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The GTPase Ypt7p of *Saccharomyces cerevisiae* is required on both partner vacuoles for the homotypic fusion step of vacuole inheritance

Albert Haas, Dietrich Scheglmann[1], Thomas Lazar[1], Dieter Gallwitz[1] and William Wickner[2]

Department of Biochemistry, Dartmouth Medical School, 7200 Vail Building, Hanover, NH 03755-3844, USA and [1]Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, PO Box 2841, 37018 Göttingen, Germany

[2]Corresponding author

In the budding yeast *Saccharomyces cerevisiae*, vacuoles are inherited by the projection of vesicles and tubules from the mother-cell vacuole into the growing daughter cell during the S phase. These vesicles then fuse and form the daughter-cell organelle. We have described previously *in vitro* reactions of the formation of vacuole-derived segregation structures and of vacuole–vacuole fusion. Homotypic vacuole fusion requires cytosol, ATP and a physiological temperature, and is sensitive to GTPase inhibitors. These reactions are divisible into early stages which require ATP and cytosol, and late stages which require neither. Here, we report that Ypt7p, a ras-like GTPase implicated previously in endocytosis in yeast, is largely localized to the vacuole and is required on both partners during the *in vitro* vacuole fusion reaction. The *in vitro* fusion reaction is inhibited either by Gdi1p, which extracts the GDP-bound form of ras-like GTPases from membranes, or by antibodies specific for Ypt7p. The presence of anti-Ypt7p during the early stages of the reaction inhibits the development of cytosol- and ATP-independent intermediates. Although cytosol and ATP are no longer needed for the late stage of vacuole inheritance *in vitro*, the inhibition of this late stage by anti-Ypt7p or Gdi1p requires the continued presence of ATP and cytosol. Ypt7p is the first GTPase for which a direct role in organelle inheritance has been established.

Keywords: homotypic fusion/organelle inheritance/vacuole/Ypt7p

Introduction

A homotypic (vacuole-to-) vacuole fusion event is the final step of the vacuole inheritance process in *Saccharomyces cerevisiae* (hereafter referred to as yeast). Cytological, biochemical and genetic studies (Weisman and Wickner, 1988, 1992; Weisman *et al.*, 1990; Gomes de Mesquita *et al.*, 1991; Shaw and Wickner, 1991; Conradt *et al.*, 1992, 1994; Raymond *et al.*, 1992b; Jones *et al.*, 1993; Haas *et al.*, 1994) have shown that the daughter cell inherits vacuole material from the mother cell via tubular and/or vesicular 'segregation' structures. Unlike constitutive intracellular trafficking, vacuole inheritance is coordinated by the cell cycle and is spatially limited to a narrow axis between mother cell and daughter cell. The segregation structures are formed at some time between early in the S phase of the cell cycle and the G_2/M phase transition. The last discernible step of vacuole inheritance is the fusion of segregation structures in the daughter cells, giving rise to one or a few larger vacuoles per cell, typical for these low copy-number organelles.

Recently we have developed cell-free assays for vacuole inheritance reactions (Conradt *et al.*, 1992, 1994; Haas *et al.*, 1994), observing segregation structure formation and homotypic vacuole fusion events microscopically, and using a biochemical assay to quantify the homotypic fusion of vacuoles. The latter assay (Haas *et al.*, 1994) is based on the maturation and activation of the unprocessed form of vacuolar alkaline phosphatase (proPho8p) by the vacuolar proteinase A (Pep4p). The homotypic fusion of vacuoles isolated from strains which carry a gene disruption in either *PHO8* or *PEP4* is quantified by measuring alkaline phosphatase activity. Vacuoles isolated from *vac2-1*, a yeast mutant normal in mitochondrial and nuclear inheritance but with defective vacuole inheritance (Shaw and Wickner, 1991), do not fuse (Haas *et al.*, 1994). Vacuoles from another vacuole inheritance mutant, *vac1-1* (*vps19*; Weisman and Wickner, 1992), fragment in the *in vitro* reactions in the presence of wild-type cytosol (Conradt *et al.*, 1992). Cytosol from a third vacuole inheritance mutant, *vac5-1*, does not support the *in vitro* fusion reaction with vacuoles isolated from wild-type cells (Nicolson *et al.*, 1995 and unpublished data). These data show a clear link between the ability of vacuoles to fuse *in vitro* and vacuole inheritance phenotype *in vivo*. The *in vitro* fusion reactions are sensitive to inhibitors of GTPases (Haas *et al.*, 1994), suggesting that such proteins have a direct role in vacuole inheritance.

In recent years, several GTPases of the *Rab/YPT* gene family have been implicated in diverse intracellular trafficking events in yeast and mammals (Gruenberg and Clague, 1992; Ferro-Novick and Novick, 1993; Novick and Brennwald, 1993; Nuoffer and Balch, 1994; Pfeffer, 1994). These monomeric GTPases serve as 'molecular switches' (Bourne, 1988) in the formation of vesicular transport intermediates and in vesicle targeting and fusion at distinct stages of the endocytic and exocytic pathways. The recent use of *in vitro* transport systems has given insight into the molecular mechanisms (Pryer *et al.*, 1992; Rothman, 1994). Monomeric GTPases are needed in intracellular trafficking events such as transport between the endoplasmic reticulum (ER) and the Golgi apparatus in yeast (Ypt1p; Bacon *et al.*, 1989; Baker *et al.*, 1990; Rexach and Schekman, 1991; Segev, 1991), in transport between the ER and the Golgi apparatus as well as within the Golgi apparatus in mammalian cells (Rab1; Plutner *et al.*, 1991; Peter *et al.*, 1994), in homotypic endosome fusion (Rab5; Gorvel *et al.*, 1991; Li and Stahl, 1993;

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Ullrich *et al.*, 1994), and in transport between late endosomes and the *trans*-Golgi network in mammalian cells (Rab9; Lombardi *et al.*, 1993; Soldati *et al.*, 1993, 1994). The exact functions of these GTPases are not yet known.

Recently, GDP dissociation inhibitor proteins (GDIs) have been used in these *in vitro* systems to study the roles of GTPases in vesicular trafficking events (Peter *et al.*, 1994). Each GDI binds to the GDP-bound form of a subset of GTPases (Araki *et al.*, 1990) and extracts them from membranes into the cytoplasm (Regazzi *et al.*, 1992; Soldati *et al.*, 1993; Ullrich *et al.*, 1993), allowing their cycling between acceptor and donor compartments (Pfeffer, 1994). Purified yeast Gdi1p (the *SEC19* gene product) extracts several GDP-bound GTPases of the Ypt/Rab family (Garrett *et al.*, 1994); purified mammalian RabGDI or *Drosophila* GDI remove not only mammalian Rab proteins but also the yeast GTPase Sec4p from membranes (Sasaki *et al.*, 1991; Garrett *et al.*, 1993). Added to an *in vitro* reaction, GDI can remove the GDP-bound forms of its cognate GTPases, rendering them unavailable for fusion reactions. Mammalian RabGDI was purified as an inhibitor of *in vitro* reactions of transport between the Golgi stacks (Elazar *et al.*, 1994). Purified (Rab)GDI inhibits both *in vitro* transport between the ER and Golgi compartments (Peter *et al.*, 1994) and traffick from late endosomes to the *trans*-Golgi network (Dirac-Svejstrup *et al.*, 1994).

Ypt7p is a prime candidate GTPase for the regulation of vacuole fusion. *Ypt7Δ* strains are viable and show normal α-factor uptake, but a severe delay in α-factor degradation, suggesting an inhibition of transport between the plasma membrane and the vacuole (Wichmann *et al.*, 1992). Further direct kinetic analyses of endocytic transport have shown that there is a transport block between late endosomes and vacuoles (Schimmöller and Riezman, 1993). In agreement with this hypothesis, hydrolases destined for the vacuole are partially secreted in *ypt7Δ* mutants (Wichmann *et al.*, 1992; data not shown). Thus, Ypt7p may play a role both in biosynthetic traffic from the Golgi to the vacuole and in the transport of endocytosed material from late endosomes to the vacuole (Wichmann *et al.*, 1992; Stack and Emr, 1993). Ypt7p-deficient cells have numerous small vacuoles (Wichmann *et al.*, 1992), which classifies *ypt7Δ* mutants as class B *vps* (vacuole protein sorting) mutants (Raymond *et al.*, 1992a). This vacuole fragmentation could be caused by (i) a lack of homotypic vacuole fusion, (ii) an intact fusion system paired with excessive vacuole fragmentation (vacuole fragmentation can be seen *in vitro* using vacuoles from inheritance-defective *vac1* or *vac2* cells; Conradt *et al.*, 1992) or (iii) biosynthetic missorting of vacuole membrane proteins which are normally required for fusion. Such missorting may be a cause of vacuole inheritance defects in many class C *vps* mutants.

Using various *YPT7* genetic constructs, polyclonal antibodies to Ypt7p and purified yeast Gdi1p, we now show that Ypt7p is directly required for the vacuole fusion reaction. Thus, Ypt7p is a central component of both endosome–vacuole transport reactions and the vacuole inheritance processes.

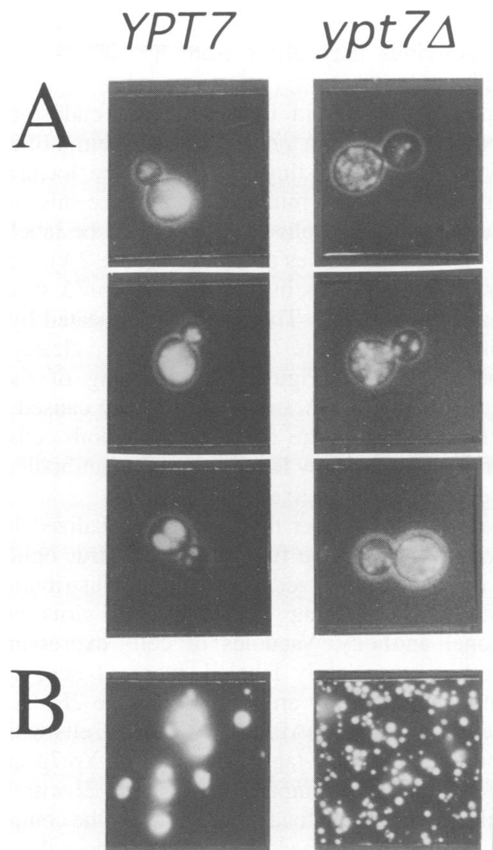

Fig. 1. Vacuoles are fragmented in *ypt7Δ* cells. (**A**) Cells were incubated with the vacuole-specific fluorophore CDCFDA (see Materials and methods). Left panels: DKY6281 (*YPT7*) cells; right panels: DKY6281-7 (*ypt7Δ*) cells. (**B**) Vacuoles isolated from DKY6281 (*YPT7*) (left panel) or DKY6281-7 (*ypt7Δ*) (right panel) cells were fluorescence-labeled *in vitro* with CDCFDA and ATP (but without cytosol) in reaction buffer (Haas *et al.*, 1994). Bar: 3 μm.

Results

Vacuoles lacking Ypt7p are fragmented in vivo

Vacuoles are usually low copy-number organelles (three to 10 per cell; Raymond *et al.*, 1992b). The daughter-cell vacuoles are assembled during cell division by fusion of the vesicles which comprise the vacuole segregation structures. In *ypt7Δ* strains (Wichmann *et al.*, 1992), vacuoles are much smaller and much more numerous than in their parent strains (Figure 1). However, these vacuole-like structures (hereafter referred to as 'vacuoles') possess several characteristic features of authentic vacuoles. They stain with the vacuole-specific fluorophores 5-(and 6-) carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA; Roberts *et al.*, 1991), fluorescein isothiocyanate–dextran (Roberts *et al.*, 1991) and FM4-64 (Vida and Emr, 1995; Figure 1A), and are also stained by an endogenous fluorophore (Weisman *et al.*, 1987) which accumulates in the vacuoles of *ade2YPT7* and *ade2ypt7Δ* cells (results not shown). Vacuoles from *ypt7Δ* strains contain the vacuole markers proteinase A (Pep4p; Figure 3B) and alkaline phosphatase (Pho8p; Figure 3B). Furthermore, vacuoles isolated from *ypt7* mutant cells float in a Ficoll gradient to the 4% Ficoll/0% Ficoll interface (results not shown), which again is typical for wild-type vacuoles. However, the concentrations of vacuolar hydrolases (e.g.

proteinase A) may be lower in *ypt7Δ* cells, and their specific activities may differ from hydrolases in wild-type cells.

Ypt7p plays a role in transport from endosomes to the vacuole (Wichmann *et al.*, 1992; Schimmöller and Riezman, 1993), suggesting that it may be localized to these organelles. Using immunofluorescence microscopy, the vacuoles in *YPT7* cells can be seen to be labeled by affinity-purified antibodies to Ypt7p (Figure 2A), whereas the fragmented vacuoles in the isogenic *ypt7Δ* strain are not labeled (Figure 2C). The nucleus, delineated by 4′,6-diamidino-2-phenylindole (DAPI) staining, clearly does not bind anti-Ypt7p (Figure 2A). Staining of the cell peripheries in Figure 2A and C is probably caused by an unspecific staining of the fixed cells, as both cells with and without antigen show labeling and the antibodies have been preincubated with fixed *ypt7Δ* cells.

To demonstrate further that Ypt7p is localized largely to the vacuoles, we have fused a Sendai virus epitope to Ypt7p and have analyzed the cellular distribution of the fusion protein using an anti-Sendai virus epitope monoclonal antibody. Vacuoles of cells expressing the virus epitope are clearly labeled, whereas neither their nuclei nor the periphery are labeled (Figure 2E, G, I and L; nuclei stained with DAPI appear blue). Cells which do not express the epitope-tagged version of Ypt7p are not labeled (Figure 2N). Comparison of Figure 2I with L (the same cell viewed in two focal planes) shows the complexity of the vacuolar arrangement. In summary, these data show that Ypt7p is highly concentrated in the yeast vacuole.

Vacuole inheritance occurs in three distinct stages: (i) segregation structure formation, (ii) movement of a stream of vesicles into the bud and (iii) fusion to form one or a few new vacuoles. *Ypt7Δ* strains are clearly defective only in the third stage. When counting the portion of newly formed buds which contain vacuoles (see Materials and methods), 94.2% of the *YPT7* cells and 90.3% of the otherwise isogenic *ypt7Δ* cells contained vacuolar material. Thus, *ypt7Δ* cells are distinct from previously studied vacuole inheritance mutants (Weisman *et al.*, 1990; Shaw and Wickner, 1991).

Ypt7p is required on both partner vacuoles for homotypic fusion in vitro

To address the question of whether the 'fragmented vacuole' phenotype in *ypt7Δ* strains results from a lack of fusion or from fusion with concomitant fragmentation, we purified vacuoles from *YPT7* cells and a *ypt7Δ* strain (Figure 1B) and measured their *in vitro* fusion (Figure 3A). Fusion required Ypt7p on each partner vacuole (Figure 3A) and was independent of whether the cytosol was from a *YPT7* or a *ypt7Δ* strain, indicating that cytosolic Ypt7p cannot restore the fusion deficiency of vacuoles from a *ypt7Δ* strain. Furthermore, we could not detect fusion in samples containing vacuoles from a *ypt7Δ* strain when adding higher concentrations of cytosol isolated from a YPT7 strain (data not shown). These data suggest that fusion-promoting Ypt7p was brought into the reaction by the vacuoles. However, *in vivo*, Ypt7p may well cycle between cytosolic and vacuole membrane pools. In parallel experiments, we could not detect any fusion by microscopic observations (data not shown). As our quantitative fusion assay is based on the maturation of proPho8p by Pep4p, we determined whether vacuoles from the *ypt7Δ* strain possessed proPho8p at a wild-type level. Although transport of Pho8p to the vacuole is slowed in *ypt7Δ* strains (Wichmann *et al.*, 1992), the steady-state levels of proPho8p were unaltered (Figure 3B), indicating that there was enough substrate for Pep4p from vacuoles isolated from a *YPT7* strain to yield a full fusion signal. Therefore, the very low fusion signal obtained with vacuoles from a *ypt7Δ* strain indicated that homotypic vacuole fusion *in vitro* requires the presence of Ypt7p on both partner vacuoles.

To test this hypothesis further, we isolated vacuoles from a strain which contains a *GAL10* promoter-driven *YPT7* gene. Ypt7p was produced at high levels when this strain was grown with galactose. After transfer to glucose, the production of Ypt7p stopped. After 24 h, Ypt7p was not detectable by immunoblot and the vacuoles had a morphology similar to vacuoles in *ypt7Δ* strains (results not shown). As vacuolar hydrolases can be stable for days *in vivo*, these vacuoles should have ample active vacuolar enzymes. To obtain the results shown in Figure 3C, vacuoles were isolated from cells grown in galactose, and then shifted to glucose for 16 h. The sizes of these vacuoles were similar to those of wild-type vacuoles. They still contained a small amount of Ypt7p (Figure 3D) and wild-type levels of Pep4p, although a small portion of Pep4p was in an abnormally processed form with an ~1 kDa higher molecular weight. Using 2 μl (0.5 μg protein) of vacuoles from DKY6281(*YPT7*), we observed almost 50% of the maximum fusion signal, whereas the addition of 2 μl (0.5 μg protein) of vacuoles from DKY6281(*GAL10–YPT7*) yielded <10% of the maximum fusion signal (Figure 3C). These data suggest that the lower alkaline phosphatase activities obtained using vacuoles from the galactose-inducible strain stem from a decreased amount of Ypt7p on these vacuoles. This again indicates that the presence of Ypt7p on both partner vacuoles is critical for fusion. The fact that ~50% of the alkaline phosphatase activity is obtained in fusion samples containing 2 μl of vacuoles from the *YPT7* strain compared with only 10% activity measured in fusion samples containing 10 μl of the Ypt7p-depleted (*GAL10–YPT7*) vacuoles suggests that multiple rounds of fusion normally occur in the *in vitro* fusion system. In the fusion samples containing only 2 μl of vacuoles with normal amounts of Ypt7p, fusion may not occur with all potential target vacuoles (containing proPho8p) because the chance of a productive encounter between the respective partner vacuoles is decreased. Therefore, the fusion signal is lower than in the samples containing 10 μl of Pep4p-containing vacuoles. Vacuoles containing less Ypt7p (from the *GAL10–YPT7* strain) may fuse very rarely with their partner vacuoles (containing proPho8p). The largest portion of alkaline phosphatase activity generated in fusion samples containing these vacuoles would then be a consequence of several rounds of fusion of few Pep4p- and Pho8p-containing vacuoles with proPho8p-containing vacuoles, leading to further proPho8p activation. These further rounds of fusion would be made possible largely by the Ypt7p contribution from the *YPT7*-derived vacuole strain. If this was true, one would expect a dramatic decrease in fusion activity with decreasing concentration

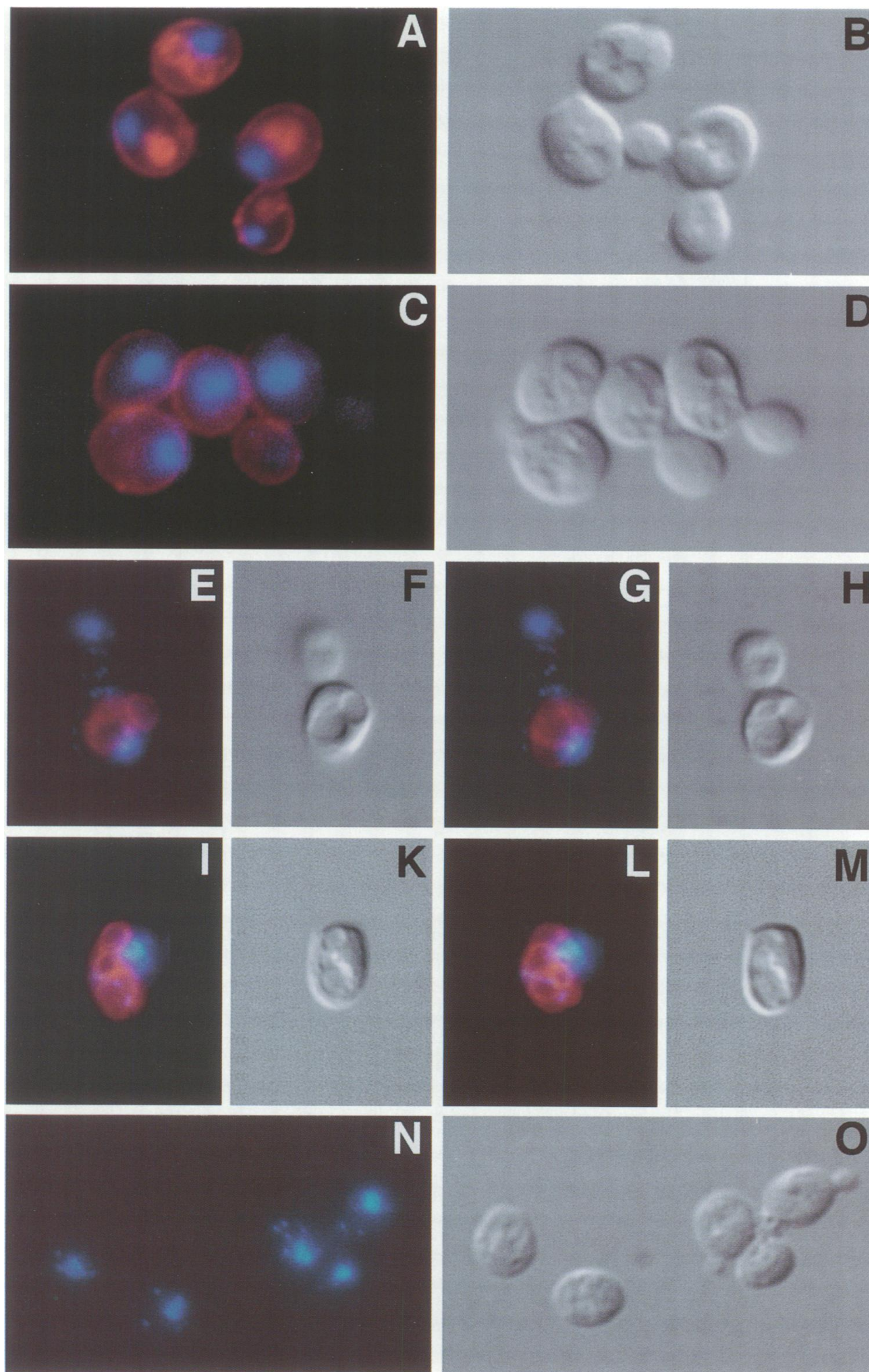

Fig. 2. An analysis of intracellular localization of Ypt7p by indirect immunofluorescence microscopy using anti-Ypt7p and an antibody to a Sendai virus epitope fused to Ypt7p. (**A–D**) Affinity-purified polyclonal Ypt7p antibodies were used to label Ypt7p. *YPT7* cells (RH270-2B) (A and B) and isogenic *ypt7Δ* cells (Y7L1) (C and D) are shown. (A and C) Double exposures showing Cy3 fluorophore signals in orange/red and the staining of nuclear DNA with DAPI in blue. (B and D) Corresponding differential interference contrast (DIC) views of the same cells. Vacuolar indentations of wild-type cells in (B) are congruent with immunostaining signals in (A). *Ypt7Δ* cells possess small vacuoles which did not stain with Ypt7p antibody (C). Some blurring of the fluorescence (A) is probably a consequence of the strong fluorescence of vacuoles in various focal planes. (**E–O**) A monoclonal antibody raised against a Sendai virus epitope was used to label the epitope-tagged Ypt7^{s}p. (E–M) The *ypt7Δ* strain Y7L1 was transformed with the plasmid pTL5 (pRS326–*YPT7^s*). (E, G, I and L) Double exposures of DAPI and antibody stainings. (E) and (F) show the same cells as (G) and (H) but in a different focal plane; (I) and (K) show the same cells as (L) and (M) but in a different focal plane. (F, H, K and M) DIC views of (E), (G), (I) and (L) respectively. (N and O) Y7L1 cells (*ypt7Δ*), untransformed controls. (N) Double exposure at the excitation wavelengths of Cy3 and DAPI. No staining with the monoclonal antibodies was visible. (O) DIC view corresponding to (N).

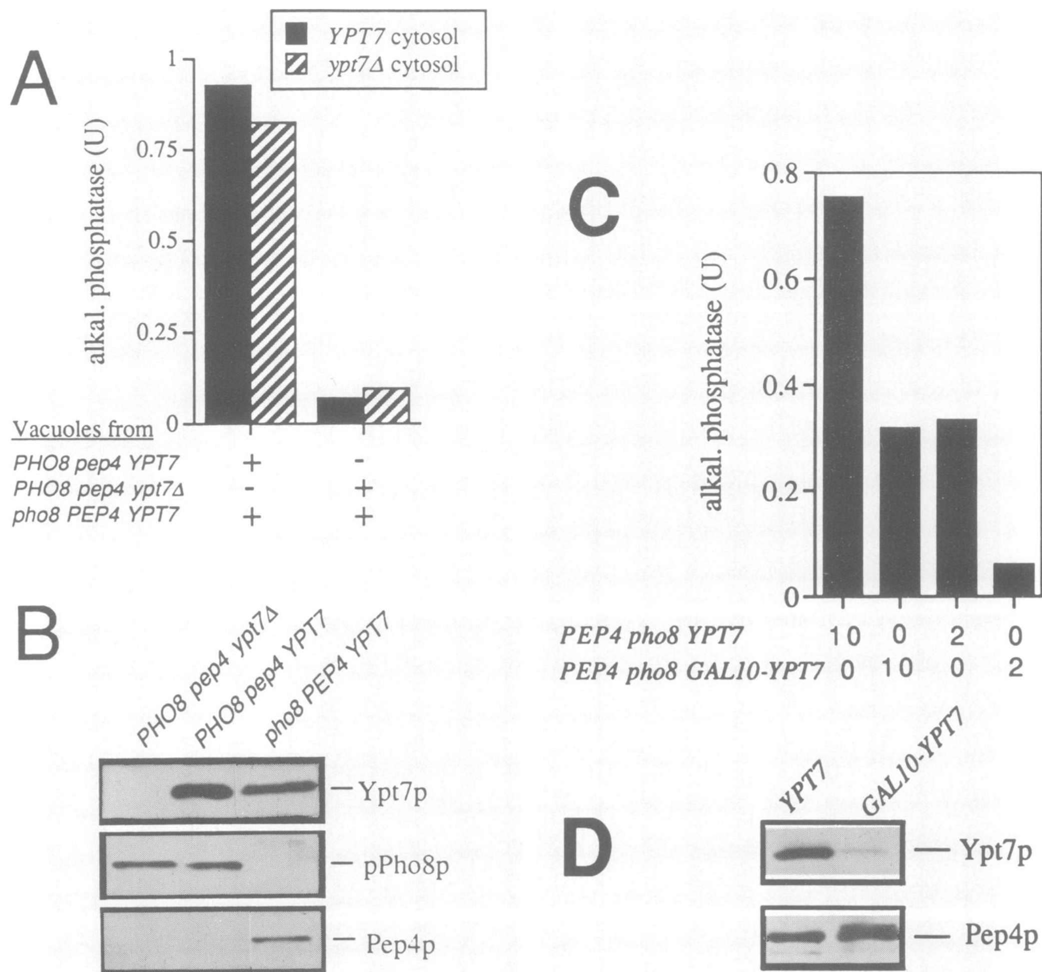

Fig. 3. Ypt7p is required on both vacuole partners for homotypic fusion. Homotypic vacuole fusion in this and the following experiments is quantitated using an assay described in Haas *et al.* (1994). Briefly, vacuoles isolated from a strain with a deletion in the proteinase A (*PEP4*) gene do not show alkaline phosphatase (Pho8p) activity because the pro-form of alkaline phosphatase must be proteolytically processed by Pep4p after transport to the vacuole to gain enzymatic activity. Vacuoles isolated from a strain with a deletion in the *PHO8* gene also do not show alkaline phosphatase activity because of a general lack of the enzyme. When these isolated vacuoles are incubated together under fusion-promoting conditions, their contents mix and proPho8p is processed to the mature and enzymatically active Pho8p. Alkaline phosphatase activity is therefore a measure of fusion frequency. (**A**) Duplicate *in vitro* fusion reactions (30 μl) were performed with cytosol from either strain RH270-2B (*YPT7*) or the isogenic strain Y7L1 (*ypt7Δ*), together with vacuoles isolated from BJ3505 (*PHO8pep4YPT7*), BJ3505-7 (*PHO8pep4ypt7Δ*), DKY6281 (*pho8PEP4YPT7*) or DKY6281-7 (*pho8PEP4ypt7Δ*). After incubation (25°C, 120 min), cytosols were removed by centrifugation (Haas *et al.*, 1994) and alkaline phosphatase activities were determined from the pellet fractions. Data were corrected for the initial alkaline phosphatase activity (0.065–0.083 U/sample). (**B**) Immunoblot analysis of vacuoles used in (A). Per lane, 30 (upper panel), 3 (middle panel) or 10 μg vacuole protein (bottom panel) were separated on a 12.5% SDS–polyacrylamide gel, transferred to a PVDF membrane, and immunodecorated with antibodies to Ypt7p, Pho8p or Pep4p. Antibody binding was visualized by chemiluminescence. *pPho8p* describes the position of proPho8p. (**C**) Duplicate *in vitro* fusion reactions (30 μl) were performed with various vacuole combinations. Vacuoles were isolated from either DKY6281 (*PEP4pho8YPT7*; grown in glucose) or DKY6281+7 (*PEP4pho8GAL10–YPT7*; grown in glucose for 16 h), and the vacuole protein concentrations in these preparations were adjusted to 0.25 mg/ml. Aliquots of 2 or 10 μl (as indicated beneath the graph) of either type of vacuole were mixed with 15 μl vacuoles isolated from BJ3505 (0.25 mg protein/ml), with salt, cytosol and an ATP-regenerating system. The reactions were then incubated at 25°C for 120 min. The cytosol used in these experiments was isolated from K91-1A (*YPT7*). Alkaline phosphatase activities were corrected for background activity (0.132 U/sample). (**D**) Vacuole samples used in (C) were analyzed by immunoblot, as described in (B).

of vacuoles from the *GAL10–YPT7* strain, as was indeed observed (Figure 3C).

Antibodies specific for Ypt7p inhibit homotypic vacuole fusion

Antibodies have been used to establish the role of various GTPases in *in vitro* transport and fusion processes (Baker *et al.*, 1990; Barlowe *et al.*, 1993; Lombardi *et al.*, 1993; Kuge *et al.*, 1994). Polyclonal antibodies against recombinant Ypt7p (Wagner *et al.*, 1992) were affinity purified on a column of immobilized Ypt7p. Anti-Ypt7p inhibited homotypic vacuole fusion, and the inhibition could be prevented completely by the addition of excess Ypt7p (Figure 4A and C, lanes 2 and 3). To demonstrate further the specificity of the inhibition of fusion by anti-Ypt7p, we used antibodies raised against Ypt1p, which is a structurally closely related GTPase involved in early steps of the secretory pathway (Schmitt *et al.*, 1986; Kibbe *et al.*, 1993). These affinity-purified antibodies, at a 60 μg/ml reaction volume, did not interfere at all with the homotypic vacuole fusion reaction (Figure 4C, lanes 1, 7 and 8), although at a concentration of only 24 μg/ml

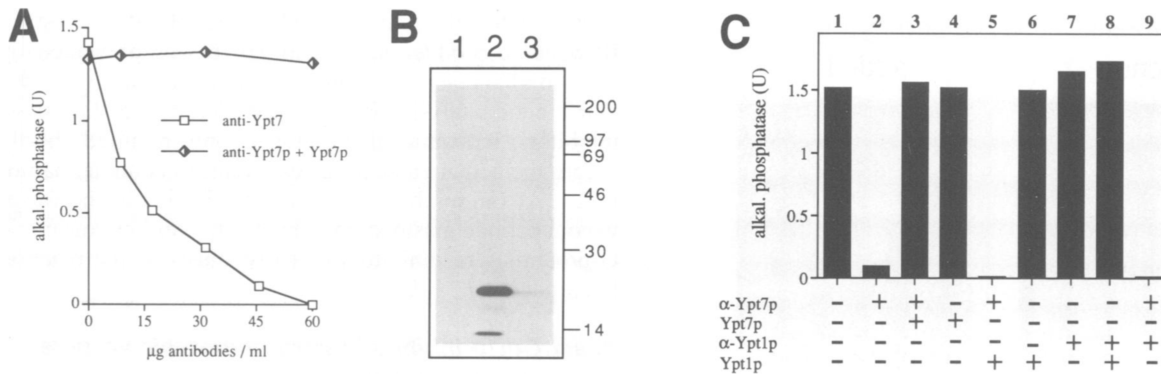

Fig. 4. Ypt7p-specific antibodies inhibit homotypic vacuole fusion. (**A**) Duplicate *in vitro* fusion reactions containing affinity-purified Ypt7p antibodies in reaction buffer (□), or a combination of the respective concentration of Ypt7p antibodies with 66 µg/ml purified recombinant Ypt7p (◆), were incubated at 25°C for 120 min followed by alkaline phosphatase activity determination. Data were corrected for background activity (0.283 U/sample). (**B**) Immunoblot analysis using affinity-purified anti-Ypt7p. 50 µg vacuolar protein isolated from DKY6281-7 (*ypt7Δ*; lane 1) or DKY6281 (*YPT7*; lane 2), or 150 µg cytosolic protein isolated from K91-1A (*YPT7*; lane 3) were separated on a 12.5% SDS–polyacrylamide gel, transferred to a PVDF membrane, and immunodecorated with affinity-purified Ypt7p antibodies. Binding was visualized by chemiluminescence. The lower molecular weight protein seen in lane 2 is a degradation product of Ypt7p, because it does not appear in the vacuole fraction of the otherwise isogenic *ypt7Δ* strain; proteolysis by vacuolar proteinases cannot always be prevented by proteinase inhibitors (Jones, 1991). The migration positions of molecular mass (×1000) marker proteins (rainbow marker, Bio-Rad) are indicated. (**C**) Duplicate *in vitro* standard fusion reactions, containing the indicated combinations of anti-Ypt7p (α-Ypt7p; 60 µg/ml), purified recombinant Ypt7p (66 µg/ml), anti-Ypt1p (α-Ypt1p; 60 µg/ml) or purified recombinant Ypt1p (66 µg/ml), were incubated on ice for 10 min, then at 25°C for 120 min, followed by alkaline phosphatase assaying. Data were corrected for background activity (0.176 U/sample). The fact that a relatively high concentration of anti-Ypt7p was needed for the complete inhibition of homotypic vacuole fusion (60 µg/ml) is probably a consequence of the affinity purification procedure. When we isolated a complete IgG fraction from the serum that was also used for affinity purification, then 70 µg total IgG/ml reaction completely inhibited the fusion reactions. Again, this inhibition could be prevented by the addition of purified Ypt7p. Most likely, a certain subspecies of antibody, that was a highly potent inhibitor of Ypt7p function, was only poorly separated on the affinity column.

reaction volume they inhibited 75% of the *in vitro* transport of ER-derived vesicles to the Golgi compartment (C.Barlowe, personal communication; fusion reactions performed as described in Barlowe *et al.*, 1994). All Ypt proteins share structurally conserved, functionally important domains (Kibbe *et al.*, 1993). Therefore, one might expect that the Ypt7p antibodies might bind not only to Ypt7p, but also to other Ypt proteins. If a Ypt protein other than Ypt7p was the actual target of the anti-Ypt7p antibody, then one would expect that the addition of any purified closely related Ypt protein would prevent anti-Ypt7p from fusion inhibition by competing with Ypt7p for the available antibodies. However, Ypt1p added to the fusion reactions at the same concentration as Ypt7p (in Figure 4C) did not compete at all with the fusion-inhibiting Ypt7p antibodies (Figure 4C, lanes 1–3 and 5). This demonstrates further the specificity of the inhibition by anti-Ypt7p.

We have also tested antibodies to Ypt51p/Vps21p in our fusion reactions (Horazdovsky *et al.*, 1994). This GTPase is probably the functional counterpart of the mammalian, endosome-located GTPase Rab5 (Horazdovsky *et al.*, 1994; Singer-Krüger *et al.*, 1994), and is therefore a Ypt protein which acts in vesicular trafficking in a step just 'upstream' of the vacuole. Although 115 µg IgG/ml reaction were tested, no inhibition of homotypic vacuole fusion could be detected. In addition, affinity-purified antibodies against the GTPase Sar1p, although added at a concentration which completely inhibits ER–Golgi transport *in vitro* (Barlowe *et al.*, 1993), did not interfere with homotypic vacuole fusion (data not shown). The inhibition of vacuole–vacuole fusion by Ypt7p antibodies was not a consequence of vacuole crosslinking via bivalent antibodies, because monovalent Fab fragments prepared from affinity-purified anti-Ypt7p IgGs inhibited the fusion reaction with an IC_{50} of 4.0 µg/ml; this inhibition could be prevented completely by the simultaneous addition of purified Ypt7p (results not shown). In addition, only a single protein reacted with anti-Ypt7p in an immunoblot of vacuole protein and of the cytosol preparation used in our fusion reactions (Figure 4B, lanes 2 and 3). No immunoreactive protein was detected in vacuoles from a *ypt7Δ* strain (Figure 4B, lane 1), further strengthening the assumption that Ypt7p antibodies are highly specific.

To determine by an independent assay whether the inhibition of proPho8p maturation by anti-Ypt7p was indeed caused directly by an inhibition of vacuole fusion, we performed a microscopic analysis of our *in vitro* reactions using the fluorophore FM4-64 (Vida and Emr, 1995). Vacuoles did not increase in size when either affinity-purified anti-Ypt7p was added or cytosol was omitted (Figure 5). When no antibodies were included or when antigen was added with the antibodies, vacuoles clearly fused (Figure 5). Thus, the low alkaline phosphatase activities measured in the presence of anti-Ypt7p were caused by an inhibition of homotypic vacuole fusion and not by an inhibition of proPho8p maturation *per se*.

Kinetics of inhibition by anti-Ypt7p

A large-scale fusion reaction (containing vacuoles, salt, an ATP-regenerating system and cytosol) was incubated at 25°C. Samples were removed at various times and mixed with anti-Ypt7p, anti-Ypt7p plus Ypt7p or reaction buffer only. By placing one set of samples on ice (to stop fusion), the extent to which fusion had occurred by the time the samples were removed can be measured (Figure 6A, ▲). Pipetting another sample into a tube with only reaction buffer allowed the demonstration that the handling procedure did not interfere with vacuole–vacuole fusion

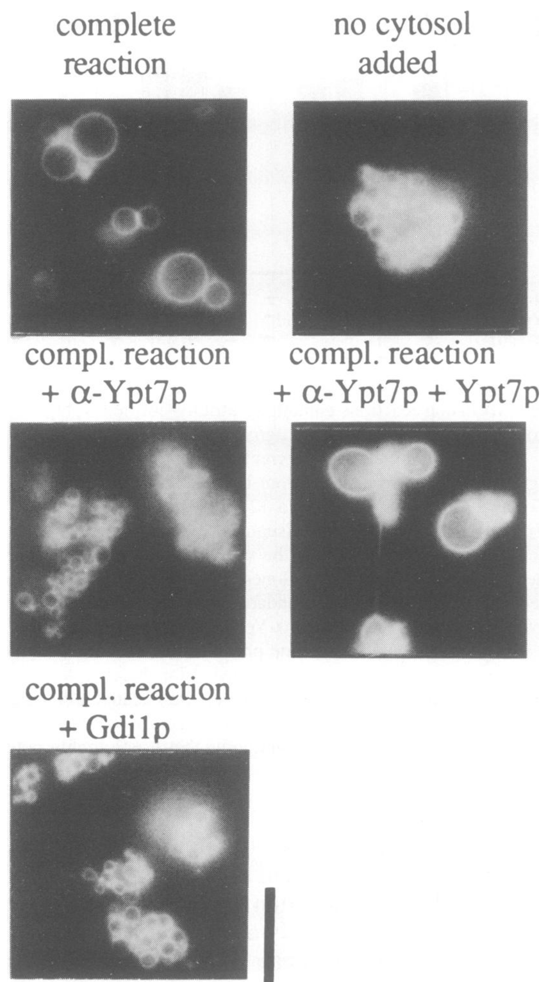

Fig. 5. Microscopic analysis of vacuole fusion reactions. Vacuole fusion reactions contained salt, cytosol and ATP (top left), salt and ATP (top right), salt, cytosol, ATP and 60 μg/ml anti-Ypt7p (middle left), a complete reaction plus 70 μg/ml anti-Ypt7p and 67 μg/ml Ypt7p (middle right), or a complete reaction plus 80 μg/ml Gdi1p (bottom left). The vacuoles were visualized by fluorescence microscopy using the vacuole membrane label FM4-64 (Materials and methods). Incubation prior to photography was at 25°C for 100 min. Bar: 6 μm.

(Figure 6A, ○). Other samples were incubated further at 25°C, allowing a measure of the inhibition by an added reagent during the subsequent incubation (Conradt *et al.*, 1994). Anti-Ypt7p inhibited the fusion reaction at every time point almost as quickly as placing the samples on ice (Figure 6A, compare □ with ▲). This indicates that the fusion reaction was blocked from reaching a stage in which vacuoles are activated for fusion, and the production of which requires the incubation of vacuoles in the presence of salt, cytosol and ATP ('stage III'). According to our previous studies (Conradt *et al.*, 1994), a vacuole fusion reaction becomes independent from salt when vacuoles are incubated briefly with moderate concentrations of potassium acetate and potassium chloride ('stage I'). In a second reaction, vacuoles can be incubated with cytosol, whereupon these 'stage II' vacuoles can fuse in the presence of ATP alone. Further incubation in the presence of ATP leads to the production of 'stage III' vacuoles which can then fuse in stage IV without further contact with salt, cytosol or ATP. The inhibition of stage III vacuole production by anti-Ypt7p was prevented by the simultaneous addition of Ypt7p (Figure 6A, ◆). Our kinetic analysis classifies anti-Ypt7p as a 'late-stage inhibitor', indicating that Ypt7p is only required shortly before the actual vacuole fusion event. In contrast, bafilomycin A_1 (an inhibitor of vacuolar ATPase) or the wasp-venom peptide mastoparan (which activates heterotrimeric G-proteins) are inhibitors of early stages of the reaction (Conradt *et al.*, 1994).

Yeast Gdi1p inhibits in vitro homotypic vacuole fusion at a late stage

Some GDIs remove certain GDP-bound small GTPases from membranes. A yeast GDI termed Gdi1p, which solubilizes the exocytosis-regulating GTPase Sec4p(GDP), has been characterized (Garrett *et al.*, 1993, 1994) and was purified from recombinant *Escherichia coli* (Figure 7B) for the experiments described here. Isolated vacuoles (at the same concentration as in the standard reaction) were incubated for 5 min at 30°C with either 70 μg Gdi1p/ml or without Gdi1p (using the Gdi1p preparation shown in Figure 7B). Vacuoles were then reisolated and their Ypt7p content determined by immunoblot. Then they were used in standard fusion reactions. Approximately 75% of Ypt7p was released from the membranes by Gdi1p, and the Gdi1p-pretreated vacuoles lost 90% of their fusion efficiency (results not shown). We also tested the Gdi1p concentrations necessary for the inhibition of homotypic vacuole fusion when added to the complete reactions. We determined an IC_{50} of 15–25 μg/ml and an IC_{100} of 60–70 μg/ml (Figure 7A). As with anti-Ypt7p, a microscopic analysis (Figure 5) confirmed that Gdi1p inhibits the fusion reaction. If Gdi1p inhibits the fusion reaction by its Ypt7p extraction activity, then one would expect that Gdi1p, like anti-Ypt7p, would inhibit a late fusion stage. A kinetic experiment (performed like the experiment describing the kinetics of anti-Ypt7p inhibition) showed that, as with anti-Ypt7p, Gdi1p stops the *in vitro* fusion reaction as rapidly as placing the samples on ice (Figure 6B, compare ● with □).

Ypt7p becomes susceptible to antibody inhibition during the in vitro reactions

We followed the kinetics with which Ypt7p can neutralize the fusion-inhibiting activity of anti-Ypt7p. Surprisingly, added Ypt7p can largely prevent further inhibition of the fusion reaction by anti-Ypt7p (Figure 8). When purified recombinant Ypt7p was added at time *t* to reactions containing Ypt7p antibodies, then its neutralization of the antibodies allowed for subsequent fusion during the interval 120–*t* (Figure 8, □), which was approximately equal to that which occurred during the interval 120–*t* in the absence of antibody or added Ypt7p (Figure 8; the difference between ○ and ▲). However, the inhibition by anti-Ypt7p is not reversible, because no protection from fusion inhibition was detectable when Ypt7p was added after ~75 min (Figure 8, □). Fusion was not disturbed by the pipetting procedures (Figure 8, ○), and only minimal fusion occurred in samples in which anti-Ypt7p was constantly present but which did not contain Ypt7p (◇, ●). There was little inhibition when vacuoles were incubated on ice with anti-Ypt7p, reisolated and then used in a

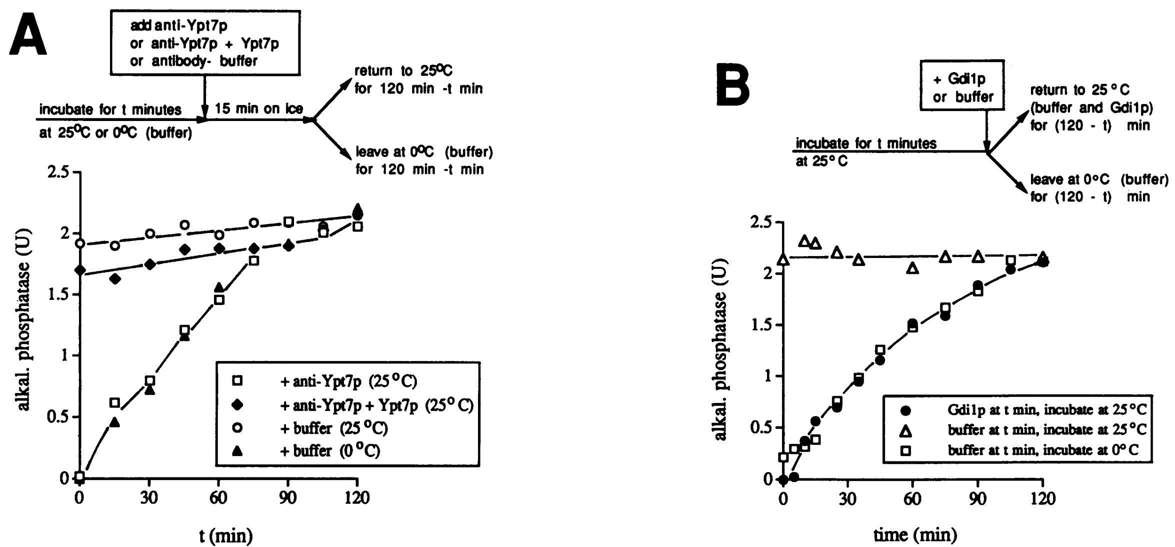

Fig. 6. Either anti-Ypt7p or Gdi1p inhibit homotypic vacuole fusion at a late stage. (**A**) A 1.35 ml standard fusion reaction was incubated at 25°C. At various times (*t* min), 30 µl samples were removed and added to fresh reaction tubes which contained, in a 2 µl volume, reaction buffer (○, ▲), anti-Ypt7p (□) or a Ypt7p/anti-Ypt7p mixture (◆). Samples were then incubated for 15 min on ice to allow for an antigen–antibody reaction, and then returned to 25°C for (120 min – *t* min) or left on ice (▲). Alkaline phosphatase activity determinations were corrected for background activity (0.353 U/sample). (**B**) Kinetics of Gdi1p-mediated inhibition. This experiment was performed as described in the legend to (A), but without an interim 15 min incubation on ice. From a 1.65 ml reaction, 30 µl samples were collected at indicated times and mixed with either 1 µl Gdi1p solution (2.14 mg/ml) or Gdi1p buffer. Incubation continued at either 25°C (●, △) or on ice (□). After a total incubation time of 120 min, alkaline phosphatase activities were determined and corrected for background activity (0.169 U/sample).

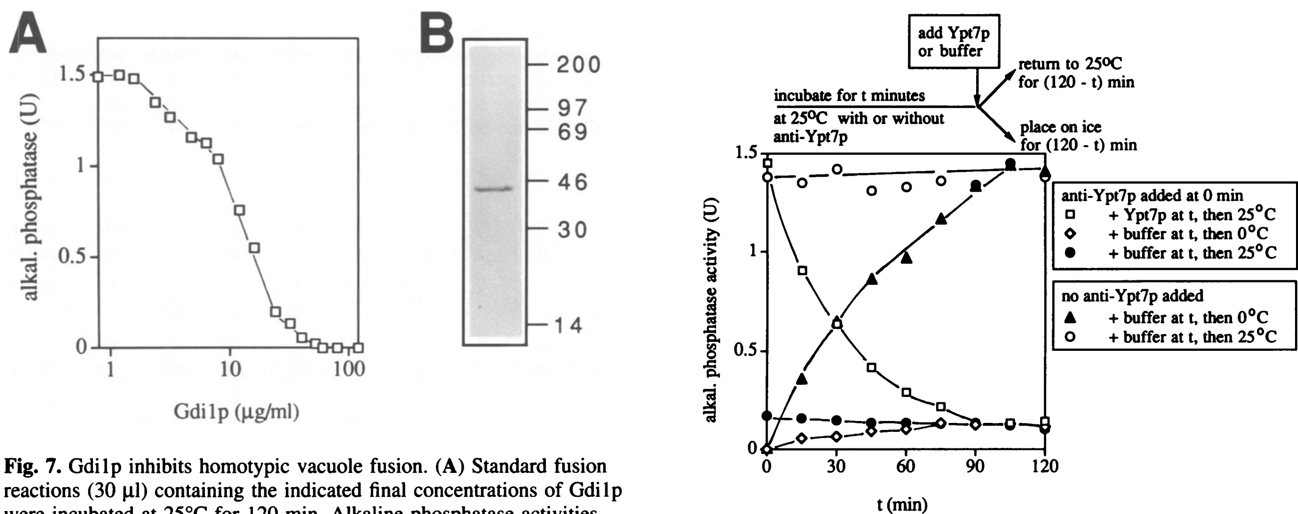

Fig. 7. Gdi1p inhibits homotypic vacuole fusion. (**A**) Standard fusion reactions (30 µl) containing the indicated final concentrations of Gdi1p were incubated at 25°C for 120 min. Alkaline phosphatase activities were determined and corrected for background alkaline phosphatase activity (0.158 U/sample). (**B**) A sample (2 µg) of the Gdi1p preparation used in (A) was separated by electrophoresis on a 12.5% polyacrylamide gel and stained with Coomassie blue. The migration positions of molecular mass (×1000) marker proteins (rainbow marker, Bio-Rad) are indicated.

Fig. 8. The inhibition of homotypic vacuole fusion by anti-Ypt7p can be prevented by the addition of pure recombinant Ypt7p. Two complete fusion reactions were prepared (first reaction), one of 0.99 ml without antibody and the other of 0.66 ml with affinity-purified anti-Ypt7p (60 µg/ml). Each was incubated at 25°C. Every 15 min, samples were removed and placed in fresh reaction tubes (second reaction) which contained (in 2 µl) either reaction buffer (◇, ●, ▲, ○) or Ypt7p (66 µg/ml; □). For the remaining time of the 2 h incubation, the removed samples either remained at 25°C (□, ●, ○) or were placed on ice (◇, ▲). After a total incubation of 120 min, alkaline phosphatase activities were determined. Data were corrected for background activity (0.162 U/sample).

fusion experiment in the absence of antibody (results not shown). These data suggest that fusion-inhibiting Ypt7p antibodies only bound to the vacuoles as the reaction progressed. However, we cannot exclude the formal possibility that the added soluble Ypt7p could compete with vacuolar Ypt7p for bound Ypt7p antibodies during an early stage of the reaction.

Maximal inhibition of the fusion reaction by anti-Ypt7p or Gdi1p requires ATP and cytosol

When vacuoles are incubated for 20–30 min at 25°C with salt buffer, cytosol and ATP (stage III vacuoles), they can complete fusion during stage IV in the absence of salt, ATP and cytosol (see above). When salt, cytosol and ATP were added to such stage III vacuoles (Figure 9A, bars with bold stripes), fusion was inhibited by anti-Ypt7p or Gdi1p during stage IV. Both cytosol and ATP were required for maximum inhibition by either anti-Ypt7p or

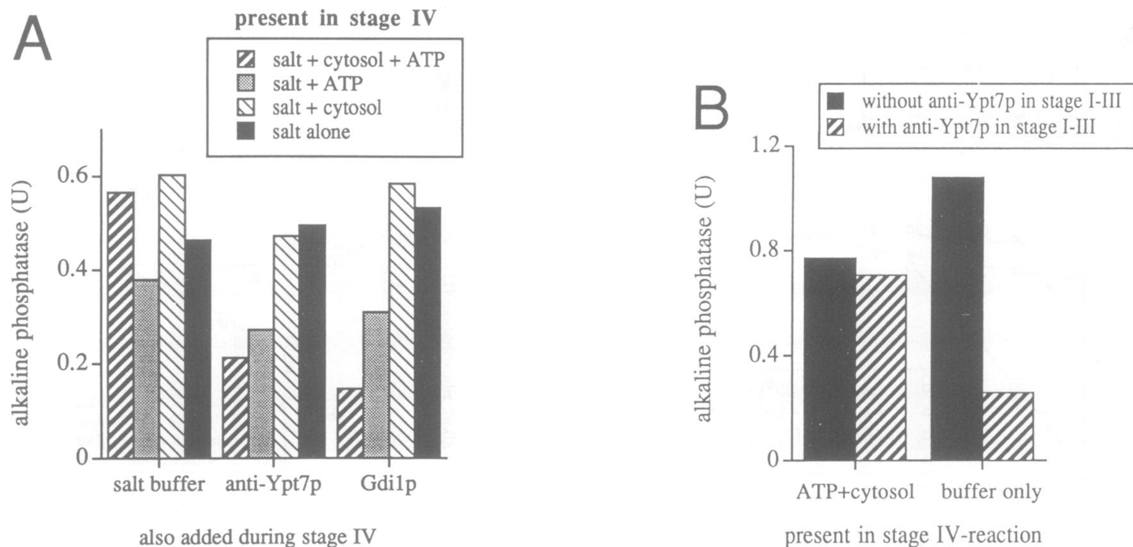

Fig. 9. (**A**) Maximum inhibition of vacuole fusion by anti-Ypt7p requires the presence of cytosol and ATP. Stage III vacuoles (Conradt *et al.*, 1994) were produced by incubating a 1.8 ml fusion reaction for 20 min at 25°C. The reaction was placed on ice for 5 min, and the vacuoles were reisolated (Beckman minifuge, 10 000 r.p.m., 4°C, 60 s) and used in duplicate stage IV reactions. Either salt (reaction buffer) with anti-Ypt7p (70 μg/ml) or with buffer and Gdi1p (69 μg/ml) was added to vacuoles with salt only, with salt, cytosol and ATP, with salt and ATP, or with salt and cytosol, as indicated at the top of the figure, and incubated for 120 min at 25°C or on ice. Alkaline phosphatase activities were determined. The alkaline phosphatase activities of the samples placed on ice after the first incubation were subtracted from the activities of the samples kept at 25°C throughout the incubations (0.473–0.715 U/sample, depending on the sample composition in stage IV). (**B**) Anti-Ypt7p inhibits the formation of stage III vacuoles. One portion of stage III vacuoles was prepared as described in (A) (black bars), and another portion contained anti-Ypt7p (70 μg/ml final concentration; hatched bars). After reisolation, these vacuoles were mixed with reaction buffer, cytosol and ATP (left) or reaction buffer only (right), and incubated for 120 min in the presence of Ypt7p (34 μg/ml) at 25°C or on ice. Alkaline phosphatase activities were determined. The alkaline phosphatase activities of those samples that were placed on ice during the second incubation were subtracted from the activities of the respective samples incubated at 25°C, yielding the alkaline phosphatase activities obtained during the stage IV reaction (the subtracted activities varied between 0.144 and 0.863 U/reaction, largely depending on whether anti-Ypt7p was present during the first incubation). The data in this figure show only the amount of fusion that occurred during stage IV (fusion which occurred during stages I–III was subtracted). Therefore, alkaline phosphatase activities are lower here than those of fusion experiments in which alkaline phosphatase activities generated during stages I–IV are shown (e.g. Figure 4A and C).

Gdi1p (Figure 9A), because the presence of salt plus ATP (dotted bars), salt and cytosol (lightly-striped bars), or salt alone (solid bars) did not significantly support fusion inhibition by either anti-Ypt7p or Gdi1p. Anti-Ypt7p also blocks the production of stage III vacuoles. In another experiment (Figure 9B), vacuoles were incubated for 20 min with salt, cytosol and ATP at 25°C in the absence or presence of anti-Ypt7p. They were then reisolated and assayed for their fusion activity during a 120 min stage IV incubation with buffer alone, or with additional ATP and cytosol. Vacuoles which had been preincubated without Ypt7p antibody (Figure 9B, solid bars) did not require ATP and cytosol for the completion of fusion. The degree of fusion observed with vacuoles that had been preincubated with antibody (Figure 9B, hatched bars) was variable but always higher in the presence of ATP and cytosol (Figure 9B; compare hatched bars), and only very low fusion activities could be observed when no cytosol and ATP were added in stage IV (Figure 9B, 'buffer only'; compare filled with hatched bars). Vacuoles which had been incubated with reaction buffer only fused well in the presence and absence of cytosol and ATP (Figure 9B).

Discussion

We have found that Ypt7p is a predominantly vacuolar membrane protein which is needed for homotypic vacuole fusion—the last step in the vacuole inheritance process. *In vivo* studies alone might have ascribed the small size of vacuoles found in *ypt7Δ* strains to a failure of these vacuoles to undergo homotypic fusion, a missorting along the secretory pathway of proteins relevant for vacuole fusion, or a constant vacuole fragmentation with concomitant fusion. However, *in vitro* inhibition by anti-Ypt7p or Gdi1p cannot be explained by protein missorting, and constant fragmentation and fusion would have mixed Pep4p with proPho8p. Therefore, our studies with anti-Ypt7p and purified Gdi1p have established that Ypt7p is directly required for vacuole fusion. In addition to Ypt1p (Schmitt *et al.*, 1986; Baker *et al.*, 1990; Rexach and Schekman, 1991; Segev, 1991), Ypt7p is the second member of the Ypt family of GTPases which has been shown in a cell-free system to be involved in an intracellular fusion event, and the first GTPase for which a direct role in organelle inheritance has been established.

The *in vitro* fusion of isolated vacuoles was inhibited even when only one of the fusion partners was prepared from *ypt7Δ* cells. In this respect, vacuoles isolated from *ypt7Δ* cells are different from vacuoles isolated from *vac2-1* cells. Intermediate fusion activities were detected using the pro-alkaline phosphatase maturation assay when vacuoles from *vac2-1* cells were mixed with vacuoles isolated from the isogenic parental strains, whereas fusion incompetence was only observed when both vacuole populations were isolated from *vac2-1* strains (Shaw and Wickner, 1991; Haas *et al.*, 1994). While Ypt7p is required on both vacuole populations for fusion, these data suggest that the presence of functional Vac2p on one fusion partner is

sufficient. It has not been determined previously if GTPases are required on both partner vesicles in any homotypic or heterotypic fusion system.

The inhibition of fusion by anti-Ypt7p IgG and Fab fragments and the selective relief of this inhibition by Ypt7p but not Ypt1p establish that the antibodies inhibit fusion merely by their binding. In contrast to fusion, segregation structure formation *in vitro* was not impaired in the presence of anti-Ypt7p (results not shown). Similar to the specificity for a particular GTPase seen in our studies, the *in vitro* homotypic fusion of early endosomes is inhibited completely by antibodies to Rab5 but not by antibodies against Rab2 or Rab7 (Gorvel *et al.*, 1991). Furthermore, transport from late endosomes to the *trans*-Golgi network in mammalian cells is inhibited completely by anti-Rab9 IgG but not by anti-Rab7 IgG (Lombardi *et al.*, 1993).

The possibility that the small size of vacuoles isolated from *ypt7Δ* strains caused the loss in fusion ability can also be ruled out because (i) Ypt7p-containing vacuoles did not fuse in the presence of anti-Ypt7p despite the normal vacuole size, (ii) vacuoles form small vesicles *in vivo* during vacuole inheritance in wild-type cells and these vesicles later fuse with each other in the bud and (iii) vacuoles harvested from *GAL10–YPT7* strains shifted for 16 h from galactose- to glucose-containing medium still had a normal size, but they contained considerably less Ypt7p than their parent *YPT7* strains and fused much less then vacuoles isolated from *YPT7* strains.

The extraction of Ypt7p by Gdi1p blocked the *in vitro* fusion reaction but not segregation structure formation. The Gdi1p concentrations needed for the inhibition of vacuole fusion ($IC_{50} \approx 20$ µg/ml) were similar to those needed in related *in vitro* fusion systems such as ER–Golgi transport ($IC_{50} \approx 4$ µg/ml; Peter *et al.*, 1994) or intra-Golgi transport ($IC_{50} \approx 16$ µg/ml; Elazar *et al.*, 1994). As Gdi1p is not specific for Ypt7p, other GTPases which could be required for an early step in the fusion reaction could be extracted too. The effects of their removal *in vitro* would be concealed by the detachment of Ypt7p by Gdi1p, because Ypt7p is required at a late stage of homotypic vacuole fusion. Because (i) the kinetics of Gdi1p- and anti-Ypt7p-mediated fusion inhibition are the same, (ii) each has similar effects, as seen in the microscopic analysis of the *in vitro* fusion reaction and (iii) stage III vacuoles are sensitive to both reagents only in the presence of cytosol and ATP, the inhibition of fusion by Gdi1p is probably caused by the inhibition of Ypt7p function. There may be a short interval in the *in vitro* reaction during which the Ypt7p antibodies block vacuole activation and thus the fusion of vacuoles (Figure 10). Once this occurs, the inhibition cannot be relieved by the addition of competing antigen (Figure 8). It is striking that, in the absence of cytosol and ATP, the stage IV fusion reaction is insensitive to anti-Ypt7p and Gdi1p. However, in the presence of cytosol and ATP, the stage IV reactions are sensitive to anti-Ypt7p and Gdi1p (Figures 9A and 10). In addition, vacuoles which have been first incubated with anti-Ypt7p in the presence of cytosol and ATP do not enter stage III, as judged by their inability to subsequently fuse in the absence of cytosol and ATP (Figures 9B and 10). Therefore we postulate that fusion complexes may cycle between active and inactive states,

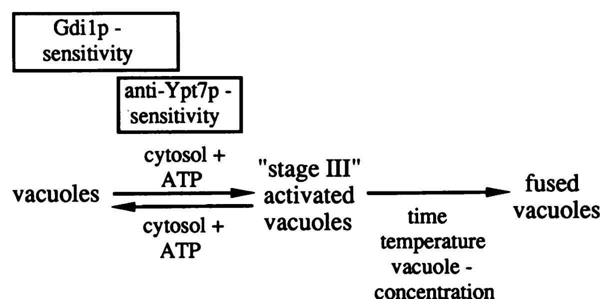

Fig. 10. Model for a possible mechanism of inhibition of homotypic vacuole fusion *in vitro* by anti-Ypt7p and Gdi1p. In the presence of salt, cytosol and ATP, vacuoles are transformed into stage III vacuoles, an activated state that requires only time, a physiological temperature and a certain vacuole concentration for fusion. Either anti-Ypt7p or Gdi1p prevents the formation of stage III vacuoles. Vacuoles alone are sensitive to Gdi1p, while sensitivity to anti-Ypt7p requires the presence of cytosol and ATP. When stage III vacuoles are incubated further with cytosol and ATP, they again become sensitive to anti-Ypt7p and Gdi1p, suggesting that the vacuoles cycle between their activated and inactivated states.

requiring cytosol and ATP in each direction. Each complete cycle would require the activation of Ypt7p for the activation of the vacuole fusion machinery (Figure 10). The addition of anti-Ypt7p would irreversibly ‘freeze’ the fusion machinery in the inactive state. In a reaction in which most of the vacuoles, although engaged in ‘cycling’, spent most of their time in stage III, the removal of cytosol would prevent the reverse reaction of cycling and thus render these vacuoles not susceptible to anti-Ypt7p and Gdi1p (Figure 10). Similarly, in a recent study, Rab3(GDP)/Rab3(GTP) cycling has been shown to be directly associated with exocytosis (Stahl *et al.*, 1994).

Our data with regard to Gdi1p are kinetically clearly different from those data obtained with *in vitro*-reconstituted ER–Golgi and intra-Golgi transport systems (Peter *et al.*, 1994), where (Rab)GDI acts on Rab1 to inhibit an early stage of vesicle budding from the ER. Although our experiments do not specifically address the question of whether Gdi1p is involved in Ypt7p cycling *in vivo*, our data do show that Ypt7p is a substrate for Gdi1p, and thus that Gdi1p may shuttle Ypt7p between vacuoles and late endosomes *in vivo*.

Because Ypt7p is directly required for homotypic vacuole fusion, we suggest that the vacuoles in a *ypt7Δ* strain are fragmented as a result of slow fusion. It has been shown previously that Ypt7p is required for α-factor degradation but not α-factor internalization (Wichman *et al.*, 1992). Further direct kinetic analyses of endocytic transport have suggested that there is a transport block between late endosomes and vacuoles (Riezman, 1993; Schimmöller and Riezman, 1993). Here, however, we present data which imply that Ypt7p is part of the vacuole inheritance machinery. To accommodate these data, we suggest that Ypt7p may take part both in transport from a prevacuolar compartment to the vacuole and in homotypic vacuole fusion. The delivery of α-factor to the vacuole would then be delayed because Ypt7p is necessary for the fusion of late endosomes with vacuoles (and possibly for the fusion of late endosomes with each other). This would explain why the delivery of vacuolar hydrolases to the vacuole is delayed in some *ypt7Δ* strains (Wichmann *et al.*, 1992; data not shown). The possibility of the

involvement of a distinct GTPase in more than one transport step has two well-studied precedents: (i) Rab1 is required not only for transport from the ER to the Golgi apparatus, but also for transport between Golgi stacks (Plutner *et al.*, 1991) and (ii) Rab5 acts in transport from the plasma membrane to early endosomes as well as in endosome–endosome fusion (Gorvell *et al.*, 1991; Bucci *et al.*, 1992).

The mammalian counterpart of Ypt7p (Rab7; 63% amino acid sequence identity) is predominantly localized to late endosomes (Chavrier *et al.*, 1990) and may play a role in transport from late endosomes to lysosomes, the functional equivalent of vacuoles in mammalian cells. It is tempting to speculate that Rab7 may also be involved in homotypic lysosome fusion (Deng and Storrie, 1988). The fact that Rab7 is found largely on late endosomes and not lysosomes (Chavrier *et al.*, 1990; Rabinowitz *et al.*, 1992) may reflect a recycling of Rab7 from lysosomes to late endosomes.

Which functional role(s) does Ypt7p play in homotypic vacuole fusion? Recent studies with other *in vitro* systems suggest that monomeric GTPases may take part in the assembly of fusion complexes on membranes (Brennwald *et al.*, 1994; Lian *et al.*, 1994; Søgaard *et al.*, 1994). The data presented here would be consistent with such a role for Ypt7p. While a fusion complex that has been formed in a Ypt7p-catalyzed reaction might be stable and fusion competent for a prolonged period of time in the absence of cytosol and ATP, their presence might lead to an accelerated disassembly or inactivation (e.g. by cytosolic protein kinases), creating a new requirement for activated Ypt7p. The *in vitro* reactions of vacuole inheritance may also allow the investigation of the possible role of other GTPases in vacuole inheritance because some intracellular trafficking reactions seem to be regulated by more than one GTPase (Schwaninger *et al.*, 1992; Fischer von Mollard *et al.*, 1994).

Materials and methods

Microbial strains and media

Saccharomyces cerevisiae strains used in this study were BJ3505 (*MATa pep4::HIS3 prb1-Δ1.6R HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can*), BJ3505-7 (*MATa pep4::HIS3 prb1-Δ1.6R HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can ypt7::URA3*), BJ5459 (*MATa ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*), DKY6281 (*MATa leu2-3 leu2-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pho8::TRP1*; from Dr D.Klionsky, University of California, Davis, CA), DKY6281-7 (*MATa leu2-3 leu2-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pho8::TRP1 ypt7::URA3*), DKY6281+7 (*MATa leu2-3 leu2-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pho8::TRP1 LEU2–GAL10–YPT7*), K91-1A (*MATa ura3 pho8::pAL134 pho13::pH13 lys1*; from Dr Y.Kaneko, Institute of Fermentation, Osaka, Japan, unpublished results), RH270-2B (*MATa Gal*$^+$ *leu2 ura3 his4 lys2 bar1-1*; from Dr Howard Riezman, Biocentre Basel, Switzerland), Y7L1 (*MATa Gal*$^+$ *leu2 ura3 his4 lys2 bar1-1 ypt7::LEU2*), YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ11*), and YPH499-7 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ11 ypt7::URA3*). The *E.coli* strains used for cloning experiments were RR1 and DH5α, or are listed below. Yeast was grown in YPD (per liter: 20 g dextrose, 20 g peptone, 10 g yeast extract) was used as a rich growth medium for yeast or, where indicated, in YPG (dextrose in YPD replaced by galactose). *E.coli* was grown in LB (per liter: 20 g dextrose, 10 g yeast extract, 10 g NaCl).

Materials

TMAXp3200 film (Kodak) was used in combination with an F3 camera (Nikon Inc., Melville, NY) and a Zeiss standard microscope. Samples were analyzed in 6 mm wells of immunofluorescence slides (teflon-coated; Polyscience, Niles, IL). For immunofluorescence analysis using antibodies (see below), Agfa 50RS Plus and Kodak Ektachrome 64 EPR films were used. CDCFDA and FM4-64 were obtained from Molecular Probes Inc. (Eugene, OR). The ECL chemiluminescence kit and hyperfilm ECL were purchased from Amersham. Protein concentrations were determined using the Bio-Rad assay reagent kit, with bovine serum albumin as a standard. Other reagents have been described in Haas *et al.* (1994) or were purchased from Sigma.

Genetic techniques

Cloning procedures and DNA manipulations were performed according to Sambrook *et al.* (1989). Disruption of the *YPT7* gene was performed as described previously (Wichmann *et al.*, 1992). To bring the *YPT7* gene under the control of a *GAL10* promoter, a *Pst*I site was inserted 22 bp upstream of the coding region into which the *Nde*I–*Sal*I fragment of the vector Yep51, containing the *LEU2* marker and the *GAL10* promoter region, was inserted. Yeast was transformed with a *Stu*I–*Pac*I linear fragment of the engineered gene, the correct integration confirmed by Southern hybridization and the inducibility of YPT7 by growth in galactose confirmed by Western blotting. An *Nde*I restriction site, including the ATG codon of the YPT7 coding region, was created by site-directed mutagenesis (Sambrook *et al.*, 1989) using the following oligonucleotide (mismatch in bold): 5′-CTAGAAGACATA**TG**TTAAT-TTGAATTGATAC-3′. The oligonucleotide coding for the peptide representing the antigenic determinant for the monoclonal antibody to a Sendai virus epitope 5′-TATGTCTGATGGTTCTTTAGGCGACATCGAACC-TTATGACTCTTCTCA-3′ was inserted into the *Nde*I site. Insertion and correct orientation were confirmed by nucleotide sequencing. A 1.7 kbp fragment containing the extended *YPT7* gene (*YPT7*^{S}) was cloned into the shuttle vector pRS326 (pRS316 harboring a 2μ sequence; Sikorski and Hieter, 1989), yielding pTL5. Yeast strains were transformed using a standard lithium acetate method and were plated on SC-URA plates. Transformants were tested for the presence of the tagged proteins by Western blotting.

Protein purification

Recombinant Gdi1p was purified from *E.coli* BL21(pNB620) using the protocol by Garrett *et al.* (1994). Recombinant Ypt1p and Ypt7p were purified from *E.coli* NM522 (pLN-Ypt1 or pLNYpt7) (Wichmann *et al.*, 1992) using the protocol by Wagner *et al.* (1992).

Antibody production and purification

Polyclonal antibodies specific for Ypt1p or Ypt7p were raised in rabbits using the complete recombinant Ypt proteins purified from *E.coli* (above). For antibody purification, each of these proteins was coupled to CNBr-activated Sepharose 4B (Pharmacia). Antibodies were bound from complete sera, washed with phosphate buffer and eluted with 200 mM glycine–HCl (pH 2.5). The solutions were neutralized immediately with Tris–Cl. IgG fractions from Ypt7p antiserum were prepared by 4-fold dilution of the serum in 20 mM potassium phosphate, pH 7.5, and mixing with 1 ml bed volume of phosphate buffer-equilibrated protein A CL4B beads (Pharmacia Inc., Piscataway, NJ) per ml serum. After 1 h at room temperature with gentle shaking, unbound proteins were eluted with 20 bed volumes of phosphate buffer, and IgG was eluted with 200 mM glycine/HCl (pH 2.5), followed by immediate neutralization with 1 M Tris–Cl, pH 8.5. The samples were concentrated in a Centricon-10 (Amicon) with repeated dilution with fusion reaction buffer. This procedure was used whenever an antibody buffer exchange was necessary. Pho8p antiserum was produced in New Zealand White rabbits using a Pho8p/T4 lysozyme (N-terminus) fusion protein (pGP102) which contained the *PHO8* portion on the *Xho*I–*Sal*I fragment (kindly donated by T.H.Stevens, Eugene, OR). The fusion protein was produced in *E.coli*, eluted from an SDS–12.5% acrylamide gel and used to immunize New Zealand White rabbits. Antiserum was produced in New Zealand White rabbits against yeast proteinase A (Pep4p; Sigma) after deglycosylation by overnight incubation with endoglycosidase H and elution of the deglycosylated protein from an SDS–polyacrylamide gel. Pep4p antibodies were affinity purified from a polyvinylidine difluoride (PVDF) membrane carrying denatured Pep4p (Sigma) using the washing and elution conditions described above for IgG purification. Fab fragments were prepared from affinity-purified Ypt7p antibodies using the Immuno-Pure Fab preparation kit from Pierce. Fab fragments were dialyzed against reaction buffer and concentrated in a Centricon-10 (Amicon).

Immunoblots and immunofluorescence

Immunoblot analysis (Conradt *et al.*, 1992) employed 12.5% polyacryl-amide–SDS gels. With vacuole preparations, 2× sample buffer was

supplemented with 2× protease inhibitor cocktail (see below) and 2 mM phenylmethylsulfonyl fluoride (PMSF), before resuspending vacuolar pellets. Samples were heated immediately for 5 min at 95°C. For immunofluorescence, yeast cells were grown to an OD_{600} of 0.7–1.2. The preparation of cells was according to Pringle *et al.* (1990) and Rose *et al.* (1991), with a total fixation time of 16–20 h. After spheroplasting with Zymolase 100T (Seikagaku; 10 µg/ml) at 30°C for 60–90 min in a sorbitol/HEPES buffer containing 70 mM β-mercaptoethanol, cells were washed in sorbitol/HEPES and treated with SDS (1% w/v final concentration) for 10 min and finally resuspended in SHA (1 M sorbitol, 0.1 M HEPES–KOH, pH 7.5, 5 mM NaN_3). Anti-Ypt7p was pre-adsorbed to fixed *ypt7Δ* cells, and Sendai virus antibodies were pre-adsorbed to fixed *YPT7* cells. Antibody binding was visualized by Cy3 (trimethine cyanine dye)-conjugated anti-rabbit antiserum (Mujumdar *et al.*, 1989; Dianova, Germany) at a 1:250 dilution (in the experiments using Sendai virus antibodies, Cy3-conjugated anti-mouse IgG was used). After staining with DAPI, slides were sealed and stored at –20°C. Photographs were taken with AGFA 50RS Plus and Kodak Ektachrome 64EPR films using a Zeiss Axiophot™ microscope and a Zeiss Zoom Optovar.

In vitro vacuole inheritance reactions and *in vivo* microscopy

Cytosol was prepared as described previously (Conradt *et al.*, 1992; Haas *et al.*, 1994), but in addition to PMSF, a protease inhibitor cocktail was added from a 50-fold concentrated stock solution during the preparation (Jones, 1991; modified by Dr Z.Xu, Dartmouth Medical School, Hanover, NH): 0.1 mM Pefablock SC (Boehringer Mannheim Inc.), 0.5 µg/ml pepstatin A, 0.1 µg/ml leupeptin and 0.5 mM phenanthroline. Vacuoles were isolated on discontinuous Ficoll gradients according to Bankaitis *et al.* (1986) and Conradt *et al.* (1992). This isolation procedure enriches vacuoles 45- to 50-fold with respect to the total cell protein applied to the gradient. Only trace amounts of cytosolic and ER markers were recovered (Bankaitis *et al.*, 1986). *In vitro* and *in vivo* microscopic analyses of vacuoles were performed, as described previously (Conradt *et al.*, 1992; Haas *et al.*, 1994).

ProPho8p maturation assays were carried out as described in Haas *et al.* (1994) with the following modifications. Standard fusion reactions were performed in 30 µl with 2.5 µg protein of each of the two vacuole fusion partners, 1–2 mg/ml cytosol, an ATP-regenerating system and a salt buffer (Haas *et al.*, 1994). Alkaline phosphatase activities were measured at pH 8.5, and the enzymatic reaction was stopped by the addition of one volume of 1 M glycine/KOH (pH 11.5). When FM4-64 (Vida and Emr, 1995) was used as a fluorescent dye *in vitro*, a 2 mM stock solution in dimethylsulfoxide was diluted to 300 µM with reaction buffer, and 1 µl was used per 30 µl fusion reaction [FM4-64 is *N*-(3-trimethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl)pyridinium dibromide]. 1 U of alkaline phosphatase activity yields 1 µmol *p*-nitrophenol/min/µg BJ3505 (or BJ5459 or BJ5459+7) vacuole protein (Haas *et al.*, 1994). All alkaline phosphatase activity data were taken spectrophotometrically against a blank containing 30 µl reaction buffer, 470 µl assay reaction solution and 500 µl 1 M glycine/KOH (pH 11.5). For 'blank', a mixture with all the components of a reaction was kept on ice for the duration of the experiment. The alkaline phosphatase activity of such a sample was the same as the sum of the alkaline phosphatase activities of the components when kept on ice. The microscopy of vacuoles in intact cells using the fluorophore CDCFDA was described in Conradt *et al.* (1992). For the determination of the fraction of buds which contain vacuoles, cells were labeled with CDCFDA and photographed. The pictures were examined for buds with ⩾25% of the mother-cell size, and the portion of buds with detectable fluorescence was determined.

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