Inhibition of the Ca^{2+}-ATPase Pmc1p by the v-SNARE protein Nyv1p

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Running Title: v-SNARE inhibits Ca^{2+}-ATPase activity in yeast

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SUMMARY

Pmc1p, the Ca$^{2+}$-ATPase of budding yeast related to plasma membrane Ca$^{2+}$-ATPases of animals, is transcriptionally up-regulated in response to signaling by the calmodulin-calcineurin-Tcn1p/Crz1p signaling pathway. Little is known about post-translational regulation of Pmc1p. In a genetic screen for potential negative regulators of Pmc1p, a vacuolar v-SNARE protein Nyv1p was recovered. Cells overproducing Nyv1p show decreased Ca$^{2+}$ tolerance and decreased accumulation of Ca$^{2+}$ in the vacuole, similar to pmc1 null mutants. Overexpression of Nyv1p had no such effects on pmc1 mutants, suggesting Nyv1p may inhibit Pmc1p function. Overexpression of Nyv1p did not decrease Pmc1p levels but decreased the specific ATP-dependent Ca$^{2+}$ transport activity of Pmc1p in purified vacuoles by at least two-fold. The effect of Nyv1p on Pmc1p function is likely to be direct because native immunoprecipitation experiments showed that Pmc1p co-precipitated with Nyv1p. Complexes between Nyv1p and its t-SNARE partner Vam3p were also isolated but these complexes lacked Pmc1p. We conclude that Nyv1p can physically interact with Pmc1p and inhibit its Ca$^{2+}$ transport activity in the vacuole membrane. This is the first example of a Ca$^{2+}$-ATPase regulation by a v-SNARE protein involved in membrane fusion reactions.
INTRODUCTION

Ca\(^{2+}\) plays an important role as a signaling molecule for cell growth. In eukaryotic cells, cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)) is typically maintained at sub-micromolar levels against millimolar free Ca\(^{2+}\) concentrations in the lumen of secretory organelles and extracellular spaces. Such steep gradients are generated and maintained by Ca\(^{2+}\) pumping ATPases and ion exchangers, which offset the effects of Ca\(^{2+}\) channels and non-specific leaks in the membrane. The regulated opening and closing of Ca\(^{2+}\) channels in cellular membranes contribute to [Ca\(^{2+}\)]\(_{c}\) modulation with intricate temporal and spatial resolution, permitting the use of cytosolic Ca\(^{2+}\) as a regulator of numerous cellular processes such as metabolism, gene expression, and membrane fusion.

In the budding yeast *Saccharomyces cerevisiae*, large lysosome-like vacuoles serve as the major intracellular Ca\(^{2+}\) reservoir, accumulating ~95% of the total cell-associated Ca\(^{2+}\) (1,2). As listed in Table 1, two vacuolar Ca\(^{2+}\) transporters have been identified, the Ca\(^{2+}\)-ATPase Pmc1p and the H\(^+/\)Ca\(^{2+}\) exchanger Vcx1p (3-5). Deletion of the *PMC1* gene decreases the ability to grow in high Ca\(^{2+}\) environments, while deletion of *VCX1* decreases Ca\(^{2+}\) tolerance only slightly, suggesting that Pmc1p normally plays a more significant role in vacuolar Ca\(^{2+}\) sequestration. Ca\(^{2+}\) stored in the vacuole can bind inorganic polyphosphates in the lumen and precipitate, thereby increasing the capacity for Ca\(^{2+}\) sequestration (2). Recently, release of Ca\(^{2+}\) from the vacuole was found to be important for homotypic fusion of vacuole membranes *in vitro* (6). Homotypic vacuole fusion involves membrane-bound SNAP receptors (SNAREs)\(^{1}\) including the v-SNAREs Nyv1p, Vti1p, and Ykt6p, the t-SNARE Vam3p, and the s-SNARE Vam7p homologous to SNAP-23/25 (7-9). At a late step in the homotypic fusion pathway, Ca\(^{2+}\) is released from the vacuole causing a local elevation in [Ca\(^{2+}\)]\(_{c}\)
which triggers fusion of docked membranes through a calmodulin-dependent process (6). However, vacuoles lacking both Pmc1p and Vcx1p were fully active in homotypic fusion assays (10). No other roles of vacuolar Ca$^{2+}$ transport have been identified to date.

The endoplasmic reticulum (ER) and the Golgi complex of yeast accumulate Ca$^{2+}$ via the Ca$^{2+}$/Mn$^{2+}$ ATPase Pmr1p which is important for a variety of secretory functions (11-15). Mutants lacking Pmr1p exhibit high rates of Ca$^{2+}$ influx and elevation of [Ca$^{2+}$]c due to stimulation of a plasma membrane Ca$^{2+}$ channel composed of Cch1p and Mid1p (16). This process resembles the capacitative calcium entry (CCE) mechanisms in animal cells where depletion of secretory Ca$^{2+}$ pools promotes Ca$^{2+}$ influx through the plasma membrane channels and refilling of the depleted organelles (17). In yeast, excessive activity of the vacuolar Ca$^{2+}$ transporters Pmc1p and Vcx1p can compete with Pmr1p for Ca$^{2+}$ and activate the CCE-like mechanism (16). Therefore, the activity of vacuolar Ca$^{2+}$ transporters must be balanced with Pmr1p activity to avoid depletion of secretory organelles and inefficient use of energy for Ca$^{2+}$ sequestration in the vacuole.

Extensive studies have revealed that all three Ca$^{2+}$ transporters in yeast are regulated by a signaling network involving calcineurin, a protein phosphatase that becomes activated by binding Ca$^{2+}$ and calmodulin upon elevation of [Ca$^{2+}$]c. Genetic studies suggest that Vcx1p may be inhibited by calcineurin at a post-translational level (4). Transcription of PMC1 and PMR1 is increased upon calcineurin-dependent activation of the transcription factor Tcn1p/Crz1p (18,19). In high Ca$^{2+}$ environments, the strong up-regulation of Pmc1p is necessary for proliferation (18). However, when environmental Ca$^{2+}$ concentrations subside, the excess Pmc1p activity might inhibit normal maintenance of Ca$^{2+}$ in secretory organelles. Negative regulation of Pmc1p...
activity may therefore be important under these and other conditions. To date, no negative regulators of Pmc1p have been identified.

In this study, we screen for negative regulators of Pmc1p in vivo and identify Nyv1p, a transmembrane v-SNARE protein in the vacuole membrane, as a likely candidate. We found no obvious role of Pmc1p or Nyv1p in vacuole morphology or fusion in vivo. Rather, Nyv1p bound to Pmc1p and inhibited its Ca\textsuperscript{2+} transport activity in vivo and in vitro. Thus, a new role of Nyv1p in yeast may be the regulation of Pmc1p activity and Ca\textsuperscript{2+} homeostasis.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Growth conditions -- Standard culture media for yeast and E. coli have been described previously (20). All yeast strains listed in Table 2 are derivatives of strain W303-1A (21) which were constructed through transformation and isogenic crosses by using standard techniques (22). The nyv1::HIS3\textsuperscript{5p} null mutation was introduced into the wild type strain K601 by transformation with a PCR product amplified from the genomic DNA of yeast strain SEY6210\textDelta nyv1 (7) using flanking primers that hybridize at nucleotide -457 and nucleotide +1109 relative to the initiation codon of NYV1. The vam3::HIS3 null mutation was introduced to strain K601 by transformation with XbaI-digested plasmid pYVQ311 (23). The mutations were confirmed by PCR analysis, western blot analysis, and/or observation of vacuole morphology.

A pmr1 cch1 strain (ELY106) was transformed with a YEp13-based library of yeast genomic DNA (gift from Kim Nasmyth, Austria) and transformants were replica
plated onto Whatman No. 3 filter papers which had been placed on the surface of agar medium containing YPD medium supplemented with 5 mM succinic acid, 10 mM CaCl₂, 10 mM MgCl₂, and 0.3 mM adenine. After growth overnight at 30°C, the filters were removed and stained for β-galactosidase activity as described previously (18). Among 8,000 transformants, 18 colonies expressed PMCl-lacZ at higher levels than controls. The plasmids were isolated from these strains and only 8 were found to be positive on re-testing. Each plasmid was sequenced from both ends of the genomic DNA insert to identify the genes included. One plasmid, pLE66, contained the TCN1/CRZ1 gene encoding the calcineurin-dependent transcription factor involved in PMCl-lacZ induction (18,19). Another plasmid pLE8 carried a genomic DNA insert spanning three complete genes SUL2, NYV1, and GIS3. In a parallel screen using a pmr1 mid1 strain and a low-copy pRS313-based library of genomic DNA (gift from David Levin, Johns Hopkins University), a plasmid carrying SUL2 and NYV1 genes was isolated as a stimulator of PMCl-lacZ expression in the pmr1 mid1 cells. Subcloning showed the NYV1 gene alone conferred the phenotype of increased PMCl-lacZ expression. The first subclone, plasmid pNYV-HIS, carried 2.5 kb of genomic DNA surrounding NYV1 (from the SmaI site at nucleotide -191 to a Sau3A site at nucleotide +2315 relative to initiation codon) ligated into the SmaI site of pYO323 (24). The second subclone, plasmid pNYV-LEU, carried a 1.4 kb SmaI-EcoRV fragment of the NYV1 locus ligated into the SmaI site of pYO325 (24). Plasmids, pPI12 and pSK60 have been described previously (25).

Measurement of Ca²⁺ pools -- Total cell-associated Ca²⁺ was determined for yeast strains grown at 30°C for 4 hr in YPD medium supplemented with 20 µCi/mL as described previously (4). The non-exchangeable Ca²⁺ pool was measured by a similar protocol except the cultures were diluted 5-fold with fresh YPD medium containing 20 mM CaCl₂ and incubated an additional 20 minutes at 30°C prior to harvesting by
filtration. The exchangeable Ca\(^{2+}\) pool represents the difference between total Ca\(^{2+}\) and non-exchangeable Ca\(^{2+}\).

**Ca\(^{2+}\) Tolerance Assays** -- Yeast cells were grown overnight at 30°C in YPD (pH 5.5) medium or half-concentrated SC-Leu medium. Saturated cell suspensions were then diluted 100-fold into 0.2 ml of the same medium supplemented with various concentrations of CaCl\(_2\) and incubated in flat-bottom 96-well dishes for 20 hr at 30 °C without shaking. Optical density of each culture was measured at 650 nm using a microplate spectrophotometer (Molecular Devices).

**Immunoprecipitation and Immunoblotting** -- One hundred µg of purified vacuoles prepared as described previously (26) were solubilized with 500 µl lysis buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.5 % Tween-20, protease inhibitors) and incubated with anti-Nyv1p antiserum (9) for 1 hr at 4 °C and then treated with protein A beads for an additional 1 hr at 4 °C. The protein A beads were collected by centrifugation and washed three times with lysis buffer. The proteins bound to the beads were separated by SDS-PAGE, transferred to PVDF membranes (Millipore), decorated with 12CA5 monoclonal antibodies specific for either the HA tag (Boehringer Mannheim) or polyclonal antibodies specific for Nyv1p or Vam3p (8). For immunoprecipitations after priming reaction with ATP, vacuoles were first suspended in 750 µl of reaction buffer containing cytosol and ATP, incubated at 27 °C, washed with 500 µl of PS buffer (10 mM PIPES-KOH pH 6.8, 200 mM sorbitol), and then solubilized, treated with antibodies to Nyv1p or Vam3p, and processed as described (8).

**Pmc1p Activity Assay** -- Purified vacuoles were suspended at 32 µg/ml in reaction buffer (10 mM PIPES-KOH pH 6.8, 200 mM sorbitol, 2 mM MgCl\(_2\), 100 mM KCl) containing 10 µCi/ml \(^{45}\)CaCl\(_2\) plus varying concentrations of non-radioactive
CaCl₂ and EGTA. Free Ca²⁺ concentrations were calculated using MaxChelator (http://www.stanford.edu/~cpatton/maxc.html). Reactions were pre-warmed to 30°C and initiated by addition of either 1 mM ATP or 2.5 mM ADP. After 1 min incubation, vacuoles were collected by rapid filtration onto 0.45 µm nitrocellulose filters (type HA, Millipore), washed three times with the ice-cold buffer A (5 mM HEPES-NaOH pH 6.5, 10 mM CaCl₂), and the associated radioactivity was determined by liquid scintillation counting. Time course experiments showed Ca²⁺ transport reactions were linear for at least 2 minutes. ATP-dependent Ca²⁺ transport was calculated by subtracting the values obtained using ADP from those obtained using ATP.

**Staining and Microscopy** -- Microscopic observation of yeast vacuoles in vivo was carried out by labeling with FM4-64 (Molecular Probes). Log-phase cells were incubated in 50 µl of YPD containing 20 µg/ml FM4-64 for 20 min at 30 °C. Cells were washed three times with fresh YPD, resuspended in 1 ml of YPD, incubated for an additional 60 min, and observed using a Zeiss Axiovert microscope equipped with 100X objectives.

**RESULTS**

**Genetic analysis of NYV1 function in Ca²⁺ homeostasis** -- Mutants lacking the Golgi Ca²⁺-ATPase Pmr1p maintain [Ca²⁺]c at elevated levels in part due to increased Ca²⁺ influx via the Chc1p-Mid1p Ca²⁺ channel, resulting in elevated expression of the calcineurin-dependent reporter gene PMC1-lacZ (16,27,28). The up-regulated Pmc1p helps lower [Ca²⁺]c to levels that permit proliferation (3). We reasoned that a hypothetical inhibitor of Pmc1p, when overexpressed in pmr1 mutants, would cause further elevation of [Ca²⁺]c and PMC1-lacZ expression and possibly decrease
proliferation. There would be no such effects in wild type cells because Pmr1p can maintain \([\text{Ca}^{2+}]_c\) and PMC1-lacZ expression at low levels even in the absence of Pmc1p and Vcx1p (4). To identify potential inhibitors of Pmc1p, a high-dosage plasmid library of yeast genomic DNA was screened for clones that elevated PMC1-lacZ expression in colonies of a pmr1 cch1 double mutant grown in optimized medium (see Experimental Procedures). The pmr1 cch1 double mutant was used instead of a pmr1 single mutant because the latter strain exhibits high background staining with X-gal whereas background was much lower in the former (16). As expected under these screening conditions, most of the colonies stained light blue with X-gal due to low expression of PMC1-lacZ whereas several stained darker blue. One of the plasmids was isolated and found to carry the NYV1 gene plus flanking genes. Subcloning revealed that the NYV1 gene itself when overexpressed was necessary and sufficient to increase PMC1-lacZ expression in pmr1 cch1 mutants. A second screen employing pmr1 mid1 double mutants also recovered a plasmid bearing NYV1 (E. G. Locke and K. W. Cunningham, unpublished). The NYV1 gene encodes a v-SNARE protein that localizes to the vacuole membrane and is required for homotypic fusion of vacuoles in vitro (7). These findings suggest Nyv1p might affect vacuolar \(\text{Ca}^{2+}\) homeostasis.

To determine whether NYV1 overexpression alters accumulation of \(\text{Ca}^{2+}\) in the vacuole, the non-exchangeable \(\text{Ca}^{2+}\) pool was quantitated in a variety of yeast strains after prolonged growth in medium containing \(45\text{Ca}^{2+}\) as a tracer. As seen previously (4), the non-exchangeable \(\text{Ca}^{2+}\) pool was very large in wild type cells and vcx1 mutants but was greatly diminished in pmc1 mutants and pmc1 vcx1 double mutants (Fig. 1). Overexpression of NYV1 significantly decreased the non-exchangeable \(\text{Ca}^{2+}\) pool in wild type and vcx1 mutants but had no significant effect in either pmc1 mutants or pmc1 vcx1 double mutants. In all these strains, there was no significant effect of NYV1 overexpression on the levels of exchangeable \(\text{Ca}^{2+}\) (data not shown). These results
suggested Nyv1p overexpression diminishes Pmc1p activity but does not abolish it. To determine whether the effect of Nyv1p required Vam3p, a vacuolar t-SNARE that forms complexes with Nyv1p (8), similar measurements of non-exchangeable Ca^{2+} pools were performed on \textit{vam3} mutants. For unknown reasons, the non-exchangeable Ca^{2+} pools were consistently elevated ~2-fold in \textit{vam3} mutants relative to wild type, independent of Vcx1p and Pmc1p (Fig. 1). Overexpression of \textit{NYV1} decreased the non-exchangeable Ca^{2+} pool in \textit{vam3 vcx1} double mutants but increased this pool in \textit{pmc1 vam3 vcx1} triple mutants. Close comparison of these two mutant strains indicates the Pmc1p-dependent activity was significantly diminished by increased \textit{NYV1} dosage.

Thus, \textit{NYV1} overexpression decreased Ca^{2+} accumulation in the vacuoles of yeast cells by a process that was independent of Vcx1p and Vam3p but dependent on Pmc1p. The results are consistent with a model where Nyv1p directly or indirectly inhibits the Ca^{2+} transport activity of Pmc1p in the vacuole independent of fusion.

Pmc1p is essential for growth in high calcium environments (3). Therefore, we tested whether \textit{NYV1} overexpression could also diminish Ca^{2+} tolerance in a Pmc1p-dependent fashion. Overexpression of \textit{NYV1} greatly decreased Ca^{2+} tolerance of \textit{vcx1} mutants almost to that of \textit{pmc1 vcx1} double mutants (Fig. 2A). Overexpression of \textit{NYV1} in \textit{pmc1 vcx1} double mutants had little effect on Ca^{2+} tolerance. As a control for specificity, we also tested the effects of overexpressing \textit{VTI1} and \textit{YKT6} genes encoding v-SNARE proteins related to Nyv1p recently implicated in vacuole fusion reactions (9). There was no detectable effect of overexpressing the related v-SNARE proteins in either \textit{vcx1} mutants or \textit{pmc1 vcx1} double mutants (Fig. 2B). Therefore, the decreased Ca^{2+} tolerance observed upon \textit{NYV1} overexpression can be mostly attributed to effects on Pmc1p function.

The above experiments show significant effects of \textit{NYV1} overexpression on
Pmc1p function. To test whether Nyv1p at native levels can also affect Pmc1p function, we analyzed the phenotype of *nyv1* knockout mutants in Ca^{2+} tolerance assays. The *nyv1* null mutant strain exhibited slightly greater Ca^{2+} tolerance than an isogenic wild type strain (Fig. 2C). These results were also reproducible in the s288c strain background obtained from Research Genetics, Inc. (data not shown). Thus, consistent with effects of Nyv1p overexpression, endogenous levels of Nyv1p appeared to limit Pmc1p function in Ca^{2+} tolerance. Growth in high Ca^{2+} conditions had no effect on the abundance of Nyv1p or the expression of a *NYV1-lacZ* reporter gene (data not shown). We suggest that a relatively static level of Nyv1p can partially inhibit Pmc1p function *in vivo* and that the up-regulation of Pmc1p in high Ca^{2+} conditions may overcome this inhibition.

*Physical interactions between Nyv1p and Pmc1p* — Because Nyv1p and Pmc1p are both components of the vacuole membrane and interact functionally, we hypothesized they might also physically interact. To test this possibility, Nyv1p was immunoprecipitated from purified vacuoles dissolved in a non-denaturing detergent and co-precipitating proteins were analyzed by western blotting. A polyclonal antibody that specifically recognizes Nyv1p but not Nyv1p-Vam3p complexes was found to co-precipitate Pmc1p-HA, a functional epitope-tagged derivative of Pmc1p (Fig. 3A, lane 1). No Pmc1p-HA co-precipitated with the antibody when *nyv1* mutants were employed (lane 2). Incubation of purified vacuoles in conditions that support homotypic fusion slightly increased the abundance of Nyv1p-Pmc1p complexes (Fig. 3B, lanes 4-6). No Vam3p was co-precipitated with the Nyv1p or Nyv1p-Pmc1p complexes using the anti-Nyv1p antibody. Furthermore, antibodies specific for Vam3p co-precipitated Nyv1p but not Pmc1p (lanes 1-3). These results show that Nyv1p-Pmc1p complexes form in the vacuole membrane and can be purified away from other components of the vacuole membrane such as Nyv1p-Vam3p complexes. Therefore, a
novel cellular role of Nyv1p may be to inhibit the Ca\textsuperscript{2+} transport activity of Pmc1p by direct or indirect physical interaction.

*Effects of Nyv1p on Pmc1p activity* -- The decreased function of Pmc1p observed upon overexpression of Nyv1p may be due to increased Pmc1p degradation, decreased catalytic activity, or a combination of these or other effects. To distinguish the possibilities, we first examined the levels of Pmc1p in whole cell extracts of strains with or without NYV1 overexpression by western blot analysis. In all cases, the levels of Pmc1p were similar (Fig 4). Thus, Nyv1p appeared to have little or no effect on Pmc1p expression. We therefore examined Ca\textsuperscript{2+} transport activity of Pmc1p in purified vacuoles isolated from *vcx1* mutants carrying or lacking the NYV1 overexpression plasmid. For these experiments, the initial rates of ATP-dependent 45Ca\textsuperscript{2+} accumulation were determined at a variety of free Ca\textsuperscript{2+} concentrations set with EGTA buffers and the data were fit to the Michaelis-Menten equation (see Experimental Procedures). ATP-dependent Ca\textsuperscript{2+} transport was undetectable in vacuoles obtained from a *pmc1 vcx1* double mutant (data not shown) but was readily detectable in the *vcx1* mutant expressing Pmc1p-HA (Fig. 5A). The apparent K\textsubscript{M} for Ca\textsuperscript{2+} was calculated to be ~4.3 µM, somewhat higher than that of Pmr1p (13). Vacuoles isolated from the same strain but overexpressing NYV1 strain also exhibited a Pmc1p- and ATP-dependent Ca\textsuperscript{2+} transport activity with apparent K\textsubscript{M} similar to the control, however the maximal transport activity was diminished to ~43%. Both vacuole preparations contained equivalent amounts of Pmc1p-HA as determined by western blotting (Fig. 5B). A similar decrease in Pmc1p specific activity was observed in 3 independent experiments. The results suggest Nyv1p decreases the Ca\textsuperscript{2+} transport activity of Pmc1p by at least two-fold.

*Vacuole Morphology in vivo* -- The above results suggest that inhibition of
Pmc1p activity is a physiological function of Nyv1p. Does Pmc1p also regulate vacuole fusion reactions? To address this question, the morphology of vacuoles in \textit{pmc1} mutants was examined after staining the vacuole membranes of live cells with the fluorescent dye FM4-64 (29). In the W303 strain background, the vacuole morphologies of \textit{nyv1} mutant cells and \textit{pmc1} mutant cells were indistinguishable from that of wild type cells (Fig. 6). In contrast, \textit{vam3} mutants exhibited fragmented vacuoles typical of defects in homotypic and heterotypic fusion. Unlike \textit{vam3} mutants, \textit{pmc1} and \textit{nyv1} mutants efficiently targeted CPY to the vacuole ((7,30); data not shown). Thus, \textit{pmc1} and \textit{nyv1} mutants had no obvious defects in either endocytosis of FM4-64 to the vacuole, trafficking of vacuolar proteins, or vacuole fusion and inheritance. That \textit{nyv1} mutants have no phenotypes other than those involving Ca$^{2+}$ homeostasis may suggest regulation of Pmc1p function is a primary cellular function of this v-SNARE protein.

**DISCUSSION**

This study reports the first evidence of a v-SNARE protein interacting physically and functionally with a Ca$^{2+}$-ATPase \textit{in vivo}. We found that Pmc1p specifically co-precipitated with Nyv1p and that the ATP-dependent Ca$^{2+}$ transport activity of Pmc1p in purified vacuole preparations was significantly decreased upon overexpression of Nyv1p. The phenotypes of yeast cells lacking or overexpressing Nyv1p confirm this model. Overexpression of Nyv1p decreased $^{45}$Ca$^{2+}$ accumulation in the non-exchangeable (vacuolar) pool and decreased Ca$^{2+}$ tolerance only when Pmc1p was present. Likewise, inactivation of Nyv1p increased Ca$^{2+}$ tolerance in a Pmc1p-dependent fashion. Taken together, the data support a model where interaction with Nyv1p decreases Pmc1p activity at least two-fold. Nyv1p appeared to affect the $V_{\text{max}}$ rather than $K_m$ for Ca$^{2+}$ in this reaction, as if it inactivates a subset of Pmc1p molecules.
The data do not rule out the alternative possibility that Nyv1p partially inhibits the activity of all Pmc1p molecules. That Nyv1p levels were constant in low and high Ca\(^{2+}\) environments (unpublished observations) suggests the strong up-regulation of Pmc1p expression during growth in high Ca\(^{2+}\) conditions may serve to overcome the inhibitory effect of Nyv1p, further promoting Ca\(^{2+}\) tolerance.

The interaction between Pmc1p and Nyv1p raises questions about the role of Ca\(^{2+}\) in fusion of vacuole membranes. Nyv1p and Vam3p were shown to be required \textit{in trans} for homotypic vacuole-vacuole fusion in a reconstituted system \cite{7}. Furthermore, one report has suggested \textit{trans}-pairing of Nyv1p and Vam3p on docked vacuoles triggers Ca\(^{2+}\) release and local elevation of \([\text{Ca}^{2+}]_{c}\) which was essential for stimulating calmodulin-dependent reactions leading to bilayer fusion \cite{6}. The vacuolar Ca\(^{2+}\) release channel has not yet been identified, but the roles of Pmc1p and Vcx1p in homotypic fusion have been examined. Vacuoles lacking both Pmc1p and Vcx1p were found to be fully competent for homotypic fusion, and even more competent for fusion than wild type vacuoles under some conditions \cite{10}. However, this observation sheds little light on the role of Ca\(^{2+}\) in fusion because the \textit{pmc1 vcx1} mutant vacuoles would likely contain lower lumenal Ca\(^{2+}\) available for triggering fusion but permit higher free Ca\(^{2+}\) levels to be reached in the suspension buffer. Our finding that Pmc1p associates with Nyv1p but not Vam3p (Fig. 3) has several possible implications for homotypic vacuole fusion. First, Pmc1p may serve as a reservoir (or buffer) for unpaired Nyv1p thus increasing (or decreasing) Nyv1p availability for eventual \textit{trans}-SNARE pairing and fusion. This role may be analogous to that of synaptophysin in synaptic vesicles which forms complexes with the v-SNARE protein synaptobrevin/VAMP (see below). Second, \textit{trans}-SNARE pairing may dissociate active Pmc1p from complexes with Nyv1p thereby increasing Ca\(^{2+}\) transport activity near the sites of membrane fusion. In this view, the cytoplasm in the vicinity of fusion sites may be rapidly restored to resting
concentrations of Ca\(^{2+}\) after fusion, or possibly depleted of Ca\(^{2+}\) just before fusion. Alternatively, the Pmc1p-Nyv1p interaction might not facilitate the fusion process to any significant degree but instead it may simply serve to regulate Ca\(^{2+}\) homeostasis. To resolve all these possibilities, it will be necessary to determine the affinity and dynamics of the Pmc1p-Nyv1p interaction under a variety of conditions and to identify its effects on sub-reactions of the fusion pathway.

Unlike \textit{vam3} mutants and all other mutants defective in homotypic fusion, \textit{nyv1} mutants fail to exhibit any significant vacuolar phenotype unrelated to Ca\(^{2+}\). Trafficking of proteins to the vacuole by any of three distinct routes was shown to be normal in \textit{nyv1} mutants but strongly disrupted in \textit{vam3} mutants (30). Furthermore, the morphology and inheritance of vacuoles in \textit{nyv1} mutants closely resembles that of wild type cells in contrast to \textit{vam3} mutants (Fig. 6; (7)). Finally, using an \textit{in vivo} assay for homotypic fusion during mating we found that fusion of vacuoles in \textit{nyv1/nyv1} zygotes was indistinguishable from wild type but disrupted in \textit{vam3/vam3} zygotes (unpublished observations). It is possible the \textit{in vivo} assay for vacuole fusion reflects a distinct form of homotypic fusion than that which has been reconstituted \textit{in vitro}. It is also possible that other v-SNARE proteins in yeast such as Vti1p and Ykt6p (9) functionally substitute for Nyv1p \textit{in vivo} but somehow fail to do so \textit{in vitro}. Currently there is little or no evidence from \textit{in vivo} studies that Nyv1p plays important roles in either homotypic or heterotypic fusion processes. The strongest phenotypes of \textit{nyv1} mutants and \textit{NYV1}-overexpressing strains to date are those reported here involving effects on Pmc1p and Ca\(^{2+}\) homeostasis.

Pmc1p belongs to the family of Ca\(^{2+}\)-ATPases that includes the plasma membrane Ca\(^{2+}\)-ATPases (PMCA) of animals (3). The animal PMCA are localized almost exclusively to the plasma membrane and carry a C-terminal auto-inhibitory
extension that can be relieved upon binding of Ca^{2+}/calmodulin (31). The homologous proteins from fungi, plants, and protozoa apparently lack this C-terminal extension and are frequently localized to vacuoles or other intracellular organelles (32,33). How the non-animal PMCA-type Ca^{2+}-ATPases are regulated in vivo remains an interesting unanswered question. Sequences homologous to Nyv1p can be found in all the species containing PMCA-type proteins, raising the possibility that the interaction identified here occurs broadly in nature.

Ca^{2+} fluxes are well known to regulate heterotypic fusion events, such as the fusion of synaptic vesicles to the plasma membrane at synapses. In this case, the N-type and P/Q-type Ca^{2+} channels in the presynaptic plasma membrane bind the t-SNARE protein syntaxin and the s-SNARE SNAP-25, which can be bound to synaptotagmin and the v-SNARE synaptobrevin/VAMP located on docked synaptic vesicles (34). A second pool of synaptobrevin/VAMP in synaptic vesicles binds to synaptophysin, a polytopic membrane protein that is thought to sequester or buffer the v-SNARE from pairing with the t-SNARE proteins (35). The interaction between Pmc1p and Nyv1p in yeast vacuoles may resemble the interaction between synaptophysin and synaptobrevin/VAMP. Currently there is no evidence that synaptobrevin/VAMP interacts with PMCAs in neurons or any other cell type. However, a large fraction of total cellular synaptobrevin/VAMP has recently been localized to the active zones of the presynaptic membrane (36) where PMCAs also reside (37). The generality of the v-SNARE interactions with Ca^{2+}-ATPases in nature remains to be determined.

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1 The abbreviations used are: SNARE, SNAP receptor; WT, wild type; PMCA, plasma membrane Ca\(^{2+}\)-ATPase, SPCA, secretory pathway Ca\(^{2+}\)-ATPase; VGCC, voltage-gated Ca\(^{2+}\) channel.

FIGURE LEGENDS
**Figure 1. Nyv1p overexpression diminishes Pmc1p-dependent Ca\(^{2+}\) accumulation in the vacuole.** The non-exchangeable Ca\(^{2+}\) pool was determined in triplicate for a variety of yeast strains with or without overexpression of Nyv1p after long-term growth in YPD medium containing \(^{45}\)Ca\(^{2+}\) as a tracer (see Experimental Procedures). Relative to control (plasmid pYO325 [-]), overexpression of Nyv1p (plasmid pNYV-LEU [2µ-NYV]) significantly decreased the non-exchangeable Ca\(^{2+}\) pool in strains containing Pmc1p (wild type, strain K601; vcx1, K661; and vcx1 vam3, YTY51) strains but not in strains lacking Pmc1p (pmc1, K605; vcx1 pmc1, K665; and vcx1 vam3 pmc1, YTY52).

**Figure 2. Nyv1p diminishes Pmc1p-dependent Ca\(^{2+}\) tolerance.** Optical density was measured at 650 nm for cultures grown for 20h in either synthetic medium (A and B) or rich medium (C) as described in Experimental Procedures. Yeast strains analyzed were vcx1 (K661), vcx1 pmc1 (K665), vcx1 nyv1 (YTY5), and vcx1 pmc1 nyv1 (YTY7) harboring plasmids pYO323 [-], pNYV-HIS [2µ-NYV], pPI12 [2µ-VTI1], pSK60 [2µ-YKT6] as indicated.

**Figure 3. Pmc1p co-immunoprecipitates with Nyv1p.** (A) Polyclonal anti-Nyv1p antibodies were employed in native immunoprecipitation experiments after detergent solubilization of isolated vacuoles. Immunoprecipitates derived from the different yeast strains were then analyzed by SDS-page and western blotting using anti-HA monoclonal antibodies to detect Pmc1p-HA (upper panel) or rabbit anti-Nyv1p polyclonal antibodies (lower panel) In (B), the purified vacuoles were incubated for 0 to 30 minutes in conditions supporting homotypic fusion, then immunoprecipitated using either anti-Vam3p or anti-Nyv1p polyclonal antibodies and processed for western blotting using either anti-HA monoclonal antibodies to detect Pmc1p-HA (top two panels, light and dark exposure), anti-Vam3p, or anti-Nyv1p polyclonal antibodies.
Each panel was obtained from the same samples loaded on separate gels. Yeast strains used to isolate vacuoles were PMC1-HA (K699), nyv1 PMC1-HA (YTY25), wild type (K601), and nyv1 (YTY1) in (A) and PMC1-HA (K699) in (B).

Figure 4. Pmc1p levels are unchanged in nyv1, wild type, and Nyv1p-overexpressing strains. Total cell extracts were prepared and analyzed by SDS-PAGE and western blotting from strain nyv1 PMC1-HA (YTY25) (lane 1), PMC1-HA (K699 [pYO323]) (lane 2), PMC1-HA 2µ-NYV1 (K699 [pNYV-HIS]) (lane 3), and wild type (K601 [pYO323]) (lane 4) that had been grown to mid-log phase in SC medium. Note that the anti-Nyv1p antibodies cross-react with unknown proteins (*) independent of Nyv1p.

Figure 5. ATP-dependent Ca^{2+} transport activity of Pmc1p in purified vacuoles is inhibited by overexpressed Nyv1p. (A) Initial rates of ATP-dependent Ca^{2+} transport were measured at a variety of free Ca^{2+} concentrations using purified vacuoles from a vcx1 PMC1-HA strain (YTY28) carrying either a pYO323 [-] or pNYV-HIS [2µ-NYV1] as described in Experimental Procedures. The apparent V_{max} was calculated to be 28.8 and 12.5 pmol Ca^{2+}/min/mg, respectively, by non-linear regression using the Michaelis-Menten equation (solid and dashed lines). (B) The samples used in (A) were analyzed for Pmc1p-HA abundance by western blotting. Ten µl of the samples were loaded. A similar decrease in Pmc1p specific activity upon Nyv1p overexpression was observed in three independent experiments.

Figure 6. Vacuole morphology is unaffected by inactivation of Nyv1p or Pmc1p but disrupted by inactivation of Vam3p. Mid-log phase cells were stained with the membrane dye FM4-64 as described in experimental procedures then visualized by epifluorescence microscopy (panels a, c, e, and g) and phase contrast microscopy.
(panels b, d, f, and h). The imaged strains were wild type (K601), pmc1 mutant (K605), nyo1 mutant (YTY1), and vam3 mutant (YTY49).
**Table 1**

*Yeast proteins, functions, and homologs*

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<tr>
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<th>function</th>
<th>Mammalian homologs</th>
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Table 2

Yeast strains used in this study

All strains are isogenic to W303-1A (21) and carry additional markers: \textit{MATa} ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1.

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Fig. 1

Takita

Non-exchangeable Ca\(^{2+}\) (nmol / 10\(^9\) cells)

- WT
- pmc1
- vcx1
- vcx1 pmc1
- vcx1 vam3
- vcx1 vam3 pmc1

Vector - 2\(\mu\)-NYV1
Fig. 2

Takita

A

Growth (OD_{650})

CaCl_2 concentration (mM)

B

Growth (OD_{650})

CaCl_2 concentration (mM)

C

Growth (OD_{650})

CaCl_2 concentration (mM)
A

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B

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Fig. 3
Taking time (min)
Fig. 4
Takita

Pmc1p →

Nyv1p →

1 2 3 4
Fig. 5
Takita

A

Ca^{2+} transport activity (pmole/min/mg protein)

free Ca^{2+} (μM)

vcx1 [-]  
vcx1 [2μ-NYV1]

B

vcx1 [-]  
vcx1 [2μ-NYV1]

Pmc1p