Autophagic Tubes: Vacuolar Invaginations Involved in Lateral Membrane Sorting and Inverse Vesicle Budding

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Abstract. Many intracellular compartments of eukaryotic cells do not adopt a spherical shape, which would be expected in the absence of mechanisms organizing their structure. However, little is known about the principles determining the shape of organelles. We have observed very defined structural changes of vacuoles, the lysosome equivalents of yeast. The vacuolar membrane can form a large tubular invagination from which vesicles bud off into the lumen of the organelle. Formation of the tube is regulated via the Apg/Aut pathway. Its lumen is continuous with the cytosol, making this inverse budding reaction equivalent to microautophagocytosis. The tube is highly dynamic, often branched, and defined by a sharp kink of the vacuolar membrane at the site of invagination. The tube is formed by vacuoles in an autonomous fashion. It persists after vacuole isolation and, therefore, is independent of surrounding cytoskeleton. There is a striking lateral heterogeneity along the tube, with a high density of transmembrane particles at the base and a smooth zone devoid of transmembrane particles at the tip where budding occurs. We postulate a lateral sorting mechanism along the tube that mediates a depletion of large transmembrane proteins at the tip and results in the inverse budding of lipid-rich vesicles into the lumen of the organelle.

Key words: microautophagocytosis • lysosome • yeast • proteolysis • budding

Introduction

Cellular organelles are usually not spheres. The ER forms large branched networks and sheets, the Golgi complex is a stack of flat, fenestrated cisternae, and mitochondria tend to be elongated, branched tubules. Their inner membrane is folded into cristae, large, sheet-like invaginations. Also, other organelles, such as endosomes and lysosomes, often deviate from a spherical shape. Many factors required for the fragmentation or fusion of organelles have been identified, such as for the Golgi complex or for vacuoles (Warren and Wickner, 1996; Warren and Malhotra, 1998). They regulate the integrity of the organelle. However, the principles organizing the shape of an intact organelle are largely unknown. Several factors which might determine organellar structure are conceivable, such as interactions with the cytoskeleton, an organellar matrix containing structural proteins, filamentous materials attached to membranes, the lateral aggregation of membrane proteins or lipids, or membrane tension (Heuser, 1989; Dai and Sheetz, 1995). Although the distinct shape of organelles is one of the most conspicuous features of the organization of eukaryotic cells, its relationship to the factors mentioned above is poorly understood on a molecular level. More information is available only for two systems. First, the budding of small transport vesicles is determined and probably driven by the apposition of coats onto the membrane (Schekman and Orci, 1996). Coat components, adaptor proteins, and cargo molecules have been identified and their cooperation in vesicle formation is being elucidated. Second, the tubular-reticular structure of the ER is at least in part determined by interactions with microtubules (Kachar et al., 1987; Dabora and Sheetz, 1988; Lee and Chen, 1988). Mutant screens have also revealed some factors influencing mitochondrial morphology (Hermann and Shaw, 1998), but their function in determining organelle shape is not well understood.

Lysosomes are dynamic organelles capable of protein uptake from the cytosol along various routes. They can translocate proteins directly through their membrane (Cuervo and Dice, 1998; Horst et al., 1999), in a similar fashion as mitochondria or the ER. In addition, they take up proteins, or even entire organelles, by means of various vesicle-mediated processes: biosynthetic transport from the Golgi complex via the carboxypeptidase Y (CPY) and al-

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kaline phosphatase (Alp) pathways (Wendland et al., 1998), the vacuolar import and degradation (Vid),[1] cytoplasm to vacuole targeting (Cvt), and autophagocytosis pathways. The Vid pathway is involved in catabolite inactivation of fructose-1,6-bisphosphatase in yeast (Chiang and Schekman, 1991; Huang and Chiang, 1997). The protein is imported into dedicated Vid vesicles which then transfer it to vacuoles. The Cvt pathway is responsible for the transport of aminopeptidase I into vacuoles (Scott and Klionsky, 1998). It operates via the formation of small double layered vesicles in the cytosol (Scott et al., 1997). These vesicles sequester aminopeptidase I and fuse with the vacuole.

Autophagocytosis is a nonselective mechanism of transferring cytosolic proteins or organelles into lysosomes (Seglen and Bohley, 1992). It operates at a constitutive level, but can be induced under conditions of stress, such as nutrient limitation, or in redifferentiation and cell death. Two different forms can be observed: macro- and microautophagocytosis. Macroautophagocytosis occurs through the formation of autophagosomes, which are specialized vesicles with double or multiple boundary membranes (Seglen and Bohley, 1992; Scott and Klionsky, 1998). During their formation, these vesicles engulf portions of cytosol or organelles such as peroxisomes. Their outer membrane fuses with lysosomes.Then, the inner membranes with their cytosolic contents (termed autophagic bodies) are degraded. The gene products required for the formation of autophagosomes largely overlap with those involved in forming Cvt vesicles (Harding et al., 1996; Scott et al., 1996). However, different sets of components are involved in fusing autophagosomes and Cvt vesicles with vacuoles (Darsow et al., 1997; Abeliovich et al., 1999). The origin of autophagosomes is unclear. The ER, the Golgi complex, and specialized structures called phagophores have been proposed as precursors (Pfeifer, 1972; Seglen, 1987; Dunn, 1990; Yamamoto et al., 1990a,b). Microautophagocytosis operates via direct invagination of lysosomes, leading to the formation of single membrane- bounded vesicles in the lysosomal lumen that are rapidly degraded (Seglen and Bohley, 1992). Also, this pathway can transfer organelles into lysosomes, as shown extensively for peroxisomes (Veenhuis et al., 1983; Tuttle et al., 1993; Tuttle and Dunn, 1995; Sakai et al., 1998). Though genetic screens have revealed many genes involved in macroautophagocytosis and in peroxisome degradation (Tsukada and Ohsumi, 1993; Thumm et al., 1994; Titorenko et al., 1995; Sakai et al., 1998; Yuan et al., 1999), much less is known about microautophagocytosis. Mutants selectively affecting this process are not available and in *Saccharomyces* a microautophagic pathway has not even been defined.

Here, we have investigated the structural dynamics of the yeast vacuole. We describe invagination processes of the vacuolar membrane and identify a novel differentiation of the vacuolar membrane, autophagic tubes. We propose that autophagic tubes are dedicated to the lateral sorting of proteins and lipids in the plane of the membrane, and thereby facilitate vesicle budding into the lumen of the vacuole.

[1]*Abbreviations used in this paper:* Apg and Aut, autophagocytosis; Cvt, cytoplasm to vacuole targeting; EF, extraplasmic fracture face; Vid, vacuolar import and degradation.

Materials and Methods

Strains

The following *Saccharomyces cerevisiae* strains were used: DBY5734 (*MATa ade2 lys2 his3 trp1 leu2 ura3 cmd1-Δ1::TRP ade3::HIS3::CMD1* (David Botstein, Stanford University, Stanford, CA); BJ3505 (*MATa HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can1 pep4::HIS3 prb1-Δ1.6R*) (Elizabeth Jones, Carnegie Mellon University, Pittsburgh, PA); DBY5734-16 (*MATa pep4Δ::LEU2; ade2; lys2; his3; trp1; leu2; ura3;cmd1-Δ1::TRP; ade3::HIS3::CMD1*) (Sattler and Mayer, 2000, this issue); and CRY1 (*MATa ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) (Trisha Davis, University of Washington, Seattle, WA). CRY1 was used for experiments involving in vivo fluorescence labeling with FM4-64 because it has larger vacuoles and virtually all cells contain only one vacuole. YTS1, YTS3, and YTS5 have been described in Sattler and Mayer (2000, this issue). YTS7 (*Δapg7*) was created in DBY5734 in the same manner using the primers 5′-TTC ATT ATA TTT CAA CAA ATA TAA GAT AAT CAA GAA TAA ACA GCT GAA GCT TCG TAC GC-3′ and 5′-TGG CAC CAC AAT ATG TAC CAA TGC TAT TAT ATG CAA AAT AGC ATA GGC CAC TAG TGG ATC TG-3.

Growth of Cells

Yeast cells were precultured in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) for 6–8 h at 30°C and then diluted for logarithmic overnight growth (14–16 h, 30°C, 225 rpm) in 100-ml Erlenmeyer flasks with 30 ml of YPD medium. For starving cells, overnight cultures were harvested at an optical density at 600 nm (OD_{600}) of 2, centrifuged (4 min, 4°C, 3,800 *g*, JLA 10.500 rotor), washed with sterile water, resuspended in 30 ml of SD(−N) (0.67% Difco yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose), and incubated (up to 3 h, 30°C, 225 rpm).

Staining of Vacuoles In Vivo

All media were supplemented with 60 mg/liter adenine sulfate. Cells were grown logarithmically in 5 ml of YPD overnight (20-ml tubes, 30°C, 225 rpm). They were reisolated (2 min, 1,300 *g*, 4°C), washed with deionized water, reisolated, and resuspended to an OD_{600} of 0.5–1 in YPD or SD(−N) medium. If desired, the cells were cultivated in these media for up to 3 h. Then, 1 ml of the suspension was supplemented with 20 μM FM4-64 (Molecular Probes) and incubated for 1 h, as described above. The cells were harvested (2 min, 1,300 *g*, 4°C), washed with 1 ml medium, pelleted as described above, resuspended in 1 ml medium without stain, and incubated for at least 30 min in the shaker. When samples were drawn, the cells were immediately chilled on ice, reisolated (2 min, 1,300 *g*, 4°C), and resuspended in medium at an OD_{600} of 3–5. 7 μl of the suspension was mixed with 7 μl of 0.4% Seaplaque agarose in 10 mM Pipes/KOH, pH 6.8, 200 mM sorbitol (kept liquid at 35°C). 12 μl of this mixture was transferred to a slide, covered immediately with a coverslip, and chilled at 4°C for 5 min to immobilize the cells. Then, the cells were investigated with a confocal microscope (Leica TCS) or a conventional fluorescence microscope. The intensity of illumination was minimized to avoid structural damage to the vacuoles that can occur at higher light intensities or after prolonged illumination of the same field. For confocal analysis, two to four images of a field were taken and averaged.

Thin Section EM

Yeast cells were cryofixed using a propane-jet freezing device (JFD 030, Bal-Tec; Balzers AG) and freeze-substituted either in 0.5% uranyl acetate in ethanol or in 0.5% osmium tetroxide and 0.5% glutaraldehyde in acetone at −90°C for 35 h, at −60°C for 4 h, and −50°C for 2 h in a freeze-substitution unit (FSU 010, Bal-Tec; Balzers AG). After washing with ethanol at –35°C or acetone at −20°C, the samples were either infiltrated with Lowicryl HM20 and UV-polymerized at −35°C for 48 h or infiltrated with Epon and polymerized at 60°C for 48 h. Ultrathin sections stained with uranyl acetate and lead citrate were viewed in a Philips CM10 electron microscope.

Freeze–Fracture Analysis

Living yeast cells or isolated vacuoles were sandwiched between thin copper sheets (Bal-Tec; Balzers AG), mounted on tweezers, and rapidly injected into melting propane (−185°C), as described previously (Gulik-

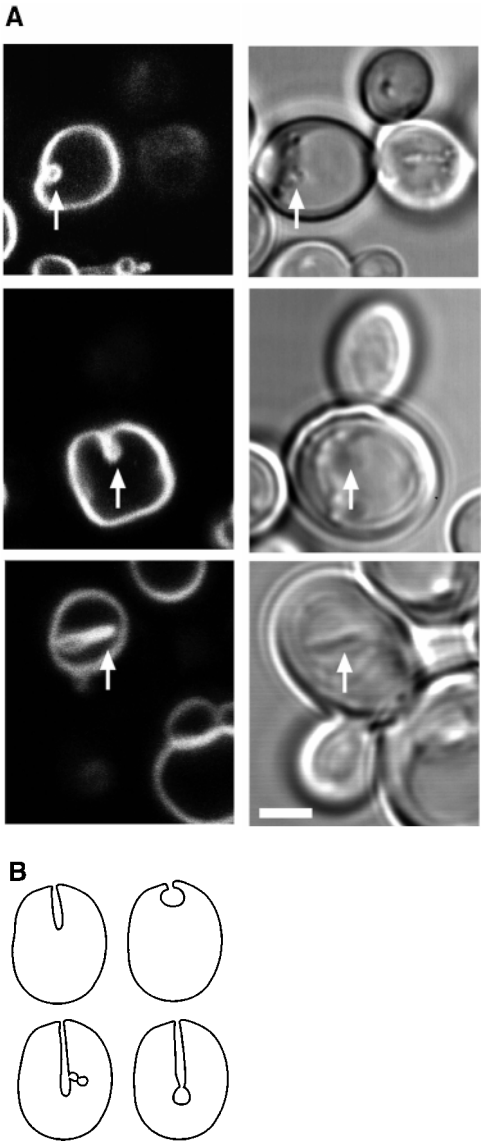

Figure 1. Tubular invaginations of vacuoles in living yeast cells. (A) CRY1 cells were grown logarithmically in YPD medium, stained with the vital dye FM4-64, and starved in SD(−N) for 1.5 h. The cells were viewed under a confocal microscope. The left images show the fluorescently stained vacuole; the right images show the same cell in transmitted light. Arrows indicate the position of the invagination. (B) Schematic summary of different shapes of tubes that can be observed. Bar, 3 μm.

Krzywicki and Costello, 1978). The sandwiches were inserted under liquid nitrogen into a freeze–fracture unit Type BAF 300 (Balzers AG), fractured at −100°C, and replicated by 45° platinum-carbon shadowing. Replicas were transferred into 2.5% SDS with 30 mM sucrose in 10 mM Tris-HCl buffer, pH 8.3. After vigorous shaking for 30 min, replicas were washed several times with distilled water and mounted onto copper grids for routine EM analysis in an EM 10 (ZEISS).

Results

We investigated structural changes of the yeast vacuole by fluorescence light microscopy. After staining the vacuoles with the vital dye FM4-64 (Vida and Emr, 1995), we detected large invaginations of the vacuolar membrane. Typically, there was only one invagination per vacuole, though in some cases up to three. The invaginations could also be seen under Nomarski optics, but they were much more difficult to detect than by fluorescence. The invaginations were tubular, variable in length, sometimes branched, and often expanded into a bubble-like structure at the tip (Fig. 1). They were dynamic, dangling back and forth in the lumen of the vacuole. The base of the invagination was more stationary. It was able to move along the boundary membrane of the vacuole, but the movement was much slower than that of the tip in the lumen of the vacuole. Extended tubes were remarkably slim. The membrane lining them usually was not able to be resolved from the lumen of the tube by fluorescence microscopy, due to the limited resolution of this method.

The invaginations were not only laterally mobile, but also able to grow and shrink rapidly (Fig. 2). We observed the formation and disappearance of tubes within as little as 10–20 s. The tubes were able to pinch off vesicles from their tip, which were then released into the lumen of the vacuole. Released vesicles moved around freely in the vacuolar lumen and at a much higher pace than the tubes, indicating that they had become fully separated from the tubes. Formation and scission of a vesicle were completed in <40 s (Fig. 2). Formed vesicles were initially labeled with FM4-64, but after pinching off into the lumen, they lost the dye rapidly. Stable staining with FM4-64 may depend on a pH or ion gradient over the membrane. Due to the lack of an energy source, it is not likely that such gradients can be maintained by vesicles inside the vacuoles, resulting in loss of the dye.

The invaginations were also investigated by EM (Fig. 3). Cells were starved for nitrogen to induce autophagocytosis. They were quick-frozen in liquid propane, freeze-substituted, embedded, and analyzed by thin section EM. Autophagic bodies, which are the vesicular products of autophagocytosis, accumulated inside the vacuoles of *Pep4* mutants (Fig. 3, A and B; compare with results from Takeshige et al., 1992). Vacuoles of such cells have reduced hydrolytic activity, which delays the degradation of autophagic bodies. The invaginations had an average diameter of 200–300 nm and frequently showed a constriction at the neck of the tube (Fig. 3, A–D, arrowheads), that is, at the interface between the invaginating membrane and the vacuolar boundary membrane. The lumen of the invaginations, as well as that of nascent vesicles at their tips, was continuous with the cytosol. Its ultrastructure was identical to that of bulk cytosol and to the lumen of autophagic bodies (arrows). Thus, budding of vesicles from the tip of the invagination must coincide with the uptake of cytosol into the lumen of the vacuole, establishing a microautophagic pathway for *S. cerevisiae*. In fluorescence analyses, we found that the frequency of vacuolar invaginations depends on the nutrition state. In rich media, only 17% of the cells showed invaginations. In this and in the other assays described below, we observed quantitative variations of the percentage of cells that showed invaginations between different wild-type strains, but no qualitative differences (not shown). Under starvation conditions, which induce autophagocytosis (Takeshige et al., 1992), the frequency of invaginations increased to 63% (Fig. 4 A). Thus, starvation induced au-

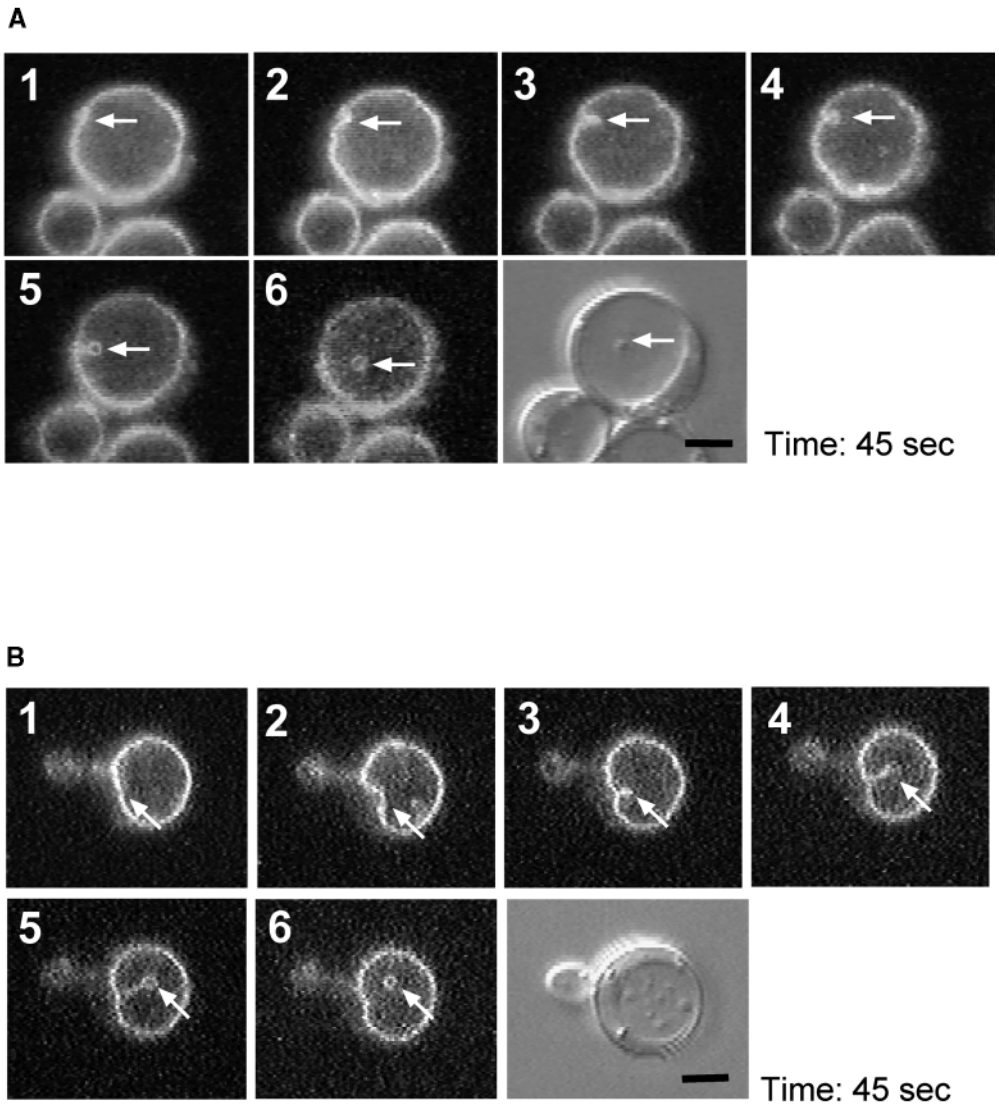

Figure 2. Budding of vesicles into the lumen of the vacuole. (A and B) CRY1 cells were grown logarithmically in YPD medium. Their vacuoles were stained with FM4-64 and the cells were starved in SD(−N) medium with 1 mM PMSF at an OD_{600} of 1 at 25°C. After 1 h of starvation they were viewed under a standard fluorescence microscope equipped with a video camera. The sequences of pictures shown were taken over a period of 45 s. The last picture shows the investigated cell under Nomarski optics at the end of the experiment. Arrows point to the nascent tube or vesicle. Bars, 5 μm.

tophagocytosis and the formation of the tubular membrane invagination of vacuoles.

Many mutants are known to affect macroautophagocytosis in yeast. We tested the effect of such mutations on the membrane invagination of vacuoles. Mutations in *Vam3* and *Vps33*, two components involved in multiple pathways of vesicular trafficking to the vacuole (Darsow et al., 1997) and in maintenance of vacuolar integrity (Wada et al., 1992), allow the formation of macroautophagosomes, but block their fusion with vacuoles (Darsow et al., 1997). We tried to analyze autophagic tubes in temperature-sensitive mutants for *Vam3* and *Vps33* after shifting to a restrictive temperature. In starvation media at 37°C, the majority of the cells showed rapid (already 15 min after the temperature shift) fragmentation of vacuoles into smaller vesicles, making it impossible to quantitate the frequency of vacuolar invaginations (not shown). Similar behavior was observed for temperature-sensitive *Sec18* and *Sec17* mutants. The *Aut* and *Apg* mutants were isolated based on their inability to transfer cytosolic proteins into the vacuole and accumulate autophagic bodies inside this organelle (Tsukada and Ohsumi, 1993; Thumm et al., 1994). A priori, the screens did not distinguish whether these autophagic bodies emanated from macro- or microautophagy. Several mutants are defective in autophagosome formation, demonstrating a function in macroautophagy. Their influence on microautophagy of soluble proteins has not been analyzed because this pathway has not been characterized in *Saccharomyces* to date. We found that in contrast to the situation in wild-type cells, invaginations were rare in mutants with deletions of genes involved in autophagocytosis, such as *Apg5, Aut1, Aut7,* and *Apg7* (Fig. 4 B). Furthermore, cytosols from these mutants showed a significantly lower activity in an in vitro assay reconstituting microautophagic vacuole invagination and uptake (Sattler and Mayer, 2000, this issue). We conclude that macroautophagocytosis and the microautophagic invaginations of yeast vacuoles are both controlled via the Aut/Apg pathway. In light of its unique structure and its functional connection to autophagocytosis, we therefore term this specialized vacuolar invagination an "autophagic tube."

What determines the formation and structure of autophagic tubes? To address this, we also detected autophagic tubes in vacuoles that had been extracted from the cells, floated in density gradients, fast frozen, and freeze–fractured (Fig. 5). The tubes found were indistinguishable

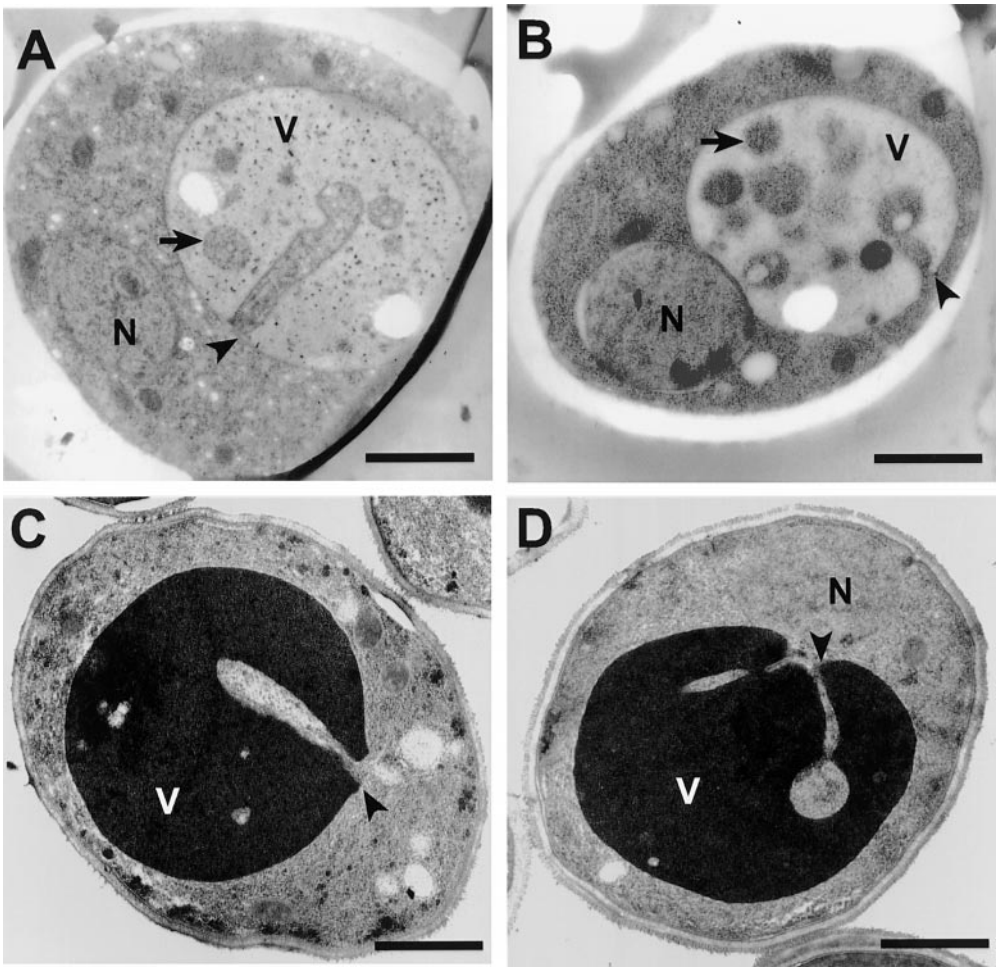

Figure 3. Tubular invaginations are filled with cytosol. Yeast cells were grown logarithmically in YPD medium, transferred to SD(−N) medium, and starved. The cells were quick-frozen in liquid propane, freeze-substituted, and embedded. Thin sections were viewed in the electron microscope. (A and B) BJ3505 (a *Pep4* mutant) starved for 1–2 h. The vacuole contains autophagic bodies (arrows). Note the high membrane curvature at the base of the tube (arrowheads). V, vacuole; N, nucleus. (C and D) DBY5734 starved for 4 h. Autophagic bodies did not accumulate because this strain is proteolytically competent. The intense dark staining of the vacuoles in C and D is a result of freeze-substitution in osmium tetroxide/acetone and embedding in Epon, whereas the cells in A and B were freeze-substituted in 0.5% uranyl acetate in ethanol and embedded in Lowicryl HM20. Bars, 1 μm.

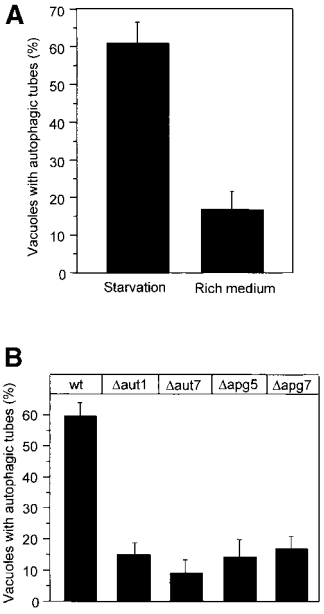

Figure 4. Frequency of autophagic tubes is influenced by starvation and the autophagy pathway. (A) DBY5734 cells were grown logarithmically overnight and then transferred to rich medium (YPD) or starved for nitrogen in SD(−N) medium. After 4 h of incubation in these media, the vital dye FM4-64 (30 μM) was added to stain the vacuoles (1 h). Then, the cells were chased in the absence of the dye (40 min). The proportion of vacuoles with autophagic tubes was determined by fluorescence microscopy. A total of 200 cells were counted from at least 10 independent fields. (B) Same as in A, but the frequency of autophagic tubes was determined in starved wild-type (wt) (DBY5734) or its derivatives with deletions in aut1 (YTS1), aut7 (YTS3), apg5 (YTS5), or apg7 (YTS7). Values in both figures are the average of five to nine independent experiments. Error bars indicate SD.

from those seen in vivo. They had the same diameter, were sometimes branched, and carried expanded termini. Also, the sharp bending of the membrane at the neck of the tube and its constriction were maintained (Fig. 5). Since authentic autophagic tubes were able to be detected after extracting the organelle from the cell, their maintenance cannot depend on an intact surrounding cytoskeletal framework. This is further supported by the observation that purified vacuoles can even form new tubes in a cell-free system (Sattler and Mayer, 2000, this issue). Therefore, we conclude that autophagic tubes are formed and maintained by the vacuolar membrane autonomously, independent of an intact cellular environment.

We extended the freeze–fracture analysis of vacuoles to compare the membrane structure of autophagic tubes with that of the vacuolar boundary membrane. In brief, this reveals either the extraplasmic fracture face (EF) or the protoplasmic fracture face (PF) of the split membrane bilayers, that is, the leaflet adhering to the vacuolar lumen or to the cytosol, respectively. Intramembranous particles, which may show up as corresponding holes on one fracture face, are known to be integral membrane proteins (Plattner and Zingsheim, 1983). There was a striking gradient of intramembranous particles along the autophagic tubes (Fig. 6). At the base of the tube, their density was high, resembling that of the vacuolar boundary membrane (asterisk). We found 510 particles/μm^2 on the EF face of

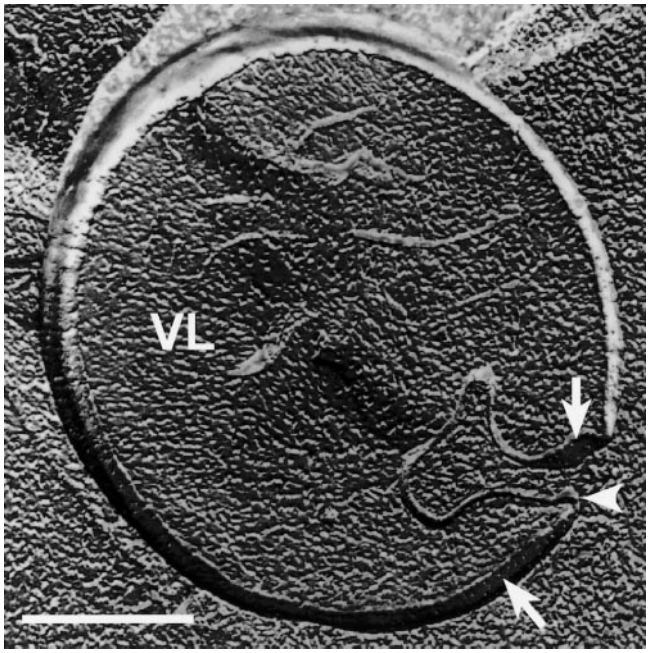

Figure 5. Tubular invaginations are maintained on isolated vacuoles. Vacuoles were isolated from logarithmically growing cells by flotation through a Ficoll gradient (Sattler and Mayer, 2000, this issue). The purified organelles were quick frozen in liquid propane, freeze–fractured, and replicated with platinum carbon for electron microscope analysis. Transmembrane particles can be seen on the limiting membrane of the vacuole and on the invaginated membrane of the tube (arrows). Note the high curvature of the membrane at the base of the tube (arrowhead). VL, vacuolar lumen. Bar, 1 μm.

the vacuolar membrane (determined from ten independent vacuoles; SD = 190 particles/μm^2). The particle density dropped significantly towards the vacuolar lumen (arrows), often resulting in a smooth, particle-free appearance at the tip of the invaginations (arrowheads). Here, we found only 17 particles/μm^2 (determined as described above; SD = 15 particles/μm^2). Areas with few or no particles were often expanded into bubble-like structures, suggesting that they were sites of vesicle formation. Autophagic bodies, the vesicular products of autophagocytosis, accumulate in cells deficient for vacuolar hydrolases (Takeshige et al., 1992). In our freeze–fracture analysis, the membrane of autophagic bodies also appeared particle free (Fig. 7; compare with results from Baba et al., 1995). The smooth membrane structure of autophagic bodies matched the situation seen on the terminal bubbles of autophagic tubes. This suggests that autophagic tubes are sites of autophagic body formation and that autophagic bodies arise from micro- as well as from macroautophagocytosis (Takeshige et al., 1992).

Further examination of the vacuolar surface in freeze–fracture replicas revealed small, particle-free areas on the vacuolar boundary membrane that often bulged inward (Fig. 8, arrows). Fig. 8, A and B, shows a concave PF face and a convex EF face, respectively. Therefore, the particle-free area in Fig. 8 B is an invagination. This indicates that particle-free zones may be early stages of autophagic tube formation. It suggests that the exclusion of large transmembrane proteins from these areas, which is probably accompanied by an enrichment of lipids in these zones, may be causal for invagination and vesicle formation.

In conclusion, we propose that lateral segregation of lipids, with local exclusion of large transmembrane proteins in the plane of the vacuolar membrane, may lead to the invagination of this membrane. The resulting autophagic tubes are organized structures serving as templates for a budding reaction into the lumen of the organelle. Budding is restricted to the laterally segregated zones depleted of transmembrane particles. It results in the formation of autophagic bodies, which are also poor in transmembrane proteins.

Discussion

We have observed a microautophagic pathway in *S. cerevisiae* that operates via autophagic tubes, which are long, highly differentiated invaginations with a narrow and constant diameter and a constriction at the neck. This distinguishes autophagic tubes from the massive vacuolar invaginations that occur during peroxisome uptake in *Pichia pastoris* or *Hansenula polymorpha* cells (Veenhuis et al., 1983; Tuttle et al., 1993; Tuttle and Dunn, 1995; Sakai et al., 1998). Since both the vacuole and peroxisomes occupy a large portion of the cell volume, it is hard to judge if first the vacuole invaginates and then the peroxisomes follow this active structural change. Alternatively, the vacuole may wrap around the peroxisomes by progressive adhesion to their surface. A comparison of both modes of microautophagic uptake will only be possible when the proteins responsible for both peroxisome uptake and the formation of autophagic tubes have been identified.

What is the function of autophagic tubes and of the microautophagic pathway, in general? Microautophagocytosis can transfer cytosol and organelles into the vacuole. However, both functions are also performed by the macroautophagic pathway. For peroxisome uptake, the balance between micro- and macroautophagocytosis is influenced by the growth conditions (Tuttle et al., 1993; Tuttle and Dunn, 1995). However, the situation is less clear for autophagocytosis of soluble cytosolic components. Could the main purpose of microautophagocytosis be fundamentally distinct from that of the macroautophagic pathway, that is, could the transfer of soluble cytosolic components by microautophagocytosis not be its main purpose? There are several possibilities. The degradation of membrane proteins might be one function of microautophagocytosis. Many transporters or receptors from the plasma membrane can become endocytosed and then degraded in vacuoles or lysosomes (Wendland et al., 1998). Although their degradation often depends on vacuolar proteases, in many cases it is unclear how degradation actually occurs. Membrane proteins could be directly transported to the vacuolar membrane by vesicular trafficking. There, they could be invaginated into the vacuolar lumen by microautophagocytosis and then degraded with the resulting autophagic bodies. These membrane proteins would have to be able to diffuse into the smooth areas of autophagic tubes, sorting them away from genuine vacuolar membrane proteins, which are excluded from these zones. Such a pathway would be functionally equivalent to the multivesicular body pathway, the invagination of endosomes, which is known to transfer membrane proteins into the lumen of endosomes. Multive-

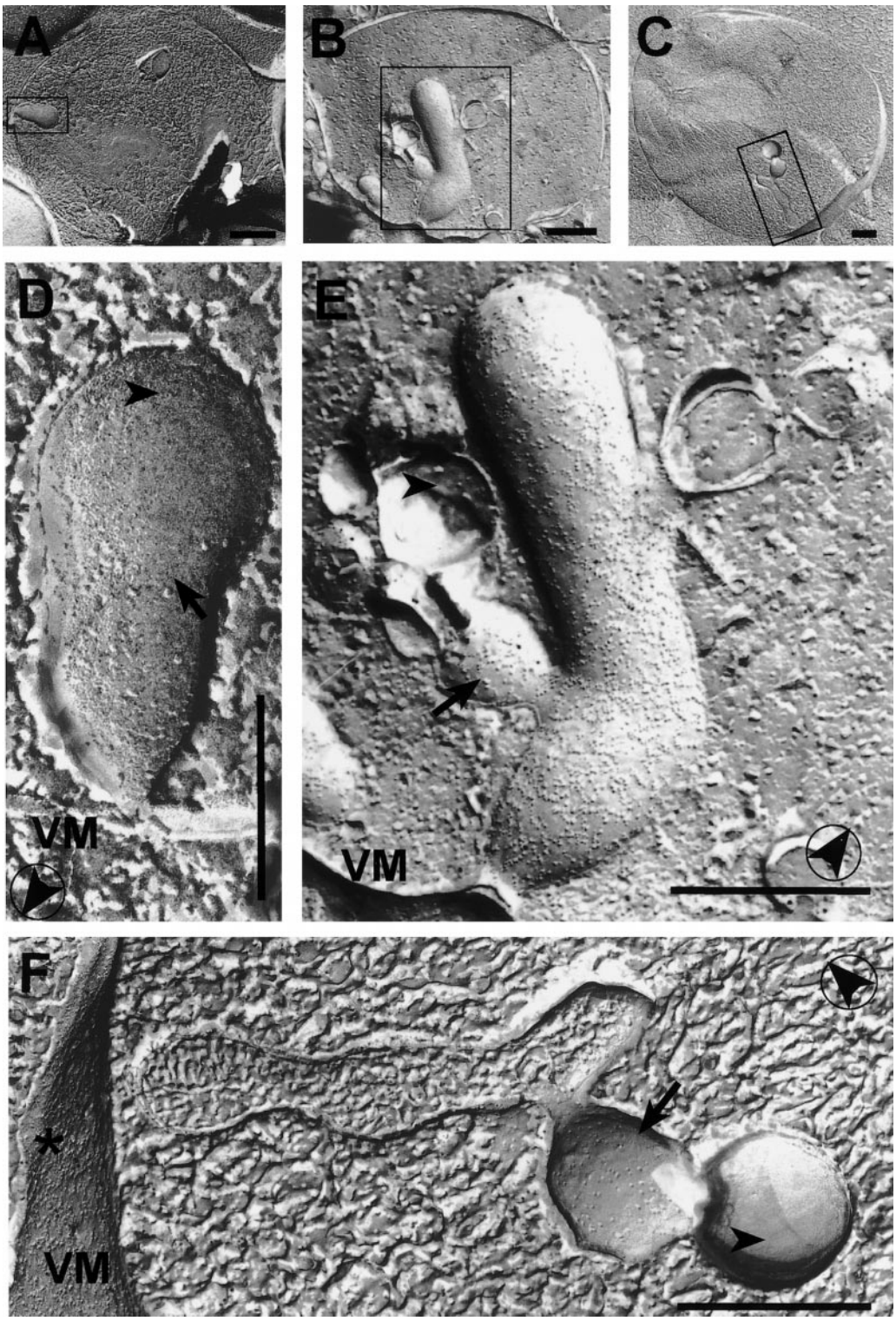

Figure 6. Distribution of transmembrane particles along autophagic tubes. (A, B, and C) Three vacuoles from DBY5734 cells processed for freeze–fracture analysis. The boxed areas are shown at higher magnifications in D, E, and F. Note the higher density of transmembrane particles at the more basal areas of the tube (arrows) and the absence of particles at the tips (arrowheads). VM, vacuolar membrane. Encircled arrowheads indicate the shadowing directions. Bars: (A–C, E, and F) 0.5 μm; (D) 0.25 μm.

sicular endosomes then fuse with vacuoles, delivering their internal vesicles into the vacuolar lumen for degradation (Odorizzi et al., 1998; Wurmser and Emr, 1998).

A major function of autophagic tubes may be the maintenance of membrane homeostasis of vacuoles and the regulation of their size. Although vacuoles are the destination of numerous routes of vesicular trafficking (Scott and Klionsky, 1998; Wendland et al., 1998), only one example for membrane retrieval from the vacuole has been found (Bryant et al., 1998). Therefore, vacuoles and lysosomes

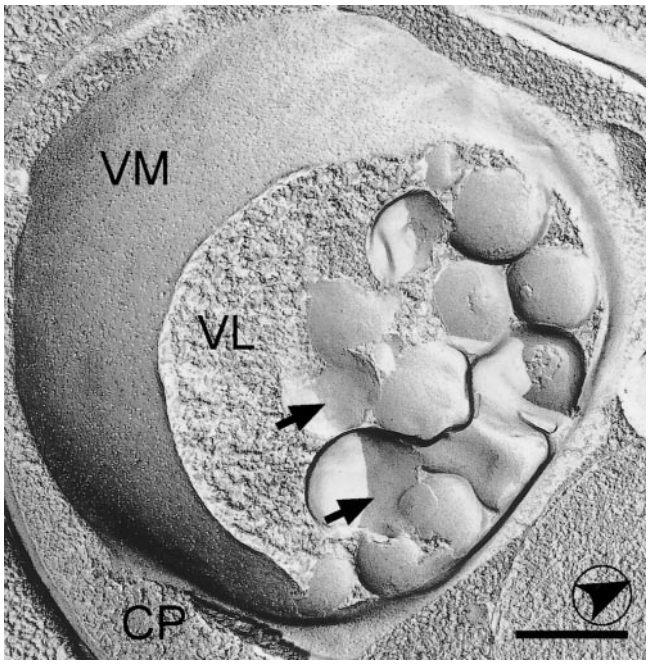

Figure 7. Autophagic bodies are also devoid of transmembrane particles. Yeast cells with a deletion of the PEP4 gene (DBY5734-16) were starved on SD(−N) medium for 3 h and then processed for freeze–fracture analysis as described in the legend to Fig. 5. The picture shows a partial view of the vacuolar membrane (VM), which is particle rich, and of the vacuolar lumen (VL) containing autophagic bodies (arrows). Note the absence of transmembrane particles in the autophagic body membrane. CP, cytoplasm. The encircled arrowhead indicates the shadowing direction. Bar, 1 μm.

may face considerable membrane influx in the absence of compensatory membrane efflux. This effect would be strongly enhanced under conditions of starvation. Macroautophagocytosis leads to a large membrane influx resulting from the fusion of vacuoles with the outer membrane of autophagosomes. The autophagosomal outer membrane also has an unusual ultrastructure and is poor in intramembranous particles (Takeshige et al., 1992; Baba et al., 1994, 1995). Accordingly, starvation leads to an increase in vacuolar size and a dilution of intramembranous particles of the vacuolar membrane (Baba et al., 1995). Therefore, there must be a compensatory mechanism that prevents the vacuolar membrane from expanding rapidly. We propose that microautophagy performs this function and guarantees membrane homeostasis. It removes membrane and, as our results show, probably lipid-rich pieces from the vacuolar surface and transfers it into the lumen for degradation. The membrane, which buds into the lumen of the vacuole, is ultrastructurally very similar to that added to the vacuoles by fusion with the outer autophagosomal membrane. The observed lateral exclusion of large transmembrane proteins from the zones of inverse budding prevents genuine vacuolar membrane components from being turned over. Therefore, we propose that microautophagic budding events may help to regulate the size and the protein to lipid ratio of the vacuole, particularly if macroautophagocytosis is induced. To achieve membrane homeostasis, the rate of microautophagic

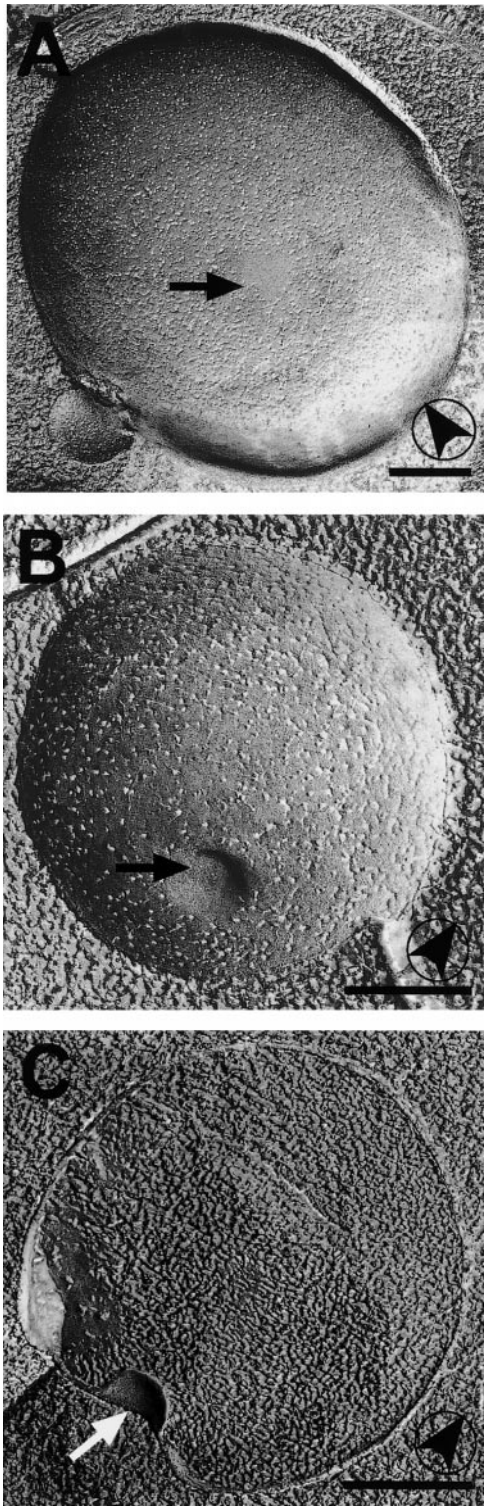

Figure 8. Smooth areas on the vacuolar membrane as precursors of tubular invaginations. Vacuoles from strain DBY5734 are shown after freeze–fracture analysis as described in the legend to Fig. 5. The arrows point to well-defined, particle-free areas. (A) Particle-free area on the vacuolar surface. (B) View of a particle-free zone on the surface in the process of invagination. (C) Same as in B, but the vacuole was fractured across its lumen. Only the invaginating smooth area was fractured along its surface. Encircled arrowheads indicate the shadowing directions. Bars, 0.5 μm.

membrane invagination must equal the rate of macroautophagic membrane influx. Then, about half of the autophagic bodies should be of microautophagic origin.

This hypothesis can also accommodate previous observations by other groups. Our microscopic analysis indicated that the size and membrane structure of autophagic bodies formed by microautophagocyosis resemble those of macroautophagosomes (Baba et al., 1994, 1995). This means that, once formed, an autophagic body cannot be morphologically distinguished if it arose from a micro or macroautophagic reaction. Are both pathways also functionally interconnected? If macro- and microautophagocytosis were independent pathways, microautophagic bodies should still accumulate when the macroautophagic pathway is blocked. However, a temperature-sensitive mutant of the target-soluble NSF attachment protein receptor (t-SNARE) Vam3p, which forms macroautophagosomes, but cannot fuse them with the vacuole, was reported not to show any autophagic bodies in the vacuolar lumen at restrictive temperature (Darsow et al., 1997). Likewise, the Aut/Apg mutants, which do not form autophagosomes efficiently (Scott and Klionsky, 1998), showed a severe reduction of autophagic tubes (Fig. 4) and do not accumulate autophagic bodies. Therefore, microautophagocytosis seems to depend on macroautophagocytosis. Our favored interpretation is that the fusion of the outer membrane of macroautophagosomes with vacuoles is needed to supply the vacuole with excess membrane as a substrate for microautophagic vacuole invagination. This means that repeated microautophagic membrane invagination is inseparably connected to macroautophagic membrane influx in vivo.

A shortage of source membrane for invagination might even explain the defect of autophagy mutants in peroxisome degradation (Hutchins et al., 1999), specifically of the *Gsa7* mutant on microautophagy of peroxisomes (Yuan et al., 1999). *P. pastoris Gsa7* is homologous to *Apg7* of *S. cerevisiae* (Kim et al., 1999; Yuan et al., 1999), which is part of the protein conjugation system that is also involved in macroautophagocytosis (Mizushima et al., 1999; Tanida et al., 1999) and autophagic tube formation. Microautophagy of peroxisomes consumes a large portion of the vacuolar membrane and must require some compensation by influx of new membrane. An *Apg7*-dependent induction of the macroautophagic pathway may be necessary to compensate for this loss of vacuolar membrane. In the absence of *Gsa7/Apg7*-dependent compensation, the shortage of vacuolar membrane may lead to the accumulation of intermediates. Peroxisomes still may be partially enwrapped by vacuoles, but the invagination may not be closed to complete uptake if the available vacuolar membrane surface is not large enough. This is consistent with the phenotype of *Gsa7* mutants described by Yuan et al. (1999). We want to stress that the interdependence of the different autophagic routes via membrane supply, which we proposed above and considered as one important connection, does, by no means, exclude additional direct roles of the Apg/Aut components in all of these pathways, as discussed in other studies (Mizushima et al., 1999; Yuan et al., 1999; Sattler and Mayer, 2000, this issue).

The peculiar appearance of autophagic tubes raises the question of how such a structure could be organized. One possibility is that the invaginations are caused by cytoskeletal filaments pushing into the vacuole. This seems unlikely for several reasons. (a) Autophagic tubes are also found in vacuoles that were isolated via flotation gradients and are thus devoid of external cytoskeletal network. (b) These isolated vacuoles are even capable of forming new autophagic tubes in vitro (Sattler and Mayer, 2000, this issue). (c) Autophagic tubes often display a striking constriction at the neck of the tube, that is, at the interface between the tube and the vacuolar boundary membrane. (d) Autophagic tubes often differentiate into a series of bubble-like structures and form branches. (e) Finally, we found that transmembrane particles are sorted along the tube, resulting in virtually particle-free areas at the tips. It is not obvious how a penetrating filament may cause these diverse and very specific effects.

We were not able to detect any indications of organization on the membrane, other than the lateral segregation into protein-rich and protein-poor areas. There were no signs of ordered arrangements of transmembrane particles, which hints at the presence of organizing larger molecules on the surface of the membrane, or at the formation of paracrystalline areas by lateral aggregation of proteins. Therefore, we currently favor the view that sorting along the tube may be a lipid-mediated process. There is accumulating evidence that domains can form within membranes and that lipids play an important role in organizing them (Brown and London, 1998). Moreover, an unusual lipid, lysobisphosphatidic acid, is enriched in the internal membranes of multivesicular endosomes (Kobayashi et al., 1998). It is still unclear, though, whether this lipid plays an active part in the invagination of endosomes. Given that the invagination of vacuoles is topologically the same reaction as that of endosomes and that the membrane of the nascent vesicles is devoid of transmembrane particles, it is tempting to speculate that lipids might be an important determinant of the budding reaction. Then, scission of the vesicle, which is topologically a homotypic fusion reaction between opposing vacuolar membranes in the autophagic tube, also may be independent of the established enzymes involved in homotypic vacuolar fusion and membrane trafficking, in general. This was in fact observed. Vesicle budding into the vacuolar lumen was found to occur independent of the vacuolar SNARE proteins Vam3p, Vam7p, and Nyv1p, and of Sec18p (yeast NSF) and Sec17p (yeast α-SNAP; Sattler and Mayer, 2000, this issue).

In any event, the formation of autophagic tubes and the inverse budding reaction must follow novel and unusual mechanisms, because they are topologically different from other vesiculation processes and yield vesicles with a unique membrane structure. A cell-free system that reconstitutes the formation of autophagic tubes, as well as the budding of vesicles into the lumen of the vacuoles (Sattler and Mayer, 2000, this issue), will be a valuable tool for the further characterization of this reaction.

We thank David Botstein and Trisha Davis for providing yeast strains.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 446; A. Mayer), from Boehringer Ingelheim Foundation (A. Mayer), and the Boehringer Ingelheim Fonds (O. Müller).

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