Organization of the Pre-autophagosomal Structure Responsible for Autophagosome Formation

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Autophagy induced by nutrient depletion is involved in survival during starvation conditions. In addition to starvation-induced autophagy, the yeast *Saccharomyces cerevisiae* also has a constitutive autophagy-like system, the Cvt pathway. Among 31 autophagy-related (Atg) proteins, the function of Atg17, Atg29, and Atg31 is required specifically for autophagy. In this study, we investigated the role of autophagy-specific (i.e., non-Cvt) proteins under autophagy-inducing conditions. For this purpose, we used *atg11Δ* cells in which the Cvt pathway is abrogated. The autophagy-unique proteins are required for the localization of Atg proteins to the pre-autophagosomal structure (PAS), the putative site for autophagosome formation, under starvation condition. It is likely that these Atg proteins function as a ternary complex, because Atg29 and Atg31 bind to Atg17. The Atg1 kinase complex (Atg1–Atg13) is also essential for recruitment of Atg proteins to the PAS. The assembly of Atg proteins to the PAS is observed only under autophagy-inducing conditions, indicating that this structure is specifically involved in autophagosome formation. Our results suggest that Atg1 complex and the autophagy-unique Atg proteins cooperatively organize the PAS in response to starvation signals.

INTRODUCTION

Macroautophagy (autophagy) is a degradation process that sequesters cytoplasmic proteins and organelles into vacuoles/lysosomes for bulk degradation (Takeshige *et al.*, 1992; Klionsky and Ohsumi, 1999). Autophagy accompanies dynamic membrane biogenesis that cannot be explained by classical secretory pathways, because it involves the formation of a unique double-membrane structure, called the autophagosome (Baba *et al.*, 1994). Although autophagic degradation is generally considered nonselective, there are several examples of selective autophagy. In *Saccharomyces cerevisiae*, the cytoplasm to vacuole targeting (Cvt) pathway is now regarded as a unique, specialized transport route, although it is not conserved in all eukaryotes (Klionsky, 2005). The Cvt pathway is responsible for delivering the vacuolar hydrolases aminopeptidase I (API) and α -mannosidase from the cytoplasm to the vacuole (Klionsky and Ohsumi, 1999). The Cvt pathway is active in cells grown under nutrient-rich conditions, whereas autophagy is suppressed to undetectable level under such conditions. In contrast, autophagy is strongly induced by starvation (Takeshige *et al.*, 1992). It has been demonstrated that the target of rapa-

mycin (TOR) signaling pathway mediates the induction of autophagy by nutrient starvation (Noda and Ohsumi, 1998; Wullschleger *et al.*, 2006).

So far, 31 autophagy-related (ATG) genes were identified and characterized. Of these, 18 ATG genes, *ATG1–ATG10*, *ATG12–ATG14*, *ATG16–ATG18*, *ATG29*, and *ATG31*, play roles in autophagosome formation (Klionsky *et al.*, 2003; Kawamata *et al.*, 2005; Kabeya *et al.*, 2007). These Atg proteins are classified into several groups by their function: Atg1 kinase and its regulatory proteins (Atg1, Atg13, Atg17), two ubiquitin-like conjugation systems (Atg12 and Atg8 systems, Atg3, Atg4, Atg5, Atg7, Atg8, Atg10, Atg12, Atg16), a phosphatidylinositol 3-kinase complex (Vps34, Vps15, Atg6/Vps30, Atg14), and a group of several other proteins (Atg2, Atg9, Atg18) (Noda *et al.*, 2002; Levine and Klionsky, 2004). Most of the Atg proteins are also required for the Cvt pathway (Noda *et al.*, 2002; Levine and Klionsky, 2004).

Sets of Atg proteins specifically required for either the Cvt or autophagic pathway have been isolated. For example, Atg11/Cvt9 and Atg19/Cvt19, the API adapter and receptor protein, respectively, function only in the Cvt pathway (Shintani *et al.*, 2002). Lack of these proteins does not affect autophagic activity at all. In contrast, three Atg proteins, Atg17, Atg29, and Atg31, are known to function specifically in autophagy but not in the Cvt (Kamada *et al.*, 2000; Kawamata *et al.*, 2005; Kabeya *et al.*, 2007). These pathway-specific proteins likely determine the distinctions between the Cvt pathway and autophagy.

Fluorescence microscopy has shown that almost all Atg proteins gather onto the pre-autophagosomal structure (PAS), which is thought to be the site of the Cvt vesicle and autophagosome formation (Noda *et al.*, 2000; Suzuki *et al.*, 2001; Suzuki *et al.*, 2007). Most Atg proteins are localized to the PAS under growth and starvation conditions. Recruitment of Atg proteins to the PAS is indispensable for forma-

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Abbreviations used: ALP, alkaline phosphatase; Cvt, cytoplasm to vacuole targeting; PAS, preautophagosomal structure.

tion of the Cvt vesicle and autophagosome (Shintani *et al.*, 2001). Recently, we performed a systematic and comprehensive analysis of Atg proteins in the PAS organization during autophagy-inducible condition, and we proposed a hierarchy map of ATG genes (Suzuki *et al.*, 2007). Localization of most Atg proteins to the PAS is impaired in the absence of ATG17, an autophagy-specific gene, thus Atg17 is situated at the basis of the PAS-organizing machinery. However, some Atg proteins are still observed at the PAS in *atg17Δ* cells, due to the ATG11-dependent Cvt pathway. The PAS completely disappeared in *atg11Δatg17Δ*, suggesting that Atg11 and Atg17 serve as alternative scaffolding proteins to organize the PAS. It is also reported that lack of the Cvt-specific factors, such as Atg11, diminished PAS localization of all Atg proteins under nutrient-rich conditions (Shintani and Klionsky, 2004). Because some Atg proteins localized to the PAS under starvation conditions in *atg11Δ* cells, PAS organization to create the Cvt vesicles requires Atg11 protein, but that to create autophagosome does not. These findings suggest that a strain lacking the Cvt pathway (such as *atg11Δ*) might give clues to understand how the PAS is generated to create autophagosomes but not the Cvt vesicles in response to starvation condition, and what are roles of the autophagy-specific Atg proteins in organization of the PAS responsible for autophagosome formation. In this study, we examined the mechanism underlying the Atg11-independent, Atg17-dependent formation of the PAS under starvation conditions.

We have previously reported the identification of Atg29 and Atg31 (Kawamata *et al.*, 2005; Kabeya *et al.*, 2007). It is reasonable to think that these proteins should have autophagy-restricted functions, which are required for autophagy but not for the Cvt pathway. Atg31 physically associates with Atg17, suggesting that Atg31 functions with Atg17 to regulate autophagy (Kabeya *et al.*, 2007). Here, we report that Atg29 physically interacts with Atg17, suggesting the cooperative function of Atg17, Atg29, and Atg31. Our data suggest that Atg proteins play an essential role(s) in organizing the PAS responsible for autophagosome formation.

MATERIALS AND METHODS

Yeast Strains and Media

Growth media, culture conditions, and genetic manipulations were used as described previously (Kaiser *et al.*, 1994; Kawamata *et al.*, 2005). The yeast strains used in this study are listed in Table 1. Unless otherwise specified, strains were generated using one-step gene disruption or replacement methods as described previously (Longtine *et al.*, 1998; Goldstein and McCusker, 1999). The *atg1Δ*, *atg2Δ*, *atg8Δ*, *atg9Δ*, *atg11Δ*, *atg13Δ*, *atg14Δ*, *atg17Δ*, *atg29Δ*, or *atg31Δ* strains were constructed as described previously (Kamada *et al.*, 2000; Shintani *et al.*, 2001; Suzuki *et al.*, 2001, 2007; Kawamata *et al.*, 2005; Kabeya *et al.*, 2007). Replacement of *kanMX* with *natMX* was performed as described previously (Goldstein and McCusker, 1999). All deletion and epitope-tagged strains constructed in this study were confirmed by polymerase chain reaction (PCR).

Plasmid Construction

The plasmids used in this study are listed in Table 2. To construct pTM20, a PCR fragment containing ATG29-13Myc was obtained by PCR using genomic DNA from TMK174 as a template, digested with SphI, and subcloned into pRS314x. To generate plasmids harboring ATG29 for two-hybrid analyses, each ATG29 variant was amplified by PCR using genomic DNA as a template and primers containing EcoRI (forward) and PstI (reverse). PCR fragments containing full-length or truncated Atg29 were digested with EcoRI and PstI, and then they were subcloned into pBGD-C2 digested with the corresponding enzymes. Similarly, plasmids harboring ATG17 were constructed by inserting fragments containing full-length ATG17 or truncated ATG17 variants into pGAD-C1 at the BamHI and ClaI sites. pTM41 was constructed by subcloning a SphI fragment bearing Gal4AD-ATG17 (Kabeya *et al.*, 2005) into pGADU-C1 digested with the corresponding enzymes. To generate point mutations in ATG29, Trp54, Pro64, Phe67, or Arg71 were changed to Arg, His, Leu, and Gly, respectively, using a QuikChange site-directed mutagenesis kit

(Stratagene, La Jolla, CA)) according to the recommendations of the manufacturer. Restriction analysis and DNA sequencing verified these different mutations. All primer sequences and detailed maps for the constructs described here can be provided upon request.

Yeast Two-Hybrid Analysis and Mutant Screening

Two-hybrid assays were carried out essentially as described previously (James *et al.*, 1996). Briefly, pGAD- and pGBD-based plasmids were cotransformed into host strain PJ69-4A. Transformant colonies were replica-plated onto either SC plates lacking tryptophan, leucine, and histidine containing 3 mM 3-aminotriazole (3-AT) or SC plates lacking tryptophan, leucine, and adenine, and then they were tested for growth.

Atg29 mutants unable to interact with Atg17 were isolated as follows. A segment of ATG29 corresponding to amino acid residues 44–104 was mutagenized by PCR in the presence of 0.5 mM MnCl₂, by using pTM34 as a template. The PCR products were cotransformed with a linearized pGBD-C2 plasmid digested with EcoRI and PstI into host strain TMK327 (PJ69-4A *atg29Δ*) harboring plasmid pTM41 (GAL4ADU-ATG17). Transformants were selected for growth on SC-Trp-Ura, and then they were replica-plated onto SC-Trp-Ura-Ade plates to identify Atg17-nonbinding mutants. Of ~300 colonies examined, 35 colonies unable to grow on SC-Leu-Trp-Ade were selected as candidates. These cells were streaked on SC-Trp plates supplemented with 5-fluoro-orotic acid at a final concentration of 1 mg/ml to select against the URA3-based ATG17 plasmid, and then they were incubated at 30°C. *atg29* mutants were recovered from these cells by isolating plasmids and determining the nucleotide sequence of *atg29* by DNA sequencing.

Fluorescence Microscopy

Intracellular localization of fluorescent proteins was examined using an inverted fluorescence microscope (IX-71 or IX-81; Olympus, Tokyo, Japan) as described previously (Suzuki *et al.*, 2001, 2007). Images were acquired using MetaMorph software (Molecular Devices, Sunnyvale, CA), and they were processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA). The percentages of cells with green fluorescent protein (GFP) fluorescent dot signals at the PAS were determined by counting 100–200 cells for each sample (Figures 1–3).

Immunoprecipitation

Immunoprecipitation of Atg29 or Atg1 were performed as described previously (Kamada *et al.*, 2000). Cells grown to mid-log phase in YEPD were treated with 0.2 μg/ml rapamycin for 3 h, collected, washed once with stop mix buffer (0.9% NaCl, 1 mM NaN₃, 10 mM EDTA, and 50 mM potassium fluoride), and suspended in ice-cold lysis buffer (1× phosphate-buffered saline [PBS], pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM Na₃VO₄, 50 mM KF, 15 mM sodium pyrophosphate, pH 7.5, 15 mM *p*-nitrophenylphosphate, 20 μg/ml leupeptin, 20 μg/ml benzamidin, 10 μg/ml pepstatin A, 40 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). An equal volume of glass beads (φ = 0.5 mm) was added to this suspension, and cells were broken by vigorous vortexing for 10 min at 4°C. A half volume of lysis buffer containing 1% Tween 20 was added to a final concentration of 0.5% Tween 20, and the mixture was incubated for 10 min with mild rotation. The beads and cell debris were removed by two consecutive 10-min centrifugations at 10,000 × *g* and 4°C. Glycerol was added to the resulting lysate to a final concentration of 30% before storage at –20°C. Protein concentrations were estimated using a bicinchoninic acid kit (Pierce Chemical, Rockford, IL). For immunoprecipitation analysis, cell lysates were brought up to 1 ml with immunoprecipitation buffer (1× PBS, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM Na₃VO₄, 50 mM KF, 15 mM NaPPi, pH 7.5, 15 mM pNPP, and 0.5% Tween 20), and then they were incubated with anti-myc (9E10, for Atg29-Myc) or anti-hemagglutinin (HA) (16B12 [Covance Research Products, Princeton, NJ] for HA-Atg1) antibodies for 1.5 h at 4°C with gentle rotation. Twenty microliters of 50% suspension of protein G-Sepharose 4 FF (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) was added to the extract/antibody mixture, followed by rotation for 1.5 h at 4°C. Immunocomplexes were washed three times with 1 ml of wash buffer (1× PBS, pH 7.4, 2 mM Na₃VO₄, and 1% NP-40). Proteins bound to the beads were suspended in 2× sample buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 0.4% bromophenol blue, and 200 mM dithiothreitol), eluted by boiling, and subjected to SDS-polyacrylamide gel electrophoresis.

Other Procedures

Immunoblot analyses were performed as described previously (Kamada *et al.*, 2000; Kabeya *et al.*, 2005). Alkaline phosphatase (ALP) assays were performed as described previously (Noda *et al.*, 1995). Pho8Δ60 was expressed from the 2μ plasmid (pTN3; Figure 1), or it was genomically expressed from the chromosome (Figure 1E).

Table 1. Yeast strains used in this study

Strain	Genotype	Source
SEY6210	<i>MATα ura3 leu2 his3 trp1 lys2 suc2</i>	Robinson <i>et al.</i> (1988)
TMK4	SEY6210 <i>atg29Δ::kanMX</i>	Kawamata <i>et al.</i> (2005)
TMK174	SEY6210 <i>ATG29-myc-kanMX</i>	This study
PJ69-4A	<i>MATa his3-200 leu2-3,112 trp1-901 ura3-52 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)
TMK327	PJ69-4A <i>atg29Δ::kanMX</i>	This study
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Giaever <i>et al.</i> (2002)
TMK181	BY4741 <i>atg29Δ::kanMX</i>	Kawamata <i>et al.</i> (2005)
TMK190	SEY6210 <i>ATG29-GFP-kanMX</i>	Kawamata <i>et al.</i> (2005)
TMK214	TMK190 <i>atg17Δ::his3MX</i>	This study
TMK400	TMK190 <i>atg11Δ::LEU2</i>	This study
TMK200	TMK190 <i>atg1Δ::his3MX</i>	This study
TMK464	TMK400 <i>atg17Δ::URA3</i>	This study
TMK553	BY4741 <i>ATG29-GFP-kanMX</i>	This study
YKY163	TMK553 <i>atg11Δ::LEU2 atg31Δ::natMX</i>	This study
TMK607	TMK400 <i>atg1Δ::his3MX</i>	This study
TMK680	TMK400 <i>atg13Δ::URA3</i>	This study
TMK698	TMK400 <i>atg2Δ::HIS3</i>	This study
TMK728	TMK400 <i>atg9Δ:: his3MX</i>	This study
TMK730	TMK400 <i>atg14Δ:: his3MX</i>	This study
YKY161	BY4741 <i>ATG31-GFP-kanMX atg11Δ::LEU2</i>	This study
YKY157	YKY161 <i>atg17Δ::HIS3</i>	This study
YKY159	YKY161 <i>atg29Δ::natMX</i>	This study
YKY155	BY4741 <i>ATG31-GFP-kanMX atg11Δ::URA3 atg1Δ::LEU2</i>	This study
YKY173	YKY161 <i>atg13Δ::URA3</i>	This study
YKY6	BY4741 <i>ATG17-GFP-his3MX</i>	Kabeya <i>et al.</i> (2007)
TMK672	YKY6 <i>atg11Δ::kanMX atg1Δ::LEU2</i>	This study
TMK576	BY4741 <i>ATG17-GFP-kanMX</i>	This study
TMK580	TMK576 <i>atg1Δ::LEU2</i>	This study
TMK600	TMK576 <i>atg29Δ::natMX</i>	This study
TMK625	TMK576 <i>atg11Δ::LEU2</i>	This study
TMK623	TMK625 <i>atg29Δ::natMX</i>	This study
YKY179	TMK625 <i>atg31Δ::natMX</i>	This study
TMK686	TMK625 <i>atg13Δ::URA3</i>	This study
TMK696	TMK625 <i>atg2Δ::HIS3</i>	This study
TMK725	TMK625 <i>atg9Δ::his3MX</i>	This study
TMK727	TMK625 <i>atg14Δ::his3MX</i>	This study
TMK689	BY4741 <i>ATG2-GFP-kanMX atg11Δ::LEU2</i>	This study
TMK617	TMK689 <i>atg29Δ::natMX</i>	This study
TMK584	BY4741 <i>ATG2-GFP-kanMX atg29Δ::natMX</i>	This study
TMK690	BY4741 <i>ATG5-GFP-kanMX atg11Δ::LEU2</i>	This study
TMK618	TMK690 <i>atg29Δ::natMX</i>	This study
TMK588	BY4741 <i>ATG5-GFP-kanMX atg29Δ::natMX</i>	This study
TMK688	BY4741 <i>ATG1-GFP-kanMX atg11Δ::LEU2</i>	This study
TMK616	TMK688 <i>atg29Δ::natMX</i>	This study
TMK731	TMK688 <i>atg13Δ::URA3</i>	This study
TMK732	TMK688 <i>atg17Δ::URA3</i>	This study
YKY175	TMK688 <i>atg31Δ::natMX</i>	This study
TMK695	BY4741 <i>ATG13-GFP-kanMX atg11Δ::LEU2</i>	This study
TMK621	TMK695 <i>atg29Δ::natMX</i>	This study
TMK765	TMK695 <i>atg1Δ::his3MX</i>	This study
TMK766	TMK695 <i>atg17Δ::his3MX</i>	This study
YKY177	TMK695 <i>atg31Δ::natMX</i>	This study
OND88	W303-1B <i>pho8::pho8Δ60</i>	This study
YYK762	OND88 <i>atg11Δ::LEU2</i>	This study
YYK865	SEY6210 <i>ATG29-YFP-his3MX atg8Δ::TRP1 atg11Δ::LEU2</i>	This study
BJ2168	<i>MATa leu2 trp1 ura3-52 prb1-1122 prc1-407 pep4-3</i>	Yeast Stock Center
YYK806	BJ2168 <i>HA-ATG1 atg29Δ::kanMX</i>	This study
YYK850	BJ2168 <i>HA-ATG1 ATG29-myc-natMX</i>	This study
YYK868	YYK850 <i>atg11Δ::LEU2</i>	This study
YYK870	YYK850 <i>atg11Δ::LEU2 atg17Δ::kanMX</i>	This study
YYK872	YYK850 <i>atg11Δ::LEU2 atg31Δ::kanMX</i>	This study
YYK874	BJ2168 <i>HA-ATG1 ATG31-myc-hphMX</i>	This study
	<i>atg11Δ::LEU2 atg29Δ::kanMX</i>	
YYK878	BJ2168 <i>HA-ATG1 ATG31-myc-hphMX</i>	This study
	<i>atg11Δ::LEU2 atg17Δ::URA3 atg29Δ::kanMX</i>	

Table 2. Plasmids used in this study

Name	Description	Source or reference
pRS313	<i>HIS3</i> , CEN6-ARSH4, amp ^r	Sikorski and Hieter (1989)
pRS314	<i>TRP1</i> , CEN6-ARSH4, amp ^r	Sikorski and Hieter (1989)
pRS316	<i>URA3</i> , CEN6-ARSH4, amp ^r	Sikorski and Hieter (1989)
pRS314x	<i>TRP1</i> , CEN6-ARSH4, amp ^r	Moriya and Isono (1999)
pTN3	pKT10, <i>PHO8Δ60</i>	Noda <i>et al.</i> (1995)
pGBD-C(X)	<i>TRP1</i> , 2μARS, <i>GAL4-BD(1-147)</i> , amp ^r	James <i>et al.</i> (1996)
pGBDU-C(X)	<i>URA3</i> , 2μARS, <i>GAL4-BD(1-147)</i> , amp ^r	James <i>et al.</i> (1996)
pGAD-C(X)	<i>LEU2</i> , 2μARS, <i>GAL4-AD(768-881)</i> , amp ^r	James <i>et al.</i> (1996)
pTM4	pRS314x, <i>ATG29</i>	This study
pTM20	pRS314x, <i>ATG29-Myc</i>	This study
pTM25	pGBD-C2, <i>ATG29(1-213)</i>	This study
pTM28	pGBD-C2, <i>ATG29(1-162)</i>	This study
pTM29	pGBD-C2, <i>ATG29(1-132)</i>	This study
pTM30	pGBD-C2, <i>ATG29(1-69)</i>	This study
pTM31	pGBD-C2, <i>ATG29(44-213)</i>	This study
pTM32	pGBD-C2, <i>ATG29(44-162)</i>	This study
pTM33	pGBD-C2, <i>ATG29(44-132)</i>	This study
pTM34	pGBD-C2, <i>ATG29(44-104)</i>	This study
pTM35	pGBD-C2, <i>ATG29(97-213)</i>	This study
pTM36	pGBD-C2, <i>ATG29(97-162)</i>	This study
pTM37	pGBD-C2, <i>ATG29(97-132)</i>	This study
pTM38	pGBD-C2, <i>ATG29(136-213)</i>	This study
pTM41	pGADU, <i>ATG17</i>	This study
pTM42	pGBD-C2, <i>ATG29^{W54R}</i>	This study
pTM43	pGBD-C2, <i>ATG29^{P64H}</i>	This study
pTM44	pGBD-C2, <i>ATG29^{F67L}</i>	This study
pTM45	pGBD-C2, <i>ATG29^{R71G}</i>	This study
pTM50	pRS316, <i>ATG29^{F67L}</i>	This study
pTM51	pRS316, <i>ATG29^{R71G}</i>	This study
pTM52	pRS314x, <i>ATG29^{W54R}-Myc</i>	This study
pTM53	pRS314x, <i>ATG29^{P64H}-Myc</i>	This study
pTM54	pRS314x, <i>ATG29^{F67L}-Myc</i>	This study
pTM55	pRS314x, <i>ATG29^{R71G}-Myc</i>	This study
pTM108	pRS313, <i>ATG29</i>	This study
pTM109	pRS313, <i>ATG29^{F67L}</i>	This study
pTM110	pRS313, <i>ATG29^{R71G}</i>	This study
pYOK6	pGAD-C1, <i>ATG17(1-417)</i>	This study
pYOK7	pGAD-C1, <i>ATG17(1-354)</i>	This study
pYOK8	pGAD-C1, <i>ATG17(1-209)</i>	This study
pYOK9	pGAD-C1, <i>ATG17(1-99)</i>	This study
pYOK10	pGAD-C1, <i>ATG17(97-417)</i>	This study
pYOK11	pGAD-C1, <i>ATG17(97-354)</i>	This study
pYOK12	pGAD-C1, <i>ATG17(97-209)</i>	This study
pYUK13	pGAD-C1, <i>ATG17(207-417)</i>	This study
pYOK14	pGAD-C1, <i>ATG17(207-354)</i>	This study
pYOK15	pGAD-C1, <i>ATG17(349-417)</i>	This study
pYOK16	pRS314 <i>ATG13(1-738)</i>	This study
pYOK17	pRS314 <i>ATG13(1-448)</i>	This study
pYOK18	pRS316, <i>ATG1</i>	This study
pYOK19	pRS316, <i>ATG1^{D211A}</i>	This study
pYOK20	pRS316, <i>ATG1^{K54A}</i>	This study
pRS314 (CFP-ATG8)	pRS314, CFP-ATG8	Suzuki <i>et al.</i> (2001)

RESULTS

Association of Atg29 with Atg17 Is Required for Autophagy

To understand the mechanism of Atg17 for the PAS assembly, we focused on Atg29, an autophagy-specific protein. We performed yeast two-hybrid analyses of the interaction between Atg29 and Atg17. Atg29 was found to associate with Atg17, suggesting that Atg29 functions with Atg17 (Figure 1A). Because our previous studies have shown that Atg17 physically associates with Atg1 and Atg13, we investigated whether Atg29 interacts with Atg1 or Atg13 by the yeast two-hybrid assay. No interaction between Atg29 and Atg1

or Atg13 was detected in these experiments, suggesting that Atg29 does not possess an intrinsic binding site for Atg1 or Atg13 (data not shown).

To determine the domain of Atg29 required for interaction with Atg17, we constructed and examined a series of truncated forms of Atg29. We found that Atg17 binds to the central region of Atg29 (residues 44-104), which contains a putative coiled-coil motif (Kawamata *et al.*, 2005) (Figure 1A). We also performed the reverse experiment, determining the domain of Atg17 required for interaction with Atg29. Atg17 possesses five putative coiled-coil domains, CC1–CC5 (Cheong *et al.*, 2005). An Atg17 fragment containing CC2

Figure 1. Atg29 interaction with Atg17 is essential for autophagy. (A) The Atg17 binding site in Atg29. The indicated segments of Atg29 were fused to the Gal4 DNA-binding domain (BD). Binding to Atg17 fused to the Gal4-activating domain was evaluated by growth on -Ade plates for 3 d. (B) The Atg29 binding sites in Atg17. The indicated segments of Atg17 were fused to the Gal4 (transcriptional) activation domain (AD). Binding to Atg29 was tested by growth on -His plates containing 3 mM 3-AT. Atg17 interacts with Atg29 via coiled-coil domain 2. (C) Mutant Atg29s (Atg29^{W54R}, Atg29^{P64H}, Atg29^{F67L}, or Atg29^{R71G}) did not associate with Atg17. Two-hybrid assays were carried out as described in A. (D) Mutant Atg29s failed to interact with Atg17 in vivo. *atg29Δ* (TMK4) cells carrying the indicated myc-tagged ATG29 plasmids (wild-type and mutants) were grown in YEPD, and then they were treated with 0.2 μ g/ml rapamycin for 3 h. Cell extracts were immunoprecipitated with anti-myc antibody. Coimmunoprecipitated proteins were analyzed by immunoblotting with anti-myc (top) or anti-Atg17 antibodies (middle). The bottom row indicates the amount of Atg17 in the total lysates. (E) The binding of Atg29 and Atg17 is essential for autophagy. Wild-type (BY4741 + pTN3) or *atg29Δ* (TMK181 + pTN3) cells carrying pTM108 (Atg29), pTM109 (Atg29^{F67L}), pTM110 (Atg29^{R71G}), or pRS313 (empty vector) were cultured in SCD, and then they were transferred to SD(-N) for 4 h, lysed, and assayed for ALP activity. The bars represent the SD of three independent experiments. (F) Atg17 is required for proper Atg29 localization to the PAS. WT (TMK190) or *atg17Δ* (TMK214) cells were grown in SD medium containing casamino acid supplemented with adenine, tryptophan, and uracil, and then they were treated with 0.2 μ g/ml rapamycin. Growing (0 h) and rapamycin-treated (3 h) cells were observed by fluorescence microscopy. The percentages of cells with fluorescent dot signals at the PAS were determined. Bar, 5 μ m. (G) Atg29 is not required for proper Atg17 localization to the PAS. WT (TMK576) or *atg29Δ* (TMK600) cells were tested as in F.

domain (residues 97-209) turned out to be sufficient to bind Gal4BD-Atg29 (Figure 1B). Together, it is likely that Atg17 and Atg29 interact with each other through their coiled-coil domains. Next, we isolated Atg29 mutants that do not bind to Atg17 by two-hybrid assay. We introduced random mutations within the coiled-coil domain region by using PCR-based mutagenesis, and we screened for mutants unable to interact with Atg17 as described in *Materials and Methods*. Among ~300 clones screened, we obtained three mutants (W54R, P64H, and F67L/R71G) that completely lost their ability to bind Atg17. We confirmed that introduction of the W54R, P64H, F67L, or R71G mutation into full-length Atg29 abolishes their interaction with Atg17 in two-hybrid analysis (Figure 1C).

We next examined the Atg17–Atg29 interaction by coprecipitation experiments. We generated a myc-tagged version of Atg29 (Atg29-myc), which was confirmed to complement the autophagic defect of *atg29Δ* cells (data not shown). Atg29-myc efficiently precipitated endogenous Atg17, confirming that Atg17 and Atg29 physically interact *in vivo* (Figure 1D). Rapamycin treatment mimics starvation condition and triggers autophagy (Noda and Ohsumi, 1998). Atg17–Atg29 interaction was detected in the cells treated with or without rapamycin. Atg29^{W54R}, Atg29^{P64H}, Atg29^{F67L}, or Atg29^{R71G} did not associate with Atg17 under either growth or starvation condition. Atg17–Atg29 association was not affected by disruption of other *ATG* genes, e.g., *ATG1*, *ATG13*, *ATG8*, or *ATG11* (data not shown). We next estimated the autophagic activity of these mutants by an ALP assay (Noda *et al.*, 1995). Wild-type Atg29 rescued the autophagic defect of *atg29Δ*, compared with vector control (Figure 1E). In contrast, autophagy was not induced by the *atg29^{F67L}* or *atg29^{R71G}* mutants, but it could be partially recovered when these mutants were overexpressed (data not shown). Atg29 localizes to the PAS (Kawamata *et al.*, 2005), and this localization required *ATG17* (Figure 1F). Atg17–GFP distribution was not affected in *atg29Δ* cells under growth or starvation conditions (Figure 1G).

We have recently reported that Atg31, another autophagy-specific protein, binds to Atg17 (Kabeya *et al.*, 2007). Atg31 also interacts with Atg29 (Kabeya and Ohsumi, unpublished data), suggesting that Atg17, Atg29, and Atg31 form a ternary complex. The PAS localization of Atg31 is also dependent on *ATG17*, but not vice versa. These results suggest that Atg29 and Atg31 associate together with Atg17 and function at the PAS to generate autophagosome.

Autophagy-Unique Atg Protein Complex Is Required for Recruitment of Atg Proteins to the PAS for Autophagosome Formation

It has been recently reported that Atg11 and Atg17 play important roles in the recruitment of other Atg proteins to the PAS (Suzuki *et al.*, 2007). *ATG11* is a Cvt-specific gene, and it is dispensable for autophagy (Kim *et al.*, 2001), and in *atg11Δ* cells the Cvt pathway is abrogated because the PAS is not organized under growth condition (Shintani and Klionsky, 2004). Thus, we used *atg11Δ* cells to learn about the targeting of autophagy-unique Atg proteins to the PAS specifically involved in autophagosome formation under starvation condition. In *atg11Δ* cells, Atg29-GFP, scattered throughout the cytosol under growth condition (Figure 6A), was recruited to the PAS after rapamycin treatment (Figure 2). The PAS localization of Atg29-GFP disappeared in rapamycin-treated *atg11Δatg17Δ* cells, confirming that Atg17 is responsible for the localization of Atg29 to the PAS in *atg11Δ* cells. Atg31 was also required for the PAS localization of

Figure 2. Deletion of *ATG29* in *atg11Δ* cells affects PAS localization of Atg proteins. Localization of GFP-tagged Atg17, Atg29, and Atg31 was analyzed in the indicated *atg* mutant cells. Similarly, localization of Atg2 and Atg5 was observed. Rapamycin-treated (3 h) cells were observed by fluorescence microscopy. The percentages of cells with fluorescent dot signals at the PAS were determined. Bar, 5 μ m.

Atg29. Because the PAS localization of Atg31-GFP was abolished in *atg11Δatg17Δ* and *atg11Δatg29Δ* cells, Atg29 was indispensable for the PAS targeting of Atg31 (Figure 2). We next examined Atg17-GFP in *atg11Δatg29Δ* and *atg11Δatg31Δ* cells. Atg29 or Atg31 was not required for the localization of Atg17 in the presence of Atg11 (Figure 1G) (Kawamata *et al.*, 2005; Kabeya *et al.*, 2007); however, unexpectedly PAS localization of Atg17-GFP disappeared in *atg11Δatg29Δ* and *atg11Δatg31Δ* mutants (Figure 2). These results suggest that Atg17, Atg29, and Atg31 are mutually essential for the PAS localization. We reported previously that any Atg protein does not target to the PAS in *atg11Δatg17Δ* cells (Suzuki *et al.*, 2007). Thus, we reexamined this using *atg11Δatg29Δ* strain. Although Atg2-GFP and Atg5-GFP normally localized to the PAS in *atg11Δ* and *atg29Δ* cells, their PAS localization was severely impaired in *atg11Δatg29Δ* cells, indicating that not only Atg17 but also Atg29 is essential for the PAS localization of Atg proteins (Figure 2). These results suggest that at least in *atg11Δ* cells the autophagy-specific protein complex plays an important role in starvation-induced assembly of Atg proteins to the PAS responsible for autophagosome formation (but not for the Cvt vesicle).

Figure 3. The PAS localization of Atg17 or Atg29 does not depend on Atg2, Atg9, or Atg14 in *atg11Δ* cells. Localization of Atg17-GFP and Atg29-GFP in the indicated *atg* mutants (treated with rapamycin for 3 h) was observed by fluorescence microscopy. The percentages of cells with fluorescent dot signals at the PAS were determined. Bars, 5 μ m.

Atg1 Complex Also Plays an Essential Role in the Localization of Atg Proteins under Starvation Condition

Next, we examined the effect of another *ATG* gene disruption on localization of Atg17 and Atg29 in *atg11Δ* cells in the presence of rapamycin. Although Atg17-GFP and Atg29-GFP localized to the PAS in *atg1Δ* and *atg11Δ* cells, none of them localized to the PAS in *atg1Δatg11Δ* cells (Figure 3). In contrast, they significantly localized to the PAS in *atg2Δatg11Δ*, *atg9Δatg11Δ*, and *atg11Δatg14Δ* cells. According to the previous report (Suzuki *et al.*, 2007), *ATG1* and *ATG9* are situated next to *ATG17* in the hierarchy of protein assembly required for PAS formation, *ATG14* is located downstream of *ATG9*, and *ATG2* is located downstream of *ATG1* and *ATG14* in the *ATG* gene hierarchy map. In addition to the autophagy-specific proteins, Atg1 plays an important role in organization of the PAS.

Because Atg1 binds to Atg13 in response to starvation (Kamada *et al.*, 2000), we tested whether Atg1–Atg13 complex is involved in the starvation-responsive recruitment of Atg proteins to the PAS. As well as *ATG1*, *ATG13* was found

Figure 4. All of five proteins (Atg1, Atg13, Atg17, Atg29, and Atg31) play important role in their localization at the Atg11-independent PAS. (A) In *atg11Δ* cells, both Atg1 and Atg13 maintain Atg17-GFP, Atg29-GFP, and Atg31-GFP at the PAS. Rapamycin-treated (3 h) cells were observed by fluorescence microscopy. (B) Localization of Atg1-GFP and Atg13-GFP in the indicated *atg* cells was examined as described in A. Bars, 5 μ m.

to be required for the PAS localization of the autophagy-specific proteins (Figure 4A). Then, we tested localization of Atg1 and Atg13 in *atg1Δatg11Δ*, *atg11Δatg13Δ*, *atg11Δatg17Δ*, *atg11Δatg29Δ*, and *atg11Δatg31Δ* mutants. The results, shown in Figure 4B, are summarized in Table 3. We found that these proteins are equally required for PAS targeting. In conclusion, these five proteins (Atg1–Atg13 complex and Atg17–Atg29–Atg31 autophagy-unique proteins) participate in the recruitment of Atg proteins to the *ATG11*-independent PAS for autophagosome formation under starvation condition.

Atg17–Atg29 and Atg1–Atg13 Interactions Are Essential for the PAS Organization

As shown above, *ATG29* and *ATG31* are necessary for the PAS localization of Atg1, Atg13, and Atg17 in *atg11Δ* cells under autophagy-inducing conditions. To further examine whether association of Atg29 to Atg17 is required for the PAS targeting, we examined the localization of Atg proteins in cells expressing Atg29^{F67L} and Atg29^{R71G}. Expression of Atg29^{WT} completely rescued the PAS localization of Atg1-GFP, Atg13-GFP, and Atg17-GFP in *atg11Δatg29Δ* cells treated with rapamycin (Figure 5A). In contrast, Atg29^{F67L} and Atg29^{R71G} failed to recruit any of these GFP-tagged Atg proteins to the PAS. Thus, the Atg17–Atg29 association is indispensable for Atg1, Atg13, and Atg17 localization.

In *atg11Δatg13Δ* cells, Atg29-GFP was dispersed in the cytosol (Figure 4A). A C-terminally truncated mutant of Atg13 (Atg13^{1–448}) lacks binding activity with Atg1, resulting

Table 3. Summary of localization of Atg1-GFP, Atg13-GFP, Atg17-GFP, Atg29-GFP, and Atg31-GFP in *atg11Δ*, *atg11Δatg1Δ*, *atg11Δatg13Δ*, *atg11Δatg17Δ*, *atg11Δatg29Δ*, or *atg11Δatg31Δ* cells under vegetative (above) or starvation (below) conditions

Strain	<i>atg11Δ</i>	<i>atg11Δatg1Δ</i>	<i>atg11Δatg13Δ</i>	<i>atg11Δatg17Δ</i>	<i>atg11Δatg29Δ</i>	<i>atg11Δatg31Δ</i>
Atg1-GFP	–	/	–	–	–	–
Atg13-GFP	–	–	/	–	–	–
Atg17-GFP	–	–	–	/	–	–
Atg29-GFP	–	–	–	–	/	–
Atg31-GFP	–	–	–	–	–	/
	<i>atg11Δ</i>	<i>atg11Δatg1Δ</i>	<i>atg11Δatg13Δ</i>	<i>atg11Δatg17Δ</i>	<i>atg11Δatg29Δ</i>	<i>atg11Δatg31Δ</i>
Atg1-GFP	+	/	–	–	–	–
Atg13-GFP	+	–	/	–	–	–
Atg17-GFP	+	–	–	/	–	–
Atg29-GFP	+	–	–	–	/	–
Atg31-GFP	+	–	–	–	–	/

+, PAS localization observed; –, no PAS localization observed.

Figure 5. Atg17–Atg29 and Atg1–Atg13 interactions are indispensable for their recruitment to the PAS. (A) Atg29 mutant (Atg29^{R71G}) and Atg29^{F67L} is defective for the ATG11-independent PAS localization of Atg1, Atg13, or Atg17. *atg11Δatg29Δ* cells expressing GFP-tagged Atg1, Atg13, or Atg17 were transformed with a plasmid expressing either Atg29^{wild-type}, Atg29^{F67L}, or Atg29^{F71G}. Cells were treated with rapamycin for 3 h before observation. (B) Atg13¹⁻⁴⁴⁸ does not recruit Atg29 to the PAS in *atg11Δ* cells. *atg11Δatg13Δ* cells expressing Atg29-GFP were transformed with a plasmid expressing either Atg13^{wild-type} or Atg13¹⁻⁴⁴⁸. Cells were observed after rapamycin treatment for 3 h. (C) Atg1 kinase activity is not essential for the PAS targeting of Atg17 and Atg29 in *atg11Δ*. *atg11Δatg1Δ* cells expressing Atg17-GFP or Atg29-GFP were transformed with a plasmid expressing either wild-type Atg1 or Atg1 kinase-deficient mutant (Atg1^{D211A} or Atg1^{K54A}). Cells were observed after rapamycin-treatment for 3 h. Bars, 5 μm.

in an autophagy defect, whereas the Cvt pathway was not affected in this mutant (Kamada *et al.*, 2000). Atg13¹⁻⁴⁴⁸ did not recruit Atg1-GFP, Atg17-GFP, or Atg29-GFP to the PAS in *atg11Δatg13Δ* cells (Figure 5B; data not shown). Therefore, the Atg1–Atg13 interaction is also essential for the PAS localization of these Atg proteins. Taking these results together, it seems likely that the PAS localization of Atg1, Atg13, Atg17, and Atg29 is mediated by the interactions among them.

Atg1 is a protein kinase essential for autophagy. We tested whether Atg1 kinase activity is required for the PAS targeting of Atg17 and Atg29 in *atg11Δ*. We observed localization of Atg17-GFP or Atg29-GFP in *atg11Δatg1Δ* cells expressing either Atg1^{WT} or a kinase-deficient Atg1^{D211A} and Atg1^{K54A} (Matsuura *et al.*, 1997; Kamada *et al.*, 2000). We confirmed that both Atg1^{K54A} and Atg1^{D211A} interact normally with Atg13 and Atg17 as well as Atg1^{WT} (Kamada and Ohsumi, unpublished results). Atg1^{WT} complemented the starvation-induced targeting of Atg17-GFP and Atg29-GFP to the PAS in *atg11Δatg1Δ* cells (Figure 5C). PAS localization of Atg17-GFP and Atg29-GFP was also observed in both *atg1^{K54A}atg11Δ* cells and *atg1^{D211A}atg11Δ* cells (Figure 5C). Thus, not the protein kinase activity but Atg1 protein itself is required for the recruitment of these two proteins. It is noteworthy that Atg17- and Atg29-GFP abnormally accumulated at the PAS in *atg1^{K54A}atg11Δ* and *atg1^{D211A}atg11Δ* than in *ATG1^{WT}atg11Δ* (Figure 5C), suggesting that the proper sub-cellular redistribution of autophagy-specific Atg proteins requires Atg1 kinase activity.

The PAS Generating Autophagosomes Is Organized in Response to Nutrient Condition

In *atg11Δ* cells, the organization of the PAS, which is specifically needed for autophagy, should be regulated by nutrient conditions. As expected, Atg17-GFP and Atg29-GFP in growing *atg11Δ* cells were recruited to the PAS only after rapamycin treatment or nitrogen depletion (Figure 6, A and B). The protein amounts of Atg17 and Atg29 were not dramatically affected by rapamycin treatment (Figure 1D), suggesting that the recruitment of Atg17 and Atg29 to the PAS is highly stimulated by starvation. We further observed that cyan fluorescent protein (CFP)–Atg8 was also recruited to the PAS in response to nitrogen starvation, colocalizing with Atg29–yellow fluorescent protein (YFP) (Figure 6C). When nitrogen-starved *atg11Δ* cells were supplied with a nitrogen source, such as amino acids and ammonium sulfate, PAS-localized Atg8 and Atg29 quickly disappeared within 10 min (Figure 6, B and C), indicating that recruitment of Atg

Figure 6. Assembly of Atg proteins to the PAS is regulated in response to nutrient conditions. (A) Recruitment of Atg17 or Atg29 to the PAS is induced by starvation in *atg11Δ* cells. Atg29-GFP and Atg17-GFP were observed in wild-type or *atg11Δ* cells under both growth and rapamycin-treated (3 h) conditions. (B) Atg29 localization in response to nutrient conditions in wild-type or in *atg11Δ* cells. Cells grown in SD medium containing casamino acid supplemented with adenine, tryptophan, and uracil were shifted to SD(-N). Nitrogen-starved cells (-N, 2 h) were supplied with 2× SD medium containing casamino acid supplemented with adenine, tryptophan, and uracil, and then they were incubated for 10 min. (C) PAS recruitment of Atg8 in response to starvation. *atg11Δatg8Δ* cells (YYK865) harboring the CFP-ATG8 (pRS314) were observed as described in B. CFP-Atg8 and Atg29-YFP were colocalized in starved cells. (Noted that CFP signal was observed in the vacuole indicating the induction of autophagy.) (D) Accumulation of Atg17 and Atg29 to the PAS in *atg11Δatg1Δ + atg1^{D211A}* is starvation specific. The localization of Atg17-GFP and Atg29-GFP was analyzed as described in B. (E) Comparison of autophagic activity of wild-type cells and *atg11Δ* cells in response to cellular nutrient condition. Wild-type (OND88) or *atg11Δ* (YYK762) cells genomically expressing Pho8Δ60 protein were cultured in YEPD, and then they were shifted to SD(-N) at time 0. After SD(-N) for 4 h, nitrogen-starved cells were supplied with nutrients as described in B. After 1 or 2 h, cells were corrected and subjected to ALP assay. The bars represent the SD of three independent experiments. Bars (A–D), 5 μm.

proteins to the PAS is tightly regulated by nutrient conditions. In *atg1^{D211A}atg11Δ* cells, the accumulated Atg17-GFP and Atg29-GFP were also quickly dispersed into the cytosol by addition of a nitrogen source within 10 min, suggesting that Atg1 kinase activity is not required for perception of nutrient signal (Figure 6D).

Based on GFP observation, Atg17 and Atg29 seemed to stay statically at the PAS irrespective of nutrient condition in wild-type (*ATG11*) cells, because the Atg11-dependent PAS was formed under nutrient-rich condition (Figure 6, A and B). However, the above-mentioned results using *atg11Δ* cells suggested that recruitment of Atg proteins to the PAS is dynamically regulated by cellular nutrient status. To examine whether Atg proteins assemble to the PAS in response to starvation even in wild-type cells, excluding the possibility that our observation is an artifact of deletion of *ATG11*, we carried out several experiments. Autophagy was induced both in nitrogen-depleted wild-type and *atg11Δ* cells (Kim *et al.*, 2001) (Figure 6E). When nutrients were supplied to the starved cells (at 4 h after starvation), autophagic activity was immediately terminated in both strains (Figure 6E). This

result indicates that the PAS responsible for autophagosome formation is organized only under starvation conditions. Furthermore, because the autophagic activity is terminated immediately after nutrient resupply, the results also imply that in wild-type (*ATG11*) cells, the autophagy-specific PAS changes to the *ATG11*-dependent, Cvt-specific PAS according to the availability of a nutrient signal.

Next, we analyzed Atg protein assembly by an immunoprecipitation experiment. When HA-tagged Atg1 was immunoprecipitated from cell lysates, coprecipitation of Atg13, Atg17, Atg29, and Atg31 was strongly enhanced by nitrogen starvation (Figure 7, A–C). When starved cells were shifted to nutrient-rich medium, these proteins quickly dissociated within 10 min. Atg1–Atg11 interaction was not affected by nutrient condition. It is noteworthy that this regulation was observed in both wild-type and *atg11Δ* cells. These results support that autophagy-specific Atg proteins assemble to and disassemble from the PAS in response to nutrient conditions, even though GFP-fusions of Atg proteins apparently stay statically at the PAS in wild-type cells. Starvation-induced Atg1 interaction toward Atg29 and Atg31 was abol-

Figure 7. Association of Atg1, Atg13, Atg17, Atg29, and Atg31 is regulated by nutrient condition. (A) Interaction of Atg1 with Atg13, 17, and Atg29 is enhanced by starvation. Wild-type (YYK850) or *atg11 Δ* (YYK868) cells grown in YEPD (lanes 1, 5, 8, and 11) were transferred to SD(-N) for 2 h (lanes 2, 3, 6, 9, and 12). Starvation was terminated by adding 2 $\times$ YEPD for 10 min (lanes 4, 7, 10, and 13). HA-Atg1 was immunoprecipitated by anti-HA ascite (Ab), and immunocomplex (lanes 1–7) was analyzed by immunoblot with the indicated antibodies. Total cell extracts also are shown (lanes 8–13). Note that all Atg proteins were chromosomally expressed under their own promoter. (B) Atg17 and Atg31 are required for starvation-induced Atg1–Atg29 association. *atg11 Δ* (YYK868), *atg11 Δ atg17 Δ* (YYK870) and *atg11 Δ atg31 Δ* (YYK872) cells were analyzed as described in A. (C) Atg1–Atg31 association is disrupted in *atg11 Δ atg17 Δ* and *atg11 Δ atg29 Δ* cells. The experiment described in A was carried out using *atg11 Δ atg29 Δ* (YYK874) harboring plasmid pTM4 (*atg11 Δ*), *atg11 Δ atg17 Δ* atg29 Δ (YYK878) harboring plasmid pTM4 (*atg11 Δ atg17 Δ*), or *atg11 Δ atg29 Δ* (YYK874).

ished in *atg11 Δ atg17 Δ* , *atg11 Δ atg31 Δ* , or *atg11 Δ atg29 Δ* cells (Figure 7, B and C). Together, these results indicate that Atg1–Atg13 and autophagy-specific Atg proteins (Atg17, Atg29, and Atg31) assemble together to the PAS in response to starvation for induction of autophagy.

DISCUSSION

In this study, we investigated PAS dynamics by observation of the GFP-tagged autophagy-specific proteins Atg17, Atg29, and Atg31. Previously, we found the PAS through the localization analysis of Atg proteins under vegetative condition (Suzuki *et al.*, 2001). Originally, the PAS was defined as a GFP–Atg8 signal a punctate structure to which almost all Atg proteins localized (Suzuki *et al.*, 2001). In *S. cerevisiae*, the Cvt pathway, which is an autophagy-related biosynthetic pathway, constitutively works in the presence of nutrients. Because most Atg proteins are required for both autophagy and the Cvt pathway, they assemble to organize the PAS under growth (i.e., nutrient-rich) conditions, and

they seem to stay at the PAS under starvation condition. Therefore, it was difficult to precisely describe the dynamics of Atg proteins under autophagy-inducing conditions, such as nitrogen starvation and rapamycin treatment. Accumulating evidence shows that Atg11 (a Cvt pathway-specific protein) is important for gathering Atg proteins to the PAS under growth conditions. For example, Atg11 has an intrinsic binding activity toward Atg17 (Cheong *et al.*, 2005), thus autophagy-specific proteins presumably localized to the Atg11-dependent PAS under nutrient-rich condition. Here, we used *atg11 Δ* cells to disconnect the autophagic pathway from the Cvt pathway. This enabled us to observe Atg proteins localizing at the PAS in an autophagy-dependent manner, specialized to form autophagosomes (but not the Cvt vesicle). Therefore, our results suggest the starvation-induced PAS responsible for autophagosome formation. They also confirm the existence of the Atg11-dependent, Cvt-specific PAS (Shintani and Klionsky, 2004). The Cvt-specific PAS seems to be present under conditions permissive for growth. So far, we know of no marker that can

distinguish the Cvt-specific PAS from the autophagy-specific PAS in wild-type cells, but the former is unable to form an autophagosome at all (Figure 6).

We found interesting characteristics of the PAS responsible for autophagosome formation. First, Atg proteins assemble to organize the PAS in response to nutrient starvation, and they quickly disassemble after resupply of nutrients (Figures 6 and 7). Our results suggest that the PAS is a dynamic structure in which many Atg proteins continuously assemble and disassemble. For example, although GFP-fusions of Atg1, Atg13, and autophagy-unique Atg protein complex (Atg17, Atg29, and Atg31) seem to statically localize at the PAS in wild-type cells, their physical association is drastically changed by nutrient conditions. Second, Atg1-Atg13 and autophagy-specific Atg proteins act as an organizing center of the starvation-induced PAS. Our previous report showed that Atg17 is the most fundamental protein in PAS organization (Suzuki *et al.*, 2007). Here, we show that Atg17 forms a ternary complex with Atg29 and Atg31 and that this association is indispensable for recruitment of Atg proteins to the PAS to generate autophagosomes. Atg1-Atg13 is also required for recruitment of autophagy-specific Atg complex to the PAS. We reported previously that the Atg17-GFP localization to the PAS is not disrupted in *atg1Δ* *ATG11* or *atg13Δ* *ATG11* cell (Suzuki *et al.*, 2007). We obtained a similar result about the PAS targeting of Atg29 and Atg31 (Figure 3; data not shown), indicating that Atg11 can recruit Atg17 and Atg29 to the PAS in an Atg1- and Atg13-independent manner. Because Atg11 has an intrinsic binding activity toward a couple of Atg proteins (e.g., Atg9 and Atg17), we assume that Atg9 and Atg17 accumulated to the (presumably Atg11-dependent) PAS in *atg1Δ* cells.

Tor signaling controls Atg1-Atg13 interaction through the phosphorylation state of Atg13. In growth condition, Atg13, when highly phosphorylated in a Tor-dependent manner, has low affinity for Atg1 (Figure 7) (Kamada *et al.*, 2000). In starvation condition, Atg13 is immediately dephosphorylated and it associates with Atg1 kinase. This is one of the earliest and most important steps in induction of autophagy. In agreement with our previous study (Kamada *et al.*, 2000), our present results indicate that assembly of Atg1, Atg13, and the autophagy-specific protein complex is tightly regulated by nutrient condition, and it is likely that Tor-dependent phosphorylation of Atg13 is responsible for this regulation. Studies using *atg13* and *atg29* mutants strongly support the idea that Atg1-Atg13 and Atg17-Atg29 associations are indispensable for the assembly of Atg proteins to the PAS. The Cvt pathway is observed in *atg13¹⁻⁴⁴⁸* cells (Kamada *et al.*, 2000), and it does not require either *ATG17*, *ATG29*, or *ATG31*, confirming that these associations are involved in the autophagy-specific process. Because Atg17-Atg29 binding is detected in *atg1Δ* and *atg13Δ* mutants (our unpublished results), and Atg1-Atg13 binding is detected in starved *atg11Δ**atg17Δ* mutants (Figure 7B), Atg17-Atg29-Atg31 complex formation and Atg1-Atg13 association are prerequisites for subsequent recruitment to the PAS.

Atg1-Atg13 association also enhances kinase activity of Atg1 (Kamada *et al.*, 2000). We have presented evidence that kinase activity of Atg1 is essential for autophagy, demonstrating autophagy is not induced in *Atg1^{K54A}* or *Atg1^{D211A}* (kinase-deficient mutants) or *Atg1^{M102A}* (ATP analogue-sensitive mutant) (Matsuura *et al.*, 1997; Kamada *et al.*, 2000; Kabeya *et al.*, 2005). In the present study, we show that Atg1 protein itself has another role in recruiting Atg proteins to the PAS to generate autophagosome. Atg17 and Atg29 accumulated to abnormally high levels at the PAS in *atg1* kinase-deficient cells (Figure 3) and recruitment of Atg2 to

the PAS requires Atg1 kinase activity (Sekito and Ohsumi, unpublished data), suggesting that kinase activity of Atg1 plays an essential role in assembly of other proteins, such as Atg2, to the PAS for autophagosome formation. We hypothesize that the kinase activity is required for the subsequent step(s) after assembly of Atg1-Atg13 and autophagy-unique Atgs, possibly phosphorylating target protein(s). Our findings clearly indicated that assembly of Atg proteins to the PAS responsible for autophagosome formation is mediated by autophagy-specific Atg proteins. Organization of the PAS should be acutely controlled in response to cellular nutrient environment.

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