Yeast Ypt51p and mammalian Rab5: counterparts with similar function in the early endocytic pathway

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SUMMARY

Ypt51p, a small GTPase of Saccharomyces cerevisiae, has been previously identified as a structural homolog of mammalian Rab5. Although disruption analysis revealed that the protein is required for endocytic transport and for vacuolar protein sorting, the precise step controlled by Ypt51p was not determined. In this work we show that by heterologous expression in animal cells Ypt51p was targeted to Rab5-positive early endosomes and stimulated endocytosis. Furthermore, two Ypt51p mutants induced similar morphological alterations as the corresponding Rab5 mutants. Also in yeast cells Ypt51p was found to be required at an early step in endocytic membrane traffic, since α-factor accumulated in an early endocytic intermediate in the absence of Ypt51p. Cell fractionation analysis revealed cofractionation of Ypt51p with endocytic intermediates, while no association with the late Golgi compartment could be detected. Indirect immunofluorescence microscopy allowed us to morphologically identify the Ypt51p-containing compartment. Similar to the mammalian system larger Ypt51p-positive structures were revealed upon expression of Ypt51p Q66L. These structures were also positive for α-factor receptor and for carboxypeptidase Y, thus providing direct evidence for their endocytic nature and for the convergence of the vacuolar biosynthetic and endocytic pathways.

Key words: endocytosis, GTPase, yeast

INTRODUCTION

In mammalian cells, morphological and biochemical studies have provided detailed insights into the organization of the endocytic pathway (for reviews see Hubbard, 1989; Kornfeld and Mellman, 1989). The best characterized pathway involves the formation of clathrin-coated vesicles that pinch off from the plasma membrane and fuse with the early endosomes after losing their clathrin coat. Early endosomes, which have a tubulo-vesicular morphology, are preferentially found at the cell periphery and play an important role in the uncoupling of ligands from their receptors and in the recycling of some receptors (e.g. transferrin receptor) back to the plasma membrane. Following sorting in early endosomes, ligands destined for degradation are next transported to late endosomes (also referred to as prelysosomes). This compartment is generally located in the perinuclear region of the cell and is enriched in lysosomal enzymes, lysosomal membrane proteins and the cation-independent mannose-6-phosphate receptor (CI-MPR). These findings led to the proposal that late endosomes correspond to the site where newly synthesized lysosomal enzymes bound to CI-MPR and lysosomal glycoproteins delivered from the trans-Golgi network, meet endocytosed material (Griffiths et al., 1988). However, more recently newly synthesized lysosomal enzymes have been shown to be transiently present in early endosomes (Ludwig et al., 1991) suggesting that this compartment represents the earliest point of convergence of the lysosomal biosynthetic and the endocytic pathways. Lysosomes serve as the terminal compartment for intracellular transport of newly synthesized hydrolases, and are generally considered as the end station of the endocytic pathway, where degradation occurs.

In contrast to higher eukaryotic cells, the compartmentalization of the endocytic pathway in the budding yeast Saccharomyces cerevisiae is still poorly understood. During the delivery from the plasma membrane to the vacuole the endocytic marker α-factor sequentially passes through two kinetically distinct membrane compartments, tentatively termed early and late endosomal fractions, with different densities on a Nycodenz density gradient (Singer and Riezman, 1990; Singer-Krüger et al., 1993). Since the biochemical and morphological characterization of the isolated fractions is presently still preliminary, it remains to be determined whether these compartments may either be equivalent to the early and late endosomes described in mammalian cells or represent transient vesicular intermediates.

While the exploration of the morphological and functional organization of the endocytic compartments in yeast is only at
its beginning, this experimental system provides the advantage of using a genetic approach to identify and characterize components that regulate endocytic transport. This approach has uncovered a number of proteins required for the initial step of endocytic internalization: clathrin heavy chain (Payne et al., 1988), the cytoskeletal elements actin and yeast fimbrin (Sac6p) (Kübler and Riezman, 1992), calmodulin (Kübler et al., 1994), and the gene products of END3, END4 (Raths et al., 1993), END8, END9, END10, and END11 (Munn and Riezman, 1994).

Small GTPases of the Rab/Ypt family have been implicated in regulating distinct steps of vesicular traffic (reviewed by Zerial and Stenmark, 1993; Pfeffer, 1994). In S. cerevisiae several Ypt proteins have been suggested to control endocytic membrane traffic. Disruption of YPT7, whose gene product is homologous to the mammalian late endosome marker Rab7 (Chavrier et al., 1990), causes pleiotropic effects on the endocytic pathway and vacuolar biogenesis (Wichmann et al., 1994). Cell fractionalization experiments suggested a role for this small GTPase in controlling membrane traffic between the temporally defined late endocytic fraction and the vacuole (Schimmöller and Riezman, 1993). Furthermore, Ypt51p (identical to Vps21p), Ypt52p and Ypt53p were previously identified as structural homologs of mammalian Rab5 (Singer-Krüger et al., 1994; Horazdovsky et al., 1994). Similar to Rab5, which is a key regulator of endocytic transport between the plasma membrane and early endosomes (Gorvel et al., 1991; Bucci et al., 1992), the yeast Ypt5 proteins were shown to be required for endocytic delivery of two endocytic markers, α-factor and Lucifer Yellow CH (LY), to the vacuole. In addition, a vacuolar protein sorting defect was revealed for mutants of the YPT51 gene (Singer-Krüger et al., 1994; Horazdovsky et al., 1994). Although the structural similarity of the Ypt5 proteins to Rab5 was intriguing and suggestive of a primary role in endocytic transport, the initial phenotypic characterization of the mutants did not reveal at which precise step in membrane traffic these small GTPases were required.

In the present paper, we have used a variety of approaches to address in more detail the role and localization of Ypt51p in endocytic transport. Although three Rab5 homologs have been identified (Singer-Krüger et al., 1994), here we focus on Ypt51p, because the ypt51 null mutant is characterized by a number of detectable phenotypes, while the other mutants behave phenotypically like wild-type cells. By heterologous expression of Ypt51p in animal cells, we provide evidence that Ypt51p displays Rab5-like properties, lending further support for the idea that Rab5 and Ypt51p are functionally conserved proteins. Furthermore, subcellular fractionation studies in combination with epistasis analysis suggest that Ypt51p is required prior to Ypt7p in transport between the kinetically defined early and late endocytic compartments and that the protein is localized to both endocytic intermediates. Using indirect immunofluorescence microscopy, we found that the Ypt51p-positive structures contain endocytosed α-factor receptor (Ste2p) and carboxypeptidase Y (CPY). These results provide direct evidence for the association of Ypt51p with an endocytic compartment at the convergence of the endocytic and vacuolar protein sorting pathways. Our results support the view that the molecular regulation of the endocytic pathway is conserved from yeast to mammals.
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Louvard et al. (1983). An aliquot (containing approximately 300 μg of peptide coupled to KLH) was mixed with complete Freund’s adjuvants to a stable emulsion and used for the initial immunization, which was applied into the popliteal lymphnodes; 250 mg of peptide-conjugate was used for the subsequent intra-dermal or intra-muscular injections using an emulsion with Freund’s incomplete adjuvant. Bleeds were taken 7-10 days after the previous injection. The sera were tested by immunoprecipitation of in vitro-translated Ypt51p, Ypt52p and Ypt53p. For this purpose, 5 μl of in vitro-translated protein (Ullrich et al., 1993) was incubated with 20 μl Protein A-Sepharose beads (loaded with 10 to 30 μl immune or preimmune serum) for 1 hour at RT and washed as described by Singer-Krüger et al. (1994). Immunoprecipitates were analyzed by PAGE and fluorography on 12% gels.

An affinity resin to purify the anti-Ypt51p antiserum was prepared with 1 ml of Affi10-gel resin and 5 μg of peptide (see above) according to the protocol provided by the manufacturer. In analogy, Affi15-gel resin and a synthetic peptide corresponding to the N-terminal sequence of Ste2p (SDAOPLSLNLNYFDPTK), was used to prepare an affinity column for the purification of the anti-Ste2p antiserum; 1 ml of anti-Ypt51p and 0.6 ml of anti-Ste2p antiserum (gratefully obtained from H. Riezman, Biozentrum, Basel, Switzerland) were added to the corresponding beads and rotated overnight at 4°C. After extensive washing with 0.1 M Hepes, pH 7.1, the bound IgGs were eluted using 0.2 M glycine, pH 2.4. After immediate neutralization with 1 M Tris-base, BSA and NaN3 were added to 1 mg/ml and 0.02%, respectively. To purify antibodies against CPY, a 0.5 cm x 7 cm nitrocellulose strip was incubated with 1.9 mg CPY (Sigma) for 4 hours and washed extensively with PBS. Subsequently, the strip was incubated with 170 μl of anti-CPY antiserum (obtained from H. Riezman). Elution was by three successive incubations with 0.2 M glycine, pH 2.4, for 5 minutes each, followed by immediate neutralization with Tris-Base. The eluates were combined and concentrated to a volume of 100 μl with Centricron-10 concentration devices. The affinity-purified antibodies were stored at 4°C or frozen at −20°C. Indirect immunofluorescence microscopy with BHK cells, overexpressing Ypt51p using the vT7 system, was performed to confirm the purification of anti-Ypt51p antibody. Western blot analysis was performed to follow the purification of anti-CPY and anti-Ste2p antibodies.

Cell fractionation

For the kinetic analysis of α-factor appearance in light and heavy endosome-enriched fractions on a Nycodenz gradient density, the wild-type (BS64) and mutant strains (BS25, BS84, RH1) were grown at 24°C to early logarithmic stage. 35 S-labeled pheromone was bound to the ferrin receptor to be transported to the plasma membrane (Bucci et al., 1992). Overexpressed proteins were labeled with: rabbit anti-Ypt7p, Rab5 and human transferrin receptor with the vT7 system, BHK-21 cells were infected with vT7 for 30 minutes at 25°C, and then transfected for 4 hours with plasmid DNA containing the gene of interest behind a T7 promoter using DOTAP (Boehringer Mannheim, Mannheim, Germany), as described by Stenmark et al. 1995. The conditions of cells infection and transfection were chosen to overexpress Rab5 ~15-fold over endogenous (Bucci et al., 1992). By western blot analysis the expression levels of the various Rab/Ypt proteins after 4 hours of transfection were analysed and found to be comparable and all within the typical range as described by Stenmark et al. (1995). Cells transfected with DNA encoding the human transferrin receptor were incubated post-transfection for 90 minutes in the presence of 10 μg/ml cycloheximide in order to allow newly synthesized receptor to be transported to the plasma membrane (Bucci et al., 1992).

For indirect immunofluorescence, cells grown on 10 mm round coverslips were permeabilized for 5 minutes with 0.05% saponin in 80 mM Hepes, pH 6.8, 5 mM EGTA, 1 mM MgCl2, and then fixed for 15 minutes with 3% paraformaldehyde as described by Zerial et al. (1992). Overexpressed proteins were labeled with: rabbit anti-Ypt51p, mouse anti-Rab5 mAb 4F11 (Bucci et al., 1994), rabbit anti-Rab5 (Chavrier et al., 1990), mouse anti-c-myc mAb 9E10 (Evan et al., 1985), and mouse anti-human transferrin receptor mAb B3-25 (Boehringer Mannheim). Endogenous Rab7 and β-COP were detected with rabbit antibodies described by Chavrier et al. (1990) and mouse anti-βCOP mAb M3A5 (Allan and Kreis, 1986), respectively. All antibodies (except mAb 4F11) were affinity purified. The secondary antibodies used were a rhodamine-labeled donkey anti-mouse antibody and a FITC-labeled donkey anti-rabbit antibody (Dianova, Hamburg, Germany). Cells were mounted with Mowiol (Merck, Darmstadt, Germany) and viewed with the EMBL confocal microscope using the excitation wavelengths 529 and 476 nm.

For analyzing HRP uptake the BHK cells, after transfection, were incubated in the presence of 1 μg/ml HRP (1,000 U/mg, Sigma) for 1 hour at 37°C and endocytosed HRP was assayed as described by
Bucci et al. (1992). Uptake values were calculated as per cent of the amount internalized in infected but untransfected cells, and corrected for differences in protein content and transfection efficiency (ranging between 50 and 80%, as determined by indirect immunofluorescence). The shown values are the average of three experiments.

Indirect immunofluorescence on yeast cells

BS64 cells (wild type), BS25 cells (Δypt51), and RH1651 cells (Δypt31) were grown overnight in YPD medium at 30°C or 24°C to approximately 0.5·10^7 cells/ml. BS64 cells carrying the CEN-plasmid pBS-ADH-Ypt51Q66L were grown at 30°C to a similar cell density in SD medium lacking uracil (for expression of Ypt51p Q66L as compared to wild-type Ypt51p see below). For labelings with anti-Ypt51p and anti-CPY antibodies cells were directly fixed and processed essentially as described by Roberts et al. (1991) and Zanolari and Riezman (1991). Fixation with 4% paraformaldehyde was for 30 minutes at 30°C and another 3-6 hours at RT.

For labelings with anti-Ste2p antibody BS64 cells harboring pBS-ADH-Ypt51Q66L were treated with 25 µg/ml cycloheximide for 1 hour at 30°C to deplete the cells of internal pools of Ste2p. The cells were cooled on ice and α-factor was added to 10^{-8} M final concentration to allow pheromone binding for 1 hour. Subsequently, cells were pelleted and resuspended in 30°C-prewarmed YPD to allow internalization of α-factor and Ste2p for 10 minutes at 30°C in the presence of 25 µg/ml cycloheximide. After that, fixative was added and cells were processed for immunofluorescence as described before.

To reduce residual background-labeling, the affinity purified anti-Ypt51p, anti-CPY and anti-Ste2p antibodies were preabsorbed against fixed BS25, RH1651, and RH449 cells, respectively, as described by Roberts et al. (1991). For single-label immunofluorescence, incubation with preabsorbed primary antibody was followed by rhodamine-labeled goat anti-rabbit IgG (diluted 1:1,000). For double-label immunofluorescence, anti-Ypt51p antibody was added first, followed by incubation with FITC-conjugated donkey anti-rabbit IgG. To block any residual binding sites of Ypt51p antibodies an incubation with an excess of unlabeled goat anti-rabbit IgG (1:50 diluted) was performed. Subsequently, cells were labeled with anti-CPY or anti-Ste2p antibodies, respectively, and with secondary rhodamine-labeled goat anti-rabbit IgG. Cells were viewed with a Zeiss Axioptih microscope equipped with appropriate Zeiss filters for rhodamine fluorescence and Nomarski interference optics and with the EMBL confocal microscope at excitation wavelengths 476 nm for FITC and 529 nm for rhodamine.

To quantitate the percentage of Ypt51p Q66L-expressing cells displaying the large Ypt51p-positive structures 82 cells viewed with the confocal microscope were analyzed. For example, those cells indicated by arrows in Fig. 6B-D were considered as positive for the large Ypt51p-positive structures, while cells with smaller punctate structures (an example marked with an arrowhead in Fig. 6D) were considered as negative for those structures.

To demonstrate that plasmid-borne Ypt51 Q66L was not strongly overexpressed as compared to endogenous wild-type Ypt51p, cell extracts of BS64 cells and BS64 cells carrying pBS-ADH-Ypt51Q66L were prepared from cells that were grown as described before. Cells (5 OD units) were incubated with 0.2 M NaOH, 0.5% β-mercaptoethanol for 10 minutes on ice and proteins were precipitated with 10% TCA as described by Singer-Krüger et al. (1994). The precipitate was collected by centrifugation, washed twice with acetone and resuspended in Laemmli sample buffer, excluding β-mercaptoethanol and Bromophenol Blue. After determining the protein concentration of both samples, β-mercaptoethanol and Bromophenol Blue were added and pairs of equal amounts of protein (50, 37.5, 25, and 12.5 mg) from wild type and Ypt51 Q66L-expressing cells were loaded onto a 12% SDS-polyacrylamide gel. After transfer of the gel onto nitrocellulose, western analysis was performed as described with the affinity-purified anti-Ypt51p antibody (at a dilution of 1:100) using the ECL system. A similar intensity of the Ypt51p-specific band was detected when 50 mg of wild-type extracts were compared to 25 mg of Ypt51Q66L containing extracts.

RESULTS

Yeast Ypt51p and mammalian Rab5 represent functional counterparts

To determine whether Ypt51p and Rab5 represent functional equivalents in yeast and mammalian cells we first analyzed whether wild-type and mutant Ypt51p could share the localization and activity of Rab5 when expressed in baby hamster kidney (BHK) cells.

Using the T7 RNA polymerase vaccinia virus (vT7) system we first determined whether Ypt51p was targeted to early endocytic compartments similar to Rab5 (Chavrier et al., 1990; Bucci et al., 1992). The intracellular distribution of Ypt51p was studied by indirect immunofluorescence using a polyclonal antibody, that was raised against a peptide corresponding to the C-terminal region of Ypt51p. As determined by immunoprecipitation experiments, the antiserum recognized specifically Ypt51p, but not the highly related Ypt52p and Ypt53p, while preimmune serum did not immunoprecipitate Ypt51p (see Materials and Methods). Furthermore, indirect immunofluorescence and western blot analysis also revealed that the affinity-purified antibody reacted specifically with vT7-expressed Ypt51p. No signal could be detected in uninfected cells (data not shown). To analyze the cellular distribution of Ypt51p and Rab5 under similar conditions, both proteins were coexpressed. Quantitative western-blotting confirmed that the experimental conditions used allowed overexpression of Rab5 ~15-fold over the endogenous level (Bucci et al., 1992; Stenmark et al., 1995) and a similar level of expression was obtained for the wild type and mutant Ypt51 proteins (see Materials and Methods). Upon staining with an anti-Rab5 monoclonal antibody (Bucci et al., 1994) a typical pattern of early endosome labeling could be observed (Fig. 1B) that largely overlapped with the Ypt51p-labeling (Fig. 1A).

When cotransfection was carried out with Ypt51p and the human transferrin receptor, again both proteins were found to colocalize (Fig. 1C,D), supporting the view that Ypt51p was associated with the early endosomal compartment.

We have recently shown that expression of two Rab5 mutants with different biochemical properties induced opposite effects on the morphology of early endosomes. Rab5 Q79L, a mutant with decreased GTPase activity, was found to induce the formation of unusually large endosomes, whereas expression of Rab5 S34N with preferential affinity for GDP led to the fragmentation of endosomes (Stenmark et al., 1994b). To test whether, in addition to targeting information, expression of Rab5 Q79L with preferential affinity for GDP led to the fragmentation of endosomes (Stenmark et al., 1994b). To test whether, in addition to targeting information, Ypt51p also shares functional properties with Rab5, we introduced the corresponding mutations into Ypt51p Q66L and S21N, respectively and analyzed their effects on the morphology of early endosomes. As shown in Fig. 1E expression of Ypt51p Q66L led to a dramatic increase in the size of the Ypt51p-positive structures. These corresponded to expanded early endosomes, since they were positive both for Rab5 (data not shown) and the human transferrin receptor (Fig. 1F). The morphological alteration of the early endosomes was comparable to that caused by Rab5 Q79L and was restricted to the early endocytic compartment (see below). In contrast,
expression of the Ypt51p S21N mutant resulted in a diffuse punctate labeling pattern that was distinct from the pattern obtained after expression of wild-type Ypt51p (data not shown). Since also in this case colocalization with the transferrin receptor was observed, expression of Ypt51p S21N is likely to induce fragmentation of early endosomes, as shown for the corresponding Rab5 S34N mutant (Stenmark et al., 1994b).

Similar experiments were carried out with myc-tagged Ypt7p to exclude the formal possibility that expression of a foreign Ypt protein resulted in association with, and/or alteration of, early endosomes unspecifically. In contrast to Ypt51p, the Ypt7p-labeling pattern was clearly distinct from the early endosome staining as determined by double-labeling with Rab5 (Fig. 2A,B) showing very little colocalization of the two chromophores. On the other hand, Ypt7p was found to be associated with Rab7-positive late endosomal structures (data not shown). These data indicate that Ypt51p and Ypt7p are specifically targeted to distinct endocytic organelles in mammalian cells. Furthermore, as revealed by double-labeling with antibodies against the Golgi-specific coat protein β-COP (Allan and Kreis, 1986) and Rab7 (Chavrier et al., 1990), the morphology of the Golgi apparatus (Fig. 2D) and late endosomes (not shown) was unaffected upon overexpression of wild-type (Fig. 2C) and mutant Ypt51p. This demonstrates that the morphological alterations caused by the mutant proteins were specific and restricted to early endosomes.

We next tested the ability of wild-type and mutant Ypt51p to stimulate endocytosis with a biochemical assay. For this the uptake of the fluid-phase marker horseradish peroxidase (HRP) was measured. As found previously (Bucci et al., 1992; Li and Stahl, 1993; Stenmark et al., 1994b), overexpression of Rab5 stimulated HRP internalization approximately two-fold as compared to untransfected cells (Fig. 3). Ypt51p shared the ability of Rab5 to stimulate HRP uptake, albeit with lower efficiency (Fig. 3). In contrast, neither overexpression of Rab7 nor of Ypt7p affected fluid-phase endocytosis although the expression levels of the two proteins were similar to that of Rab5 and Ypt51p. Conversely, expression of Ypt51p S21N inhibited HRP uptake (Fig. 3), as previously found for the corresponding Rab5 mutant (Li and Stahl, 1993; Stenmark et al., 1994b). These results support the idea that, in addition to Rab5-like targeting information, Ypt51p also contains Rab5-specific regulatory activity.

Given the Rab5-like functional properties of Ypt51p, we also undertook the reverse approach and analyzed whether Rab5 can complement the loss of Ypt51p in ypt51 null cells.

### Table 1. Strains used

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>BS25</td>
<td>Mata his4 ura3 leu2 lys2 ypt51::LYS2 bar1-1</td>
<td>Singer-Krüger et al., 1994</td>
</tr>
<tr>
<td>BS64</td>
<td>Mata his4 ura3 leu2 lys2 bar1-1</td>
<td>Singer-Krüger et al., 1994</td>
</tr>
<tr>
<td>BS84</td>
<td>Mata his4 ura3 leu2 lys2 ypt51::LYS2 ypt7::URA3 bar1-1</td>
<td>This study</td>
</tr>
<tr>
<td>RH1</td>
<td>Mata his4 ura3 leu2 ypr7::URA3 bar1-1</td>
<td>D. Gallwitz, Göttingen</td>
</tr>
<tr>
<td>RH449</td>
<td>Mata his4 ura3 leu2 lys2 pep4::URA3 bar1-1</td>
<td>H. Riezman, Basel</td>
</tr>
<tr>
<td>RH732</td>
<td>Mata his4 ura3 leu2 lys2 pep4::URA3 bar1-1</td>
<td>H. Riezman</td>
</tr>
<tr>
<td>RH1651</td>
<td>Mata his4 ura leu2 prc1::LEU bar1-1</td>
<td>H. Riezman</td>
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![Image](image.png)
whether expression of Rab5 could restore this defect. Although the Rab5-transformants generated larger colonies on plates after growth at 37°C in comparison to control cells carrying the vector alone, a difference in the growth-rate at 37°C could not be observed in liquid culture. Therefore, we did not assess the putative complementation by Rab5 with other assays.

Role of Ypt51p at an early stage of endocytic transport in yeast

Previous studies have demonstrated that loss of Ypt51p resulted in inhibition of α-factor degradation and lack of accumulation of Lucifer Yellow in the vacuole. These phenotypes were consistent with a defect in endocytosis, but did not discriminate between a fucntion of Ypt51p in early or late steps of endocytic transport (Singer-Krüger et al., 1994). Based on the ability to regulate early endocytic traffic in mammalian cells we tested the hypothesis that Ypt51p may also function in the early endocytic pathway in yeast. We used a previously described cell fractionation approach to separate two kinetically defined endocytic intermediates (Singer-Krüger et al., 1993). Thus, this fractionation method can be conveniently used to investigate whether the endocytic trace α-factor is able to gain access to early and late compartments in cells depleted of Ypt51p.

35S-labeled α-factor was internalized into wild-type and ∆ypt51 cells for 3, 15 and 40 minutes at 30°C and a high speed pellet fraction (P3), that was enriched for endosomes, was obtained. Subsequent density gradient centrifugation on Nycodenz gradients of the P3 fraction allowed the separation of the early and late endocytic fractions. Consistent with previous findings, in wild-type cells a sequential passage of α-factor from the plasma membrane (Fig. 4, fraction no. 12, after 3 minutes of uptake) to the early (fraction no. 7, 8, after 3 minutes of uptake) and the late endocytic fraction (fraction no. 4, 5, after 15 minutes of uptake) could be observed after increasing times of internalization. In contrast, the appearance of α-factor in the late fraction was strongly inhibited in the ∆ypt51 mutant (Fig. 4). After 15 minutes of internalization the majority of the pheromone was still associated with the early fraction, whereas in wild-type cells it was mainly found in the late fraction. Remarkably, even after 40 minutes of endocytosis in ∆ypt51 cells no change in the distribution of α-factor could be observed. On the other hand, wild-type cells revealed a strong decrease of α-factor in the fractions from the gradient suggesting that the pheromone was delivered to the vacuole (not enriched in the P3 fraction applied to the gradient; Singer and Riezman, 1990).

A similar cell fractionation approach has been previously employed to show that the ypt7 null mutant accumulates endocytosed α-factor in a gradient fraction corresponding to the late endocytic intermediate and to propose a function for Ypt7p in transport from the late compartment to the vacuole (Schimmöller and Riezman, 1993). To determine whether Ypt51p and Ypt7p regulate endocytic membrane traffic in a successive fashion we carried out the analysis in the ypt51ypt7 double mutant as well as in the ypt7 mutant. Since both of the mutants had a similar genetic background to the wild type and the ypt51 strain this comparison was possible. Most likely as a result of
the Pep4+-phenotype of our Δypt7 strain background, we did not observe a substantial accumulation of α-factor in the late endocytic fraction of the Δypt7 mutant (Fig. 4), in agreement with Schimmöller and Riezman (1993). This is explained by the finding that in a PEP4 background α-factor is subject to degradation in the late endocytic intermediate. Most importantly, in Δypt51/Δypt7 cells the gradient profiles of α-factor after the various times of internalization were very similar to those of the ypt51 and not of the ypt7 mutant (Fig. 4). Therefore, this epistasis experiment further suggests that Ypt51p is required prior to Ypt7p in transport to the vacuole, most likely between the kinetically defined early and late endocytic intermediates.

**Ypt51p is localized to endocytic organelles but not to the late Golgi**

If the transport block observed is a direct consequence of the loss of Ypt51p, one would expect this small GTPase to be localized to the endocytic compartments. Furthermore, the vacuolar protein sorting defect displayed by the Δypt51 mutant (Singer-Krüger et al., 1994; Horazdovsky et al., 1994) led to the proposal that Ypt51p may play a role in transport between a late Golgi subcompartment and the late endosome (Horazdovsky et al., 1994). To shed further light onto this question, we also analyzed whether Ypt51p cofractionates with the late Golgi compartment.

The proteins and pheromone 35S-α-factor were followed in wild-type cells during a three-step isolation procedure, which has been recently developed to further purify the early and late endocytic intermediates isolated from the Nycodenz gradient. This method consists of an additional density gradient centrifugation step on a sucrose/D2O gradient (Singer-Krüger et al., 1993). Importantly, this procedure allows the separation of the late Golgi subcompartment, as defined by the presence of the marker Kex2p, from the early endocytic intermediate.

As shown in Fig. 5, Ypt51p cofractionated with α-factor associated with both the early and late endocytic intermediates on sucrose/D2O gradients. In contrast, no Ypt51p could be detected in the fractions (#9-13) that were highly enriched for Kex2p. When the specific activity of Ypt51p was determined during the purification, it became apparent that it was mostly enriched in the late endocytic intermediate (960-fold over lysate), while the enrichment in the early intermediate was lower (100-fold over lysate).

In conclusion, the cofractionation of Ypt51p with both endosomal intermediates is most consistent with the localization on these compartments and with the observed role of Ypt51p in controlling membrane traffic between these two compartments. While our data do not support an association of Ypt51p with the late Golgi compartment positive for Kex2p, we cannot rule out an association with other compartments, for example Golgi-derived vesicles, due to the lack of markers to follow their distribution during cell fractionation.

**Appearance of enlarged Ypt51p-positive structures upon expression of mutant Ypt51p Q66L**

Despite the existence of endocytic transport intermediates, the vacuole is the only organelle of the endocytic pathway in wild-type cells that has been morphologically visualized so far (Riezman, 1985). We therefore attempted the morphological characterization of the Ypt51p-containing endocytic compart-

![Fig. 4](image-url)  
**Fig. 4.** Endocytic marker, 35S-labeled α-factor, accumulates in an early endocytic fraction in ypt51 as well as in ypt51/Δypt7 mutants. After growth at 24°C the wild-type and mutant strains were incubated with 35S-α-factor for 1 hour at 0°C to allow pheromone binding. The cells were collected, resuspended in 30°C-prewarmed medium and incubated at 30°C for 3, 15 and 40 minutes to allow internalization of α-factor. The cells were then transferred to ice and NaN3 and NaF were added to stop further endocytosis. The cells were lysed at 4°C and a high speed pellet fraction (P3), obtained by differential centrifugation, was subjected to flotation centrifugation on a Nycodenz gradient. After centrifugation 15 fractions were collected from the top and aliquots from each fractions were analyzed for the presence of 35S-α-factor by liquid scintillation counting.
late endocytic intermediate (Singer-Krüger et al., 1993), it was only the one presented in B was loaded with the late fraction. Since loaded with the early endocytic fraction from the Nycodenz gradient, activity (see Materials and Methods). The gradient shown in A was subjected to Nycodenz density gradient centrifugation. The early and late endocytic fractions were subfractionated on sucrose/D₂O gradients, loaded either with the early or the late endocytic fraction from the Nycodenz gradient. Cell fractionation analysis to follow Ypt51p and Kex2p in comparison to endocytic marker α-factor was performed with YPT51 cells (RH732). The cells were incubated with ³⁵S-labeled α-factor for 20 minutes at 15°C to accumulate the pheromone in the early and late endocytic fractions. A P3 fraction was generated by differential centrifugation and subjected to Nycodenz density gradient centrifugation. The early and late endocytic fractions were subfractionated on sucrose/D₂O gradients, as described in Materials and Methods; 18 fractions (2 ml) were collected from the top and the pellet, which formed during centrifugation, was resuspended in 2 ml sorbitol medium (fraction 19). α-factor in each of the fractions was followed by scintillation counting. Ypt51p by western blotting and fluorography using the ECL-system. Kex2p localization was determined by its enzyme activity (see Materials and Methods). The gradient shown in A was loaded with the early endocytic fraction from the Nycodenz gradient, the one presented in B was loaded with the late fraction. Since Kex2p is largely present in fractions containing the early but not the late endocytic intermediate (Singer-Krüger et al., 1993), it was only followed on the gradient shown in A.

Fig. 5. Ypt51p cofractionates with ³⁵S-α-factor, but not with Kex2p, on sucrose/D₂O gradients, loaded either with the early or the late endocytic fraction from the Nycodenz gradient. Cell fractionation analysis to follow Ypt51p and Kex2p in comparison to endocytic marker α-factor was performed with YPT51 cells (RH732). The cells were incubated with ³⁵S-labeled α-factor for 20 minutes at 15°C to accumulate the pheromone in the early and late endocytic fractions. A P3 fraction was generated by differential centrifugation and subjected to Nycodenz density gradient centrifugation. The early and late endocytic fractions were subfractionated on sucrose/D₂O gradients, as described in Materials and Methods; 18 fractions (2 ml) were collected from the top and the pellet, which formed during centrifugation, was resuspended in 2 ml sorbitol medium (fraction 19). α-factor in each of the fractions was followed by scintillation counting. Ypt51p by western blotting and fluorography using the ECL-system. Kex2p localization was determined by its enzyme activity (see Materials and Methods). The gradient shown in A was loaded with the early endocytic fraction from the Nycodenz gradient, the one presented in B was loaded with the late fraction. Since Kex2p is largely present in fractions containing the early but not the late endocytic intermediate (Singer-Krüger et al., 1993), it was only followed on the gradient shown in A.

ments by indirect immunofluorescence microscopy. Due to the relatively low abundance of Ypt51p (Singer-Krüger et al., 1994) and the presumptive small size of the endosomes, we initially attempted to detect the small GTPase under conditions which, in animal cells, resulted in enlarged endocytic structures (Stenmark et al., 1994b, and see above). This was done by expressing the mutant Ypt51p Q66L, which leads to enlarged endosomal structures when transiently expressed in BHK cells (Fig. 1). To avoid mislocalization of the protein by overproduction, the mutant gene was placed behind the ADH-promoter on a centromere-based plasmid and expressed in wild-type cells. In fact, as determined by quantitative western blotting, this resulted in only a 2- to 3-fold increased level of mutant Ypt51p over wild-type protein (see Materials and Methods). For immunofluorescence, the affinity purified antibody used for the labeling was additionally pre-absorbed against fixed Δypt51 cells lacking the Ypt51p protein. This step efficiently removed background staining, as shown in the case of Δypt51 cells (Fig. 6A). In contrast, yeast cells expressing Ypt51p Q66L revealed a very bright fluorescent signal, consisting of usually one to three larger structures per focal plane (Fig. 6B-F, arrows) and a number of smaller structures (for example Fig. 6D, arrowhead). Although the Ypt51p-positive large structures were frequently found in the proximity of the vacuole identified by Nomarski optics (Fig. 6E,F), they did not correspond to that compartment. Also nuclei and mitochondria, seen by DAPI staining of the DNA, showed distinct and nonoverlapping distributions in comparison to the Ypt51p labeling (data not shown). Quantification revealed a high percentage of cells (approximately 55%) containing the large Ypt51p-positive structures (as indicated by arrows in Fig. 6), of which approximately 30% appeared to contain one, 18% two, and 7% three large structures.

We next investigated the localization of Ypt51p in wild-type yeast cells. Interestingly, these cells did not reveal the large punctate structures but numerous smaller entities, that were distributed throughout the cell (Fig. 6G,H, indicated by arrowheads).

The Ypt51p-positive compartment is endocytic in nature and localized at the intersection with the vacuolar protein sorting pathway

To provide further evidence that the enlarged structures labeled by the anti-Ypt51p antibody upon expression of Ypt51p Q66L were endosomal in origin, double-labeling with an endocytic marker was attempted. Since so far we have not been able to detect the pheromone α-factor by immunolocalization techniques, the α-factor receptor (Ste2p) was followed. This seven-transmembrane protein is internalized via endocytosis and delivered to the vacuole, where it gets rapidly degraded (Davis et al., 1993). Due to the presence of the receptor also along the secretory pathway we examined its location after preventing protein synthesis with cycloheximide and allowing pre-existing Ste2p protein to be transported to the plasma membrane. Following this treatment, pheromone was bound at 4°C and receptor-mediated endocytosis allowed for 10 minutes at 30°C before the cells were fixed with paraformaldehyde and prepared for immunofluorescence microscopy. Ste2p was detected with an affinity-purified antibody, raised against a Ste2p-specific peptide (Zanolari et al., 1992), which was pre-absorbed against fixed α cells that do not express Ste2p. Since both the anti-Ypt51p and the anti-Ste2p antibodies were from rabbit, conditions were established for double-labeling experiments to independently detect each primary antibody with FITC- and rhodamine-conjugated secondary antibodies, respectively. This was achieved by an incubation step in the presence of an excess of unlabeled goat anti-rabbit IgG following labeling with the first primary and secondary antibodies (see Materials and Methods).

Although some residual diffuse background labeling was seen with the purified anti-Ste2p antibody preparation (Fig. 7B), a punctate staining was only observed in Ste2p-contain-
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ing cells (Fig. 7A,D). Since synthesis of Ste2p was inhibited, these structures are likely to represent endocytic organelles. When double immunofluorescence analysis was performed with the anti-Ypt51p and anti-Ste2p antibodies, several of the labeled structures were positive for both antigens (Fig. 7C,D, indicated by arrows). This finding strengthens the idea that the structures containing Ypt51p do represent endocytic elements. In a control experiment to demonstrate the lack of cross-reactivity, after the first round of labeling the second primary antibody was omitted and rhodamine-labeled goat anti-rabbit IgG was added (for details see Fig. 7 and Materials and Methods). Had the aforementioned blocking step been inefficient, then labeling with rhodamine should have been observed. As shown in Fig. 7E-F, structures FITC-labeled for Ypt51p were visible (Fig. 7E) whereas under these conditions the rhodamine signal was hardly detectable (Fig. 7F). Furthermore, to test whether the excitation wavelength selected for observing the FITC signal could result in some rhodamine-derived signal (and vice versa) single-labeled samples were viewed at the wavelength of the complementary chromophore. This analysis revealed that no fluorescent signal could be detected under these conditions (data not shown).

In addition to inhibition of endocytic transport, the Δypt51 mutant was characterized by a defect in the vacuolar protein sorting pathway. Maturation of the Golgi-modified form of CPY was delayed and a fraction of the protein was missorted to the extracellular space (Singer-Krüger et al., 1994; Horazdovsky et al., 1994). These findings were explained by a putative intersection of the endocytic pathway with the vacuolar protein sorting pathway. An implication of this idea is that CPY would be expected to localize, at least partially, to the Ypt51-containing compartment. To test this, we analyzed whether the structures, labeled by the anti-Ypt51p antibody, also contained CPY. Using an affinity-purified, pre-absorbed antibody against CPY, which resulted in low background labeling with cells deleted for PRC1 (the gene encoding CPY) (Fig. 7H), in cells expressing Ypt51p Q66L a punctate labeling was predominant (Fig. 7G,K). The finding that the vacuole was not labeled can most likely be explained by the antibodies detecting preferentially the precursor form of CPY. This was supported by the observation that in cells deleted for PEP4 (thereby lacking thezymogen proteinase A) vacuolar staining was clearly detectable (data not shown).

Double-labeling experiments in cells expressing Ypt51p Q66L to detect Ypt51p and CPY clearly revealed a high degree of colocalization of the two proteins (Fig. 7J,K). This finding provides direct evidence for the idea that CPY transits through the compartment which is involved in endocytic transport of α-factor and that contains Ypt51p as a regulatory component. It would follow, that the additional structures occasionally seen only with α-CPY but not with α-Ypt51p antibodies might represent CPY en route through the secretory pathway (Fig. 7K, indicated by arrows).

**DISCUSSION**

While previous studies identified Ypt51p of *S. cerevisiae* as a structural homolog of mammalian Rab5, the initial phenotypic characterization of *YPT51*-disrupted cells revealed pleiotropic defects in endocytosis and vacuolar protein sorting, but did not allow us to determine at which precise step in membrane traffic Ypt51p is required (Singer-Krüger et al., 1994). In this paper, we have applied a variety of experimental approaches to study in more detail the role and localization of Ypt51p in intracellular membrane traffic. We show that Ypt51p controls early
steps of endocytosis in yeast and mammals and morphologically identify the Ypt51-containing endocytic compartments of yeast. Our results are consistent with the idea that Ypt51p and Rab5 are functional counterparts in yeast and mammals.

**Rab5 and Ypt51p are counterparts with a similar function in the early endocytic pathway**

Heterologous expression of Ypt51p in mammalian cells provided evidence that these two small GTPases represent functional equivalents. First, similar to *S. pombe* ypt5 (Armstrong et al., 1993), transiently expressed wild-type Ypt51p in mammalian cells colocalized with Rab5 and the transferrin receptor indicating a specific association with early endosomes. Second, Ypt51p was found to be able to control Rab5-specific fusion events. Expression of wild-type Ypt51p specifically stimulated endocytosis, while Ypt7p and Rab7

**Fig. 7.** Double-label immunofluorescence microscopy reveals that the Ypt51p-containing structure is also positive for α-factor receptor and CPY. BS64 cells expressing Ypt51p Q66L (A,C-F) were collected after growth at 30°C and treated with 25 μg/ml cycloheximide for 1 hour in YPD medium to prevent new synthesis of α-factor receptor (Ste2p) and to deplete the cells of internal pools of Ste2p. Subsequently, cells were cooled to 4°C and incubated in the presence of pheromone for 1 hour on ice. After collecting the cells and resuspending them in 30°C prewarmed YPD (containing cycloheximide), endocytosis was allowed for 10 minutes at 30°C. After that cells were fixed and processed for immunofluorescence microscopy as described in Fig. 7. Cells in B (RH449, mating type α), H (RH1651, Δprc1), and G, J, K (BS64 carrying pBS-ADH-Ypt51Q66L) were directly fixed after growth at 30°C. Single-label immunofluorescence was performed with anti-Ste2p antibody on cells shown in A and B, and with anti-CPY antibody on cells shown in G and H. Double-label immunofluorescence was performed in C,D, E,F and J,K. In C, E, and J the primary antibody detected was rabbit anti-Ypt51p. In D the primary antibody detected was rabbit anti-Ste2p. In F cells are shown, where the primary antibody anti-Ste2p was omitted. In K the primary antibody detected was rabbit anti-CPY. Cells were viewed using the EMBL confocal microscope. Bar, 5 μm. Arrowheads point to structures that were double-labeled by antibodies against Ypt51p and Ste2p (C,D) or Ypt51p and CPY (J,K), respectively. Arrows in K indicate structures labeled by the anti-CPY antibody that were not labeled by the anti-Ypt51p antibody. Anti-Ypt51p antibodies were visualized with FITC-conjugated donkey anti-rabbit IgG, whereas anti-CPY or anti-Ste2p antibodies were detected with secondary rhodamine-labeled goat anti-rabbit IgG (see Materials and Methods).
were shown to have no effect. Analogous to Rab5 S34N, shown to exist preferentially in the GDP-bound form and to inhibit endocytic fusion events (Stenmark et al., 1994b), the yeast counterpart Ypt51p S21N inhibited endocytosis in BHK cells. Third, the expression of Ypt51p mutants resulted in morphological alterations of the early endosome compartment similar to those caused by the corresponding Rab5 mutants (Stenmark et al., 1994a).

What is the molecular basis for the targeting of *S. cerevisiae* Ypt51p (and *S. pombe* ypt5) to the early endosomes and for its function in early endocytic membrane traffic in mammalian cells? Earlier work from our laboratory and others has demonstrated that distinct structural motifs confer localization and functional specificity to Rab/Ypt proteins (Chavrier et al., 1990; Brennwald and Novick, 1993; Stenmark et al., 1994a). Our results suggest that Ypt51p is sufficiently conserved to interact with the Rab5-regulatory factors and effectors. For example, the effector domain and part of the α2-helix (LAPMYYR), which have been shown to determine Rab5-specific activity, are highly conserved in Ypt51p. The C terminus of Ypt51p, ypt5, and Rab5, which contains targeting information, displays a low sequence conservation. This might suggest that the localization signal is either a short sequence or a structural motif with no specific sequence requirements, as previously suggested (Armstrong et al., 1993; Stenmark et al., 1994a). Furthermore, it is possible that the other conserved functional determinants may contribute to the targeting process.

**Role of Ypt51p in endocytic traffic in yeast**

Cell fractionation analysis in yeast cells lacking Ypt51p revealed an accumulation of α-factor in the early endocytic intermediate and indicated that transport to the late endocytic compartment was inhibited. The finding that Ypt51p is localized to both endocytic intermediates argues that the effects of the protein on endocytic traffic are not indirect but reflect a functional requirement of this small GTPase in transport between the two endocytic compartments. In particular, the fact that Ypt51p was mostly enriched in the late fraction suggests that this may represent its acceptor compartment. These data confirm our previous hypothesis, which postulated that the lack of α-factor degradation in the various ypt mutants is due to inhibited transport to the vacuole rather than to vacuolar protein mis-sorting and lack of vacuolar protease activity (Singer-Krüger et al., 1994).

Previous studies with the Δypt7 mutant suggested that the yeast Rab7-homolog functions more distal in the endocytic pathway towards the vacuole in comparison to Ypt51p. First, α-factor degradation is less strongly inhibited in ypt7 than in ypt51 mutants. Second, in the ypt7 mutants endocytic markers accumulate in the late endocytic fraction (Schimmöller and Riezman, 1993), whereas in Δypt51 cells transport is blocked in the early intermediate. While these phenotypes per se indicate that Ypt51p and Ypt7p act in a sequential fashion, the observation that the endocytic phenotype of the ypt51Δypt7 double mutant is similar to that of the ypt51 mutant provides further evidence for this interpretation and strengthens the idea of a molecular regulation similar to animal cells. Nevertheless, we cannot exclude the possibility that Ypt51p and Ypt7p function at the same step, although one would have to consider that the ypt51 phenotype appears to be prevalent.

**Equivalence of yeast and mammalian endocytic organelles?**

In mammalian cells Rab5 was found to be present at the plasma membrane, clathrin coated vesicles and early endosomes (Chavrier et al., 1990; Bucci et al., 1992) and to regulate the kinetics of endocytosis (Bucci et al., 1992) as well as the homotypic fusion between early endosomes (Gorvel et al., 1991; Stenmark et al., 1994b; Barbieri et al., 1994). In the yeast system, the present data imply a role of Ypt51p in transport between an early and a late endocytic intermediate. How can this apparent difference between the two systems be reconciled? One explanation could be that a fundamental difference exists between the mammalian and the yeast pathways, and, although Ypt51p also appears to control early endocytic traffic in mammalian cells, it may regulate a different step of transport in yeast. This possibility would be consistent with the finding that no effect on the rate of endocytic internalization of surface-bound α-factor could be observed upon disruption of YPT51 (Singer-Krüger et al., 1994). However, it is worth considering that the early and late "endosomes" in yeast have been named due to their sequential acquisition of the endocytic marker α-factor and, so far, have only been described from a kinetic point of view (Singer-Krüger et al., 1993). Except for the existence of two endocytic intermediates reminiscent of the situation in mammalian cells, there is no experimental evidence indicating that these particular fractions correspond to the early and late endosomes of animal cells. It is conceivable that one of the fractions may rather represent an endocytic transport vesicle. The finding that in ypt51 mutants small vesicular structures accumulate (Singer-Krüger et al., 1994) supports this hypothesis. For example, the yeast early endosomal intermediate, though clearly distinct from the bulk of the plasma membrane (Singer and Riezman, 1990), may represent a specialized plasma membrane domain or an endocytic vesicle derived from it. Recently, finger-like invaginations of the plasma membrane, around which actin filaments and actin binding proteins were organized, have been identified by electron microscopy (Mulholland et al., 1994). Since the actin cytoskeleton has been recently implicated in the control of endocytic internalization in *S. cerevisiae* (Kübler and Riezman, 1993), these plasma membrane domains may constitute active sites of endocytosis, where actin-cables may be involved to drive scission of endocytic vesicles. It could be imagined that after or during this step Ypt51p is recruited onto this intermediate to control the fusion of these vesicles with the acceptor compartment. Since, in addition to the kinetic definition, the endocytic intermediates can now be defined on the basis of their molecular composition and regulation, the analysis of the compartmental organization of the endocytic pathway will hopefully be facilitated.

**Ypt51p may not directly be involved in Golgi to endosome transport but is localized at the intersection of the endocytic and vacuolar protein sorting pathways**

In a model to explain the vacuolar protein sorting defect upon disruption or mutation of the YPT51/VPS21 locus (Singer-Krüger et al., 1994; Horazdovsky et al., 1994), it was speculated that Ypt51p (Vps21p) could regulate transport between the late Golgi compartment (the site where sorting of vacuolar
enzymes from the secretory pathway occurs; Graham and Emr, 1991) and endosomes (Horazdovsky et al., 1994). This would imply that the endocytic transport defect to the vacuole might be caused indirectly. Alternatively, it could be envisaged that the vacuolar sorting defect itself may arise as an indirect consequence of inhibition of endocytic transport to the vacuole. Our subcellular fractionation studies revealed cofractionation of Ypt51p with the early and late endocytic fractions, but not with the late Golgi compartment, suggesting that this small GTPase is not associated with the latter organelle. The association of Ypt51p with an endocytic compartment was independently confirmed by double labeling immunofluorescence showing colocalization of Ypt51p and the α-factor receptor. Therefore, our results do not support a direct role for Ypt51p in Golgi to endosome traffic. On the other hand, the finding that Ypt51p partially colocalized with CPY provided direct experimental evidence for the idea that the endocytic and vacuolar protein sorting pathways converge at least at the stage of the Ypt51p-containing compartment. Indirect evidence for the latter idea was previously provided by various observations (Vida et al., 1993; Davis et al., 1993), the most compelling being that endocytosed α-factor can be degraded by vacuolar hydrolases before the delivery to the vacuole (Schimmöller and Riezman, 1993). The fact that vacuolar enzymes transit through a Ypt51p-containing compartment implies that the defect in vacuolar protein sorting shown by the ypt51 mutant may arise as an indirect consequence of a defect in endocytosis.

**Morphological identification of the Ypt51p-containing compartment**

Immunofluorescence microscopy enabled us to identify the Ypt51p-containing structure in wild-type cells and in cells expressing the Ypt51p Q66L mutant. While endogenous Ypt51p in wild-type cells could be localized to numerous punctate structures, which were evenly distributed throughout the cytoplasm, expression of Ypt51p Q66L resulted in the appearance of less numerous (most frequently one to three) and larger Ypt51p-positive structures. Consistent with a role for Ypt51p in the early endocytic pathway, the appearance of the vacuole was unaffected upon expression of the Ypt51p Q66L mutant. Therefore, the morphological alteration of Ypt51p-containing structures is similar to that previously observed upon expression of either Rab5 Q79L or Ypt51p Q66L in mammalian cells. This implies that Ypt51p can stimulate endocytic fusion events in yeast cells similar to the mammalian counterpart.

In wild-type cells this is the first time that endocytic transport intermediates besides the vacuole have been morphologically visualized in vivo. Interestingly, in a search for mutants that are impaired in endocytosis of a α-factor receptor, ren1-1 (a mutant of REN1, identical to VPS2) was identified and found to accumulate the receptor in a compartment localized in the proximity of the vacuole. This compartment is reminiscent of the Ypt51p-containing structures appearing upon expression of Ypt51p Q66L. In fact, a pre-vacuolar compartment with similar morphological characteristics was originally described by Raymond et al. (1992). Because it was found in a number of vacuolar protein sorting mutants, it was defined as the class E compartment. This pre-vacuolar structure was characterized by the presence of CPY and vacuolar ATPase, and by a lack of alkaline phosphatase, another vacuolar marker, which was mostly present in the vacuole. However, yps21 (which is a mutant allele of YPT51; Horazdovsky et al., 1994) was not classified as a class E mutant. This mutant displayed rather a diffuse staining of one subunit of the vacuolar ATPase (Raymond et al., 1992). We believe that the different, sometimes opposing, morphological alterations of endocytic/pre-vacuolar compartments can be explained as the result of distinct mutations in one and the same gene product, as has been shown for two different Rab5 mutants (Stenmark et al., 1994b), and not as the consequence of the inactivation of the gene product per se.

In conclusion, this work establishes Ypt51p as a marker protein for the early yeast endocytic pathway. Together with Ypt7p, this small GTPase should hopefully facilitate the biochemical and morphological characterization of this transport route. One major aim towards this direction is the reconstitution of the Ypt51p-controlled transport step in vitro, by which we hope to further identify and characterize the molecular requirements for endocytic traffic in yeast.

We would like to thank Howard Riezman for providing the anti-Ste2p and anti-CPY antisera and a number of yeast strains; Dieter Gallwitz for providing the ypt7 strain, plasmids and antisera; and Frauke Schimmöller for help with the densitometric analysis. We gratefully acknowledge Valerie Doye, Bernard Hoflack, Meltje de Hoop, Rob Parton and Kai Simons for critical reading of the manuscript. This research was supported by a grant from the Human Frontier Science Program (to M.Z.). During part of the work B.S.-K. has been a recipient of a postdoctoral long-term EMBO fellowship, and H.S. of a postdoctoral fellowship from the Research Council of Norway.

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(Received 22 February 1995 - Accepted 4 August 1995)