Presence of the Fps1p aquaglyceroporin channel is essential for Hog1p activation, but suppresses Slt2(Mpk1)p activation, with acetic acid stress of yeast

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When grown at pH 4.5, *Saccharomyces cerevisiae* acquires a resistance to inhibitory acetic acid levels (~0.1 M) by destabilizing Fps1p, the plasma membrane aquaglyceroporin that provides the main route for passive diffusional entry of this acid into the cell. Acetic acid stress transiently activates Hog1p mitogen-activated protein (MAP) kinase, which, in turn, phosphorylates Fps1p in order to target this channel for endocytosis and degradation in the vacuole. This activation of Hog1p is abolished with the loss of Fps1p, but is more sustained when cells express an open Fps1p channel refractory to destabilization. At neutral pH, much higher levels of acetate (~0.5 M) are needed to inhibit growth. Under such conditions, the loss of Fps1p does not abolish, but merely slows, the activation of Hog1p. Acetate stress also activates the Slt2(Mpk1)p cell integrity MAP kinase, possibly by causing inhibition of glucan synthase activity. In pH 4.5 cultures, this acetate activation of Slt2p is strongly enhanced by the loss of Fps1p and is dependent upon the cell surface sensor Wsc1p. Lack of Fps1p therefore exerts opposing effects on the activation of Hog1p and Slt2p in yeast exposed to acetic acid stress.

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INTRODUCTION

Hog1p mitogen-activated protein (MAP) kinase directs a number of the adaptive responses to stress in *Saccharomyces cerevisiae*. Activated in response to hyperosmotic stress (Hedfalk *et al.*, 2004; Pettersson *et al.*, 2005; Tamas *et al.*, 2003), arsenite (Thorsen *et al.*, 2006) and acetic acid (Mollapour & Piper, 2006), it induces physiologically very different responses under these diverse conditions of stress. Only with acetic acid, not hyperosmotic or arsenite stress, has the active Hog1p been shown to trigger endocytosis of Fps1p, the major plasma membrane channel for glycerol, arsenite and undissociated acetic acid (Mollapour & Piper, 2007). In contrast, in cells shifted to high osmolarity, Fps1p is not destabilized, but undergoes rapid, Hog1p-independent channel closure in response to the altered cell turgor (Hedfalk *et al.*, 2004; Pettersson *et al.*, 2005; Tamas *et al.*, 2003). Furthermore, in low-pH cultures the Hog1p activated by acetic acid stress does not cause either *GPD1* gene induction or glycerol production, events that are directed by this same MAP

kinase when it is activated by hyperosmotic stress (Mollapour & Piper, 2006).

Osmoadaptation involves the altered expression of a large number of genes, representing ~10% of the total yeast genome (O'Rourke & Herskowitz, 2004). To a large extent this occurs through the Hog1p activated by osmostress being translocated to the nucleus where, by phosphorylation of a number of proteins with transcriptional activity (Proft & Struhl, 2004), it triggers roughly half of all of the gene induction or gene repression events in osmostressed cells (O'Rourke & Herskowitz, 2004). In contrast, acetic acid resistance involves a transcription factor for which there is no evidence of Hog1p regulation (Haalp; Fernandes *et al.*, 2005) and is completely unaffected by the loss of Nmd5p, the importin essential for the translocation of the activated Hog1p to the nucleus (our unpublished observations).

An unexpected similarity has emerged, though, with regard to these different Hog1p-directed stress responses of *S. cerevisiae*. They all seem to be critically dependent on the active Hog1p being able to rapidly phosphorylate one or more key activities of the plasma membrane. A subfraction of the Hog1p of unstressed yeast exists bound to the amino-terminal, cytosolic domain of Fps1p, thereby being 'poised' to achieve an almost instant destabilization of this aquaglyceroporin, and therefore the alleviation of acetic

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Abbreviations: HOG, high-osmolarity glycerol; MAP kinase, mitogen-activated protein kinase.

acid stress, upon activation of Hog1p (Mollapour & Piper, 2007). This is analogous to the way that Hog1p alleviates salt stress, a situation where this same MAP kinase – but now the Hog1p subfraction in association with certain plasma membrane ion transporters (Nha1p and Tok1p) – generates an instant relief of NaCl stress [Hog1p stimulation of Nha1p Na^+/H^+ antiporter activity following salt stress is practically instant, preceding any Hog1p-dependent changes in gene transcription (Proft & Struhl, 2004)]. Hog1p also directly phosphorylates Fps1p in the response to arsenite stress, though unlike with acetic acid treatment, it appears that here it causes the closure, not the destabilization, of this channel (Thorsen *et al.*, 2006). Fps1p channel closure is also triggered by osmotic stress, but this is a turgor-, rather than a Hog1p-mediated event and extremely rapid [Fps1p-mediated glycerol flux diminishes within seconds following a shift to high osmolarity and increases over an apparently similar timescale when cells are then shifted to low osmolarity (Luyten *et al.*, 1995; Tamas *et al.*, 1999)]. In contrast, the Hog1p-dependent endocytosis of Fps1p in acetic acid-stressed pH 4.5 cultures is relatively slow, occurring over a timescale of several minutes (Mollapour & Piper, 2007).

In the presence of fermentable sugars, *S. cerevisiae* cannot assimilate acetate as a carbon source (Casal *et al.*, 1996; Paiva *et al.*, 2004). When glucose is repressed, this yeast is therefore inhibited by high acetic acid levels, especially at low pH when the acid is substantially in the undissociated state that readily enters the cell through the Fps1p channel (Mollapour & Piper, 2007). By abolishing the major route of entry into the cell for this acid, Hog1p-directed endocytosis of Fps1p generates a resistance to levels of acetic acid that would otherwise prove toxic (Mollapour & Piper, 2007). In this study we have investigated how Fps1p itself influences Hog1p activation by acetate stress. The data indicate that Fps1p initiates the events that lead to its own demise in acetic acid-stressed yeast, allowing the acid entry that, in turn, activates Hog1p. Fps1p also affects acetate activation of another MAP kinase, Slt2(Mpk1)p, which, unlike Hog1p, is not required for acetate resistance (Mollapour & Piper, 2006). The effects of Fps1p loss on acetate-induced phosphorylation of Slt2p are profoundly different from the effects on Hog1p, an indication that acetic acid stress activates these two MAP kinases by fundamentally different mechanisms.

METHODS

Yeast strains and yeast growth. BY4741-derived *hog1Δ*, *fps1Δ* and *hog1Δ fps1Δ* *S. cerevisiae* strains and plasmid pUG23FPS1-C-GFP were as previously described (Mollapour & Piper, 2007). *HIS3MX6* cassette deletion of *SLN1* sequences in the *hog1Δ* and *hog1Δ fps1Δ* mutants was used to generate the *hog1Δ sln1Δ* double and *hog1Δ fps1Δ sln1Δ* triple mutant strains. YEpFPS1 and YEpfps1-Δ1 were as previously described (Luyten *et al.*, 1995; Tamas *et al.*, 2000, 2003).

Yeast was grown on YPD [2 % (w/v) bacto-peptone, 1 % yeast extract, 2 % glucose, 20 mg adenine l^{-1}]. Selective growth was on dropout 2 % glucose (DO) medium (Adams *et al.*, 1997). The medium pH was

adjusted to pH 4.5 or pH 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 OD₆₀₀ 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 3–5 days at 30 °C.

Protein analysis and immunoblots. Total protein extracts were prepared and analysed by Western blotting, as described by Martin *et al.* (2000). Western blot analysis of yeast Hog1p, Slt2p and Sba1p used, as primary antibody, rabbit polyclonal antisera raised against these proteins. Slt2p and Hog1p were analysed with polyclonal anti-Mpk1p (Y-244) and anti-Hog1 (Y-215) antibodies (Santa Cruz Biotechnology), respectively. The secondary antibody was horseradish peroxidase-anti-rabbit or -anti-mouse IgG (Amersham) diluted 2000-fold. Analysis of the active forms of Hog1p and Slt2p used antibodies against the dually phosphorylated (Thr¹⁸⁰/Tyr¹⁸²)-p38 MAP kinase or anti-(Thr²⁰²/Tyr²⁰⁴)-p44/42 MAP kinase (New England Biolabs). These recognize, respectively, the dually Thr¹⁷⁴/Tyr¹⁷⁶-phosphorylated Hog1p and the Thr¹⁹⁰/Tyr¹⁹²-phosphorylated Slt2p in yeast extracts (Martin *et al.*, 2000; Siderius *et al.*, 1997). Enhanced chemiluminescence reagents (Amersham) were used for detection. To help to ensure that the results in Figs 1, 2 and 3 were a true indication of the magnitude and kinetics of Hog1 phosphorylation, the blots shown in Fig. 1(a–c) were combined for the probing with each antiserum in turn, so as to ensure identical conditions of antibody detection. This also applied to those shown in Figs 2(a) and 3(a, b).

Northern blot analysis of transcript levels and fluorescence microscopy. These were performed as previously described (Mollapour & Piper, 2006, 2007).

RESULTS

At low pH, Fps1p is essential for acetate activation of Hog1p

Earlier we had shown that culture pH strongly affects the kinetics of Hog1p activation by 0.1 M acetic acid in wild-type *S. cerevisiae* (Mollapour & Piper, 2006). In pH 4.5 cultures, Hog1p activation was apparent by 5 min and maximal after 10–40 min, whereas at pH 6.8 this activation was much slower (Fig. 1a, b). Taken in conjunction with measurements showing that acetate labelling of cells is much slower at the higher pH (Mollapour & Piper, 2007), these observations suggested that an intracellular pool of free acetate might be generating the Hog1p induction signal.

The Fps1p channel is the major route for entry of acetic acid into these cells (Mollapour & Piper, 2007). We therefore investigated whether it influences this Hog1p activation in response to acetic acid stress, analysing Hog1p activation by 0.1 M acetic acid in pH 4.5 and pH 6.8 cultures of *fps1Δ* cells that contained either an empty vector (YEp195), a plasmid-borne gene for the wild-type Fps1p, or the gene for an unregulated Fps1p (*fps1-Δ1*). The cells transformed with the native *FPS1* gene plasmid displayed the transient Hog1p phosphorylation (*FPS1*⁺; Fig. 1a, b) that we had observed in earlier work (Mollapour & Piper, 2006, 2007). In contrast, the cells devoid of Fps1p

Fig. 1. Presence of Fps1p is required for Hog1p phosphorylation in response to 0.1 M acetate. (a, b) Kinetics of Hog1p phosphorylation at 30 °C following a 0.1 M acetate challenge of pH 4.5 (a) and pH 6.8 (b) cultures; BY4741*fps1Δ* transformed with empty vector (*fps1Δ*), a plasmid containing the full-length *FPS1* gene (*FPS1+*) or a plasmid bearing the gene for an unregulated Fps1p (*fps1-Δ1*). (c) Kinetics of Hog1p phosphorylation following a 0.1 M NaCl challenge of the pH 4.5 cultures in (a). The blots were probed for dually phosphorylated Hog1p (Hog1p-TP,YP), total Hog1p and Sba1p (the latter a loading control).

(*fps1Δ* transformed with the empty YEp195 vector) lacked discernible Hog1p phosphorylation in response to 0.1 M acetate, both at pH 4.5 and at pH 6.8 (*fps1Δ*; Fig. 1a, b). The cells expressing *fps1-Δ1* [the gene for Fps1-Δ1p, an unregulated, constitutively open form of Fps1p (Luyten *et al.*, 1995; Tamas *et al.*, 2000, 2003)] displayed a Hog1p phosphorylation that had slightly higher basal levels and was also seemingly more sustained than that observed in the cells expressing the wild-type *FPS1* (Fig. 1a, b). Unlike wild-type Fps1p, Fps1-Δ1p is not destabilized by acetate activation of Hog1p, its persistence as an open aquaglyceroporin channel in the plasma membrane causing higher levels of acetic acid uptake and sensitivity relative to the cells that express a wild-type Fps1p (Mollapour & Piper, 2007; and unpublished data).

Hog1p activation in the cells expressing either a normal or an unregulated Fps1p channel was slower at pH 6.8, as compared to pH 4.5, but distinctly more sustained with Fps1-Δ1p present (*FPS1+*, *fps1-Δ1*; Fig. 1a, b). This is

consistent with much slower acetic acid labelling of cultures at the higher pH, and also with the higher levels of acetate labelling with an unregulated Fps1p present in the cells (Mollapour & Piper, 2007).

Fig. 1 reveals that it is the presence of the Fps1p channel that determines whether Hog1p is transiently activated by 0.1 M acetic acid. In the absence of Fps1p, cells of pH 4.5 cultures accumulate much less acetate label (Mollapour & Piper, 2007) and Hog1p is not activated (Fig. 1a, b). With the wild-type Fps1p present, Fps1p is slowly – over a time-course of several minutes – endocytosed in response to the Hog1p activation, thereby downregulating the entry of acetate into the cell (Mollapour & Piper, 2007). In contrast, when Fps1p is refractory to this degradation (*fps1-Δ1* expression), the Hog1p activation is more sustained (Fig. 1a, b). This indicates that acetate entry into the cell leads to the Hog1p induction signal, consistent with this signal being caused by an intracellular pool of free acetate (see Discussion).

Fig. 2. Acetate concentration affects the kinetics and Fps1p dependence of Hog1p and *GPD1* activation by acetate. (a) Analysis of the kinetics of Hog1p phosphorylation. (b) Northern blot analysis of *GPD1* transcript levels following challenge of pH 6.8 wild-type (wt) and *fps1Δ* cultures with either 0.1 or 0.5 M acetate, or with 0.5 or 1.0 M NaCl [the 0.5 M acetate result being that in (Mollapour & Piper 2006)]. U, untreated. (c) Visualization of Fps1-GFP in wild-type cells following (top to bottom) 0, 60 or 120 min of inhibitory 0.5 M acetate challenge at pH 6.8. Unlike with inhibitory acetate stress at pH 4.5 (Mollapour & Piper, 2007), Fps1-GFP is not endocytosed to the vacuole at this pH but remains at the plasma membrane. (d) The BY4741 wild-type (wt) and *hog1Δ*, *fps1Δ*, *hog1Δ fps1Δ*, *hog1Δ sln1Δ*, and *hog1Δ fps1Δ sln1Δ* mutants spotted in 1 : 10 dilution series onto pH 6.8 YPD plates, lacking or containing 0.5 M acetic acid, and then grown for 3 days at 30 °C.

Hog1p activation by osmotic stress is primarily a turgor-sensing process, similar in wild-type and *fps1Δ* mutant cells (Tamas *et al.*, 2000) (see below). It has been reported, though, that cells that express a non-regulated Fps1p also

exhibit more sustained Hog1p activation by osmotic stress (Hedfalk *et al.*, 2004; Tamas *et al.*, 2000). We therefore analysed Hog1p activation by mild salt (0.1 M NaCl) stress of our pH 4.5 cultures (Fig. 1c). With this 0.1 M NaCl

Fig. 3. Fps1p and Hog1p exert opposing influences on Slt2p phosphorylation in response to acetate stress. (a) Levels of phosphorylated (Slt2p-TP,YP) and total Slt2p (Slt2p) in pH 4.5 cultures of BY4741 wild-type, *fps1Δ* and *hog1Δ* single mutants, and a *fps1Δ hog1Δ* double mutant, measured either before (–) or 20 min after (+) the addition of 0.1 M acetate. (b) Slt2p phosphorylation in pH 4.5 cultures of the BY4741 wild-type and BY4741-derived mutants lacking either membrane sensors (Wsc1–4p, Mid2p or Mtl1p) or Rom2p, before (–) and after (+) 60 min exposure to 0.1 M acetate. (c) BY4741 wild-type (wt) and *fks1Δ*, *slt2Δ*, *wsc1Δ* mutant cells spotted in 1:10 dilution series onto pH 4.5 YPD plates, lacking or containing acetic acid, and then grown for 3 days at 30 °C.

addition, Hog1p activation was extremely transient and appreciably more rapid than with 0.1 M acetic acid. Consistent with the earlier findings (Hedfalk *et al.*, 2004; Tamas *et al.*, 2000), the presence of the unregulated Fps1p appeared to be causing the Hog1p activation by 0.1 M NaCl to be more sustained, while slight Hog1p activation by 0.1 M NaCl was now apparent even in the absence of Fps1p (Fig. 1c).

At pH 6.8, when much higher concentrations of acetate are needed to inhibit growth, Fps1p still partially influences acetate resistance and the acetate activation of Hog1p

The above findings reveal that the Hog1p activation by 0.1 M acetic acid is, whether studied at pH 4.5 or pH 6.8, slow and Fps1p-dependent. This contrasts with the Hog1p activation by 0.4 or 0.8 M salt (NaCl) stress, which is rapid (complete within 1–5 min) and largely independent of Fps1p (Tamas *et al.*, 1999, 2000). Even with a mild salt stress (0.1 M NaCl), Hog1p activation is at least partially independent of Fps1p (Fig. 1c). Furthermore, an inhibitory (0.1 M) acetic acid stress at pH 4.5 does not – unlike a salt

stress – cause the induction of *GPD1* gene transcripts or intracellular glycerol (Mollapour & Piper, 2006). At pH 6.8, when acetate enters the cells much more slowly than at pH 4.5 (Mollapour & Piper, 2007), a moderate *GPD1* gene induction in response to 0.1 M acetate was apparent, but this, like the Hog1p activation, was relatively slow and Fps1p-dependent (Fig. 2b). In this situation there are also increases in intracellular glycerol (Mollapour & Piper, 2006).

We found these events of Hog1p and *GPD1* activation in pH 6.8 cultures to be appreciably more rapid using 0.5 M, rather than 0.1 M, acetate as the inducer (Fig. 2). At pH 6.8, 0.5 M acetate is needed to achieve an appreciable inhibition of growth, whereas at pH 4.5 – when acetic acid is a much more effective inhibitor – just 0.1 M acetate is inhibitory (Mollapour & Piper, 2006). With such inhibitory (0.5 M) acetate treatment at pH 6.8, Fps1-GFP is not endocytosed but, even in *HOG1*⁺ cells, remains at the plasma membrane, adopting a more punctate appearance in response to the stress (Fig. 2c). Fps1-GFP adopting a punctate appearance is also seen in cells exposed to 1 M NaCl stress (Mollapour & Piper, 2007).

Unlike with 0.1 M acetate at pH 6.8, this more severe (inhibitory, 0.5 M) acetate stress generated an appreciable Hog1p activation and *GPD1* gene induction, even in cells lacking Fps1p (compare *fps1Δ* and wild-type, Fig. 2 a, b). The loss of Fps1p was still exerting an effect, though, acting to slow these events of Hog1p and *GPD1* activation (Fig. 2 a, b). Therefore, Hog1p and *GPD1* activation are both slow and completely Fps1p-dependent when induced by 0.1 M acetate at pH 6.8 (Figs 1a, b; Fig. 2), but much more rapid and only partially influenced by the loss of Fps1p when induced by higher, inhibitory acetate stress at this same pH (0.5 M; Fig. 2a, b). *hog1Δ* and *hog1Δ sln1Δ* mutants are sensitive to a 0.5 M acetate stress at pH 6.8 (Fig. 2d). However, the loss of Fps1p provided a significant rescue of these enhanced sensitivities of *hog1Δ* and *hog1Δ sln1Δ* cells to 0.5 M acetate (compare *hog1Δ* to *hog1Δ fps1Δ*; also *hog1Δ sln1Δ* to *hog1Δ fps1Δ sln1Δ* cells; Fig. 2d).

These influences of loss of the Fps1p channel suggest that, with relatively mild (0.1 M) acetate stress at pH 6.8, the acid must first undergo Fps1p-mediated entry into the cell in order to generate the Hog1p activation signal. Activation is slower at pH 6.8 compared to pH 4.5 (Fig. 1a) due to the slower entry of the acid into the cell [a much smaller fraction of the acetic acid (pK_a 4.76) is now in undissociated form]. At pH 6.8, the higher acetate levels needed to inhibit growth generate a more rapid Hog1p activation that is now only partly dependent on Fps1p (Fig. 2a), a Hog1p activation that – except in those cells lacking Fps1p – contributes to resistance (Fig. 2d). The Fps1p-independent component of this Hog1p phosphorylation (Fig. 2a) most probably corresponds to a turgor-sensing activation, as analysed in the numerous studies of the classical hyperosmotic stress response (Hohmann *et al.*, 2007).

Fps1p and Hog1p also influence Slt2p phosphorylation under acetate stress

Both the Hog1p and Slt2p MAP kinases of *S. cerevisiae* are activated by acetic acid stress, but only Hog1p is required for acetic acid resistance (Mollapour & Piper, 2006). Also, while culture pH strongly affects the kinetics of Hog1p phosphorylation in response to acetic acid stress (Fig. 1a, b), it has a much smaller effect on the commensurate phosphorylation of Slt2p (see Fig. 4a of Mollapour & Piper, 2006).

Fig. 3 shows the effects of loss of Hog1p and/or Fps1p on Slt2p phosphorylation in response to 0.1 M acetic acid at pH 4.5. Phosphorylation of Slt2p was moderately reduced by the lack of Hog1p, but dramatically enhanced by the absence of Fps1p. In contrast to Hog1p, which was not activated by acetic acid in pH 4.5 *fps1Δ* mutant cultures (Fig. 1), Slt2p displayed an appreciable basal activation and elevated activation by acetate stress in *fps1Δ* cells (Fig. 3). Other studies have identified that the *fps1Δ* mutant has unusually high levels of Slt2p phosphorylation, even in the absence of stress (Davenport *et al.*, 1995; Tamas *et al.*,

1999). The activity of Slt2p does appear to be essential in the *fps1Δ* mutant, as *fps1Δ* is synthetically lethal with *slt2Δ* (Philips & Herskowitz, 1997; Tong & Boone, 2006). However, while the unusually high acetic acid activation of Slt2p in *fps1Δ* cells (Fig. 3a) might be thought to contribute to the enhanced acetate resistance displayed by this mutant (Mollapour & Piper, 2007), this seems unlikely as excessive Slt2p activity is generally highly detrimental in yeast (Martin *et al.*, 2000).

In both *fps1Δ* and *FPS1⁺* cells, loss of Hog1p reduced the enhanced Slt2p phosphorylation of unstressed *fps1Δ* cultures, as well as the acetate-induced increase in levels of phosphorylated Slt2p (compare *fps1Δ HOG1⁺* and *fps1Δ hog1Δ*; Fig. 3a). Previous studies (Davenport *et al.*, 1999; Garcia-Rodriguez *et al.*, 2005; Hahn & Thiele, 2002; Mager & Siderius, 2002) have indicated a substantial degree of cross-talk between the cell integrity (Slt2p) and osmosensing (Hog1p) MAP kinase pathways. Despite this, it would appear that a fraction of the Slt2p activation by acetate stress is independent of Hog1p (Fig. 3a).

Wsc1p is the main membrane sensor for the acetate activation of Slt2p

The Slt2 MAP kinase pathway is involved in cell wall remodelling and the maintenance of cell integrity (reviewed by Levin, 2005). The Wsc1–4p, Mid2p and Mtl1p membrane sensors are upstream components of this pathway. These, in some cases, have been shown to signal through the Rho1p GTPase. Analysing mutants lacking each of these sensors in turn for acetic acid-induced phosphorylation of Slt2p, it was apparent that the major effect was with the loss of Wsc1p (Fig. 3b). In *wsc1Δ* cells there was no acetate activation of Slt2p (Fig. 3b). Unexpectedly, both basal and acetate-induced activations of Slt2p were enhanced by the loss of Wsc2p or Rom2p (the latter a GDP/GTP exchange protein that converts the GDP-bound inactive form of Rho1p and Rho2p to the GTP-bound active form) (*wsc2Δ* and *rom2Δ*; Fig. 3b). The C-terminal cytoplasmic domains of Wsc1p (also Mid2p) interact with Rom2p (Philip & Levin, 2001). Acetate-induced activation of cell integrity signalling is therefore strongly dependent on the Wsc1p sensor and amplified with the loss of negative regulation of Rho1p GTPase activity. The *wsc1Δ* and *slt2Δ* mutants (defective in cell integrity signalling with acetate) were found not to be acetate sensitive (Fig. 3c), though appreciable acetate sensitivity was apparent in a mutant with lowered activity of glucan synthase (*fks1Δ*; Fig. 3c).

DISCUSSION

In low-pH cultures, acetic acid is largely in the undissociated state, a form that can readily traverse the Fps1p channel (Mollapour & Piper, 2007). Following its entry to the cell, this acid dissociates in the higher pH environment of the cytosol. Since glucose-repressed *S. cerevisiae* cannot

readily assimilate acetate into acetyl-CoA (see Introduction) and the charged acetate anion cannot readily diffuse from the cell, this influx of acid causes cells to accumulate a large pool of free acetate. It is this that probably leads to both the transient activation of Hog1p (Fig. 1) and, at still higher levels, to an apoptotic cell death (Giannattasio *et al.*, 2005; Ludovico *et al.*, 2001).

Hog1p activation by acetic acid (Fig. 1a, b), heat (Winkler *et al.*, 2002) or osmotic stress (Tamas *et al.*, 2000) is transient, due to the high-osmolarity glycerol (HOG) signalling pathway being controlled by strong feedback regulation. Depending on the nature of the stress, such feedback regulation may be acting on both the events of HOG-pathway activation and the Hog1p-deactivating phosphatases. Studies of the activation of this pathway by the membrane-bound Sln1p and Sho1p osmosensors, also in *fps1Δ* mutants (Maeda *et al.*, 1994; Tamas *et al.*, 2000; Tao *et al.*, 1999), have revealed the Hog1p activation by osmostress to be largely independent of Fps1p. Instead, it involves the sensing of a change in membrane tension, i.e. turgor stress.

It would appear that, when acetate is the inducer, this system for Hog1p activation may operate only at high concentrations. Even with inhibitory (0.5 M) acetate at pH 6.8, Fps1p still strongly influences the kinetics of Hog1p activation (Fig. 2a), this Hog1p – except in cells lacking Fps1p – then contributing to acetate resistance (Fig. 2d). In contrast, the activation of this MAP kinase by lower (0.1 M) acetate level is, whether monitored at pH 4.5 or pH 6.8, completely dependent upon the presence of Fps1p; therefore Fps1p-facilitated entry of the acetate into the cell (Figs 1 and 2). The more sustained acetate accumulation (Mollapour & Piper, 2007) and Hog1p activation (Fig. 1) when cells express a constitutively open Fps1p channel, refractory to degradation (*fps1-Δ1*), is also consistent with the signal for the Hog1p induction being intracellular accumulation of acetate.

Hydration of a large intracellular pool of the acetate anion also has the potential to create a high intracellular turgor pressure, more likely to cause water entry into the cell (as with hypoosmotic stress), rather than the water loss of hyperosmotic stress. Hypotonic shock does indeed cause a very fast, transient activation of Slt2p (Davenport *et al.*, 1995). In contrast, the phosphorylation of Slt2p with acetate stress is slow (Mollapour & Piper, 2006), as is acetate labelling of these cells (Mollapour & Piper, 2007). That this Slt2p phosphorylation is greatly enhanced by the loss of Fps1p (Fig. 3) is an indication that – unlike activation of HOG pathway signalling – activation of cell integrity signalling is suppressed by Fps1p-facilitated entry of the acetate into the cell, and therefore intracellular acetate accumulation.

Acetate-induced activation of Slt2p is dependent on Wsc1p (Fig. 3), the sensor that also signals activation of this MAP kinase in response to high temperature (Gray *et al.*, 1997; Verna *et al.*, 1997), alkaline pH (Serrano *et al.*, 2006) and

the 1,3-β-glucan synthase inhibitor caspofungin (Reinoso-Martin *et al.*, 2003). *S. cerevisiae* has two 1,3-β-glucan synthases, encoded by *FKS1* (expressed during vegetative growth) and *FKS2* (mainly expressed during sporulation). Cells of the *fks1Δ* mutant show an elevated basal level of phospho-Slt2p (Reinoso-Martin *et al.*, 2003) and compensate for their reduced cell wall glucan content by increasing their chitin content as well as the expression of the second glucan synthase gene, *FKS2*. This has raised the suggestion of a signalling feedback from the cell wall via Wsc1p to monitor the activity of Fks1p (Zhao *et al.*, 1998). Therefore, the Wsc1p-dependent phosphorylation of Slt2p in response to acetate stress (Fig. 3b) may reflect this stress, causing the inhibition of glucan synthase activity. Wsc1p colocalizes with Fks1p (Delley & Hall, 1999), and *fks1Δ* mutant cells are sensitive to acetate stress, even though the *wsc1Δ* and *slt2Δ* mutants are not acetate sensitive (Fig. 3c). It therefore appears that, while survival of acetate stress does not require an activation of Slt2p cell integrity pathway signalling, it may require the maintenance of glucan synthase activity and cell integrity.

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