CELL STRUCTURE AND FUNCTION 28: 49–54 (2003)

The Early Secretory Pathway Contributes to Autophagy in Yeast

Maho Hamasaki[1,2], Takeshi Noda[1,2], and Yoshinori Ohsumi[1,2]*

[1]*National Institute for Basic Biology, Department of Cell Biology, 38 Nishigonaka, Myodaiji-cho, Okazaki, 444-8585, Japan and* [2]*Department of Molecular Biomechanics, School of Life Science, The Graduate University for Advanced Studies*

ABSTRACT. **Autophagy is a starvation response in eukaryotes by which the cell delivers cytoplasmic components to the vacuole for degradation, and is mediated by a double membrane structure called the autophagosome. We have previously proposed that the specific combination of COPII like components, including Sec24p, is required for autophagy (Ishihara, N. *et al*. (2001) *Mol. Biol. Cell*, 12: 3690-3702). The autophagic defect in *sec24* deleted mutant cells was, however, suppressed upon the recovery of its secretory flow by the overexpression of its homologue, Sfb2p. We have also reported that the autophagic defect is not observed in *sec13* and *sec31* mutants, a phenomenon that can be explained by the fact that starvation stress suppresses the secretory defect of these mutants. These observations indicate that the active flow in the early secretory pathway plays an important role in autophagy; that is, autophagy proceeds in the presence, but not in the absence of the early secretory flow. Both autophagy and its closely related cytoplasm to vacuole-targeting (Cvt) pathway occur through a pre-autophagosomal structure (PAS), and since the PAS and the functional Cvt pathway exist in all *sec* mutants, the early secretory pathway must be involved specifically in autophagy, subsequent to PAS formation.**

Key words: autophagy/COPII/Cvt pathway/early secretory pathway/Sec

Autophagy is one of the cellular responses to starvation stress, in which cytoplasmic components are delivered to the vacuole for degradation thus producing the free amino acid pool (Klionsky and Ohsumi, 1999). To carry out this process, the formation of a double membrane organelle called the autophagosome is required. We and an other group have isolated a series of *apg/aut* mutants that show defects in the autophagosome formation in the yeast, *Saccharomyces cerevisiae* (Thumm *et al.*, 1994; Tsukada and Ohsumi, 1993). Most of the *apg/aut* mutants also have defects in another cellular process called the Cvt (cytoplasm to vacuole targeting) pathway that transports specific vacuolar enzymes, API (aminopeptidase I) and α-mannosidase, to the vacuole (Harding *et al.*, 1996). In contrast to autophagy, the Cvt pathway is a constitutively ongoing process and is independent of starvation stress (Scott *et al.*, 1997). It also employs an autophagosome-like structures called the Cvt vesicles which are of much smaller size (Baba *et al.*, 1997).

In addition to Apg proteins, we have recently identified additional cellular factors involved only in autophagy (Ishihara *et al.*, 2001). Autophagosome formation is completely blocked when some early Sec proteins lose their function (Ishihara *et al.*, 2001). Early Sec proteins are involved in the formation of the COPII coated vesicles that travel from ER to Golgi (Antonny and Schekman, 2001). The formation of the COPII coated vesicles is initiated by the recruitment of GTP-Sar1p to the ER membrane via Sec12p, followed by the sequential recruitment of the Sec23p/Sec24p subcomplex and the Sec13p/Sec31p subcomplex (Kuehn *et al.*, 1998). The defect in autophagosome formation was shown to be in *sec23/24*, however, and not in the *sec13/31* mutant cells (Ishihara *et al.*, 2001). This unanticipated result forced us to revise our thinking on whether only specific COPII components are involved in autophagy. By focusing our attention on the early secretory flow and not on the specific role of each COPII component, we hoped to clarify their relations. Here we suggest that the active flow in the early secretory pathway contributes to autophagy in *S. cerevisiae*.

*To whom correspondence should be addressed: Prof. Yoshinori Ohsumi, Department of Cell Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaiji-cho, Okazaki, 444-8585, Japan.
Tel: +81–564–55–7515, Fax: +81–564–55–7516
E-mail: yohsumi@nibb.ac.jp

Abbreviations: ALP, alkaline phosphatase; API, aminopeptidase I; COP, coatmer protein; CPY, carboxypeptiase Y; Cvt, cytoplasm to vacuole targeting; ER, endoplasmic reticulum; GFP, green fluorescent protein; PAS, pre-autophagosomal structure.

Materials and Methods

Strains, Media and Growth Conditions

Saccharomyces cerevisiae strains used in this study were constructed by using standard yeast genetic methods for gene disruption and transformation (Adams *et al.*, 1997). YPH499 and KVY55 have been described previously (Ishihara *et al.*, 2001; Sikorski and Hieter, 1989). CKY496 (*sec24-1*; (Kurihara *et al.*, 2000)), RSY1780 (Δ*iss1*; (Kurihara *et al.*, 2000) (27)) and RSY1004 (*sec31-1*; (Salama *et al.*, 1997)) were a gift from Dr. R. Schekman (University of California Berkeley, CA). YKH4 (Δ*sec24::LEU2* [pAN1(*TRP1 SEC24 CEN*)]; (Higashio *et al.*, 2000)) and YKH6 (Δ*sec24::LEU2* [pSF11(*TRP1 SFB2 2*μ)]; (Higashio *et al.*, 2000)) were a gift from Dr. K. Kohno (Nara Institute for Science and Technology, Nara). MTsec12, MTsec13 and MTsec23 were generated by crossing the original *sec* mutants with YPH499 cells four times. MHY20 (*MAT*α *leu2 ura3 trp1 his3 lys2 suc2 pho8::PHO8*Δ*60 iss1-*Δ*2::TRP*) was constructed from KVY55. MHY24 (*MAT*α *leu2 ura3 trp1 his3 ade2 pho8::PHO8*Δ*60 sec24-1*), MHY98 (YKH4 *pho8::PHO8*Δ*60*) and MHY99 (YKH6 *pho8::PHO8*Δ*60*) were constructed by disrupting *PHO8* with *PHO8*Δ*60* using pTN3 as described previously (Noda *et al.*, 1995). Cells were grown either in YPD medium (1% yeast extract, 2% peptone and 2% glucose) or in synthetic medium (SD) containing nutritional supplements. For starvation condition, SD(-N) medium (0.17% yeast nitrogen base with 2% glucose without amino acid and ammonium sulfate) or YPD containing 0.5 μg/ml rapamycin was used (Noda and Ohsumi, 1998).

Plasmids pTN3 and pRS316 GFP-AUT7 used in this study have been described previously (Noda *et al.*, 1995; Suzuki *et al.*, 2001).

Microscopy

Fluorescence microscopy was performed using a DeltaVision microscope (Applied Precision) as described previously (Suzuki *et al.*, 2001).

Pulse-Chase Experiments

Cell cultures grown in SD(-met) were used to examine the API transport in early sec mutants at non-permissive temperature. For starvation experiment, cells cultured in YPD medium were cultured overnight at 23°C and grown to OD_{600} 1, collected by centrifugation (1,000×*g* for 2 min), washed once with SD(-N) medium and incubated in SD(-N) medium for 1 hr. Cells were then preincubated at 37.5°C for 15 min, pulse labeled by adding 200 μCi of [^{35}S]methionine and chased by adding 40 μl of chase solution (0.4% methionine and 0.3% cysteine in distilled water). One ml of each sample was collected at the indicated time, 10 μl of 6.5% azide was added and mixed with alkali-lysis solution (0.2 M NaOH and 1/100 β-mercaptoethanol). Proteins were precipitated by adding 100 μl of 20% trichloroacetic acid, washed once with 100% acetone, and extracted with 50 μl of STE (1% SDS, 50 mM Tris-Cl (pH 7.5) 1 mM EDTA) and boiled for 5 min. Samples were diluted to 1 ml in Triton X-100 buffer (50 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 0.15 M NaCl and 2% Triton X-100) and centrifuged at 10,000×*g* for 10 min to remove insoluble materials. 950 μl of samples were removed, anti-CPY antibody (0.5 μl) or anti-API (2 μl) and 20 μl of protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) were added and incubated at room temperature for 1 hr. Samples were centrifuged for 20 seconds and precipitated beads were washed twice with Triton X-100 buffer and once with 10 mM Tris-Cl (pH 7.5). Proteins were boiled in 100 μl of STE for 5 min to elute, halved, and immunoprecipitated again with anti α-1,6-mannose antibody as above. Proteins were again eluted in 30 μl of denaturing buffer 0.75% SDS, 1% β-mercaptoethanol with 5 min boil. Samples were analyzed by SDS-PAGE and autoradiographed with the BioImage BAS2000 analyzer (Fuji Photo Film, Tokyo, Japan).

ALP assay

Alkaline phosphatase (ALP) assay was performed as previously described (Noda *et al.*, 1995; Noda and Ohsumi, 1998).

Results and Discussion

In order to monitor the autophagic process in temperature sensitive (*ts*) *sec* mutant cells, we observed the localization of GFP-Aut7p, the marker commonly used to follow this process (Kirisako *et al.*, 1999; Suzuki *et al.*, 2001). Wild-type and *sec* mutant cells expressing GFP-Aut7p were grown to an early-log phase in YPD medium at permissive temperature and then treated with rapamycin to induce autophagy. Rapamycin is known to evoke starvation response in cells by specifically inhibiting Tor kinase, a negative regulator of autophagy (Noda and Ohsumi, 1998). By rapamycin treatment, the localization of GFP-Aut7p changes from the cytosol to the vacuole by autophagic process in wild-type cell (Fig 1A a, b and c). Likewise, autophagy was shown to be normal in *sec* mutant cells at permissive temperature (Fig. 1A, b, e and h). We next monitored the effect of the block of early secretory pathway on autophagy. The temperature shift to 37°C had an effect on GFP-Aut7p localization in *sec12* and *sec24* mutant cells; it remained cytosolic (Fig. 1A f and not shown). On the contrary, GFP-Aut7p was found in the vacuole in *sec31* mutant cells (Fig. 1A i), thus autophagy proceeded in *sec31* mutant cells, but not in *sec12* and *sec24* mutants. GFP-Aut7p was also seen as one or two bright dots next to the vacuole, called the pre-autophagosomal structure (PAS), which is thought to be the initial site for both autophagosome and the Cvt vesicle formation (Suzuki *et al.*, 2002; Suzuki *et al.*, 2001). The dot structures of GFP-Aut7p around the vacuole were observed in *sec12* and *sec24* mutant cells although the number seemed slightly increased. Similar images were also observed with GFP-Apg5p (not shown), another PAS marker, implying that these structures were the PAS. It was reported that the Cvt pathway in *sec12* and *sec23* mutants is normal (Ishihara *et al.*, 2001; Klionsky *et al.*, 1992). The

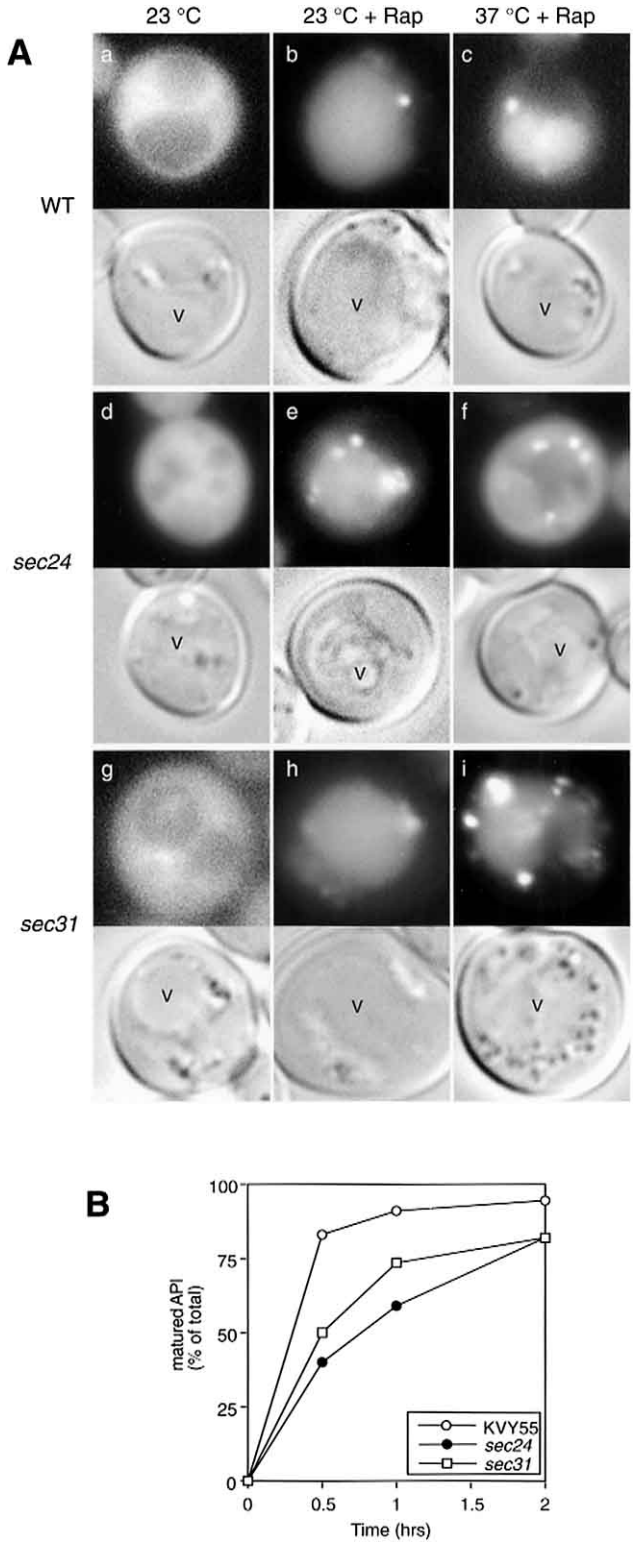

Fig. 1.

intact Cvt pathway was also seen in *sec24* and *sec31* mutants (Fig. 1B) indicated that the Cvt pathway was functional in all early sec mutants even at the non-permissive temperature. These findings suggest that the PAS capable of forming Cvt vesicles exists in these early *sec* mutants.

As the above early Sec proteins play an important role in the transport from the ER (Barlowe *et al.*, 1994; Kaiser and Schekman, 1990), we next investigated the effect of starvation stress on anterograde flow. The flow from the ER was observed by following the fate of carboxypeptidase Y (CPY), a vacuolar soluble protein. Wild-type and early *sec* mutant cells grown to an early-log phase in SD(-met) were further incubated in the nitrogen-depleted medium at permissive temperature (23°C) for 1 hr, shifted to non-permissive temperature (37.5°C) for 15 min, then pulse labeled for 15 min with [^{35}S]methionine and chased up to 30 min. CPY was immunoprecipitated with anti-CPY antibody and the precipitants were analyzed by SDS-PAGE and autoradiography (Fig. 2). Newly synthesized CPY is first modified in the ER (p1 form, 67 kDa), further mannosylated in the Golgi to become the p2 form (69 kDa), and then finally proteolytically processed in the vacuole to the mature form (61 kDa) (Stack *et al.*, 1995). In wild-type cells, the majority of labeled CPY was converted to the mature form within 30 min of the chase period in SD medium (Fig. 2 top). The same result was observed in the starvation medium (Fig. 2 bottom). The mature form of CPY was also detected in all of early *sec* mutant cells within 30 min of chase in SD medium at the permissive temperature, but only the p1 form was found upon the shift to non-permissive temperature (Fig. 2 top and not shown) indicating that the early secretory flow in these sec mutants was indeed arrested. To our surprise, the conversion of the p1 form of CPY to the mature form was observed in *sec13* and *sec31* mutant cells when placed under starvation condition (Fig. 2 bottom). This phenomenon was specific only to the *sec13* and *sec31* mutants as it was not observed in *sec12*, *sec23* and *sec24* mutant cells under the same condition (Fig. 2 bottom). We next examined whether this mCPY went through or bypassed the Golgi by observing the α1,6-mannosylation of mCPY, the hallmark of Golgi transit (Nakayama *et al.*, 1992). The

Fig. 1. (A) Localization of GFP-Aut7p in early *sec* mutant cells. Wild-type (KVY55, a–c), *sec24* (MHY24, d–f) and *sec31* (SRY1004, g–i) cells haboring pRS316 GFP-Aut7p were used. GFP-Aug7p was visualized under growing condition (a, d and g), starvation condition at permissive temperature (b, e and h) and starvation condition at non-permissive temperature (c, f and i). The vacuole is seen as a spherically-shaped structure denoted as "v" in the Nomarski images. (B) API transport in early secretory mutants. Wild-type (KVY55), *sec24* (MHY24) and *sec31* (SRY1004) grown to an early-log phase in SD(-met) at 23°C were shifted to 37°C for 15 min, pulse labeled with [^{35}S]methionine for 15 min then chased for the indicated time. Immunoprecipitation was carried out using anti-API antibody as described in Materials and Methods.

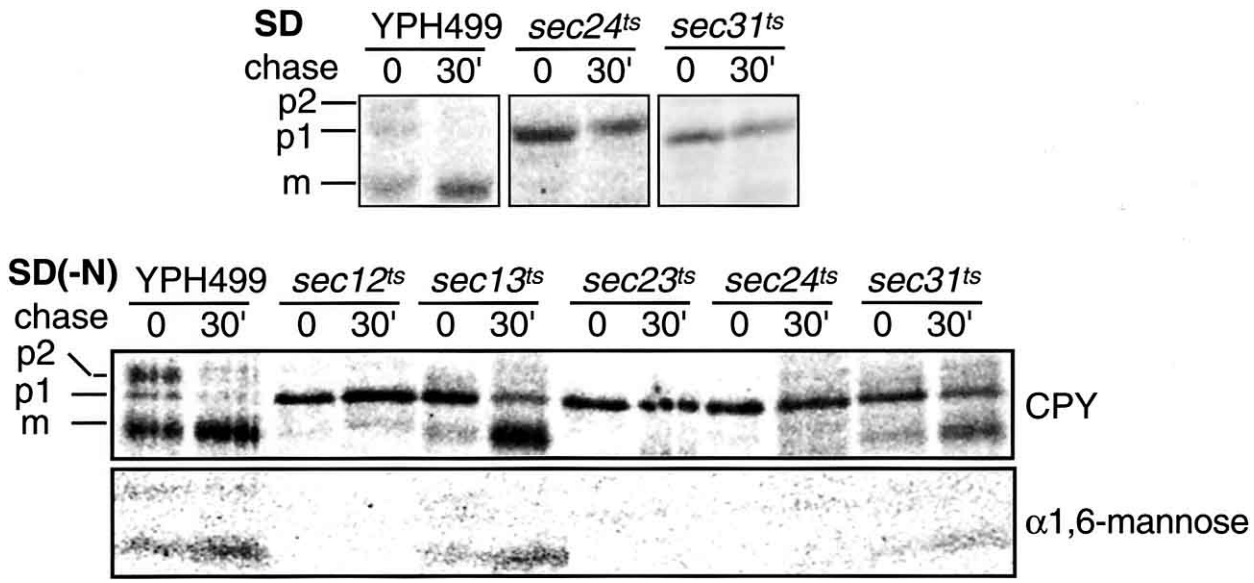

Fig. 2. CPY transport in early *sec* mutant cells under starvation condition. (top panel; in SD) Wild-type cells (YPH499), *sec24*^{ts} (MHY24) and *sec31*^{ts} (RSY1004) mutant cells grown to an early log phase at 23°C were preincubated at 37.5°C for 15 min, pulse labeled with [^{35}S]methionine for 15 min and then chased in SD medium for 30 min. The extracts were prepared and subjected to immunoprecipitation with anti-CPY antiserum. (bottom panel; in SD(-N)) Wild-type cells (YPH499), *sec12*^{ts} (MTsec12), *sec13*^{ts} (MTsec13), *sec23*^{ts} (MTsec23), *sec24*^{ts} (MHY24) and *sec31*^{ts} (RSY1004) mutant cells grown to an early log phase at 23°C were placed in SD(-N) for 1 hr, preincubated at 37.5°C for 15 min, pulse labeled with [^{35}S]methionine for 15 min and then chased in SD(-N) for 30 min. The extracts were prepared and subjected to immunoprecipitation with anti-CPY antiserum. The bound proteins were immunoprecipitated again with anti-α1,6 mannose antibodies. Proteins were eluted and analyzed by SDS-PAGE followed by autoradiography. p1, ER-precursor form; p2, Golgi-precursor form; m, mature form of CPY.

mature CPY was found to be re-precipitated with anti-α1,6-mannose antibody, suggesting that mCPY was delivered to the vacuole through the Golgi (Fig. 2 bottom). This indicated the block in the anterograde flow in *sec13* and *sec31* mutant cells was suppressed with the starvation stress, but the precise mechanism is not clear. Sec13p and Sec31p are known as a subcomplex of the COPII coat, and they form an outer core of the coat (Lederkremer *et al.*, 2001; Matsuoka *et al.*, 2001). Interestingly, Sec13p is reported to be dispensable when Bst1p/Bst2p/Bst3p, the bypass of sec thirteen, is mutated (Elrod-Erickson and Kaiser, 1996). A similar state may be produced by the starvation stress, thus the subcomplex may become dispensable. These findings suggest that autophagy takes place only when the early secretory flow from the ER exists.

We have proposed that the involvement of certain COPII components, including Sec24p, in autophagy (Ishihara *et al.*, 2001). Further study using *sec24* mutant cells was carried out to determine whether the COPII components themselves or the active flow in the early secretory pathway as stated above plays a crucial role in autophagy. Sfb2p, also called Iss1p, is a Sec24p related protein sharing 56% identity with it (Peng *et al.*, 2000). Although its native role is not clearly identified, overexpression of this protein can suppress the growth defect of Δ*sec24* mutant cells when cultured at 30°C, but not at 23°C or 37°C (Higashio *et al.*, 2000). First we tested to see if Sfb2p itself is involved in autophagy. To measure autophagic activity, we employed the ALP assay using cells expressing Pho8Δ60p, a truncated form of the vacuolar alkaline phosphatase in the cytosol, which becomes activated upon delivering to the vacuole via autophagy (Noda *et al.*, 1995). ALP activity was increased in *sfb2* deletion mutant cells under starvation, implying that Sfb2p itself is not required for autophagy (Fig. 3A). Next, we performed the time course assessment of the ALP assay in Δ*sec24* (*SFB2*, *2*μ) cells and its control cells. These cells grown to an early-log phase were incubated in nitrogen-depleted medium for the time indicated. In *sec24 ts* mutant cells, the increment of ALP activity was observed at permissive temperature but not at the non-permissive one (Fig. 3B). A similar result was observed in Δ*sec24* (*SFB2*, *2*μ) cells, that is, the ALP activity was increased at the same rate as in wild-type cells (Fig. 3B). These findings indicated that the requirement is not a specific component like Sfb2p or Sec24p, but the active flow in the early secretory pathway in autophagy. The lag time before the onset of autophagy observed in Δ*sec24* (*SFB2*, *2*μ) cells was not seen in Δ*sec24* cells which harbors a wild type *SEC24* on its centromeric plasmid (Fig. 3B). It is reported that Sec24p, which is involved in the cargo selection and the transport of specific proteins such as Gas1p, is impaired in Δ*sec24* (*SFB2*, *2*μ) cells while transport of most of the protein is restored

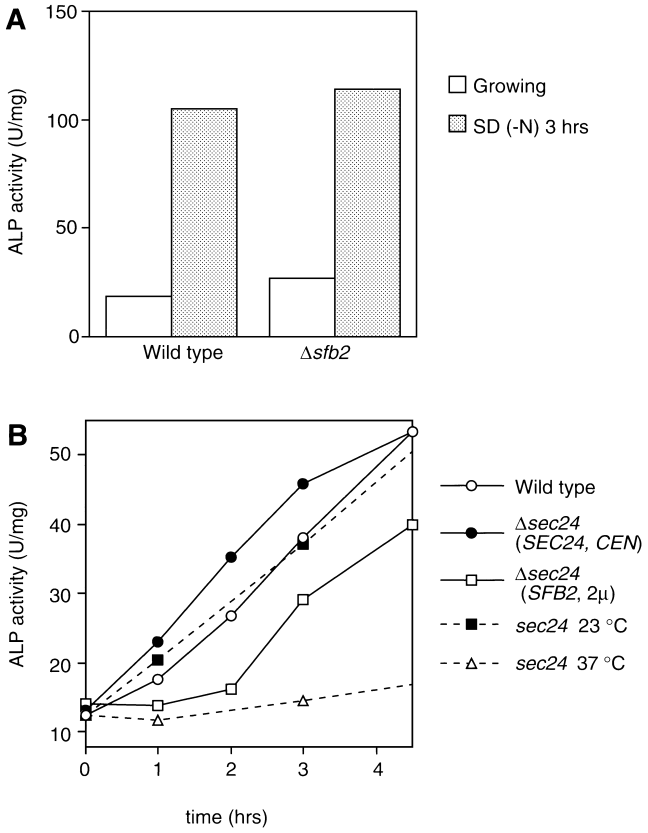

Fig. 3. Suppression of autophagic defect in Δ*sec24* cells by multi-copy expression of *SFB2*. (A) Autophagic activity in Δ*sfb2* cells. Wild-type and Δ*sfb2* cells expressing Pho8Δ60 were cultured to an early log phase in YPD and then transferred to SD(-N) for 3 hr, lysed and assessed for ALP activity as described in Materials and Methods. (B) The autophagic activity in *sec24* mutant cells. Wild-type (KVY55), Δ*sec24* (*SEC24, CEN*) (MHY98) and Δ*sec24* (*SFB2, 2*μ) (MHY99) cells expressing PHO8Δ60 were grown in YPD to an early log phase at 30°C, were shifted to SD(-N) and cultured for the indicated time. *sec24*^{ts} (MHY24) was grown at 23°C, shifted to SD(-N) and incubated at 23°C or 37°C. The ALP activity of the lysates was measured to estimate the autophagic activity. The graph is the representative result of several independent experiments.

(Higashio *et al.*, 2000). This lag time may be due to the partial complementation of *sec24* mutant defect.

In this study, we presented the clear correlation between the active flow in the early secretory pathway and autophagy for the first time. Autophagy does not occur when the flow is blocked as seen in *sec12, sec16, sec23* and *sec24* cells, while autophagy occurs when the flow exists as seen in *sec13, sec31* and Δ*sec24* (*SFB2, 2*μ) cells under non-permissive temperature. Our results suggest that the flow itself is the prerequisite for autophagy and not the COPII like vesicles specific to autophagy as discussed in our previous report (Ishihara *et al.*, 2001). Further studies are required to more precisely discuss the involvement of flow past Golgi in autophagy although the autophagic defect was not seen in late *sec* mutant cells, *sec4* and *sec15*, which blocks the transport between the Golgi and the plasma membrane (Ishihara *et al.*, 2001). An obvious possible role of the active flow described in this study is to supply the lipid source for the autophagosome. Another possibility is that the flow is required to maintain the functional ER involved in autophagy. Further studies may lead to determining the membrane source of the autophagosome.

Acknowledgments. We wish to thank Dr. R. Schekman (University of California, Berkeley) and Dr. K. Kohno (Nara Institute For Science and Technology, Nara) for the yeast strains and members of the Ohsumi lab for their constructive comments. This work was supported in part by Grants-in-Aids for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. M. Hamasaki was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

Reference

Adams, L.J., Gottschling, D.E., Kaiser, C.A., and Stearns, T. 1997. Methods in Yeast Genetics. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Antonny, B. and Schekman, R. 2001. ER export: public transportation by the COPII coach. *Curr. Opin. Cell Biol.*, **13**: 438–443.

Baba, M., Osumi, M., Scott, S.V., Klionsky, D.J., and Ohsumi, Y. 1997. Two distinct pathways for targeting proteins from the cytoplasm to the vacuole/lysosome. *J. Cell Biol.*, **139**: 1687–1695.

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M.F., Ravazzola, M., Amherdt, M., and Schekman, R. 1994. COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell.*, **77**: 895–907.

Elrod-Erickson, M.J. and Kaiser, C.A. 1996. Genes that control the fidelity of endoplasmic reticulum to Golgi transport identified as suppressors of vesicle budding mutations. *Mol. Biol. Cell*, **7**: 1043–1058.

Harding, T.M., Hefner-Gravink, A., Thumm, M., and Klionsky, D.J. 1996. Genetic and phenotypic overlap between autophagy and the cytoplasm to vacuole protein targeting pathway. *J. Biol. Chem.*, **271**: 17621–17624.

Higashio, H., Kimata, Y., Kiriyama, T., Hirata, A., and Kohno, K. 2000. Sfb2p, a yeast protein related to Sec24p, can function as a constituent of COPII coats required for vesicle budding from the endoplasmic reticulum. *J. Biol. Chem.*, **275**: 17900–17908.

Ishihara, N., Hamasaki, M., Yokota, S., Suzuki, K., Kamada, Y., Kihara, A., Yoshimori, T., Noda, T., and Ohsumi, Y. 2001. Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for vacuolar fusion. *Mol. Biol. Cell*, **12**: 3690–3702.

Kaiser, C.A. and Schekman, R. 1990. Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell*, **61**: 723–733.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. 1999. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J. Cell Biol.*, **147**: 435–446.

Klionsky, D.J., Cueva, R., and Yaver, D.S. 1992. Aminopeptidase I of *Saccharomyces cerevisiae* is localized to the vacuole independent of the secretory pathway. *J. Cell Biol.*, **119**: 287–299.

Klionsky, D.J. and Ohsumi, Y. 1999. Vacuolar import of proteins and organelles from the cytoplasm. *Annu. Rev. Cell Dev. Biol.*, **15**: 1–32.

Kuehn, M.J., Herrmann, J.M., and Schekman, R. 1998. COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. *Nature*, **391**: 187–190.

Kurihara, T., Hamamoto, S., Gimeno, R.E., Kaiser, C.A., Schekman, R.,

and Yoshihisa, T. 2000. Sec24p and Iss1p function interchangeably in transport vesicle formation from the endoplasmic reticulum in *Saccharomyces cerevisiae*. *Mol. Biol. Cell*, **11**: 983–998.

Lederkremer, G.Z., Cheng, Y., Petre, B.M., Vogan, E., Springer, S., Schekman, R., Walz, T., and Kirchhausen, T. 2001. Structure of the Sec23p/24p and Sec13p/31p complexes of COPII. *Proc. Natl. Acad. Sci. USA*, **98**: 10704–10709.

Matsuoka, K., Schekman, R., Orci, L., and Heuser, J.E. 2001. Surface structure of the COPII-coated vesicle. *Proc. Natl. Acad. Sci. USA*, **98**: 13705–13709.

Nakayama, K., Nagasu, T., Shimma, Y., Kuromitsu, J., and Jigami, Y. 1992. OCH1 encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides. *Embo J.*, **11**: 2511–2519.

Noda, T., Matsuura, A., Wada, Y., and Ohsumi, Y. 1995. Novel system for monitoring autophagy in the yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.*, **210**: 126–132.

Noda, T. and Ohsumi, Y. 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.*, **273**: 3963–3966.

Peng, R., De Antoni, A., and Gallwitz, D. 2000. Evidence for overlapping and distinct functions in protein transport of coat protein Sec24p family members. *J. Biol. Chem.*, **275**: 11521–11528.

Salama, N.R., Chuang, J.S., and Schekman, R.W. 1997. Sec31 encodes an essential component of the COPII coat required for transport vesicle budding from the endoplasmic reticulum. *Mol. Biol. Cell.*, **8**: 205–217.

Scott, S.V., Baba, M., Ohsumi, Y., and Klionsky, D.J. 1997. Aminopeptidase I is targeted to the vacuole by a nonclassical vesicular mechanism. *J. Cell. Biol.*, **138**: 37–44.

Sikorski, R.S. and Hieter, P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**: 19–27.

Stack, J.H., Horazdovsky, B., and Emr, S.D. 1995. Receptor-mediated protein sorting to the vacuole in yeast: roles for a protein kinase, a lipid kinase and GTP-binding proteins. *Annu. Rev. Cell Dev. Biol.*, **11**: 1–33.

Suzuki, K., Kamada, Y., and Ohsumi, Y. 2002. Studies of cargo delivery to the vacuole mediated by autophagosomes in *Saccharomyces cerevisiae*. *Dev. Cell*, **3**: 815–824.

Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. 2001. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *Embo J.*, **20**: 5971–5981.

Thumm, M., Egner, R., Koch, B., Schlumpberger, M., Straub, M., Veenhuis, M., and Wolf, D.H. 1994. Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*. *FEBS Lett.*, **349**: 275–280.

Tsukada, M. and Ohsumi, Y. 1993. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett.*, **333**: 169–174.

(*Received for publication, December 6, 2002*
and in revised form, December 25, 2002)