

NBD-labeled phosphatidylcholine enters the yeast vacuole via the pre-vacuolar compartment

Pamela K. Hanson, Althea M. Grant and J. Wylie Nichols

Department of Physiology, 615 Michael St, 605G Whitehead Building, Emory University School of Medicine, Atlanta, GA 30322, USA

Author for correspondence (e-mail: wnichols@physio.emory.edu)

Accepted 1 April 2002

Journal of Cell Science 115, 2725-2733 (2002) © The Company of Biologists Ltd

Summary

At low temperature, the short-chain fluorescent-labeled phospholipids, 1-myristoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminocaproyl]-phosphatidylcholine (M-C₆-NBD-PC) and its phosphatidylethanolamine analog, M-C₆-NBD-PE, are internalized by flip across the plasma membrane of *S. cerevisiae* and show similar enrichment in intracellular membranes including the mitochondria and nuclear envelope/ER. At higher temperatures (24–37°C), or if low temperature internalization is followed by warming, M-C₆-NBD-PC, but not M-C₆-NBD-PE, is trafficked to the lumen of the vacuole. Sorting of M-C₆-NBD-PC to the vacuole is blocked by energy-depletion and by null mutations in the *VPS4* and *VPS28* genes required for vesicular traffic from the pre-vacuolar compartment (PVC) to the vacuole. This sorting is not blocked by a

temperature-sensitive mutation in *SEC12*, which inhibits ER to Golgi transport, a null mutation in *VPS8*, which inhibits Golgi to PVC transport, or temperature-sensitive and null mutations in *END4*, which inhibit endocytosis from the plasma membrane. Monomethylation or dimethylation of the primary amine head-group of M-C₆-NBD-PE is sufficient for sorting to the yeast vacuole in both wild-type yeast and in strains defective in the phosphatidylethanolamine methylation pathway. These data indicate that methylation of M-C₆-NBD-PE produces the crucial structural component required to sort these phospholipid analogues to the vacuole via the PVC.

Key words: Phosphatidylcholine, Phosphatidylethanolamine, Vacuole, Fluorescence, Yeast

Introduction

To date it remains unclear how membrane lipids, which are synthesized primarily at the endoplasmic reticulum (ER), become so highly organized at both the inter- and intra-organellar level. Among the classic examples of interorganellar lipid organization is the enrichment of sterols and phosphatidylserine (PtdSer) in the plasma membrane compared with the ER (Zambrano et al., 1975). This non-random distribution of lipids between membranous organelles requires active sorting mechanisms and presumably reflects a functional requirement for different lipid milieus at various sites throughout the cell. For example, the regulation and formation of cholesterol and sphingolipid rafts at the plasma membrane may be critically important for proper receptor activation and function (Brown and London, 1998).

Much of the trafficking involved in the generation of the plasma membrane's unique lipid composition is thought to take place in the Golgi apparatus because its lipid composition reflects properties of both the ER and the plasma membrane (Zambrano et al., 1975). In addition to helping establish interorganellar differences in lipid composition, the Golgi is a prime example of intraorganellar lipid organization. If the membranous stacks of the cis- and trans-Golgi are compared, there is a dramatic increase in the content of cholesterol as well as sphingolipids in the trans-Golgi network, making these membranes more plasma membrane-like (Orci et al., 1981).

Another example of intraorganellar lipid organization is at the plasma membrane of eukaryotic cells. The evidence currently available is consistent with aminophospholipids

being sequestered to the cytoplasmic leaflet of the membrane, whereas choline lipids are enriched in the outer leaflet (reviewed by Devaux and Zachowski, 1994). It is well established that loss of this asymmetric distribution, resulting in exposure of PtdSer in the outer leaflet of the plasma membrane, is required for activation of the coagulation cascade and can serve as a signal for clearance of aging red blood cells and apoptotic cells (reviewed by Zwaal and Schroit, 1997). Despite these advances in our understanding of the existence and significance of non-random lipid distributions within cells, the dynamic processes involved in generating and maintaining this level of organization remain poorly understood.

Historically, fractionation studies have proven informative by establishing the steady-state distribution of lipids both between and within organelles. However, these approaches do not address the dynamics of lipid movement. To investigate such issues, a variety of additional tools have been developed including short-chain, labeled lipids (reviewed by Daleke and Lyles, 2000). These lipid analogs have proven useful for studying lipid transport and trafficking events. For example, in many cell lines, phosphatidylethanolamine (PtdEtn) and PtdSer analogs are internalized rapidly by flip whereas phosphatidylcholine (PtdCho) is internalized by flip slowly, if at all (reviewed by Devaux and Zachowski, 1994; Zwaal and Schroit, 1997). These dynamic differences are thought to account for the asymmetric distribution of endogenous phospholipids at the plasma membrane. Furthermore, synthetic lipids appear to be sorted in a backbone and head-group specific manner within cells. In fact, fluorescent labeled

ceramide is commonly used as a Golgi marker (Lipsky and Pagano, 1985b) and can be metabolized into other fluorescent sphingolipid analogs (Lipsky and Pagano, 1985a) indicating that this fluorescent probe is a suitable substrate for endogenous lipid modifying enzymes.

To study the establishment and maintenance of inter- and intra-organellar lipid organization, we have examined the trafficking of fluorescent labeled phospholipids in the genetically tractable yeast, *Saccharomyces cerevisiae*. In our initial studies we found that short-chain fluorescent-labeled 1-myristoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminocaproyl]-phosphatidyl-ethanolamine (M-C₆-NBD-PE) is taken up by yeast at normal growth temperature and labels predominantly the mitochondria and nuclear envelope/endoplasmic reticulum without being metabolized to an appreciable extent (Kean et al., 1997). By contrast, 1-myristoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminocaproyl]-phosphatidylcholine (M-C₆-NBD-PC) predominantly labels the lumen of the vacuole where it is catabolized (Kean et al., 1993). In *pep4* mutants, which do not process vacuolar hydrolases to their active form, M-C₆-NBD-PC still labels the lumen of the vacuole, but its catabolism is dramatically inhibited, indicating that intact M-C₆-NBD-PC is trafficked to the lumen of the vacuole prior to its breakdown (Kean et al., 1993).

These and other observations led to the hypothesis that M-C₆-NBD-PE was internalized by a non-endocytic mechanism, presumably protein-mediated, inward-directed flip across the plasma membrane, whereas M-C₆-NBD-PC was thought to be internalized by endocytosis and subsequently trafficked to the vacuole (Kean et al., 1993). However, our more recent studies showed that mutants defective for the internalization step of endocytosis are not defective for the net uptake of M-C₆-NBD-PC or M-C₆-NBD-PE (Grant et al., 2001). These data, combined with the fact that yeast can internalize both lipids to a similar extent at 2°C, when vesicular trafficking is inhibited, support the model that M-C₆-NBD-PC as well as M-C₆-NBD-PE are internalized predominantly by a non-endocytic mechanism, presumably inward-directed transbilayer translocation or flip, across the plasma membrane (Grant et al., 2001; Hanson and Nichols, 2001). Furthermore, we have shown that internalization of both M-C₆-NBD-PC and M-C₆-NBD-PE is dependent on the plasma membrane proton

electrochemical gradient and is inhibited in anterograde *sec* mutants, suggesting that flip of these phospholipid analogs across the plasma membrane requires continuous transport of a proton-coupled flippase to the cell surface (Grant et al., 2001).

In addition to decreasing fluorescent lipid internalization we found that one *sec* mutant, *sec18*, inhibited vacuolar localization of M-C₆-NBD-PC (Grant et al., 2001). Because *SEC18* is the yeast homologue of the mammalian NEM-sensitive factor (NSF) required for membrane fusion in numerous vesicle trafficking steps (Burd et al., 1997; Graham and Emr, 1991; Hicke et al., 1997), we hypothesized that vesicular traffic is necessary for vacuolar localization of M-C₆-NBD-PC. Having ruled out endocytosis as the primary means for M-C₆-NBD-PC uptake, we examined which of the remaining vesicular trafficking steps were required for M-C₆-NBD-PC sorting to the vacuole and showed that trafficking from the pre-vacuolar compartment to the vacuole is necessary for its access to the lumen of the vacuole. Furthermore, we demonstrated that addition of even a single methyl group to the M-C₆-NBD-PE head group was sufficient for its sorting to the vacuole.

Materials and Methods

Materials

Yeast media was obtained from Difco Laboratories (Detroit, MI). 4',6-diamidino-2 phenylindole (DAPI) and all other reagents, unless otherwise noted, were purchased from Sigma (St Louis, MO). M-C₆-NBD-PE and M-C₆-NBD-PC were from Avanti Polar Lipids (Alabaster, AL). Phospholipids were stored at -20°C and periodically monitored for purity by thin-layer chromatography. Phospholipid concentrations were determined by lipid phosphorous assay (Ames and Dubin, 1960) or by absorbance at 466 nm ($\epsilon_{466}=21,000$ in methanol).

Yeast strains and cultures

The *S. cerevisiae* strains used in this study are shown in Table 1. All temperature-sensitive strains were assayed for the inability to grow at 37°C on YPAD (1% yeast extract, 2% peptone, 0.004% adenine, 2% glucose) plates prior to lipid trafficking assays. For mutants without a temperature-sensitive growth defect, other mutation-related phenotypes, such as the mislocalization of FM4-64 were confirmed.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
CRY3	<i>MATα Sec⁺ can1-100 ade2-1 his2-11,15 leu2-3-112 trp1-1 ura3-1/ MATα can1-100 ade2-1 his2-11,15 leu2-3-112 trp1-1 ura3-1</i>	R.S. Fuller*
NY13	<i>MATα Sec⁺ ura3-52</i>	P. Novick†
NY431	<i>MATα ura3-52 sec18-1</i>	P. Novick†
X2180-1A	<i>MATα Sec⁺ SUC2 mal gal2 CUP1</i>	Yeast Genetic Stock Center
SF266-1C	<i>MATα SUC2 mal gal2 CUP1 sec12-4</i>	Yeast Genetic Stock Center
SEY6210	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	S. Emr‡
SEY6210 <i>Δvps4</i>	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps4::TRP1</i>	S. Emr‡
SEY6210 <i>Δvps8</i>	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps8::LEU2</i>	S. Emr‡
RH1800	<i>END4</i>	H. Riezman§
RH1800 <i>end4-1</i>	<i>end4-1</i>	H. Riezman§
RH448	<i>MATα his4 leu2 ura3 lys2 bar1</i>	H. Riezman§
RH1966/299-1D	<i>MATα end4::LEU2 his4 leu2 ura3 lys2 bar1</i>	H. Riezman§

*University of Michigan, Ann Arbor, MI.

†Yale University, New Haven, CT.

‡University of California - San Diego, La Jolla, CA.

§University of Basel, Basel, Switzerland.

For all experiments, early log-phase cultures ($OD_{600}=0.2-0.4$) were grown in SDC [0.67% yeast nitrogen base, 2% glucose, and complete amino acid supplement, as described (Sherman et al., 1986)] from overnight cultures. $SCNaN_3$ is SDC media lacking glucose but containing 2% sorbitol and 20 mM sodium azide. $SCNaN_3 + NaF$ is SDC media lacking glucose but containing 2% sorbitol, 10 mM sodium fluoride and 10 mM sodium azide. Temperature-sensitive strains were grown at a permissive temperature of 23°C and internalization assays were performed at either 23°C or 37°C, as indicated.

Lipid preparation

To prepare dimethyl sulfoxide-solubilized stocks, aliquots of NBD-phospholipids dissolved in chloroform were dried under a stream of nitrogen, desiccated under vacuum for at least 1 hour, and resuspended in the appropriate amount of DMSO to obtain a 1000× stock concentration. To prepare large unilamellar vesicles, phospholipids dissolved in chloroform were mixed in a 4:6 mole ratio of NBD-phospholipid to dioleoylphosphatidylcholine, dried under a stream of nitrogen, and desiccated under vacuum for at least 1 hour. Desiccated phospholipids were resuspended by vortex mixing with SDC and the mixture was passed 5-8 times through a Lipex Extruder (Lipex Biomembranes, Vancouver, BC, Canada) equipped with 0.1 μm filters to produce a solution of vesicles with 1 mM total phospholipid. Phospholipid preparations were made fresh for each experiment.

Synthesis of monomethyl and dimethyl M-C₆-NBD-PE

Monomethyl M-C₆-NBD-PE (1-myristoyl-2-[6-(NBD) aminocaproyl]-phosphatidylmonomethylethanolamine) and dimethyl M-C₆-NBD-PE (1-myristoyl-2-[6-(NBD) aminocaproyl]-phosphatidyl-dimethylethanolamine) were synthesized from M-C₆-NBD-PE by phospholipase D-catalyzed based exchange (Comfurius and Zwaal, 1977). M-C₆-NBD-PE was reacted with phospholipase D from cabbage (Sigma) in the presence of either 2-dimethylethanolamine or 2-(methylamino)ethanol (Acros Organics). Reaction products were purified by preparative TLC. Appropriate bands were identified by their predicted mobility in relation to M-C₆-NBD-PE and M-C₆-NBD-phosphatidate standards, scraped, and eluted into chloroform/methanol. Purified products yielded a single spot by TLC.

Internalization of phospholipid and dyes into yeast cells

For analysis of wild-type and deletion strains at ambient temperature, cultures were grown to early log phase in SDC at 30°C. DMSO-solubilized lipid was added to a final concentration of 5 μM and vortex mixed. After 45-minute incubation with the lipid at 30°C, cells were harvested and washed twice with SDC at room temperature. To label mitochondrial and nuclear DNA, the yeast were resuspended in 1 ml of SDC, and 1 μl of DAPI was added from a 1 mg/ml stock solution in water. After 15 minutes of incubation with DAPI at 30°C, cells were washed three times with ice-cold $SCNaN_3$ and imaged by fluorescence microscopy.

For labeling experiments performed at 2°C, cells were grown to early log phase in SDC and labeled with DAPI as described above. Yeast were then cooled to 2°C for 15 minutes prior to the addition of lipid. Lipid was added to a final concentration of 20 μM by pipetting an aliquot from the stock solution while vortex mixing the cells. After addition of lipid, cells were incubated at 2°C for 90 minutes. Cells were then either washed with ice-cold $SCNaN_3$ or treated as described in Fig. 4 legend.

For labeling experiments performed on temperature-sensitive strains, the mutants and their isogenic parental

strains were grown to early-log phase in SDC at 23°C. The incubation temperature was then shifted to 37°C for 30 minutes prior to the addition of phospholipid vesicles to a final concentration of 50 μM total phospholipid. Cells were incubated for 45 minutes with the vesicles before adding DAPI to a final concentration of 1 μg/ml. After an additional 15 minute incubation with both DAPI and NBD-phospholipids, the cells were washed three times with two volumes of ice-cold $SCNaN_3$ and imaged by fluorescence microscopy.

Fluorescence microscopy

Fluorescence microscopy was performed on a Zeiss Axiovert 135 microscope equipped with barrier filters that eliminate detectable crossover of NBD and DAPI fluorescence. The fluorescent images were enhanced with a SIT66 image-intensifying camera (DAGE-MTI, Michigan City, IN), digitized, and stored using Metamorph software (Universal Imaging).

Results

M-C₆-NBD-PC but not M-C₆-NBD-PE is trafficked to the vacuole

When labeling wild-type yeast at ambient temperature (24°-37°C) we consistently observe dramatic differences in the localization of M-C₆-NBD-PC and M-C₆-NBD-PE. M-C₆-NBD-PC predominantly labels the vacuole, whereas M-C₆-NBD-PE is excluded from the vacuole and enriched in the mitochondria and nuclear envelope/ER [Fig. 1 (Kean et al., 1993)]. We have demonstrated previously that the majority of cell-internalized M-C₆-NBD-PE remains intact (Kean et al., 1997), whereas the majority of M-C₆-NBD-PC is degraded to water soluble fluorescent products after its traffic to the vacuole (Kean et al., 1993).

Vacuolar localization of M-C₆-NBD-PC is not mediated by endocytosis from the plasma membrane

The localization of M-C₆-NBD-PC to the vacuole is one of

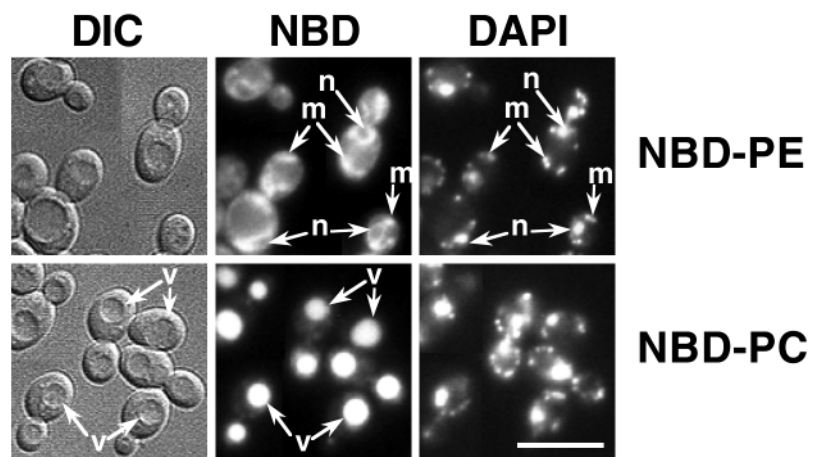


Fig. 1. NBD-PC but not NBD-PE is trafficked to the vacuole (v) in yeast. The wild-type diploid strain CRY2 was grown to early log phase in SDC at 30°C. Cells were then labeled with 5 μM DMSO-solubilized lipid for 45 minutes at 30°C. After being washed twice with room temperature SDC, DAPI was added to label nuclear (n) and mitochondrial (m) DNA. Cells were incubated at 30°C for 15 additional minutes, harvested, and washed twice with ice-cold $SCNaN_3$ prior to analysis and imaging by fluorescence microscopy.

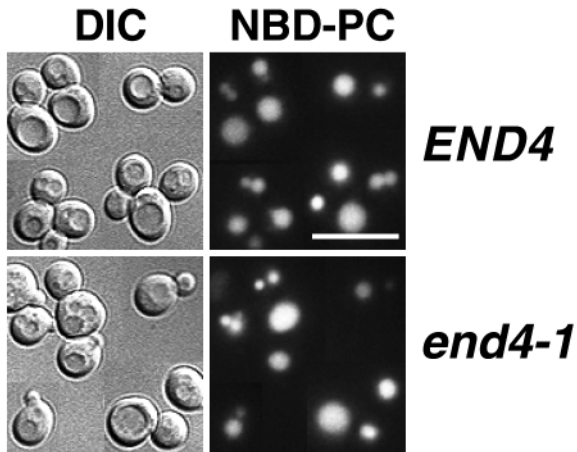


Fig. 2. M-C₆-NBD-PC is not trafficked to the vacuole via the endocytic pathway. Strains were grown to early log phase in SDC at 23°C. After a 30 minute incubation at 37°C, cells were labeled for 1 hour with 50 μM of M-C₆-NBD-PC-containing vesicles as described in the Materials and Methods. The cells were then washed three times with ice-cold SCN₃ and harvested prior to imaging by fluorescence microscopy. The cells shown were labeled at the restrictive temperature, 37°C. When labeled at the permissive temperature, 23°C, both strains also exhibited vacuolar labeling (data not shown).

several pieces of evidence that led to the hypothesis that M-C₆-NBD-PC is internalized by endocytosis. However, upon further examination, we found that strains defective for the internalization step of endocytosis were not defective in the uptake of M-C₆-NBD-PC or M-C₆-NBD-PE (Grant et al., 2001). Here we show that, in addition to being unaffected in the net uptake of NBD-phospholipids, *end4* mutants (Raths et al., 1993) display no defect in sorting M-C₆-NBD-PC to the vacuole (Fig. 2). These data indicate that the standard pathway for endocytosis and recycling of plasma

membrane components is not required for the uptake or proper localization of M-C₆-NBD-PC.

NBD-PC is not trafficked to the vacuole at low temperature

Although significantly slower, the non-endocytic mechanism of M-C₆-NBD-PC and M-C₆-NBD-PE uptake – presumably inward-directed transbilayer transfer or flip – still functions when cells are incubated at low temperature, even on ice (Grant et al., 2001; Hanson and Nichols, 2001). However, under these conditions M-C₆-NBD-PC is not trafficked to the vacuole (Grant et al., 2001). In fact, at low temperature, M-C₆-NBD-PC and M-C₆-NBD-PE exhibit the same labeling pattern, with both lipids enriched in the mitochondria, nuclear envelope/endoplasmic reticulum and perhaps other intracellular organelles [Fig. 3 (Grant et al., 2001; Hanson and Nichols, 2001)]. This labeling pattern is thought to be achieved through a passive mechanism because it is not disrupted in respiration-defective petite strains that exhibit dramatic reductions in mitochondrial membrane potential (Hanson and Nichols, 2001).

Because these fluorescent phospholipids have a shortened acyl chain at the *sn*-2 position, it is possible that once flipped to the inner leaflet of the plasma membrane, M-C₆-NBD-phospholipids can passively diffuse through the cytosol and eventually be enriched in the cytoplasmic leaflet of membranes with the most favorable partitioning properties. This default labeling pattern can, of course, be disrupted if the cell actively sorts some lipids into the lumen of an organelle, thereby effectively trapping even short-chain lipids.

M-C₆-NBD-PC is trafficked from intracellular membranes to the vacuolar lumen by a vesicle-mediated, energy-dependent mechanism

Given that our data indicate that the early stages of endocytosis are not required for the internalization and distribution of M-C₆-NBD-PC to the vacuole (Fig. 2), we predicted that cells in which the intracellular organelles were labeled with M-C₆-NBD-PC at 2°C would traffic this probe to the vacuole upon warming to 30°C. The images presented in Fig. 4 demonstrate that this is in fact the case. Following labeling of the nuclear envelope/ER, mitochondria, and perhaps other undetermined intracellular membranes with M-C₆-NBD-PC at 2°C, cells were warmed to 30°C for 60 minutes and imaged. During the 30°C chase period M-C₆-NBD-PC is trafficked to the vacuole lumen (Fig. 4A, left panel). As this trafficking occurs, the cell-associated fluorescence becomes noticeably dimmer for two reasons – some of the lipid is effluxed out of the cell and either degraded or washed away (Kean et al., 1997), and the lipid that is trafficked to the vacuole is degraded to water-soluble products with low quantum yield (Monti et al., 1977; Nichols, 1987). Traffic to the vacuole, as well as efflux across the plasma membrane (Hanson and Nichols, 2001), is blocked upon warming, when the cells are depleted of cellular energy by incubation with azide- and

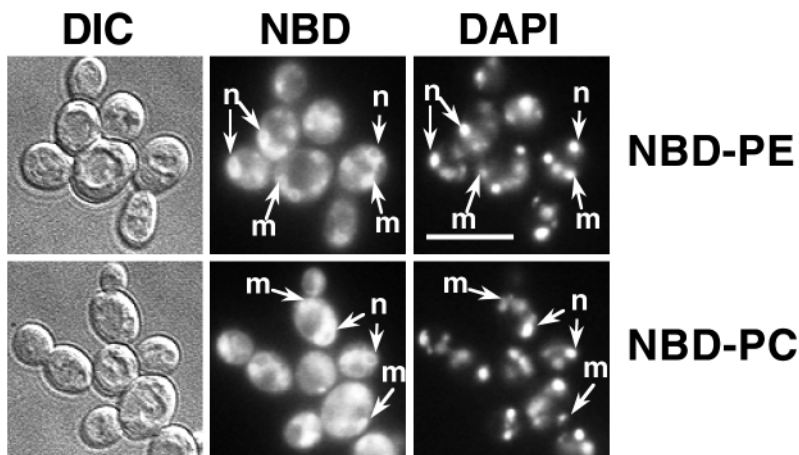


Fig. 3. M-C₆-NBD-PC and M-C₆-NBD-PE label mitochondria (m) and the nuclear envelope (n) at low temperature. The wild-type diploid strain CRY2 was grown to early log phase in SDC at 30°C. Cells were then labeled with DAPI for 10 minutes prior to being placed on ice and chilled. After approximately 10 minutes on ice, cells were labeled with 5 μM DMSO-solubilized lipid for 90 minutes. Cells were then harvested and washed three times with ice-cold SCN₃ prior to analysis and imaging by fluorescence microscopy.

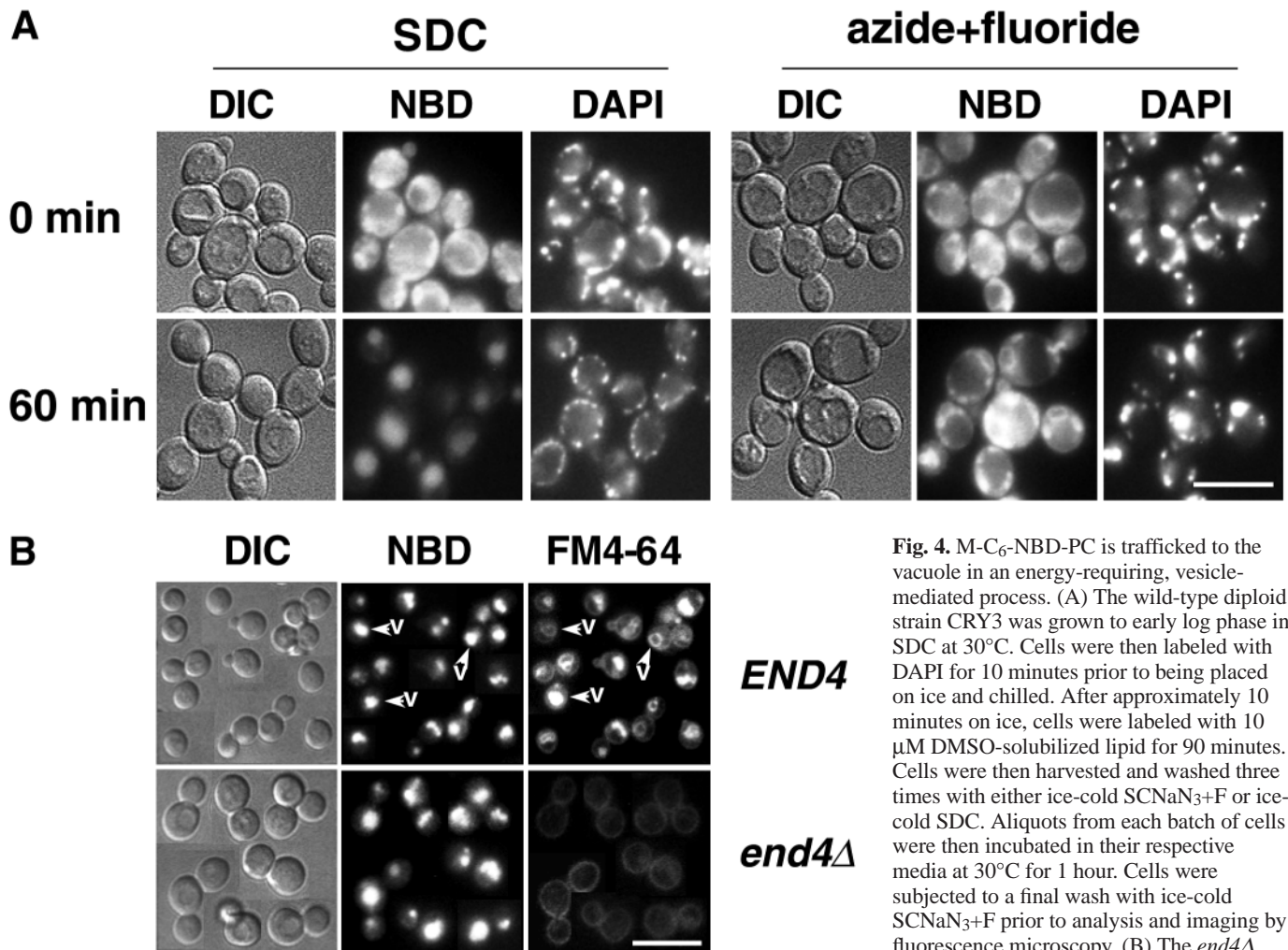


Fig. 4. M-C₆-NBD-PC is trafficked to the vacuole in an energy-requiring, vesicle-mediated process. (A) The wild-type diploid strain CRY3 was grown to early log phase in SDC at 30°C. Cells were then labeled with DAPI for 10 minutes prior to being placed on ice and chilled. After approximately 10 minutes on ice, cells were labeled with 10 μM DMSO-solubilized lipid for 90 minutes. Cells were then harvested and washed three times with either ice-cold SCNaN₃+F or ice-cold SDC. Aliquots from each batch of cells were then incubated in their respective media at 30°C for 1 hour. Cells were subjected to a final wash with ice-cold SCNaN₃+F prior to analysis and imaging by fluorescence microscopy. (B) The *end4Δ* mutant strain and its isogenic parent strain

were grown to early log phase in SDC at 30°C. The isogenic parent and *end4Δ* strains were labeled with DMSO-solubilized M-C₆-NBD-PC (1.0 μM and 0.5 μM, respectively) on ice for 60 minutes, followed by washing twice in ice-cold SDC. Cells were warmed to 30°C and labeled with FM4-64 (40 μM) for 15 minutes, washed twice in SDC (30°C) and resuspended in SDC (30°C) for 60 minutes. Cells were centrifuged, then resuspended in ice-cold SCNaN₃ prior to imaging by fluorescence and differential interference contrast (DIC) microscopy.

fluoride-containing media following the internalization period and during the 30°C chase period (Fig. 4A, right panel).

Given that M-C₆-NBD-PC internalized at low temperature, in addition to its traffic to the vacuole, is effluxed across the plasma membrane and degraded, it is possible that the NBD fluorescence observed in the vacuole results from the endocytosis of M-C₆-NBD-PC or its degradative products from the plasma membrane. To address this possibility, the warm-up experiment was repeated in an *end4Δ* strain, which is blocked in both fluid-phase and receptor-mediated endocytosis from the plasma membrane (Wesp et al., 1997). A commonly used marker of endocytosis, FM4-64 (Vida and Emr, 1995), was used to confirm defective endocytosis in the mutant strain. The images in Fig. 4B illustrate that M-C₆-NBD-PC is trafficked to the vacuole upon warming following low temperature labeling in both the *end4Δ* and its isogenic parent strain. However, FM4-64 is internalized to the vacuole only in the parent and not the *end4Δ* strain. Thus, following its internalization to intracellular membranes at low temperature, M-C₆-NBD-PC is trafficked independently from M-C₆-NBD-

PE to the vacuole by an energy-dependent mechanism that does not require endocytosis from the plasma membrane.

Previous results demonstrated that a mutant strain carrying a temperature-sensitive, loss-of-function mutation in the *SEC18* gene does not traffic M-C₆-NBD-PC to the vacuole (Grant et al., 2001). *SEC18* encodes the yeast homologue of the NEM-sensitive fusion protein, NSF, which is required for fusion of vesicles with target membranes at many sites throughout the cell (Burd et al., 1997; Graham and Emr, 1991; Hicke et al., 1997). These data suggest that at least one of the energy-dependent steps in M-C₆-NBD-PC traffic to the vacuole is dependent on NSF-mediated vesicle fusion.

Class E vps mutants do not sort M-C₆-NBD-PC to the vacuole

Having established that sorting of M-C₆-NBD-PC to the vacuole was mediated by vesicular trafficking, but not endocytosis from the plasma membrane, we next hypothesized that M-C₆-NBD-PC was traveling through the vacuolar protein

sorting (vps) pathway in order to reach the lumen of the vacuole and that its topological reorientation from the cytoplasmic to luminal compartments was a necessary step at some point along the pathway. A logical place for this to occur would be at the ER. Because M-C₆-NBD-PC readily labels the nuclear envelope/ER, an organelle that is known to exhibit flippase activity in yeast (Nicolson and Mayinger, 2000) as well as in mammalian cells (Bishop and Bell, 1985), this lipid could be preferentially flipped from the cytoplasmic leaflet to the lumen of the ER and subsequently shunted through the vps pathway to the vacuole. To test whether this occurred exclusively in the ER, we examined the labeling pattern of a *sec12-4* mutant that, when incubated at the non-permissive temperature, is defective in the guanine nucleotide exchange activity required for vesicular traffic from the ER to the Golgi apparatus (Nishikawa and Nakano, 1993; Novick et al., 1980). If flip across the ER membrane was the only site for M-C₆-NBD-PC to enter the luminal compartment of the vps pathway, then blocking ER to Golgi vesicle traffic would prevent its vacuolar localization. As seen in Fig. 5A, vacuolar localization of M-C₆-NBD-PC is not perturbed in the *sec12-4* mutant at the restrictive temperature. Thus, although M-C₆-NBD-PC flip is likely to occur across the ER membrane (Nicolson and

Mayinger, 2000), we concluded that neither flip across the ER membrane nor ER to Golgi traffic is required for M-C₆-NBD-PC transport to the vacuole.

The next major vesicular trafficking step in the vps pathway is from the Golgi to the pre-vacuolar compartment (PVC), and this event can be disturbed by disruption of the *VPS8* gene (Horzodovsky et al., 1996). If M-C₆-NBD-PC entered the vps pathway exclusively by flip into the lumen of the Golgi and subsequent traffic to the vacuole, then disruption of Golgi to PVC traffic would prevent vacuolar labeling by M-C₆-NBD-PC. This hypothesis seemed plausible based on the reported flippase activity in the Golgi apparatus (Chen et al., 1999). However, as seen in Fig. 5B, the lumen of the vacuole is brightly labeled in strains disrupted at the *VPS8* locus, which indicates that neither Golgi to PVC vesicle traffic nor flip into the Golgi lumen is necessary for vacuolar localization of M-C₆-NBD-PC.

The final vesicular trafficking step in the vps pathway is from the PVC to the vacuole itself. Class E vps mutants are defective in this membrane trafficking event and exhibit an exaggerated PVC full of small vesicles (Raymond et al., 1992; Rieder et al., 1996; Wurmser and Emr, 1998). These vesicles are thought to result from invaginations of the PVC membrane

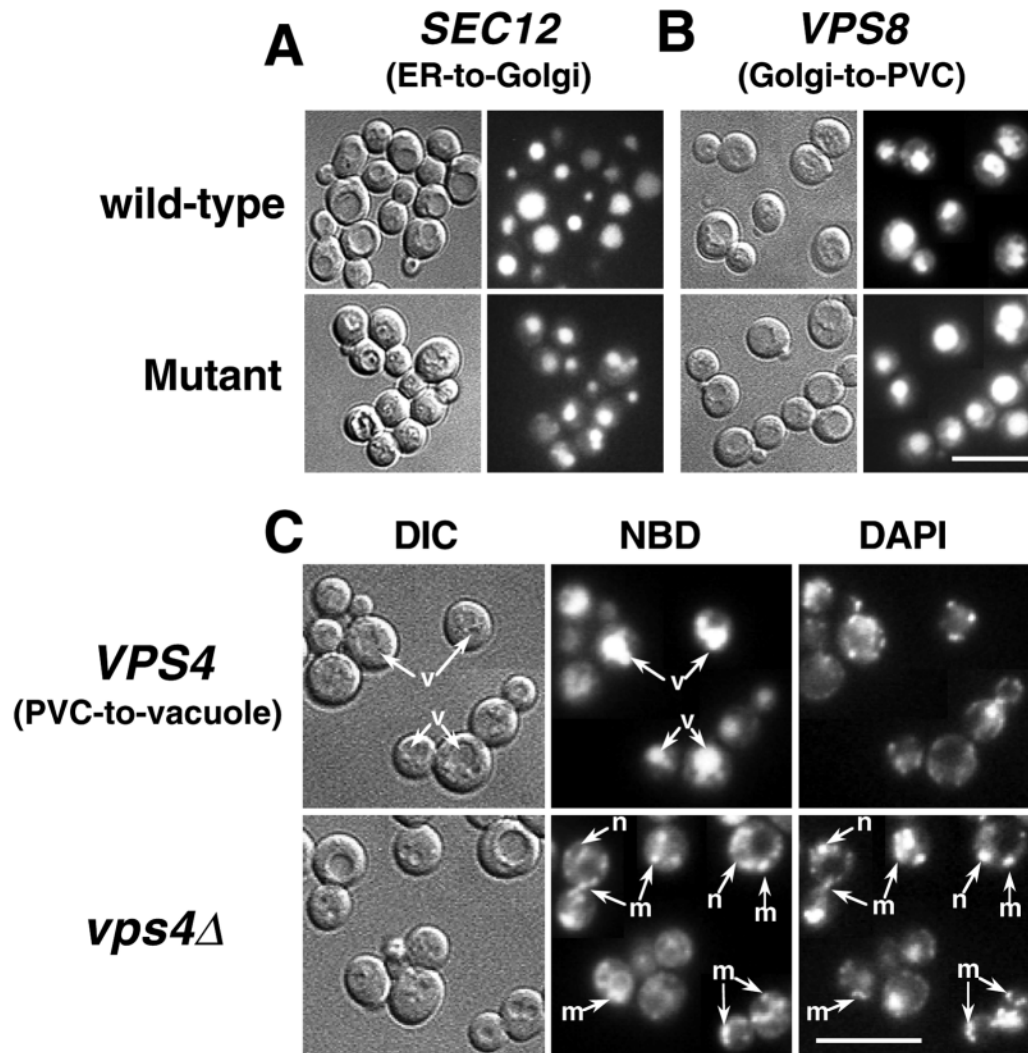


Fig. 5. Trafficking of M-C₆-NBD-PC to the vacuole requires vesicular transport from the PVC to the vacuole. (A) The *sec12-4* mutant strain and its wild-type parent were labeled as described in Fig. 2. (B,C) The wild-type and deletion strains were grown to early log phase in SDC at 30°C and labeled as described in Fig. 1.

that are pinched off and subsequently trafficked to the vacuole, where they are degraded (Wurmser and Emr, 1998). In fact, this mode of lipid turnover is hypothesized to be the major mechanism for PtdIns(3)*P* degradation (Wurmser and Emr, 1998). Thus, the invagination of vesicles and/or flippase activity at the PVC provide two potential mechanisms for the topological reorientation necessary for M-C₆-NBD-PC traffic to the vacuolar lumen. If either or both of these mechanisms were present in the PVC, but not in the vacuole, class E mutants would not exhibit M-C₆-NBD-PC vacuolar labeling. In fact, this was observed. Fig. 5C illustrates that M-C₆-NBD-PC is not localized in the vacuole, but instead is enriched in the mitochondria of a strain carrying a disruption in the *vps4* gene which encodes an AAA ATPase essential for PVC to vacuole trafficking (Finken-Eigen et al., 1997). In addition, another class E mutant disrupted at the *VPS28* locus (Rieder et al., 1996) exhibited mitochondrial rather than vacuolar labeling by M-C₆-NBD-PC (data not shown).

Monomethyl and dimethyl M-C₆-NBD-PE are sorted to the vacuole

Given the intriguing differences between the trafficking of M-C₆-NBD-PC and M-C₆-NBD-PE, lipids that differ structurally by only three methyl groups, we chose to examine the trafficking of monomethyl and dimethyl M-C₆-NBD-PE – the NBD analogues of the two intermediates in the methylation pathway for PtdCho synthesis from PtdEtn (Kanipes and Henry, 1997). As seen in Fig. 6, both monomethyl and dimethyl M-C₆-NBD-PE label the vacuole suggesting that even a single methylation event is sufficient for targeting of PtdEtn to the vacuole. Since M-C₆-NBD-PE is not converted to detectable amounts of NBD-labeled monomethyl PtdEtn, dimethyl PtdEtn, or PtdCho in wild-type cells (Kean et al., 1997), it is unlikely that the monomethyl and dimethyl analogues are first converted to M-C₆-NBD-PC prior to traffic to the vacuole. However, to be certain that this was not occurring, we repeated these experiments in a mutant strain in which both PtdEtn methyl transferase genes, *PEM1/CHO2* (Greenberg et al., 1983; Kodaki and Yamashita, 1987) and *PEM2/OPI3* (Kodaki and Yamashita, 1987; Summers et al., 1988), were deleted and found the same result (data not shown). Thus, it is unlikely that traffic of the NBD-labeled monomethyl and dimethyl PtdEtn to the vacuole depends on their metabolism to M-C₆-NBD-PC, and we concluded that the sorting mechanism that distinguishes PtdEtn from PtdCho for transport can also recognize the monomethyl and dimethyl ethanolamine head groups.

Discussion

Our laboratory has consistently observed that M-C₆-NBD-PE labels the mitochondria and nuclear envelope of yeast, whereas the comparable PtdCho analog labels the vacuole at normal growth temperature (Grant et al., 2001; Kean et al., 1993). Because of the intriguing similarities in the passive localization of M-C₆-NBD-PC and -PE to the mitochondria at low temperature, and the contrasting active sorting of M-C₆-NBD-PC to the vacuole at 30°C, we examined the basic structural requirements needed for cells to separate these phospholipids from one another. We

monitored the localization of NBD-labeled analogs of the two intermediates in the methylation pathway for PtdCho synthesis from PtdEtn – monomethyl and dimethyl M-C₆-NBD-PE – in both wild-type and PtdEtn-methylation-defective strains, and determined that addition of a single methyl group to the primary amine of M-C₆-NBD-PE is sufficient for targeting to the lumen of the vacuole (Fig. 6).

Although methylation has long been recognized as a modifier of protein function (reviewed by Aletta et al., 1998), few reports, if any, have indicated that methylation can so dramatically alter the trafficking of membrane lipids. However, as with any study employing labeled lipids, extrapolation to the movement of endogenous lipids must be made with caution. Clearly, endogenous PtdCho will be subject to a different set of trafficking 'rules' because its two long acyl chains will prevent it from exchanging freely between membranous organelles in the absence of vesicular traffic or phospholipid transfer proteins. Regardless, the ability of cells to target lipids with methylated amine head-groups for degradation in the vacuole could provide a highly specific and efficient means for maintaining proper PtdCho or lyso-PtdCho homeostasis within the cell, as well as a pathway for recycling choline under conditions of choline deprivation (Walkey et al., 1998). The selective targeting of the methylated products of PtdEtn to the vacuole for degradation appears to be analogous to the finding that functional PVC to vacuole trafficking is required for the proper turnover of PtdIns(3)*P* (Wurmser and Emr, 1998).

Analysis of vacuolar localization of M-C₆-NBD-PC in mutants defective in various vesicular trafficking events throughout the *vps* pathway demonstrated that trafficking from the PVC to the vacuole is the only step required for proper localization. The observed block of M-C₆-NBD-PC traffic to the vacuole in the class E *vps* mutants (Fig. 5C) identifies the *vps* pathway as the predominant mode of M-C₆-NBD-PC traffic to the vacuole, since protein traffic via the alternative

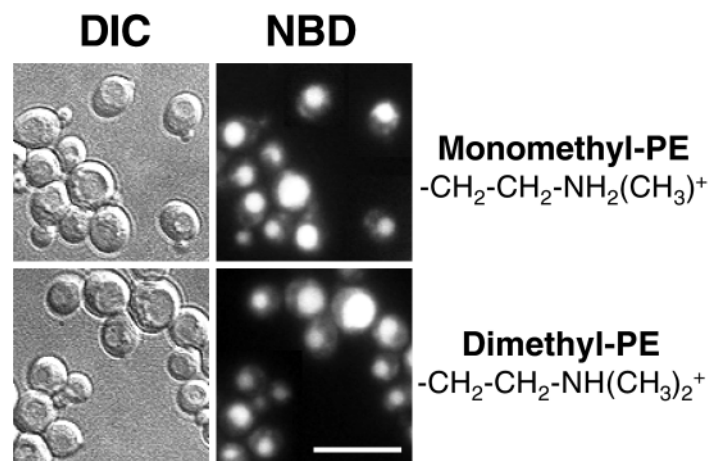


Fig. 6. Both monomethyl and dimethyl M-C₆-NBD-PE are trafficked to the vacuole in yeast. The wild-type diploid strain CRY3 was grown to early log phase in SDC at 30°C. Cells were then labeled with 5 μM DMSO-solubilized lipid for 45 minutes at 30°C. After being washed twice with room temperature SDC, DAPI was added to label nuclear and mitochondrial DNA. Cells were incubated at 30°C for 15 additional minutes, harvested and washed twice with ice-cold SCN₃Na prior to analysis and imaging by fluorescence microscopy.

ALP pathway bypasses the PVC and is not inhibited in class E mutants (Cowles et al., 1997; Piper et al., 1997). However, the observed block of M-C₆-NBD-PC vacuolar transport in the class E vps mutants does not rule out the possibility that M-C₆-NBD-PC can move from a cytoplasmic topological location to the lumen of organelles at prior steps in the vps pathway. Although the exact mechanism of M-C₆-NBD-PC movement to the lumen of the vacuole has yet to be determined, the requirement for trafficking from the PVC to the vacuole would appear to rule out the direct flip or invagination of M-C₆-NBD-PC into the vacuole. However, a block of vesicular traffic from the PVC to the vacuole might preclude the transport of a vacuolar flippase or proteins required for invagination to their proper location in the vacuole, thereby preventing M-C₆-NBD-PC uptake from the cytosolic compartment into the lumen of the vacuole. If this were true, mutants defective in trafficking from the ER to Golgi (*sec12-4*) or from the Golgi to PVC (*vps8Δ*) should also block traffic of the newly synthesized essential proteins to the vacuole and thus also block M-C₆-NBD-PC vacuolar labeling. Since M-C₆-NBD-PC is trafficked to the vacuole in both the *sec12* and *vps8Δ* mutant strains (Fig. 5A,B), we excluded the possibility that M-C₆-NBD-PC is internalized directly into the vacuole.

The diagram presented in Fig. 7 summarizes the steps involved in trafficking M-C₆-NBD-PC from the plasma membrane to the vacuole and illustrates two alternative models for the internalization of M-C₆-NBD-PC into the lumen of the PVC. In the first model, M-C₆-NBD-PC and methylated M-C₆-NBD-PE gain access to the luminal topological compartment of the vps pathway via a methylated PtdEtn-specific flippase in the PVC. In the second model, invagination of the PVC as described by Emr and colleagues (Rieder et al., 1996; Wurmser and Emr, 1998) is proposed to explain the movement of M-C₆-NBD-PC from a cytoplasmic topology into the lumen of the vacuole. According to this model, the PVC continually forms invaginations that are subsequently pinched off as intra-PVC vesicles (Rieder et al., 1996; Wurmser and Emr, 1998). These small vesicles are then packaged into larger vesicles that are trafficked to the vacuole for degradation (Wurmser and Emr, 1998). Under this paradigm, M-C₆-NBD-PC could insert into the outer leaflet of the PVC and get trapped within a vesicle formed by invagination. As part of this vesicle, M-C₆-NBD-PC would then be trafficked to the lumen of the vacuole and degraded (Fig. 7).

Both models are consistent with the observations that M-C₆-NBD-PC trafficking to the vacuole is blocked in the class E vps mutants, *vps4Δ* and *vps28Δ*, which are blocked in vesicular transport from the PVC to the vacuole, and the *sec18-1* mutant, which is blocked at multiple vesicular trafficking steps. If the PVC invagination model were accurate, one might expect to see M-C₆-NBD-PC labeling of an exaggerated PVC in the class E vps mutants. Although this labeling pattern was observed occasionally (data not shown), most cells exhibited M-C₆-NBD-PC enrichment in the nuclear envelope/ER and mitochondria, as seen in Fig. 5B. The lack of consistent labeling of an exaggerated PVC in the class E mutants remains

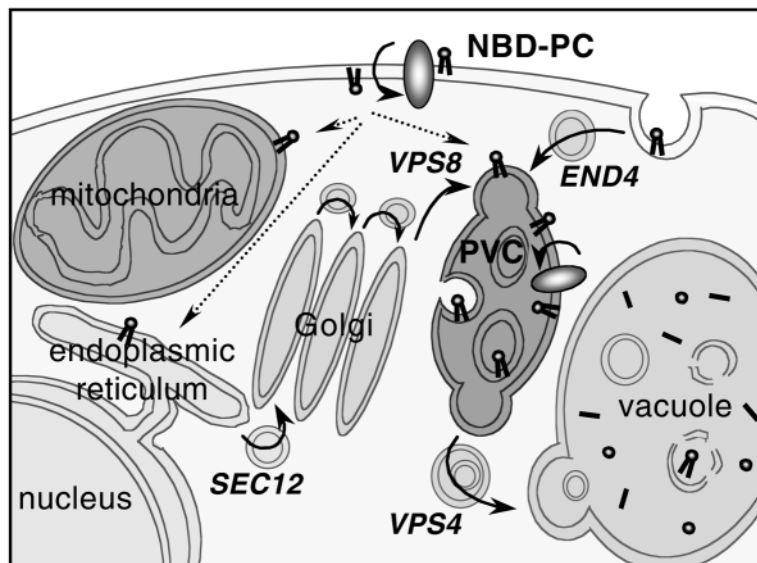


Fig. 7. After internalization by flip, M-C₆-NBD-PC is sorted to the vacuole and must pass through the PVC. Based on analysis of membrane trafficking mutants deficient in the steps shown in the figure, we determined that M-C₆-NBD-PC must pass through the PVC in order to gain access to the lumen of the vacuole. The key result leading to this model is that class E vps mutants, which are defective in vesicular transport from the PVC to the vacuole, do not sort M-C₆-NBD-PC to the vacuole. Two models of how M-C₆-NBD-PC might gain access to the lumen of organelles in the vps pathway are illustrated – namely, internalization into the PVC by either invagination of the PVC or by a PVC-localized flippase (see Discussion for details).

unclear, although it may reflect a requirement for continued vesicular flux through the PVC for the internalization of M-C₆-NBD-PC.

The data presented here do not distinguish the PVC-localized flippase model from the invagination model. However, the data clearly demonstrate the existence of a sorting process that distinguishes PtdCho and its methylated precursors from PtdEtn. This head group-dependent sorting may either be achieved by a specific binding site on the flippase or by a molecular sieve inherent in the budding machinery that allows selective lateral diffusion of methylated PtdEtn products into the emerging bud. Furthermore, both mechanisms may be functionally interdependent in that an energy-dependent invagination process might require selective flippase activity to accommodate the geometric constraints inherent in producing small diameter buds, or conversely, an energy-dependent flippase activity might generate the unequal surface pressure between the two leaflets of the PVC bilayer required to drive the invagination of small buds (Devaux and Zachowski, 1994). In addition to clarifying this mechanistic ambiguity, it remains to be determined whether the primary function of selective vacuolar transport and degradation of PtdCho is metabolic, in that it results in the selective turnover and recycling of choline perhaps for reincorporation into PtdCho via the CDP-choline pathway, or whether its transport and degradation are byproducts of its primary function as an essential step in the membrane invagination and budding process.

The authors thank Robert Fuller, Peter Novick, Scott Emr and Howard Riezman for the generous gift of mutant yeast strains, and

Lynn Malone for expert technical assistance. This research was supported in part by a National Institutes of Health grant GM52410 and a grant from the University Research Committee of Emory University (to J.W.N.), a National Institutes of Health Minority Predoctoral Fellowship (to A.M.G) and a National Institutes of Health predoctoral training grant (to P.K.H.).

References

- Aletta, J. M., Cimato, T. R. and Ettinger, M. J. (1998). Protein methylation: a signal event in post-translational modification. *Trends Biochem. Sci.* **23**, 89-91.
- Ames, B. N. and Dubin, D. T. (1960). The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *J. Biol. Chem.* **235**, 769-775.
- Bishop, W. R. and Bell, R. M. (1985). Assembly of the endoplasmic reticulum phospholipid bilayer: the phosphatidylcholine transporter. *Cell* **42**, 51-60.
- Brown, D. A. and London, E. (1998). Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14**, 111-136.
- Burd, C. G., Peterson, M., Cowles, C. R. and Emr, S. D. (1997). A novel Sec18p/NSF-dependent complex required for Golgi-to-endosome transport in yeast. *Mol. Biol. Cell* **8**, 1089-1104.
- Chen, C. Y., Ingram, M. F., Rosal, P. H. and Graham, T. R. (1999). Role for Drs2p, a P-type ATPase and potential aminophospholipid translocase, in yeast late Golgi function. *J. Cell Biol.* **147**, 1223-1236.
- Comfurius, P. and Zwaal, R. F. A. (1977). The enzymatic synthesis of phosphatidylserine and purification by cm-cellulose column chromatography. *Biochim. Biophys. Acta* **488**, 36-42.
- Cowles, C. R., Snyder, W. B., Burd, C. G. and Emr, S. D. (1997). Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. *EMBO J.* **16**, 2769-2782.
- Daleke, D. L. and Lyles, J. V. (2000). Identification and purification of aminophospholipid flippases. *Biochim. Biophys. Acta* **1486**, 108-127.
- Devaux, P. F. and Zachowski, A. (1994). Maintenance and consequences of membrane phospholipid asymmetry. *Chem. Phys. Lipids* **73**, 107-120.
- Finken-Eigen, M., Rohrer, R. A. and Kohrer, K. (1997). The *VPS4* gene is involved in protein transport out of a yeast pre-vacuolar endosome-like compartment. *Curr. Genet.* **31**, 469-480.
- Graham, T. R. and Emr, S. D. (1991). Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (NSF) mutant. *J. Cell Biol.* **114**, 207-218.
- Grant, A. M., Hanson, P. K., Malone, L. and Nichols, J. W. (2001). NBD-labeled phosphatidylcholine and phosphatidylethanolamine are internalized by transbilayer transport across the yeast plasma membrane. *Traffic* **2**, 37-50.
- Greenberg, M. L., Klig, L. S., Letts, V. A., Loewy, B. S. and Henry, S. A. (1983). Yeast mutant defective in phosphatidylcholine synthesis. *J. Bacteriol.* **153**, 791-799.
- Hanson, P. K. and Nichols, J. W. (2001). Energy-dependent flip of fluorescence-labeled phospholipids is regulated by nutrient starvation and transcription factors, *PDR1* and *PDR3*. *J. Biol. Chem.* **276**, 9861-9867.
- Hicke, L., Zanolari, B., Pypaert, M., Rohrer, J. and Riezman, H. (1997). Transport through the yeast endocytic pathway occurs through morphologically distinct compartments and requires an active secretory pathway and Sec18p/N-ethylmaleimide-sensitive fusion protein. *Mol. Biol. Cell* **8**, 13-31.
- Horazdovsky, B. F., Cowles, C. R., Mustol, P., Holmes, M. and Emr, S. D. (1996). A novel RING finger protein, Vps8p, functionally interacts with the small GTPase, Vps21p, to facilitate soluble vacuolar protein localization. *J. Biol. Chem.* **271**, 33607-33615.
- Kanipes, M. I. and Henry, S. A. (1997). The phospholipid methyltransferases in yeast. *Biochim. Biophys. Acta* **1348**, 134-141.
- Kean, L. S., Fuller, R. S. and Nichols, J. W. (1993). Retrograde lipid traffic in yeast: Identification of two distinct pathways for internalization of fluorescently-labeled phosphatidylcholine from the plasma membrane. *J. Cell Biol.* **123**, 1403-1419.
- Kean, L. S., Grant, A. M., Angeletti, C., Mahe, Y., Kuchler, K., Fuller, R. S. and Nichols, J. W. (1997). Plasma membrane translocation of fluorescently-labeled phosphatidylethanolamine is controlled by transcription regulators, *PDR1* and *PDR3*. *J. Cell Biol.* **138**, 255-270.
- Kodaki, T. and Yamashita, S. (1987). Yeast phosphatidylethanolamine methylation pathway. Cloning and characterization of two distinct methyltransferase genes. *J. Biol. Chem.* **262**, 15428-15435.
- Lipsky, N. G. and Pagano, R. E. (1985a). Intracellular translocation of fluorescent sphingolipids in cultured fibroblasts: endogenously synthesized sphingomyelin and glucocerebroside analogues pass through the Golgi apparatus en route to the plasma membrane. *J. Cell Biol.* **100**, 27-34.
- Lipsky, N. G. and Pagano, R. E. (1985b). A vital stain for the Golgi apparatus. *Science* **228**, 745-747.
- Monti, J. A., Christian, S. T., Shaw, W. A. and Finley, W. H. (1977). Synthesis and properties of a fluorescent derivative of phosphatidylcholine. *Life Sci.* **21**, 345-356.
- Nichols, J. W. (1987). Binding of fluorescently-labeled phosphatidylcholine to rat liver nonspecific lipid transfer protein. *J. Biol. Chem.* **262**, 14172-14177.
- Nicolson, T. and Mayinger, P. (2000). Reconstitution of yeast microsomal lipid flip-flop using endogenous aminophospholipids. *FEBS Lett.* **476**, 277-281.
- Nishikawa, S. and Nakano, A. (1993). Identification of a gene required for membrane protein retention in the early secretory pathway. *Proc. Natl. Acad. Sci. USA* **90**, 8179-8183.
- Novick, P., Field, C. and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21**, 205-215.
- Orci, L., Montesano, R., Meda, P., Malaisse-Lagae, F., Brown, D., Perrelet, A. and Vassalli, P. (1981). Heterogeneous distribution of filipin-cholesterol complexes across the cisternae of the Golgi apparatus. *Proc. Natl. Acad. Sci. USA* **78**, 293-297.
- Piper, R. C., Bryant, N. J. and Stevens, T. H. (1997). The membrane protein alkaline phosphatase is delivered to the vacuole by a route that is distinct from the VPS-dependent pathway. *J. Cell Biol.* **138**, 531-545.
- Raths, S., Rohrer, J., Crausaz, F. and Riezman, H. (1993). *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. *J. Cell Biol.* **120**, 55-65.
- Raymond, C. K., Howald-Stevenson, I., Vater, C. A. and Stevens, T. H. (1992). Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. *Mol. Biol. Cell* **3**, 1389-1402.
- Rieder, S. E., Banta, L. M., Kohrer, K., McCaffery, J. M. and Emr, S. D. (1996). Multilamellar endosome-like compartment accumulates in the yeast *vps28* vacuolar protein sorting mutant. *Mol. Biol. Cell* **7**, 985-999.
- Sherman, F., Fink, G. R. and Hicks, J. B. (1986). *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Summers, E. F., Letts, V. A., McGraw, P. and Henry, S. A. (1988). *Saccharomyces cerevisiae cho2* mutants are deficient in phospholipid methylation and cross-pathway regulation of inositol synthesis. *Genetics* **120**, 909-922.
- Vida, T. A. and Emr, S. D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* **128**, 779-792.
- Walkey, C. J., Yu, L., Agellon, L. B. and Vance, D. E. (1998). Biochemical and evolutionary significance of phospholipid methylation. *J. Biol. Chem.* **273**, 27043-27046.
- Wesp, A., Hicke, L., Palecek, J., Lombardi, R., Aust, T., Munn, A. L. and Riezman, H. (1997). End4p/Sla2p interacts with actin-associated proteins for endocytosis in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **8**, 2291-2306.
- Wurmser, A. E. and Emr, S. D. (1998). Phosphoinositide signaling and turnover: PtdIns(3)P, a regulator of membrane traffic, is transported to the vacuole and degraded by a process that requires luminal vacuolar hydrolase activities. *EMBO J.* **17**, 4930-4942.
- Zambrano, F., Fleischer, S. and Fleischer, B. (1975). Lipid composition of the Golgi apparatus of rat kidney and liver in comparison with other subcellular organelles. *Biochim. Biophys. Acta* **380**, 357-369.
- Zwaal, R. F. and Schroit, A. J. (1997). Pathophysiological implications of membrane phospholipid asymmetry in blood cells. *Blood* **89**, 1121-1132.