Formation of the ~350-kDa Apg12-Apg5-Apg16 Multimeric Complex, Mediated by Apg16 Oligomerization, Is Essential for Autophagy in Yeast*

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Akiko Kuma‡§, Noboru Mizushima‡, Naotada Ishihara‡, and Yoshinori Ohsumi§**

From the ‡Department of Cell Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaiji, Okazaki 444-8585, Japan, the §Department of Biomechanics, School of Life Science, the Graduate University for Advanced Studies, Okazaki 444-8585, Japan, and ¶Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Corporation, Kawaguchi 332-0012, Japan

Autophagy, responsible for the delivery of cytoplasmic components to the lysosome/vacuole for degradation, is the major degradative pathway in eukaryotic cells. This process requires a ubiquitin-like protein conjugation system, in which Apg12 is covalently bound to Apg5. In the yeast Saccharomyces cerevisiae, the Apg12-Apg5 conjugate further interacts with a small coiled-coil protein, Apg16. The Apg12-Apg5 and Apg16 are localized in the cytosol and pre-autophagosomal structures and play an essential role in autophagosome formation. Here we show that the Apg12-Apg5 conjugate and Apg16 form a ~350-kDa complex in the cytosol. Because Apg16 was suggested to form a homo-oligomer, we generated an in vitro system that allowed us to control the oligomerization state of Apg16. With this system, we demonstrated that formation of the ~350-kDa complex and autophagic activity depended on the oligomerization state of Apg16. These results suggest that the Apg12-Apg5 conjugate and Apg16 form a multimeric complex mediated by the Apg16 homo-oligomer, and formation of the ~350-kDa complex is required for autophagy in yeast.

In eukaryotic cells, the majority of intracellular bulk degradation occurs in the lysosome/vacuole, an acidic compartment that contains various hydrolytic enzymes. Autophagy is the major pathway by which the cell delivers cytoplasmic components to the vacuole for degradation (1–3). In this process, cytoplasmic constituents, including organelles, are sequestered nonselectively by double-membrane structures termed autophagosomes, which subsequently fuse with the vacuole. The released inner membrane and sequestered components in the vacuolar lumen are degraded for reuse. Autophagy is a cellular survival response to starvation and plays an important role in developmental processes and cell differentiation.

By genetic screens in the yeast Saccharomyces cerevisiae, the APG and AUT genes involved in autophagy were isolated (4, 5). We found previously that four of the Apg proteins constitute a novel protein conjugation system, the Apg12 system (6). In this ubiquitin-like system, the carboxyl-terminal glycine of Apg12 (molecular mass is 21 kDa) is bound covalently to Lys149 at the center of Apg5 (33 kDa). This conjugating reaction is catalyzed by Apg7 and Apg10. Apg7 is the Apg12-activating enzyme (7–9), and Apg10 functions as an Apg12-conjugating enzyme (10). Human homologs of Apg12 and Apg5 have been identified and undergo a similar covalent linkage (11), indicating that this conjugation system is conserved in mammalian cells. Analysis of an apg5 null mutant and a temperature-sensitive mutant suggested that Apg5 is required for formation or completion of sequestering vesicles in yeast (12). Recent morphological observation revealed that in yeast, most Apg5 exists diffusely in the cytoplasm, whereas only a small portion localizes to the pre-autophagosomal structures (13). Similarly, in a study of Apg5 in mouse embryonic stem cells, it was demonstrated that the Apg12-Apg5 conjugate is targeted from the cytoplasm to the autophagic isolation membranes during autophagosome formation (14). Immediately before or after completion of autophagosome formation, the Apg12-Apg5 conjugate dissociates from the isolation membrane. The Apg12-Apg5 conjugate is required for elongation of the isolation membrane. The Apg12-Apg5 conjugate is also required for association of Aut7/LC3 with the pre-autophagosomal membranes in both yeast and mammalian cells (13–15). But, although the Apg12-Apg5 conjugate is considered to be of vital importance for autophagosome formation, its molecular function is still poorly understood.

Apg16 was originally obtained by a two-hybrid screen using Apg12 as bait and was found to interact with the Apg12-Apg5 conjugate (16). It was then determined that Apg16 interacts directly with Apg5 but not with Apg12. Apg16 is a 150-amino acid protein (17 kDa) that contains a carboxyl-terminal coiled-coil motif (residues 58–123) and associates with Apg5 at its amino-terminal region. It was suggested that Apg16 forms an oligomer through the coiled-coil region and functions as a linker to form the Apg12-Apg5-Apg16 multimeric complex (16). Because Apg16 is the only molecule thus far identified to interact with the Apg12-Apg5 conjugate, further characterization of Apg16 would provide valuable insights into the molecular role of the Apg12-Apg5 conjugate in autophagosome formation. In this study, we characterize the Apg12-Apg5-Apg16 protein complex and show, by use of a regulated oligomerization system, that formation of the ~350-kDa complex is required for functioning of the Apg12-Apg5 conjugate in the autophagic pathway.

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** ¶ Present address: Dept. of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan.

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EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The S. cerevisiae strains used in this study were SEY6210 (MATa his3-d200 leu2-d3, 112 lys2-s301 trp1-d901 ura3-52 suc2-n5 Dα GAL), KVY115 (MATα his3-d200 leu2-d3, 112 lys2-s301 trp1-d901 ura3-52 suc2-n5 Dα GAL Δapg12-HIS3), KVY117 (MATα his3-d200 leu2-d3, 112 lys2-s301 trp1-d901 ura3-52 suc2-n5 Dα GAL Δapg12-HIS3 ΔLEU2), and KVY118 (MATα his3-d200 leu2-d3, 112 lys2-s301 trp1-d901 ura3-52 suc2-n5 Dα GAL Δapg12-HIS3 ΔLEU2). The other αgα disruptants used in Fig. 1A were also created with SEY6210 (13). Cells were grown either in YPD (1% yeast extract, 2% peptone, 2% glucose) medium or in SD medium containing nutritional supplements. For nitrogen starvation, SD (–N) medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose) was used.

Preparation—To create glutathione S-transferases (GST)-tagged Apg12, GST-Apg16, and GST-Apg5 fusion constructs, the open reading frames of Apg12 and Apg16 were cloned into the blunt-ended EcoRI and Smal sites of pGEX-3X (Amersham Biosciences) to yield pGEX-3X-Apg12 and pGEX-3X-Apg16, respectively. The Apg5 open reading frame was cloned into the BamHI site of pGEX-2T to generate pGEX-2T-Apg5. For expression of His6-tagged Apg12, the Apg16 open reading frame was first cloned into the EcoRI site of pENT3C from the Gateway cloning system (Invitrogen). A His6-Apg16 expression plasmid (pDEST17-Apg16) was then generated according to the manufacturer’s instructions. To make the Apg12-2FKBP39 expression constructs, we first created BamHI sites within the carboxyl-terminal coiled-coil region of Apg12 (after the amino acid residue 65 and thereafter) by mutagenizing pApg12, a pBluescript plasmid containing the Apg12 cDNA (16), using the QuikChange site-directed mutagenesis kit (Stratagene). The fragment containing two tandem copies of FKBP39 was PCR amplified from the plasmid pC4-Fv2E5 (provided by ARIAD Pharmaceuticals, Inc.) with primers that were designed to have in-frame BamHI sites in their 5′-ends: Fv2E5N, 5′-ATCGGATCCAGGCGTCCAAGTCGAAACCA-3′; and Fv2E5C, 5′-GATGGATCCGGCGTCCAAGTCGAAACCA-3′; and Fv2E5C, 5′-GATGGATCCGGCGTCCAAGTCGAAACCA-3′. The resulting fragment was digested with BamHI and inserted into the mutated pApg12 plasmids, either into one of the two BamHI sites or between them, to generate pApg12-2FKBP39, pApg16-2FKBP39, and pApg5-2FKBP39 plasmids.

Antibodies—Anti-Apg12, anti-Apg16, and anti-Apg5 antibodies were prepared as follows. The pGEX-3X-Apg12, pGEX-3X-Apg16, and pGEX-2T-Apg5 plasmids were transformed into Escherichia coli XL1 Blue (Stratagene), and transformants were grown up to 0.5 A600 unit/ml and, if necessary, induced to express Apg12 or Apg16—

Characterization of Endogenous Apg12-Apg5 Conjugate and Apg16—We generated polyclonal antibodies against Apg12 and Apg16 to examine the state of the endogenous Apg12-Apg5 conjugate and Apg16. Upon immunoblotting, most endogenous Apg12 exists in the form of the conjugate with Apg5 in wild-type cells (Fig. 1A). This is true in apg mutants except for Δapg5, Δapg7, and Δapg10. In these three apg mutants, which are defective in Apg12 conjugation (6), Apg12 was present exclusively in the unconjugated form. Apg16 was detected at the expected molecular size with affinity-purified anti-Apg16 antibody (Fig. 1B). When Apg16 was overexpressed, an additional band (* in Fig. 1B) was observed as reported previously (16), although it has not yet been characterized. The amounts of unconjugated Apg12, Apg12-Apg5 conjugates, and Apg16 did not change during nitrogen starvation (Fig. 1B and data not shown).

We next examined the subcellular distribution of the endogenous Apg12-Apg5 conjugate and Apg16 by differential centrifugation. Previous studies demonstrated that the distribution of the hemagglutinin (HA) epitope-tagged Apg12-Apg5 conjugate depends on lysis buffer conditions (12, 16). Thus, we tested different lysis buffer conditions and confirmed that the distribution of the endogenous Apg12-Apg5 conjugate was indeed affected considerably by the salt concentration of the lysis buffer. Yeast spheroplasts were homogenized with the lysing buffer and subjected to physiological concentrations of salts. After removal of cell debris, the lysates were centrifuged at 13,000 × g for 20 min to generate a pellet (P13) fraction. The resulting supernatant was centrifuged again at 100,000 × g for 1 h to further separate it into pellet (P100) and supernatant (S100) fractions. Each fraction was subjected to immunoblotting with anti-Apg12 and anti-Apg16 antibodies. In buffer containing 150 mM NaCl, the Apg12-Apg5 conjugate was found primarily in the S100 fraction, whereas in the absence of salt it was found mainly in the P100 fraction (Fig. 1C). Apg16 displayed a similar distribution pattern (Fig. 1C). Because the distribution we observed using the salt-containing buffer is more consistent with our morpho-
logical observations that most Apg5 is distributed throughout the cytoplasm in both yeast and mammalian cells (13, 14), we used buffer containing physiological salt concentrations in the following experiments.

Cytosolic Apg12-Apg5 Conjugate and Apg16 Form a ~350-kDa Complex—Our previous study suggested that Apg16 forms an oligomer and cross-links the Apg12-Apg5 conjugate (16). To characterize the Apg12-Apg5-Apg16 complex further, we performed gel filtration analysis. The S100 fraction was subjected to gel filtration analysis using a Superdex 200 column and subsequently immunoblotted with anti-Apg12 and anti-Apg16 antibodies. The Apg12-Apg5 conjugate eluted mainly in a single peak in fractions corresponding to ~350 kDa. Most Apg16 was also recovered in these fractions (Fig. 2A). Coelution of Apg12-Apg5 and Apg16 indicates that most of the Apg12-Apg5 conjugate and Apg16 form a ~350-kDa protein complex. Monomeric Apg12-Apg5 conjugate and Apg16 were scarcely detected. The ~350-kDa complex was already formed under nutrient conditions, and the elution profile was not affected by nitrogen starvation (Fig. 2B). In contrast, in Δapg16 cells, the ~350-kDa complex was not observed (Fig. 3A), indicating that the formation of this complex depends on Apg16. The Apg12-Apg5 conjugate was found in ~60-kDa fractions, corresponding to the sum of the molecular masses of Apg12 (21 kDa) and Apg5 (33 kDa) (Fig. 3A). We expected that Apg16 may cross-link two Apg12-Apg5 conjugates (16), but the size of the resulting complex was significantly larger than what would be expected from two sets of Apg12, Apg5, and Apg16. One possible explanation is that there may be additional components. However, as far as we examined by immunoprecipitation analysis using anti-Apg12 antibody, we could not detect any protein other than Apg12, Apg5, and Apg16 (data not shown). Furthermore, our observation that the main Apg12-Apg5 conjugate peak was shifted to fractions much smaller than half of 350 kDa in Δapg16 cells (Fig. 3A) suggests that the ~350-kDa complex is not dimeric. Therefore, we believe that the ~350-kDa complex represents an Apg12-Apg5-Apg16 multimeric complex, most probably tetrameric, the formation of which is mediated by Apg16 oligomerization.

We also performed gel filtration analysis on Δapg5 cells. In Δapg5 cells, the ~350-kDa complex was not present, and the Apg12 and Apg16 peaks were detected in fractions of low molecular size (Fig. 3B). The eluting peak position of Apg16 (fractions 11 and 12) was larger than the expected molecular size of the Apg16 monomer. When we performed gel filtration analysis using Δapg5 Δapg16 cells expressing both Apg16 and HHApg16, the tagged Apg16 was recovered in the same fractions (data not shown). Coimmunoprecipitation analysis revealed that the amount of the HHApg16 oligomerization. Apg16 might not be a globular protein and behave aberrantly in size exclusion chromatography. Taken together, these results suggest that the Apg5-Apg16 interaction would be important for Apg16 to form an oligomer. In contrast, in Δapg12 cells, Apg5 and Apg16 were mainly coeluted in ~250-kDa fractions, which would represent the complex made up of four sets of Apg5 and Apg16 (Fig. 3C). Therefore, even in the absence of Apg12, Apg5 and Apg16 could form the tetrameric complex.

To confirm that formation of the ~350-kDa complex is mediated by Apg16, we reconstituted the complex formation using two different cell lysates: one was prepared from Δapg16 cells, in which the Apg12-Apg5 conjugate existed as a monomeric form (Fig. 4A), and the other was from Δapg5 Δapg12 cells overexpressing Apg16, in which most Apg16 was monomeric (Fig. 4B). These two cell lysates were mixed, incubated at 4 °C overnight, and subjected to gel filtration analysis. In contrast to the premixed sample (Fig. 4A), the ~350-kDa complex appeared clearly in the incubated mixture (Fig. 4C). We could not detect the shift of Apg16 probably because it was below the detectable level by the antibody, judging from the band intensity of overproduced Apg16 (data not shown). We also observed a small but significant shift of the Apg12-Apg5 fractions after a 1-h incubation of the lysates (data not shown).

Apg16 Oligomerization Is Required for Formation of the ~350-kDa Apg12-Apg5-Apg16 Complex—Because the above experiments suggested that formation of the ~350-kDa complex is mediated by the Apg16 homo-oligomer, we next attempted to control the oligomerization state of Apg16 to determine whether the formation of this complex is required for autophagy. We employed a regulated oligomerization system, which allows drug-induced multimerization of proteins of interest (18, 19). This system is based on FKBP and its small ligand FK506. A bivalent drug AP20187, which is created by chemically linking two FK506 derivatives, is able to cross-link fusion proteins containing the FKBP(A36V) domain, in which Phe36 of wild-type
FKBP is replaced with valine. AP20187 has a 1,000-fold higher affinity for FKBP36V than for wild-type FKBP. Thus, a fusion protein containing two copies of FKBP F36V is capable of forming oligomers in the presence of AP20187. Because Apg16 is suggested to form an oligomer through its carboxyl-terminal coiled-coil region (16), we modified Apg16 by inserting two tandem copies of FKBP36V after amino acid 65 or 118 (described as Apg1665-2FKBP36V and Apg16118-2FKBP36V, respectively) or by using it to replace nearly the entire coiled-coil region (Apg1665-118-2FKBP36V), where it would disrupt the coiled-coil region and thereby inhibit natural Apg16 oligomerization (Fig. 5A). We then tested whether each Apg16-2FKBP36V fusion protein oligomerized in an AP20187-dependent manner. Apg16 cells expressing each Apg16-2FKBP36V fusion protein were treated with or without 0.1 mM AP20187, and cell lysates were separated on a Superdex 200 column as described in Fig. 2. Each fraction was subjected to immunoblot analysis using anti-Apg12, anti-Apg5, and anti-Apg16 antibodies.

**FIG. 2.** Apg12-Apg5 and Apg16 form a ~350-kDa complex in the cytosol. The S100 fractions were prepared from wild-type cells that were growing (A) or starved for 3 h in SD (−N) medium (B) and separated by size exclusion chromatography on a Superdex 200 column. Each fraction was subjected to immunoblotting using anti-Apg12 and anti-Apg16 antibodies. Positions of molecular mass standards (in kDa) are shown. V, void fraction.

**FIG. 3.** The ~350-kDa complex is not formed in Δapg16, Δapg5, and Δapg12 cells. The S100 fractions from Δapg16 (A), Δapg5 (B), and Δapg12 (C) cells were subjected to gel filtration analysis using a Superdex 200 column as described in Fig. 2. Each fraction was subjected to immunoblot analysis using anti-Apg12, anti-Apg5, and anti-Apg16 antibodies.

**FIG. 4.** The ~350-kDa complex is formed in vitro using Δapg16 and Δapg5Δapg12 cell lysates. The S100 fractions from Δapg16 cells (A) and Δapg5Δapg12 cells (YNM117) overexpressing Apg16 from a 2 μ plasmid (B) were prepared. Equal volumes of these two cell lysates were mixed, incubated at 4 °C overnight, and subjected to gel filtration analysis using a Superdex 200 column. Each fraction was analyzed by immunoblotting using anti-Apg12 (A and C) and anti-Apg16 antibodies (B).
AP20187, the Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> was detected in fractions corresponding to ∼200 kDa (Fig. 5Ba). In this peak, Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> associated with the Apg12-Apg5 conjugate because Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> was detected at ∼55 kDa in Δapg5 cells, which was probably monomeric Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> (Fig. 5Be). Because the sum of the molecular masses of Apg12, Apg5, and Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> is about 100 kDa, our data suggest that dimeric Apg12-Apg5-Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> could be formed even in the absence of AP20187. The remaining coiled-coil region of Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> might function partially (see “Discussion”). After AP20187 treatment, the peak of Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> was clearly shifted to fractions corresponding to ∼400 kDa (Fig. 5Bc). This change of peak indicates that Apg16 oligomerizes upon AP20187 treatment. When the Apg16<sup>115-</sup>-2FKBP<sup>F36V</sup> fusion protein, in which 2FKBP<sup>F36V</sup> was inserted at almost the end of the coiled-coil region, was expressed in Δapg16 cells, the ∼400-kDa complex was present irrespective of the AP20187 treatment (data not shown). Expression of the Apg16<sup>115-</sup>-2FKBP<sup>F36V</sup> was too low to be evaluated (data not shown).

Using this controlled Apg16 oligomerization system with the Apg16<sup>115-</sup>-2FKBP<sup>F36V</sup> construct, we examined the complex state of the Apg12-Apg5 conjugate. In the absence of AP20187, the Apg12-Apg5 conjugate was eluted mainly at ∼60 kDa, equivalent to the molecular mass of the conjugate (Fig. 5Bb). After treatment with AP20187, the Apg12-Apg5 conjugate was found to elute in ∼400-kDa fractions (Fig. 5Bd), together with oligomerized Apg16<sup>115-</sup>-2FKBP<sup>F36V</sup>. This peak corresponds to the wild-type ∼350-kDa Apg12-Apg5-Apg16 complex; insertion of 2FKBP<sup>F36V</sup> (about 24 kDa) into Apg16 most likely accounts for the difference. These results suggest that formation of the ∼350-kDa Apg12-Apg5-Apg16 complex requires Apg16 oligomerization and that we were effectively able to regulate the formation of the complex with this AP20187-dependent oligomerization system.

The ∼350-kDa Complex Is Required for Autophagy—Next, we used the oligomerization system to determine whether the formation of the ∼350-kDa complex is necessary for autophagy. Δapg16 cells expressing Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> were incubated with AP20187 overnight and then starved with nitrogen-free medium containing 1 mM phenylmethanesulfonyl fluoride and 0.1 μM AP20187 for 8 h. Cells were then examined by light microscopy for accumulation of autophagic bodies in vacuoles (20). Typical accumulation of autophagic bodies was observed in AP20187-treated cells but was rarely detected in untreated cells (Fig. 6A).

Induction of autophagy was also confirmed by examining the maturation of a vacuolar enzyme, API. In S. cerevisiae, API is synthesized in a pro-form (prAPI) and delivered from cytoplasm to vacuole via the cytoplasm to vacuole targeting pathway, an autophagy-related pathway (21). During starvation, API is transported to the vacuole via the autophagic pathway. Delivery to the vacuole leads to maturation of API into its active form (mAPI). Δapg5 cells expressing Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> were grown in medium with or without 0.1 μM AP20187 and starved for 3 h. Lysates were prepared and subjected to immunoblotting using anti-API antibody. Although most API was processed to the mature form in wild-type cells (Fig. 6B, lane 3), mature API was scarcely detected in Δapg16 cells expressing Apg16<sup>65-</sup>-2FKBP<sup>F36V</sup> (Fig. 6B, lane 1). As expected, maturation of API was restored after treatment with AP20187 (Fig. 6B, lane 2) in a concentration-dependent manner (Fig. 6C). Taken together, the results of these experiments suggest that formation of the ∼350-kDa Apg12-Apg5-Apg16 complex is required for autophagy.

**DISCUSSION**

In the present study we have demonstrated that Apg12, Apg5, and Apg16 form a ∼350-kDa multimeric complex that exists stably in the cytosol. Our approach, utilizing the regu-
lated oligomerization system, demonstrated concretely that formation of the 350-kDa complex, which depends on oligomerization of Apg16, is required for autophagy. Our study also demonstrated that this oligomerization system works well in yeast cells in vivo.

In our previous experiment using HA-tagged Apg12 (HA-Apg12), less than half of the Apg12 was found conjugated to Apg5 (6), whereas most of endogenous Apg12 was found attached to Apg5 (Fig. 1A). It is conceivable that plasmid-derived expression of HA-Apg12 affects the conjugation efficiency. HA tagging may also affect the subcellular distribution determined from differential centrifugation experiments. As shown in Fig. 1C, the endogenous Apg12-Apg5 conjugate was recovered primarily in the cytosolic fraction, in contrast to previous reports showing that most HA-tagged Apg12-Apg5 was pelletable (16), even when lysis buffer containing salt was used (12). Combined with our recent morphological studies demonstrating that most Apg12-Apg5 is found in the cytoplasm (13, 14), we concluded that this is the primary localization of endogenous Apg12-Apg5. Under the present conditions, a small portion of Apg12-Apg5 was still observed in the pellet fractions (Fig. 1C). However, its amount was not changed during nitrogen starvation or in Δapg6 cells, in which the pre-autophagosomal structures were not formed (13). Therefore, it is dubious that the conjugate in the pellet fractions represents any real physiological localization.

Upon gel filtration analysis, we found that almost all of the Apg12-Apg5 conjugate and Apg16 are included in the 350-kDa complex, whereas the monomeric forms of each component were scarcely detected. This is not simply because each monomer is unstable; free Apg12-Apg5 conjugate and Apg16 were easily detected in Δapg16 and Δapg5 cells, respectively, neither of which contain the 350-kDa complex (Fig. 3). In the previous report, we suggested that Myc-Apg16 interacts efficiently with Apg12-HA-Apg5 but inefficiently with unconjugated HA-Apg5 (16). However, gel filtration analysis of endogenous proteins showed that Apg16 could interact quite well with unconjugated Apg5, though still somewhat weaker than with conjugated Apg5 (Fig. 3C). Therefore, the primary role of Apg12 conjugation would not be to strengthen the Apg5-Apg16 association. It is also unknown which takes place earlier in vivo, Apg12-Apg5 conjugation or Apg5-Apg16 interaction. Al-
though both are possible, these two molecular interactions do not depend on each other, i.e., the Apg12-Apg5 conjugate is generated in ∆apgs cells (Fig. 1A), and Apg5 could form a complex with Apg16 in the absence of Apg12 (Fig. 1C).

From our analysis, it is most likely that the ∼350-kDa complex consists of four sets of Apg12-Apg5-Apg16 proteins. Using the regulated oligomerization system, we showed successfully that formation of the ∼350-kDa complex is mediated by Apg16 homo-oligomer. Although Apg1665-2FKBP36V and Apg12-Apg5 were clearly shifted to ∼400 kDa by AP20187 treatment, it was intriguing that these proteins were detected at the fraction corresponding to ∼200 kDa in the absence of AP20187. Because 2FKBP36V was inserted immediately after the beginning of the coiled-coil region, the remaining coiled-coil region of Apg16 might mediate formation of dimeric Apg12-Apg5-Apg1665-2FKBP36V complex. If that is the case, it was conceivable that our system regulates tetramer formation by cross-linking the dimeric Apg1665-2FKBP36V. We demonstrated that the ∼400-kDa Apg12-Apg5-Apg1665-2FKBP36V complex formed by such a manner was functional, suggesting that the wild-type ∼350-kDa complex might be also formed by dimer-dimer association. In general, tetramer formation mediated by dimer-dimer contact is possible as observed in the cases of Eps15 (24), F1-ATPase inhibitor protein IF1 (25), and Mnt associated by dimer-dimer interaction is possible as observed in the cases of Eps15 (24), F1-ATPase inhibitor protein IF1 (25), and Mnt (26). In four-stranded coiled-coils have been reported (27). We also tried to test whether Apg16 inserted with a single copy of FKBP36V domain at the same site was able to function. However, the resulting Apg1665-1FKBP36V was still functional in the absence of AP20187, probably because of insufficient interference of the tetramer formation, and thus could not be evaluated (data not shown).

The regulated oligomerization system used in this study is based on the interaction between FK506 and FKBP. Another natural ligand of FKBP is rapamycin, which is known to induce FKBP complex inhibiting Tor-mediated pathway was also rejected because AP20187 and nitrogen starvation had such strong additive effects on autophagy and API maturation (Fig. 6).

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Formation of the ~350-kDa Apg12-Apg5-Apg16 Multimeric Complex, Mediated by Apg16 Oligomerization, Is Essential for Autophagy in Yeast
Akiko Kuma, Noboru Mizushima, Naotada Ishihara and Yoshinori Ohsumi

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