Apg14p and Apg6/Vps30p Form a Protein Complex Essential for Autophagy in the Yeast, *Saccharomyces cerevisiae*

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Mutation in the *Saccharomyces cerevisiae* APG14 gene causes a defect in autophagy. Cloning and structural analysis of the *APG14* gene revealed that *APG14* encodes a novel hydrophilic protein with a predicted molecular mass of 40.5 kDa, and that Apg14p has a coiled-coil motif at its N terminus region. We found that overproduction of Apg14p partially reversed the defect in autophagy induced by the *apg6-1* mutation. The *apg6-1* mutant was found to be defective not only in autophagy but also in sorting of carboxypeptidase Y (CPY), a vacuolar-soluble hydrolase, to the vacuole. However, overexpression of *APG14* did not alter the CPY sorting defect of the *apg6-1* mutant, nor did the *APG14* null mutation affect the CPY sorting pathway. Structural analysis of *APG6* revealed that *APG6* is identical to *VPS30*, which is involved in a retrieval step of the CPY receptor, Vps10p, to the late-Golgi from the endosome (Seaman, M. N. J., Marcusson, E. G., Cereghino, J. L., and Emr, S. D. (1997) *J. Cell Biol.* 137, 79–92). Subcellular fractionation indicated that Apg14p and Apg6p peripherally associated with a membrane structure(s). Apg14p was co-immunoprecipitated with Apg6p, suggesting that they form a stable protein complex. These results imply that Apg6p/Vps30p has two distinct functions in the autophagic process and the vacuolar protein sorting pathway. Apg14p may be a component specifically required for the function of Apg6p/Vps30p through the autophagic pathway.

Cell growth is governed by a fine-tuned balance between the synthesis and degradation of proteins. In mammalian cells, both lysosomal and nonlysosomal protein degradation mechanisms are responsible for turnover of endogenous proteins. Intracellular proteolytic activity is essential for cells to survive in various extracellular environments. Autophagy is the bulk degradation of cytoplasmic components in the lysosome/vacuole (1–4). Under serum starvation conditions, animal cells induce autophagy to supply amino acids (5). Autophagy starts with formation of the autophagosome, a cytoplasmic membrane structure surrounding cytosolic components or organelles.

outer membrane of the autophagosome subsequently fuses with the lysosomal membrane, and the contents are degraded in a lysosomal proteinase-dependent manner (6). Although mammalian autophagy has been characterized with morphological and biochemical approaches, the molecular basis of each process is still unclear.

Recent studies revealed that autophagy takes place in the budding yeast, *Saccharomyces cerevisiae*, in a similar manner to that of higher eukaryotes (7, 8). Several lines of investigation showed that yeast autophagy is composed of the processes as follows: (i) starvation signaling, (ii) formation of autophagosome, (iii) targeting of autophagosome to the vacuole, (iv) fusion of the outer membrane of the autophagosome to the vacuolar membrane and release of the autophagic body in the vacuole, and (v) degradation of the autophagic body in the vacuole (4, 7–9). To elucidate the complex phenomena at a molecular level, autophagy-defective mutants (*apg* mutants) were isolated, and at least 14 *APG* genes were shown to be essential for yeast autophagy (10). The phenotypic similarities among the 14 *apg* mutants suggest that the Apg proteins are involved in close processes in the autophagic pathway. Previous morphological studies showed that none of the *apg* mutants could form autophagosomes, suggesting the *APG* products function at autophagosome formation or at earlier steps of autophagic processes. So far, most of the *APG* genes have been cloned and characterization of the Apg proteins is underway (11–13). However, their functions in the autophagic pathway remain to be elucidated.

In this study, we report the structural and functional analyses of Apg14p and Apg6p. *APG6* were identical to *VPS30*, which is involved in the vacuolar protein sorting pathway (14, 15). Our findings suggest that Apg6p/Vps30p has two distinct functions, both on autophagy and vacuolar protein sorting pathways, and that Apg14p is specifically required for the function of Apg6p/Vps30p in autophagy.

EXPERIMENTAL PROCEDURES

*Strains, Media, and Genetic Methods—* Yeast strains used (Table I) were derived from X2180-1A and X2180-1B (Yeast Genetic Stock Center, Berkeley, CA). The media used for yeast were described previously (11). Standard genetic methods were performed as described previously (16). Yeast transformation was carried out as described elsewhere (17).

*Alkaline Phosphatase Assay—* Assay of alkaline phosphatase was performed as described previously (9). Protein concentration of the cell lysate was examined with Bradford’s method (18).

*Isolation, Sequencing, and Disruption of APG14 and APG6—* Standard molecular biological techniques for the manipulation of DNA were used throughout this study (19). Yeast strain MT54-4-2 was transformed with a yeast genomic library based on YCP50. Approximately 3,200 Ura+ transformants were replica-plated to 0.17% yeast nitrogen base without amino acids and ammonium sulfate and 2% glucose (SD–N) plates including 5 μg/ml phloxine B and incubated for 48–72

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**Apg14-Apg6 Protein Complex Required for Autophagy in Yeast**

**TABLE I**

| Strains | Genotypes | Source |
|---------|------------|--------|
| YWS5–1B | MATa leu2 ura3 trp1 | Y. Wada |
| YNY1   | MATaMATa leu2/leu2 ura3 ura3 trp1/trp1 | Y. Wada |
| TN124  | MATa ura3 trp1 leu2 PHO8::PHO8 ∆60 pho13::LEU2 | Noda et al. (24) |
| M19–4–3 | MATa apg6–1 ura3 | M. Tsukada |
| MT37–4–3 | MATa apg6–1 ura3 | M. Tsukada |
| SKV6DP  | MATa apg6–1 ura3 PHO8::PHO8 ∆60 | This work |
| SKG6DP  | MATa apg6–1 LEU2 ura3 leu2 trp1 | This work |
| SKD6–1D | MATa apg6::LEU2 ura3 leu2 trp1 | This work |
| MT54–1–2 | MATa apg6–1 ura3 | M. Tsukada |
| SKD14–1O | MATa apg6::LEU2 ura3 leu2 trp1 | This work |
| SKD14–4F | MATa apg14::LEU2 ura3 leu2 trp1 | This work |
| SKDV29L | MATa ∆apg6::LEU2 ura3 leu2 trp1 | This work |
| SKDV35L | MATa ∆psp35::LEU2 ura3 leu2 trp1 | This work |

h at 30 °C. Seven white colonies were picked up and subsequently examined for their autophagic activity by a morphological assay as described previously (4, 10). Four autophagy-positive (Agp+) clones were obtained, and the genomic fragments were isolated from them. Partial sequencing analysis showed that the 9-kb genomic fragment obtained was identical to a region on chromosome II, and the 2.0-kb EcoRI-ClaI fragment containing a sole open reading frame YBR128c was found to complement the apg14-1 mutation. The DNA region was introduced into pRS306 (20), and subsequently integrated at the AFG14 locus (data not shown), confirming that it contains the authentic AFG14 gene.

Plasmid pYAPG1411 containing the 1.6-kb EcoT22I-ClaI fragment in pBluescript II SK+ (Stratagene) was digested with EcoRI, and ligated with the 2.0-kb SalI fragment from plJZ283 (21). The resultant plasmid pYAPG14DL1 was digested with BamHI and HindIII and introduced into a diploid strain YNY1, and Leu+ transformants were obtained. Correct integration of the apg14-1::LEU2 fragment into the chromosomal APG14 locus was verified by Southern hybridization (data not shown). One Leu+ transformant was selected and sporulated; then tetrads were dissected and germinated on YEPD plates.

Cloning of the APG6 gene was performed as described above except that we used a yeast genomic library based on the YEp13 vector plasmid. After integration mapping and subcloning, we obtained the 3.8-kb PvuII fragment that contains the APG6 gene. For nucleotide sequencing, the PvuII fragment was inserted into pBluescript II SK+ at the SalI site; then nested deletion series were constructed by treatment with exonuclease III and mung bean nuclease (22). The single strand DNA was isolated by infecting helper phage M13KO7 and used for templates for DNA sequencing. To make the APG6 deletion mutant, the 3.8-kb PvuII fragment was cloned into pBluescript II SK+ at the PvuII site, and the 1.1-kb EcoRI-XhoI region was replaced with the 2.0-kb SalI fragment from plJZ283. The resultant plasmid pYAPG6DL1 was digested with PstI and the apg6::LEU2 fragment was used for disruption of the chromosomal APG6 locus.

**Isolation of apg6-1 Mutant Allele**—The apg6-1 mutant allele was recovered as described previously (23). In brief, genomic DNA was prepared from mutant strain M79-4-4 as described elsewhere (16), and the mutant allele was amplified by a polymerase chain reaction using primers 5′-AGGATTCGCTGAGGCGAACATGT-3′ and 5′-AAGGATCCTTGGCATGCTTCTTCT-3′ ex Taq DNA polymerase (Takara, Japan). The apg6 disruptant cells were co-transformed with both the amplified DNA fragment of 1.6 kb and pYAPG650 (CEN APG6) predigested at Sphi, which is the sole site in the APG6 open reading frame. Obtained transformants were examined for Apg phenotype by checking for the accumulation of AB+ (see above). From Apg+ transformants, the plasmid DNA was recovered and the mutation site was determined.

**Production of Anti-Apg6p Antibody**—The 1.4-kb EcoRI-HindIII fragment of APG6 was subcloned into pUR292 to generate a plasmid pLAC-APG6EH. The β-galactosidase-Apg6p fusion protein was expressed in E. coli JM109 and purified with electrodution from SDS-polyacrylamide gel electrophoresis gel. The purified β-galactosidase-Apg6p fusion protein was used for preparation of polyclonal antibody in a rabbit. Immunization was carried out at Shibayagi Co. (Maebashi, Japan).

**Immunoblotting Analyses, Vacular Protein Sorting, Subcellular Fractionation, and Differential Solubilization**—Total yeast lysate was prepared by mixing cells with glass beads as described previously (25). Immunoblotting analyses were performed as described previously (24, 25). Vacular sorting of carboxypeptidase Y (CPY) was analyzed by the method previously reported (15). Immunoprecipitation of CPY and alcohol dehydrogenase in the cellular and medium fractions and fluorography were done as described previously (26, 27).

Subcellular fractionation by differential centrifugation was performed as described elsewhere (25) with spheroplasting media YEPD-SPM (1% yeast extract, 2% peptone, 0.5% glucose, 50 mM Tris-Cl (pH 7.5), 1.2 mM sorbitol, 40 mM β-mercaptoethanol) for vegetatively growing cells and SPM (Tris-Cl (pH 7.5), 1.2 mM sorbitol, 40 mM β-mercaptoethanol) for starved cells. For cell lysis, spheroplasts were suspended in ice-cold lysis buffer (0.2 mM sorbitol, 20 mM triethanolamine (pH 7.2), 1 mM EDTA, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 μg/ml aproin). After centrifugation at 500 × g for 3 min, the cleared cell lysate (total lysate) was subsequently centrifuged at 10,000 × g for 20 min to generate a low speed pellet and low speed supernatant, then the low speed supernatant was further centrifuged at 100,000 × g for 1 h to generate a high speed pellet and high speed supernatant. Each fraction was then subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotting was carried out as described above.

For the differential solubilization experiment, cell lysates were prepared as described above and treated with buffer, 1 mM NaN3, 0.1 mM Na2CO3 (pH 11.0), 2 mM urea, and 2% Triton X-100 on ice for 30 min, and centrifuged at 100,000 × g for 1 h. The resultant pellet and supernatant fractions were subjected to immunoblotting analysis.

**Immunoprecipitation of Apg6p**—Immunoprecipitation of Apg6p was performed essentially as described elsewhere (28) with slight modification.

In brief, yeast cells were collected, washed with distilled water containing 10 mM NaCl, then converted to spheroplasts and lysed osmotically in IP-lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, 1 mg/ml pepstatin A, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After centrifugation at 500 × g for 3 min twice, the protein concentration was determined. The sample was adjusted to a protein concentration of 2 mg/ml with ice-cold IP-lysis buffer. To the sample, 0.1 volume of 10% Nonidet P-40 and 0.05 volume of protein A-Sepharose 4B (50% slurry, Amersham Pharmacia Biotech) were added, and preabsorption was performed at 4 °C for 1 h. To remove the protein A-Sepharose, the sample was microcentrifuged at 5000 rpm for 15 s, and the supernatant was recovered. Then the first antibody was added (3.5 μl of anti-Apg6p antiserum per 1 mg of lysate) was used for immunoprecipitation of Apg6p to the preabsorbed lysate and incubated at 4 °C for 12 h. Forty μl of protein A-Sepharose (50% slurry) were added and incubated at 4 °C for 2 h to recover the IgG and washed with IP-lysis buffer containing 1% Nonidet P-40 four times, and the immunoprecipitated proteins were obtained by boiling the protein A-Sepharose with SDS-polyacrylamide gel electrophoresis buffer for 5 min.

**Disruption of VPS35 and VPS29**—To obtain DNA regions of VPS35 and VPS29, the gene fragments were amplified from genomic DNA with 5′-TTTACCCGGGACATACTTCTCT-3′ (35-R), 5′-TGTGAGGGCCACAGCAT-3′ (35-R), and 5′-CAGATGTTAGTTGAGG-3′ (29-F), 5′-ATTAGCTTAGGAGGAG-3′ (29-R) primer pairs, respectively. For disruption of VPS35, the 3.0-kb EcoRV fragment was subcloned.

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2 The abbreviations used are: AB, autophagic body; kb, kilobase pair(s); PMSF, phenylmethylsulfonyl fluoride; CPY, carboxypeptidase Y; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; APl, aminopeptidase I.
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RESULTS

apg14 Mutant Exhibits a Defect in Autophagy—Previously we had isolated the apg14 mutant which is defective in autophagy (10). When wild-type cells were incubated in a nitrogen-depleted medium containing PMSF, the final membrane structure of autophagy, ABs, are accumulated in the vacuole (Fig. 1A). However, the apg14-1 mutant shows no ABs accumulating in the vacuole, indicating that the mutation causes a defect in autophagy. Moreover, the mutant showed loss of viability under nitrogen starvation conditions and was also defective in autophagy-dependent alkaline phosphatase processing (Fig. 1B). These results indicate that APG14 is essential for autophagy.

Apg14p Is Peripherally Associated with Membrane Fractions—To identify the gene product of APG14, an epitope-tagged version of Apg14p was constructed by introducing the c-Myc 11 amino acids epitope (EQKLISEEDLN). Six repeats of Myc were inserted into the N terminus of Apg14p to obtain a significant signal for characterization of Apg14p. A deduced molecular mass of the Myc-tagged Apg14p was 49.4 kDa (Fig. 2A). A plasmid pYAPGS316-6m14 (CEN 6 × MYC-APG14) complemented the defect in autophagy of the apg14 disruptant, indicating the Myc-tagged Apg14p was functional in vivo (data not shown). Using anti-Myc monoclonal antibody 9E10 (30) and antibodies that recognize the Myc epitope sequences, a 52-kDa protein was detected in yeast whole cell extract by immunoblot analysis. The Myc-Apg14p was found to be expressed constitutively in growing cells and the amount of the product did not change during nitrogen-starvation (Fig. 2A).

The intracellular localization of Myc-Apg14p was investigated by subcellular fractionation experiments. We found that most of Myc-Apg14p was detected in precipitable fractions, mainly in the low speed pellet fraction (Fig. 2B).

To examine the nature of the association in the precipitable fractions, differential solubilization experiments were performed. As shown in Fig. 3C, Myc-Apg14p was solubilized completely with alkali-treatment, and partially with urea or Triton X-100 treatment. This suggests that Myc-Apg14p associates with a membrane fraction and the association must be peripheral, because Apg14p does not have apparent transmembrane domains or sites for lipid modification.

Overproduction of Apg14p Suppresses the Autophagic Defect of the apg6-1 Mutant—The quite similar phenotypes exhibited by the apg mutants suggested that some of the Apg proteins act together in the autophagic processes. Moreover, Myc-Apg14p was found to peripherally associate with a membrane, suggesting there are other components which act with Apg14p. To test this, we looked for genetic interaction between APG14 and other APG genes. We found that apg6-1 cells carrying pKTm14, a multiple copy plasmid harboring APG14 downstream of the GAPDH promoter, accumulated ABs in the vacuole (Fig. 3A). The result indicates that the autophagic defect of the apg6-1 mutant was suppressed with APG14 overexpression, whereas the apg14-1 mutation was not suppressed with APG6 overexpression (data not shown).

Structural Analysis of APG6 and apg6-1 Mutant Allele—For

FIG. 1. Phenotype of autophagy defective mutants. A, apg14 and apg6 are defective in accumulation of autophagic bodies in the vacuole. Cells of wild-type (YWS-1B; a and b), apg14-1 (MT54-4-2; c), and apg6-1 (MT9-4-4; d) were grown to 2 × 10⁶ cells/ml in YEPD and incubated in SDX (−N) in the presence (b—d) or absence (a) of 1 mM PMSF for 4.5 h. The ABs were observed with phase-contrast microscopy. ABs are indicated with arrowheads. Bar, 2 µm. B, apg14 and apg6 show loss of viability under starvation. Vegetatively growing cells of YWS-1B (closed circles), MT54-4-2 (open circles), MT9-4-4 (open squares), and MT37-4-3 (apg5-1; open triangles) in YEPD were transferred to SDX (−N) and incubated at 30 °C. At the time points indicated, viability of the cells was examined as described previously (11).
characterization of the APG6 gene, cloning of authentic APG6 was carried out (see “Experimental Procedures”). APG6 encodes a protein with expected molecular mass of 63.3 kDa and has a heptad repeat of leucine residues. The central portion of Apg6p is strongly predicted to form coiled-coil structures (29). No other remarkable features including hydrophobic cluster for transmembrane region, signal sequences, or sites for post-translational modifications were found. To determine the mutation site of the apg6-1 allele, the mutant allele was cloned from mutant genomic DNA and sequencing was carried out (see “Experimental Procedures”). Structural analysis of the apg6-1 mutant allele revealed that a single nucleotide substitution (C to T) at base position 1805 caused a nonsense mutation at glutamine residue 269 in the apg6-1 mutant (Fig. 6A, asterisk). Next, to analyze the physiological roles of APG6, the genomic APG6 locus was disrupted as described under “Experimental Procedures” (Fig. 6B). The disruptant strain SKD6-1D was viable, indicating that Apg6p function is not essential for vegetative growth in yeast. Δapg6 cells showed a defect in autophagy (Fig. 6C) and loss of viability under nitrogen starvation conditions similar to the APG14 disruptant (data not shown).

Genetic Interaction between APG14 and APG6—To analyze the effects of the mutants on autophagic expression, SKY6DP (apg6-1) and SKD6DP (Δapg6) were transformed with pKTm14. Autophagic activity of the apg6-1 mutant was rescued approximately 35% by overexpression of APG14. However, the suppression was not observed in the Δapg6 disruptant (Fig. 5B). Immunoblot analysis showed that the apg6-1 mutant gene product was stably expressed as a protein of 35 kDa (data not shown). These results suggest that the existence of the truncated form of Apg6p is necessary for reversal of autophagy by Apg14p overproduction. Furthermore, overproduction of the mutant Apg6p from the apg6-1 allele weakly complemented the autophagic defect of the Δapg6 disruptant (data not shown), suggesting the N-terminal half of the protein contains an essential function for Apg6p.

Subcellular Localization of Apg6p—To detect the APG6 gene product, we generated a polyclonal antibody against Apg6p (see “Experimental Procedures”). The antibody recognized a 65-kDa

FIG. 3. Gene disruption of APG14. A, scheme of APG14 deletion. A restriction map of the 2.0-kb genomic DNA fragment containing the APG14 locus. The APG14 coding sequence is indicated by a black arrow; C, ClaI; D, DraI; H, HindIII; Hc, HinII; N, NcoI; RV, EcoRV. B, Δapg14 is defective in accumulation of ABs. Wild-type cells (YW5-1B; a) and Δapg14 (SKD14-1C; b) were grown in YEPD to mid-log phase and transferred to SD(2N) containing 1 mM PMSF. After incubation at 30 °C for 6 h, the cells were observed with phase contrast microscopy. Bar, 2 μm. C, autophagic activity of SKD14 and Δapg14 cells. Wild-type (TN124) and Δapg14 (SKD14PD) carrying pRS316 (vector) or pYAPG1405 (APG14) were grown in YEPD and transferred to SD(2N) at 30 °C for 0 (open bars) and 4.5 h (solid bars), alkaline phosphatase (ALP) activity of Pho8Δ360p in each cell extract was measured.
protein in the total lysate of wild-type cells. Since the protein was not detected in the \textit{apg6} deletion mutant, we concluded that this 65-kDa protein is the \textit{APG6} gene product. Subcellular localization of Apg6p was determined by subcellular fractionation (see “Experimental Procedures”). As a result, Apg6p was fractionated in precipitable fractions and was detected mainly in the 10,000 g pelletable fraction (Fig. 7B, LSP). As shown in Fig. 7C, Apg6p was solubilized by treatment with the reagents indicated. Treatment with 1 M NaCl, 0.1 M Na$_2$CO$_3$, or Triton X-100 effectively solubilized Apg6p. This suggests that Apg6p peripherally associates with some membrane structure(s).

Apg14p and Apg6p Are Part of a Protein Complex—The \textit{apg14} and \textit{apg6} mutants have similar phenotypes, and over-
production of Apg14p suppresses the autophagic defect of the apg6-1 mutant (Fig. 5). Furthermore, Apg14p and Apg6p appear to be associated with membrane fractions (Figs. 4 and 7). These results strongly suggest that Apg14p and Apg6p physically interact and act together through autophagic processes. To address this possibility, immunoprecipitation of Apg6p was carried out from SKD14-1C cells expressing Myc-Apg14p. Spheroplasts were gently lysed and the extract was subjected to immunoprecipitation with anti-Apg6p antiserum. The immunoprecipitants were used for immunoblot analysis with anti-Myc monoclonal antibody. As shown in Fig. 8, Myc-Apg14p was precipitated and subjected to SDS-polyacrylamide gel electrophoresis.

APG6 Is Identical to VPS30, Involved in the Vacular Protein Sorting Pathway—Structural analysis of APG6 revealed that it is identical to the VPS30 gene. Recently, the VPS30 gene, which is involved in sorting the vacuolar soluble protease, CPY, was cloned and characterized (15, 31). In vps30 mutant cells, Vps10p is thought to be a Golgi resident CPY receptor and required for transport of CPY to the endosome from the late-Golgi. Emr and colleagues proposed a scheme that Vps30p is identical to the VPS30 gene, subcellular fractionation of Apg6p and C, differential solubilization of Apg6p were performed with wild-type (YWS5-1B) cells as described in Fig. 4. The asterisk indicates nonspecific signal detected in the high speed supernatant (HSS). LSP and HSP, low and high speed pellet, respectively.

FIG. 7. Apg6p is a membrane bound protein. A, immunoblot of Apg6p. Cell extracts from wild-type (W.T.) (YWS5-1B) and Δapg6 (SKD6-1D) were prepared, and Apg6p was detected by immunoblotting with anti-Apg6p antibody. B, subcellular fractionation of Apg6p and C, differential solubilization of Apg6p were performed with wild-type (YWS5-1B) cells as described in Fig. 4. The asterisk indicates nonspecific signal detected in the high speed supernatant (HSS). LSP and HSP, low and high speed pellet, respectively.

FIG. 8. Apg14p and Apg6p form a protein complex. Δapg14 (SKD6-1C) and Δapg6 (SKD6-1D) cells carrying pYAPG6m14 (6 × Myc-APG14) were grown in YEPD to mid-log phase, transferred to SD (–Nt), and incubated at 30°C for 3 h. Before and after starvation, cells were collected and immunoprecipitation of Apg6p was carried out. The precipitants were subjected to immunoblotting analysis of Myc-Apg14p with anti-Myc monoclonal antibody (9E10).
from its defect in the vacuolar protein sorting pathway, we examined the involvement of VPS35 and VPS29 in autophagy. As shown in Fig. 10, these vps mutants accumulated ABs in their vacuoles as observed in wild-type cells, whereas the Apg6/VPS30 disruptant did not accumulate ABs. This indicates that these VPS genes are not essential for the autophagic process and the autophagic defect in the apg6-1 mutant is not caused by mislocalization of the CPY receptor and subsequent CPY missorting.

**DISCUSSION**

Previously, a series of autophagy defective mutants (apg mutants) was isolated and so far 14 APG genes were shown to be involved in autophagy in yeast (10). Under nutrient starvation conditions, all the apg mutants show defects in autophagy, indispensable for bulk protein degradation and survival under adverse environmental conditions (10). Electron microscopic analyses of these apg mutants showed that none of them accumulated autophagosomes, suggesting that the Apg proteins are involved in early steps of autophagy, such as the formation of the autophagosome.

In this report, we characterized a novel factor Apg14p, which is essential for autophagy. Apg14p is a hydrophilic protein with coiled-coil regions at its N terminus. Overexpression of Apg14p suppressed the autophagic defect of the apg6-1 mutant. Structural analysis revealed that Apg6 is identical to VPS30, which is involved in the vacuolar protein sorting pathway (15). Apg6 encodes a hydrophilic protein with a leucine zipper motif. Apg14p and Apg6p form a protein complex, and they are associated in precipitable fractions. However, their intracellular localization is still unclear.

**Apg14p and Apg6p Form a Protein Complex That Peripherally Associated with a Membrane**—Since Apg14p and Apg6p form a heteromeric protein complex, it is likely that they function together in the autophagic pathway. A large portion of this complex is peripherally associated with membrane structure(s). However, a differential solubilization experiment showed that Apg14p was not solubilized with 1 M NaCl treatment, whereas most of Apg6p was solubilized. Moreover, Apg14p and Apg6p were still detected in precipitable fractions in Apg6 and Apg14 cells, respectively. These indicate that other components are included in the Apg14-Apg6 protein complex.

A previous report on Apg6/Vps30p showed that this protein is partly associated with membrane, while most (~80%) was fractionated in the supernatant (15). We performed the same experiment several times and we found Apg6p in precipitable fractions reproducibly as shown here. Although we tried the experiment with their wild-type cells and their experimental conditions, Apg6p fractionated in the pellet. When we performed the same experiment with Myc-tagged Apg6p, the results were the same. At this moment, we have no explanation for the discrepancy of the results found between the two laboratories.

Sequential centrifugation and differential solubilization experiments revealed that Apg14p and Apg6p are associated with membrane(s) (Figs. 4 and 7). To address intracellular localization of these proteins, we performed iodixanol density gradient centrifugation and found that these gene products are co-fractionated in a fraction of 1.16–1.18 g/cm³. And the fraction did not correspond to the peak fractions of other organelle marker proteins, such as alkaline phosphatase (vacuolar membrane), Kex2p (late-Golgi), Sec12p (endoplasmic reticulum), F₁F₀-ATPase β-subunit (mitochondria), or Pma1p (plasma membrane) (data not shown). These results imply that the Apg14-Apg6 protein complex is localized to an unknown membrane structure.

The apg6-1 mutant gene product was analyzed. We found that the C terminus truncated form of Apg6p is stably expressed and was fractionated in the 100,000 x g supernatant (data not shown). We presume that the C-terminal region is responsible for localization of Apg6p to precipitable fractions, and the association with the membrane fraction(s) is required for Apg6p function through autophagy and CPY sorting.

**Apg14-Apg6 Protein Complex Is Required for Aminopeptidase I (API) Transport**—API is a vacuolar soluble hydrolase. It is constitutively synthesized as an inactive precursor form (proAPI) in the cytosol and the precursor is targeted to the vacuole. In contrast to other vacuolar proteins, maturation of proAPI was shown to be independent of the secretory pathway (34, 35). All the apg mutants were defective in proAPI maturation, indicating that autophagy and API targeting utilize overlapping molecular machinery (36, 37). Recent studies on the API targeting mechanism showed that proAPI is sequenced by an autophagosome-like double membrane structure and targeted to the vacuole (38, 39). These results strongly suggest that API transport to the vacuole is mediated by an autophagy-related mechanism. Apg14 and Apg6 disruptant cells are defective in maturation of proAPI (data not shown). Moreover, Apg14p and Apg6p are shown to be constitutively expressed, and they form a protein complex in vegetatively growing cells. These results indicate that the Apg14-Apg6 protein complex also has a crucial function for the API transport pathway in growing cells.

**Cross-talk between Protein Transport Pathway through Endosome and Autophagy**—A sequential analysis of Apg6 revealed that Apg6 was identical to VPS30. Mutation in Apg6/VPS30 caused a defect in autophagy and CPY sorting, and as reported previously, Vps10p, the late-Golgi resident CPY receptor, is mislocalized to the vacuole in the apg6/vps30 mutant (15). Moreover, another VPS gene, VPS4/CSC1 was recently found to be involved in autophagy (27). We showed that depletion of VPS4/CSC1 resulted in a severe defect in autophagy, and the vps4Δ229K mutant allele caused constitutive autophagy (27). Vps4p has been shown to be required for protein transport from the endosome to the late-Golgi and the vacuole (40).

So far, Seaman et al. (15) showed that some other VPS genes,
such as VPS35 and VPS29 are involved in localization of Vps10p. Vps35p cofractionated with Vps10p by sucrose density gradient centrifugation, and the subcellular localization of Vps35p was affected in the vps6/vps30 disruptant (15). These data strongly suggest that Vps35p and Apg6/Vps30p are involved in Vps10p localization at very close steps. However, deletion of the VPS35 and VPS29 genes did not affect autophagy at all, suggesting that the CPY sorting machinery is not required for autophagy. We also observed that Δapg12, Δend3, Δvps21, Δvps8, and Δapg12 Δend3 double null mutants accumulated autophagic bodies in the vacuole in response to starvation. These results suggest that protein flow through the endosome is not essential for autophagy (41–45).

**Apog6p/Vps30p May Have Two Distinct Functions through Autophagy and the Vacuolar Protein Sorting Pathway**—The apg6 mutant is defective both in autophagy and the CPY sorting pathway (Figs. 5 and 9). We showed that overexpression of APG14 suppressed only the autophagic defect in the apg6-1 mutant, but not the CPY sorting defect. Moreover, APG14 itself is not required for CPY transport. Taken together, these data suggest that Apg6p/Vps30p presumably has two distinct functions through autophagy and the CPY sorting pathway, and Apg14p could be an autophagy-specific component of the protein complex.

Recent study revealed that the autophagic pathway and endocytic pathway converge in mammalian cells (46). Previous functional analyses on Vps30p and Csc1p/Vps4p revealed that these proteins are closely related with endosomal functions (15, 27, 40). These observations suggest that an unknown endosomal function is required for autophagy in yeast. Further investigation of the Apg14-Apg6 protein complex will provide novel insights into the molecular mechanisms of autophagy and the requirement of endosomal function in this complex phenomenon.

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