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Acidification of Vacuoles Is Required for Autophagic Degradation in the Yeast, *Saccharomyces cerevisiae*[1]

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Acidification inside vacuoles has been shown to play a key role in a number of physiologically important cellular events. We studied the role of vacuolar membrane H^+-ATPase in the autophagic process of *Saccharomyces cerevisiae*. Mutants lacking *VMA* genes which encode their subunits of the vacuolar H^+-ATPase accumulated autophagic bodies in vacuoles on starvation. *vma* mutants also had a defect in protein degradation induced by starvation. In *vma* mutants, the activities of vacuolar proteases were remarkably lower than those of the wild-type. Overexpression of vacuolar proteases did not overcome the defect in the disintegration of autophagic bodies in *vma* mutant, even the overexpressed proteinase A and proteinase B being substantially localized to the vacuolar compartment and undergoing proper proteolytic maturation. Our results showed that the acidification of vacuoles is not required for the formation and delivery of autophagosomes to vacuoles, but is essential for the disintegration of autophagic bodies.

Key words: autophagy, vacuolar H^+-ATPase, vacuole, yeast.

The yeast vacuole is an acidic compartment that contains a wide variety of hydrolases (*1*). Acidification inside a vacuole is implicated in the expression of a number of its functions such as chemiosmotic regulation of cytosolic ionic homeostasis, efficient sorting of vacuolar proteins to the vacuole, and so on (*2-7*). The acidic environment in the vacuolar lumen is maintained by the vacuolar membrane H^+-ATPase, the subunits of which are encoded by a set of *VMA* and *VPH* genes (*4, 5, 8*).

The vacuole plays a role in the degradation of macromolecules. The degradation in the vacuole is vitally important for the maintenance of intracellular homeostasis. One of the major vacuolar/lysosomal degradation systems is thought to be autophagy. Autophagy is a ubiquitous vacuolar/lysosomal proteolytic process seen in many cell types (*9*). We showed previously that yeast vacuoles also serve a function in autophagic protein degradation (*10*). On nutrient starvation, a double-membrane bound structure (autophagosome) enclosing a portion of the cytosol and organelles appears in the cytoplasm, and then fuses with the vacuole. As a result, a spherical structure (autophagic body, AB) encircled by a single membrane is delivered to the lumen of the vacuole. Finally, the AB and its contents are degraded by the set of hydrolases in the vacuole. Immunoelectron microscopic and biochemical analyses showed that autophagosomes sequester cytoplasmic components nonselectively. Thus, autophagic degradation provides a nonselective pathway for bulk turnover of the cytoplasmic components in yeast (*10, 11*).

Genetic selection has been used to identify many mutants that have a defect in autophagy (*12, 13*). These autophagy-defective mutants (*apg* and *aut* mutants) have a lesion in the mechanisms involved in steps before the fusion of an autophagosome to a vacuole, and analysis of these mutants should ultimately reveal the molecular components and mechanisms necessary for the induction of autophagy, formation of autophagosomes, and delivery of ABs to the vacuoles. For instance, it was found that one of the *APG* genes, *APG1*, encodes a serine/threonine protein kinase essential for autophagy (Matsuura *et al.*, in preparation). In contrast, the mechanism of the degradation of ABs is poorly understood. We showed that the activities of proteinase A (PrA; *PEP4/PRA1* gene product) and proteinase B (PrB; *PRB1* gene product) are essential for the disintegration of ABs in the vacuoles since *pep4* or *prb1* mutants accumulate ABs in the vacuoles on starvation (*10*). Because an AB is limited by a membrane, we presumed the existence of a mechanism for disintegration of the membrane allows the vacuolar hydrolases access to the contents of ABs. To elucidate the mechanism for the disintegration of ABs, we screened mutants which exhibit defects in this process (see "EXPERIMENTAL PROCEDURES"), and identified 5 recessive mutations in 4 complementation groups. We designated the genes as *ABD* for autophagic body disintegration. During phenotypic analyses of the mutants, we found that *abd1*, *abd2*, and *abd3* were defective in vacuolar acidification. Complementation tests among *abd* mutants and *vma* mutants showed that the *abd1*, *abd2*, and *abd3* mutations

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Abbreviations: AB, autophagic body; CPY, carboxypeptidase Y; PrA, proteinase A; PrB, proteinase B.

are allelic to *vma11*, *vma1*, and *vma3*, respectively. This implied that the vacuolar acidification plays an important role in the disintegration of ABs. We refer to *abd1-1*, *abd2-1*, and *abd3-1* as *vma11-101*, *vma1-101*, and *vma3-101*, respectively, and report in this paper that the function of vacuolar H^+-ATPase is required for the disintegration of ABs.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Media—The yeast strains used in this study are listed in Table I. Yeast cells were grown in YPD or SD supplemented as needed (*14*). The nitrogen or carbon starvation medium was described previously (*12, 15*). We introduced the *Δapg1::LEU2* fragment (Matsuura *et al.*, unpublished) into strains YW5-1B, STY3, and NNY5142 to obtain NNY20, NNY23, and NNY26, respectively. The disruption of the chromosomal *APG1* gene was confirmed by Southern blotting analyses. A plasmid, pPRA1-1, was isolated from a yeast genomic library in the yeast plasmid, YEp13, by complementation of *pep4-3*. The restriction analyses confirmed that pPRA1-1 carries the *PEP4* gene (*16, 17*, Wada, unpublished). A multicopy plasmid containing the *PRB1* gene, YEpPRB1-1, was constructed as follows. A 3.8 kb *Xho*I-*Sac*I fragment containing the *PRB1* gene (*18*) was inserted into the *Xho*I-*Sac*I sites of multicopy plasmid pMRU1, which was constructed by introducing the 2 μm into pRS306 (Matsuura, unpublished).

Isolation of Mutants Defective in the Disintegration of ABs—YW5-1B cells were mutagenized with ethylmethane sulfonate to 30% viability. The mutagenized cells were spread on YPD plates and incubated for 4 days. Then the colonies were replica-plated onto nitrogen-starved solid medium containing phloxine B, which stains inviable cells, as previously described (*12*). Colonies stained red were selected, grown again on YPD plates, and then transferred to nitrogen starvation medium. After 3-4 h incubation, cells were observed under a light microscope. Mutants which accumulated an AB-like structure in their vacuoles were kept. Mutants carrying a *pep4* or *prb1* lesion were excluded by complementation analyses with the *pep4* or *prb1* strain. The isolated mutants were backcrossed at least five times.

Immunoblotting, Protease Activity Assays, and Preparation of Vacuoles—Cells were harvested, washed, and disrupted with glass beads in 0.1 M Tris-Cl, pH 7.6. The cell extract was clarified by centrifugation at 12,500 × *g* for 30 min at 4°C, and then used for immunoblotting analyses (*19*) and determination of protease activities (*20*). The vacuoles were prepared as described previously (*10*). *vma1* cells (NNY5142), and *vma1* cells harboring pPRA1-1 and pPRB1-1 were grown in SD containing appropriate supplements to a density of 2×10^7 cells/ml. The cells were harvested and converted to spheroplasts. The spheroplasts were washed twice and then lysed in buffer A [10 mM MES-Tris, pH 6.9, 0.1 mM $MgCl_2$, and 12% (w/v) Ficoll 400] with a Dounce homogenizer. The lysate was transferred to centrifuge tubes and an equal volume of buffer B [10 mM MES-Tris, pH 7.5, 0.5 mM $MgCl_2$, and 8% (w/v) Ficoll 400] was layered on top, followed by centrifugation at 30,000 × *g* for 30 min. The white layer at the top of the tube was collected and resuspended in buffer B. This crude vacuole suspension was transferred to centrifuge tubes and an equal volume of buffer C [10 mM MES-Tris, pH 7.5, 0.5 mM $MgCl_2$, and 4% (w/v) Ficoll 400] was layered on top, followed by centrifugation at 10,000 × *g* for 30 min. Vacuoles were collected from the top of the buffer C.

PrA activity assay: The reaction mixture was prepared by mixing 400 μl of 2% acid-denatured hemoglobin (Sigma), 400 μl of 0.2 M glycine-Cl, pH 3.2, and 100 μl of the clarified cell extract. After 30 min incubation at 37°C, 200 μl of the reaction mixture was withdrawn, and added to 100 μl of 1 M perchloric acid prechilled on ice, followed by centrifugation at 1,650 × *g* for 5 min. The supernatant (100 μl) was neutralized with 100 μl of 0.5 M NaOH, and then the amount of generated peptides in 100 μl of the solution was determined with BCA protein assay reagent (Pierce).

PrB activity assay: A 200 μl sample of the clarified cell extract was incubated in a final volume of 740 μl of buffer (0.1 M Tris-Cl pH 7.6, 1% SDS, and 0.17% Triton X-100) containing 20 mg of Azocoll (Calbiochem) at 37°C for 30 min, and the reaction was terminated by rapid cooling on ice and the addition of 1 ml of ice-cold distilled water. After centrifugation for 5 min at 1,650 × *g*, the A_{520} of the supernatant was determined with a spectrophotometer (Hitachi U-2000).

CPY activity assay: A 100 μl sample of the clarified cell extract was incubated with 4.8 mM *N*-benzoyl-L-tyrosine *p*-nitroanilide (Sigma) in dimethylformamide in a final 300 μl of 0.1 M Tris-Cl pH 7.6, for 30 min at 37°C. Then, $HgCl_2$ and SDS were added to terminate the reaction in a final volume of 980 μl to give final concentrations of 0.6 mM and 1.6%, respectively. The A_{410} of the mixture was determined.

Measurement of Protein Degradation—Exponentially growing cells were harvested, washed, incubated in SD medium containing [^{14}C]leucine (ICN) at a final concentration of 0.011 MBq/ml for 60 min, and then chased for 90 min by adding 2 volumes of YPD. The cells were washed and incubated in the carbon starvation medium. Samples (100 μl) were withdrawn at 0, 2, 4, 6, or 8 h, added to 1 ml of 11% TCA, and kept at 4°C overnight. The suspensions were centrifuged at 12,500 × *g* and the radioactivity in the supernatants was determined with a liquid scintillation counter (Beckman LS5800). Protein degradation was expressed as the ratio of the TCA-soluble radioactivity to the initial total cell-associated radioactivity.

RESULTS

Vacuolar H^+-ATPase Deficient Mutants Accumulate AB-Like Structures in Their Vacuoles—In yeast autophagy, the ABs disintegrated rapidly after they reached the vacuoles, and thus ABs were not accumulated in the vacuoles on starvation (Fig. 1, WT). We showed previously that the activity of PrB is required for the disintegration of ABs (*10*). *Δprb1* cells could not disintegrate the ABs, and thus ABs accumulated in their vacuoles, as can be seen in Fig. 1 (*Δprb1*). Deletion of the *VMA1*, *VMA3*, or *VMA11* gene resulted in the accumulation of spherical structures in the vacuoles (Fig. 1). We concluded that they are ABs for following reasons. Firstly, these spherical structures were morphologically indistinguishable from ABs. Secondly, the spherical structures appeared only when the cells were subjected to starvation. Thirdly, they gradually increased

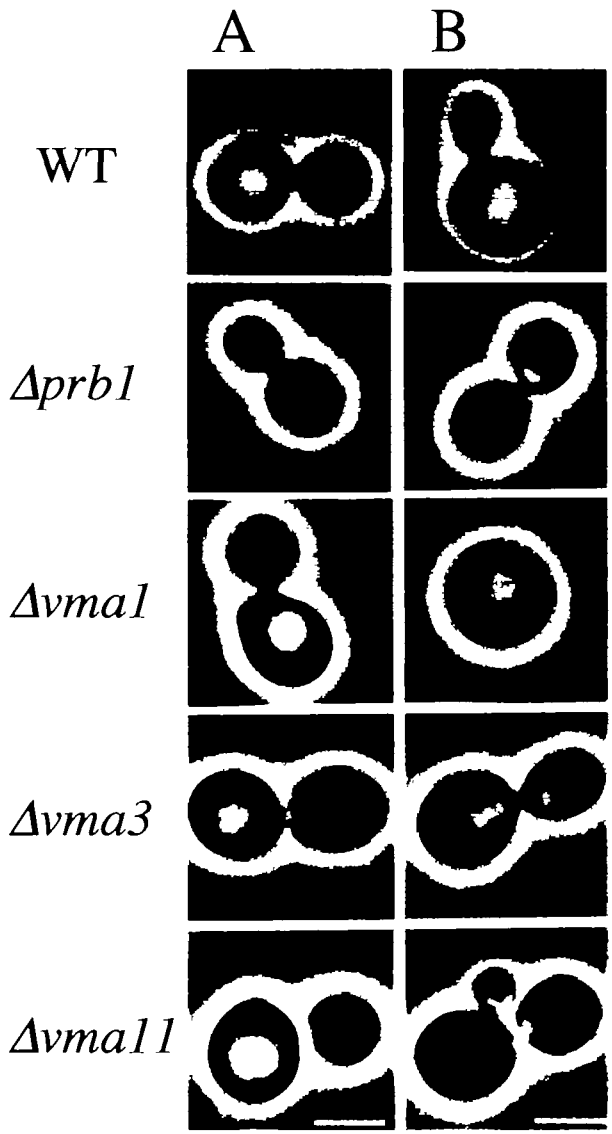

Fig. 1. **Vacuolar H⁺-ATPase deficient mutants accumulate AB-like structures in their vacuoles on nitrogen starvation.** Cells were grown to the early exponential phase in YPD, transferred to nitrogen starvation medium, and incubated for 0 h (A) or 3.5 h (B). Then the cells were observed under a phase contrast microscope. The strains were: WT, YPH499; *Δprb1*, STY99; *Δvma1*, RH104; *Δvma3*, DU3T-A(a); *Δvma11*, YRH11a. Bars indicate 5 μm.

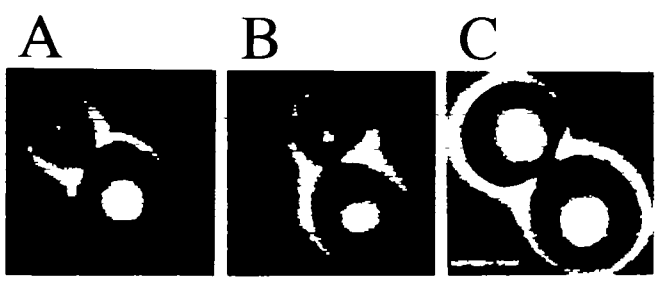

Fig. 2. **The *Δvma1 Δapg1* mutant did not accumulate AB-like structures in the vacuole.** Cells were subjected to nitrogen starvation and then observed as described in Fig. 1. The strains were: NNY20 (*Δapg1*) (A), NNY23 (*Δpep4 Δapg1*) (B), NNY26 (*vma1-101 Δapg1*) (C). The bar indicates 5 μm.

in number and finally filled the vacuoles during starvation. Finally, we found that *vma1-101* cells lacking the *APG1* function, which is required for the autophagic process (*12*), did not accumulate the spherical structures (Fig. 2).

vma Mutants Are Defective in Protein Degradation Induced by Starvation—Yeast cells exhibit accelerated protein degradation and protein turnover on starvation, reflecting rearrangement and reconstruction of the cellular components to adapt to the nutrient stress (*21, 22*). We previously showed that most of this starvation-induced protein degradation is due to autophagy (*12*). If vacuolar acidification is required for the disintegration of ABs, *vma* mutants may exhibit a defect in protein degradation. To test this, we examined the protein degradation in *vma* mutants. Total cellular proteins were metabolically labeled, and then the time-course of their breakdown *in vivo* on starvation was determined by measuring the TCA-soluble radioactivity (see "EXPERIMENTAL PROCEDURES"). When labeled wild-type cells were transferred to the starvation medium, the radioactivity in the TCA-soluble fraction increased, reflecting gross protein degradation on starvation (Fig. 3). In contrast, the protein degradation in *Δvma* mutants decreased to 31% (*Δvma1*), 58% (*Δvma3*), or 11% (*Δvma11*) of the wild-type level (Fig. 3). In the *Δprb1* mutant, the radioactivity in the TCA-soluble fraction decreased to 61% of the wild-type level on 8 h incubation in the starvation medium (Fig. 3). These results indicate that loss of these *VMA* genes causes a significant reduction in the protein degradation induced by starvation.

Maturation and Localization of Vacuolar Proteases in vma Mutants on Nitrogen Starvation—We examined the maturation of the vacuolar proteases in *vma* mutants in the steady state by immunoblotting analysis. Vacuolar proteases undergo a number of compartment-specific modifications as they pass through the early stages of the secretory pathway (*23, 24*). This modification reflects the localization of the vacuolar proteases, and each form can be determined from its migration on SDS-PAGE analysis. The mature forms of PrA, PrB, and CPY are 43, 30, and 61 kDa, respectively, while their Golgi forms (p2 or pro-form) are

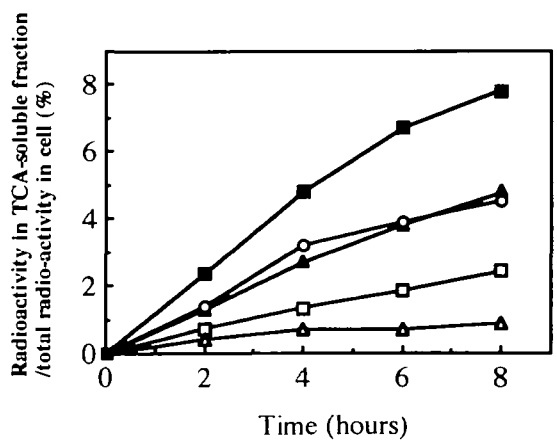

Fig. 3. ***vma* mutants have a defect in starvation-induced protein degradation.** Radioactivity in the TCA soluble fraction and total cells was measured as described under "EXPERIMENTAL PROCEDURES." The strains were: (■) YPH499 (*VMA*), (▲) STY99 (*Δprb1*), (□) RH104 (*Δvma1*), (○) DU3T-A(a) (*Δvma3*), (△) YRH11a (*Δvma11*).

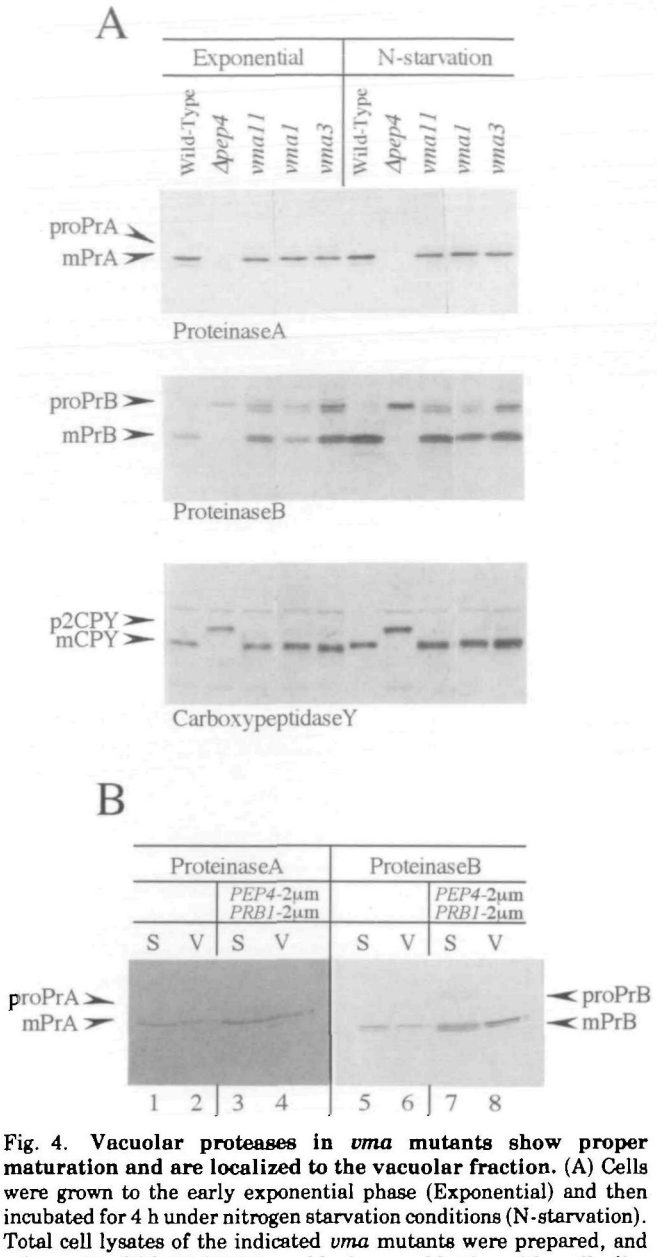

Fig. 4. **Vacuolar proteases in *vma* mutants show proper maturation and are localized to the vacuolar fraction.** (A) Cells were grown to the early exponential phase (Exponential) and then incubated for 4 h under nitrogen starvation conditions (N-starvation). Total cell lysates of the indicated *vma* mutants were prepared, and subjected to SDS-PAGE followed by immunoblotting with antibodies directed against either PrA, PrB, or CPY. The migration positions of proPrA (Golgi form), mPrA (mature form), proPrB (Golgi form), mPrB (mature form), p2CPY (Golgi form), and mCPY (mature form) are indicated. The strains were: Wild-type (YW5-1B), *Δpep4* (STY3), *vma1* (NNY5142), *vma3* (NNY1349), *vma11* (NNY4143). (B) *vma1* (NNY5142) cells, and *vma1* cells harboring pPRA1-1 and pPRB1-1 were grown in SD containing appropriate supplements, and then vacuoles were prepared. The sample in each lane was normalized as to vacuolar membrane α-mannosidase activity, and then subjected to immunoblotting with anti-PrA and anti-PrB antibodies. Lanes 1, 2, 3, and 4, probed with anti-PrA antibodies; lanes 5, 6, 7, and 8, probed with anti-PrB antibodies; lanes 1, 2, 5, and 6, *vma1* (NNY5142) cells; lanes 3, 4, 7, and 8, *vma1* cells harboring pPRA1-1 and pPRB1-1; lanes 1, 3, 5, and 7, spheroplast lysates; lanes 2, 4, 6, and 8, vacuolar fractions.

52, 41, and 69 kDa on SDS-PAGE, respectively. In wild-type cells, PrA, PrB, and CPY appeared as mature forms, indicating that they reached the vacuoles and were processed properly under both exponential growth and starvation conditions (Fig. 4A). In *vma* mutants, a small portion of these proteases was remained as their Golgi forms, proPrA, proPrB, and p2CPY, indicating a decreased rate of maturation of these proteases, as reported previously (*6, 25*). However, PrA, PrB, and CPY were mostly found as their mature forms in the *vma* mutants under both exponential growth and starvation conditions (Fig. 4A). We also examined the localization of PrA and PrB in the *vma1* mutant. Vacuoles were isolated from *vma1* cells, and the amounts of PrA and PrB in the vacuoles were determined by immunoblotting (Fig 4B; lanes 1, 2, 5, and 6). The proforms of these proteinases observed in the spheroplast lysate were not found at all in the vacuole fraction, and approximately 81 and 73% of mature PrA and PrB, respectively, were cofractionated with a vacuolar marker, α-mannosidase, suggesting that mature PrA and PrB were properly localized in the vacuole. We also examined the activities of PrA, PrB, and CPY in the *vma* mutants. The levels of most vacuolar hydrolase activities vary with the growth stage and nutritional availability. The vacuolar proteases are known to be induced by limited glucose or nitrogen sources (*26-29*). The wild-type, *Δpep4*, and *vma* mutants grown in YPD were transferred to nitrogen starvation medium, and incubated for 4 h. In wild-type cells, the protease activities increased several fold on nitrogen starvation compared to those in the exponentially growing phase (Table II). The PrB and CPY activities in *Δpep4* cells were at the background levels (Table II), since the activation of both PrB and CPY requires the PrA function (*30-34*). The protease activities in the exponentially growing *vma* mutants were similar to those in the wild-type. However, they remained at considerably lower levels upon nitrogen starvation, as compared with the increase in the protease activities observed in the wild-type cells (Table II). In wild-type cells, nitrogen starvation resulted in 4.5-, 13-, and 2.6-fold increases in the PrA, PrB, and CPY activities, respectively (Table II). However, these protease activities in the *vma* mutants on starvation were remarkably lower than those in the wild-type. The levels of PrA, PrB, and CPY activities in the *vma* mutants on nitrogen starvation were only 28-47, 13-19, and 41-77% of the wild-type levels, respectively. This shows that the loss of the *VMA* function causes a defect in the expression of the PrA, PrB, and CPY activities on nitrogen starvation.

Overexpression of PrA and PrB in the vma1 Mutant—The accumulation of ABs in the vacuoles of *vma* mutants may reflect the insufficient expression of PrA and PrB activities. To examine this possibility, we introduced the *PEP4* and *PRB1* genes on a multicopy plasmid into *vma1* cells. The PrA and PrB activities of *vma1* cells overexpressing PrA and PrB increased to 2.9- and 2.5-fold those of untransformed *vma1* cells, and 2.4- and 1.1-fold those of wild-type cells, respectively (Table III). Approximately 87% and 77% of the mature PrA and PrB expressed by the multicopy plasmids, respectively, were cofractionated with the vacuoles (Fig. 4B; lanes 3, 4, 7, and 8). However, *vma1* cells overexpressing PrA and PrB still accumulated ABs in their vacuoles (Table III). This indicates that overexpression of PrA and PrB did not compensate for the defect in the disintegration of ABs. Therefore, the insufficiency of the PrA and PrB activities alone is not the reason why the ABs were not disintegrated in the *vma* mutants.

TABLE I. **Strains used in this study.**

Strain	Genotype	Source
YW5-1B	*MATa leu2-3,112 trp1 ura3-52*	Takeshige *et al.*, 1992 (*10*)
YPH499	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Y.G.S.C.
STY99	*MATa prb1::TRP1 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Takeshige *et al.*, 1992 (*10*)
STY3	*MATa pep4::URA3 leu2-3,112 ura3-52*	This study
NNY4143	*MATa abd1-1(vma11-101) leu2-3,112 ura3-52*	This study
NNY5142	*MATa abd2-1(vma1-101) leu2-3,112 ura3-52*	This study
NNY1349	*MATa abd3-1(vma3-101) ura3-52*	This study
NNY20	*MATa apg1::LEU2 leu2-3,112 trp1 ura3-52*	This study
NNY23	*MATa apg1::LEU2 pep4::URA3 leu2-3,112 ura3-52*	This study
NNY26	*MATa abd2-1(vma1-101) apg1::LEU2 leu2-3,112 ura3-52*	This study
RH104	*MATa vma1::TRP1 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Y.A.
DU3T-A(a)	*MATa vma3::TRP1 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Y.A.
YRH11a	*MATa vma11::TRP1 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Y.A.

Y.G.S.C.: Yeast Genetic Stock Center. Y.A.: kindly provided by Yasuhiro Anraku (The University of Tokyo).

TABLE II. **Vacuolar protease activities in the *vma* mutants.**

Strain	PrA activity (ΔA_{562}/min/mg protein)		PrB activity (ΔA_{520}/min/mg protein)		CPY activity (ΔA_{410}/min/mg protein)	
	Expon.	N-starv.	Expon.	N-starv.	Expon.	N-starv.
WT	2.8	12.7	1.7	21.8	3.8	9.7
Δpep4	0.4	0.1	0.0	0.0	0.0	0.0
vma1	3.2	5.3	1.8	3.4	2.5	4.0
vma3	5.2	6.0	2.0	2.8	5.5	7.5
vma11	4.3	3.5	1.3	4.2	2.7	5.0

The protease activities of exponentially growing cells in YPD (Expon.) and cells incubated in nitrogen starvation medium for 4 h (N-starv.) were determined. The strains were: WT (YW5-1B), *Δpep4* (STY3), *vma1* (NNY5142), *vma3* (NNY1349), *vma11* (NNY4143).

TABLE III. **Overexpression of PrA and PrB in *vma1* mutant.**

Strain	PrA activity (ΔA_{562}/min/mg protein)	PrB activity (ΔA_{520}/min/mg protein)	Accumulation of ABs
WT	11.4	28.1	no
vma1	9.3	12.4	yes
vma1/*PEP4*-2 μm *PRB1*-2 μm	27.3	30.8	yes

YW5-1B (WT), NNY5142 (*vma1*), and NNY5142 harboring both pPRA1-1 and YEpPRB1-1 cells grown in SD containing appropreate supplements, were transferred to nitrogen starvation medium, and then incubated for 4 h. The PrA and PrB activities in the cell lysate, and the accumulation of ABs were examined.

DISCUSSION

The vacuolar proton-translocating ATPase mediates the active transport of protons into the lumen of vacuoles, thus it provides the proton motive force across the vacuolar membrane (*4, 5*). The proton motive force generated by the H^+-ATPase is converted to chemical potential difference for protons through the function of anion and cation transport activities equipped in the vacuolar membrane (*4, 7, 35, 36*). The acidification inside the vacuoles is involved in the expression of certain vacuolar functions. The diversity of vacuolar functions has been underscored by the pleiotropic phenotypes of *vma* mutant cells lacking the vacuolar H^+-ATPase (*4, 5*). The *vma* mutants exhibit increased sensitivity to divalent cations and the inability to grow on nonfermentable carbon sources or in media buffered above neutral pH. In this study, we revealed a novel phenotype of *vma* mutant cells: the *vma* mutants are defective in the bulk protein degradation on starvation. They accumulate ABs in their vacuoles on starvation, indicating that they are defective in the disintegration of ABs in their vacuoles.

We concluded that vacuolar acidification is required for the disintegration of ABs from the several lines of evidence we have described. The spherical structures in the *vma1*, *vma3*, and *vma11* cells were indistinguishable from the ABs in wild-type cells in morphological characteristics. These structures did not appear unless the cells were subjected to nutrient starvation and they increased in number during starvation, sharing common features with the ABs in wild-type cells. Their accumulation required the functional *APG1* gene. The bulk protein degradation induced by starvation decreased in *vma* cells to the same level as or less than that in *prb* cells, whereas there were some differences in the degradation rate among three *Δvma* mutants, the reason for this difference remaining unclear. As previously reported by Umemoto *et al.* (*37*), we have also confirmed that these *vma* mutants show a defect in the acidification of their vacuoles using a weakly basic dye, quinacrine (data not shown). We previously observed that Prb^- cells accumulate ABs in their vacuoles in the presence of bafilomycin A_1, a potent and specific inhibitor for the vacuolar H^+-ATPase, indicating that the formation, delivery, and fusion of autophagosomes with the vacuoles are not dependent on the acidification inside the vacuoles. This observation is well consistent with our current results for the *vma* mutant cells. We have also examined other *vma* mutant cells (*vma2, vma4, vma5, vma6, vma12*, and *vma13*), and found that they also accumulated ABs in their vacuoles on starvation (data not shown). Yamamoto *et al.* (*38, 39*) previously reported that the fusion of autophagosomes with lysosomes/vacuoles requires the H^+-ATPase function in mammalian cells, since, in presence of bafilomycin A_1, autophagosomes accumulate in the cytosol. This may reflect the differences in the mechanism of fusion between yeast and mammalian cells, and suggest that the mammalian cells utilize more complicated mechanisms which need the acidification.

It has been shown that *vma* mutants missort a part of the soluble vacuolar proteases to the medium (*3, 6, 25*). However, the amounts of secreted vacuolar proteases are significantly lower than those of the cell-associated ones, and the intracellular vacuolar proteases are mostly converted to their mature forms. In agreement with the report

of Yaver *et al.* (*25*), we also think that the maturation of the vacuolar proteases in the *vma* mutants occurs in the vacuoles because only the mature forms of the vacuolar proteases were found in the purified vacuoles. Since only the mature forms of the vacuolar proteases exhibit activity, we conclude that the protease activities in Tables II and III are due to the mature forms in the vacuoles. However, we still could not rule out the possibility that the high pH in the vacuoles deviates from pH optima of the vacuolar proteases for disintegration of ABs.

There are several possibilities why the loss of the H^+-ATPase function results in a defect in AB disintegration. (1) The acidic environment in the vacuoles may be essential for the activation or activities of components for AB disintegration; (2) the acidic pH in the vacuoles may act directly upon the membrane structures of ABs; and (3) the proper ionic environment in the vacuoles may be required for the disintegration of ABs.

An AB is enclosed by a single membrane, and thus disintegration of the membrane structure is required prior to the degradation of its contents by vacuolar hydrolases, otherwise, the hydrolases responsible for the degradation of the contents can not gain access to them. We previously showed that the disintegration of ABs takes place rapidly (*10*). This implies there should be certain mechanism(s) for rapid disintegration of the membrane structures, for example, unknown hydrolase activities, such as that of lipase, in the vacuoles. We also suggest the possibility that the acidic pH in the lumen of the vacuoles may influence the membrane structure of ABs, thereby allowing disintegration of ABs by proteinases. The other possibility is that alteration of the ionic concentrations and ionic strength in the vacuoles may result in the loss of ability to disintegrate ABs. The proton gradient generated by the vacuolar H^+-ATPase provides the primary driving force for the transport of most metabolites and ions across the vacuolar membranes. A number of solute transport systems in the vacuolar membrane regulate the membrane potential, and the concentrations of metabolites and ions (*4, 7, 35, 40, 41*). The loss of the vacuolar H^+-ATPase function certainly affects the regulation of these transport systems, thus leading to a failure to provide the co-factors for the disintegration of ABs.

We previously showed that autophagic degradation is essential for cells to survive starvation. We found that the *APG* genes are required for the process preceding the fusion of the autophagosomes with the vacuoles. Here, we showed that the disintegration of ABs requires acidification of the vacuoles, a final step of autophagic degradation. Further genetic and biochemical analyses of the *APG, VMA*, and *ABD* genes will be important for further understanding of autophagy at the molecular level.

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