t-SNARE Phosphorylation Regulates Endocytosis in Yeast

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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 necessitates 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 (Brenwald et al., 1994; Couve and Gerst, 1994; Rossi et al., 1997). Deletion of the SNC genes results in the intracellular accumu-
t-SNARE Phosphorylation and Endocytosis

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 (C₄-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 μM. 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 C₄-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 μM C₄-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 snc¹¹⁴₆₃ and snc¹¹⁴₆₃ tlg cells, cells were grown to log phase on glucose-containing medium at 26°C. Cells then were seeded at an OD₆₀₀ of 0.05 into prewarmed glucose-containing medium either containing or lacking C₄-ceramide (10 μM) 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 pTLG1 and pTLG2T (Gurunathan et al., 2000). Transformants were selected and the disruptions verified by PCR analysis. To create snc¹¹⁴₆₃ tlg yeast, snc tlg cells (JC9-TLG1 or JC9-TLG2) were transformed with either plasmid pLADH-SNC¹¹⁴₆₃ or pLADH-SNC¹¹⁴₆₃t. Transformants were cured of their GAL-SNC1-containing plasmids on synthetic medium at 26°C. For cells expressing STE2-GFP constructs that bear the kasr resistance marker, strains were transformed with YCPK-STEG2-GFP and selected for on synthetic medium containing 100 μg/ml gentamicin.

Plasmids

Previously described SNC expression plasmids included the following: pADH-SNC1 (Gerst et al., 1992); pADH-HASNC1 and pTGA-SNC1 (Protopopov et al., 1993); and pAHGAL-SNC2 (David et al., 1998). Plasmids pRS314STE2-GFP and pRS314ste2Δtail-GFP, which express the STE2-GFP or STE2-tail-GFP gene fusions (Stefan and Blumer, 1999), respectively, were provided by K. Blumer (Washington University School of Medicine). Other plasmid expression constructs included: pADH-SNC1₆₄₋₈₂, pTADH-SNC1₆₄₋₈₂; and pLADH-SNC1¹¹⁴₆₃ (Gurunathan et al., 2000). TGL1.2 disruption constructs, pTLG1L and pTGL2T, respectively, were described previously, as was a CEN plasmid that expresses STE2HA, pLADH-STEG2HA (Gurunathan et al., 2000). Plasmids for the expression of SIT4 (YEpSIT4)
Plasmids created for this study

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Gene expressed</th>
<th>Vector</th>
<th>Sites of cloning</th>
<th>Oligo name a</th>
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<td>EcoRI and Xhol</td>
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* 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 kam into pRS314STE2-GFP.

e This plasmid was created by subcloning a blunt-ended EcoRV and SalI fragment encoding kam into pADH-SNC1.
buffer, heated for 15 min at 37°C, and resolved on 10% SDS-PAGE gels. For experiments involving snc1\textsuperscript{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 μM), ATP-S (20 μM), 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 μl per reaction) and detection (1:2000). The amount of Tlg1 present in heteromeric complexes with Tlg2 was determined using the anti-Tlg2 antisera for IP and detection with the anti-Tlg1 antisera. The presence of Tlg1 complexes with Tlg2 was determined using the anti-Tlg2 antisera for detection (1:2000). The amount of Tlg1 present in heteromeric complexes with Tlg2 was determined using the anti-Tlg2 antisera for IP and detection with the anti-Tlg1 antisera. The presence of Tlg1 or Tlg2 in homomeric complexes was 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-Tlg1\textsuperscript{1–206} and GST-Tlg2\textsuperscript{206–317} were phosphorylated in vitro by Tpk1, using the procedure described previously for GST-Sso1\textsuperscript{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 μg) or GST-Sit4 (2 μg) 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-Tlg1\textsuperscript{1–206} and GST-Tlg2\textsuperscript{206–317} proteins were mixed together at a ratio of 1:1 (5 μg 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 μl 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\textsuperscript{ala43} and snc1\textsuperscript{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 effects on snc tlg1 and snc tlg2 yeast, which lack one of the TGL 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 ~2.3 h, respectively. Introduction of a vbm1/ela3 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 VBM1 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 TGL1 disruption is more severe than that of TGL2 (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

<table>
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<td>N.D.\textsuperscript{a}</td>
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<td>SNC1 tlg2</td>
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<td>2.1</td>
</tr>
<tr>
<td>SNC1 (37°C)</td>
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<tr>
<td>snc\textsuperscript{ala43} (37°C)</td>
<td>N.A.\textsuperscript{b}</td>
<td>3.0</td>
</tr>
<tr>
<td>snc\textsuperscript{ala43} tlg1 (37°C)</td>
<td>N.A.</td>
<td>3.2</td>
</tr>
<tr>
<td>snc\textsuperscript{ala43} tlg2 (37°C)</td>
<td>N.A.</td>
<td>3.1</td>
</tr>
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\textsuperscript{a} Not determined.

\textsuperscript{b} Not applicable, fails to grow at 37°C.
results) this value remained basically the same (2.1 h for both tlg1 and tlg2 yeasts). Because the growth defects in tlg yeast are mild, it is likely that CAPP activation has only little effect.

The snc1ala43 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, snc1ala43 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 snc1ala43 in snc tlg cells does not rescue their growth defects, suggesting that the TLG gene products are essential for Snc1ala43 function and/or recycling (Gurunathan et al., 2000). We next examined the effects of ceramide addition on snc1ala43 and snc1ala43 tlg cells that were shifted to the restrictive temperature (37°C; Table 3). In the absence of ceramide, we found that snc1ala43 and snc1ala43 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 snc1ala43 tlg yeast grew slightly slower than their snc1ala43 counterparts (Table 3), but reached a similar final density (our unpublished results). Thus, ceramide treatment appears to restore the functionality of Snc1ala43.

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 µm², respectively, which was similar to control snc cells (17.0 ± 0.7 vesicles per µm²). However, upon treatment with ceramide, the number of vesicles per µm² declined by >60%; snc tlg1 and snc tlg2 had 9.0 ± 0.7 and 7.0 ± 0.6 vesicles per µm², respectively. Control snc cells were found to have 5.0 ± 0.9 vesicles per µm² 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 snc1ala43 and snc1ala43 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 snc1ala43 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 functioning (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 snc1ala43 cells (Figure 1C). Indeed, we found that staining of vacuoles was normal in snc1ala43 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 (Sso1ala79; 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 Sso1ala79, 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).
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 sncala43 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 sncala43 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 µg/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 snc tlg1 and snc tlg2 cells shifted to restrictive temperatures. snc cells expressing sncala43 (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.
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 snc1ala43 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, snc1ala43 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 snc1ala43 cells.

**Ste2 Is Degraded in Ceramide-treated snc and Temperature-shifted snc1ala43 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 snc1ala43 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 snc1ala43 cells (Gu-
Figure 3. Ligand-mediated delivery of Ste2 to the vacuole is restored in \textit{snc}, \textit{snc tlg}, and temperature-shifted \textit{snc}^{ala43} cells by CAPP activation. (A) Delivery of Ste2-GFP, but not Ste2-tailGFP, to the vacuole is restored in ceramide-treated \textit{snc} cells. \textit{snc} cells (JG8) expressing either Ste2-GFP (\textit{snc} \textit{STE2GFP}) or a truncated form of Ste2-GFP (\textit{snc} \textit{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 (\(\text{+C}_2\)) or without (\(-\text{C}_2\)) added \(\text{C}_2\)-ceramide (10 \(\mu\text{M}\)) 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 \(\alpha\)-factor (1 \(\mu\text{M}\)) and processed immediately for microscopy. \textit{snc} cells expressing \textit{SNC1} constitutively (\textit{SNC1} \textit{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 \textit{snc tlg} cells. \textit{snc tlg1} (JG9-TLG1) and \textit{snc tlg2} cells (JG9-TLG2) expressing Ste2-GFP (\textit{snc tlg1} \textit{STE2GFP} or \textit{snc tlg2} \textit{STE2GFP}) from the \textit{kan}’ plasmid were grown in galactose-containing medium at 26°C, before being shifted to glucose-containing medium with (\(\text{+C}_2\)) or without (\(-\text{C}_2\)) \(\text{C}_2\)-ceramide (10 \(\mu\text{M}\)). After 24 h, cells were labeled with FM4-64, treated with \(\text{\textit{\alpha}}\)-factor and processed for microscopy. (C) Ligand-mediated delivery of Ste2-GFP to the vacuole is restored in \textit{snc} cells bearing a mutation in \textit{VBM1}. \textit{snc vbm1} cells (MM1) expressing either Ste2-GFP (\textit{snc vbm1} \textit{STE2GFP}) or the truncated form of Ste2-GFP (\textit{snc vbm1} \textit{STE2-tailGFP}) were grown to log phase at 26°C in glucose-containing medium, before labeling with FM4-64, treatment with \(\text{\textit{\alpha}}\)-factor, and processing for microscopy. (D) Ligand-mediated delivery of Ste2-GFP to the vacuole occurs in temperature-shifted \textit{snc}^{ala43} cells upon ceramide treatment. \textit{snc}^{ala43} cells (JG8-\textit{SNC1A43L}) expressing Ste2-GFP were grown at 26°C in glucose-containing medium with (\(\text{+C}_2\)) or without (\(-\text{C}_2\)) \(\text{C}_2\)-ceramide (10 \(\mu\text{M}\)). Half of each culture was shifted to prewarmed medium (37°C), for 1 h before FM4-64 labeling and treatment with \(\text{\textit{\alpha}}\)-factor at the restrictive temperature.
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 communoprecipitation (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 pathways 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 (Sso1ala79) 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 Sso1ala79, does not influence Tlg SNARE assembly (our unpublished results) or endocytosis per se (Figure 2A).

Because CAPP activation confers endocytosis to snc 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.

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.
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 Sso1ala79 (Figure 6B). Thus, the presence of Sso1ala79 and TLG1ala31 or Sso1ala79 and TLG2ala90 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 (snc1ala43, snc1ala44 tlg1, and snc1ala4 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 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 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-

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**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, TLG1ala31, or TLG2ala90 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, TLG1ala31, TLG2ala90, SSO1ala79, or both SSO1ala79 and TLG1ala31 or SSO1ala79 and TLG2ala90 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.
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 Slt4 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 phosphofingosine lyase (Dlp1) as a multicopy suppressor of the snc phenotype. Overexpression of Dlp1 also rescued endocytic defects resulting from the Snc1ala43 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 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.

Thus, Sit4 alone regulates both endo- and exocytic assembly, whereas the other PP2A catalytic subunits act more specifically (and, apparently, in a contrary manner) at the endocytic level. Because Sit4 dephosphorylates 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 (Holtluis 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.

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