Induction of vacuolar Ca^{2+}-ATPase and H^+/Ca^{2+} exchange activity in yeast mutants lacking Pmr1, the Golgi Ca^{2+}-ATPase

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Abstract **We have analyzed Ca^{2+} transport activity in defined subcellular fractions of an isogenic set of wild-type and mutant yeast. The results, together with measurements of polypeptide expression levels and promoter::reporter gene activity, show that the Golgi Ca^{2+}-ATPase, Pmr1, is the major Ca^{2+} pump under normal growth conditions. In the absence of Pmr1, we show a massive, calcineurin-dependent compensatory induction of the vacuolar Ca^{2+}-ATPase, Pmc1. In addition, H^+/Ca^{2+} exchange activity, that may be distinct from the vacuolar exchanger Vcx1, is also increased.**

Key words: Calcium homeostasis; Ca^{2+}-ATPase; *PMR1* gene; *PMC1* gene; H^+/Ca^{2+} exchanger; *Saccharomyces cerevisiae*

1. Introduction

In eukaryotic cells, complex and fine-tuned calcium signalling gives rise to diverse phenomena such as cell division, muscle contraction and gene transcription. A fundamental prerequisite for calcium signalling is the maintenance of submicromolar cytoplasmic calcium levels by an array of calcium pumps and exchangers, distributed at the cell surface or on intracellular membranes. In higher eukaryotes, cell and tissue-specific expression of different combinations of transport proteins, and their isoforms and splice variants, allows for differential regulation and control of calcium homeostasis. However, much remains to be learned about the individual contributions of these transport proteins and their roles in normal and diseased states. Thus, in Brody disease, the functional 'knockout' of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (*SERCA1*) gene encoding the fast twitch muscle isoform of the sarco/endoplasmic reticulum Ca^{2+}-ATPase, leads to impairments in skeletal muscle relaxation, stiffness and cramps [1,2]. In normal fast twitch muscle, *SERCA1* is believed to be essential for pumping back the Ca^{2+} released from the sarcoplasmic reticulum to initiate muscle relaxation, prior to commencing another cycle of excitation and contraction [3]. It is clear, however, that Brody patients have partially compensated for the loss of *SERCA1* since muscle relaxation does occur, albeit at a reduced rate. A variety of alternative mechanisms have been suggested to contribute to the compensatory removal of Ca^{2+} in the diseased cells. These include the upregulation of plasma membrane Ca^{2+}-ATPase (PMCA) or Na^+/Ca^{2+} exchangers, proliferation of reticular membranes containing other SERCA isoforms or refilling of stores through capacitative Ca^{2+} entry [4].

In the yeast *Saccharomyces cerevisiae*, the complete inventory of the genome and availability of sequence information, combined with a relative ease of genetic and biochemical manipulation, will ultimately allow the emergence of a comprehensive picture of the molecular mechanisms involved in cellular calcium regulation. Here, we describe a first step towards such an elucidation, by showing that calcium pumps and exchangers are differentially expressed or activated under different cellular conditions. Although previous reports have identified the individual calcium transporters that control cellular calcium levels [5–8], a systematic assay of calcium transport activity into defined subcellular fractions derived from an isogenic set of wild-type and mutant yeast has not previously been reported. Using such an approach, we arrive at the novel conclusion that Pmr1, the Golgi Ca^{2+}-ATPase [9–11], is the principal calcium pump expressed under normal growth conditions and that knockout of the *PMR1* gene results in large compensatory increases in the expression of a second calcium pump, Pmc1 [5], and in the activity of one or more H^+/Ca^{2+} exchangers.

2. Materials and methods

2.1. Media and strains

Cultures were grown in defined minimal media containing yeast nitrogen base (6.7 g/l, Difco), dextrose (2%) and supplements as needed. Where indicated, $CaCl_2$ was added to the media. All strains used in this study are isogenic, derived from W303-1A and are completely described elsewhere [5,6]. The following strains, with the relevant genotypes indicated, were used: K601 (wild-type), K605 (*pmc1::TRP1*), K610 (*pmr1::HIS3*), K612 (*pmr1::HIS3 cnb1::LEU2*), K616 (*pmr1::HIS3 pmc1::TRP1 cnb1::LEU2*), K693 (*pmr1::HIS3 vcx1Δ*). Strains K698 and K699 have the *PMC1* and *VCX1* genes tagged with the hemagglutinin epitope (HA), respectively [6], and were used to derive the isogenic *pmr1*::LEU2 disruption using the method of one-step gene disruption [12], with plasmid pAA106 [10].

2.2. β-Galactosidase assays

Construction of the *PMR1*, *PMC1* and *VCX1* promoter::*lacZ* reporter genes has been described earlier [6]. Plasmids pKC190, pKC199 and pKC200, carrying the above reporter gene constructs, were introduced separately into each of the yeast strains K601, K605 and K610 by lithium acetate transformation [13]. Assays of cellular β-galactosidase activity were performed essentially as described [14].

2.3. Cell fractionation and biochemical assays

Yeast cultures were grown to the late logarithmic stage, treated with yeast lytic enzyme (ICN Biomedicals) and lysed by Dounce homogenization in minimal volumes (3–6 ml) under hypotonic condi-

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HA, hemagglutinin

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tions, as described [11]. The clarified lysate was subjected to centrifugation on a 10-step sucrose gradient (18–54% w/w), exactly as described [11]. It is noteworthy that in order to maintain organellar integrity, membranes in the lysate were not subjected to high speed centrifugation prior to fractionation on the density gradient. The protein concentration in the fractions was determined by a modified Lowry assay [15], following precipitation of samples containing sucrose by 10% trichloroacetic acid and using bovine serum albumin as standard. Assays of α-mannosidase, NADPH cytochrome *c* reductase and GDPase have been previously described [11]. Plasma membrane ATPase activity was measured in buffer containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid/Tris, pH 6.0, 5 mM $MgCl_2$, 5 mM ATP, 5 mM NaN_3 and 50 mM KNO_3 in the presence and absence of 100 μM orthovanadate. Release of inorganic phosphate was quantitated as described [16]. ATP-dependent ^{45}Ca transport assays of sucrose gradient fractions and pooled Golgi membranes were done by rapid filtration, exactly as described previously [11]. ^{45}Ca was used at 0.4 μCi/ml (6.73 Ci/g, ICN Radiochemicals). Where indicated, bafilomycin A (Sigma, 10 nM) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (25 μM) were added to eliminate activity of vacuolar H^+/Ca^{2+} exchange.

2.4. Gel electrophoresis and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described previously [16]. Samples were prepared for electrophoresis by precipitating with trichloroacetic acid to a final concentration of 10% by volume, followed by microcentrifugation at 4°C, as described [11]. Antibodies against the C-terminal one-third of PMR1 have been described previously [11]. Monoclonal 12CA5 anti-HA antibodies were purchased from Boehringer Mannheim and used at a dilution of 1:5000.

3. Results

3.1. Differential expression of Ca^{2+} pump activity in yeast

We took a biochemical approach toward determining the number and distribution of Ca^{2+}-ATPases in yeast by assaying ATP-driven Ca^{2+} transport in defined subcellular fractions derived from an isogenic set of wild-type and mutant strains. Yeast lysates were separated on sucrose density gradients (18–54% w/w) and individual fractions were analyzed for the localization of organellar markers, as previously described [11]. Table 1 shows the peak distribution of markers for the vacuole (α-mannosidase), Golgi (GDPase), plasma membrane (vanadate-sensitive ATPase) and endoplasmic reticulum (NADH cytochrome *c* reductase), in a typical fractionation of a wild-type strain. H^+-driven Ca^{2+} transport was abolished by the addition of the protonophore CCCP and bafilomycin, a specific inhibitor of the vacuolar H^+-ATPase. This allowed for the determination of Ca^{2+} pump activity in the absence of H^+/Ca^{2+} exchange.

In fractions derived from wild-type yeast, we observed a broad peak of Ca^{2+} pump activity overlapping with the Golgi marker, followed by a trailing shoulder in the denser half of the gradient (Fig. 1A). Deletion of *PMC1*, a gene having a high level of identity with the mammalian PMCAs, reduces the activity found in the shoulder to levels indistinguishable from the background (absence of ATP, not shown). The Ca^{2+} transport activity remaining in the *Δpmc1* mutant was likely due to the Golgi-localized Pmr1 pump (Table 1) [10,11]. To verify this, it seemed reasonable to analyze a *pmr1* null mutant. Surprisingly, Ca^{2+} transport activity in the *Δpmr1* mutant was substantially increased in the denser half of the gradient, strongly pointing to the induction of one or more Ca^{2+}-ATPases (Fig. 1B). A similar induction of Ca^{2+} pump activity was observed upon addition of extracellular calcium (50–200 mM) to wild-type cells, indicating that the induced pump(s) played a role in calcium detoxification (Fig. 1B). Cunningham and Fink have shown a calcineurin-dependent induction of Pmc1 in response to extracellular calcium [5,6]. In Fig. 1C, we show that a null allele in the regulatory subunit of calcineurin (*CNB1*) prevents induction of Ca^{2+} pump activity in the *Δpmr1* null mutant. Since deletion of both *PMR1* and *PMC1* is lethal [5], we show that the viable triple mutant *Δpmr1Δpmc1Δcnb1*, in which H^+/Ca^{2+} exchange activity is activated ([5,6] and see ahead), is devoid of Ca^{2+} pump activity.

Thus, our data show that all of the observed Ca^{2+} pump activity in yeast is derived from the two Ca^{2+}-ATPases, Pmr1 and Pmc1. Expression of *PMC1* from the galactose-inducible *GAL1* promoter in a *Δpmc1* strain resulted in a substantial

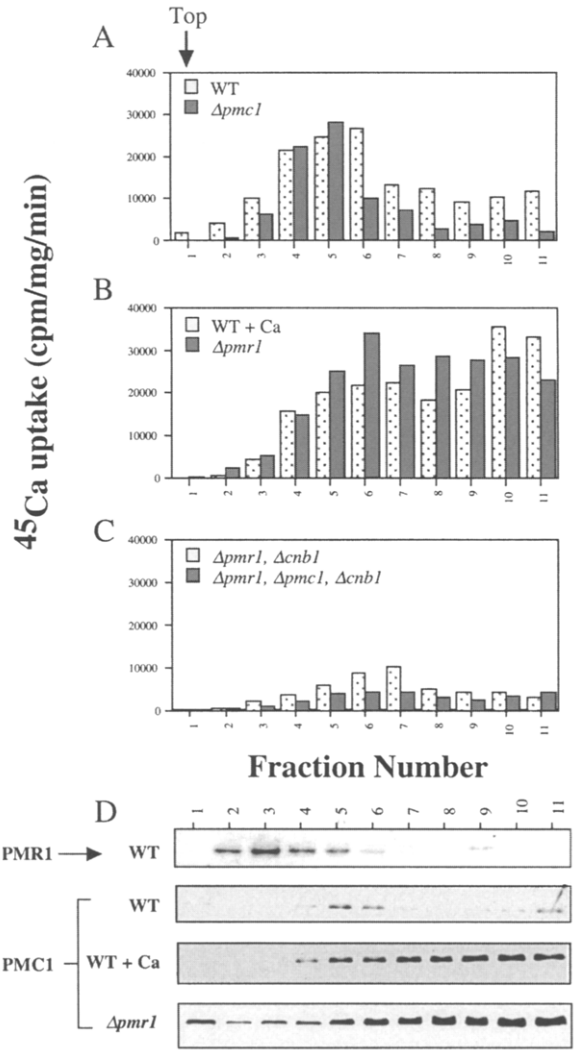

Fig. 1. Differential expression of Pmr1 and Pmc1 Ca^{2+}-ATPases in yeast. (A–C) ^{45}Ca transport was assayed in the presence of ATP and inhibitors of vacuolar H^+/Ca^{2+} exchange [11], in the individual fractions of a sucrose density gradient separation of yeast lysates from the strains shown. Where indicated, 50 mM $CaCl_2$ was added to the growth medium. *CNB1* is the regulatory subunit of the protein phosphatase calcineurin. Data are representatives of two or more independent experiments. (D) Western blots of individual gradient fractions from wild-type (*PMC1::HA*) or *Δpmr1(PMC1::HA)* strains, as indicated. 100 μg (for Pmr1) or 20 μg (for Pmc1) protein was separated by SDS-PAGE and the immunoblots were probed with antibodies against Pmr1 or HA epitope.

Table 1
Distribution of organelles on sucrose gradient fractionation of yeast lysates

Organelle	Marker enzyme	Peak fractions	Peak activity (% of total)
Vacuole	α-mannosidase	1–4	75%
Golgi	GDPase	2–5	62%
Endoplasmic reticulum	NADPH cytochrome *c* reductase	6–10	65%
Plasma membrane	vanadate-sensitive ATPase	6–9	63%

Individual fractions (1–11) from a sucrose density gradient separation of wild-type yeast lysates were assayed for organellar markers as described under Section 2. The distribution of the peak, in each case, is shown as the percentage of total activity over all fractions.

(ca. 300%) increase of Ca^{2+} pump activity, which was distributed in the same fractions observed in the *Δpmr1* strain (not shown). These results lead to the novel conclusion that Ca^{2+}-ATPase activity encoded by *PMC1* is induced in the absence of *PMR1*.

The distribution and expression levels of Pmr1 and Pmc1 polypeptides were examined by Western analysis of individual gradient fractions (Fig. 1D). Polyclonal antibodies raised against the C-terminal one-third of Pmr1 detected a 105 kDa band in Golgi-containing fractions, that was missing in the *Δpmr1* null strain [11]. A cross-reacting polypeptide of a slightly higher molecular weight found in plasma membrane-containing fractions corresponded to the Pma1 H^+-ATPase, as determined separately using Pma1-specific antibody (not shown). We showed earlier that overexpression of the *PMR1* gene from the strong heat shock-inducible promoter resulted in a substantial increase of the 105 kDa polypeptide in the Golgi-containing fractions [11]. Here, we show that chromosomally encoded HA-tagged Pmc1 was found in fractions corresponding to the trailing shoulder of Ca^{2+} pump activity. Addition of extracellular calcium (50–200 mM), or isogenic deletion of *PMR1*, led to a large increase in expression levels of Pmc1 in the denser half of the gradient (Fig. 1D). From this, we conclude that the two Ca^{2+}-ATPases, Pmr1 and Pmc1, are differentially expressed in yeast.

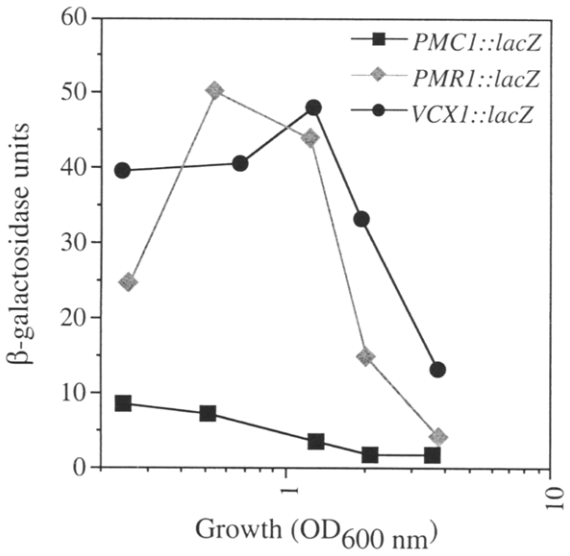

Fig. 2. Differential promoter activity of PMR1, PMC1 and VCX1 genes. β-Galactosidase activity was monitored in cultures of wild-type yeast transformed with the promoter::reporter constructs indicated, at different stages of growth. Averages of dupliate determinations are shown.

3.2. Activity of promoter-lacZ fusions from three Ca^{2+} transporters in yeast

Promoter activity of the two Ca^{2+}-ATPase genes, *PMR1* and *PMC1*, and the vacuolar H^+/Ca^{2+} exchanger *VCX1* was monitored using fusions with the reporter gene *lacZ*. As shown in Fig. 2, *lacZ* expression was found to be highly dependent on the growth phase of the culture, with sharp declines upon approach of the stationary phase. In wild-type cells, *PMR1* promoter activity increased during the early stages of growth to a peak at the mid-logarithmic phase, while *VCX1* promoter activity was maintained at high levels at all stages of growth, before declining in the stationary phase. In contrast, only low levels of *PMC1* promoter activity were observed, consistent with relatively low levels of Pmc1 expression and Ca^{2+}-ATPase activity (Fig. 1). Promoter activity of the *PMC1* and *VCX1* genes was also measured in the *Δpmr1* null mutant. Table 2 shows β-galactosidase activity measured at the late logarithmic stage in wild-type and *pmr1* mutant strains. There was a 5-fold increase in the promoter activity of the *PMC1* gene, consistent with the induction of Pmc1 expression and activity observed in the *pmr1* mutant. As in the wild-type, promoter activity was also dependent on the growth phase, peaking at the late logarithmic stage, followed by a decrease in the stationary phase (not shown). Interestingly, we observed a corresponding 5-fold decrease in expression from the *VCX1* promoter, relative to wild-type, in the *Δpmr1* strain.

3.3. H^+/Ca^{2+} exchange activity in wild-type and mutant yeast

Our initial observation that H^+/Ca^{2+} exchange activity was substantially higher in the triple mutant *Δpmr1Δpmc1Δcnb1* led to a systematic analysis of protonophore-sensitive Ca^{2+} transport activity in wild-type and isogenic mutant strains. Individual fractions from sucrose gradients were assayed for ATP-dependent ^{45}Ca accumulation in the presence and absence of CCCP and bafilomycin. A large increase in ΔpH-dependent Ca^{2+} transport was observed in the *pmr1* null mutant (Fig. 3A), but not in the *pmc1* null strain (not shown).

Table 2
Comparison of promoter activities of *PMR1*, *PMC1* and *VCX1*

Strain	Promoter::*lacZ*fusion	β-Galactosidase activity (U)
Wild-type	*PMR1*	43.8
	PMC1	3.6
	VCX1	48.2
Δpmr1	*PMC1*	17.9
	VCX1	10.1

The promoters of *PMC1*, *PMR1* and *VCX1* genes were fused to the lacZ reporter gene and expressed in wild-type or *ΔPMR1* strains. Yeast cultures were grown to the late logarithmic stage in minimal medium and β-galactosidase levels were assayed as described under Section 2. Averages of duplicate determinations are shown, which varied by $< 5\%$.

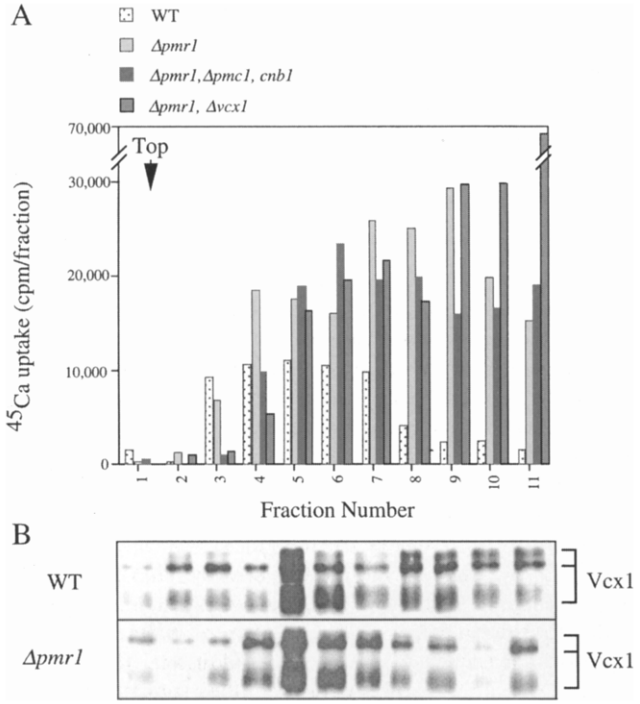

Fig. 3. H^+/Ca^{2+} exchange activity in wild-type and *Δpmr1* yeast. (A) H^+/Ca^{2+} exchange activity was determined as protonophore- and bafilomycin-sensitive ^{45}Ca transport, as described in the text, in individual fractions of sucrose density gradient separations of yeast lysates. Data are representatives of two or more independent experiments. (B) Western blots of 5 μg of gradient fractions from wild-type *(VCX1::HA)* and *Δpmr1(VCX1::HA)* yeast probed with anti-HA antibody.

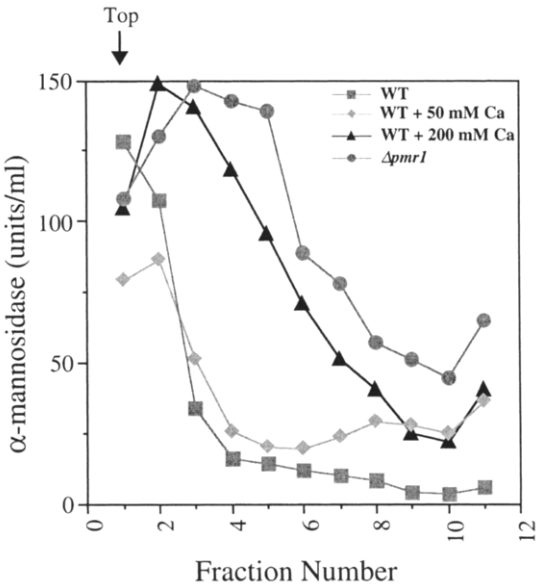

Fig. 4. Distribution of α-mannosidase activity on sucrose density gradients. Wild-type or *Δpmr1* strains of yeast were fractionated on sucrose density gradients and individual fractions analyzed for the vacuolar membrane marker α-mannosidase, as described under Section 2. Where indicated, 50 or 200 mM $CaCl_2$ was added to the culture.

The induced activity remained in the absence of both Pmr1 and Pmc1 pumps. In contrast to the induction of Pmc1, calcineurin was not required for the observed H^+/Ca^{2+} exchange activity. Previous studies have shown that Vcx1/Hum1 contributes to H^+/Ca^{2+} exchange in isolated vacuoles [7]. Measurements of Ca^{2+} tolerant growth and of non-exchangeable Ca^{2+} pools in intact cells have shown that calcineurin inhibits Vcx1 activity in vivo [6,7]. Thus, it would appear that although Vcx1 activity might account for the increased H^+/Ca^{2+} exchange activity in the *Δpmr1Δpmc1Δcnb1* triple mutant, the presence of activated calcineurin in the *Δpmr1* strain would be expected to inhibit Vcx1. This would suggest the existence of one or more additional H^+-dependent Ca^{2-} transport mechanisms in yeast that are induced in the absence of Pmr1. Alternatively, calcineurin-mediated inhibition of Vcx1 activity may be lost upon fractionation of cell membranes. In support of the former hypothesis, however, H^+/Ca^{2+} exchange activity remains high in a *pmr1vcx1* double null mutant (Fig. 3A).

Fig. 3B shows the distribution of chromosomally encoded, epitope-tagged Vcx1 protein in sucrose gradient fractionations of wild-type and *Δpmr1* strains, which was similar to the distribution of Pmc1. As expected from measurements of *VCX1* promoter activity, there was no increase in expression levels of HA-tagged Vcx1 in *Δpmr1* strains, rather, a small decrease in expression levels was noted.

3.4. Heterogeneous distribution of vacuolar markers in subcellular fractions of yeast

Both Vcx1 and Pmc1 have been localized by immunological methods to the vacuolar membrane in intact cells [5,6]. As shown in Table 1, the activity of the vacuolar membrane marker α-mannosidase peaked in the upper half of the sucrose gradient. Thus, the anomalous distribution of Ca^{2+} transport activity and immunoreactive Pmc1 and Vcx1 polypeptides in the denser half of the sucrose gradient warranted further study. We determined the distribution of various organellar markers in the different strains and culture conditions used in this study. With the exception of the vacuolar marker, we observed no significant change in the localization of organellar markers on the sucrose density gradients. Fig. 4 shows that there was a substantial redistribution of α-mannosidase to the denser sucrose gradient fractions upon induction of vacuolar Ca^{2+} transport. However, it is noteworthy that the bulk of the α-mannosidase marker remains separate from fractions showing peak Ca^{2+} transport activity. We also observed distribution of Vph1, a 100 kDa integral membrane subunit of the vacuolar H^+-ATPase, to be similar to that of Vcx1 and Pmc1 on Western blots (not shown). Taken together, these results are suggestive of transporter-rich subdomains of the vacuole, that are considerably denser than membranes containing α-mannosidase.

4. Discussion

4.1. On the number and identity of Ca^{2+}-ATPases in yeast

The number of potential Ca^{2+}-ATPases in yeast has been proposed to range from four or more, based on the biochemical assay of Ca^{2+} transport in subcellular fractions [17], to 11, based upon the number of P-type ATPase genes of unknown function [18]. On the other hand, only two genes have been definitively assigned a Ca^{2+}-ATPase function based on direct measurements of ATP-dependent Ca^{2+} transport activity, *PMR1* and *PMC1* [5,11]. Furthermore, genetic analysis of yeast mutants shows that the double knockout of the

PMR1 and *PMC1* genes is not viable, suggesting that these are the only two Ca^{2+}-ATPases in yeast [5]. Thus, a genetic screen for yeast genes which, upon overexpression, could compensate for the failure of *pmr1* mutants to grow on Ca^{2+}-deficient media yielded *PMC1* [19]. However, genetic screens can identify candidate proteins that do not directly participate in calcium transport. For example, overexpression of Vps10, a receptor for vacuolar sorting, can also suppress some *pmr1* phenotypes [19]. Therefore, a direct analysis of Ca^{2+} transport properties in membranes derived from wild-type and mutant yeast seemed worthwhile. We observe that all of the ATP-dependent, protonophore-insensitive calcium transport activity measured in yeast can be accounted for by the Pmr1 and Pmc1 ATPases. Thus, yeast differs from mammalian cells in the absence of specialized Ca^{2+}-ATPases in the endoplasmic reticulum. One interpretation is that the latter may have arisen later in evolution. In earlier work [11], we have predicted the widespread distribution of Pmr1 homologues, suggesting that the Golgi Ca^{2+}-ATPases constitute a separate and possibly ancient class of calcium pumps. We now note the emergence of numerous Pmr1 homologues in human, rodent, worm and other fungi in the DNA sequence databases. Curiously, the sequence similarity between Pmc1 and the mammalian plasma membrane Ca^{2+}-ATPases suggests that sequestration of calcium within the yeast vacuole is topologically equivalent to transport into the extracellular milieu.

4.2. *Pmr1 is the principal Ca^{2+}-ATPase in yeast whereas Pmc1 is induced upon calcium overload*

Phenotypic observations of *pmr1* and *pmc1* mutants led to the recognition that the roles of Pmr1 and Pmc1 in calcium homeostasis are not equivalent. Thus, cells lacking Pmr1 cannot grow under low Ca^{2+} conditions and have a 4–5-fold elevation of cellular Ca^{2+} levels [8], while the *Δpmc1* strain is sensitive to high environmental Ca^{2+} and has cellular Ca^{2+} levels 2–3-fold lower than normal [5]. Because the vacuole is the primary reservoir (>90%) of stored Ca^{2+} in yeast, it would appear that vacuolar Ca^{2+} transporters dominate over others in maintaining cellular Ca^{2+} homeostasis. Indirect evidence for the homeostatic role of the Golgi Ca^{2+}-ATPase came from observations that in a vacuolar biogenesis (*Δvps33*) mutant, deletion of *PMR1* resulted in hypersensitivity to extracellular Ca^{2+} [20]. Here, we show by measurements of promoter activity, polypeptide expression levels and Ca^{2+}-ATPase activity that Pmr1 is the principal Ca^{2+}-ATPase under normal growth conditions. In *pmr1* mutant cells, Halachmi and Eilam [8] observed a much higher rate of cellular Ca^{2+} uptake, accompanied by a massive accumulation of Ca^{2+} within an intracellular store, which was proposed to be the vacuole. Our evidence for a large induction of the vacuolar Ca^{2+} pump, Pmc1, in the *pmr1* mutant provides the molecular basis for these observations. We propose that in wild-type cells, Ca^{2+} transport into the Golgi via Pmr1 is the primary route for Ca^{2+} exit. Thus, Ca^{2+} efflux rates in a *pmr1* mutant are extremely low [8], likely contributing to increased cytoplasmic Ca^{2+} levels. Ca^{2+}/calcineurin-dependent induction of Pmc1 in the *pmr1* mutant, together with an increased activity of one or more H^+/Ca^{2+} exchangers, results in a greatly increased uptake into the vacuolar compartment. The increased partitioning of Ca^{2+} into an intracellular organelle would contribute to the net increase in cellular Ca^{2+} influx observed [8]. In extremely high extracellular Ca^{2+} (>100 mM), the capacity of the Golgi efflux mechanism would be exceeded and cytoplasmic Ca^{2+} would rise. Under these conditions, calcium homeostasis is restored via calcineurin-dependent transcriptional induction of Pmc1 [6], resulting in the elevated Ca^{2+} transport activity described here.

4.3. *H^+/Ca^{2+} exchange activity is induced in the absence of Pmr1*

Analysis of ATP-dependent Ca^{2+} transport activity that was sensitive to protonophores and inhibitors of the vacuolar H^+ pump provided a measure of H^+/Ca^{2+} exchange activity. Here, we show a large increase in H^+/Ca^{2+} exchange activity in the *Δpmr1* mutant, similar in distribution to Pmc1-mediated protonophore-independent Ca^{2+} pump activity. There was no concomitant increase in expression levels of Vcx1, the vacuolar H^+/Ca^{2+} exchanger, in the *Δpmr1* mutant, rather, a decrease in *VCX1* promoter activity was observed. Furthermore, the presence of activated calcineurin in the *Δpmr1* mutant would be expected to inhibit the activity of Vcx1 in vivo [6,7]. Finally, H^+/Ca^{2+} exchange activity remained elevated in the double mutant *Δpmr1Δvcx1*. Taken together, our data suggest the existence of one or more additional H^+/Ca^{2+} exchangers that remain to be identified. One candidate is a gene, YNL321w, which has a 22% identity with Vcx1 in the C-terminal half of its predicted polypeptide sequence.

4.4. *Increased density of transporter-enriched domains of the vacuolar membrane*

The anomalous distribution of vacuolar Ca^{2+} transport to denser fractions of the sucrose gradient, away from the bulk of the vacuolar membrane marker, α-mannosidase, is noteworthy. Interestingly, we observed an increased distribution of α-mannosidase in the denser gradient fractions with induction of vacuolar Ca^{2+} transport, resulting in some degree of colocalization of the vacuolar marker with Pmc1 and Vcx1. The data suggest the existence of subdomains of vacuolar membrane that are enriched in vacuolar transport proteins. Membrane vesicles derived from such subdomains might have different densities, possibly due to accumulation of Ca^{2+} and other ions, resulting in different migration properties on the density gradient. Such a hypothesis is not without precedence: plant cells have been shown to contain two functionally distinct vacuolar compartments, separately specialized for storage of proteins and for acid-activated hydrolytic enzymes [21]. The proposed heterogeneity of the vacuolar compartment in yeast warrants further study.

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