MOLECULAR AND CELLULAR BIOLOGY, Oct. 2005, p. 8683–8692 Vol. 25, No. 19
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.19.8683–8692.2005

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

Lynne Yenush,* Stephanie Merchan, James Holmes,† and Ramón Serrano

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, 46022 Valencia, Spain

Received 3 May 2005/Returned for modification 27 May 2005/Accepted 5 July 2005

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 only 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 Mg^{2+} 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, 38, 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 dra-

* Corresponding author. Mailing address: Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera s/n, 46022 Valencia, Spain. Phone: 34 96 387 7860. Fax: 34 96 387 7859. E-mail: lynne@ibmcp.upv.es.

† Present address: Warwick Horticulture Research International, Wellesbourne, Warwick, CV35 9EF, United Kingdom.

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 Ppz1-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 4 ml of an alkaline magnesium mixture (0.3 M $MgCl_2$, 1.9 M NH_4Cl, 100 ml/liter of concentrated NH_4OH) to 100 ml of the standard yeast extract-peptone mixture in order to precipitate the inorganic phosphate. The precipitate was removed by filtration, the pH was adjusted to 6.0, glucose was added, and the medium was sterilized.

Plasmids and gene insertions. The inducible, hemagglutinin (HA)-tagged version of *TRK1* was constructed by inserting a PCR-generated 1.7-kb NH_2-terminal fragment into pBluescript using a primer-derived XhoI site and the endogenous PstI site and subsequently cloning a 2.0-kb COOH-terminal fragment using the endogenous PstI site and a primer-derived NotI site. The *TRK1* coding sequence was then excised as a PmeI/NotI fragment and inserted into the pCM262 vector. This vector is derived from pCM190 (20), and it contains tetracycline-regulatable promoter and three copies of the HA epitope fused to the COOH terminus of the target gene. Expression was reduced to minimal levels by the addition of doxycycline (Sigma) to a final concentration of 20 μg/ml. A centromeric version of the Trk1-HA fusion was constructed using a similar two-step cloning strategy but replacing the synthetic restriction sites at the ends with SpeI and NdeI and inserting this fragment into a modified version of YCp414 lacking the *N*-ubiquitin sequence (27, 55). The same vector was digested with SalI and XhoI, and a PCR-generated fragment corresponding to the ORF of green fluorescent protein (GFP) was inserted in frame to generate the Trk1-GFP fusion protein. The strategy used for the construction of the GFP fusion of Ppz1p was based on that reported by Venturi et al. (56).

Flotation gradients and raft isolation. Flotation gradients to purify raft proteins were performed essentially as described previously (3). The modified fractionation experiments were identical, except that extracts, treated or not with Triton X-100 as indicated, were subjected to only one round of centrifugation for 4 h at 200,000 × *g*. In both cases, fractions were taken from the top and proteins were precipitated with trichloroacetic acid for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blots were performed using the monoclonal 12CA5 anti-HA antibody (Roche Molecular Biochemicals), anti-Pma1p (50), anti-Hal3p (16), or anti-Ppz1p rabbit polyclonal antibodies (8). Immunoreactive bands were visualized using the ECL-Plus chemiluminescence system and horseradish peroxidase-conjugated secondary antibodies (Amersham).

Fluorescence microscopy. Fluorescence images were obtained for live cells grown to exponential phase in minimal medium using a Leica TCS SL inverted confocal microscope with a 40×/1.25-numerical-aperture planapochromat oil objective. Excitation was at 488 nm, and detection was between 500 and 530 nm. Vacuolar membranes were stained using the FM4-64 vital stain (Molecular Probes) according to the previously reported protocol (57). Excitation was at 488 nm, and detection was between 620 and 660 nm.

Immunoprecipitation of Trk1p and coimmunoprecipitation of Trk1p and Ppz1p. The immunoprecipitation of the Trk1-HA fusion protein was carried out overnight, using the anti-HA high-affinity antibody (Roche) and protein G-agarose (Roche). For in vitro kinase assays, extracts were prepared by vortexing (in the presence of glass beads) cell pellets resuspended in RIPA buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% SDS, 1% [wt/vol] deoxycholic acid [sodium salt; Sigma], 1% Triton X-100, and protease inhibitor cocktail [Roche]). For coimmunoprecipitation experiments, cell pellets (10 to 12 optical density at 66 nm [OD_{660}] units) were resuspended in phosphate-buffered saline (supplemented with protease inhibitor cocktail [Roche]) and vortexed in the presence of glass beads. Insoluble material was removed by centrifugation for 5 min at 500 × *g* (2,000 rpm). Cross-linking was performed using dithiobis[succinimidylpropionate] (Pierce Biotechnology) as described by the manufacturer. The insoluble material was collected and homogenized in TNEGlyc buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 10% glycerol, protease inhibitor cocktail [Roche]). Proteins were solubilized by incubating them with 3 mM Zwittergent TM314 (Calbiochem) for 15 min at 28°C, and immunoprecipitations of the soluble material were carried out as described above. Identical experiments were conducted for the immunoprecipitation of Nha1p (strain MAP 73) (Table 1) and the myc-tagged versions of both Trk1p and Cch1p (strains AM300 and ELY242, respectively), except that where indicated, 3 μg of anti-myc (clone 9E10; Roche) was used in place of anti-HA.

In vitro kinase assay. Immunoprecipitations were performed as described above, and the Trk1-HA-containing protein G agarose was resuspended in 30 μl of kinase reaction buffer (50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 μCi [γ-^{32}P]ATP [Amersham]), with or without the addition of 50 mM EDTA, and incubated for 30 min at 28°C. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were exposed overnight to visualize the incorporated ^{32}P and then processed for Western analysis as described above using the 12CA5 anti-HA antibody.

In vivo labeling. The indicated strains were grown in low-phosphate medium (50 ml/strain) to an OD_{660} of 0.4. Cells were collected by centrifugation and resuspended in 5 ml of low-phosphate medium, 250 μCi of [^{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.

In vitro binding and phosphatase assays. In vitro binding assays and phosphatase assays were performed as described previously, except that 50 mM BIS-TRIS {[*bis*-(2-hydroxyethyl)-imino]-*tris*-(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 to an OD_{660} 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 dithioerythritol, 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 × *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

TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Reference[a]
W303-1A	*mat***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	61
WΔ3	W303-1A *trk1*::*LEU2 trk2*::*HIS3*	36
LY 185	WΔ3 [YCp414]	
LY 236	WΔ3 [YCp414-*TRK1-HA*]	
LY 247	WΔ3 [YCp414-*TRK1-HA-GFP*]	
LY 152	WΔ3 [pCM262]	
LY 161	WΔ3 [pCM262-*TRK1-HA*]	
LY 139	W303-1A *ppz1*::*URA3 ppz2*::*TRP1*	61
LY 166	W303-1A *ppz1*::*URA3 ppz2*::*TRP1 PPZ1-GFP*::*LEU2*	
LY 140	W303-1A *trk1*::*LEU2 trk2*::*HIS3 ppz1*::*URA3 ppz2*::*TRP1*	61
JM 76	W303-1A [YEp351-*HAL5*]	36
SKY684	W303-1A *hal3*::*LEU2*	16
DBY746	*mat*α *ura3-52 leu2-3 leu2-112 his3-*Δ*1 trp1-289*	A. Rodriguez-Navarro
LY 78	DBY746 [pRS699]	61
LY 79	DBY746 [pRS699-*HAL3*]	61
LY 83	DBY746 *ppz1*::*URA3 ppz2*::*TRP1*	10
MAP 73	W303-1A *NHA1*-3xHA-Kan^r	43
AM300	PSY 316 *TRK1*-13xmyc-Kan^r	38
ELY242	W303-1A *CCH1*-13xmyc-*TRP1*	30

[a] Strains not otherwise indicated are from this study.

(Beckman). The particulate fraction was resuspended by homogenization. Approximately 50 μg of protein/lane was separated on 8% SDS-PAGE. The immunodetection of Hal3p was conducted as described above.

RESULTS

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 purified by applying Triton-treated extracts to flotation gradients. To determine whether Trk1p is present in rafts, we employed a strain harboring an inducible plasmid encoding *TRK1* fused to three tandem copies of the HA epitope at the COOH terminus. Since the overproduction of protein products is known to produce artifacts in subcellular-localization assays, fractionation experiments were performed under conditions where the *TRK1*-HA fusion was expressed at very low levels (in the presence of 20 μg/ml of doxycycline). We observed that both the Trk1-HA fusion protein and Pma1p were highly enriched in the lightest density fractions, corresponding to the purified lipid rafts (Fig. 1). We subsequently confirmed this lipid raft association for Trk1p expressed from a centromeric plasmid under the control of the *ADH1* promoter or under the control of its own promoter by using a genomically tagged version of *TRK1* (strains LY 236 and AM300) (data not shown). These results establish Trk1p as yet another yeast transporter present in lipid rafts. Interestingly, a previous report suggested that Trk2p is also present in these microdomains, although in that case, the protein was highly overexpressed, complicating the interpretation of these data (62).

Ppz1p is peripherally localized at the plasma membrane in vivo and physically associates with Trk1p. Several lines of evidence have suggested that the activity of Trk1p is modulated by the type 1-related phosphatases encoded by the *PPZ1* and *PPZ2* genes (35, 61). As a first step in determining whether Trk1p is a direct substrate for these enzymes in vivo, we investigated the subcellular localization of the endogenous Ppz1 protein. We constructed a strain in which a fusion of *PPZ1* with GFP was integrated into the genome, thus providing endogenous levels of Ppz1-GFP under the control of its own promoter. The function of this fusion protein was confirmed by complementation of the growth defect of the *ppz1* and *-2* mutant on alkaline medium or medium containing caffeine (data not shown). As shown in Fig. 2, Ppz1-GFP is mostly

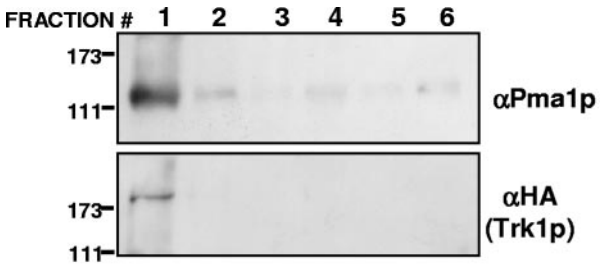


FIG. 1. Trk1p is localized to plasma membrane rafts. Rafts were purified from the WΔ3 [pCM262-*TRK1*] yeast strain (grown in the presence of 20 μg/ml doxycycline) as described in Materials and Methods. The presence of Pma1p and the HA-tagged version of Trk1p in the various fractions was analyzed by Western blotting. Identical results were observed in four different experiments. The numbers above the lanes indicate the fraction number starting from the top of the gradient (lowest to highest density).

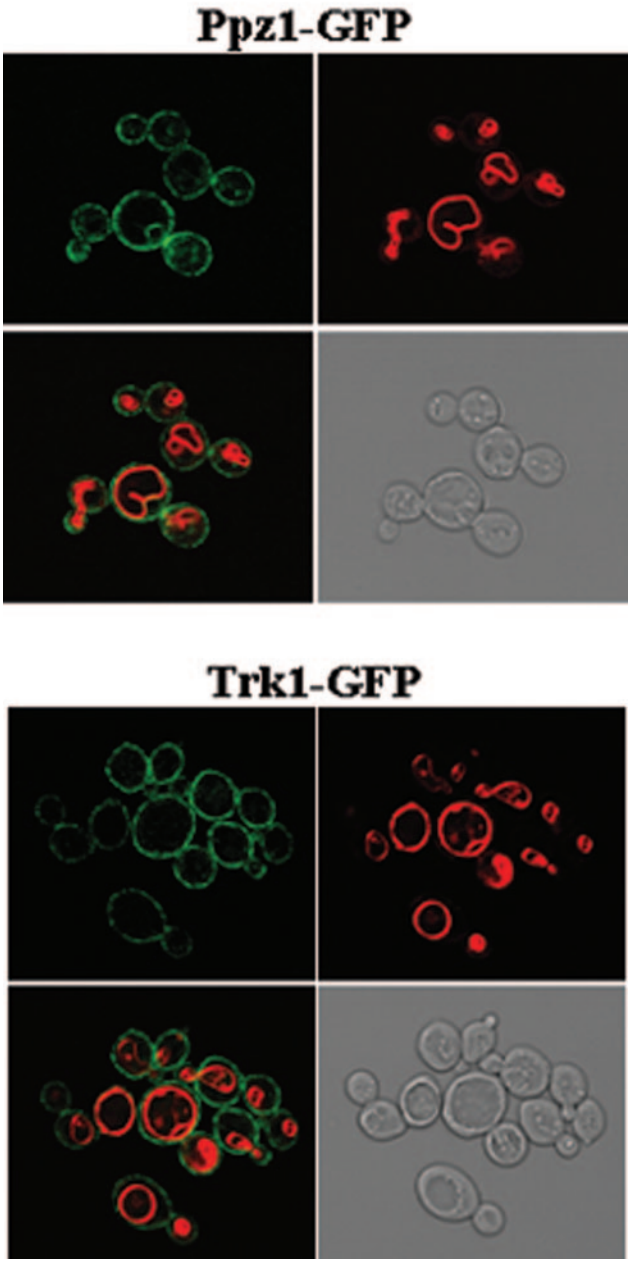


FIG. 2. Localization of the Ppz1-GFP fusion protein. The strains LY 166 and LY 247 were analyzed by confocal microscopy, as described in Materials and Methods. The localization of the fusion proteins is shown in green. The FM4-64-stained vacuolar membranes are shown in red. Overlay and gray scale images are also shown.

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.

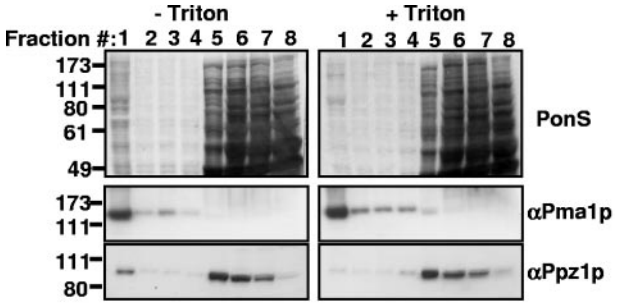


FIG. 3. Ppz1p partially associates with the lowest-density fraction in flotation gradients. Proteins were extracted from the W303-1A strain and processed as described in Materials and Methods. The Ponceau S staining of the membranes is shown in the top panel, as a control for protein loading. Fraction numbers are indicated above each lane (1 indicates the lowest-density and 8 indicates the highest-density fraction).

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 *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 *TRK1* and *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 *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-

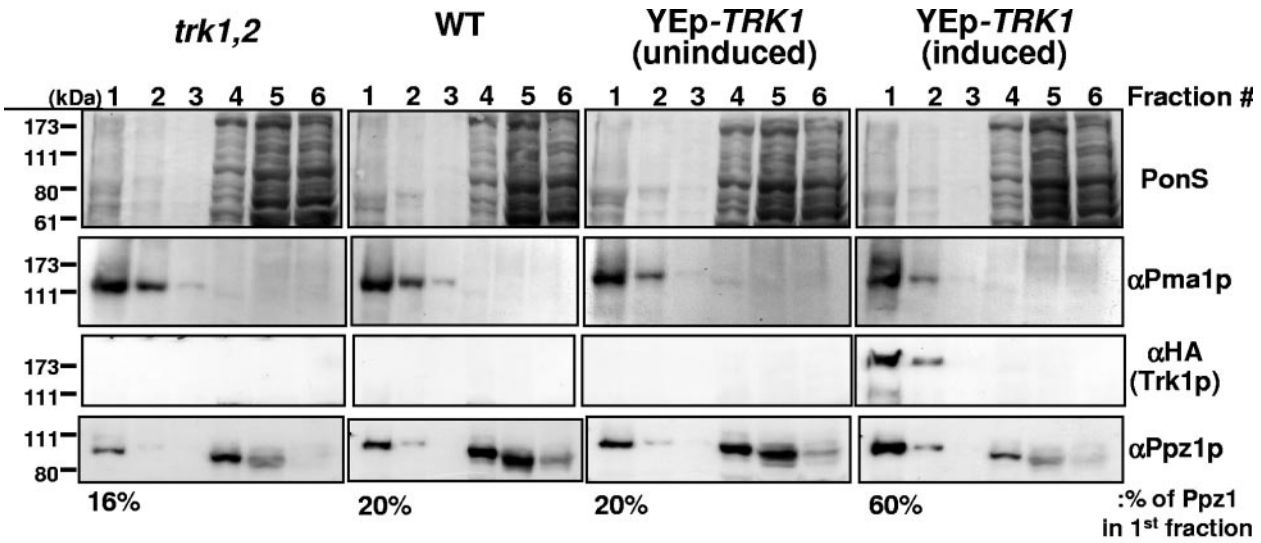


FIG. 4. Redistribution of Ppz1p upon *TRK1* overexpression. The indicated yeast strains (WΔ3, W303-1A, LY 152, and LY 161) were processed for flotation gradients as described in Materials and Methods. The top row shows the Ponceau S staining of the membranes as a control for protein loading. The amount 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.

iments. To this end, we constructed strains in which the only source of Trk proteins was provided by a centromeric plasmid harboring an HA-tagged version of *TRK1*. Figure 5A shows the results of the coimmunoprecipitation experiments in which we specifically detected Ppz1p in Trk1p immunoprecipitates. We confirmed the reproducibility and specificity of this interaction by performing coimmunoprecipitation experiments between Ppz1p and versions of Nha1p, Cch1p, and Trk1p fused to either the HA or myc epitope using in-frame genomic insertions (Fig. 5B and C). In all three experiments, regardless of the epitope fused to Trk1p and the expression context (centromeric plasmid or genomic insertion), Ppz1p was copurified with Trk1p, but not with the Na^+/H^+ antiporter, Nha1p, or a subunit of the high-affinity plasma membrane Ca^{2+} uptake system, Cch1p.

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-^{32}P]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 Mg^{2+}-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 ^{32}P-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 phosphorylated 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 [^{32}P]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 ^{32}P 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

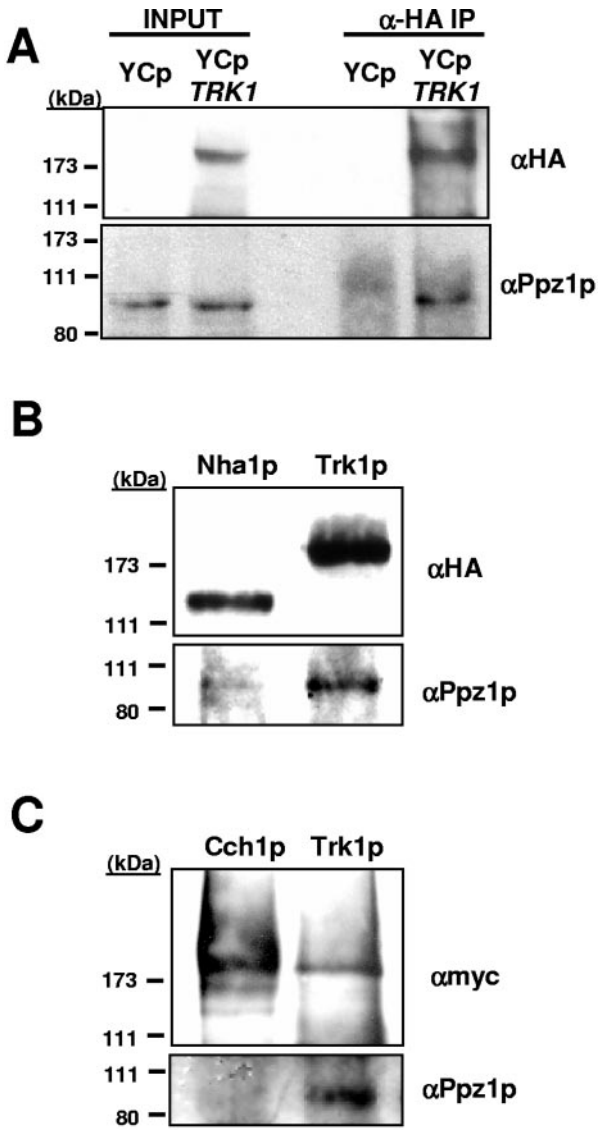


FIG. 5. Ppz1p coimmunoprecipitates with Trk1p. Proteins were extracted from the indicated yeast strains, cross-linked, and processed for immunoprecipitation experiments as described in Materials and Methods. The proteins recovered by the affinity resin were analyzed by Western blotting using the indicated antibodies. In panel A, the first two lanes contain an aliquot of the crude extracts, and the last two lanes contain the immunoprecipitated material. In panels B and C, the lanes correspond to the immunoprecipitated material from the indicated strain.

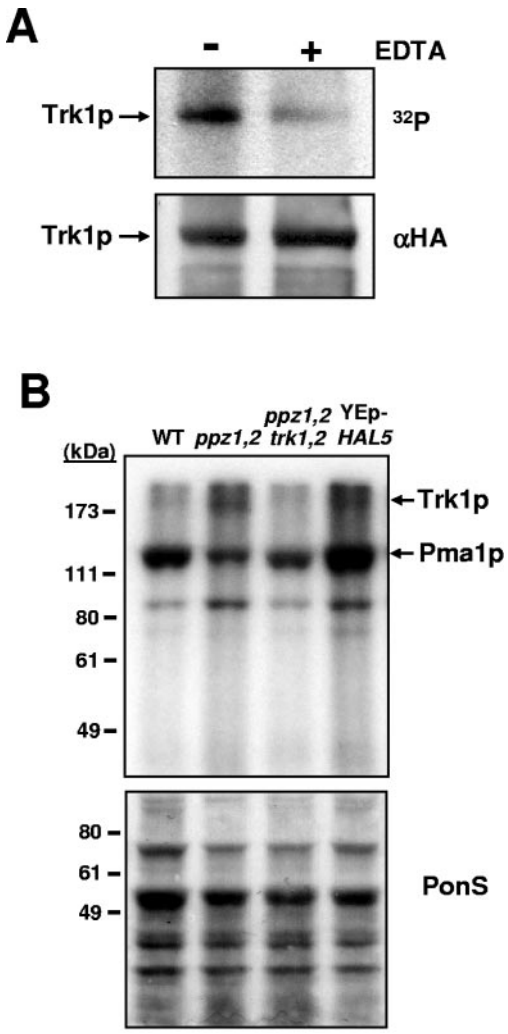


FIG. 6. Phosphorylation of Trk1p in vitro and in vivo. (A) Proteins extracted from the yeast strain LY 236 were processed for immunoprecipitation experiments, followed by in vitro kinase assays, as described in Materials and Methods. On top are shown the results of the autoradiography, and the results of the Western blot used as a control of protein loading are below. Similar results were observed in three separate experiments. (B) The indicated strains were grown in low-phosphate media, and phosphorylated proteins were radioactively labeled in vivo and processed for raft purification, as described in Materials and Methods. Proteins present in the top fraction of the second gradient were separated by SDS-PAGE and analyzed by autoradiography (top). On the bottom is a Ponceau S-stained membrane showing equal amounts of protein present in each sample extracted just before labeling. Similar results were observed in three different experiments. WT, wild type.

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-

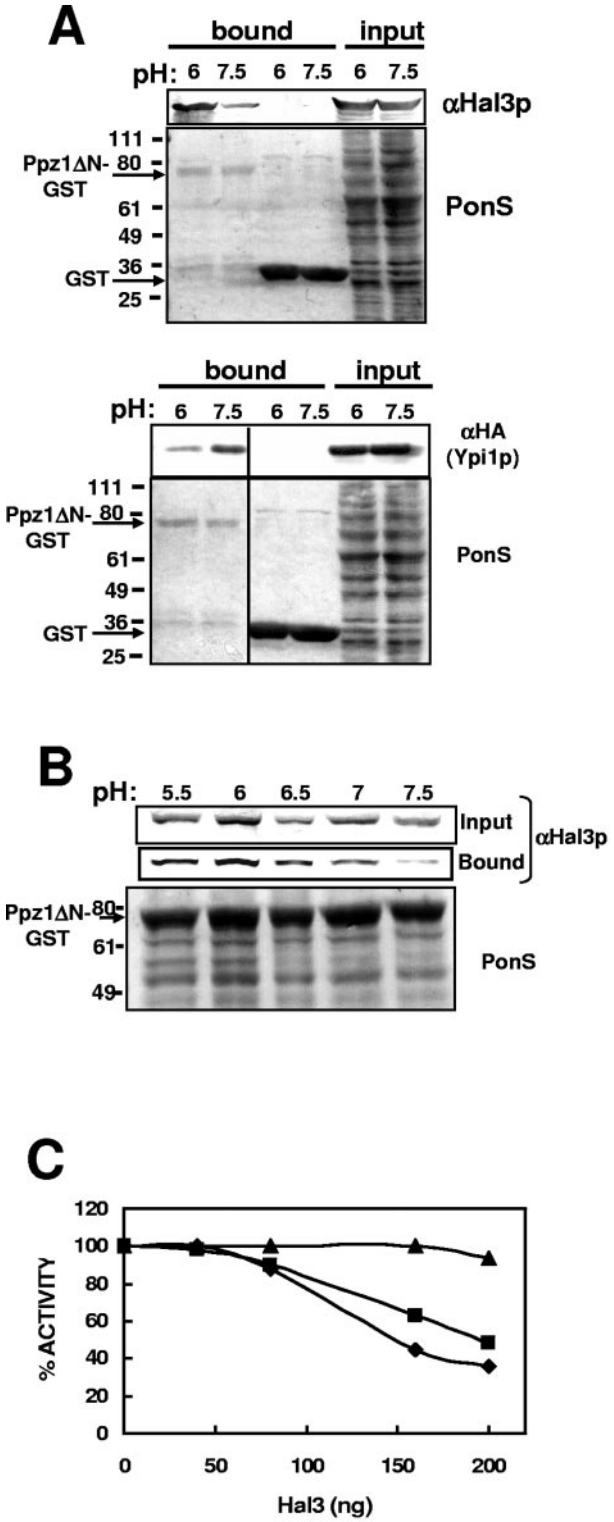
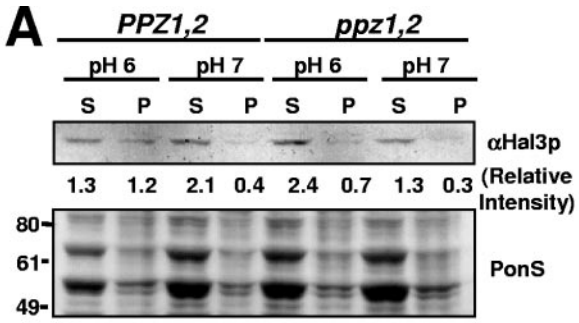


FIG. 7. The in vitro interaction between Ppz1p and Hal3p is pH dependent. (A) Representative Western blot showing the amount of Hal3p (top) or Ypi1p (bottom) bound by the Ppz1ΔN-GST affinity resin at the pH indicated and the amount of Hal3p or Ypi1p in the starting extracts (input). The specificity of the binding interactions is demonstrated by the lack of binding to GST alone. Ponceau S-stained nitrocellulose membranes demonstrating equal protein loading are shown. The positions of GST and the Ppz1ΔN-GST fusion protein are indicated. Similar results were observed in two different experiments. (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).

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 *ppz1* and *-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, *hal3* strains are more sensitive to acidic pH, while



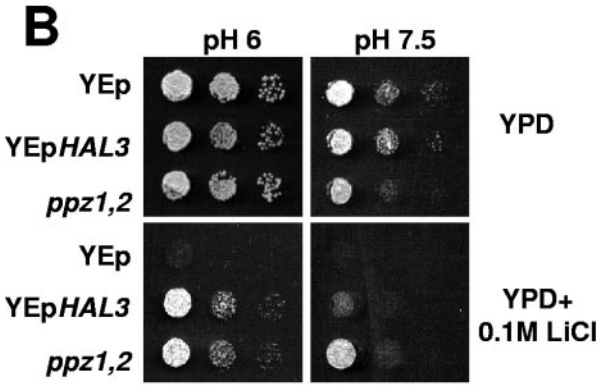


FIG. 8. The pH dependence of the Ppz1-Hal3 interaction can be observed biochemically and phenotypically in vivo. (A) The amount of Hal3p recovered in the insoluble fraction was assayed as described in Materials and Methods. (Top) Representative Western analysis of the amount of Hal3p present in the indicated 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.

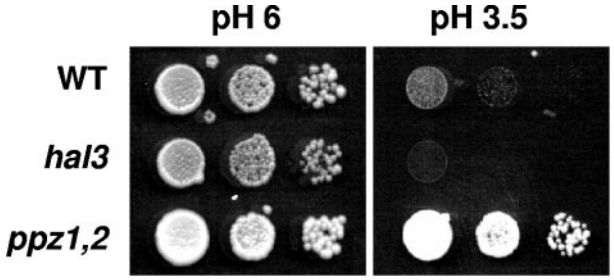


FIG. 9. Both Hal3p and the Ppz phosphatases are involved in the adaptation of yeast cells to acidic pH. The indicated strains were grown to saturation in rich media, serially diluted in water, and spotted on the indicated solid media. Growth was recorded after 48 to 72 h of incubation at 28°C. WT, wild type.

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 *HAL3* and a relative accumulation of potassium versus sodium in *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 *TRK1* and *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-

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 Tok1p (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 *HAL5* 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 profile of Ppz1p inhibition of these mutations is not straightforward and suggests a complex interaction between the two proteins. In any case, the observation that this system is regulated by increases in internal pH makes intuitive sense; as potassium accumulates in the cytosol, the internal pH will increase as protons are extruded in order to maintain electrical neutrality. However, as mentioned earlier, accumulation of potassium can be very detrimental to the cell, causing a large increase in turgor pressure and the risk of cell lysis. Therefore, the cell can use this concomitant increase in intracellular pH to signal the down-regulation of potassium transport. In the model presented here, this mechanism would involve the destabilization of the interaction between Hal3p and Ppz1p at relatively alkaline pH, thus allowing the phosphatase to act on Trk1p and decrease the potassium uptake into the cell. Our experiments with the Ppz1-GFP fusion protein suggest that this phosphatase is localized at the plasma membrane under normal growth conditions and that this localization pattern is not changed by varying the external pH or by removing *TRK1* and *TRK2* (data not shown). These results may 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).

ACKNOWLEDGMENTS

This work was supported by grants PB98-0565, BMC2000-1129, and BCM2002-04011 from the Spanish Ministries of Education and Culture and General Promotion of Knowledge and of Science and Technology (Madrid). L.Y. is an investigator sponsored by the Ramón y Cajal Program (Spanish Ministry of Science and Technology, Madrid, Spain). S.M. is supported by a predoctoral grant from the Ministry of Education (Madrid, Spain).

We thank Joaquin Ariño for providing Ppz1p antibodies; Kyle Cunningham, Markus Proft, Pascual Sanz, and Anne Rosenwald for providing yeast strains and plasmids; and Dolores Bernal and José Ramón Murguía for helpful discussions. We also thank María Dolores Gómez and Huw Jenkins for excellent technical assistance with the confocal microscopy.

REFERENCES

1. **Antz, C., T. Bauer, H. Kalbacher, R. Frank, M. Covarrubias, H. R. Kalbitzer, J. P. Ruppersberg, T. Baukrowitz, and B. Fakler.** 1999. Control of K+ channel gating by protein phosphorylation: structural switches of the inactivation gate. Nat. Struct. Biol. **6:**146–150.
2. **Bagnat, M., A. Chang, and K. Simons.** 2001. Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast. Mol. Biol. Cell **12:**4129–4138.
3. **Bagnat, M., S. Keranen, A. Shevchenko, A. Shevchenko, and K. Simons.** 2000. Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. Proc. Natl. Acad. Sci. USA **97:**3254–3259.
4. **Benito, B., F. Portillo, and R. Lagunas.** 1992. In vivo activation of the yeast plasma membrane ATPase during nitrogen starvation. Identification of the regulatory domain that controls activation. FEBS Lett. **300:**271–274.
5. **Bianchini, L., G. L'Allemain, and J. Pouyssegur.** 1997. The p42/p44 mitogen-activated protein kinase cascade is determinant in mediating activation of the Na+/H+ exchanger (NHE1 isoform) in response to growth factors. J. Biol. Chem. **272:**271–279.
6. **Bierman, A. J., E. J. Cragoe, Jr., S. W. de Laat, and W. H. Moolenaar.** 1988. Bicarbonate determines cytoplasmic pH and suppresses mitogen-induced alkalinization in fibroblastic cells. J. Biol. Chem. **263:**15253–15256.
7. **Chang, A. and C. W. Slayman.** 1991. Maturation of the yeast plasma membrane [H+]ATPase involves phosphorylation during intracellular transport. J. Cell Biol. **115:**289–295.
8. **Clotet, J., F. Posas, E. de Nadal, and J. Arino.** 1996. The NH_2-terminal extension of protein phosphatase PPZ1 has an essential functional role. J. Biol. Chem. **271:**26349–26355.
9. **De Craene, J. O., O. Soetens, and B. Andre.** 2001. The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease. J. Biol. Chem. **276:**43939–43948.
10. **de Nadal, E., J. Clotet, F. Posas, R. Serrano, N. Gomez, and J. Arino.** 1998.

The yeast halotolerance determinant Hal3p is an inhibitory subunit of the Ppz1p Ser/Thr protein phosphatase. Proc. Natl. Acad. Sci. USA **95:**7357–7362.
11. **Di Como, C. J., R. Bose, and K. T. Arndt.** 1995. Overexpression of SIS2, which contains an extremely acidic region, increases the expression of SWI4, CLN1 and CLN2 in sit4 mutants. Genetics **139:**95–107.
12. **Durell, S. R., and H. R. Guy.** 1999. Structural models of the KtrB, TrkH, and Trk1,2 symporters based on the structure of the KcsA K^+ channel. Biophys. J. **77:**789–807.
13. **Epel, D. and F. Dubé.** 1987. Intracellular pH and cell proliferation. *In* A. L. Boynton and H. L. Leffert (ed.), Control of animal cell proliferation. American Press, New York, N.Y.
14. **Eraso, P., and F. Portillo.** 1987. Activation of yeast plasma membrane ATPase by acid pH during growth. FEBS Lett. **224:**187–192.
15. **Esguerra, M., J. Wang, C. D. Foster, J. P. Adelman, R. A. North, and I. B. Levitan.** 1994. Cloned Ca^{2+}-dependent K^+ channel modulated by a functionally associated protein kinase. Nature **369:**563–565.
16. **Ferrando, A., S. J. Kron, G. Rios, G. R. Fink, and R. Serrano.** 1995. Regulation of cation transport in *Saccharomyces cerevisiae* by the salt tolerance gene HAL3. Mol. Cell. Biol. **15:**5470–5481.
17. **Forment, J., J. M. Mulet, O. Vicente, and R. Serrano.** 2002. The yeast SR protein kinase Sky1p modulates salt tolerance, membrane potential and the Trk1,2 potassium transporter. Biochim. Biophys. Acta **1565:**36–40.
18. **Fuglsang, A. T., S. Visconti, K. Drumm, T. Jahn, A. Stensballe, B. Mattei, O. N. Jensen, P. Aducci, and M. G. Palmgren.** 1999. Binding of 14-3-3 protein to the plasma membrane H^+-ATPase AHA2 involves the three C-terminal residues Tyr(946)-Thr-Val and requires phosphorylation of Thr(947). J. Biol. Chem. **274:**36774–36780.
19. **Garcia-Gimeno, M. A., I. Munoz, J. Arino, and P. Sanz.** 2003. Molecular characterization of Ypi1, a novel *Saccharomyces cerevisiae* type 1 protein phosphatase inhibitor. J. Biol. Chem. **278:**47744–47752.
20. **Gari, E., L. Piedrafita, M. Aldea, and E. Herrero.** 1997. A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. Yeast **13:**837–848.
21. **Gillies, R. J., R. Martinez-Zaguilan, G. M. Martinez, R. Serrano, and R. Perona.** 1990. Tumorigenic 3T3 cells maintain an alkaline intracellular pH under physiological conditions. Proc. Natl. Acad. Sci. USA **87:**7414–7418.
22. **Gillies, R. J., K. Ugurbil, J. A. den Hollander, and R. G. Shulman.** 1981. 31P NMR studies of intracellular pH and phosphate metabolism during cell division cycle of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **78:**2125–2129.
23. **Gottlieb, R. A., J. Nordberg, E. Skowronski, and B. M. Babior.** 1996. Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. Proc. Natl. Acad. Sci. USA **93:**654–658.
24. **Grinstein, S., D. Rotin, and M. J. Mason.** 1989. Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. Biochim. Biophys. Acta **988:**73–97.
25. **Haro, R. and A. Rodriguez-Navarro.** 2002. Molecular analysis of the mechanism of potassium uptake through the TRK1 transporter of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **1564:**114–122.
26. **Hayashi, H., K. Szaszi, and S. Grinstein.** 2002. Multiple modes of regulation of Na^+/H^+ exchangers. Ann. N. Y. Acad. Sci. **976:**248–258.
27. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff.** 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2:**163–167.
28. **Hoffman, J. F.** 1964. The cellular functions of membrane transport. Prentice-Hall Inc., Englewood Cliffs, N.J.
29. **Liu, K. H., and Y. F. Tsay.** 2003. Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. EMBO J. **22:**1005–1013.
30. **Locke, E. G., M. Bonilla, L. Liang, Y. Takita, and K. W. Cunningham.** 2000. A homolog of voltage-gated Ca^{2+} channels stimulated by depletion of secretory Ca^{2+} in yeast. Mol. Cell. Biol. **20:**6686–6694.
31. **Ludovico, P., M. J. Sousa, M. T. Silva, C. Leao, and M. Corte-Real.** 2001. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology **147:**2409–2415.
32. **Madrid, R., M. J. Gomez, J. Ramos, and A. Rodriguez-Navarro.** 1998. Ectopic potassium uptake in *trk1 trk2* mutants of *Saccharomyces cerevisiae* correlates with a highly hyperpolarized membrane potential. J. Biol. Chem. **273:**14838–14844.
33. **Maser, P., B. Eckelman, R. Vaidyanathan, T. Horie, D. J. Fairbairn, M. Kubo, M. Yamagami, K. Yamaguchi, M. Nishimura, N. Uozumi, W. Robertson, M. R. Sussman, and J. I. Schroeder.** 2002. Altered shoot/root Na^+ distribution and bifurcating salt sensitivity in *Arabidopsis* by genetic disruption of the Na^+ transporter AtHKT1. FEBS Lett. **531:**157–161.
34. **Mendoza, I., F. Rubio, A. Rodriguez-Navarro, and J. M. Pardo.** 1994. The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. J. Biol. Chem. **269:**8792–8796.
35. **Merchan, S., D. Bernal, R. Serrano, and L. Yenush.** 2004. Response of the *Saccharomyces cerevisiae* Mpk1 mitogen-activated protein kinase pathway to increases in internal turgor pressure caused by loss of Ppz protein phosphatases. Eukaryot. Cell **3:**100–107.
36. **Mulet, J. M., M. P. Leube, S. J. Kron, G. Rios, G. R. Fink, and R. Serrano.** 1999. A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. Mol. Cell. Biol. **19:**3328–3337.
37. **Munoz, I., A. Ruiz, M. Marquina, A. Barcelo, A. Albert, and J. Arino.** 2004. Functional characterization of the yeast Ppz1 phosphatase inhibitory subunit Hal3. A mutagenesis study. J. Biol. Chem. **279:**42619–42627.
38. **Munson, A. M., D. H. Haydon, S. L. Love, G. L. Fell, V. R. Palanivel, and A. G. Rosenwald.** 2004. Yeast ARL1 encodes a regulator of K^+ influx. J. Cell Sci. **117:**2309–2320.
39. **Murguia, J. R., L. De Vries, L. Gomez-Garcia, A. Schonthal, and R. Perona.** 1995. Activation of the c-*fos* promoter by increased internal pH. J. Cell Biochem. **57:**630–640.
40. **Padan, E., T. Tzubery, K. Herz, L. Kozachkov, A. Rimon, and L. Galili.** 2004. NhaA of *Escherichia coli*, as a model of a pH-regulated Na^+/H^+antiporter. Biochim. Biophys. Acta **1658:**2–13.
41. **Perona, R. and R. Serrano.** 1988. Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump. Nature **334:**438–440.
42. **Pollard, T. D., and W. C. Earnshaw.** 2002. Cell biology, p. 149–174. Saunders, Philadelphia, Pa.
43. **Proft, M., and K. Struhl.** 2004. MAP kinase-mediated stress relief that precedes and regulates the timing of transcriptional induction. Cell **118:**351–361.
44. **Rios, G., A. Ferrando, and R. Serrano.** 1997. Mechanisms of salt tolerance conferred by overexpression of the HAL1 gene in *Saccharomyces cerevisiae*. Yeast **13:**515–528.
45. **Ruiz, A., M. del Carmen Ruiz, M. A. Sanchez-Garrido, J. Arino, and J. Ramos.** 2004. The Ppz protein phosphatases regulate Trk-independent potassium influx in yeast. FEBS Lett. **578:**58–62.
46. **Ruiz, A., I. Munoz, R. Serrano, A. Gonzalez, E. Simon, and J. Arino.** 2004. Functional characterization of the *Saccharomyces cerevisiae* VHS3 gene: a regulatory subunit of the Ppz1 protein phosphatase with novel, phosphatase-unrelated functions. J. Biol. Chem. **279:**34421–34430.
47. **Ruiz, A., L. Yenush, and J. Arino.** 2003. Regulation of ENA1 Na^+-ATPase gene expression by the Ppz1 protein phosphatase is mediated by the calcineurin pathway. Eukaryot. Cell **2:**937–948.
48. **Rus, A., S. Yokoi, A. Sharkhuu, M. Reddy, B. H. Lee, T. K. Matsumoto, H. Koiwa, J. K. Zhu, R. A. Bressan, and P. M. Hasegawa.** 2001. AtHKT1 is a salt tolerance determinant that controls Na^+ entry into plant roots. Proc. Natl. Acad. Sci. USA **98:**14150–14155.
49. **Serrano, R.** 1991. Transport across yeast vacuolar and plasma membranes. *In* J. R. Broach, J. R. Pringle, and E. W. Jones (ed.), The molecular and cellular biology of the yeast *Saccharomyces*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
50. **Serrano, R., B. C. Monk, J. M. Villalba, C. Montesinos, and E. W. Weiler.** 1993. Epitope mapping and accessibility of immunodominant regions of yeast plasma membrane H^+-ATPase. Eur. J. Biochem. **212:**737–744.
51. **Serrano, R., J. M. Mulet, G. Rios, J. A. Marquez, I. F. de Larrinoa, M. P. Leube, I. Mendizabal, A. Pascual-Ahuir, M. Proft, R. Ros, and C. Montesinos.** 1999. A glimpse of the mechanisms of ion homeostasis during salt stress in plants. J. Exp. Bot. **50:**1023–1036.
52. **Serrano, R. and A. Rodriguez-Navarro.** 2001. Ion homeostasis during salt stress in plants. Curr. Opin. Cell Biol. **13:**399–404.
53. **Shieh, C. C., M. Coghlan, J. P. Sullivan, and M. Gopalakrishnan.** 2000. Potassium channels: molecular defects, diseases, and therapeutic opportunities. Pharmacol. Rev. **52:**557–594.
54. **Shrode, L. D., E. A. Rubie, J. R. Woodgett, and S. Grinstein.** 1997. Cytosolic alkalinization increases stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) activity and p38 mitogen-activated protein kinase activity by a calcium-independent mechanism. J. Biol. Chem. **272:**13653–13659.
55. **Stagljar, I., C. Korostensky, N. Johnsson, and S. te Heesen.** 1998. A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. Proc. Natl. Acad. Sci. USA **95:**5187–5192.
56. **Venturi, G. M., A. Bloecher, T. Williams-Hart, and K. Tatchell.** 2000. Genetic interactions between GLC7, PPZ1 and PPZ2 in *Saccharomyces cerevisiae*. Genetics **155:**69–83.
57. **Vida, T. A., and S. D. Emr.** 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J. Cell Biol. **128:**779–792.
58. **Wakabayashi, S., M. Shigekawa, and J. Pouyssegur.** 1997. Molecular physiology of vertebrate Na^+/H^+ exchangers. Physiol. Rev. **77:**51–74.
59. **Wang, Y. T., X. M. Yu, and M. W. Salter.** 1996. Ca^{2+}-independent reduction of *N*-methyl-D-aspartate channel activity by protein tyrosine phosphatase. Proc. Natl. Acad. Sci. USA **93:**1721–1725.
60. **Weiger, T. M., A. Hermann, and I. B. Levitan.** 2002. Modulation of calcium-activated potassium channels. J. Comp. Physiol. A **188:**79–87.
61. **Yenush, L., J. M. Mulet, J. Arino, and R. Serrano.** 2002. The Ppz protein phosphatases are key regulators of K^+ and pH homeostasis: implications for salt tolerance, cell wall integrity and cell cycle progression. EMBO J. **21:**920–929.
62. **Zeng, G. F., M. Pypaert, and C. L. Slayman.** 2004. Epitope tagging of the yeast K^+ carrier Trk2p demonstrates folding that is consistent with a channel-like structure. J. Biol. Chem. **279:**3003–3013.