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# Assortment of Phosphatidylinositol 3-Kinase Complexes—Atg14p Directs Association of Complex I to the Pre-autophagosomal Structure in *Saccharomyces cerevisiae*

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In the yeast *Saccharomyces cerevisiae*, two similar phosphatidylinositol 3-kinase complexes (complexes I and II) function in distinct biological processes, complex I in autophagy and complex II in the vacuolar protein sorting via endosomes. Atg14p is only integrated into complex I, likely facilitating the function of complex I in autophagy. Deletion analysis of Atg14p revealed that N-terminal region containing the coiled-coil structures was essential and sufficient for autophagy. Atg14p localized to pre-autophagosomal structure (PAS) and vacuolar membranes, whereas Vps38p, a component specific to complex II, localized to endosomes and vacuolar membranes. Vps34p and Vps30p, components shared by the two complexes, localized to the PAS, vacuolar membranes, and several punctate structures that included endosomes. The localization of these components to the PAS was Atg14p dependent but not dependent on Vps38p. Conversely, localization of these proteins to endosomes required Vps38p but not Atg14p. Vps15p, regulatory subunit of the Vps34p complexes, localized to the PAS, vacuolar membranes, and punctate structures independent of both Atg14p and Vps38p. Together, these results indicate that complexes I and II function in distinct biological processes by localizing to specific compartments in a manner mediated by specific components of each complex, Atg14p and Vps38p, respectively.

## INTRODUCTION

Membrane trafficking events should occur in a spatiotemporally regulated manner to deliver vesicles and their cargo proteins to the correct destination. The class III phosphatidylinositol 3-kinase (PI3-kinase; hereafter PI3-kinase indicates this class III kinase) participates in the regulation of membrane trafficking. PI3-kinase is a lipid kinase that specifically phosphorylates phosphatidylinositol at the D-3 position of the inositol ring to produce phosphoinositide 3-phosphate (PI3-P) (Schu *et al.*, 1993; Stack and Emr, 1994). The generated PI3-P is recognized by downstream effectors; these effector molecules concentrate to a restricted area of the membrane where the subsequent membrane trafficking events occur (reviewed in Martin, 1998; Odorizzi *et al.*, 2000). PI3-kinase is involved in membrane trafficking events, including endocytosis, the sorting of soluble vacuolar proteins, and autophagy (Schu *et al.*, 1993; Munn and Riezman, 1994; Kihara *et al.*, 2001). Currently, the mechanisms by which PI3-kinase mediates these diverse functions while maintaining the fidelity of each membrane-trafficking event remains poorly understood.

The involvement of PI3-kinase in membrane trafficking has been extensively studied in yeast. Vps34p, the only

PI3-kinase encoded by yeast, is required for the sorting of nascent soluble vacuolar hydrolases, such as carboxypeptidase Y (CPY), via endosomes (Robinson *et al.*, 1988; Schu *et al.*, 1993). Yeast cells harboring point mutations in the Vps34p lipid kinase domain missort CPY into the secretory pathway. The function of Vps34p is dependent on the protein kinase activity of Vps15p, a serine/threonine kinase; therefore,  $\Delta vps15$  cells display a similar phenotype to that seen for  $\Delta vps34$  cells (Herman and Emr, 1990; Herman *et al.*, 1991a, b; Stack *et al.*, 1993, 1995). PI3-kinase is also involved in protein transport in mammalian cells. Proteins resident in lysosomes, an organelle functionally equivalent to the yeast vacuole, are erroneously secreted after treatment with PI3-kinase inhibitors (Brown *et al.*, 1995; Davidson, 1995), suggesting a conserved function for PI3-kinases in protein sorting into the vacuole/lysosome in both yeast and mammalian cells. The human homologue of Vps15p associates with the Vps34p homologue in vivo (Volinia *et al.*, 1995).

Autophagy is another major pathway delivering proteins into the vacuole/lysosome. Using this process, cells degrade large quantities of their cytoplasmic content to recycle the components in response to severe shortages of nutrients. This process is composed of multiple steps that are well conserved among eukaryotes. On induction of autophagy, the cytoplasmic contents are enclosed into a double membrane structure called an autophagosome. The autophagosome then fuses with a vacuole/lysosome; the inner membrane structures and packaged cytoplasmic macromolecules are degraded within this compartment (Baba *et al.*, 1994; Mizushima *et al.*, 2001; reviewed in Klionsky and Ohsumi, 1999). Microscopic analysis suggested that the majority of autophagy-related (Atg) proteins localize to a perivacuolar structure called pre-autophagosomal structure (PAS) at which autophagosomes are thought to originate (Suzuki *et al.*,

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Abbreviations used: ALP, alkaline phosphatase; API, aminopeptidase I; CPY, carboxypeptidase; PAS, preautophagosomal structure; PI3-kinase, phosphatidylinositol 3-kinase; PI3-P, phosphoinositide 3-phosphate; WT, wild-type.

**Table 1.** Strains used in this study

Strain	Genotype	Source
SEY6210	<i>MATα leu2 ura3 his3 trp1 lys2 suc2</i>	Robinson <i>et al.</i> (1988)
KVY55	SEY6210 <i>pho8::PHO8Δ60</i>	Kirisako <i>et al.</i> (1999)
YOK9	KVY55 <i>Δatg14::kanMX</i>	This study
YOK359	KVY55 <i>Δatg11::LEU2</i>	This study
YOK360	KVY55 <i>Δatg11::LEU2 Δatg14::kanMX</i>	This study
YOK135	SEY6210 <i>Δvps38::HIS3</i>	This study
GYS115	SEY6210 <i>Δatg14::LEU2</i>	Suzuki <i>et al.</i> (2001)
BJ2168	<i>MATα leu2 trp1 ura3 pep4-3 prb1-1122 prc1-407</i>	Yeast Genetic Stock Center
STY103	BJ2168 <i>Δatg14::kanMX</i>	This study
YOK219	SEY6210 <i>Δatg14::LEU2 Δvps38::HIS3</i>	This study
YOK358	KVY55 <i>Δatg14::kanMX Δvps38::HIS3</i>	This study
YOK435	<i>MATα Δatg14::LEU2/Δatg14::LEU2 Δvps38::HIS3/Δvps38::HIS3 API-mDsRed::natMX/API</i>	This study
YOK89	<i>MATα Δvps38::LEU2/VPS38-YEGFP::natMX API-mDsRed::kanMX/API</i>	This study
YOK152	<i>MATα VPS34/VPS34-YEGFP::kanMX API-mDsRed::natMX/API</i>	This study
YOK153	YOK152 <i>Δatg14::LEU2/Δatg14::LEU2</i>	This study
YOK154	<i>MATα VPS34/VPS34-YEGFP::kanMX API-mDsRed::kanMX/API Δvps38::TRP1/Δvps38::HIS3</i>	This study
YOK173	YOK152 <i>Δatg14::LEU2/Δatg14::LEU2 Δvps38::HIS3/Δvps38::TRP1</i>	This study
YOK179	<i>MATα VPS30/VPS30-YEGFP::kanMX API-mDsRed::natMX/API</i>	This study
YOK180	YOK179 <i>Δatg14::LEU2/Δatg14::HIS3</i>	This study
YOK181	<i>MATα VPS30/VPS30-YEGFP::kanMX API-mDsRed::kanMX/API Δvps38::TRP1/Δvps38::HIS3</i>	This study
YOK172	<i>MATα VPS30-YEGFP::kanMX Δatg14::HIS3 Δvps38::TRP1</i>	This study
YOK190	<i>MATα VPS15-YEGFP::kanMX/VPS15-YEGFP::kanMX API-mDsRed::natMX/API</i>	This study
YOK191	YOK190 <i>Δatg14::LEU2/Δatg14::LEU2</i>	This study
YOK192	YOK190 <i>Δvps38::TRP1/Δvps38::HIS3</i>	This study
YOK193	YOK190 <i>Δatg14::LEU2/Δatg14::LEU2 Δvps38::HIS3/Δvps38::TRP1</i>	This study

2001). Because subcellular fractionation and electron microscopic studies have revealed that this poorly characterized structure does not contain vacuolar, endoplasmic reticulum (ER), Golgi, or late-endosomal markers, autophagosome formation likely involves a novel process that is distinct from the mechanisms governing the classical endomembrane system (reviewed in Noda *et al.*, 2002). PI3-kinase also functions in the membrane dynamics of autophagy (Kiel *et al.*, 1999). *Δvps34* and *Δvps15* mutant cells exhibited a complete loss of autophagic activity as seen for other autophagy-defective mutants (*atg* mutants) (Tsukada and Ohsumi, 1993; Thumm *et al.*, 1994; Kihara *et al.*, 2001). Two additional genes essential for autophagy, *ATG14* and *ATG6/VPS30*, are subunits of a PI3-kinase complex (Kihara *et al.*, 2001). In addition, autophagy is suppressed in mammalian cells treated with PI3-kinase inhibitors (Petiot *et al.*, 2000), confirming the conserved role of PI3-kinase in autophagy from yeast to mammals.

Although only one functional lipid kinase is encoded by yeast, we have previously identified two distinct PI3-kinase complexes, complexes I and II, in this organisms (Kihara *et al.*, 2001). Complex I functions in autophagy, whereas complex II functions in vacuolar protein sorting. Both of these complexes contain three common subunits, Vps15p, Vps34p, and Vps30p. Vps15p tethers the complex to the membrane; although this protein is myristylated at its N terminus, membrane association of the complex is not dependent solely on myristoylation (Herman *et al.*, 1991b). Vps34p is recruited to the membrane and activated by Vps15p (Stack *et al.*, 1995). The protein kinase activity of Vps15p is essential for complex formation; the lipid kinase activity of Vps34p is not. Whereas the function of Vps30p within these PI3-kinase complexes is not well understood, the homologous protein Beclin 1 has also been implicate in autophagy in both animals and plants (Liang *et al.*, 1999; Liu *et al.*, 2005). In addition to the common subunits, each complex also contains a unique factor. Atg14p is integrated into complex I, whereas Vps38p is specific for complex II. These additional

subunits may act as connector molecules, bridging Vps30p and Vps34p to allow complex formation (Kihara *et al.*, 2001). These two complexes function in distinct biological processes; disruption of *ATG14* does not affect vacuolar protein sorting, whereas deletion of *VPS38* does not affect autophagy (Kametaka *et al.*, 1998; Kihara *et al.*, 2001). The mechanisms determining the specificity of these PI3-kinase complex functions have not been examined. Because both complexes exhibit PI3-kinase activity (Kihara *et al.*, 2001), we assume that the primary functions of the two complexes are the same, namely, the production of PI3-P. Additional factors are likely necessary to direct each PI3-kinase complex to function in distinct biological processes, despite generating a common primary product. Atg14p and Vps38p may be these key factors, both conferring upon each complex a specificity of function and connecting Vps30p and Vps34p.

In this study, we investigated the function of Atg14p by deletion analysis. We also monitored the intracellular localization of the components of individual PI3-kinase complexes. Based on these analyses, we demonstrated the function of Atg14p in autophagy. These results led to our proposal of a model detailing the mechanisms by which PI3-kinase complexes I and II acquire distinct functions.

## MATERIALS AND METHODS

### Yeast Strains and Media

The *S. cerevisiae* strains used in this study were derived from SEY6210 (Robinson *et al.*, 1988) or BJ2168 (Yeast Genetic Stock Center, University of California, Berkeley, CA) strains, as specified in Table 1. We used standard methods and media for yeast manipulation (Kaiser *et al.*, 1994). *Δatg14::kanMX* mutants were constructed by amplifying the region containing the disruption marker and the flanking sequence by PCR from genomic DNA prepared from the BY4741 *Δatg14::kanMX* strain. The *ATG14* locus was replaced with the amplified fragment. *Δvps38::HIS3*, *Δvps38::TRP1*, and *Δvps38::LEU2* mutants were constructed by amplifying the regions containing the disruption marker and the flanking sequences by PCR using pRS313, pRS314, and pRS315 (Sikorski and Hieter, 1989) as templates, respectively. These amplified disruption cassettes replaced *VPS38*. Disruption of *ATG11* was performed as

described previously (Kim *et al.*, 2001). Green fluorescent protein (GFP) was integrated into the genomic DNA as follows. A region containing GFP (S65T), the *ADH1* termination sequence, and the *kanMX6* marker was amplified by PCR from pFA6a-GFP(S65T)-*kanMX6* (Longtine *et al.*, 1998) using primer sets containing the flanking regions of the target genes and then inserted directly into the chromosomes. Vps15-GFP, Vps30-GFP, and Vps34-GFP fusion proteins were functional for the maturation of API and growth at 37°C. *API-RFP::kanMX6* cells were constructed as follows. Sequence encoding red fluorescent protein (RFP) (monomeric RFP [mRFP]; Campbell *et al.*, 2002) was amplified by PCR from the *RFP* sequence on pRSETB vector obtained from Carlsberg Research Center (Copenhagen, Denmark), to have *PacI* and *AscI* sites at 5' and 3' end, respectively. Amplified fragment was ligated into the *PacI/AscI* site of pFA6a-GFP (S65T)-*kanMX6* to replace the GFP sequence. From this plasmid, the region containing *RFP*, *ADH1* termination sequence, and *kanMX6* marker was amplified by PCR with primer sets containing flanking regions of the target genes. Amplified cassettes were inserted directly into the chromosome. *API-RFP::natMX6* cells were constructed by replacing the *kanMX6* marker with a PCR-amplified *natMX6* fragment. All primer sequences are available upon request.

Autophagy was induced by transferring the cells into nitrogen-depleted medium SD (-N), or S (-NC) medium in which both nitrogen and carbon have been depleted.

### Plasmid Construction

The promoter region of *ATG14*, a hemagglutinin (HA)-GFP sequence, and the 3' untranslated region of *ATG14* were cloned in tandem into pRS316 to generate the pOK4 vector. Linker sequences (two repeats of the sequence encoding GlyGlyGlySer) were added before the coding sequences for HA and GFP. Sequences encoding the Atg14p deletion series were ligated in frame into the site between the promoter sequence and HA-GFP in pOK4. To construct multicopy plasmids overexpressing the variants, the *KpnI/SacI* fragment derived from these plasmids was ligated into the *KpnI/SacI* site of pRS426. *VPS38-HA-GFP* constructs were generated in the same manner. Successful plasmid construction was confirmed by sequencing. All primer sequences and plasmid maps are available upon request.

### Estimation of Autophagic Activity

To quantify the extent of autophagy, we performed alkaline phosphatase (ALP) assays as described previously (Noda and Ohsumi, 1998). Cell viability in SD (-N) medium was measured by counting the number of dead cells stained with phloxine B (final concentration 2 µg/ml) that exhibited bright fluorescence (Onodera and Ohsumi, 2004). Maturation of aminopeptidase I (API) was estimated by immunoblotting with an anti-API antibody. The accumulation of autophagic bodies was examined by phase-contrast microscopy (model IX-71; Olympus, Tokyo, Japan). Images were acquired with MetaMorph software (Universal Imaging, Downingtown, PA).

### Coimmunoprecipitation

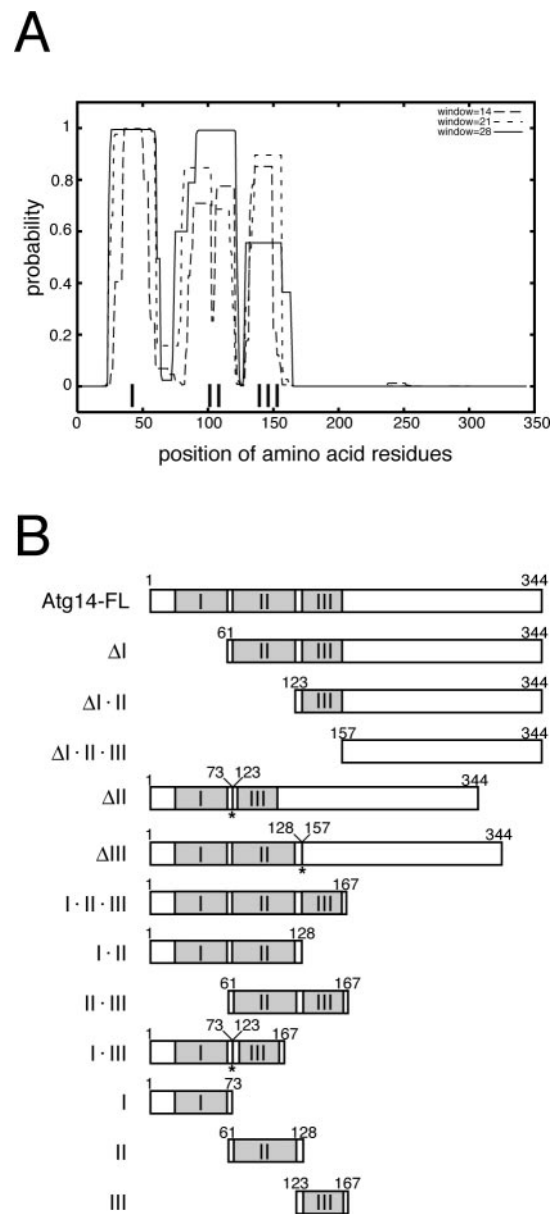
Spheroplasts were lysed by osmotic shock and then solubilized for 30 min at 4°C in immunoprecipitation (IP) buffer (50 mM HEPES-NaOH, pH 8.0, 200 mM sorbitol, 150 mM NaCl, 10 mM 2-mercaptoethanol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF]), 40 µg/ml aprotinin, 10 µg/ml pepstatin A, 20 µg/ml leupeptin, 40 µg/ml benzamidin, and protease inhibitor cocktail (Complete, EDTA-free; Roche Diagnostics, Indianapolis, IN). After the removal of cell debris by centrifugation at 1500 × g for 5 min, samples were centrifuged at 100,000 × g for 1 h. Supernatants were incubated with anti-GFP (rabbit polyclonal antibody; Molecular Probes, Eugene, OR) or anti-Atg6 (Kihara *et al.*, 2001) antibodies at 4°C for 2 h. After the addition of protein G-Sepharose beads, samples were incubated for an additional hour at 4°C. Beads were washed four times with IP buffer. Bound proteins were eluted with SDS sample buffer and separated by SDS-PAGE.

### Immunoblotting

Immunoblotting was performed using anti-HA (HA-7; Sigma-Aldrich, St. Louis, MO), anti-API, anti-Vps15p, affinity-purified anti-Vps30p, and affinity-purified anti-Vps34p (Kihara *et al.*, 2001) antibodies. Immunodetection used an ECL system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) with a bioimaging analyzer (LAS1000; Fujifilm, Tokyo, Japan). To detect Atg14p variants and Vps15p, immunoreaction enhancer (Can Get Signal; Toyobo Engineering, Osaka, Japan) was used according to the manufacturer's instructions.

### Subcellular Fractionation

Yeast spheroplasts were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM sorbitol, 1 mM PMSF, 40 µg/ml aprotinin, 10 µg/ml pepstatin A, 20 µg/ml leupeptin, 40 µg/ml benzamidin, and protease inhibitor cocktail) by extrusion through a polycarbonate 3-µm filter (Vida and Gerhardt, 1999). To remove cell debris, the filtrate was centrifuged at 500 × g for 5 min. The supernatant (total) was subsequently centrifuged at 13,000 × g for 15 min to

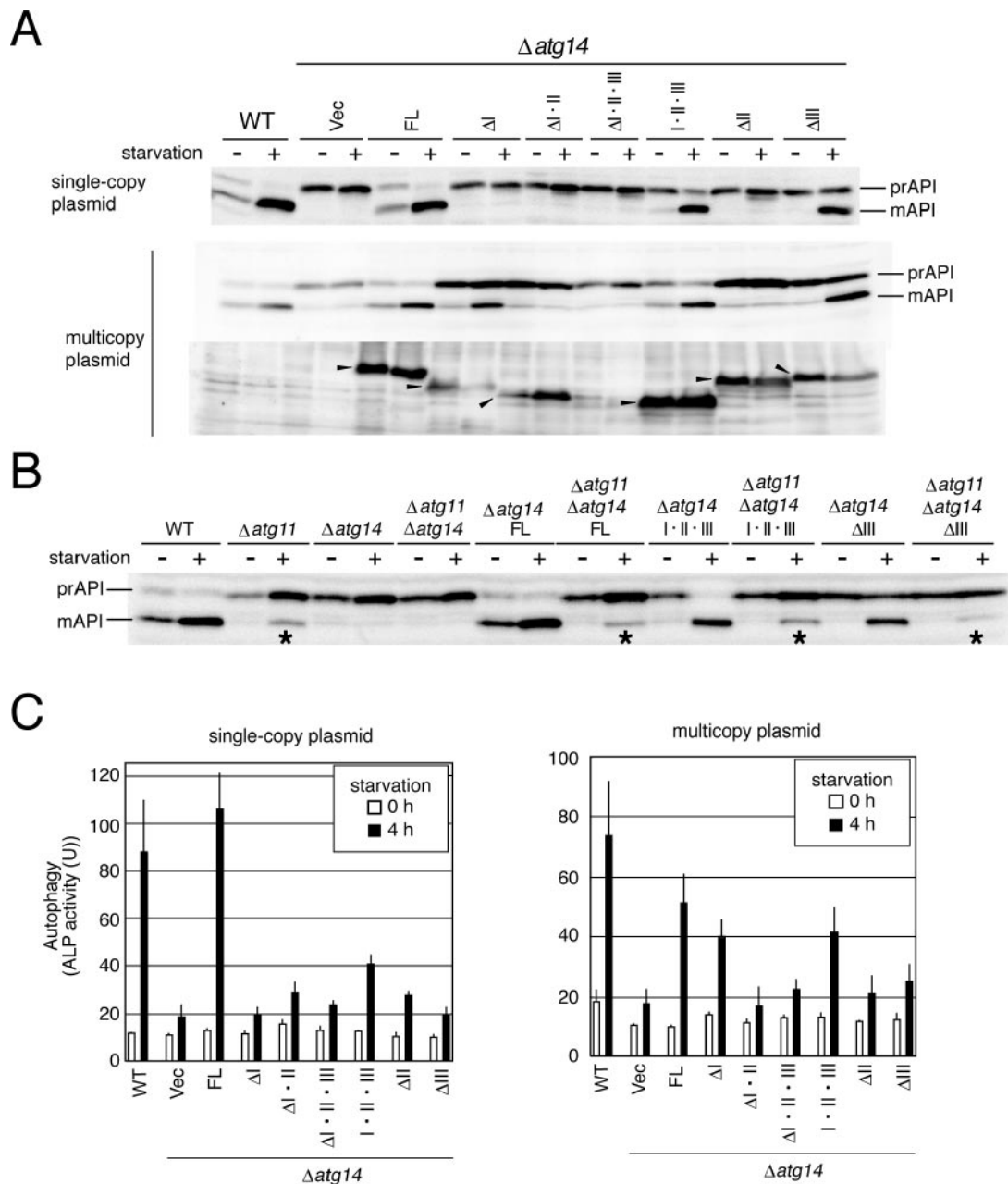


**Figure 1.** Atg14p deletion series. (A) The presence of three coiled-coil regions (I, II, and III) were predicted within Atg14p (COILS; [http://www.ch.embnet.org/software/COILS\\_form.html](http://www.ch.embnet.org/software/COILS_form.html)). (B) The deleted variants of Atg14p are shown schematically. HA-GFP, with a linker sequence (two repeats of GlyGlyGlySer) before both the HA and GFP sequences was fused to the C terminus of each variant. The coiled-coils are shown in gray. Two amino acid residues (SerSer, corresponding to the ligation site, *XbaI/SpeI*) were inserted at the position denoted by the asterisks.

generate a low-speed pellet (LSP) and low-speed supernatant. The supernatant was further centrifuged at 100,000 × g for 1 h to generate a high-speed pellet (HSP) and high-speed supernatant (HSS).

### Microscopy

The intracellular localization of GFP-tagged proteins and RFP-tagged API was observed using inverted fluorescence microscopes (IX-71 and IX-81; Olympus) equipped with cooled charge-coupled device cameras (CoolSNAP HQ; Nippon Roper, Tokyo, Japan). Images were acquired using MetaMorph software (Universal Imaging) and processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA).



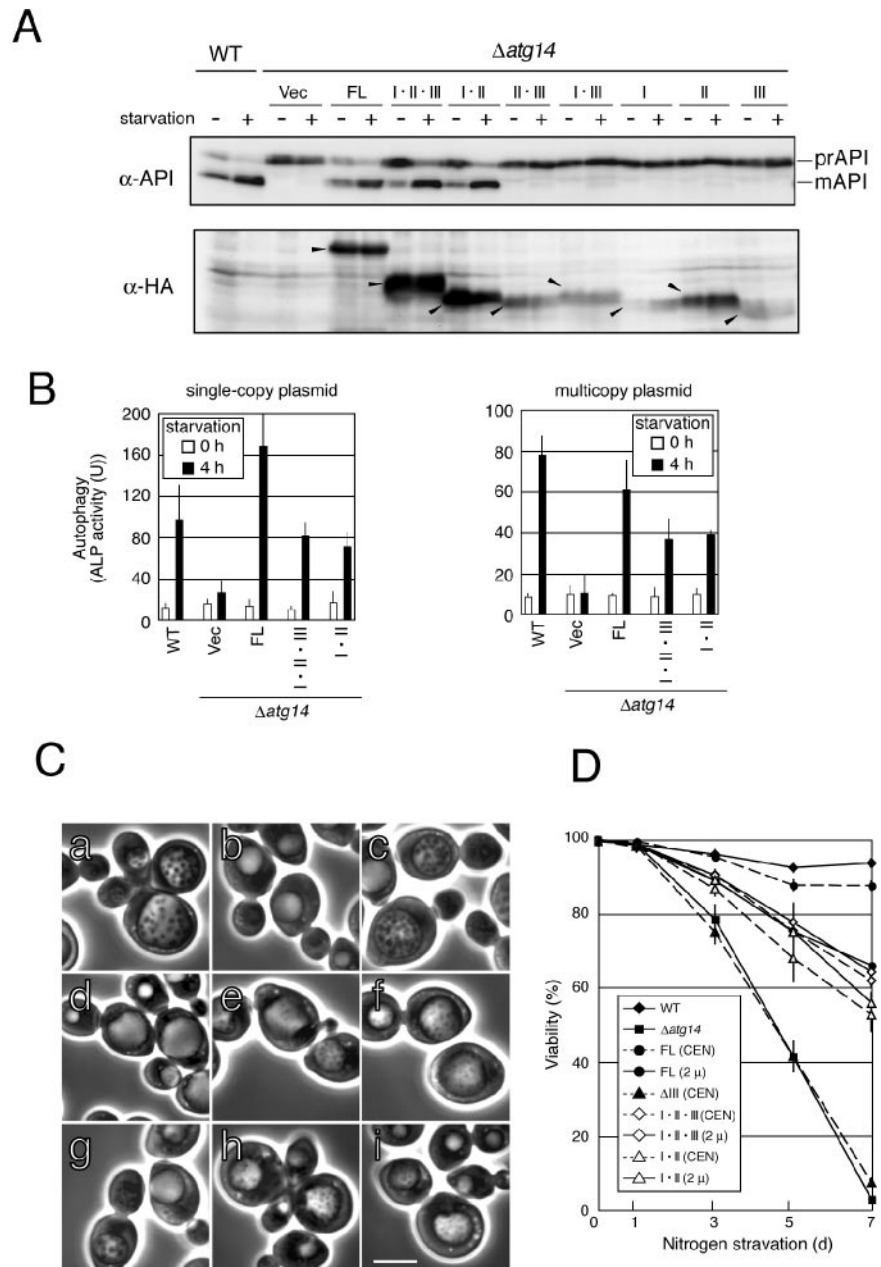
**Figure 2.** Autophagic activity of cells expressing deleted variants of Atg14p. Cells grown in SD + CA + AdeTrp medium at 30°C were collected during logarithmic phase growth (starvation -) and at 4 h after transfer to SD (-N) medium (starvation+). Lysates were prepared by the alkaline-trichloroacetic acid (TCA) method (A and B) or using glass beads (C). (A) Maturation of API was monitored by immunoblotting with an anti-API antibody. The amount of each Atg14p variant (arrowheads) was estimated by immunoblotting with an anti-HA antibody (HA-7). (B) Autophagy-dependent API maturation was estimated in cells lacking *ATG11*. Each variant was expressed from a single-copy plasmid. The asterisks indicate autophagy-dependent API maturation. (C) Autophagic activity was estimated using an ALP assay. The means  $\pm$  SD of results from triplicate measurements are shown.

## RESULTS

### *Coiled-Coils at the N-Terminal Half of Atg14p Are Essential for Autophagy*

Structurally, Atg14p was predicted to possess three coiled-coil regions within the N-terminal half of the protein (Figure 1A). No prominent motifs could be identified within the C-terminal half of Atg14p. To determine which regions of Atg14p were critical, we performed deletion analysis of

Atg14p based on these predictions. A deletion series for Atg14p lacking one to three of the coiled-coil regions or the C-terminal half were constructed and designated as follows: for example, full-length of Atg14p, Atg14p lacking coiled-coil I, and Atg14p containing the three coiled-coil regions but not the C-terminal half were termed Atg14-FL, Atg14- $\Delta I$ , and Atg14-I·II·III, respectively (Figure 1B). The C terminus of each variant was fused to an HA-GFP tag by a linker sequence (two repeats of GlyGlyGlySer), present before both



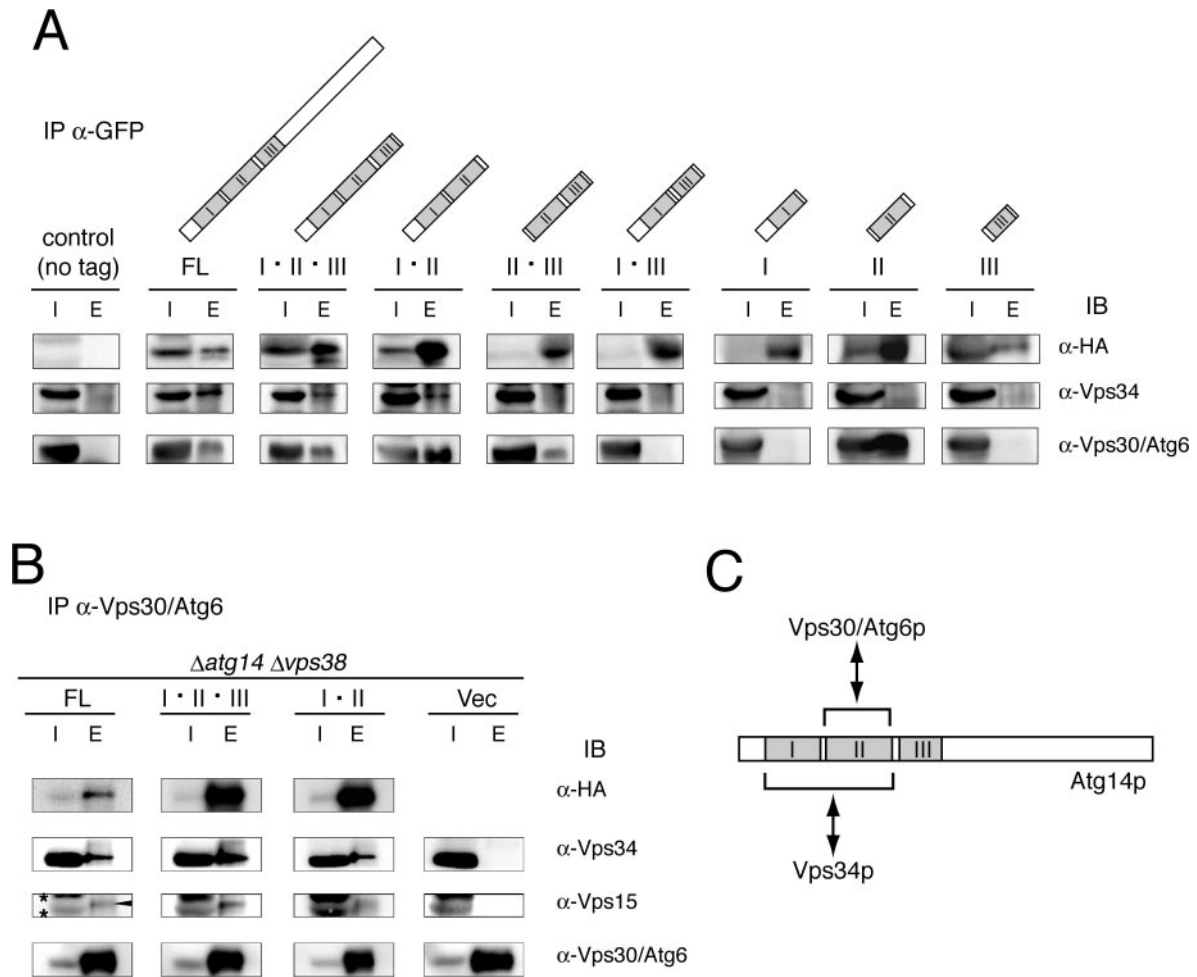
**Figure 3.** Minimal region essential for Atg14p function in autophagy. Cells grown in SD + CA + AdeTrp medium at 30°C were collected during logarithmic growth (starvation-) and 4 h after transfer to SD (-N) (starvation+) (A and B). Lysates were prepared by the alkaline-TCA method (A) or using glass beads (B). (A) Maturation of API was monitored by immunoblotting with an anti-API antibody. The amount of each Atg14p variant (arrowheads) expressed from a multicopy plasmid was estimated by immunoblotting with an anti-HA antibody (HA-7). (B) The extent of autophagy was estimated by ALP assay. The means  $\pm$  SD of results from triplicate measurements are shown. (C) The accumulation of autophagic bodies was examined on the BJ2168 background, which lacks vacuolar proteases. Cells in logarithmic phase growth were transferred to S (-NC) medium. After 4 h of culture in S (-NC) medium, cells were observed by phase-contrast microscopy. WT cells bearing an empty vector (a) and  $\Delta atg14$  cells bearing an empty vector (b) or expressing Atg14-FL from a single-copy plasmid (c), Atg14- $\Delta III$  from a single-copy plasmid (d), Atg14-I-II-III from a single-copy plasmid (e), Atg14-FL from a multicopy plasmid (g), Atg14-I-II-III from a multicopy plasmid (h), or Atg14-I-II from a multicopy plasmid (i) are shown. Bar, 5  $\mu$ m. (D) Cell viability under nitrogen-starvation conditions was measured. Cells were grown to logarithmic phase in SD + CA + AdeTrp and then transferred to SD (-N) medium. Cell viability was determined by phloxine B staining, as described in *Materials and Methods*.

the HA and GFP coding sequences. We confirmed that HA-GFP-tagged Atg14p was functional for autophagy (our unpublished data).

These variants were expressed in  $\Delta atg14$  cells from single- or multicopy plasmids under the control of the *ATG14* promoter. Protein functionality was assayed first by monitoring the maturation of proform of API, which is transported into vacuoles via the cytoplasm-to-vacuole targeting (Cvt) pathway or by autophagy under nutrition-rich and starvation conditions, respectively. The protein is then processed into a mature form within vacuoles. API maturation was enhanced, especially under starvation conditions, in cells expressing Atg14-FL, Atg14-I-II-III, or Atg14- $\Delta III$  from single-copy plasmids (Figure 2A). API maturation was also restored in cells overexpressing Atg14- $\Delta I$  from a multicopy plasmid. None of the other variants were functional, even when expressed from multicopy plasmids. The amounts of the variants ex-

cept Atg14- $\Delta I$ -II-III were at least at several times higher levels than that of endogenous Atg14p when expressed from multicopy plasmids, although only Atg14-FL and Atg14-I-II-III were detectable when expressed from single-copy plasmids (our unpublished data). We next assayed autophagy-dependent API-maturation in  $\Delta atg11$  cells, which lacks one of the factors required for the Cvt pathway, but not for autophagy (Kim *et al.*, 2001) (Figure 2B). Levels of autophagy-dependent API maturation was similar to that in  $\Delta atg11$  cells, expressing endogenous Atg14p, in  $\Delta atg11 \Delta atg14$  cells expressing exogenous Atg14-FL, Atg14-I-II-III, or Atg14- $\Delta III$ .

Next, autophagic activity was estimated by measuring the activity of ALP, which was expressed in the cytosol as a truncated proform and delivered to and activated in vacuoles in an autophagy-dependent manner (Noda *et al.*, 1995). After both single-copy and multicopy expression, autophagic activity in Atg14-I-II-III cells under starvation conditions



**Figure 4.** Interaction of Atg14p with Vps30p and Vps34p is mediated by coiled-coils I and II. Proteins were coimmunoprecipitated from the supernatants of Triton X-100-treated lysates using anti-GFP (A) and anti-Vps30 (B) antibodies. Isolated proteins (E, elution fraction) were resuspended in 1/10 (A) or 1/40 (B) volume of the starting supernatant fraction (I, input); equal (A) or 1.5 $\times$  volume (B) was loaded onto SDS-PAGE. (A) Atg14p interacts with Vps30p and Vps34p at coiled-coil I and II regions. Atg14-I, Atg14-II, and Atg14-III were expressed from multicopy plasmids, and the other variants were expressed from single-copy plasmids. (B) Vps34p and Vps15p coimmunoprecipitated with Vps30p in a manner dependent on the variants of Atg14p expressed from single-copy plasmids in  $\Delta vps38\Delta atg14$  cells. Arrow and asterisks indicate Vps15p and nonspecific bands, respectively. (C) In this schematic representation of Atg14p-mediated complex formation, the regions required for this interaction are indicated.

was significantly higher than that in  $\Delta atg14$  cells but lower than that in wild-type (WT) cells (Figure 2C). Results of ALP assays represent the activities of bulk and nonselective degradation of the cytoplasm via autophagy (Noda *et al.*, 1995). It is a less sensitive system to detect partial autophagic activities than API maturation assay, which represents a selective transport activity of a large complex of API via both Cvt pathway and autophagy. The lower ALP activity in Atg14-I-II-III cells than in WT, in spite of the normal API maturation activity, may result from the difference in the sensitivity of the assays used. These results indicate that the N-terminal half of Atg14p containing the three coiled-coil structures is functional for autophagy, although it is not fully active. In contrast, the C-terminal half of Atg14p is not essential for autophagy.

Cells expressing Atg14-FL from a single-copy plasmid possessed higher autophagic activity under starvation conditions than WT cells. This is likely because of the higher expression of the protein from a single-copy plasmid than endogenous expression (our unpublished data), which sug-

gests that the amount of Atg14p may be an important factor limiting the extent of autophagy. Overexpression of Atg14-FL from a multicopy plasmid, however, yielded lower autophagic activity than that seen in WT cells, suggesting that excess amount of Atg14p affects autophagy to some extent and that the proper amount of Atg14p is required for normal autophagy.

Because the three coiled-coil regions within the N-terminal half of Atg14p are essential for autophagy, whereas the C-terminal half is dispensable, we further dissected the N-terminal region containing the three coiled-coils. We examined cells expressing further deleted variants for autophagic activity. Atg14-I-II-expressing cells exhibited similar autophagic activity as those expressing Atg14-I-II-III (Figure 3, A and B), whereas none of the other further deleted variants exhibited any function to induce API maturation. Therefore, Atg14-I-II is the minimal region essential for autophagy.

Cells expressing Atg14-I-II, Atg14-FL, or Atg14-I-II-III accumulated autophagic bodies under starvation conditions (Figure 3C). The autophagic bodies accumulated in cells

expressing either Atg14-I-II-III or Atg14-I-II, however, were smaller than those seen in WT and Atg14-FL cells. This change in autophagosome size may be responsible for the reduction in autophagic activity monitored by ALP assay. Activity of PI3-kinase complex I might influence the size of the autophagosomes. Cells overexpressing Atg14-FL from a multicopy plasmid accumulated normal size autophagic bodies, although the population of cells accumulating autophagic bodies was reduced from that of WT cells (our unpublished data), which may explain the reduced autophagic activity measured.

Cells expressing Atg14-I-II-III and Atg14-I-II maintained significantly higher viabilities in nitrogen-starved medium than autophagy-defective cells such as  $\Delta atg14$  and Atg14- $\Delta$ III cells, although these levels could not reach those of WT and Atg14-FL (single-copy plasmid) cells. These results correlate well with the fact that these cells exhibited partial autophagic activity, monitored by ALP assay, and accumulated smaller autophagic bodies. Together, the N-terminal region containing coiled-coils I and II is essential and sufficient for autophagy.

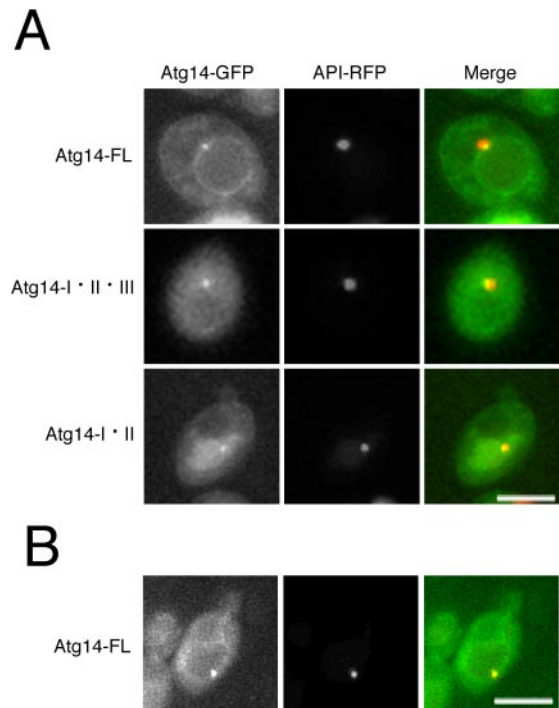
#### Atg14p Binds to Vps30p and Vps34p at the Coiled-Coil I and II

Because coiled-coil regions often mediate protein-protein interaction, we investigated the associations of Atg14p variants with other components of PI3-kinase complex I by coimmunoprecipitation. HA-GFP-tagged Atg14p variants were immunoprecipitated with an anti-GFP antibody; coimmunoprecipitates were examined by immunoblotting. In cells expressing Atg14-FL, Vps34p and Vps30p were coimmunoprecipitated (Figure 4A). Atg14-I-II-III and Atg14-I-II also interacted with Vps34p and Vps30p. The region containing only coiled-coil II was sufficient to interact with Vps30p.

To determine whether Atg14p, Vps34p, and Vps30p were isolated as part of a single complex or as the combination of Atg14p-Vps34p and Atg14p-Vps30p complexes, we further attempted immunoprecipitation with an anti-Vps30 antibody. We used  $\Delta vps38$  cells to exclude any potential interactions between Vps34p and Vps30p that were mediated by Vps38p. Vps34p coimmunoprecipitated with Vps30p in the presence of Atg14-FL, Atg14-I-II-III, or Atg14-I-II (Figure 4B). This interaction was completely dependent on the presence of an Atg14p variant, indicating that Vps30p, Atg14p, and Vps34p are integrated into a single complex within these cells. Vps15p was also coimmunoprecipitated with Vps30p in the presence of Atg14-FL, Atg14-I-II-III, or Atg14-I-II, indicating that all subunits of complex I form a complex in these cells.

#### Atg14p Localizes to the PAS and the Vacuolar Membrane

Atg14p is known to localize to the PAS (Strømhaug *et al.*, 2004), which we confirmed by examining the colocalization of Atg14p with RFP-tagged API, a PAS marker. We also detected Atg14-FL, expressed from single-copy plasmid, on the vacuolar membrane (Figure 5A). Similarly, integrated Atg14-GFP localized to the PAS and vacuolar membrane (our unpublished data). Because Atg14p is unstable, making it easily degraded to undetectable levels in the absence of interactions with Vps30p and Vps34p (Kihara *et al.*, 2001), Atg14p at these compartments is integrated into PI3-kinase complex I, as confirmed by immunoprecipitation experiments (Figure 4). Both Atg14-I-II-III and Atg14-I-II retained the ability to localize to the PAS, although localization to the vacuolar membrane was significantly reduced to nearly undetectable levels. It is unclear whether the reduced vacuolar



**Figure 5.** PAS-localization of Atg14p variants. (A) Atg14-FL, Atg14-I-II-III, and Atg14-I-II expressed from single-copy plasmids colocalized with API, a PAS marker. Vacuolar fluorescence was significantly reduced in cells expressing either Atg14-I-II-III or Atg14-I-II. (B) Atg14-FL expressed from a single copy-plasmid in  $\Delta atg14\Delta vps38$  cells (YOK435). Bars, 5  $\mu$ m.

fluorescence is the cause of the reduced autophagic activity. The localization pattern of Atg14-FL was not affected by the disruption of *VPS38* (Figure 5B). The same was true in the case of Atg14-I-II-III and Atg14-I-II (our unpublished data).

Atg14-I-II, which interacts with both Vps30p and Vps34p, localized to the PAS and was able to function in autophagy (Table 2), confirming that this is the minimum essential for the function in autophagy. These results indicate that the critical functions of Atg14p are mediated by protein-protein interactions of coiled-coils I and II.

#### Complexes I and II Localize to Distinct Compartments Dependent on Their Specific Components

Previous studies demonstrated that disruption of *ATG14* did not affect vacuolar protein sorting and that disruption of *VPS38* did not affect autophagic activity. These results indicate that complexes I and II function in distinct biological processes (Kametaka *et al.*, 1998; Kihara *et al.*, 2001). We have confirmed this concept by additional criteria. Autophagic bodies were accumulated normally in  $\Delta vps38$  cells (Figure 6). Overexpression of Vps38-HA-GFP did not restore API maturation and the accumulation of autophagic bodies in  $\Delta atg14$  cells, although it could reverse the defect in vacuolar protein sorting in  $\Delta vps38$  cells. In conjunction with previous studies, these results clearly indicate that the functions of complexes I and II do not overlap.

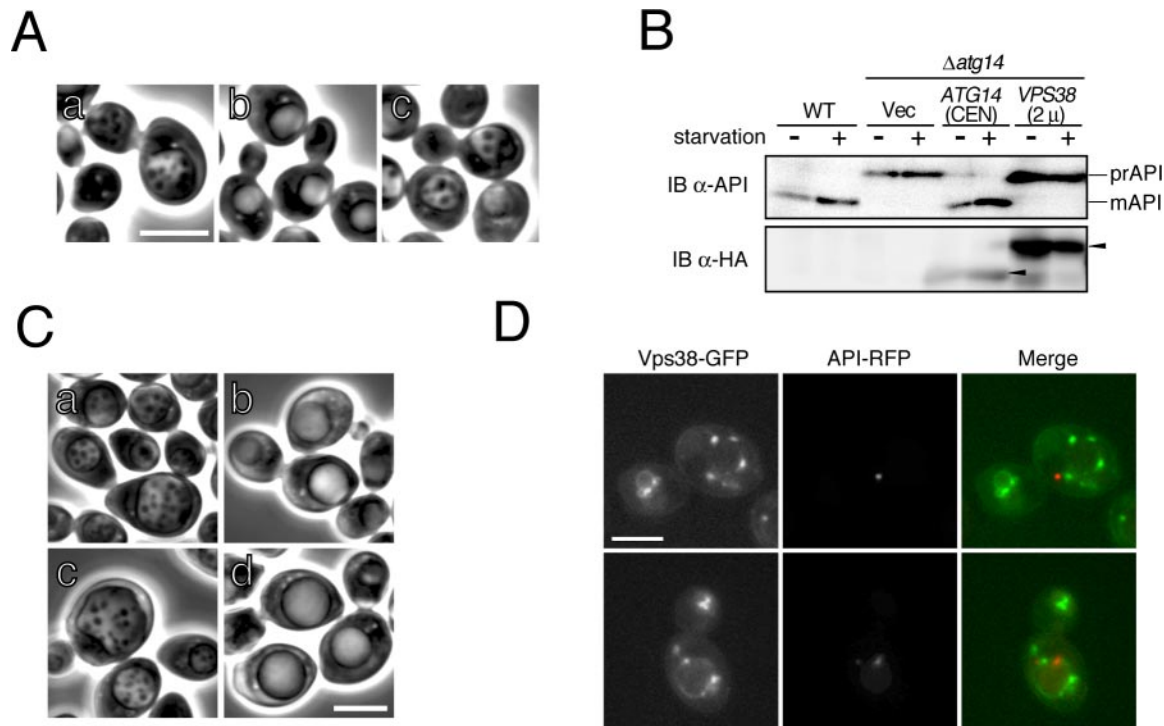
We examined the specificity of PI3-kinase complex function by investigating the intracellular localization of each complex. As described above, Atg14p localized to the PAS and the vacuolar membrane (Figure 5). We next investigated the localization of Vps38p, the factor specific for complex II. Vps38p localized to the vacuolar membrane and a subset of



**Table 2.** Summary of Atg14p deletion analysis

Variant	Autophagy	PAS localization	Binding to Vps30p	Binding to Vps34p
Atg14-FL	+	+	+	+
Atg14-I-II-III	+	+	+	+
Atg14-I-II	+	+	+	+
Atg14-II-III	–	N.D.	+	–
Atg14-I-III	–	N.D.	–	–

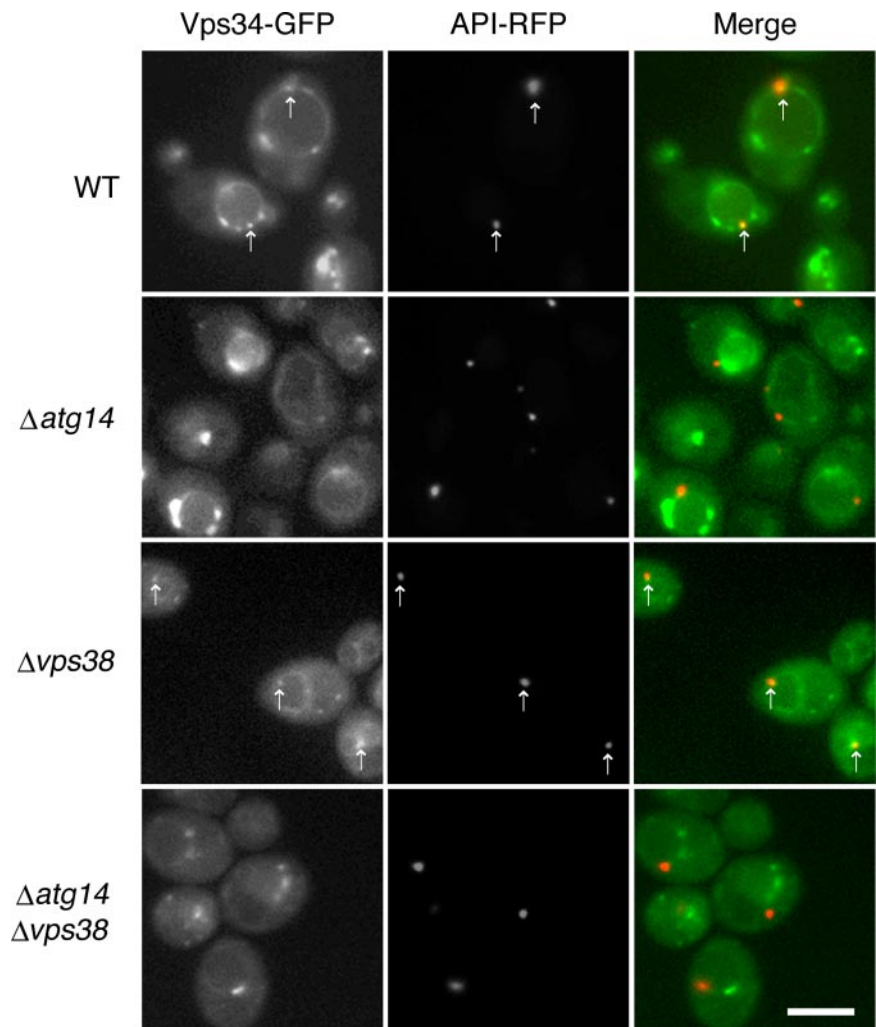
N.D., not determined.



**Figure 6.** Vps38p and autophagy. (A) SEY6210 strain cells at logarithmic phase growth were transferred to S (-NC) medium in the presence of 1 mM PMSF. After 4 h of culture in S (-NC) medium, WT cells (a),  $\Delta atg14$  cells (b), and  $\Delta vps38$  cells (c) were observed by phase-contrast microscopy. (B) API maturation was monitored in  $\Delta atg14$  cells overexpressing Vps38-HA-GFP. Cells, grown in SD + CA + AdeTrp medium at 30°C, were collected during logarithmic growth (starvation -) or 4 h after transfer to SD (-N) medium (starvation +). Lysates were subjected to immunoblotting with anti-API and anti-HA (HA-7) antibodies. (C) The accumulation of autophagic bodies was examined in Vps38-HA-GFP-overexpressing  $\Delta atg14$  cells, on the BJ2168 background which lacks vacuolar proteases. WT cells (a),  $\Delta atg14$  cells (b),  $\Delta atg14$  cells expressing Atg14-FL from a single-copy plasmid (c), and  $\Delta atg14$  cells expressing Vps38-HA-GFP from a multicopy plasmid (d) were transferred to S (-NC) medium. After 4 h of culture in S (-NC) medium, cells were observed by phase-contrast microscopy. (D) We investigated the localization of Vps38p. Diploid cells expressing chromosomal Vps38-GFP and API-RFP (YOK89) were cultured in SD + CA + AdeTrpUra medium and subjected to fluorescence microscopy. Two representative fields are shown. Bars, 5  $\mu m$ .

punctate structures unique from the PAS (Figure 6). These punctate structures are endosomes, because integrated Vps38p is known to colocalize with an endosome marker, Snf7p (Huh *et al.*, 2003). We have confirmed the colocalization of Vps38p with Snf7p (our unpublished data). To avoid confusion, fluorescent punctate structures will hereafter be described as “endosomes” when they are Vps38p positive or Vps38p dependent. In addition to the endosomes, Vps34p and Vps15p localized to additional punctate structures (see below) that are described as “dot structures” for convenience. Because Vps38p is essential for the formation of complex II (Kihara *et al.*, 2001), the absence of detectable Vps38p at the PAS indicates that complex II does not accumulate at the PAS.

We also examined the localization of Vps34p, Vps30p, and Vps15p in WT,  $\Delta atg14$ ,  $\Delta vps38$ , and  $\Delta atg14\Delta vps38$  cells. Vps34p localized to the PAS, the vacuolar membrane, endosomes, and dot structures (Figure 7). The PAS localization of Vps34p was abolished in  $\Delta atg14$  cells, indicating that targeting of this molecule is dependent on Atg14p. In contrast, Vps34p staining of endosomes, dot structures, and vacuolar membranes was retained in  $\Delta atg14$  cells. In  $\Delta vps38$  cells, a significant amount of Vps34p was dispersed throughout the cytoplasm. Vps34p was still detected at the PAS, the vacuolar membrane, and dot structures, indicating that localization of Vps34p to these structures does not require Vps38p. Vps34p localization to dot structures and vacuolar membranes could be detected even in  $\Delta atg14\Delta vps38$  cells,



**Figure 7.** Localization of Vps34p. Diploid cells expressing chromosomal Vps34-GFP and API-RFP were observed by fluorescence microscopy. The arrows indicate the structures to which Vps34-GFP and API-RFP colocalize. Bar, 5  $\mu$ m.

although the fluorescence from the vacuolar membranes was significantly reduced.

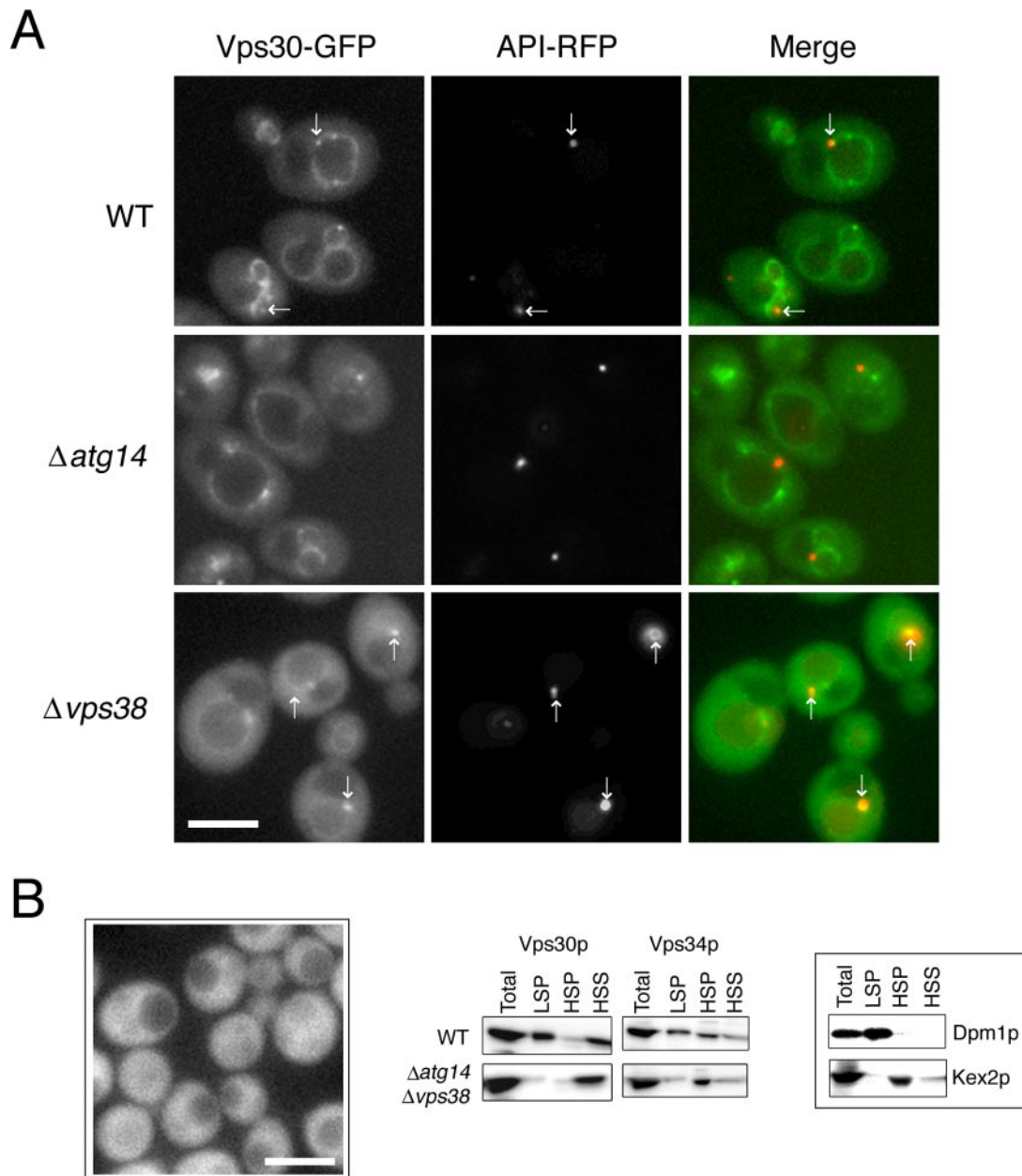
Vps30p was localized to the PAS, vacuolar membranes, and endosomes (Figure 8). As seen for Vps34p, Atg14p was required for the PAS localization of Vps30p but dispensable for the localization of Vps30p to endosomes. The PAS localization of Vps30p did not require Vps38p, because Vps30p remains localized to the PAS in  $\Delta vps38$  cells. In contrast, Vps30p disappeared from endosomes, leaving the majority of Vps30p dispersed throughout the cytoplasm in  $\Delta vps38$  cells, demonstrating an essential role for Vps38p in the endosomal localization of Vps30p. In  $\Delta atg14\Delta vps38$  cells, Vps30p was distributed evenly throughout the cytoplasm with no apparent compartment. This result was consistent with the cell fractionation experiments, demonstrating that the majority of Vps30p was found in the postlysis supernatants of  $\Delta atg14\Delta vps38$  cells (Figure 8B).

Similar to Vps34p, Vps15p localized to the PAS, the vacuolar membrane, endosomes, and dot structures (Figure 9). In contrast to the results seen for Vps34p and Vps30p, however, the PAS localization of Vps15p was Atg14p independent. In addition, Vps38p was not required for the localization of Vps15p to the PAS. In  $\Delta atg14\Delta vps38$  cells, Vps15p remained localized to dot structures (see *Discussion*), vacuolar membranes, and the PAS, although the fluorescence from the vacuolar membranes was significantly reduced.

A summary of the microscopy is detailed in Figure 10. The PAS and vacuolar membranes were sites to which all components of complex I, including Atg14p, localized. PAS localization of Vps34p and Vps30p was Atg14p dependent but Vps38p independent. In contrast, endosomes and vacuolar membranes were the sites to which the components of complex II localized. Localization of these components to endosomes required Vps38p but not Atg14p. In addition to a shared distribution to vacuolar membranes, both complexes I and II had an additional specific site of intracellular localization, complex I to the PAS and complex II to endosomes. This localization was specified by the components exclusive to each complex. Vps15p and Vps34p also were observed on dot structures; this localization required neither Atg14p nor Vps38p.

## DISCUSSION

In this study, we focused on Atg14p, a specific component of PI3-kinase complex I that is essential for autophagy. Analyses using a deletion series of Atg14p indicated that the region containing coiled-coils I and II within the N-terminal half were essential and sufficient for the function in autophagy. This region was minimum essential for the localization of Atg14p to the PAS. The two coiled-coil structures in this region were responsible for interactions with Vps34p and

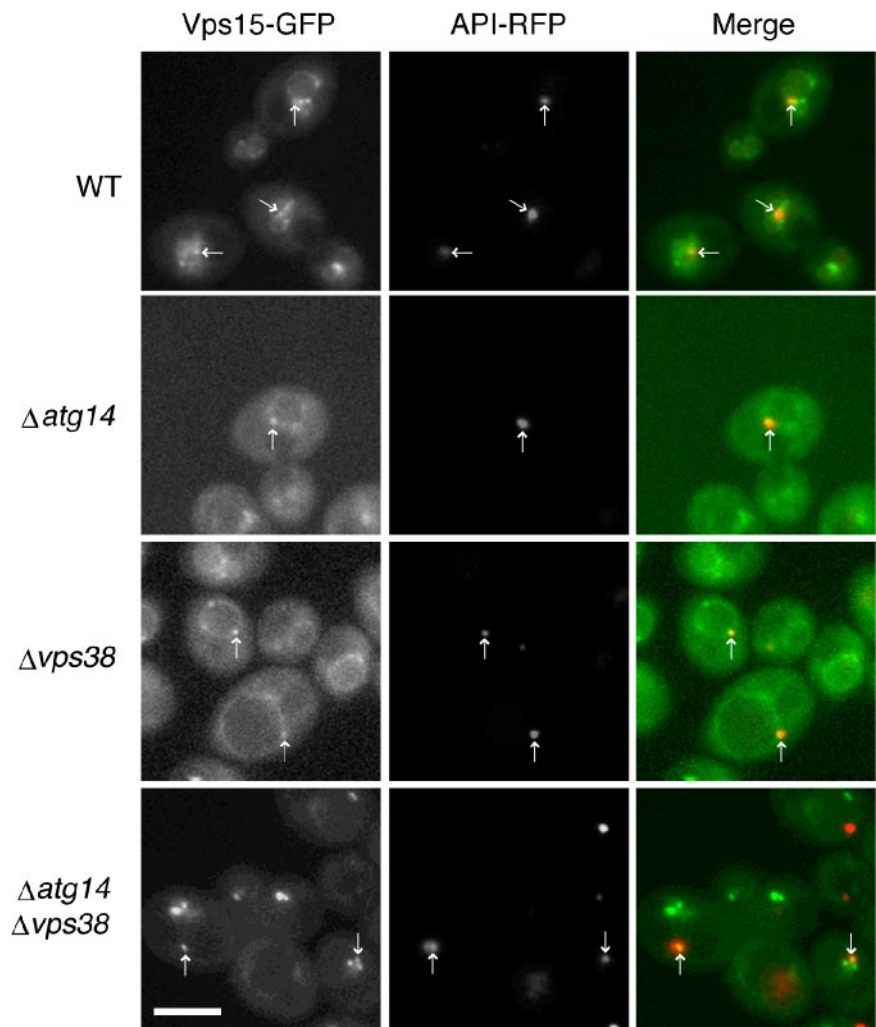


**Figure 8.** Localization of Vps30p. (A) Diploid cells expressing chromosomal Vps30-GFP and API-RFP were observed by fluorescence microscopy. The arrows indicate the structures to which Vps30-GFP and API-RFP colocalize. (B) Haploid cells expressing chromosomal Vps30-GFP (left). Vps30-GFP was dispersed throughout the cytoplasm, which was confirmed by cell fractionation analysis of WT and  $\Delta atg14 \Delta vps38$  cells expressing an untagged Vps30p (right). Dpm1p and Kex2p were used as markers for the ER and late Golgi, respectively. Bars, 5  $\mu$ m.

Vps30p to form PI3-kinase complex I. These results indicate that the primary function of Atg14p is mediated by protein-protein interactions of the coiled-coils.

PI3-kinase complex I is essential for autophagy but dispensable for vacuolar protein sorting. Conversely, complex II functions in the sorting of vacuolar proteins but is dispensable for autophagy. How this specificity of the function is determined has not been elucidated. In this study, we investigated the intracellular localization of the two complexes by microscopy, which provided several important discoveries. All the components of complex I were present at the PAS. The localization of Vps34p and Vps30p to the PAS

was dependent on the expression of Atg14p, indicating an essential role for Atg14p in the accumulation of PI3-kinase complex I at the PAS. Atg14p, however, was not essential for the localization of Vps34p and Vps30p to vacuolar membranes and endosomes. Vps38p, a specific component of complex II, did not localize to the PAS, but localized to endosomes and vacuolar membranes. Vps38p was not required for the PAS localization of Vps34p, Vps30p, and Vps15p but was essential for their localization to endosomes. Therefore, Vps38p plays a crucial role in the association of complex II with endosomes. From these results, we concluded that only complex I can accumulate at the PAS,



**Figure 9.** Localization of Vps15p. Within the fluorescence images of diploid cells expressing chromosomal Vps15-GFP and API-RFP, the arrows indicate the structures at which Vps15-GFP and API-RFP colocalize. Bar, 5  $\mu$ m.

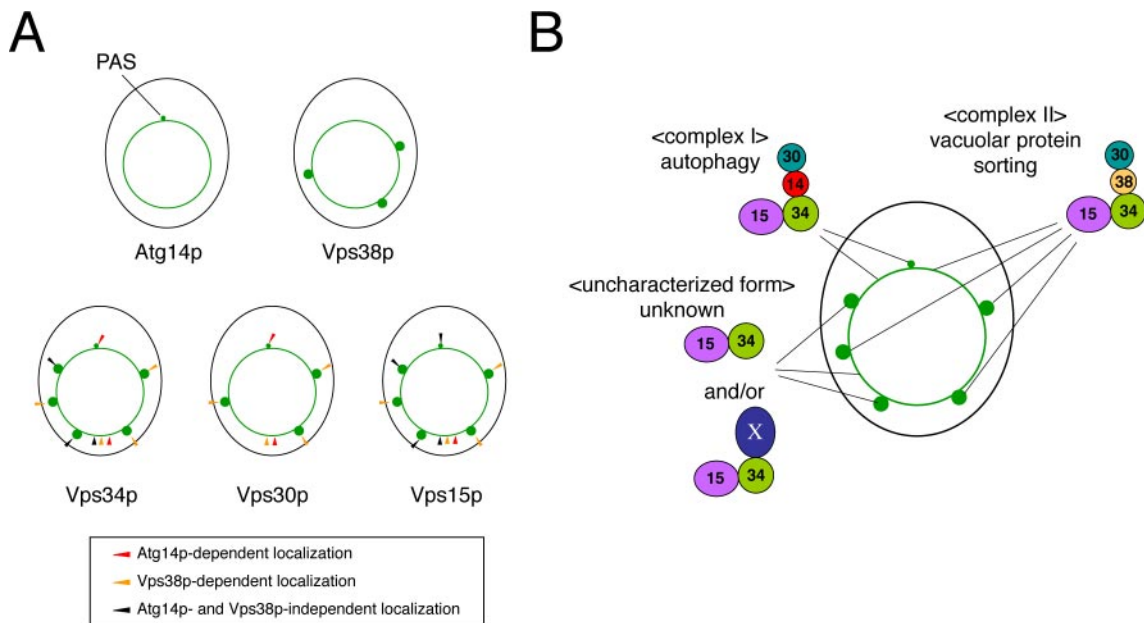
whereas only complex II is found at endosomes. These unique localization patterns are mediated by the specific components of each complex, Atg14p and Vps38p. Here, we propose a model that the distinct functions of PI3-kinase complexes I and II are acquired by the specific association of each complex to a distinct compartment that is mediated by the specific components.

Unlike Vps34p and Vps30p, Vps15p localized to the PAS in the absence of Atg14p (Figure 9). The mechanism by which Vps15p localizes to the PAS is currently unknown. This Atg14p-independent localization of Vps15p suggests a potential mechanism by which Atg14p can promote the accumulation of complex I at the PAS. Atg14p may recruit Vps34p and Vps30p to the PAS, forming a complex with preexisting Vps15p on the membrane. In this way, Atg14p would not have to bring a preformed complex containing Vps15p to the PAS. Alternatively, Atg14p could prevent the dissociation of complex I, stabilizing it at the PAS. In any case, the interaction between certain PAS-localized proteins and the complex I subunits may be critical.

All of the components of PI3-kinase complexes I and II localized to the vacuolar membrane. At present, it is not clear whether the PI3-kinase complexes on the vacuolar membrane are functional. Vesicles undergoing anterograde transport finally fuse with vacuoles. Similarly, autophagosomes finally fuse with vacuoles (Baba *et al.*, 1994; Kirisako

*et al.*, 1999). Therefore, it remains possible that the presence of complexes I and II at vacuolar membranes simply represents the consequence of membrane-trafficking events. Fab1p, a phosphoinositide kinase that phosphorylates PI3-P to generate phosphoinositide-3,5-diphosphate, is involved in the regulation of vacuolar morphology (Yamamoto *et al.*, 1995; Bonangelino *et al.*, 1997; Gary *et al.*, 1998; Dove *et al.*, 2002, 2004). Alternatively, PI3-kinase complexes at vacuolar membranes may function in the maintenance of vacuolar homeostasis in combination with Fab1p and its possible effectors. It will be interesting to examine the vacuolar membrane localization of PI3-kinase complex subunits in various mutants defective in anterograde or retrograde transport, retrieval of the vacuolar membrane components, and autophagy (Tsukada and Ohsumi, 1993; Yamamoto *et al.*, 1995; Bryant *et al.*, 1998; Gary *et al.*, 1998; Dove *et al.*, 2002, 2004).

Vps34p and Vps15p remain localized to dot structures and vacuolar membranes in  $\Delta atg14 \Delta vps38$  cells (Figures 7 and 9). These GFP-positive signals may represent Vps34p-Vps15p complex and/or a novel PI3-kinase complex containing Vps34p, Vps15p, and potentially novel undefined components. The existence of PI3-kinase complex(es) unique from both complexes I and II has been suggested (Kihara *et al.*, 2001). Given that ~80% of WT PI3-kinase activity is retained in  $\Delta vps30$  cells and that  $\Delta vps34$  and  $\Delta vps15$  cells



**Figure 10.** Distinct distribution of the two PI3-kinase complexes. (A) The intracellular localization of each component of the PI3-kinase complexes is shown in green, whereas the outline of the cell is detailed in black. (B) The distribution of the PI3-kinase complexes is summarized. “X” represents an unknown component(s).

have additional phenotypes beyond that of  $\Delta vps30$  cells (Robinson *et al.*, 1988; Raymond *et al.*, 1992; Kihara *et al.*, 2001), the putative novel complex likely does not contain Vps30p. This result would be in good agreement with our microscopy and cell fractionation data demonstrating that Vps30p was dispersed throughout the cytosol and did not localize to a specific compartment in  $\Delta atg14\Delta vps38$  cells (Figure 8).

To date, genes with significant homology with *ATG14* and *VPS38* have not been found in animals and plants. Given the conserved roles of class III PI3-kinase in autophagy and protein sorting to the vacuole/lysosome from yeast to mammals (Petiot *et al.*, 2000; Brown *et al.*, 1995; Davidson, 1995), it would be interesting to search the functional homologues of Atg14p and Vps38p that potentially function to connect Vps34p and Vps30p in mammals.

We demonstrated that PI3-kinase complex I accumulates at the PAS in a manner dependent on Atg14p. What is the function of PAS-localized complex I in autophagy? In mammals, wortmannin and LY294002, two inhibitors of PI3-kinase, inhibit autophagy (Petiot *et al.*, 2000), suggesting that the lipid kinase activity of a Vps34p homologue is required for this process. We have confirmed that the lipid kinase activity of Vps34p is also essential for autophagy in yeast (our unpublished data). In a variety of biological processes, PI3-P marks specific membrane sites to which downstream molecules need to be recruited (Martin, 1998; Odorizzi *et al.*, 2000). One possible role of PI3-kinase complex I in autophagy may be to produce PI3-P at the PAS to recruit PI3-P-binding molecules, which in turn recruit additional downstream molecules to the PAS. Atg14p is required for the localization of Atg2p, Atg5p, and Atg8p to the PAS (Shintani *et al.*, 2001; Suzuki *et al.*, 2001). Therefore, complex I may act primarily at a relatively early point in the autophagosome formation, potentially during formation and/or maintenance of the PAS through the production of PI3-P.

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