Analysis of the Membrane Structures Involved in Autophagy in Yeast by Freeze-Replica Method

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ABSTRACT. Under starvation conditions, the yeast S. cerevisiae sequesters its own cytoplasmic components by forming autophagosomes with double membrane in the cytoplasm. The autophagosome then fuses with the vacuolar membrane and delivers its own cytoplasmic components into the vacuole in the form of an autophagic body with a single membrane (Baba, M., Takeshige, K., Baba, N., and Ohsumi, Y. 1994. J. Cell Biol., 124: 903–913). We examined membrane structures involved in the autophagy induced by nitrogen-starvation by using freeze-replica method. The most conspicuous characteristic of the autophagic body is that the intramembrane particles were rarely detected on either the protoplasmic or exoplasmic face of its fracture membrane. This morphological feature of the fractured face was clearly different from other intracellular organelles. Next we examined the autophagosomal membrane. The inner membrane of autophagosome was also intramembrane particle-free, and its morphological feature was identical to the membrane of autophagic body. At the fusion site between autophagosome and vacuole we obtained direct evidence that two different membranes, the outer membrane of autophagosome and vacuolar membrane, became continuous by using freeze-etching technique. From these results we concluded that the autophagic body originated from the inner membrane of the autophagosome, and its membrane reflects an intrinsic feature of autophagosomal membrane. The outer membrane of autophagosome had only a few intramembrane particles and may be differentiated from the inner membrane. In cells under nitrogen-starvation condition, the density of intramembrane particles of vacuolar membrane decreased beyond that in control cells.

Autophagy is a major protein degradation process which occurs in response to nutrient-deprivation in eukaryotic cells (7, 12). In autophagy, dynamic membrane flow occurs in the cells to deliver their own cytosolic components non-selectively into lytic compartments (2, 8, 10, 15). Recently we reported that, in the yeast S. cerevisiae, autophagy is induced under various starvation conditions (13). This whole process is equivalent to the lysosomal protein degradation process in mammalian cells (1). When yeast cells are shifted from growing condition to starvation condition, a portion of the cytosol is enclosed with a membrane sac, and then an autophagosome, double membrane structure is formed in the cytoplasm. The autophagosome immediately fuses with the vacuolar membrane, delivering a single membrane organelle, autophagic body to the vacuole. The autophagic body is quickly disintegrated in the vacuole of wild-type cells. However, in vacuolar protease-deficient mutants many autophagic bodies accumulated in the vacuoles. One of the important problems to be solved is from what and how autophagosomes are formed under nutrient-starvation conditions. In mammalian cells, morphological studies have revealed that the limiting membranes of autophagosome are originated from ribosome-free regions of the rough ER (3, 4, 5, 17) or post-Golgi membrane (18, 19). The sequestering mechanism has not been resolved yet, partly because of the complexity of the membrane system in mammalian cells.

In vacuolar protease-deficient mutants the latest step of autophagy is blocked. By this genetic dissection the morphological analysis of the intermediate structures during autophagy, such as autophagic body and autophagosome, became possible. We investigated these structures by using ultrathin sectioning (1). The membranes of these structures showed morphologically unique characteristics, that is, they seemed to be thinner than those of any other intracellular organelles such as the vacuole, rER, or Golgi body. Histochemical staining for polysaccharides with PATAg showed that auto-
phagosomal membranes were stained with PATAg, but more weakly than the membranes of vacuole or Golgi bodies. Immunoelectron microscopy using antiserum against α1-3 mannose residues supported this result, that is, the autophagosomal membrane had less carbohydrate content than the vacuolar membrane.

In this report, to define the membrane structures involved in the autophagic pathway, we tried to observe membrane of the intermediate structures, such as the autophagic body and autophagosome, induced under nitrogen-starvation condition by freeze-replica method. We show in this report that the membranes of the autophagic body and autophagosome are smooth and are almost free of intramembrane particles.

MATERIALS AND METHODS

Yeast strain. The strain of Saccharomyces cerevisiae used in this work was STY99 (MATα / ura3-52 lys2-801 ade2-101 trpl-Δ163 his3-Δ200 leu2-Δ1 prb1::TRP1).

Media and Growth conditions. The nutrient medium (YEPD) and nitrogen-starvation medium (SD(-N)) were as described previously (13). Cells grown in YEPD at 23°C or 30°C to the logarithmic phase, were collected by centrifugation (1,600 g x 3 min), washed twice with nitrogen-starvation medium, resuspended in the same medium and incubated for 2.5-3.5 h at 23°C or 30°C.

Light Microscopy. Cells were examined under an Olympus BH2 microscope (Tokyo, Japan) with a 100 x oil-immersion objective for phase contrast optics. Images were photographed with T-MAX ASA 400 film with a T-Max developer (Eastman Kodak Co., Rochester, NY).

Chemical Fixation and Freezing. Cells were fixed with 2.5% glutaraldehyde (EM science) in 50 mM sodium phosphate buffer (pH 7.4). The fixed cells were then washed three times in the same buffer and were glycerinated stepwise to 30%.

Harvested cells were mounted on a hat-shaped specimen holder and were immediately immersed into liquid Freon 22 at −160°C.

Rapid-freezing for unfixed cells. For rapid-freezing, a drop of unfixed cell pellet was put on a copper disc and was immediately brought into contact with a copper stage pre-cooled to liquid nitrogen temperature.

Freeze-fracture. Freeze-fracturing was performed in a Balzers High Vacuum Freeze-Etch Unit BAF301 at −110°C. Etching was done at −100°C for 1 min. Platinum-carbon was evaporated onto the specimen rotating at 70 rpm at an angle of 25°, followed by carbon evaporation at an angle of 90°, using an electron beam gun EK552. The platinum-carbon and carbon were 2 and 20 nm in thickness, respectively.

The replicas were cleaned with household bleach and washed five times in distilled water, and then mounted on grids.

The replicas were observed with a JEM-1200EXII electron microscope at 100 kV and a Hitachi H-800 electron microscope at 200 kV.

RESULTS

Morphological characteristics of the membrane of autophagic body. To characterize the membrane structures involved in the autophagy induced by nutrient-starvation, we used haploid strain STY99 which is a disruptant of PRB1 gene encoding vacuolar proteinase B.

STY99 cells grown in YEPD (Fig. 1a) were transferred to a nitrogen-deficient medium and incubated at 23°C or 30°C for 2.5-3.5 h. Under these conditions most cells had vacuoles filled with many autophagic bodies as shown in Fig. 1b (arrow).

First, to examine the membrane of autophagic bodies

![Image](image-url)
in the vacuoles, cells were rapidly frozen without chemical fixation, and were observed using freeze-etching method. A typical cross-fractured cell is shown in Fig. 2. Vacuoles were observed in a central region of the cell. The higher magnification images of vacuoles are shown in Fig. 3. Within the fractured vacuoles autophagic bodies were observed as perfectly spherical structures, and were randomly distributed (Fig. 3a). The sizes of these structures ranged from about 400 to 800 nm which correspond to those of autophagic bodies observed in the ultrathin sections (1). The interior of a cross fractured autophagic body gave the same image as that of the cytosol (Fig. 3b, arrow), and occasionally and autophagic body contained cytoplasmic structures such as a small vesicle (Fig. 3b, arrowhead). The most characteristic features of autophagic bodies observed by freeze-etching method are that they are smooth and round, and that both of their fractured faces, that is, protoplasmic (PF) and exoplasmic face (EF), are free of intramembrane particles. Only a few autophagic bodies showed a quite small number of intramembrane particles on the PF of the membrane (Fig. 3c, arrowheads).

**Characterization of the membrane of autophagosome.** Previously by ultrathin sectioning we found an autophagosome, a unique double membrane structure in the cytosol of the cells under starvation conditions. Serial sectioning and three-dimensional reconstruction images of the starved cells showed that the number of autophagosomes in a cell is quite small and they are localized next to the vacuolar membrane. Autophagosome might be a transient intermediate structure in the autophagic process (1).

By freeze-replica method we detected smooth-surface structures near the vacuole in the cytoplasm (Fig. 4). The size of these structures corresponded to that of autophagic bodies in the vacuole. Fig. 4a showed the cross-fractured image of the smooth surface-structure, which clearly showed that the inside is similar to the cytosol and was clearly distinguishable from lipid granules (Fig. 4b and d). These smooth surface-structures were observed only in the cytoplasm of cells incubated under starvation conditions, but not in cells growing in a nutrient rich medium such as YEPD. As an autophagosome consists of double-membrane structures (1), the fracture plane may run through the inner or outer membrane. In many cases the smooth surface-structures appeared as multilamellar structures (Fig. 4c and e). These structures must be autophagosomes. The PF of the inner membrane of autophagosome was very smooth, and intramembrane particles were rarely detected (Fig. 4c arrow). Fig. 4e shows the concave image of autophagosome, and as indicated by single arrows the EF of the inner membrane showed no intramembrane particles at all.

These results indicated that the inner membrane of autophagosome was almost free of intramembrane particles and that this morphological feature was identical to that of the membrane of autophagic bodies. Therefore the characteristic feature of autophagic body reflects the intrinsic morphology of the autophagosomal membrane.

On the EF of the outer membrane of autophagosome, a few intramembrane particles were present as shown in Fig. 4c (arrowheads) and 4d (arrowheads). They were dispersed randomly on the membrane, however, the density of these intramembrane particles was extremely low. These results suggested that both membranes of autophagosome had specialized morphology, and it is likely that the outer membrane contains more intramembrane particles and differentiates from the inner membrane.

**Fusion of autophagosome with vacuole.** In ultrathin sections, we obtained an image of fusion between the outer membrane of autophagosome and vacuolar membrane (1). By histochemical staining with PATAg for polysaccharide we also showed that the two different types of membranes became continuous at the fusion point.
Fig. 3.
We thoroughly examined where the autophagosomes came into contact with the vacuoles by freeze-etching method, and detected the fusion image of them shown in Fig. 4f. In the replica image the outer membrane of autophagosome with only a few intramembrane parti-
Fig. 5. The exoplasmic face of the vacuolar membrane. Glutaraldehyde fixed cell. (a) YEPD grown cell. (b) A cell incubated in SD(-N) medium for 2.5 h at 23°C.

icles (arrowheads) was continuous with the vacuolar membrane which had many intramembrane particles (small arrows). The inner membrane of the autophagosome is just being exposed to the vacuolar sap at the point indicated by double arrow. This freeze-replica image strongly suggests that double membrane structure in the cytosol is autophagosome and provides direct evidence that the outer membrane of autophagosome fuses with the vacuolar membrane.

Morphology of vacuolar membrane under starvation conditions. Under starvation condition cells sequester their own cytosolic components to the vacuole one after another by fusion of autophagosomes. The vacuolar membrane was examined in the cells grown in YEPD (Fig. 5a) and incubated in SD(-N) (Fig. 5b) by freeze-replica method. Both vacuolar membranes showed many randomly-distributed intramembrane particles, but apparently the starved cells contained less particles. We counted the intramembrane particles on the EF of each membrane, because the PF of vacuolar membrane contained too many intramembrane particles. This quantitative analysis revealed that the density of intramembrane particles on the vacuolar membrane of starved cells decreased to about 10% as compared with control cells.

This result is consistent with the idea that autophagosomes, which are almost free of intramembrane particles, fused with the vacuolar membrane. But further analysis is required to make this conclusive.

Fig. 4. Autophagosome in cytoplasm of an STY99 cell incubated in SD(-N) medium for 2.5–3.5 h at 23°C. (a) A cross-fractured autophagosome. (b) A lipid granule. (c) An autophagosome fractured through the inner and outer membrane (arrow). Arrowheads indicate the intramembrane particles on the outer membrane. (d) An autophagosome contacted with vacuolar membrane (arrow). Double arrow indicates the convex image of autophagosome near the vacuole. Arrowheads indicate intramembrane particles on the outer membrane of autophagosome. (e) The concave image of autophagosome. Single arrow indicates the EF of the inner membrane. Double arrow indicates the PF of the outer membrane. (f) Fusion image between autophagosome and vacuole. Unfixed cells. Large arrow indicates the outer membrane of autophagosome. Double arrow indicates the inner membrane of autophagosome. Arrowheads indicate intramembrane particles on the autophagosomal membrane. Small arrows indicate intramembrane particles on the vacuolar membrane. (a)–(e) Glutaraldehyde fixed cell. (d)–(f) Photograph was reversed.

DISCUSSION

In this paper we studied the membranes involved in the autophagy of yeast induced by nitrogen-starvation by using freeze-replica method. Autophagic body and autophagosome are distinctive intermediates of the process of the autophagy (1, 13). First we demonstrated that the membrane of autophagic body is mostly free of intramembrane particles. Analysis of autophagosomes then revealed that the density of intramembrane particles is also extremely low in this membrane and that the inner membrane is especially lacking in them. These membranes are apparently different from those of any other organelles such as vacuole, nucleus, mitochondrion, rER, or plasma membrane, which have significant numbers of intramembrane particles on their fractured faces. These results strongly support that the autophagic body is originated from the structure enclosed by the inner membrane of autophagosomes, and that the specific feature of the autophagic body's membrane is not due to the digestion of its membrane proteins in the vacuoles.

It is likely that autophagosomal membrane contains less protein, especially multi-membrane spanning proteins, and may be highly specialized just for delivering cytosolic components to the vacuoles. It may have only targeting and fusion machinery to the vacuole but not energy transducing system such as H⁺-pump or various types of transporter, which are multi-membrane spanning proteins.

The origin and formation of autophagosome are controversial and still unclear in mammalian cells. So far we have no biochemical or histochemical marker on this membrane in yeast. The morphological feature demonstrated in this study will provide a good marker for further studies on the mechanism of formation of autophagosome.

We detected intramembrane particles in the outer membrane of the autophagosome, although the density was extremely low. The outer membrane should play important roles in the targeting and fusion event, and may differentiate from the inner membrane. There are two reports on the drug-induced autophagocytosis in mammalian cells studied by freeze-replica method (6, 11). Both authors showed that the membrane of early autophagic vacuoles had only a few intramembrane particles, and also that their two limiting membranes are apparently
different. It is an important but totally unresolved problem how these two membranes differentiate in the process of formation.

During starvation, the density of intramembrane particles on the vacuolar membrane clearly decreased. Similar results were obtained with wild type cells (data not shown). By the consequence of fusion of many autophagosomes to the vacuoles, intramembrane particles may be diluted in the membrane. Consistent with this result, we reported that size of vacuole becomes larger during starvation (13). But further quantitative analysis is necessary for the fate of autophagosomal membrane fused with the vacuoles.

Recently many mutants which are defective in autophagy were isolated (9, 14, 16). The genetic analysis of these mutants may provide us with further information on the dynamics of the membrane in the autophagic pathway.

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REFERENCES


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