Yeast Cls2p/Csg2p localized on the endoplasmic reticulum membrane regulates a non-exchangeable intracellular Ca^{2+} pool cooperatively with calcineurin

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Abstract ***Saccharomyces cerevisiae CLS2*** **gene product (Cls2p) that is localized on the endoplasmic reticulum is important for the regulation of intracellular Ca^{2+} in a compartment distinct from the vacuole. Using a *vma3* mutation that impairs the Ca^{2+} sequestering activity into the vacuole, we have shown that the *cls2* mutation results in 3.4-fold increase in the Ca^{2+} pool that is not exchangeable with extracellular Ca^{2+}. Accumulation of Ca^{2+} within the *cls2* cells is synergistically elevated by the addition of immunosuppressant, FK506. Moreover, in the *vma3* background, toxicity caused by the *cls2* mutation is greatly enhanced by FK506. Given that FK506 inhibits the calcineurin activity, Cls2p likely functions in releasing Ca^{2+} flux from the endoplasmic reticulum, somehow cooperating with calcineurin.**

Key words: Calcium homeostasis; *CLS2*; FK506; Calcineurin; Endoplasmic reticulum; *vma* mutant

1. Introduction

In the budding yeast *Saccharomyces cerevisiae*, Ca^{2+} is essential for cell growth [1,2]. In order to understand the cellular Ca^{2+} function and/or Ca^{2+} homeostasis in this organism, Ohya et al. (1986) isolated a number of Ca^{2+}-sensitive mutants (*cls*; calcium sensitive) that were unable to grow in the presence of 100 mM $CaCl_2$ [3]. Based on calcium contents of whole cells and initial Ca^{2+} uptake activities, the mutants were classified into four types. Among 18 complementation groups of the mutants, type III *cls* mutants (*cls1, cls2,* and *cls3* mutant) contain elevated calcium contents but exhibit normal initial rates of the Ca^{2+} uptake. Molecular analysis of one of the type III mutants have shown that *CLS2* encodes a hydrophobic polypeptide of the 410 amino acid residues [4]. The *cls2* mutants were recently isolated by another group, and also referred to *csg2* [5]. In the presence of high concentration of Ca^{2+} (10–50 mM $CaCl_2$), the *cls2/csg2* mutant cells accumulate a much higher amount of Ca^{2+} presumably in a non-vacuolar compartment [5]. This is consistent with our observation that epitope-tagged Cls2p/Csg2p is predominantly localized on the ER membrane [4].

Type IV *cls* mutants, including *cls7, cls8, cls9, cls10,* and *cls11* exhibit elevated initial rates of the Ca^{2+} uptake [3]. Molecular/biochemical analysis of the type IV *cls* mutants revealed that the mutations resulted in the lesion of the subunits or the assembly factors of the vacuolar membrane H^+-ATPase, and were therefore renamed as *vma* (vacuolar membrane ATPase) (*cls7* = *vma3*, *cls8* = *vma1*, *cls9* = *vma11*, *cls10* = *vma12* and *cls11* = *vma13*) [6]. *VMA3, VMA1, VMA11* and *VMA13* encode the 17 kDa, 69 kDa, 17 kDa and 54 kDa subunits, respectively, and *VMA12* encodes an assembly factor [6–9]. All of the *vma* mutants impair generation of the proton motive force across the vacuolar membrane, and are unable to sequester Ca^{2+} into the vacuole, a major Ca^{2+} pool in yeast cells [10–17]. Hence, the amount of Ca^{2+} in the total cellular compartments in *cls7/vma3* cells is dramatically decreased [6,18].

Recent study has indicated that the *vma* mutants are more sensitive than the wild-type strain to the immunosuppressants, FK506 and cyclosporin A (CsA) [18]. The *vma* mutants do not grow on YPD plates containing 1 μg/ml FK506 or 50 μg/ml CsA, although the wild-type strain can grow on the same plates. Evidence has been accumulated in yeast and higher eukaryotic cells that both FK506 and CsA inhibit the activity of a Ca^{2+}/calmodulin dependent phosphoprotein phosphatase, calcineurin, and that the inhibition by the drugs depends on its own cytosolic receptors, FKBP-12 (Fkb1p) and Cyp-18 (Cyp1p) [19–22]. We found that inhibition of the calcineurin activity results in increase of a non-exchangeable (compartmentalized) Ca^{2+} pool, which is distinct from the vacuole, causing decrease of $[Ca^{2+}]_c$ in *cls7/vma3* cells [18]. This result indicates that calcineurin controls intracellular calcium homeostasis by somehow repressing Ca^{2+} flux into a non-vacuolar component.

We have noticed the coincidence that both Cls2p/Csg2p and calcineurin regulates a non-vacuolar compartmentalized Ca^{2+} pool. In this study we present a synergistic relationship between the function of calcineurin and Cls2p/Csg2p in regulating the intracellular Ca^{2+} homeostasis of yeast cells.

2. Materials and methods

2.1. Yeast strains, media and growth conditions

YPD medium contained 1% (w/v) Bacto-yeast extract (Difco), 2% (w/v) Bacto-peptone, and 2% (w/v) glucose. YPD pH 5.0 medium was buffered with 50 mM succinate/NaOH at pH 5.0. FK506 (0.1–1 μg/ml) and $CaCl_2$ (1–100 mM) were supplemented in the medium when indicated in the text. The SD media for auxotroph selection were described in Sherman et al. [23].

Strains used in this study are listed in Table 1. All yeast strains used were derivatives of YPH499, YPH500 or YPH501 [24], and constructed by transformation using the lithium acetate method or by standard genetic crosses. For the disruption of the *CLS2* gene, pYT24 [4] was linearized with *Bam*HI and used to transform haploid strains, YPH499

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Abbreviations: ER, endoplasmic reticulum; Cls2p, a *CLS2* gene product; FKBP-12, FK506 binding protein; CsA, Cyclosporin A; Cyp-18, cyclophilin A; YPD, 1% yeast extract–2% peptone–2% dextrose; $[Ca^{2+}]_c$, intracellular free Ca^{2+} concentration.

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and YPH500, to construct *cls2* null mutants, DCLS2-A1 and DCLS2-B1, respectively as described in Takita et al. [4].

2.2. Recombinant DNA

All procedures for recombinant DNA were carried out with *Escherichia coli* strain XL1-blue (Stratagene) grown in Luria Broth medium with appropriate antibiotics [25]. pBluescriptII[KS+] was bought from Stratagene.

2.3. Measurement of exchangeable and non-exchangeable Ca^{2+} pools

Measurement of cellular Ca^{2+} pools was described in Tanida et al. [18]. During this experiment, we monitored the cell viability of the *vma3* mutant and the *cls2 vma3* double mutant in the presence of FK506. When *vma3* and *cls2 vma3* cells were cultured in YPD pH 5.0 medium containing 1 μg/ml FK506 at 30°C for 8 h, more than 95% of the cells was still viable.

3. Results

A previous report has indicated that when the *cls2/csg2* cells are exposed to high concentration of extracellular Ca^{2+} (10–50 mM), the cells accumulate higher levels of intracellular Ca^{2+} that is exchangeable with extracellular Ca^{2+} [5]. Our study was undertaken in order to investigate the function of Cls2p/Csg2p under a normal, relatively low Ca^{2+} condition (c.a. 200 μM Ca^{2+} included in YPD pH 5.0 medium). When the wild-type cells were cultured in YPD pH 5.0 medium, most (91%) of the Ca^{2+} pool was non-exchangeable with extracellular Ca^{2+}, largely representing the vacuolar Ca^{2+} pool (Fig. 1). The *vma3* cells, instead, contained the dramatically reduced non-exchangeable Ca^{2+} pool (13% compared with the wild-type control), because the *vma3* mutant impaired the Ca^{2+} sequestering activity into the vacuole. In this strain background, the *cls2/csg2* mutation resulted in 3.4 fold increase in the non-exchangeable Ca^{2+} pool (Fig. 1), suggesting that Cls2p/Csg2p regulates the non-exchangeable Ca^{2+} pool under the normal condition. The *cls2/csg2* mutation alone slightly affected the non-exchangeable Ca^{2+} pool, probably due to the fact that high Ca^{2+}-sequestering activity of the vacuole decreased the difference.

Using compounds (FK506 and cyclosporin A) that inhibit the calcineurin activity in yeast cells, we have shown that calcineurin regulates a non-exchangeable Ca^{2+} pool, which is distinct from the vacuole [18]. In order to examine whether calcineurin regulates the same Ca^{2+} pool as the one affected by the

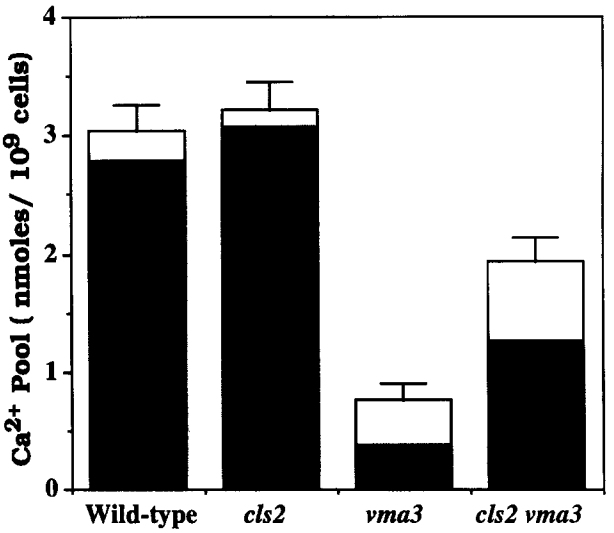

Fig. 1. Ca^{2+} compartmentalization in the wild-type, *cls2*, *vma3* and *cls2 vma3* strains. The exchangeable (open box) and non-exchangeable (*solid box*) pools of cell-associated Ca^{2+} were measured in strains YPH499 (Wild-type), DCLS2-A1 (*cls2*), DV3T-A (*vma3*), and DV3DCLS2-1 (*cls2 vma3*) cultured in YPD pH 5.0 medium at 30°C. Bars indicate the variation of individual values of the total Ca^{2+} pools from the mean (n = 2).

cls2/csg2 mutation, we tested the sensitivity of the *cls2 vma3* cells to FK506. The theory of this experiment is that if calcineurin and Cls2p/Csg2 cooperatively regulates the same Ca^{2+} compartments, inhibition of the calcineurin activity would cause synergistic effects on the *cls2 vma3* cells. Here we used the *cls2 vma3* double mutant, because phenotype of *cls2/csg2* was enhanced in the *vma3* background (Fig. 1). We found that even a small amount of FK506 (0.1 μg/ml) inhibited the growth of the *cls2 vma3* double mutant (Fig. 2). The control *vma3* cells were still able to grow in the presence of the same concentration of FK506. Binding of FK506 to FKBP-12 (Fkb1p) results in inhibition of calcineurin activity [19,20]. In order to confirm whether binding of the drug to FKBP-12 results in the growth inhibition in the *cls2 vma3* cells, we constructed the *cls2 vma3 fkb1* triple mutant lacking FKBP-12. We found that the *cls2*

Table 1
List of yeast strains

Strains	Genotype	Source
YPH499	*MATa leu2 ade2 lys2 his3 trp1 ura3*	Sikorski and Hieter (1989)
YPH500	*MATα leu2 ade2 lys2 his3 trp1 ura3*	Sikorski and Hieter (1989)
DV3T-A	*MATa leu2 ade2 lys2 his3 trp1 ura3 vma3::TRP1*	Tanida et al. (1995)
DV3T-B	*MATα leu2 ade2 lys2 his3 trp1 ura3 vma3::TRP1*	Tanida et al. (1995)
DCLS2-A1	*MATa leu2 ade2 lys2 his3 trp1 ura3 cls2::LEU2*	This study[a]
DCLS2-B1	*MATα leu2 ade2 lys2 his3 trp1 ura3 cls2::LEU2*	This study[a]
DF1	*MATα leu2 ade2 lys2 his3 trp1 ura3 fkb1::URA3*	Tanida et al. (1995)
DV3C1-11C	*MATa leu2 ade2 lys2 his3 trp1 ura3 vma3::TRP1 cyp1::NEO*	Tanida et al. (1995)
DV3DCLS2-1	*MATa leu2 ade2 lys2 his3 trp1 ura3 vms3::TRP1 cls2::LEU2*	This study[b]
DV3DCLS2-2	*MATa leu2 ade2 lys2 his3 trp1 ura3 vma3::TRP1 cls2::LEU2*	This study[b]
DV3DCLS2DF1-1	*MATa leu2 ade2 lys2 his3 trp1 ura3 vma3::TRP1 cls2::LEU2 fkb1::URA3*	This study[c]
DV3DCLS2DF1-2	*MATa leu2 ade2 lys2 his3 trp1 ura3 vma3::TRP1 cls2::LEU2 fkb1::URA3*	This study[c]
DV3DCLS2DC1-1	*MATa leu2 ade2 lys2 his3 trp1 ura3 vma3::TRP1 cls2::LEU2 cyp1::NEO*	This study[d]
DV3DCLS2DC1-2	*MATa leu2 ade2 lys2 his3 trp1 ura3 vma3::TRP1 cls2::LEU2 cyp1::NEO*	This study[d]

[a] This strain was constructed as described in section 2.
[b] One of the segregants from DCLS2-A1 × DV3T-B.
[c] One of the segregants from DV3DCLS2-1 × DF1.
[d] One of the segregants from DV3C1-11C × DCLS2-B1.

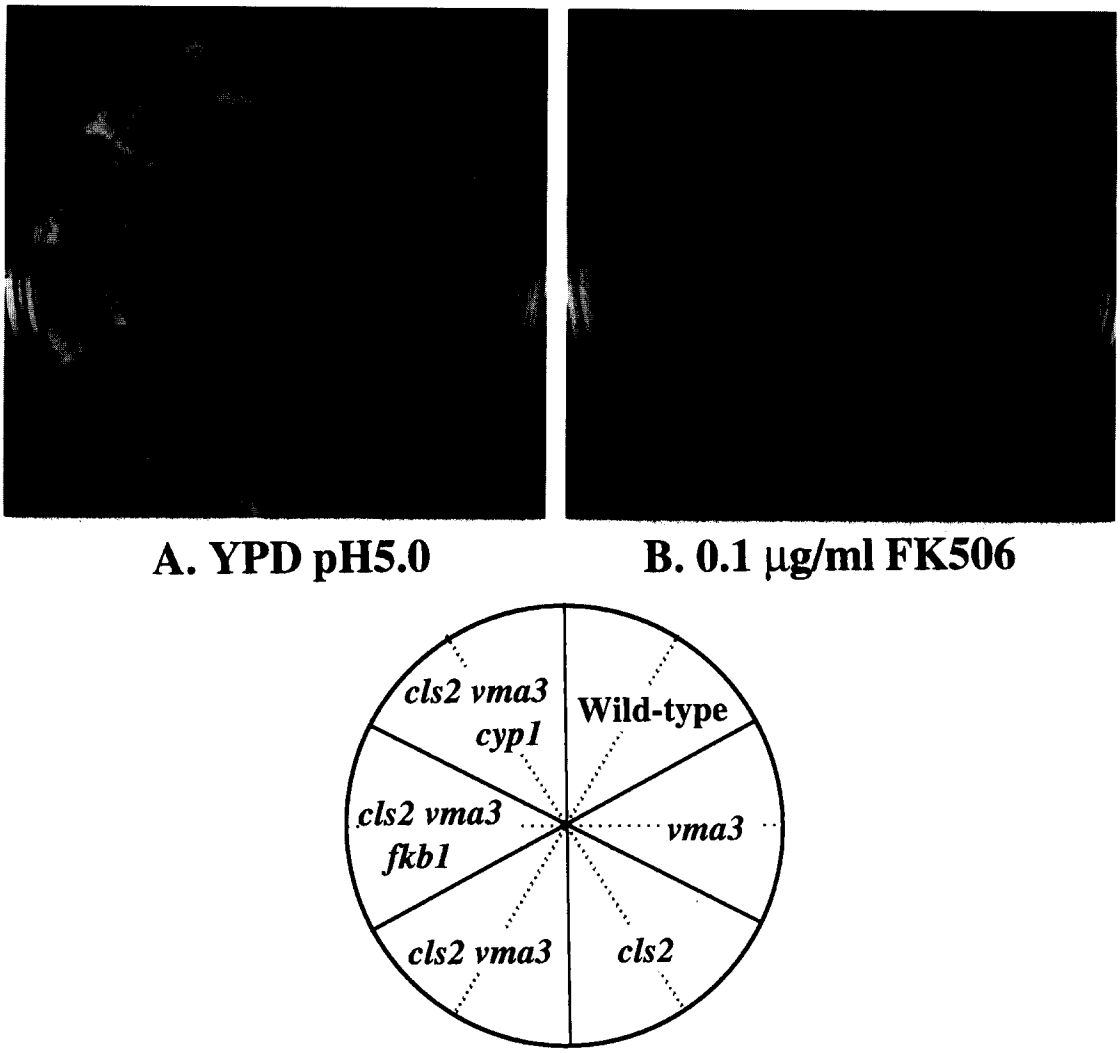

Fig. 2. FK506 causes a severe growth defect of the *cls2 vma3* mutant. Yeast strains were spread onto the surface of YPD pH 5.0 agar medium (A) or YPD pH 5.0 agar medium with 0.1 μg/ml FK506, and incubated at 30°C for 3 days. Strains clockwise from the top were YPH499 (Wild-type), YPH500 (Wild-type), DV3T-A (*vma3*), DV3T-B (*vma3*), DCLS2-A1 (*cls2*), DCLS2-B1 (*cls2*), DV3DCLS2-1 (*cls2 vma3*), DV3DCLS2-2 (*cls2 vma3*), DV3DCLS2DF1-1 (*cls2 vma3 fkb1*), DV3DCLS2DF1-2 (*cls2 vma3 fkb1*), DV3DCLS2DC1-1 (*cls2 vma3 cyp1*), DV3DCLS2DC1-2 (*cls2 vma3 cyp1*).

vma3 fkb1 triple mutant grew in the presence of 0.1 μg/ml FK506. The effect of CsA-Cyp-18 complex on the growth of the *cls2 vma3* mutant was similar to the case of FK506 (data not shown). Together with the fact that FK506-FKBP-12 complex inhibits calcineurin activity in yeast cells, we concluded that inhibition of the calcineurin activity results in a severe growth defect of the *cls2 vma3* mutant.

Since significant increase of the non-exchangeable Ca^{2+} pool may account for the severe growth defect of the *cls2 vma3* mutant, we examined the effect of FK506 on cellular Ca^{2+} pools in the mutant cells. Fig. 3 shows that addition of 0.1 μg/ml FK506 resulted in 6-fold increase of the non-exchangeable Ca^{2+} pool of the *cls2 vma3* cells: 1.0 μg/ml FK506 had more pronounced effect. FK506 had no effect on the Ca^{2+} pool of the *cls2 vma3 fkb1* cells lacking FKBP-12, confirming that formation of FK506-FKBP-12 was required for elevation of a Ca^{2+} pool. Under these conditions, no quinacrine accumulation into the vacuoles of the *cls2 vma3* cells was observed (data not shown), indicating that vacuolar acidification no longer occurred in the *cls2 vma3* mutant. The synergistic effect on the non-exchangeable Ca^{2+} pool in the *VMA* cells was also observed. Addition of 1 μg/ml FK506 induced a dramatic increase in the non-exchangeable Ca^{2+} pool of the *cls2* single mutant, being 2.2 fold higher than that of the wild-type under the same condition (Fig. 3). These results suggested that both Cls2p and calcineurin synergistically control the non-exchangeable Ca^{2+} pool probably in the ER.

4. Discussion

In the present study, we show evidence suggesting that Cls2p regulates the non-vacuolar compartmentalized Ca^{2+} pool of the ER under the low Ca^{2+} condition. This is consistent with the previous report that Ca^{2+} is accumulated in the non-vacuolar organelle when the cells are exposed in 10–50 mM Ca^{2+} [5]. Cls2p is a hydrophobic protein localized on the ER membrane

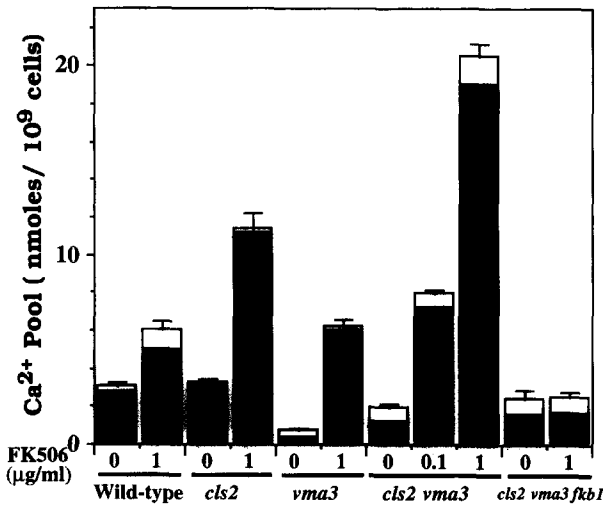

Fig. 3. The effect of FK506 on the cellular Ca^{2+} pools in yeast cells. The exchangeable (open box) and non-exchangeable (solid box) pools of cell-associated Ca^{2+} were measured in strains cultured in YPD pH 5.0 medium at 30°C without FK506 (0), with 0.1 μg/ml (0.1) or 1 μg/ml FK506 (1). Strains indicated were YPH499 (Wild-type), DCLS2-A1 (*cls2*), DV3T-A (*vma3*), DV3DCLS2-1 (*cls2 vma3*), and DV3DCLS2DF1-1 (*cls2 vma3 fkb1*). Bars indicate the variation of individual values of the total Ca^{2+} pools from the mean (n = 2).

with a putative Ca^{2+} binding site possibly exposed to the cytoplasmic side [4,5]. Although Cls2p/Csg2p is not a homolog of Ca^{2+}-ATPase or calcium channel such as the ryanodine-receptor or the IP_3-receptor in mammalian cells [26–34], Cls2p/Csg2p likely possess an important function in Ca^{2+} homeostasis of the ER.

The *cls2/csg2* mutation and inhibition of calcineurin activity synergistically increased the intracellular non-exchangeable Ca^{2+} pool. Even if Cls2p/Csg2p and calcineurin independently control the Ca^{2+}-flux into the different organelle, the increase of the non-exchangeable Ca^{2+} pool might be additive. In consideration with the synergistic growth inhibition, we propose a model as shown in Fig. 4. In this model, Cls2p functions in releasing Ca^{2+} from the lumen of the ER. When the Ca^{2+} efflux from the ER is blocked by the *cls2* mutation, the Ca^{2+} pool in the ER increased. The *cls2* mutant is not able to grow in the presence of high concentration (100 mM) of $CaCl_2$, probably due to the extraordinary high concentration of Ca^{2+} in the ER. In this model, calcineurin also regulates the Ca^{2+} pool in the ER by repressing Ca^{2+} influx into the lumenal space. Inhibition of calcineurin activity by addition of 0.1 μg/ml FK506 induced further increase of the compartmentalized Ca^{2+} pool. In the consequence, the *cls2 vma3* double mutant becomes more sensitive to immunosuppressants than the *vma3* single mutant. In this model, however, we do not exclude the possibilty that calcineurin also regulates the Ca^{2+} pool in the other non-vacuolar compartment(s). At any rate, since calcineurin is a cytosolic protein complex, there must be a target protein of calcineurin on the ER membrane. Genetic study is now under way in our laboratory to identify the target protein(s) involved in Ca^{2+} flux/homeostasis of the ER.

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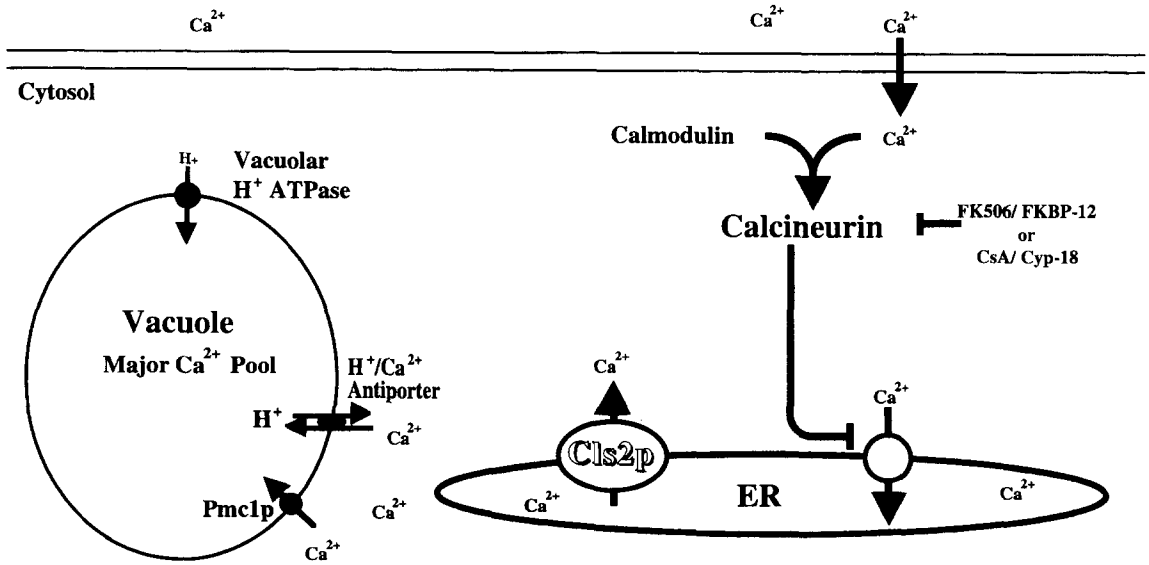

Fig. 4. A working model for the function of Cls2p and calcineurin in yeast intracellular Ca^{2+} homeostasis. It is believed that cytosolic free Ca^{2+} is mainly sequestered into the vacuole by a Ca^{2+}/H^+ antiporter [11] and Pmc1p, a homolog of SERCA family Ca^{2+}-ATPase localized on the vacuole [35]. Involvement of the ER in the yeast intracellular Ca^{2+} homeostasis is discussed in the text.

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