The yeast endosomal Na⁺/H⁺ exchanger, Nhx1, confers osmotolerance following acute hypertonic shock

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Osmotolerance in yeast is regulated by at least two distinct mechanisms. The acquired response occurs following long-term exposure to hypertonic medium and requires the induction of the HOG-MAP (high-osmolarity glycerol mitogen-activated protein) kinase cascade to increase levels of the osmolyte glycerol. The acute response occurs following sudden exposure to high osmotica and appears to be dependent on normal vacuole function. In this study it is reported that the yeast endosomal/prevacuolar Na⁺/H⁺ exchanger Nhx1 contributes to osmotolerance following sudden exposure to hyperosmotic media. Vacuolar shrinkage and recovery in response to osmotic shock was altered in the $\Delta nhx1$ null mutant. Our results also show that the osmotolerance conferred by Nhx1 contributes to the postdiauxic/stationary-phase resistance to osmotic stress and allows for the continued growth of cells until the acquired osmotolerance response can occur.

Keywords: Na⁺/H⁺ exchanger, osmotolerance, hypertonic shock, vacuole, *Saccharomyces cerevisiae*

INTRODUCTION

Saccharomyces cerevisiae responds to hyperosmotic stress by increasing the production and retention of glycerol (Brown *et al.*, 1986; Blomberg *et al.*, 1988; Blomberg & Adler, 1992; Albertyn *et al.*, 1994a, b). The intracellular glycerol concentration can rise to over 2 M and is largely dependent on the induction of NADH-dependent glycerol-3-phosphate dehydrogenase (GPDH), the activity of which can rise 30-fold (Blomberg & Adler, 1992; Albertyn *et al.*, 1994a; Serrano, 1996). The induction of GPDH is regulated by the high-osmolarity glycerol (HOG) response pathway (Brewster *et al.*, 1993). Since significant glycerol production does not begin for several hours following exposure to the osmotic stress, it has been proposed that another regulatory mechanism must occur to protect cells from an immediate osmotic challenge (Blomberg *et al.*, 1988; Latterich & Watson, 1993).

The yeast vacuole is involved in osmoregulation (Matile,

1978; Banta *et al.*, 1988) and may play a role in protecting cells from a sudden osmotic challenge. Many vacuole mutants that have altered morphology, or have defects in protein sorting, are also sensitive to osmotic stress (Banta *et al.*, 1988; Latterich & Watson, 1991, 1993). Thus a yeast mutant defective in vacuole biogenesis loses viability within 10 s exposure to 1.5 M NaCl (Latterich & Watson, 1993).

Tolerance to osmotic shock is dependent on the physiological state of the cells. Exponential-phase cells are much more sensitive to osmotic shock relative to the slow-growing postdiauxic-phase cells or non-growing stationary-phase cells (Brown *et al.*, 1986; Mackenzie *et al.*, 1986; Blomberg *et al.*, 1988; Mager & Varela, 1993; Werner-Washburne *et al.*, 1993). Although this phenomenon remains largely unexplained, the non-reducing disaccharide trehalose may partially contribute toward osmotolerance; thus osmoresistance in yeast correlates with an increase in the intracellular content of this sugar, which accumulates during stationary phase (Crowe *et al.*, 1984; Hottiger *et al.*, 1987). However, trehalose levels alone may not be sufficient to counter the large increase in osmotolerance (Andre *et al.*, 1991).

Higher eukaryotes regulate intracellular ion concentrations in response to an immediate osmotic challenge. The vacuole in plant cells is involved in regulating

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Abbreviations: GPDH, NADH-dependent glycerol-3-phosphate dehydrogenase; HOG, high-osmolarity glycerol; MAP, mitogen-activated protein.

turgor pressure (Zimmermann, 1978; Boller & Wiemken, 1986). In halophytes, Na^+ accumulation (with Cl^- and water) in the vacuoles of the leaf protects the cells from the detrimental effects of high salt and osmolarity (Flowers *et al.*, 1986). The transport of Na^+ into the tonoplast is believed to be mediated via a Na^+/H^+ antiporter, which is driven by the H^+ gradient generated by the vacuolar H^+ -ATPase (Blumwald & Poole, 1987; Garbarino & DuPont, 1988; Barkla & Blumwald, 1992).

In mammalian cells, plasma membrane Na^+/H^+ exchangers are activated in response to both short- and long-term hyperosmotic stress (Grinstein *et al.*, 1989; Soleimani *et al.*, 1995; Wakabayashi *et al.*, 1997). Although the exact molecular mechanisms involved in exchanger activation are unknown, the exchanger is believed to become activated in response to cell shrinkage (Donowitz *et al.*, 1996; Wakabayashi *et al.*, 1997). The decrease in the cell volume causes the exchanger to become more sensitive to intracellular protons, and the activation of the exchanger causes an increased influx of Na^+ (and Cl^-). With the obligatory influx of water, cell volume is restored (Grinstein *et al.*, 1985, 1992; Hoffmann & Simonsen, 1989).

We have recently identified a novel intracellular Na^+/H^+ exchanger in yeast, Nhx1, which contributes to cellular Na^+ homeostasis (Nass *et al.*, 1997). We have localized the exchanger to the late endosome/prevacuolar compartment (PVC) and have proposed that it may be involved in Na^+ transport, water movement and vesicle volume regulation (Nass & Rao, 1998). Consistent with these functions, we have shown that the exchanger colocalizes with Gef1, the yeast homologue of the CLC family of chloride channels, and is required for salt amelioration conferred by overexpression of an *Arabidopsis* vacuolar H^+ pyrophosphatase in yeast (Gaxiola *et al.*, 1999). Considering the important role of Na^+/H^+ exchangers in the osmoregulatory response of plants and mammalian cells, and the similarities in ion homeostasis in plants and yeast, we asked whether Nhx1 contributes to osmotolerance. Our results indicate that Nhx1 plays a role in the recovery of cell growth following exposure to hyperosmotic media. Furthermore, we show that the osmotolerance conferred by Nhx1 contributes to the postdiauxic-phase resistance to osmotic stress, and allows for the continued growth of cells until acquired osmotolerance can occur.

METHODS

Yeast strains and media. The two strains used in this study, K601 (wild-type) and R100 ($\Delta nhx1$) are isogenic to W303 and have been described previously (Nass *et al.*, 1997). APG is a synthetic minimal medium containing 10 mM arginine, 8 mM phosphoric acid, 2% glucose, 2 mM MgSO_4 , 1 mM KCl, 0.2 mM CaCl_2 and trace minerals and vitamins, as described previously (Nass *et al.*, 1997). APGal medium is identical to APG except that 2% galactose is substituted for glucose. Where indicated, sorbitol was added or the pH was adjusted by the addition of acetic acid.

Growth assays. Seed cultures of K601 and R100 were grown at 30 °C in APG medium, adjusted to pH 4 with dilute acetic acid. The cultures were diluted tenfold and grown for 72 h in fresh medium. Postdiauxic-phase cells were then diluted 100-fold in APG medium, pH 4, and growth for 60 h on a shaker at 290 r.p.m. in the absence or presence of 1 M sorbitol was monitored at 600 nm. Where indicated, cultures were grown to the appropriate growth phase and then equivalent numbers of cells were inoculated into 1 ml medium containing a range of sorbitol concentrations in a multiwell plate. Growth was monitored by measuring OD_{600} after culturing for the indicated times at 30 °C. Growth assays shown are representative of one of at least three independent experiments. Relative growth is the OD_{600} at any sorbitol concentration, divided by the OD_{600} of the culture grown in the absence of sorbitol, expressed as percentage.

Confocal imaging. Cultures of K601 and R100 were grown to late exponential phase, loaded with the styryl dye FM 4-64 and examined by confocal microscopy as described previously (Nass & Rao, 1998). Cells were collected by centrifugation for 20 s at 5000 r.p.m., resuspended in APG medium, pH 4, containing 2 M sorbitol and incubated at 23 °C for the indicated times.

RESULTS

Nhx1 confers osmotolerance following hypertonic stress

Hypertonic stress alters the specific activity of Na^+/H^+ exchangers in mammalian cells. A rise in external osmolarity increases the activity of NHE1 and NHE4, while the activity of NHE3 is inhibited (Bookstein *et al.*, 1994; Grinstein *et al.*, 1992; Nath *et al.*, 1996; Soleimani *et al.*, 1995). The activity of NHE2 is also affected by exposure to hyperosmotic medium in a manner dependent on the expression system (Kapus *et al.*, 1994; Nath *et al.*, 1996). To determine if the yeast Na^+/H^+ exchanger Nhx1 also contributes to osmotolerance, we examined the growth of the $\Delta nhx1$ mutant and its isogenic wild-type control in response to increasing osmotic stress in APG medium, pH 4, using varying concentrations of sorbitol. The wild-type control attained up to threefold higher cell density relative to the $\Delta nhx1$ mutant under the hyperosmotic conditions tested (Fig. 1). Half-maximal growth (IC_{50}) was observed at 1 M sorbitol for the $\Delta nhx1$ strain and 1.3 M sorbitol for wild-type. In medium adjusted to pH 7, there was no effect of the $\Delta nhx1$ mutation on osmotolerance (K. Wells & R. Rao, unpublished observations), suggesting that Nhx1-mediated osmotolerance requires a pH gradient. We have previously noted a similar absence of the salt-tolerant phenotype of Nhx1 at neutral pH (Nass *et al.*, 1997).

Nhx1-mediated osmotolerance occurs on a derepressing carbon source, and in stationary-phase cells but not in exponential-phase cells

Intracellular glycerol is believed to be the major osmolyte in protecting cells from long-term exposure to osmotic stress (Blomberg & Adler, 1989; Albertyn *et al.*,

Fig. 1. *Nhx1* contributes to osmotolerant growth in hypertonic medium. Postdiauxic-phase cultures of K601 (□) and R100 ($\Delta nhx1$; ◇) were generated as described in Methods and inoculated into 1 ml cultures of APG medium, pH 4, supplemented with varying amounts of sorbitol as shown. Growth was monitored at 600 nm following incubation at 30 °C for 33 h; relative growth is expressed as a percentage of control (no sorbitol added).

1994a). To reduce the contribution of glycerol, we examined cell growth in the presence of the derepressing sugar galactose, which limits glycerol production (Fraenkel, 1982; Rios *et al.*, 1997). Consistent with previous observations, cells inoculated in galactose media were more sensitive to osmotic stress, with half-maximal growth occurring at 800 mM sorbitol (Fig. 2a) (Rios *et al.*, 1997). However, as in glucose-containing medium, wild-type cells were more osmotolerant than the isogenic *nhx1* null mutant (up to fourfold higher cell density relative to the $\Delta nhx1$ mutant; Fig. 2b). These results indicate that the osmotolerance conferred by *Nhx1* is not directly dependent on glycerol production and can occur in medium containing a derepressing sugar.

Tolerance to various physical and environmental stresses has been correlated to the growth phase of yeast cells. Thus, slow growing postdiauxic-phase cells, or non-proliferating stationary-phase cells, often show increased osmotolerance relative to actively dividing exponential-phase cells (Schenberg-Frascino & Moustacchi, 1972; Elliott & Futcher, 1993; Werner-Washburne *et al.*, 1993). A similar effect of growth phase has been observed following exposure of cells to sudden osmotic shock (Mackenzie *et al.*, 1986; Blomberg *et al.*, 1988; Werner-Washburne *et al.*, 1993). Fig. 2 shows that *Nhx1* confers significant osmotolerance when the inoculum contains postdiauxic-/stationary-phase cells but not with exponential-phase cells. Similar results were seen in cultures grown in glucose-containing medium (data not shown). These results indicate that *Nhx1* plays a significant role in the recovery of postdiauxic-

Fig. 2. Effect of growth phase and derepressing carbon source on osmotolerant growth. Seed cultures of K601 (□) and R100 ($\Delta nhx1$; ◇) were either in exponential phase (OD_{600} 0.2–0.3; a) or stationary phase (OD_{600} 1.7–2.7; b). Equal numbers of cells were inoculated into APG medium containing 2% galactose in place of glucose. Growth was monitored at 600 nm after 48 h of growth at 30 °C; relative growth is expressed as a percentage of control (no sorbitol added).

/stationary-phase cells following exposure to sudden osmotic shock.

***Nhx1* plays a role in the short-term response to hyperosmotic shock but not in long-term acquired osmotolerance**

To determine whether *Nhx1* may play a role in protecting cells from sudden exposure to osmotic dehydration, we followed duplicate growth curves in APG, pH 4, and monitored growth at or before 40 h (short-term) and at 90 h (long-term). The inoculum contained postdiauxic-phase cells. As shown earlier (Fig. 1), there was a difference in cell density of the $\Delta nhx1$ mutant relative to wild-type after 40 h. However, this difference in growth was completely eliminated

Fig. 3. For legend see facing page.

after 90 h, indicating that the absence of Nhx1 does not affect viability or long-term acquired osmotolerance (data not shown). For example, in cultures containing 1.4 M sorbitol, the cell density of the mutant was threefold lower than wild-type after 40 h of growth, but was identical to wild-type after 90 h. We conclude that Nhx1 plays a significant role in the events following sudden exposure to hyperosmotic media.

Nhx1 contributes to vacuolar shrinkage upon exposure to hypertonic stress

Yeast cells can alter their cell volume in response to osmotic challenge, decreasing volume in response to hypertonic stress and increasing volume in the presence of hypotonic stress (Niedermeyer *et al.*, 1977; Morris *et al.*, 1986; Blomberg & Adler, 1992). These responses are reversible, and in the case of dehydration, occur within a minute (Morris *et al.*, 1986). The vacuolar size also decreases following exposure to hypertonic solutions (Morris *et al.*, 1986; Blomberg & Adler, 1992). To determine if Nhx1 contributes to changes in vacuolar volume, we directly visualized vacuole size *in vivo*, by confocal microscopy, after acute exposure to hyperosmotic medium. Vacuoles in living cells were labelled with the styryl dye FM 4-64, which intercalates into the plasma membrane and is internalized by endocytosis, accumulating in the acidic vacuolar compartment (Vida & Emr, 1995). In the absence of hypertonic stress, vacuoles from wild-type or the $\Delta nhx1$ mutant had a similar appearance (Fig. 3a–f). Upon exposure to hypertonic medium, vacuoles of wild-type cells shrank within 6 min, with the surface rapidly changing from smooth to crenellated (Fig. 3g–i). Longer-term exposure to sorbitol (Fig. 3m–o) or growth for several days in 1 M sorbitol (data not shown) largely reversed these changes, with persistent staining of perivacuolar vesicles that are likely to represent the prevacuolar compartment as observed previously (Nass & Rao, 1998). In contrast, the majority of $\Delta nhx1$ cells showed significantly smaller changes in vacuolar volume and appearance in the short-term response to hyperosmotic challenge (Fig. 3j–l). Furthermore, the changes in vacuolar structure persisted for longer times in the mutant (Fig. 3p–r), suggesting that the response to osmotic stress was delayed or compromised in the absence of Nhx1. These observations suggest that Na^+/H^+ exchange by Nhx1 may be involved in regulating vacuolar volume in response to hypertonic stress.

Deletion of Nhx1 increases lag time and decreases exponential growth under osmotic stress

Slow-growing cells are often more resistant to environmental stresses relative to fast-growing cells (Elliott & Futcher, 1993). However, the rate of growth and its

Fig. 4. Altered growth profile of $\Delta nhx1$ cells in hyperosmotic medium. Seed cultures of K601 and R100 were grown for 48 h in APG medium, pH 4, diluted tenfold and grown for an additional 72 h in fresh medium. The postdiauxic-phase cells (OD_{600} 1.0–1.1) were then diluted 100-fold in APG medium in the absence or presence of 1 M sorbitol. Growth was monitored at 600 nm for 60 h. $\square$, wild-type, no sorbitol; $\diamond$, $\Delta nhx1$, no sorbitol; $\circ$, wild-type, 1 M sorbitol; $\triangle$, $\Delta nhx1$, 1 M sorbitol.

relation to osmotolerance is debatable. To determine the effects of osmotic stress on the growth rate, we examined growth of wild-type and $\Delta nhx1$ strains in APG medium, pH 4, in the presence and absence of 1 M sorbitol. The growth rates and growth profiles of wild-type and $\Delta nhx1$ cells were very similar under non-stressed conditions but dramatically different in 1 M sorbitol (Fig. 4). As expected, growth in 1 M sorbitol increased the time it took for wild-type cells to enter exponential phase, consistent with other results (Chowdhury *et al.*, 1992; Albertyn *et al.*, 1994a). However, in both media, wild-type cells entered exponential phase at an OD_{600} of 0.1 and passed through the diauxic shift at an OD_{600} of 0.8. In contrast, the $\Delta nhx1$ strain had a prolonged lag phase and a period of about 10 h of extremely slow growth in hyperosmotic medium. Furthermore, relative to wild-type, the mutant cells entered exponential phase later (at an OD_{600} of 0.3), went through little more than one doubling to an OD_{600} of 0.7 before the diauxic transition and reached saturation at a lower cell density than wild-type. These results reiterate that Nhx1 plays a significant role in the response of postdiauxic/stationary-phase cells to osmotic stress, dramatically decreasing their growth rate over much of their early life cycle. Thus, even though the $\Delta nhx1$ strain grew more slowly than wild-type, it was not more osmotolerant; in fact it was more osmo-sensitive. These results are consistent with the data in Figs 1 and 2: the mutant takes a much longer time to acclimatize to the osmotic stress and growth resumes at a slower rate.

Fig. 3. Effect of hyperosmotic shock on vacuolar morphology. Confocal microscopy of living yeast from wild-type (a–c, g–i, m–o) and $\Delta nhx1$ (d–f, j–l, p–r) cells as described in Methods. Images are of cells in APG medium (a–f), or following incubation in APG medium containing 2 M sorbitol for 4–5 (g–i) or 102–105 min (m–r). (a, d, g, j, m, p) Contrast images of yeast cells; (b, e, h, k, n, q), vacuoles stained red with FM 4-64; (c, f, i, l, o, r) overlays. Bars, 5 μm .

DISCUSSION

Yeast deal with an osmotically challenging environment via at least two separate mechanisms, depending on whether the challenge is chronic or acute (Latterich & Watson, 1993). The chronic response, or acquired osmotolerance, is primarily due to the activation of the HOG-MAP (mitogen-activated protein) kinase cascade with increased production and specific activity of GPDH (Brewster *et al.*, 1993; Latterich & Watson, 1993; Serrano, 1996). This enzyme is responsible for production of the osmolyte glycerol, which counters long-term osmotic stress and is therefore essential for growth under high osmotic conditions (Blomberg & Adler, 1992; Albertyn *et al.*, 1994b; Serrano, 1996). The acute response occurs during sudden exposure of non-osmotically challenged cells to an osmotically stressful environment (Blomberg *et al.*, 1988; Latterich & Watson, 1993). The acute response does not appear to be connected with the chronic response since cells retain little or no glycerol during non-osmotically stressed conditions, and the activity and cellular content of glycerol or GPDH does not correlate with the ability of cells to respond to a sudden osmotic shock (Blomberg *et al.*, 1988; Latterich & Watson, 1993). Previously, no molecular mechanism has been identified in yeast that is directly involved in the acute response to osmotic shock.

In this study we examined the role of the endosomal Na^+/H^+ exchanger Nhx1 under osmotically stressful conditions. Following inoculation of wild-type and $\Delta nhx1$ cells from a postdiauxic-phase culture into sorbitol-supplemented medium, the mutant showed a reduction in osmotolerant growth, indicating that Nhx1 does play a role in the response to hyperosmotic stress. In the absence of osmotic stress, the wild-type and $\Delta nhx1$ mutant grew to similar cell densities; this is similar to the effect of null mutants of the MAP kinase cascade required for glycerol accumulation in acquired osmotolerance (*HOG1* or *PBS2*; Brewster *et al.*, 1993), and a null mutant required for vacuole biogenesis and the acute response to hyperosmotic shock (*ssv1-2*; Latterich & Watson, 1993). Interestingly, the reduction in growth of the $\Delta nhx1$ mutant in sorbitol-containing medium was observed within 30–40 h post-inoculation, but upon further growth to saturation, similar cell densities were observed for both mutant and wild-type. These results show that the $\Delta nhx1$ mutant is compromised in its ability to initially respond to the osmotic stress, but following prolonged exposure can apparently acclimatize and begin growing to levels similar to wild-type.

The activity and induction of the glycerol-producing enzyme GPDH is highest when cells use glucose as the carbon source (Rios *et al.*, 1997; Serrano, 1996). Thus, carbon catabolite repression conditions, in which cells are grown in glucose, produce the greatest amounts of glycerol (Serrano, 1996). We examined the contribution of Nhx1 to osmotolerance using the partially derepressing carbon source galactose (Fraenkel, 1982) to minimize the contribution of high intracellular glycerol

concentrations. Consistent with previous conclusions (Rios *et al.*, 1997), galactose-grown cultures of both wild-type and the $\Delta nhx1$ mutant were significantly more sensitive to osmotic stress, and similar to glucose-grown cultures, $\Delta nhx1$ cells were more osmotically sensitive relative to the isogenic control.

Yeast cells pass through distinct physiological phases during growth in glucose media (Blomberg *et al.*, 1988; Werner-Washburne *et al.*, 1993). It has long been observed that postdiauxic-phase and stationary-phase cells are more tolerant to many environmental stresses, including osmotic stress (Schenberg-Frascino & Moustacchi, 1972; Mackenzie *et al.*, 1986; Blomberg *et al.*, 1988; Elliott & Fitcher, 1993; Werner-Washburne *et al.*, 1993). We show here that the physiological state of the cells affects Nhx1-mediated osmotolerance. Exponential-phase cells have greater sensitivity to sorbitol, as observed by the lower IC_{50} of 900 mM in wild-type (Fig. 2a). Furthermore, Nhx1 appears to contribute to osmotolerance only when the cells are in the postdiauxic/stationary phase upon exposure to sorbitol. It is possible that induction of Nhx1 activity or expression may occur at, or following, the diauxic shift, or that there are other mechanisms that contribute to osmotolerance in exponential-phase cells which compensate for the absence of Nhx1.

The yeast vacuole plays an important role in pH and ion homeostasis and contributes to protein trafficking, sorting and degradation (Banta *et al.*, 1988; Klionsky *et al.*, 1990; Bryant & Stevens, 1998). Osmotolerance has been directly linked to normal vacuole morphology and function (Banta *et al.*, 1988; Mager & Varela, 1993; Latterich & Watson, 1993). We show here that the recently identified late endosomal/prevacuolar Na^+/H^+ exchanger Nhx1 contributes to osmotolerance (Nass *et al.*, 1997; Nass & Rao, 1998). Under normal culture conditions, it is likely that Nhx1-mediated sequestration of Na^+ in the late endosome/vacuole provides an osmoticum that serves to retain water in the vacuoles (Morris *et al.*, 1986; Serrano, 1996). The maintenance of turgor pressure in the late endosome and vacuole may be important for the proper trafficking of essential proteins required for osmotic tolerance or normal cellular function.

Yeast cells respond to an immediate hyperosmotic stress by decreasing cell size, possibly through activation of plasma membrane mechanosensitive ion channels (Gustin *et al.*, 1988) and water loss. It is possible that cell-shrinkage-mediated activation of the Na^+/H^+ exchange activity of Nhx1 may allow exit of Na^+ ions, and consequently water, from the vacuoles, contributing to the observed changes in vacuolar morphology (Fig. 4). The flow of water from the vacuole to the cytoplasm may be an important initial response to counter hyperosmotic cell shrinkage. In the long-term, transport of Na^+ into intracellular organelles by Nhx1 would protect sodium-sensitive proteins in the cytoplasm and contribute to the accumulation of osmotic equivalents in the vacuole; this may explain the delayed cell growth and

short exponential phase of the $\Delta nhx1$ mutant. Future studies will need to address the molecular mechanisms of osmosensing and signal transduction involved in the Nhx1-mediated response to hyperosmotic challenge.

ACKNOWLEDGEMENTS

This work was supported by a National Institutes of Health Grant GM 52414 to R.R.

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Received 22 April 1999; revised 29 June 1999; accepted 9 July 1999.