**Saccharomyces cerevisiae** glucose signalling regulator Mth1p regulates the organellar Na⁺/H⁺ exchanger Nhx1p

Keiji MITSUI, Masafumi MATSUSHITA and Hiroshi KANAZAWA

Department of Biological Sciences, Graduate School of Science, Osaka University 1-1 Machikaneyama-cho, Toyonaka City, Osaka 560-0043, Japan

INTRODUCTION

In all eukaryotic cells, the lumina of organelles, such as the Golgi, endosomes, secretory vesicles and lysosomes (vacuoles in fungi and plants) are maintained at distinct acidic pHs. These distinct pH values are important for a variety of physiological processes, such as intracellular transport of small molecules [1,2], activation of lysosomal (vacuolar) hydrolases, post-translational modifications and processing of secreted proteins [3], and trafficking of endocytosed ligands or newly synthesized proteins to their functional sites [4].

V-ATPase (vacuolar H⁺-ATPase) is an ATP-driven proton pump that is found in most organelar membranes and plays an essential role in the acidification of the organelle [5]. However, V-ATPase alone does not account for the differences in pH among various organelles. One possibility is that an organelle-specific proton leakage system regulates luminal pH. In fact, the addition of a V-ATPase inhibitor, such as bafilomycin or concanamycin, results in immediate alkalization of organelles [6,7], supporting the presence of a proton leak system across the organelle membranes. One candidate for this leakage system is an organellar-type NHE (Na⁺/H⁺ exchanger).

NHEs are ubiquitous proteins in the membranes of cells of various species from yeast to humans and higher plants [8]. The NHE proteins are predicted to have 12 transmembrane segments and a hydrophilic tail region [9–16]. The NHEs exchange Na⁺ (or K⁺) for H⁺ across the cell membranes and play an important physiological role in the regulation of intracellular pH and Na⁺ concentration [9–15]. Nine isoforms (NHE1–NHE9) have been described in mammalian cells. NHE1–NHE5 are mainly localized to the plasma membranes [9–13] and NHE6–NHE9 are distributed to the organellar membranes; NHE6 and NHE9 are in early/recycling endosomes, NHE7 is in the trans-Golgi network and NHE8 is in the mid-Golgi stacks [14–16]. We found previously that organelles in mammalian cells transiently overexpressing NHE8 or NHE9 have higher luminal pH than control cells [15]. Knockdown or overexpression of NHE6 causes a decrease or increase of the endosomal pH respectively [17]. Knockdown of the gene encoding a NHE6-interacting protein, RACK1 (receptor for activated C-kinase 1), increases endosomal NHE6 levels and results in alkalization of endosomes [18]. These findings suggest that mammalian organelle-type NHEs contribute to organellar pH homeostasis by a proton-leak system from the organellar lumen in cooperation with the V-ATPase.

The budding yeast *Saccharomyces cerevisiae* also has an organelle-type NHE, Nhx1p. Nhx1p has a primary sequence similar to mammalian NHE6 and is localized in the late endosomes (pre-vacuolar compartments) [19,20]. Yeast cells lacking the NHX1 gene show impaired cell growth and acidification of their vacuolar lumen relative to the wild-type cells when the cells are grown in medium at an acidic pH (pH < 4) [21]. These findings suggest that yeast Nhx1p also contributes to the regulation of the luminal pH of the organelles, and that Nhx1p is a functional yeast orthologue of mammalian NHE6.

Organellar pH is always influenced by changes in intracellular metabolite levels, which in turn depend on carbon sources in the culture medium and uptake of extracellular solutes through endocytosis [22]. Living yeast cells are believed to have mechanisms to maintain their pH homeostasis against such changes. In this regard, it has been shown that V-ATPase...
activity is tightly regulated, depending on the carbon source in the culture medium [23,24]. One mechanism regulating the V-ATPase activity is reversible dissociation of its peripheral-membrane V$_1$ subunit from its membrane-integrated V$_0$ subunit, depending on the presence of carbon sources such as glucose or galactose [23–25]. This mechanism is a rapid and effective way to reduce or increase proton pump activity. Unlike V-ATPase, the dependence of the regulatory mechanisms for Nhxl1p on carbon sources in the culture medium has not been investigated.

We have shown that the mammalian organellar-type NHE6 is regulated by binding of the scaffold protein RACK1 to the hydrophilic tail region [18]. Thus this hydrophilic tail region is believed to play an important role in its regulation [26–30]. On the basis of this finding, we hypothesized that a putative protein interacts with the hydrophilic tail region of yeast Nhxl1p to regulate its activity. By using a yeast two-hybrid technique, we found a new binding partner for Nhxl1p. This protein, Mth1p, has been described as a negative transcriptional regulator of an extracellular glucose-sensing mechanism [31]. In the present study we show that Mth1p regulates Nhxl1p activity, probably by modulating its ion transport activity in response to extracellular carbon sources. This regulation of Nhxl1p activity by Mth1p may contribute to maintain the luminal pH of endosomes regardless of changes in extracellular carbon sources.

**Experimental**

**Strains and culture conditions**

Yeast _S. cerevisiae_ strains MKY05121, MKY0813 and MKY0814, bearing deletions of _NHX1_, _MTH1_, and both _NHX1_ and _MTH1_ respectively, were derived from W303–1B (MATa ade2–1 trp1–1 can1–100 leu2–3/112 his3–11/15 ura3–1). Deletion of _NHX1_ or _MTH1_ was performed by one-step gene replacement [32]. The LEU2 or _S. chromosomogenes_ pombe his5 gene was amplified by PCR from pUG73 or pUG27 [33] using the following primer sets respectively: forward #1 (5'-ATGCTATCCAGGTATGTCGAATATACTTCAAAGGTGCGAGCTGTAAGCTTGCAGTCGAC-3') and reverse #1 (5'-CTAGTGTTTGGAGGGAAGGAAATGCTCAGGATGGCTGACATAGGCGCAGTCTGGAGTTGAGC-3') for _NHX1_ knockout; and forward #2 (5'-GAATTCTTATTCCACGCGCATAGTACACACACTAAGGACAGCTGATGTCGAC-3') and reverse #2 (5'-TCTGAGAGCTCAAAAACCATCGGGAAGGTTTCTTTAATGATCGATGCCACTAGTGGATCTG-3') for _MTH1_ knockout. The PCR products were introduced into yeast cells and the resulting Leu$^+$ or His$^+$ transformants were selected on synthetic medium lacking leucine or histidine. Gene knockout was confirmed by PCR amplification of the genomic DNA.

Standard yeast culture medium and genetic manipulations were as described by Sherman [34]. Transformation of yeast cells was performed by the lithium acetate method [34]. All yeast strains were routinely cultured at 30°C in YPAD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 40 mg/l adenine and 2% (w/v) glucose], SD medium [0.17% yeast nitrogen base without ammonium sulfate or amino acids, 0.5% ammonium sulfate and 2% (w/v) glucose] or SCD medium [0.17% yeast nitrogen base without ammonium sulfate or amino acids, 0.5% ammonium sulfate, 0.5% casamino acids and 2% (w/v) glucose] supplemented with appropriate nucleotides and amino acids. SGal and SCGal medium have 2% (w/v) galactose to replace the glucose in SD and SCD medium. SRAf and SGly/Eth medium have 2% (w/v) raffinose, and 3% (v/v) glycerol and 2% (v/v) ethanol respectively to replace the glucose in SD and SCD medium. _Escherichia coli_ strains JM109 and BL21(DE3) were used to propagate the plasmids and to express various proteins. _E. coli_ cells were cultured in lysogeny broth with an antibiotic appropriate for selection of transformants, as described previously [35].

**Plasmid construction for expression of recombinant peptides in _E. coli_ cells**

For expression of recombinant GST (glutathione transferase)–NHX1ct (full length), GST–NHX1ct (489–560), GST–NHX1ct (561–633), GST–NHX1ct (524–604) or MBP (maltose-binding protein)–MTH1 proteins, the corresponding DNA fragments were amplified by PCR from yeast genomic DNA using the following primers: forward #3 (5'-TTCCGATCC-AAGACTGGTTGCTAATGTAAGAG-3') and reverse #3 (5'-GGACTGTGACACCTGTTTGGGAAAAGAAATC-3'), forward #3 and reverse #4 (5'-AGACGTCGACCTAATCIAAACGAATGTTG-3') and reverse #3, forward #5 (5'-CGGATCCGGCCCAATATTTGGCAACAA-3') and reverse #5 (5'-AGACGTCGACCTAATCIAAACGAATGTTG-3'), forward #6 (5'-GGGATCCCTGGGAATGATCAGCTT-3') and reverse #6 (5'-TCACTGACGTTGGAATGATCAGCTT-3'). The resulting PCR products were cloned into pGEX4T-2 (GE Healthcare) or pMALcri (New England Biolabs) using the BamHI/SalI or BamHI/PstI sites respectively.

**Plasmid construction for expression of recombinant peptides in yeast cells**

To obtain plasmids encoding GAL4-BD (binding domain)–NHX1ct, GAL4-BD–NHX1ct (489–560), GAL4-BD–NHX1ct (561–633), GAL4-BD–NHX1ct (524–604) and GAL4-AD (activation domain)–MTH1 for the yeast two-hybrid assay, corresponding DNA fragments were amplified by PCR from yeast genomic DNA using the following primers: forward #7 (5'-GGGATCCGGCTAATTGAACATGGTGAAGAGAG-3') and reverse #3, forward #7 and reverse #4, forward #8 (5'-GGGATCCGGCTAATTGAACATGGTGAAGAGAG-3') and reverse #3, forward #9 (5'-GGGATCCGGCTAATTGAACATGGTGAAGAGAG-3') and reverse #5, and forward #6 and reverse #6 respectively. The PCR product was cloned into pGPT9 or pGAD424 (Clontech) using the EcoRI/SalI or BamHI/PstI sites. For expression of wild-type Nhxl1p–GFP (green fluorescent protein) and Nhxl1p–GFP mutants with the truncation of the C-terminus (Δ604, Δ560, Δ523 and Δ488), the corresponding DNA fragments were amplified by PCR from yeast genomic DNA using the following primers: forward #10 (5'-GAACGGTACCTAAATCCAAATGTTTGGGAGGAAGGTTTCTCTTTTATGATCGATGGACTAGTGGATCTG-3') and reverse #11 (5'-GAACGGTACCTAAATCCAAATGTTTGGGAGGAAGGTTTCTCTTTTATGATCGATGGACTAGTGGATCTG-3') for _NHX1_ knockout. The PCR products were introduced into yeast cells and the resulting Leu$^+$ or His$^+$ transformants were selected on synthetic medium lacking leucine or histidine. Gene knockout was confirmed by PCR amplification of the genomic DNA.

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**Yeast two-hybrid screening**

_GAL4_–based yeast two-hybrid screening was performed using a peptide from the Nhxl1p C-terminus (residues 489–633) as bait. A plasmid encoding GAL4-BD–NHX1ct was introduced into
yeast strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, lys2::GAL1-UAS-GAL1-TATA-HIS3, gal2::GAL2-UAS-GAL2-TATA-ADE2, ura3::MEL1-UAS-MEL1-TATA-lacZ; Clontech) was incubated at room temperature (25°C) for 4 h. The cells were harvested, suspended in PBS buffer (7.81 mM Na2HPO4, 1.47 mM KH2PO4, 137 mM NaCl, and 2.68 mM KCl) containing 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin, and then disrupted with a French pressure cell press. Triton X-100 was added to the cell lysates at a final concentration of 0.5%, and then the lysate was centrifuged at 12000 g for 30 min. The resulting supernatants were incubated with glutathione–Sepharose (GE Healthcare) or amylose–agarose (New England Biolabs) resins for 4 h at 4°C. The resins were washed with PBS buffer, and bound proteins were eluted with PBS buffer containing 20 mM glutathione or 10 mM maltose.

**Expression and purification of GST and MBP fusion proteins**

*E. coli* BL21(DE3) cells transformed with an expression plasmid were induced to express the fusion proteins with 0.4 mM IPTG (isopropyl β-D-thiogalactopyranoside) at 30°C for 4 h. The cells were harvested, suspended in PBS buffer (7.81 mM Na2HPO4, 1.47 mM KH2PO4, 137 mM NaCl, and 2.68 mM KCl) containing 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin, and then disrupted with a French pressure cell press. Triton X-100 was added to the cell lysates at a final concentration of 0.5%, and then the lysate was centrifuged at 12000 g for 30 min. The resulting supernatants were incubated with glutathione–Sepharose (GE Healthcare) or amylose–agarose (New England Biolabs) resins for 4 h at 4°C. The resins were washed with PBS buffer, and bound proteins were eluted with PBS buffer containing 20 mM glutathione or 10 mM maltose.

**In vitro pull-down assay**

MBP–MTH1 protein (0.5 μg) or 100 μg of lysate from yeast cells expressing Mth1p–GST was incubated at room temperature (25°C) for 1 h with 1.0 μg of GST, GST–NHX1Ct (full length), GST–NHX1Ct (489–560), GST–NHX1Ct (561–688) or GST–NHX1Ct (524–604) proteins immobilized to glutathione–Sepharose. After extensive washes with PBS buffer, bead-bound fractions were resolved by SDS/PAGE (10% gels), and then GST, MBP or GST fusion proteins were detected by immunoblotting with anti-GST, anti-MBP or anti-GFP (GF200; Nacalai Tesque) antibodies respectively.

**Immunoblotting experiments and antibodies**

Protein samples to be analysed were subjected to SDS/PAGE (10% gels). The separated proteins were transferred on to a hydrophobic PVDF membrane (Millipore). The membranes were incubated with 10% (w/v) skimmed milk powder in PBST (PBS with Tween 20; 7.81 mM Na2HPO4, 1.47 mM KH2PO4, 137 mM NaCl, 2.68 mM KCl and 0.1% Tween 20) and then treated with primary antibodies. After washes with PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized by means of the enhanced chemiluminescence method (Amersham Pharmacia Biotech). The intensity of immunoreactive bands was quantified using Image J. The results shown are the means ± S.D. for at least three independent experiments. The rabbit polyclonal anti-GST antibody was prepared by immunizing a rabbit with the bacterially expressed GST followed by affinity purification. The mouse anti-MBP antibody was purchased from Sigma–Aldrich. Rabbit anti-GFP serum and mouse anti-Vph1p antibodies were purchased from Invitrogen Molecular Probes. Horseradish peroxidase-conjugated secondary antibodies against rabbit and mouse IgG were purchased from Vector Laboratories.

**Fluorescence microscopy**

Yeast cells expressing Nhx1p–GFP or Mth1p–GFP were grown in SCD or SCGal medium at 30°C to the exponential phase of growth and observed under a fluorescence microscope (BX51; Olympus) equipped with a NIBA filter. Images were recorded using an ORCA-ER1394 digital camera (Hamamatsu Photonics).

**Cell growth assay**

Yeast cells were grown in YPAD or SCD medium at 30°C to the exponential phase of growth and then diluted serially as indicated. The cells were spotted on to SD or SGal plates (made with 20 mM Mes/Tris, pH 5.5) supplemented with hygromycin. The plates were incubated for 3–6 days at 30°C. For the growth assay in acidic pH medium, SD or SGal plates adjusted to pH 2.5

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Fig. 1 Screening of NHX1Ct-binding partners by a yeast two-hybrid assay

Upper panel: a schematic illustration of the secondary structure of Nhx1p. The hydrophilic tail region (residues 489–633) of Nhx1p was fused with GAL4-BD and used in a two-hybrid assay as bait. Lower panel: yeast AH109 strains transformed with plasmid GAL4-BD–NHX1Ct (full length) alone, GAL4-AD–MTH1 alone, and both GAL4-BD–NHX1Ct (full-length) and GAL4-AD–MTH1 were grown in SD medium lacking tryptophan and leucine. The transformed cells (diluted 10−1, 10−2 or 10−3 as indicated) were spotted on to SD plates lacking tryptophan, leucine, adenine and histidine, and were then incubated for 3 days at 30°C. As the positive control, GAL4-AD and GAL-BD, fused with the α and β subunit from *E. coli* F1Fo-ATPase [56] respectively, were used.
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Figure 2 Identification of Mth1p-binding region within the Nhxl1p C-terminus by a yeast two-hybrid assay

(A) Schematic diagram of GAL4-BD fusions of the various NHX1Ct fragments; numbers indicate amino acid positions. (B) Yeast AH109 strains expressing a combination of GAL4-BD fusions with various NHX1Ct fragments and GAL4-AD–MTH1, as indicated, were grown in SD medium lacking tryptophan and leucine. The transformed cells were spread on to SD plates lacking tryptophan, leucine, adenine and histidine, and then incubated for 6 days. As the positive control, GAL4-AD and GAL4-BD, fused with the α and β subunit from E. coli F1Fo ATPase respectively, were used.

with phosphoric acid were used. Cell growth was monitored spectrophotometrically (D600) and the relative growth rate was expressed as a percentage of the rate of growth in the absence of hygromycin or in culture medium adjusted to pH 5.5. The results are means ± S.D. for at least three independent experiments.

Other procedures and materials

DNA manipulations were performed in accordance with procedures published previously [37]. Protein levels were measured by the Bradford assay as described previously [38]. Restriction enzymes, Thermococcus kodakaraensis DNA polymerase and T4 DNA ligase were purchased from Toyobo and Takara. Oligomer primers used in the present study were synthesized by Invitrogen. Other materials were of the highest commercially available grade.

RESULTS

Mth1p interacts with the hydrophilic tail of Nhxl1p

To survey binding partners of Nhxl1p, we performed a yeast two-hybrid screening with the hydrophilic tail of Nhxl1p [residues 489–633; NHX1Ct (full length)] as bait. We obtained 514 positive clones from 3.4×10⁶ independent clones in an S. cerevisiae cDNA library and then sequenced the plasmid DNA carried by 94 randomly selected positive clones. Two of the sequenced clones carried a plasmid with a gene encoding MTH1 [39]. These plasmids complemented the Ade− His− phenotype of the yeast mutant cells in the presence of a plasmid carrying GAL4-BD fused NHX1Ct (full length) (Figure 1).

We analysed further the interaction of Nhxl1p and Mth1p using a yeast two-hybrid system and identified a specific region within the C-terminal tail of Nhxl1p required for interaction with Mth1p (Figure 2). For this, we expressed fragments containing the N-terminal (residues 489–560), central (residues 524–604), or C-terminal (residues 561–633) region of NHX1Ct (Figure 2A), together with GAL4-AD–MTH1. Yeast cells expressing each fragment alone or co-expressing the N-terminal region with GAL4-AD–MTH1 did not grow on the selection plates during incubation for 6 days (Figure 2B, numbers 4, 5, 6 and 8), whereas the cells co-expressing the central or C-terminal regions with GAL4-AD–MTH1 showed obvious cell growth on the selection plates after 6 days of incubation (Figure 2B, numbers 7 and 9). From the growth rate of yeast cells on the selection plates, the C-terminal region was estimated to interact with Mth1p more weakly than the central region, and the N-terminal region interacted very little.

We next confirmed the interaction between recombinant GST–NHX1Ct, a fusion of GST and the hydrophilic tail of Nhxl1p, and MBP–MTH1 produced in E. coli cells and purified, by means of an in vitro pull-down assay. GST–NHX1Ct clearly bound to MBP–MTH1, whereas GST alone did not (Figure 3A). This result indicates that the hydrophilic tail of Nhxl1p directly interacts with Mth1p. The GST–NHX1Ct (489–633) also interacted with Mth1p–GFP expressed in yeast cells (Figure 3B). Moreover, Mth1–GFP specifically bound to GST–NHX1Ct (524–604), but not efficiently to GST–NHX1Ct (489–560) and GST–NHX1Ct (561–633) peptide, confirming the results of the two-hybrid assay. These results strongly suggested that the central region of Nhxl1p hydrophilic domain (residues 489–560) is responsible for binding Mth1p.

Mth1p is a negative regulator of Nhxl1p activity

Glucose induces expression of HXTs (hexose transporters) [40,41]. Mth1p localizes mainly in the nucleus in the absence of glucose, but a small fraction of total Mth1p is also present in the plasma membranes due to interactions with the plasma membrane glucose sensor Snf3p/Rgt2p [41,42]. Mth1p can shuttle between nucleus and cytoplasm. However, in culture medium containing glucose, Mth1p associated with glucose sensor in the plasma membrane is ubiquitylated in response to a glucose signal and rapidly degraded [42]. Thus the protein level of
Mth1p is low in the presence of glucose [41,43]. Removal of glucose prevents this degradation of Mth1p and permits its translocation into the nucleus. In the nucleus, Mth1p bound to the repressor protein Rgt1p suppresses the expression of the HXTs [31,41,44,45]. These findings indicate that Mth1p is a negative transcriptional regulator of an extracellular glucose-sensing mechanism. As reported previously [46], we found that a Mth1p–GFP fusion, tagged with GFP chromosomally at its C-terminus, was present in both the nucleus and cytoplasm in the cells cultured in medium containing 2 % (w/v) galactose (results not shown) and was detected at an expected size by immunoblotting using an anti-GFP antibody (Figure 3B, input). In contrast, a only very weak Mth1p–GFP signal was detected in the cells cultured in medium containing 2 % (w/v) glucose by both of microscopic observation (results not shown) and immunoblotting (Figure 3B, control). Thus the majority distribution of Mth1p in yeast cells was not identical to that of Nhx1p (i.e., at the endosomal membranes), implying that the interaction between these two proteins takes place for small fractions of Nhx1p and Mth1p, and is transient.

To clarify the physiological significance of the binding of Mth1p to Nhx1p, we analysed the effect of MTH1-knockout on Nhx1p-activity-mediated resistance to hygromycin. Deletion of NHX1 retarded cell growth on agar plates containing hygromycin and 2 % (w/v) galactose (Figure 4A). This increase in sensitivity to hygromycin upon NHX1-knockout was also observed on glucose/agar plates (Figure 4B). On agar plates containing 2 % (w/v) galactose, growth of cells with a disrupted MTH1 gene (mth1Δ) was substantially more resistant to hygromycin than wild-type cells (Figure 4A). However, mth1Δ cells showed a resistance to hygromycin similar to that of the wild-type cells on agar plates containing 2 % (w/v) glucose (Figure 4B), consistent with Mth1p being degraded in the wild-type cells on glucose plates. In a liquid culture assay, deletion of the MTH1 gene caused higher resistant to hygromycin than that of the wild-type cells in galactose-containing medium, consistent with the results of the agar-plate assay (Figure 4C). These results suggest that deletion of MTH1 elevated Nhx1p activity, suggesting that Mth1p is a negative regulator of Nhx1p. Cells with a double deletion of NHX1 and MTH1 grew at a rate similar to that of nhx1Δ cells in the presence of hygromycin (Figures 4A–4C), also supporting the notion that Mth1p is a negative regulator of Nhx1p.

S. cerevisiae utilizes a variety of sugars as carbon and energy sources. Whereas glucose enters the glycolysis pathway directly, galactose, glycerol and ethanol are converted into intermediate metabolites before entering the glycolysis pathway. Outside of the cells yeast hydrolyse raffinose (a trisaccharide composed of glucose, galactose and fructose) by glycosidases, such as invertase and melibiase, secreted from cells in order to obtain glucose [46,47]. Therefore we analysed the effect of other carbon sources, such as raffinose or glycerol/ethanol, on the increase in resistance to hygromycin seen upon MTH1 knockout. Cell growth of mth1Δ cells showed that the resistance to hygromycin was similar to that of the wild-type cells on agar plates containing 2 % (w/v) raffinose (Figure 4D), whereas mth1Δ cells showed more resistance to hygromycin than that of the wild-type cells on plates containing 3 % glycerol and 2 % (v/v) ethanol (Figure 4E). These findings strongly suggest that regulation of Nhx1p activity by Mth1p depends on the extracellular glucose level.

Another known phenotype of nhx1Δ cells is that they grow more slowly than the wild-type cell in acidic medium, possibly due to a defect in the regulation of vacuolar or cytoplasmic pH [21]. To confirm a functional interaction between Nhx1p and Mth1p, we also analysed the role of Mth1p in cells growth at pH 2.5. Indeed, nhx1Δ cells grew more slowly than wild-type...
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Figure 4 Growth of MTH1-knockout cells is more resistant to hygromycin than that of wild-type cells in medium containing galactose

(A and B) Yeast strains (wild-type, mth1Δ, nhx1Δ and nhx1Δ mth1Δ) were grown in YPAD medium. The cells (diluted 10⁻¹, 10⁻² or 10⁻³ as indicated) were spotted on to (A) SGal plates, containing 2 % (w/v) galactose, or (B) SD plates, containing 2 % (w/v) glucose, and were then incubated for 3–5 days. The cell growth assay was performed three times and essentially similar results were obtained each time. (C) The wild-type (black bar), mth1Δ (grey bar), nhx1Δ (white bar) and nhx1Δ mth1Δ (hatched bar) were inoculated in 5 ml of SD medium supplemented with 100 μg/ml hygromycin and cultured at 30°C with vigorous shaking. The growth rate was spectrophotometrically determined after 16 h (glucose) or 24 h (galactose) of incubation. The relative growth rate was calculated as described in the Experimental section and is presented as the percentage of the growth rate of cells cultured in medium without hygromycin. The results are means ± S.D. for at least three independent experiments. (D and E) Yeast strains (wild-type, mth1Δ, nhx1Δ, and nhx1Δ mth1Δ) were grown in YPAD medium. The cells (diluted 10⁻¹, 10⁻² or 10⁻³ as indicated) were spotted on to (D) SRaf plates containing 2 % (w/v) raffinose or (E) SGly/Eth plates containing 3 % (w/v) glycerol and 2 % (v/v) ethanol, and were then incubated for 3–6 days. The cell growth assay was performed three times and essentially similar results were obtained each time.

cells on agar plates and liquid cultures at pH 2.5, whereas mth1Δ and wild-type cells showed similar growth on culture medium adjusted to pH 5.5 (Figure 5). When yeast cells were grown in medium containing galactose as the sole carbon source, mth1Δ cells showed a slightly higher growth rate on agar plates adjusted to pH 2.5 than the wild-type cells (Figure 5A). In contrast, nhx1Δ mth1Δ cells showed a growth defect similar to that of the nhx1Δ cells (Figure 5A). As expected, deletion of MTH1 as a control had no effect on cell growth in glucose medium at acidic pH (Figure 5B). The results of a liquid-culture assay were essentially the same as those with agar plates (Figure 5C). These results also strongly suggest that the interaction with Mth1p regulates Nhx1p activity in response to extracellular carbon sources.

Next, in order to test whether the increasing resistance to hygromycin observed in mth1Δ cells is due to up-regulation of Nhx1p activity, we examined how cells expressing mutant Nhx1p lacking the binding region for Mth1p, lacking the binding region for Mth1p, grow in medium containing hygromycin. To do this, we constructed a series of Nhx1p mutants with various deletions from the C-terminus of the hydrophilic region (Figure 6A). All Nhx1p mutants were...
expressed and localized at the endosomes, like the wild-type Nhx1p (Figure 6B). Expression of the wild-type Nhx1p in the nhx1Δ cells restored the hygromycin-sensitivity of nhx1Δ cells to the wild-type level on both galactose- (Figure 6C) and glucose- (Figure 6D) containing agar plates. As shown in Figure 6(C), the Nhx1p mutant with a deletion of the C-terminal 40 amino acids (Δ604) showed slightly lower resistance to hygromycin than the wild-type Nhx1p on agar plates containing 2% (w/v) galactose. Moreover, nhx1Δ cells expressing Nhx1p mutants with a deletion of the C-terminal 80 and 120 amino acids (Δ560 and Δ523 respectively), regions that include the Mth1p-binding region, showed higher resistance to hygromycin than the Δ604 mutant, whereas a mutant Nhx1p with a complete deletion of the hydrophilic region of Nhx1p (Δ488) caused severe retardation of cell growth in the presence of hygromycin (Figure 6C). In addition, on glucose/agar plates, deletion of the C-terminal 40 amino acids (Δ604) caused a slight decrease of cell growth in the presence of hygromycin (Figure 6D). However, the Δ560 and Δ523 Nhx1p mutants showed essentially the same level of cell growth in the presence of hygromycin as the Δ604 mutant (Figure 6D). These results suggest that the increasing resistance to hygromycin on galactose plates is due to impairment of the interaction between Nhx1p and Mth1p. Moreover, these results also indicate that residues within the regions 489–523 and 605–633 play an important role in Nhx1p activity, whereas residues 524–604, which include the Mth1p-binding region, are not involved in the regulation of Nhx1p activity on glucose/agar plates.

**Mth1p does not affect the expression level of Nhx1p**

To analyse the down-regulation of Nhox1 activity by Mth1p, we tested whether deletion or overexpression of *MTH1* affects the expression level of Nhox1p. When these strains were grown in medium containing 2% (w/v) galactose (Figure 7A) or 2% (w/v) glucose (Figure 7B), Nhox1p–GFP fusions in both mth1Δ cells and *MTH1*-overexpressing cells were expressed at the same level as in wild-type cells. These results suggest that the apparent activation of Nhox1p activity by the loss of *MTH1* is not due to an increase in the expression of Nhox1p in cells.

**DISCUSSION**

In the present study we found that the yeast endosomal NHE Nhox1p interacts with Mth1p, a transcriptional regulator mediating a signal of extracellular carbon sources, especially in the presence or absence of glucose. Mth1p negatively regulates Nhox1p activity when yeast cells are grown in medium containing galactose or glycerol/ethanol, but not glucose or raffinose. This is the first study showing that Nhox1p activity is regulated by interaction with a transcriptional regulator in response to changes in extracellular carbon sources. Nhox1p activity contributes to the regulation of organellar pH. Therefore we propose that the regulation of Nhox1p function by Mth1p is required to maintain the pH of organellar lumina regardless of changes in extracellular carbon source.

Yeast V-ATPase activity is tightly controlled by the extracellular glucose level [23–25]. In medium containing 2% (w/v) glucose, most Vᵦ subunits of V-ATPase are assembled with Vᵥ subunits, but after 5 min of glucose depletion, only 20% of Vᵥ subunits are bound to Vᵦ subunits [23–25]. The dissociated Vᵥ subunit shows very little ATPase activity, and the Vo subunit does not appear to form an open proton pore [48, 49]. Thus this dissociation between the Vᵦ and Vᵥ subunits of V-ATPase impairs the coupling of ATP hydrolysis and proton transport, leading to alkalinization of the
Figure 6 Disruption of the Nhxl–Mthlp interaction increases resistance to hygromycin

(A) Schematic diagram of Nhxl constructs with various truncations in the C-terminal region. (B) Subcellular localization of mutant Nhxl–GFPs with truncated C-terminal regions. Yeast cells (nhxlΔ) expressing Δ604, Δ560, Δ523 and Δ488 Nhxl–GFP mutants were grown to the exponential phase of cell growth in SCGal medium, containing 2 % (w/v) galactose, lacking tryptophan and then observed by fluorescence microscopy. Scale bars, 5 μm. (C and D) Yeast strains (nhxlΔ) expressing various Nhxl mutants (Δ604, Δ560, Δ523 and Δ488) were grown in SD medium lacking tryptophan. The cells (diluted as indicated) were spotted on to (C) SGal plates, containing 2 % (w/v) galactose, or (D) SD plates, containing 2 % (w/v) glucose, and were then incubated for 3–5 days. The cell growth assay was performed three times and essentially similar results were obtained each time.

Accordingly, we propose that the interaction with Mthlp inhibits the antiporter activity of Nhxl, and that Nhxl is constitutively activated in mthlΔ cells. Although we measured the vacuolar pH of the wild-type and mthlΔ cells grown in medium containing glucose or galactose, by using the pH-sensitive fluorescent dye BCECF [2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein] trapped in yeast vacuoles as described previously [21], we did not detect a significant difference in the vacuolar pHs between the wild-type and mthlΔ cells (results not shown). This result suggested that the difference in vacuolar pH between the wild-type and mthlΔ cells is very small or transient. We also investigated the effect of MTH1-deletion on CPY (carboxypeptidase Y) secretion, defects in which are a known phenotype of nhxlΔ cells [20]. The mthlΔ cells showed the same level of CPY secretion as the wild-type cells in medium containing glucose or galactose (results not shown). This result also suggests the change of vacuolar (or endosomal) pH by MTH1 deletion is very small or transient.

It has been shown previously that yeast Nhxl also interacts with Gyp6p, a GTPase-activating protein for the yeast Rab family member Ypt6p [53]. Gyp6p also interacts with the C-terminal region (residues 607–633) of the Nhxl C-terminus,

organellar lumen [48–51]. In this situation, it would be reasonable to prevent alkalinization in the organellar lumen by decreasing Nhxl activity through the actions of Mthlp, as we found in the present study. Regulation of V-ATPase activity by glucose has also been observed in mammalian cells [52]. Therefore mammalian organellar-type NHEs may also be controlled in response to extracellular glucose, as yeast Nhxl is. However, because Mthlp is not conserved in mammalian cells, mammalian organellar-type NHEs may not be regulated in exactly the same way as yeast Nhxl.

We have shown that Mthlp does not affect the stability of Nhxl in cells (Figure 7). Deletion or overexpression of MTH1 also did not cause mislocalization of Nhxl–GFP fusions within cells grown in medium containing either 2 % (w/v) glucose or galactose (results not shown). These findings indicate that the apparent activation of Nhxl upon the loss of MTH1 (Figures 4 and 5) is not due to an increase in the expression level or change of the intracellular localization of Nhxl in cells. Moreover, overexpression of MTH1 in wild-type (Nhxl+) cells decreased the resistance to hygromycin, although no increase in sensitivity to hygromycin was observed in nhxlΔ cells (results not shown).
but in a different area to the Mth1p-binding site, so Gyp6p may not compete with the binding of Mth1p to Nhx1p. The ability of Nhx1p to bind Gyp6p and Mth1p suggests that there are at least two independent regulatory mechanisms. ∆488 or ∆604 Nhx1p mutants showed retardation of cell growth in the presence of hygromycin relative to that of the ∆523 mutant and wild-type Nhx1p respectively (Figure 6). Therefore we conclude that the N-terminal and C-terminal halves (residues within 489–523 and 604–633 respectively) of the Nhx1p C-terminal hydrophilic region also play important roles in Nhx1p activity regardless of the extracellular carbon source. Both ∆488 and ∆604 Nhx1p mutants were stably expressed and localized at dot-like structures, similar to the wild-type Nhx1p (Figure 6B), suggesting that these regions are involved in the antiporter activity of Nhx1p, not in endosomal targeting of Nhx1p. Although the exact roles of these regions in Nhx1p activity remains unknown, these results might indicate that some Nhx1p-enhancing factors besides Mth1p and Gyp6p interact with the Nhx1p C-terminus.

In mammalian cells, Chp1 (calcineurin homologous protein 1) interacts with an more N-terminal membrane-proximal region in the hydrophilic tail of NHE1 [29]. This binding plays an essential role in stabilizing the functional NHE1 molecule with the antiporter activity [29,30]. However, because Chp1 does not bind to the Nhx1p orthologue NHE6 [29], an alternative molecule, similar to Chp1, might be involved in Nhx1p function. The N-terminal half of the C-terminal hydrophilic region of the yeast plasma membrane NHE Nha1p is also essential for ion transport [54] and is capable of binding Cos3p, which enhances the antiporter activity [55]. These findings together with the present results suggest that the N-terminal half or membrane-proximal region of the C-terminal hydrophilic region of NHEs is generally important for their function and regulation.

REFERENCES


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