Ultrastructural Analysis of the Autophagic Process in Yeast: Detection of Autophagosomes and Their Characterization

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Abstract. Under nutrient-deficient conditions, the yeast *S. cerevisiae* sequesters its own cytoplasmic components into vacuoles in the form of "autophagic bodies" (Takeshige, K., M. Baba, S. Tsuboi, T. Noda, and Y. Ohsumi. 1992. *J. Cell Biol.* 119:301–311). Immunoelectron microscopy showed that two cytosolic marker enzymes, alcohol dehydrogenase and phosphoglycerate kinase, are present in the autophagic bodies at the same densities as in the cytosol, but are not present in vacuolar sap, suggesting that cytosolic enzymes are also taken up into the autophagic bodies. To understand this process, we performed morphological analyses by transmission and immunological electron microscopies using a freeze-substitution fixation method. Spherical structures completely enclosed in a double membrane were found near the vacuoles of

protease-deficient mutant cells when the cells were shifted to nutrient-starvation media. Their size, membrane thickness, and contents of double membrane-structures corresponded well with those of autophagic bodies. Sometimes these double membrane structures were found to be in contact with the vacuolar membrane. Furthermore their outer membrane was occasionally seen to be continuous with the vacuolar membrane. Histochemical staining of carbohydrate strongly suggested that the structures with double membranes fused with the vacuoles. These results indicated that these structures are precursors of autophagic bodies, "autophagosomes" in yeast.

All the data obtained suggested that the autophagic process in yeast is essentially similar to that of the lysosomal system in mammalian cells.

RECENT studies on the mechanism of protein degradation have revealed that autophagy is a ubiquitous and physiologically important cellular activity in eukaryotic cells. On nutrient deprivation, the protein turnover of cells increases and required nutrients are acquired by the degradation of components of the cytoplasm or cellular activities are reduced (Glaumann and Ballard, 1987; for review see Kovács and Rég, 1989). The autophagic process of protein degradation consists of sequestration of cytosol, fusion of primary lysosomes, and subsequent degradation of proteins in autophagosomes (Dunn, 1990*a,b*; Kopitz et al., 1990; Rabouille et al., 1993).

Previously we reported that yeast cells show extensive activity for sequestering cytoplasmic components in vacuoles (Takeshige et al., 1992). The vacuoles in yeast contain various kinds of hydrolases and have been considered to be a lytic compartment analogous to lysosomes in animal cells (Matile, 1975; Wiemken et al., 1979). Under starvation con-

ditions, many spherical structures surrounded by a single unit membrane appear in the vacuoles. We refer to those structures as autophagic bodies. Electron microscopic observations showed that the autophagic bodies have quite similar morphological characteristics to the cytosol: they contain ribosomes, RER, small vesicles, glycogen granules, and also occasionally mitochondria.

Wild-type cells accumulate autophagic bodies in the presence of PMSF, but after removal of PMSF they are rapidly degraded. Autophagic bodies may be intermediate structures of protein degradation in the vacuole.

In this paper we examined the changes of intracellular membranes during nutrient-starvation by electron microscopy. We found structures with double membranes in the cytosol that interact with the vacuolar membrane. These findings suggest that autophagy in yeast is similar to that in mammalian cells.

Materials and Methods

Yeast Strains

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1990). Strain BJ926, constructed by E. Jones (1977), was obtained from the Yeast Genetic Stock Center, Berkeley, CA.

Media and Growth Conditions

The compositions of the nutrient medium (YEPD) and synthetic media (SG and SD[−N]) used were as described in the previous paper (Takeshige et al., 1992).

Cells were grown in YEPD at 30°C until the late logarithmic phase, collected by centrifugation (1,600 g × 3 min), washed once with distilled water and resuspended in starvation medium. For carbon-starvation, cells were incubated for 3 h at 30°C in SG medium. For nitrogen-starvation, cells were incubated for 2 h at 30°C in SD(−N) medium. The cells were then harvested by centrifugation (1,600 g × 3 min) or filtration on a glass fiber filter, GF/C (Whatman, Maidstone, England).

Electron Microscopy

The harvested cells were fixed by a freeze-substitution fixation method as described previously (Baba and Osumi, 1987). Ultrathin sections were examined with a Hitachi H-500H electron microscope at 100 kV.

Polysaccharides were detected histochemically by the periodic acid-thiosemicarbazide-silver proteinate (PATAg)[1] method described by Thiéry (1967).

Preparation of Antibodies

Commercially available alcohol dehydrogenase (ADH, Sigma Chem. Co., St. Louis, MO) and phosphoglycerate kinase (PGK, Sigma Chem. Co.) were purified by SDS-PAGE, and used for immunization of rabbits. Antiserum against $\alpha 1 \rightarrow 3$ mannose residues was prepared by the method of Nakajima and Ballou (1975).

SDS-PAGE and Western Blotting

Whole-cell lysates of BJ926 cells were prepared as described by Wada et al. (1990) and subjected to SDS-PAGE (Laemmli, 1970). Proteins in the gel were transferred electrophoretically to a nitrocellulose sheet, blocked with 10% nonfat dry milk, treated with anti ADH or PGK antibody, and then detected with alkaline phosphatase–conjugated antibodies against rabbit IgG.

Immunoelectron Microscopy

Fixation and embedding of yeast cells were done by a modified freeze-substitution fixation method (Baba and Osumi, 1987). Yeast cells were sandwiched between a copper and a molybdenum disk, and were plunged into liquid nitrogen-cooled Freon 22. Cells attached to a molybdenum disk were transferred to 0.5% formaldehyde in cold-absolute acetone kept below −80°C. Substitution fixation was carried out at −80°C for 2 d. The specimens were warmed gradually (at −20°C for 2 h and at 0°C for 2 h), and washed with three changes of absolute acetone, and then with three changes of absolute ethanol. They were infiltrated with LR White resin (London Resin, Hampshire, England). The resin was polymerized at −20°C for 48 h under ultraviolet irradiation. Ultrathin sections were prepared with a Reichert Ultracut microtome and placed on nickel grids.

Ultrathin sections on nickel grids were incubated on drops of staining buffer (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 1% BSA for 15 min at room temperature for blocking non-specific binding sites. Then they were incubated in the staining buffer containing 0.1% BSA with primary antibody against ADH (1:500 dilution), PGK (1:400 dilution), or antiserum against $\alpha 1 \rightarrow 3$ mannose residues (1:50 dilution) for 2 h at room temperature. The grids were then washed with the staining buffer containing 0.1% BSA three times, and placed on drops of 10-nm colloidal gold conjugated protein A solution (Amersham, England, 1:50 dilution) for 30 min at room temperature. Then they were washed with the staining buffer and distilled water, and dried. The sections were stained with 4% uranyl acetate.

As controls, the preincubated sections were incubated directly with protein-A gold without reaction with primary antibodies, or diluted primary antibodies were absorbed with the purified antigens, and then used for the primary antibodies reaction.

Three-dimensional Reconstruction

Three-dimensional reconstruction of solid models was performed by computer graphics as described previously (Baba, 1992). The sections were estimated to be 80–90-nm thick from their interference color.

Results

Cytosolic Enzymes Are Sequestered in Vacuoles in the Form of Autophagic Bodies

Vacuoles were isolated from vacuolar proteinase-deficient cells cultured under carbon-starvation conditions. During starvation, the latent activity of the cytosolic marker enzyme glucose-6-phosphate dehydrogenase increased (Takeshige et al., 1992). First we examined the localization of cytosolic enzymes in the vacuoles in situ by immunological electron microscopy. Throughout this work, we used a multiple vacuolar proteinase-deficient mutant strain BJ926 (*pep4 prb1 prc1*). Thin sections of the cells fixed by the freeze-substitution fixation method were treated with a specific antibody against ADH or PGK, and labeled with 10-nm gold-labeled protein A. The antibodies used were affinity-purified and recognized single bands on Western blotting (data not shown). In preliminary experiments, non-specific binding of gold particles was found to be negligible, and specificity of the labeling was confirmed by immunoabsorption. In control cells grown in YEPD medium, gold particles associated with ADH were found in the cytosol, but were excluded from the vacuoles (Fig. 1 a). During incubations for 2 and 3 h in nitrogen- and carbon-starvation media, respectively, the cells accumulated autophagic bodies in the vacuoles. Immunological staining of thin sections of these cells revealed that the autophagic bodies in vacuoles were also stained with gold particles, while scarcely any gold particles were found in the vacuolar sap (Fig. 1 b, and see Fig. 3 a). The density of gold particles in the autophagic bodies (18.6 particles/0.1 μm^2) was similar to that in the cytosol (18.8 particles/0.1 μm^2), suggesting that both compartments contain similar densities of ADH. Antibody against PGK also gave almost the same staining pattern in the cells (data not shown). These data indicate that cytosolic enzymes were not imported directly into vacuolar sap, but were sequestered non-selectively into vacuoles in the form of autophagic bodies.

Novel Double Membrane-Structure Appears under Nutrient-Starvation Conditions

We examined the ultrastructure of the cytoplasm of BJ926 cells under starvation conditions by electron microscopy. A unique structure surrounded with double membranes was found in the cytosol of the cells under the carbon-starvation conditions (Fig. 2 a, arrow). Serial sections revealed that this structure was completely encompassed by a double membrane (Fig. 2, c–g). The sizes of these spherical structures coincided with those of the autophagic bodies in the vacuoles (400 nm–900 nm, Takeshige et al., 1992). As shown in Fig. 2 e, both the outer and inner membranes of these structures were thinner than those of other intracellular membranes (Fig. 2, h–k, arrowheads). This morphological characteris-
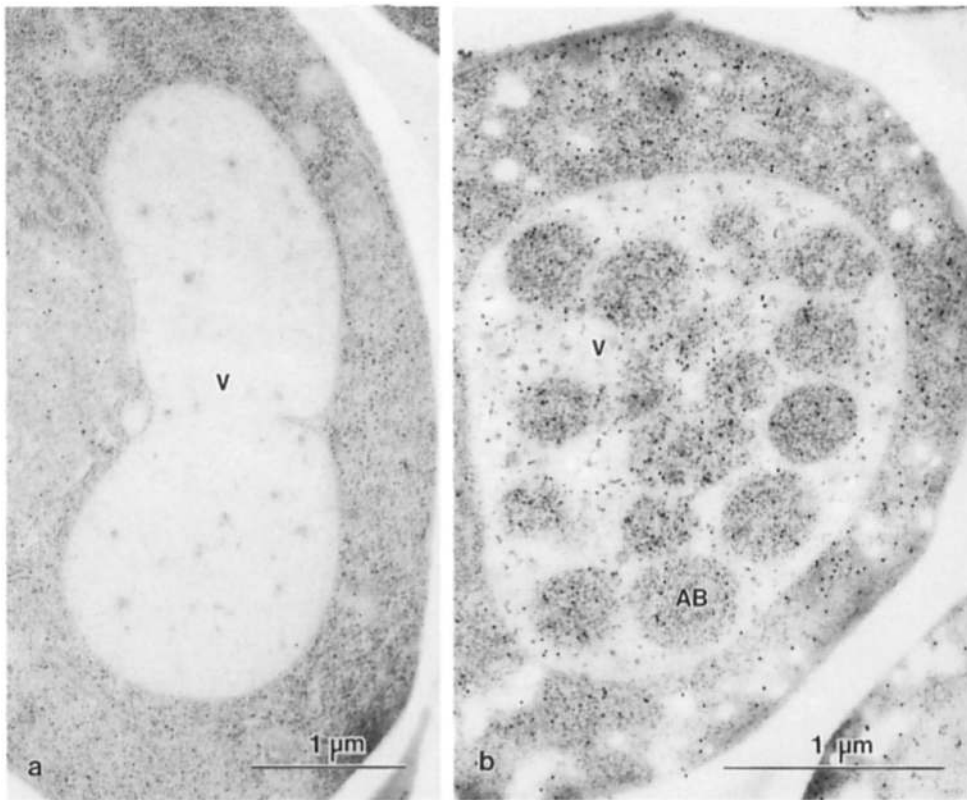

Figure 1. Immunological detection of the cytosolic marker enzyme, ADH. Thin sections were immunolabeled with anti-ADH antibodies followed by 10-nm colloidal gold-conjugated protein A. (*a*) A BJ926 cell in YEPD. (*Control*). (*b*) A BJ926 cell incubated in SD(−N) medium for 2 h. *AB*, autophagic body; *V*, vacuole.

tic also coincided with that of the membrane of autophagic bodies (Takeshige et al., 1992). As shown in Fig. 2 *b* (*arrow*), similar structures were found in the cells under nitrogen-starvation conditions. But we did not observe similar double membrane-structures in cells grown in YEPD medium.

The inside of the double membrane structure was morphologically identical to the cytosol or the contents of autophagic bodies, containing similar densities of ribosomes. Immunoelectron microscopy demonstrated the cytosolic enzyme ADH inside the double membrane structures (Fig. 3, *a* and *b*). Another cytosolic enzyme, PGK, was also demonstrated in these structures using specific anti-PGK antibody (data not shown). Sometimes autophagic bodies appeared as double or even multi-lamella structures (Fig. 2 *b*, *arrowhead*). Multi-lamella structures were also found in the cytosol (Fig. 2 *b*, *double arrows*). We have reported that mitochondria are taken up into vacuoles through autophagic bodies (Takeshige et al., 1992). Fig. 3, *c* and *d* showed a double membrane structure containing mitochondrion in the cytosol. The same cell contains three autophagic bodies including mitochondria were seen (Fig. 3, *c* and *d*, *arrowheads*).

The double membrane structures in the cytosol have morphological similarities to autophagic bodies in the vacuoles. Therefore, we refer to these double membrane structures in yeast as "autophagosomes".

Autophagosomes Are Few in Number

In spite of intensive accumulation of autophagic bodies in the

vacuoles, only a few random sections of nutrient-starved cells showed autophagosomes, suggesting that the number of autophagosomes in the cytosol is quite low. To obtain more detailed information on the location of autophagosomes in cells, we examined serial sections. In carbon-starved cells, most autophagosomes were seen separately as single forms near the vacuole (Fig. 2 *a*). The number of autophagosomes was also low in the early stage of nitrogen-starvation (1 h). After 2 h starvation, when many autophagic bodies had accumulated in the vacuoles, autophagosomes increased slightly in number and were seen in restricted regions of the cytosol. Two examples of autophagosomes in nitrogen-starved cells are shown in Fig. 4. In the cell shown in Fig. 4, *a–f*, two multilamellar autophagosomes are located next to the vacuolar membrane. Serial sections indicated that they were not in contact with each other, or with the vacuolar membrane. The other cell had five autophagosomes in a restricted region (Fig. 4, *g–j*). Fig. 4 *g*, *h*, *i*, and *j* show the appearance of the 6th, 7th, 9th, and 10th of 13 serial sections, respectively. In Fig. 4, *i* and *j*, two autophagosomes are seen connected with each other.

The Autophagosomal Membrane Differs from the Vacuolar Membrane

To characterize intracellular membranes histochemically, we used the PATAg method described by Thiéry (1967) to stain polysaccharides. By this method, the outer layer of the cell wall, glycogen granules, Golgi bodies, and secretory vesicles were stained heavily (Fig. 5). The vacuolar membrane was also stained well (Fig. 5 *a*), reflecting the existence of polysaccharide chains on the vacuolar membrane
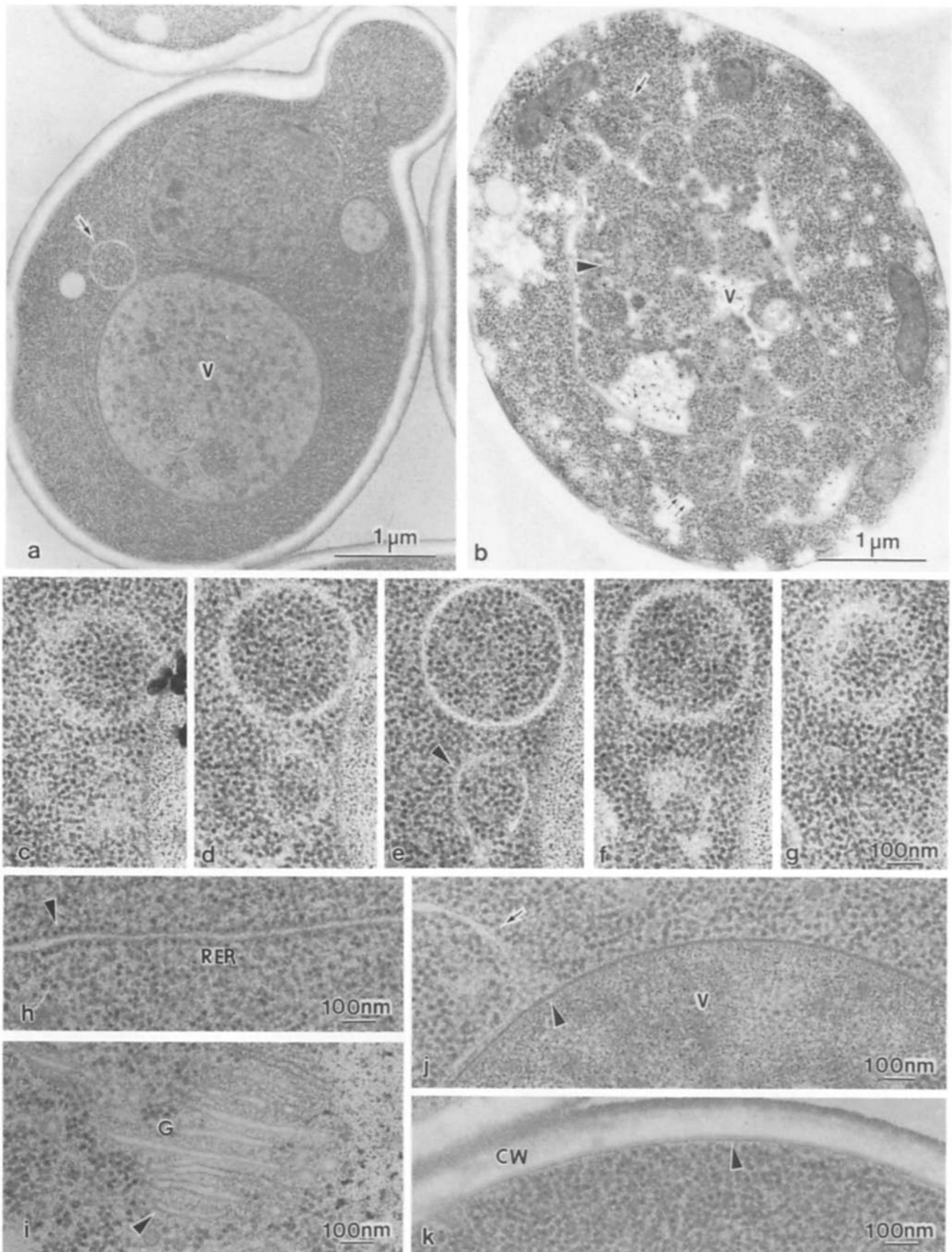
Figure 2. Double membrane structures appearing in the cytosol under nutrient starvation conditions. (*a*) A BJ926 cell incubated in SG medium for 3 h. The arrow indicates double membrane structure. (*b*) A BJ926 cell incubated in SD(−N) medium for 2 h. The vacuole contains many autophagic bodies. The arrowhead indicates an autophagic body with a multi-lamellar membrane. The arrow indicates dou-
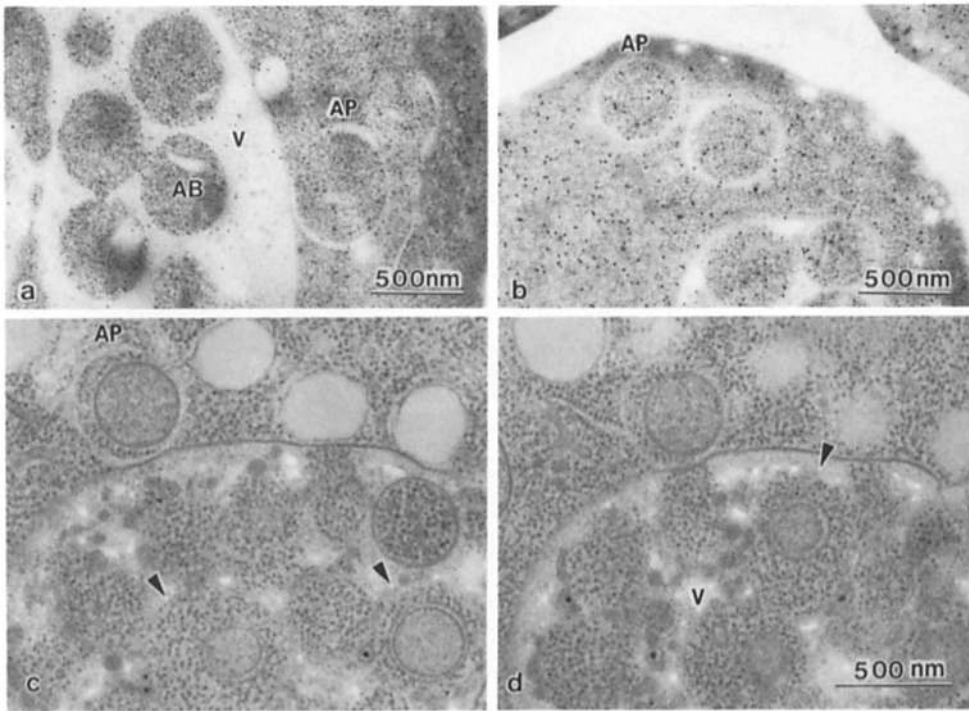
Figure 3. Detection of a cytosolic enzyme and mitochondrion in the double membrane structure. (*a* and *b*) Thin sections were immunolabeled with anti-ADH antibody followed by 10-nm colloidal gold-conjugated protein A. A BJ926 cell incubated in SG medium for 3 h (*a*) A BJ926 cell incubated in SD(−N) medium for 2 h (*b*). (*c* and *d*) Micrographs of serial sections of a BJ926 cell incubated in SD(−N) medium for 2 h. Arrowheads indicate three autophagic bodies containing mitochondria. *AB*, autophagic body; *AP*, autophagosome; *V*, vacuole.

proteins (Nishikawa et al., 1990). Glycogen granules induced by nitrogen-starvation accumulated in the cytosol, and they provided an appropriate marker of the cytosol. Spherical structures containing glycogen granules were judged to be autophagosomes. Autophagosomal membranes were stained with PATAg, but more weakly than vacuolar membranes (Fig. 5 *b*, arrow).

We also labeled ultrathin sections with antiserum against α1→3 mannose residues. Both vacuolar membranes and vacuolar sap were stained heavily (Fig. 6, *a* and *b*), indicating that soluble and membrane vacuolar proteins have extensive mannose α1→3 linkages (Hasilik and Tanner, 1978; Onishi et al., 1979), but the autophagosomal membrane and its lumen were not labeled with antiserum against α1→3 mannose residues (Fig. 6 *c*). The membrane of autophagic bodies seemed to be less densely stained than the vacuolar membrane, though heavy staining of vacuolar sap interfered with precise analyses.

These results indicate that the autophagosomal membrane differs from the vacuolar membrane not only in its morphological appearance but also in its carbohydrate content.

Autophagosomes Interact with the Vacuoles

Careful examination of the peripheral region of the vacuoles showed an autophagosome in contact with the vacuole. In most cases the vacuolar membrane becomes more concaved at the contact site, as shown in Fig. 7 *a* (*arrow*). Furthermore we found images showing the outer membrane of autophago-

somes continuous with the vacuolar membrane (Fig. 7 *b*, arrows) and a part of the inner membrane exposed to the vacuolar sap (Fig. 7 *b*, arrowhead).

In cells under nitrogen-starvation, clustered autophagosomes interacting with the vacuole were found by serial sectioning (Fig. 7, *c–f*). Three-dimensional images were reconstructed from 17 serial sections. Fig. 7, *g–i* clearly show that a restricted region of the vacuolar membrane had many spherical protrusions, while the rest of the membrane had a quite normal morphology. Only one free autophagosome that did not contact to the vacuole was observed in this cluster (Fig. 7, *c*, *h*, and *i*, arrows). Seven autophagosomes were in contact with the vacuoles and the outer membranes of some of them were even continuous with the vacuolar membrane.

By PATAg staining, we found that the heavily stained vacuolar membrane was interrupted by a weakly stained bubble-like structure that contained several glycogen granules (Fig. 8, *arrow*). This image strongly suggests that the two different types of membranes had become continuous in this region.

The Autophagosome Structure Differs Slightly with Starvation Conditions

Though the intracellular milieu must vary depending upon the starvation conditions, the structure of autophagic bodies in the vacuoles in carbon- and nitrogen-starved cells appeared quite similar. However, we noticed some difference

ble membrane structure. Double arrows indicate multilamellar structures. (*c–g*). Five serial section images of a double membrane structure. The arrowhead indicates a cup-shaped structure. (*h–k*). Other organelle membrane in the same strain. (*h*) RER. (*i*) Golgi body. (*j*) Vacuole. (*k*) Plasma membrane. *h* and *k* were from YEPD grown cells. *CW*, cell wall; *G*, Golgi body; *RER*, rough endoplasmic reticulum; *V*, vacuole.
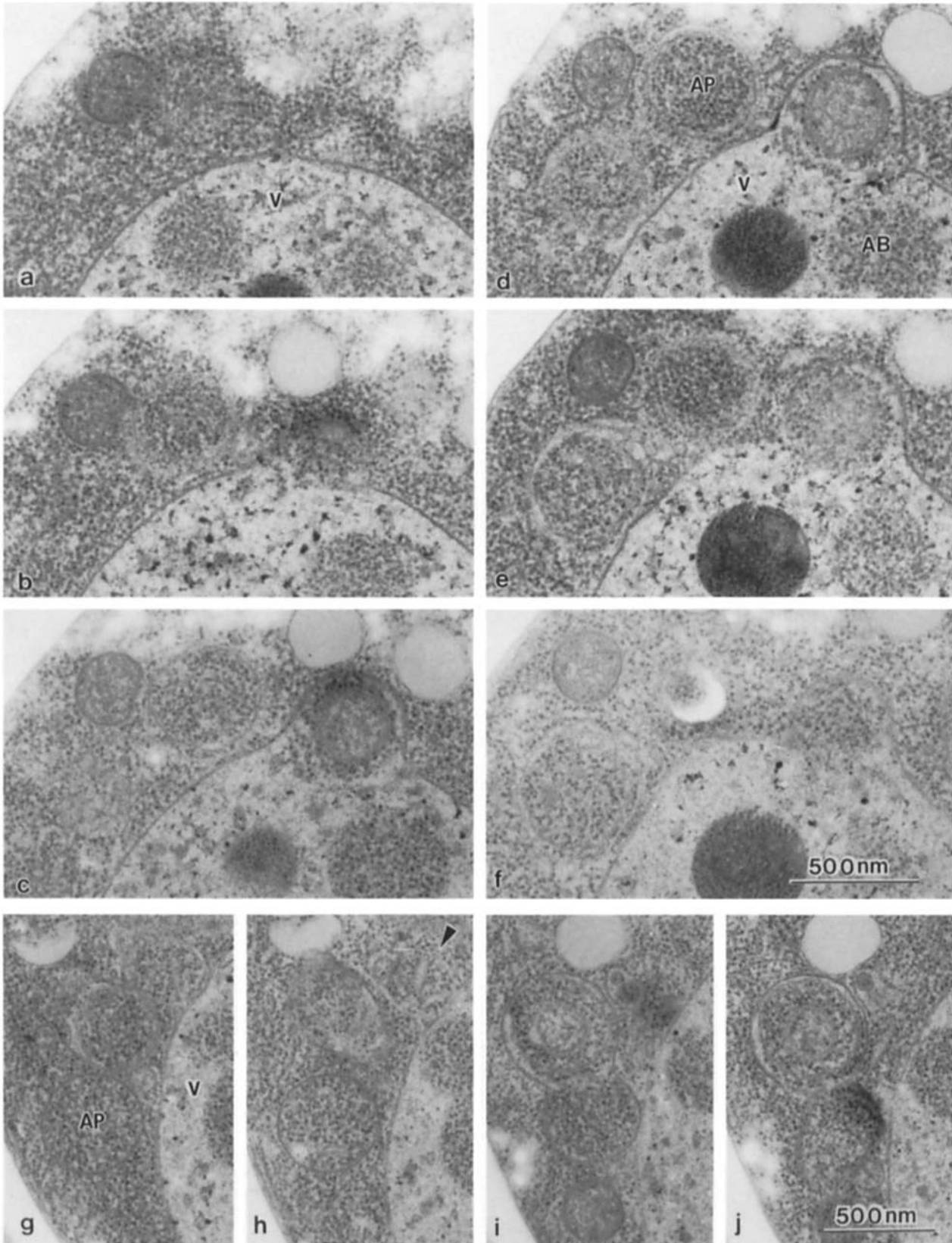
Figure 4. Autophagosomes in cells in SD(−N) medium. (*a–f*) Six serial section images of autophagosomes. (*g–j*) Four section images of autophagosomes. The arrowhead indicates a cup-shaped structure. *AB*, autophagic body; *AP*, autophagosome; *V*, vacuole.
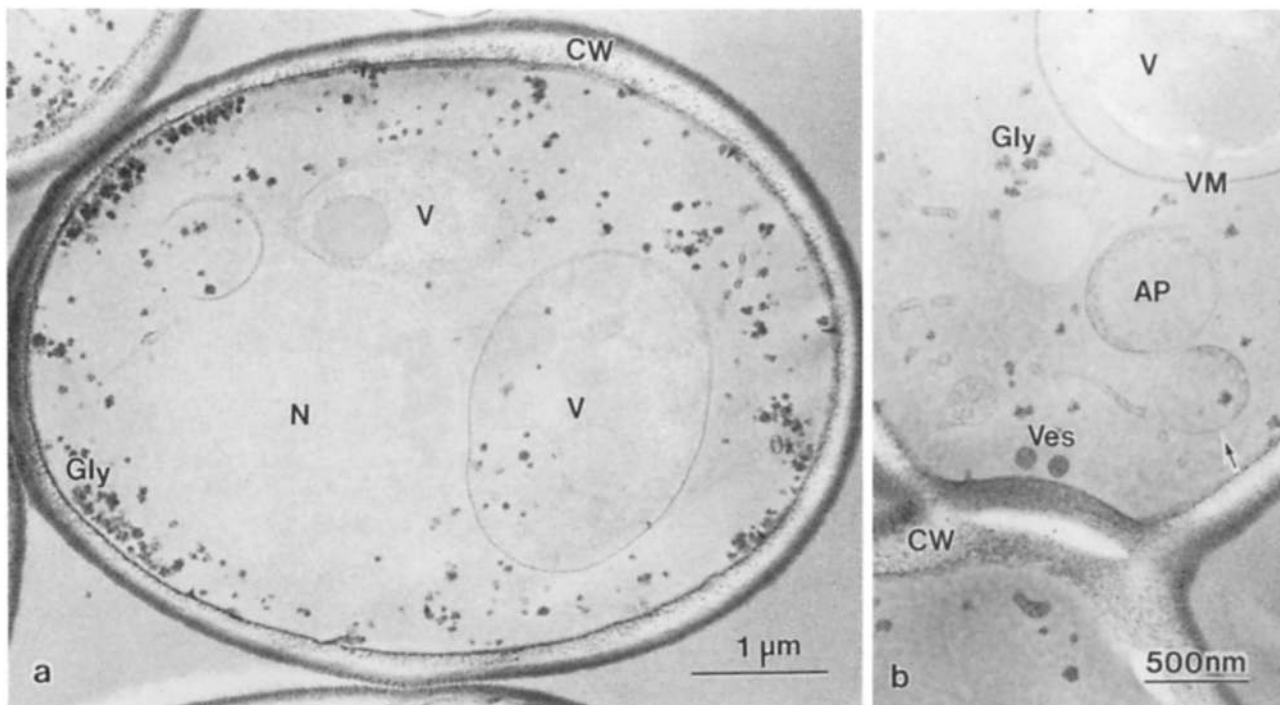
Figure 5. Histochemical staining of polysaccharides with PATAg. (*a*) A BJ926 cell incubated in SD(−N) medium for 2 h. (*b*) Part of the cell at higher magnification image. The arrow indicates a weakly stained autophagosome (*AP*). *CW*, cell wall; *Gly*, glycogen granules; *N*, nucleus; *V*, vacuole; *Ves*, vesicles; *VM*, vacuolar membrane.

in the structure of autophagosomes depending on the starvation conditions. In the carbon-starved cells double membranes of the autophagosome were usually separated by a narrow electron-translucent space (Fig. 2 *a*), whereas the autophagosomes in nitrogen-starved cells had a thinner lumenal space. The double membranes of autophagosomes in contact with the vacuolar membrane were in close contact with each other, in contrast to those of free autophagosomes (see Fig. 4 *d*). In nitrogen-starved cells the morphology of autophagosomes was more complex (see Fig. 4) and the frequency of multilamellar autophagosomes was much higher than that in carbon-starved cells.

Autophagosomes Accumulate in Cells Lacking a Vacuolar Compartment

Many mutants with defective vacuolar morphology have been isolated (for reviews see Klionsky et al., 1990; Raymond et al., 1992*a,b*). One Class I *vam* mutant (Class C *vps* mutant), *slp1* (*vam5/vps33*) completely lacks a vacuolar compartment (Kitamoto, 1988; Wada, 1990). YW10-2B, a gene disruptant of *SLP1* was examined under nitrogen-starvation conditions. The cytosol was examined under nitrogen-starvation conditions. The cytosol of Δ*slp1* cells grown in YEPD had a normal appearance, although they contained small vesicles in the cytosol more than wild-type cells (Kitamoto et al., 1988; Wada et al., 1990). After 3 h incubation in SD(−N) medium, single membrane bound vesicular structures appeared in the cytosol (Fig. 9 *b, arrowheads*) and double membrane structures accumulated in the cytosol (Fig. 9, *a* and *b, arrows*). These structures could be observed only when the cells were starved. The size, mem-

brane-thickness, and contents of double membrane structures were similar to those of autophagosomes of *SLP1* cells. Immunoelectron microcopy showed that they also contained the cytosolic enzymes ADH and PGK, and their membrane was not labeled with antiserum against α1→3 mannose residues (data not shown). These double membrane structures also contained various cytosolic structures non-selectively, and occasionally enclosed single membrane bound vesicular structures (Fig. 9 *b*). These similarities suggest that these structures are autophagosomes.

The number of autophagosomes in these cells in SD(−N) medium was much higher than that in BJ926 cells. Serial sectioning of six cells revealed that in this condition, cells contained an average of ∼20 autophagosomes (see Fig. 9, *c* and *d*). Three-dimensional reconstruction images of these cells clearly indicated that the autophagosomes were present in a single cluster (Fig. 9, *c* and *d*) or a small number of clusters (data not shown).

Discussion

In a previous paper we described novel membrane structures, autophagic bodies, which deliver cytosolic components to vacuoles (Takeshige et al., 1992). In the present study we found by immunological electron microscopy that soluble enzymes in the cytosol are also taken up by the vacuoles through autophagic bodies. One obvious question is how these autophagic bodies accumulate in the vacuoles. There are at least two possible mechanisms by which a single membrane structure containing cytosol accumulates in the
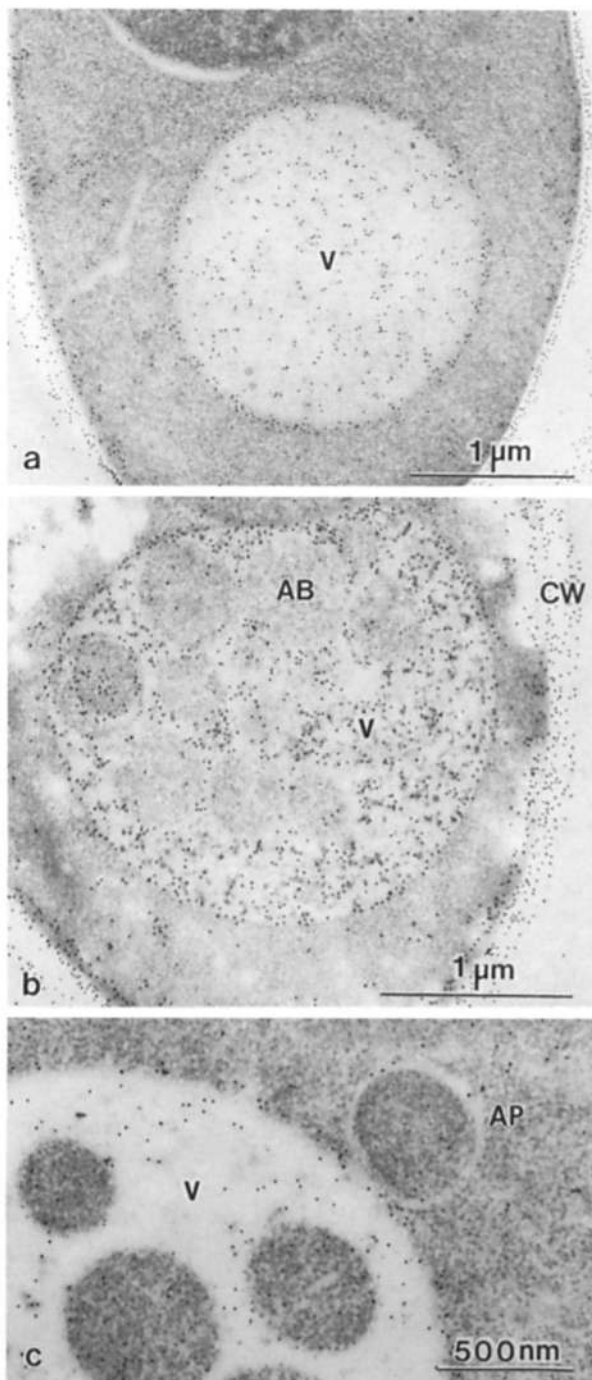
Figure 6. Immunolabeling of $\alpha1\rightarrow3$ mannose residues. Thin sections were incubated with antiserum against $\alpha1\rightarrow3$ mannose residues followed by 10-nm colloidal gold-conjugated protein A. (*a*) A BJ926 cell grown in YEPD. (*b*) A BJ926 cell incubated in SD(−N) medium. (*c*) A BJ926 cell incubated in SG medium. *AB,* autophagic body; *AP,* autophagosome; *CW,* cell wall; *V,* vacuole.

bodies differs morphologically from the vacuolar membrane. The membrane of autophagic bodies is also distinguishable from the vacuolar membrane by PATAg staining or reactivity with antiserum against $\alpha1\rightarrow3$ mannose residues. Therefore, this mechanism seems unlikely.

Another possible mechanism is that single membrane cisternae enclose a portion of the cytosol, resulting in structures surrounded by double membranes. The outer membranes of these structures may then fuse with the vacuole, and consequently deliver a single membrane structure to the vacuoles. In this study we found that double membrane structures and autophagosomes appeared specifically when cells were transferred to nutrient-starvation media. The size, membrane thickness, and contents of these double membrane structures corresponded well with those of autophagic bodies. Furthermore some autophagosomes were found to be in contact with the vacuolar membrane. The outer membrane of a few autophagosomes was seen to be continuous with the vacuolar membrane. Moreover, carbohydrate staining clearly demonstrated two different types of membranes in continuity in a protrusion (Fig. 8). This microscopic image is not consistent with the idea that the bubble-like structure observed resulted from budding off from the vacuolar membrane, but our results are consistent with the idea that the outer membranes of autophagosomes fuses with the vacuolar membrane and autophagic bodies originate from autophagosomes. Thus the autophagic process in yeast appears to consist of essentially the same membrane events as that in mammalian cells.

We found that the autophagosomes are not uniformly distributed in the cytosol, but are localized in a few limited regions and interact with a restricted area of the vacuolar membrane. One possible explanation for this finding is that although autophagosomes are formed throughout the cytosol, they gathered to fuse with a specific region of the vacuole. An alternative possibility is that clustering of autophagosomes is due to their formation in restricted regions. We often detected a cup-shaped structure within or next to clusters of autophagosomes (Figs. 2 *e* and 4 *h, arrowhead*). Serial sectioning showed that this was an open spherical structure. The cup-shaped structure enclosing a portion of cytosol had an electron translucent lumen, but the width of the lumen was not uniform, part of it being expanded. Golgi-like membrane cisternae and small vesicles were found near this structure. This structure might be an autophagosome in the process of formation. In mutants without vacuoles, many autophagosomes accumulated as a small number of clusters, probably because of the absence of a vacuole to fuse with. Thus autophagosomes may be formed in a few regions of the cytosol, and rapidly fuse with the vacuolar membrane.

Another problem is the origin of the autophagosomal membrane. This is still a disputable problem in mammalian cells. There are many reports that the autophagosomal membrane originates from the smooth ER (Dunn, 1990*a,b*). However, Yamamoto et al. (1990*a,b*) concluded that it originates from the post-Golgi compartment. The autophagosomal membrane in yeast differs morphologically from those of the vacuole, RER, or Golgi body, and is stained by the PATAg method, but not with anti $\alpha1\rightarrow3$ mannose residue antiserum. But for further analyses, it will be necessary to identify a specific marker of autophagosome.
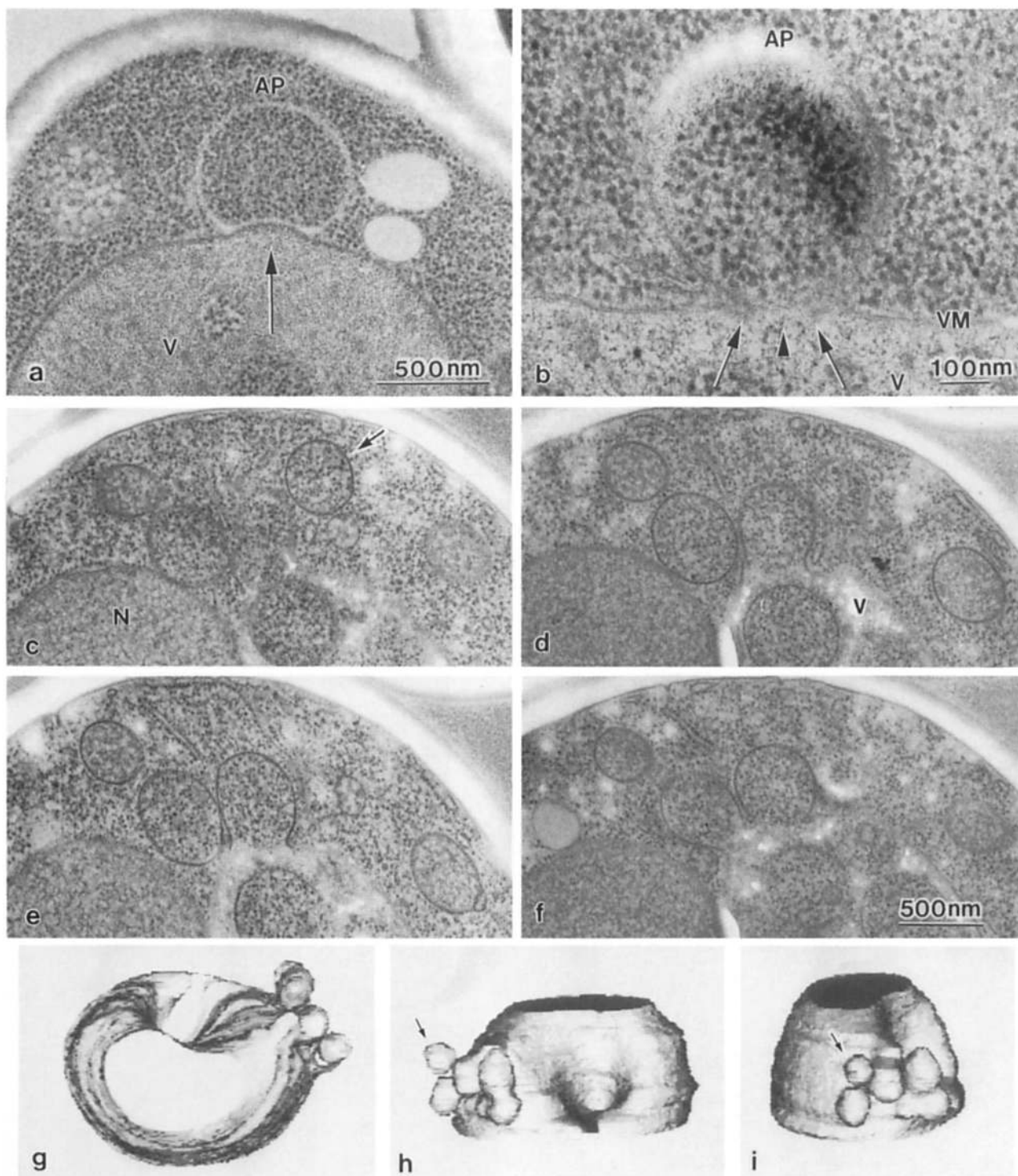
vacuoles. One is that the vacuolar membrane may invaginate, and pinch off a part of the cytosol. In this case, the membrane of autophagic bodies would be formed from the vacuolar membrane. However, the membrane surrounding autophagic

Figure 7. Interaction of autophagosomes with vacuoles. (*a* and *b*) BJ926 cells were incubated in SG medium. Arrowhead indicates autophagosomal membranes exposed to vacuolar sap. Arrows indicate continuity of the outer membrane of an autophagosome with the vacuolar membrane. (*c–f*) Four of 17 serial section images of a BJ926 cell incubated in SD(−N) medium. The arrow indicates a free autophagosome near the fusion site. (*g–i*). Three-dimensional reconstructed images from 17 serial sections corresponding to the vacuole shown in *c–f*. (*g*) Top view. (*h* and *i*) Side views. *AP*, autophagosome; *N*, nucleus; *V*, vacuole; *VM*, vacuolar membrane.

The membrane system involved in autophagy in yeast must be much simpler than that in mammalian cells, and autophagy in yeast can be induced quite synchronously in al-most all cells. Genetic and biochemical dissection of the yeast system should provide many clues to the process of autophagy at a molecular level.
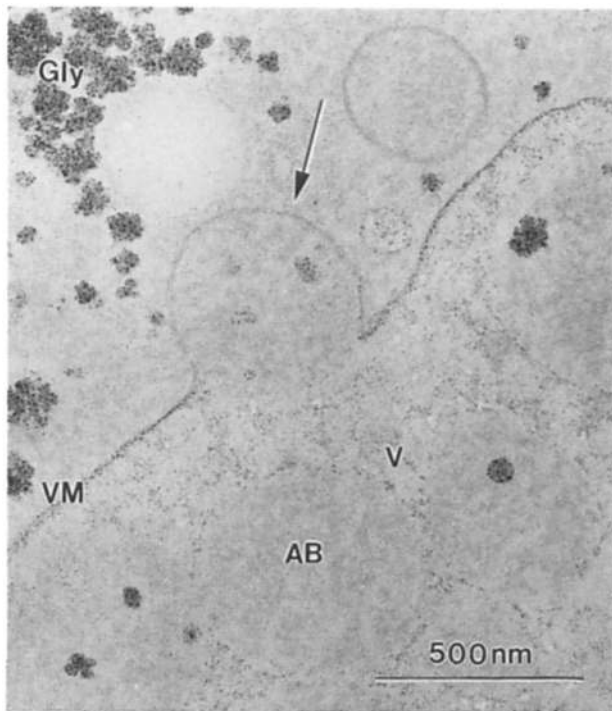
References

Baba, M., and M. Osumi. 1987. Transmission and scanning electron microscopic examination of intracellular organelles in freeze-substituted *Kloeckera* and *Saccharomyces cerevisiae* yeast cells. *J. Electron Microsc. Techn.* 5:249–261.

Baba, N. 1992. Computer-aided three-dimensional reconstruction from serial section images. *In* Image Analysis in Biology. D. P. Hader, editor. CRC Press, London. 251–270.

Figure 8. Histochemical staining with PATAg of the site of interaction of an autophagosome with a vacuole. The arrow indicates the interacting autophagosome. *AB*, autophagic body; *Gly*, glycogen granules; *V*, vacuole; *VM*, vacuolar membrane.
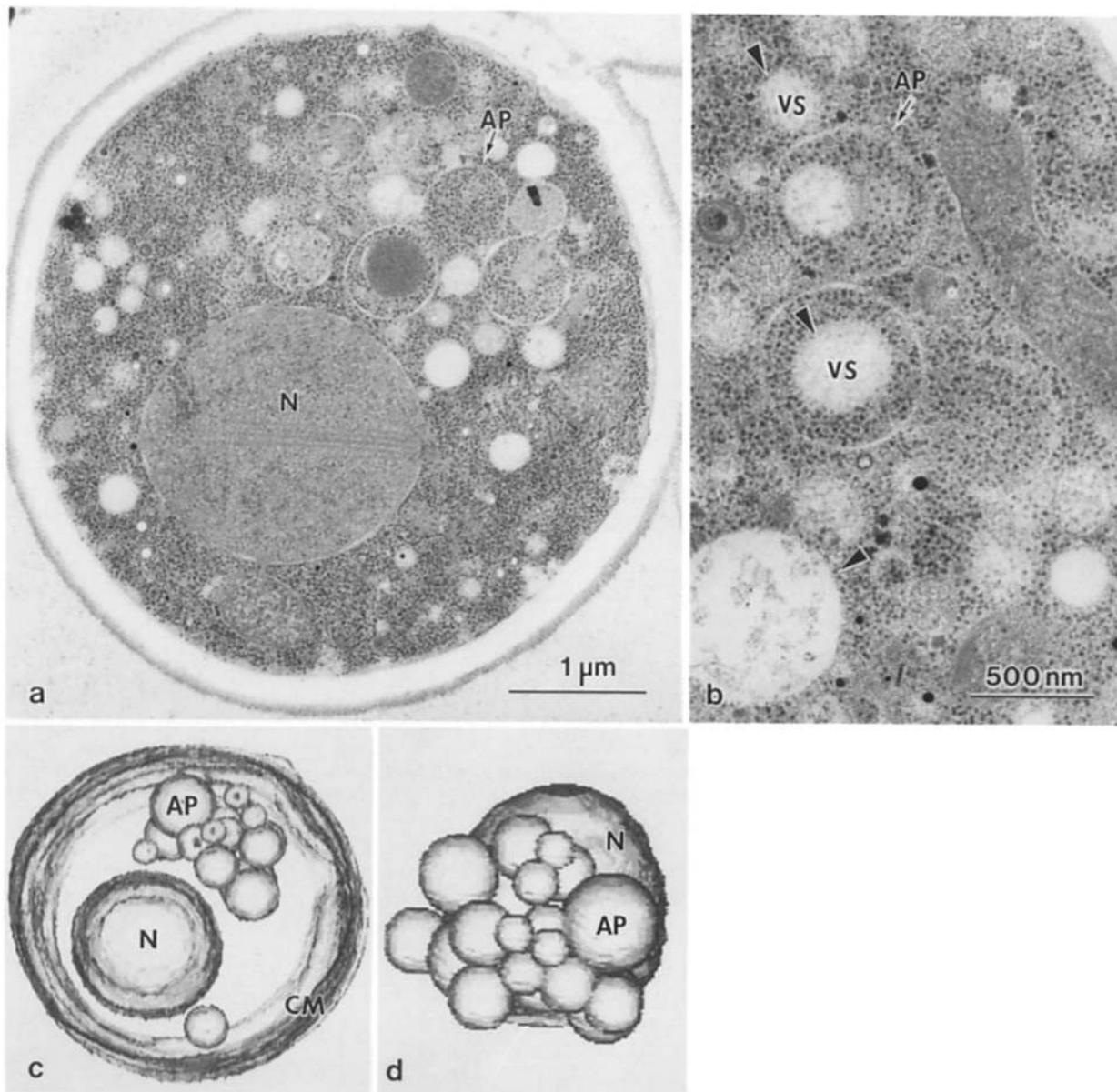
Dunn, Jr., W. A. 1990a. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J. Cell Biol.* 110:1923–1933.

Dunn, Jr., W. A. 1990b. Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. *J. Cell Biol.* 110:1935–1945.

Glaumann, H., and F. J. Ballard. 1987. *In* Lysosomes: their role in protein breakdown. Glaumann, H., and F. J. Ballard, editors. Academic Press Inc., London. 737 pp.

Hasilik, A., and W. Tanner. 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Conversion of precursor into the enzyme. *Eur. J. Biochem.* 85:599–608.

Jones, E. W. 1977. Proteinase mutants of *Saccharomyces cerevisiae. Genetics.* 85:23–33.

Kitamoto, K., K. Yoshizawa, Y. Ohsumi, and Y. Anraku. 1988. Mutants of *Saccharomyces cerevisiae* with defective vacuolar function. *J. Bacteriol.* 170:2687–2691.

Klionsky, D. J., P. K. Herman, and S. D. Emr. 1990. The fungal vacuole: composition, function, and biogenesis. *Microbiol. Rev.* 54:266–292.

Kopitz, J., G. Ø. Kisen, P. B. Gordon, P. Bohley, and P. O. Seglen. 1990. Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes. *J. Cell Biol.* 111:941–953.

Kovács, J., and C. Réz. 1989. Quantitative aspects of cellular autophagocytosis. Morphometric and cell fractionation studies. *In* RBC. Cell Biology Reviews. Knecht, E., and S. Grisolia, editors. Springer International. 20:63–78.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Matile, P. 1975. The lytic compartment of plant cells. Cell Biology Monographs. *In* Vol. 1. The lytic compartment of plant cells. Springer Verlag, Wien, New York. 1–175.

Nakajima, T., and C. E. Ballou. 1975. Yeast manno-protein biosynthesis. solubilization and selective assay of four mannosyltransferases. *Proc. Natl. Acad. Sci. USA.* 72:3912–3916.

Nishikawa, S., N. Umemoto, Y. Ohsumi, A. Nakano, and Y. Anraku. 1990. Biogenesis of vacuolar membrane glycoproteins of yeast *Saccharomyces cerevisiae. J. Biol. Chem.* 265:7440–7448.

Onishi, H. R., J. S. Tkacz, and J. O. Lampen. 1979. Glycoprotein nature of yeast alkaline phosphatase. *J. Biol. Chem.* 254:11943–11952.

Rabouille, C., G. J. Strous, J. D. Crapo, H. J. Geuze, and J. W. Slot. 1993. The different degradation of two cytosolic proteins as a tool to monitor autophagy in hepatocytes by immunocytochemistry. *J. Cell Biol.* 120:897–908.

Raymond, C. K., C. J. Roberts, K. E. Moore, I. H. Stevenson, and T. H. Stevens. 1992a. Biogenesis of the vacuole in *Saccharomyces cerevisiae. Int. Rev. Cytol.* Jeon, K. W., and M. Friedlander, editors. Academic Press Inc. London. 139:59–120.

Raymond, C. K., I. H. Stevenson, C. A. Vater, and T. H. Stevens. 1992b. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E *vps* mutants. *Mol. Biol. Cell.* 3:1389–1402.

Takeshige, K., M. Baba, S. Tsuboi, T. Noda, and Y. Ohsumi. 1992. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J. Cell Biol.* 119:301–311.

Thiéry, J. P. 1967. Mise en evidence des polysaccharides sur coupes fines en microscopie electronique. *J. Microscopie (Paris).* 6:987–1018.

Tsukada, M., and Y. Ohsumi. 1993. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae. FEBS (Fed. Eur. Biochem. Soc.) Lett.* 333:169–174.

Wada, Y., K. Kitamoto, T. Kanbe, K. Tanaka, and Y. Anraku. 1990. The *SLP1* gene of *Saccharomyces cerevisiae* is essential for vacuolar morphogenesis and function. *Mol. Cell. Biol.* 10:2214–2223.

Wiemken, A., M. Schellenberg, and K. Urech. 1979. Vacuoles: the sole compartments of digestive enzymes in yeast (*Saccharomyces cerevisiae)? Arch. Microbiol.* 123:23–35.

Yamamoto, A., R. Masaki, and Y. Tashiro. 1990a. Characterization of the isolation membranes and the limiting membranes of autophagosomes in rat hepatocytes by lectin cytochemistry. *J. Histochem. Cytochem.* 38:573–580.

Yamamoto, A., R. Masaki, Y. Fukui, and Y. Tashiro. 1990b. Absence of cytochrome P-450 and presence of autolysosomal membrane antigens on the isolation membranes and autophagosomal membranes in rat hepatocytes. *J. Histochem. Cytochem.* 38:1571–1581.

Figure 9. Autophagosomes accumulated in a mutant without vacuoles. (*a* and *b*) A YW10-2B cell incubated in SD(−N) medium for 3 h. The arrows indicate autophagosomes. The arrowheads indicate vesicular structure. (*c* and *d*) Three-dimensional reconstructed images of autophagosomes. (*c*) Top view. (*d*) Side view. *AP,* autophagosome; *CM,* cell membrane; *N,* nucleus; *VS,* vesicular structure.