

# Hog1 Mitogen-Activated Protein Kinase Phosphorylation Targets the Yeast Fps1 Aquaglyceroporin for Endocytosis, Thereby Rendering Cells Resistant to Acetic Acid<sup>∇</sup>

Mehdi Mollapour and Peter W. Piper\*

Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court,  
Western Bank, Sheffield S10 2TN, England

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**Aquaporins and aquaglyceroporins form the membrane channels that mediate fluxes of water and small solute molecules into and out of cells. Eukaryotes often use mitogen-activated protein kinase (MAPK) cascades for the intracellular signaling of stress. This study reveals an aquaglyceroporin being destabilized by direct MAPK phosphorylation and also a stress resistance being acquired through this channel loss. Hog1 MAPK is transiently activated in yeast exposed to high, toxic levels of acetic acid. This Hog1 then phosphorylates the plasma membrane aquaglyceroporin, Fps1, a phosphorylation that results in Fps1 becoming ubiquitinated and endocytosed and then degraded in the vacuole. As Fps1 is the membrane channel that facilitates passive diffusional flux of undissociated acetic acid into the cell, this loss downregulates such influx in low-pH cultures, where acetic acid (pK<sub>a</sub>, 4.75) is substantially undissociated. Consistent with this downregulation of the acid entry generating resistance, sensitivity to acetic acid is seen with diverse mutational defects that abolish endocytic removal of Fps1 from the plasma membrane (loss of Hog1, loss of the soluble domains of Fps1, a T231A S537A double mutation of Fps1 that prevents its *in vivo* phosphorylation, or mutations generating a general loss of endocytosis of cell surface proteins [*doa4Δ* and *end3Δ*]). Remarkably, targeting of Fps1 for degradation may be the major requirement for an active Hog1 in acetic acid resistance, since Hog1 is largely dispensable for such resistance when the cells lack Fps1. Evidence is presented that in unstressed cells, Hog1 exists in physical association with the N-terminal cytosolic domain of Fps1.**

Baker's yeast (*Saccharomyces cerevisiae*) is extensively used as a model for studying how cells adapt to and survive different forms of stress. Its responses to hyperosmotic stress have been the subject of extensive investigations (11, 12, 21, 26). Important for an adaptation to hyperosmotic conditions is counteracting the water loss from the cell, which is achieved in yeast by accumulating a high intracellular pool of glycerol. This glycerol acts as a compatible solute, ensuring that the proteins in the intracellular environment remain hydrated and protected. Osmostress adaptation also involves the activation of the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) signaling cascades, which generate an activation of a multifunctional Hog1 MAPK. This activated Hog1 then translocates to the nucleus, where, by the phosphorylation of at least three separate transcription factors (Sko1, Hot1, and Smp1), it can generate an altered regulation of >10% of the total yeast genome (21). Active Hog1 has recently been found to exert important actions, much more instant than its effects on transcription, at the plasma membrane, where it directly phosphorylates certain of the membrane ion transporters in osmotressed cells in order to rapidly readjust the transmembrane fluxes of Na<sup>+</sup> and K<sup>+</sup> (26). In this work, we show that activated Hog1 can also phosphorylate a plasma membrane aquaglyceroporin, in order to trigger the endocytosis and degradation

of this Fps1 channel. This Fps1 destabilization is seen, though, in response to a different condition of Hog1-activating stress: a high acetic acid level, not hyperosmotic stress. In addition, we describe how this targeting of Fps1 for degradation is important for the acquisition of acetic acid resistance.

Our attention was initially drawn to the *S. cerevisiae* acetic acid response by a discovery that in cultures growing at slightly acid pH (pH 4.5), this stress response involves the activation of HOG pathway signaling, the same pathway that is activated by osmotic stress, but without the strong *GPD1* gene or intracellular glycerol inductions that are hallmarks of Hog1 becoming activated by a hyperosmotic stress (19). It appeared, therefore, that the Hog1 MAPK activated by acetic acid stress might be initiating a response rather different from the Hog1 activated by hyperosmotic stress. We show here that the Hog1 activated by acetic acid stress generates endocytosis and degradation of the Fps1 aquaglyceroporin. Such Fps1 destabilization does not occur when Hog1 is activated by hyperosmotic stress. In low-pH yeast cultures this loss of Fps1 is important for the acquisition of resistance to acetic acid, as it eliminates the channel for the passive diffusional entry of this acid into cells. In nature this response may help yeast survive in environments where competitor organisms (e.g., *Acetobacter* spp.) are excreting large amounts of acetic acid.

Aquaporins and aquaglyceroporins (also called the major intrinsic proteins) are integral membrane channels that facilitate an energy-independent transmembrane transport of small molecules such as water, glycerol, glyceraldehyde, glycine, and urea (2, 11, 14). As such, they are important mediators of the water and solute fluxes in both prokaryotes and eukaryotes.

\* Corresponding author. Mailing address: Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, United Kingdom. Phone: 44-114-222-2851. Fax: 44-114-222-2800. E-mail: peter.piper@sheffield.ac.uk.

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Their proper functioning and regulation are vital for several aspects of cellular physiology, with an altered functioning of these channels now being implicated in a number of diverse disease disorders such as congestive heart failure, glaucoma, and brain edema (2, 14). These channels are also important in toxicology, as they often facilitate the entry/exit of small toxic compounds to/from the cell. Though we focus in this study on the importance of Fps1 for acetic acid resistance, as the channel that facilitates the entry of this acid into cells, the same aquaglyceroporin has also been studied from the standpoint of its capacity to facilitate the exit of toxic methylamine from (37) or the entry of toxic metalloids to (33, 36) yeast.

## MATERIALS AND METHODS

**Strains and plasmids.** The yeast strains used in this study (BY4741, BY4741 *fps1Δ kanMX4*, and BY4743) were from Euroscarf (www.uni-frankfurt.de/fb15/mikro/euroscarf/), except for the *hog1Δ fps1Δ* strain (generated by *hphMX4* cassette [8] deletion of the *HOG1* gene in BY4741 *fps1Δ*). YEpFPS1, YEp*fps1-ΔI*, and YEpFPS1-*cmyc* (16, 28, 30) were generously provided by S. Hohmann. YEpFPS1-*AN-cmyc* (deletion of amino acids 13 to 230) was made by replacing the Sall-PstI fragment from YEpFPS1-*cmyc* with the Sall-PstI-truncated fragment from YEp*fps1-ΔI*. YEpFPS1-*ΔC-cmyc* (Fps1 lacking amino acids 534 to 650) was generated by removing the KpnI-XbaI fragment from the YEpFPS1-*cmyc* plasmid and replacing it with the PCR-amplified truncated Fps1 lacking amino acids 534 to 650 amino acid fragment, the latter digested with KpnI-XbaI.

Fps1 was C-terminally green fluorescent protein (GFP) tagged using pUG23 (20), with Fps1 without the stop codon being ligated to the SpeI-Sall-cut vector to generate pUG23FPS1-C-GFP. Mutant forms of YEpFPS1-*cmyc* and pUG23FPS1-C-GFP were derived by site-directed mutagenesis of these vectors and checked by DNA sequencing. N-Fps1-His<sub>6</sub> and C-Fps1-His<sub>6</sub>, C-terminally hexahistidine (His<sub>6</sub>)-tagged forms of Fps1(1–255) and Fps1(531–669), respectively, were generated by PCR. N-Fps1-His<sub>6</sub> and C-Fps1-His<sub>6</sub>, as well as their mutant derivatives N-Fps1<sup>T231A</sup>-His<sub>6</sub> and C-Fps1<sup>S537A</sup>-His<sub>6</sub>, were then ligated to PstI-XhoI-cut YEp81Met (this plasmid, a gift of Frank Cooke, is YEplac181 [7] with an insert containing the *MET25*-inducible promoter and the transcription termination site from *PGKI*, separated by a multiple cloning site).

pES86-HA-HOG1 (a hemagglutinin [HA]-tagged *HOG1* coding sequence under *ADHI* promoter control) and various mutant derivatives of this plasmid were gifts of David Engelberg. P<sub>GAL1</sub>-PBS2DD in pYES2 was from Francesc Posas.

**Growth conditions.** Yeast was grown on YPD (2% [wt/vol] Bacto peptone, 1% yeast extract, 2% glucose, 20 mg/liter adenine). Selective growth was on dropout 2% glucose (DO) medium (1). The medium pH was adjusted to 4.5 or 6.8 with either HCl or NaOH before autoclaving. Acetic acid was added from an 8.7 M stock acetic acid solution titrated to pH 4.5 with NaOH. For agar growth acetic acid sensitivity assays, overnight pH 4.5 YPD cultures were diluted to an optical density at 600 nm of 0.5, and ~5-μl aliquots of a 10-fold dilution series were spotted onto YPD (pH 4.5)–1.5% agar plates supplemented with the indicated level of acetic acid. Growth was monitored over 3 to 5 days at 30°C.

**Acetic acid uptake measurements.** Cultures in exponential growth at 30°C (5 × 10<sup>7</sup> cells ml<sup>-1</sup>) on pH 4.5 DO or YPD medium were transferred to medium of the same pH plus 100 mM acetic acid. Accumulation of radiolabeled acetic acid was determined essentially as described previously for measurements of the uptake of radiolabeled benzoic acid (10, 23). Fifty-milliliter mid-exponential-phase cultures, grown at 30°C on pH 4.5 DO or YPD medium, were harvested and resuspended (5 × 10<sup>7</sup> cells ml<sup>-1</sup>) in 6 ml of medium of the same pH containing 100 mM acetic acid, which was labeled with 25 μCi (15) (Amersham, United Kingdom). Intracellular versus extracellular radiolabeled acetic acid was then measured at different times during subsequent 30°C maintenance by rapidly filtering 0.5 ml of culture and then briefly washing the filters with ice-cold water. Filters were air dried and weighed, and their radioactivity was determined by liquid scintillation counting. Each data point is the mean of three separate determinations at each time point using the same culture.

**Protein analysis and immunoblots.** Total protein extracts were prepared and analyzed by Western blotting, as described previously (18). Western blot analysis of total Hog1 used polyclonal anti-Hog1 (Y-215) antibody (Santa Cruz Biotechnology). Analysis of the active form of Hog1 used anti-dually phosphorylated (Thr<sup>180</sup>/Tyr<sup>182</sup>) p38 MAPK antibody (New England Biolabs). As a loading control, Sba1 levels were measured (17). His<sub>6</sub>-tagged full-length Fps1 or the His<sub>6</sub>-tagged N- and C-terminal fragments of Fps1 were detected with monoclonal

anti-tetra-His antibody (QIAGEN), and GFP-tagged Fps1 was detected with monoclonal anti-GFP antibody (Roche). *cmyc*-tagged Fps1 detection used a monoclonal anti-*c-myc* (9B11) antibody (New England Biolabs), and HA-tagged Hog1 detection used a monoclonal anti-HA (HA.11) antibody (Convance). Fps1 ubiquitination was detected with monoclonal antiubiquitin (P4D1) antibody (Santa Cruz Biotechnology). Detection of Fps1 phosphorylation was with anti-phosphoserine or antiphosphothreonine monoclonal antibodies (QIAGEN). Secondary antisera were horseradish peroxidase–anti-rabbit, –anti-goat or –anti-mouse immunoglobulin G (Amersham) diluted 2,000-fold. Enhanced chemiluminescence reagents (Amersham) were used for detection.

**Binding assays.** Interaction between Fps1 and Hog1 was analyzed by in vivo coimmunoprecipitation. Fps1-*cmyc* was purified using protein A/G-agarose plus (Santa Cruz Biotechnology). Interactions of His<sub>6</sub>-tagged N- or C-Fps1 fragments with Hog1 were analyzed in vitro by incubating 50 μg of the total protein lysate from wild-type and *hog1Δ* cells with 50 μl of Talon beads (Clontech) with the N-Fps1-His<sub>6</sub> or C-Fps1-His<sub>6</sub> bound. Incubation was at 4°C for 30 min in a buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and protease inhibitor cocktail (Roche). The beads were washed three times with the same buffer on Coster spin filters. Twenty microliters of sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer was added to the beads, and 15 μl was loaded on sodium dodecyl sulfate-polyacrylamide gels for blotting onto nitrocellulose membranes.

**In vitro kinase assay.** The N-Fps1-His<sub>6</sub> and the mutant N-Fps1<sup>T231A</sup>-His<sub>6</sub> were expressed in *hog1Δ* cells and purified using Talon beads (Clontech). Hog1-HA was expressed and purified from exponentially grown cells subjected to brief acetic acid stress (100 mM, 10 min). Hog1-HA was immunoprecipitated from 2 mg yeast protein extract using monoclonal anti-HA-agarose conjugate clone HA-7 (Sigma). Hog1-HA kinase reactions were essentially as previously described (25).

**Fluorescence microscopy.** Fluorescent and Nomarski images were acquired using a Leica DMLB microscope equipped with a GFP and red filter set and Nomarski objectives, and images were captured using OpenLab imaging software (Improvision Ltd.).

**Two-hybrid analysis.** The two-hybrid analysis was essentially as described previously (17, 18). Genes for amino acids 1 to 255 and 531 to 669 of Fps1 fused at their C terminus to the Gal4 binding domain (BD) were generated by gap repair using vector pBDC (18) and strain PJ694α (34). Wild-type, nonphosphorylatable (NP), and kinase-inactive mutant (K52R) activator domain (AD)-Hog1 fusions were constructed by gap repair using vector pADC and strain PJ694α (34). PJ694α and PJ694a strains were then mated, with the diploids being selected on DO lacking tryptophan and leucine. Protein-protein interactions were checked by spotting these diploids onto DO lacking leucine, tryptophan, and histidine and supplemented with increasing concentrations (0 to 6 mM) of 3-aminotriazole. Growth on these selective plates was scored after 4 days at 30°C.

## RESULTS

**Loss of Fps1 influences acetic acid uptake and resistance in yeast.** Accumulation of a high intracellular glycerol pool by osmostressed yeast cells reflects both increased glycerol synthesis and an increased capacity of the cell to retain this glycerol, rather than lose it to the culture medium. The increased glycerol retention is achieved by turgor-mediated closure of the plasma membrane aquaglyceroporin Fps1, a closure that prevents glycerol diffusion through this channel and therefore the glycerol loss from the cell (9, 11, 12, 22). Though the acetic acid response of yeast grown at slightly acid pH (pH 4.5) does not involve increases in intracellular glycerol (19), we found that Fps1 was still a major factor in acetic acid resistance. With Fps1 loss, the cells were even more resistant to acetic acid than normal (compare wild-type and *fps1Δ* mutant cells in Fig. 1a). In addition, whereas Hog1 MAPK is normally essential for acetic acid resistance (19), this activity was rendered almost completely dispensable for this resistance by the loss of Fps1 (compare wild-type, *hog1Δ*, and *fps1Δ* single gene deletion mutant and *fps1Δ hog1Δ* double gene deletion mutant cells in Fig. 1a).

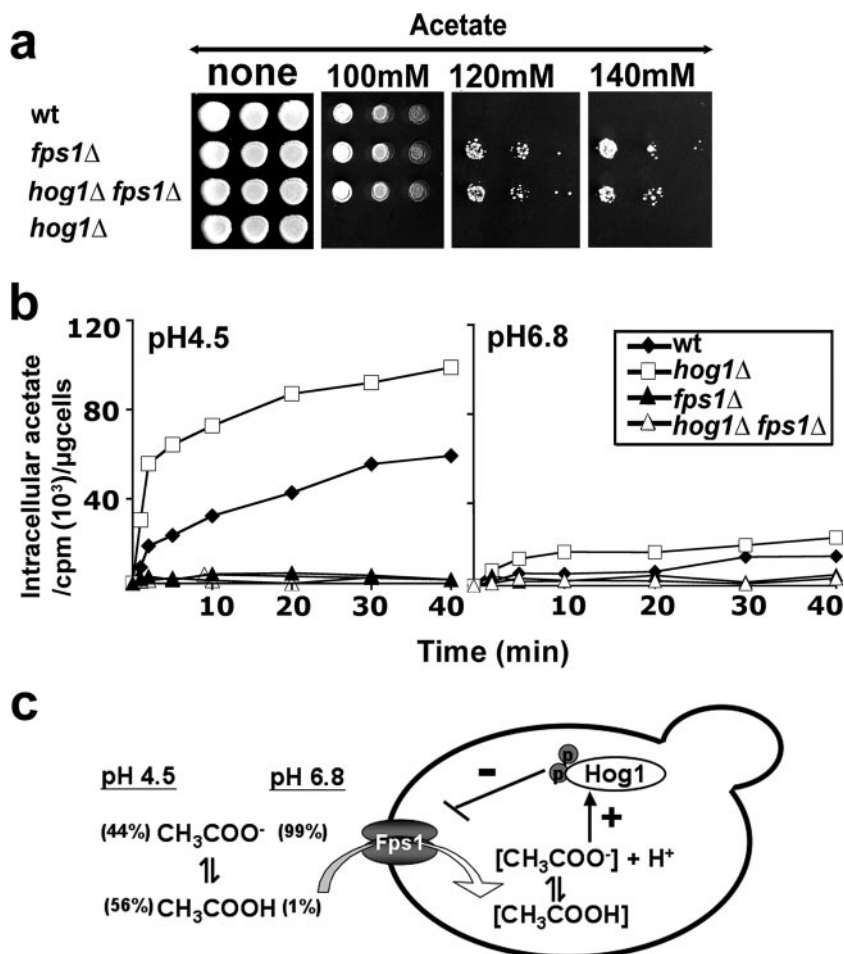


FIG. 1. (a) Loss of Fps1 enhances acetate resistance and suppresses the acetate sensitivity generated by the loss of Hog1. Growth of wild-type (wt), *fps1*Δ and *hog1*Δ single mutant, and *fps1*Δ *hog1*Δ double mutant cells (a 1:10 dilution series grown [3 days, 30°C] on pH 4.5 YPD agar containing the indicated level of acetic acid) is shown. (b) Acetic acid accumulation by pH 4.5 and pH 6.8 YPD cultures of the strains in panel a, measured over the initial 40 min following the addition of 100 mM acetic acid. (c) A working model to explain the results in panels a and b. Entry of undissociated acetic acid into the cell is Fps1 facilitated, with the acid that enters the cell in this way then dissociating in the cytosol (where the pH is close to neutral) so as to generate an intracellular pool of the acetate anion. This acetate then activates Hog1, an activity that in turn downregulates the Fps1-mediated acid influx into the cell.

**Fps1 mediates the diffusional entry of undissociated acetic acid to glucose-repressed yeast.** Before investigating further this apparent linkage between the requirement for Hog1 MAPK in acetic acid resistance and the presence of Fps1, we first had to establish whether the Fps1 aquaglyceroporin could facilitate the diffusion of acetic acid across the cell membrane. Acetic acid accumulation was measured in cells suddenly exposed to the highest acetic acid level that would enable the growth of glucose-repressed, wild-type cultures at pH 4.5 (100 mM), with this acid being labeled to low specific activity with  $^{14}\text{C}$ . The initial rate of acetic acid accumulation by these cells, suddenly exposed to such a high level of acetic acid, is essentially a measure of their acetic acid uptake (*S. cerevisiae* does not use acetic acid as a carbon source in the presence of high glucose levels). These measurements indicated a relatively slow equilibration of the intracellular and extracellular acetic acid pools (Fig. 1b). The yeast cell membrane is therefore not freely permeable to acetic acid (in contrast to what is observed with more lipophilic carboxylic acids, compounds that equili-

brate much more rapidly across this membrane [e.g., benzoic acid]) (10, 23). Importantly, the loss of Fps1 essentially eliminated acetic acid accumulation by these acid-challenged cells (compare wild-type and *fps1*Δ mutant cells in Fig. 1b), revealing that the entry of this acid into glucose-repressed, wild-type yeast is mainly by passive diffusion through the Fps1 channel. When cultures were exposed to the same level of acetic acid (100 mM) but at pH 6.8, when the acetic acid ( $\text{pK}_a$  4.75) in the medium will be almost completely dissociated, cellular accumulation of acetate was greatly reduced (compare pH 4.5 versus pH 6.8 cultures in Fig. 1b). We interpret this as the open Fps1 channel facilitating the transmembrane flux of only the uncharged, undissociated acetic acid ( $\text{CH}_3\text{COOH}$ ) (Fig. 1c), not the acetate anion ( $\text{CH}_3\text{COO}^-$ ). The Fps1 pore is structurally similar to that of bacterial GlpF (5, 13). It is therefore too small to readily accommodate the hydrated acetate anion. Since Fps1 therefore facilitates a substantial acetic acid entry to cells only at low pH (Fig. 1b), all of the experiments described below on the interplay between acetic acid and Fps1

analyzed the effects of 100 mM acetic acid challenge in pH 4.5 cultures (a regimen hereafter termed “acetic acid stress”). At pH 6.8 considerably higher acetate levels, effectively a high osmstress generated by a high acetate salt concentration, are needed in order to achieve any comparable degree of growth inhibition (19).

Remarkably, the loss of Hog1 MAPK generated a higher-than-normal acetic acid accumulation in pH 4.5 cultures (compare wild-type and *hog1Δ* deletant strains in Fig. 1b). This enhanced acetic acid uptake by the *hog1Δ* mutant was Fps1 mediated, as uptake was almost completely abolished in the double *fps1Δ hog1Δ* deletion strain (Fig. 1b). Hog1, an activity required for resistance to these conditions of acetic acid stress (19) (Fig. 1a), therefore appeared, from these acetate accumulation measurements, to be downregulating Fps1-facilitated acetic acid entry into the cell.

Figure 1c shows the model that was indicated by these acetic acid accumulation measurements, the model on which we based our subsequent experimentation. In this, the initial acetic acid entry to the cell is an Fps1-facilitated diffusional entry of the undissociated acid, and this generates an intracellular acetate anion pool that then provides the signal for transient Hog1 activation (pH 4.5 *fps1Δ* cultures lack any acetic acid-induced activation of Hog1 [unpublished observations]). This activated Hog1 then downregulates the Fps1-mediated influx of the acid into the cell (Fig. 1b and c). When cultures are exposed to 100 mM acetic acid, but at the higher pH of 6.8, both the uptake of the acid (Fig. 1b) and the Hog1 activation (19) are much slower than at pH 4.5, since a much smaller fraction of the acid is now undissociated and therefore able to traverse the Fps1 pore (Fig. 1b and c).

**Hog1 MAPK activation by acetic acid stress directs endocytosis and degradation of Fps1.** To determine how Hog1 MAPK might be downregulating the Fps1-facilitated entry of acetic acid into the cell, we initially measured whether Fps1 levels were affected by acetic acid stress. Western blot analysis of a functional *cmyc*-tagged Fps1 (Fps1-*cmyc*) (28) expressed as the sole form of Fps1 channel in pH 4.5 *HOG1*<sup>+</sup> and *hog1Δ* cultures revealed that this Fps1-*cmyc* was being degraded when *HOG1*<sup>+</sup>, but not *hog1Δ*, cells were exposed to acetic acid (Fig. 2a). The Hog1 that is transiently activated by and which confers resistance to these conditions of acetic acid stress (19) (Fig. 1a) appeared therefore to be directing the destabilization of Fps1 (Fig. 2a). Importantly, no Fps1-*cmyc* loss could be observed when, instead of the 100 mM acetic acid addition, the same pH 4.5 cultures were challenged by different conditions of osmstress, irrespective of the presence or absence of Hog1 (the effects of a 1 M NaCl addition are shown in Fig. 2a). Furthermore, addition of 1 M NaCl 10 min prior to addition of 100 mM acetic acid also prevented the Fps1-*cmyc* degradation seen with the application of just the latter stress alone (data not shown). It is possible, therefore, that only the open-channel state of Fps1 is destabilized by an active Hog1, not the closed conformation that is rapidly adopted by this plasma membrane channel in cells exposed to osmstress (see Discussion).

The experiments in Fig. 2a indicated, therefore, that the downregulation of acetic acid influx into wild-type yeast is due to Hog1-dependent loss of the channel that mediates this influx (*hog1Δ* cells, where Fps1 does not degrade [Fig. 2a], exhibit a higher acid accumulation [Fig. 1b]). To obtain further

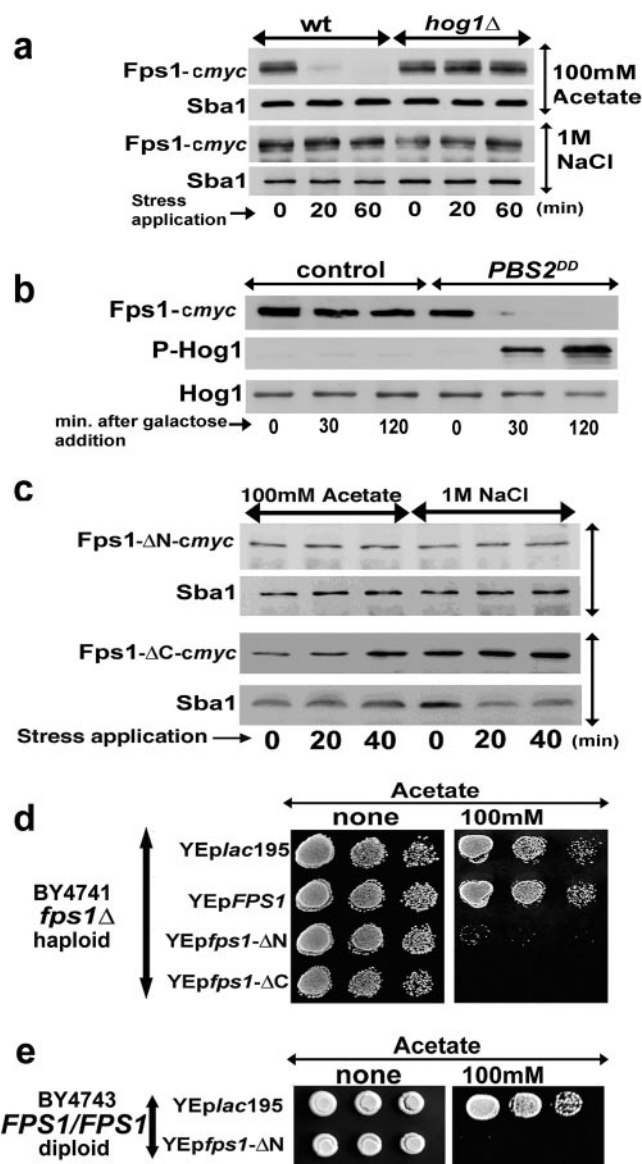


FIG. 2. (a) Measurements of Fps1-*cmyc*, expressed as the sole form of Fps1 channel in *fps1Δ* (wt) and *fps1Δ hog1Δ* (*hog1Δ*) mutant cells, at time points following the addition of either 100 mM acetic acid or 1 M NaCl. (b) Fps1-*cmyc* is degraded in unstressed cells in response to the active form of Hog1 (P-Hog1), the latter generated by galactose-inducible expression of the hyperactive *PBS2<sup>DD</sup>* allele. (c) Forms of Fps1-*cmyc* that lack the amino-terminal or carboxy-terminal cytosolic domain of the channel (Fps1- $\Delta$ N-*cmyc* and Fps1- $\Delta$ C-*cmyc*) did not undergo the degradation in response to acetic acid stress shown for the full-length Fps1-*cmyc* in panel a. (d) Acetic acid sensitivity of BY4741 *fps1Δ* cells transformed, as indicated, with either empty YEplac195 vector, a plasmid for full-length Fps1 expression (YEplFPS1), or plasmids for expression of Fps1 forms that lack either the amino-terminal or the carboxy-terminal cytosolic domain (YEplfps1- $\Delta$ N and YEplfps1- $\Delta$ C). (e) Acetic acid sensitivity of a BY4743 (*FPS1/FPS1*) diploid transformed, as indicated, with either empty YEplac195 vector or plasmid YEplfps1- $\Delta$ N containing the gene for an unregulated Fps1. In panels d and e, cells were spotted in a 1:10 dilution series onto pH 4.5 DO plates lacking leucine and lacking or containing 100 mM acetic acid and then were grown for 3 days at 30°C.

evidence that it is indeed the activation of Hog1 that provides the Fps1 degradation signal we studied the effects of inducing, in the absence of applied stress, a hyperactive allele of the MAPK activator of Hog1, Pbs2 (*Pbs2<sup>DD</sup>*) (24). Fps1-*cmyc* was

rapidly destabilized in response to a *GAL1* promoter-directed induction of this *Pbs2<sup>DD</sup>* allele, with this destabilization of Fps1-*cmyc* by *Pbs2<sup>DD</sup>* expression occurring in the absence of either acetic acid stress or osmotic stress (Fig. 2b).

Fps1 has cytosolic domains at its amino and carboxy termini, both of which are crucial for its regulation. Loss of either cytosolic domain generates a channel protein with constitutive, unregulated glycerol transport activity, whose in vivo expression causes an inability to retain glycerol and accumulate an osmolyte pool and therefore a sensitivity to hyperosmotic stress (9, 28–30). Fps1-*cmyc* forms lacking either of these cytosolic domains (Fps1- $\Delta$ N-*cmyc* and Fps1- $\Delta$ C-*cmyc*) were found not to degrade in response to acetic acid stress (Fig. 2c), their presence creating a sensitivity to this stress (Fig. 2d and e). Plasmids for expression of the wild-type Fps1 or an N-terminally truncated, unregulated Fps1 (YE*pFPS1* and YE*pfps1- $\Delta$ 1*) (28) were also transformed into the *FPS1/FPS1* diploid strain BY4743. As expected for the acetate sensitivity with expression of an N-terminally truncated Fps1 corresponding to a gain of function (the presence of an unregulated, permanently open Fps1 channel), the capacity of the *fps1- $\Delta$ 1* allele to confer acetate sensitivity was genetically dominant (Fig. 2d).

**Fps1-GFP undergoes Hog1-dependent endocytosis in response to acetic acid but not salt stress.** We next observed the fate of a functional Fps1-GFP fusion, expressed as the sole form of Fps1 in *HOG1<sup>+</sup>* and *hog1 $\Delta$*  cells. This Fps1-GFP was placed under *MET25* promoter control so as to enable its expression to be switched off by addition of methionine 2 h prior to stress and therefore observations of the fate of the Fps1-GFP preexisting in the cells at the time of the stress application. Unstressed cells showed a uniform Fps1-GFP distribution in the plasma membrane, irrespective of the presence or absence of Hog1 (Fig. 3a). Upon application of acetic acid stress, this Fps1-GFP was endocytosed to the vacuole in 80 to 90% of the *HOG1<sup>+</sup>* cells examined (Fig. 3a). Simultaneously it was also degraded (Fig. 3c). In contrast, in the *hog1 $\Delta$*  mutant subjected to an identical acetic acid stress, Fps1-GFP remained at the plasma membrane and intact (Fig. 3a and c). Both the Fps1-*cmyc* degradation (Fig. 2) and the Fps1-GFP endocytosis/degradation (Fig. 3a) in response to acetic acid stress are therefore Hog1-dependent events.

When, instead of being subjected to acetic acid stress, these Fps1-GFP-expressing *HOG1<sup>+</sup>* and *hog1 $\Delta$*  cultures were subjected to 1 M NaCl stress, their Fps1-GFP remained at the cell membrane and intact (Fig. 3b and c), moving rapidly from a uniform distribution in this membrane into dot-like structures (Fig. 3b). Though the nature of the Fps1-GFP in these dot-like structures was not investigated further, this Fps1-GFP was observed to return subsequently to a uniform plasma membrane distribution when these salt-stressed cells were reshifted from high to low osmolarity (data not shown).

**Fps1 undergoes Hog1-dependent phosphorylation in response to acetic acid stress.** The next question we addressed is whether Hog1 directly binds to and phosphorylates Fps1 or whether its action in targeting this channel for degradation is indirect, for example, mediated through an intermediary protein kinase. Lack of the Fps1 degradation in response to acetic acid stress generates a sensitivity to this stress (*hog1 $\Delta$*  cells) (Fig. 1a and 2a), but our recent screen for such sensitivity

among the collection of strains lacking nonessential yeast protein kinases uncovered only the kinases of the HOG signaling cascade as being important for acetic acid resistance (19).

Upon immunoprecipitating Fps1-*cmyc* from extracts of unstressed cells or extracts of cells exposed, very briefly, to 100 mM acetic acid (a 10-min treatment; longer stress would have caused Fps1-*cmyc* degradation), we made two important observations: first, that Fps1-*cmyc* was acquiring Hog1-dependent phosphorylations on threonine and on serine in vivo in response to the acid stress (Fig. 4a) and second, that appreciable amounts of Hog1 were coprecipitated with this Fps1-*cmyc*, with the levels of this coprecipitated Hog1 being unaffected by the brief in vivo acetic acid treatment prior to extract preparation (Fig. 4b). Further evidence of a direct Hog1-Fps1 association is presented later in this report.

As proline-directed protein kinases, MAPKs phosphorylate their substrates at TP/SP motifs (32). There are two such motifs (corresponding to T231 and S537 in the *S. cerevisiae* Fps1) within two 12-amino-acid regions previously identified as being important for Fps1 channel regulation, regions that are on the cytosolic surface but located immediately adjacent to the amino-terminal and carboxy-terminal transmembrane domains of the channel (9, 28). These TP/SP motifs are also conserved among Fps1 aquaglyceroporins of diverse yeasts (<http://www.gmm.gu.se/groups/hohmann/fungalMIP/index.htm>). If Hog1 phosphorylates Fps1 at T231 and/or S537 in order to initiate the Hog1-dependent endocytosis seen in Fig. 3a, then conservative mutation of these residues should render Fps1 refractory to this endocytosis.

We mutated both T231 and S537 in the Fps1-GFP fusion to either nonphosphorylatable alanine residues (Fps1<sup>T231A S537A</sup>-GFP) or phosphomimetic amino acid residues (Fps1<sup>T231E S537D</sup>-GFP). The Fps1<sup>T231A S537A</sup>-GFP fusion was both localized correctly at the plasma membrane (Fig. 3d) and functional as an osmogated glycerol channel (data not shown). Despite this, when expressed as the sole Fps1 channel of *HOG1<sup>+</sup>* cells, this Fps1<sup>T231A S537A</sup>-GFP remained at the plasma membrane and was stable under conditions of acetic acid stress where the wild-type Fps1-GFP fusion was endocytosed to the vacuole and degraded (Fig. 3d). In contrast, the mutation of T231 and S537 in the Fps1-GFP fusion to phosphomimetic residues resulted in a substantial reduction of the GFP signal at the plasma membrane, even in unstressed cells (Fps1<sup>T231E S537D</sup>-GFP) (Fig. 3d), a result consistent with phosphorylation of Fps1 at T231 and/or S537 in such cells normally providing a signal for channel endocytosis.

The T231A S537A double mutant Fps1-*cmyc* (Fps1<sup>T231A S537A</sup>-*cmyc*), expressed as the sole Fps1 channel in *HOG1<sup>+</sup>* cells, was found to lack the acetic acid-induced in vivo phosphorylation (Fig. 4a) and in vivo degradation (Fig. 4c) exhibited by the wild-type Fps1-*cmyc*. Furthermore, the expression of T231A S537A double mutant Fps1 forms as the sole Fps1 channel of *fps1 $\Delta$  HOG1<sup>+</sup>* or *fps1 $\Delta$  hog1 $\Delta$*  deletion strains generated (like the loss of Hog1 MAPK in cells with normal Fps1 [Fig. 1a and b]) a hypersensitivity to acetic acid (Fig. 4d) and a higher-than-normal acetic acid accumulation (Fig. 4e), results that are consistent with the wild-type Fps1-GFP being endocytosed but the mutant Fps1<sup>T231A S537A</sup>-GFP remaining at the plasma membrane during acid stress (Fig. 3a and d). Conversely, expression of the phosphomimetic (T231E S537D) double mutant Fps1-

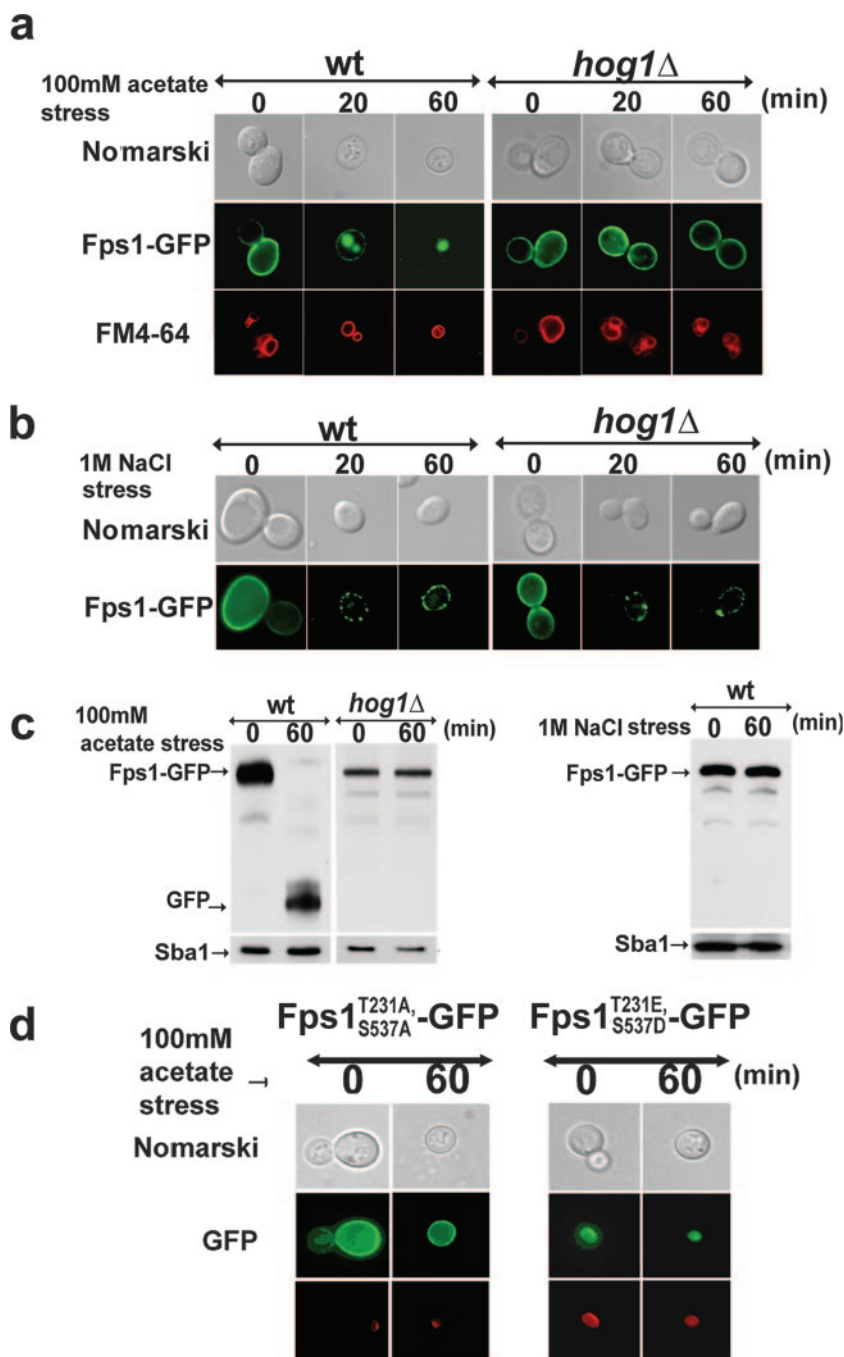


FIG. 3. (a and b) Visualization of Fps1-GFP expressed as the sole form of Fps1 channel in *fps1* $\Delta$  (wt) and *fps1* $\Delta$  *hog1* $\Delta$  (*hog1* $\Delta$ ) mutant cells stressed for 0, 20, or 60 min either with 100 mM acetic acid (a) (vacuoles revealed by FM4-64 staining) or 1 M NaCl (b). (c) An analysis of Fps1-GFP fusion integrity in these same cultures by Western blotting. The blots were probed using anti-GFP and anti-Sba1 antisera (the latter as a loading control). (d) Expressed as the sole Fps1 of *HOG1*<sup>+</sup> cells, a nonphosphorylatable T231A S537A double mutant Fps1-GFP was correctly plasma membrane localized but was not endocytosed under the conditions of acetate stress where the wild-type Fps1-GFP is endocytosed to the vacuole (a). In contrast, very little of a phosphomimic T231E S537D double mutant Fps1-GFP was plasma membrane localized, even in the absence of stress.

GFP (a form substantially not plasma membrane localized even in unstressed cells [Fig. 3d]) had no discernible effect on the levels of acetate resistance displayed by the *fps1* $\Delta$  *HOG1*<sup>+</sup> and *fps1* $\Delta$  *hog1* $\Delta$  deletion strains (Fig. 4d).

In vivo, therefore, double T231A S537A mutation of Fps1

abolishes the Hog1-dependent phosphorylation and the endocytosis of this aquaglyceroporin in response to acetic acid stress. As a result, this channel now remains in the membrane, where it generates a sensitivity to acetic acid by providing an open channel for acid entry into the cell. In contrast, the

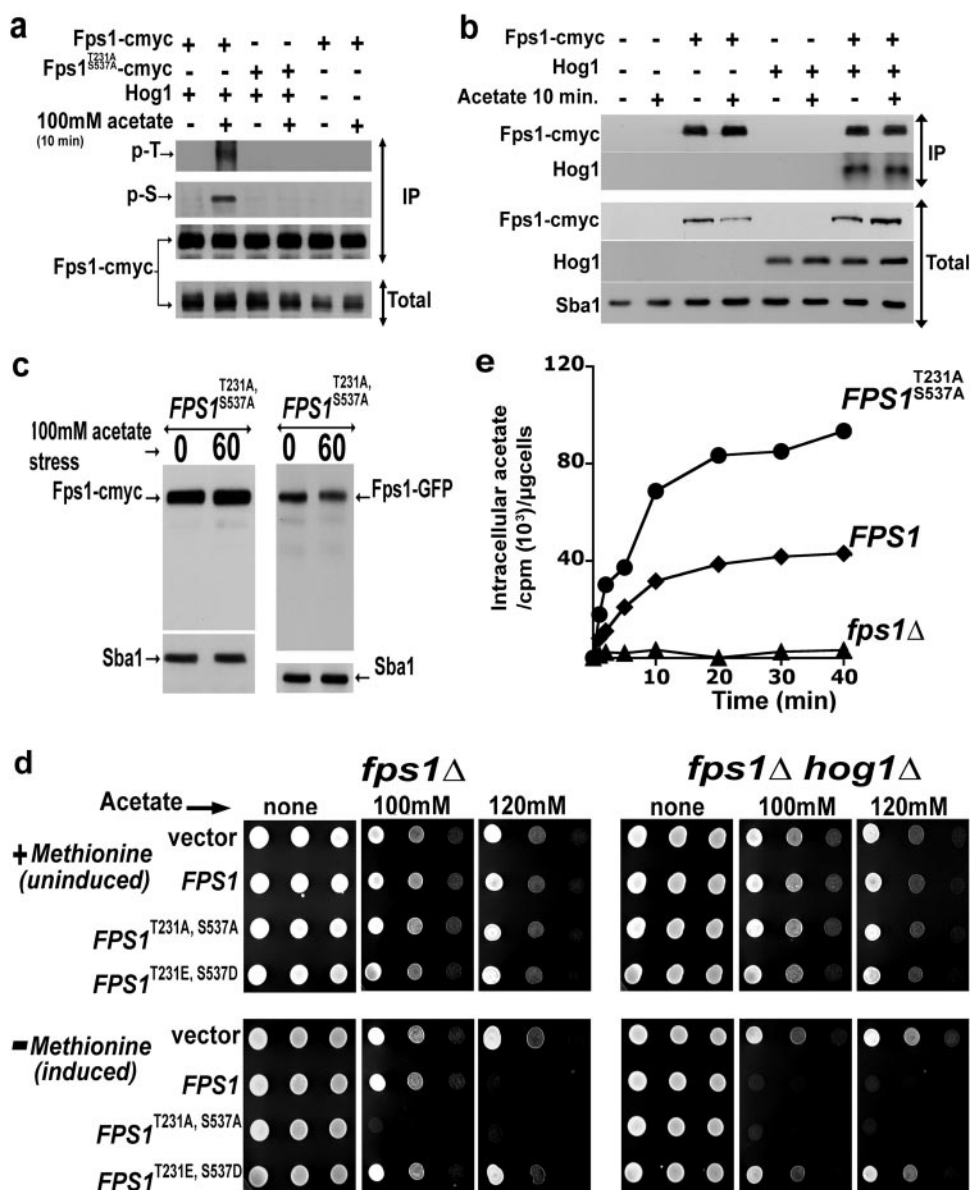


FIG. 4. (a) Brief acetic acid stress leads to *in vivo* phosphorylation of Fps1-*cmyc* on both threonine (p-T) and serine (p-S), phosphorylations that are abolished by the lack of Hog1 or the expression of T231A S537A double mutant Fps1-*cmyc*. (b) Immunoprecipitated (IP) Fps1-*cmyc* coprecipitates Hog1. (c) T231A S537A double mutant forms of Fps1-*cmyc* and Fps1-GFP are refractory to acetate-induced degradation under conditions where the wild-type forms become degraded (Fig. 2d and 3c). (d) The acetate sensitivity generated by *MET25* promoter-regulated induction of a functional wild-type Fps1-GFP fusion (*FPS1*), expressed as the sole Fps1 channel in *fps1Δ* and *fps1Δ hog1Δ* mutant cells, is made more severe by T231A S537A double mutation of this Fps1-GFP, whereas the induction of a phosphomimic (T231E S537D) Fps1-GFP has no influence over acetate resistance. (e) Expression of a T231A S537A double mutant Fps1-*cmyc* as the sole Fps1 of *HOG1*<sup>+</sup> cells leads to enhanced acetate accumulation (measured as for Fig. 1c, except that cultures were maintained on pH 4.5 DO medium lacking uracil).

corresponding phosphomimic (T231E S537D) mutant form of Fps1 is constitutively delocalized from the plasma membrane, such that its expression does not compromise the acetate resistance intrinsic to *fps1Δ* deletion strains, irrespective of the presence or absence of Hog1 (Fig. 4d).

**Acetic acid induces a transient Fps1 ubiquitination that is Hog1 dependent.** Ubiquitination of cell surface proteins is generally a key step in triggering their internalization by endocytosis (4, 35). In yeast exposed to a brief acetic acid stress, ubiquitinated forms of Fps1-*cmyc* were readily detectable, with

their appearance being abolished by either the loss of Hog1 or the expression of the T231A S537A double mutant Fps1-*cmyc* (Fig. 5a). Hog1-dependent phosphorylation of Fps1 appears therefore to be the signal for this aquaglyceroporin to become ubiquitinated prior to its endocytosis and degradation in the vacuole (Fig. 6d).

*doa4Δ* and *end3Δ*, two mutations causing a general loss of endocytosis of cell surface proteins, also caused Fps1-GFP to remain stable and plasma membrane localized under those conditions of acetic acid stress where it is normally endocytosis-

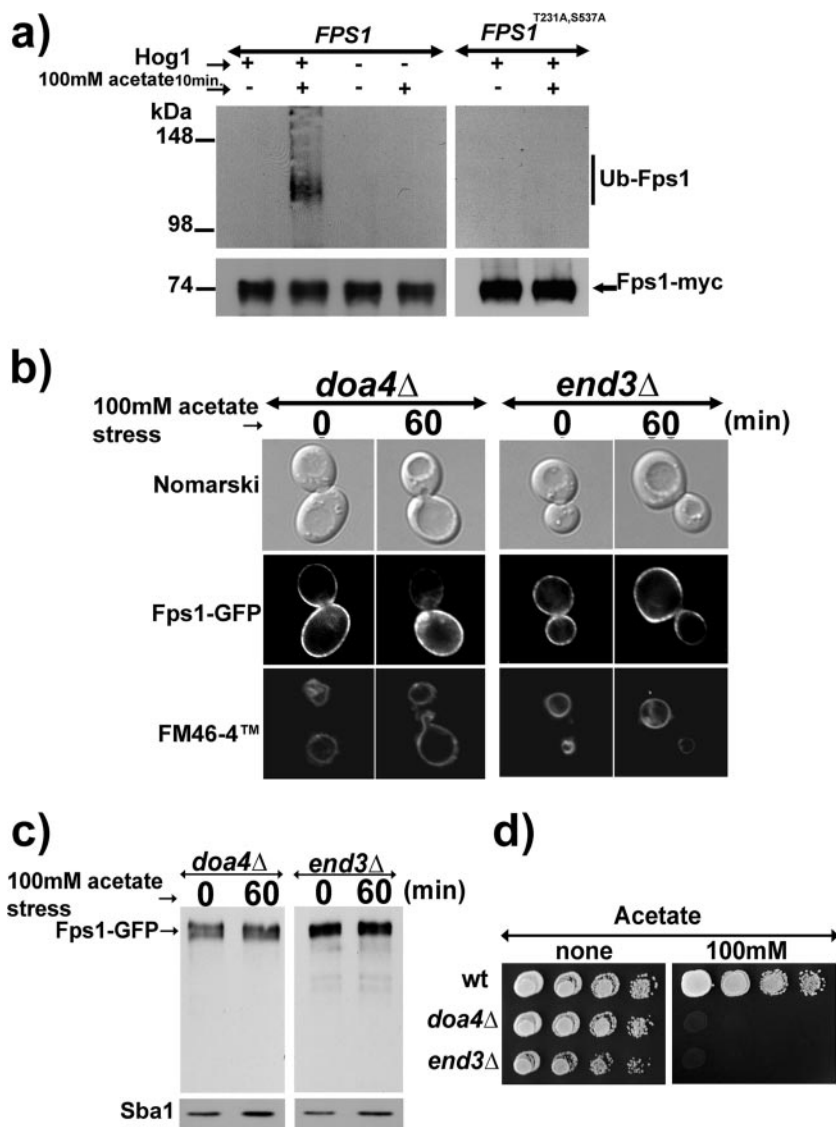


FIG. 5. (a) Transient, Hog1-dependent ubiquitination of wild-type but not T231A S537A double mutant Fps1-*myc* in cells exposed to a brief (10-min) acetate stress (protein was immunoprecipitated from cell extracts and then detected by immunoblotting using either antiubiquitin [upper image] or anti-*c-myc* [lower image] antisera). (b and c) In the *doa4Δ* and *end3Δ* mutants, Fps1-GFP remained at the plasma membrane (b) and was stable (c) under conditions of acetic acid stress where in wild-type cells it was endocytosed and degraded (Fig. 2c and d). (d) The *doa4Δ* and *end3Δ* mutants are acetic acid sensitive (growth was as for Fig. 1a).

tosed (Fig. 5b and c). In addition, these mutants displayed an acetic acid sensitivity phenotype (Fig. 5d). The *doa4Δ* and *end3Δ* mutants are, respectively, defective in Doa4, a ubiquitin-protein hydrolase important in recycling ubiquitin from proteolytic substrates destined for degradation by the 26S proteasome or the vacuole (3, 27), and End3, a component of a multiprotein complex required for the internalization step of endocytosis (31).

**Hog1 binds the N-terminal regulatory domain of Fps1, with the active form of this MAPK phosphorylating threonine 231 of this domain, both in vivo and in vitro.** The experiments described above reveal that the active Hog1 MAPK generated by acetic acid stress directs phosphorylation, ubiquitination, and endocytosis of Fps1, thereby removing from the plasma membrane the major route for diffusional entry of acetic acid

into the cell (Fig. 1 and 6). Removal of this channel is important for resistance to toxic levels of acetic acid, since acid sensitivity is apparent with diverse defects that abolish this loss of Fps1. Thus, defective Fps1 endocytosis and acid sensitivity are apparent with the loss of Hog1 (Fig. 1a), with the loss of the amino- or carboxy-terminal cytosolic domain of Fps1 (Fig. 2c-e), with T231A S537A double mutation of Fps1 (Fig. 3d and 4d), or with mutations causing a general loss of endocytosis of cell surface proteins (*doa4Δ* and *end3Δ*) (Fig. 5b to d). Figure 4a reveals that the in vivo Hog1-dependent phosphorylation of Fps1 involves two residues (T231 and S537), both of which correspond to SP/TP motifs such as are recognized by the MAPKs.

While these results are fully consistent with the signal for the endocytosis of Fps1 being a direct Hog1 phosphorylation of



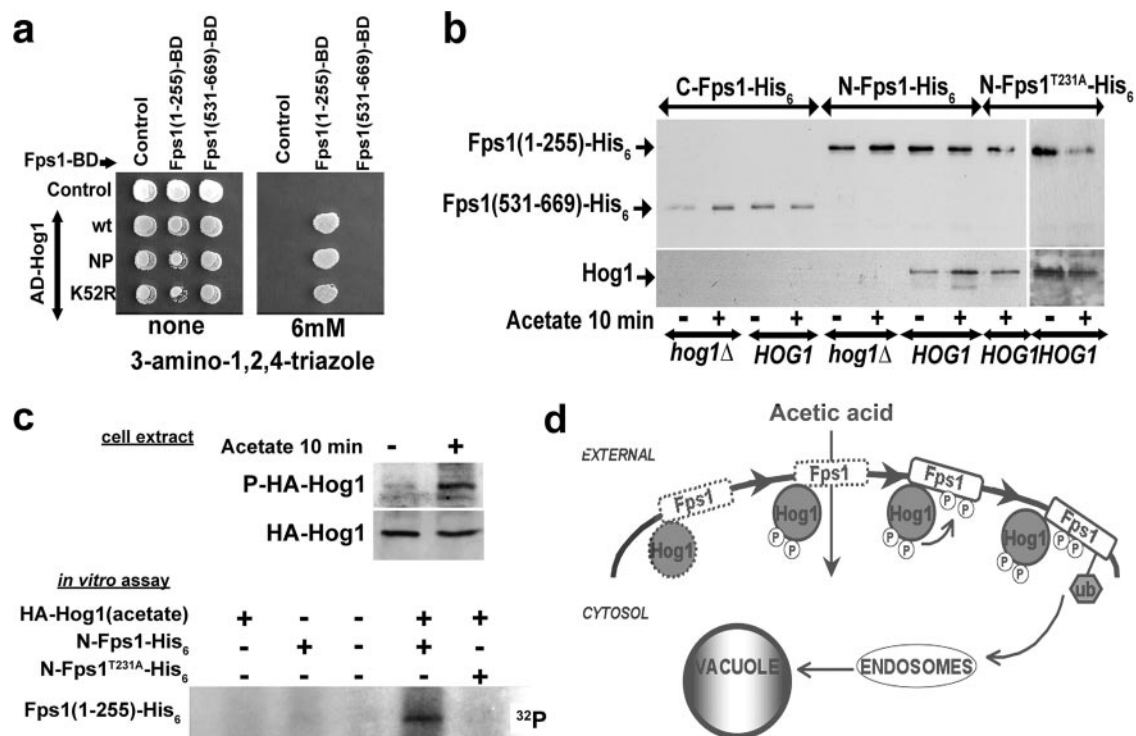


FIG. 6. (a) A functional AD-Hog1 fusion interacts with Fps1(1–255)-BD but not Fps1(531–669)-BD in the two-hybrid system; this interaction is unaffected by mutations that render this AD-Hog1 either nonphosphorylatable by Pbs2 (NP) or inactive (K52R). Interaction was detected as yeast growth in the absence of histidine and in the presence of 3-amino-1,2,4-triazole; the latter is an inhibitor of the *HIS3* product. (b) Hog1 binds Fps1(1–255)-His<sub>6</sub> but not Fps1(531–669)-His<sub>6</sub> in cell extracts, with Hog1 binding to the former fragment being unaffected by the T231A mutation. (c) HA-Hog1 immunoprecipitated from extracts of briefly acetate-stressed cells is in a phosphorylated, active form (P-HA-Hog1) and in an in vitro kinase assay phosphorylates the Fps1(1–255)-His<sub>6</sub> fragment at T231 (the controls in lanes 2 and 3 were immunoprecipitates from non-HA-Hog1-expressing cells). (d) Model of the acetic acid stress response. Hog1 is constitutively bound to the open Fps1 channel and thereby is poised to achieve an almost instant phosphorylation of the latter whenever there is activation of HOG pathway signaling. Such phosphorylation in turn causes Fps1 to be ubiquitinated and endocytosed to the vacuole.

this channel protein, we sought further evidence that Hog1 interacts with, and phosphorylates, Fps1. Finding that appreciable amounts of Hog1 coprecipitated with the Fps1-*myc* immunoprecipitated from yeast cell extracts (Fig. 4b), we next used two-hybrid analysis to probe for an in vivo Hog1 interaction with the amino- or carboxy-terminal hydrophilic region of Fps1 (amino acids 1 to 255 and 531 to 669, respectively). These are regulatory domains exposed on the cytosolic face of the cell membrane in the open-channel state of this aquaglyceroporin (9, 28). Interaction of Fps1(1–255) and Fps1(531–669), each fused to the Gal4 BD, was tested with fusions of the Gal4 AD to the native Hog1 MAPK, a nonactivatable (T180AY182F double mutant) Hog1 (NP), and a kinase-dead (K52R) Hog1. The Fps1(1–255)-BD “bait,” but not Fps1(531–669)-BD, exhibited strong two hybrid interaction with the wild-type AD-Hog1, as well as with both the NP and K52R mutant forms of this AD-Hog1 “prey” fusion (Fig. 5a).

This Fps1(1–255)-Hog1 two-hybrid interaction was then confirmed in studies of in vitro protein binding. His<sub>6</sub>-tagged versions of Fps1(1–255) and Fps1(531–669) were expressed separately in the *fps1Δhog1Δ* yeast mutant and then isolated from extracts of these cells using nickel affinity resin (see Materials and Methods). These soluble subdomains of the aquaglyceroporin were then incubated with extracts from non-stressed or briefly acetic acid-stressed wild-type or *hog1Δ* cells.

Hog1 MAPK was found to be associated with the Fps1(1–255)-His<sub>6</sub> fragment but not with Fps1(531–669)-His<sub>6</sub> (Fig. 6b), its binding to the former fragment being unaffected by whether or not the wild-type cells used for extract preparation had been preconditioned by brief, Hog1-activating (Fig. 6c) acetic acid stress. Together, the experiments in Fig. 4b and 6a and b indicate that Hog1 binds to the amino-terminal cytosolic domain of Fps1, irrespective of the activation state or activity of this MAPK, and also that in unstressed yeast cells, significant amounts of Hog1 are associated with the plasma membrane Fps1. Hog1 has traditionally been thought to exist mainly in association with its MAPK activator, Pbs2 (32). Hog1 is, though, considerably more abundant than Pbs2 in yeast (6).

Using an HA epitope-tagged Hog1 (HA-Hog1) immunoprecipitated from extracts of unstressed or briefly acetic acid-stressed yeast, we investigated whether Hog1 would directly phosphorylate the amino- or carboxy-terminal hydrophilic regions of Fps1 in vitro. As substrates, we added Fps1(1–255)-His<sub>6</sub> (either the wild-type or T231A mutant form) and Fps1(531–669)-His<sub>6</sub>, which were previously expressed in and then purified from *fps1Δhog1Δ* mutant cells. HA-Hog1 phosphorylated the Fps1(1–255)-His<sub>6</sub> fragment (Fig. 6c) but not Fps1(531–669)-His<sub>6</sub> (not shown). Furthermore, in vitro phosphorylation of the former fragment was much more efficient using HA-Hog1 purified from briefly acetate-stressed cells

(consistent with the greater pool of active Hog1 MAPK in these cells [19] [Fig. 6c]). The T231A mutation in Fps1(1–255)-His<sub>6</sub> abolished this *in vitro* phosphorylation by HA-Hog1 (Fig. 6c) but not Hog1 binding to this fragment (Fig. 6b). *In vivo*, the Hog1-dependent threonine phosphorylation of the full-length Fps1-*cmyc* in response to acetic acid stress was similarly abolished by the T231A S537A double mutation of this Fps1-*cmyc* (Fig. 4a). Therefore, Hog1 binds the region from amino acid 1 to 255 of Fps1, phosphorylating this region at T231 (Fig. 4a and 6c). Thorsen et al. have also recently presented independent evidence for Fps1 being regulated by a Hog1-dependent phosphorylation of T231, though under a different stress condition (arsenite, not acetate, stress) (33) (see Discussion).

Hog1 may also phosphorylate Fps1 on the S537 residue of the latter, since acetic acid-induced, Hog1-dependent phosphorylation of Fps1-*cmyc* on serine was detected *in vivo*, with this serine phosphorylation being abolished by the T231A S537A double mutation of Fps1-*cmyc* (Fig. 4a). The Fps1(531–669)-His<sub>6</sub> soluble subdomain of the channel that contains this S537 was not phosphorylated by HA-Hog1 in our *in vitro* kinase assays, possibly due to a lack of Hog1 binding to this subfragment (Fig. 6c). Thus, our *in vitro* assays were able to confirm direct Hog1 phosphorylation only of the T231 Fps1 residue.

## DISCUSSION

In this study we show that acetic acid enters glucose-repressed yeast cells primarily by facilitated diffusion of the undissociated acid through the Fps1 aquaglyceroporin channel (Fig. 1). When cells are challenged with inhibitory concentrations of acetic acid, there is a transient activation of Hog1 (19). This MAPK then directly phosphorylates Fps1 on T231 and also probably on S537 (Fig. 4a and 6), a phosphorylation that is the signal for this channel to be ubiquitinated and endocytosed to the vacuole. Fps1 is degraded even when Hog1 is activated in the absence of stress (Fig. 2b). Double T231A S537A mutation of Fps1 abolishes this phosphorylation by Hog1, as well as the Hog1-dependent ubiquitination and endocytosis (Fig. 3d, 4a, and 5a), generating a hypersensitivity to acetic acid (Fig. 4d) that appears to reflect a higher-than-normal acid entry into cells (Fig. 4e). In contrast, a phosphomimic mutant Fps1<sup>T231E S537D</sup>-GFP fusion is substantially delocalized from the plasma membrane, even in the absence of stress (Fig. 3d), and cannot confer Fps1 function (Fig. 4d and data not shown).

This removal of Fps1 from the plasma membrane appears to be essential for downregulating the acetic acid influx to the cell (Fig. 1b and 4e), since the acetic acid stress alone appears not to cause the complete closure of the Fps1 channel (Fps1-dependent acetic acid uptake is not immediately arrested in stressed wild-type cells; also, it is enhanced should this channel remain in the plasma membrane, as occurs in the *hog1Δ* mutant [Fig. 1b and 3a] or with the expression of the T231A S537A double mutant Fps1 [Fig. 3d and 4e]). Indeed, targeting Fps1 for degradation may be the major requirement for an active Hog1 in acetic acid resistance (19), since, remarkably, Hog1 is largely dispensable for this resistance when the cells lack Fps1 (Fig. 1a). Total loss of Fps1 also creates an acetate-resistant phenotype (the *fps1Δ* mutant; Fig. 1a), probably as

this loss eliminates the major source of the acetic acid flux into the cell.

Activation of HOG MAPK pathway signaling is apparent both with osmostress (9, 12, 22) and with acetic acid stress (19), but Fps1 is destabilized only with the latter and not with the former condition of stress (Fig. 2a). According to current models, Fps1 refolds to a closed-channel conformation within seconds in cells shifted to high osmolarity, a Hog1-independent response to an altered cell turgor (9, 12, 22). With osmostress Fps1-*cmyc* and Fps1-GFP were not destabilized in *HOG1*<sup>+</sup> cells (Fig. 2a and 3b and c), their stability under these conditions contrasting with their destabilization when the same *HOG1*<sup>+</sup> transformants were exposed, instead, to acetic acid (Fig. 2a and 3a and c). Fps1 is therefore either unstable or stable under different conditions of HOG pathway-activating stress. Measurements of the acetic acid accumulation by the *hog1Δ* mutant (Fig. 1b) indicate that should this channel remain in the plasma membrane (Fig. 2a and 3a), it does not close completely in response to acetic acid stress. It appears, therefore, that an active Hog1 MAPK may target only the open-channel state of Fps1 for degradation, not the closed-channel conformation that is rapidly adopted by Fps1 in cells shifted to high osmolarity.

The Fps1 channel is also the route whereby toxic metalloids, such as arsenite and antimonite, enter yeast (36). Recently, Hog1 was shown to mediate a protective response to these metalloids, acting to downregulate their entry into the cell through this channel (33). There are some striking parallels with this metalloid response and the acetic acid response that we have been studying (notably, Hog1-dependent downregulation of the entry of the xenobiotic compound to the cell via Fps1 and also the requirement for the T231 residue of Fps1 in this downregulation). There appears, though, to be an important difference between the response to arsenite and the acetic acid response that we describe here. Unfortunately, Thorsen et al. did not investigate the *in vivo* localization of Fps1 in their study, but it is evident from their data that Fps1 is not becoming degraded in arsenite-treated cells but appears instead to be adopting a closed-channel conformation in response to the arsenite-triggered, Hog1-dependent phosphorylation (33). High intracellular levels of arsenite may be strongly inhibitory to the events of endocytosis that we observe. It is also possible that the Hog1 phosphorylation of Fps1 in response to acetic acid (Fig. 4a) generates closure of the Fps1 channel but that, because these same phosphorylations also target the channel for endocytosis, it is the latter events of endocytosis that are observed as the dominant phenotype in the case of our acetic acid-stressed cells.

Furthermore, our results indicate that Hog1 is bound constitutively to the amino-terminal cytosolic domain of Fps1 (Fig. 6). This domain is thought to “dip” into the membrane during the turgor-mediated channel closure (9, 22), whereupon Hog1 may dissociate (an aspect not investigated here). Our data reveal that Fps1 engages in an association with Hog1 irrespective of the activation state or the activity of this MAPK (Fig. 6). Hog1 is thereby “poised” to achieve almost instant Fps1 phosphorylation, and thereby an altered stability (and possibly conformation) of this aquaglyceroporin, in response to the activation of HOG pathway signaling.

As far as we are aware this is the first demonstration of the

destabilization of an aquaglyceroporin being dependent upon direct MAPK phosphorylation and of a resistance being acquired through the selective degradation of such a channel protein. It also appears to be the first evidence for the MAPK that regulates the activity and stability of an aquaglyceroporin also being engaged in a strong, constitutive association with this target aquaglyceroporin.

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