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The yeast mutant vps5Δ affected in the recycling of Golgi membrane proteins displays an enhanced vacuolar Mg\(^{2+}\)/H\(^{+}\) exchange activity

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Growth of the yeast vacuolar protein-sorting mutant vps5Δ affected in the endosome-to-Golgi retromer complex was more sensitive to Mg\(^{2+}\)-limiting conditions than was the growth of the wild-type (WT) strain. This sensitivity was enhanced at acidic pH. The vps5Δ strain was also sensitive to Al\(^{3+}\), known to inhibit Mg\(^{2+}\) uptake in yeast cells. In contrast, it was found to be resistant to Ni\(^{2+}\) and Co\(^{2+}\), two cytotoxic analogs of Mg\(^{2+}\). Resistance to Ni\(^{2+}\) did not seem to result from the alteration of plasma-membrane transport properties because mutant and WT cells displayed similar Ni\(^{2+}\) uptake. After plasma-membrane permeabilization, intracellular Ni\(^{2+}\) uptake in vps5Δ cells was 3-fold higher than in WT cells, which is consistent with the implication of the vacuole in the observed phenotypes. In reconstituted vacuolar vesicles prepared from vps5Δ, the rates of H\(^{+}\) exchange with Ni\(^{2+}\), Co\(^{2+}\), and Mg\(^{2+}\) were increased (relative to WT) by 170%, 130%, and 50%, respectively. The rates of H\(^{+}\) exchange with Ca\(^{2+}\), Cd\(^{2+}\), and K\(^{+}\) were similar in both strains, as were \(\alpha\)-mannosidase and H\(^{+}\)-ATPase activities, and SDS/PAGE patterns of vacuolar proteins. Among 14 other vacuolar protein-sorting mutants tested, only the 8 mutants affected in the recycling of trans-Golgi network membrane proteins shared the same Ni\(^{2+}\) resistance phenotype as vps5Δ. It is proposed that a trans-Golgi network Mg\(^{2+}\)/H\(^{+}\) exchanger, mislocalized to vps5Δ vacuole, could be responsible for the phenotypes observed in vivo and in vitro.

Protein sorting pathways from the trans-Golgi network (TGN) to the vacuole have been extensively studied through a large number of yeast mutants (reviewed in refs. 1 and 2). To date, two main intracellular routes have been identified, exemplified by the respective targeting pathways of carboxypeptidase Y (CPY) and alkaline phosphatase (ALP). CPY travels through a prevacuolar/endosomal compartment (PVC) and subsequently onto the vacuole, whereas ALP bypasses the PVC to reach the same organelle. Numerous dedicated proteins localized in transport vesicles or target membranes take part in this process, and the recycling of some of these proteins is important for the efficiency of some trafficking steps. In particular, a membrane-coat complex, designated as the retromer complex, is essential for the PVC-to-Golgi retrograde vesicular transport (3). The retromer complex assembles from two subcomplexes composed of Vps35p/Vps29p/Vps26p and Vps5p/Vps17p, respectively. The first subcomplex is thought to select cargo for retrieval, whereas the second would promote vesicle formation (3–5). Mutation of VPS35, a nonessential gene, causes the secretion of CPY (6, 7). Indeed, Vps5p is required for the PVC-to-Golgi retrieval of Vps10p, a membrane protein that ensures binding and Golgi-to-PVC transport of CPY. Consequently, Vps10p is misrouted to the vacuole membrane in vps5 mutants, whereas CPY accumulates in the Golgi and is then secreted.

In this study, a vps5Δ strain is shown to display new growth phenotypes toward Mg\(^{2+}\)-limiting media or media containing the Mg\(^{2+}\) cytotoxic analogs Ni\(^{2+}\) and Co\(^{2+}\). Together with in vitro assays of Ni\(^{2+}\) uptake and in vivo assays of vacuolar C\(^{2+}\)-divalent cation/H\(^{+}\) exchange, our data suggest that the phenotypes originate from an enhanced vacuolar compartmentalization of C\(^{2+}\) mediated by a Mg\(^{2+}\)/H\(^{+}\) exchange mechanism.

Materials and Methods

Strains, Media, and Spot Assays. The haploid Saccharomyces cerevisiae wild-type (WT) strain Hansen BY4741 (ref. 8; genotype: MATα his3-1 leu2-0 met15-0 ura3-0) was obtained from the American Type Culture Collection, and the vps mutants were obtained from Research Genetics (Huntsville, AL). Yeast strains were propagated in standard yeast nitrogen base (YNB) minimal medium (9). To screen heavy metal resistance phenotypes, medium was prepared from a YNB powder containing ammonium sulfate but no C\(^{2+}\) (Bio 101). The YNB powder was complemented with glucose and Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\) up to standard concentrations (9), except for Mg\(^{2+}\), which was adjusted to 100 μM. A supplemental mixture (CSM, Bio 101) was added according to manufacturer’s instructions, and agar was used as the gelling agent (14 gliter\(^{-1}\)). After autoclaving, heavy metals to which resistance was tested were added in the medium at the desired concentrations. Sensitivity to Mg\(^{2+}\)-limiting conditions was studied on a similar YNB minimal medium, except that agar was replaced by a low-Mg\(^{2+}\)-containing agarose (12 gliter\(^{-1}\); Low EEO, Sigma). This medium did not allow yeast growth without Mg\(^{2+}\) supplementation (data not shown). Magnesium was added at the indicated concentrations after autoclaving. The pH of YNB minimal media was 4.3. When required, the pH was adjusted before autoclaving with HCl to pH 3.0, with 50 mM succinate/KOH to pH 4.3 or 5.6, or with 50 mM Hepes/KOH to pH 7.5.

Spot assays were performed with strains grown up to stationary phase in standard YNB minimal medium (OD\(_{600}\) = 6). Ten microliters of diluted cultures (OD\(_{600}\) = 0.02) was laid on plates containing the selective media, and growth phenotypes were documented daily with a video imaging system. A wide range of ion conditions was tested; only representative conditions were chosen for the figures presented below. For Ni\(^{2+}\) uptake or tonoplast isolation, strains were grown to mid-logarithmic phase.

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Abbreviations: TGN, trans-Golgi network; CPY, carboxypeptidase Y; PVC, prevacuolar compartment; C\(^{2+}\), divalent cation; WT, wild type; AGMA, 9-amino-6-chloro-2-methoxy-acridine; \(\text{Zn}^{2+}\) and \(\text{Co}^{2+}\), resistant and sensitive.

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Vacuole Isolation, Biochemical Assays. According to Roberts et al. (10), yeast cells were resuspended in Zymolyase-containing buffer to be converted to spheroplasts, and then osmotic lysis in the presence of a yeast protease inhibitor mixture (Sigma), vacuoles were purified by flotation on a discontinuous Ficoll gradient. After elimination of lipid particles (11), vacuoles were fragmented, and tonoplast vesicles were pelleted and resuspended in 2 mM Tris-HCl, pH 7.5/250 mM sorbitol/2 mM DTT/20% (vol/vol) glycerol and stored in liquid nitrogen. V-ATPase and α-mannosidase activities were measured as described by Lietch et al. (12) and Roberts et al. (10). SDS/PAGE of tonoplast proteins was performed according to Laemmli (13). Gels were silver-stained (14) and analyzed with IMAGEMASTER image analysis software (Amersham Pharmacia). Cell fractionation experiments using differential centrifugation to separate P150 and P13 membrane fractions from the cytosolic fraction (S150) were performed essentially as described (15). First, the purity of vacuolar membrane preparations was assessed from the ATPase activities of plasma membrane and mitochondria, according to Landolt-Marticorena et al. (16). Second, Western blotting was performed by using monoclonal antibodies (Molecular Probes) against Vph1p (dilution 1:2,000), Pep12p (1:1,000), and Vpp10p (1:400). Proteins were immunodetected by using a chemiluminescent assay (Aurora, ICN). Protein concentrations were determined by the Schaffner and Weissman method (17), using BSA as a standard.

C2+/H+ Exchange In Vitro. Tonoplast proteins were inserted into soybean liposomes essentially as described (18), except for the following. The detergent octyl glucoside was used as described by Sephadex G-50 minicolumns (Amersham Pharmacia) (19). Reconstitution was performed at a lipid/protein ratio of 30 (wt/wt). The reconstitution buffer contained 5 mM EDTA (Na+ free), adjusted to pH 5.5 with 1,3-bis[tris(hydroxymethyl)methylamino]propane and then adjusted to pH 7.0 with NH₄OH, 0.4 M sorbitol, and glycerol to a final concentration of 20% (vol/vol). Reconstituted tonoplast vesicles were diluted 200-fold in a stirred cuvette (1 ml) containing a NH₄⁺-free assay buffer (5 mM LiHepes, pH 7.5/0.4 M sorbitol) and the fluorescent permeant pH probe 9-amino-6-chloro-2-methoxyacridine (ACMA) at 1 μM. The NH₄⁺ dilution resulted in an acid-loading of the vesicles, caused by the outward diffusion of NH₃ (20). Transmembrane ΔpH was monitored by the fluorescence quenching of ACMA measured at 415 nm. Thereafter, quasi-infinite inward gradients of the indicated C₂⁺ were imposed by adding C₂⁺ to the outside. C₂⁺/H⁺ exchangers used this gradient to generate divalent influxes coupled to H⁺ efflux, estimated by the initial rate of dissipation of the ACMA quenching (determined from the derivation of the kinetics). This initial rate, named V₄₁＿, was linear with the protein concentration (data not shown) and is expressed in %·min⁻¹ per mg of protein.

63Ni²⁺ Uptake. The yeast pellet was resuspended (final OD₆₆₀ ≈ 4) in 5 mM Tris-succinate, pH 4.3/2% (wt/vol) glucose/50 mM KCl. This suspension (900 μl) was incubated for 30 min at 30°C under constant agitation (80 rpm). Nickel uptake was initiated by the addition of 63Ni (6.14 MBq·mM⁻¹/63NiCl₂, DuPont) at 3.5 μM final concentration and 250 μM NiCl₂. The uptake was stopped at different times by the filtration of 100-μl aliquots on 0.45-μm pore nitrocellulose membranes (Millipore). Filters were washed three times with 10 ml of 40 mM NiCl₂. Radioactivity retained on filters was measured by using a liquid scintillation analyzer. 63Ni²⁺ contents were expressed in mmol per mg⁻¹ dry weight. 63Ni²⁺ uptake experiments on permeabilized yeast cells were performed at pH 6.9 as described (21, 22) and in the same conditions as above.

Results

On Mg²⁺ nonlimiting solid media, the vps5Δ and WT strains displayed a similar growth capacity at any pH tested (pH 3.0 to 7.5; Fig. 1). In contrast, on Mg²⁺-limiting media, the growth capacity of both strains was impaired upon medium acidification. Importantly, vps5Δ exhibited a stronger sensitivity than WT to such acidification in low-Mg²⁺ conditions. This phenotype is called low-Mg²⁺ in the rest of this paper.

The vps5Δ strain also showed contrasting phenotypes in response to heavy metals in nonlimiting Mg²⁺ conditions (Fig. 1B). Relative to WT, the vps5Δ strain was resistant to Ni²⁺ and to Co²⁺ (Ni²⁺ and Co²⁺ phenotypes) but sensitive to Al³⁺ and Cd²⁺ (Al³⁺ and Cd²⁺ phenotypes). The kinetics of 63Ni²⁺ uptake by vps5Δ and WT cells were similar (Fig. 2A). However, after plasma-membrane permeabilization (21, 22), the intracellular 63Ni²⁺ uptake by vps5Δ cells reached a level 3-fold higher after 6 hr (Fig. 2B). Tonoplast vesicles were prepared from Ficoll gradient-purified vacuoles as indicated in Materials and Methods. The isolation yields of tonoplast proteins per g fresh weight of the two strains were similar, as well as the activities of the tonoplast enzyme markers V-ATPase and α-mannosidase (Fig. 3A). Inhibition of the total ATP hydrolysis activity by bafilomycin was about 70%, whereas inhibition by oligomycin and vanadate was less than 10%, indicating minimal contamination
by mitochondrial and plasma membranes, respectively. SDS/PAGE patterns of tonoplast proteins were indistinguishable (Fig. 3B). Fig. 3C shows Western blot analyses performed on vacuolar membrane preparations and subcellular P15, P150, and S150 fractions from WT and mutant by using monoclonal antibodies directed against membrane markers of the tonoplast (Vph1p), mitochondria (CoxIIIp), endoplasmic reticulum (Dpm1p), PVC (Pep12p), and TGN (Vps10p). These data indicate that the vacuolar preparations are highly enriched in vacuolar membrane proteins, which is in agreement with previous studies (11). Contaminations with endoplasmic reticulum and PVC were minor and comparable for both strains, whereas contaminations with mitochondria and TGN were not detectable.

Initial trials (not shown) showed that the filling kinetics of native tonoplast vesicles with C2+ in exchange with H+ were too fast to determine the initial rate of the exchange (VH+). Therefore, the latter was assayed on reconstituted vesicles to slow down the transport reaction (23). We showed that a similar reconstitution of a plant tonoplast Mg2+/H+ exchanger allowed for the recovery of the same affinity for Mg2+ and sensitivity to inhibitors as at the native membrane level (18). VH+ was measured after an initial acid-loading step was performed as described in Materials and Methods. The initial fluorescence quenching of the pH probe ACMA could be totally dissipated by NH4+ addition. This result ascertained the instantaneous establishment of a stable transmembrane ΔpH across the membrane (Fig. 4). In control liposomes, the addition of Ni2+ (or other cations used in this study) to the outside caused a negligible dissipation of ΔpH, as compared with that observed with reconstituted tonoplast vesicles (Fig. 4). Relative to WT, a 2.7-fold higher initial rate of Ni2+/H+ exchange was observed with reconstituted vesicles from vps5Δ.

Selectivity of C2+/proton exchange of reconstituted tonoplast vesicles from vps5Δ and WT was compared (Fig. 5). In vps5Δ vesicles, VH+ for Mg2+, Co2+, or Ni2+ increased by 50%, 130%, and 170%, respectively, compared with WT. In contrast, VH+ with Ca2+,Cd2+, or K+ was similar for both strains.

To determine the kinetic parameters of the Mg2+/H+ exchange, VH+ was measured at various Mg2+ concentrations (Fig. 6A). Scatchard plots were linear (Fig. 6B), indicating that the facilitated exchange reaction could be fitted to the classical Michaelian model. The enhanced VH+ in vps5Δ-reconstituted tonoplast vesicles resulted from an increase of the Vmax parameter relative to that of WT, whereas Km was found at about 1 M for both strains.

Finally, Ni2+ growth phenotypes of 14 other yeast mutants of the vacuolar protein sorting pathway were determined (Fig. 7). Eight of these mutants also were shown to be defective in the retention of TGN membrane proteins: two other retromer complex mutants vps17Δ and vps29Δ (3), vps54Δ (24), vps1Δ (25), vps8Δ (26), tlg2Δ (27), vps13/soi1Δ (15), and grd19Δ (28). All these mutants displayed a NiR phenotype. In contrast, six
other mutants, for which no alteration of this TGN retention process was described, showed a Ni²⁺ phenotype: vps3Δ (29), vps4Δ (30), vamΔ (31), vam7Δ (32), vps18Δ (33), and apl6Δ (34).

Discussion

The vps5Δ Mutant Strain Displays an Enhanced Vacuolar Mg²⁺ (Ni²⁺, Co²⁺)/H⁺ Exchange Activity. The vps5Δ strain belongs to class B vps mutants (35) and displays fragmented vacuoles (refs. 6 and 7; data not shown). However, no gross alteration of functional properties of the vps5Δ vacuole could be observed at the cellular level (35, 36). The present study confirms this conclusion at the biochemical level: the isolated vacuole membranes of WT and vps5Δ exhibit the same SDS/PAGE protein profiles, α-mannosidase and V-ATPase activities, and H⁺ exchange activities with K⁺, Ca²⁺, and Cd²⁺.

In contrast, we found that the vacuole membrane isolated from vps5Δ displays a higher H⁺ exchange activity with Mg²⁺, Ni²⁺, and Co²⁺. Ni²⁺ and Co²⁺ are two Mg²⁺ analogs commonly used to trace Mg²⁺ transport (37–42). They are not required for normal yeast growth and are usually omitted from standard growth media. Moreover, significant H⁺ exchange activities with these cytotoxic heavy metals were observed only at high concentrations.

Fig. 4. Activity of Ni²⁺/H⁺ exchange in reconstituted tonoplast vesicles from vps5Δ and WT. Ammonium-containing vesicles were diluted 200-fold in the assay buffer free of NH₄⁺, causing vesicle acid-loading and instant quenching of the permeant pH probe ACMA. Fluorescence recovery after the addition of 0.5 mM Ni²⁺ to reconstituted tonoplast vesicles, not observed on control liposomes, ascertained the facilitated exchange activity. The final addition of NH₄⁺ (15 mM) to the outside allowed the total dissipation of the pH gradient.

Fig. 5. Selectivity of cation/proton exchange across reconstituted tonoplast vesicles from vps5Δ or WT. Cation concentration was 0.5 mM, except for Cd²⁺, which was 0.2 mM. Exchange rates (Vmax) are presented as mean Vmax ± SE (n = 3), except for Mg²⁺ (n = 5). Exchange rates were significantly different between vps5Δ and WT for Mg²⁺, Ni²⁺, and Ni²⁺, but not for Cd²⁺, Ca²⁺, and K⁺ (Student’s t tests, data not shown).

Fig. 6. Mg²⁺/H⁺ exchange rate of reconstituted tonoplast vesicles from vps5Δ (○) or WT (□) as a function of Mg²⁺ concentration. (A) Exchange rates (Vmax) were determined from experimental kinetics illustrated in Fig. 4 and described in Materials and Methods. Values are presented as mean Vmax ± SE (n = 5). (B) Scatchard plot: Km values derived from linear regression of data in A are 0.96 ± 0.02 mM and 0.92 ± 0.01 mM for vps5Δ and WT strains, respectively; Vmax are 4.53 ± 0.36 and 2.93 ± 0.26%·min⁻¹ per mg of protein.

Fig. 7. Ni²⁺ growth phenotypes of yeast mutants of the vacuolar protein-sorting pathway. Mutants were grown on agar/YNB minimal medium containing 0.1 mM Mg²⁺, with (+) or without (−) 0.2 mM Ni²⁺. Phenotypes were recorded after 4 days. Class (34) and name of each mutant are indicated. ? indicates class not determined or not available in the literature. Among these mutants, only Ni²⁺-resistant mutants were found to be defective in the retention of Golgi membrane proteins. We refer the reader to the Yeast Proteome Database (http://www.proteome.com) for further information about the mutants and the corresponding genes (including alternative names and functional details).
concentrations (0.1 to 1 mM, data not shown), indicating that vacuolar Co²⁺/H⁺ and Ni²⁺/H⁺ exchanges are unlikely to play a physiological role in yeast in standard growth conditions. Conversely, the affinity of the H⁺ exchange with Mg²⁺ seems physiologically sound because it displayed a Kₘ for Mg²⁺ at about 1 mM, to the reported activity of Mg²⁺ in the yeast cytosol (43). Moreover, the yeast vacuole has been described as an essential Mg²⁺ reservoir (44). The present work provides direct evidence for the activity of a Mg²⁺/H⁺ transport system at the tonoplast of S. cerevisiae. The plant vacuolar protein AtMHX was proposed to be an Mg²⁺/H⁺ exchanger (45).

Nevertheless, no clear homologue could be detected in yeast. C²⁺/H⁺ exchangers have been shown to promote a net vacuolar uptake of C²⁺, driven by the exergonic efflux of H⁺ out of acidic vacuoles in yeast cells (46–48). Thus, the rate of vacuolar uptake by C²⁺/H⁺ exchangers is expected to depend on the size of the ΔpH. In the present study, WT and vps5Δ exhibited similar vacuolar H⁺-ATPase activities. Furthermore, their growth capacity at pH 7.5, known to depend on the presence of a functional vacuolar H⁺-ATPase (49, 50), was similar on standard media. In agreement with published data (6, 35), the vacuole should be energized by similar ΔpH in both strains.

In conclusion, the vacuole membrane of the vps5Δ strain was found to exhibit similar biochemical and functional features compared with those of the WT strain, except for an enhanced Mg²⁺ (Ni²⁺, Co²⁺)/H⁺ exchange activity. The latter is expected to mediate a higher vacuolar uptake of C²⁺ in mutant yeast cells. Consistently, Ni²⁺ uptake was 3-fold higher in vps5Δ cells than in WT cells after plasma-membrane permeabilization. By comparison, Ni²⁺ uptakes by nonpermeabilized mutant cells and WT cells measured in short-term experiments were the same, indicating that both strains would display similar transport properties at the plasma-membrane level.

The vps5Δ Mutant Displays New Growth Phenotypes Likely Related to its Enhanced Vacular Mg²⁺ (Ni²⁺, Co²⁺)/H⁺ Activity. In the present study, a new selectable growth phenotype is presented for mutants altered in the recycling of Golgi membrane proteins: all mutants tested were resistant to Ni²⁺ (51, 52). As mentioned earlier, vps5Δ displays a fragmented vacuole composed of a large number of minivacuoles. Unexpectedly, the amounts of tonoplast isolated per g fresh weight of vps5Δ and WT were the same, suggesting that vacuoles of both strains might share the same tonoplast surface area despite their different morphologies. With the same areas but smaller vacuoles, a reduction of the vacuome volume might be expected in mutant cells, which would supposedly be detrimental for metal tolerance. Consistently, the Cd³⁺ phenotype of vps5Δ was shared by vps41Δ (data not shown) and vps5ΔΔ (53), two class B mutants showing fragmented vacuoles (30, 35, 53). In addition, vps18Δ, a class C mutant lacking any structure resembling a normal vacuole (35), also displays a Cd³⁺ phenotype (54). Thus, a reduction of the vacuole volume might be responsible for the Cd³⁺ phenotype of vps5Δ.

Along the same lines, the sensitivity of vps41Δ and vps18Δ to Ni²⁺ could be related as well to such a reduction of the vacuole volume. Indeed, the vacuole has been described also as a major site for Ni²⁺ detoxification (48, 55, 56). Because class B mutants, showing fragmented vacules, displayed either Ni²⁺ (vps18Δ, vps39Δ, vam3Δ, vam7Δ) or Ni²⁺ (vps5Δ, vps17Δ, vps5ΔΔ) phenotypes (Fig. 7), the assumed reduction in size of their vacuome cannot account for the phenotype of Ni²⁺ B mutants. Therefore, we propose that the Ni²⁺ (and Co²⁺) phenotypes of vps5Δ would result from the marked enhancement of its vacuolar Ni²⁺ (Co²⁺)/H⁺ exchange activity. This transport activity would improve Ni²⁺ (Co²⁺) detoxification by increasing their vacuolar compartmentalization in vps5Δ (as shown for Ni²⁺, Fig. 2).

In contrast, vacuolar sequestration of Mg²⁺ by Mg²⁺/H⁺ exchange should be responsible for both WT and vps5Δ growth improvement to Ni²⁺ in acidic pH in Mg²⁺-limiting media (Fig. 1A). Importantly, acidic conditions alone were not responsible for this effect because the latter was not observed on media containing nonlimiting Mg²⁺ concentrations. On the other hand, the lower the pH in the medium, the lower the pH in the vacuole (57), and thus, the higher the ΔpH energizing vacuolar ion uptake by cation/proton exchangers. In the present study, an increase of the vacuolar Mg²⁺ uptake is expected at acidic pH. In this condition, the increase of the vacuolar uptake would exceed the low cellular Mg²⁺ uptake occurring in low-Mg²⁺ media, thereby impairing cytosolic Mg²⁺ homeostasis. This result would explain the decrease of yeast growth at acidic pH in low-Mg²⁺ (but not in nonlimiting Mg²⁺) media, depicted horizontally in Fig. 1A. At a given pH, i.e., at a constant vacuolar ΔpH, cellular Mg²⁺ uptake would decrease below vacuolar uptake upon Mg²⁺ deprivation. This imbalance would also impair Mg²⁺ cytotoxic homeostasis and would explain the yeast growth decrease depicted vertically in Fig. 1A.

In this context, the enhanced vacuolar Mg²⁺/H⁺ activity of vps5Δ, relative to that of WT, should be responsible for the higher sensitivity of the mutant to the conditions described above. The Al³⁺ phenotype of vps5Δ could be interpreted along the same line, because Al³⁺ toxicity was shown to result from the Al³⁺-inhibition of Mg²⁺ cellular uptake in yeast (42, 58), thereby mimicking Mg²⁺-limiting conditions. Finally, it should be noted that the low-Mg⁶ growth phenotype of vps5Δ is clearly observable at acidic pH relative to neutral pH, which is consistent with the implication of the vacuole energization in this phenotype.

The vps5Δ strain was resistant to Co²⁺, but sensitive to Cd³⁺, Al³⁺, and Mg²⁺-limiting media. The vacuole has been described as a major site for Cd³⁺ detoxification (51, 52). As mentioned earlier, vps5Δ displays a fragmented vacuole composed of a large number of minivacuoles. Unexpectedly, the amounts of tonoplast isolated per g fresh weight of vps5Δ and WT were the same, suggesting that vacuoles of both strains might share the same tonoplast surface area despite their different morphologies. With the same areas but smaller vacuoles, a reduction of the vacuome volume might be expected in mutant cells, which would supposedly be detrimental for metal tolerance. Consistently, the Cd³⁺ phenotype of vps5Δ was shared by vps41Δ (data not shown) and vps5ΔΔ (53), two class B mutants showing fragmented vacuoles (30, 35, 53). In addition, vps18Δ, a class C mutant lacking any structure resembling a normal vacuole (35), also displays a Cd³⁺ phenotype (54). Thus, a reduction of the vacuome volume might be responsible for the Cd³⁺ phenotype of vps5Δ.

Missorting of a TGN Mg²⁺/H⁺ Exchanger to the Vacuole Membrane of vps5Δ: A Molecular Hypothesis. One straightforward interpretation of the increase of vacuolar C²⁺/H⁺ exchange activity in vps5Δ would be that the endogenous vacuolar exchanger is more active in mutant cells. However, deregulation of this exchanger (resulting, for example, in an increase of the exchanger density at the membrane surface or of the proportion of exchangers in the active state) is expected to increase the Vₚmax of the transport reaction without modification of its selectivity. Compared with WT, vps5Δ exhibited an increase of the Vₚmax, but also a marked alteration of ion selectivity: the selectivity sequence was Mg²⁺ > Co²⁺ > Ni²⁺ for WT and Co²⁺ > Mg²⁺ > Ni²⁺ for vps5Δ (Fig. 5). In addition, yeast vps3 mutants, like vps17 and vps29 mutants of the retromer complex and other mutants altered in the recycling of Golgi membrane proteins (vps54Δ, vps7Δ, vps8Δ, vps25A, vps13A, and vps92A), have been reported to missort various membrane proteins from the TGN (proteinase Kex2p, Vps10p, or dipeptidyl aminopeptidase A) to the tonoplast (3, 15, 24–28). In our study, all these mutants displayed a Ni²⁺ phenotype, whereas other vacuolar protein sorting mutants not affected in this recycling process displayed a Ni²⁺ phenotype. In this context, it may be hypothesized that vps5Δ, and possibly other mutants in retention of Golgi membrane proteins, would also missort a TGN Mg²⁺/H⁺ exchanger to the vacuole membrane of mutant cells. It is noteworthy that the literature data indicate that TGN and endosomal compartments sustain high ΔpH generated by the V-ATPase (59, 60).

In conclusion, our data argue in favor of the coexistence of two kinds of exchangers with distinct selectivities at the vacuolar membrane of vps5Δ (and possibly of other Golgi retention-defective mutants). Validation of this working hypothesis will require the molecular identification of yeast Mg²⁺/H⁺ exchang-
ers. In wild-type cells, the TGN Mg\(^{2+}\)/H\(^{+}\) exchanger would be recycled between the PVC and the TGN by the retromer complex and could be involved in the pH regulation of the TGN or Golgi-derived vesicles. Such a regulation is thought to be important for the sorting of secretory cargo and the retrieval of components of the biosynthetic pathway (59, 61). Recently, it has been shown that the VPS44 gene actually encodes the Nhx1p Na\(^{+}\)/H\(^{+}\) exchanger of the PVC and that it is required for endosomal protein trafficking (62). It is noteworthy that neither Na\(^{+}\)/H\(^{+}\) nor Ca\(^{2+}\)/H\(^{+}\) exchanges were found to be involved in the regulation of the acidification of TGN vesicles (59).

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