A Novel Fluorescence-activated Cell Sorter-Based Screen for Yeast Endocytosis Mutants Identifies a Yeast Homologue of Mammalian eps15

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Abstract. A complete understanding of the molecular mechanisms of endocytosis requires the discovery and characterization of the protein machinery that mediates this aspect of membrane trafficking. A novel genetic screen was used to identify yeast mutants defective in internalization of bulk lipid. The fluorescent lipophilic styryl dye FM4-64 was used in conjunction with FACS® to enrich for yeast mutants that exhibit internalization defects. Detailed characterization of two of these mutants, dim1-1 and dim2-1, revealed defects in the endocytic pathway. Like other yeast endocytosis mutants, the temperature-sensitive dim mutants were unable to endocytose FM4-64 or radiolabeled α-factor as efficiently as wild-type cells. In addition, double mutants with either dim1-Δ or dim2-1 and the endocytosis mutants end4-1 or act1-1 displayed synthetic growth defects, indicating that the DIM gene products function in a common or parallel endocytic pathway. Complementation cloning of the DIM genes revealed identity of DIM1 to SHE4 and DIM2 to PAN1. Pan1p shares homology with the mammalian clathrin adaptor-associated protein, eps15. Both proteins contain multiple EH (eps15 homology) domains, a motif proposed to mediate protein–protein interactions. Phalloidin labeling of filamentous actin revealed profound defects in the actin cytoskeleton in both dim mutants. EM analysis revealed that the dim mutants accumulate vesicles and tubulo-vesicular structures reminiscent of mammalian early endosomes. In addition, the accumulation of novel plasma membrane invaginations where endocytosis is likely to occur were visualized in the mutants by electron microscopy using cationized ferritin as a marker for the endocytic pathway. This new screening strategy demonstrates a role for She4p and Pan1p in endocytosis, and provides a new general method for the identification of additional endocytosis mutants.

The plasma membrane of cells acts as an interface between the cell and the extracellular environment, and serves as an important site for communication and transduction of environmental stimuli into appropriate cellular responses. The regulation of exocytosis and endocytosis is crucial for maintaining the appropriate composition, function, and surface area of the plasma membrane during the continuous trafficking of lipids and proteins to and from this essential cellular domain.

In mammalian cells, endocytosis is responsible for both internalization of nutrients such as iron (Hemmaplardh and Morgan, 1976; Karin and Mintz, 1981) and cholesterol (Anderson et al., 1977), as well as downregulation of activated receptor–ligand complexes. There are believed to be at least three forms of endocytosis: clathrin dependent, clathrin independent, and phagocytosis (Lamaze and Schmid, 1995). The best characterized mechanism is clathrin-dependent endocytosis, in which receptors are first concentrated into clathrin-coated pits via an interaction between the cytoplasmic tail of the receptor and clathrin adaptor proteins, followed by the association of clathrin triskelions with the adaptor proteins (reviewed in Pearse and Crowther, 1987). The clathrin triskelions can self-assemble in vitro, and this property is thought to provide the mechanical force that bends the plasma membrane into a budding coated vesicle. After budding is completed, the coat is disassembled, and the adaptors and clathrin triskelions are recycled back through the cytoplasm to reassemble at a new coated pit. The uncoated vesicle then fuses with an endosome before the contents are ultimately delivered to the lysosome, and the vesicular membrane is degraded or recycled to other membrane destinations. Clathrin-independent endocytosis is less well understood, but may be mediated by two classes of vesicles: “noncoated” vesicles and caveolae (also known as plasmaleminal vesicles; Palade, 1953; Rothberg et al., 1992). Finally, phagocytosis is a more specialized form of endocytosis that involves the bulk internalization of particles by an actin-dependent engulfment process (West et al., 1989; Hewlett et al., 1994). In addition to the well-characterized function of clathrin in endocytosis, a general role for actin in the internalization
brane uptake, a common feature of all pathways of endocytosis. FACS®-based approach for a detailed analysis of endocytosis mutants in yeast, using the lipophilic styryl dye N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64) to follow bulk internalization of plasma membrane ATPase. Methanesulfonate; FM-64, N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64) in DMSO) and a 1-h chase. The cells were then subjected to a FM4-64 labeling of mutants generally have assayed for defects in the internalization of mating phenome-receptor complexes, a process that at least partially depends on the function of clathrin heavy chains (Tan et al., 1993). However, yeast cells deleted for the gene encoding clathrin heavy chain are viable, (Payne and Scheckman, 1985), but these strains internalize mating phenome receptors at a much reduced rate relative to wild-type cells (Payne et al., 1988). This suggests that, as in mammalian cells, clathrin-independent modes of endocytosis exist in yeast.

Flow cytometry was used previously to describe the endocytosis kinetics of fluorescently labeled histone in CHO cells (Murphy et al., 1982), and more recently, in a screen for endocytosis mutants in mammalian cells using fluorescently labeled transferrin as a marker for recycling endosomes (Cain et al., 1991). These studies demonstrate the use of a FACS®-based approach for a detailed analysis of endocytosis in a population of cells, as well as for the isolation of mutants in the endocytic pathway. We report a novel screen for endocytosis mutants in yeast, using the lipophilic styryl dye N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64) to follow bulk internalization of plasma membrane (Vida and Emr, 1995). Unlike previous selections, this screen was designed to isolate mutants defective in membrane uptake, a common feature of all pathways of endocytosis. FM4-64 is a suitable probe for this screen because it is an endocytic tracer that travels from the plasma membrane to the vacuolar membrane via punctate cytoplasmic intermediates in a time-, temperature-, and energy-dependent manner (Vida and Emr, 1995). We report the isolation of many new candidate endocytosis mutants, as well as the detailed characterization of two of these mutants, dim1-1 and dim2-1. Both characterized dim mutants exhibit reduced internalization of FM4-64 and the mating pheromone α-factor; additionally, actin localization is disrupted in both dim mutants.

Cloning and sequencing revealed identity of DIM1 with SHE4 and of DIM2 with PAN1. So far, no function has been described for PAN1, but the sequence contains motifs that are present in the mammalian clathrin adaptor-associated protein eps15, as well as in yeast End3p, another protein that is required for endocytosis (Raths et al., 1993; Benedetti et al., 1994). The dim mutants were found to accumulate aberrant membranous structures, including vesicles and tubules that might represent exaggerated intermediates in an endocytic pathway. Preliminary studies examining the internalization of cationized ferritin highlight a route of endocytosis via plasma membrane invaginations that is highly exaggerated in the dim mutants.

Materials and Methods

Strains and Media

Bacterial strains were grown on standard media (Miller, 1972). The genotypes of Escherichia coli strains used were XL1Blue [supE44 thi-l lac endA1 gyrA96 relA1 F' proAB lacP15 lacZAM15 proAB] (Ya- nisch-Perron et al., 1985). Saccharomyces cerevisiae yeast strains used (Table 1) were grown in standard YPD, SD minimal, and sporulation media (Sherman et al., 1979).

Materials

N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64) and rhodamine phalloidin were purchased from Molecular Probes, Inc. (Eugene, OR). 35S[γ-ATP] and Trans35S label were obtained from ICN Radiochemicals (Irvine, CA). Restriction enzymes, T4 DNA ligase and polymerase, Klenow enzyme, and alkaline phosphatase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) or New England Biolabs (Beverly, MA). Sequence-sequencing kits 5-bromo-4-chloro-3-indolyl-β-D-galactoside and isopropyl-β-D-thiogalactoside were from U.S. Biochemical Corp. (Cleveland, OH), and [α-35S]ATP was acquired from Amer sham Corp. (Ar lington Heights, IL). Glusulase was from DuPont (Wilmington, DE), and Zymolyase-100T was purchased from Seikagaku Kogyo (Tokyo, Japan). Sephacryl S-1000 was from Pharmacia (Uppsala, Sweden). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Mutagenesis, FM4-64 Labeling, and FACS® Analysis

Ethyl methanesulfonate (EMS) mutagenesis of wild-type SEY6210 was performed as described (Rose et al., 1990), and 22–25% viability was obtained using this procedure. The mutagenized cells were diluted in YPD and grown overnight at 22°C. A 1-h preincubation at 38°C was followed by a 10-min labeling with 16 μM FM4-64 (1:10 dilution of stock; 1.6 μM FM4-64 in DMSO) and a 1-h chase. The cells were then subjected to a sterile sort on a FACStar® fluorescence-activated cell sorter (Becton Dickinson & Co., Mountain View, CA), and cells of low fluorescent intensity were collected and plated on YPD at 26°C. Replica plating to 38°C permitted analysis of the percent of surviving colonies that were temperature sensitive for growth. Individual colonies were labeled with FM4-64 and chased at 38°C, allowing identification of dim mutants. After several back-crosses to SEY6210 and SEY6211, strains BWY1, BWY2, BWY10, BWY11, and BWY16 were generated carrying dim1-1 or dim2-1 alleles. FM4-64 labeling of dim mutants was performed as described (Vida and Emr, 1995), except that cells were grown in YNB plus appropriate amino acids and were diluted 1:1 with YPD before the temperature shift to 38°C. FM4-64 was used at 16 and 32 μM for 38°C and 26°C labeling, respectively. Images were acquired under identical conditions using a CCD camera (model 4995; COHU; San Diego, CA), an integrator box (model 4401A; Scientific Colorado Video Inc., Boulder, CO), and an LG-3 Frame Grabber (Scion Corp., Frederick, MD). The software used was NIH Image 1.55 and Adobe Photoshop 3.0.

Cloning of the DIM1 and DIM2 Genes

For cloning of the DIM1 gene, a LEU2-CEN S. cerevisiae genomic library

1. Abbreviations used in this paper: EH, eps15 homology; EMS, ethyl methanesulfonate; FM-64, N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide; v-ATPase, vacuolar ATPase.
(in the E. coli yeast shuttle vector YCp50, CEN4 ARS1 URA3; Rose et al., 1987) was transformed into BWY1, replica plated to 38°C, and colonies that were temperature resistant were selected for plasmid rescue. Eight colonies representing three different genetic inserts were isolated from BWY1 temperature-resistant cells and retransformed into BWY1 to confirm plasmid linkage. Restriction mapping and subcloning narrowed the region of the open reading frame (pDIM1, also known as pSHE4, BglII-BamHI fragment in pRS414 TRPC1 CEN) that was sequenced on both strands using the Sequenase kit, revealing a 2,367-bp open reading frame predicting a 789-amino acid protein. 321 bp of the 5’ end of the neighboring PEP12 gene was also included in this fragment. A deletion construct was generated by cloning the BglII-BamHI fragment into the BamHI site of pBluescript KSII (+) (Stratagene, La Jolla, CA) and inserting the HIS3 gene into the ClaI and BamHI sites, which removes 92% of the open reading frame (pDIM1A1). This construct was linearized with PvuII and BstYI (which cuts at the site generated by the BglII-BamHI ligation), and was transformed into the wild-type strains SEY6210, SEY6211, and SEY6210.5, generating BWY6, BWY7, and BWY5, respectively. PCR analysis using oligonucleotides outside the linearization sites confirmed the deletion of the DIM1 gene. An additional deletion replacing the EcoR1HI-Nsal fragment with LEU2 (pDIM1A2) removed 17% of the open reading frame and also resulted in a null phenotype. The integrative mapping construct was generated by cloning the BglII-BamHI fragment into pRS304 (pDIM1-MAP), which contains the TRP1 gene and no sequences for maintenance of plasmid replication in yeast. This construct was linearized with Nsal, transformed into BWY3 (generating BWY4), sporulated, and dissected.

To clone the DIM2 gene, a LEU2-CEN S. cerevisiae genomic library was transformed into BWY10, replica plated to 38°C, and colonies that were temperature resistant were selected for plasmid rescue. Three colonies representing two different genetic inserts were isolated from temperature-resistant BWY10 colonies and retransformed into BWY10 cells to confirm plasmid linkage. Restriction mapping and subcloning narrowed the complementing region to an XmnI-DraI fragment (pDIM2, also known as pPAN1, the XmnI-DraI fragment in pRS415 LEU2 CEN). Both ends of two EcoRI fragments were sequenced that revealed identity of the DIM2 gene with PANI. Using oligonucleotides and subcloning for further sequencing across regions of incongruity between PANI and YIR006c (the same genomic region identified by the yeast genome project) showed identity with YIR006c. For the PANI disruption construct (pPANI-KO), the 4.4-kb SalI-BamHI fragment was subcloned into pBluescript cut with Sall and BamHI, followed by insertion of the HIS3 gene into the EcoRV sites of PANI and linearization with Sall and BamHI for integration into SEY6210.5, generating BWY14. Integrative mapping was done using the BglII fragment subcloned into pRS303 cut with BamHI to generate pPANI-MAP, which was linearized using NcoI and transformed into BWY12 to produce BWY13.

**α-Factor Internalization and Degradation**

Radiolabeled α-factor was produced according to the method of Dulic et al. (1991) and used in internalization and degradation assays performed essentially as described, with the following exceptions: unbound α-factor was removed with one wash of binding buffer at 4°C, followed by a preincubation at 26°C or 38°C for 20-30 min. To initiate internalization, the cells were diluted to 30 OD/ml by the addition of 4 vol of prewarmed 1.25x YPD and 5% glucose. Experiments were performed two to three times with each strain. Strains BWY17, BWY15, and BWY9 were used. BWY15 was generated by crossing BWY10 with a wild-type strain harboring a bari-1 allele.

**Generation of Double Mutants for Genetic Interaction Studies**

For vma4Δ dim double mutants, KMY10004s was mated with BWY1 and BWY10, sporulated, and dissected onto YPD-MES, pH 5.5, plates for germination. For other double mutants, spores were germinated on YPD plates at 26°C, except for end4 doublets, which were germinated at room temperature. DBY1691 was mated with BWY6 and DBY1692 with BWY10 to generate acl1::LEU2 dim double mutants. RH268-1C was crossed to BWY6 and BWY10 to produce end4::LEU2 dim double mutants. DIM1 deletions were introduced into YAS1114, YAS1115, GPY1000s, and GPY418 by integrating pDIM1Δ1(LEU2) (10-20 tetrads were dissected in each case, and the identification of alleles contained within a germinated spore was confirmed by back-crossing with parental strains and testing resultant diploids for homozygosity.

**Phalloidin Labeling of Filamentous Actin and Bud Scar Staining**

Mid-log phase cells SEY6210.5, BWY8, BWY12, SEY6210, BWY6, YAS1, YAS1114, and YAS1115 were grown at 26°C, a portion shifted to 38°C for 2.5 h, and then fixed and stained with Calcefluor White MZM (Sigma) or phalloidin as described (Beneditti et al., 1994).

**Purification of Clathrin-coated Vesicles**

Vesicles were isolated from SEY6210 and BWY6 as described (Mueller and Emr, 1984). 1 ml of every fifth fraction was precipitated with 10% TCA with the addition of insulin as carrier. The proteins were resolved by SDS-PAGE, immunoblotted, and Chc1p was detected using the mAb SKL1 (Lemmon et al., 1988) at a 1:2,000 dilution. The data represent at least two independent experiments for each strain. The indicated fractions were fixed with 3% glutaraldehyde, pelleted at 100,000 g for 1 h, and processed for routine morphology EM as described below.

**EM and Cationized Ferritin Internalization**

Conventional EM for routine morphology was performed as described (Rieder et al., 1996) on SEY6210, BWY6, and BWY16 grown at 26°C, and on a portion shifted to 38°C for 90 min. The same strains, as well as RH268-1C, YAS1114, and YAS1115, were used for cationized ferritin internalization. 15-OD cells were grown to mid-log phase, washed twice (1.2 M sorbitol, 100 mM Tris, pH 7.5, 10 mM CaCl2, 2 mM DTT, 0.6 mM PMSF, 0.1-1.0 mg/ml Zymolyase-100T), washed twice (1.2 M sorbitol, 100 mM MES, pH 6.5, 0.5 mM MgCl2, 1 mM EGTA, 0.2 mM DTT, 0.6 mM PMSF), and resuspended in 1.1× internalization buffer (1.1× YNB, 2.22% glucose, 1.33 M sorbitol, 1.1× amino acids) at 8.3 OD/ml. A preincubation at the appropriate temperature for 30 min was followed by the addition of 0.2 ml 10 mg/ml cationized ferritin and continued incubation for 30 min. The cells were gently pelleted, resuspended in fixative (2% glutaraldehyde, 1.2 M sorbitol, 5 mM MgCl2, 5 mM CaCl2, 97 mM cacodylate, pH 7.4), and processed as described above for EM with the exception of poststaining with uranyl acetate and lead citrate.

**Results**

**FACS® Isolation of dim Mutants**

The lipophilic dye FM4-64 has been shown to be a useful probe for endocytosis in yeast (Vida and Emr, 1995). We exploited these observations in an enrichment protocol to identify endocytic mutants: the endocytosis mutants end3-1 and end4-1 were found to internalize less fluorescent dye than wild-type cells (two- to fourfold reduction in fluorescent signal in the vacuolar membrane, data not shown), and therefore endocytosis mutants could be easily sorted by FACS®.

As an initial test of the feasibility of this approach, we tested whether cells that bind but do not endocytose the dye could be stripped of uninternalized, plasma membrane—localized FM4-64. Cells were incubated with FM4-64 at 0°C to allow insertion of the dye into the plasma membrane, followed by washing at 0°C. For comparison, cells were also incubated with FM4-64 at 30°C, followed by a wash and a 60-min chase period, during which FM4-64 is internalized and transported to the vacuole (Vida and Emr, 1995). The samples were then subjected to analysis by FACS®. The cells that were labeled and washed at 0°C (Fig. 1A) revealed a low level of fluorescence that was nearly identical to that observed in unincubated cells (data not shown). The cells with internalized FM4-64 displayed ~10 times greater fluorescence intensity (Fig. 1B), which
bated at 38°C for 1 h, labeled with FM4-64 for 10 min, and chased from the plasma membrane to the vacuole, followed by FACS® analysis. (A) Wild-type SEY6210 was labeled and chased at 30°C to allow internalization from the plasma membrane to the vacuole, followed by FACS® analysis. (B) SEY6210 was labeled with FM4-64 on ice to restrict the dye to the plasma membrane, and was then washed four times with ice-cold medium to extract the dye, followed by FACS® analysis. (C) SEY6210 cells were mutagenized with EMS, incubated at 38°C for 1 h, labeled with FM4-64 for 10 min, and chased for 1 h. A FACS® sort was performed, and cells falling into the “dim” window were collected for further analysis.

was equivalent to the signal observed in cells labeled at 0°C and not washed (data not shown). This demonstrated that plasma membrane–localized FM4-64 could be efficiently stripped from cells, and that FACS® analysis can be used to discriminate cells that endocytose FM4-64 versus those that do not.

To screen for endocytosis mutants, wild-type cells were mutagenized with EMS and were allowed to recover in rich medium at 22°C for 12 h. The level of EMS mutagenesis used resulted in an ~80% kill rate, and, of the survivors, 10% were temperature sensitive for growth. The mixed population of cells was then incubated at 38°C for 1 h, followed by a 10-min incubation with FM4-64 and a 1-h chase period at 38°C. Under these conditions, wild-type cells internalize FM4-64 to the vacuole (see below). The temperature shift was performed to allow for the isolation of temperature-conditional alleles of genes required for endocytosis and possibly also for growth, and does not exclude the isolation of genes that are not essential. The labeled and chased cells (1 × 10⁷) were then subjected to FACS® and selected from a window of low fluorescent intensity (Fig. 1 C, sort window). Approximately 0.05% of the total cells (4,894) were selected by this window, of which 602 yielded viable colonies. 25% of cells selected by FACS® were temperature sensitive for growth, a greater than twofold enrichment relative to the unsorted population.

100 colonies were tested individually for the ability to internalize FM4-64 after a 1-h preshift to 38°C. 10 colonies displayed less intense labeling of the vacuole, referred to as the “dim” phenotype. Two of these mutants that also were temperature sensitive for growth were characterized further. In both, the “dim” phenotype and the temperature sensitivity cosegregated through three back-crosses to the parental strain. The two mutants contained mutations in distinct loci: a diploid strain generated by mating the two mutants resulted in wild-type levels of FM4-64 internalization, whereas homozygous mutant diploids were “dim” (data not shown).

dim1 and dim2 Cells Exhibit Endocytosis Defects

The fluorescent lipophilic steryl dye FM4-64 is useful as a marker to observe the function of the endocytic pathway in yeast. Reduced fluorescent intensity in the vacuolar membrane would presumably correspond to a reduction in endocytic flow from the plasma membrane to the vacuole. We therefore analyzed uptake of FM4-64 in the dim mutants at both 26°C and 38°C in cells that either did or did not harbor single-copy plasmids encoding wild-type copies of the DIM genes (see below). Identical exposure parameters were used when acquiring images to allow fluorescent intensity comparisons. When the labeling was performed at 26°C, vacuolar labeling in dim1-A and dim2-1 cells was slightly less than wild-type levels (Fig. 2, 26°C). Labeling that was performed after a 30-min preincubation at 38°C followed by 45 min of chase revealed diminished vacuolar staining and the appearance of punctate structures (Fig. 2, 38°C, 30 min). When a 1-h preshift to 38°C preceded the labeling, the majority of the signal was restricted within punctate structures (Fig. 2, 38°C, 60 min). Even after 2.5 h of chase, the signal was still observed in the punctate structures and did not chase to the vacuolar membrane (data not shown). A temperature-dependent phenotype for a deletion strain was not expected, but has been reported for other deletions (e.g., temperature-sensitive polarized secretion in rpm1-A; Liu and Bretscher, 1992). In addition, when the dim mutants are incubated at elevated temperatures, only a single large vacuole is seen rather than the three to five smaller vacuoles that are observed in wild-type and complemented mutant cells (Fig. 2). However, processing and sorting of the soluble vacuolar hydrolase carboxypeptidase Y is normal in both dim mutants (data not shown), indicating that their ER to vacuole transport pathway is unaffected. As anticipated, based on the selection scheme used for their isolation, dim1-A and dim2-1 cells exhibited approximately two- to fourfold reduced fluorescent intensity of FM4-64 at 38°C when compared to
Figure 2. FM4-64 labeling of wild-type and dim mutants. SEY6210 (wild-type), BWY6 (dim1-Δ) with or without complementing pDIM1 plasmid, and BWY10 (dim2-1) with or without complementing pDIM2 plasmid cells were labeled at 26°C for 20 min, followed by a 90-min chase, and were preincubated at 38°C for 30 min, labeled for 10 min, and chased for 45 min; or were preincubated at 38°C for 1 h, labeled for 10 min, and chased for 45 min. Images were acquired under identical conditions to compare fluorescent intensities. Vacuoles (V) are indicated in Nomarski panels of the uncomplemented dim mutants. Bar, 5 μm.

the intensity of wild type cells, and this internalization defect was rescued by the presence of the DIM1 or DIM2 cloned genes, respectively.

The dim phenotype is consistent with defects in the trafficking of bulk lipid. As an alternative assay for endocytic defects in dim1-Δ and dim2-1 mutants, internalization of the yeast-mating pheromone α-factor serves as a simple measure of receptor-mediated endocytosis (Dulic et al., 1991). In this experiment, α-factor was bound to the surface of the cells, and internalization was initiated at time 0 by the addition of glucose. At 38°C, dim1-Δ cells had a two to threefold reduction in the kinetics of α-factor internalization relative to that observed in dim1-Δ cells at 26°C or in wild-type cells (Fig. 3, A and B). The initial rate of internalization appeared to be even slower in the dim2-1 mutant at 38°C (Fig. 3 C). These defects were rescued to nearly wild type kinetic rates when dim1-Δ or dim2-1 cells harboring complementing plasmid were assayed (data not shown).

Further analysis of α-factor internalization in dim1-Δ cells involved examining the fate of internalized α-factor. In wild type cells, internalized α-factor is delivered to the vacuole, where it is degraded by vacuolar proteases (Singer and Riezman, 1990). Consequently, the arrival of α-factor in the vacuole is monitored by assessing its degradation. Internalization of radiolabeled α-factor experiments were performed, followed by processing for TLC to visualize α-factor degradation. In dim1-Δ cells, at a permissive temperature, cell-associated α-factor was cleared/internalized nearly as rapidly as in wild-type cells (Fig. 4, A and B, lanes 1–8). When the cells were incubated at 38°C for 30 min before internalization, however, the α-factor in dim1-Δ cells was stabilized in the intact form (Fig. 4, A and B, lanes 11 and 12) and very little α-factor was observed in
Figure 3. α-factor internalization in wild-type and dim mutants. (A) BWY17 (wild-type), (B) BWY9 (dim1-Δ), and (C) BWY15 (dim2-1) cells were incubated with radiolabeled α-factor at 0°C to allow binding to surface receptors, washed of unbound label, preincubated at 26°C or 38°C for 30 min, and glucose was added at time 0 to initiate internalization. At the indicated time points, aliquots were withdrawn and diluted in ice-cold buffer at pH 6.2 (total α-factor, surface-bound plus internalized) or pH 1.1 (internalized α-factor, acid wash to dissociate surface-bound α-factor). The samples were then filtered, counted by scintillation, and the results were plotted as the ratio of pH 1/pH 6 counts for each time point to represent the percent of internalized α-factor. The data represent the average of two experiments.

Figure 4. Degradation of internalized α-factor. (A) BWY17 (wild-type) or (B) BWY9 (dim1-Δ) cells were treated as indicated in Fig. 3, but rather than filtering the samples for scintillation, they were pelleted, washed, and lysed to recover internalized α-factor. The samples were resolved by thin-layer chromatography to differentiate mature from degraded α-factor.

The internal fraction of these cells (Fig. 4 B, lanes 13–16). Together, these experiments indicate that dim mutants exhibit a significant reduction in the endocytosis of both bulk lipids and α-factor, and that these defects are exaggerated at a high temperature.

Cloning and Characterization of the DIM1 and DIM2 Genes: Identity with SHE4 and PAN1

The DIM1 gene was cloned by complementation of the temperature sensitive for growth phenotype of the dim1-1 mutant. To do this, dim1-1 cells were first transformed with a genomic CEN-LEU2 library (Rose et al., 1987) and plated at 26°C. Colonies were then replica plated and grown at 26°C or 38°C. Colonies that could survive at 38°C were chosen, and plasmid DNA was rescued from them. Three distinct but overlapping library plasmids that were capable of rescuing the growth defect were subjected to restriction mapping, and a region of overlap was determined. A minimum complementing BglII/BamH1 fragment (Fig. 5 A) rescued both the growth and the FM4-64 internalization defects of dim1-1 cells. Both strands of this fragment were sequenced, and a single large open reading frame of 789 amino acids was detected.

Analysis of the DIM1 gene predicted a hydrophilic protein sequence. Database searches indicated small regions of similarity to the yeast dynamin-like protein Dnm1p (Gammie et al., 1995) and also to the carboxy-terminal region of the Caenorhabditis elegans open reading frame yk44f2.5, which predicts a 961-amino acid protein with 38% identity to heat shock protein-binding immunophilins (Callebaut et al., 1992) in the amino-terminal 43–115 residues. During the course of this work, SHE4, isolated as a gene necessary for Swi5p-dependent HO expression, was reported in the database (Jansen et al., 1996), as was the chromosome XV open reading frame OR26.26 (Saccharomyces Genome Database). Both are identical to DIM1, with the exception that SHE4 is predicted to encode a 791-amino acid protein. This difference is likely caused either by strain variations or sequencing errors. Hereafter, DIM1 will be referred to as SHE4 and the dim1-1 allele as she4-1.

Integrative mapping confirmed that the SHE4 gene corresponded to the she4-1 locus. To determine if SHE4 was essential, a disruption construct in which 92% of the SHE4 gene was replaced by the HIS3 gene was generated (Fig. 5 A). The construct was used to disrupt one genomic copy of SHE4 in a wild-type diploid strain. The resulting strain was then sporulated, and 20 tetrads were dissected. Germination of the meiotic products at 30°C yielded four viable haploid colonies (data not shown), indicating that SHE4 is not an essential gene. We also constructed haploid she4-Δ null strains that, like she4-1, are temperature sensitive for growth (Fig. 5 B) and display the dim pheno-
Figure 5. Analysis of the SHE4/DIM1 gene. (A) A 10-kb genomic fragment that complemented the growth defect of BWY1 (dim1-1) cells was isolated. The 2,367-bp open reading frame is shown contained within the minimum complementing BgII/BamHI fragment. A deletion construct replacing 92% of the open reading frame with the HIS3 gene is schematized. Bg, BgII; C, ClaI; X, XhoI; Bs, BstBI; P, PvuII; B, BamHI. (B) SEY6210 (wild-type) and BWY6 (dim1-1) with or without complementing pDIM1 plasmid were grown on YPD plates at 30°C or 37°C for 3 d.

type (Fig. 2). The growth defect and dim phenotypes are rescued in she4-Δ cells that harbor a complementing plasmid (Figs. 2 and 5 B).

The DIM2 gene was isolated by complementation of the growth defect in dim2-1 cells at 38°C. Two distinct library plasmids that rescued the growth defect were analyzed by restriction mapping and contained a common region of overlapping sequence. The largest clone, which fully complemented the mutant, is schematized in Fig. 6 A. Several regions of the clone were sequenced. Comparison with the database revealed identity to a previously characterized essential gene of unknown function, PAN1 (Sachs and Deardorff, 1992; Boeck et al., 1996), and also to the chromosome IX open reading frame YIR006C (Voss et al., 1995). PAN1 was originally thought to encode a poly-A-specific nuclease, but was recently reported to be a contaminant in the purification of Pan2p, the true nuclease (Boeck et al., 1996). PAN1 has also been isolated recently in other screens for mitochondrial protein–targeting mutants (Zoladek et al., 1995) and rapid-death mutants with cdc28-4 (Tang and Cai, 1996). Five regions of discrepancy between PAN1 and open reading frame YIR006C have been reported. Sequencing of these regions in our library plasmid indicated identity with the open reading frame YIR006C sequence, which predicts a 1,480-amino acid protein. The sequence variations may be the result of strain differences, recombination events, or sequencing errors. The temperature sensitivity and dim phenotype of the dim2-1 cells was rescued by a plasmid containing the minimum comple-
We constructed a deletion of YBL047c, which has no assigned function, and have found that the deletion strain is viable and has no obvious growth defects have been detected (our unpublished data).

**SHE4 and PAN1 Interact with Other Genes Involved in Endocytosis**

Munn and Riezman (1994) recently described a screen in which endocytosis mutants were isolated by selecting for mutants that exhibited synthetic lethality in combination with vacuolar ATPase (v-ATPase) mutants. This screen was based on the fact that v-ATPase mutants require acidic media for viability and the supposition that viability is dependent on vacuole acidification via endocytosis of protons (Nelson and Nelson, 1990). We asked whether the dim mutants also fell into this class of endocytosis mutants. VMA4 encodes a subunit of the v-ATPase (Ho et al.,

**Table II. Genotypes of Yeast Strains**

| Strain | Genotype |
|--------|----------|
| SEY6210 | MATα ura3-52 his3-d200 trp1-A901 leu2-3, 112 lys2-801 suc2-Δ9 |
| SEY6211 | MATα ura3-52 his3-d200 trp1-A901 leu2-3, 112 ade2-101 suc2-Δ9 |
| SEY6210.5 | MATα/MATα ura3-52/ura3-52 his3-d200 ade2-101 trp1-A901/trp1-A901 leu2-3, 112/112 ade2-101, 112 suc2-Δ9/suc2-Δ9 |

**Table II. Growth Phenotypes of Double Mutants**

| Strain | Growth Phenotype |
|--------|------------------|
| se4-Δ +/+- | -/+- |
| pan1-20 +/+- | +/- |

Strains that contained two mutant alleles were constructed, and their growth phenotypes at various temperatures were tested. +, wild-type growth; +, growth that is slower than wild type; +/-, very slow growth; --/+ , almost no growth; and --, complete lethality. All strains were tested on rich YPD plates, except the strains containing vma4-Δ alleles, which were grown on MES-buffered YPD plates, pH 5.5. In each case, the single mutants grew nearly as well as the wild type at all temperatures reported in this table.
1993), and a \textit{vma4-Δ} strain is deficient in \textit{v}-ATPase function. \textit{she4-Δ} \textit{vma4-Δ} and \textit{panl-20} \textit{vma4-Δ} heterozygous diploids were sporulated and dissected, and synthetic lethality was observed in spores that were deduced to harbor both mutations, consistent with the endocytosis defects observed in the \textit{dim} mutants (Table II).

Synthetic growth defects often are used as an argument to place gene products on the same or parallel pathways. We observed synthetic growth defects in strains harboring both \textit{she4-Δ} and \textit{panl-3} (Sachs and Deardorff, 1992), and also with \textit{she4-Δ} and \textit{cht-ts} (Tan et al., 1993; Table II in this paper). These data are indicative of a role for the \textit{SHE4} and \textit{PAN1} gene products in endocytosis (also see Discussion).

\textbf{Actin Cytoskeleton Structure Is Altered in \textit{she4-Δ} and \textit{panl-20} Cells}

The actin cytoskeleton is intimately involved in the process of endocytosis in yeast (Kubler and Riezman, 1993; Munn et al., 1995). Actin localization in yeast cells changes as a function of cell cycle progression, and patches of filamentous actin (actin cortical dots) at the plasma membrane coincide with sites of synthesis of new cell wall material in budding cells and with secretion of \textit{a}-agglutinin in shmooping (mating) cells (Adams and Pringle, 1984; Kilman and Adams, 1984). The normal polarized localization of actin is disrupted in many previously described endocytosis mutants (Benedetti et al., 1994; Munn et al., 1995), so we examined the structure and polarity of the actin cytoskeleton in the \textit{she4-Δ} and \textit{panl-20} cells.

Wild-type, \textit{she4-Δ}, and \textit{panl-20} diploid cells were first fixed with formaldehyde, and then incubated with rhodamine-phalloidin to label filamentous actin. In \textit{she4-Δ} cells, we observed a loss of polarity of actin localization at both 26°C (Fig. 7 A) and 38°C (data not shown). The cells also adopted a round morphology at all temperatures, similar to that seen with \textit{actl-1} mutant cells (Novick and Botstein, 1985), and consistent with a loss of polarized secretion and a switch to isotropic growth. Normal elongated cell morphology and polarity of actin cortical spots were restored in a \textit{she4-Δ} strain harboring a complementing plasmid (Fig. 7 A).

\textit{panl-20} diploid cells revealed slightly disrupted actin polarity at 26°C, with a few more cortical spots found in the mother cells relative to wild-type cells (data not shown), and the cells were rounder than wild-type cells, consistent with the mislocalization of actin spots. After incubation at 38°C for 2.5 h, the morphology of the actin cytoskeleton became markedly perturbed: thick, curved actin bundles appeared to be adjacent to the cell periphery, and polarity of the cortical spots was lost (Fig. 7 B). Mislocalization of actin in a \textit{panl} allele was also recently reported by Tang and Cai (Tang and Cai, 1996). In addition, actin structures did not display the wild type double band of cortical dots at the neck during cytokinesis and instead adopted a filamentous appearance with a larger amount of actin directed toward the smaller (presumably daughter) cell. Actin polarity and cell shape were restored to a wild-type appearance when \textit{panl-20} cells harbored a complementing plasmid (Fig. 7 B).

Budding yeast exhibit stereotypic patterns of bud site selection: haploid cells bud in an axial pattern in which the next budding event occurs adjacent to the previous one, whereas diploid cells can bud from either pole of their el-

Figure 7. Localization of filamentous actin. (A) Diploid SEY6210.5 (wild-type) and BWY8 (\textit{she4-Δ}) with or without complementing plasmid were grown at 26°C, fixed with formaldehyde, and labeled with rhodamine-conjugated phalloidin to visualize filamentous actin. (B) Diploid SEY6210.5 (wild-type) and BWY12 (\textit{panl-20}) with or without complementing plasmid were grown at 26°C, shifted to 38°C for 2.5 h before fixation with formaldehyde, and labeled with rhodamine-conjugated phalloidin to visualize filamentous actin. Bar, 5 μm.
lipsoidal shape (Chant and Pringle, 1995). Haploid she4-Δ and pan1-20 cells were capable of normal axial budding as seen in wild-type cells; however, in diploid she4-Δ and pan1-20 cells, the budding pattern became random (data not shown). The same diploid-specific alterations in bud site selection are also observed in other endocytosis mutants such as end3 (Benedetti et al., 1994), rvs161/end6 (Sivadon et al., 1995), and rvs167 (Bauer et al., 1993).

**Ultrastructural Analysis of she4-Δ and pan1-20 Cells Reveals Vesicles and Tubulovesicular Membranes**

When the dim mutants are examined with the light microscope for FM4-64 internalization and localization of filamentous actin, defects that may correlate with morphological aberrations are observed. EM was performed to examine more closely the structure of these mutants. In the she4-Δ cells, EM revealed the presence of 100-nm vesicles in the cytoplasm at both 26°C (Fig. 8, B and C) and 38°C (Fig. 8, D–G), whereas in wild-type cells, such structures are rarely detected (Fig. 8 A). Additional tubulovesicular compartments reminiscent of early endosomes in mammalian cells (Helenius et al., 1983), as well as 40–50-nm vesicles, appeared upon incubation at 38°C. These structures may correspond to the punctate staining that is observed in FM4-64 labeling after a temperature shift, and they may represent endocytic intermediate compartments. While fewer 50- and 100-nm vesicles were observed, similar endosomal-like structures were also seen in the pan1-20 cells after incubation at 38°C, whereas they were absent in pan1-20 cells containing a complementing plasmid (data not shown).

In many cases, the 100-nm vesicles observed in the she4-Δ cells at 26°C appeared to have associated coats (Fig. 8 C). These vesicles are unlikely to correspond to classical post-Golgi secretory vesicles because sorting and processing of the vacuolar hydrolase carboxypeptidase Y (Stevens et al., 1982) and secretion of the heat shock protein Hsp150p (Russo et al., 1992) did not differ from wild-type cells at 26°C or 30°C (data not shown). A partial kinetic defect in secretion of Hsp150p and the periplasmic enzyme invertase (Gascon et al., 1968) was observed at 38°C; however, this may be caused by lack of growth at 38°C (data not shown).

**she4-Δ Vesicles Are Unlikely to Be Clathrin-coated Vesicles**

Given the established role for clathrin heavy chain in endocytosis (Tan et al., 1993), we tested if the 100-nm coated vesicles observed in the she4-Δ cells were clathrin coated. After the protocol of Mueller and Branton (1984), light membranes isolated from wild-type or she4-Δ cells grown at 30°C were fractionated by size on an S-1000 Sephacryl column (Fig. 9, A and B). A 280-nm absorbance peak was observed in fractions 33–38 from she4-Δ membranes that was not observed from wild-type membranes, suggesting that this peak might correspond to the observed vesicles. To determine the elution position of clathrin-coated vesicles, every fifth fraction was assayed by immunoblotting with antibodies to the heavy chain of the clathrin triskelion (Chclp). The peak of Chclp elution was not coincident with the large absorbance peak (Fig. 9 B). For further analysis, membranes from fractions 36–37 and 45–50 were concentrated and prepared for examination by conventional EM (Fig. 9, C and D). Fractions 36–37 contained a fairly homogeneous population of 100-nm vesicles consistent with the size of vesicles observed in sections of spheroplasted cells (Fig. 8, B and C). In contrast, fractions coincident with clathrin immunoreactivity contained a heterogeneous population of vesicles, including many with coats exhibiting a classic clathrin cagelike coat (Fig. 9 D, arrowheads). Thus it is unlikely that the vesicles in the she4-Δ cells represent clathrin-coated vesicles, although they could be vesicles that have lost their coats, vesicles coated with a nonclathrin coat, or endocytic vesicles of an unknown origin.

**Cationized Ferritin Internalization Reveals Endocytic Structures at the Plasma Membrane**

To examine the structure and morphology of the endocytic pathway at the ultrastructural level, internalization of cationized ferritin was assayed. This is a commonly used method for following the endocytic pathway in mammalian cells (Ottosen et al., 1980; Herzog and Farquhar, 1983), but had not yet been used in yeast. Yeast cells were spheroplasted in an osmotically supported environment and preincubated at permissive or nonpermissive temperatures for 30–60 min, followed by incubation with cationized ferritin for 30 min. Ferritin accumulation was observed in numerous plasma membrane invaginations in she4-Δ cells at 30°C (Fig. 10, A and B) and 38°C (Fig. 10 C) and in pan1-3 (Fig. 10 D) and end4-1 cells at 38°C (data not shown). In wild-type cells, roughly half as many invaginations (0.46 invaginations per micrometer of plasma membrane, wild type; 0.81 invaginations per micrometer of plasma membrane, she4-Δ) that were significantly shallower (0.08 +/- 0.03 μm, wild type; 0.16 +/- 0.04 μm, she4-Δ) and that only rarely contained cationized ferritin labeling were observed. In addition, in she4-Δ cells at 30°C, membranous compartments within the cell that may represent endocytic intermediates that appear to have separated from the cell surface were observed (Fig. 10 B). As seen in wild-type cells, end4-1 at a permissive temperature exhibited fewer, rarely labeled membrane invaginations and membranous compartments (data not shown). In pan1-3 cells, compared to the isogenic wild-type strain at elevated temperature, we observed five times as many invaginations (0.55 invaginations per micrometer of plasma membrane, pan1-3; 0.11 invaginations per micrometer of plasma membrane, PAN1) that extended >10 times deeper into the cell (0.43 +/- 0.15 μm, pan1-3; 0.03 +/- 0.02 μm, PAN1). In general, the membrane invaginations in the mutants were deeper and often more elaborate at the high temperature than at the lower, permissive temperatures. The cationized ferritin–labeled plasma membrane invaginations have a morphology that appears to be distinct from that of actin cortical dots (Mulholland et al., 1994).

**Discussion**

The dim mutants described here were identified in a novel screen for mutants defective in bulk lipid endocytosis. Two mutants (dim1/she4, dim2/pan1) were characterized in de-
Figure 8. EM of wild-type and she4-Δ cells. (A) SEY6210 (wild-type) and (B and C) BWY6 (she4-Δ) cells were grown at 26°C, and (D–G) a portion shifted to 38°C for 90 min before glutaraldehyde fixation. M, mitochondria; N, nucleus; V, vacuole; arrowheads, 100-nm vesicles; small arrows, 40–50-nm vesicles; asterisks, tubulovesicular compartments. Bar in A and B, 0.5 μm; C–G, 0.1 μm.
These two temperature-sensitive dim mutants exhibit defects in internalization of the lipophilic dye FM4-64, they display reduced kinetics of α-factor internalization as compared to wild-type cells, and they also exhibit altered actin localization. Previously characterized mutants in the early steps of endocytosis share these general phenotypes with the dim mutants. Synthetic growth defects between the dim mutants and other endocytosis mutants are consistent with these gene products acting at similar stages in endocytosis. Both dim mutants also accumulated vesicles and aberrant membranous compartments with morphology reminiscent of mammalian early endosomes. In contrast, neither mutant yielded significant defects in secretory function. This new screening technique has thus allowed the identification of new genes involved in endocytosis. In addition, the usefulness of FM4-64-based FACS® screening for mutants in yeast was also recently demonstrated by Wang et al. (1996) in a search for mutants defective in vacuolar inheritance.

The yeast actin cytoskeleton is known to play an important role in endocytosis. Endocytosis mutants almost invariably exhibit disorganized actin cytoskeletons; and not surprisingly, many mutants with a perturbed actin cytoskeleton exhibit defects in endocytosis (Kubler and Riez-
Figure 10. Cationized ferritin internalization in dim mutants. (A–C) BWY6 (she4Δ) and (D) YAS1115 (pan1-3) cells were spheroplasted in 1.2 M sorbitol for osmotic support and preincubated at 30°C (A and B) or 38°C (C and D) for 30 min, followed by incubation with 1 mg/ml cationized ferritin (arrowheads) for 30 min. The cells were then fixed and examined by EM. Arrowheads, internalized cationized ferritin; small arrows, surface-bound ferritin; V, vacuole. Bar, 0.1 μm.

man, 1993; Munn et al., 1995). In yeast, the actin cytoskeleton is comprised of fine cytoplasmic cables and cortical actin dots that correlate with sites of secretion and cell growth (Adams and Pringle, 1984). The identity of the thick actin bundles observed in the pan1-20 cells at 38°C and their possible relationship to actin cortical dots or other actin-based complexes remains to be clarified. The structure of actin cortical dots, which has been described as a fingerlike invagination of plasma membrane surrounded by actin filaments (Mulholland et al., 1994), and perhaps the structure of other membrane invaginations are suggestive of their role as a reservoir of membrane that can be used for secretion, endocytosis, and responses to osmotic changes. The observation that dim and end mutant cells exhibit an enlarged and rounded morphology suggests that the restricted sites for exocytosis and/or the normal balance between exocytosis and endocytosis are perturbed. It has been proposed that these two processes are coordinated through the homeostasis of membrane tension (Sheetz and Dai, 1996).

The actin cytoskeleton is important for establishing the distinction between mother and daughter cells during budding. This developmental asymmetry is exemplified by the process of mating-type switching: only mother cells are capable of mating-type switching in wild-type strains (Nasmyth, 1993), while she mutants exhibit defects in this asymmetry and in mating-type switching (Jansen et al., 1996). The fact that mutations in SHE4/DIM1 cause defects both in mating-type switching and in endocytosis (Figs. 2–4) may reflect the importance of the actin cytoskeleton in each of these processes. In addition, the identification of SHE1 as MYO4 suggests a role for actin/myosin interactions in directing traffic from the mother cell to the daughter cell (Jansen et al., 1996; see also Johnston et al., 1991; Govindan et al., 1995). It is possible that the block of mating-type switching in the she4 mutant is an indirect consequence of the mislocalization of actin cables and cortical spots. Actin polarization may be required for the proper asymmetric localization of factors that regulate mating-type switching (Jansen et al., 1996).

While presently there are no known homologues to She4p, there are several proteins in yeast and mammalian cells with very significant homology to Panlp/Dim2p, including eps15 (Wong et al., 1995). The mammalian protein eps15 was first discovered as a phosphorylated substrate of the EGF receptor tyrosine kinase (Fazioli et al., 1993). Eps15 is comprised of three domains: three imperfect tandem repeats in the amino terminus (EH domains), a central domain rich in heptad repeats that are predicted to form coiled coils, and a carboxy-terminal proline-rich region, a portion of which has been shown to interact with the SH3 domains contained within the signaling molecule crk (Schumacher et al., 1995). The carboxy terminus of eps15 also contains a 72-amino acid domain that is distinct from the crk-binding motif, which has been demonstrated to interact constitutively with the plasma membrane adaptor complex AP2 (Benmerah et al., 1995, 1996). The interaction between eps15 and AP2 is intriguing because AP2 interacts with clathrin to promote clathrin-coated pit assembly at the plasma membrane (Smythe et al., 1992), thus
providing a connection between eps15 and endocytosis. Panlp and the yeast YBL047c protein share extensive structural similarity with eps15, each containing coiled-coil and proline-rich domains (Fig. 6). The proline-rich domains of Panlp and the YBL047c protein are likely to interact with SH3 domain-containing proteins (Ren et al., 1993; Yu et al., 1994); several candidates include proteins associated with the actin cytoskeleton (e.g., Abp1p [Drubin et al., 1990] and Slalp [Holtzman et al., 1993]) and proteins implicated in endocytosis (e.g., Rvs167p [Bauer et al., 1993; Munn et al., 1995] and Myo3p [Goodson and Spudich, 1995; Géli and Riezman, 1996]). An additional correlation between eps15 and endocytosis is provided by the presence of EH domains in the yeast proteins End3p and Panlp (Benedetti et al., 1994; Wong et al., 1995). EH domain-containing proteins have been proposed to function in endocytosis or at other sites of membrane trafficking (Seaman et al., 1996). Clearly, an area of great interest is the identification of other molecules that interact with each of these structural motifs.

Endocytosis in yeast has been particularly difficult to study morphologically, partly because of the uncharacterized structure of the endosomal membrane system within this organism. It is interesting to speculate on the identity of the tubulovesicular structures that accumulate in the dim mutants. These structures accumulated after shifts to elevated temperatures (Fig. 8), correlating well with the appearance of punctate staining in FM4-64-labeling experiments (Fig. 2). Further studies will be necessary to equate these two compartments unambiguously. It is intriguing to compare these structures with the tubulovesicular early endosomes that are observed in mammalian cells (Helensus et al., 1983). Our results with cationized ferritin provide a view of early internalization structures in yeast (Fig. 10). However, additional high resolution probes and techniques will be required to define each of the endocytic intermediates in yeast.

Several lines of evidence in both yeast and mammalian cells suggest the presence of two or more endocytic pathways (Sandvig and van Deurs, 1994). It has been shown in mammalian cells that different receptors rely upon distinct pathways for their endocytosis: the EGF receptor enters cells via clathrin-coated pits (Willingham and Pastan, 1982), while the folate receptor is internalized through a clathrin-independent pathway (Rothberg et al., 1990). It has also been shown that mammalian cells disrupted in the dynamin/clathrin-coated pit pathway upregulate an alternate endocytic pathway (Damke et al., 1995). In yeast, chcl-1Δ strains are viable and endocytose FM4-64 (data not shown) and α-factor receptor (Payne et al., 1987, 1988), suggesting that two or more endocytic pathways in yeast may also exist. In addition, a dim phenotype was observed when the end3-1 and end4-1 strains were tested for FM4-64 internalization (data not shown). This suggests that a block in internalization of mating pheromone receptor is not sufficient to completely block all flow from the plasma membrane into the cell. The synthetic growth defects we observed in double endocytosis mutants are also consistent with a multiple endocytic pathway model. Additionally, because act1-1 displays both endocytic and late secretory defects, it remains possible that the synthetic growth defects observed in act1-1/dim mutants are in part caused by perturbations of secretory function in the dim mutants. chcl-1ts mutants also display multiple membrane trafficking defects, including deficiencies in vacuolar protein sorting and endocytosis. However, since the vacuolar protein sorting pathway is normal in dim mutants, the synthetic defect observed in chcl-1ts/dim double mutants is a likely result of combined endocytosis deficits. Finally, synthetic defects between secretory and endocytic mutants in general might be expected if these two processes are tightly coupled.

As increasing numbers of proteins that mediate endocytosis in yeast are discovered, a need arises for an understanding of the precise functions of each of these proteins, how they are regulated, and how they interact with one another. Future studies will include the use of this FM4-64 screening strategy for the identification of additional endocytosis mutants. Genetic and two-hybrid screens will shed light on interacting partners that might establish pathways in endocytic membrane traffic. In vitro assays should allow for the purification of activities that are necessary for internalization and subsequent stages of membrane traffic. Through the use of these screens and other approaches, it should be possible to identify biologically significant interactions among relevant gene products, and to decipher molecular mechanisms for the control of endocytosis in eukaryotic cells.

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