Retrograde Lipid Traffic in Yeast: Identification of Two Distinct Pathways for Internalization of Fluorescent-labeled Phosphatidylcholine from the Plasma Membrane

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Abstract. Digital, video-enhanced fluorescence microscopy and spectrofluorometry were used to follow the internalization into the yeast Saccharomyces cerevisiae of phosphatidylcholine molecules labeled on one acyl chain with the fluorescent probe 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD). Two pathways were found: (1) transport by endocytosis to the vacuole and (2) transport by a non-endocytic pathway to the nuclear envelope and mitochondria.

The endocytic pathway was inhibited at low temperature (<2°C) and by ATP depletion. Mutations in secretory (SEC) genes that are necessary for membrane traffic through the secretory pathway (including SEC1, SEC2, SEC4, SEC6, SEC7, SEC12, SEC14, SEC17, SEC18, and SEC21) almost completely blocked endocytic uptake. In contrast, mutations in the SEC63, SEC65, or SEC11 genes, required for translocation of nascent secretory polypeptides into the ER or signal peptide processing in the ER, only slightly reduced endocytic uptake. Phospholipid endocytosis was also independent of the gene encoding the clathrin heavy chain, CHC1. The correlation of biochemical analysis with fluorescence microscopy indicated that the fluorescent phosphatidylcholine was degraded in the vacuole and that degradation was, at least in part, dependent on the vacuolar proteolytic cascade.

The non-endocytic route functioned with a lower cellular energy charge (ATP levels 80% reduced) and was largely independent of the SEC genes. Non-endocytic transport of NBD-phosphatidylcholine to the nuclear envelope and mitochondria was inhibited by pretreatment of cells with the sulfhydryl reagents N-ethylmaleimide and p-chloromercuribenzenesulfonic acid, suggesting the existence of protein-mediated transmembrane transfer (flip-flop) of phosphatidylcholine across the yeast plasma membrane.

These data establish a link between lipid movement during secretion and endocytosis in yeast and suggest that phospholipids may also gain access to intracellular organelles through non-endocytic, protein-mediated events.

Although membranes of eukaryotic cells are composed of numerous classes of lipids, no membrane system can synthesize all of its lipid components (Pagano, 1990a). Thus, the transport and sorting of lipid molecules are important processes in the cell, and can affect both the homeostasis of organelles, and the regulation of other cellular processes such as endocytosis and secretion. Transport and sorting of lipids occurs via many intracellular pathways (for reviews see Pagano, 1990a,b; Pagano and Sleight, 1985; Bishop and Bell, 1988; van Meer, 1990). Anterograde transport pathways direct newly synthesized lipids to proper cellular destinations. These pathways can be either vesicular or non-vesicular, following the same pathways as protein secretion in some instances and independent pathways in others (Novick and Schekman, 1979; Sleight and Pagano, 1983; Urbani and Simoni, 1990). Transport of lipids also occurs through recycling pathways. One membrane recycling pathway that may carry out unique cellular functions is the transport of lipids from the plasma membrane to the intracellular organelles. Retrograde lipid transport can occur either by endocytosis, which allows both membrane-bound and soluble molecules to reach intracellular organelles (Koval and Pagano, 1990; Sleight and Pagano, 1984; Sleight and Abanto, 1989), or by protein-mediated transmembrane transport (flip-flop) followed by diffusion, perhaps facilitated by lipid transfer proteins, to intracellular organelles (van Meer and Op den Kamp, 1982; Sleight and Pagano, 1985; Martin and Pagano, 1987). Transmembrane flip-flop of lipids is important for the creation and maintenance of an asymmetric lipid composition across the bilayer. Such asymmetry has been shown to be an important indicator of the state of activation of platelets, in that the transbilayer distribution of the amino-phospholipids changes dramatically when platelets undergo...
their characteristic morphological and functional changes during activation (for review see Schroit and Zwaal, 1991). The proteins responsible for both types of retrograde lipid flow promise to be important players in many cellular sorting pathways, but, by and large, remain to be identified.

In mammalian cells, retrograde lipid transport pathways have been studied using fluorescent phospholipid analogs (Pagano and Sleight, 1985). Diacylated lipids with a fluorescent, 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled aminocaproic acid in place of one fatty-acyl chain can be transferred to the plasma membrane at low temperature and thus serve as powerful tools for dissecting both the endocytic and non-endocytic phospholipid internalization pathways (Pagano, 1990a; Struck and Pagano, 1980). Internalization of fluorescent phospholipid depends both on their polar head-group and on cell-type. NBD-labeled phosphatidylycholine is internalized exclusively through endocytosis in untransformed mammalian cell lines, and is targeted either to the Golgi apparatus, or to endosomes (Sleight and Pagano, 1984; Sleight and Abanto, 1989). In VA-2 cells, which are SV40-transformed human lung fibroblasts, NBD-labeled phosphatidylycholine is internalized both by endocytosis to the Golgi apparatus, and by flip-flop from the outer to inner leaflets of the plasma membrane before transferring to the nuclear envelope and mitochondria (Sleight and Abanto, 1989). In Chinese hamster V79 fibroblasts, NBD-labeled phosphatidylethanolamine is internalized both by endocytic and protein-mediated, non-endocytic mechanisms (Sleight and Pagano, 1985; Martin and Pagano, 1987). Endocytosis delivers this molecule to the Golgi apparatus. The non-endocytic pathway targets the lipid to both the nuclear envelope and the mitochondria. In contrast, NBD-labeled phosphatidylserine is internalized exclusively through protein-mediated flip-flop, and is transported to the nuclear envelope and mitochondria of V79 fibroblasts (Martin and Pagano, 1987). Thus, pathways taken by phospholipids in mammalian cells appear to be both head-group specific and cell-type specific. One general rule appears to be followed, namely, endocytic uptake delivers lipids to the Golgi and endosomal/lysosomal system, whereas flip-flop-mediated uptake principally targets lipids to the nuclear envelope and mitochondria.

The importance of lipid trafficking events for cell viability has been highlighted recently by studies of Saccharomyces cerevisiae (yeast). Riezman (1985) has shown that yeast internalize the aqueous marker, Lucifer yellow, by fluid phase endocytosis and transport it specifically to the vacuole (the yeast lysosome). Endocytosis of this "content-marker" depends on several of the essential SEC genes that secretory vesicles from the Golgi (Novick et al., 1981; Stevens et al., 1982) encodes a phosphatidylcholine-phosphatidylinositol transfer protein (Bankaitis et al., 1990), suggesting that the maintenance of the correct organellar lipid composition may be essential for viability. SEC14 mutations also inhibit the delivery of Lucifer yellow to the vacuole by ~50% (Riezman, 1985), suggesting that regulation of lipid composition and trafficking may be crucial for both endo- and exocytosis. The fact that many mutants originally isolated for their secretory defects are also deficient in fluid phase endocytosis suggests either that such proteins function in both pathways, or that secretory and endocytic events are obligatorily coupled.

Recently, mutations in two genes, END3 and END4, have been isolated for their defect in receptor-mediated endocytosis of the mating pheromone α-factor into MA1a haploids. Although mutations in these genes also inhibit endocytosis of Lucifer yellow, they have no effect on secretion (Raths et al., 1993). Thus the pathways of receptor-mediated endocytosis and secretion do exhibit some independence.

In this study, we have used fluorescent-labeled phosphatidylycholine (M-C6-NBD-PC) to follow retrograde lipid transport in S. cerevisiae. We present evidence for two distinct lipid traffic pathways. One has the characteristics of a classic endocytic pathway, results in phospholipid transport to the vacuole, and requires the activities of at least 10 SEC genes. The other pathway is largely SEC gene-independent, and transports phospholipids to the nuclear envelope and mitochondria, characteristic of a transmembrane transport (flip-flop) process.

**Materials and Methods**

**Materials**

Yeast media was from Difco Labs. (Detroit, MI). Unless otherwise noted, all other materials were purchased from Sigma Chem. Co. (St. Louis, MO).

**Yeast Strains and Culture**

The S. cerevisiae strains used are shown in Table I. For all experiments, early to mid log-phase cultures (OD600 = 0.2-1) were grown from either overnight cultures or fresh plates in synthetic complete media (SDC) as described (Sherman et al., 1986). Wild-type strains were typically grown at 30°C. Temperature-sensitive (Ts-) strains were grown at a permissive temperature of 22-24°C.

**Vesicle Preparation**

1-myristoyl-2-[6-(NBD)amino]caproyl]phosphatidylcholine (M-C6-NBD-PC), dioleoylphosphatidylcholine (DOPC), and dioleoylphosphatidylethanolamine were from Avanti Polar Lipids Inc. (Alabaster, AB). N-Rhodamine-dioleoylphosphatidylethanolamine (N-Rh-DP) was synthesized and purified as described (Struck et al., 1981). Phospholipids were stored at -20°C, periodically monitored for purity by thin-layer chromatography, and repurified when necessary. Phospholipid concentrations were determined by the lipid phosphorous assay (Amer and Dubin, 1960). To prepare vesicles, lipids were first mixed in desired proportions and the chloroform was transferred to the plasma membrane at low temperature and periodically monitored for purity by thin-layer chromatography, and repurified when necessary. Phospholipid concentrations were determined by the lipid phosphorous assay (Amer and Dubin, 1960). To prepare vesicles, lipids were first mixed in desired proportions and the chloroform was removed by evaporation under nitrogen followed by overnight vacuum desiccation. Desiccated lipids were then solubilized in SDC medium lacking glucose and the mixture passed seven times through a Lipex Extruder (Lipex Biomembranes Inc., Vancouver, BC, Canada) equipped with 0.1-μm filters to produce vesicles. Total lipid concentration in the stock vesicle preparation was 1 mM. Typically, proportions were 40 mol% M-C6-NBD-PC, 2 mol% N-Rh-DP, 58 mol% DOPC.
Table 1. Yeast Strains Used in This Study

| Strain | Genotype | Source                |
|--------|-----------|-----------------------|
| CRY3*  | (MATa Sec+ can-1-100 ade2-1 his2-1-15 leu2-3-112 trp1-1 ura3-1) | R. S. Fuller lab collection |
|        | (MATa Sec+ can-1-100 ade2-1 his2-1-15 leu2-3-112 trp1-1 ura3-1) | R. S. Fuller lab collection |
| CRY1*  | (MATa Sec+ can-1-100 ade2-1 his2-11,15 leu2-3-112 trp1-1 ura3-1) | R. S. Fuller lab collection |
| CBO18* | (MATa Sec+ can-1-100 ade2-1 leu2-3-112 trp1-1 ura3-1 pep4Δ::His3 prb1a::His G prc1A::hisG) | R. S. Fuller lab collection |
| KRY30-2C* | (MATa sec1-1 can-1-100 ade2-1 his2-11,15 leu2-3-112 trp1-1) | R. S. Fuller lab collection |
| KRY31-3C* | (MATa sec1-1 sec1-1 can-1-100 ade2-1 his2-11,15 leu2-3-112 trp1-1) | R. S. Fuller lab collection |
| CWS11-2* | (MATa sec11 ade2-1 his2-11,15 leu2-3-112 trp1-1 ura3-1) | R. S. Fuller lab collection |
| CRY2*  | (MATa Sec+ can-1-100 ade2-1 his2-11,15 leu2-3-112 trp1-1 ura3-1) | R. S. Fuller lab collection |
| CRY33-2C* | (MATa sec14 can-1-100 ade2-1 his2-11,15 leu2-3-112 trp1-1 ura3-1) | R. S. Fuller lab collection |
| CWS18-1* | (MATa sec18 can-1-100 ade2-11,15 leu2-3-112 trp1-1 ura3-1) | R. S. Fuller lab collection |
| X2180-1A | (MATa Sec+ SUC2 mal2 gal2 CUP1) | Yeast Genetic Stock Center |
| HMF5106 | (MATa sec2-56 SUC2 mal2 gal2 CUP1) | Yeast Genetic Stock Center |
| HMF13 | (MATa sec4-2 SUC2 mal2 gal2 CUP1) | Yeast Genetic Stock Center |
| SF266-1C | (MATa sec12-4 SUC2 mal2 gal2 CUP1) | Yeast Genetic Stock Center |
| HMF175 | (MATa sec17-1 SUC2 mal2 gal2 CUP1) | Yeast Genetic Stock Center |
| HMF180 | (MATa sec21-1 SUC2 mal2 gal2) | Yeast Genetic Stock Center |
| X2180-1B | (MATa Sec+ SUC2 mal2 gal2 CUP1) | Yeast Genetic Stock Center |
| RSY153 | (MATa sec63-1 leu2-3-112 ura3-52) | R. Schekman, UC Berkeley |
| RSY457 | (MATa sec65 ade2 his3 leu2-3-112 trp1-1 ura3-52) | R. Schekman, UC Berkeley |
| NY13 | (MATa; Sec+ ura3-52) | P. Novick, Yale U. |
| NY17 | (MATa; sec6-4 ura3-52) | P. Novick, Yale U. |
| GPY55 | (MATa CHC1 leu2-3-112 ura3-52 his4 trp1 prb1) | G. Payne, UCLA |
| GPY184 | (MATa chcl-52 leu2-3-112 ura3-52 his4 trp1 prb1) | G. Payne, UCLA |

* Strains were derived from a nominally isogenic pair of strains, W303-1A (MATa can-1-100 ade2-1 his2-11,15 leu2-3-112 trp1-1 ura3-1) and W303-1B (W303-1A MATa) originally obtained from R. Rothstein, Columbia University.

Internalization of Phospholipids into Yeast Cells

For internalization assays using non-Ts− strains, logarithmically growing cells were harvested by centrifugation and washed twice with either SDC or SCNaN3 (SDC medium lacking glucose but containing 2% sorbitol and 20 mM sodium azide). In the azide-containing cultures, cells were incubated with shaking at 30°C to deplete cellular ATP stores. For fluorescence microscopy, 1.5 ml of cells was mixed with vesicles (50 μM total lipid concentration) and incubated at 30°C or 2°C for 1.5-2 h with constant (30°) or intermittent (2°) shaking. Cells were then rapidly moved to an ice-water bath, washed rapidly four times with two volumes ice-cold SCNaN3 and viewed under the microscope. For experiments with Ts− strains, both growth to log-phase and ATP depletion were carried out at 37°C. Warming to 37°C was accomplished by shifting the temperature of the water bath up to 37°C, resulting in a complete change of temperature in ~10 min. After shifting the temperature for 30 min, vesicles were added and incubated at 37°C for continued 1 h. Cells that were kept at the permissive temperature (24°C) were incubated with vesicles for 2 h. Cells were then plunged into an ice-water bath and washed rapidly with ice-cold SCNaN3 in preparation for microscopy. In experiments involving Ts− mutants, control strains were handled identically to the mutants. For fluorometry, 1.9 ml of cells was placed in a fluorescence cuvette (pre-equilibrated to the proper temperature) and stirred continuously with a magnetic stirrer. Vesicles were then added (50 μM total lipid) and the change in fluorescence was monitored continuously.

Measurement of ATP Levels

ATP was measured using the Luciferase assay on cells incubated in either glucose-rich medium (SDC) or ATP depletion medium (SCNaN3). After ATP depletion, cells were centrifuged for 25 min at 1,500 g to yield a well-packed cell-paste, 50 μl of which was diluted into 0.9 ml of 7% PCA and incubated on ice for 15 min. Extracts were centrifuged for 5 min at 4°C in a microfuge at maximum speed. 100 μl of the supernatant was added to 0.5 ml luciferase assay buffer (5 mM sodium arsenate, 4 mM magnesium sulfate, 20 mM glycylglycine, pH 8.0) and the pH was adjusted to 8-9 by adding 1.47 N KOH in 25 mM Tris, pH 8.0 (~90 μl). 50 μl of pH-adjusted extract was added to 0.9 ml of luciferase assay buffer, followed by the addition of 50 μl of luciferase/luciferin reagent (supplied at 20 mg/ml). Luminescence was measured using a Packard TriCarb Liquid Scintillation Spectrometer.

Catabolism of M-γ-NBD-PC in Yeast

To study the degradation of M-γ-NBD-PC, internalization assays were performed as described above. Equal numbers of cells were harvested and frozen at ~80°C for at least 1 h. Extraction of cellular lipids was performed as described (Hjelmstad and Bell, 1987). After extraction, the aqueous and organic phases were evaporated to dryness and then taken up in chloroform/methanol, 1:1 (aqueous phase). A portion of this was separated by silica gel TLC (Merck Inc., Germany) in chloroform/methanol ace tone/acetic acid/water, 5:1:1:0.5 (Struck and Pagano, 1980), and viewed on an ultraviolet transilluminator.

Relative Internalization of M-γ-NBD-PC and N-Rh-DOPe

Lipids were extracted from yeast cells as described above, the organic phase was evaporated, and the lipids were reconstituted in 2 ml chloroform/methanol, 1:1. NBD and rhodamine fluorescence was measured using an SLM 8000C spectrophotometer as described below. To quantify internalization of NBD-labeled lipids, the NBD signal was compared to a standard curve of M-γ-NBD-PC in chloroform/methanol, 1:1.

N-ethylmaleimide and para-chloromercuribenzenesulfonic Acid Sensitivity Experiments

Mid-log phase cells were prepared as described above, cooled to 2°C, har-
vested by centrifugation and resuspended in buffered media (SDC containing 50 mM sodium phosphate buffer, pH 7.5) containing either 2 mM N-ethylmaleimide (NEM), 10 mM para-chloromercuribenzenesulfonyl acid (pCMBS), or 50 mM pCMBS. Cells were incubated on ice for 30 min and were shaken after the first 15 min. Cells recovered by centrifugation were resuspended in buffered medium (for pCMBS experiments), or buffered medium containing 2 mM dithiothreitol (for NEM experiments), and incubated on ice for 5 min. Cells were pelleted again, washed once with buffered medium, and resuspended in buffered media with 50 μM M-Ca-NBD-PC-containing vesicles. Cells were incubated on ice for 1.5 h, and washed three times with ice-cold SCNaN4N buffered with 50 mM sodium phosphate (pH 7.5) before fluorescence microscopy.

**Fluorescence Measurements**

Fluorescence was recorded using a MPF-44F fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) or from an SLM 8000C spectrophuorometer (SLM Instruments Inc., Urbana, IL). Temperature was controlled with a circulating water bath. Analogue output from the Perkin-Elmer fluorometer was digitized and stored on an IBM-XT. Digital output from the SLM 8000C was stored on an IBM-AT-compatible computer. NBD fluorescence was monitored by exciting the fluorophore at 590 nm. DAPI-labeled nuclei were observed with an SIT camera.

**Fluorescence Microscopy**

Fluorescence microscopy was performed on a Zeiss Axiovert microscope equipped with barrier filters that allowed no crossover of NBD and rhodamine fluorescence. For localization of 4',6-diamidino-2-phenylindole (DAPI) fluorescence, barrier filters were used that did not allow crossover of NBD and DAPI fluorescence. The fluorescence image was enhanced with a SIT 66 image-intensifying camera (DAGE-MTI Inc., Michigan City, IN), digitized, and stored on magnetic disks using an IBM-AT-compatible computer. Contrast enhancement and pixel brightness analysis of the digital images was performed with Image-IT software. Average pixel brightness was measured from a rectangle (1 x 3 pixels) in the middle of vacuoles or a rectangle (10 x 12 pixels) surrounding the nuclear envelope. Pixel brightness analysis of DOPC vesicles containing known amounts of M-Ca-NBD-PC confirmed that these measurements fell within the linear range of the SIT camera.

**Statistical Analysis**

Statistical analysis was performed with the InStat statistics software from GraphPad Inc. (San Diego, CA). χ² analysis and two-tailed P values were obtained for the vesicular-transport sec mutants after creating a 3 x 2 contingency table to compare the vacuolar pixel brightness values of the sec mutants and their isogenic Sec+ alleles. All pixel brightness measurements from the nonpermissive temperature (37°C) were corrected for strain-to-strain variations in vacuolar pixel brightness at the permissive temperature (24°C). The contingency tables were split into three pixel brightness bins, in which vacuolar pixel brightness scores were divided. The bins were for vacuolar pixel brightness values of 0, >0-20, and >20. These bins were chosen after examination of the pixel brightness ranges of both the vesicular-transport sec mutants and their isogenic Sec+ alleles. Many vesicular-transport sec mutants had vacuolar brightness values of 0 (their vacuoles were not fluorescent). The remaining cells had vacuoles with wide-ranging vacuolar pixel brightness, but the vast majority did not exceed a pixel brightness value of 20. The Sec+ strains had widely ranging pixel brightness values, with an average vacuolar pixel brightness of ~100. Because the ER sec mutants did not have any cells with vacuolar fluorescence of 0 (see Fig. 7), it was impossible to use the 3 x 2 contingency tables to perform the χ² test and Fisher's exact test was employed instead. For those experiments which yielded normal (Gaussian) pixel brightness distributions, the Student's t test was used to compare the means and calculate a two-tailed P value.

**Results**

**Uptake of M-Ca-NBD-PC into Multiple Intracellular Organelles**

When the diploid wild-type (wt) strain, CRY3, was incubated at 30°C in the presence of vesicles (50 μM total lipid concentration) containing 40 mol% M-Ca-NBD-PC, 2 mol% N-Rh-DOPE and 58 mol% DOPC, only the exchangeable fluorescent lipid, M-Ca-NBD-PC (Nichols, 1985), was internalized. M-Ca-NBD-PC was transported to the vacuole, identified by colocalization of NBD fluorescence with either the fluorescent vacuoles of ade2 yeast (L. S. Weisman et al., 1987) (data not shown) or with vacuoles visualized by differential interference contrast (DIC) optics (Fig. 1, A, B, and D). A smaller amount of M-Ca-NBD-PC was incorporated into the nuclear envelope and the mitochondria, as identified by colocalization with vital DAPI-staining of DNA in these organelles (Fig. 1, A and C). Although the vacular fluorescence shown in Fig. 1A obscures the mitochondrial fluorescence, in other experiments (data not shown), colocalization of NBD fluorescence with DAPI-labeled mitochondria was clear (see Fig. 3). The NBD-fluorescence of nuclei and mitochondria photobleached much more rapidly than did the vacuolar fluorescence. Fluorescent nuclei and mitochondria could be photographed after ~2 s of exposure to the excitation light (Fig. 1 A) but had faded almost entirely by 20 s of exposure, leaving the vacuole as the sole fluorescent organelle (Fig. 1 B). In fact, even when photographed after ~2 s, significant photobleaching of the nuclear envelope and mitochondria had occurred. Thus, although the fluorescence patterns shown in Fig. 1 A demonstrated that M-Ca-NBD-PC was transported both to the vacuole and to the nuclear envelope, the vacuolar destination is emphasized due to the fact that the vacuolar fluorescence photobleached more slowly than the fluorescence in the nuclear envelope and mitochondria.

**Internalization Occurs after Incorporation of M-Ca-NBD-PC Monomers into the Plasma Membrane**

Three possible mechanisms could account for the ability of cells to internalize M-Ca-NBD-PC. (1) The lipid could be internalized as part of an intact donor vesicle during endocytosis, or through fusion of the donor vesicles with the plasma membrane. (2) The lipids could be internalized as soluble monomers in the aqueous phase after dissociation from donor vesicles, and thus serve only as a content marker for endocytic events. (3) The lipids could be internalized as monomers after insertion into the plasma membrane of the cell, and thus serve as a marker for lipid traffic via both endocytic and non-endocytic pathways.

The first possibility was ruled out by the differential partitioning experiment shown in Table II. Previous studies have shown that while M-Ca-NBD-PC monomers can rapidly partition from donor vesicles (t₅₀ for dissociation ~1 s), N-Rh-DOPE dissociates from vesicles very slowly (t₅₀ for dissociation ~ days) (Nichols, 1985; Nichols and Pagano, 1983). Thus, internalization of M-Ca-NBD-PC monomers after incorporation into the plasma membrane can be distinguished from internalization of intact donor vesicles or fusion of donor vesicles with the plasma membrane by the extent to which M-Ca-NBD-PC is enriched in cells relative to N-Rh-DOPE. The extent of enrichment was determined from the ratio of NBD to rhodamine (Rh) fluorescence measured in a total lipid extract from cells vs the ratio measured in the extracted donor vesicles. As can be seen in Table II, the ratio of NBD to Rh fluorescence increased from 7.8 (in
vesicle extracts) to 330 (in cellular extracts) when CRY3 cells were incubated with vesicles under conditions that allowed internalization. Thus, at least ~98% of the fluorescence seen in CRY3 was due to the uptake of M-C6-NBD-PC monomers.

As will be discussed in detail below, when total cellular lipids were extracted from CRY3 cells and separated by TLC, significant degradation of M-C6-NBD-PC was observed only under conditions (incubation at 30°C in SDC medium) that allowed transport to the vacuole (Fig. 5). Because this degradation resulted in the production of molecules that did not partition into the organic phase of a lipid extraction (Table III), the NBD fluorescence signal used to determine the fold-enrichment of NBD fluorescence relative to Rh fluorescence after phospholipid internalization (which was measured from the organic phase of a lipid extraction of CRY3 cells) might be artifactually low. The experiment was repeated, therefore, with the strain CB018 (Pep-), which is isogenic to CRY3 (Pep+) except that it is a MATa haploid and lacks the three major vacuolar peptidases, proteases A and B and carboxypeptidase Y (CPY). The vacuoles of Pep- strains are largely devoid of hydrolytic activities, presumably including lipases (Jones, 1991; Takeshige et al., 1992). In this experiment, the ratio of NBD to Rh fluorescence was 3.3 for vesicles and 1900 for CB018 cells. Therefore, in CB018, at least 99.8% of M-C6-NBD-PC uptake into the cells was independent of the uptake of intact donor vesicles.

The second possibility, that the fluorescence observed in the microscope was due to internalization of soluble M-C6-NBD-PC monomers as part of the aqueous content of endocytic vesicles seems highly unlikely, based on the following experiment. Internalization assays were performed on CRY3 and CB018 under conditions allowing maximum lipid internalization. Total cellular lipids were then extracted, and the NBD fluorescence was compared to a standard curve for NBD fluorescence vs moles of M-C6-NBD-PC. The total amount of NBD-labeled lipid internalized per OD unit for CRY3 and CB018 was 0.37 nmol and 0.65 nmol, respectively.
Table III. Degradation of M-C6-NBD-PC Is Partially Inhibited in a Pep4+ Prb1- Prc1- Strain

| Strain     | Relevant genotype                | Vacular fluorescence (Avg. pixel brightness of vacuoles in digital images) (arbitrary units) | Relative fluorescence of lipids separated by TLC* |
|------------|----------------------------------|------------------------------------------------------------------------------------------|--------------------------------------------------|
| CRY3       | pep4Δ::HIS3, prb1Δ::hisG, prc1Δ::hisG | 47                                                                                       | M-C6-NBD-PC | 1                                                                 |
| CB018      |                                  | 96                                                                                       | major aqueous catabolite | 1.5                                                              |

Internalization assays were performed on Pep+ (CRY3) or pep- (CB018) cells. The amount of vacuolar fluorescence was measured by quantifying the pixel brightness of the vacuoles seen in the digital images using the Image1/AT software system. Also, lipids were extracted from the cells, and separated by thin-layer chromatography (TLC). The spots corresponding to the starting material (M-C6-NBD-PC) and the major aqueous catabolite were scraped, extracted from the silica support, and quantified spectrophotometrically.

* The fluorescence intensities shown for the TLC-separated products are relative to the fluorescence intensity measured for M-C6-NBD-PC from CRY3.

As described previously (Nichols, 1985), if we assume that all of the fluorescence that was measured arose from monomers of M-C6-NBD-PC internalized in the aqueous phase, the equivalent number of internalized cell volumes required to account for the amount of uptake can be calculated from the following equation:

$$N_{vi} = (n/L_{wm})/V_c$$

where $N_{vi}$ = number of cellular volumes internalized, $n$ = nmol of M-C6-NBD-PC per OD unit, $L_{wm}$ = concentration of fluorescent lipid in the aqueous phase, and $V_c$ = the volume of packed cells per OD unit. The concentration of the fluorescent phospholipids in the aqueous phase in equilibrium with vesicles can be calculated by dividing the mole fraction of M-C6-NBD-PC in the donor vesicles (0.4) by the equilibrium constant for its association with these vesicles (9.8 × 10^-4 M^-1) (Nichols, 1985). Thus, the concentration of M-C6-NBD-PC in the aqueous phase at equilibrium ($L_{wm}$) for a typical experiment is 4 × 10^-4 M. Given that the volume of packed cells per OD unit was 3.9 μl (CRY3) and 3.4 μl (CB018), we can calculate that the two strains would have had to internalize a minimum of 2400 (CRY3) and 4800 (CB018) times their cellular volumes in the two hour recording time (20 cell vol per min and 40 cell vol per min, respectively) if the intracellular fluorescence signal arose from the internalization of lipid monomers in the aqueous phase. It seems highly improbable that yeast turn-over their cellular volume at such a high rate, and thus highly unlikely that the fluorescence signal is due to internalization of lipid monomers in the aqueous phase. Thus, having eliminated the possibility that cells internalize M-C6-NBD-PC (1) as part of an intact donor vesicle, or (2) as a monomer in the aqueous phase, the most plausible mechanism for the uptake of M-C6-NBD-PC by yeast cells is internalization of lipids that have inserted into the plasma membrane after dissociating from donor vesicles.

**Temperature and Energy Dependence Distinguish Two Import Pathways**

The effects of temperature and intracellular ATP levels on the internalization of M-C6-NBD-PC were investigated. Microscopic analysis revealed that vacuolar accumulation of NBD fluorescence was blocked at 2°C, but labeling of the mitochondria and nuclear envelope still occurred (Fig. 2, D, E, and F, and Fig. 3, A, B, and C). Incubation of CRY3 in medium lacking a carbon source and containing 20 mM sodium azide (SCNaN3 medium) for 45-50 min at 30°C decreased the intracellular ATP pools by 80% as determined by luciferase assays (data not shown). At 30°C, this degree of ATP-depletion inhibited traffic of M-C6-NBD-PC to the vacuole, but did not interfere with transport to the nuclear envelope and mitochondria (Fig. 2, A, B, and C and Fig. 3, D, E, and F). As can be seen in Fig. 2, B and E mitochondria could not be labeled with DAPI when it was added either after ATP depletion (Fig. 2 B) or during incubation in SDC at 2°C (Fig. 2 E), although labeling of the nucleus still occurred. To confirm that M-C6-NBD-PC was transported to the mitochondria under these incubation conditions, we first pretreated cells with DAPI at 30°C in SDC (which allowed both the nuclei and mitochondria to be labeled) before depleting ATP stores or lowering the incubation temperature to 2°C and adding M-C6-NBD-PC-containing donor vesicles. With this procedure, we were able to confirm the nuclear envelope- and mitochondrial-location of the NBD fluorescence both when the incubation temperature was lowered to 2°C and under conditions of ATP depletion (Fig. 3).

When the incubation temperature was lowered to 2°C in cells that had undergone ATP depletion, traffic not only to the vacuole, but also to the nuclear envelope and mitochondria was completely blocked (Fig. 2, G, H, and I). Inhibition of traffic to the vacuole with continued traffic to the nuclear envelope and mitochondria when cells were incubated at 30°C in SCNaN3 or at 2°C in SDC suggested that internalization to the vacuole occurred by an endocytic pathway (Anderson et al., 1977; Draper and Simon, 1980; Helenius et al., 1980), and internalization to the nuclear envelope and the mitochondria resulted from non-endocytic events.

Since a fluorescence signal occurred only upon internalization of M-C6-NBD-PC, the rate of internalization could be measured as the rate of increase in fluorescence in real time in the spectrofluorometer. Fig. 4 shows that this increase in fluorescence signal with time depended on temperature and intracellular ATP. Incubation of cells at 30°C in normal growth medium (shown in the trace labeled "30°C, SDC") in Fig. 4), resulted in a rapid increase in fluorescence.
(G–I) Cells incubated at 2°C after incubation in SCNaN₃ medium to deplete ATP. (A) NBD fluorescence; (B) DAPI fluorescence; (C) DIC optics; (D) NBD fluorescence; (E) DAPI fluorescence; (F) DIC optics; (G) NBD fluorescence; (H) DAPI fluorescence; (I) DIC optics. Open arrowheads point to representative vacuoles, arrows point to representative nuclei. Bar, 5 μm.
Figure 3. Digital image of CRY3 cells in which M-C6-NBD-PC internalization assays were performed at 2°C in SDC and at 30°C in SCNaN3. The cells were preincubated with 2.5 μM DAPI for 1 h at 30°C in SDC before shifting the temperature to 2°C or depleting ATP levels and adding M-C6-NBD-PC-containing vesicles. This pretreatment enhanced the fluorescence of DAPI-labeled mitochondria, and thus allowed the confirmation of the localization of NBD fluorescence to the nuclear envelope and mitochondria. (A–C) Cells incubated at 2°C in SDC. (D–F) Cells incubated at 30°C in SCNaN3. (A) NBD fluorescence; (B) DAPI fluorescence; (C) DIC optics; (D) NBD fluorescence; (E) DAPI fluorescence; (F) DIC optics. Closed arrowheads point to representative mitochondria, arrows point to representative nuclei. Bar, 5 μm.

Figure 4. Spectrofluorometric analysis of M-C6-NBD-PC internalization into CRY3. Internalization assays were performed on the wild-type strain, CRY3. NBD fluorescence was monitored continuously by exciting the fluorophore at 475 nm and monitoring emission at 530 nm. Cell densities were also monitored by reading the OD at 600 nm at regular intervals. Incubation conditions are as shown in the figure. When vesicles were initially added to an early-log phase cell suspension. Under conditions of continued logarithmic growth, this increase in fluorescence continued for ~30 min, after which it began to plateau. As shown in Table III, the plateau can be explained, at least in part, by degradation of M-C6-NBD-PC to less fluorescent, aqueous metabolites. When ATP was depleted before incubation at 30°C (trace labeled “NaNaN3, 30°C”, Fig. 4) the fluorescence increase was reduced significantly (by ~90% at 30 min after the addition of donor vesicles). At 2°C in SDC medium a dramatic decrease was observed in the rate and amount of fluorescence increase; both were inhibited by ~87% at 30 min. Extraction of cellular lipids and separation by TLC showed no degradation of the M-C6-NBD-PC molecule when incubation was in SCNaN3 medium at 30°C or in SDC at 2°C (Fig. 5). This lack of degradation correlates with the continual increase in cell-associated fluorescence shown in the respective spectrofluorometer traces (Fig. 4). At 2°C with ATP depletion (trace labeled “NaNaN3, 2°C”, Fig. 4), the fluorescence increase was almost completely blocked (~97%). Presumably, the reduced fluorescence observed in

Figure 5. TLC analysis of fluorescent lipids extracted from CRY3 and CBO18 cells. Internalization assays were performed on CRY3 (Pep+) and CBO18 (Pep−) strains. Lipids were extracted from the cells as described in Materials and Methods. Solvent was evaporated under a nitrogen stream, and the lipids that partitioned into the organic phase of the extraction were resuspended in chloroform. Lipids from equal numbers of cells were spotted onto a TLC plate which was developed in chloroform/methanol/acetic acid/water, 5:1:2:1:0.5. The lanes show: (1) CBO18 cells incubated with donor vesicles in SCNaN3 medium at 2°C. (2) CBO18 incubated with vesicles in SDC at 2°C. (3) CBO18 incubated with vesicles in SCNaN3 at 30°C. (4) CBO18 incubated with vesicles in SDC at 30°C. (5) CBO18 in SDC at 30°C, incubated without vesicles. (6) CRY3 cells incubated with donor vesicles in SCNaN3 at 2°C. (7) CRY3 incubated with vesicles in SDC at 30°C. (8) CRY3 incubated with vesicles in SCNaN3 at 30°C. (9) CRY3 incubated with vesicles in SDC at 30°C. (10) CRY3 in SDC at 30°C, incubated without vesicles. (11) M-C6-NBD-PC treated with phospholipase A2. (12) M-C6-NBD-phosphatidylethanolamine standard. (13) M-C6-NBD-phosphatidic acid standard. (14) M-C6-NBD-diaclylglycerol standard. (15) M-C6-NBD-aminoglycoside standard. (16) NBD-amine standard. (17) N-Rh-DOPE standard.
the spectrofluorometer when cells were incubated at 2°C in SDC medium, or at 30°C with ATP depletion correlates with what is seen in the microscope, namely, continued uptake into the nuclear envelope and mitochondria and elimination of vacuolar uptake (Fig. 2, A and D and Fig. 3). Similarly, the drastically reduced fluorescence signal in the spectrofluorometer for cells incubated at 2°C with ATP depletion correlates with the blockage of both pathways observed microscopically (Fig. 2 G).

**Degradation of the Internalized M-C6-NBD-PC Occurs in the Vacuole**

The fate of the internalized phosphatidylcholine was monitored by extracting cells and separating lipids by TLC (Fig. 5). Cells were incubated either at 30°C or at 2°C, under conditions that either maintained or depleted cellular ATP stores. After allowing transport of lipid molecules to occur for 2 h, the cells were extracted with organic solvents, and the aqueous and organic phases were analyzed by TLC. In the Pep+ strain, CRY3, there was significant degradation of M-C6-NBD-PC only under the condition (30°C, SDC) in which vacuolar fluorescence was seen in the microscope (Fig. 5, lane 9). Under this condition, four predominant breakdown products appeared in the organic phase. Two of these comigrated with monoaoyl-C6-NBD-phosphatidylcholine and the C6-NBD-fatty acid markers that were produced by treatment of M-C6-NBD-PC with phospholipase A2 (Fig. 5, lane 1I). Another breakdown product comigrated with an M-C6-NBD-diaclyglycerol marker and thus could have been formed by phospholipase C-dependent degradation of M-C6-NBD-PC molecules (Fig. 5, lane 1J). (The fourth breakdown product occurred under all incubation conditions, and is unidentified at this time.) One predominant (unidentified) breakdown product occurred in the extracted aqueous phase (Table III). Importantly, no significant degradation of M-C6-NBD-PC occurred when cells were incubated at 2°C or under conditions of ATP depletion (Fig. 5, lanes 6–8). Together with the microscopic analysis (Figs. 1, 2, and 3), these data argue strongly that metabolism of M-C6-NBD-PC occurs only in the vacuole.

To examine further the vacuolar location of the phospholipid breakdown, the same extraction protocol was applied to the Pep− strain, CB018. Although the monoaoyl-C6-NBD-phosphatidylcholine and the C6-NBD-fatty acid metabolites were also present in CB018 (Fig. 5, lane 4) (consistent with the presence of a phospholipase A2 activity in the mutant cells), there was a significant difference between the two strains. Although equal numbers of cells were extracted and equal volumes of lipid were spotted onto the TLC plate, there was significantly (~5-fold) more intact M-C6-NBD-PC present in CB018 than in CRY3 (compare lanes 4 and 9 of Fig. 5). There was a concomitant 7.5-fold reduction in the amount of aqueous break-down product (Table III), consistent with a reduced capacity to metabolize M-C6-NBD-PC in the Pep− strain. These results also indicated that the site of degradation of M-C6-NBD-PC was the vacuole.

Quantitation of the fluorescence intensity of the vacuoles observed microscopically by pixel analysis yielded consistent results. In CB018 cells, the average vacuolar pixel brightness was twice that seen for CRY3 (Table III) or for the isogenic Pep+ MALα haploid, CRY1 (data not shown).

The NBD fluorophore exhibits exquisite sensitivity to the polarity of its environment (Monti et al., 1977). For example, NBD fluorescence is 100 times higher in a phospholipid bilayer than in aqueous environments (Nichols, 1987). Therefore, the fact that degradation of M-C6-NBD-PC yields products that preferentially partition into the aqueous phase, together with the observation that the vacuoles were twice as bright in CB018 cells as compared to either CRY3 or CRY1 cells, lends further support to the conclusion that less degradation of M-C6-NBD-PC occurs in Pep− cells.

**sec Mutations Block Endocytic Phospholipid Uptake But Not Trafficking to the Nuclear Envelope and Mitochondria**

To determine whether there were genetic links between the forward, secretory movement of lipids and the retrograde movement from the plasma membrane demonstrated here, we performed the internalization assay using M-C6-NBD-PC on yeast strains carrying Ts− mutations in selected secretory (SEC) genes (Novick and Schekman, 1979; Novick et al., 1980; Novick et al., 1981). The SEC genes can be broadly categorized as those that are required for the translocation of secretory proteins into the ER, or signal peptide processing in the ER (referred to here as "ER sec mutants," exemplified by the SEC63, SEC65, and SEC11 genes) (Bohni et al., 1988; YaDeau et al., 1991; Stirling and Hewitt, 1992; Stirling et al., 1992; Sadler et al., 1989), and those that are required for vesicular transport from the ER to the various secretory organelles (referred to here as "vesicular-transport sec mutants," exemplified by the SEC1, SEC2, SEC4, SEC6, SEC7, SEC12, SEC14, SEC17, SEC18, and SEC21 genes) (Novick and Schekman, 1979; Novick et al., 1981; Novick and Schekman, 1983). After internalization assays were performed on these strains, two methods of data analysis were employed. First, the morphology of fluorescence accumulation was documented by creating digital images of representative fields of cells (Fig. 6). Also, by using Image-I/AT image analysis software, the fluorescence intensities of the vacuoles visualized in the digital images of both the Sec+ and Sec− strains were determined. These intensities are expressed as average pixel brightness, and range on an arbitrary scale between 0 and 255.

When the internalization assay was performed on the sec mutant strains at a temperature permissive for growth (24°C), both the fluorescence patterns observed in the microscope (Fig. 6) and the pixel brightness of the vacuoles (not shown) were identical to those observed for their isogenic Sec+ strains, and indistinguishable from that observed with CRY3 (Fig. 1) and CB018. However, when the internalization assay was performed at the restrictive temperature (37°C), a functional distinction between the two classes of sec mutants was revealed. Mutants that block vesicular transport from the ER through the secretory pathway were also required for lipid endocytosis to the vacuole. These included the secretory-vesicle-accumulating mutants sec1, sec2, sec4 (a yeast ras-like protein) (Novick and Schekman, 1979; Saminen and Novick, 1987), and sec6, which showed almost complete inhibition of lipid flow to the vacuole, although lipid flow to the nuclear-envelope and mitochondria still occurred (Fig. 6, E–H). The sec7 and sec14 mutants, which affect protein transport out of the Golgi complex (No-
Figure 6. Effect of sec mutations on the internalization of M-C6-NBD-PC. Internalization assays were performed on a variety of wild-type strains and on isogenic strains bearing ts mutations in one of the SEC genes. All strains were grown to mid-log phase at 24°C. They were then split, and either shifted to 37°C for 30 min, or kept at 24°C. Internalization assays were then performed, followed by fluorescence microscopy. The panels show: (A) CRY1, 37°C, NBD fluorescence (NBD). (B) CRY1, 37°C, DIC optics (DIC). (C) CRY1, 25°C, NBD.
vick et al., 1981; Achstetter et al., 1988; Franza and Schekman, 1989; Bankaitis et al., 1990), also blocked internalization to the vacuole (Fig. 6, I-L). At the restrictive temperature, Ts alleles of the SEC18 gene (which encodes the yeast homolog of the mammalian transport factor NSF [Eakle et al., 1988; Graham and Emr, 1991; Wilson et al., 1989]), of SEC17 (which encodes the yeast α-SNAP homolog [Clary et al., 1990]), and of SEC21 (which encodes the yeast homolog of the non-clathrin coat protein, β-COP [Hosobuchi et al., 1992]) reduced phospholipid internalization to the same extent as the late acting sec mutants (Fig. 6, R, S, T, and V).

The lack of endocytosis in the vesicular-transport sec mutant strains can be seen clearly in the digital images in Fig. 6. A statistical analysis of the inhibition of endocytosis is shown in Fig. 7. Because many of the vesicular-transport sec mutants had vacuolar pixel brightness values of 0 (their vacuoles were not fluorescent at all), a normal (gaussian) distribution of pixel brightness did not occur, and thus parametric comparison of the means was not possible. For those that had measurable fluorescence, the pixel brightness values ranged widely, but the vast majority did not exceed a value of 20% of the mean of the Sec+ strains. Very few cells from Sec+ strains had vacuoles that were non-fluorescent. The fluorescence intensities for these cells also ranged widely, with mean pixel brightness being >100. To determine if the distribution of pixel brightness for the Sec− strains was statistically different from the Sec+ strains, a 3 × 2 contingency table was created with bins of 0, >0-20, and >20, and χ² analysis was performed. P values were determined, which reflect the statistical significance of the sec mutant pixel brightness distributions as compared to their isogenic Sec+ strains. This analysis, shown in Fig. 7, demonstrated that the vacuolar pixel brightness in all of the vesicular-transport sec mutants was statistically different from the pixel brightness in the isogenic Sec+ strains to at least the 0.0003 level. Thus, the SEC genes that affect membrane traffic during secretion are also required for retrograde lipid traffic during endocytosis. It is possible that the inhibition of endocytic uptake of M-C₆-NBD-PC by the vesicular transport class of sec mutants may not be due to direct involvement of each of these gene products in the endocytic pathway. For example, endocytic uptake might be tightly coupled to lipid exocytosis by a regulatory mechanism (see Discussion).

The results with the vesicular transport sec mutants sharply contrast those obtained with sec mutants that control the translocation of nascent secretory proteins into the ER, or processing in the ER. Mutations in SEC11 (which encodes a component of the signal peptidase) (Bohni et al., 1988; YaDeau et al., 1991), SEC63 (a dna1 homolog) (Sadler et al., 1989), or SEC65 (which encodes a component of the signal recognition particle) (Stirling and Hewitt, 1992; Stirling et al., 1992), allowed far greater vacuolar accumulation of M-C₆-NBD-PC than did the other sec mutants (Fig. 6). In fact, when contingency table statistical analysis was performed on the pixel brightness distributions from these strains, these distributions were found to be not statistically different from the pixel brightness distributions of their isogenic Sec+ alleles (Fig. 7). These strains also differed from the vesicular-transport sec mutants in that there was a normal distribution to their pixel brightness values, thus allowing

Figure 7. Graphical representation of a 3 × 2 contingency table to test the statistical difference of the distribution of vacuolar pixel brightness values between the sec mutant strains and their isogenic Sec+ alleles. 0, >0-20, and >20 are the three pixel brightness ranges that served as statistical bins for the contingency table. The sec mutants in this figure are grouped under their respective Sec+ strains. Clear bars represent the % of cells with pixel brightness values of exactly 0. Stippled bars represent the % of cells with pixel brightness values of >0-20. Filled bars represent the % of cells with pixel brightness values of >20. The χ² contingency test was used to test whether the pixel brightness distributions of the sec strains differed from their isogenic Sec+ alleles. *signifies sec strains where P < 0.0001. # signifies sec strains where P < 0.0003. ns signifies sec strains whose distributions were not significantly different from their isogenic Sec+ alleles. The number of cells scored is shown in parentheses. Because sec11, sec63, and sec65 had no cells with pixel brightness values of 0, 3 × 2 contingency tables could not be made. Instead, 2 × 2 tables were made, and the Fisher's Exact statistical test was performed. With this type of statistical analysis, the pixel brightness distributions for these strains were judged to be not significantly different from their isogenic Sec+ strains. However, unlike the other sec mutants, there was a normal distribution of pixel brightness values in the sec11, sec63, and sec65 strains. Therefore, the Student's t test was performed and is shown in Table IV. Abbreviations used in this figure: NSF, NEM-sensitive factor; PI/PC, phosphatidylinositol/phosphatidylcholine; prot, protein; SRP, signal recognition particle.

(D) CRY1, 25°C, DIC. The morphology shown for the Sec+ strain, CRY1, is representative of the other Sec+ strains, CRY2, CRY3, NY13, X2180-1A, and X2180-1B. (E) sec1, 37°C, NBD. (F) sec1, 37°C, DIC. (G) sec1, 25°C, NBD. (H) sec1, 25°C, DIC. The morphology shown for the SEC11 ts strain is representative of strains that carry ts mutations in the SEC2, SEC4, and SEC6 genes. (I) sec7, 37°C, NBD. (J) sec7, 37°C, DIC. (K) sec7, 25°C, NBD. (L) sec7, 25°C, DIC. The morphology shown for the SEC7 ts strain is representative of the strain that carries a ts mutation in the SEC7 gene. (M) sec11, 37°C, NBD. (N) sec11, 37°C, DIC. (O) sec11, 25°C, NBD. (P) sec11, 25°C, DIC. The morphology shown for the SEC11 ts strain is representative of the strains that carry ts mutations in the SEC63 and SEC65 genes. (R) sec18, 37°C, NBD. (S) sec18, 37°C, DIC. (T) sec18, 25°C, NBD. (V) sec18, 25°C, DIC. The morphology shown for the SEC18 ts strain is representative of the strains that carry ts mutations in the SEC12, SEC17, and SEC21 genes. Bar, 5 μm.

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that were left untreated. sec IV) revealed that in cycloheximide-treated cells lipid endocytosis, inhibiting endocytic lipid flow by 39–55% (Table IV). However, as seen in Fig. 6, even with this level of inhibition, the ER sec mutants still carry out substantial lipid endocytosis.

These results suggest that the effects of the sec mutations on endocytic phospholipid transport are predominantly due to the inhibition of membrane flow, rather than cargo flow. This interpretation is supported by internalization experiments performed after pretreating cells for 1 h with cycloheximide, a treatment expected to eliminate the newly synthesized protein cargo in transport vesicles, but, at least in the short term, not to eliminate the trafficking of these vesicles along the secretory pathway. When assayed microscopically (data not shown, but comparable to Fig. 6 M), cells pretreated with cycloheximide still carried out endocytosis of M-C₆-NBD-PC to a substantial extent, and just as with the ER sec mutants, deposited the fluorescent phospholipid into the vacuole. Pixel brightness analysis of vacuoles (Table IV) revealed that in cycloheximide-treated cells lipid endocytosis was affected to much the same degree as with the ER sec mutants, being inhibited by ~36% as compared to cells that were left untreated.

Although the endocytic pathway was dependent on many SEC genes, it was not dependent on a functional clathrin heavy chain. Thus, in the strain GPY184, having a ts-clathrin heavy chain gene (chcl-52), endocytic lipid traffic continued even after incubation of these cells for 1.5 h at the restrictive temperature (Table V). This was in sharp contrast to the rapid effects seen in this strain (within 15 min) on proper retention of Golgi-resident enzymes and maturation of pro-CPY (Seeger and Payne, 1992a,b).

Although none of the other 12 sec mutants tested had any inhibitory effect on the non-endocytic still carry out transport of M-Ç-NBD-PC to the nuclear envelope and mitochondria, we did observe a reproducible inhibition of this traffic in sec18 cells (Fig. 6 R). Although a comparison of pixel brightness values showed that this inhibition was less that the sec18-mediated inhibition of endocytic lipid flow (~55% compared to ~98%), a requirement for the sec18 gene product in non-endocytic lipid traffic is suggested by this data. The sec mutant experiments provide further evidence that two distinct pathways exist for retrograde lipid traffic, because whereas lipid flow to the vacuole was blocked by many sec mutations, with the exception of sec18, lipid flow to the nuclear envelope and mitochondria was not. However, although non-endocytic lipid traffic was largely independent of SEC genes, it did appear to be protein-mediated, due to its inhibition by the two (chemically distinct) sulfhydryl reagents, NEM, and pCMBS (Table VI). It was inhibited by 89% when cells were pretreated with 2 mM NEM, by 40% when pretreatment was with 10 mM pCMBS, and by 50% when pretreatment was with 50 mM pCMBS.

### Discussion

**Two Pathways of Lipid Flow Occur during the Internalization of M-C₆-NBD-PC**

The data presented here have provided evidence for two previously undocumented lipid transport pathways in yeast (Fig. 8). First, plasma membrane phospholipids can be actively internalized by an endocytic mechanism, concentrated and degraded in the vacuole (Fig. 8 A). A second pathway, which is largely independent of SEC gene function, deposits plasma membrane phospholipids in the nuclear envelope and mitochondria (Fig. 8 B). This pathway resembles the non-endocytic phospholipid flip-flow pathways that have been observed in mammalian systems.

The two pathways shown in Fig. 8 share a common first step: insertion of the M-C₆-NBD-PC molecules into the yeast plasma membrane. The yeast plasma membrane appears to have a lower affinity for NBD-labeled phospholipids than do the plasma membranes of mammalian cells. "Ring

### Table IV. Effect of ER sec mutants and Cycloheximide on Endocytosis of M-C₆-NBD-PC

| Strain          | Mean vacuolar pixel brightness ± std. dev. (n. of cells analyzed) | % Inhibited compared to isogenic Sec+ strain |
|-----------------|---------------------------------------------------------------|---------------------------------------------|
| CRY1            | 76 ± 34 (20)                                                  |                                              |
| sec11           | 34 ± 20 (41)*                                                 | 55%                                         |
| X2180-1B        | 130 ± 73 (29)                                                 |                                              |
| sec63           | 79 ± 32 (32)*                                                 | 39%                                         |
| sec65           | 75 ± 34 (19)*                                                 | 42%                                         |
| CRY3            | 76 ± 20 (42)                                                  |                                              |
| CRY3 (cycloheximide) | 49 ± 21 (30)*                                               | 36%                                         |

Internalization assays were performed on the strains as described in Materials and Methods. Vacular pixel brightness values were measured using Image1/AT image analysis software. Using the Student's t test to determine statistically different means. By this method, the ER sec mutants were found to have a modest, but statistically significant affect on lipid endocytosis, inhibiting endocytic lipid flow by 39–55% (Table IV). However, as seen in Fig. 6, even with this level of inhibition, the ER sec mutants carry out substantial lipid endocytosis.

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### Table V. Effect of a Clathrin Heavy Chain ts Allele on Endocytosis of M-C₆-NBD-PC

| Strain          | Relevant phenotype | Mean vacuolar pixel brightness ± std. dev. (n. of cells analyzed) |
|-----------------|--------------------|---------------------------------------------------------------|
| GPY55-15B       | CHC1 (wt)          | 77 ± 55 (40)                                                |
| GPY184          | chc1 (ts)          | 66 ± 50 (34)*                                               |

Internalization assays were performed on the two strains as described in Materials and Methods. Vacular pixel brightness values were measured using Image1/AT image analysis software.

* The mean vacuolar pixel brightness for the wt and ts CHC1 alleles were analyzed using the Student's t test, and were found to be not statistically different from one another (p < 0.37).
Table VI. Effect of NEM and pCMBS on the Internalization of M-C6-NBD-PC to the Nuclear Envelope at 2°C

| Strain        | Nuclear envelope pixel brightness ± std. dev. (n. of cells analyzed) | % Inhibition |
|---------------|---------------------------------------------------------------------|--------------|
| CRY3          | 5.8 ± 1.2 (23)                                                      | -            |
| CRY3 (pretreated with 2 mM NEM) | 0.65 ± 0.29 (34)*                                                 | 89%          |
| CRY3          | 7.33 ± 1.67 (18)                                                   | -            |
| CRY3 (pretreated with 10 mM pCMBS) | 4.20 ± 1.38 (28)*                                                 | 42%          |
| CRY3          | 10.88 ± 4.66 (17)                                                  | -            |
| CRY3 (pretreated with 50 mM pCMBS) | 5.51 ± 5.32 (14)*                                                 | 50%          |

Cells were either pretreated for 30 min with 2 mM NEM, 10 mM pCMBS, 50 mM pCMBS (as described in Materials and Methods), or left untreated. Internalization assays were then performed at 2°C, and the fluorescence in the nuclear envelope was determined as an average pixel brightness value using ImageJ/AT image analysis software. The Student's t test was used to determine the statistical significance of the mean pixel brightness from treated or untreated cells. The two means were statistically different from each other. * = p < 0.0001, † = p < 0.0056. A % inhibition by NEM or pCMBS was then determined from the mean pixel brightness values.

Cells were either pretreated for 30 min with 2 mM NEM, 10 mM pCMBS, 50 mM pCMBS (as described in Materials and Methods), or left untreated. Internalization assays were then performed at 2°C, and the fluorescence in the nuclear envelope was determined as an average pixel brightness value using ImageJ/AT image analysis software. The Student's t test was used to determine the statistical significance of the mean pixel brightness from treated or untreated cells. The two means were statistically different from each other. * = p < 0.0001, † = p < 0.0056. A % inhibition by NEM or pCMBS was then determined from the mean pixel brightness values.

Figure 8. Working model of the two internalization pathways for M-C6-NBD-PC into yeast cells.
branched, the unusually high turgor pressure of the yeast plasma membrane (Gustin et al., 1988) or by constraints imposed by cytoskeletal elements (Chowdhury et al., 1992) that limit membrane expansion required for lipid insertion. Recent investigations into the fluidity of the yeast plasma membrane employing FRAP indicate that the lateral mobility of lipids in the yeast membrane is significantly lower than that observed in mammalian cells (Greenberg and Axelrod, 1993). The constraints responsible for this reduced lateral mobility are also likely to reduce the extent to which exogenous phospholipids can be inserted into the plasma membrane.

Retrograde transport of NBD-labeled phospholipids into cultured mammalian cells is usually monitored after the removal of exogenous donor vesicles after preloading the plasma membrane with the fluorescent phospholipid (Sleight and Pagano, 1984). In contrast, the low level of M-C6-NBD-PC insertion into the yeast plasma membrane required continual incubation in the presence of vesicles to accumulate measurable levels of intracellular fluorescence. Control experiments (Table II) confirmed that, even in the constant presence of donor vesicles, internalization occurred only after M-C6-NBD-PC monomers dissociated from these vesicles and inserted into the plasma membrane. Additionally, because cell-associated fluorescence increased only as a result of lipid-internalization, we could estimate the relative rates of the two internalization pathways by monitoring cell-associated fluorescence continuously in the spectrofluorometer (Fig. 4).

Under normal growth conditions, the majority (>70%) of M-C6-NBD-PC molecules were internalized by endocytosis, and were transported to the vacuole (Fig. 8 A). The vacuolar pathway has been termed "endocytic" for three reasons. First, low temperatures and decreased cellular ATP levels, two classical conditions in which endocytosis is inhibited (Anderson et al., 1977; Draper and Simon, 1980; Helenius et al., 1980), both completely inhibited transport of M-C6-NBD-PC to the vacuole. Second, a number of ts see mutations (that are known to inhibit the vesicular traffic involved in protein secretion at the restrictive temperature) also blocked transport of M-C6-NBD-PC to the vacuole. Third, the target organelle of this traffic, the vacuole (yeast lysosome), is the terminal destination for the vacuolar pathway, and is inhibited (Kean, L. S., R. S. Fuller, J. W. Nichols, unpublished results). This suggests that the level of M-C6-NBD-PC in the ER is low.

The non-endocytic pathway is inhibited by the sulfhydryl reagents NEM and pCMBS, and thus appears to be protein-mediated. While NEM has been shown to be membrane-permeant at 37°C (Abbot and Schacter, 1976; May, 1985; D'Amore and Lo, 1986), pCMBS has been shown to be membrane impermeant in a variety of cell systems (Rothstein, 1970; May, 1985; D'Amore and Lo, 1986). While both reagents inhibit the non-endocytic pathway at 2°C (Table VI), NEM inhibition (89%) is more extensive than pCMBS inhibition (40–50%). Two possible explanations exist for the greater inhibition by NEM. NEM may simply modify (and inhibit) a cell-surface protein more effectively than pCMBS, and thereby lead to greater inhibition of the non-endocytic pathway. Alternatively, NEM may also modify intracellular proteins which are important for specific traffic to the nuclear envelope and mitochondria. Inhibition of these intracellular proteins might further shut down the flux of M-C6-NBD-PC and therefore lead to a more complete reduction in the intracellular fluorescence accumulation.

The non-endocytic trafficking of M-C6-NBD-PC to the nuclear envelope and mitochondria in yeast resembles the non-endocytic trafficking of NBD-phosphatidylethanolamine and NBD-phosphatidylserine in untransformed mammalian cell lines (Martin and Pagano, 1987; Sleight and Pagano, 1985), and of NBD-phosphatidylcholine in virally transformed human lung fibroblasts (Sleight and Abanto, 1989) which has been shown to occur by protein-mediated flip-flop (Martin and Pagano, 1987; Sleight and Pagano, 1985; Sleight and Abanto, 1989). Inhibition of this putative yeast flip-flop activity by NEM suggests that it may be more closely related to the aminophospholipid flip-flop activities than to the NEM-resistant phosphatidylcholine flip-flop activity found in virally transformed human lung fibroblasts. An additional similarity between the yeast non-endocytic transport pathway and the mammalian flip-flop pathway is the selectivity that these pathways display towards phospholipids bearing different head-groups. In mammalian cells, lipid sorting occurs at the plasma membrane, such that the aminophospholipids (phosphatidylethanolamine and phosphatidylserine) are preferentially internalized by the non-endocytic (flip-flop) pathway (Sleight and Pagano, 1985; and Martin and Pagano, 1987), while phosphatidylcholine is preferentially internalized by the endocytic pathway in untransformed cell lines (Sleight and Pagano, 1984; Sleight and Abanto, 1989). We have found that lipid sorting also occurs in yeast. M-C6-NBD-phosphatidylethanolamine and M-C6-NBD-phosphatidylserine are internalized into yeast almost exclusively via the non-endocytic pathway, and are trafficked by this pathway to the ER, nuclear envelope and mitochondria, but not to the vacuole (Kean, L. S., R. S. Fuller, and J. W. Nichols, unpublished results). This is in striking con-
that has been shown to be required in all steps of vesicular
zation pathway (Fig. 1). It is possible that a fusion reaction is
also required during the non-endocytic targeting of lipids,
and that SEC18 plays a role in this process.

Flip-flop activities have been observed in the membranes
of many cell systems, and have been implicated in main-
ing lipid asymmetry across the membrane bilayers (Rousse-
let et al., 1976; van Meer and Op den Kamp, 1982). Asym-
metry generated by the combined action of flip-flop proteins
and phospholipid transfer proteins could play a role in
differentiating organelles, or in targeting proteins to subdo-
mains of a continuous lipid bilayer. The selective transport
of M-Cr-NBD-PC to the nuclear envelope and mitochon-
drial membranes by the non-endocytic route suggests the ex-
istence of specific intracellular transport pathways, perhaps
relying on specific lipid transfer proteins. The phosphatidyli-
ositol-phosphatidylycholine transfer protein encoded by the
SEC14 gene would not be implicated in these transfer reac-
tions (at least for the transfer of M-Cr-NBD-PC), since non-endocytic phospholipid transport is not blocked in a
sec14 mutant assayed at the non-permissive temperature.

Despite the proposed importance of lipid flip-flop proteins
for a variety of membrane traffic events, the proteins respon-
sible for transblayer lipid movement remain to be identified.
Having an assay for flip-flop in yeast should assist the
identification of this class of proteins, and the genes that en-
code them.

Two Models to Explain the Link between Secretion and
Lipid Endocytosis

As is shown in Figs. 6 and 7 and Table IV, the SEC genes
required for proper membrane flow during secretion were
also required for lipid endocytosis. These vesicular-trans-
port SEC genes were distinguished from genes such as
SEC63, SEC65, and SEC11, which are required very early in
the secretory pathway for translocation of proteins into and
processing in the ER, and were not required for endocytic
lipid traffic. Two distinct models can account for the striking
interconnection that we have noticed between endocytic lipid
transport and vesicular-transport SEC gene function.

One model states that the SEC gene products required for
vesicular transport are also directly and functionally re-
quired for endocytic events. Given the large number of sec
mutants that also inhibit lipid endocytosis, this model
predicts that the biochemical links between endocytosis and
secretion must be extensive. The second model assumes no
direct requirement for SEC gene products in the mechanism
of lipid internalization, but rather, a regulatory coupling of
the two pathways. Exocytic trafficking might require retro-
grade transport of excess phospholipid from the plasma
membrane back to intracellular organelles. Analogous retro-
grade vesicular transport pathways have been hypothesized
both on theoretical (Rothman, 1981) and experimental
grounds (Lippincott-Schwartz et al., 1989; Dean and Pel-
ham, 1990; Hsu et al., 1991). Indirect evidence for such a
process in yeast is found in the analysis of inositol auxo-
trophs (ino1 mutants) under conditions of inositol starvation.
Such cells exhibit a rapid cessation of membrane growth, but
secretion appears to continue unabated (Henry et al., 1977;
Atkinson and Ramirez, 1984). Thus, retrograde transport of
lipids delivered to the plasma membrane by secretory vesicle
fusion could complete a "closed cycle" of lipid transport,
effectively coupling exocytosis and lipid endocytosis. Cons-
istent with this model, preliminary quantitative measure-
ments of the rate of internalization of M-Cr-NBD-PC by
the endocytic pathway suggest that significantly more than
one plasma membrane-equivalent is internalized per cell di-
vision cycle (Kean, L. S., R. S. Fuller, and J. W. Nichols,
unpublished data). Thus, instead of there being a specific re-
quirement for the individual proteins encoded by the various
SEC genes, there could be a regulatory coupling of the two
processes to ensure plasma membrane homeostasis. In
effect, failure in either process would generate an inhibitory
signal that would shut off the other process. A key question
raised by the regulatory coupling of the two processes is how
signaling between the two pathways occurs. One possibility
might be that a mechanism exists that measures the flux of
exocytic membrane traffic directly and communicates this
information to the endocytic apparatus. A second possibility
is that there is a system capable of sensing changes in the
physical state of the plasma membrane that occur upon a
reduction of the rate of exocytosis (Gustin et al., 1988; Mar-
tinac et al., 1990; Brewster et al., 1993).

The data presented in this paper do not distinguish be-
tween the two models for explaining the linkage of endo- and
exo-cytosis. However, the isolation of single mutations that
affect lipid exocytosis or lipid endocytosis exclusively would
be incompatible with the regulatory coupling hypothesis.

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