Atg18 phosphoregulation controls organellar dynamics by modulating its phosphoinositide-binding activity

Naoki Tamura,1 Masahide Oku,1 Moemi Ito,2 Nobuo N. Noda,4 Fuyuhiko Inagaki,3 and Yasuyoshi Sakai1,2

1Division of Applied Life Sciences, Graduate School of Agriculture; and 2Research Unit for Physiological Chemistry, the Center for the Promotion of Interdisciplinary Education and Research, Kyoto University, Kyoto 606-8502, Japan
3Department of Structural Biology, Faculty of Advanced Life Science, Hokkaido University, Sapporo 060-0808, Japan
4Institute of Microbial Chemistry, Tokyo 141-0021, Japan

The PROPPIN family member Atg18 is a phosphoinositide-binding protein that is composed of a seven β-propeller motif and is part of the conserved autophagy machinery. Here, we report that the Atg18 phosphorylation in the loops in the propellar structure of blade 6 and blade 7 decreases its binding affinity to phosphatidylinositol 3,5-bisphosphate in the yeast *Pichia pastoris*. Dephosphorylation of Atg18 was necessary for its association with the vacuolar membrane and caused septation of the vacuole. Upon or after dissociation from the vacuolar membrane, Atg18 was rephosphorylated, and the vacuoles fused and formed a single rounded structure. Vacular dynamics were regulated according to osmotic changes, oxidative stresses, and nutrient conditions inducing micropexophagy via modulation of Atg18 phosphorylation. This study reveals how the phosphoinositide-binding activity of the PROPPIN family protein Atg18 is regulated at the membrane association domain and highlights the importance of such phosphoregulation in coordinated intracellular reorganization.
the conventional Atg proteins, Atg11 and Atg30 (Farré et al., 2008; Oku and Sakai, 2010). P. pastoris Atg18 (PpAtg18) is necessary for VSM formation (Guan et al., 2001); however, its involvement in MIPA formation has not yet been reported. In contrast, in the budding yeast, Saccharomyces cerevisiae, Atg18 is required for the regulation of vacuolar shape via its PI(3,5)P2-binding activity. Vacuolar fission is induced under certain environmental stresses, such as hyperosmotic pressure and high glucose concentrations. PI(3,5)P2, synthesized by the PI(3)P 5-kinase, Fab1, is required for this process (Dove et al., 1997, 2009). By regulating vacuolar shape, Atg18 is suggested to function as an effector of PI(3,5)P2 signaling (Dove et al., 2004).

Collectively, these studies suggested that Atg18 has dual physiological functions, i.e., autophagy regulation mediated by PI(3)P signaling, and control of vacuolar shape via PI(3,5)P2 signaling. However, the molecular mechanisms underlying Atg18’s role in these signaling pathways were not known. In this paper, two phosphorylation regions were found in the loops in the β-propeller structures. Based on the recently revealed molecular structure of the PROPPIN family member Hsv2, from the yeast Kluyveromyces lactis (KlHsv2), the two phosphorylation sites were thought to play critical roles in the protein’s affinity for phosphoinositides (Baskaran et al., 2012; Krick et al., 2012; Watanebe et al., 2012). However, phosphorylation of PpAtg18 was found to decrease the PI-binding activity of PpAtg18. Remarkably, the level of PpAtg18 phosphorylation in the cells correlated with the intracellular localization of PpAtg18. Further analyses revealed that the phosphorylation of PpAtg18 governed vacuolar shape during adaption to various environmental stresses, including situations that induced micropexophagy.

**Results**

PpAtg18 is phosphorylated in *P. pastoris*

We overproduced GST-tagged PpAtg18 under the control of the constitutive GAPDH promoter in the original host *P. pastoris*. The purified fraction from a Glutathione Sepharose 4B (GS 4B) column exhibited two distinct bands corresponding to the molecular size of the GST-PpAtg18 protein as determined by Coomassie brilliant blue staining of SDS-PAGE (Fig. 1 A). Because phosphatase treatment abolished the upper band, leaving a single band on the gel, we concluded that the purified GST-PpAtg18 protein contained both phosphorylated and nonphosphorylated forms (Fig. 1 A and B).

To exclude the possibility that phosphorylation of PpAtg18 was caused by overexpression of the protein, we expressed functional PpAtg18 tagged with five repeats of the Flag peptide (5xFlag) under the control of the original PpATG18 promoter. Again, we observed two bands via immunoblot analysis of cell-free extracts from *P. pastoris* (grown in glucose). Phosphatase treatment of the cell-free extract yielded a single band that was cross-reactive with the Flag tag (Fig. 1 C). These experiments indicated that PpAtg18 was present in both phosphorylated and nonphosphorylated forms in *P. pastoris* under physiological conditions.

Phosphorylation of PpAtg18 inhibits PI(3,5)P2-binding activity

Atg18 proteins were reported to bind to PI(3)P and PI(3,5)P2 (Dove et al., 2004). Purified GST-PpAtg18 protein containing both phosphorylated and nonphosphorylated isoforms was subjected to a variety of lipid binding tests, including the phosphatidylinositol phosphate (PIP) strip assay, the PIP array assay, a liposome pull-down assay, and surface-plasmon resonance analysis to determine the specificity of lipid binding. The PIP strip, PIP array, and liposome pull-down assays identified significant PI(3,5)P2-binding and weaker PI(3)P-binding activities (Fig. 1, D–F). However, we could not detect significant PI(3)P binding activity using Biacore surface-plasmon resonance analysis (Fig. S2 B). These data were in accord with previous results obtained using Atg18 from *S. cerevisiae* (Dove et al., 2004).

Next, we treated the purified PpAtg18 fraction (including both phosphorylated and nonphosphorylated forms) with phosphatase (Fig. 1 A, lane 2), and PI-binding activities were compared between phosphatase-treated and nontreated samples. As determined using the PIP strip assay, phosphatase treatment dramatically increased PpAtg18 binding activity toward PI(3,5)P2 as compared with PI(3)P (Fig. 1 D). This increase in affinity to PI(3,5)P2, after dephosphorylation was confirmed using the PIP array and the liposome-binding assay (Fig. 1, E and G). These experiments suggested that the affinity of PpAtg18 toward PI(3,5)P2 is reduced by phosphorylation. However, the affinity of PpAtg18 toward PI(3)P was too weak to draw comparisons between phosphatase-treated and nontreated samples.

PpAtg18 has two distinct phosphorylation regions in the loops of blades 6 and 7 that affect PI binding activity

MS analysis of purified PpAtg18 protein identified two phosphorylated peptides, one with two putative phosphorylation sites. Further MS/MS analyses identified these as 387-SSTTS-391 and 492-SSTS-495 (Fig. S1, A and B). Next, we compared the sequence around the phosphorylated sites with those of other PROPPIN family members by BLAST analysis. The crystal structure of KlHsv2 revealed consensus sequences for each phosphorylated region in blade 6 and 7 that affect PI binding activity.

To confirm the negative effect of phosphorylation in vivo, the putative phosphorylated residues of Ser and Thr were mutated to Ala and expressed in *P. pastoris* (Table 1). Cell-free extracts were subjected to normal and Phos-tag SDS-PAGE. Both PpAtg18 SA and AS mutations caused increased mobility of bands in both normal and Phos-tag SDS-PAGE; the PpAtg18 AA mutant produced the fastest migrating band among the tested
subjected to SDS-PAGE. The purified PpAtg18 DD protein migrated slower and the PpAtg18 AA protein faster, the former and the latter nearly corresponding to the mobility of the phosphorylated and dephosphorylated forms, respectively (Fig. S2 A). Next, these samples were subjected to lipid binding experiments (Fig. 1, D–G; and Fig. S2 B). Both the PIP array and the liposome pull-down assay revealed that the phosphorylation-defective mutant had a higher affinity to PI(3,5)P2, whereas the phosphorylation mimic PpAtg18 DD protein showed an affinity for PI(3)P, PI(3,5)P2, and PI(3,4,5)P3. Therefore, we concluded that both the Ser and Thr residues at positions 387–391 and 491–495 were phosphorylated in P. pastoris. Similarly, we also constructed PpAtg18 mutants that mimicked phosphorylation by replacing Ser or Thr residues within these regions with Asp (Table 1).

We purified the phosphorylation-defective PpAtg18 AA protein and the phosphorylation mimic PpAtg18 DD protein as GST fusion proteins, along with the wild-type PpAtg18 protein from P. pastoris cells. Molar equivalents of each sample were subjected to SDS-PAGE. The purified PpAtg18 DD protein migrated slower and the PpAtg18 AA protein faster, the former and the latter nearly corresponding to the mobility of the phosphorylated and dephosphorylated forms, respectively (Fig. S2 A). Next, these samples were subjected to lipid binding experiments (Fig. 1, D–G; and Fig. S2 B). Both the PIP array and the liposome pull-down assay revealed that the phosphorylation-defective mutant had a higher affinity to PI(3,5)P2, whereas the phosphorylation mimic PpAtg18 DD protein showed an affinity for PI(3)P, PI(3,5)P2, and PI(3,4,5)P3.
After SDS-PAGE with or without Phos-tag (Fig. S2 D). As a result, we identified four amino acid residues critical for Atg18 phosphorylation: two in the loop of blade 6, S388 and S391; and two in the loop of the C terminus, S492 and S495 (Fig. 2). Phosphorylation of PROPPIN, as observed for PpAtg18, may mediate the physiological signal specific to each PROPPIN by modulating its PI-binding activity.

Phosphorylation of PpAtg18 correlates with vacuolar shape under various environmental stresses
Atg18 was previously shown to regulate vacuolar shape during adaptation to various environmental stresses; e.g., nutrient and osmotic change. Furthermore, this regulation was mediated by the PI-binding activity of Atg18 (Dove et al., 2004; Krick et al., 2006; Efe et al., 2007; Obara et al., 2008). In S. cerevisiae, hypo-osmotic stress and carbon-source limitation lead to homotypic vacuolar fusion. In contrast, hyperosmotic stress and high glucose concentrations lead to vacuolar fission (Dove et al., 2009; Li and Kane, 2009).

The physiological significance of PpAtg18 phosphorylation was sought. Cells expressing PpAtg18-5×Flag were adapted to various environmental conditions, and the phosphorylation level of PpAtg18-5×Flag was assessed (Fig. 4 A).
Phosphoregulation of Atg18 modulates lipid binding • Tamura et al.

These results were consistent with a correlation between PpAtg18 phosphorylation levels and vacuolar shape in P. pastoris. Dephosphorylation of PpAtg18 regulates vacuolar fission by increasing PI(3,5)P2-binding activity. Fab1 synthesizes PI(3,5)P2 from PI(3)P. In Ppfab1Δ, PpAtg18-YFP, which is present on the vacuolar membrane in the wild-type strain when cells are cultured in glucose media or under hyperosmotic conditions, seemed to be released to the cytosol (Fig. 5 A). The finding that PpAtg18-YFP was recruited to the vacuolar membrane in a PI(3,5)P2-dependent manner led us to test whether PpAtg18 phosphorylation-defective mutants were localized differently as compared with wild-type protein. The phosphorylation-defective mutant PpAtg18AA-YFP showed stronger fluorescence at the vacuolar membrane in hypo-osmotic (SD + 0.9 M NaCl) or control (SD) conditions. In contrast, localization of the PpAtg18DD-YFP mutant, which retained PI(3,5)P2-binding activity, was similar to the wild-type PpAtg18 (Fig. 5 B). However, both PpAtg18 AA and DD mutant proteins were released into the cytosol under vacuolar fusion conditions (SD-glucose); the cells in these experiments displayed a single spherical vacuole. To investigate whether PpAtg18AA localized to the vacuolar membrane affected vacuolar shape, we next assessed the level of vacuolar fission as the number of vacuolar compartments per cell. As a result, PpAtg18AA displayed more vacuolar fission than the wild-type and PpAtg18DD when cultured both in SD media and under hyperosmotic conditions (Table 2). Together with the results from the in vitro PI(3,5)P2-binding experiment (Fig. 1 and Fig. 3), we concluded that dephosphorylation of PpAtg18 positively regulates vacuolar fission by increasing binding to PI(3,5)P2.

Next, we observed vacuolar morphology and the localization of PpAtg18 under these various environmental conditions. PpAtg18 tagged with the fluorescent protein YFP was expressed under its own promoter. Under vacuolar-fission conditions, the vacuole became fragmented, and PpAtg18-YFP was recruited to the vacuolar membrane, especially to the sites of vertex domains (Fig. 4 B). Alternatively, under vacuolar fusion conditions, PpAtg18-YFP was released from vacuolar membrane, and diffused throughout the cytosol. In the control experiment, PpAtg18 was weakly detected on the vacuolar membrane, and the level of vacuolar fusion (counted as the vacuolar number) seemed to be intermediate between that observed under hyperosmotic and hypo-osmotic stress (Fig. 4 B). We also tested other stress conditions that induce changes in vacuolar morphology (e.g., heat shock and ethanol stress), and observed that PpAtg18 phosphorylation levels increased under vacuolar fusion conditions and decreased under fission conditions.

Interestingly, PpAtg18 was highly phosphorylated when cells were exposed to oxidative stresses that induced vacuolar fusion: the oxidants, H2O2, tBOOH, and diamide (Fig. 4, D and E). We further tested other environmental stresses (reducing agents, exogenous calcium levels) that did not change either PpAtg18 phosphorylation levels or vacuolar shape. These results were consistent with a correlation between PpAtg18 phosphorylation levels and vacuolar shape in P. pastoris.

Figure 3. PpAtg18 is phosphorylated at two distinct sites in vivo. (A) The designated PpAtg18 phosphorylation mutants were grown in SD, collected at the exponential phase, and analyzed (5 µg) by immunoblotting. (B) Analysis of PpAtg18 variants using Phos-tag. The samples are the same as those used in Fig. 3 A. (C) PIP array analysis. Membranes were incubated with 1.0 µg/ml protein and detected simultaneously to ensure equal exposure times. (D) Liposome pull-down assay of PpAtg18 phosphorylation-defective mutants. The samples were subjected to immunoblotting and the bands were analyzed by densitometry. Error bars indicate mean ± SEM. ***, P < 0.1.
Figure 4. PpAtg18 phosphorylation correlates with the vacuolar dynamics observed under a variety of environmental conditions. (A) PpAtg18-5×Flag from cells grown under a variety of conditions was detected by immunoblotting with the anti-FLAG antibody. Cells were shifted from SD (0 min) to SD medium containing 0.9 M NaCl or 20% glucose, to water, to YNB medium without amino acids (~glucose), to methanol-containing medium, or (as a control) to SD medium for the indicated times (10, 20, 40, or 60 min). (B) Fluorescence microscopy analysis of PpAtg18 localization and vacuolar morphology. Growing conditions were the same as in Fig. 4 A. PpAtg18-tagged YFP was expressed under the original promoter and vacuoles were stained with FM 4-64. (C) Pulse-chase experiment. Cells were shifted from SD medium to either SD + 0.9 M NaCl or SD-glucose, and then shifted again into the opposite medium (see arrows). Analysis was performed by immunoblotting. (D) Oxidative stress induces PpAtg18 phosphorylation. t-BOOH, t-butylhydroperoxide. Analysis was performed by immunoblotting. (E) Morphological analysis and morphometric analysis of vacuoles in cells with or without oxidants. Cells...
Phosphoregulation of PpAtg18 modulates lipid binding

Increased its vacuolar association, this result may represent the fact that phosphorylated PpAtg18 is mainly localized to the vacuolar membrane.

Phosphoregulation of PpAtg18 during adaptation to methanol medium, and during micro- and macropexophagy

Regulation of vacuolar shape. Previously, we reported that vacuoles fused to form a single spherical vacuole in the wild-type strain. The level of PpAtg18 phosphorylation increased under vacuolar fusion conditions, and decreased under vacuolar fission conditions. Interestingly, the level of PpAtg18 phosphorylation was lower in the PpAtg18 FTTG mutant (Dove et al. 2004; Krick et al. 2006; Obara et al. 2008) as compared with the wild-type protein (Fig. 5 C). In these mutants, however, the level of dephosphorylation was comparable to that observed in wild type (Fig. 5 C). Because the PI(3,5)P2-binding activity of PpAtg18 increased its vacuolar association, this result may represent the fact that phosphorylated PpAtg18 is mainly localized to the vacuolar membrane.

In the wild-type strain, the level of PpAtg18 phosphorylation increased under vacuolar fusion conditions, and decreased under vacuolar fission conditions. Interestingly, the level of PpAtg18 phosphorylation was lower in the PpAtg18 FTTG strain and the PpAtg18 FTTG mutant (Dove et al. 2004; Krick et al. 2006; Obara et al. 2008) as compared with the wild-type protein (Fig. 5 C). In these mutants, however, the level of dephosphorylation was comparable to that observed in wild type (Fig. 5 C). Because the PI(3,5)P2-binding activity of PpAtg18 increased its vacuolar association, this result may represent the fact that phosphorylated PpAtg18 is mainly localized to the vacuolar membrane.

Phosphoregulation of PpAtg18 during adaptation to methanol medium, and during micro- and macropexophagy

Regulation of vacuolar shape. Previously, we reported that vacuoles fused to form a single spherical vacuole in a
Collectively, these data suggest that VSM formation during PI(3,5)P2-binding activity, showed a drastic decrease in VSM PpVac14 formation. In contrast, neither VSM nor MIPA were found in the PpPatg18 mutant (PpATG18AA) grown in nutrient starvation–induced autophagy (unpublished data). Vac14 and Fab1 are components of the PI(3)P 5-kinase complex. Both kinase component mutants, PpFab1Δ, and PpVac14Δ, along with the PpPatg18 mutant that was impaired in PI(3,5)P2-binding activity, showed a drastic decrease in VSM formation (unpublished data).

Table 2. **Septation of vacuoles during hyperosmotic adaptation**

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The difference in the average of total vacuoles per cell between PpAtg18Wt and PpATG18AA was marginally significant (P < 0.1). Wt, wild type.

*PpAtg18Wt, AA, or DD tagged 5×Flag were expressed under the PpATG18 promoter in PpAtg18Δ. Cells were grown in SD medium, and shifted to SD or SD + 0.9 M NaCl for 1 h.

†Vacular fission indicated as the population of the cells [%] having the indicated number of vacuoles in a cell.

PpAtg8-dependent manner during early periods of adaptation from glucose to methanol medium concomitant with peroxisome proliferation (Tamura et al., 2010). During subsequent adaptation to ethanol medium that induced macrophagocytosis, PpAtg18 was expected to be responsible for pexophagosome formation, but vacuolar shape did not change significantly. However, during adaptation to glucose medium that induced macrophagocytosis, PpAtg18 may have another function in addition to the formation of MIPA (Fig. 6 A). Specifically, because PpAtg18 is reported to be required for the early stage of macrophagocytosis, we speculate that PpAtg18 is involved in the formation of VSM concomitant with vacuolar fission (Guan et al., 2001). Thus, the two distinct processes, i.e., VSM formation and MIPA formation, should be coordinately regulated. Therefore, we wanted to understand the mechanisms underlying PpAtg18 regulation of vacuolar dynamics, including the formation of autophagic membranes during methanol adaptation and pexophagic conditions.

First, we examined MIPA and VSM formation during macrophagocytosis in the Ppatg18Δ strain (Fig. 6 B). In the wild-type strain, VSMs labeled with FM 4-64 budded from maternal vacuoles and YFP-PpAtg8 formed a cuplike structure representing MIPA formation. In contrast, neither PpAtg8 nor MIPA were observed in the Ppatg18Δ strain. In the Ppfab1Δ cells, MIPA-like structures were formed, but VSM formation was not observed. We performed morphometric analysis of VSM formation by counting the number of vacuoles during macrophagocytosis (unpublished data). Vac14 and Fab1 are components of the PI(3)P 5-kinase complex. Both kinase component mutants, PpFab1Δ and PpVac14Δ, along with the PpAtg18 mutant that was impaired in PI(3,5)P2-binding activity, showed a drastic decrease in VSM formation (unpublished data).

Next, we investigated the intracellular localization of PpAtg18-YFP. In cells grown in methanol medium (at both late log phase and early lag phase) or under macrophagocytic conditions, PpAtg18-YFP localized to the cytosol (Fig. 6 C). In contrast, during macrophagocytosis, PpAtg18-YFP was recruited to the vacuolar membrane. This vacuolar recruitment was lost in the PpFab1Δ strain (Fig. 6 C), which indicates that PpAtg18 was recruited to the vacuolar membrane in a Pp(3,5)P2-dependent manner during macrophagocytosis. Collectively, these data suggest that VSM formation during microphagocytosis required the PI(3,5)P2-dependent recruitment of PpAtg18 to the vacuolar membrane.

Then, we examined the phosphorylation levels of PpAtg18 under various conditions. At early phases of methanol adaptation (1–2 h), the phosphorylated form of PpAtg18 became dominant concomitant with vacuolar fusion, and PpAtg18-YFP diffused into the cytosol (Fig. 4, A and B). After 8–12 h of methanol adaptation, we could detect both phosphorylated and dephosphorylated forms of PpAtg18 and observed huge peroxisomes (Fig. 6 D). Therefore, although PpAtg18 was phosphorylated upon vacuolar fusion at early log phase, part of the phosphorylated PpAtg18 became dephosphorylated during cellular proliferation. This may reflect the fact that vacuolar inheritance from mother to daughter cells includes both vacuolar fusion and fission, which may be controlled by phosphoregulation of PpAtg18 (Weisman, 2006; Tamura et al., 2010).

Cells cultured in methanol at late-log phase were induced to undergo micro- and macrophagocytosis by transfer to glucose and ethanol medium, respectively. Then, PpAtg18 phosphorylation and localization (using PpAtg18-YFP) were observed. When cells were shifted to ethanol medium to induce macrophagocytosis, the phosphorylated form became dominant and the dephosphorylated form decreased significantly, suggesting that the phosphorylated form of PpAtg18 functioned in pexophagosome formation (Fig. 6 D). In contrast, both the phosphorylated and dephosphorylated forms of PpAtg18 were observed during macrophagocytosis, which included both MIPA formation and vacuolar fission for VSM formation (Fig. 6 D).

Regulation of autophagy. As shown in Fig. 6 B, PpAtg18 was necessary for MIPA formation in macrophagocytosis. In nutrient starvation–induced autophagy, PpAtg18 was reported to be recruited to the preautophagosomal structure (PAS), represented by PpAtg8 dots, and this localization required Atg2 and Pp(3)P (Suzuki et al., 2007). To visualize such colocalization in P. pastoris, we expressed CFP-tagged PpAtg18 under the PpACT1 promoter, and coexpressed YFP-PpAtg8 under its original promoter. Fluorescence microscopy revealed that CFP-PpAtg18 was colocalized with the PAS (Fig. S3 A) during macrophagocytosis. However, we also observed that PpAtg26, which is necessary for pexophagy, was dispensable for the recruitment of PpAtg18 to the PAS.
Figure 6. Phosphorylation of PpAtg18 regulates VSM formation in micropexophagy. (A) Intracellular structures observed during micropexophagy and macropexophagy. MIPA, micropexophagy-specific membrane apparatus; VSM, vacuolar sequestering membrane. (B) Fluorescence microscopy analysis during micropexophagy. Cells were shifted from methanol medium to glucose medium for 30–60 min to induce micropexophagy. Vacuoles were stained with FM 4-64, and MIPA was visualized by YFP-tagged PpAtg8 expressed under the PpATG8 promoter. (C) Intracellular localization of PpAtg18. YFP C-terminally tagged with PpAtg18 was expressed in Ppatg18Δ under the PpATG18 promoter. Cells were shifted from methanol medium to ethanol or glucose medium for 30–60 min to induce macropexophagy or micropexophagy, respectively. (D) Immunoblot detection of PpAtg18-5×Flag. PpAtg18-5×Flag was expressed in Ppatg18Δ under the PpATG18 promoter. Cells were shifted from methanol medium to ethanol or glucose medium to induce macropexophagy or micropexophagy, respectively. This blot was incubated with the anti-FLAG antibody. (E) Intracellular localization of PpAtg18 AA and DD during micropexophagy. YFP-tagged PpAtg18 mutants were expressed under the PpATG18 promoter. Cells were shifted from methanol medium to glucose medium for 60 min to induce micropexophagy. Bars, 2 µm. (F) PpPex12 degradation assay to assess micropexophagy and macropexophagy activity in PpAtg18 mutants. Strains were grown in methanol medium and then shifted to glucose or ethanol medium to induce micropexophagy or macropexophagy, respectively. Cell-free extracts were prepared and analyzed by immunoblotting with anti-PpPex12 or anti-Pgk1 antibodies. Optical density measurements showed no significant difference in growth between these mutants.
In contrast to the VSM formation during micropexophagy and in the phosphorylation of PpAtg18. Although the phosphorylation levels of PpAtg18 (Fig. S3, A and C) and the vacuolar shape did not change significantly. We suggested that mutations in the phosphorylated regions did not influence nitrogen starvation–induced autophagy using the YFP-Atg18 promoter in Ppa18B. Micropexophagy was induced by adapting cells from methanol to glucose medium for 30 min

Deletion of PpAtg2 had no effect on VSM formation (Fig. S3 A) or on Atg18 phosphorylation (Fig. S3 D). Also, the association of PpAtg18 to the vacular membrane was normal. Because both PpAtg18 AA and DD mutants exhibited autophagic activities in starvation-induced autophagy and pexophagy, phosphoregulation of PpAtg18 is unlikely to be involved in the formation of the Atg18–Atg2 complex.

Among various autophagic processes, PpAtg18 and its phosphoregulation were found to play a critical role during micropexophagy, i.e., the autophagic process concomitant with dynamic changes in vacuolar shape and MIPA formation. The dephosphorylated PpAtg18 with higher affinity for PI(3,5)P2 was necessary for association of PpAtg18 with the vacular membrane and VSM formation that mediates incorporation of peroxisomes to the vacule. Conversely, cytosolic phosphorylated PpAtg18 with decreased affinity for PI(3,5)P2 was suggested to be responsible for MIPA formation after complexing with PpAtg2 at the PAS.

**Discussion**

In this study, we observed phosphorylation of PpAtg18 in the yeast P. pastoris, and biochemically characterized the molecular function of these phosphate groups with respect to PI binding and the intracellular localization of PpAtg18. We further showed the physiological roles of this phosphoregulation of PpAtg18 by using various mutant strains that mimicked PpAtg18-phosphorylated or -dephosphorylated forms and were grown under various environmental stresses and conditions.

**Phosphorylation of PpAtg18 interferes with the interactions of PpAtg18 and PI(3,5)P2 in vitro**

One of the important findings in this study is that the dephosphorylation of PpAtg18 increases its affinity for PI. Atg18 is a member of the PROPPIN family of proteins that bind to PI(3)P and PI(3,5)P2 via a seven β-propeller motif (Dove et al., 2004). Recently, the crystal structure of KHVsv2 was solved, representing the first PROPPIN family member to be molecularly characterized at that level. Hsv2 binds PI2 specifically at two distinct sites: site 1 on blade 5 and site 2 on blade 6 (Baskaran et al., 2012; Krick et al., 2012; Watanabe et al., 2012). Specificity to PI was mediated by ionic interactions between the negative charge of the PI head group and the basic amino acid residues.

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<td>Wt</td>
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<td>DD</td>
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The difference in the average of VSMs per cell between PpAtg18Wt and PpATG18AA was significant (P < 0.05). Wt, wild type.

VSM formation is indicated as the population of the cells (%) having the indicated number of vacuoles in a cell.

**Table 3. VSM formation during micropexophagy in PpAtg18 AA and DD mutant strains**

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of sites 1 or 2. Furthermore, the hydrophobic loop of blade 6, conserved in all PROPPINs, was able to embed in the membrane, thus facilitating the interactions between PROPPIN and the membrane (Baskaran et al., 2012).

Our results indicated that dephosphorylation of either region, in blade 6 or blade 7, was sufficient to increase the PI-binding activity (Fig. S2 C). The higher affinity of PpAtg18 AA protein toward PI(3,5)P2 compared with PpAtg18 DD protein (Fig. 3) suggested that the charged moieties of the phosphorylated regions inhibited the lipid-binding activity. One plausible explanation is that the negative charge of the phosphorylated residues simply impeded the hydrophobic interactions between the hydrophobic loop of blade 6 and the membrane (Fig. 7). Although the lipid-binding PH domain of protein kinase B was known to be phosphoregulated (Powell et al., 2003), to our knowledge, this is the first study that addresses, at molecular and biochemical levels, how phosphorylation of a protein modulates its binding to PIs.

Figure 7
Phosphoregulation of PpAtg18 for maintenance of vacuolar shape.

Role of PpAtg18 phosphoregulation in the maintenance of vacuolar shape
When the dephosphorylated form of PpAtg18 increased and PpAtg18 was recruited to the vacuolar membrane by its PI(3,5)P2-binding activity, vacuolar fission occurred. Phosphorylation-defective PpAtg18 AA mutants exhibited more separted vacuoles than did the cells expressing wild-type PpAtg18. In contrast, when the phosphorylated form of PpAtg18 increased and PpAtg18 dissociated from the vacuolar membrane, the vacuoles fused and formed a single rounded compartment. From these results, we conclude that the phosphorylation of PpAtg18 is correlated to its intracellular localization and vacuolar shape.

Atg18 is thought to be a negative regulator of PI(3,5)P2 synthesis, and the localization cycle of Atg18 between the vacuolar membrane and the cytosol regulates PI(3,5)P2 levels at the vacuolar membrane (Dove et al., 2004, 2009; Efe et al., 2007). Under hyperosmotic conditions, the cytosolic phosphorylated form of PpAtg18 was dephosphorylated, and recruited to the vacuolar membrane by binding vacular PI(3,5)P2. Once PpAtg18 is recruited to the vacuolar membrane, the PI(3,5)P2 levels on the vacuolar membrane may be decreased. When the levels of PI(3,5)P2 on the vacuolar membrane are decreased and/or PpAtg18 is phosphorylated, PpAtg18 will be released from the vacuolar membrane. We think that dephosphorylation of PpAtg18 mediates a signal inducing vacuolar fission, as PpAtg18 AA mutation was sufficient to induce and enhance the vacuolar fission. This result also indicates that the vacuolar PI(3,5)P2 level is sufficient to recruit PpAtg18 (Dove et al., 2004; Krick et al., 2006; Efe et al., 2007). However, for dissociation of PpAtg18 from the vacuolar membrane to occur, their interactions should weaken. It was difficult to determine whether the phosphorylation of PpAtg18 or the decrease in vacuolar PI(3,5)P2 was the key event allowing the dissociation of PpAtg18 and the signaling of vacuolar fusion, because low vacuolar PI(3,5)P2 levels were difficult to follow experimentally, and the phosphorylation mimic PpAtg18 DD mutant behaved like the wild type, and did not induce vacuolar fusion. Although the detailed mechanism for vacuolar fission itself should be investigated further, this study first clarified that vacuolar fission is regulated by phosphorylation of PpAtg18 through its association to the vacuolar membrane.

We tried to identify the upstream kinases that were responsible for PpAtg18 phosphorylation. Our results suggested that phosphorylation occurred at the vacuolar membrane. Specifically, both the Ppfα14 mutant and the PpAtg18 FTTG mutant, in which PpAtg18 was not recruited to the vacuolar membrane, had dephosphorylated PpAtg18 under low glucose conditions (Fig. 5 C), whereas wild-type cells had only phosphorylated PpAtg18 under the same conditions (Fig. 4 A). P. pastoris mutants with a single disruption of the kinases PpAtg1, PpTor1, PpHog1, or PpVps15 retained phosphorylation of PpAtg18 (Fig. S3 E). Depletion of yeast casein kinase PpYck3 caused a partial defect in PpAtg18 phosphorylation. It is noteworthy that, in contrast to PpAtg18, in S. cerevisiae, Vps41, a component of a tethering complex for vacuolar homotypic fusion (HOPS family), is phosphorylated by Yck3 under vacuolar fission conditions and released into the cytosol (LaGrassa and Ungermann, 2005; Cabrera et al., 2009). Vps41 contains an amphipathic lipid-packing sensor (ALPS) motif, which interacts with highly curved membranes, and the phosphorylation of this motif prevents membrane recruitment of Vps41 (Cabrera et al., 2010). Vps41 seems to serve as a counterpart to Atg18 in regulating vacuolar shape.
membrane, does not seem to directly affect the PI(3)P-binding activity of PpAtg18 necessary for autophagy. It may be noteworthy to point out that the phosphorylated form PpAtg18 still binds to PI3, which may be sufficient to execute macroautophagy.

Phosphoregulation of PpAtg18 was necessary for optimal execution of micropexophagy, which involved formation of both the VSM and MIPA (Fig. 6A), but was not necessary for other autophagic processes. During micropexophagy, although VSM formation was enhanced in the phosphorylation-defective PpAtg18 AA mutant, PpPex12 degradation was retarded. This result likely reflected the fact that efficient recruitment of the PpAtg18 AA mutant to the vacuolar membrane reduced the amount of PpAtg18 in the PAS, which is necessary for MIPA formation. The efficiency of MIPA formation could not be assessed using the PpAtg18 AA mutant, as micropexophagy was induced after glucose adaptation and nascent pexophagosomes could not be distinguished from MIPA. We suggest that, during micropexophagy, dephosphorylated and phosphorylated forms of PpAtg18 function in VSM and MIPA formation, respectively, and that PpAtg18 phosphorylation is regulated accordingly. Although a defect caused by impaired phosphoregulation was not observed in macroautophagy, PpAtg18 was observed to be mainly in a phosphorylated form, which suggests that the dissociation of PpAtg18 from the vacuolar membrane enhances the recruitment of PpAtg18 to PAS for autophagosome formation.

Phosphoregulation of PpAtg18 governs the intracellular re-arrangement and coordinated dynamics of multiple organelles, i.e., vacuoles, autophagosomes, MIPA, and peroxisomes, in response to various environmental conditions and stresses. An example of such a highly coordinated event may be seen in fungi living in nature. Previously, we showed that the proper execution of pexophagy was necessary for fungal cells to proliferate on the leaves of living plants by showing that the pexophagy-defective PpAtg26 and PpAtg30 mutants lose normal growth and differentiation properties (Asakura et al., 2009; Kagawuchi et al., 2011). In nature, cells have to adapt not only to nutrient stresses leading to autophagy, but also to oxidative and osmotic stresses leading to changes in vacuolar shape. It is likely that, in P. pastoris, micropexophagy is the biological process through which cells adapt simultaneously to both nutritional and environmental stresses, whereby phosphoregulation of Atg18 plays a critical role under natural conditions.

Materials and methods

Strains, media, and antibodies

The strains used in this study are listed in Table S1. Yeast extract/peptone/dextrose (YPD) medium consisted of 1% yeast extract, 2% Bacto Peptone, and 2% glucose. SD medium contained 6.7% yeast nitrogen base without amino acids and 2% glucose. Synthetic minimal (SM) medium contained 6.7% yeast nitrogen base without amino acids and 0.8% methanol. These synthetic media were supplemented with the appropriate amino acids (100 μg/ml arginine and 100 μg/ml histidine) for auxotrophic strains. Anti-FLAG (Wako), anti-GFP (Molecular Probes), anti-GST (GE Healthcare), anti-Pgk1 (Molecular Probes), anti–mouse-HRP (EMD Millipore), anti–goat-HRP (Abcam), and anti–rabbit-HRP antibodies (Rockland Immunochemicals, Inc.) were used in this study. The anti-PpPex12 antibody was a generous gift from S. Subramani.

Protein purification from P. pastoris

For purification of PpAtg18 variants, we used P. pastoris cells expressing GST-tagged proteins. Cells were cultivated in YPD media up to stationary growth and harvested by centrifugation at 2,000 g for 5 min. Cells were washed with 1x PBS buffer once, and then suspended in lysis buffer (PBS, 1 mM MOPS, 10 mM MgCl2, 2 μg/ml Pepstatin A, 10 μg/ml Leupeptin, 0.05% [w/v] Tween 20, and phosphatase inhibitor PhosSTOP). Next, cells were lysed by sonication and centrifuged at 6,000 g for 15 min to yield cell lysates. Cell lysates were incubated with GS 4B (GE Healthcare) at RT for 30 min. The column was washed with 2x PBS multiple times and the captured proteins were eluted with reduced glutathione (for PIP strips, PIP arrays, and liposome pull-downs), or cleaved with PreScission Protease (for surface plasmogen resonance; GE Healthcare). In the phosphatase treatment experiment, we incubated the captured GST-PpAtg18 with λ-phosphatase for 2 h on the column. Then, to remove λ-phosphatase from the sample, the column was washed with 1x PBS buffer multiple times before exchanging the buffer with liposome binding assay buffer: LB buffer, 10 mM Hesper- KOH, pH 8.0, and 50 mM NaCl.
Lipid dot blot analysis
PIP strip and PIP array assays were performed according to the protocols of Echelon Bioscience, Inc. For the PIP strip, membranes were incubated with 0.5 µg/ml purified proteins for 1 h. For the PIP array, membranes were incubated with 1.0 µg/ml protein for 3 h. Anti-GST antibody diluted 1:5,000 was incubated for 1 h and anti-goat-HRP diluted 1:10,000 was incubated for 1 h. For both PIP strip and PIP array experiments, the signals were acquired using the same exposure time.

Liposome preparation
Lipids used in this study were purchased from Sigma-Aldrich: DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; DSPE, 1,2-Distearyloyl-sn-glycero-3-phosphoethanolamine; DPPS, 1,2-Dipalmityloyl-sn-glycero-3-phospho-L-serine; PI(3)P, 1-O-phosphatidylinositol 3-monophosphate diyl; PI(4)P, 1-O-phosphatidylinositol 4-monophosphate diyl; PI(3,5)P2, 1-O-phosphatidylinositol 3,5-bisphosphate diyl; and PI(4,5)P2, 1-O-phosphatidylinositol 4,5-bisphosphate diyl. For the liposome preparation, 200 µl of LB buffer containing 2.0 × 10−3 M NaCl, 1 µg/ml of the purified GST-PpAtg18 variants were incubated with 50 µl of liposomes (Avanti Polar Lipids, Inc.) with a 100-nm pore size filter to make liposomes.

For PIP strip and PIP array experiments, the lipid-containing mixture was passed through a Mini-Extruder set (Avanti Polar Lipids, Inc.) and the liposomes were acquired using the same exposure time.

LC-MS/MS
LC-MS/MS analysis of the digested samples was performed with an RP-LC system (Paradigm MS4B; Michrom BioResources, Inc.) interfaced with a hybrid mass spectrometer (LTQ-Orbitrap; Thermo Fisher Scientific) using a nanoelectrospray ionization device (AMR). Samples were loaded onto the trap cartridge and washed with mobile phase A (98% H2O, 2% acetonitrile, and 0.1% formic acid) for concentration and desalting. Subsequently, peptides were eluted over 20 min from the analytical column via the trap cartridge using a linear gradient of 5–45% mobile phase B (10% H2O, 90% acetonitrile, and 0.1% formic acid) at a flow rate of 1 µl/min. To survey the phosphorylated peptides in each sample, the mass spectrometer was operated in the data-dependent mode to automatically switch between high-resolution MS scan (resolution, 60,000; scan range, m/z 400–1,600) by the Orbitrap hybrid mass spectrometer and up to three concurrent MS/MS scans in the LTQ for the three most intense peaks selected from each MS scan. For determination of the phosphorylation sites on the phosphorylated peptides, the mass spectrometer was operated in the targeted MS/MS scan mode to switch between high-resolution MS and high-resolution MS/MS scans (resolution, 60,000); both were acquired using the Orbitrap in profile mode. Mascot software (version 2.1.1, Matrix Science) was used for matching searches against the PpAtg18 sequence without an initial Met and with an artificial Gly-Pro-Leu-Gly-Ser sequence at the N terminus. Peptide mass tolerance was 20 ppm, fragment mass tolerance was 0.8 D, and trypsin specificity was applied with a maximum of two missed cleavages. Carbamidomethylation of Cys was allowed as a fixed modification, whereas oxidation of Met and phosphorylation of Ser and Thr were allowed as variable modifications. Phosphorylation sites were determined by manually comparing datasets of product ions acquired from Orbitrap MS/MS spectra, and theoretical fragment ions were estimated using the MSProduct tool on the ProteinProspector web site (http://prospector.ucsf.edu/prospector/mshome.htm).

Online supplemental material
Fig. S1 shows identification of phosphorylated regions by LC-MS/MS, Fig. S2 shows purification of PpAtg18 proteins from P. pastoris, and Fig. S3 shows the role of PpAtg18 in autophagic pathways. Table S1 lists yeast strains used in this study. Table S2 lists oligonucleotides used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201302067/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201302067.dv.

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