t-SNARE Phosphorylation Regulates Endocytosis in Yeast

Sangiliyandi Gurunathan, Michael Marash, Adina Weinberger, and Jeffrey E. Gerst*

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

Submitted November 7, 2001; Revised January 28, 2002; Accepted February 1, 2002
Monitoring Editor: Randy Schekman

Earlier we demonstrated that activation of a ceramide-activated protein phosphatase (CAPP) conferred normal growth and secretion to yeast lacking their complement of exocytic v-SNAREs (Snc1,2) or bearing a temperature-sensitive mutation in an exocytic t-SNARE (Sso2). CAPP activation led to Sso dephosphorylation and enhanced the assembly of t-SNAREs into functional complexes. Thus, exocytosis in yeast is modulated by t-SNARE phosphorylation. Here, we show that endocytic defects in cells lacking the v- and t-SNAREs involved in endocytosis are also rescued by CAPP activation. Yeast lacking the Tlg1 or Tlg2 t-SNAREs, the Snc v-SNAREs, or both, undergo endocytosis after phosphatase activation. CAPP activation correlated with restored uptake of FM4-64 to the vacuole, the uptake and degradation of the Ste2 receptor after mating factor treatment, and the dephosphorylation and assembly of Tlg1,2 into SNARE complexes. Activation of the phosphatase by treatment with C₂-ceramide, *VBM/ELO* gene inactivation, or by the overexpression of *SIT4* was sufficient to confer rescue. Finally, we found that mutation of single PKA sites in Tlg1 (Ser31 to Ala31) or Tlg2 (Ser90 to Ala90) was sufficient to restore endocytosis, but not exocytosis, to *snc* cells. These results suggest that endocytosis is also modulated by t-SNARE phosphorylation in vivo.

INTRODUCTION

Intracellular protein and lipid trafficking involves the selective transfer of cargo molecules from one compartment to the next. This is accomplished by the fusion of membranes derived from the donor compartment with those belonging to a specific target. A number of proteins are involved in the tethering, docking, and fusion steps that confer bilayer fusion and subsequent protein transport. Among those, SNAREs comprise three evolutionarily conserved families (e.g., the syntaxin, synaptobrevin/VAMP, and SNAP-25 families) of membrane-associated proteins that are required for the docking and fusion steps (Ferro-Novick and Jahn, 1994; Rothman and Warren, 1994). 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). The SNARE complex usually involves three or four SNARE molecules, each contributing one or two α -helical cores to the formation of a four-helix coiled-coil bundle (Sutton *et al.*, 1998; Katz *et al.*, 1998). Formation of this complex is necessary and sufficient for membrane fusion in vitro but ne-

cessitates interactions between specific and precisely arrayed SNARE partners (Weber *et al.*, 1998; McNew *et al.*, 2000).

Because SNAREs play a pivotal role in the fusion process, it is likely that they receive input from the cellular environment to either enhance or impede membrane trafficking events. In particular, kinases involved in growth control are likely to modulate SNARE functions, presumably by the posttranslational modification of residues that participate in SNARE partnering or the binding of SNARE regulatory proteins (reviewed in Gerst, 1999; Turner *et al.*, 1999; Klenchin and Martin, 2000). Numerous studies have outlined the possible involvement of kinases in the regulation of SNARE protein interactions (Hirling and Scheller, 1996; Shimazaki *et al.*, 1996; Foster *et al.*, 1998; Shuang *et al.*, 1998; Risinger and Bennett, 1999; Chung *et al.*, 2000) as well as the availability of SNAREs to participate in membrane trafficking events (Cabaniols *et al.*, 1999; Foletti *et al.*, 2000; Kataoka *et al.*, 2000). Therefore, a role for signal-induced protein kinases in regulating SNARE complex assembly is likely and offers a potential mechanism for coordinating the dynamics of cell growth with cell cycle control.

In yeast, the Snc v-SNAREs (Gerst *et al.*, 1992; Protopopov *et al.*, 1993) assemble into fusion complexes with the Sso and Sec9 t-SNAREs to form the exocytic SNARE complex (Brennwald *et al.*, 1994; Couve and Gerst, 1994; Rossi *et al.*, 1997). Deletion of the *SNC* genes results in the intracellular accu-

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.01-11-0541. Article and publication date are at www.molbiol-cell.org/cgi/doi/10.1091/mbc.01-11-0541.

* Corresponding author. E-mail address: jeffrey.gerst@weizmann.ac.il.

mulation of secretory vesicles, in an inhibition in protein secretion, and in various conditional lethal phenotypes (Protopopov *et al.*, 1993; David *et al.*, 1998). Mutations in either of two genes that encode homologous ER-localized proteins (Vbm1,2/Elo2,3), which are involved in long-chain fatty acid (LCFA) elongation, result in the intracellular accumulation of phytosphingosine and rescue *snc* cells (David *et al.*, 1998). This occurs via activation of a sphingoid base/ceramide-activated phosphatase (CAPP), Sit4, and the subsequent dephosphorylation of discrete PKA sites in the Sso t-SNAREs (Marash and Gerst, 2001). Thus, two signal transduction pathways in yeast converge upon the exocytic t-SNAREs and have opposing effects on their ability to form SNARE complexes. The PKA pathway, which is involved in growth control, phosphorylates Sso proteins and inhibits their assembly into SNARE complexes in vitro and in vivo. In contrast, a CAPP signaling pathway, which mediates cellular stress responses, activates Sit4 and dephosphorylates the Sso t-SNAREs. Dephosphorylation of the t-SNAREs enhances SNARE complex assembly and led to restored secretion in cells bearing mutations in the Snc v-SNAREs or Sso t-SNAREs (Marash and Gerst, 2001).

The Snc exocytic v-SNAREs also mediate endocytosis (Gurunathan *et al.*, 2000). In *snc* null cells or cells possessing a temperature-sensitive allele of *SNC1* (*snc1^{ala43}*) that were shifted to restrictive temperatures, uptake of the soluble dye FM4-64 and the Ste2 mating factor receptor to the vacuole is abolished. Thus, Snc v-SNAREs confer both anterograde and retrograde transport events between the Golgi and the plasma membrane. Appropriately, their endocytic functions are likely to be dependent on two different t-SNAREs, Tlg1 and Tlg2, which are *trans*-Golgi and endosomal t-SNAREs involved in endosomal protein sorting (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a; Seron *et al.*, 1998; Coe *et al.*, 1999; Abeliovich *et al.*, 1999). This is because Snc proteins coimmunoprecipitate with the Tlg t-SNAREs (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a); *Snc1^{ala43}* is nonfunctional in their absence (Gurunathan *et al.*, 2000); and *snc tlg1* and *snc tlg2* strains show synthetically enhanced phenotypes (Gurunathan *et al.*, 2000). Moreover, Tlg1 and 2 have been recently shown to bind Snc v-SNAREs and to mediate an in vitro fusion event similar to endocytosis (Paumet *et al.*, 2001).

Here we show that CAPP activation also confers normal endocytic functioning to cells lacking the Snc v-SNAREs. Rescue occurs by either the exogenous treatment of cells with active ceramide analogs, by *VBM* gene inactivation, or by overexpression of *SIT4* itself. Interestingly, endocytic defects in cells lacking either Tlg1 or Tlg2 alone or in combination with deletions in the *SNC* genes are also rescued by CAPP activation. In all cases, rescue correlated with the dephosphorylation of Tlg1 and Tlg2 and their assembly into complexes both in vivo and in vitro. Finally, we demonstrate that the mutation of single PKA sites in the putative NH₂-terminal auto-inhibitory domains of Tlg1 and Tlg2 results in constitutively activated SNAREs that confer endocytosis in the absence of CAPP activation. Thus, both the PKA and CAPP signaling pathways modulate endocytosis by regulating phosphorylation of the Tlg t-SNAREs and their ability to assemble into SNARE complexes.

MATERIALS AND METHODS

Media, DNA, and Genetic Manipulations

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

Growth Tests

Yeast were grown on synthetic and rich growth media (Rose *et al.*, 1990). In experiments involving ceramide, *N*-acetyl-D-erythro-sphingosine (C₂-ceramide; Sigma, St. Louis, MO) was dissolved in ethanol (at 15 mM or 1 mg/ml) and added to the media to a final concentration of 10 μ M. For growth tests on plates, yeast were counted using a hemocytometer, diluted serially, and plated by drops onto solid medium preincubated at different temperatures. For growth of cells in liquid culture, cells were cultured overnight in medium containing C₂-ceramide.

For growth tests involving *snc* and *snc tlg* mutants, which carry a galactose-inducible form of *SNC1*, cells were grown to log phase on galactose-containing synthetic medium. Next, cells were shifted to glucose-containing medium (36 h) to induce the *snc* phenotype. Cells were then seeded at an optical density (OD₆₀₀) of 0.05 into fresh glucose-containing medium at 26°C with or without the addition of 10 μ M C₂-ceramide. Cells were monitored for growth (OD measured at 600 nm) every 3 h up to 36 h. Saturation of the culture (with or without ceramide) was reached usually by 27 h. OD₆₀₀ values were plotted on graphs versus time to show the kinetics of saturation as well as on logarithmic graphs to calculate cell division times for the different strains.

For growth tests involving *snc1^{ala43}* and *snc1^{ala43} tlg* cells, cells were grown to log phase on glucose-containing medium at 26°C. Cells were then seeded at an OD₆₀₀ of 0.05 into prewarmed glucose-containing medium either containing or lacking C₂-ceramide (10 μ M) and grown at 37°C. Cells were monitored for growth as described above.

Yeast Strains

Yeast strains used are listed in Table 1. To create *snc tlg vbm1* yeast, the *snc vbm1* strains DD1 and MM1 were cured of their *GAL-SNC1*-containing plasmids and transformed with linearized DNA fragments isolated from the enzymatic digestion of pTLG1L and pTLG2T (Gurunathan *et al.*, 2000). Transformants were selected and the disruptions verified by PCR analysis. To create *snc1^{ala43} tlg* yeast, *snc tlg* cells (JG9-TLG1 or JG9-TLG2) were transformed with either plasmid pLADH-SNC1^{ala43} or pTADH-SNC1^{ala43}. Transformants were cured of their *GAL-SNC1*-containing plasmids on synthetic medium at 26°C. For cells expressing STE2-GFP constructs that bear the *kan^r* resistance marker, strains were transformed with YCpK-STE2-GFP and selected for on synthetic medium containing 100 μ g/ml geneticin.

Plasmids

Previously described *SNC* expression plasmids included the following: pADH-SNC1 (Gerst *et al.*, 1992); pADH-HASNC1 and pTGAL-SNC1 (Protopopov *et al.*, 1993); and pAHGAL-SNC2 (David *et al.*, 1998). Plasmids pRS314STE2-GFP and pRS314ste2 Δ tail-GFP, which express the STE2-GFP or STE2-tailGFP gene fusions (Stefan and Blumer, 1999), respectively, were provided by K. Blumer (Washington University School of Medicine). *snc1^{ala43}* expression constructs included: pADH-SNC1^{ala43}; pTADH-SNC1^{ala43}; and pLADH-SNC1^{ala43} (Gurunathan *et al.*, 2000). TLG1,2 disruption constructs, pTLG1L and pTLG2T, respectively, were described previously, as was a *CEN* plasmid that expresses STE2HA, pLADH-STE2HA (Gurunathan *et al.*, 2000). Plasmids for the expression of *SIT4* (YEpsIT4)

Table 1. Strains used in this study

Name	Genotype	Source
JG8	<i>MATa can1 his3 his4 leu2 trp1 snc1::URA3 snc2::ADE8 pTGAL-SNC1</i> or <i>pLGAL-SNC1</i>	J. Gerst
DD1	<i>MATa can1 his3 his4 leu2 trp1 snc1::URA3 snc2::ADE8 vbm1::TRP1 pLGAL-SNC1</i>	J. Gerst
MM1	<i>MATa can1 his3 his4 leu2 trp1 snc1::URA3 snc2::ADE8 vbm1::LEU2 pTGAL-SNC1</i>	This study
JG8 SNC1A43L	<i>MATa can1 his3 his4 leu2 trp1 snc1::URA3 snc2::ADE8 pLADH-SNC1^{ala43}</i>	J. Gerst
JG8 SNC1A43T	<i>MATa can1 his3 his4 leu2 trp1 snc1::URA3 snc2::ADE8 pTADH-SNC1^{ala43}</i>	J. Gerst
JG9-TLG2	<i>MATa can1 his3 his4 trp1 snc1::URA3 snc2::ADE8 tlg2::LEU2 pTGAL-SNC1</i>	J. Gerst
JG9-TLG2,VBM1	<i>MATa can1 his3 his4 trp1 snc1::URA3 snc2::ADE8 tlg2::LEU2 vbm1::TRP1</i>	This study
JG9-TLG2,SNC1A43	<i>MATa can1 his3 his4 trp1 snc1::URA3 snc2::ADE8 tlg2::LEU2 pTADH-SNC1^{ala43}</i>	J. Gerst
JG9-TLG1	<i>MATa can1 his3 his4 leu2 snc1::URA3 snc2::ADE8 tlg1::TRP1 pLGAL-SNC1</i>	J. Gerst
JG9-TLG1,VBM1	<i>MATa can1 his3 his4 leu2 snc1::URA3 snc2::ADE8 tlg1::TRP1 vbm1::LEU2</i>	This study
JG9-TLG1,SNC1A43	<i>MATa can1 his3 his4 leu2 snc1::URA3 snc2::ADE8 tlg1::TRP1 pLADH-SNC1^{ala43}</i>	J. Gerst
SP1	<i>MATa can1 his3 leu2 trp1 ura3 ade8</i>	M. Wigler
NY778	<i>MATα ura3-52 leu2-3,112 sec6-4</i>	P. Novick

in yeast and *TPK1* (pGEX-TPK1) in bacteria were described previously (Marash and Gerst, 2001). Plasmids created for this study are listed in Table 2.

Microscopy

Cells were labeled with FM4-64 and monitored for fluorescence, as described (Gurunathan *et al.*, 2000). GFP fluorescence was visualized via the FITC channel in strains expressing the appropriate GFP fusion proteins. Thin sectioning and electron microscopy was performed essentially as described by Zelicof *et al.* (1996). Quantification of the vesicles per unit area was calculated by counting the number of vesicles in photos of thin-sectioned cells. Next, the total area counted was determined by weighing cutouts of the cells and dividing them by the weight of a cutout equivalent to 1 μm^2 .

Dividing the former by the latter yields the number of vesicles per μm^2 . At least 30 cells per strain were used in the measurements.

Metabolic Labeling

Pulse-chase studies for Ste2 uptake, using [³⁵S]methionine (Amersham, Amersham, United Kingdom), were performed essentially as described previously (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 [³⁵S]methionine (0.1 mCi/OD₆₀₀ unit). After a 30-min chase in medium containing 5 mM methionine and cysteine, cells were treated with α -factor (1 μM) for up to 60 min. Cell extracts were prepared, and Ste2HA was immunoprecipitated using anti-HA antibodies. Precipitated Ste2HA was solubilized in SDS-containing sample

Table 2. Plasmids created for this study

Plasmid name	Gene expressed	Vector	Sites of cloning	Oligo name ^a
pGST-SIT4	<i>SIT4</i>	pGEX4T-3	<i>EcoRI</i> and <i>XhoI</i>	Sit4-f, Sit4-r
pGST-TLG1 ¹⁻²⁰⁶	<i>TLG1¹⁻²⁰⁶</i>	pGEX4T-3	<i>EcoRI</i> and <i>SalI</i>	Tlg1-f, Tlg1-r
pGST-TLG2 ¹⁻³¹⁷	<i>TLG2¹⁻³¹⁷</i>	pGEX4T-3	<i>EcoRI</i> and <i>SalI</i>	Tlg2-f, Tlg2-r
pADH-mycTLG1	<i>myc-TLG1</i>	pAD6	<i>SalI</i> and <i>SacI</i>	Tlg1-f, Tlg1-r
pADH-mycTLG2	<i>myc-TLG2</i>	pAD6	<i>SalI</i> and <i>SacI</i>	Tlg2-f, Tlg2-r
pADH-mycTLG1 ^{ala31}	<i>myc-TLG1^{ala31}</i>	pAD6	<i>SalI</i> and <i>SacI</i>	Tlg1A31-f, Tlg1A31-r
pADH-mycTLG2 ^{ala6}	<i>myc-TLG2^{ala6}</i>	pAD6	<i>SalI</i> and <i>SacI</i>	Tlg2A6-f, TlgA6-r
pADH-mycTLG2 ^{ala15}	<i>myc-TLG2^{ala15}</i>	pAD6	<i>SalI</i> and <i>SacI</i>	Tlg2A15-f, TlgA15-r
pADH-mycTLG2 ^{ala54}	<i>myc-TLG2^{ala54}</i>	pAD6	<i>SalI</i> and <i>SacI</i>	Tlg2A54-f, TlgA54-r
pADH-mycTLG2 ^{ala90}	<i>myc-TLG2^{ala90}</i>	pAD6	<i>SalI</i> and <i>SacI</i>	Tlg2A90-f, TlgA90-r
pADH-mycTLG2 ^{ala101}	<i>myc-TLG2^{ala101}</i>	pAD6	<i>SalI</i> and <i>SacI</i>	Tlg2A101-f, TlgA101-r
pADH-mycTLG2 ^{ala138}	<i>myc-TLG2^{ala138}</i>	pAD6	<i>SalI</i> and <i>SacI</i>	Tlg2A138-f, TlgA138-r
pGEM-SIT4	<i>SIT4</i>	pGEM-T	<i>SalI</i>	Sit4-2-f, Sit4-2-r
YE ⁺ TSIT4 ^b	<i>SIT4</i>	pRS424	<i>SalI</i>	
pTADH-mycTLG1 ^c	<i>myc-TLG1</i>	pRS424	<i>BamHI</i>	
YCpKSTE2-GFP ^d	<i>STE2-GFP</i>	pRS314STE2-GFP	<i>EcoRI</i>	
pKADH-SNC1 ^e	<i>SNC1</i>	pADH-SNC1	<i>ClaI</i>	

^a Oligonucleotide sequences will be provided upon request.
^b This plasmid was created by subcloning a *SalI* fragment from pGEM-SIT4 into pRS424.
^c This plasmid was created by subcloning a *BamHI* fragment from pADH-mycTLG1 into pRS424.
^d This plasmid was created by subcloning a blunt-ended *EcoRV* and *SalI* fragment encoding *kan^r* into pRS314STE2-GFP.
^e This plasmid was created by subcloning a blunt-ended *EcoRV* and *SalI* fragment encoding *kan^r* into pADH-SNC1.

buffer, heated for 15min at 37°C, and resolved on 10% SDS-PAGE gels. For experiments involving *snc1^{ala43}* cells at 37°C, strains were shifted to 37°C 1 h before labeling.

SNARE Complex Measurement from Cell Lysates

SNARE complexes present in cell lysates were monitored by the immunoprecipitation (IP) of SNAREs from cell extracts, as described in Couve and Gerst (1994). However, the following additions to the lysis and IP buffers were made: 0.5% NP-40 (instead of Triton X-100); MG132 (100 μ M), ATP γ S (20 μ M), EDTA (2 mM), and *n*-ethylmaleimide (1 mM), to inhibit SNARE complex dissociation and degradation. Anti-Tlg1 and anti-Tlg2 antibodies (gifts of H. Pelham and H. Abeliovich) were used for IP (1 μ l per reaction) and detection (1:2000). The amount of Tlg1 present in heteromeric complexes with Tlg2 was determined using the anti-Tlg2 antiserum for IP and detection with the anti-Tlg1 antiserum. The presence of Tlg1 or Tlg2 in homomeric complexes were determined by expressing myc-tagged Tlg1 or Tlg2 in *snc tlg2* and *snc tlg1* cells, respectively. IP was performed using anti-myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and subsequent detection of both tagged and untagged proteins was performed using anti-Tlg1 or anti-Tlg2 antibodies. Samples of TCLs, and immunoprecipitates were resolved by electrophoresis and detected by Western blotting. Detection was performed using chemiluminescence (ECL).

Measurement of Tlg Phosphorylation and SNARE Assembly In Vitro

Recombinant GST fusions of Tlg1 and 2, Tpk1, and Sit4 were expressed separately in BL21 *Escherichia coli* and purified over glutathione-Sepharose beads, using standard procedures. Affinity-purified GST-Tlg1¹⁻²⁰⁶ and GST-Tlg2¹⁻³¹⁷ were phosphorylated in vitro by Tpk1, using the procedure described previously for GST-Sso1¹⁻²⁶⁵ (Marash and Gerst, 2001). Labeled proteins were detected in SDS-PAGE gels using autoradiography or by protein-dye staining using Coomassie blue (Bio-Rad Laboratories, Richmond, CA). To measure dephosphorylation, recombinant GST-Sit4 (2 μ g) or GST-Sit4 (2 μ g) with C₂-ceramide (0.05 mM) was added. To measure the association of Tlg1 and Tlg2, affinity-purified in vitro phosphorylated or non-phosphorylated GST-Tlg1¹⁻²⁰⁶ and GST-Tlg2¹⁻³¹⁷ proteins were mixed together at a ratio of 1:1 (5 μ g each) in buffer containing 0.5% Triton X-100 in PBS and allowed to incubate overnight at 4°C. After which, the proteins were IP'd with anti-Tlg1 (1 μ l per IP reaction), resuspended in sample buffer, and resolved by SDS-PAGE. Detection and quantification of the proteins using either anti-Tlg1 or -Tlg2 antibodies (1:2000 dilution) was performed using ECL. ECL signals were measured over a linear range of activity, as determined using known quantities of recombinant GST-Tlg proteins as standards in Western blots.

RESULTS

vbm Mutation or the Exogenous Addition of C₂-ceramide Rescue Growth Defects in *snc*, *snc tlg*, and Temperature-shifted *snc1^{ala43}* and *snc1^{ala43} tlg* Cells

Previous work demonstrated that defects in exocytosis in *snc* null cells or cells bearing a temperature-sensitive mutation in the Sso2 t-SNARE can be rescued by the exogenous addition of active sphingoid bases and ceramide analogs to the growth medium. These molecules activate CAPP and mimic the effects seen upon *vbm* gene mutation in *snc* yeast, leading to dephosphorylation of the Sso t-SNAREs. Because the Snc v-SNAREs are also involved in endocytosis, we determined whether the exogenous addition of C₂-ceramide, an active form of ceramide, or *vbm* gene mutation can restore normal endocytic trafficking in *snc* cells. We also wanted to test its

Table 3. Cell division times

Cell type	Time (h)	Time (h)
	– C ₂ -ceramide	+ C ₂ -ceramide
<i>SNC1</i>	2.0	2.1
<i>snc</i>	4.0	2.4
<i>snc tlg1</i>	4.4	3.1
<i>snc tlg2</i>	4.1	3.0
<i>snc vbm1</i>	2.0	N.D. ^a
<i>snc tlg1 vbm1</i>	2.2	N.D.
<i>snc tlg2 vbm1</i>	2.0	N.D.
<i>SNC1 tlg1</i>	2.2	2.1
<i>SNC1 tlg2</i>	2.2	2.1
<i>SNC1</i> (37°C)	2.0	2.1
<i>snc1^{ala43}</i> (37°C)	N.A. ^b	3.0
<i>snc1^{ala43} tlg1</i> (37°C)	N.A.	3.2
<i>snc1^{ala43} tlg2</i> (37°C)	N.A.	3.1

^a Not determined.

^b Not applicable, fails to grow at 37°C.

effects on *snc tlg1* and *snc tlg2* yeast, which lack one of the *TLG* genes and are more growth deficient than *snc* cells. Because the *snc* and *tlg* mutations each block endocytic functioning, it is likely that the synthetic phenotypes observed in the triple mutants result from an additional role for Tlg1 and 2 in anterograde transport (Gurunathan *et al.*, 2000).

First, it was important to determine the effects of ceramide addition or *vbm* mutation on the growth of *snc tlg* cells to show whether cells lacking both v- and t-SNAREs are rescued (Table 3). Cells were grown at 26°C in liquid culture in glucose-containing medium in order to shut off *SNC1* expression from under a galactose-inducible promoter. In the absence of *SNC1* expression, *snc* and *snc tlg* cells grew slowly and had doubling times of ~4 h, whereas *snc* cells constitutively expressing *SNC1* double every 2 h. In contrast, *snc* and *snc tlg* cells treated with ceramide had shorter doubling times of 2.4 h and ~3 h, respectively. Introduction of a *vbm1/elo3* mutation in *snc tlg* yeast had a similar effect and lowered the doubling time to ~2 h. Both *snc tlg vbm1* cells and ceramide-treated *snc tlg* cells reached the same level of saturation as *snc* cells constitutively expressing *SNC1* (our unpublished results). Finally, we found that *snc tlg* cells treated with ceramide grew much better at other temperatures (i.e., 15°C, 30°C, 35°C, and 37°C), including *snc tlg1* cells, which normally do not grow at 15°C and at temperatures > 26°C (Gurunathan *et al.*, 2000; our unpublished results). Thus, both ceramide treatment and *VBM* gene disruption, which activate CAPP, restore growth in *snc tlg* cells, as shown previously for *snc* cells. We note that the growth of cells bearing the *tlg1* mutation was slightly slower than that of *snc tlg2* cells, even in the presence of ceramide or after the disruption of *VBM1*. This corresponds with earlier results that indicate *TLG1* disruption is more severe than that of *TLG2* (Gurunathan *et al.*, 2000).

We also tested whether the growth of *tlg1* and *tlg2* deletion mutants at 26°C is improved upon ceramide treatment or the overexpression of *SIT4*. We found that *tlg* cells have a doubling time of ~2.2 h, whereas after ceramide treatment (Table 3) or the overexpression of *SIT4* (our unpublished

results) this value remained basically the same (2.1 h for both *tlg1* and *tlg2* yeast). Because the growth defects in *tlg* yeast are mild, it is likely that CAPP activation has only little effect.

The *snc1^{ala43}* allele encodes a thermosensitive v-SNARE (Gurunathan *et al.*, 2000) that is defective specifically in its retrieval and recycling from the plasma membrane (Grote *et al.*, 2000; Gurunathan *et al.*, 2000; Lewis *et al.*, 2000). When expressed from a single-copy plasmid, *snc1^{ala43}* confers the growth of *snc* cells up to 35°C, although the growth rate is slower than that conferred by native *SNC1* (Gurunathan *et al.*, 2000). Interestingly, the expression of *snc1^{ala43}* in *snc tlg* cells does not rescue their growth defects, suggesting that the *TLG* gene products are essential for *Snc1^{ala43}* function and/or recycling (Gurunathan *et al.*, 2000). We next examined the effects of ceramide addition on *snc1^{ala43}* and *snc1^{ala43} tlg* cells that were shifted to the restrictive temperature (37°C; Table 3). In the absence of ceramide, we found that *snc1^{ala43}* and *snc1^{ala43} tlg* cells were unable to grow altogether, as shown previously (Gurunathan *et al.*, 2000). However, after ceramide treatment all strains grew robustly at 37°C and had doubling times of ~3 h. Treated *snc1^{ala43} tlg* yeast grew slightly slower than their *snc1^{ala43}* counterparts (Table 3), but reached a similar final density (our unpublished results). Thus, ceramide treatment appears to restore the functionality of *Snc1^{ala43}*.

Ceramide Treatment Inhibits Vesicle Accumulation in *snc tlg* Cells

Secretory vesicles (SVs) are hardly seen in thin-sectioned wild-type yeast but are common in *snc* cells growing at permissive temperatures (Protopopov *et al.*, 1993; David *et al.*, 1998). We next examined the accumulation of 100–120 nm SVs in ceramide-treated and untreated *snc* and *snc tlg* cells by electron microscopy. We counted the number of SVs present in thin-sectioned *snc tlg1* and *snc tlg2* cells and found they had 24 ± 1 and 19.0 ± 0.5 vesicles per μm^2 , respectively, which was similar to control *snc* cells (17.0 ± 0.7 vesicles per μm^2). However, upon treatment with ceramide, the number of vesicles per μm^2 declined by >60%; *snc tlg1* and *snc tlg2* cells had 9.0 ± 0.7 and 7.0 ± 0.6 vesicles per μm^2 , respectively. Control *snc* cells were found to have 5.0 ± 0.9 vesicles per μm^2 after treatment, as shown previously (Marash and Gerst, 2001). Thus, although *snc tlg* cells accumulate more SVs than *snc* cells, their numbers are also greatly reduced upon ceramide treatment.

Delivery of the Soluble Dye FM4-64 to the Vacuole Is Restored in *snc*, *snc tlg*, and Temperature-shifted *snc1^{ala43}* and *snc1^{ala43} tlg* Cells upon Ceramide Treatment or *vbm* Mutation

Because ceramide treatment has pronounced effects on exocytosis (i.e., cell growth and SV accumulation) in both *snc* and *snc tlg* yeast, we determined whether endocytosis is restored in these cells by the exogenous addition of C₂-ceramide. Previously, we have shown that these strains are defective in the uptake of the vacuolar staining dye, FM4-64 (Gurunathan *et al.*, 2000). Labeling of the cytoplasm and small vacuolar bodies was shown to occur in glucose-shifted *snc* cells and temperature-shifted *snc1^{ala43}* cells as a function of time. *snc tlg* cells also are labeled in a similar manner,

even when expressing *SNC1*, due to the loss in *Tlg* function-ing (Gurunathan *et al.*, 2000).

snc and *snc tlg* strains were shifted to glucose-containing medium for 24 h to induce the *snc* phenotype. Cells were incubated with FM4-64 for short periods and examined *in vivo* using fluorescence and visual (Nomarski) microscopy (Figure 1A). As expected, untreated *snc* cells had a hazy staining of the cytoplasm and small, fragmented, vacuolar bodies. Likewise, *snc tlg1* and *snc tlg2* cells had an identical staining pattern, unlike control *snc* cells expressing *SNC1*. Ceramide treatment restored FM4-64 staining of the vacuole not only in *snc* cells, but also in *snc tlg* cells. Moreover, large round vacuoles were apparent in the treated cells, as observed using Nomarski optics. These results suggest that ceramide treatment restores dye trafficking and vacuole biogenesis to *snc* cells, even in the presence of *tlg* mutations.

Similarly, we found that the mutation of *VBM1* in *snc tlg* cells also restored FM4-64 staining of the vacuole as well as conferring normal vacuole biogenesis (Figure 1B). Thus, both ceramide treatment and *vbm* mutation have identical effects. Finally, we tested whether ceramide treatment could restore vacuolar staining in temperature-shifted *snc1^{ala43}* cells (Figure 1C). Indeed, we found that staining of vacuoles was normal in *snc1^{ala43}* cells shifted to 37°C for 2 h. Therefore, defects that arise from inactivating mutations in the v- and t-SNAREs involved in endocytosis are corrected upon CAPP activation.

Because ceramide treatment or *vbm* mutation result in the dephosphorylation of Sso and rescue *snc* and *sso2-1* cells (Marash and Gerst, 2001), it was possible that the enhanced rate of exocytosis alone might confer an improvement in endocytosis. To test this, we expressed a constitutively activated form of Sso1 (*Sso1^{ala79}*; Marash and Gerst, 2001), which rescues *snc* cells in the absence of CAPP activation, in *snc* yeast and examined the uptake of FM4-64. In contrast to ceramide-treated cells, FM4-64 was unable to label vacuoles or vacuolar bodies in *snc* yeast expressing *Sso1^{ala79}*, and the labeling was identical to that seen in control *snc* cells (Figure 2A). Thus, the effects of ceramide or *vbm* mutation on endocytosis are direct and not a result of enhanced exocytosis.

Overproduction of *Sit4* Confers FM4-64 Labeling of the Vacuole in *snc* and *tlg* Null Cells

Rescue of growth and exocytosis in *snc* cells by either ceramide treatment or *vbm* mutation necessitates the activation of the catalytic subunit of CAPP, *Sit4* (Nickels and Broach, 1996), whose overexpression can also rescue *snc* yeast (Marash and Gerst, 2001). Therefore, we next examined whether *SIT4* overexpression alone could confer the normal uptake of FM4-64 to the vacuole.

We found that *snc* cells transformed a plasmid expressing *SIT4* gave normal staining of the vacuole by FM4-64, after being shifted from galactose-containing medium to glucose-containing medium (Figure 2B). This rescue was apparent up to 36 h after the shift. However, at later time points it also became apparent that this restoration was temporal, because hazy staining of the cytoplasm and vacuolar fragmentation became more obvious (our unpublished results). This time-dependent decrease in the ability of *SIT4* overexpression to restore FM4-64 trafficking could be reversed upon the addition of ceramide into the medium (our unpublished results).

Figure 1. Delivery of FM4-64 to the vacuole is restored in *snc*, *snc tlg*, and temperature-shifted *snc1^{ala43}* cells by CAPP activation. (A) Delivery of FM4-64 to the vacuole is restored in *snc tlg* cells. *SNC1*, *snc* (JG8), *snc tlg1* (JG9-TLG1), and *snc tlg2* (JG9-TLG2) cells were grown to log phase on galactose-containing medium, before being shifted to glucose-containing medium at 26°C for 36 h, with (+C₂) or without (−C₂) C₂-ceramide. Cells were incubated with FM4-64 (1

Finally, we examined whether ceramide addition or *SIT4* overexpression could confer normal FM4-64 staining of the vacuole in *tlg1* and *tlg2* mutant cells (Figure 2C). As shown above for *snc* and *snc tlg* cells, defects in FM4-64 labeling of the vacuole in *tlg* cells were abolished upon ceramide addition or *SIT4* overexpression.

Ligand-mediated Delivery of the Ste2 Mating Factor Receptor to the Vacuole Is Restored in *snc*, *snc tlg*, and Temperature-shifted *snc1^{ala43}* Cells by Ceramide Addition or *vbm* Mutation

Because defects in FM4-64 delivery to the vacuole in *snc* and *tlg* mutant yeast are restored independently by CAPP activation, we examined whether ligand-mediated uptake of a cell surface receptor is also rescued. Previously, we demonstrated that uptake of a Ste2-GFP fusion protein (Stefan and Blumer, 1999) to the vacuole (after α -factor treatment) was blocked in *snc* and temperature-shifted *snc1^{ala43}* cells (Gurunathan *et al.*, 2000).

In *snc* cells (JG8) shifted to glucose-containing medium for 36 h, we found both Ste2-GFP and Ste2-tailGFP present primarily on the plasma membrane in both untreated and α -factor-treated cells (Figure 3A), as described previously. The Ste2-tail GFP protein is deficient in its ability to be endocytosed (Stefan and Blumer, 1999) and served as a control. In contrast to the *snc* control cells, *snc* cells expressing *SNC1* showed a time-dependent uptake of Ste2-GFP upon α -factor treatment, as described (Gurunathan *et al.*, 2000). We then checked whether ceramide addition had an effect on Ste2 uptake in α -factor-treated *snc* yeast. Addition of ceramide restored the time-dependent uptake of Ste2-GFP, but not of Ste2-tailGFP, to the vacuole (Figure 3A). The disappearance of Ste2-GFP in ceramide-treated *snc* cells was similar to that seen for *snc* cells expressing *SNC1*, upon α -factor addition. Thus, ceramide treatment restored endocytic uptake of a membrane protein in *snc* cells.

Because ceramide treatment restored FM4-64 labeling of the vacuole in *snc tlg* cells, we examined the uptake of Ste2-GFP in these strains (Figure 3B). As can be seen, ceramide-treated *snc tlg1* and *snc tlg2* cells responded to α -factor addition and Ste2-GFP was delivered to the vacuole in a time-dependent manner. In contrast, no change in Ste2-GFP localization was observed in untreated cells, after α -factor addition. Thus, ceramide treatment restores Ste2 uptake in cells lacking one of the *TLG* genes as well as in cells lacking both *SNC* genes.

Next, we examined whether Ste2-GFP uptake in *snc* cells is restored by *vbm* mutation. We measured Ste2-GFP uptake

(μ g/ml) and processed for fluorescence and light (Nomarski) microscopy. (B) Delivery of FM4-64 to the vacuole is restored in *snc vbm1* (MM1), *snc tlg1 vbm1* (JG9-TLG1, VBM1), and *snc tlg2 vbm1* (JG9-TLG2, VBM1) cells. Cells were grown on glucose-containing medium for 24 h at 26°C, incubated with FM4-64, and processed for microscopy. (C) Delivery of FM4-64 to the vacuole is restored in ceramide-treated *snc1^{ala43}* cells shifted to restrictive temperatures. *snc* cells expressing *snc1^{ala43}* (JG8-SNC1A43L) were grown in glucose-containing medium at 26°C and either maintained at 26°C during labeling or shifted for 0.5 h to 37°C, before labeling with FM4-64 at the restrictive temperature. Some cells were treated with C₂-ceramide (+C₂) during growth at 26°C.

Figure 2. *SIT4*, but not *SSO1*^{ala79}, overexpression restores the delivery of FM4-64 to the vacuole in *snc* cells. (A) Expression of *SSO1*^{ala79} does not restore FM4-64 labeling of the vacuole. *snc* yeast (JG8) transformed with a plasmid expressing *SSO1*^{ala79} were grown on galactose-containing medium at 26°C, before being shifted to glucose-containing medium for 36 h to deplete Snc1. Cells were incubated with FM4-64 at 26°C and processed for microscopy. (B) *SIT4* overexpression restores FM4-64 labeling of the vacuole. *snc* cells (JG8) transformed with a plasmid expressing *SIT4* were grown and labeled with FM4-64 as described above. (C) Ceramine treatment and *SIT4* overexpression restore FM4-64 labeling of the vacuole in *tlg* yeast. *tlg1* and *tlg2* yeast treated with (+C₂) or without (−C₂) ceramide, or overexpressing *SIT4*, were labeled with FM4-64 and processed for microscopy.

after α -factor addition to *snc vbm1* cells (Figure 3C). As expected Ste2-GFP, but not Ste2-tailGFP, was delivered to the vacuole as shown above for ceramide-treated *snc* cells or *snc* cells expressing *SNC1* (Figure 3, A and B).

Finally, we examined the uptake of Ste2-GFP in *snc1*^{ala43} cells that were shifted to the restrictive temperature (37°C), before the addition of α -factor (Figure 3D). We found that Ste2-GFP was delivered to the vacuole in ceramide-treated, but not untreated, *snc1*^{ala43} cells at 37°C. In contrast, Ste2-GFP was taken up in the absence of ceramide at 26°C. Thus, ceramide treatment can confer the uptake and delivery of Ste2 to the vacuole in *snc*, *snc tlg*, and in temperature-shifted *snc1*^{ala43} cells.

Ste2 Is Degraded in Ceramide-treated snc and Temperature-shifted snc1^{ala43} Cells

In yeast, some membrane proteins undergo ubiquitin-dependent endocytosis that delivers the internalized protein to the vacuole for degradation (Hicke, 1999). To verify that Ste2 is degraded in ceramide-treated *snc* and *snc1*^{ala43} cells, as predicted by the experiments shown in Figure 3, we used an HA-tagged form Ste2 (Ste2HA) in pulse-chase labeling experiments. Previously, we demonstrated that Ste2HA is stable for extended periods in its higher molecular weight, presumably ubiquitinated/phosphorylated form, in α -factor-treated *snc* and temperature-shifted *snc1*^{ala43} cells (Gu-

Figure 3. Ligand-mediated delivery of Ste2 to the vacuole is restored in *snc*, *snc tlg*, and temperature-shifted *snc1^{ala43}* cells by CAPP activation. (A) Delivery of Ste2-GFP, but not Ste2-tailGFP, to the vacuole is restored in ceramide-treated *snc* cells. *snc* cells (JG8) expressing either Ste2-GFP (*snc STE2GFP*) or a truncated form of Ste2-GFP (*snc STE2-tailGFP*), which does not undergo endocytosis, were grown to log phase in galactose-containing medium at 26°C, before being shifted to glucose-containing medium with (+C₂) or without (−C₂) added C₂-ceramide (10 μM) for 36 h, and before labeling with FM4-64. After labeling, cultures were split, whereby half was mounted in soft agar containing no additions, whereas the other half was mounted in agar containing α-factor (1 μM) and processed immediately for microscopy. *snc* cells expressing *SNC1* constitutively (*SNC1 STE2GFP*) were used as control. Cells were monitored by confocal microscopy as a function of time. Separate untreated cells (untreated) were used as controls. (B) Delivery of Ste2-GFP to the vacuole is restored in ceramide-treated *snc tlg* cells. *snc tlg1* (JG9-TLG1) and *snc tlg2* cells (JG9-TLG2) expressing Ste2-GFP (*snc tlg1 STE2GFP* or *snc tlg2 STE2GFP*) from the *kan^r* plasmid were grown in galactose-containing medium at 26°C, before being shifted to glucose-containing medium with (+C₂) or without (−C₂) C₂-ceramide (10 μM). After 24 h, cells were labeled with FM4-64, treated with α-factor and processed for microscopy. (C) Ligand-mediated delivery of Ste2-GFP to the vacuole is restored in *snc* cells bearing a mutation in *VBM1*. *snc vbm1* cells (MM1) expressing either Ste2-GFP (*snc vbm1 STE2GFP*) or the truncated form of Ste2-GFP (*snc vbm1 STE2-tailGFP*) were grown to log phase at 26°C in glucose-containing medium, before labeling with FM4-64, treatment with α-factor, and processing for microscopy. (D) Ligand-mediated delivery of Ste2-GFP to the vacuole occurs in temperature-shifted *snc1^{ala43}* cells upon ceramide treatment. *snc1^{ala43}* cells (JG8-SNC1A43L) expressing Ste2-GFP were grown at 26°C in glucose-containing medium with (+C₂) or without (−C₂) C₂-ceramide (10 μM). Half of each culture was shifted to prewarmed medium (37°C), for 1 h before FM4-64 labeling and treatment with α-factor at the restrictive temperature.

Figure 4. Ceramide treatment restores the degradation of Ste2 in *snc* and temperature-shifted *snc1^{ala43}* cells. *snc* (JG8) and *snc1^{ala43}* (JG8-SNC1A43) cells expressing Ste2HA were grown to log phase in glucose-containing medium at 26°C with (+C₂) or without (–C₂) C₂-ceramide. *snc* cells expressing *SNC1* constitutively (*SNC1*) were grown without ceramide and were used as control. Cells were pulse-labeled with [³⁵S]methionine and chased for 30 min in medium containing 5 mM methionine/cysteine, before treatment with α -factor (1 μ M). For *snc1^{ala43}* cells, half of the cultures were shifted to prewarmed medium (37°C) for 1 h before labeling. At various times after treatment with α -factor (2–60 min), samples were removed, extracts were prepared, and Ste2HA was IP'd using anti-HA antibodies. Ste2HA was detected by autoradiography.

runathan *et al.*, 2000). We next tested whether ceramide treatment could restore the normal degradation of Ste2HA in these cells after α -factor treatment (Figure 4).

We found that Ste2HA is stable for up to 60 min in α -factor-treated *snc* and temperature-shifted *snc1^{ala43}* cells that were not treated with ceramide (Figure 4), as was shown previously. Both the lower (47 kDa) and higher (54 kDa) molecular weight forms were apparent, the lower form being present before α -factor addition. In contrast, C₂-ceramide treatment restored the degradation of Ste2 in both cell types (Figure 4). The higher molecular weight form apparent after 2 min was found to disappear between 20 and 40 min after the addition of α -factor. The rate of degradation was similar to that seen in *snc* yeast expressing *SNC1*. Thus, as suggested by the GFP fluorescence data (Figure 3), ceramide treatment restores Ste2 degradation in *snc* and temperature-shifted *snc1^{ala43}* cells upon α -factor addition.

Ceramide Treatment Enhances Tlg Assembly into Complexes in *snc* and *snc tlg* Cells

Our results show that CAPP activation normalizes both the exocytic (Marash and Gerst, 2001) and endocytic pathways (this study). Previously, we demonstrated that rescue of the exocytic path depends on dephosphorylation of the Sso1,2 t-SNAREs, which assemble into t-t-SNARE complexes with Sec9 to, presumably, form the functional *trans* complex (Marash and Gerst, 2001). The question arises as to which t-SNAREs mediate the endocytosis of materials from the cell surface and whether they also undergo Sit4-dependent dephosphorylation.

Physical and genetic studies show that the Tlg t-SNAREs are involved in endocytosis and interact with the Snc v-SNAREs (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a; Seron *et al.*, 1998; Grote and Novick, 1999; Gurunathan *et al.*, 2000). Thus, it is likely that Tlg2, a syntaxin-like t-SNARE, and Tlg1, a SNARE light-chain, form complexes with Snc1,2 at endosomes or the *trans*-Golgi (TGN). Although Tlg2 has been proposed to act at early endosomes (Abeliovich *et al.*, 1998; Seron *et al.*, 1998), it overlaps with Tlg1, whose placement is broader and encompasses the TGN (Holthuis *et al.*, 1998a, 1998b; Coe *et al.*, 1999). Thus, we conjectured that Tlg1 and 2 could be partners, along with Snc1 or Snc2, in SNARE complexes relevant to endocytic functioning. Although not the focus of this work, a fourth helix is probably contributed to the complex by other SNAREs involved in endosomal functioning, i.e., Vti1.

Both Tlg1 and Tlg2 are phosphorylated *in vivo* (our unpublished results); thus, we examined whether these t-SNAREs are present in complexes in *snc* cells and whether the amounts of these complexes increase upon ceramide treatment. By performing coimmunoprecipitation (coIP) experiments, we found that Tlg1 precipitates from *snc* cell lysates with anti-Tlg2 antibodies and that the amount of Tlg1 in a complex with Tlg2 goes up by substantially after ceramide treatment (Figure 5A). Thus, the assembly of Tlg1 and 2 into SNARE complexes is modulated by CAPP activation. Next, we performed a complimentary experiment whereby we examined whether PKA overexpression affects the assembly of Tlg1 and Tlg2 into complexes in wild-type cells. We found that *TPK1* overexpression lowered the amount of Tlg1 and Tlg2 present in complexes by half (Figure 5B). Thus, both the CAPP and PKA signaling paths modulate Tlg SNARE assembly *in vivo*.

Interestingly, CAPP activation did not block the effect of *TPK1* overexpression in wild-type cells (Figure 5B) and did not increase Tlg assembly in *snc* cells expressing *SNC1* (our unpublished results). This suggests that the A kinase signaling pathway has a dominant effect in the wild-type background. We also examined whether expression of the activated form of Sso1 (Sso1^{ala79}) affects Tlg1–Tlg2 assembly in *snc* cells; however, we found no increase in the level of complex formation (our unpublished results). Thus, an enhancement in exocytosis, as mediated by Sso1^{ala79}, does not influence Tlg SNARE assembly (our unpublished results) or endocytosis *per se* (Figure 2A).

Because CAPP activation confers endocytosis to *snc tlg* and *tlg* cells, which lack one of the Tlg proteins, we examined whether ceramide treatment increases assembly of the remaining Tlg into multimeric complexes. This phenomenon was observed previously with the Sso t-SNAREs after ceramide treatment (Marash and Gerst, 2001). We found that ceramide treatment increased the coIP of native Tlg1 with myc-Tlg1 by about twofold in lysates prepared from *snc tlg2* cells that express a myc epitope-tagged form of Tlg1 (Figure 5C). Likewise, ceramide treatment increased the coIP of native Tlg2 with myc-Tlg2 by twofold in lysates prepared from *snc tlg1* cells expressing myc-Tlg2 (Figure 5D). Thus, individual Tlg t-SNAREs multimerize in response to CAPP activation and, presumably, dephosphorylation.

Figure 5. Ceramide treatment promotes Tlg assembly into Tlg–Tlg SNARE complexes in vivo, whereas Tlg phosphorylation by Tpk1 inhibits assembly in vitro and in vivo. (A) C₂-ceramide treatment promotes Tlg1–Tlg2 complex assembly in *snc* cells. *snc* cells (JG8) were grown to log phase with (+C₂) or without C₂-ceramide (–C₂; 10 μM). Cell extracts were prepared and Tlg1–Tlg2 SNARE complexes were IP'd using purified anti-Tlg2 antibodies (abs) and detected using either anti-Tlg1 or anti-Tlg2 abs. TCL, total cell lysate (100 μg protein/lane). A histogram of the IP data, after normalization for the expression of *TLG1* and *TLG2*, and subtraction of the background, is shown. Density is given in arbitrary units. (B) *TPK1* overexpression inhibits the assembly of Tlg1 and Tlg2 in wild-type cells. Lysates were prepared from SP1 cells that either expressed *TPK1* (+TPK1) or not (–TPK1) and had been treated either with (+C₂) or without C₂-ceramide (–C₂). Tlg1–Tlg2 complexes were IP'd using anti-Tlg2 abs, and Tlg1 was detected in immunoblots using anti-Tlg1 abs. Data are represented in histograms, as described in A. (C) Ceramide treatment promotes formation of a Tlg1–Tlg1 complex in *snc tlg2* cells. *snc tlg2* cells expressing myc-Tlg1 were grown to log phase with (+C₂) or without C₂-ceramide (–C₂; 10 μM). Cell extracts were prepared and myc-Tlg1 was IP'd using anti-myc abs and detected using anti-Tlg1 abs. TCL, total cell lysate (100 μg protein/lane); IP, immunoprecipitate. Data are represented in histograms, as described in A. (D) Ceramide treatment promotes formation of a Tlg2–Tlg2 complex in *snc tlg1* cells. *snc tlg1* cells expressing myc-Tlg2 were grown to log phase with (+C₂) or without C₂-ceramide (–C₂; 10 μM). Cell extracts were prepared and myc-Tlg2 was IP'd using anti-myc abs and detected using anti-Tlg2 abs. TCL, total cell lysate (100 μg protein/lane); IP, immunoprecipitate. Data are represented in histograms, as described in A. (E) In vitro phosphorylation of GST-Tlg1^{1–206} and GST-Tlg2^{1–317} by Tpk1 and dephosphorylation by Sit4. GST-Tlg1^{1–206} and GST-Tlg2^{1–317} were phosphorylated in vitro using Tpk1 and radiolabeled ATP for 1 h at 30°C with or without added Sit4 or Sit4 and C₂-ceramide. Proteins were resolved on SDS-PAGE gels and detected by autoradiography or by staining with Coomassie blue. A histogram of the autoradiography data is shown. (F) Tlg–Tlg assembly is inhibited by phosphorylation. Recombinant GST-Tlg1^{1–206} and GST-Tlg2^{1–317} were phosphorylated in vitro using recombinant Tpk1 and ATP. After phosphorylation, the proteins were mixed and IP'd using anti-Tlg1 abs. In parallel, unphosphorylated GST-Tlg1^{1–206} and GST-Tlg2^{1–317} were also mixed and IP'd using anti-Tlg1 abs. GST-Tlg2^{1–317} that coIP'd with GST-Tlg1^{1–206} was detected using anti-Tlg2 abs. Data are represented in a histogram, as described in A.

Tlg Phosphorylation In Vitro by Tpk1 Inhibits Assembly into Complexes

To examine whether Tlg phosphorylation also inhibits assembly in vitro, we expressed recombinant Tlg1 and Tlg2 (lacking their membrane-spanning domains) as GST-fusion proteins in bacteria. Affinity-purified proteins were phosphorylated in vitro with Tpk1 (Marash and Gerst, 2001) and

used in subsequent binding reactions. Both recombinant Tlg1 and Tlg2 underwent Tpk1-dependent phosphorylation as well as Sit4-dependent dephosphorylation (Figure 5E). The latter was markedly enhanced by the addition of C₂-ceramide into the assay mixture. Binding assays using either phosphorylated or unphosphorylated proteins revealed that the unphosphorylated Tlg t-SNAREs formed two- or three-

fold more heteromeric complexes than the phosphorylated proteins (Figure 5F). Thus, these endosomal t-SNAREs appear to undergo the same types of regulation shown for the Sso exocytic t-SNAREs, both in vivo and in vitro (Marash and Gerst, 2001).

Mutations in Putative PKA Sites in Tlg1 and 2 Result in Activated t-SNAREs that Confer Endocytosis, but not Exocytosis in *snc* Cells

Because the restoration of endocytosis by CAPP activation correlates with Tlg dephosphorylation and assembly, we examined potential sites for phosphorylation in Tlg1 and Tlg2. As shown previously (Marash and Gerst, 2001), mutation of a PKA site (ser-79) in the autoinhibitory domain (Habc) of Sso confers full exocytic functioning in the absence of CAPP activation. Therefore, we mutated putative PKA sites in the same region of Tlg1 (e.g., thr-31) and Tlg2 (e.g., thr-6, -15, -54, -138, and ser-90, -101) by site-directed mutagenesis. We tested these mutants for their ability to confer the uptake of FM4-64 in *snc* yeast in the absence of CAPP activation (Figure 6A and our unpublished results). We found that alanine substitutions at position 31 of Tlg1 and position 90 of Tlg2 restored FM4-64 uptake to the vacuole in *snc* cells (Figure 6A). In contrast, the other alanine substitutions in Tlg2 were unable to do so (our unpublished results).

When tested for conferring growth to *snc* cells, we found that expression of these mutant Tlg proteins had no significant effect alone, but did enhance the rescue mediated by Sso1^{ala79} (Figure 6B). Thus, the presence of Sso1^{ala79} and Tlg1^{ala31} or Sso1^{ala79} and Tlg2^{ala90} restored normal growth (exocytosis) and endocytosis in the absence of CAPP activation. Moreover, their growth appeared more robust than that of *snc* cells expressing SNC1. Thus, abolishment of these PKA sites leads to dominant effects on the exo- and endocytic pathways in *snc* yeast.

DISCUSSION

Earlier we demonstrated that yeast lacking the SNC genes (*snc*, *snc tlg1*, and *snc tlg2* cells) or bearing a temperature-sensitive allele of SNC1 (*snc1^{ala43}*, *snc1^{ala4}tlg1*, and *snc1^{ala4}tlg2* cells) are deficient in the endocytic uptake of components from the cell surface (Gurunathan *et al.*, 2000). Thus, the Snc exocytic v-SNAREs are actively involved in endocytosis. More recent work suggests that CAPP activation allows *snc* and *sso2-1* cells to overcome blocks in exocytosis, via dephosphorylation of the Sso t-SNAREs and their subsequent assembly into SNARE complexes with Sec9 (Marash and Gerst, 2001). Thus, we examined whether the endocytic defects present in *snc* and *snc tlg* cells are also ameliorated upon CAPP activation. Our results show that ceramide treatment, VBM inactivation, or SIT4 overexpression (all of which lead to CAPP activation) confer normal endocytic functioning to yeast lacking the endocytic v-SNAREs (*snc* cells), endocytic t-SNAREs (*tlg1* and *tlg2* cells), or both (*snc tlg1* and *snc tlg2* cells; Figures 1–3). We had expected *snc tlg* cells to be even more inhibited in endocytic functions than *snc* cells, because of the loss of an additional component of the endocytic fusion complex. Nevertheless, CAPP activation also rescues these triple mutants. Thus, CAPP has a prominent role in the control of endocytic functions as well

Figure 6. Mutation of thr-31 in Tlg1 and ser-90 in Tlg2 is sufficient to restore endocytosis, but not exocytosis, to *snc* yeast. (A) Mutation of thr-31 in Tlg1 and ser-90 in Tlg2 restores endocytosis to *snc* yeast. *snc* cells (JG8) expressing a control plasmid (*snc*) or overexpressing SNC1, Tlg1, Tlg2, Tlg1^{ala31}, or Tlg2^{ala90} were grown, shifted to glucose-containing medium for 24 h, and labeled with FM4-64. Cells were visualized using Nomarski optics (Light) or via the rhodamine channel (FM4-64). (B) Mutation of thr-31 in Tlg1 and ser-90 in Tlg2 does not restore growth to *snc* yeast. *snc* cells (JG8) expressing a control plasmid (*snc*) or overexpressing SNC1, Tlg1^{ala31}, Tlg2^{ala90}, SSO1^{ala79}, or both SSO1^{ala79} and Tlg1^{ala31} or SSO1^{ala79} and Tlg2^{ala90} were grown, shifted to glucose-containing medium for 24 h, diluted serially and plated onto galactose-containing medium (SC+GAL) at 26°C, glucose-containing medium (SC+GLU) at 26, 35.5, or 37°C, or amino acid-rich medium (YPD) at 26°C. Cells were grown for 48 h.

as exocytic functions, as described previously (Marash and Gerst, 2001).

The restoration of endocytosis also involves t-SNARE dephosphorylation and assembly, as ceramide-treated *snc* cells showed a large increase in Tlg1–Tlg2 complex formation (Figure 5A), whereas *snc tlg1* and *snc tlg2* cells showed an increase in the formation of Tlg2–Tlg2 or Tlg1–Tlg1 complexes, respectively (Figures 5, C and D). Correspondingly, recombinant Tlg t-SNAREs phosphorylated in vitro showed a reduced ability to assemble into complexes (Figure 5F). Thus, phosphorylation appears to modulate Tlg SNARE assembly, as shown previously for the Sso t-SNAREs (Marash and Gerst, 2001). Interestingly, endocytosis could be restored completely in *snc* cells expressing mutant Tlg proteins that bear alanine substitutions in specific PKA phosphorylation sites (Figure 6A). Thus, the Tlg endosomal t-

SNAREs are likely receivers of regulatory signals arising from the PKA and CAPP pathways, as shown previously for the Sso1,2 exocytic t-SNAREs (Marash and Gerst, 2001).

It is clear from our ongoing work that t-SNARE phosphorylation plays a prominent role in controlling SNARE assembly, particularly in cells that have transport defects. Signaling via the Sit4 catalytic subunit of CAPP relieves environmental stresses placed on the cell (Hannun and Luberto, 2000) as well as blocks in protein trafficking and cell growth (Marash and Gerst, 2001 and this study). These blocks can be at various points in the endo- and exocytic pathways and are modulated by PKA, which confers growth signals based on nutrient availability. Thus, signaling paths that inhibit the cell cycle in wild-type cells, such as the CAPP pathway, and those that stimulate entry, such as the RAS-adenylyl cyclase-PKA pathway, meet at the level of vesicle trafficking. The interactions between these pathways, clearly evident in yeast secretion mutants, are also likely to be operative in wild-type cells although the physiological basis remains undefined. At this point we do not believe that t-SNAREs undergo phosphorylation and dephosphorylation during each round of SNARE assembly, because alanine substitutions (which prevent phosphorylation) in the relevant PKA sites of Sso and Tlg actually enhance protein trafficking (Figure 6 and Marash and Gerst, 2001). It is more likely that phosphorylation regulates the availability of t-SNAREs to participate in trafficking events, although additional work is required to understand this important mechanism.

Other groups have shown that sphingoid bases are involved in endocytosis. Zanolari *et al.* (2000) demonstrated that sphingoid base synthesis is required for internalization in *Saccharomyces cerevisiae*, as a *lcb1* mutant (involved in serine palmitoyltransferase activity) is defective in endocytic uptake. Moreover, Grote *et al.* (2000) identified a phosphosphingosine lyase (Dlp1) as a multicopy suppressor of the *snc* phenotype. Overexpression of Dlp1 also rescued endocytic defects resulting from the Snc1^{ala43} protein at restrictive temperatures. From the mechanistic point of view, it would appear that Dlp1 elevates the intracellular levels of sphingosine, which we have shown to activate CAPP and lead to the dephosphorylation of Sso (Marash and Gerst, 2001) and Tlg (this study). Interestingly, Friant *et al.* (2000) demonstrated that the inactivation of other PP2A catalytic subunits (including Pph21, Pph22, and Pph3, but not Sit4) abrogates the sphingoid base requirement in endocytosis. However, these other PP2A subunits had no effect on blocks in exocytosis and could not rescue *snc* cells (Marash and Gerst, 2001). Thus, Sit4 alone regulates both endo- and exocytosis, whereas the other PP2A catalytic subunits act more specifically (and, apparently, in a contrary manner) at the endocytic level. Because Sit4 dephosphorylates t-SNAREs that have been phosphorylated by PKA, it is likely that the other PP2A activities modulate endocytosis in a different manner.

Ceramides also act as regulators of intracellular trafficking events in mammalian cells (Hannun and Luberto, 2000). For example, the production of ceramide by sphingomyelinase elicits phagocytosis (Grassme *et al.*, 1997; Hinkovska-Galcheva *et al.*, 1998; Zha *et al.*, 1998). Likewise, ceramide treatment has been shown to induce the formation of endocytic vesicles (Li *et al.*, 1999). Thus, it is possible that mammalian

CAPP acts on protein trafficking in a similar manner as shown for yeast.

Another finding to come from this work is that ceramide treatment bypasses the deletion of either *TLG* gene and confers endocytosis. Thus, the rescue mechanism by which CAPP acts is operative in the absence of the endosomal v- or t-SNAREs alone, or in combination. Deletion of either *TLG* gene is known to affect intracellular protein trafficking and endocytic functions to a degree and in some cell types leads to lethality (Coe *et al.*, 1999), although conditional lethal strains bearing deletions in both genes have been reported (Holthuis *et al.*, 1998a). In our strain background, *TLG* deletions have prominent effects on endocytosis, but only small effects on cell growth (Gurunathan *et al.*, 2000). Based on the work shown here, it is likely that the deletion of individual *TLG* genes is tolerated in *snc* cells, perhaps because of the ability of Tlg proteins to form homomeric complexes (Figure 5, C and D). Although this, by itself, is insufficient to confer endocytosis, CAPP activation by ceramide treatment, *VBM/ELO* inactivation, or *SIT4* overexpression, clearly overcomes any inhibition placed on the existing Tlg t-SNARE. This inhibition is likely to result from Tlg phosphorylation by PKA, based on the in vitro and in vivo experiments performed here (Figures 5 and 6). Thus, as shown earlier for the Sso t-SNAREs (Marash and Gerst, 2001), phosphorylation of the Tlg t-SNAREs modulates their ability to confer endocytic trafficking by inhibiting SNARE assembly. Although the constituents of the endocytic SNARE complex have not been completely resolved, combinations involving Tlg1 and Tlg2 with the Snc v-SNAREs as well as other endosomal SNAREs (i.e., Vti1) are likely to occur. Very recently, two studies demonstrated that Tlg1 and 2 assemble into complexes along with Vti1 to mediate the fusion of *trans*-Golgi membranes and liposomes in vitro (Brickner *et al.*, 2001; Paumet *et al.*, 2001). Moreover, liposome fusion was dependent on the presence of a Snc v-SNARE, suggesting that a Tlg1–Tlg2–Vti1–Snc complex mediates endosomal trafficking (Paumet *et al.*, 2001). Whether this complex forms in wild-type yeast in vivo has yet to be determined. Moreover, the constituents of the SNARE complexes formed in the two *snc tlg* strains (which become competent for endocytosis upon CAPP activation) are unknown. Perhaps, a functional t-t-SNARE complex, like that proposed to exist at the exocytic level (Marash and Gerst, 2001), can also confer endocytosis in the absence of the Snc v-SNAREs.

ACKNOWLEDGMENTS

The authors thank Hagai Abeliovich, Kendall Blumer, Hugh Pelham, and Michael Wigler for the generous gifts of antibodies and reagents and Vera Shindler for electron microscopy. This work was generously supported by a grant from the Minerva Foundation, Germany. J.E.G. holds the Henry Kaplan Chair in Cancer Research.

REFERENCES

- 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.
- 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.

- 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.
- Brickner, J.H., Blanchette, J.M., Sipos, G., and Fuller, R.S. (2001). The Tlg SNARE complex is required for TGN homotypic fusion. *J. Cell Biol.* 155, 969–978.
- Cabaniols, J.-P., Ravichandran, V., and Roche, P.A. (1999). Phosphorylation of SNAP-23 by the novel kinase SNAK regulates t-SNARE complex assembly. *Mol. Biol. Cell* 10, 4033–4041.
- Chung, S.H., Polgar, J., and Reed, G.L. (2000). Protein kinase C phosphorylation of syntaxin 4 in thrombin-activated human platelets. *J. Biol. Chem.* 275, 25286–25291.
- 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.
- 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.
- Ferro-Novick, S., and Jahn, R. (1994). Vesicle fusion from yeast to man. *Nature* 370, 191–193.
- Foletti, D.L., Lin, R., Finley, M.A., and Scheller, R.H. (2000). Phosphorylated syntaxin 1 is localized to discrete domains along a subset of axons. *J. Neurosci.* 20, 4535–4544.
- Foster, L.J., Yeung, B., Mohtashami, M., Ross, K., Trimble, W.S., and Klip, A. (1998). Binary interactions of the SNARE proteins syntaxin-4, SNAP23, and VAMP-2 and their regulation by phosphorylation. *Biochemistry* 37, 11089–11096.
- Friant, S., Zanolari, B., and Riezmann, H. (2000). Increased protein kinase or decreased PP2A activity bypass sphingoid base requirement in endocytosis. *EMBO J.* 19, 2834–2844.
- 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.
- Gerst, J.E. (1999). SNAREs and SNARE regulators in membrane fusion and exocytosis. *Cell. Mol. Life. Sci.* 55, 707–734.
- Grassme, H., Gulbins, E., Brenner, B., Ferlin, K., Sandhoff, K., Harzer, K., Lang, F., and Meyer, T.F. (1997). Acidic sphingomyelinase mediates entry of *N. gonorrhoeae* into nonphagocytic cells. *Cell* 91, 605–615.
- Grote, E., and Novick, P.J. (1999). Promiscuity in Rab-SNARE interactions. *Mol. Biol. Cell* 10, 4149–4161.
- Grote, E., Vlachich, G., Pypaert, M., and Novick, P.J. (2000). A *snc1* endocytosis mutant. Phenotypic analysis and suppression by overproduction of dihydrosphingosine phosphate lyase. *Mol. Biol. Cell* 11, 4051–4065.
- Gurunathan, S., Chapman- Shimshoni D., Trajkovic S., and Gerst, J.E. (2000) Yeast exocytic v-SNAREs confer endocytosis. *Mol. Biol. Cell* 11, 3629–3643.
- Hannun, Y.A., and Luberto, C. (2000). Ceramide in the eukaryotic stress response. *Trends Cell Biol.* 10, 73–80.
- Hicke, L., and Riezman, H. (1996). Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell* 84, 277–287.
- Hicke, L. (1999). Getting down with ubiquitin: turning of cell-surface receptors, transporters and channels. *Trends Cell Biol.* 1, 1–39.
- Hinkovska-Galcheva, V., Kjeldsen, L., Mansfield, P.J., Boxer, L.A., Shaymann, J.A., and Suchard, S.J. (1998). Activation of a plasma membrane-associated neutral sphingomyelinase and concomitant ceramide accumulation during IgG-dependent phagocytosis in human polymorphonuclear leukocytes. *Blood* 91, 4761–4769.
- Hirling, H., and Scheller, R.H. (1996). Phosphorylation of synaptic vesicle proteins: modulation of the α -SNAP interaction with the core complex. *Proc. Natl. Acad. Sci. USA* 93, 11945–11949.
- 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.
- Katz, L., Hanson, P.I., Heuser, J.E., and Brennwald, P. (1998). Genetic and morphological analyses reveal a critical interaction between the C-termini of two SNARE proteins and a parallel four helical arrangement for the exocytic SNARE complex. *EMBO J.* 17, 6200–6209.
- Kataoka, M., Kuwahara, R., Iwasaki, S., Shoji-Kasai, Y., and Takahashi, M. (2000). Nerve growth factor-induced phosphorylation of SNAP-25 in PC12 cells. a possible involvement in the regulation of SNAP-25 localization. *J. Neurochem.* 74, 2058–2066.
- Klenchin, V.A., and Martin, T.E. (2000). Priming in exocytosis. attaining fusion-competence after vesicle docking. *Biochimie* 82, 399–407.
- 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.
- Li, R., Blanchette-Mackie, J., and Ladisch, S. (1999). Induction of endocytic vesicles by exogenous C₆-ceramide. *J. Biol. Chem.* 274, 21121–21127.
- Marash, M., and Gerst, J.E. (2001). t-SNARE dephosphorylation promotes SNARE assembly, and exocytosis in yeast. *EMBO J.* 20, 411–421.
- McNew, J.A., Parlati, F., Fukuda, R., Johnston, R.J., Paz, K., Paumet, F., Sollner, T.H., and Rothman, J.E. (2000). Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* 404, 153–159.
- Nickels, J.T., and Broach, J.R. (1996). A ceramide-activated protein phosphatase mediates ceramide-induced G1 arrest of *Saccharomyces cerevisiae*. *Genes Dev.* 10, 382–394.
- Paumet, F., Brugger, B., Parlati, F., McNew, J.A., Sollner, T.H., and Rothman, J.E. (2001). A t-SNARE of the endocytic pathway must be activated for fusion. *J. Cell Biol.* 155, 961–968.
- 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.
- Risinger, C., and Bennett, M.K. (1999). Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. *J. Neurochem.* 72, 614–624.
- 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., and Warren, G. (1994). Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr. Biol.* 4, 220–233.
- Seron, K., Tieaho, V., Prescianotto-Bachong, C., Aust, T., Blondel, M.-O., Guillaud, P., Devilliers, G., Rossanese, O.W., Glick, B.S., Riezman, H., Keranen, S., and Haguenaue-Tsapis, R. (1998). A yeast t-SNARE involved in endocytosis. *Mol. Biol. Cell* 9, 2873–2889.
- Shimazaki, Y., Nishiki, T., Omori, A., Sekiguchi, M., Kamata, Y., Kozaki, S., and Takahashi, M. (1996). Phosphorylation of 25-kDa synaptosome-associated protein. Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. *J. Biol. Chem.* 271, 14548–14553.
- Shuang, R., Zhang, L., Fletcher, A., Groblewski, G.E., Pevsner, J., and Stuenkel, E.L. (1998). Regulation of Munc-18/syntaxin 1A interaction by cyclin-dependent kinase 5 in nerve endings. *J. Biol. Chem.* 273, 4957–4966.
- 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.
- 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 Å resolution. *Nature* 395, 347–353.
- Turner, K.M., Burgoyne, R.D., and Morgan, A. (1999). Protein phosphorylation and the regulation of synaptic membrane traffic. *Trends Neurosci.* 22, 459–464.
- Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H., and Rothman, J.E. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* 92, 759–772.
- Zanolari, B., Friant, S., Funato, K., Sutterlin, C., Stevenson, B.J., and Riezman, H. (2000). Sphingoid base synthesis requirement for endocytosis in *Saccharomyces cerevisiae*. *EMBO J.* 19, 2824–2833.
- Zelico, 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.
- Zha, X., Pierini, L.M., Leopold, P.L., Skipa, P.J., Tabas, I., and Maxfield, F.R. (1998). Sphingomyelinase treatment induces ATP-independent endocytosis. *J. Cell Biol.* 140, 39–47.