Nichols and Pelham, 1998). Since no other v-SNAREs, except for Snc1 and Snc2, are operant on the Golgi-to-PM trafficking pathway, we examined whether they also are involved in endocytic protein sorting in yeast. Here, we demonstrate that the Snc v-SNAREs are likely to play an important role in endocytic events. We have shown, both in cells lacking the *SNC* genes (JG8 strain) as well as in *snc* cells possessing a temperature-sensitive allele of *SNC1* (Figure 1), that delivery of a lipophilic dye from the cell surface to the vacuole is greatly impaired (Figures 2 and 3). More importantly, we show that ligand-mediated delivery of the Ste2 mating factor receptor from the cell surface to the vacuole also is impaired, if not completely blocked, in both *snc* (JG8) and temperature-shifted *SNC1*^{ala43}cells (Figures 3 and 4). Thus, we conclude that both constitutive and regulated endocytic processes are deficient in the absence of Snc v-SNAREs.

In *snc* and temperature-shifted *SNC1*^{ala43} cells, FM4–64 labeling leads to hazy staining of the cytoplasm areas as well as fragmented vacuoles. Vacuolar fragmentation could imply a role for these v-SNAREs in the fusion of vacuolar bodies, although more work will be required to prove this point. Complete fragmentation of the vacuole might also lead to the cytoplasmic staining pattern observed in these cells, although we believe this is not the case. This is because vacuoles remain distinguishable in *snc* cells, using either light (Figure 2, A and B) or electron microscopy (Protopopov *et al.*, 1993), and since CPY processing appears to be normal (David *et al.*, 1998). It seems more likely then that FM4–64 is labeling the vesicles that accumulate in both *snc* (Protopopov *et al.*, 1993) and temperature-shifted *SNC1*^{ala43} cells (Figure 1). These may be endocytic vesicles that fail to undergo docking and fusion with endosomes or, more likely, secretory vesicles that accumulate in the cytoplasm. If the latter is indeed correct, it may point toward the involvement of endosomes as a site of secretory vesicle biogenesis, of which there is some initial evidence (Yuan *et al.*, 1997; Holthuis *et al.*, 1998b, Luo and Chang, 2000).

Our data imply that Snc v-SNAREs mediate the proper delivery of endocytosed components from the cell surface. This contrasts with other late-acting secretory mutants, such as *sec4* or *sec9*, which are not deficient in α-factor internalization and, thus, are likely to act exclusively on exocytosis (Hicke *et al.*, 1997). As vital dyes like FM4–64 (Figures 2 and 3) and lucifer yellow (our unpublished results) enter *snc* yeast, but not the Ste2 mating factor receptor (Figures 3 and 4), it is likely that there is more than one endocytic route into the cell. This idea is supported by studies showing that mutations in the *END* genes, which are blocked in α-factor and α-factor receptor uptake, do not block FM4–64 entry (Vida and Emr, 1995). It is also supported, at least tangentially, by the fact that multiple trafficking routes to the cell surface are operant in yeast (Harsay and Bretscher, 1995; David *et al.*, 1998).

As Ste2 fails to reach the vacuole in *snc* yeast (evidenced by the lack of retrieval of Ste2-GFP from the PM [Figure 3] and the impaired delivery of Ste2HA to the vacuole [Figure 4]), it may be that biogenesis of the Ste2-containing class of endocytic vesicles is deficient in the absence of these v-SNAREs. This is supported by electron microscopy studies performed earlier on *snc* cells (Protopopov *et al.*, 1993) in which we were unable to identify the small 30–50-nm vesicles that have been proposed to result from a block in vesicle fusion with endosomes (Prescianotto-Baschong and Riezman, 1998; Seron *et al.*, 1998). However, we have noted the presence of 30–50-nm vesicles in the dense fractions of density gradients used to resolve the 100–120-nm LDSV and HDSV classes of secretory vesicles (David and Gerst, unpublished data). Although present in very low numbers (relative to secretory vesicles) they were not found in membrane preparations derived from temperature-shifted late-acting *sec* mutants. Thus, it is possible that some nonsecretory vesicles accumulate in *snc* mutants and may be one possible source for FM4–64 entry into the cell.

We have shown that FM4–64 is able to enter the cell via a route that is initially independent of the Snc v-SNAREs but at some point requires them for proper targeting to the vacuole. This trafficking step is likely to involve Tlg2, given the following: 1) its role in endocytosis (Abeliovich *et al.*, 1998; Seron *et al.*, 1998); 2) the similar FM4–64 staining patterns observed in *snc*, *tlg2* (*snc tlg2* cells on galactose-containing medium), *snc tlg2*, and *SNC1*^{ala43} *tlg2* cells; and 3) the genetic interactions observed between the *SNC* and *TLG2* genes (Figures 2, 3, 5; our unpublished results). Yet, given the genetic and physical linkage between Snc1,2 and Tlg1 (and the similar mislocalization of FM4–64 in *tlg1* [i.e., *snc tlg1* cells on galactose-containing medium], *snc tlg1*, and *SNC1*^{ala43} *tlg1* cells) (Figure 5; our unpublished results), we also envisage Snc proteins as mediating the fusion of a class of endosomally derived vesicles with either a late endosomal or Golgi compartment. Both hypotheses are illustrated in a schematic (Figure 6). However, due to conflicting evidence regarding the actions and localization of Tlg1 (Holthuis *et al.*, 1998; Coe *et al.*, 1999; Lewis *et al.*, 2000), it is possible that this t-SNARE is involved in membrane fusion between more than one compartment.

Several parameters indicate that the *SNC* and *TLG1,2* gene products interact. First, Snc proteins were shown by others to coimmunoprecipitate with Tlg1,2 from yeast cell lysates (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a). Although no specific function could be ascribed at that time, it did suggest that these t-SNAREs might mediate the docking and fusion of a vesicle population that bears the Snc v-SNAREs. Second, genetic studies performed here (Figure 5) demonstrate that *snc tlg2* and *snc tlg1* cells show additive defects, in

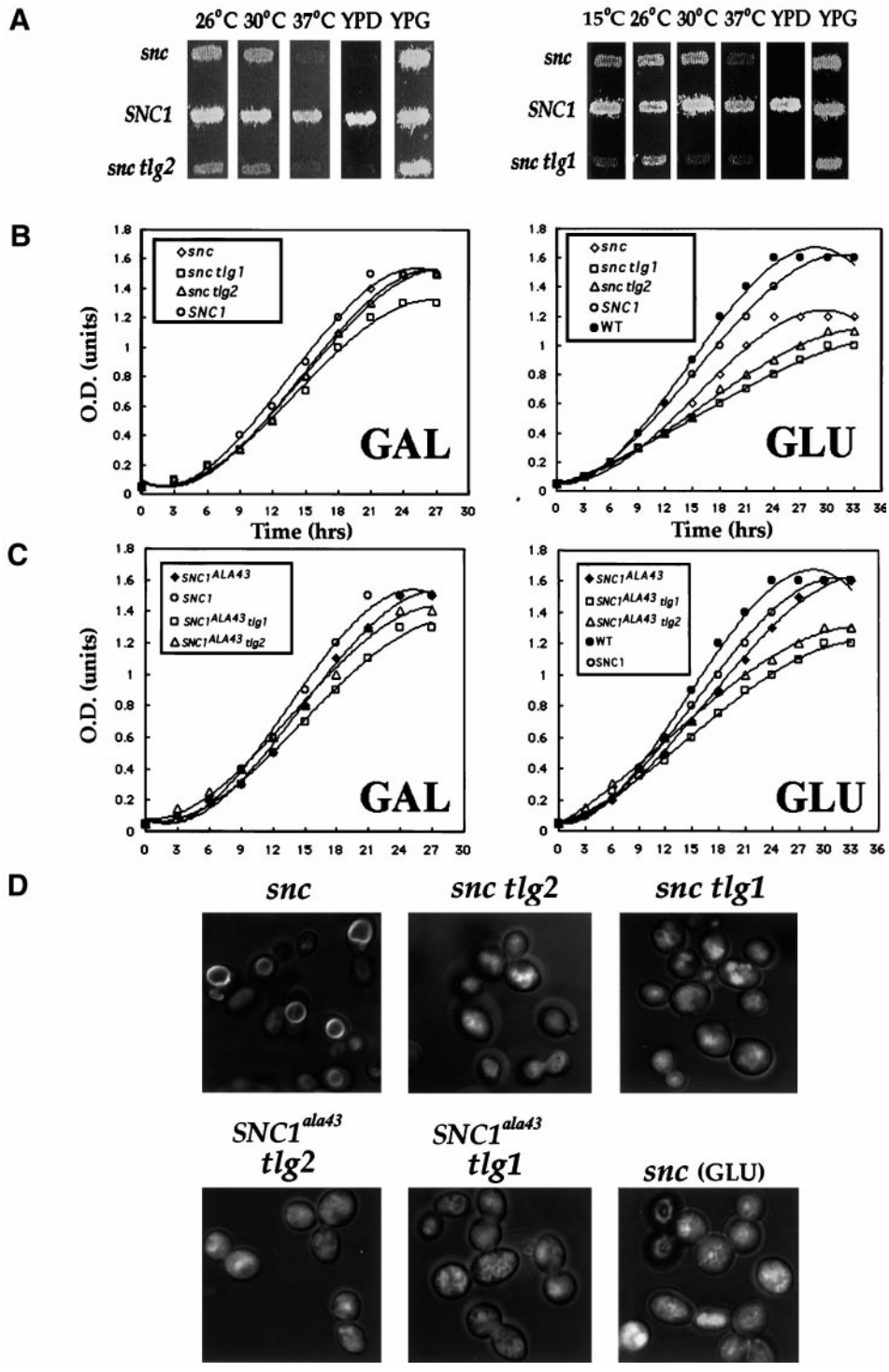

Figure 5. Genetic interactions between *TLG* and *SNC* genes in yeast. (A) Disruption of *TLG1* or *TLG2* in *snc* cells (JG8) slows cell growth and results in conditional lethality. Left panel: *snc* cells (JG8) transformed with a control plasmid (*snc*), a plasmid expressing *SNC1* constitutively (*SNC1*), or *snc tlg2* cells. Right panel: *snc* cells (JG8) transformed with a control plasmid (*snc*), a plasmid expressing *SNC1* constitutively (*SNC1*), or *snc tlg1* cells. All cells first were grown at 26°C on medium containing galactose, before replica plating onto medium containing glucose to deplete Snc1. After 24 h, patches were replica-plated onto the following: glucose-containing plates preequilibrated to 15°C, 26°C, 30°C, and 37°C; rich medium (YPD); and rich medium containing galactose (YPG). Plates were incubated for 30 h. (B) Growth curves of *snc tlg* cells on galactose- and glucose-containing medium. Left panel: *snc* (JG8), *snc* cells expressing *SNC1*, *snc tlg1*, and *snc tlg2* cells were grown to log phase at 26°C on galactose-containing medium (to induce *SNC1*). Equal amounts of cells then were seeded into fresh

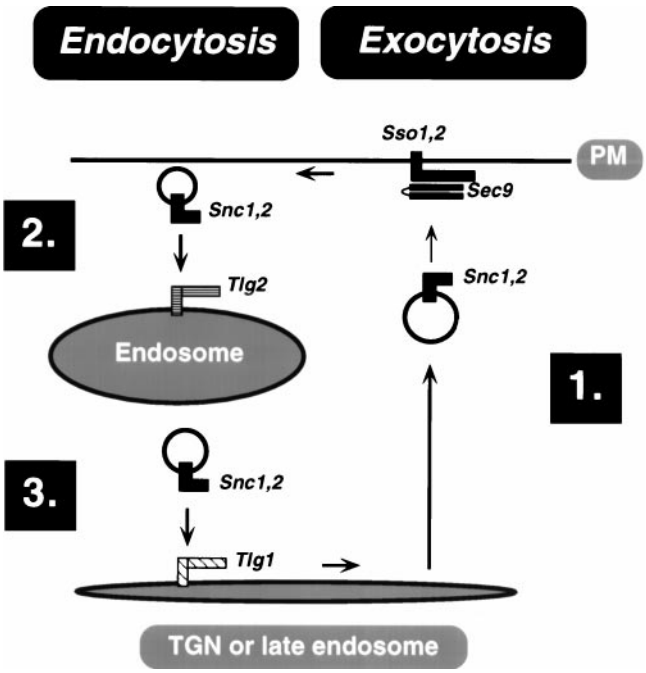

Figure 6. A model for Snc v-SNARE functioning in exocytosis and endocytosis. (1) Secretory vesicles derived from the *trans* Golgi (TGN) or, perhaps, a late endosomal sorting compartment undergo docking and fusion at the PM. Snc v-SNAREs confer the docking and fusion of two classes of secretory vesicles by forming functional SNARE complexes in *trans* with partner t-SNAREs from the PM, Sso1,2, and Sec9. (2) After membrane fusion and subsequent disassembly of the *cis* SNARE complexes (catalyzed by Sec17/18 [not illustrated]), Snc proteins are recruited to newly forming endocytic vesicles. Next, they interact with Tlg2 at an early endosomal compartment and mediate fusion of the endocytic vesicles, as suggested from this and other studies (see DISCUSSION). (3) Finally, Snc proteins are retrieved back to the Golgi by entering an endosome-derived vesicle and subsequently conferring its ability to fuse with the TGN. This last step is probably mediated by Tlg1, which has been shown to localize to that compartment. Given the long half-life of Snc proteins (approximately 8 h; Couve *et al.*, 1995), it is likely that these v-SNAREs participate in many rounds of exocytic and endocytic vesicle trafficking.

particular an impaired rate of cell growth. Third, the presence of the *SNC1*^{ala43} allele in *snc tlg* cells confers no additional benefit (Figure 5). The latter findings not only imply a functional linkage between these gene products, but also suggest that recycling of the mutant v-SNARE is linked to its ability to confer exocytosis. An alternative explanation is that secreted proteins traverse a Golgi-to-endosome sorting route, before being trafficked to the cell surface via secretory vesicles. If such a route exists, then Snc-Tlg v-SNARE–t-SNARE interactions could be involved, in addition to their role downstream of the Snc proteins in endocytic uptake. If so, this could explain why combined *snc* and *tlg* mutations do not have an additive effect on FM4–64 delivery, while clearly having additive effects on cell growth.

Combined *snc* and *tlg* mutations led to a significant increase in cell division time and conditional lethality. Yet, this contrasts with the unconditional lethality observed in cells bearing both *snc* null mutations and a temperature-sensitive mutation in *SSO2* (*sso1*Δ *sso2–1*) (David *et al.*, 1998), *SEC9* (*sec9–4*) (Couve and Gerst, 1994), or *SEC17* (*sec17–1*) (Gerst, 1997), which encode proteins involved in the exocytic fusion step. Therefore, we presume that Snc–Tlg interactions are not essential for cell viability but are necessary for normal growth and endocytic functioning. As neither *TLG1* nor *TLG2* are essential in most backgrounds, and even *tlg1 tlg2* cells are viable (Holthuis *et al.*, 1998a), it appears that blocks in the endocytic pathway are tolerated more than those on the exocytic route. This is substantiated by fact that *sec* mutations are confined entirely to ER-Golgi-PM transport routes (Novick *et al.*, 1980). Likewise, studies on vacuolar protein sorting reveal that blocks in endosomal sorting do not result in lethality (Bryant and Stevens, 1998). Based on knowledge concerning the role of Tlg2 in endocytosis (Abeliovich *et al.*, 1998; Seron *et al.*, 1998), Tlg1 in Golgi–endosome function (Holthuis *et al.*, 1998a; Coe *et al.*, 1999), and the experiments shown here, it is likely that Snc proteins mediate at least one endocytic transport step, if not more. Notably, a recent study suggested that the *TLG* gene products mediate delivery of Snc v-SNAREs to the Golgi (Lewis *et al.*, 2000), implying a passive role for these v-SNAREs as retrieved components. Yet, taken together with the experiments performed here, it would seem far more likely that they are active components of the endosomal protein-sorting machinery.

As no other v-SNARE is known to operate on membrane transport from the cell surface to early endosomes in yeast, we propose that the Snc proteins fulfill this function. If so, these v-SNAREs may complete a dynamic cycle of trafficking steps. Beginning with the docking and fusion of the two classes of exocytic vesicles with the PM (Protopopov *et al.*, 1993; David *et al.*, 1998), we propose that they also mediate the docking and fusion of newly formed endocytic vesicles with early endosomes (Figure 6), in conjunction with Tlg2. From there, they are likely to be delivered back to the

Figure 5 (cont). galactose-containing medium (GAL), and optical density (measured at 600 nm) at 26°C was monitored as a function of time. Right panel: Wild-type (WT), *snc* (JG8), *snc* cells expressing *SNC1*, *snc tlg1*, and *snc tlg2* cells were grown to log phase at 26°C on galactose-containing medium before transfer to glucose-containing medium for 24 h to deplete Snc1. Equal amounts of cells then were seeded into glucose-containing medium (GLU) and optical density (measured at 600 nm) at 26°C was monitored as a function of time. (C) *snc tlg* cells expressing *SNC1*^{ala43} are not rescued for growth defects. Left panel: *snc* (JG8), *snc* cells expressing *SNC1*, *snc tlg1*, and *snc tlg2* cells were grown to log phase at 26°C on galactose-containing medium (to induce *SNC1*). Equal amounts of cells then were seeded into fresh galactose-containing medium (GAL) and optical density (at 600 nm) at 26°C was monitored as a function of time. Right panel: WT, *snc* (JG8), *snc* cells expressing *SNC1*, *SNC1*^{ala43} *tlg1*, and *SNC1*^{ala43} *tlg2* cells were grown to log phase at 26°C on galactose-containing medium before transfer to glucose-containing medium for 24 h to deplete Snc1. Equal amounts of cells then were seeded into fresh glucose-containing medium (GLU) and optical density (at 600 nm) at 26°C was monitored. (D) FM4–64 labeling of vacuoles is impaired in *snc tlg* and *SNC1*^{ala43} *tlg* cells. *snc* (JG8), *snc tlg1*, and *snc tlg2* cells were grown continually on galactose-containing medium at 26°C before labeling with FM4–64 (top three panels). *snc* (JG8), *SNC1*^{ala43} *tlg1*, and *SNC1*^{ala43} *tlg2* cells were grown continually on glucose-containing medium at 26°C before labeling with FM4–64 (bottom three panels).

compartment of origin of the exocytic classes of vesicles (Figure 6) via an endosome-derived vesicle. Since Tlg1 has been shown to localize to both the late Golgi as well as prevacuolar/endosomal compartments, we presume that it is the t-SNARE that functions in this step. The circuit traversed by the Snc v-SNAREs allows them to confer the anterograde transport of secreted proteins to the cell surface, as well as the retrograde transport of relevant cargo proteins (including themselves) back to the Golgi, via the endosomal sorting pathway. Since the yeast and mammalian proteins are conserved structurally and functionally in evolution, their homologues/orthologues in higher organisms may traverse similar routes.

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REFERENCES

Abeliovich, H., Darsow, T., and Emr, S.D. (1999). Cytoplasm to vacuole trafficking of aminopeptidase I requires a t-SNARE-Sec1p complex composed of Tlg2p and Vps45p. EMBO J. *18*, 6005–6016.

Abeliovich, H., Grote, E., Novick, P., and Ferro-Novick, S. (1998). Tlg2p, a yeast syntaxin homolog that resides on the Golgi and endocytic structures. J. Biol. Chem. *273*, 11719–11727.

Banfield, D.K., Lewis, M.J., Rabouille, C., Warren, G., and Pelham, H.R. (1994). Localization of Sed5, a putative vesicle targeting molecule, to the cis-Golgi network involves both its transmembrane and cytoplasmic domains. J. Cell Biol. *127*, 357–371.

Brennwald, P., Kearns, B., Champion, K., Keranen, S., Bankaitis, V., and Novick, P. (1994). Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. Cell *79*, 245–258.

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.

Coe, J.G., Lim, A.C., Xu, J., and Hong, W. (1999). A role for Tlg1p in the transport of proteins within the Golgi apparatus of *Saccharomyces cerevisiae*. Mol. Biol. Cell *10*, 2407–2423.

Couve, A., and Gerst, J.E. (1994). Yeast Snc proteins complex with Sec9: functional interactions between putative SNARE proteins. J. Biol. Chem. *269*, 23391–23394.

Couve, A., Protopopov, V., and Gerst, J.E. (1995). Yeast synaptobrevin homologs are modified posttranslationally by the addition of palmitate. Proc. Natl. Acad. Sci. USA *92*, 5987–5991.

David, D., Sundararabu, 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*, 1176–1182.

Fasshauer, D., Sutton, R.B., Brunger, A.T., and Jahn, R. (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc. Natl. Acad. Sci. USA *95*, 15781–15786.

Fischer von Mollard, G.F., Nothwehr, S.F., and Stevens, T.H. (1997). The yeast v-SNARE Vti1p mediates two vesicle transport pathways through interactions with the t-SNAREs Sed5p and Pep12p. J. Cell Biol. *137*, 1511–1524.

Fischer von Mollard, G.F., and Stevens, T.H. (1999). The *Saccharomyces cerevisiae* v-SNARE Vti1p is required for multiple membrane transport pathways to the vacuole. Mol. Biol. Cell *10*, 1719–1732.

Gerst, J.E. (1997). Conserved α-helical segments on yeast homologs of the synaptobrevin/VAMP family of v-SNAREs mediate exocytic function. J. Biol. Chem. *272*, 16591–16598.

Gerst, J.E. (1999). SNAREs and SNARE regulators in membrane fusion and exocytosis. Cell Mol. Life. Sci. *55*, 707–734.

Gerst, J.E., Ferguson, K., Vojtek, A., Wigler, M., and Field, J. (1991). CAP is a bifunctional component of the *Saccharomyces cerevisiae* adenylyl cyclase complex. Mol. Cell. Biol. *11*, 1248–1257.

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.

Goldstein, A., and Lampen, J.O. (1975). β-D-fructofuranoside fructohydrolase from yeast. Methods Enzymol. *42*, 504–511.

Gotte, M., and Fischer von Mollard, G. (1998). A new beat for the SNARE drum. Trends Cell. Biol. *8*, 215–218.

Grote, E., Hao, J.C., Bennett, M.K., and Kelly, R.B. (1995). A targeting signal in VAMP regulating transport to synaptic vesicles. Cell *81*, 581–589.

Grote, E., and Kelly, R.B. (1996). Endocytosis of VAMP is facilitated by a synaptic vesicle targeting signal. J. Cell Biol. *132*, 537–547.

Hardwick, K.G., and Pelham, H.R. (1992). SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. J. Cell Biol. *119*, 513–521.

Harsay, E., and Bretscher, A. (1995). Parallel secretory pathways to the cell surface in yeast. J. Cell Biol. *131*, 297–310.

Hay, J.C., and Scheller, R.H. (1997). SNAREs, and NSF. in targeted membrane fusion. Curr. Opin. Cell Biol. *9*, 505–512.

Hicke, L. Zanolari, B., Pypaert, M., Rohrer, J., and Riezman, H. (1997). Transport through the yeast endocytic pathway occurs through morphologically distinct compartments and requires an active secretory pathway and Sec18p/*N*-Ethylmaleimide-sensitive fusion protein. Mol. Biol. Cell *8*, 13–31.

Hicke, L., and Riezman, H. (1996). Ubiquitination of a yeast plasma membrane receptor signals its ligand- stimulated endocytosis. Cell *84*, 277–287.

Holthuis, J.C., Nichols, B.J., Dhruvakumar, S., and Pelham, H.R. (1998a). Two syntaxin homologues in the TGN/endosomal system of yeast. EMBO J. *17*, 113–126.

Holthuis, J.C., Nichols, B.J., and Pelham, H.R. (1998b). The syntaxin Tlg1p mediates trafficking of chitin synthase III to polarized growth sites in yeast. Mol. Biol. Cell *9*, 3383–3397.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene *77*, 61–68.

Lewis, M.J., Nichols, B.J., Prescianotto-Baschong, C., Riezman, H., and Pelham, H.R. (2000). Specific retrieval of the exocytic SNARE Snc1p from early yeast endosomes. Mol. Biol. Cell *11*, 23–38.

Lewis, M.J., and Pelham, H.R. (1996). SNARE-mediated retrograde traffic from the Golgi complex to the endoplasmic reticulum. Cell *85*, 205–215.

Lewis, M.J., Rayner, J.C., and Pelham, H.R. (1997). A novel SNARE complex implicated in vesicle fusion with the endoplasmic reticulum. EMBO J. *16*, 3017–3024.

Lian, J.P., and Ferro-Novick, S. (1993). Bos1p, an integral membrane protein of the endoplasmic reticulum to Golgi transport vesicles, is required for their fusion competence. Cell *73*, 735–745.

Lian, J.P., Stone, S., Jiang, Y., Lyons, P., and Ferro-Novick, S. (1994). Ypt1p implicated in v-SNARE activation. Nature *372*, 698–701.

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.

Lupashin, V.V., Pokrovskaya, I.D., McNew, J.A., and Waters, M.G. (1997). Characterization of a novel yeast SNARE protein implicated in Golgi retrograde traffic. Mol. Biol. Cell *8*, 2659–2676.

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.

Munn, A.L., Silveira, L., Elgort, M., and Payne, G.S. (1991). Viability of clathrin heavy-chain-deficient *Saccharomyces cerevisiae* is compromised by mutations at numerous loci: implications for the suppression hypothesis. Mol. Cell. Biol. *11*, 3868–3878.

Nichols, B.J., and Pelham, H.R.B. (1998). SNAREs and membrane fusion in the golgi apparatus. Biochim. Biophys. Acta *1404*, 9–31.

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.

Pelham, H.R.B. (1997). SNAREs and the organization of the secretory pathway. Eur. J. Cell Biol. *65*, 311–314.

Pfeffer, S.R. (1996). Transport vesicle docking: SNAREs and associates. Annu Rev. Cell Dev. Biol. *12*, 441–461.

Prescianotto-Baschong, C., and Riezman, H. (1998). Morphology of the yeast endocytic pathway. Mol. Biol. Cell *9*, 173–189.

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.

Rose, M.D., Winston, F., and Hieter, P. (1990). Methods in Yeast Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Rossi, G., Salminen, A., Rice, L.M., Brunger, A.T., and Brennwald, P. (1997). Analysis of a yeast SNARE complex reveals remarkable similarity to the neuronal SNARE complex and a novel function for the C terminus of the SNAP-25 homolog, Sec9. J. Biol. Chem. *272*, 16610–16617.

Rothman, J.E. (1996). The protein machinery of vesicle budding and fusion. Protein Sci. *5*, 185–194.

Rothman, J.E., and Warren, G. (1994). Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. Curr. Biol. *4*, 220–233.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Seron, K. *et al*. (1998). A yeast t-SNARE involved in endocytosis. Mol. Biol. Cell *9*, 2873–2889.

Søgaard, M., Tani, K., Ye, R.R., Geromanos, S., Tempst, P., Kirchhousen, T., Rothman, J.E., and Söllner, T. (1994). A rab protein is required for the assembly of SNARE complex in the docking of transport vesicles. Cell *78*, 937–948.

Spang, A., and Schekman, R. (1998). Reconstitution of retrograde transport from the Golgi to the ER in vitro. J. Cell Biol. *143*, 589–599.

Stefan, C.J., and Blumer, K.J. (1999). A syntaxin homolog encoded by *VAM3* mediates down-regulation of a yeast G protein-coupled receptor. J. Biol. Chem. *274*, 1835–1841.

Stone, S., Sacher, M., Mao, Y., Carr, C., Lyons, P., Quinn, A.M., and Ferro-Novick, S. (1997). Bet1p activates Bos1p. Mol. Biol. Cell *8*, 1175–1181.

Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature *395*, 347–353.

Vida, T.A., and Emr, S.D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J. Cell Biol. *128*, 779–792.

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. 1997 *272*, 25787–25793.

Zelicof, A., Protopopov, V., David, D., Lin, X.Y., Lustgarten, V., and Gerst, J.E. (1996). Two separate functions are encoded by the carboxyl-terminal domains of the yeast cyclase-associated protein and its mammalian homologs: dimerization and actin binding. J. Biol. Chem. *271*, 18243–18252.