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ARTICLE *in* YEAST · JUNE 2001

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Yeast 2001; **18**: 759–773.
DOI: 10.1002/yea.726

Research Article

Identification and characterization of *Saccharomyces cerevisiae* mutants defective in fluid-phase endocytosis

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Received: 16 October 2000
Accepted: 3 January 2001

Abstract

A mutant library generated by the European Functional Analysis Network (EUROFAN) was screened for strains defective in fluid-phase endocytosis. Accumulation of Lucifer yellow in the vacuole was used as a marker for efficient endocytosis. Fourteen mutants, including *ede1*Δ, *rcy1*Δ, *sys1*Δ and *tlg2*Δ, previously described to be involved in membrane trafficking, were identified in this screen. α-Factor uptake, endocytosis of FM4-64, carboxypeptidase Y secretion, vacuolar morphology, and a *vma2* synthetic growth defect were used as criteria to characterize the endocytic defect of the mutant strains obtained. Accordingly, eight mutant strains have endocytic phenotypes in addition to their defect in Lucifer yellow accumulation. These fluid-phase endocytosis mutants are defective at different steps of the endocytic pathway. Interestingly, only two mutants were defective for internalization, two for vacuolar protein sorting and four mutants had aberrant vacuolar morphologies. Some of the mutants identified in this screen that sort carboxypeptidase Y correctly may affect endocytosis at an early post-internalization step before the intersection of the endocytic with the vacuolar protein-sorting pathway.

Keywords: **endocytosis; Lucifer yellow; FM4-64; EUROFAN; yeast**

Introduction

The process of membrane trafficking from the plasma membrane through membraneous compartments to lysosomes has been termed 'endocytosis'. In the first step of endocytosis, portions of the plasma membrane are invaginated before their internalization. During the endocytic uptake step, plasma membrane proteins and lipids are taken up, together with extracellular components such as fluid, low molecular weight compounds, proteins and even large particles. Depending on the size of surface membrane and the type of endocytic content internalized, the different types of endocytosis have been named 'pinocytosis' (fluid-phase endocytosis), 'receptor-mediated endocytosis' or 'phagocytosis' (uptake and degradation of large particles) (Mellman, 1996).

Scientists have exploited the property of cells to take up molecules and particles from their environment to follow both internalization and transport of endocytic material within the cell. A large variety of tracers, such as horseradish peroxidase, labelled proteins, growth factors and even virus particles, have been used for this purpose (Steinman *et al.*, 1983).

The study of endocytosis in *Saccharomyces cerevisiae* has been hindered by the presence of a cell wall, which prevents the access of tracer proteins to the plasma membrane. To circumvent these problems a low molecular weight fluorescent compound, Lucifer yellow (LY), was used to follow endocytosis in this organism (Riezman, 1985). As a result of fluid-phase endocytosis, the dye accumulates in the vacuole. The fluorescence is strong enough to allow detection of LY in the large lumen of the vacuole but most often not in smaller endocytic compartments. LY is a highly soluble dye that cannot cross biological membranes. Its accumulation in the vacuole therefore depends entirely on endocytic membrane trafficking (Riezman, 1985).

Since this first demonstration of endocytosis in yeast a large number of plasma-membrane proteins have been shown to be transported along the

endocytic pathway to be degraded in the vacuole (Geli and Riezman, 1998; D'Hondt *et al.*, 2000). As in mammalian cells, endocytosis in yeast helps to regulate the presence and abundance of membrane proteins at the cell surface. When nutrient conditions change, endocytosis can mediate the degradation of permeases that are no longer needed. Endocytosis can also mediate the downregulation of pheromone receptors in response to ligand binding. Our group has focused its studies mainly on the endocytosis of the pheromone α-factor and its receptor Ste2p. Upon binding to Ste2p, α-factor is internalized and then transported to the vacuole. Inside the cell, α-factor is degraded by vacuolar proteases. The α-factor can thus be used to monitor both internalization and post-internalization steps of endocytosis (Singer and Riezman, 1990; Munn and Riezman, 1994; Hicke and Riezman, 1996).

Several screens have been performed in *S. cerevisiae* to identify endocytosis mutants now known as end, dim, sop and svl mutants (Raths *et al.*, 1993; Munn and Riezman, 1994; Wendland *et al.*, 1996; Luo and Chang, 1997; Zheng *et al.*, 1998). From such screens, protein components of the endocytic machinery and factors that regulate endocytosis have been identified. For instance, the role of EH-domain proteins such as End3p and Pan1p in internalization has first been noted because strains defective in these genes were identified in endocytosis screens; e.g. *end3* was identified in a screen for mutants unable to internalize α-factor (Raths *et al.*, 1993); Dim mutants such as *pan1* can not efficiently take up the dye FM4-64 (Wendland *et al.*, 1996). More recently, roles for different lipids in endocytosis have been revealed by the identification of *end8* (identical to *lcb1,* catalysing the first step of sphingoid base synthesis) and *end11* (identical to *erg2*, required for one of the final steps of ergosterol synthesis) in a screen for *vma2* synthetic lethality (Zanolari *et al.*, 2000; Munn *et al.*, 1999; Munn and Riezman, 1994).

Most of these screens were either designed to identify only internalization mutants or proved to select mainly for mutants that affect this first step of endocytosis (Raths *et al.*, 1993; Munn and Riezman, 1994; Wendland *et al.*, 1996). Independently, screens for vacuolar protein sorting (vps) mutants have isolated strains that affect endocytosis at a post-internalization step (Bryant and Stevens, 1998; Raymond *et al.*, 1992). The biosynthetic pathway to the vacuole and the endocytic pathway intersect at the prevacuolar compartment (PVC). Mutants that inhibit membrane traffic from the PVC to the vacuole therefore also inhibit endocytic membrane traffic (Piper *et al.*, 1995; Munn and Riezman, 1994). Internalization mutants and specific vps mutants comprise the majority of the endocytosis mutants identified to date. Mutants that affect endocytosis at an early post-internalization step are scarce, suggesting that previous screens have missed a whole group of endocytic mutants affecting the endocytic pathway before its intersection with the vacuolar protein sorting pathway or that most of these mutants, like *sec18* (Hicke *et al.*, 1997) affect various other steps of membrane trafficking as well.

To identify novel mutants affecting membrane trafficking at specific steps along the endocytic pathway we have tested mutant strains for their ability to accumulate LY in the vacuole. The yeast library that was used for this purpose contains 700 yeast strains generated by the European Function Analysis Network (EUROFAN; Oliver, 1996). Each of the strains is disrupted for a single open reading frame (ORF). The strains are disrupted for ORFs that were identified during the yeast genome sequencing project in European laboratories. ORFs with limited or no homology to previously studied genes at the time this project was started were disrupted and the mutants were assembled into this collection. The systematic screen for endocytosis mutants presented here has yielded 14 mutants. The different mutants exhibit more or less severe fluid-phase endocytosis defects. We have used α-factor uptake, FM4-64 endocytosis, vacuolar morphology and a *vma2* synthetic growth defect as additional criteria to characterize the endocytic defect. Several of the mutants have additional phenotypes that suggest a role of the corresponding proteins in membrane trafficking. During this screen we have identified mutants that inhibit endocytosis at different steps along the pathway. Several of them may belong to a new set of endocytosis mutants that inhibit endocytosis at an early post-internalization step upstream of the PVC.

Materials and methods

Yeast strains, media and reagents

Yeast strains created or used in this study are listed in Table 1. A complete list of the EUROFAN yeast

Table 1. Yeast strains used in this study

Strain	Genotype	Source
RH144-3D	*Mat***a** *his4 leu2 ura3 bar1*	Raths *et al.* (1993)
RH1604	*Matα vma2::LEU2 ura3 leu2*	Laboratory strain
RH2180	*Matα vps1-Δ2 pep4 his4 leu2 ura3 ade6*	T. Stevens
RH2604	*Mat***a** *end13-1/vps4 his4 leu2 ura3 lys2 bar1*	Munn *et al.* (1994)
RH4344	*Mat***a** *rcy1::kanMX4 his4 leu2 ura3 lys2 bar1*	Wiederkehr *et al.* (2000)
RH5021	*Mat***a** *sys1::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5023	*Mat***a** *ynl177c::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5025	*Mat***a** *ydl231c::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5026	*Mat***a** *sds24::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5027	*Mat***a** *tlg2::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5028	*Mat***a** *fth1::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5029	*Mat***a** *ydr266c::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5030	*Mat***a** *ydr036c::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5031	*Mat***a** *ynr075w::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5033	*Mat***a** *vps53::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5035	*Mat***a** *ynl297c::kanMX4 his4 leu2 ura3 lys2 bar1*	This study
RH5042	*Mat***a** *ynl227c::kanMX4 his4 leu2 ura3 lys2 bar1*	This study

strains screened in this study is available at **www.wiley.com/products/subject/life/genetics/genetics_yeast** and **www.biozentrum.unibas.ch/personal/riezman/**. Yeast cells were grown in YPUAD (2% casein peptone, 1% yeast extract, 2% glucose, 40 mg/l adenine, 20 mg/l uracil and 20 mg/l tryptophan). To select for disruptant strains containing the kanMX-module (Wach *et al.*, 1994), cells were grown on YPUAD plates containing 285 μg/ml Geneticin (Gibco BRL). To allow for growth of *vma2*Δ strains, the YPUAD medium was adjusted with HCl to pH 5.2 (Munn and Riezman, 1994). The minimal SD medium was prepared as described (Dulic *et al.*, 1991). Solid media contained 2% Bactoagar (Difco). Lucifer yellow CH was purchased from Fluka (Buchs, Switzerland), FM4-64 from Molecular Probes (Leiden, The Netherlands) and Zymolyase 20T from the Seikagaku Corporation (Tokyo, Japan). Biosynthetically, $^{35}SO_4$-α-factor was purified using the amberlite resin, CG-50 (Serva, Heidelberg, Germany) followed by C18 reverse-phase HPLC, as described in detail previously (Dulic *et al.*, 1991).

DNA constructs and molecular biology techniques

The plasmids carrying the deletion cassettes (derived from pBluescript II) and the wild-type genes cloned into pRS416 (centromeric plasmid; URA3 marker) were obtained from EUROFAN (Oliver, 1996). The restriction enzyme NotI was used to release the different disruption cassettes. Gel purified inserts were used to transform yeast strains. The *sys1*Δ::kanMX cassette was amplified by polymerase chain reaction (PCR) from the EUROFAN knockout strain FY10603B, using the following pair of primers: 5′-TCTAGGCGGCC GCGTGTACGGCCATAACCCGGC-3′ and 5′-CT AGAGCGGCCGCCGCTAGAGAAGTACCTCA GC-3′ The replacement of yeast genes by kanMX was confirmed by PCR. Geneticin-resistant cells were digested with Zymolyase 20T (120 U/ml) for 15 min at 37°C. The cells were collected and the pellet heated for 5 min at 92°C. The cell pellet was then re-suspended in PCR reaction buffer.

Yeast techniques

Standard yeast techniques were used for mating, sporulation, dissection and marker analysis. For the transformation of yeast cells a lithium acetate method was used (Gietz *et al.*, 1995). Cells were grown to a density of 10^7 cells/ml. The cells were washed and then re-suspended in a small volume of 100 mM lithium acetate, 10 mM Tris, 1 mM EDTA, pH 7.5. 10^8 yeast cells in a volume of 100 μl were mixed with 10 μl carrier DNA (10 μg/μl salmon sperm DNA) and 1 μg of the gel-purified disruption cassette. For plasmid transformations only 500 ng DNA was used per transformation. Polyethyleneglycol 4000 (70% in 10 mM Tris, 1 mM EDTA, pH 7.5) was added to a final concentration of 35%. The cells were then incubated at room temperature

for 30 min and then at 42°C for 15 min. The cell suspension was washed once to remove the polyethyleneglycol and the cells were plated directly on appropriate selective media. For the selection of kanMX disruption strains, the cells were re-suspended in YPUAD and incubated for 3 h at 24°C. After this recovery step the cells were collected and plated on YPUAD plates containing 285 μg/ml geneticin. Replacement of the gene by kanMX was confirmed by PCR.

Lucifer yellow accumulation in the vacuole

Fluid-phase endocytosis was assayed using the dye LY (Riezman, 1985; Dulic *et al.*, 1991). Cultures of yeast cells were grown to logarithmic phase ($OD_{600} = 0.2 - 0.5$) in YPUAD at 24°C. The cells were concentrated to 2–5 OD_{600} U/ml in 1.5 ml tubes. The cell suspension (100 μl) was incubated in the presence of 4 mg/ml LY for 1 h at 24°C. Holes were pierced through the top of the tubes to allow aeration of the cells during the LY uptake step. The cells were washed three times in 1 ml ice-cold 50 mM sodium phosphate, 10 mM sodium azide and 10 mM sodium fluoride buffer, pH 7. Samples were viewed on an Axiophot fluorescence microscope (objective lens 100×; NA 1,3) (Zeiss, Germany). A fluorescein filter set was used (excitation 450–490 nm; emission > 520 nm). During the primary screen for endocytosis mutants, sets of 10–20 strains were assayed in parallel. Strains with reduced amounts of LY in the vacuole were re-examined together with the next set of mutants. Exposure times for fluorescence pictures were identical within a set of mutants but varied 8–12 s between different sets of mutants.

Internalization of α-factor

α-Factor internalization was measured as described by Dulic *et al.* (1991). Cells were grown overnight in YPUAD at 24°C. Logarithmically growing cells (about 10^9) were harvested, re-suspended in 1 ml YPUAD and preincubated on ice for 10 min. [^{35}S]α-factor was then bound to the cells for 45 min on ice. Uptake of α-factor was initiated by re-suspending the cells in YPUAD pre-warmed to 24°C. The percentage of internalized α-factor at the given time-points was calculated by dividing the internalized counts (counts after washing cells in a 50 mM citrate buffer, pH 1.1) by the total cell bound counts (cells washed in 50 mM potassium phosphate buffer, pH 6) and multiplying by 100.

FM4-64 staining

FM4-64 was used to stain vacuoles and endosomes as described previously (Vida and Emr, 1995), with some modifications. Yeast cells were grown to logarithmic phase in YPUAD at 24°C. The cells were harvested and re-suspended in ice-cold YPUAD (10^8 cells/ml). FM4-64 was added at a final concentration of 40 μm to the cells and left on ice for 30 min. The cells were then washed once with ice-cold YPUAD and harvested. Uptake of FM4-64 was initiated by re-suspending the cells in pre-warmed YPUAD (24°C). After 45 min the cells were washed twice in cold YPUAD containing 15 mM sodium azide and 15 mM sodium fluoride. The samples were visualized using rhodamine optics.

Colony blot assay to detect CPY secretion

Yeast cells were grown overnight on YPUAD plates and further grown in contact with a 0.45 μm nitrocellulose filter (Schleicher and Schuell, Dassel, Germany) at 24°C. After 24 h the filter was removed and adherent yeast cells were eluted with water. CPY bound to the filter was detected with a polyclonal CPY antibody as described (Munn and Riezman, 1994).

Results

Identification of yeast mutants defective in fluid-phase endocytosis

A systematic screen in the yeast *S. cerevisiae* was performed to identify mutants that are defective for endocytosis. The yeast library that was used for this purpose contains about 700 yeast strains generated by EUROFAN (Oliver, 1996). In each strain a single ORF is replaced with the selectable marker kanMX (for a list of all strains tested, see **www.wiley.com/products/subject/life/genetics/genetics_yeast** and **www.biozentrum.unibas.ch/personal/riezman**). Each of the 700 EUROFAN strains was incubated in a medium containing LY and analysed under the microscope for its ability to accumulate the dye in the vacuole as described (Riezman, 1985; see also Materials and methods). In this primary screen, 18 of the knock-out yeast strains showed clearly reduced levels of LY in the vacuole. In addition, four of the 18 mutants showed alterations in their vacuolar morphology. To confirm these results, the genes were also disrupted in our strain background (for strains,

see Table 1). Replacement of the ORFs with the kanMX-cassette was confirmed by PCR on the DNA of geneticin-resistant colonies (data not shown). Four of the initial 18 mutants are not further described because they show, if anything, only a minor defect in LY accumulation in our strain background and behave like the wild-type cells for all the other endocytic assays tested (see below). Fourteen mutants had a defect in our strain background (Table 2; Figure 1). The corresponding wild-type gene on a centromere-containing vector could complement the endocytic defect in all of these strains (data not shown). Therefore, the endocytic defect is linked to the disruption we have introduced. The 14 mutants were categorized into four groups according to their defect in fluid-phase endocytosis and vacuolar morphology (Table 2).

Half of the mutants obtained in this screen belong to the first group of mutants that were strongly defective for the accumulation of LY in the vacuole (Figure 1A). Compared to the strong staining of wild-type vacuoles, the vacuoles of these mutants were only faintly stained, even though vacuolar morphology was indistinguishable from those of wild-type cells.

Smaller endocytic compartments cannot be visualized as discrete compartments using LY as a marker dye (Riezman, 1985). However, there was no detectable obvious increase in the LY signal in the cytoplasm surrounding the vacuole (Figure 1). On the other hand, LY gives quite a high background staining of the cell wall. This background was approximately equally strong for mutant and wild-type cells (Figure 1A) and underscores the relative absence of LY in mutant vacuoles. The endocytic defects of the *rcy1*Δ, *ede1*Δ and *tlg2*Δ mutants using LY have recently been published and are therefore not shown here (Abeliovich *et al*., 1998; Seron *et al*., 1998; Wiederkehr *et al*., 2000; Gagny *et al*., 2000). *SYS1* has been isolated as a multicopy suppressor of the *ypt6* mutation, but a possible involvement of Sys1p in endocytosis was not described (Tsukada and Gallwitz, 1996). *YNL177c*, *YNL227c* and *YNR075w* belong to the group of previously uncharacterized genes. *YNL227c* encodes a protein with several interesting protein motifs, including a DNA J-domain (see Discussion).

The second group of mutants identified in this screen had a less severe endocytic phenotype (Figure 1B; Table 2). The LY signal in the vacuole

Table 2. Overview of mutants and phenotypes

ORF	Group	*vma2*	Internalization	FM4-64	CPY	Further description
*sys1*Δ	1	No	Normal	No	Weak	Tsukada *et al.* (1996)
ynl177c	1	No	Reduced	No	No	
ynl227c	1	vsgd	Normal	No	No	J-domain
ynr075w	1	No	Normal	No	No	
*ede1*Δ	1	No	Reduced	ND	ND	Gagny *et al.* (2000); EH-domain
*tlg2*Δ	1	vsl	Normal	ND	Weak	Abeliovich *et al.* (1998); t-SNARE
*rcy1*Δ	1	ND	Normal	Endosome	No	Wiederkehr *et al.* (2000); Wysocki *et al.* (1999); F-box
sds24	2	No	Normal	No	No	
ydr036c	2	No	Normal	No	No	
fth1	2	No	Normal	No	No	Urbanowski *et al.* (1999) Rodriguez-Navarro *et al.* (1999)
ybr266c	3	No	Normal	Endosome	No	Sanjuan *et al.* (1999)
ydl231c	3	No	Normal	Vacuoles	No	Brefeldin A sensitivity; Muren *et al.* (2000)
ynl297c	3	No	Normal	Endosome?	No	Brefeldin A sensitivity; Muren *et al.* (2000)
vps53	4	vsgd	Normal	Vacuoles	No	Conibear *et al.* (2000)

Group 1: strong fluid-phase endocytosis defect; normal vacuolar morphology. Group 2: less severe fluid-phase endocytosis defect; normal vacuolar morphology. Group 3: fluid-phase endocytosis defect; aberrant vacuolar morphology. Group 4: vma2 synthetic growth defect but no defect in fluid phase endocytosis. vsgd/vsl: *vma2* synthetic growth defect/*vma2* synthetic lethality. Endosome: retention of FM4-64 in endosomes. Vacuole: vacuolar morphology phenotype. CPY: secretion of CPY.

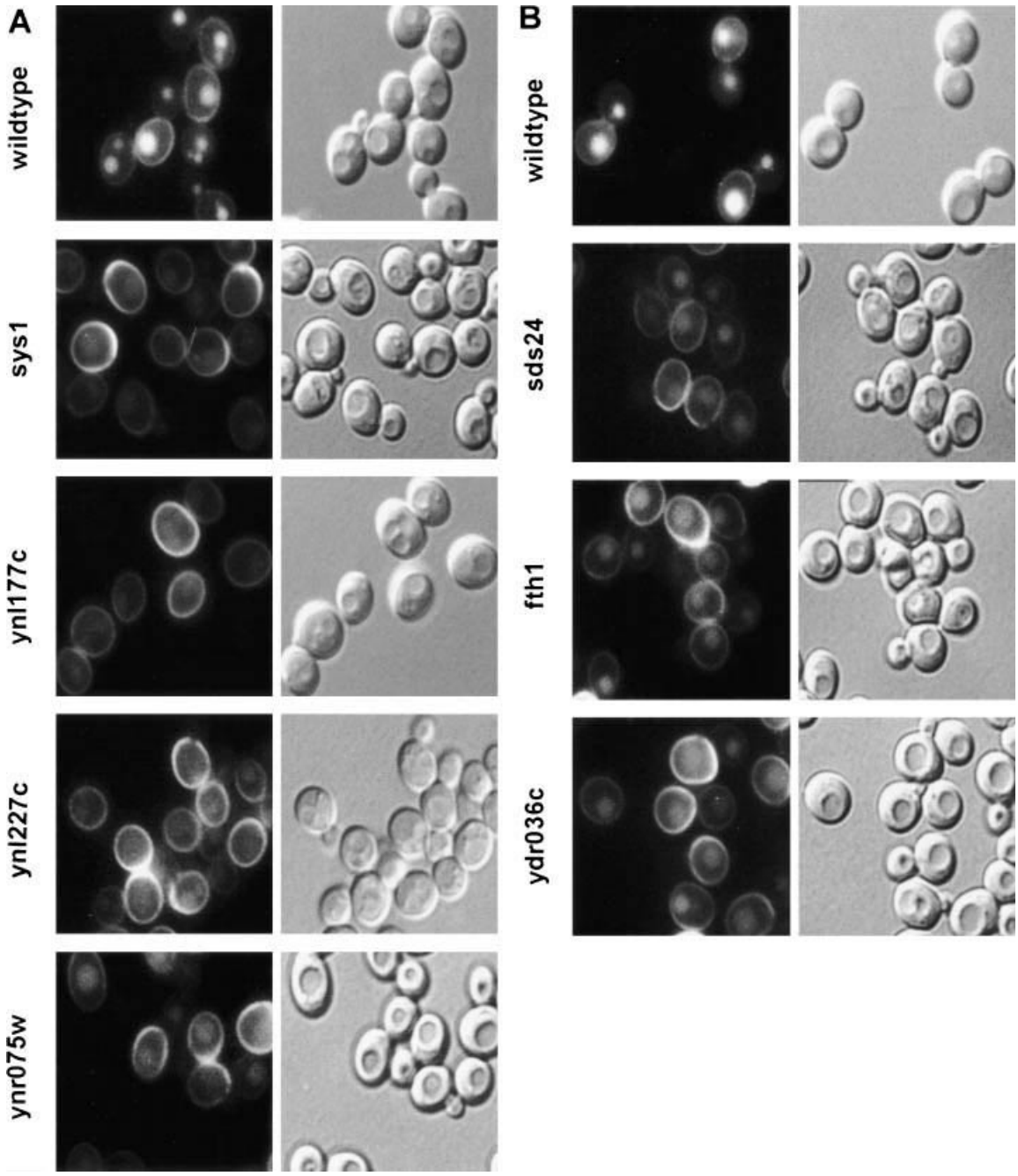

Figure 1. Continues on next page

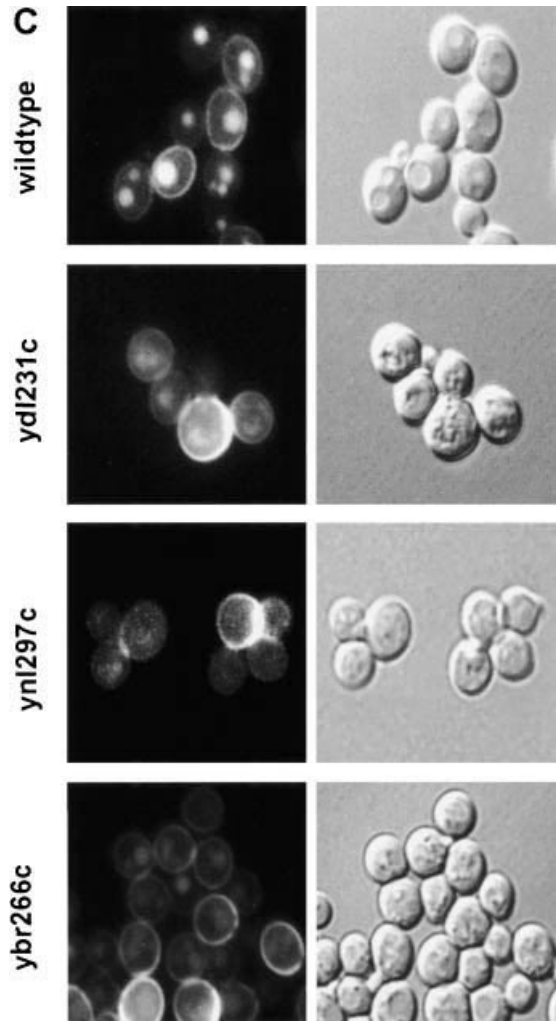

Figure 1. Continued
Accumulation of LY in the vacuoles of mutants and corresponding wild-type cells. Yeast cells were incubated for 1 h at 24°C in YPUAD medium containing LY (4 mg/ml). The washed cells were viewed with a fluorescence microscope using a fluorescein filter set (left) and Nomarski optics (right). Mutant cells with a strong defect in fluid-phase endocytosis (A), with a less severe defect (B) and those with an additional vacuolar phenotype (C) are shown in separate panels. LY assays shown in individual panels were performed in parallel

of these mutant cells was still clearly visible but, compared to the wild-type strain, the three mutants *fth1*Δ, *sds24*Δ, and *ydr036c* failed to accumulate LY efficiently. The morphology of their vacuoles is normal. The *fth1*Δ mutant has been previously characterized (Urbanowski and Piper, 1999; Rodriguez-Navarro *et al*., 1999). Fth1p is an iron transporter that localizes to vacuolar membranes. Sds24p has been named due to its homology to sds23 from *Schizosaccharomyces pombe* and Ydr036c is homologous to a family of enoyl-CoA hydratase/isomerase proteins.

The third group of mutants with a defect in fluid-phase endocytosis has an additional vacuolar phenotype (Figure 1C; Table 2). The vacuoles of *ydl231c* mutant cells were strongly fragmented but still visible by Nomarski optics. Weak LY staining was observed when large vacuolar fragments were present. The reduced signal observed in *ydl231c* mutant cells is not likely to be the consequence of the fragmentation of the vacuoles alone, since several of the mutants of the EUROFAN strain collection had fragmented vacuoles, and were still able to accumulate LY efficiently (data not shown). The vacuoles of *ynl297c* mutant cells were too small in size to be clearly identified by either fluorescence or Nomarski optics (Figure 1C) and the cell wall also showed reduced staining. Almost no intracellular LY-positive areas could be observed. The vacuolar phenotype of *ybr266c* cells was less striking. Individual cells contained a larger number of vacuoles that were smaller than those in wild-type cells (Figure 1C). Accumulation of LY in the vacuoles of *ybr266c* cells was reduced to a similar extent as for the mutants shown in Figure 1B.

The *vps53*Δ mutant has been isolated previously as a vacuolar protein-sorting mutant. Its defects in membrane trafficking and vacuolar morphology have been described in detail elsewhere (Conibear and Stevens, 2000). The mutant *vps53*Δ has been identified during our primary screen but in our genetic background endocytosis of LY was close to normal (data not shown). Still, *vps53*Δ was included (Table 2; group 4) because of its genetic interaction with *vma2*Δ (see below).

Mutants with *vma2* synthetic growth defect

An earlier genetic screen performed in our laboratory has identified six endocytosis mutants on the basis of their synthetic lethality with *vma2*Δ (Munn and Riezman, 1994). Vma2p is a subunit of the vacuolar ATPase essential for its function (Nelson and Klionsky, 1996). The *vma2*Δ mutant is inviable when grown in media buffered at pH 7 demonstrating that acidification of the vacuolar system is an essential process. However, in media of low pH

(here pH 5.2) the *vma2*Δ mutant cells can still grow. Under these conditions acidification of the vacuolar system may depend upon endocytosis of protons from the medium. To test for *vma2* synthetic lethality, the above-described mutants were crossed with our *vma2*Δ*::LEU2* strain (Table 1). Diploid strains heterozygous for *vma2*Δ and a gene of interest were sporulated and the resulting tetrads dissected. Surprisingly, most of the mutants identified here showed no synthetic growth defect with *vma2*Δ. As mentioned above, the *vps53*Δ mutation, despite its very weak effect on LY accumulation, had a strong growth defect when combined with *vma2*Δ (Figure 2B). Among the mutants with a strong defect in fluid-phase endocytosis shown in Figure 1A, only *ynl227c* showed a strong growth defect with *vma2*Δ (Figure 2C). The *ynl227c vma2*Δ double mutant grew slower than either of the corresponding single mutant haploid cells. In summary, we have not observed a correlation between the severity of the fluid-phase endocytosis defect and the *vma2* synthetic growth defect. Only three of our mutants showed genetic interactions with *vma2*Δ.

Defect in internalization of α-factor pheromone

To characterize the endocytic defect further, we distinguished whether the newly identified mutants were defective for internalization or subsequent delivery of endocytic material to the vacuole. To measure internalization we followed the uptake of the pheromone α-factor. Upon binding of α-factor, the receptor Ste2p undergoes ligand-stimulated endocytosis. In wild-type cells radio α-factor is internalized with a half-time of 7 min (Figure 3;

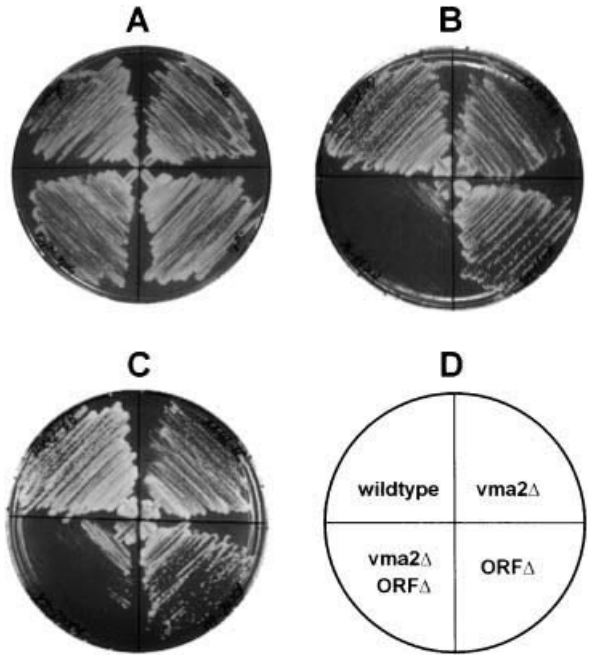

Figure 2. *vma2* synthetic growth defects. The different kanMX disruptants were crossed with a *vma2*::*LEU2* strain. The heterozygous diploids were sporulated and dissected on YPUAD plates pH 5.2. After assigning the markers strains derived from a tetrad were streaked out on YPUAD plates pH 5.2 as shown in panel D. The cells were allowed to form colonies at 24°C. Strains derived from heterozygous diploids *vma2/VMA2* in combination with *sys1*Δ*/SYS1* (A), *vps53*Δ*/VPS53* (B) or *ynl227c/YNL227c* (C) are shown

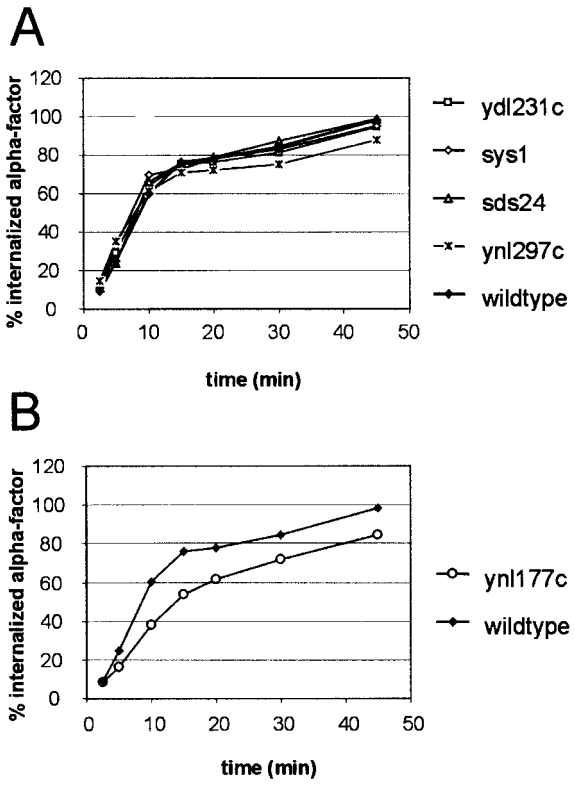

Figure 3. α-Factor internalization. Radio α-factor was bound to the different mutant cells on ice. At time-point 0, the cells were resuspended in YPUAD (24°C). The percentage of internalized α-factor at the indicated time-points was calculated as described in Materials and methods. (A) Mutants with wild-type kinetics for α-factor internalization. (B) Internalization of α-factor is slower in *ynl177c* mutant cells. Each time-point shows the average of two independent experiments

Raths *et al*., 1993). With the exception of ynl177c, all the mutants internalized α-factor with similar kinetics to the corresponding wild-type strain. Several uptake curves for mutants with wild-type internalization kinetics are shown in Figure 3A. α-Factor internalization by *ynl177c* mutant cells in comparison with wild-type cells is shown in Figure 3B. During the initial phase of the assay (up to 10 min), α-factor internalization occurs at a constant rate. This internalization rate can be used to quantify the defect observed in the *ynl177c* mutant. Under our experimental conditions the *ynl177c* mutant strain internalizes 4% of the α-factor/ min and is therefore 41% slower than the corresponding wild-type ($6.8 \pm 0.3\%$ SEM). The kinetics of the internalization defect of *ynl177c* mutant strain is very similar to the defect that has been described for the *ede1*Δ mutant, which was also identified in this screen (Table 2; Gagny *et al*., 2000). Therefore, two of a total of 14 mutants identified in this screen were defective for the internalization step of endocytosis.

Secretion of carboxypeptidase Y

Vps mutants comprise the large majority of post-internalization endocytosis mutants identified to date. The vps and endocytosis pathway intersect at the PVC, which explains the strong dependence of effective endocytosis on a functional vps pathway. For example, mutants that block membrane trafficking between the PVC and the vacuole affect both pathways (Piper *et al*., 1995; Bryant and Stevens, 1998). One of the characteristics of vps mutants is that a fraction of the newly synthesized vacuolar protease carboxypeptidase Y (CPY) is mis-sorted and as a consequence secreted from the yeast cells. Secretion of CPY from the mutant cells was tested using a colony blot assay (see Materials and methods). Surprisingly, only the *sys1*Δ and *tlg2*Δ mutants secreted enough CPY to be detected by this assay (Figure 4). Secretion of CPY from *sys1*Δ, and *tlg2*Δ mutant cells has been noted earlier (Abeliovich *et al*., 1998; Tsukada and Gallwitz, 1996). The positive controls *vps1* and *vps4* gave a very strong signal with this assay. These mutants are known to secrete 80% (*vps1*) and 45% (*vps4*) of their newly synthesized CPY (Raymond *et al*., 1992). The *sys1*Δ and *tlg2*Δ mutant cells secreted clearly less CPY than *vps4*. The low levels of secreted CPY observed for the *sys1*Δ and tlg2Δ strains is in agreement with previous reports that had found only about 10% of newly synthesized CPY in the extracellular fraction for sys1Δ (Tsukada and Gallwitz, 1996) and 20% for tlg2Δ (Abeliovich *et al*., 1998). Unexpectedly, in our strain background the vps53Δ mutant cells did not secrete CPY (Figure 4).

Detection of endosomes using FM4-64

To distinguish between the different mutants that are likely to be blocked at a post-internalization step of endocytosis, we used the dye FM4-64. This dye is strongly fluorescent when bound to membranes and has been used to label the vacuole in yeast (Vida and Emr, 1995). The strong signal obtained with this dye also allows the visualization of much smaller compartments, such as endosomes (Vida and Emr, 1995; Wiederkehr *et al*., 2000). In our hands, FM4-64 is less convenient to observe kinetic delays in endosomal transport to the vacuole, as even mutants with strong endocytic defects are able to transport this dye to the vacuole (Vida and Emr, 1995). Possible differences in the amount of FM4-64 in the vacuole are difficult to judge at the single-cell level. For the above reasons, we have primarily used FM4-64 to visualize the vacuole and to identify endosomal compartments where FM4-64 may be retained in the different mutant strains. The cells were allowed to take up the dye for 45 min at room temperature, as described in Materials and methods. After this incubation time, all of the mutants studied here were able to accumulate some of the dye in vacuoles (Figure 5; data not shown). For the *ynl297c* mutant it was not clear whether the FM4-64-positive compartments were endosomes or highly fragmented vacuoles. In contrast to LY, FM4-64 easily allowed the detection of compartments of the vacuolar system in the *ynl297c* mutant cells (cf. Figures 1C, 5). Consistent with our observations from Nomarski pictures, FM4-64 staining nicely revealed the highly fragmented vacuoles of *ydl231c* and the less aberrant vacuoles of *ybr266c* (Figure 5). For the *ybr266c* mutant cells we noted strongly stained small compartments close to the vacuoles that could still be observed after long chase times. The distribution and size of these compartments suggests that they may be late endosomes.

In summary, eight of the newly identified mutants have additional endocytic phenotypes, such as

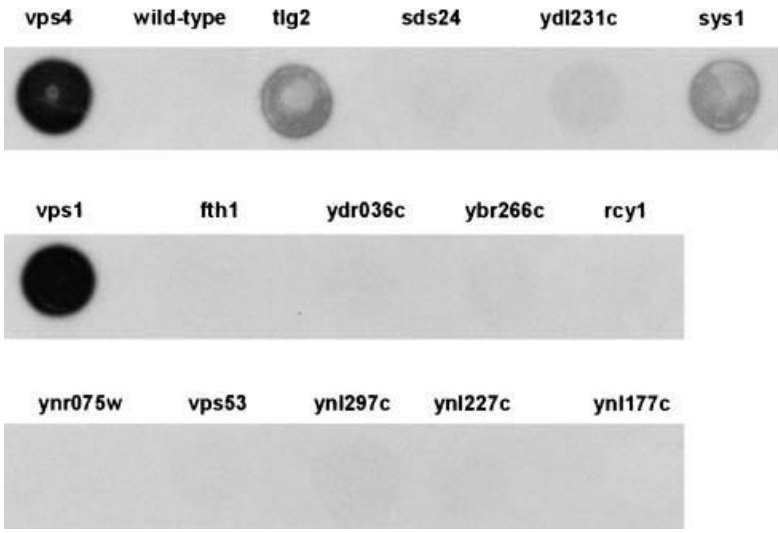

Figure 4. Filter immunoblot for the detection of secreted CPY. The indicated mutant strains were grown on YPUAD plates at 24°C and then incubated in contact with the nitrocellulose filter for 24 h as described in Materials and methods. The filter was probed with a rabbit antiserum raised against CPY. The shown results were obtained from a single blot (chemiluminescence Western blotting detection: exposure time 3.0 min)

reduced α-factor internalization, secretion of CPY, accumulation of FM4-64 in endosomes or a vacuolar phenotype.

Discussion

From a yeast library of 700 strains we have identified 14 mutants defective for fluid-phase endocytosis. Each of the strains tested was disrupted for a single non-essential ORF that had been identified during the sequencing of the yeast genome (Oliver, 1996). As 2% of the disruptant strains were defective in the accumulation of LY, it can be extrapolated that, in the entire yeast genome, there should be approximately 110 non-essential genes present that are necessary for efficient fluid-phase endocytosis. Earlier mutant analysis has shown that yeast strains disrupted for non-essential genes can be strongly defective for endocytosis (Munn and Riezman, 1994; Babst *et al.*, 1997). It was therefore likely to find new endocytosis mutants in the EUROFAN collection of mutants disrupted for non-essential genes. An obvious advantage of such a screen is that there is no need to subsequently identify the defective gene and clone the wild-type copy by phenotypic complementation. Therefore, this is a very efficient screen for systematically identifying the non-essential yeast genes involved in endocytosis.

Mutants along the endocytic pathway

Two main groups of endocytosis mutants are known: mutants that are defective for the internalization step and those that are inhibited at a post-internalization step of endocytosis. *Ede1*Δ and *ynl177c* identified in this screen belong to the internalization mutants, as they showed reduced kinetics for α-factor internalization. The majority of the newly identified mutants, however, are likely to be inhibited at a post-internalization step of endocytosis.

Most endocytosis mutants affected at a post-internalization step of endocytosis have been identified in the extensive screens for vps mutants (Bryant and Stevens, 1998). As the endocytic and vps pathways intersect, mutations of VPS genes often cause inhibition of the endocytic pathway. With the exception of *sys1*Δ and *tlg2*Δ, which secrete low levels of CPY, the newly identified mutants do not secrete CPY to the extent that it could be detected by colony blot analysis (Figure 4). Another indication for possible defects in vacuolar protein sorting is changes in vacuolar morphology. This is one criterion that has been used to classify the vps mutants; notably, three mutants, *ybr266c*,

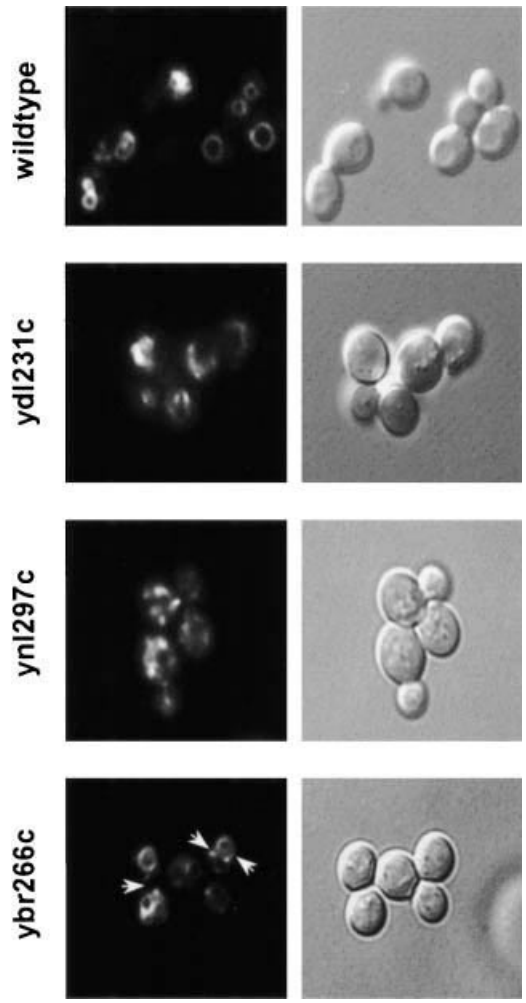

Figure 5. Visualization of vacuoles and/or endosomes using FM4-64. Wild-type and mutant cells were allowed to internalize FM4-64 for 45 min at 24°C as described in Materials and methods. The cells were visualized under the fluorescence microscope using a rhodamine filter set (left) or Nomarski optics (right). Using FM4-64 the aberrant endosomal and vacuolar compartment of *ydl231c*, *ynl297c* and *ybr266c* strains can be seen in more detail. The compartment in *ynl297c* cells that were not observed with LY or Nomarski could be endosomes. Arrows indicate the small FM4-64-positive structures close to the vacuole of *ybr266c* cells

ydl231c and *ynl297c* (not including *vps53*Δ), identified here have abnormal vacuoles. Following CPY maturation and the detection of CPY in the extracellular fraction by pulse-chase analysis, it will be necessary for these mutants to judge the effect of the mutation on vacuolar protein sorting. The extent of CPY secretion appears to vary with strain background (unpublished results). In our strain background, inhibition of specific steps along the vacuolar protein-sorting pathway may lead to the secretion of CPY (see Figure 4; vps4/end13), whereas other defects in the transport between the Golgi apparatus and the vacuole may not (see Figure 4; vps53).

Only very few mutants have been isolated in the past that block an early post-internalization step of endocytosis. As such mutants act upstream of the PVC, they may leave the vps pathway unaffected. Several of the mutants identified in this screen were not defective for the internalization step of endocytosis and did not secrete CPY, neither could we observe any vacuolar abnormalities. The phenotype of these newly identified fluid-phase endocytosis mutants therefore suggests that, like *rcy1*Δ, they may belong to endocytosis mutants that affect endocytosis at a step before the intersection with the vps pathway (Wiederkehr *et al.* 2000). This mutant screen may have uncovered a set of mutants affecting transport steps of the endocytic pathway that have obtained less attention in the past.

Vma2 synthetic growth defect

One of the surprising results in this screen is that most of the mutants have no synthetic growth defect in combination with *vma2*Δ. The *vma2*Δ mutant does not acidify the lumen of the vacuolar membrane system (Nelson and Klionsky, 1996) and has been proposed to grow only on acidic medium because it becomes dependent on endocytosis of protons from the medium for viability. In a genetic screen for endocytosis mutants 18 mutants were isolated as *vma2*Δ synthetically lethal; seven were strongly defective for the accumulation of LY in the vacuole (Munn and Riezman, 1994). Endocytosis mutants were therefore strongly enriched ($\approx$40%) in a set of strains pre-selected for *vma2* synthetic lethality. Here, the mutants *tlg2*Δ, *vps53*Δ and *ynl227c* led to a strong growth defect or even synthetic lethality (Figure 2; Seron *et al.*, 1998) when combined with *vma2*Δ. This result suggests a role for Tlg2p, Vps53p and Ynl227cp in fluid phase transport (proton transport) to the compartment of the vacuolar system that needs to be acidified. Unfortunately, it is not known which compartment this is and recent results point to an alternative, NH^{4+} dependent pathway for vacuolar acidifica-

tion (Plant *et al.*, 1999). The lack of genetic interaction of our with *vma2*Δ could be due to a partial block in endocytosis. The lack of correlation between the strength of the LY accumulation defect and the *vma2* synthetic growth defect argues against this explanation. It is therefore more likely that inhibition of specific membrane trafficking steps may cause *vma2* synthetic growth defects. For instance, inhibition of endocytosis at a post-internalization step may be circumvented, as the transported material may be able to take different routes within the endosomal system. In contrast, inhibition of the internalization step of endocytosis should prevent the acidification of any intracellular compartments via endocytosis. Indeed, most of the *vma2* synthetically lethal endocytosis mutants are known to affect the internalization step of endocytosis (Munn and Riezman, 1994). The internalization mutant *ede1*Δ and *ynl177c* identified in this screen were probably not strongly enough inhibited (about 35%) to cause *vma2* synthetic lethality. Of course, the possibility remains that the lack of endocytosis is not the true reason for the expression of *vma2* synthetic lethality.

New fluid-phase endocytosis mutants

Since the creation of the EUROFAN mutant collection and during the course of this study, four of the fluid-phase endocytosis mutants identified were shown to be involved in membrane trafficking. The fact that mutants such as *ede1*Δ, *rcy1*Δ, *tlg2*Δ and *sys1*Δ were identified, underlines the usefulness of this screen to obtain new endocytosis mutants. *Ede1*Δ, *rcy1*Δ and *tlg2*Δ mutants were identified in this screen, but their endocytic defect has been characterized in detail and presented elsewhere (Seron *et al.*, 1998; Wiederkehr *et al.*, 2000; Gagny *et al.*, 2000). In particular, this screen permitted the isolation of the *rcy1*Δ mutant, which revealed a novel endocytic phenotype because this mutant accumulates a compartment that is most likely involved in endocytic recycling (Wiederkehr *et al.*, 2000).

SYS1 has been isolated as a multicopy suppressor of *ypt6*Δ (suppressor of ypt six) (Tsukada and Gallwitz, 1996). The role of Sys1p and Ypt6p in membrane trafficking is still elusive. Ypt6p is a member of the Ypt/Rab family of small GTPases involved in specific vesicle transport and fusion steps. Ypt6p may function in the cycling of proteins such as Kex2p and Vps10p between endosomes and the late Golgi apparatus. However, no obvious defect in secretion and only a minor defect in vacuolar protein sorting were seen for the *sys1*Δ mutant (Tsukada and Gallwitz, 1996; Figure 4). Here, we show that *sys1*Δ cells are strongly inhibited for fluid-phase endocytosis. Together, these results suggest that Sys1p may primarily play a role in endocytosis.

For the other nine mutants defective for fluid-phase endocytosis, no membrane-trafficking phenotype has been noted earlier. However, several of them have additional phenotypes or encode proteins with known domains that may give information on the role of these genes in endocytosis. The two mutant strains with strongly fragmented vacuoles, *ynl297c* and *ydl231c,* have also been picked up in a screen for Brefeldin A-sensitive yeast mutant strains (Muren, 2000). Brefeldin A is a fungal toxin that can bind to some Sec7 domain proteins and thereby inhibits their GTP exchange activity for ADP-ribosylation factor (ARF) (Peyroche *et al.*, 1996; Jackson and Casanova, 2000). Treatment with Brefeldin A leads to a block in many membrane transport steps and to the disassembly of the Golgi complex. In yeast, Gea1p, Gea2p and Sec7p are GTP exchange factors for ARF and are sensitive to Brefeldin A. Wild-type yeast cells, however, are rather insensitive to this drug, despite the presence of Brefeldin A-sensitive factors. Mutations that render yeast cells more permeable for the drug can increase the sensitivity for Brefeldin A (Vogel *et al.*, 1993). It is also possible that hypersensitivity could arise because of a decrease in export of Brefeldin A, as occurs for many other drugs (Balzi *et al.*, 1994). Another possible mechanism to explain increased sensitivity to Brefeldin A is that the presence of a low intracellular level of Brefeldin A inhibits sec7-domain proteins partially. Partial inhibition by Brefeldin A on top of defect in membrane trafficking caused by the mutation may render the cells unable to grow. It is not clear by what mechanism the *ydl231c* or the *ynl297c* mutations result in Brefeldin A sensitivity. *Ynl297c* has homologies to the Golgi localization domain of BIG1, a human GEF (Mansour *et al.*, 1999). BIG1, BIG2, Gea1p and Gea2p are large sec7-domain proteins 150–200 kDa in size that are subunits of multiprotein complexes of about 600 kDa (Peyroche *et al.*, 1996; Yamaji *et al.*, 2000). *YNL297c* encodes

a putative protein of 190 kDa (1635 amino acids) and may be a component of such a complex. As Ynl297c protein itself is unlikely to be a GEF, because it has no sec7 domain, it may assist in the regulation or localization of such an activity.

From what is known about the group of mutants with weaker defects in fluid-phase endocytosis (*fth1*, *sds24* and *ydr036c*), it can be speculated that their role in endocytosis is only indirect. Fth1p transports Fe^{3+} from stores in the vacuolar lumen to the cytosol (Urbanowski and Piper, 1999). Sds24p is 34% identical along its entire length with the *Sz. pombe* protein sds23p, which may be involved in cell cycle progression (Ishii *et al*., 1996). Due to its homologies, *Ydr036c* has been classified as a member of the enoyl-CoA hydratase/isomerase protein family.

Ynl227c belongs to the group of mutants strongly affected for fluid-phase endocytosis. In addition, it is the only mutant that has a strong synthetic growth defect with *vma2* (Figure 2C). Ynl227c protein has a 70 amino acid motif called the J-domain, named after DNAJ, an *E. coli* protein that belongs to the family of Hsp40 co-chaperones (Kelley, 1998). Like Ynl227c protein, Hsp40 family proteins have an N-terminal J-domain and zinc-finger motifs. The role of Hsp40 proteins is to stimulate ATP hydrolysis of Hsp70, the actual chaperone. The role of chaperones is to assist in protein folding or degradation or to mediate conformational changes of proteins. The J-domain of Ynl227c protein is highly identical to well-described J-domain proteins (55% identity to J-domain of Ydj1p) and has the conserved HPD sequence required for co-chaperone activity (Tsai and Douglas, 1996). Ynl227c might recruit Hsp70-like activity to specific sites where chaperone function is required for membrane function. Cytoplasmic Hsp70 activity has been implicated, for example, in the uncoating of clathrin-coated vesicles in mammalian cells (Ungewickell *et al*., 1995).

Conclusion

The different mutants isolated here have defects in endocytosis of variable severity and therefore could be essential components for specific membrane trafficking steps or have regulatory functions. Alternatively, it is possible that some of the mutations affect functions unrelated to membrane trafficking that indirectly cause endocytosis to be inhibited. Our initial characterization of the mutants shows that several of the mutants have additional phenotypes consistent with a role for the gene products in membrane trafficking. Previous analysis in yeast has repeatedly demonstrated that different membrane-trafficking pathways are interconnected (Riezman, 1985; Hicke *et al*., 1997; Wooding and Pelham, 1998). For instance, blocking the early secretory pathway can cause a strong inhibition of endocytosis at a post-internalization step (Hicke *et al*., 1997). Therefore, for the mutants described here that affect fluid phase endocytosis, a careful characterization of not only the endocytic defect but also the secretory and vacuolar protein-sorting pathway will be necessary. Further analysis of these mutants and the molecular role of the cognate proteins should be good starting points to uncover new concepts in endocytosis and membrane trafficking.

Acknowledgements

We would like to thank Rosine Haguenauer-Tsapis and Sandrine Avaro for interesting comments on the manuscript and for generously sharing results prior to publication. For EUROFAN plasmids and strains, we thank Peter Koetter and other members of EUROSCARF. This work was funded by a grant from the Swiss National Science Foundation and the Bundesamt für Bildung und Wissenschaft (Switzerland).

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