Apg2p Functions in Autophagosome Formation on the Perivacuolar Structure*

Received for publication, March 15, 2001, and in revised form, April 27, 2001
Published, JBC Papers in Press, May 29, 2001, DOI 10.1074/jbc.M102346200

Takahiro Shintani‡, Kuninori Suzuki‡, Yoshiaki Kamada§, Takeshi Noda§, and Yoshinori Ohsumi§

From the Department of Cell Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan and the Department of Molecular Biomechanics, School of Life Science, The Graduate University for Advanced Studies, Okazaki, 444-8585, Japan.

Autophagy is a degradative process in which cytoplasmic components are non-selectively sequestered by double-membrane structures, termed autophagosomes, and transported to the vacuole. We have identified and characterized a novel protein Apg2p essential for autophagy in yeast. Biochemical and fluorescence microscopic analyses indicate that Apg2p functions at the step of autophagosome formation. Apg2p localizes to some membranous structure distinct from any known organelle. Using fluorescent protein-tagged Apg2p, we showed that Apg2p localizes to a dot structure close to the vacuole, where Apg8p also exists, but not on autophagosomes unlike Apg8p. This punctate localization of Apg2p depends on the function of Apg1p kinase, phosphatidylinositol 3-kinase complex and Apg9p. Apg2p, encoded by an apg2-2 allele, shows a severely reduced activity of autophagy and a dispersed localization in the cytoplasm. Overexpression of the mutant Apg2p lessens the defect in autophagy. These results suggest that the dot structure is physiologically important. Apg2p and Apg8p are independently recruited to the structure but coordinate function there to form the autophagosome.

The lysosome/vacuole is a central organelle for macromolecular turnover in eukaryotic cells, where various hydrolytic enzymes reside (1, 2). These enzymes are transported to the vacuole via the secretory or the cytoplasm-to-vacuole targeting (Cvt)1 pathway. On the other hand, their substrates to be degraded are delivered from outside of the cells and the plasma membrane through the endocytic pathway or from the cytoplasm by autophagy. Macroautophagy is a cellular mechanism for bulk degradation and recycling of cytoplasmic components, which may be important for cellular remodeling during development and differentiation (3). Genetic and morphological studies revealed an interesting fact that the biosynthetic Cvt pathway shares the overlapping mechanistic features with autophagy despite the differences in their cellular functions (4–7).

Macroautophagy is a dynamic process involving changes in membrane topology. During the autophagic process, cytoplasmic components including macromolecules and organelles are enveloped by an isolation membrane to form a double membrane-bound structure, termed the autophagosome. Subsequently, its outer membrane fuses to the membrane of the lysosome/vacuole to release a single membrane vesicle into the lumen. Finally, this single membrane structure, termed the autophagic body, is degraded in a protease-dependent manner (8–10). The process of autophagosome formation should require several events such as the supply of lipids or membranes to the site of formation and expansion of the isolation membrane.

Many efforts have been made to understand the molecular mechanism of autophagy. In those processes, a lot of proteins essential for autophagy, such as Apg, Aut, and Cvt proteins, have been identified and characterized (11). We have isolated the APG genes and characterized their gene products, and most of the Apg proteins are now classified into several groups by their functions. The Apg1p kinase complex is comprised of Apg1p, Apg13p, and Apg17p, and the enhancement of its kinase activity is necessary for the induction of autophagy, which is controlled by Apg13p and Apg17p (12, 13). Apg6p/Vps30p and Apg14p are constituents of the autophagy-specific PI 3-kinase complex together with Vps34p and Vps15p (14). The autophagy/Cvt pathway requires two ubiquitination-like systems. The first ubiquitin-like protein, Apg12p, is conjugated to Apg5p via Apg7p (E1) and Apg10p (E2), and the conjugation facilitates the binding of Apg5p to Apg16p (15–18). Apg8p/Aut7p is the second ubiquitin-like protein of which the exposed C-terminal glycine is covalently attached to the amino group of phosphatidyethanolamine. This process is catalyzed by the Apg5p/Aut2p protease, Apg7p (E1), and Apg3p/Aut1p (E2) (19, 20). All these Apg proteins are suggested to function in autophagosome formation, and most of them are associated with certain membranes (13, 14, 21, 22, 24). It was also reported that Apg8p is a potential tracer of the autophagic process. Immuno-EM study revealed that Apg8p localizes to the autophagosomes, the autophagic bodies, and the isolation membranes near the vacuole (23).

Fluorescence microscopic analysis
showed that GFP-fused Apg5p/Aut7p localizes to the punctate structures proximal to the vacuole in addition to autophagic bodies (24). This punctate localization of GFP-Apg5p/Aut7p needs the Apg12p-Apg5p conjugation and Apg5p lipidsation system, suggesting that the structure is physiologically important.

As described above, in the past few years the molecular characterization of Apg proteins have proceeded and allowed their classification into several functional groups. For the further understanding of the molecular mechanism of autophagy, it becomes important to elucidate how these proteins participate in autophagosome formation. However, the APG2 gene has not yet been identified and its identification is essential for an overall understanding of autophagy. In this study, we report the cloning and characterization of Apg2p. By fluorescence microscopic analysis, Apg2p co-localizes with Apg5p to the dot structure close to the vacuole. Its localization is perturbed in some apg mutants and by its own point mutation. These suggest that the structure is crucial for autophagy.

### EXPERIMENTAL PROCEDURES

#### Strains and Media, Genetics, and Molecular Biological Techniques—

The yeast strains used in this study are listed in Table I. Yeast cells are grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose), synthetic complete (SC) medium containing nutritional supplements, or SCD medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acid, and 2% glucose) supplemented with 0.002% adenine sulfate, 0.002% uracil, and 0.002% tryptophan if necessary. For nitrogen starvation, SD-N) medium (0.17% yeast nitrogen base without ammonium sulfate and amino acids and 2% glucose) was used. Standard genetic manipulations were performed as described by Adams et al. (25). DNA manipulations were performed using standard methods (26).

#### Cloning and Disruption of APG2 Gene—The APG2 gene was cloned by complementing the spore morphology-negative phenotype of the apg2-1 diploid strain YTS19 (apg2-1/apg2-1 ADE2/ade2 ura3/ura3) on the basis of random spore analysis as described by Adams et al. (25). YTS19 cells were transformed with a Yep24-based yeast genomic library, spread and grown on SC-Ura plates at 30 °C for 3 days. About 60,000 transformants on 3 plates were collected and pooled as a frozen stock. SC-Ura plates were incubated at 30 °C for 12 h. Then cells were collected from the plate, washed with sterile water three times, and resuspended in 2.5 ml of sporulation medium (1% potassium acetate, 0.025% glucose). After an 8-day incubation at 30 °C, sporulated culture was treated with 1 mg/ml Zymolyase 100T (Seikagaku kogyo) at 30 °C for 1 h and sonicated moderately to kill non-spore cells and disrupt ascii to scatter the spores. One-hundred microliters of the spore suspension was then spread onto SC-Ura plates and incubated at 30 °C for 5 days. The red colonies derived from the ade2 ascospores were picked to exclude the surviving ADE2/ade2 diploid cells (white), and then checked their accumulation of autophagic bodies in SD-N containing 1 mM phenylmethylsulfonyl fluoride (PMSF).

Plasmids were recovered from the positive clones and sequenced with the pBluescript oligonucleotides. Partial sequences were analyzed with the Saccharomyces Genome Data base (genome-www.stanford.edu/Saccharomyces) and the plasmids were found to contain one ORF, YNL242w. The 5.8-kb SphI-KpnI fragment containing the entire YNL242w ORF was subcloned into pUC18 to generate pTS101. The 2.0-kb XbaI-PstI LEU2 fragment from pJJ282 (27) was ligated to XbaI-PstI digested pTS101 to generate pTS104. The 1.75-kb SmaI-HincII HIS3 fragment from pJJ215 (27) was ligated to the Neo-BamHI digested and blunted pTS101 to generate pTS105. The 4.4-kb Sphl-KpnI fragment from pTS105 was used for transformation of KA311A and the 4.05-kb Sphl-KpnI fragment from pTS104 was for transformation of YW5-1B and TN125. The disruption of the APG2 gene was verified by PCR.

### Plasmid Construction—The 5.8-kb Sphl-KpnI fragment containing Apg2p gene was subcloned into YCplac33 and Yep352 to generate pTS102 and pTS103, respectively. The DNA fragments encoding GFP-tagged Apg2p proteins were constructed as follows. The 1.4-kb Sphl-EcoRV fragment from pTS104 was subcloned into pUC18 to generate pTS108. The BamHI site was introduced just after the initiation codon of the Apg2p gene on pTS108 using a QuikChange™ Site-directed Mutagenesis Kit (Stratagene) and the following primers: 5'-TGATT-TGATACAATGGGATCCCATTTTGGTTACCTCA-3' and 5'-TGAG-GTACCAAAATCCCGATTCATTATTGGTACTCCA-3', to generate pTS109. The DNA fragment encoding GFP (S65T) with BamHI site on both sites was then ligated to the BamHI site of pTS108 to generate pTS109. The 5.05-kb BglII-KpnI fragment from pTS109 was then ligated into the BglII-KpnI site of pTS110 to generate pTS111. Finally, pTS112 (GFP-APG2 on YCplac33) was constructed by subcloning the 6.5-kb Sphl-KpnI fragment of GFP-APG2 into YCplac33. pTS114 (YFP-APG2 on YCplac33) was constructed by the same procedure with GFP-APG2 using YFP fragments. For the construction of pTS119 (apg2-2 on YCplac33) and pTS120 (apg2-2 on Yep352), the 5.8-kb Sphl-KpnI fragment containing the apg2-2 allele was amplified from MT82 genomic DNA by PCR and then cloned to YCplac33 and Yep352. The mutation site was verified by DNA sequencing analysis. Site-directed mutagenesis with primers 5'-CCTAACCAGAAGCTGTCGATCGTCTTAAAAGC-3' and 5'-CTTAAACCGAAGCTGTCGATCGTCTTAAAAGC-3' was used to change the glycine at position 83 in APG2 to a glutamate (QuikChange™ Site-directed Mutagenesis Kit, Stratagene) to generate pTS121 (GFP-apg2-2 CEN).

### Antibodies—Antibody to Apg2p was prepared against the recombinant protein corresponding to the S24–1952 amino acid residues of Apg2p (Apg2p-C). To construct the plasmid for bacterial expression of Apg2p-C, a DNA fragment encoding Apg2p-C was amplified by PCR.
Using following primers: 5'-AGGCAGATCTTCTTCTAAAGGGCAATA-CAC-3' and 5'-GTCTGCAAATTTTTAAGATCTGATCATCGATGGA-TTG-3'. The resulting PCR product was digested with BglII and then ligated into the BamHI site of pET15b (Novagen, Madison, WI) to generate pET130. Escherichia coli BL21(DE3) cells transformed with pET130. Escherichia coli BL21(DE3) cells transformed with pET130 were grown up to \(A_{600} = 0.5\) and then incubated in Luria-Bertani medium containing 0.5% L-tryptophan and 0.2 M sorbitol, 5 mM MgCl\(_2\) and 1x protease inhibitor mixture (Complete, EDTA-free; Roche Molecular Biochemicals) with or without 0.5% Triton X-100 and 5 mM CaCl\(_2\). The resulting PCR product was digested with HindIII (Promega, Madison, WI) and BamHI (Promega, Madison, WI). The final product was ligated into the HindIII and BamHI sites of pET15b (Novagen, Madison, WI) to generate pET130. For the protection assay, the P5 fraction equivalent to 32000 cells was suspended in 400 μl of PSM200 buffer, and divided into four aliquots (100 μl). Each aliquot was diluted 2-fold in 100 μg/ml proteinase K, 100 μg/ml protease K plus 0.5% Triton X-100, 0.5% Triton X-100, or distilled water. The samples were incubated for 30 min on ice, and then 200 μl of 20% trichloracetic acid was added to terminate reactions. After centrifugation and two washes with cold acetone, the samples were dissolved in 200 μl of SDS loading buffer, and subjected to immunoblot analysis with anti-API antibody.

**Subcellular Fractionation and Extraction Studies—** Yeast cells were grown in YPD and then incubated in SDS-N, and then converted to spheroplasts. The spheroplasts were lysed in Lysis buffer (20 mM PIPES-KOH, pH 6.8, 0.2 mM sorbitol, 50 mM sodium acetate, 1 mM EDTA, 1 mM PMSF, and 4x protease inhibitor mixture) by extrusion through a polycarbonate filter with 3-μm diameter pores. After removing cell debris during a low-speed spin (5000 g for 5 min), the supernatant (Total) was centrifuged at 13,000 g for 15 min to separate into low-speed supernatant (LSS) and pellet (LSP) fractions. The LSS fraction was then centrifuged at 100,000 x g for 1 h to obtain high-speed supernatant (HSS) and pellet (HSP) fractions. The total membrane fraction was prepared by centrifugation of cell lysate at 100,000 x g for 30 min. This fraction (1 ml) was layered onto an Optiprep (NYCOMED PHARMA AS, Oslo, Norway) step gradient in Lysis buffer (0.5 ml of 50%, 1 ml of 40%, 1 ml of 30%, 1 ml of 25%, 2 ml of 20%, 2 ml of 15%, and 1.5 ml of 10% w/v Optiprep) and centrifuged at 174,000 x g for 16 h at 4 °C in a PS40T rotor (Hitachi). Fourteen
Apg2p Functions in Autophagosome Formation

Apg2p Functions in Autophagosome Formation

30455

RESULTS

Isolation of the APG2 Gene—We isolated and characterized an apg2-1 mutant (31). Wild-type cells accumulated autophagic bodies in their vacuoles in a nitrogen-starvation medium containing 1 mM PMSF, while not in the apg2-1 mutant cells (Fig. 1A). The mutation severely caused loss of viability under starvation conditions (Fig. 1C). Precursor aminopeptidase I (proAPI) is transported to the vacuole by the Cvt pathway and processed to a mature form in a protease A-dependent manner (7). The apg2-1 mutant strain also showed the defect in API maturation in both rich and starvation media, whereas the maturation of other vacuolar enzymes, carboxypeptidase Y and proteinase A, was normal (Fig. 1B), indicating that Apg2p functions in the Cvt pathway but not in the Vps pathway.

All of apg mutants show a defect in sporulation (31). We used this phenotype for cloning of the apg2 gene. Yeast gene libraries were introduced into the apg2-1/apg2-1 diploid strain (ADE2/ade2) and the transformants were subjected to sporulation conditions. A procedure that kills non-spore cells, the spores were grown on SC-Ura plates. The ade2 ascospores show red pigment, so that they can be distinguished from the white colonies including the ADE2/ade2 or ADE2 cells. Therefore, the red colonies were picked to exclude the surviving diploid cells from the screening. More than 500 red colonies were obtained and 16 clones of them were checked for restoration of autophagy. Twelve clones accumulated autophagic bodies in their vacuoles in SD-N containing 1 mM PMSF. Finally, two kinds of plasmodia were yielded from those clones, containing 8.6- and 8.5-kb genomic fragments, respectively. Sequencing and data base analyses revealed that both DNA fragments contained only one entire ORF, YNL242w. It was reported that a Dnl242w homologous diploid cell was unable to sporulate (32), corresponding with our observation for the apg2-1 mutant. The 5.8-kb Sph1-Kpn1 fragment containing the entire ORF was subcloned to YCplac33 and introduced into the apg2-1 cell. A single copy of YNL242w was sufficient to complement the mutation (Fig. 1). Next, to determine whether YNL242w was the authentic APG2 gene, this ORF was disrupted by replacing with a LEU2 or HIS3 gene. The disruptants exhibited the same phenotypes as the apg2-1 mutant for autophagy, activation of proAPI, and viability under starvation conditions (Fig. 1). A diploid cell obtained by crossing apg2-1 and Dnl242w cells was also defective in autophagy (data not shown). We, therefore, concluded that YNL242w is the authentic APG2 gene.

APG2 is a novel gene and encodes a hydrophilic protein of 1,592 amino acids with a predicted molecular mass of 178 kDa. The amino acid sequence of Apg2p provided no insight into its function. A BLAST search identified proteins closely related to Apg2p in human (KIAA0404), Drosophila melanogaster (CG1241), Caenorhabitis elegans (M03A2.2), and Schizosaccharomyces pombe (SPBC31E1.01c). Their functions are not characterized yet, but are expected to be involved in autophagy, as is the case with ApG8t and Apg12p (33, 34).

We generated polyclonal antibody against the C-terminal half of Apg2p protein produced bacterially. As shown in Fig. 2A, the affinity-purified anti-Apg2p antibody specifically recognized a 150-kDa protein in crude yeast extracts, which corresponds well to the predicted molecular mass of Apg2p. This band was not detected in the strain deleted for APG2, and therefore represents Apg2p. The expression level of Apg2p was unaffected by starvation or treatment with the immunosuppressant drug rapamycin, a specific inhibitor of Tor, which...
Apg2p functions in autophagosome formation. Autophagic activities can be measured by monitoring autophagy-dependent processing of the cytotoxic form of alkaline phosphatase Pho8Δ60, which represents nonselective transport of cytoplasmic materials (28, 30). On the other hand, API transport is a highly selective and rapid process compared with macroautophagy. Therefore, API maturation can be a more sensitive index than autophagy that is measured by the activation of Pho8Δ60. Accordingly, autophagy is almost completely defective in all of these strains (Fig. 2B). The G83E mutation seems to cause a severe defect in the Apg2p function, but the excess amount of the protein can compensate its defect in API transport (which can be compensated by the excess amount of the protein only for the defect in API transport).

Apg2p function is required for completion of autophagosome formation—Apg8p is incorporated in autophagosomes, and as a consequence into the vacuolar lumen (22–24). We attempted to understand the functional step of Apg2p by using GFP-Apg8p. In the wild-type cells, GFP-Apg8p stained the dot structures proximal to the vacuole as described by Kim et al. (24). Rapamycin treatment, which mimics starvation conditions, gave the additional vacuolar stain, indicating the transport of Apg8p to the vacuole occurred via autophagy (Fig. 3A). In the Δapg2 cells, GFP-Apg8p still gave one or a few prominent dot stains in both conditions, although no vacuolar stain was observed (Fig. 3A). These results demonstrated that Apg2p is not required for the localization of Apg8p to the dot structure but is important for its transport to the vacuole. The localization of GFP-Apg8p was also observed in the Δypt7-background strain. Ypt7p is a Rab guanosine triphosphatase involved in vesicle fusion to the vacuole in both the carboxypeptidase Y and ALP sorting pathways (35) as well as homotypic vacuole fusion (36). Ypt7p is also required for the fusion of the Cvt vesicle/autophagosome to the vacuole (23, 35, 37). Therefore, in Δypt7 cells autophagosomes accumulate in the cytosol under starvation conditions. One or a few prominent Apg8p dots were observed in growing conditions in Δypt7 cells, too (Fig. 3A). After rapamycin treatment, in addition to the prominent dots, a number of weak GFP-Apg8p dots emerged (Fig. 3A), which represent autophagosomes (23). These dots were not observed in Δypt7Δapg2 cells (Fig. 3A). The results suggest two possibilities. One is that autophagosomes are not formed in the Δapg2 cells. The other is that autophagosomes are formed but Apg8p is not correctly delivered to the autophagosome. The later possibility is based on the fact that even in the absence of Apg8p small autophagosomes are formed (38).

To test these possibilities, we performed the biochemical assay below. It is known that proAPI is transported to the vacuole by macroautophagy under starvation conditions (6). Therefore, by using API as a marker, we determined the step at which the autophagic process was blocked in the Δapg2 cells. First, we examined whether the Δapg2 cells accumulated proAPI in a membrane-associated form under starvation conditions. The Δapg7 cells were used as a control, because Apg7p is shown to act at the step of vesicle formation (36). The lysates from Δapg7 and Δapg2 cells were centrifuged at 5,000 × g to generate the pellet (P5) and supernatant (S5) fraction. About 50% of proAPI was found in the P5 fraction in both cells, while most of ADH, a cytosolic marker protein, was found in the S5 fraction (Fig. 3B). When fractionation was performed in the presence of 0.25% Triton X-100, most proAPI was found in the S5 fraction (Fig. 3B), implying that the sedimentation of proAPI into the P5 fraction was due to a membrane association.

Next, to elucidate whether proAPI was sequestered in mem-

mimics starvation and induces autophagy (data not shown), indicating that Apg2p was constitutively expressed in both growing and starvation conditions.

Using this antibody for immunoblot analysis, we surveyed the expression of Apg2 protein in the original 13 Δapg2 mutants. Among them, only one strain, Δapg2-2 mutant, expressed Apg2p with full size. Sequence analysis revealed that Δapg2-2 contains a single amino acid change from glycine to glutamate at the 83rd amino acid of Apg2p. We cloned the mutant Δapg2 gene, Δapg2-2, and introduced it into the Δapg2 strain. The Δapg2 strain showed a complete defect in API transport under both growing and starvation conditions. Single copy Δapg2-2 did not complement the defect under growing conditions, however, under starvation conditions proAPI was partially matured (Fig. 2C, column 3). This partial maturation was much enhanced when Δapg2-2 was expressed via a multicopy plasmid (Fig. 2, column 5). Thus, the mutation does not cause complete loss of

**Fig. 4.** Biochemical characterization of Apg2p. A, wild-type cells (YW5-1B) grown in YPD or incubated in SD(-N) for 4 h were converted to spheroplasts, lysed, and fractionated by differential centrifugation as described under “Experimental Procedures.” Total cell lysate (Total), low speed pellet (LSP), high speed pellet (HSP), and high speed supernatant (HSS) fractions were subjected to SDS-PAGE, followed by immunoblot analysis using anti-Apg2p and anti-ADH antibodies. B, a total membrane fraction was isolated from the lysate of wild-type cells grown in YPD by centrifugation at 100,000 × g for 30 min. The membrane fraction was incubated in buffer, 2% Triton X-100, 1 M NaCl, 0.1 M Na2CO3, pH 11.5, and 6 M urea on ice for 20 min, before centrifugation at 100,000 × g for 30 min and separation into pellet (P) and supernatant (S) fractions. Equivalent amounts (relative to the starting material) of each fraction were subjected to SDS-PAGE, followed by immunoblot analysis using anti-Apg2p, anti-Kex2p, and anti-ALP antibodies. C, the total membrane fraction from wild-type cells was laid onto the Optiprep density gradient ranging from 10 to 50% (w/v), and centrifuged at 174,000 × g for 16 h as described as described under “Experimental Procedures.” Fractions were collected from the top (fraction 1) to the bottom (fraction 14). Each fraction was subjected to SDS-PAGE and immunoblot analysis with antibodies against Apg2p, ALP (vacuole), Kex2p (late Golgi), Pep12p (endosome), and Sec12p (ER).
brane-bound structures in Δapg2 cells, a protease-protection assay was performed. As a control, we utilized Δypt7 cells. The P5 fractions were prepared from the Δypt7 or Δapg2 cells as described above, and then treated with proteinase K in the absence or presence of 0.25% Triton X-100. It was found that most of proAPI in the Δypt7 cells was resistant to exogenously added proteases and was digested only in the presence of detergent (Fig. 3C). These results indicate that proAPI is sequestered by membrane-bound structures, presumably autophagosomes. On the other hand, proAPI in the Δapg2 cells was completely digested even in the absence of detergent (Fig. 3C). Taken together with the results of fractionation, proAPI accumulated in a membrane-associated but protease-accessible state in the Δapg2 cells. These results suggested that Apg2p plays a role(s) at the step prior to the completion of autophagosome formation.

Apg2p Is Associated to the Membranous Structure Distinct from Any Known Organelle—We then investigated the biochemical property of Apg2p. Lysate from wild-type cells grown in YPD or starved in SD(-N) was centrifuged at 13,000 × g for 15 min to separate into LSS and LSP fractions, and the LSS fraction was further centrifuged at 100,000 × g for 1 h to generate HSS and HSP fractions. Apg2p was mainly fractionated into the HSP and HSS fractions in both growing and starvation conditions, while only a small amount appeared in the LSP fraction. In contrast, ADH, a cytosolic marker protein, was detected only in the HSS fraction (Fig. 4A). Next, to determine the nature of Apg2p association with the membrane, we performed extraction studies with detergent, high salt, high pH, or protein denaturant (Fig. 4B). Triton X-100 should extract both integral and peripheral membrane proteins, and only peripheral membrane proteins could be released from membrane by high salt, high pH, or protein denaturant (Fig. 4B). Triton X-100 should extract both integral and peripheral membrane proteins, and only peripheral membrane proteins could be released from membrane by high salt, high pH, and protein denaturant. Kex2p and ALP are integral membrane proteins resident in the late Golgi and the vacuole, respectively. ALP was partially solubilized by 2% Triton X-100, while Kex2p was fully released from the pellet. In this condition, Apg2p was partially released similar to ALP. Other reagents such as NaCl, Na₂CO₃, and urea efficiently released Apg2p from the pellet, while none of them released Kex2p and ALP from membranes. Thus we concluded that a part of Apg2p behaves as a peripheral membrane protein.

Next, we examined the subcellular localization of Apg2p by Optiprep density gradients (Fig. 4C). A total membrane fraction from wild-type cells under growing condition was applied to the top of an Optiprep step gradient (10–50% (w/v)). After
centrifugation at 174,000 × g for 16 h, 14 fractions were collected from the top of the gradient and examined by immunoblot analysis. Apg2p appeared broadly distributed in fractions 8–12 and did not comigrate with any organelle marker proteins including Sec12p (endoplasmic reticulum), Kex2p (late Golgi), Pep12p (endosome), and ALP (vacuole). Although Apg2p showed a partial cofractionation with Sec12p, it seemed not to reside to the endoplasmic reticulum because most of Sec12p was found in the LSP fraction by differential centrifugation (data not shown). These results indicate that Apg2p localizes to a membrane structure distinct from any known organelle. The distribution profiles of the proteins from the nitrogen-starved cells were essentially identical to those from growing cells (data not shown).

**Apg2p Localizes on the Dot Structures in the Perivacuolar Region**—To examine the localization of Apg2p in a living cell, an N-terminal GFP-tagged Apg2p was constructed. The expression of GFP-Apg2p from a CEN plasmid with the authentic promoter showed partial but significant complementation to the autophagic defect of Δapg2 strain. The defect in proAPI maturation in vegetative growth was fully restored by GFP-Apg2p (Fig. 5D). These results strongly suggest that the localization of Apg2p to the dot structure is tightly coupled with the function of Apg2p. Overexpression of Apg2pG83E should raise the efficiency of functional localization of the protein. Therefore, the structure seems to be important for autophagosome formation.

**The Localization of Apg2p Requires Some Apg Proteins**—Apg proteins are classified into several groups by their functions. To examine whether they are involved in the localization of Apg2p, we performed the microscopic observation in several apg mutant strains representative of each group. The Δapg2-background mutant strains were transformed with the GFP-APG2 plasmid. After the cells were treated with or without rapamycin for 2 h, the localization of GFP-Apg2p was analyzed (Fig. 6). The staining patterns of GFP-Apg2p in these cells were divided into two groups. One group including the Δapg5, Δapg8, and Δapg16 strains showed the same pattern as the wild-type cells: one or a few prominent dots were detected proximal to the vacuole after the treatment with rapamycin (Fig. 5C). The subcellular fractionation by the Optiprep density gradient showed that Apg2p did not comigrate with ALP, a vacuolar membrane protein (Fig. 4C), indicating that the punctate structures on which Apg2p localized were not on the vacuolar membrane.

When GFP-APG2 was introduced via multicopy plasmid into the Δapg2 strain, the signal is evenly detected throughout the cytoplasm (data not shown). This indicated that the localization of Apg2p to the structure would be determined by unknown saturable factors. GFP-Apg2pG83E expressed from a single copy plasmid displayed an essentially cytoplasmic pattern, while a very faint signal was observed at low frequency (Fig. 5D). These results strongly suggest that the localization of Apg2p to the dot structure is tightly coupled with the function of Apg2p. Overexpression of Apg2pG83E should raise the efficiency of functional localization of the protein. Therefore, the structure seems to be important for autophagosome formation.

**Fig. 6. Localization of GFP-Apg2p in apg mutants.** Δapg2 (YTS21), Δapg2Δapg1 (YTS27), Δapg2Δapg5 (YTS28), Δapg2Δapg6 (YTS29), Δapg2Δapg8 (YTS30), Δapg2Δapg9 (YTS31), Δapg2Δapg14 (YTS32), and Δapg2Δapg16 (YTS33) cells harboring GFP-APG2 (pTS112) were grown in SCD medium, and treated with 0.2 μg/ml rapamycin (Rap) for 2 h. Bar, 5 μm.

**Fig. 7. Co-localization of Apg2p with Apg8p.** Δapg2 (GYS104) (A) and Δapg7Δapg2 (YAK2) (B) cells harboring both YFP-APG2 (pTS114, CEN) and CFP-APG8 (CEN) were grown in SCD medium. After treatment with 0.2 μg/ml rapamycin for 5 h, cells were observed under a fluorescence microscope. Bar, 2 μm.
Apg2p Functions in Autophagosome Formation

We hypothesized that both of them might co-localize to a similar distribution pattern of GFP-Apg2p with GFP-Apg8p (Fig. 5) made us hypothesize that both of them might co-localize to the same structure. To test this idea, we examined the double structures proximal to the vacuole (Ref. 24, Fig. 3A). The similar distribution pattern of GFP-Apg2p with GFP-Apg8p (Fig. 5) made us hypothesize that both of them might co-localize to the same structure. To test this idea, we examined the double staining by using YFP-Apg2p and CFP-Apg8p. The Δapg2 strain co-transformed with the YFP-APG2 and CFP-APG8 plasmids was analyzed. As expected, YFP-Apg2p co-localized with CFP-Apg8p on the dot structures close to the vacuole in this strain (Fig. 7A).

GFP-Apg8p was shown to be on autophagosomes accumulated in the Δapg7 cells (Fig. 3A). If Apg2p also localizes to the autophagosomes, the signals of YFP-Apg2p would overlap with all of the CFP-Apg8p signals. To test this hypothesis, the Δapg7Δapg2 cells expressing both YFP-Apg2p and CFP-Apg8p were observed. A number of CFP-Apg8p dots emerged in this strain after rapamycin treatment, while YFP-Apg2p still stained one or a few dots where CFP-Apg8p localized (Fig. 7B), showing the absence of Apg2p on autophagosomes. Taken together, these results indicated that Apg2p and Apg8p co-localize on the dot structures that may be involved in autophagosome formation.

**DISCUSSION**

Here we found that Apg2p localizes to some membranous structure. Identity of the structure is still obscure, but we reasoned that the localization of Apg2p to the structure is important for its function from several lines of evidence. First, Apg8p, engaged in autophagosome formation, also localizes to the structure (Fig. 7). Second, the Apg2p localization is impaired in some apg mutants. In the Δapg6 and Δapg14 strains, Apg2p shows the dispersed distribution in the cytoplasm (Fig. 6). Apg6p and Apg14p are constituents of an autophagy-specific PI 3-kinase complex together with Vps34p and Vps15p (14). Apg9p is also required for the proper localization of Apg2p. How these proteins participate in the autophagosome formation is still to be determined, but at least the mislocalization of Apg2p is partly assigned to these defects. Third, a mislocalization of a point mutant Apg2pG83E seems to be correlated with the decreased activity of its function. In the apg2-2 (apg2-G83E) mutant, API is partially matured under starvation conditions, while null mutant completely blocks its maturation. Furthermore, this partiality was improved by the overexpression of the mutant protein (Fig. 2C), suggesting that the mutation causes a decreased efficiency of some Apg2p function. GFP-Apg2pG83E basically shows the dispersed localization but a small portion is associated to the structure (Fig. 5D). This is consistent with the idea that the localization of Apg2p to the structure is crucial for its function. When Apg2pG83E is over-produced, the amount of protein on the structure might increase, however, we could not test it because overproduction itself causes the overflow of Apg2p to the cytoplasm.

It has already been reported that Apg8p localizes to the dot structure close to the vacuole (23, 24). The dot localization of Apg8p requires the Apg12p-Apg5p conjugation system (24). From the report, however, we are unable to judge whether the conjugation system is involved in the dot structure formation or the targeting of Apg8p to the structure. Our result that Apg2p shows the normal localization even in the Δapg5 or Δapg16 strain provides the evidence for the existence of the structure (Fig. 6). Thus the conjugation is necessary for the membrane association of Apg8p but not for the formation of the structure. In addition, Apg8p is dispensable for the localization of Apg2p (Fig. 6), indicating that the localization of Apg2p to the structure is independent of two ubiquitination-like systems. Apg8p is modified by phosphatidylethanolamine via the Apg8p lipidation pathway, which is required for its localization (19, 20, 24). The Apg8p localization is also unaffected by the loss of Apg2p (Fig. 3A), which corresponds with the fact that Apg2p is not involved in both the Apg8p lipidation and the Apg12p-Apg5p conjugation (15). Taken together, it is suggested that Apg2p and Apg8p are recruited to a certain pre-existing structure independently. The forming autophagosome is heavily stained by an antibody against an HA-tagged Apg8p, implying that Apg8p functions during autophagosome formation (23). Some Apg8p is then trapped within the autophagosomes, while Apg2p is not (Fig. 7B). Apg2p might act prior to the completion of autophagosome formation.

The structure where Apg2p localizes seems to be a nonconventional structure, because Apg2p and known organelle marker proteins did not comigrate in the Optiprep density gradient centrifugation (Fig. 4C). In addition to the membrane-associated population, the soluble Apg2p also exists (Fig. 4A). Overexpression of GFP-Apg2p increases the cytosolic staining (data not shown), suggesting that Apg2p binds to some saturable factors. The dot signals of GFP-Apg2p appear to become prominent by starvation (Fig. 5, C and D), although its total amount does not increase. We hypothesize that Apg2p exists in equilibrium between membrane-bound and dissociated states, and the membrane-bound population increases upon induction of autophagy. Unexpectedly, however, the pelletable Apg2p did not increase even during starvation in our subcellular fractionation conditions (Fig. 4A). In addition, Apg2pG83E is still partially pelletable in the fractionation study as well as the wild-type Apg2p (data not shown), although GFP-Apg2pG83E shows a dispersed distribution (Fig. 5D). These results suggest that all of membrane-bound form does not localize to the dot structure. In the subcellular fractionation, the ratio of the membrane-bound population of Apg2p is varied by salt concentration of lysis buffer, suggesting that the association to the membrane is at least partly defined by the electrostatic interaction. We hypothesize that Apg2p binds to some factor(s) on the membrane through the domain containing the glycine 83 residue. One such candidate is Apg1p. In the apg1 null mutant, the signal of GFP-Apg2p was diffused throughout the cytoplasm, but in the cells expressing Apg1pK54A, a kinase-negative derivative, the distribution of GFP-Apg2p are normal (data not shown). This result suggests that Apg1p itself but not its kinase activity is crucial for the Apg2p localization. We also observed the co-localization of Apg2p and Apg1p on the dot structures near the vacuole (data not shown). Apg1p may interact directly or indirectly to Apg2p to recruit and/or stabilize it. It is unclear whether Apg2p directly interacts with Apg1p kinase complex at the present time. Co-immunoprecipitation of Apg2p and Apg1p has been unsuccessful (data not shown).

Apg2p is a novel protein of which amino acid sequence does not provide any insight into its function. However, the homology search reveals that Apg2p counterparts are widely distributed in eukaryotes. Here we found that Apg2p plays a crucial...
role in yeast autophagy. Therefore, in most eukaryotic cells, its counterparts would function in autophagy.

Acknowledgments—We thank Dr. Daniel J. Klionsky (University of Michigan) for anti-API and anti-Pep12p antibodies. We also thank Akiko Kuma for constructing the strain.

REFERENCES

1. Jones, E. W., Webb, G. C., and Hiller, M. A. (1997) in The Molecular and Cellular Biology of the Yeast Saccharomyces: Cell Cycle and Cell Biology (Pringle, J. R., Broach, J. R., and Jones, E. W., eds) pp. 363–470, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

2. Bryant, N. J., and Stevens, T. H. (1998) Microbiol. Mol. Biol. Rev. 62, 230–247

3. Klionsky, D. J., and Ohsumi, Y. (1999) Annu. Rev. Cell Dev. Biol. 15, 1–32

4. Harding, T. M., Hefner-Gravink, A., Thumm, M., and Klionsky, D. J. (1996) J. Biol. Chem. 271, 17621–17624

5. Scott, S. V., Hefner-Gravink, A., Morano, K. A., Noda, T., Ohsumi, Y., and Klionsky, D. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12304–12308

6. Baba, M., Osumi, M., Scott, S. V., Klionsky, D. J., and Ohsumi, Y. (1997) J. Cell Biol. 138, 37–44

7. Takeshige, K., Baba, M., Tsuboi, S., Noda, T., and Ohsumi, Y. (1992) J. Cell Biol. 119, 301–311

8. Baba, M., Takeshige, K., Baba, N., and Ohsumi, Y. (1994) J. Cell Biol. 124, 903–913

9. Baba, M., Osumi, M., and Ohsumi, Y. (1995) Cell Struct. Funct. 20, 465–471

10. Kim, J., and Klionsky, D. J. (2000) Annu. Rev. Biochem. 69, 303–342

11. Kim, J., and Ohsumi, Y. (2000) J. Cell Biol. 150, 1507–1513

12. Scott, S. V., Nice, D. C., 3rd, Nau, J. J., Weisman, L. S., Kamada, Y., Keizer-Gunnink, I., Funakoshi, T., Veenhuis, M., Ohsumi, Y., and Klionsky, D. J. (2000) J. Biol. Chem. 275, 25840–25849

13. Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000) J. Cell Biol. 151, 263–276

14. Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimizu, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000) Nature 408, 488–492

15. George, M. D., Baba, M., Scott, S. V., Mizushima, N., Garrison, B. S., Ohsumi, Y., and Klionsky, D. J. (2000) Mol. Biol. Cell 11, 903–912

16. Huang, W. P., Scott, S. V., Kim, J., and Klionsky, D. J. (2000) J. Biol. Chem. 275, 5845–5851

17. Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999) J. Cell Biol. 147, 435–446

18. Kim, J., Huang, W. P., and Klionsky, D. J. (2001) J. Cell Biol. 152, 51–64

19. Adams, A., Gottschling, D. E., Kaiser, C. A., and Stearns, T. (1998) Methods in Yeast Genetics, A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

21. George, M. D., Baba, M., Scott, S. V., Mizushima, N., Garrison, B. S., Ohsumi, Y., and Klionsky, D. J. (1999) Mol. Biol. Cell 11, 903–908

22. Huang, W. P., Scott, S. V., Kim, J., and Klionsky, D. J. (1996) J. Biol. Chem. 271, 17621–17624

23. Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999) J. Cell Biol. 147, 435–446

24. Kim, J., Huang, W. P., and Klionsky, D. J. (2001) J. Cell Biol. 152, 51–64

25. Adams, A., Gottschling, D. E., Kaiser, C. A., and Stearns, T. (1998) Methods in Yeast Genetics, A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

27. Jones, S. J., and Prakash, L. (1990) Yeast 6, 363–366

28. Baba, M., Osumi, M., Ohsumi, Y., and Klionsky, D. J. (1998) J. Biol. Chem. 273, 395–400

29. Baba, M., Osumi, M., Ohsumi, Y., and Klionsky, D. J. (1998) J. Biol. Chem. 273, 5845–5851

30. Baba, M., Osumi, M., Ohsumi, Y., and Klionsky, D. J. (1998) J. Biol. Chem. 273, 395–400

31. Tsukada, M., and Ohsumi, Y. (1998) FEBS Lett. 433, 169–174

32. Saiz, J. E., Santos, M. A., Vazquez de Aldana, C. R., and Revuelta, J. L. (1999) Yeast 15, 155–164

33. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) EMBO J. 19, 5720–5728

34. Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kaheya, Y., Suzuki, K., Tokuniwa, T., Ohsumi, Y., and Yoshimori, T. (2003) J. Cell Biol. 152, 657–667

35. Wurmser, A. E., Sato, T. K., and Emr, S. D. (2000) J. Cell Biol. 151, 551–562

36. Haas, A., Schegelmilch, D., Lazay, T., Gallwitz, D., and Wickner, W. (1995) EMBO J. 14, 5258–5270

37. Kim, J., Dalton, V. M., Eggerton, K. P., Scott, S. V., and Klionsky, D. J. (1999) Mol. Biol. Cell 10, 1337–1351

38. Abeliovich, H., Dunn, W. A., Jr., Kim, J., and Klionsky, D. J. (2000) J. Cell Biol. 151, 1025–1034

39. Vida, T. A., and Emr, S. D. (1995) J. Cell Biol. 126, 779–792

40. Irie, K., Takase, M., Lee, K. S., Levin, D. E., Araki, H., Matsumoto, K., and Oshima, Y. (1993) Mol. Biol. Cell 13, 3076–3083

41. Abeliovich, H., Darsow, T., and Emr, S. D. (1999) EMBO J. 18, 6005–6016
Apg2p Functions in Autophagosome Formation on the Perivacuolar Structure
Takahiro Shintani, Kuninori Suzuki, Yoshiaki Kamada, Takeshi Noda and Yoshinori Ohsumi

J. Biol. Chem. 2001, 276:30452-30460.
doi: 10.1074/jbc.M102346200 originally published online May 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102346200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 28 of which can be accessed free at
http://www.jbc.org/content/276/32/30452.full.html#ref-list-1