pH-Responsive, Posttranslational Regulation of the Trk1 Potassium Transporter by the Type 1-Related Ppz1 Phosphatase

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Intracellular pH and K+ concentrations must be tightly controlled because they affect many cellular activities, including cell growth and death. The mechanisms of homeostasis of H+ and K+ are partially understood. In the yeast *Saccharomyces cerevisiae*, proton efflux is mediated by the Pma1 H+-ATPase. As this pump is electrogenic, the activity of the Trk1 and -2 K+ uptake system is crucial for sustained Pma1p operation. The coordinated activities of these two systems determine cell volume, turgor, membrane potential, and pH. Genetic evidence indicates that Trk1p is activated by the Hal4 and -5 kinases and inhibited by the Ppz1 and -2 phosphatases, which, in turn, are inhibited by their regulatory subunit, Hal3p. We show that Trk1p, present in plasma membrane “rafts,” physically interacts with Ppz1p, that Trk1p is phosphorylated in vivo, and that its level of phosphorylation increases in *ppz1* and -2 mutants. Interestingly, both the interaction with and inhibition of Ppz1p by Hal3p are pH dependent. These results are consistent with a model in which the Ppz1-Hal3 interaction is a sensor of intracellular pH that modulates H+ and K+ homeostasis through the regulation of Trk1p activity.

Ion homeostasis is a fundamental property of living cells. The intracellular concentrations of the major monovalent cations (H+, K+, and Na+) must be tightly regulated because they influence the activities of many cellular systems that require a narrow range of ion concentrations for operation (28, 51). The homeostasis of monovalent cations is maintained primarily by systems that extrude H+ and Na+ and import K+. Cellular volume, turgor, electrical membrane potential, and ionic strength depend mostly on intracellular K+ concentrations. In animal cells, Na+ extrusion is crucial for cell volume regulation (28), but in fungi and plants, this cation is only toxic, probably by antagonizing Mg2+ in the active sites of some key enzymes (51). Intracellular pH, on the other hand, modulates the activities of many cellular systems, including those regulating cell growth (13, 22, 41) and death (23, 31).

The important roles of cellular H+ and K+ concentrations raise the unanswered question of whether these parameters are used as second messengers of external signals or are simply maintained within permissive ranges for the functionality of sensitive cellular systems (6, 21, 24, 39, 54). In any case, the regulation of H+, Na+, and K+ transport is crucial for cellular physiology, and defects in these systems have a wide array of consequences in medicine and agriculture, which range from diseases related to the nervous system, muscle, kidney, and heart (42) to sensitivity to low-pH and high-Na+ environments of agriculturally important crop plants (51, 52).

The remarkable capability of living cells to adjust intracellular H+ is not completely understood. Known mechanisms include the pH dependence of H+ transporters, such as the bacterial (40) and animal (58) Na+/H+ exchangers, and the activation of the animal Nhe1 exchanger by a mitogen-activated protein kinase cascade in response to growth factors (5).

In the yeast *Saccharomyces cerevisiae*, a model system for ion homeostasis studies (52), intracellular-pH regulation is partially explained by the pH dependence of the plasma membrane H+-pumping ATPase, Pma1p (49). This major transport system has an optimal pH of 6.5 and therefore is well suited to set the intracellular pH at a neutral value (14). However, the activity of the enzyme is regulated by glucose metabolism (which promotes acidification and fast growth) and by acid pH, suggesting an additional mechanism of pH regulation based on some unknown pH sensor that modulates the phosphorylation and activity of the ATPase (4). In addition, the activity of the major K+ transporters, Trk1p and Trk2p, are important to set the intracellular pH. H+ pumping by Pma1p is electrogenic, and K+ transport via Trk1p (and/or Trk2) is the major return current in yeast. Therefore, the regulation of Trk activity affects not only intracellular K+ and turgor (35), but also electrical membrane potential (32, 36) and intracellular pH (32, 61).

Genetic and phenotypic analyses have identified several proteins implicated in the regulation of potassium transport in yeast, including the protein phosphatases Ppz1p, Ppz2p, and calcineurin; the protein kinases Hal4p, Hal5p, and Sky1p; a protein of unknown function, Hal1p; and the G protein of the Ras superfamily, Arl1p (17, 34, 36, 44, 61). The exact mechanisms of action of these proteins with respect to potassium homeostasis are largely unknown, but some have been suggested to directly regulate the activity of the Trk1p transporter (Hal4p, Hal5p, and calcineurin), while others appear to act indirectly (Hal1p, Sky1p, and Arl1p).

In the case of the Ppz phosphatases, several lines of evidence indicate that these proteins are involved in determining the upper limits of potassium accumulation in the cell in a largely (but not exclusively) Trk1p-dependent manner (35, 45, 47, 61). This alteration in the regulation of potassium transport dru-
matically affects the internal pH homeostasis due to the strict requirement for maintaining electrical neutrality during H\(^+\) pumping by the Pma1p ATPase. Specifically, in the \(ppz1\) and -2 mutant grown under normal conditions, potassium accumulates and the internal pH is increased by approximately 0.4 pH units (61).

Protein phosphatases are often associated with regulatory subunits that provide substrate specificity, determine subcellular localization, or modulate the activity of the enzyme. To date, only one type of regulatory subunit has been described as regulating the activity of the Ppz phosphatases in vivo: Hal3p and a less active homologue encoded by the \(VHS3\) gene (10, 46). The \(HAL3\) gene was identified several years ago based on its impact on both toxic-cation tolerance and cell cycle progression (11, 16). It was then shown, in vitro and in vivo, to be a negative regulatory subunit of the Ppz phosphatases, thus explaining the observed phenotypes associated with the overexpression or disruption of the \(HAL3\) gene (10, 47). However, little information is available regarding the physiological role and the nature of the signal transduced by the Ppz1p-Hal3 regulatory complex.

Due to the important implications of the Trk1- and -2-dependent effects observed in turgor, internal pH homeostasis, and cell cycle progression for the \(ppz1\) and -2 mutant, we have investigated whether the Ppz1p phosphatase binds to and modulates the phosphorylation levels of the Trk1p transporter. Furthermore, we present evidence for a novel mechanism of regulation of the activity of the Trk1p transporter based on a pH-dependent interaction of the Ppz1p phosphatase with its inhibitory subunit, Hal3p. The model that is suggested by previous reports and data presented here contends that Trk1p has a higher activity when phosphorylated in vivo and that Ppz1p decreases the phosphorylation levels of this transporter when potassium levels are high to avoid overaccumulation. This deactivation mechanism appears to be triggered by increases in the internal pH, which destabilize the interaction between Ppz1p and its inhibitory subunit, Hal3p, suggesting that the Hal3-Ppz1 complex acts as a pH sensor within the cell.

MATERIALS AND METHODS

**Yeast culture conditions.** Rich (yeast extract-peptone-dextrose) and minimal (SD) media were prepared as described previously (61). Low-phosphate medium was prepared by adding 8 ml of an alkaline magnesium mixture (0.3 M MgCl\(_2\) (SD) media were prepared as described previously (61). Low-phosphate medium was added, and the medium was sterilized. The precipitate was removed by filtration, the pH was adjusted to 6.0, glucose yeast extract-peptone mixture in order to precipitate the inorganic phosphate.

**PP1 and Ppz1p overexpression.** The indicated strains were grown in low-phosphate medium (30 ml/strain) to an OD\(_{600}\) of 0.4. Cells were collected by centrifugation and resuspended in 5 ml of low-phosphate medium, 250 \(\mu\)l of 4% [\(\gamma\)-\(^{32}\)P]orthophosphate (Amersham) was added, and incubation was continued for 1 h at 28°C with rotation. Cell pellets were collected by centrifugation and then lysed and processed for raft purification as described above. After the second flotation gradient, the lightest density fraction was precipitated with trichloroacetic acid, resuspended in Laemmli sample buffer, and separated on 8% SDS-PAGE. Gels were dried and exposed to X-ray film for 30 min to 1 h.

**PP1 and Ppz1p overexpression.** In vitro kinase assays. The indicated strains were grown in low-phosphate medium (30 ml/strain) to an OD\(_{600}\) of 0.4. Cells were harvested by centrifugation and resuspended in 5 ml of low-phosphate medium, 250 \(\mu\)l of 4% [\(\gamma\)-\(^{32}\)P]orthophosphate (Amersham) was added, and incubation was continued for 1 h at 28°C with rotation. Cell pellets were collected by centrifugation and then lysed and processed for raft purification as described above. After the second flotation gradient, the lightest density fraction was precipitated with trichloroacetic acid, resuspended in Laemmli sample buffer, and separated on 8% SDS-PAGE. Gels were dried and exposed to X-ray film for 30 min to 1 h.

**PP1 and Ppz1p overexpression.** In vitro binding assays and phosphatase assays. In vitro binding assays and phosphatase assays were performed as described previously, except that 50 mM BIS-TRIS (\(\{\{(\text{2-hydroxyethyl})-iminoo\}\}-(\text{hydroxymethyl})\)-methane), (pK\(_a\) 6.5; CalBiochem) was used to buffer the pH of the interaction and reaction buffers (19).

**Fractionation assays.** The indicated yeast strains were grown in rich media adjusted to the indicated pH for an OD\(_{600}\) of 0.5 to 0.6, and cells were harvested by centrifugation and frozen at -70°C. The cells were resuspended in homogenization buffer (50 mM Tris, pH 8.0, 0.1 M KCl, 5 mM EDTA, 5 mM dithio-erythritol, 20% sucrose [wt/vol], protease inhibitor cocktail) and lysed by vortexing them with glass beads. The lysate was collected after centrifugation for 5 min at 500 \(\times\) g (2,000 rpm). The crude extract was separated into soluble and particulate fractions by centrifugation for 30 min at 30,000 rpm in a Ti70 rotor.
Trk1p is localized to plasma membrane rafts. In order to establish the possible biochemical link between Trk1p and Ppz1p, we first wanted to further explore the subcellular localization of both proteins. Recent studies have shown that rafts, sphingolipid-rich microdomains of the plasma membrane initially identified in mammalian systems, are present in yeast and are important for delivery to and stability of proteins at the cell surface, especially the plasma membrane proton ATPase, Pma1p, which is a resident raft protein (2, 3). Since Pma1p and the high-affinity potassium transporters Trk1p and Trk2p are proposed to be the major determinants of yeast membrane potential and internal pH and thus may be coordinately regulated, we reasoned that they might exist in these microdomains of the plasma membrane.

Lipid rafts are operationally defined as protein-lipid complexes of low buoyant density that are resistant to extraction with nonionic detergents, such as Triton X-100, at low temperatures. These plasma membrane microdomains can be highly enriched by applying Triton-treated extracts to flotation gradients of low buoyant density that are resistant to extraction with nonionic detergents, such as Triton X-100, at low temperatures. These plasma membrane microdomains can be highly enriched by applying Triton-treated extracts to flotation gradients (lowest to highest density).

RESULTS

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localized to the plasma membrane, although a fraction of the protein is present in internal nonvacuolar membranes, likely representing the endoplasmic reticulum and/or the nuclear membrane. As expected, the Trk1-GFP fusion protein expressed from a centromeric vector was exclusively present in the plasma membrane (Fig. 2). The fluorescence pattern observed for Ppz1p demonstrates that, in live cells, the majority of this protein is localized to the plasma membrane, thus satisfying an important requirement necessary to establish this phosphatase as a physiologically important regulator of Trk1p.

To further examine the subcellular localization of Ppz1p and to determine if Ppz1p is also present in plasma membrane rafts, we performed various fractionation experiments. We observed that, although Ppz1p is not present in rafts purified in the presence of Triton X-100 using a modified flotation gradient protocol, Ppz1p partially associated with the lowest-density fraction in flotation gradients not subjected to extraction with Triton X-100 (Fig. 3). This observation suggests that the association of Ppz1p with the plasma membrane observed with the GFP fusion protein is peripheral, because it is not resistant to treatments with detergents, such as Triton X-100.

Interestingly, we observed a marked redistribution of the Ppz1 protein upon Trk1p overproduction in the modified flotation gradients without Triton X-100 (Fig. 4). Approximately 20% of the total endogenous Ppz1p is present in the lowest-density fraction in strains transformed with an empty vector or transformed with the \textit{TRK1} overexpression vector under conditions where the promoter driving expression is not induced (Fig. 4). This percentage appears to be only slightly decreased in strains lacking \textit{TRK1} and \textit{TRK2}, suggesting that Trk1p and/or Trk2p is not necessary for this small fraction of Ppz1p to be present in this part of the gradient. However, the amount of Ppz1p found in the lowest-density fraction was dramatically increased (60% of the total) when expression of \textit{TRK1} was transiently induced, suggesting that the presence of excess Trk1p recruits and/or stabilizes Ppz1p in this lipid-associated fraction of the gradient. The lightest density fraction in this type of flotation gradient is postulated to contain, in addition to raft proteins, non-raft-resident plasma membrane proteins and plasma membrane-associated proteins, but it may also contain lipid-associated proteins from other organelles, such as the Golgi apparatus or endoplasmic reticulum. Thus, although we cannot formally discard the possibility that this Ppz1p recruitment by Trk1p takes place in lipid-associated organelles in addition to, or other than, the plasma membrane, the combined interpretation of this experiment with the localization of the Ppz1-GFP fusion protein strongly argues that this phosphatase is peripherally associated with the plasma membrane and suggests that Ppz1p functionally interacts with Trk1p.

In order to determine if in fact Ppz1p physically interacts with Trk1p, we next performed coimmunoprecipitation exper-
results demonstrate increases in the steady-state phosphorylation in the
HAL5 mutant cannot be explained by increased transcription of the gene encoding the transporter or accumulation of the protein, as no changes in gene expression

FIG. 4. Redistribution of Ppz1p upon TRK1 overexpression. The indicated yeast strains (W303-1A, LY 152, and LY 161) were processed for flotation gradients as described in Materials and Methods. The top row shows the Ponceau S staining of the membranes as a control for protein loading. The amount of Ppz1p in each lane was estimated in digitally converted images using the MacBAS software. The LY 161 strain was grown in the presence (uninduced) or absence (induced) of doxycycline (20 μg/ml) to control TRK1 expression. Similar results were observed in two separate experiments. WT, wild type.

Phosphorylation of Trk1p in vitro and in vivo. The association of Trk1p and Ppz1p suggests that Trk1p may be regulated by phosphorylation. However, to date, no biochemical evidence has been presented. To establish whether Trk1p is indeed phosphorylated, we performed in vitro kinase reactions with purified Trk1p. We clearly observed radioactive labeling of the immunoprecipitated Trk1 protein after a 30-min incubation with [γ-32P]ATP (Fig. 6A). The radioactive labeling of Trk1p is largely inhibited by the addition of EDTA, suggesting that, as expected, it is the result of an Mg2+-dependent chemical reaction catalyzed by an endogenous kinase associated with Trk1p. Studies are under way to identify this kinase.

To directly examine changes in the steady-state phosphorylation levels of the Trk proteins in vivo, we purified rafts from 32P-labeled strains grown in low-phosphate media. We chose this method, as opposed to immunoprecipitation of Trk1p, for several reasons: the ability to assay endogenous proteins without any need for overexpression or epitope tagging; the high degree of purification and complete recovery of the Trk1 protein by using this method; and the rapid separation of membrane proteins from the rest of the extract, conserving possible posttranslational modifications. Autoradiography of the purified raft proteins, after separation by SDS-PAGE, showed that only four proteins in the molecular mass range examined were phosphorlated under the tested conditions (Fig. 6B). In addition to two unidentified lower-molecular-mass bands, we observed a phosphorylated protein at approximately 111 kDa, tentatively identified as Pma1p, according to its previous identification as a phosphoprotein, its abundance in the raft fractions, and its observed molecular mass (7). We also observed a phosphoprotein whose apparent molecular mass (approximately 180 kDa) is consistent with its identification as Trk1p (Fig. 6B). This high-molecular-mass band incorporates more [32P]orthophosphate in strains lacking PPZ1 and PPZ2 than wild-type strains. Importantly, genetic evidence suggests that this phosphorylated protein is Trk1p, since it is not observed in ppz1 and -2 mutant strains lacking TRK1 and TRK2 (Fig. 6B, lane 2 versus lane 3). Interestingly, the band corresponding to Pma1p appears to incorporate less 32P in ppz1 and -2 strains than in the wild-type control. This observation is consistent with our previous results demonstrating a lower in vivo activity for Pma1p in this mutant and may reflect the observed increase in intracellular pH, which is known to down-regulate Pma1p activity (61).

In addition, we observed increased phosphorylation of this high-molecular-mass band in strains overexpressing HAL5 (Fig. 6B, lane 4). This gene encodes a kinase genetically established as a positive regulator of Trk1p activity (36). These results demonstrate increases in the steady-state phosphorylation levels of Trk1p in strains lacking PPZ1 and PPZ2 or overexpressing HAL5. The observed increase in Trk1p phosphorylation in the ppz1 and -2 mutant cannot be explained by increased transcription of the gene encoding the transporter or accumulation of the protein, as no changes in gene expression...
or protein levels were detected for TRK1 in Ppz-deficient strains (data not shown).

**The interaction between Hal3p and Ppz1p is pH responsive.** Having established a physical interaction between Ppz1p and Trk1p and modulation of Trk1p phosphorylation levels in ppz1 and -2 mutants, we were further interested in investigating how Ppz1p may be regulated under physiological conditions. Several lines of evidence have established Hal3p as a negative regulator of the Ppz phosphatases (10, 47). Based on the marked increase in the internal pH of the ppz1 and -2 mutant and our proposed model for Ppz1p function, we reasoned that the activity of this phosphatase would be vital when the internal pH increases (61). Therefore, we investigated the possibility that the interaction between Hal3p and Ppz1p may be affected by pH. In vitro binding experiments were performed using the catalytic domain of Ppz1p fused to glutathione-S-transferase (GST), as this domain was previously reported to be sufficient for Hal3p binding (10). As shown in Fig. 7A, much more Hal3p can be retained by the Ppz1p-containing affinity resin at pH 6.0 than at pH 7.5. The specificity of this modulation in the Hal3-Ppz1 interaction was investigated by compar-
ing the interaction profile of the catalytic domain of Ppz1p with that of Ypi1p. The Ypi1 protein is a recently identified inhibitor of the protein phosphatase Glc7p capable of binding both Glc7p and Ppz1p in vitro (19). As shown in Fig. 7A, Ypi1p appears to have a slightly higher affinity for Ppz1p at pH 7.5 versus pH 6, suggesting that the differences observed for Hal3p and Ppz1p are not the result of nonspecific changes in the binding interaction due to the change in pH employed. Further experiments over a larger pH range (5.5 to 7.5) showed that maximal Hal3p binding was observed at pH 6.0 (Fig. 7B). This observation was further supported by results obtained using in vitro phosphatase assays, in which we observed that the Hal3p-mediated inhibition of Ppz1p activity was markedly decreased at pH 7.5 compared to pH 6 (Fig. 7C).

Due to its association with the plasma membrane, the interaction between Hal3p and Ppz1p has proven to be difficult to analyze in vivo using two-hybrid or coimmunoprecipitation techniques. Therefore, in order to corroborate the pH dependence of this interaction within the cell, we performed crude fractionation experiments on wild-type and ppz1Δ-2 mutant strains grown in media buffered to pH 6 or pH 7. As previously reported, in this type of crude fractionation procedure, we observed that approximately 15% of the total amount of Hal3p present in the cell can be recovered in the insoluble fraction under normal growth conditions (pH 6.0) (16). However, in strains grown at more alkaline pH or in strains lacking PPZ1 and PPZ2, very little Hal3p (less than 5%) is found in the insoluble fraction (Fig. 8A). These results suggest that the amount of Hal3p recovered in the insoluble fraction is dependent on the presence of the Ppz proteins (which are found exclusively in the insoluble fraction using this type of crude fractionation protocol) and on the intracellular pH. Evidence demonstrating the influence of pH on this protein-protein interaction in vivo is provided by the analysis of HAL3-dependent LiCl tolerance, varying the external pH. In this case, we compared the lithium tolerance derived from Ppz inhibition either by gene disruption or by overexpression of the Hal3 inhibitory subunit. If the interaction between Hal3p and Ppz1p and/or Ppz2p is destabilized in vivo by increased pH, we would predict that overexpression of HAL3 would be less active as a Ppz inhibitor at increased external pH. Accordingly, we observed that, in contrast to strains lacking PPZ1 and PPZ2, strains overexpressing HAL3 confer much less tolerance to LiCl at a more alkaline pH of 7.5 (Fig. 8B), suggesting that at higher internal pH, the ability of Hal3p to bind to and inhibit Ppz1p inside the cell is also reduced.

Finally, to establish the physiological relevance of this pH-sensitive regulatory mechanism, we compared the growth of strains lacking HAL3 or PPZ1 and PPZ2 at acidic pH. As expected, hal3Δ strains are more sensitive to acidic pH, while (B) The same experiment as in panel A was performed at the indicated pHs. Shown are Western analysis with Hal3p-specific antibodies of the starting material (top) and the affinity-purified material (middle) and the Ponceau S-stained nitrocellulose membrane (bottom). (C) In vitro phosphatase assays were performed as described in Materials and Methods. The results are expressed as the percentage of phosphatase activity observed in the absence of Hal3p and represent the average of duplicate determinations (diamonds, pH 6.5; squares, pH 7.0; triangles, pH 7.5).
ppz1 and -2 strains are more tolerant (Fig. 9). These results demonstrate that both Hal3p and the Ppz phosphatases are important for the adaptation of the cell to acidic pH and are consistent with the proposed model for their function. As predicted from this model, we previously reported both a decrease in Trk1p activity (as measured by rubidium uptake) in strains lacking \( \text{HAL3} \) and a relative accumulation of potassium versus sodium in \( \text{HAL3} \)-overexpressing strains grown under NaCl stress (16, 61).

**DISCUSSION**

The maintenance of ionic homeostasis in response to changes in the environment is vital to all living cells. In many cases, the delicate balance of ions is determined by the dynamic and/or cell-type-specific expression of genes encoding channels, pumps, and transporters. However, an important role for the posttranslational modification, in the form of protein phosphorylation, of these plasma membrane proteins has emerged over the last few years (18, 26, 43, 60). There are several examples of phosphorylation-dependent regulation of the activities of channels and pumps, but in the case of transporters, much less is known (1, 15, 59). Recently, it was reported that the nitrate transporter CHL1 from *Arabidopsis thaliana* is converted from low to high affinity by phosphorylation of a threonine residue (29). This role for protein phosphorylation in the regulation of ion homeostasis implies the existence of signal transduction routes involving kinases and phosphatases that act on substrates in response to a stimulus perceived by the cell. For the most part, these signal transduction routes are uncharacterized, but they are likely to be very important in maintaining the ionic homeostasis of the cell, especially in response to transient changes in the external environment.

In this report, we provide evidence for a pH-responsive, phosphorylation-dependent regulation of the Trk1p potassium transporter. Our results suggest that the activity of the Trk1p transporter is increased by phosphorylation. As Trk1p is known to have two activity (and affinity) states, phosphorylation of the transporter may be involved in this switch, as has been previously proposed (25). Another possibility is that the phosphorylation of Trk1p controls transporter trafficking and/or stability, as has been described for other yeast plasma membrane proteins, like Gap1 (9). Both of these possibilities are under investigation.

A genetic link between the Ppz phosphatases and the Trk potassium transporters has been established (35, 61). The majority of the phenotypes observed for the ppz1 and -2 mutant, including that associated with cell cycle progression, depend on the presence of the \( \text{TRK1} \) and \( \text{TRK2} \) genes. Here, we report the subcellular localization of the phosphatase at the plasma membrane, the physical interaction between Ppz1p and Trk1p by both immunoprecipitation and fractionation techniques, and the increased in vivo phosphorylation of Trk1p in ppz1 and -2 strains. Although we do not discard the existence of additional Ppz substrates, many lines of evidence, including Rb\(^+\) uptake and K\(^+\) accumulation measurements, potassium sensitivity phenotypes, and increases in internal pH, suggest that the Ppz1p phosphatase acts to down-regulate the activity of the Trk1p potassium transporter by modulating its phosphorylation state. The data presented here do not unequivocally identify Trk1p as a direct substrate for Ppz1p, although all of the results presented are consistent with this hypothesis. However, the results of the in vitro kinase assays suggest that several interacting proteins are present in the Trk1p immunoprecipi-

**FIG. 8.** The pH dependence of the Ppz1-Hal3 interaction can be observed biochemically and phenotypically in vivo. (A) The amount of Hal3p recovered in the insoluble fraction was assayed as described in Materials and Methods. (Top) Representative Western analysis of the amount of Hal3p present in the indicated fractions. (Bottom) Ponceau S staining of the nitrocellulose filter as a control for equal protein loading in comparable fractions. (B) The indicated strains (LY 78, LY 79, and LY 83) were grown to saturation in rich media, serially diluted in water, and spotted on the indicated solid media. Growth was recorded after 48 to 72 h of incubation at 28°C.

**FIG. 9.** Both Hal3p and the Ppz phosphatases are involved in the adaptation of yeast cells to acidic pH. The indicated strains were grown to saturation in rich media, serially diluted in water, and spotted on the indicated solid media. Growth was recorded after 48 to 72 h of incubation at 28°C. WT, wild type.
tate. Therefore, we cannot rule out the possibility that Ppz1p acts on one or more proteins present in this complex in order to modify the phosphorylation state of Trk1p.

Studies to identify both the specific phosphorylation sites and the kinase associated with the Trk1p transporter are under way but will be technically challenging. Trk1p is a large protein with a calculated molecular mass of 140 kDa (apparent molecular mass in SDS-PAGE, 180 kDa) that is not highly expressed. Structural modeling suggests that it adopts a 4-MPM channel-like conformation and that it contains intracellular extensions at both the NH$_2$ and COOH termini and three intracellular loops of various lengths (12). There are more than 80 serine and threonine residues within these domains that are predicted phosphorylation sites (NetPhos 2.0 prediction software CBS; Technical University of Denmark). However, attempts to phosphorylate the cytosolic domains individually as GST fusion proteins in vitro or in vivo have been unproductive so far (L. Yenush, unpublished data). These results suggest that localization to the plasma membrane and/or the tertiary structure of the protein may be required for interaction with the kinase.

There are several obvious candidate Trk1p kinases, including Hal4p, Hal5p (36), Sky1p (17), and Hog1p, which was recently shown to phosphorylate Nha1p and Tpk1p (43). Biochemical studies are in progress to investigate the role of each of these kinases in the regulation of Trk1p. Our results show that more phosphorylated Trk1p is observed upon overexpression, but the levels of the other phosphorylated proteins, including Pma1p, are also increased, suggesting that other approaches are necessary to clarify the physiological role of Hal5p in Trk1p regulation. Interestingly, in preliminary experiments, very low levels of phosphorylation of all four raft-associated proteins were observed in strains lacking both HAL4 and HAL5 (L. Yenush, unpublished observations). Studies are under way to investigate this important point.

One of the novel aspects of the results reported here is the pH-responsive component of this regulatory mechanism of potassium homeostasis. To our knowledge, this is the first example of the interaction between a phosphatase and an inhibitory subunit responding to changes in pH. The molecular details of how changes in pH regulate this interaction will require analysis of the atomic structure of the protein complex. Recent mutagenesis analysis of Hal3p has identified a complex interaction between Hal3p and Ppz1p (37). Although many of the Hal3p residues identified as being important for the interaction map to a region of approximately 30 amino acids, the predicted phosphorylation sites (NetPhos 2.0 prediction software CBS; Technical University of Denmark) are not changed by varying the external pH or by removing TRK1 and TRK2 (data not shown). These results suggest that the key regulatory step is the Hal3p-Ppz1p interaction and would provide a rapid response mechanism to quickly inhibit high-affinity potassium uptake in response to transient increases in pH.

Studies of transporters similar to Trk1p, like the HKT transporters from plants, will reveal if this phosphorylation-dependent mechanism for maintaining potassium homeostasis is conserved evolutionarily and whether this process is influenced by pH. As these plant transporters are proposed to be important determinants of salinity tolerance, elucidation of these types of regulatory mechanisms will have an important impact in agricultural biotechnology (33, 48). Similarly, since the regulation of both potassium transport and internal pH homeostasis has been implicated in a wide range of diseases, including cancer, investigation of similar signal transduction pathways connecting pH and potassium homeostasis represents an exciting area of investigation (53).

REFERENCES


