Golgi-to-Late Endosome Trafficking of the Yeast Pheromone Processing Enzyme Ste13p Is Regulated by a Phosphorylation Site in its Cytosolic Domain

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This study addressed whether phosphorylation regulates trafficking of yeast membrane proteins that cycle between the *trans*-Golgi network (TGN) and endosomal system. The TGN membrane proteins A-ALP, a model protein containing the Ste13p cytosolic domain fused to alkaline phosphatase (ALP), and Kex2p were found to be phosphorylated *in vivo*. Mutation of the S₁₃ residue on the cytosolic domain of A-ALP to Ala was found to block trafficking to the prevacuolar compartment (PVC), whereas a S₁₃D mutation generated to mimic phosphorylation accelerated trafficking into the PVC. The S₁₃ residue was shown by mass spectrometry to be phosphorylated. The rate of endoplasmic reticulum-to-Golgi transport of newly synthesized A(S₁₃A)-ALP was indistinguishable from wild-type, indicating that the lack of transport of A(S₁₃A)-ALP to the PVC was instead due to differences in Golgi/endosomal trafficking. The A(S₁₃A)-ALP protein exhibited a TGN-like localization similar to that of wild-type A-ALP. Similarly, the S₁₃A mutation in endogenous Ste13p did not reduce the extent of or longevity of its localization to the TGN as shown by α -factor processing assays. These results indicate that S₁₃ phosphorylation is required for TGN-to-PVC trafficking of A-ALP and imply that phosphorylation of S₁₃ may regulate recognition of A-ALP by vesicular trafficking machinery.

INTRODUCTION

Resident *trans*-Golgi network (TGN) membrane proteins of the yeast *Saccharomyces cerevisiae* frequently cycle between the TGN and endosomal system. Ste13p (dipeptidyl aminopeptidase A) and the endopeptidase Kex2p are TGN resident enzymes with single transmembrane spanning domains that process the mating pheromone α -factor in the TGN. These proteins are delivered to a prevacuolar endosomal compartment (PVC) with a half-time of ~60 min as determined using the Ste13p-based reporter protein A-ALP (Bryant and Stevens, 1997; Nothwehr *et al.*, 1999). Once at the PVC, Ste13p and Kex2p are packaged into retrograde vesicles for delivery to the TGN. This transport step is dependent on a peripheral membrane complex called the retromer that may function as a vesicle coat (Seaman *et al.*, 1997, 1998; Nothwehr *et al.*, 1999). Retromer-based sorting of Ste13p/A-ALP involves the association of retromer subunit Vps35p with an aromatic amino acid-based sorting signal (FXFXD) in the cytosolic domain of Ste13p (Nothwehr *et al.*, 2000). Kex2p and Vps10p also contain aromatic PVC re-

trieval signals. Mutation of these signals, or loss of retromer function, causes rapid default transport of these cargo to the vacuole (Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993; Cereghino *et al.*, 1995; Cooper and Stevens, 1996).

In addition to a cycling itinerary that includes the PVC, TGN residents seem to also visit early endosomal compartments as shown by several recent studies. For example, Kex2p and Ste13p seem to localize to a certain degree with early endosomes. This has been shown by colocalization with early endosomal markers Tlg1p, Chs3p, and Snc1p, a secretory v-SNARE that transits through the early endosomal system on its journey from the plasma membrane to the TGN (Ziman *et al.*, 1996; Santos and Snyder, 1997; Holthuis *et al.*, 1998; Lewis *et al.*, 2000). Both Ste13p and Kex2p contain poorly defined cytosolic domain signals that when mutated accelerate trafficking into the PVC (Brickner and Fuller, 1997; Bryant and Stevens, 1997), suggesting that either they function as static retention signals in the TGN or regulate trafficking through the early endosomal system. Mutation of the yeast synaptojanin Inp53p also caused A-ALP (as a model for Ste13p) and Kex2p to be more rapidly delivered to the PVC; however, trafficking of Vps10p was unaffected (Ha *et al.*, 2001). The phenotypes associated with a loss of Inp53p function and a loss of the AP-1 adaptor complex share similarities in that both types of lesions exhibit synthetic growth defects when combined with mutations in GGA1 and GGA2 (Costaguta *et al.*, 2001; Ha *et al.*, 2001, 2003). GGA1 and GGA2 encode adaptor proteins necessary for clathrin-mediated transport from the TGN directly to the PVC (Black and Pelham, 2000; Costaguta *et al.*, 2001; Scott *et al.*, 2004). The synthetic growth defects obtained with mutations in the AP-1 adaptor complex or Inp53p with mutations in the GGAs suggest that AP-1 and Inp53p probably mediate a

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Abbreviations used: ALP, alkaline phosphatase; ER, endoplasmic reticulum; PVC, prevacuolar/endosomal compartment; TGN, *trans*-Golgi network.

Table 1. Yeast strains used in this study

Strain/plasmid	Description	Origin or reference
SHY35	<i>MATα leu2-3112 ura-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2</i>	Ha <i>et al.</i> , 2001
SHY38	<i>SHY35 inp53Δ::LEU2</i>	Ha <i>et al.</i> , 2001
SNY148	<i>SHY35 vps4Δ::NatR</i>	This study
SHY39	<i>SHY35 vps1Δ::LEU2</i>	This study
SNY36-9A	<i>MATα leu-3112 ura-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2</i>	Nothwehr <i>et al.</i> , 1995
LSY2	<i>SNY36-9A pep4Δ::TRP1</i>	Spelbrink and Nothwehr, 1999
PBY33	<i>LSY2 vps4Δ::KanR</i>	This study
SNY17	<i>MATα leu-3112 ura3-52 his3-Δ200 trp1-Δ901 lys-801 suc2-Δ9 pho8Δ::LEU2</i>	Nothwehr <i>et al.</i> , 1995
SNY37	<i>SNY17 chc-521^{ts}</i>	Ha <i>et al.</i> , 2003
AHY4-13D	<i>MATα leu-3112 ura-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2 pep4Δ::LEU2 kex2Δ::LEU2</i>	This study
SNY9-9D	<i>MATα leu-3112 ura-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pho8Δ::ADE2 vps1Δ::LEU2 end3-ts</i>	Nothwehr <i>et al.</i> , 1999
W30-1B	<i>MATα leu-3112 ura-1 his-11 trp-1 ade-1</i>	R. Rothstein, Columbia University, New York, NY
HJY3	<i>W303-1B dap2Δ::HIS3</i>	This study
HJY4	<i>HJY3 ste13::GAL1-STE13</i>	This study
HJY5	<i>HJY3 ste13::GAL1-ste13 (F_{85A}; F_{87A})</i>	This study
HJY6	<i>HJY3 ste13::GAL1-ste13 (S_{13A})</i>	This study
HJY7	<i>HJY3 ste13::GAL1-ste13 (S_{13A}; F_{85A}; F_{87A})</i>	This study
AHY62	<i>MATα ura-1 leu-3112 his-11,15 trp-1 ade-1 can-100</i>	Ha <i>et al.</i> , 2001
JHRY2-2Ca	<i>MATα leu-3112, ura-52, his3-Δ200</i>	T. Stevens, University of Oregon, Eugene, OR

distinct pathway into the endosomal system, presumably to early endosomes. This assertion also is supported by the observation that trafficking of Chs3p and Tlg1p between the TGN and early endosomes is disrupted by mutations in AP-1 (Valdivia *et al.*, 2002). Finally, mutation of *SOI3/RAV1*, which is required for efficient transport between the early endosomes and the PVC, reduced the rate of trafficking of Kex2p to the PVC (Sipos *et al.*, 2004). Together, these results suggest that Ste13p and Kex2p cycle between the TGN and early endosome and also reach the PVC via transport from the early endosome.

With yeast TGN resident proteins engaging in such a complex trafficking itinerary, there is clearly more to learn regarding the regulation of trafficking between the different compartments. Phosphorylation of membrane proteins that cycle within the TGN/endosomal system of mammalian cells is known to influence their trafficking. In some cases, phosphorylation of a serine or threonine influences the activity of a nearby sorting signal, whereas in other cases phosphorylation generates a new sorting signal that functions by binding to an accessory protein (Bonifacino and Traub, 2003; Hinners and Tooze, 2003). For example, TGN localization of the Kex2p homologue furin is dependent on an acidic cluster motif in its cytosolic domain (Schäfer *et al.*, 1995; Takahashi *et al.*, 1995; Voorhees *et al.*, 1995). This motif contains a serine that can be phosphorylated by casein kinase II (Jones *et al.*, 1995). In response to phosphorylation, furin is thought to cycle between a TGN/endosomal loop and also between an early endosome/plasma membrane loop (for review, see Molloy *et al.*, 1999). Transport in each cycling loop relies on binding of the PACS-1 adaptor to the phosphorylated form of furin and PACS-1, in turn, associates with the clathrin-associated sorting machinery (Wan *et al.*, 1998; Crump *et al.*, 2001). Dephosphorylation by protein phosphatase 2A of furin causes movement of furin from one loop to another (Molloy *et al.*, 1998).

In yeast, phosphorylation has been shown to be important for routing of cell surface transporters and pheromone re-

ceptors into ubiquitin-dependent degradative vacuolar pathways (Hicke *et al.*, 1998; Feng and Davis, 2000; Marchal *et al.*, 2000). However, the role of phosphorylation in sorting of cargo that cycle between the Golgi and endosomes is unknown. In this study, we have investigated the role of potentially phosphorylatable residues in the cytosolic domain of Ste13p, both in the Ste13p and A-ALP contexts. We show for both proteins that mutation of a phosphorylation site prevents delivery into the PVC, indicating that phosphorylation regulates trafficking of A-ALP/Ste13p.

MATERIALS AND METHODS

General Methods and Antibodies

The production of minimal (synthetic dextrose) and rich (YPD) yeast media, the genetic manipulation of yeast strains, and all general molecular biology methods were performed as described previously (Ausebel *et al.*, 2000) or as otherwise noted. Rabbit polyclonal antibodies against alkaline phosphatase (ALP) and Kex2p have been described previously (Nothwehr *et al.*, 1996; Spelbrink and Nothwehr, 1999). Rabbit polyclonal antibodies against rabbit anti-Och1p were a gift from Vladimir Lupashin (University of Arkansas, Fayetteville, AR). Mouse antibodies against Vma2p, Vph1p, and Dpm1p were from Molecular Probes (Eugene, OR) and rabbit anti-hemagglutinin epitope (HA) antibodies were from Covance (Richmond, CA).

Plasmids and Yeast Strains

Plasmids pSN34, pSN55, pSN100, pAH16, and pAH49 have been described previously (Nothwehr *et al.*, 1993, 1999). pSN55-PS1 to pSN55-PS11 were made using site-directed mutagenesis (Kunkel *et al.*, 1987) to introduce point mutations (detailed below) into pSN55, a pRS316 derivative containing a *STE13-PHO8* gene fusion. A *TRP1*-based version of pSN55-PS2, called pSN397, was made by subcloning the 2.3-kbp *EagI-EcoRI* fragment from pSN55-PS2 into the *EagI-EcoRI* sites of pRS314. Plasmids pHJ63, pHJ73, pHJ74, and pHJ75, which are derivatives of pSN55 containing various combinations of Δ 2-11, S_{13A}, S_{13D}, F_{85A}, and F_{87A} mutations, were made using the "megaprimer method" (Tyagi *et al.*, 2004). Primer sequences are available upon request. The mutations were verified by DNA sequencing. Yeast strains used in this study are listed in Table 1.

Plasmid pSN286, used to introduce a *GAL1-STE13* allele at the *STE13* locus, consists of pRS306 (Sikorski and Hieter, 1989) containing an insert with the following elements in the order given: a 1.9-kbp *BamHI-EagI* fragment from the 5' untranslated region of *STE13*, a 1.1-kbp *EagI-BamHI* fragment containing the *GAL1* promoter, and a 2.7-kbp *EcoRI-KpnI* fragment containing the

STE13 open reading frame and 3' untranslated region. Three derivatives of pSN286 contain the following point mutations in the *STE13* coding region: F₈₅A, F₈₇A (pHJ68), S₁₃A (pHJ69), and S₁₃A, F₈₅A, F₈₇A (pHJ70). These four *GAL1*-*STE13* plasmids were introduced into yeast HJY3 by first linearizing them at the unique *Sma*I site that lies in the 5' untranslated region of *STE13*; transforming them into yeast; selecting transformants on media lacking uracil; and finally, looping out intervening DNA on media containing 5-fluoroorotic acid.

A *GAL1* promoter-driven A-ALP construct tagged with two copies of the IgG-binding Z domain was generated by ligating the 2.25-kbp *Eag*I-*Sal*I fragment from pAH98 (Nothwehr *et al.*, 2000) and a 1.2-kbp *Sal*I-*Xba*I fragment, consisting of a 3' fragment of the *PHO8* open reading frame (ORF) fused in-frame to the green fluorescent protein (GFP) ORF, into the *Eag*I/*Xba*I sites of pRS316. The resulting plasmid, (pSN332), consisting of a *GAL1*-*STE13*-*PHO8*-GFP construct, was digested with *Bam*HI, blunted with T4 DNA polymerase, and digested with *Pac*I to release the GFP region. Plasmid pFA6a-ZZ (a gift from Per Stromhaug, University of Missouri, Columbia, MO) was digested with *Pst*II, blunted as described above, digested with *Pac*I, and the 0.75-kbp insert was ligated to the pSN332 vector backbone fragment described above, resulting in the *GAL1*-*STE13*-*PHO8*-ZZ construct pCF7.

Radioactive Labeling, Immunoprecipitation, and Western Blot Analysis

The procedure for immunoprecipitation of wild-type and mutant A-ALP from [³⁵S]methionine/cysteine-labeled cells was performed as described previously (Nothwehr *et al.*, 1993). Radioactively labeled proteins were quantified from gels using a PhosphorImager system (Fuji Photo Film, Tokyo, Japan). For calculation of the half-time of A-ALP processing, the log of the percentage of A-ALP that was unprocessed at each time point was plotted as a function of time, and the plots were analyzed by linear regression analysis.

For assessment of *in vivo* phosphorylation, cultures were grown for several doublings in phosphate-depleted rich (YPD) media (Warner, 1991) to log phase. Fifty to 100 μ Ci of [³²P]P_i was then added to 0.5 OD₆₀₀ units of cells, and the culture was incubated at 30°C for 45 min. The cells were then spheroplasted, lysed, and subjected to A-ALP immunoprecipitation as described previously (Nothwehr *et al.*, 1993). After separation by SDS-PAGE, the proteins were electroblotted onto nitrocellulose, and the relative extent of radioactive labeling quantified as described above. To quantify the level of recovery of wild-type and mutant A-ALP proteins on the blot, immunoblotting by using a rabbit anti-ALP antibody was carried out followed by incubation with ALP-conjugated anti-rabbit secondary antibodies and chemiluminescent detection using the Lumi-Phos substrate (Pierce Chemical, Rockford, IL). The blots were quantified and imaged using a Fuji LAS-1000 CCD camera and ImageReader LAS-1000 1.2 software (Fuji Photo Film).

Subcellular Fractionation

Subcellular fractionation of [³⁵S]-labeled cells by differential centrifugation was carried out as described previously (Nothwehr *et al.*, 1999), except that the immunoprecipitations were performed using anti-ALP antibody, and the pulse was for 10 min and the chase was for 0, 20, and 40 min.

The nonradioactive subcellular fractionation experiment performed by differential centrifugation was carried out as described previously (Ha *et al.*, 2001), except centrifugation at 13,000 $\times$ g was used instead of 15,000 $\times$ g. Equivalent percentages of the P13, P200, and S200 fractions were subjected to SDS-PAGE followed by blotting to nitrocellulose. The blots were probed with the indicated primary antibodies followed by incubation with ALP-conjugated anti-rabbit or anti-mouse secondary antibodies and were detected, quantified, and imaged as described above. Images were further adjusted and formatted using Adobe Photoshop 7.0.

To perform the assay for plasma membrane localization, 12 OD₆₀₀ units of cells labeled with [³⁵S]methionine/cysteine were washed with 10 mM Na₃N₃ and 10 mM NaF and resuspended in 550 μ l of ice-cold lysis buffer [5% sucrose (wt/wt), 20 mM triethanolamine, pH 7.2, and 1 mM EDTA] supplemented with protease inhibitors (Roche Diagnostics, Mannheim, Germany). Cells were lysed by vortexing with glass beads, and lysates were centrifuged at 500 $\times$ g to pellet unlysed cells. Then, 350 μ l of the 500-g supernatant was loaded on top of a sucrose step gradient made in 20 mM triethanolamine, 1 mM EDTA. The percentage of sucrose and volume of each step were as follows from bottom to top: 350 μ l of 50%, 875 μ l of 45%, and 700 μ l of 35%. The gradients were centrifuged at 44,000 rpm in a SW 55 Ti rotor (Beckman Coulter, Fullerton, CA) for 17 h at 4°C. Eight fractions of 284 μ l each were removed from the top and were subjected to immunoprecipitation of A-ALP, A(S₁₃A)-ALP, Pma1-HA, and Och1p. Radioactively labeled proteins were quantified from gels using a PhosphorImager system (Fuji Photo Film).

Fluorescence Microscopy

The procedures for preparation of fixed spheroplasted yeast cells and attachment to microscope slides were described previously (Roberts *et al.*, 1991). All secondary antibodies were diluted 1:500 before use. Simultaneous detection of wild-type and mutant A-ALP and Vma2p was performed as described previously (Ha *et al.*, 2003). Yeast cells were photographed using a BX-60

fluorescence microscope (Olympus, Lake Success, NY) equipped with a C4742-95 digital camera (Hamamatsu, Bridgewater, NJ). Images were initially captured using Openlab 3.1.7 software (Improvision, Lexington, MA) and were processed into figures using Adobe Photoshop 7.0.

Mating Assay to Measure the Onset of Sterility

The mating efficiency of *MAT α* yeast strains at various times after terminating expression of *STE13* and mutant derivatives under control of the *GAL1* promoter was determined using a published assay (Hartwell, 1980; Wilcox *et al.*, 1992). Briefly, cells were grown synthetic galactose media for several generations to log phase and were then switched to synthetic glucose (SD) media. After various times of growth in glucose, 0.25 OD₆₀₀ units of cells ($\sim 5 \times 10^6$ cells) were mixed with 0.75 OD₆₀₀ units of cells ($\sim 1.5 \times 10^7$ cells) of *MAT α* mating partner strain JHRY20-2Ca, and the cells were adhered onto a 2.4-cm HATF filter (Millipore, Billerica, MA). The filters were incubated cell side up on YPD plates at 30°C for 4 h, the cells were resuspended off the filter, and the dilutions of cells were plated on SD-his media to select for *MAT α* and diploid cells and onto SD-his-trp to select for diploids only. Mating efficiency is expressed as the number of diploids divided by the number of diploids plus *MAT α* haploids.

Mass Spectrometry Analysis of Ste13p Cytosolic Domain Phosphorylation

The A-ALP protein fused to two copies of the IgG-binding Z domain was purified from yeast strain AHY62 carrying plasmid pCF7. In total, 100 OD₆₀₀ units of cells was spheroplasted and lysed by incubating in 2.5 ml of 5% SDS/8 M urea at 100°C for 5 min in the presence of protease inhibitors (see above). The yeast extract was then diluted into 50 ml of immunoprecipitation (IP) buffer lacking SDS (10 mM Tris, pH 8.0, 0.1% Triton X-100, and 2 mM EDTA), and a 0.5-ml bed volume of Sepharose beads was added. After incubating for 1 h at 4°C, the beads were sedimented, and the supernatant was then added to 0.1-ml bed volume of IgG-Sepharose beads. After incubating for 2 h at 4°C, the beads were washed four times with 50-ml volumes of IP buffer containing 0.1% SDS (Nothwehr *et al.*, 1993), incubated with SDS-PAGE sample buffer at 100°C, and the eluted protein separated by SDS-PAGE and stained with Coomassie Brilliant Blue. A gel band corresponding to full-length A-ALP-ZZ was subjected to in-gel trypsin digestion according to (Shevchenko *et al.*, 1996), and peptides were purified and de-salted using Zip Tip columns (Millipore) according to the instructions of the manufacturer.

Nanospray quadrupole time of flight (TOF) time mass spectrometry and tandem mass spectrometry (MS/MS) analyses of the obtained peptides were performed with a QSTAR/PULSARI instrument (Applied Biosystems, Foster City, CA) fitted with a nanospray source (Proxeon, Odense, Denmark). A stable spray was achieved at 800 V in the presence of nitrogen curtain gas. Positive ion spectra were acquired in the profile MCA mode at a pulser frequency of 6.99 kHz. Collision-induced dissociation spectra (MS/MS) were acquired for peptides selected by the first quadrupole and fragmented in the collision quadrupole at appropriate nitrogen collision gas pressures and collision energy settings.

RESULTS

Yeast Resident TGN Proteins Are Phosphorylated

As a first step to investigate whether phosphorylation of TGN resident proteins is important for their trafficking, we assessed whether the model TGN protein A-ALP is phosphorylated. A-ALP is a model TGN-membrane protein consisting of the N-terminal cytosolic domain of Ste13p fused to the transmembrane and luminal domains of ALP, the *PHO8* gene product (Nothwehr *et al.*, 1993). A failure to retain A-ALP in the TGN/endosomal system results in its delivery to the vacuole where its C-terminal propeptide is proteolytically removed, resulting in a mobility shift on SDS-PAGE. However, to simplify the phosphorylation analysis, *pep4* yeast strains deficient in vacuolar protease activity were used to prevent any vacuolar proteolytic processing from occurring. A yeast strain expressing A-ALP was grown in phosphate-depleted media for several generations and cells were then incubated with [³²P]P_i for 45 min followed by immunoprecipitation of A-ALP. A band of the expected size for A-ALP was obtained, whereas this band was missing in a strain expressing an A-ALP mutant lacking residues 2–100 of the cytosolic domain (Figure 1A, lanes 1 and 4). However, a very faint band at the size expected for A(Δ 2-100)-ALP was

observed. The extent of ^{32}P incorporation into wild-type and mutant A-ALP was quantified and normalized to the amount of A-ALP protein immunoprecipitated (Figure 1B). These results indicated that phosphorylation of the A(Δ 2-100)-ALP mutant was reduced to <20% of that of A-ALP. We thus conclude that A-ALP is phosphorylated and that most, if not all, of the phosphorylation occurs on the cytosolic domain. In this regard it is worth noting that the truncated cytosolic domain in A(Δ 2-100)-ALP contains two Ser residues (Figure 2A). Kex2p (Figure 1A) and Vps10p (our unpublished data) also were demonstrated to be phosphoproteins.

We next addressed whether trafficking defects would affect phosphorylation of A-ALP. A mutant form of A-ALP, A(F₈₅A; F₈₇A)-ALP, defective for retrieval from the PVC (Nothwehr *et al.*, 1993; Bryant and Stevens, 1997) was phosphorylated to a similar degree as A-ALP (Figure 1). Phosphorylation of A-ALP expressed in the class E *vps* mutant *vps4* Δ also was analyzed. In class E mutants, transport into the PVC occurs normally but both retrograde and anterograde traffic out of the PVC is blocked (Piper *et al.*, 1995; Babst *et al.*, 1997; Finkeneigen *et al.*, 1997). Thus, in class E cells A-ALP rapidly becomes trapped in the PVC (Bryant and Stevens, 1997). However, loss of Vps4p function did not seem to affect the extent of phosphorylation of A-ALP (Figure 1B).

A S₁₃A Mutation in the Cytosolic Domain of A-ALP Blocks Its Delivery to the PVC

The 24 potentially phosphorylatable Ser, Thr, and Tyr residues in the cytosolic domain of Ste13p/A-ALP were systematically mutated to determine whether they have a role in trafficking of A-ALP. In total, 11 mutant forms of A-ALP were generated by site-directed mutagenesis of one to four residues at a time (Figure 2A). These mutants, called PS1–PS11, and controls were expressed in a wild-type strain, cells were pulsed for 10 min, chased for 150 min, lysed, and then immunoprecipitated using anti-ALP antibody. Little or no vacuolar processing was observed, with the exception of PS3 that exhibited weak processing (Figure 2B). A defect in retrieval of A-ALP from the PVC results in processing with a half-time of ~60 min (Nothwehr *et al.*, 1993; Bryant and Stevens, 1997); thus, the mutations apparently do not have a marked effect on PVC-to-TGN retrieval.

To determine whether the mutations affect the rate of transport into the PVC, we expressed wild-type and mutant A-ALP proteins in the class E *vps* mutant *vps4* Δ that trap TGN resident proteins in the PVC. In contrast to wild-type cells, the PVC in such strains contains substantial vacuolar protease activity. Therefore, the rate of processing of newly synthesized A-ALP in class E mutants reflects the rate of transport from its site of synthesis at the endoplasmic reticulum (ER) to the PVC (Bryant and Stevens, 1997; Ha *et al.*, 2001). *vps4* Δ cells carrying wild-type and mutant A-ALP proteins were pulsed for 10 min and chased for 60 min. Under these conditions, 71% of wild-type A-ALP was processed (Figure 2C). A mutant form of A-ALP lacking amino acids 2–11, A(Δ 2-11)-ALP, was more extensively processed (90%), consistent with previous work showing that this deletion accelerates trafficking into the PVC (Bryant and Stevens, 1997; Ha *et al.*, 2001). Most of the PS mutants were processed to a similar degree as wild-type, suggesting that the mutated residues play little or no role in trafficking. Notable exceptions were the PS2 mutant (S₁₃A) that exhibited little or no processing, and the PS3 (S₂₁A, S₂₂A, S₂₄A, S₂₅A) and PS6 (T₅₇A, T₆₀A, T₆₄A) mutants whose processing was somewhat accelerated (82% processing). Because the

Figure 1. The cytosolic domains of both A-ALP and Kex2p are phosphorylated *in vivo*. (A) Yeast strains were grown for several doublings in phosphate depleted media and were then continuously labeled with [^{32}P]P_i for 45 min. In lanes 1–7 strains LSY2/pSN55, LSY2/pSN55-PS2, LSY2/pSN100, LSY2/pSN34, PBY33/pSN55, LSY2, and AHY48-13D were analyzed, respectively, by immunoprecipitation with anti-ALP antibodies (lanes 1–5) or with anti-Kex2p antibodies (lanes 6 and 7). (B) The strains analyzed in lanes 1–5 of A were labeled with ^{32}P and subjected to immunoprecipitation of wild-type and mutant A-ALP proteins as described above. After separation by SDS-PAGE, the relative amounts of purified wild-type and mutant A-ALP proteins was determined by immunoblotting, and the relative amounts of ^{32}P incorporated was determined by PhosphorImager analysis as described under *Materials and Methods*. Relative phosphorylation is the extent of ^{32}P incorporation divided by the level of wild-type or mutant A-ALP protein on the blot. Phosphorylation of wild-type A-ALP expressed in a wild-type strain was arbitrarily set at 100%. The average and SD of three independent data points for each strain are shown.

S₁₃A mutation caused such a striking effect on A-ALP trafficking, we compared A-ALP and A(S₁₃A)-ALP expressed in a *vps4* Δ strain in a more extensive pulse-chase time course (Figure 2D). No processing of A(S₁₃A)-ALP was observed in the 80-min time course, whereas wild-type was processed with a half-time of 46 min (Table 2).

Figure 2. Mutation of the S_{13} residue of A-ALP blocks its processing in class E vps cells. (A) The sequence of the cytosolic domain of Ste13p is shown. All potentially phosphorylatable residues (bold) in the cytosolic domain were mutated in the context of A-ALP carried in pSN55 (in groups of 1–4 mutations) by site-directed mutagenesis resulting in 11 mutants named PS1 to PS11. (B–D) Wild-type and mutant A-ALP proteins were immunoprecipitated from ^{35}S -labeled cell extracts and were analyzed by SDS-PAGE. (B) Wild-type strains (SHY35) carrying plasmids pSN55-PS1-pSN55-PS11, pSN55 (WT; A-ALP) and pAH49 [$\Delta 2-11$; A($\Delta 2-11$)-ALP] were pulsed for 10 min with [^{35}S]methionine/cysteine and chased for 150 min. (C) The *vps4* Δ mutant SNY148 carrying the same plasmids as in B was pulsed for 10 min and chased for 60 min. The percentage of A-ALP processing is indicated below each lane. (D) SNY148 cells carrying pSN55, pSN55-PS2, and pHJ56 [A(S_{13} D)-ALP] were pulsed for 10 min and chased for 0, 20, 40, and 80 min.

To determine whether the $S_{13}\text{A}$ mutation blocks delivery of A-ALP to the PVC in wild-type cells, we introduced this mutation into the A($F_{85}\text{A}$; $F_{87}\text{A}$)-ALP construct that is incapable of being retrieved from the PVC. The rate of processing of A-ALP, A($F_{85}\text{A}$; $F_{87}\text{A}$)-ALP, A($S_{13}\text{A}$)-ALP, and A($S_{13}\text{A}$; $F_{85}\text{A}$; $F_{87}\text{A}$)-ALP in wild-type cells was determined. Whereas retrieval-defective A($F_{85}\text{A}$; $F_{87}\text{A}$)-ALP was processed with a half-time of 59 min, introduction of the $S_{13}\text{A}$ mutation into this protein dramatically delayed its processing with just a hint of processing at the 180-min time point (Figure 3 and Table 2). Consistent with the results of Figure 2B, no processing of the A($S_{13}\text{A}$)-ALP single mutant was observed.

The S_{13} Residue of A-ALP Is Phosphorylated

Given that A-ALP is phosphorylated and that the $S_{13}\text{A}$ mutation severely retards delivery to the PVC, we next attempted to assess whether the S_{13} residue is phosphorylated. If S_{13} is phosphorylated, then mutating it to a D residue to mimic the phosphorylated state would be expected to give a much different phenotype than the $S_{13}\text{A}$ mutation, which would mimic the nonphosphorylated state. Consistent with S_{13} phosphorylation, the A($S_{13}\text{D}$)-ALP mutant was indeed found to be processed significantly faster than A-ALP in *vps4* Δ cells (Figure 2D and Table 2). As another approach, we directly assessed the extent of *in vivo* phosphorylation of A($S_{13}\text{A}$)-ALP compared with wild-type. We have repeatedly observed a modest decrease in the extent of phosphorylation due to the $S_{13}\text{A}$ mutation although the extent of this decrease varies from experiment to experiment. In the experiment shown in Figure 1B, this difference fell within the SD due to quantitative limitations in the assay. If S_{13} is phosphorylated it is clearly not the only phosphorylation site in A-ALP, although the other uniden-

tified site(s) do not seem to dramatically influence trafficking.

Given this uncertainty, mass spectrometry was used to address whether the S_{13} residue was phosphorylated. A-ALP fused to two copies of the IgG-binding Z domain was expressed in yeast, purified, and the protein contained within a one-dimensional gel band was trypsinized. Single-charge positive ions corresponding to the phosphorylated residue 12–19 peptide (at 1078.5 Da) and phosphorylated 11–19 or 12–20 peptides (at 1206.6 Da) were observed in the matrix-assisted laser desorption ionization/TOF MS analysis of both the nonbound and bound peptide fractions from a C18 matrix used for desalting the in-gel tryptic digest (our unpublished data). These ions were observed at significant signal-to-noise ratios only in the presence of the matrix 2,5-dihydroxybenzoic acid [at 10 mg/ml in 500:500:10 (vol/vol/vol) acetonitrile/water/*o*-phosphoric acid] and not with the matrix alpha-cyano-4-hydroxycinnamic acid. Subsequent nanospray quadrupole time of flight mass spectrometry analysis revealed a triple-charge ion at a mass/charge ratio (m/z) of 402.9 that corresponded well to the expected m/z for a phosphorylated peptide corresponding to residues 11–19. The fragmentation spectrum of this peptide showed a complete y-ion series consistent with phosphorylation at S_{13} that included both phosphorylated fragments and the respective fragment ions resulting from the loss of phosphoric acid (Figure 4). For example, the triple-charge y_9^* ion at $m/z = 370.2$ corresponds to the fragment with the loss of phosphoric acid ($402.9 \times 3 - 370.2 \times 3 = 98$, the mass of H_3PO_4). Thus, we conclude that the S_{13} residue of A-ALP is phosphorylated as well as one or more unidentified sites within the cytosolic domain. Together, these results strongly suggest that phosphorylation at S_{13} controls delivery of A-ALP/Ste13p to the PVC.

Table 2. Rate of processing of wild-type and mutant A-ALP proteins in various strain backgrounds

Strain	Form of A-ALP	Half-time of processing	
Wild type	Wild type	$\gg 80$	
Wild type	F ₈₅ A; F ₈₇ A	59 ± 4	n = 2
Wild type	S ₁₃ A	$\gg 80$	
Wild type	S ₁₃ A; F ₈₅ A; F ₈₇ A	$\gg 80$	n = 2
Wild type	S ₁₃ D; F ₈₅ A; F ₈₇ A	48	
<i>inp53Δ</i>	F ₈₅ A; F ₈₇ A	35	
<i>inp53Δ</i>	S ₁₃ A; F ₈₅ A; F ₈₇ A	$\gg 80$	
<i>inp53Δ</i>	S ₁₃ D; F ₈₅ A; F ₈₇ A	28 ± 3	n = 2
<i>vps1Δ</i>	wild type	62 ± 3	n = 2
<i>vps1Δ</i>	S ₁₃ A	$\gg 80$	n = 2
<i>vps4Δ</i>	Wild type	46 ± 4	n = 5
<i>vps4Δ</i>	S ₁₃ A	$\gg 80$	n = 5
<i>vps4Δ</i>	S ₁₃ D	32 ± 1	n = 5
<i>vps4Δ</i>	Δ2-11	22 ± 8	n = 2
<i>vps4Δ</i>	Δ2-11; S ₁₃ A	23 ± 6	n = 2
<i>vps4Δ</i>	Δ2-11; S ₁₃ D	19 ± 3	n = 2

Strains of the indicated genotype (SHY35, SHY38, SHY39, and SNY148) carrying plasmids for expression of wild-type (pSN55), F₈₅A; F₈₇A (pSN100), S₁₃A (pSN55-PS2), S₁₃A; F₈₅A; F₈₇A (pHJ63), S₁₃D; F₈₅A; F₈₇A (pHJ75), Δ2-11 (pAH49), Δ2-11; S₁₃A (pHJ73), and Δ2-11; S₁₃D (pHJ74) forms of A-ALP were analyzed. Strains were pulsed with [³⁵S]methionine/cysteine for 10 min and chased for a total of four time points at 30°C. After each time point, wild-type or mutant A-ALP proteins were immunoprecipitated, separated by SDS-PAGE, the percentage of processing quantified by PhosphorImager analysis, and the processing half-time calculated (see *Materials and Methods*). For data points derived from multiple experiments (n > 1), the average and SD are indicated.

The A(S₁₃A)-Alkaline Phosphatase Mutant Exhibits a TGN/Endosomal-like Distribution

The results mentioned above suggested that the S₁₃ residue is required for trafficking of A-ALP into the PVC. To explore the alternative possibility that the S₁₃A mutation converts A-ALP into an unsuitable substrate for vacuolar processing, we assessed the localization of A(S₁₃A)-ALP in wild-type and *vps4Δ* cells by immunofluorescence microscopy (Figure 5). In wild-type cells, both A-ALP and A(S₁₃A)-ALP exhibited punctate staining patterns typical of localization to the yeast Golgi/endosomal system. However, extensive analysis of many fields of cells indicated that the staining pattern of A(S₁₃A)-ALP was subtly different in that it decorated structures that seemed slightly smaller and more numerous than A-ALP (our unpublished data). The exaggerated PVC

(class E compartment) in *vps4Δ* cells is marked by Vma2p, a component of the vacuolar ATPase (Raymond *et al.*, 1992). Although wild-type A-ALP clearly colocalizes with Vma2p in *vps4Δ* cells, indicating that it is trapped in the exaggerated PVC, A(S₁₃A)-ALP remains punctate in the *vps4Δ* cells. Thus, the lack of processing of A(S₁₃A)-ALP in *vps4Δ* cells is due to its lack of transport to the PVC.

The punctate staining pattern suggested that A(S₁₃A)-ALP had reached the TGN/endosomal system; however, an alternative possibility was that this mutant protein was trapped in the ER. To explore this issue, we used a differential centrifugation approach to fractionate organelles from lysates derived from wild-type cells expressing A-ALP and cells expressing A(S₁₃A)-ALP. Lysates were centrifuged at $13,000 \times g$ to generate a pellet (P13) and supernatant (S13) fraction. The S13 fraction was then centrifuged at $200,000 \times g$ to generate P200 and S200 fractions. Under these conditions A-ALP, A(S₁₃A)-ALP, and Kex2p were mainly found in the P200 fraction with a minor amount in the P13 fraction (Figure 6). In contrast, the ER marker Dpm1p and vacuolar marker Vph1p were clearly enriched in the P13 fraction. These results indicated that A(S₁₃A)-ALP was not localized to the ER in the steady state and were consistent with a Golgi/endosomal localization.

The S₁₃A Mutation Does Not Slow the Rate of ER-to-Golgi Transport of A-ALP

Although A(S₁₃A)-ALP was not localized to the ER under steady-state conditions, it was possible that newly synthesized A(S₁₃A)-ALP transited slowly through the early part of the secretory pathway. If so, this could partially account for the slow rate of transport into the PVC. To investigate this issue, we performed subcellular fractionation on strains expressing A-ALP and A(S₁₃A)-ALP that had been pulse labeled for 10 min and chased for 0, 20, and 40 min (Figure 7). By the earliest time point measured (0 min), the majority of A-ALP and A(S₁₃A)-ALP were already found in the P150 fraction with only minor amounts in the P13 fraction. As in Figure 6, the ER marker Dpm1p in this experiment was confined almost completely to the P13 fraction, with little or no Dpm1p in the P150 and S150 fractions (Figure 7B). Proceeding through the 0- to 40-min time course, little or no additional shift of A-ALP and A(S₁₃A)-ALP from the P13 to the P150 fraction was observed. Although this type of experiment is not capable of ruling out very minor differences in ER-to-Golgi trafficking rates, it is clear that both proteins were transported from the ER to the Golgi in <10 min after being synthesized. Together, these results indicate that the block into transport of A(S₁₃A)-ALP into the PVC must be due to an effect on intra-Golgi or post-Golgi trafficking.

The S₁₃A Mutation Suppresses the Accelerated Depletion of PVC Retrieval-Defective Ste13p from the α-Factor Processing Compartment

The S₁₃A mutation could cause A-ALP to limit its cycling to a TGN/early endosome itinerary. Alternatively, A(S₁₃A)-ALP could be localized to a post-TGN non-PVC endosomal organelle distinct from the α-factor processing compartment. To investigate this issue, and to extend our analysis to endogenous Ste13p, we used a functional assay that measures the persistence of wild-type and mutant forms of Ste13p in the α-factor processing compartment (the TGN and/or early endosome). We hypothesized that retrieval-defective Ste13p (F₈₅A; F₈₇A) would be depleted from the TGN/early endosome more rapidly after its synthesis was shut-off than wild-type Ste13p but that the S₁₃A mutation would block entry into the PVC and thus suppress this

Figure 3. The S₁₃A mutation blocks delivery of A-ALP to the PVC in wild-type cells. Strain SHY35 carrying pSN55 (A-ALP), pSN100 [A(F₈₅A; F₈₇A)-ALP], pSN55-PS2 (A(S₁₃A)-ALP), and pHJ63 [A-(S₁₃A; F₈₅A; F₈₇A)-ALP] were pulsed for 10 min and chased for 0, 45, 90, and 180 min. Little or no processing of the double mutant is seen, even after 180 min, suggesting that the S₁₃A mutation blocks delivery into the PVC in wild-type cells.

Figure 4. The S_{13} residue in the Ste13p cytosolic domain is phosphorylated. The MS/MS fragmentation spectra and amino acid sequence of a tryptic-miscleaved peptide from the Ste13p cytosolic domain beginning with Lys11 is shown. The intact 3+ charged parent ion (y_9) had a mass/charge ratio (m/z) of 402.86. Peaks corresponding to the y (C-terminal) and b (N-terminal) fragment ions (inset) are labeled on the plot. An exception is the relatively small peak for the y_6 ion with an m/z of 399.22 that could only be discerned on an expanded version of the plot. The ion fragments with an expected mass resulting from neutral loss of H_3PO_4 are marked with asterisks. The y_3 fragment was identified in two distinct peaks with one corresponding to a 1+ charged ion ($m/z = 400.24$) and the other to a 2+ charged ion ($m/z = 200.62$) species.

effect. Strains were constructed in which the endogenous *STE13* gene was replaced with constructs containing the galactose-inducible *GAL1* promoter fused to the following alleles of the *STE13* gene: 1) wild-type, 2) $F_{85}A$; $F_{87}A$, 3) $S_{13}A$, and 4) $S_{13}A$; $F_{85}A$; $F_{87}A$. These strains were grown on galactose media to induce expression of wild-type and mutant Ste13p, switched to glucose media for various times,

and then were tested for their ability to mate with a *MATa* strain (Figure 8). The efficiency of mating of the $F_{85}A$; $F_{87}A$ mutant did indeed drop off more quickly than wild type after the shift to glucose, as can be seen at the 6- and 12-h time points. The Ste13 ($S_{13}A$) single mutant activity persisted in the TGN/early endosome to a similar degree as wild type over the time course. In addition, the $S_{13}A$ muta-

Figure 5. A($S_{13}A$)-ALP mutant seems to localize to the TGN/early endosome with little or no cycling through the late endosome. Strains LSY2/pSN55, LSY2/pSN55-PS2, PBY33/A-ALP and PBY33/A($S_{13}A$)-ALP (shown in columns from left to right) were fixed and spheroplasted. The cells were then costained for A-ALP/Vma2p or A($S_{13}A$)-ALP/Vma2p as described under *Materials and Methods*. The cells were viewed by differential interference contrast optics (bottom row) and by epifluorescence with filters specific for each fluorochrome (top and center rows).

Figure 6. A(S₁₃A)-ALP fractionates similarly to wild-type A-ALP by differential centrifugation of cell lysates. Strains SHY35/pSN55 and SHY35/pSN55-PS2 were spheroplasted, lysed, and lysates were subjected to centrifugation at $13,000 \times g$ to generate pellet (P13) and supernatant (S13) fractions. The S13 fraction was the centrifuged at $200,000 \times g$ to generate P200 and S200 fractions. Equivalent percentages of the three fractions from each strain were run on SDS-PAGE, gels were transferred to nitrocellulose, and blots were probed using antibodies to detect the indicated proteins.

tion suppressed the effect of the F₈₅A, F₈₇A mutations with the triple mutant persisting similarly to wild-type Ste13p. These data indicate that Ste13 (S₁₃A) is localized to the TGN/early endosomal compartment with similar efficiency to that of wild-type Ste13p where it can participate in α -factor processing. Furthermore, the suppression of the F₈₅A and F₈₇A mutations by S₁₃A indicates that, like A-ALP, the delivery of Ste13p to the PVC relies on phosphorylation of S₁₃.

The S₁₃ Phosphorylation Site Is Necessary for Plasma Membrane-mislocalized A-ALP to Reach the PVC

Loss of function of clathrin heavy chain, Chc1p, and the dynamin homolog Vps1p block direct Golgi-to-endosome trafficking of A-ALP. In *chc1-521^{ts}* and *vps1 Δ* mutants, A-ALP is initially mislocalized to the plasma membrane and is then delivered to the vacuole in a manner dependent on the End3 and Sla2/End4 proteins (Nothwehr *et al.*, 1995; Ha *et al.*, 2003). End3p and Sla2p are required for the internalization step of endocytosis (Raths *et al.*, 1993; Bénédicti *et al.*, 1994). Presumably in *vps1* and *chc1* mutants A-ALP vacuolar delivery from the plasma membrane occurs via the early endosome and endosome-to-TGN retrieval is compromised in these mutants. We assessed whether A(S₁₃A)-ALP could access the PVC/vacuole after it was mislocalized to the plasma membrane in *chc1-521^{ts}* and *vps1 Δ* mutants. Wild-type A-ALP was clearly processed in *vps1 Δ* cells and in *chc1-521^{ts}* mutants at the nonpermissive temperature within the 80-min time course (Figure 9, A and B) as has been observed previously (Nothwehr *et al.*, 1995; Ha *et al.*, 2003). However, processing of A(S₁₃A)-ALP was blocked in these mutants.

A pool of A-ALP is trapped on the plasma membrane in *vps1 Δ* cells with a loss of End3p or Sla2p function (Nothwehr *et al.*, 1995, 1999). If A(S₁₃A)-ALP also is mislocalized to the plasma membrane in *vps1 Δ* cells, it should be trapped there in *vps1 Δ end3-*ts** cells. To investigate this, we pulse labeled a *vps1 Δ end3-*ts** strain at the nonpermissive temperature for 10 min and chased for 60 min. Cell lysates were loaded at the top of a sucrose equilibrium gradient designed to separate

Figure 7. The S₁₃A mutation does not alter the kinetics of A-ALP trafficking from the ER to the Golgi. (A) Strains SHY35/pSN55 (A-ALP) and SHY35/pSN55-PS2 [A(S₁₃A)-ALP] were spheroplasted and pulse labeled for 10 min and were chased for 0, 20, and 40 min. After each chase period, the cells were lysed and lysates were centrifuged at $13,000 \times g$ to generate pellet (P13) and supernatant (S13) fractions. The S13 fraction was then centrifuged at $150,000 \times g$ to generate P150 and S150 fractions. A-ALP or A(S₁₃A)-ALP were immunoprecipitated from each fraction, analyzed by SDS-PAGE, and quantified by PhosphorImager analysis. The numbers below each panel represent the amount of the A-ALP or A(S₁₃A)-ALP present in the P13 and P150 fractions as a percentage of the sum total present in the P13 and P150 fractions. At each time point, the amount of A-ALP or A(S₁₃A)-ALP found in the S150 was <5% of the total found in the P13, P150, and S150 fractions combined. (B) Equivalent percentages of the 0 min P13, P150, and S150 fractions (nonimmunoprecipitated) were run on SDS-PAGE and were Western-blotted for Dpm1p. Fractions from strain SHY35/pSN55 and strain SHY35/pSN55-PS2 are shown in lanes 1–3 and 4–6, respectively.

plasma membrane from internal membranes. Both A-ALP and A(S₁₃A)-ALP exhibited a bimodal distribution with peaks around fractions 3 and 8 (Figure 9, C and D). The Golgi marker Och1p peaked around fraction 3, whereas the plasma membrane marker Pma1p peaked in fraction 8. These data indicate that pools of both A-ALP and A(S₁₃A)-ALP are localized to the plasma membrane in the *vps1 Δ end3-*ts** strain. These results suggest that A-ALP lacking the S₁₃ phosphorylation site is unable to traffic to the PVC once it has been mislocalized to the plasma membrane.

Accelerated Transport of A-ALP to the PVC in *inp53/sjl3* Mutants Is Blocked by the S₁₃A Mutation

Loss of function of the synaptojanin homolog Inp53/Sjl3p causes A-ALP transport into the PVC to be accelerated (Ha *et al.*, 2001, 2003). To investigate the epistatic relationship between *INP53* and the S₁₃ phosphoserine, we analyzed the rate of processing of retrieval defective A(F₈₅A; F₈₇A)-ALP in *inp53 Δ* cells with or without the S₁₃A mutation. As observed previously, A(F₈₅A; F₈₇A)-ALP was processed faster in *inp53 Δ* cells (35 min) than in wild-type cells (59 min; Table 2). However, processing of the F(S₁₃A; F₈₅A; F₈₇A)-ALP mutant was blocked in *inp53 Δ* cells as well as in wild-type cells. Interestingly, the acceleration of trafficking into the PVC caused by the S₁₃D mutation was additive to that caused by the *inp53 Δ* mutation with the processing half-time

Figure 8. The $S_{13}A$ mutation suppresses the accelerated loss of retention in the α -factor processing compartment observed for Ste13p carrying the $F_{85}A$; $F_{87}A$ mutations. Strains HJY4, HJY5, HJY6, and HJY7 carrying wild-type and mutant forms of the *STE13* under control of the *GAL1* promoter were grown on galactose for several generations. The strains were then shifted to glucose-containing media to turn off expression of the wild-type and mutant *STE13* constructs. At the indicated time points after shifting to glucose the ability of the cells to mate was measured to generate an index of mating efficiency (for details, see *Materials and Methods*). The data shown are the average of three independent data points and the error bars indicate the SD.

for the $S_{13}D$; $F_{85}A$; $F_{87}A$ triple mutant in wild-type cells (48 min) decreasing in *inp53* Δ cells (28 min). These data indicate that Inp53p mediates trafficking of A-ALP in a manner dependent on the phosphoserine.

The Block in A-ALP Trafficking Caused by the $S_{13}A$ Mutation Requires the 2–11 Region

Deletion of residues 2–11 of A-ALP accelerates its transport into the PVC (Bryant and Stevens, 1997; Ha *et al.*, 2001); thus, this region may contain a signal to slow transport into the PVC. The $S_{13}D$ mutation constructed to mimic hyperphosphorylation accelerates A-ALP trafficking to the PVC, whereas the $S_{13}A$ mutation that mimics unphosphorylated serine blocks this trafficking step. Given the contrast in phenotype between the $\Delta 2$ –11 and $S_{13}A$ mutants, we tested the possibility that the phosphoserine and 2–11 motifs antagonize each other.

The rate of processing of single and double mutant A-ALP constructs was assessed in the *vps4* Δ mutant that provides a measure of the rate of trafficking into the PVC. Interestingly, in *vps4* Δ cells the $S_{13}A$; $\Delta 2$ –11 double mutant was processed more rapidly than wild-type, with kinetics similar to the $\Delta 2$ –11 single mutant (Table 2). Similar results were obtained for the $\Delta 2$ –11; $S_{13}D$ double mutant. Therefore, these double mutant experiments indicate that the $\Delta 2$ –11 mutation neutralizes the effect that mutating S_{13} has on the rate of trafficking into the PVC, consistent with the idea that the phosphoserine may act by antagonizing the 2–11 signal.

DISCUSSION

This study demonstrates for the first time that phosphorylation influences trafficking of a yeast resident TGN membrane protein. Mutation of S_{13} to A in the cytosolic domain of A-ALP did not slow trafficking to the Golgi. However, this mutation dramatically inhibited the normal delivery of A-ALP to the PVC under several different conditions. The notion that the $S_{13}A$ mutation simply converts A-ALP to an unsuitable proteolytic substrate can be ruled out because the A($S_{13}A$)-ALP was clearly shown to exhibit a non-PVC, Golgi-like localization pattern in class E *vps4* cells, whereas wild-type A-ALP was predominantly associated with the PVC in *vps4* mutants. In addition, we demonstrate that the phosphorylation state of S_{13} regulates A-ALP trafficking. First, analysis of peptides containing S_{13} by mass spectrometry demonstrated that this serine residue is indeed phosphorylated. Second, mutation of S_{13} to D to mimic phosphorylation accelerated trafficking into the PVC, whereas the $S_{13}A$ mutation to mimic unphosphorylated serine retarded trafficking into the PVC.

New insights into how yeast TGN membrane proteins reach the PVC have been gained in recent years, and these are relevant to understanding the role of S_{13} phosphorylation. A variety of evidence suggests that TGN membrane proteins reach the PVC via an early endosomal compartment (see Introduction). Whether A-ALP and Kex2p also use the GGA-dependent direct TGN-to-PVC pathway is less clear. A loss of function of the GGA proteins clearly delays vacuolar processing of Cps1p (Costaguta *et al.*, 2001) consistent with the idea that Cps1p relies on the direct TGN-to-PVC pathway. However, the loss of GGA protein function does not affect the rate of trafficking of A($F_{85}A$; $F_{87}A$)-ALP trafficking into the PVC/vacuole in *gga1,2* Δ mutants (Ha *et al.*, 2003). Trafficking of A($F_{85}A$; $F_{87}A$)-ALP into the PVC/vacuole in *gga1,2* Δ mutants does not occur via the plasma membrane because it is not blocked by an *end3-ts* mutation (Foote and Nothwehr, unpublished data). Together, these results suggest either

Figure 9. Unlike A-ALP, A(S₁₃A)-ALP is not mislocalized to the vacuole in *vps1Δ* and *chcl-ts* cells. (A and B) Cells were pulsed with [³⁵S]methionine/cysteine for 10 min and chased for 0, 20, 40, and 80 min before being lysed and subjected to immunoprecipitation by using an anti-ALP antibody to analyze the indicated proteins. (A) Strains SHY39/pSN55 and SHY39/pSN55-PS2 were propagated at 30°C for the entire experiment. (B) Strains SNY37/pSN55 and SNY37/pSN55-PS2 were propagated for several generations at 24°C. Ten minutes before the pulse and chase, the strains were shifted to 37°C or were allowed to continue growth at 24°C, as indicated. Analysis of *vps1Δ end3-ts* strains SNY96-9D/pWQ13/pAH16 (C) and SNY96-9D/pWQ13/pSN397 (D) expressing HA-Pma1p and either A-ALP (C) or A(S₁₃A)-ALP (D) is shown. The strains were propagated for several generations at permissive temperature (24°C), shifted to nonpermissive temperature (37°C) for 15 min, pulsed for 10 min, and chased for 60 min. Cell lysates were fractionated on sucrose equilibrium gradients and fractions were analyzed by SDS-PAGE and quantitative immunoblotting for the following proteins: A-ALP or A(S₁₃A)-ALP (black solid line), the Golgi marker Och1p (gray line), and the plasma membrane marker HA-Pma1p (dotted line).

that A-ALP and Kex2p do not normally use the GGA-dependent direct TGN-to-PVC pathway or they do use this pathway to a limited degree but alternatively use the TGN-to-early endosome pathway if the GGA pathway is blocked. A definitive resolution to this issue will require thorough analysis of the transport vesicles from these pathways and their contents.

To gain clues about the function of the S₁₃ phosphoserine, we attempted to determine where the A(S₁₃A)-ALP protein was localized. A(S₁₃A)-ALP exhibited a punctate staining appearance typical of Golgi/endosomal proteins. However, the structures containing A(S₁₃A)-ALP seemed slightly smaller and more numerous than those containing A-ALP. Nevertheless, equilibrium density gradient analysis demonstrated that A(S₁₃A)-ALP associated with membranes having a density indistinguishable from that of A-ALP (Johnston and Nothwehr, unpublished data). Thus, the basis for the slightly different staining pattern of A-ALP and A(S₁₃A)-ALP is presently unclear. In this regard, the S₁₃A mutation does not preclude localization to the compartment(s) where α -factor maturation occurs because Ste13p carrying the S₁₃A mutation exhibited α -factor processing efficiency that was similar to wild-type Ste13p. Furthermore, the rates of depletion of wild-type and S₁₃A mutant Ste13p out of the processing compartment after their synthesis was stopped were very similar. Consistent with the A-ALP processing results, the S₁₃A mutation in Ste13p suppressed the effect of the F₈₅A; F₈₇A mutations, indicating that the S₁₃A mutation inhibited delivery of Ste13p into the PVC. Together, the results imply for A-ALP/Ste13p that both the wild-type and S₁₃A mutants populate the TGN/early endosome. Rather, the difference between the wild-type and mutant proteins seems to involve the wild-type protein fre-

quently cycling via the PVC, whereas the S₁₃A mutant does not.

A loss in Inp53p function causes accelerated transport of A-ALP into the PVC (Ha *et al.*, 2001). Epistasis analysis between the S₁₃ mutations and the *inp53Δ* mutation was carried out to gain clues about the relative order of function of the phosphoserine and Inp53p. Like wild-type cells, *inp53Δ* cells exhibited a block in delivery of A(S₁₃A)-ALP to the PVC. Interestingly, a small additive effect was observed between S₁₃D and *inp53Δ*. These data suggest that the acceleration of A-ALP through the pathway that occurs due to loss of Inp53p function occurs before (or at) the step in which the phosphoserine is required. A previous study showed that processing of retrieval-defective A-ALP was severely retarded in an *inp53Δ gga1Δ gga2Δ* triple mutant (Ha *et al.*, 2003). A model proposed in that study was that A-ALP reached the PVC more rapidly in *inp53Δ* single mutants because it used the direct TGN-to-PVC pathway by default. The implication was that Inp53p was needed to restrict trafficking to the TGN-to-early endosome pathway. However, the present study would seem to rule out such a model. The S₁₃A mutation seems to affect trafficking at the early endosome (see below). Therefore, if A-ALP were using the direct TGN-to-PVC pathway in *inp53Δ* mutants then the S₁₃A mutation would not be expected to have an effect on A-ALP trafficking in *inp53Δ* cells. The data suggest instead that loss of Inp53p function could reduce static retention in the TGN/early endosome so that A-ALP rapidly enters the PVC in a phosphoserine-dependent manner. The reason for the delay in retrieval defective A-ALP processing in the *inp53Δ gga1Δ gga2Δ* triple mutant is not known but these mutations also caused a severe growth defect and the slow

trafficking could be due to more broad-ranged defects in the TGN/endosomal system.

To learn more about the trafficking step affected by the $S_{13}A$ mutation, we tested whether trafficking of A($S_{13}A$)-ALP from the plasma membrane to the PVC could occur. This was accomplished using the *vps1Δ* and *chc1-521^{ts}* mutations that mislocalize A-ALP to the plasma membrane whereupon A-ALP is then delivered to the vacuole, presumably via the PVC. The A($S_{13}A$)-ALP was shown to mislocalize to the plasma membrane, but unlike A-ALP, it was not subsequently delivered to the vacuole. Together with the other results of this study, the most straightforward interpretation of this result is that the early endosome-to-PVC transport step is abolished for A($S_{13}A$)-ALP. These results imply that the phosphoserine is needed at the level of an early endosomal compartment for subsequent delivery to the PVC.

Two sorting signals have previously been characterized in the A-ALP cytosolic domain. The FFXFD motif associates with the retromer to mediate PVC-to-TGN retrieval (Nothwehr *et al.*, 2000), whereas a poorly characterized signal in the 2–11 region somehow regulates entry into the PVC. The 2–11 signal was identified based on the observation that deletion of this region accelerated A-ALP transport into the PVC (Bryant and Stevens, 1997). A similar region in Kex2p (TLS2) has been proposed to function by regulating the partitioning of Kex2p between the TGN-to-early endosome (EE) pathway and the direct TGN-to-PVC pathway (Sipos *et al.*, 2004). According to this model, mutation of TLS2 causes Kex2p to predominantly use the direct TGN-to-PVC pathway. Recent data seem to argue against this type of role for the 2–11 signal of A-ALP. Analysis of trafficking of A-ALP and A(Δ 2–11)-ALP into the PVC in a *gga1,2Δ end3-ts* strain, which should limit trafficking to the TGN/early endosome/PVC route, revealed that deletion of 2–11 accelerated trafficking into the PVC (Nothwehr, unpublished data). Thus, it is plausible that the 2–11 signal could function in static retention at the TGN/early endosome or in retrieval from the early endosome back to the TGN (but see below).

Although the 2–11 region is necessary to slow delivery into the PVC, it is not known whether this region is sufficient for this function or whether other motifs may regulate its function. Thus, it is possible that a true signal slowing transport into the PVC might lie outside of this region. One interesting possibility is that the 2–11 region and phosphorylated S_{13} might play antagonistic roles. To test this, we analyzed the phenotype of double mutant combinations of $S_{13}A$, $S_{13}D$, and Δ 2–11 mutations. Interestingly, we found in a *vps4Δ* background that the S_{13} mutations had no effect in the context of A(Δ 2–11)-ALP. The finding that the 2–11 region is needed for the S_{13} mutations to have an impact on trafficking suggests that the phosphoserine might antagonize the 2–11 signal. Thus, it seems likely that S_{13} and Δ 2–11 mutations affect the same transport step. A role for the phosphoserine at an early endosome to encourage transport to the PVC, as the *vps1Δ* results imply (Figure 9), would be consistent with a model in which the 2–11 region mediates phosphoserine-regulated retrieval from an early endosome. In this case, the extent of wild-type A-ALP flux through the PVC could be increased by S_{13} phosphorylation that would make retrieval less efficient (Figure 10, step 1). Thus, the lack of transport of A($S_{13}A$)-ALP into the PVC could be a reflection of enhanced early endosome-to-TGN retrieval compared with wild-type A-ALP. A variation of this basic model is that the 2–11 signal could be a static retention signal for the early endosome and thus would tend to inhibit early endosome-to-PVC transport. Antagonism of the 2–11 signal

Figure 10. Potential membrane trafficking steps affected by the phosphorylated S_{13} residue. Data presented here are consistent with a model in which the phosphorylated S_{13} residue of A-ALP/Ste13p functions at the early endosome to either reduce the efficiency of early endosome-to-TGN retrieval (1) or enhance transport to the PVC (2).

by the phosphoserine would promote early endosome-to-PVC transport (Figure 10, step 2). Deletion of 2–11 or phosphorylation of S_{13} would thus enhance the rate of transport into the PVC.

Several animal cell cargo proteins contain phosphorylation sites embedded with an acidic cluster motif that, when phosphorylated, enhance binding of adaptors and other sorting machinery. In addition to the furin example (see Introduction), phosphorylation of the mannose 6-phosphate receptor increases affinity for the GGA proteins by approximately threefold (Kato *et al.*, 2002). Similarly, AP-1 and AP-2 binding to the T-cell coreceptor CD4 via a dileucine motif is dramatically enhanced by phosphorylation at nearby Ser residues (Pitcher *et al.*, 1999). However, models discussed here for the S_{13} residue of Ste13p/A-ALP instead posit that phosphorylation may decrease affinity for a sorting factor. In this regard it is worth noting that the 2–11 region is highly basic. Thus, the efficiency of interaction between a sorting factor and the basic 2–11 region might rely on electrostatic interactions could be reduced by the presence of a negatively charged phosphoserine at position 13.

What purpose might be served by using phosphorylation as way of regulating trafficking of a TGN membrane protein? An interesting possibility is that this may allow the cell to alter trafficking patterns of certain proteins as a function of environmental or intrinsic conditions e.g., stress, nutrient availability, pheromone response, cell cycle etc. A few examples of this type of regulation are known in yeast. For example, cell surface receptors such the uracil permease and a-factor receptor also are phosphorylated which positively regulates their ubiquitin-mediated internalization (Hicke *et al.*, 1998; Marchal *et al.*, 2000, 2002). The localization of the yeast glycosyltransferase Mnn1p to early Golgi compartments is controlled by the high osmolarity glycerol mitogen-

activated protein kinase pathway (Reynolds *et al.*, 1998), presumably to integrate osmotic stress responses with localization of enzymes such as Mnn1p that are important for cell wall biosynthesis. Moreover, osmotic stress also causes yeast chitin synthase Chs3p to be transported from an early endosomal compartment to the plasma membrane, an event regulated by a protein kinase C pathway (Valdivia and Schekman, 2003). Thus, it is possible that the pheromone processing enzymes Ste13p and Kex2p also might be regulated by cell signaling pathways that could affect the production of mature pheromone or other proteins that are dependent on processing by these enzymes. Testing of this idea awaits identification of the kinase and phosphatase for the S₁₃ residue of Ste13p/A-ALP and characterization of the upstream regulatory machinery.

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