BIOCHEMICAL PROPERTIES OF VACUOLAR ZINC TRANSPORT SYSTEMS OF
SACCHAROMYCES CEREVISIAE

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SUMMARY

The yeast vacuole plays an important role in zinc homeostasis by storing zinc for later use under deficient conditions, sequestering excess zinc for its detoxification, and buffering rapid changes in intracellular zinc levels. The mechanisms involved in vacuolar zinc sequestration are only poorly characterized. Here we describe the properties of zinc transport systems in yeast vacuolar membrane vesicles. The major zinc transport activities in these vesicles were ATP-dependent, requiring a H+ gradient generated by the V-ATPase for function. One system we identified was dependent on the ZRC1 gene, which encodes a member of the CDF family of metal transporters. These data are consistent with the proposed role of Zrc1 as a vacuolar zinc transporter. Zrc1-independent activity was also observed that was not dependent on the closely related vacuolar Cot1 protein. Both Zrc1-dependent and independent activities showed a high specificity for Zn2+ over other physiologically relevant substrates such as Ca2+, Fe2+, and Mn2+. Moreover, these systems had high affinities for zinc with apparent K_m’s in the 100-200 nM range. These results provide biochemical insight into the important role of Zrc1 and related proteins in eukaryotic zinc homeostasis.
INTRODUCTION

Zinc is an essential catalytic component of over 300 enzymes, and a critical component of structural motifs such as zinc fingers. Zinc is estimated to be required for the function of more than 3% of the yeast proteome (1) and, therefore, cells must possess mechanisms to obtain sufficient quantities of this important nutrient. Although zinc is not redox active under physiological conditions, excess zinc can be toxic to cells. Zinc toxicity may be mediated via binding of the cation to inappropriate sites in proteins or co-factors. For example, excess zinc can interfere with mitochondrial aconitase activity and impair respiration (2). The essential but potentially toxic nature of zinc necessitates precise homeostatic control mechanisms. In E. coli, zinc homeostasis is accomplished largely through the transcriptional control of zinc uptake and efflux transporters (3,4). Recent studies of the regulatory zinc sensors that control expression of these transporters suggest that E. coli cells strive to maintain essentially no labile zinc in their cytoplasm under steady state growth conditions (5). Similarly, in eukaryotic cells, labile zinc levels are estimated to be in the low nM range under steady state conditions (6).

Studies of the yeast Saccharomyces cerevisiae have uncovered many aspects of zinc homeostasis in this eukaryotic organism. One major component is the transcriptional regulation of the Zrt1 and Zrt2 zinc uptake transporters. Expression of these proteins is induced under zinc limiting conditions by the Zap1 transcriptional activator (7). A second important mechanism of zinc homeostasis is the post-translational inactivation of Zrt1 by high zinc. When zinc-limited cells are transferred to zinc-replete conditions, zinc rapidly accumulates in cells due to the high level expression of the plasma membrane transporters. We refer to this condition as “zinc shock.” In response to zinc shock, Zrt1 is ubiquitinated and removed from the plasma membrane.
by endocytosis (8,9). This results in a rapid decrease in zinc uptake activity that helps limit zinc overaccumulation under these conditions.

Yet another critical mechanism of zinc homeostasis is the sequestration of zinc in the vacuole. Several studies suggest that the vacuole is a storage/detoxification site for excess zinc under steady state high zinc conditions. First, mutations that disrupt vacuolar biogenesis or activity of the V-ATPase, which acidifies the vacuole, are hypersensitive to zinc treatment (10,11). Second, mutations that disrupt vacuolar accumulation of polyphosphate, a potential zinc-binding component in the vacuole, are also zinc sensitive (12,13). Third, several potential zinc transporters, Zrc1, Cot1, and Zrt3, have been implicated in vacuolar zinc sequestration. Zrt3 is a member of the ZIP family, which also includes the Zrt1 and Zrt2 transporters, and has been proposed to efflux stored zinc from the vacuole to the cytosol during the transition from high to low zinc (14). Zrc1 and Cot1 are members of the cation diffusion facilitator (CDF) family, a ubiquitous group of transporter proteins found in prokaryotes and eukaryotes (15-17). Many CDF proteins have been implicated in zinc efflux or compartmentalization, and Zrc1 and Cot1 are thought to transport zinc into the vacuole. Both proteins have been localized to the vacuole membrane (18,19), although these observations must be interpreted with some caution as they were obtained using cells that overexpressed these proteins. The overexpression of Zrc1 or Cot1 confers zinc tolerance (20,21), which in the case of Zrc1 is associated with increased zinc accumulation in a compartment that is not accessible to the Zap1 transcription factor1. These observations suggest that Zrc1 and Cot1 mediate the sequestration of excess zinc in the vacuole. Consistent with this model, the zrc1 and cot1 mutations are associated with reduced total cellular zinc accumulation and increased zinc sensitivity1 (14,20,22).
Interestingly, although the zinc sensitivity of yeast is significantly increased by the zrc1 mutation, such mutant strains are still tolerant to mM concentrations of zinc, which are unlikely to be encountered in the natural environment. Our recent studies suggest that an important physiological role of Zrc1 is to tolerate the rapid influx of zinc which results from exposing zinc-deficient yeast to zinc-replete conditions (zinc shock)\(^1\). Despite the transcriptional and post-translational control of plasma membrane zinc transporters, zinc shock still results in the overaccumulation of large quantities of zinc (8). The \textit{ZRC1} gene is transcriptionally induced by Zap1 in zinc-deficient cells (23,24) and this up-regulation is required to provide tolerance to zinc shock\(^1\). Mutation of the \textit{ZRC1} gene renders cells extremely sensitive to zinc shock, as does the disruption of \textit{ZRC1} induction by Zap1. Hence, the vacuole appears to play a role in buffering cytoplasmic zinc against changes in zinc availability.

To fully understand the role of the vacuole in zinc homeostasis, a biochemical characterization of the zinc transporter activities in this compartment is essential. Two previous studies have addressed this issue. Okorokov et al. (25) were the first to assay zinc transport activity in yeast vacuolar vesicles. These investigators characterized a transport system with an apparent \(K_m\) of 55-170 \(\mu\)M. However, this apparent low affinity for substrate is unlikely to be of physiological significance given the low amounts of labile zinc (i.e. nM) now thought to be present in cells. Similarly, White and Gadd (26) noted zinc transport activity in vacuole vesicles, but this activity was not further described. Because of these considerations, we initiated this study of the biochemical properties of zinc transport activities in vacuolar vesicles. Furthermore, using genetic manipulations in combination with our biochemical assays, we investigated the contribution of the CDF family proteins and the V-ATPase to vacuolar zinc transport.
EXPERIMENTAL PROCEDURES

Growth conditions and general methods - Yeast were routinely grown in YPD or in synthetic defined (SD) medium with 2% glucose and necessary auxotrophic supplements. LZM was prepared as previously described (8,27). Low zinc YPD (LZ-YPD) medium was prepared by treating standard YPD medium (1 l) with 25 g Chelex 100 resin for 12 h at 4°. The resin was removed and the medium supplemented with MgCl₂ (4 mM), CaCl₂ (100 µM), MnCl₂ (25 µM), FeCl₃ (10 µM) and CuCl₂ (2 µM). LZ-YPD had a zinc content of less than 1 µM as determined by atomic absorption spectroscopy (data not shown). Na-citrate (20 mM, pH 4.2) and EDTA (1 mM) were added to further limit zinc bioavailability, and the medium was filter-sterilized. Cell number/ml of yeast suspensions was determined by measuring the optical density at 600 nm (A₆₀₀) and comparing with a standard curve. E. coli and yeast transformations were performed using standard methods. Immunoblot analysis and indirect immunofluorescence was performed as previously described (9,14). Antibodies were obtained from Molecular Probes (Eugene, OR) and Pierce (Rockford, IL). Affinity purified rabbit Kex2 antibody was a gift of Steve Nothwehr.

Yeast plasmids and strains used - The ZRC1 gene was amplified from yeast genomic DNA (DY1457) using oligonucleotides designed to include 1000 bp of the promoter and 500 bp of the terminator regions. The resulting DNA fragment was cloned into the EcoRI and PstI sites of pFL38 (28) to generate YCpZRC1. An epitope-tagged ZRC1 allele was generated by overlap PCR (29) adding three copies of the H. influenzae hemagglutinin (HA) epitope to the C-terminus of Zrc1. The PCR product was then inserted into BamHI-, HpaI-digested YCpZRC1 by gap repair to generate YCpZRC1-HA. Yeast strains CM100 (ZRC1 COT1), CM102 (zrc1::HIS3), CM103 (cot1::URA3) and CM104 (zrc1::HIS3 cot1::URA3) were previously described (14). All...
newly constructed strains were isogenic to CM100. CM142 (ZRC1\(^{HA}\)) was constructed by transformation of CM104 with the insert of YCpZRC1-HA. Transformants were selected by complementation of the zinc-sensitive phenotype of CM104, and the mutations verified using PCR. The \(cot1\) mutation was removed by backcrossing to a wild-type strain. Yeast strain MM112 \((vph1::LEU2\ stv1::LYS2)\) (30) was a gift of Rob Piper.

**Vacuole vesicle preparation**- Yeast vacuoles were prepared using the method of Kakinuma et al. (31) with the following modifications. Yeast cells were grown to late log phase in YPD medium with 4\% glucose, then washed once with water and once with buffer A (100 mM KPO\(_4\) buffer pH 7.0, 1.2 M sorbitol). The cells were resuspended in 3x the pellet volume in buffer A with 10 mM DTT, 1\% glucose, and 50 units zymolyase/ml. Cells were incubated for 1-2 hrs at 30 degrees with gentle shaking. Spheroplasts were washed twice with buffer A and resuspended in 5 ml lysis buffer (10 mM MES-Tris pH 6.9, 0.1 mM MgCl\(_2\), 12\% Ficoll 400). Most cells lysed after resuspension in this buffer, and cell clumps were broken up by 15 strokes in a Dounce homogenizer. The lysate was then processed as previously described (31), except that membrane wafers were resuspended by repeated pipetting. The final wafer of vacuoles was resuspended in 5 ml 1 x buffer C (10 mM MES-Tris pH 6.9, 25 mM KCl, 4 mM MgCl\(_2\)) and dispersed by repeated pipetting. Vesiculated vacuole membranes were collected by centrifugation at 60,000 x g for 30 min, resuspended in buffer C + 10\% glycerol and frozen in liquid nitrogen prior to storage at -80 C. Protein content of vacuole preparations was assayed using a Bradford assay kit (Bio-Rad). Efforts to reduce the degree of Zrc1 degradation during vesicle preparation using various protease inhibitor mixtures were unsuccessful.
**Zinc uptake and ATPase assays** - Accumulation of $^{65}\text{Zn}^{2+}$ by vacuole vesicles was assayed in 10 mM MES-Tris buffer (pH 6.9) with 4 mM MgCl₂ and 25 mM KCl. Vacuole vesicles (routinely 5 µg protein) were added to 200 µl of this buffer with or without 1 mM ATP and the indicated $^{65}\text{Zn}^{2+}$ concentration and incubated at 30°C. Reactions were stopped by addition of 4.5 ml cold wash buffer (10 mM MES-Tris pH 6.9, 25 mM KCl, 1 mM Tris-buffered EDTA) and incubation on ice. The vesicles were collected on nitrocellulose filters (Millipore, 0.45 µm pore size) and washed three times with 5 ml cold wash buffer. Radioactivity retained on the filters was quantified in a gamma counter. Michaelis-Menten constants were determined by nonlinear interpolation of the data using Prism (version 3.0a for Macintosh, GraphPad Software, San Diego). Adenosine-triphosphatase activity of isolated vacuole membranes was assayed in 10 mM Tris-MES buffer (pH 6.9) containing 5 mM MgCl₂, 25 mM KCl and 1 mM Mg²⁺-ATP. Aliquots of 500 µL buffer were incubated at 30°C and vacuole vesicles (5 µg protein) were added to start the assay. The assay was terminated by addition of 1 ml stop buffer (2% H₂SO₄, 0.5% NH₄MoO₄, 0.5% SDS) and color developed by addition of 10 µL of freshly prepared 10% Na ascorbate. After 10 min color development, samples were read at 750 nm against a blank with no protein added. Na-orthovanadate was prepared by boiling a 100 mM stock of Na-vanadate for 10 min and used immediately.

To assay changes in the internal pH of vacuole vesicles, vacuole membranes (10 µg protein) were added to 1 ml ATPase assay buffer in a spectrofluorimeter cuvette at 30°C. ACMA (9-amino-6-chloro-2-methoxyacridine) was added to a final concentration of 2 µM. Dye fluorescence was excited by light of wavelength 410 nm and the emission at 482 nm was continuously recorded using a Hitachi F3010 spectrofluorimeter. When a stable emission signal was obtained, ATP (1 mM) was added and quenching of the dye fluorescence was recorded.
RESULTS

Zinc accumulation by isolated yeast vacuole vesicles- Many genetic studies have implicated the importance of vacuolar zinc transport in zinc homeostasis. To examine this process at a biochemical level, vacuolar vesicles were isolated from yeast cells for in vitro zinc transport studies. The purity of these preparations was assessed by immunoblot analysis using antibodies against marker proteins of known compartments. Compared to whole cell lysates, vacuole vesicles from wild-type cells were enriched approximately 4-fold in two vacuolar marker proteins, Cpy and Vma1 (Figure 1). The levels of marker proteins for other compartments such as mitochondria, Golgi, and endoplasmic reticulum, as well as a cytoplasmic enzyme, were at similar or reduced levels in the vacuole fraction. These data indicated that the isolation procedure resulted in a significant enrichment of vacuole vesicles. Similar results were obtained with vesicles isolated from zrc1 mutant cells (Figure 1). Experiments using vesicles from this mutant strain will be described later in this report. From previous reports of this isolation procedure, it was known that vacuole membrane vesicles were primarily obtained in the right-side-out orientation, making them suitable for studies of vacuolar cation import (31,32). The observed enrichment of CPY, a lumenal vacuolar protein, indicated that a significant fraction of the vesicles isolated were right-side-out. This conclusion was also supported by our observation that significant V-ATPase activity was detected in nonpermeabilized vesicles (see below).

To determine if vacuole vesicles could accumulate zinc, membranes isolated from a wild-type strain were incubated with radioactive $^{65}$Zn with or without Mg-ATP as an energy source. Zinc accumulation was assessed over time by collecting the vesicles on nitrocellulose filters, washing them to remove surface-bound zinc, and then measuring the level of accumulated isotope. In the absence of ATP, only a low level of zinc accumulation was observed. In the
presence of ATP, however, a high level of zinc uptake activity was detected (Figure 2A). This ATP-dependent zinc uptake was rapid and essentially complete after 2 minutes of incubation with 1 µM $^{65}$Zn. This plateau was likely due to an equilibrium effect (i.e. high lumenal zinc inhibiting additional uptake) because assays using higher concentrations of zinc resulted in longer periods of increasing accumulation and higher final levels of zinc (data not shown). Zinc accumulation was not stimulated by ADP or a non-hydrolyzable analogue of ATP (AMP-PNP) indicating a requirement for hydrolysis of the $\gamma$-phosphate of ATP. That the vesicle-associated zinc was actually in the vesicle lumen rather than bound to the membrane surface was confirmed using pyrithione, a zinc ionophore. The addition of pyrithione to vesicles after zinc accumulation caused a rapid decrease in vesicle-associated $^{65}$Zn. This effect was not simply due to pyrithione chelating and removing surface-bound zinc, because the membrane impermeable chelator EDTA was routinely used to wash the vesicles after $^{65}$Zn treatment, and had no effect on the pyrithione-accessible zinc. These observations indicated that $^{65}$Zn was accumulated within a membrane-bound compartment. Because pyrithione treatment caused a net release of accumulated zinc, these data also indicated that the vesicles were capable of generating a zinc concentration gradient.

**Mechanism of ATP-dependent zinc transport by vacuole vesicles** - The accumulation of ions and metabolites in yeast vacuoles is often mediated by secondary active transport systems, which drive solute transport using the stored energy of a proton concentration gradient generated by the vacuolar V-type H⁺-ATPase (33). The vacuole vesicles isolated in this study were capable of generating a proton gradient when supplied with ATP, as determined by quenching of the pH-sensitive fluorescent indicator, ACMA (Figure 2B). Generation of this proton gradient was also
dependent on a hydrolyzable ATP substrate as was observed for the zinc uptake assays. To
determine if this proton gradient was required for zinc uptake, the effect of compounds that
dissipate pH gradients was examined (Table I). As a control, the effect of these inhibitors on
total ATPase activity of the extracts and vesicle acidification was also determined. Addition of
the protonophore FCCP at the start of the zinc uptake assay severely inhibited this activity. This
compound also effectively dissipated the proton gradient generated by adding ATP to ACMA-
treated vesicles (Figure 2B). Consistent with its mode of action, FCCP did not inhibit ATPase
activity. In fact, FCCP stimulated this activity, an effect that is consistent with the uncoupling of
ATP hydrolysis and vesicle acidification mediated by this inhibitor. Nigericin, an ionophore that
dissipates proton gradients via K⁺/H⁺ exchange, also inhibited zinc uptake and stimulated ATPase
activity. We also tested the effect of valinomycin, a potassium ionophore that dissipates
gradients of K⁺ and electrical potential gradients but does not affect proton gradients.
Valinomycin treatment did not inhibit zinc uptake, indicating the uptake was not dependent on a
K⁺ gradient nor an electrical potential gradient generated by ATPase activity. The above
observations indicated that the ATP-dependent accumulation of zinc by vacuole vesicles
required a proton concentration gradient. As expected, nigericin dissipated vesicle protein
gradient while valinomycin did not (data not shown).

To determine which enzyme was responsible for generating this proton gradient, we
tested the effect of inhibitors of the V-type H⁺-ATPase (concanamycin A), P-type H⁺-ATPases
(vanadate) and the mitochondrial F₁Fₒ H⁺-ATPase (oligomycin) on zinc uptake and ATPase
activity (Table I). Of the three inhibitors used, concanamycin A most strongly inhibited zinc
accumulation. Concanamycin A at a concentration of 100 nM or above maximally inhibited
approximately 60% of the ATPase activity in yeast vacuole preparations. The remaining
concanamycin A-insensitive ATPase activity is likely due to vacuolar phosphohydrolases or from contamination of the preparations with other organelles (Figure 1). ATP hydrolysis by this concanamycin A-insensitive activity was not coupled to vesicle acidification; 100 nM concanamycin A completely prevented the acidification of vacuole vesicles as determined using ACMA (Figure 2B). In contrast, oligomycin and vanadate had far lesser effects on vesicle acidification. Taken together, these data indicate that ATP-dependent zinc accumulation in vacuole vesicles is mediated by secondary active transport systems that are dependent on the proton gradient generated by the V-ATPase. Further evidence for the involvement of the V-ATPase in zinc transport was obtained by assays of vesicles from a vph1 stv1 mutant strain, which lack V-ATPase activity due to loss-of-function mutations in the two V-ATPase 100 kDa subunits (see below).

Intracellular glutathione concentrations are in the mM range suggesting that much of the labile cytosolic zinc in cells may be present in the form of Zn-glutathione complexes. Surprisingly, high levels of reduced glutathione (1 mM GSH with 1 µM ^{65}Zn, data not shown) did not inhibit ATP-dependent zinc uptake in vitro despite glutathione’s ability to bind zinc with high affinity (34). These results suggest that Zn-GSH complexes may be a labile source of Zn^{2+} for transport in vivo.

**Subcellular localization of Zrc1** - The observation that vacuole vesicles contained zinc transport activity led us to investigate which proteins might be responsible for this activity. Two obvious candidates were the CDF proteins Zrc1 and Cot1; both proteins are likely to be zinc transporters, although direct evidence supporting this hypothesis has not been reported. To determine if Zrc1 contributes to zinc uptake by vacuole vesicles, we first confirmed the subcellular location of this
protein. Zrc1 had previously been localized to the vacuole membrane when overexpressed (18,19), but the overexpression of membrane proteins can lead to their mislocalization. For this reason, we examined the subcellular location of an epitope-tagged Zrc1 protein expressed at normal levels from its own promoter. This tagged protein includes three repeats of the hemagglutinin antigen (HA) tag at the C-terminus, and was fully functional as determined by complementation of the zinc sensitivity associated with a \textit{zrc1} mutation (data not shown).

Because an important physiological role of Zrc1 is the resistance of zinc-deficient cells to sudden increases in zinc availability\(^1\), Zrc1 location was determined in cells grown under zinc-limited (LZM + 1 \(\mu\)M zinc) as well as zinc-replete (LZM + 1000 \(\mu\)M zinc) conditions. As shown in \textbf{Figure 3A}, Zrc1 was clearly located in the vacuolar membrane in both high and low-zinc grown cells. We also confirmed that the vacuole vesicle preparations were enriched for Zrc1 (\textbf{Figure 1}). While some degradation of the protein had occurred during vesicle isolation, there was a significant level of apparently full-length protein present in the preparations. (The predicted molecular mass of Zrc1-HA is 49 kDa.) The subcellular localization of Cot1 at normal expression levels was not examined.

\textbf{Effect of zrc1 and cot mutations on zinc uptake by vacuole vesicles-} To determine if either Zrc1 or Cot1 play a role in the zinc transport activity observed in vacuolar vesicles, zinc uptake activity was assayed in vesicles isolated from wild-type, \textit{zrc1}, \textit{cot1}, and \textit{zrc1 cot1} strains. As before, wild-type vesicles had a high level of ATP-dependent zinc uptake activity and a low level of ATP-independent activity. Results with \textit{zrc1} vesicles demonstrated that maximal ATP-dependent uptake activity in the wild type was dependent on Zrc1; vesicle zinc uptake activity was reduced approximately 75% in the \textit{zrc1} mutant (\textbf{Figure 4A}). In contrast, the \textit{cot1} mutation
had little or no effect on ATP-dependent zinc uptake, either alone or in combination with the zrc1 mutation. Thus, the ATP-dependent, Zrc1-independent activity was not Cot1-dependent. Consistent with the proposed role of the V-ATPase in driving transport activity, no ATP-dependent zinc accumulation was observed in vesicles prepared from a vph1 stv1 mutant. Although V-ATPase mutants are known to have defects in protein targeting to the vacuole (35), the vph1 stv1 mutations had no effect on the location of Zrc1 as determined by indirect immunofluorescence (Figure 3B), so the reduced transport activity associated with vph1 stv1 mutations could not be attributed to Zrc1 mislocalization. Neither Zrc1 nor Cot1 were required for ATP-independent zinc accumulation (Figure 4A).

Comparison of total ATPase activity between wild-type and mutant strains showed that vesicles from the zrc1 mutant had consistently lower concanamycin A-sensitive (i.e. V-ATPase) activity (~50% of wild type) (Figure 4B). This difference did not result from a reduced purity of the vacuole vesicles because a similar enrichment of the Vma1 and Cpy proteins was obtained for vacuole vesicles from wild-type and zrc1 mutant strains (Figure 1). Although Zrc1-dependent zinc transport by vacuole vesicles was dependent on V-ATPase activity, the reduced activity of the V-ATPase in zrc1 vesicles was unlikely to have caused the decrease in zinc uptake observed. This conclusion is based on the observation that addition of 10 nM concanamycin A to wild-type vacuole preparations reduced V-ATPase activity and vesicle acidification by ~95%, but did not decrease zinc uptake (data not shown, Figure 2B). Thus, V-ATPase activity was present in excess of that required to drive maximal zinc uptake.

Because Zrc1 protein levels are induced in zinc-deficient cells, we also assayed zinc uptake in vacuole vesicles isolated from zinc-deprived cultures (Figure 4C). Wild-type vacuole vesicles isolated from zinc-deficient cells exhibited consistently less zinc uptake activity than
those isolated from zinc-replete cells. This reduction in zinc transport could result from high expression of the putative zinc efflux system Zrt3 in vacuoles of zinc deficient yeast, which would counteract zinc accumulation. This was apparently not the case as zinc uptake in vesicles isolated from zinc-replete and zinc-limited zrt3 mutants was similar to the wild type results (data not shown). Alternatively, this decrease could be a consequence of some physiological stress suffered by zinc-deficient cells. Immunoblots indicated that the degree of Zrc1 proteolysis occurring during vesicle preparation increased when membranes were isolated from zinc-limited cells and this could explain the lower activity. The zrc1 mutation also reduced zinc uptake by vacuole vesicles isolated from zinc-deficient cells. Interestingly, ATP-dependent zinc uptake under these conditions was almost entirely dependent on Zrc1, with very little residual activity remaining in the mutant vacuoles. Thus, these data are consistent with the increased expression of Zrc1 under zinc-limiting conditions leading to a greater proportion of Zrc1-dependent activity in isolated vesicles.

**Kinetic analysis of zinc transport by vacuole membranes** - Although our preceding results suggested that Zrc1 was directly responsible for the Zrc1-dependent transport activity observed, alternative explanations were also possible. For example, Zrc1 may somehow stimulate the activity of the Zrc1-independent, ATP-dependent activity detected in our assays. Therefore, we compared the biochemical properties of the Zrc1-dependent and independent zinc transport activities in vacuole vesicles. First, we characterized the substrate concentration-dependence of ATP-dependent zinc uptake in wild-type and zrc1 vacuoles. With 1 μM $^{65}$Zn as substrate, ATP-dependent uptake by vacuole vesicles derived from both strains was linear for at least 40 seconds (Figure 5A). The effect of zinc concentration on this initial rate of uptake was then determined
for vesicles isolated from the two strains (Figure 5B), and non-linear analysis was used to derive apparent $K_m$ and $V_{max}$ constants (Figure 5C). The contribution of the ATP-independent zinc uptake activity, although small, was subtracted from the data before analysis. Vacuole vesicles from the wild-type strain mediated zinc uptake with a high affinity for substrate (apparent $K_m$ of 150 nM). The $zrc1$ mutation did not significantly alter the apparent $K_m$ value for zinc transport, but had a major negative effect on $V_{max}$. The contribution of the Zrc1-dependent transport to the total activity was calculated by subtracting the activity measured in the $zrc1$ mutant from the wild-type activity (Figure 5B, dashed line). The apparent $K_m$ calculated from these data did not differ markedly from that determined for the Zrc1-independent activity measured in the $zrc1$ mutant. Therefore, from these data alone, it was not possible to clearly distinguish the Zrc1-dependent and Zrc1-independent activity detected in the $zrc1$ mutant strain. In contrast, the ATP-independent activity had a much lower affinity for zinc, with an apparent $K_m$ of 5 µM (data not shown).

**Metal ion sensitivity and pH optimum for vacuole zinc uptake**—If two separate ATP-dependent zinc transport systems exist in the yeast vacuole membrane (one Zrc1-dependent and another system), it might be possible to distinguish these by their relative sensitivity to potential inhibitors of zinc transport. In addition, we were interested in determining how specific these systems were for zinc over other potential substrate cations that might compete for transport. To address these questions, the effect of various divalent metal ions on $^{65}$Zn transport by vacuole vesicles was determined. Zinc uptake was assayed using 1 µM $^{65}$Zn$^{2+}$ with or without a 10-fold molar excess of other metal ions. ATP-independent uptake was subtracted from the total activity, and the results were normalized to the control value with no inhibitor added. Because
cytoplasmic iron would likely be present in its reduced form, the effectiveness of this metal as an inhibitor was determined in the presence of 1 mM ascorbate to reduce Fe\(^{3+}\) to Fe\(^{2+}\). Control experiments showed no effect of ascorbate alone on vesicle zinc transport (data not shown). With wild-type vesicles, non-radioactive Zn\(^{2+}\), Ni\(^{2+}\), and Cd\(^{2+}\) inhibited isotope accumulation to the greatest degree (Figure 6A). Little or no effects were noted with Ca\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), and Fe\(^{2+}\). These treatments had no effect on V-ATPase activity (data not shown), indicating that any effects on zinc transport were not a consequence of the inhibition of this enzyme. When uptake by zrc1 vacuole vesicles was compared to wild type, the results showed nearly identical sensitivity to inhibition by metal ions. Hence, we could not distinguish the residual activity in zrc1 mutants from the Zrc1-dependent activity on this basis. In contrast, the ATP-independent activity could be distinguished from active transport; the ATP-independent activity was sensitive to Zn\(^{2+}\) and Cd\(^{2+}\), but Ni\(^{2+}\) had no effect on this system (data not shown).

As an additional test to determine if the Zrc1-dependent activity could be distinguished from the Zrc1-independent activity, we determined the pH optimum for each component (Figure 6B). Zrc1-dependent, ATP-dependent zinc transport in wild-type vacuoles exhibited optimal activity at pH 6.5. In contrast, the Zrc1-independent activity exhibited a more complex pH profile, with two possible peaks in activity at pH 6 and 7.5. Therefore, taken together, the data in Figures 5 and 6 indicate that the Zrc1-dependent and independent transport activities may be closely related but different systems. The ATP-dependent transport activities could also be distinguished from ATP-independent transport on the basis of pH optimum, as the ATP-independent system showed maximal activity at pH 8.5 or higher (Figure 6B).
DISCUSSION

In this study, we identified two general types of zinc transport activities in isolated vacuole vesicles. One type of activity was apparently passive transport that occurred in the absence of ATP. The second type was active transport dependent on ATP hydrolysis. We focused our efforts on characterizing the ATP-dependent transport components for two reasons. First, because of the important role the vacuole plays in zinc homeostasis, we were primarily interested in characterizing the zinc transport systems in the membrane of this organelle. The ATP-dependent activities are likely to be vacuolar in origin because of their strict dependence on V-ATPase activity. In contrast, it was unclear if the ATP-independent activity we observed was vacuolar given that our vesicle preparations contained some membranes derived from other compartments (e.g. endoplasmic reticulum and mitochondria). Second, we predicted that efficient transport from the cytoplasm and accumulation in the vacuole would require an active transport system to work against the zinc concentration gradient likely to be generated when zinc accumulates in this compartment.

Active transport systems are of two basic types: primary active transport systems, many of which directly utilize the energy of ATP-hydrolysis to drive transport, and secondary active transport systems, which harness the energy stored in solute concentration gradients. Several lines of evidence suggested that the ATP-dependent accumulation of zinc by vacuole vesicles is a secondary active transport process coupled to the vacuolar proton gradient. First, previous studies have documented the increased zinc sensitivity of mutants defective for V-ATPase function (10,11). This sensitivity is consistent with a role of the V-ATPase in generating a H⁺ gradient required to drive vacuolar zinc uptake. Second, in our in vitro studies, zinc uptake was dependent on ATP hydrolysis and was not stimulated by ADP or an ATP analogue that is not a
substrate for the V-ATPase. Third, zinc transport was eliminated by a specific inhibitor of the V-ATPase (concanamycin A), but less affected by inhibitors of other primary active transport systems such as P-type ATPases. Fourth, ATP-dependent zinc transport was absent in vitro with vesicles obtained from a vph1 stv1 mutant strain that lacks V-ATPase activity. Finally, agents that dissipate proton gradients (FCCP and nigericin) also inhibited vesicle zinc accumulation in vitro. One mechanism of how the vacuolar proton gradient generated by the V-ATPase could drive zinc uptake into the vacuole is through Zn$^{2+}$/H$^+$ antiport. To test this hypothesis, we attempted to detect zinc-stimulated proton efflux in vesicles loaded with the pH-sensitive fluorophore, ACMA. Vesicles were rapidly acidified when supplied with ATP as determined by quenching of ACMA fluorescence. However, the addition of 100 nM concanamycin A to the vesicles to prevent further acidification and stabilize the internal pH revealed a rapid proton leak, which made it impossible to measure any additional effect of zinc on dissipation of the gradient (Figure 2B). We also could not detect acidification of zinc-loaded vacuoles incubated in the absence of ATP probably due to the proton leak effect. Nevertheless, the dependence of zinc uptake on vacuole acidification and V-ATPase activity is consistent with the hypothesis that vacuolar zinc uptake is mediated by a Zn$^{2+}$/H$^+$ antiport mechanism. This conclusion is supported by the recent studies of Guffanti et al. (36) on the CzcD protein of the prokaryotic Ralstonia sp. strain CH34. czcD encodes a member of the CDF family related to Zrc1. Their studies indicated that CzcD transported zinc in exchange for K$^+$ and H$^+$.

Many previous genetic studies have suggested an important role for Zrc1 in vacuolar zinc sequestration. The tentative localization of Zrc1 to the vacuole under overexpression conditions (18,19) supported this hypothesis and this localization was confirmed here using a functional epitope-tagged allele expressed at normal levels from its own promoter. Our transport studies
also support the hypothesis that Zrc1 is critical for efficient transport of zinc into the vacuole; Zrc1 was required for $\geq 75\%$ of ATP-dependent activity. A key remaining question is whether Zrc1 is the transporter per se or if it alters the activity of another protein that serves as the transporter. Evidence supporting a direct role of Zrc1 in zinc transport comes from Zrc1’s ability to suppress the zinc sensitivity phenotype of a *Ralstonia czcD* mutant (37). Expression of Zrc1 in *Ralstonia* also inhibited accumulation of Zn. These data suggested that Zrc1 is capable of mediating zinc efflux when expressed in this heterologous system. The dramatic decrease in zinc uptake activity we observed with *zrc1* vacuole vesicles is also consistent with Zrc1 being a transporter, as is our ability to distinguish between Zrc1-dependent and independent activities based on pH optima. Ultimately, however, the definitive test of Zrc1 as a zinc transporter will require transport studies using liposomes reconstituted with the purified protein. Surprisingly, overexpression of Zrc1 had no consistent effect on vacuolar zinc transport despite a large increase in Zrc1 protein level (data not shown). If Zrc1 is indeed the transporter, these data argue that some other factor, e.g. a cytosolic protein contaminating our vacuolar vesicles, may be limiting Zrc1’s activity in our *in vitro* assays. Precedence for this model comes from studies of CzcD. The CzcO protein is not predicted to be a membrane protein but is required for full activity of CzcD (36). We were also surprised to find no effect of the *cot1* mutation on vacuolar zinc transport given that Cot1 had been localized to this compartment when overexpressed (18) and provided zinc tolerance (21,22). This result may be due to the localization of Cot1 to a different cellular compartment under normal expression conditions. Alternatively, the conditions of our *in vitro* assay may not be optimized to detect Cot1-dependent activity.

Our studies of Zrc1-dependent uptake activity may provide some insight into the mechanism of transport used by other CDF family members. Members of this family have been
implicated in zinc efflux and sequestration in bacteria, fungi, plants, and mammals but we still know little about their mechanisms of action. Consistent with a H⁺-dependent transport mechanism, the mammalian Znt-2 protein was found to colocalize with zinc in the late endosome, an acidic compartment much like the yeast vacuole (38,39). In contrast, the Arabidopsis ZAT protein promoted the association of zinc with proteoliposomes independent of a pH gradient (22). Moreover, accumulation by these liposomes was not saturable even with substrate concentrations as high as 1 mM Zn²⁺, suggesting that ZAT behaves more like a zinc channel than a carrier protein. Therefore, different members of the CDF family may mediate zinc transport through very different mechanisms. Our studies provide an experimental system to define these potential differences.

Recent studies have suggested that E. coli cells strive to maintain a perfect balance of zinc accumulation and utilization such that there is essentially no labile zinc in the cytosol during cell growth (5). The apparent Kₘ of vacuolar zinc uptake may shed some light on the physiological levels of labile cytosolic zinc in yeast cells. In a previous study, we estimated that the minimum total zinc content for growth of a yeast cell is about 4 x 10⁶ atoms per cell, and the optimum zinc content for growth is ~1.5 x 10⁷ atoms per cell (14). We predict that this is the amount of zinc required to populate all of the zinc-binding sites (i.e. zinc-dependent enzymes, etc.) required for optimal metabolism. Our studies indicated that vacuolar zinc sequestration becomes evident when the total cytosolic zinc levels exceed that optimum number (14). The ATP-dependent activities we have studied have apparent Kₘ’s of ~150 nM. Assuming approximately 10% of the total cell volume is cytosolic [the yeast vacuole alone can occupy greater than 50% of the cell volume], this zinc content translates into a cytosolic Zn²⁺ concentration of ~450 atoms per cell. Our estimate of labile zinc also does not consider the
excluded volume effects of macromolecular crowding in the cytosol (40) and therefore likely represents an overestimate of labile zinc levels that may be present. The buffering capacity of vacuolar zinc transport will be most effective between 0.1-10-fold of the $K_m$ level (see Figure 5 for illustration) which represents 45-4,500 labile zinc atoms per cell. These values are consistent with the estimated low levels of labile cytosolic zinc (as opposed to total zinc) found in eukaryotic cells (6,38). They are also consistent with the hypothesis that vacuolar zinc transport helps maintain the very low cytosolic labile zinc levels in cells.

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**FOOTNOTES**

1 C. W. MacDiarmid and D. Eide, manuscript in preparation
FIGURE LEGENDS

Figure 1. Immunoblot analysis of isolated vacuole vesicles.

Immunoblot analysis of cell lysate (L) and isolated vacuoles (V) from zinc-replete CM142 (WT, expressing Zrc1-HA) and CM102 (zrc1) strains. Vacuoles were prepared as described in Materials and Methods. Equal amounts of protein (2.5 µg) was fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with antibodies directed against markers for the vacuole (Cpy, Vma1), cytoplasm (Pgk1), trans-Golgi network (Kex2), endoplasmic reticulum (Dpm1), mitochondria (Omp2) and Zrc1-HA (α-HA). For the α-HA blot, bands corresponding to full length (arrow) and partially degraded (*) Zrc1 are indicated. The positions of molecular mass markers are shown on the Zrc1 blot.

Figure 2. 65Zn2+ uptake and acidification by vacuolar vesicles

A. Aliquots of uptake buffer containing 65Zn2+ (0.5 µM) and either no ATP or 1 mM of the indicated nucleotides were prepared. At T₀, vacuolar vesicles isolated from zinc-replete wild-type cells were added. An aliquot (5 µg protein) was immediately removed and added to 4.5 ml cold wash buffer to stop the reaction. Subsequent aliquots were removed at the indicated times. Vesicle-associated zinc was determined as described in Materials and Methods. Pyrithione was added to a +ATP reaction at 3 min 10 sec into the time course (arrow). A control with no membranes added showed no significant vesicle retention of zinc (not shown). This experiment was performed three times with similar results; the results of a single representative experiment are shown. B. Acidification of vacuolar vesicles. Vacuole vesicles (10 µg protein) were assayed for acidification using ACMA as described in Experimental Procedures. Some assays were performed in the presence of oligomycin (10 µg/ml), vanadate (1 mM), or the indicated
concentration of concanamycin A. Timing of later additions are indicated by the vertical lines; ATP, ADP, or AMP-PNP (each at 1 mM), FCCP (5 μM), or concanamycin A (100 nM) were added at those times. The instantaneous reduction in fluorescence observed upon addition of ATP and other compounds is due to a quenching artifact and was not reversed by addition of FCCP or nigericin. Representative experiments are shown.

Figure 3. Zrc1 is a vacuolar membrane protein
A. Yeast strains CM142 (expressing an integrated Zrc1-HA construct) and CM100 (expressing untagged Zrc1) were grown in low-zinc (LZM + 1 μM zinc) or high zinc (LZM + 1000 μM zinc) media. The cells were fixed, probed for the HA epitope, and viewed by DIC optics or epifluorescence (Epi). B. Yeast strain MM112 (vph1 stv1) was transformed with an epitope-tagged version of Zrc1 (YCpZRC1-HA, tagged) or the control vector YCpZRC1 (untagged) and the transformants were grown in zinc-deficient medium (LZM + 2 μM zinc). Cells were then processed and viewed as in panel A.

Figure 4. Effect of the zrc1 and cotl mutations on zinc uptake by vacuole vesicles
A. Vacuole vesicles were prepared from zinc-replete CM100 (WT), CM102 (zrc1), CM103 (cotl), CM104 (zrc1 cotl), and MM112 (vph1 stv1) strains, and zinc uptake rate was assayed. B. ATPase activity in vacuolar vesicles. Reactions contained 1 mM ATP with or without addition of 100 nM concanamycin A. C. Zinc uptake by vesicles isolated from zinc-deficient cells. CM100 (WT) or CM102 (zrc1) cells were grown in LZ-YPD medium supplemented with 1 μM zinc and isolated vacuole vesicles. For all experiments shown, data points are means of three replicates and error bars represent 1 S.D.
Figure 5. Time- and concentration-dependence of zinc uptake in wild-type and zrc1 vacuole vesicles.

A. Time course of zinc uptake in zinc-replete wild-type (WT, CM100) and zrc1 (CM102) mutant vacuole vesicles showing linearity of uptake. B. Effects of zinc concentration on initial rate of ATP-dependent zinc uptake. Vacuole membrane vesicles (10 µg protein) were added to uptake buffer with the indicated zinc concentration, with or without 1 mM ATP. Reactions were incubated at 30 °C for 20 s, then halted by addition of cold wash buffer. The experiment was repeated twice and ATP-independent uptake was subtracted to give the data shown. The apparent contribution of Zrc1 (Zrc1-dependent) was calculated by subtracting the data for the zrc1 mutant from the wild-type data. C. The WT, zrc1 and Zrc1-dependent data were subjected to non-linear analysis to calculate apparent $K_m$ (µM) and $V_{max}$ (nmol $^{65}$Zn/min/mg protein) constants. These values are shown ± 1 S.E., which was calculated from the fit of the data to the ideal curve shown.

Figure 6. Effect of potential metal ion inhibitors and pH on zinc transport

A. Effect of divalent metal cations on ATP-dependent zinc uptake by vacuole vesicles. Vacuoles (5 µg protein) prepared from zinc-replete wild-type (WT, CM100) and zrc1 mutants (CM102) were added to uptake buffer containing 1 µM $^{65}$Zn$^{2+}$ with or without 1 mM ATP. Except for control assays (C), uptake buffer also contained 10 µM of the indicated metal cation. Reactions were incubated for 1 min and stopped by addition of cold wash buffer. ATP-independent uptake was subtracted from the ATP-dependent data and normalized to the control value (control values for ATP-dependent transport by WT and zrc1 mutants were 5.9 and 1.8
nmol $^{65}$Zn/mg protein/min respectively). Reactions containing Fe$^{3+}$ also contained 1 mM ascorbate to maintain Fe in a reduced state. B. pH-dependence of zinc uptake. Vacuole vesicles (5 µg protein) were added to 200 µl aliquots of assay buffer containing 1 µM $^{65}$Zn$^{2+}$ at the pH indicated, with or without 1 mM ATP. Assays were incubated for 1 min before addition of wash buffer. The pH of the uptake buffer was varied by using 10 mM MES (pH 5.5-6.5), TES (pH 7-7.5), or Tris (pH 8-8.5) buffer, adjusted to the required pH using Tris-base.
Table I. Effect of inhibitors on zinc uptake and ATPase activity of vacuole vesicles.

Zinc uptake assays were performed as described in the Materials and Methods (1 min incubation with 0.5 µM $^{65}$Zn$^{2+}$). Reactions contained ATP (1 mM), or ATP and either FCCP (5 µM), nigericin (5 µM), valinomycin (5 µM), oligomycin (10 µg/ml), concanamycin A (100 nM), or Na vanadate (1 mM). The effect of these inhibitors on total ATPase activity in vacuolar membrane vesicles was also determined. ATPase assay reactions were incubated for 8 min prior to stopping the reactions. Identical ATP and inhibitor concentrations were used for both zinc uptake and ATPase assays.

| Treatment | $^{65}$Zn uptake rate$^a$ | Relative $^{65}$Zn uptake rate$^b$ | ATPase specific activity$^c$ | Relative ATPase specific activity$^d$ |
|-----------|--------------------------|-----------------------------------|-----------------------------|-----------------------------------|
| - ATP     | 0.8 ± 0.2                | 5.0 ± 1.0                         | NA                          | NA                                |
| + ATP     | 16.8 ± 0.7               | 100.0 ± 4.2                       | 1.6 ± 0.1                   | 100.0 ± 3.0                       |
| FCCP      | 4.0 ± 0.7                | 23.6 ± 4.0                        | 4.6 ± 0.1                   | 295.2 ± 1.6                       |
| Nigericin | 2.4 ± 0.2                | 14.5 ± 1.3                        | 4.1 ± 0.1                   | 260.2 ± 8.3                       |
| Valinomycin| 17.8 ± 0.3              | 105.7 ± 1.5                       | 1.6 ± 0.1                   | 100.2 ± 2.4                       |
| Oligomycin| 13.4 ± 0.9              | 80.0 ± 5.0                        | 1.2 ± 0.1                   | 75.1 ± 0.4                        |
| Concanamycin A | 3.1 ± 0.2 | 18.5 ± 1.0              | 0.6 ± 0.1                   | 40.0 ± 2.1                        |
| Vanadate  | 16.4 ± 0.5              | 97.3 ± 3.2                        | 0.4 ± 0.1                   | 26.4 ± 3.0                        |

$^a$ (nmol/mg protein/min)

$^b$ (% of + ATP value)

$^c$ (nmol Pi/µg protein/min)

$^d$ (% of + ATP value)
Figure 1

|   | WT |   | zrc1 |
|---|----|---|------|
| L |    | L |      |
| V |    | V |      |

- Cpy
- Vma1
- Pgk1
- Kex2
- Dpm1
- Omp2

α-HA (Zrc1)
Figure 4

A

$^{65}$Zn uptake rate (nmol/mg protein/min)

|          | +ATP | -ATP |
|----------|------|------|
| WT       |      |      |
| zrc1     |      |      |
| cot1     |      |      |
| zrc1 cot1|      |      |
| vph1     |      |      |
| stv1     |      |      |

B

ATPase specific activity (nmol P1/ug protein/min)

|          | -ConA | +ConA |
|----------|-------|-------|
| WT       |       |       |
| zrc1     |       |       |
| cot1     |       |       |
| zrc1 cot1|       |       |
| vph1     |       |       |
| stv1     |       |       |

C

$^{65}$Zn uptake rate (nmol/mg protein/min)

|          | +ATP | -ATP |
|----------|------|------|
| WT       |      |      |
| zrc1     |      |      |
Figure 5

C

| Values (± SE)          | WT       | zrc1    | Zrc1-dependent |
|------------------------|----------|---------|----------------|
| $V_{\text{max}}$      | 41.9 ± 1.1 | 6.3 ± 0.4 | 35.6 ± 1.4     |
| $K_{m}$               | 0.15 ± 0.01 | 0.12 ± 0.02 | 0.16 ± 0.02    |
Biochemical properties of vacuolar zinc transport systems of Saccharomyces cerevisiae

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