

# A New Vital Stain for Visualizing Vacuolar Membrane Dynamics and Endocytosis in Yeast

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**Abstract.** We have used a lipophilic styryl dye, *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl) pyridinium dibromide (FM 4-64), as a vital stain to follow bulk membrane-internalization and transport to the vacuole in yeast. After treatment for 60 min at 30°C, FM 4-64 stained the vacuole membrane (ring staining pattern). FM 4-64 did not appear to reach the vacuole by passive diffusion because at 0°C it exclusively stained the plasma membrane (PM). The PM staining decreased after warming cells to 25°C and small punctate structures became apparent in the cytoplasm within 5–10 min. After an additional 20–40 min, the PM and cytoplasmic punctate staining disappeared concomitant with staining of the vacuolar membrane. Under steady state conditions, FM 4-64 staining was specific for vacuolar membranes; other membrane structures were not stained. The dye served as a sensitive reporter of vacuolar dynamics, detecting such events as segregation structure formation during mitosis, vacuole fission/fusion events, and vacuolar morphology in different classes of vacuolar protein sorting (*vps*) mutants. A particularly striking pattern

was observed in class E mutants (e.g., *vps27*) where 500–700 nm organelles (presumptive prevacuolar compartments) were intensely stained with FM 4-64 while the vacuole membrane was weakly fluorescent. Internalization of FM 4-64 at 15°C delayed vacuolar labeling and trapped FM 4-64 in cytoplasmic intermediates between the PM and the vacuole. The intermediate structures in the cytoplasm are likely to be endosomes as their staining was temperature, time, and energy dependent. Interestingly, unlike Lucifer yellow uptake, vacuolar labeling by FM 4-64 was not blocked in *secl8*, *secl4*, *end3*, and *end4* mutants, but was blocked in *secl* mutant cells. Finally, using permeabilized yeast spheroplasts to reconstitute FM 4-64 transport, we found that delivery of FM 4-64 from the endosome-like intermediate compartment (labeled at 15°C) to the vacuole was ATP and cytosol dependent. Thus, we show that FM 4-64 is a new vital stain for the vacuolar membrane, a marker for endocytic intermediates, and a fluor for detecting endosome to vacuole membrane transport in vitro.

**S**ECRETION and endocytosis are major mechanisms of membrane flow to and from the plasma membrane (PM)<sup>1</sup> in eukaryotic cells. Plasma membrane equilibrium is maintained by the addition of secretory vesicles after fusion and subtraction of endocytic vesicles after invagination. Thus, eukaryotic cells must balance secretory and endocytic traffic to maintain the appropriate protein and lipid content in the PM. Studies on the secretory and endocytic pathways have contributed greatly to our present understand-

ing of eukaryotic membrane biology. Both pathways consist of vectorial events that encompass a series of vesicular transfers from one organelle to another. However, requirements of the secretory pathway have been characterized in greater detail than the requirements for incoming endocytic membrane traffic (Pryer et al., 1992; Rothman and Orci, 1992).

Recently, genetic analysis of the endocytic pathway in yeast has resulted in the isolation of mutants defective in endocytosis. The *end3* and *end4* mutants were isolated with defects in  $\alpha$ -factor internalization and subsequent degradation in the vacuole (Raths et al., 1993). Another mutant *ren1*, defective in receptor endocytosis accumulates the internalized  $\alpha$ -factor receptor in a prevacuolar compartment (Davis et al., 1993). These genetic approaches in yeast are beginning to complement studies in higher eukaryotic cells. For example, yeast temperature-sensitive actin mutants are defective for internalization of a  $\alpha$ -factor (Kubler and Riezman, 1993), which is consistent with depolymerization of

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1. **Abbreviations used in this paper:** CDCFDA, 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate; DASPMI, 2-(4-dimethylaminostyryl)-*N*-methylpyridinium iodide; D, 2% dextrose; DIC, differential interference contrast; FM 4-64 *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl) pyridinium dibromide; LY, Lucifer yellow carbohydrazide; P, 2% bactopectone; PM, plasma membrane; and Y, 1% yeast extract.

actin filaments blocking endocytosis at the apical surface of polarized epithelial cells (Gottlieb et al., 1993).

One drawback, however, in the use of yeast as a model system for studying endocytosis is the lack of morphological and biochemical approaches that are already well established in animal cell systems (Gruenberg and Howell, 1989; Salzman and Maxfield, 1993). *Saccharomyces cerevisiae* does not internalize a variety of ligands that can be used as morphological tracers to visualize intermediates between the PM and the vacuole. Furthermore, no biochemical assay for any endocytic fusion or transport event has been reconstituted with yeast membranes in vitro.

In this report, we have used a morphological and biochemical approach to examine endocytosis in yeast with the styryl dye *N*-(3-thiethylammoniumpropyl)-4-(*p*-diethylaminophenyl)hexatrienyl pyridinium dibromide (FM 4-64). Styryl dyes are sensitive to membrane potentials and have been useful in studying mitochondria (Bereiter-Hahn, 1976) and synaptic vesicle recycling in vitro (Betz et al., 1992a). We show that FM 4-64 initially stains the yeast PM, then small cytoplasmic compartments, and finally the vacuolar membrane. This process is time, temperature, and energy dependent. As a vital stain, FM 4-64 allows us to follow transport from the PM to the vacuole membrane in real time.

Yeast mutants defective in ER to Golgi transport (*sec18*), Golgi transport (*sec14*), and receptor-mediated endocytosis (*end3* and *end4*) did not block vacuolar membrane labeling by FM 4-64. However, yeast mutants that accumulate secretory vesicles (i.e., *sec1*) did block vacuolar labeling by FM 4-64. Additionally, we have reconstituted transport of FM 4-64 from the cytoplasmic intermediates to the vacuole. The data suggest that FM 4-64 enters the yeast cell by an endocytic mechanism, which may be: (a) visualized with fluorescence microscopy; (b) distinct from receptor-mediated events; and (c) amenable to biochemical analysis.

## Materials and Methods

### Strains and Media

The *S. cerevisiae* strains used in these studies are listed in Table I. All strains were maintained on rich media plates composed of 1% yeast extract (Y), 2% bactopectone (P), 2% dextrose (D), and 2.5% agar. Liquid YPD media was used for culturing all strains prior to labeling with fluorescent dyes.

### Staining with Vital Dyes

All strains were grown at 23° or 30°C to an OD<sub>600</sub> U between 0.8 and 1.6. For FM 4-64 (Molecular Probes Inc., Eugene, OR) staining, cells were har-

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
BHY10.5	<i>MATa/MATa leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 trp1-Δ901/trp1-Δ901 lys2-801 ade2-101 suc2-Δ9/suc2-Δ9 leu2-3,112::pBHY11 (CPY-Inv-LEU2)/Leu2-3,112::pBHY11 (CPY-Inv-LEU2) GAL/GAL</i>	(Paravicini et al., 1992)
SEY6210	<i>MATa ura3-52 his3-Δ200 trp1-Δ200 trp1-Δ901 leu2-3,112 lys2-801 suc2-Δ9 GAL</i>	(Robinson et al., 1988)
SEY6211	<i>MATa ura3-52 his3-Δ200 trp1-Δ901 leu2-3,112 ade2-101 suc2-Δ9 GAL</i>	(Robinson et al., 1988)
TVY614	<i>MATa ura3-52 his3-Δ200 trp1-Δ901 leu2-3,112 lys2-801 suc2-Δ9 GAL Δpep4::LEU2 Δprc1::HIS3 Δprb1::HISG</i>	(this study)
RH144-3D	<i>MATa his4 leu2 ura3 bar1-1</i>	(Raths et al., 1993)
RH266-1D	<i>MATa his4 leu2 ura3 bar1-1 end3</i>	(Raths et al., 1993)
RH268-1C	<i>MATa his4 leu2 ura3 bar1-1 end4</i>	(Raths et al., 1993)
SEY5107	<i>MATa leu2-3,112 ura3-52 suc2-Δ9 sec1-1</i>	S. D. Emr lab collection
SEY5187	<i>MATa leu2-3,112 ura3-52 suc2-Δ9 sec18-1</i>	S. D. Emr lab collection
Sf292-1A	<i>MATa sec14-3</i>	Yeast Genetic Stock Center
GPY1135	SEY6211 <i>vps35Δ1::HIS3</i>	(Paravicini et al., 1992)
KKY10	SEY6210 <i>vps17Δ1::HIS3 leu2-3,112::pBHY11</i>	(Kohrer and Emr, 1993)
JSR18D1	SEY6210 <i>vps18Δ1::TRP1</i>	(Robinson et al., 1991)
PHY112	SEY6210 <i>vps15Δ1::HIS3</i>	(Herman et al., 1990)
SEY27-1	SEY6210 <i>vps27-1</i>	(Robinson et al., 1988)
SEY1-3	SEY6211 <i>vps1-3</i>	(Robinson et al., 1988)

vested and resuspended at 20–40 OD<sub>600</sub> U/ml in YPD media. FM 4-64 was added to 20–40 μM from a stock solution of 16 mM in DMSO. The cells were then incubated with shaking for 30–60 min if performed at 0°C or 10–15 min at 23° and 30°C. After this preliminary labeling step, the cells were harvested at 700 g for 3 min at room temperature or 4°C for the 0°C experiments. The cells were then resuspended in fresh YPD media at 10–20 OD<sub>600</sub> U/ml, incubated with shaking for various times in kinetic studies or for 30–60 min for steady state experiments. After this “chase” period, the cells were again harvested at 700 g for 3 min, resuspended at 25–40 OD<sub>600</sub> U/ml in fresh YPD media, placed on standard slides, and viewed. For CDCFDA (Molecular Probes Inc.) staining, cells were incubated in YPD media containing 0.1 M citrate-KOH, pH 4.0, for 20 min at 23° or 30°C with 10 μM CDCFDA added from a 10 mM stock solution in DMF. The cells were harvested at 700 g for 3 min, resuspended at 25–40 OD<sub>600</sub> U/ml in fresh YPD media, placed on standard slides, and viewed. For DASPMI (Molecular Probes Inc.) staining, cells were incubated in YPD media with 5–10 μg/ml of DASPMI (added from a 50 μg/ml stock solution in water) for 5–10 min at 23°C, placed on standard slides, and viewed. To measure cell associated FM 4-64, stained cells were converted to spheroplasts (Vida et al., 1993) and then lysed in a buffer containing 25 mM Tris-HCl, pH 7.5, 25% methanol, 0.5% Triton X-100, and 0.5% SDS. After clarifying the lysate with centrifugation at 13,000 g for 3 min, the fluorescence of the supernatant was determined in a Perkin Elmer 650-10S fluorescence spectrophotometer with excitation and emission wavelengths of 515 and 640 nm, respectively.

### Photomicroscopy

A Nikon Microphot-SA microscope equipped with a UFX-DX metering system was used for all photomicrographs. A 100× CF N Plan DIC achromatic objective (NA 1.25) was used for all photomicrographs occasionally using a 1.5× magnification changer. A 546 nm filter was used for FM 4-64 fluorescence and a 450–490 nm filter was used for CDCFDA and DASPMI fluorescence. Fluorescence exposures ranged from 8 to 45 s, DIC exposures were 2–5 s (through a No. 1 green filter), all using Kodak T-Max 400 film. To immobilize cells, 5–7 μl of a 1 mg/ml solution of concanavalin A (Sigma Chemical Co., St. Louis, MO) in water was air dried on 1 × 75 × 25 mm slides. 4 μl of a cell suspension was applied and a 22 × 22 mm No. 1 coverslip was gently layered on top of the cells. These conditions resulted in flat, evenly spread cells and the slide preparations were suitable for viewing up to 30 min.

### Reconstitution with Permeabilized Cells

All procedures for preparing cytosol and cytosol-free permeabilized yeast spheroplasts were identical to previously described methods (Vida et al., 1990, 1993). Cytosolic extracts were made from TVY614 spheroplasts with the 150,000 g supernatant having a protein concentration of 29 mg/ml.

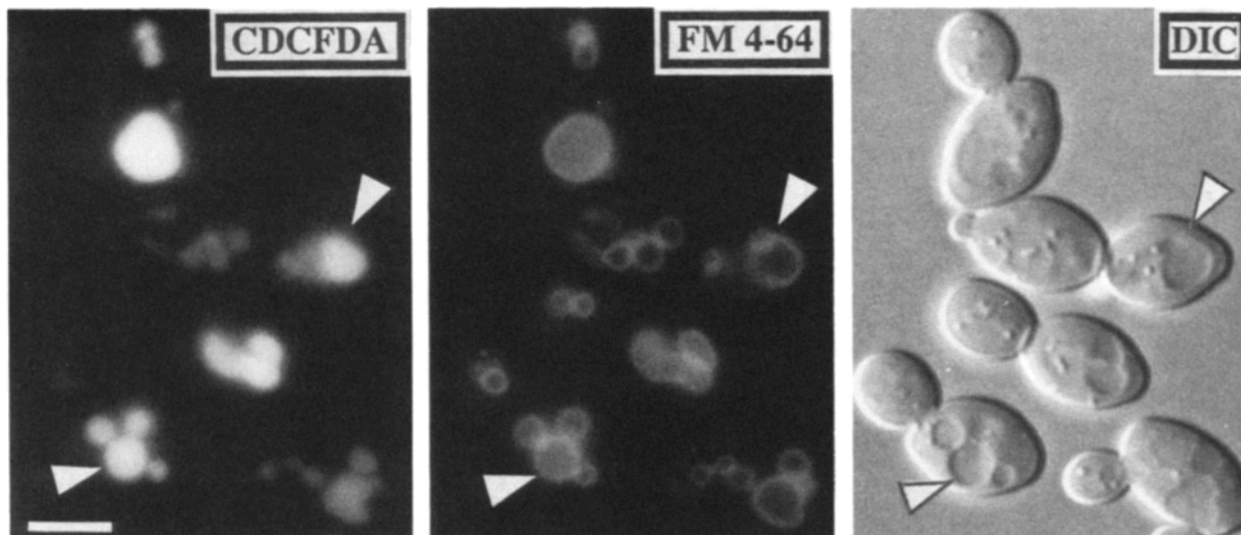
## Results

### The Styryl Dye, FM 4-64, Is a Vital Stain for the Vacuolar Membrane in Yeast

Contractile vacuoles of *Dictyostelium discoideum* ameba have been vitally stained with the styryl dye FM 4-64 (Heuser et al., 1993). To determine if this potential sensitive dye would stain the vacuolar membrane of *S. cerevisiae*, we treated a diploid strain (BHY10.5) with FM 4-64. This dye selectively labeled the membrane of structures within the cell that corresponded to the vacuole as viewed by differential interference contrast optics (Fig. 1). To confirm that the vacuole membrane was stained with FM 4-64, double labeling with CDCFDA, a vital dye for the vacuole lumen (Pringle et al., 1989), was used to mark the vacuoles with luminal fluorescence. The apparent membrane ring staining of FM 4-64 was coincident with the internal fluorescence of CDCFDA in the vacuole lumen (Fig. 1). From this double-labeling analysis, we concluded that FM 4-64 was a specific and sensitive dye for the vacuolar membrane in yeast.

### An Endocytic Mechanism Transports FM 4-64 from the PM to the Vacuolar Membrane

Temperature has played a key role in dissecting receptor-mediated endocytic events in animal cells. Reduced temperatures (<4°C) block ligand internalization while ligand

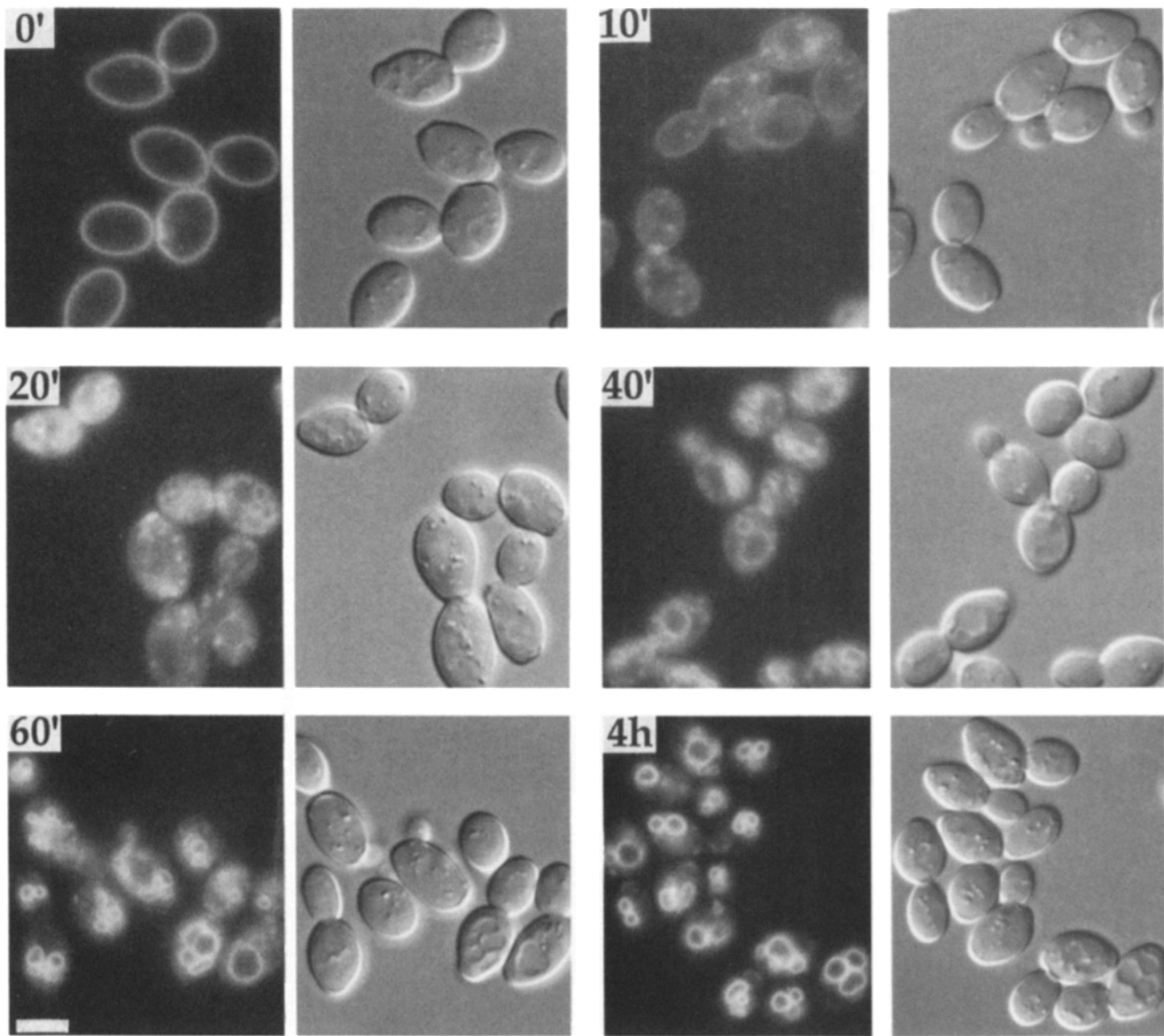


**Figure 1.** The styryl dye, FM 4-64, stains the vacuolar membrane in yeast. A diploid strain (BHY10.5) was incubated at 30°C for 15 min with 40 μM FM 4-64 in YPD media. The cells were harvested with centrifugation, the free dye was removed, fresh YPD media was added, and the cells were incubated further at 30°C. After 30 min, the media pH was adjusted to 4.0, CDCFDA was added (10 μM final concentration), and the cells were incubated an additional 15 min at 30°C. The cells were then viewed under a fluorescence microscope with filters for CDCFDA, FM 4-64, or DIC optics as indicated. Arrows indicate examples of coincident staining between CDCFDA and FM 4-64. Bar, 5 μm.

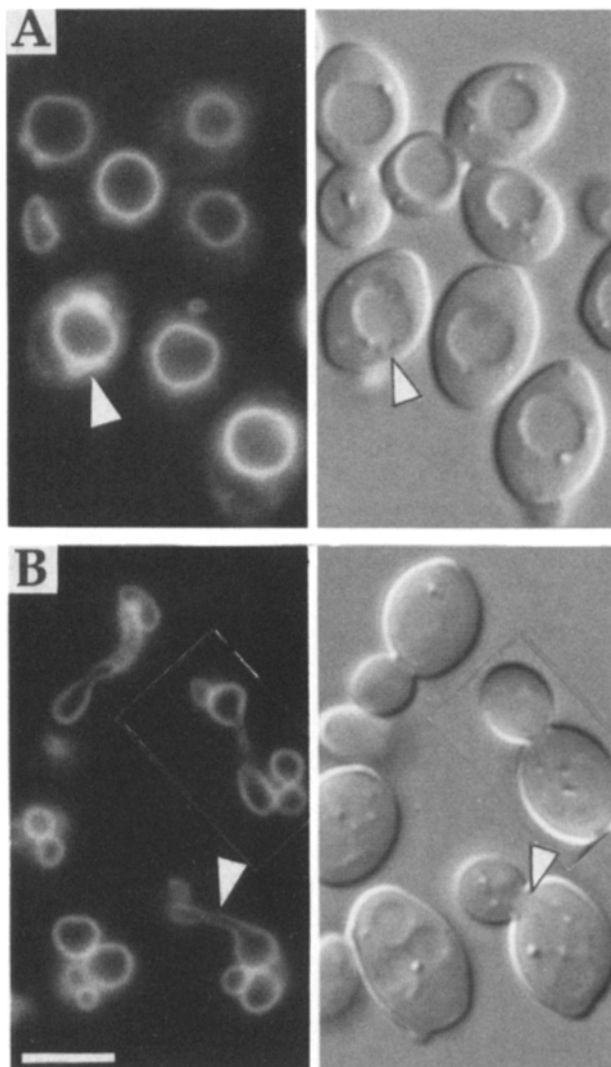
binding is unaffected. The earliest characteristics of PM internalization can be observed in cells after warming. To determine if an endocytic mechanism was used for internalization of FM 4-64 in yeast, we stained cells at 0°C, warmed them to 25°C, and followed dye transport over time with fluorescence microscopy. After 30 min at 0°C, FM 4-64 exclusively stained the cell perimeter, consistent with its possible insertion into the PM (Fig. 2, 0'). Washing away the free FM 4-64 followed by warming the cells allowed us to perform pulse/chase experiments and track internalization of the dye over time. After just 10 min at 25°C, the PM signal of FM 4-64 decreased while small discrete fluorescent dots appeared in the cytoplasm (Fig. 2, 10'). Within the next 10 min of incubation at 25°C, these structures were still visible (Fig. 2, 20'). After 40 min at 25°C, the number of small

punctate structures decreased concomitant with vacuolar membrane staining, which increased even further after 60 min (Fig. 2, 40' and 60'). The vacuolar membrane staining by FM 4-64 persisted for more than 4 h of growth (almost three generations, Fig. 2, 4h). This kinetic analysis of dye uptake closely resembled the expected pattern of an endocytic pathway. Particularly striking was the appearance of the presumptive endocytic intermediates, which previously had not been observed in yeast. These fluorescent dots did not appear to exhibit the punctate nature of the Golgi complex in yeast (Franzusoff et al., 1991), or the polarized pattern observed during endocytic uptake of the *STE2* gene product, the  $\alpha$ -factor receptor (Jackson et al., 1991).

FM 4-64 showed remarkable stability in the vacuolar membrane, persisting for as long as 4 h in an environment



**Figure 2.** Kinetics of FM 4-64 internalization from the PM and transfer to the vacuole. BHY10.5 cells were continuously shaken with 80  $\mu$ M FM 4-64 for 30 min at 0°C in YPD media. An aliquot was removed and immediately photographed (indicated as 0'). The remaining cells were washed, resuspended in fresh media, placed at 25°C, and aliquots were removed and photographed at the times indicated. The left side of every panel is FM 4-64 fluorescence while the right side is DIC optics. Bar, 5  $\mu$ m.



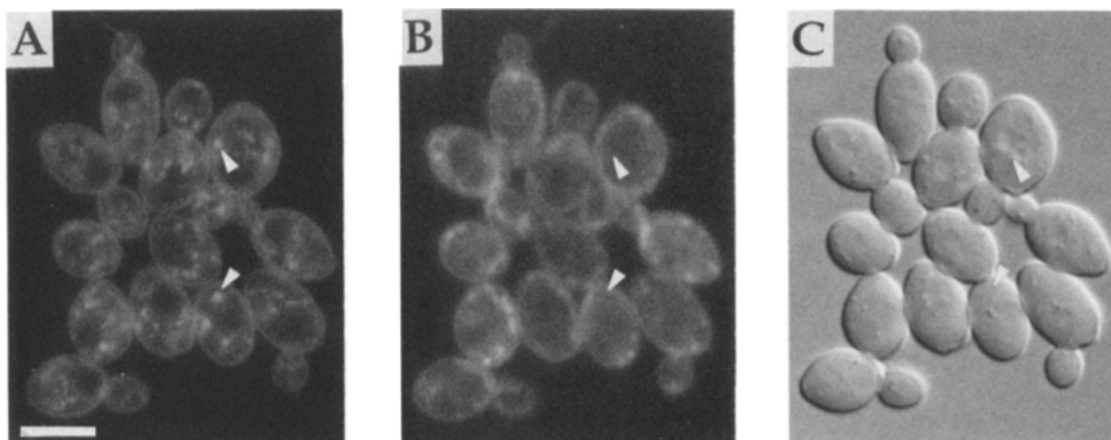
**Figure 3.** FM 4-64 is stable and follows vacuolar membrane dynamics. BHY10.5 cells were stained with FM 4-64 as described previously (see Fig. 1). (A) After incubation for 45 min at 30°C,

rich in hydrolytic enzymes (Fig. 2, 4h). To further study the stability of FM 4-64, we stained cells and then allowed the culture to reach stationary phase. After 24 h (~16 cell generations), the vacuoles, extremely spherical due to the starved condition of the cells, remained brightly fluorescent indicating that the dye was not degraded (Fig. 3 A). However, within 30 min of restoring the cells to fresh media, vacuole morphology changed dramatically showing fragmentation and segregation structure formation (Fig. 3 B). The FM 4-64-staining persisted during these events and served as a sensitive, stable marker following these physiologically dynamic processes of the vacuolar membrane.

#### **FM 4-64 Does Not Stain Mitochondria**

The apparent specificity of FM 4-64 for the vacuolar membrane was unexpected. Depending on growth conditions, yeast mitochondria can appear as dots or tubules near the cell periphery (McConnell et al., 1990). Because FM 4-64 is a membrane potential-sensitive styryl dye, mitochondria were one possibility for the identity of the punctate dot-like structures seen early after warming cells to 25°C (Fig. 2, 10'). Additionally, fluorescently labeled phosphatidylcholine can stain mitochondria in yeast (Kean et al., 1993). To examine this further, we compared FM 4-64 staining to the staining pattern of DASPMI, a membrane potential sensitive mitochondrial dye (Bereiter-Hahn, 1976). Cells were stained at 0°C with FM 4-64 and warmed to 25°C for 10–15 min, to observe the punctate pattern of discrete dots (Fig. 4 A). This pattern was distinct from the tubule mitochondrial structures stained with DASPMI (Fig. 4 B). These double staining experiments revealed no overlaps between FM 4-64 and DASPMI in the same cell. Furthermore, mitochondrial staining by DASPMI (Fig. 4 B) was observed even at 0°C, conditions where FM 4-64 exclusively labeled the PM (Fig.

the cells were placed at 23°C for 24 h and photographed. (B) Fresh media was added to the cells in A, they were incubated at 30°C for 30 min, and photographed. The left side of each panel is FM 4-64 fluorescence while the right side is DIC optics. Bar, 5 μm.



**Figure 4.** FM 4-64 is not internalized from the PM to mitochondrial membranes. BHY10.5 cells were first stained at 23°C for 3 min with DASPMI. The cells were then stained at 0°C for 30 min with FM 4-64. An aliquot was removed, allowed to warm-up on a microscope slide for 10 min, and photographed. (A) FM 4-64 staining; (B) DASPMI staining; and (C) DIC optics. Arrows point to representative examples of the noncoincident staining patterns. Bar, 5 μm.

2, 0). From this analysis, we concluded that FM 4-64 does not stain mitochondrial membranes and it enters the cell by a different mechanism than DASPMI.

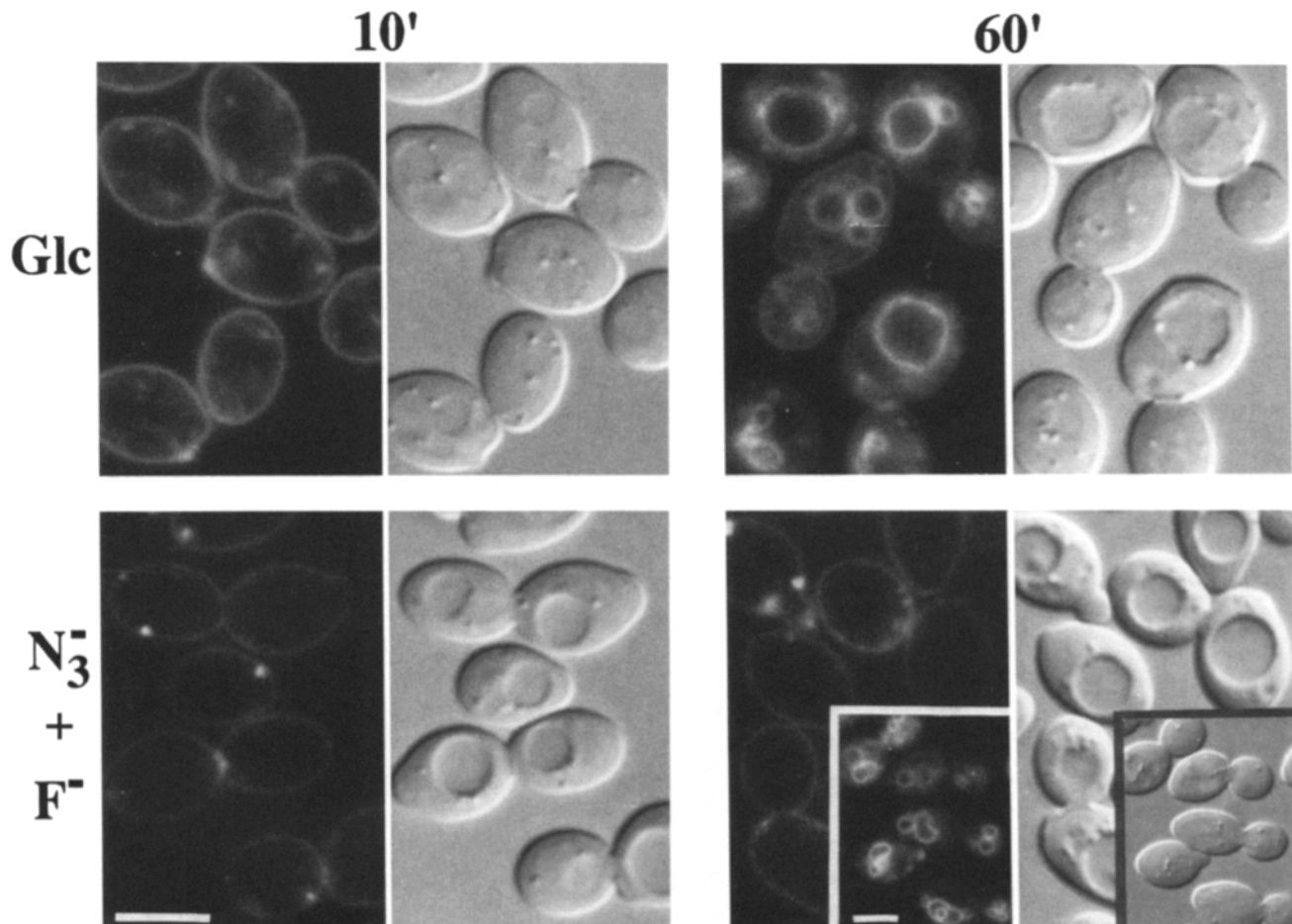
#### FM 4-64 Uptake from the PM Is Energy Dependent

To further characterize the apparent endocytic behavior of FM 4-64 uptake, we determined if FM 4-64 transport from the PM, to the small punctate organelles, and subsequently to the vacuolar membrane was energy dependent. Cells were incubated at 30°C in rich media with glucose or in rich media with 10 mM NaN<sub>3</sub>, 10 mM NaF as energy poisons to block all ATP production. After 10 min in the glucose media, transport from the PM to the small punctate structures was observed, which eventually gave rise to vacuolar membrane staining within 60 min (Fig. 5). In contrast, when energy poisons were present, only PM staining was observed (Fig. 5). Dot-like staining and vacuolar membrane staining was completely inhibited even after 60 min in the presence of energy poisons (Fig. 5). Additionally, the energy-depleted cells showed a marked decrease in fluorescence intensity. This is most likely due to rapid photobleaching when the membrane potential of the PM was lost as a result of energy

depletion. To test this, cells were lysed after 60 min of NaN<sub>3</sub>/NaF treatment and the associated FM 4-64 was measured in a spectrofluorometer. Energy starved cells still bound as much as 79% of the dye compared to cells treated with glucose, suggesting the decrease in staining intensity (Fig. 5) was not due to less cell associated FM 4-64. Vacuole morphology was also sensitive to the loss of cellular ATP. The organelle appeared as a single large spherical compartment (Fig. 5) similar to that seen in starved cells (Fig. 3 A). Importantly, the presence of energy poisons was not lethal to the cells even after 60 min of treatment. When the cells were washed free of NaN<sub>3</sub>/NaF and supplied with fresh glucose media, normal vacuolar membrane staining of FM 4-64 was observed within another 60 min of incubation (Fig. 5, inset). Therefore, as expected for an endocytic uptake process, membrane delivery of FM 4-64 from the PM to the vacuole was energy dependent.

#### Internalization at 15°C Allows Trapping FM 4-64 in Endocytic Intermediates

Another characteristic of endocytosis is the effect intermediate temperatures have on transport rates from the PM to in-



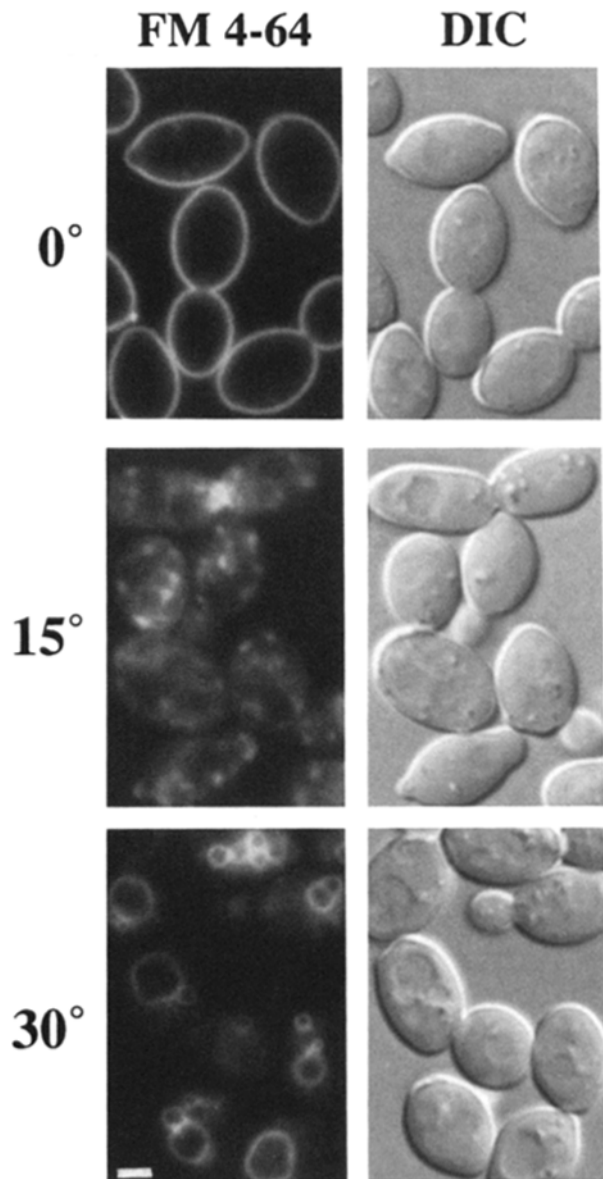
**Figure 5.** Uptake of FM 4-64 and vacuolar membrane staining is energy dependent. A culture of BHY10.5 was harvested, washed once with water, once with YP media, resuspended in YP media, and divided in two equal cultures. One culture received glucose at 2% final concentration (*Glc*) and the other received NaN<sub>3</sub> plus NaF, each at 10 mM final concentration (*N<sub>3</sub><sup>-</sup> + F<sup>-</sup>*). FM 4-64 was added and the cultures were incubated at 30°C. Aliquots from each culture were removed and photographed at 10' and 60' as indicated. The left side of each time point panel is FM 4-64 fluorescence while the right side is DIC optics. Inset shows reversal of the NaN<sub>3</sub> and NaF block after washing away the poisons and restoring the cells to fresh glucose for 60 min. Bar, 5 μm.



tracellular destinations. In yeast, the transport rate of internalized  $\alpha$ -factor is sufficiently slowed at 15°C as to allow significant trapping of  $\alpha$ -factor in an endocytic compartment between the PM and the vacuole (Singer and H. Riezman, 1990). We tested these temperature conditions on FM 4-64 internalization and vacuolar membrane delivery. After loading the PM with FM 4-64 at 0°C (Fig. 6, 0°), cells were shifted to 15°C for 25 min resulting in the apparent accumulation of intermediate transport structures (Fig. 6, 15°). This appearance was not observed after shifting the cells from 0° to 30°C for 25 min where the vacuolar membrane was stained (Fig. 6, 30°). If cells were placed back at 0°C after the 15°C incubation, the fluorescent intermediates between the PM and the vacuole remained stable and discrete for periods of up to 2 h (data not shown). This permitted a detailed kinetic analysis of FM 4-64 delivery from the 15°C compartment to the vacuole within the same cell. After shifting cells from 15° to 0°C, they were placed on a microscope slide and observed to contain numerous small fluorescent structures throughout the cytoplasm (Fig. 7, 0'). Within 2 min of warming to 25°C, some of these structures disappeared concomitant with the vacuole membrane becoming weakly fluorescent (Fig. 7, 2'). This observation continued during the next 2 min and by 6 min very few of the 15°C intermediates were visible leaving only vacuole membrane fluorescence (Fig. 7, 6'). After 12 min of incubation, the 15°C intermediates were undetectable, leaving the vacuole membrane exclusively fluorescent (Fig. 7, 12'). From this kinetic analysis, we concluded that FM 4-64 could mark two endocytic steps in yeast; (a) transport from the PM to the cytoplasmic intermediate endosomes; and (b) transport from these intermediate structures to the vacuole.

#### FM 4-64 Staining in the Morphological Classes of Vacuolar Protein Sorting (*vps*) Mutants

The morphology of the yeast vacuole changes continually due to the dynamic behavior of this organelle throughout the cell cycle. Mutants defective for vacuolar protein sorting, *vps*, in addition to mislocalizing vacuolar hydrolases also show defects in proper maintenance of vacuole morphology (Banta et al., 1988). The *vps* mutants were first classified into three morphological groups (A–C) with the use of vital stains and electron microscopy (Banta et al., 1988). Immunofluorescence localization of two vacuolar membrane proteins, alkaline phosphatase and the H<sup>+</sup>-ATPase, divided the mutants into three additional morphological groups (D–F) (Raymond et al., 1992). The wild-type (class A) morphology was observed in *vps35* (Fig. 8 A); fragmented vacuoles (class B) were seen in *vps17* (Fig. 8 B); very small cytoplasmically dispersed “specks” of fluorescence (class C) were detected in *vps18* (Fig. 8 C); a single enlarged vacuole (class D) was visible in *vps15* (Fig. 8 D); a mix of fragmented vacuoles and one to three larger vacuoles (class F) were stained in *vps1* mutants (Fig. 8 F). In a class E *vps* mutant, *vps27*, the vacuole was only weakly stained but an ~750 nm membrane-enclosed structure was intensely stained (Fig. 8 E). This organelle is thought to correspond to a prevacuolar intermediate compartment that becomes exaggerated in class E mutants (Raymond et al., 1992). Additionally, class E *vps* mutants have been shown to accumulate the  $\alpha$ -factor receptor (*STE3* gene product) in this compartment (Davis et al., 1993;

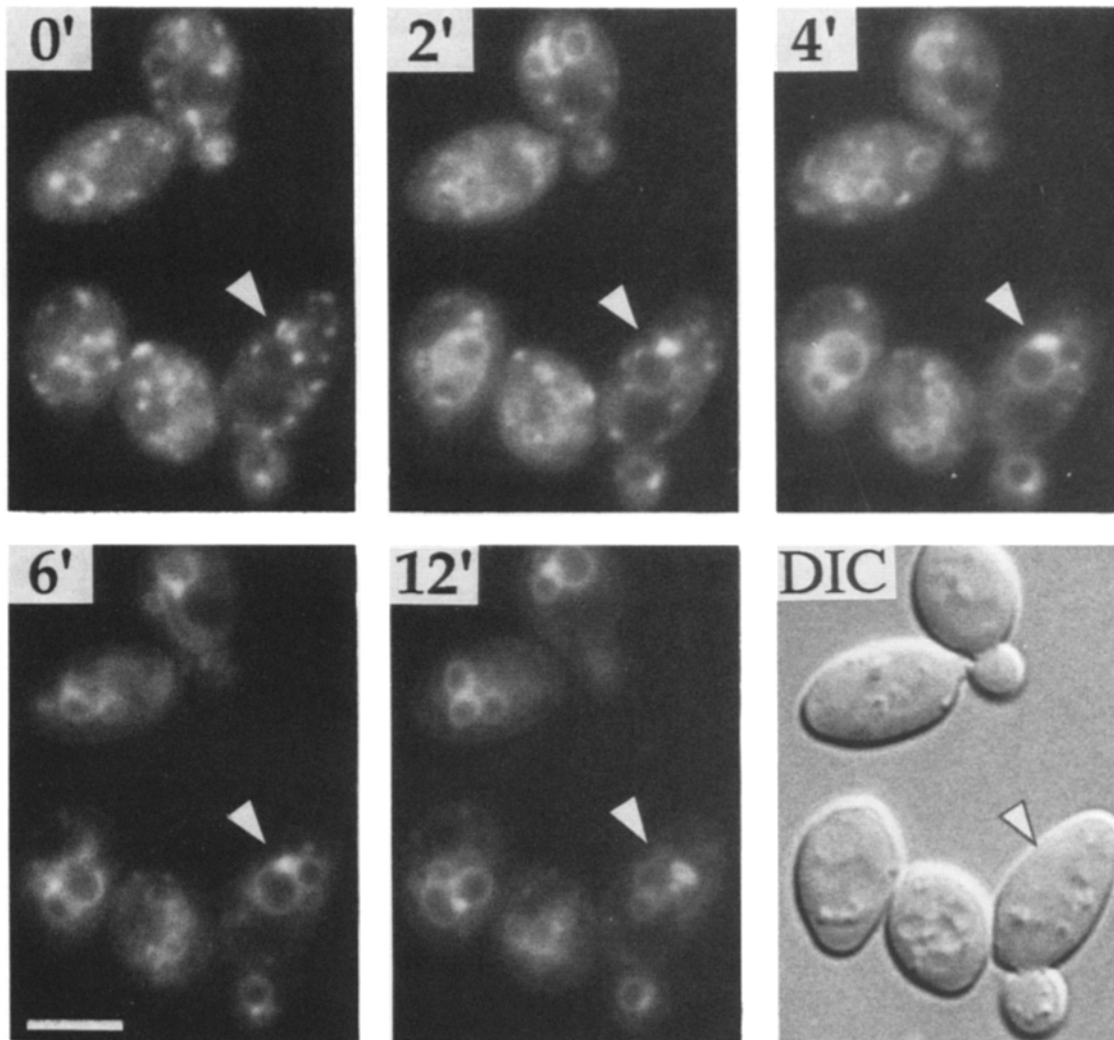


**Figure 6.** Internalization at 15°C traps FM 4-64 in endosomal intermediates. BHY10.5 cells were stained with FM 4-64 for 30 min at 0°C. An aliquot was removed and immediately photographed (0°C). The remaining cells were harvested, resuspended in fresh media, and split in half. One half was incubated at 30°C while the other was incubated at 15°C (as indicated) for 25 min. The sample at 15°C was placed back at 0°C (to stop all membrane transport) before photography for FM 4-64 fluorescence and DIC optics as indicated. Bar, 2.5  $\mu$ m.

Raths et al., 1993). Thus, in class E *vps* mutants, FM 4-64 appears to become trapped in the same endocytic intermediate that contains internalized  $\alpha$ -factor receptor molecules.

#### Effects of *sec* and *end* Mutants on FM 4-64 Internalization

In all *vps* mutants, FM 4-64 labeled the vacuole with an expected pattern, suggesting that internalization from the PM was not severely impaired. We examined certain of the secre-



**Figure 7** Transport kinetics from the 15°C intermediate compartment to the vacuole. BHY10.5 cells were stained with FM 4-64 at 0°C and internalized at 15°C as described previously (see Fig. 6). An aliquot was removed from ice to a microscope slide and photographs were taken at two min intervals as indicated. The arrows mark disappearance of fluorescence in the intermediate “dot-like” compartments and appearance of fluorescence in the vacuole membrane within a single cell. Bar, 5  $\mu\text{m}$ .

tion defective (*sec*) yeast mutants to determine if they showed defects in internalization or localization of FM 4-64 to the vacuolar membrane. After preshifting these temperature-sensitive mutants to the restrictive temperature (37°C) for 30 min, they were incubated with FM 4-64. Neither the *sec18*, (blocked in ER-to-Golgi transport) nor the *sec14* (Golgi blocked) mutants inhibited vacuolar staining by FM 4-64 at 37°C (Fig. 9). The *sec18* mutant did show reduced intensity of vacuolar membrane staining, suggesting that the overall transport efficiency of FM 4-64 may have been affected. In contrast, the *sec1* mutant (blocked in vesicle-to-PM transport) did not show vacuolar staining at 37°C but instead exhibited diffuse cytoplasmic staining with some prominent punctate dots (Fig. 9). At 23°C, the vacuolar membrane was stained normally, in *sec1* mutant cells (Fig. 9). The block of vacuolar membrane fluorescence at 37°C in the *sec1* mutant was reversible. After shifting cells back to 23°C, vacuolar membrane staining was observed (data not shown). Similar results were obtained with the *sec6*, and *sec15* mutants that also block secretory vesicles from fusing with the PM (data not shown). From this analysis, no *sec*

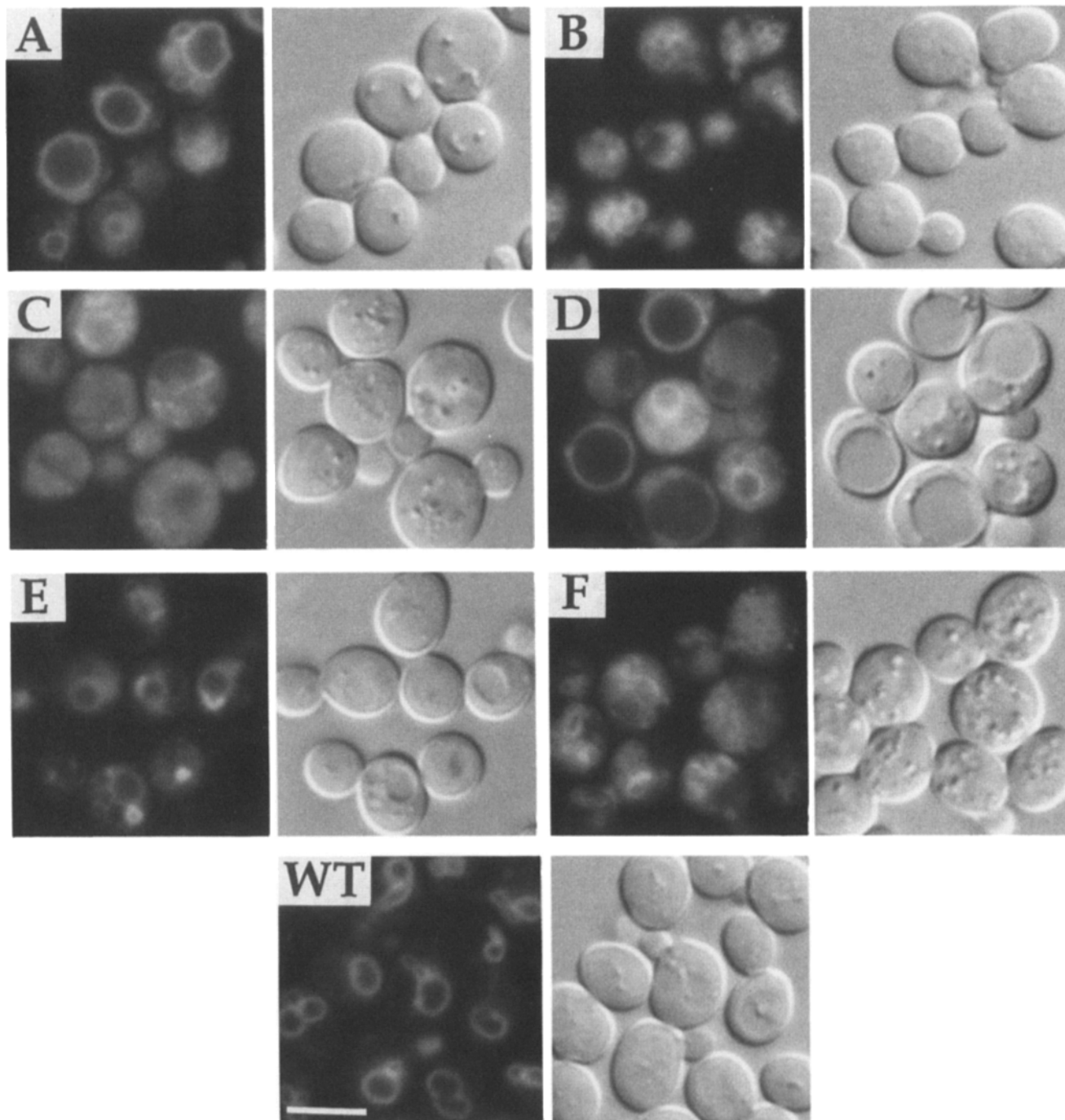
mutant blocked internalization of FM 4-64 from the PM and only late-acting *sec* mutants blocked vacuolar delivery of internalized FM 4-64.

Recently, two yeast mutants, *end3* and *end4*, were isolated that specifically show defects in  $\alpha$ -factor internalization as well as an apparent defect in fluid-phase endocytosis of Lucifer yellow (Raths et al., 1993). To determine if the *end3* and *end4* mutants also exhibited defects in FM 4-64 uptake or vacuolar membrane staining, we stained these mutant cells at the permissive (23°C) and restrictive (37°C) temperatures. Both mutants did not show defects in vacuolar membrane staining and appeared identical to wild-type cells under the same temperature conditions (Fig. 10). This suggested that FM 4-64 could enter the cell independent of *END3* and *END4* gene functions.

#### ***In Vitro* Reconstitution of Transport from the 15°C Intermediate Compartment to the Vacuolar Membrane**

Internalization of PM-bound FM 4-64 at 15°C for 25 min resulted in accumulation of the dye in an intermediate com-

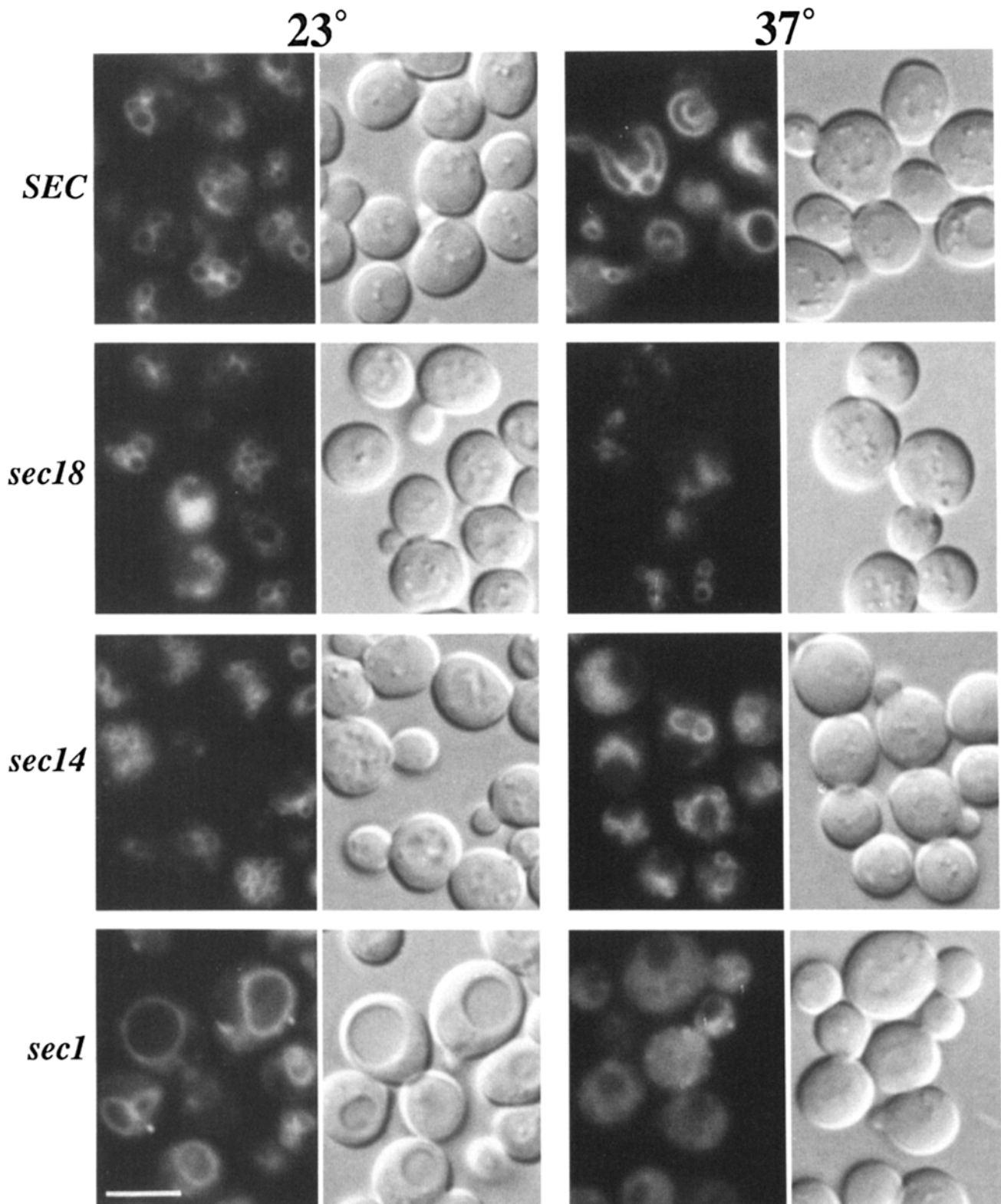




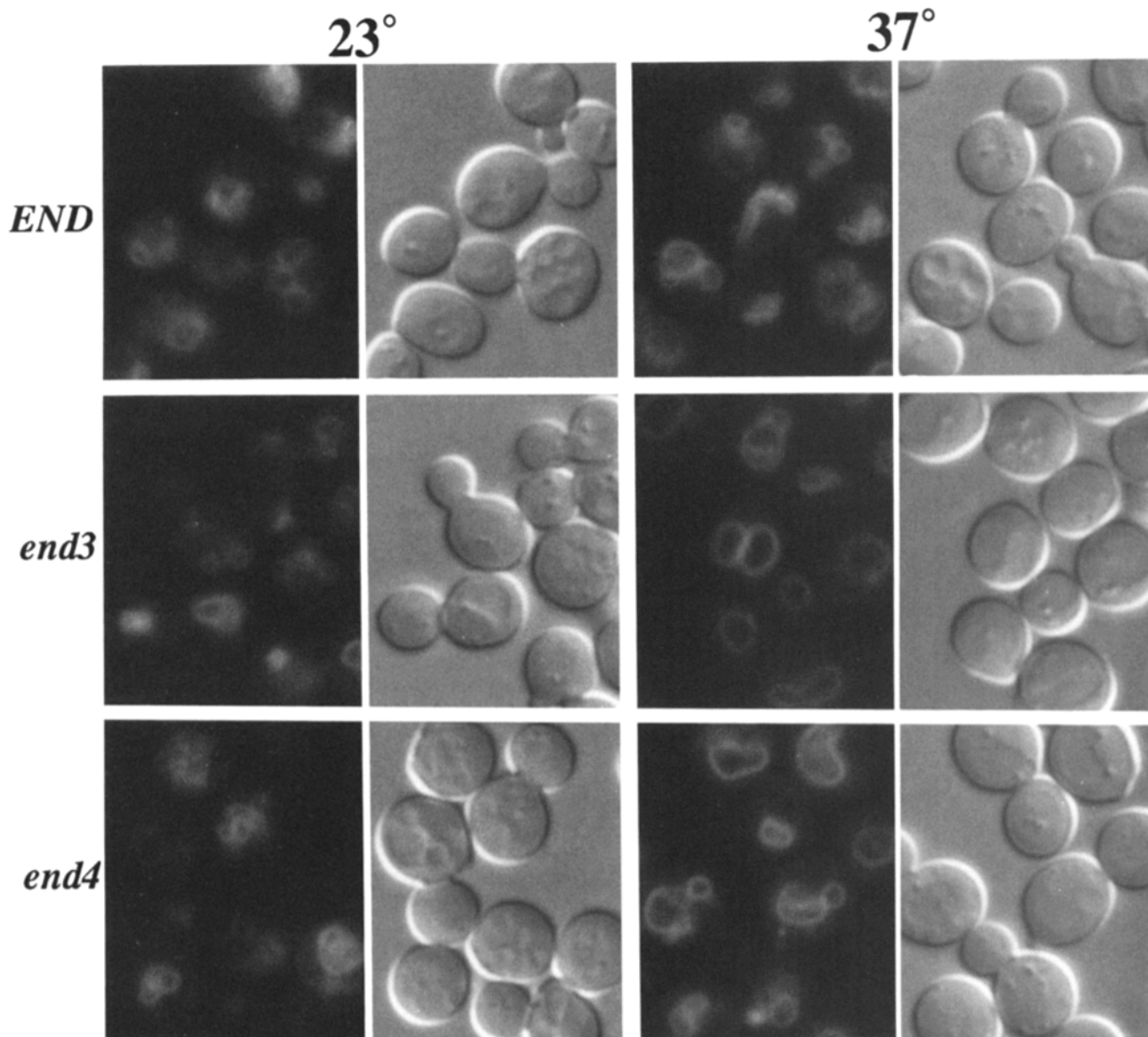
**Figure 8.** FM 4-64 staining in the morphological classes of *vps* mutants. A representative allele of each *vps* mutant class was pulsed 15 min with FM 4-64 followed with a 45 min chase in YPD media. (A) Class A, GPY1135  $\Delta vps35$ ; (B) class B, KKY10  $\Delta vps17$ ; (C) class C, JSRY18-1,  $\Delta vps18$ ; (D) class D, PHY112,  $\Delta vps15$ ; (E) class E, SEY27-1, *vps27*; (F) class F, SEY1-3, *vps1*; and (WT) SEY6210. Bar, 5  $\mu\text{m}$ .

partment (see above). The transfer from this intermediate to the vacuole upon warming occurred within 2–6 min and appeared to represent a vesicular transport event (Fig. 7). To further test the apparent vesicular nature of this reaction, we determined if this process could be reconstituted *in vitro*. We have previously reconstituted intercompartmental protein transport to the vacuole from the secretory pathway using a permeabilized yeast cell system (Vida et al., 1990). To test for transport of FM 4-64 in permeabilized cells, spheroplasts were first loaded with FM 4-64 at 0°C, internalized at 15°C,

and then subjected to permeabilization by freeze/thaw and a series of hypotonic washes. After spheroplasts were washed under conditions that removed >98% of the cytosol (Vida et al., 1990, 1993), they were incubated at 25°C for 60 min. A diffuse cytoplasmic pattern of FM 4-64 staining was observed (Fig. 11). The FM 4-64 staining was distinct from the vacuolar staining observed with CDCFDA under identical permeabilization conditions (Fig. 11). When the permeabilized cells were incubated with exogenous cytosolic protein extracts or ATP, the diffuse FM 4-64 staining pattern ap-



**Figure 9.** Late acting but not early acting *sec* mutants block vacuolar membrane staining of FM 4-64. Wild-type and *sec* mutant strains (as indicated) were grown at 23°C and the cultures were split in half. One half remained at 23°C while the other was shifted to 37°C for 30 min. The cultures were then pulsed for 15 min with FM 4-64 and chased for 45 min at either 23° or 37°C. Photographs were then taken, keeping the restrictive cultures at 37°C until just prior to slide preparation. The left side of each temperature panel is FM 4-64 fluorescence while the right side is DIC optics. Bar, 5  $\mu$ m.



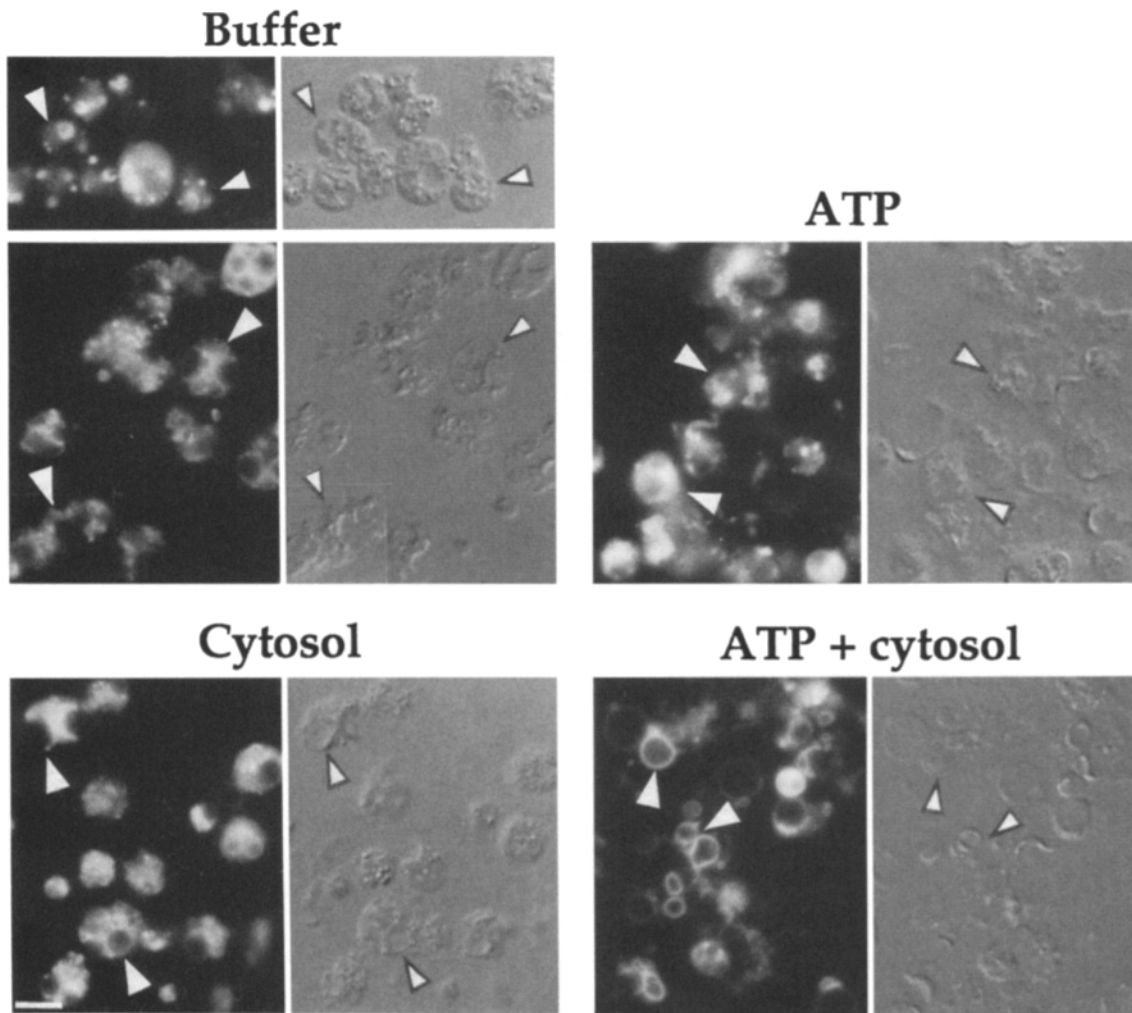
**Figure 10.** Two *end* mutants do not block vacuolar membrane staining of FM 4-64. Wild-type and *end* mutant strains (as indicated) were grown at 23°C and the cultures were split in half. One half remained at 23°C while the other was shifted to 37°C for 10 min. The cultures were then pulsed for 15 min with FM 4-64 and chased for 45 min at either 23° or 37°C. Photographs were then taken, keeping the restrictive cultures at 37°C until just prior to slide preparation. The left side of each temperature panel is FM 4-64 fluorescence while the right side is DIC optics. Bar, 5  $\mu$ m.

peared similar to incubation in buffer alone (Fig. 11). However, when ATP and cytosol were added together, the FM 4-64 fluorescence redistributed to the vacuolar membrane (Fig. 11). This suggested that *in vitro* transport of FM 4-64 from the intermediate compartment to the vacuole requires both energy and cytosolic protein and thus may correspond to a vesicle-mediated transport event.

### Discussion

In this report, we have explored the staining and transport properties of a styryl dye, FM 4-64, in yeast cells for the first time. Previously, FM 4-64 has been used to follow synaptic vesicle recycling in the frog neuromuscular junction (Betz et al., 1992a) and osmotic regulation in *D. discoideum* (Heuser

et al., 1993). Under steady-state conditions in yeast, FM 4-64 specifically stains the vacuolar membrane. Delivery of the dye to the vacuole appears to follow an endocytic transport route. During a time course of FM 4-64 staining, we observed: (a) initial PM staining followed by; (b) cytoplasmic punctate staining, which finally chased to; (c) staining of the vacuole membrane. Similar to other vesicle-mediated transport events, the internalization of FM 4-64 and transport of the dye to the vacuole exhibited energy, time, and temperature dependence. Energy depletion lead to PM staining exclusively (Fig. 5). At 0°C, FM 4-64 accumulated in the PM while at 15°C the dye accumulated in small punctate intermediate compartments that do not correspond to the vacuole or other previously characterized organelles in yeast. Other vital stains like DASPMI or CDCFDA show no



**Figure 11.** Reconstitution of membrane transport from the 15°C intermediate compartment(s) to the vacuole with permeabilized cells in vitro. Spheroplasts from BHY10.5 were stained with FM 4-64 using the same conditions as described previously (see Fig. 6, and Materials and Methods). The cells were then subjected to permeabilization with one freeze-thaw cycle and four different washes to remove endogenous cytosol. Incubations with buffer alone, ATP, exogenous cytosol, and ATP plus exogenous cytosol were performed for 60 min as indicated. All ATP samples included a regeneration mix and cytosolic protein was added back at 5 mg/ml. The upper panel (*Buffer*) shows the same cells stained with CDCFDA demonstrating the presence of vacuoles, all other panels show FM 4-64 fluorescence. Bar, 5  $\mu$ m.

temperature dependent characteristics. These observations support a model in which FM 4-64 staining of the vacuole membrane is mediated by endocytic uptake of the dye and vesicle-mediated transport via an endosome-like intermediate organelle. Thus, FM 4-64 represents a useful marker in yeast to visualize bulk lipid transport from the PM to the vacuole.

The unexpected specificity of FM 4-64 for vacuolar membranes and not other organelles underlies its use as a precise intracellular marker. This specificity also implies that FM 4-64 mirrors a natural pathway for endocytosis of endogenous PM lipids and proteins. Using fluorescent-labeled phosphatidylcholine, an endocytic pathway to the vacuole and a nonendocytic pathway to mitochondria have been described in yeast (Kean et al., 1993). FM 4-64 appears to follow an endocytic pathway. However, other possibilities could explain the characteristics of FM 4-64 internalization. For instance, the cytoplasmic intermediate structures could be lipid droplets or large complexes of lipid carrier proteins.

These would need to selectively extract components out of the PM (along with FM 4-64) and specifically shuttle them to the vacuole. These two characteristics argue against a lipid droplet but a specific carrier protein complex could possibly perform such a task (Pagano, 1990). This putative carrier protein would need to transfer significant amounts of phospholipid to maintain FM 4-64 fluorescence in the cytoplasm because styryl dyes like FM 4-64 are preferentially fluorescent in lipid bilayers (Betz et al., 1992a). Furthermore, the fluorescent intermediates trapped at 15°C were not washed out of the cytoplasm under conditions that remove >98% of cytosolic protein when making permeabilized cells (Vida et al., 1990), which further argues against a carrier protein. This makes a carrier protein mechanism somewhat unlikely. The data favor a model in which endosomes or clusters of endocytic vesicles function as intermediates in transport of FM 4-64 from the PM to the vacuole.

Until recently, endocytosis has been difficult to study in yeast. Yeast, unlike mammalian cells, do not internalize a

wide array of physiological ligands that can serve as endocytic markers for receptor-mediated or fluid-phase events. For this reason, enveloped viruses (Makarow, 1985a),  $\alpha$ -amylase, fluorescent dextrans (Makarow, 1985b), and LY have all been used to study endocytic events in yeast (Riezman, 1985). Receptor-mediated internalization of the  $\alpha$ - and  $\alpha$ -factor mating pheromones is the most reliable measure for biologically relevant endocytic events in yeast. Two mutants defective in receptor-mediated internalization of  $\alpha$ -factor (*end3* and *end4*) have been reported (Raths et al., 1993). The *end3* and *end4* mutants also show defects in LY uptake, suggesting that they also affect fluid-phase endocytosis (Raths et al., 1993). Other yeast mutants like *secl8*, *sec7*, and *secl4*, have also been reported to block LY from staining the vacuole (Riezman, 1985). However, these mutants did not completely block FM 4-64 uptake and transport to the vacuole membrane. This might suggest that FM 4-64 enters the cell by a non fluid-phase uptake mechanism. It should be noted, however, low rates of fluid phase endocytosis could go undetected with LY as a fluid phase marker (Riezman, H., personal communication). Thus, the lack of a detectable LY signal in the vacuole of the *end3* and *end4* mutants may not indicate a complete block in fluid phase uptake.

Surprisingly, FM 4-64 was blocked from reaching the vacuole in the late acting *sec* mutants, which accumulate Golgi-derived secretory vesicles. In these late *sec* mutants (e.g., *secl*) FM 4-64 was observed as diffuse punctate staining in the cytoplasm, suggesting that internalization of FM 4-64 is not blocked in late *sec* mutants but transfer of the dye to the vacuole from these cytoplasmic intermediates is blocked. These observations suggest that certain of the late acting *SEC* gene products may also function in the early endocytic pathway (Riezman, 1985). Distinct gene products may facilitate the pathway for FM 4-64 uptake and transport to the yeast vacuole.

The selective vacuole membrane staining by FM 4-64 provides a major improvement in the visual detection of vacuolar organelle morphology and dynamics. Vital stains like FITC, the endogenous fluorophore in *ade2* mutants, and CDCFDA have played important roles in studying vacuole morphology and inheritance (Weisman et al., 1987). However, these dyes share similar disadvantages in that they are lumenally sequestered and all passively diffuse across membranes into the vacuole independent of vesicle carriers. FM 4-64 is the first vital stain specific for the vacuolar membrane and its use allows unencumbered views of organelle structure. For instance, with luminal stains, the distinct identity of small vacuoles clustered together is often difficult to observe. However, with just the vacuole membrane stained by FM 4-64, these types of structural variations are easily resolved and identified.

The morphological classes of *vps* mutants also support the vacuolar membrane specificity of FM 4-64. With certain *vps* mutants, the vacuole follows predictable albeit aberrant structural changes, which is most severe in class B and C *vps* mutants (Banta et al., 1988; Raymond et al., 1992). FM 4-64 staining in all *vps* mutants tested gave the predicted pattern of change observed for each mutant class. Most interesting was the class E morphology where a distinct organelle proximal to the vacuole stained intensely with FM 4-64. This is very similar in appearance to the initial demonstration of this compartment using antibodies against the vacuo-

lar  $H^+$ -ATPase for immunofluorescence staining (Raymond et al., 1992). Remarkably, FM 4-64 localization in a class E *vps* mutant was nearly identical in appearance to the localization pattern observed for the  $\alpha$ -factor receptor (*STE3* gene product) in the class E *ren1* (*vps2*) mutant, defective in receptor endocytosis and turnover (Davis et al., 1993). The apparent increased concentration of FM 4-64 in this compartment most likely reflects decreased membrane transport to the vacuole or fluorescent instability due to membrane potential decrease of the vacuolar membrane. Thus, in class E *vps* mutants, transport to the vacuole appears to be impaired.

Using FM 4-64 as a marker, a new pathway in yeast membrane traffic has been opened up to biochemical and genetic analysis. The permeabilized cell system we developed to study biosynthetic protein traffic to the vacuole (Vida et al., 1990, 1993) and formation of vacuole segregation structures (Conradt et al., 1992) is now useful for studying transport from the 15°C endocytic intermediate compartment to the vacuole (Fig. 11). Internalized label was not washed out of the cell along with cytosolic proteins, indicating the intermediate compartment retains strong association with permeabilized cells, possibly to cytoskeletal components. The ATP and cytosol dependence of in vitro vacuolar membrane staining with FM 4-64 indicates that at the simplest level, a fusion reaction must take place between the intermediates and the vacuole. It seems more likely, however, that vesicles bud from the intermediate compartment(s) and mediate transport of dye to the vacuole. Further evidence will be required to prove that a vesicle-mediated transport reaction is reconstituted between the 15°C endocytic intermediate compartment and the vacuole. Using recently developed procedures to purify endocytic intermediates from yeast (Vida et al., 1993; Singer-Kruger et al., 1993), it may be possible to isolate and characterize the putative vesicles that bud from FM 4-64-labeled intermediates. Additionally, FM 4-64 could be a useful marker for fluorescence activated cell sorting to isolate yeast mutants that cannot take up the dye.

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