Calcineurin-dependent Growth Control in *Saccharomyces cerevisiae* Mutants Lacking *PMC1*, a Homolog of Plasma Membrane Ca^{2+} ATPases

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Abstract. Ca^{2+} ATPases deplete the cytosol of Ca^{2+} ions and are crucial to cellular Ca^{2+} homeostasis. The *PMC1* gene of *Saccharomyces cerevisiae* encodes a vacuole membrane protein that is 40% identical to the plasma membrane Ca^{2+} ATPases (PMCAs) of mammalian cells. Mutants lacking *PMC1* grow well in standard media, but sequester Ca^{2+} into the vacuole at 20% of the wild-type levels. *pmc1* null mutants fail to grow in media containing high levels of Ca^{2+}, suggesting a role of *PMC1* in Ca^{2+} tolerance. The growth inhibitory effect of added Ca^{2+} requires activation of calcineurin, a Ca^{2+} and calmodulin-dependent protein phosphatase. Mutations in calcineurin A or B subunits or the inhibitory compounds FK506 and cyclosporin A restore growth of *pmc1* mutants in high Ca^{2+} media. Also, growth is restored by recessive mutations that inactivate the high-affinity Ca^{2+}-binding sites in calmodulin. This mutant calmodulin has apparently lost the ability to activate calcineurin in vivo. These results suggest that activation of calcineurin by Ca^{2+} and calmodulin can negatively affect yeast growth. A second Ca^{2+} ATPase homolog encoded by the *PMR1* gene acts together with *PMC1* to prevent lethal activation of calcineurin even in standard (low Ca^{2+}) conditions. We propose that these Ca^{2+} ATPase homologs are essential in yeast to deplete the cytosol of Ca^{2+} ions which, at elevated concentrations, inhibits yeast growth through inappropriate activation of calcineurin.

Ca^{2+} plays a key role in the transduction of external signals through the cytoplasm of eukaryotic cells. Fluctuations in the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]c$,[1] directly elicit a cellular response by altering the function of Ca^{2+}-binding proteins and their targets. Resting cells maintain $[Ca^{2+}]c$ at very low levels against large gradients of compartmentalized and extracellular Ca^{2+}. A variety of stimuli can trigger the opening of Ca^{2+}-specific channels in plasma membrane or endoplasmic reticulum causing massive Ca^{2+} influx and accumulation in the cytoplasm. After stimulation, the basal $[Ca^{2+}]c$ levels are restored by Ca^{2+} ATPases and antiporters that transport Ca^{2+} from the cytoplasm through the plasma membrane and several internal membranes. To regulate $[Ca^{2+}]c$ and effect the appropriate responses to Ca^{2+} signals, cells utilize a wide array of ion transporters and sensory factors.

Ca^{2+} signaling plays an important role in the activation of T cells (Gardner, 1989). Binding of antigens to specific receptors at the surface of quiescent T cells triggers the opening of Ca^{2+} channels in the endoplasmic reticulum and plasma membrane, leading to rapid elevation in $[Ca^{2+}]c$. The rise in $[Ca^{2+}]c$ is necessary for induction of many genes including IL-2 which produces an autocrine factor required for T cell proliferation. Ca^{2+} dependent transcription is blocked by the immunosuppressive drugs cyclosporin A and FK506 that act together with their respective binding proteins, cyclophilin and FKBP-12, as potent inhibitors of calcineurin (Liu et al., 1991*a*). A direct role for calcineurin in IL-2 expression is supported by the observation that overproduction of calcineurin partially bypasses the requirement for elevated $[Ca^{2+}]c$ and decreases the effectiveness of FK506 and cyclosporin A (Clipstone and Crabtree, 1992; O'Keefe et al., 1992). These findings strongly suggest that Ca^{2+}-dependent activation of calcineurin is a crucial step in the activation of T cells.

Recent reports have extended the range of cell types and species that respond to these drugs, suggesting that calcineurin may be a widespread component of Ca^{2+}-signaling mechanisms. Studies in the budding yeast *Saccharomyces cerevisiae* indicate that calcineurin is involved in the response to mating pheromone. Yeast cells respond to mating pheromones by arresting cell cycle progression transiently in G1 phase and inducing expression of many genes involved in conjugation. Cells that have not mated after prolonged pheromone exposure can recover and proliferate as long as sufficient Ca^{2+} is supplied in the medium (Iida et al., 1990). Recovery is inefficient in mutants lacking calcineurin activity (Cyert et al., 1991; Cyert and Thorner, 1992). The recovery defect of calcineurin mutants can be mimicked in

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1. *Abbreviations used in this paper*: $[Ca^{2+}]c$, cystolic free Ca^{2+} concentration; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; PMCA, plasma membrane Ca^{2+} ATPase.

wild-type strains by addition of cyclosporin A or FK506 (Foor et al., 1992). Although calcineurin is required for recovery from pheromone arrest, it is not essential for vegetative growth under standard conditions (Cyert et al., 1991; Liu et al., 1991*b*; Kuno et al., 1991; Cyert and Thorner, 1992).

Yeast calmodulin is essential for viability (Davis et al., 1986), but its ability to bind Ca^{2+} with high affinity is not necessary for vegetative growth (Geiser et al., 1991). These findings can be explained by proposing that any positive functions of Ca^{2+}/calmodulin and its targets such as calcineurin are redundant with those of other cellular factors during standard growth conditions. Alternatively, the vegetative functions of Ca^{2+}/calmodulin and calcineurin could have gone undetected because the mutants were analyzed under conditions where $[Ca^{2+}]c$ is low, ~0.1–0.3 μM (Halachmi and Eilam, 1989; Ohya et al., 1991).

The functions of calmodulin and calcineurin could be better analyzed if $[Ca^{2+}]c$ was experimentally elevated above the basal levels. A way to accomplish this is by genetically manipulating the ion transporters involved in Ca^{2+} homeostasis. The *PMR1* gene product, a member of the sarco/endoplasmic reticulum (SERCA) family of Ca^{2+} ATPases (Serrano, 1991), is thought to directly transport Ca^{2+} into the Golgi complex to support a variety of secretory functions (Rudolph et al., 1989; Antebi and Fink, 1992). The ability of *pmr1* mutants to tolerate variations in external Ca^{2+} suggests that additional Ca^{2+} transporters might be more important for controlling $[Ca^{2+}]c$. The *PMR2* gene product identified previously (Rudolph et al., 1989) is not related to known Ca^{2+} ATPases any more than H^+ or Na^+/K^+ ATPases (Serrano, 1991) and is required for Na^+ tolerance (Haro et al., 1991) but not Ca^{2+} tolerance (K. W. Cunningham, unpublished data). However, a low-affinity H^+/Ca^{2+} antiport activity is present in isolated vacuole membranes (Ohsumi and Anraku, 1983). Most eukaryotic cells also express a plasma membrane Ca^{2+} ATPase (PMCA) that is primarily responsible for maintaining $[Ca^{2+}]c$ submicromolar levels (Carafoli, 1992).

This study reports the identification of the *PMC1* gene, which encodes a homolog of mammalian PMCAs. Genetic analysis suggests that the product of *PMC1* (Pmc1p) transports Ca^{2+} into the vacuole and participates in the control of $[Ca^{2+}]c$ together with Pmr1p. High external Ca^{2+} inhibits the growth of *pmc1* mutants because calcineurin becomes activated by Ca^{2+}/calmodulin. Thus, Ca^{2+}/calmodulin and calcineurin perform at least one function that prevents cell proliferation under these conditions. Genetic manipulation of *PMC1* provides a valuable new approach to resolve the nature and functions of Ca^{2+} signals.

Materials and Methods

Recombinant DNA

All procedures with recombinant DNA were performed using *Escherichia coli* strain DH5α grown in Luria Broth medium with appropriate antibiotics (Maniatis et al., 1982). Protocols used for polymerase chain reaction (Boehringer-Mannheim, Mannheim, Germany) and sequencing (United States Biochemicals, Cleveland, OH) were performed according to manufacturer's directions. Plasmid vectors were obtained from P. Hieter (Sikorski and Hieter, 1989).

The *PMC1* gene was identified by PCR amplification of genomic DNA

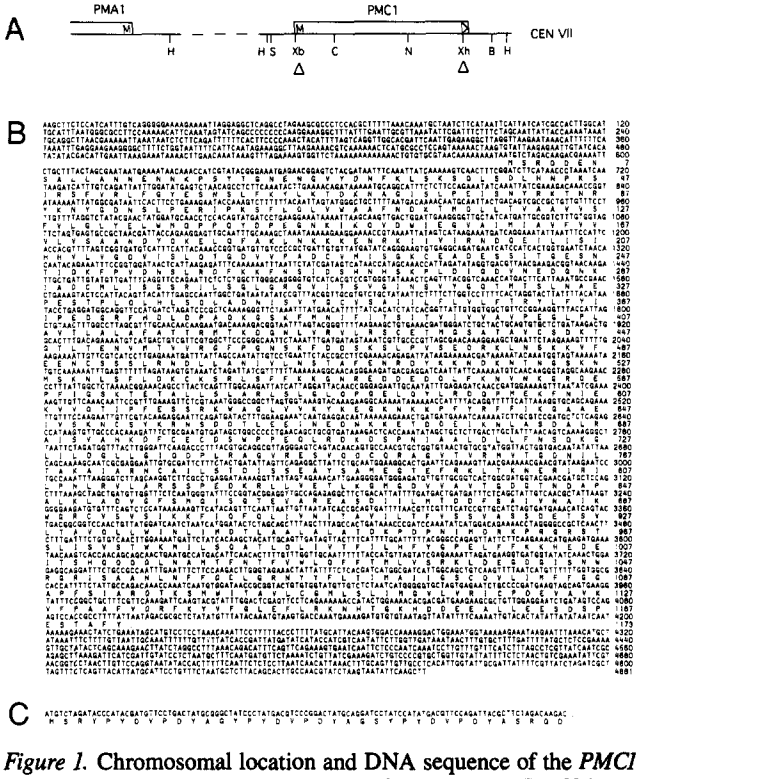


Figure 1. Chromosomal location and DNA sequence of the *PMC1* gene. (*A*) A schematic representation of the genomic *PMC1* locus showing the *PMC1*- and *PMA1*-coding regions (boxes) and partial restriction map. *B*, BglII; *H*, HindIII; *Xb*, XbaI; *Xh*, XhoI; Δ, sites used for constructing *pmc1::LEU2* and *pmc1::TRP1* mutants. Approximately 2 kb of unsequenced DNA between *PMA1* and *PMC1* is indicated by a dashed line (Serrano et al., 1986). (*B*) DNA sequence of the 4.881-kb HindIII fragment containing *PMC1* and predicted amino acid sequence of Pmc1p. Initiation and termination codons are predicted at nucleotides 579 and 4,098. A potential TATA box (TATATA) occurs at nucleotide 481. (*C*) DNA and deduced protein sequence of the *PMC1::HA* open reading frame which contains a 96-bp insertion in the XbaI site at codon +2. The sequence data are available from EMBL/GenBank/DDBJ under accession number U03060.

from the *pmr1::LEU2 pmr2::URA3* strain L4420 using degenerate oligonucleotides corresponding to the most highly conserved regions of known P-type ATPases (Fig. 1). 30 cycles of amplification (94, 45, and 72°C, each for 1 min) using the primer families A1 (CGGGATCCGTNATNTGYWSNGAYAARACNGGNAC) and B1 (CGGAATTCGSRTCRTTNRYNCCRTCNCCNG) produced a novel 1.2-kb product distinct from the 0.8-kb product from *PMA1* and *PMA2* (Serrano et al., 1986; Schlesser et al., 1988). This product was cloned into the EcoRV site of pRS303 (*HIS3*) forming plasmid pKC38. Partial sequencing of this fragment revealed an open reading frame homologous to Ca^{2+} ATPases but not identical to any previously reported yeast gene. This fragment was used to map the *PMC1* locus near the *PMA1* gene on chromosome VII (see Fig. 1) by hybridization to an ordered library of genomic DNA (Riles et al., 1993). The entire *PMC1* locus was subcloned from plasmid B1157 containing *PMA1* (Serrano et al., 1986) as a 4.9-kb HindIII fragment into pRS315 (*CEN LEU2*) forming pKC44 and pKC45 (both orientations). A series of nested deletions in these plasmids were generated by limited ExoIII/ExoVII nuclease digestion according to manufacturer's specifications (GIBCO-BRL, Gaithersburg, MD).

The integrating plasmid pKC55, used to create a chromosomal *PMC1::HA* epitope-tagged allele, was constructed by transferring a 1.7-kb fragment of pKC45 (containing the 5' end of *PMC1*) into pRS306 (*URA3*) and subsequently inserting a 96-bp fragment (Fig. 1 *C*) encoding three tandem repeats of the HA epitope YPYDVPDYA recognized by 12CA5 monoclonal antibody into the XbaI site at codon +2. The construction was verified by

DNA sequencing, linearized by digestion with ClaI, and integrated into the chromosomal *PMC1* locus leaving full-length *PMC1::HA* adjacent to a truncated *pmc1::URA3* allele. Plasmid pKC60 contains the complete epitope-tagged *PMC1::HA* gene (from HindIII to BglIII at position 4,630) in the high copy vector B2205 which is a derivative of pRS306 containing a 2-μm origin of replication. Both single copy and high copy *PMC1::HA* alleles produced immunoreactive polypeptides and functionally complemented *pmc1* mutations for growth in high Ca^{2+} media. The plasmid pKC11 contains the *PMR1* locus (4.4-kb PvuII to SpeI fragment) from pL149 (Rudolph et al., 1989) inserted into B2205 (2 μ *URA3*) digested with SmaI plus XbaI.

The plasmids pKC52 and pKC59 used to replace the chromosomal *PMC1* gene with *pmc1::LEU2* and *pmc1::TRP1*, respectively, were constructed by three part ligations of the 0.58-kb HindIII/XbaI fragment and 0.88-kb XhoI/HindIII fragment of pKC45 into pRS305 and pRS304 digested with XbaI and XhoI. The plasmids were linearized before yeast transformation using HindIII (pKC52) or using StuI plus BglII (pKC59). pKC38 was linearized with NruI to form the *pmc1::HIS3* disruption.

The plasmid pKC73 is a derivative of pRS316-Gal (*CEN ARS URA3*) containing the *GAL1* promoter region fused to the truncated *CNA1ΔC* allele which was amplified by PCR from W303-1A genomic DNA (from nucleotide −10 to nucleotide +1,350 relative to the initiation codon), terminating after codon 450 just before the putative calmodulin binding and autoinhibitory domains (amino acid residues 454–553) (Cyert et al., 1991; Liu et al., 1991*b*). The natural COOH-terminal domain was restored in pKC74 by replacing the 3′ segment from SalI to HindIII in the polylinker with a fragment from pCNA1-201 (Cyert et al., 1991) from SalI to HindIII in the 3′ noncoding region. Both pKC73 and pKC74 complemented *cna1 cna2* double mutants in media containing galactose but not glucose (see Fig. 8).

Yeast Strains, Media, and Growth Conditions

Yeast strains were grown in standard YPD medium (2% Difco yeast extract, 1% bacto-peptone, 2% dextrose) or YPD, pH 5.5, containing 5 mM succinic acid and supplemented with 200 mM $CaCl_2$ when indicated in the text. SC-ura and SCGal-ura media (Sherman et al., 1986) were supplemented with 400 mM $CaCl_2$ when indicated in the text.

Strain L4420 was derived from a cross between strains AA274 (Antebi and Fink, 1992) and YR86 (Rudolph and Fink, 1990). All yeast strains listed in Table I are derived from the isogenic strains W303-1A and W303-1B (Wallis et al., 1989) by transformation using the lithium acetate procedure or by standard genetic crosses among isogenic derivatives (Sherman et al., 1986). The *pmc1::HIS3*, *pmc1::LEU2*, and *pmc1::TRP1* null alleles were produced in W303-1A or W303-1B as described above from linearized plasmids pKC38, pKC52, and pKC59. All three alleles produced identical phenotypes and are referred to as *pmc1* in the text.

The *pmr1::HIS3* null allele was introduced using pL117, a derivative of pL119 (Antebi and Fink, 1992). Construction of *cna1::hisG*, *cna2::HIS3*, and *cnb1::LEU2* null mutants was performed as described (Cyert et al., 1991; Cyert and Thorner, 1992). The *fpr1::URA3* allele was introduced using plasmid pYJH30 (Heitman et al., 1991). Strains JGY41 (*cmd1-3*) and JGY148 (*cmd1-6*) containing the 3E-V+3D-N, and 3E-V mutations, respectively (Geiser et al., 1991), were derived from W303-1A and kindly provided by J. Geiser and T. Davis, Univ. of Washington, Seattle, WA. Standard genetic crosses were used to create some strains containing multiple mutations. In such crosses, the phenotypes of all ascospores in a tetrad were tabulated and found to be uniformly consistent for each genotype.

Spontaneous revertants of *pmc1* were obtained by selection for growth of K473 (*MATα pmc1::LEU2*) and K482 (MATα *pmc1::TRP1*) on YPD pH 5.5, agar medium supplemented with 200 mM $CaCl_2$. Independent revertants were picked after 5 d at 30°C, purified, and placed into complementation groups by mating the K473 and K482 derivatives in all combinations, selecting for diploids on SC-Leu-Trp, and assaying for growth on YPD, pH 5.5, plus 200 mM $CaCl_2$ or SC-Leu-Trp plus 400 mM $CaCl_2$. In this way, three complementation groups were identified among 42 independent recessive revertants. Based on genetic linkage and complementation testing, the first group of calcium-resistant mutants, termed *crm1*, defines the *CNB1* gene (Cyert and Thorner, 1992). The *crm2* and *crm3* groups (11 and 3 members, respectively) are not complemented by pTD59 containing the *CMD1* gene (Geiser et al., 1991) and will be described in future reports.

Measurement of Exchangeable and Nonexchangeable Ca^{2+}

Yeast cultures growing exponentially in YPD medium containing 0.18 mM Ca^{2+} (Ohya et al., 1984) were shifted to fresh YPD medium supplemented with $^{45}Ca^{2+}$ (41 cpm/pmol) and grown at 30°C for 6.5 h to a final OD_{600} of 1.5 (7.5×10^7 cells/ml). The total cell-associated Ca^{2+} was calculated by measuring the radioactivity recovered from 0.1-ml culture aliquots which were diluted into 5 ml ice-cold buffer A (5 mM Na-Hepes, pH 6.5, 10 mM $CaCl_2$), filtered rapidly onto 24 mm Whatman GFF filters using a multiple vacuum filtration unit (model HV224, Hoefer Sci. Instrs., San Francisco, CA), washed three times with ice cold buffer A, dried in vacuo, and

Table I. List of Yeast Strains

Strain	Genotype*	Source‡
W303-1A	*MATa*	Wallis et al., 1989
W303-1B	*MATα*	Wallis et al., 1989
JGY41	*MATa cmd1-3*	Geiser et al., 1991
JGY148	*MATa cmd1-6*	Geiser et al., 1991
K444	*MATa pmc1::HIS3*	W303-1A
K445	*MATa cmd1-6 pmc1::HIS3*	JGY148
K470	*MATa PMC1::HA (pmc1::URA3)*	W303-1A
K473	*MATa pmc1::LEU2*	W303-1A
K473-a7	*MATa pmc1::LEU2 cnb1-1*	K473
K482	*MATα pmc1::TRP1*	W303-1B
K484	*MATa pmc1::LEU2 fpr1::URA3*	K473
K510	*MATa pmc1::LEU2 cmd1-3*	
K541	*MATa pmc1::LEU2 cna2::HIS3*	
K557	*MATa pmc1::LEU2 cna1::hisG*	
K559	*MATa pmc1::LEU2 cna1::hisG cna2::HIS3*	
K603	*MATa cnb1::LEU2*	
K605	*MATa pmc1::TRP1*	
K607	*MATa pmc1::TRP1 cnb1::LEU2*	
K610	*MATα pmr1::HIS3*	
K612	*MATα pmr1::HIS3 cnb1::LEU2*	
K614	*MATα pmr1::HIS3 pmc1::TRP1*	
K616	*MATα pmr1::HIS3 pmc1::TRP1 cnb1::LEU2*	

* All strains harbor the following additional mutations: *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*.
‡ Strains K444 through K484 were constructed by transformation of the source strain. Strains K510 through K616 were produced by crossing derivatives of W303-1A, W303-1B, and JGY41 which contained mutations in *cna1*, *cna2*, or *cnb1* that were introduced by transformation.

processed for liquid scintillation counting. The nonexchangeable Ca^{2+} pools were determined by the same procedure except that each culture was first diluted 10-fold into prewarmed YPD medium supplemented with 20 mM $CaCl_2$ (a 1,000-fold isotopic dilution) and incubated an additional 20 min before filtration. The radioactivity released from the cells by this equilibration procedure represents the exchangeable Ca^{2+} pool, which was calculated as the difference between the total cell-associated Ca^{2+} and the nonexchangeable Ca^{2+}. All measurements were performed in duplicate and averaged.

Miscellaneous Procedures and Reagents

DAPI and immunofluorescent stainings were performed as described (Pringle et al., 1991) on early log phase cultures growing in SC-ura medium at 30°C. SDS gel electrophoresis (Laemmli, 1970) and Western blots (Johnson et al., 1984) were performed on total cell lysates (Reid and Schatz, 1982) prepared from equivalent numbers of log phase cells grown in SC-ura medium. $CaCl_2$ (C-7902; Sigma Immunochemicals, St. Louis, MO) and $^{45}CaCl_2$ (Amersham Corp., Arlington Heights, IL) were used to supplement growth medium. FK506 was a generous gift from Vertex Pharmaceuticals, Inc. (Cambridge, MA). Cyclosporin A was provided by H. Lodish (Whitehead Institute, Cambridge, MA). All other reagents were commercially available.

Results

Cloning and Expression of a Yeast Homolog of PMCA

Our strategy to control calcium signaling in yeast was to identify and manipulate the genes encoding the Ca^{2+} transporters. Ca^{2+} ATPases are members of a highly conserved family of P-type ion pumps, all of which contain highly conserved phosphorylation and ATP-binding domains (Fig. 2). At least five different yeast genes contain both these sequence motifs, as suggested by PCR analysis (see Materials and Methods). Four of these PCR products encode portions of the previously identified *PMA1*, *PMA2*, *PMR1*, and *PMR2* genes. A novel 1.2-kbp PCR product that comigrates with the PMR2 product was cloned after amplification of genomic DNA from a *pmr1 pmr2* deletion strain. This clone contained an open reading frame highly homologous to a segment of mammalian PMCAs. The DNA segment was mapped by hybridization to chromosome VII very close to the *PMA1* gene (Fig. 1 *A*), and defined a new gene termed *PMC1* based on its homology to PMCAs.

The entire *PMC1* gene was subcloned from plasmid B1157 (see Fig. 1 *A*), which had been isolated previously in this laboratory during the cloning of *PMA1*, the major H^+ ATPase in the plasma membrane (Serrano et al., 1986). A long open reading frame of 1,173 codons encompassing the PCR product (Fig. 1 *B*) predicts a 131-kD polypeptide showing 41% identity with rat PMCA1a (Shull and Greeb, 1988), about 40% identity with other PMCA isoforms, and less than 24% identity with SERCA and other known P-type ion pumps. Alignment of the predicted protein sequence Pmc1p with rat PMCA1a (Fig. 2) shows similarity through all 10 putative transmembane domains (M1–M10) and most hydrophilic regions except for the NH_2- and COOH-terminal domains as well as several apparent insertions and deletions. The major structural differences occur in regulatory regions that are not well conserved among the known PMCA isoforms (Carafoli, 1992). The large insertion and deletion in *PMC1* between M2 and M3 occur in a phospholipid binding regulatory region that is subject to alternative splicing in various PMCA isoforms. Interestingly, a COOH-terminal domain that is present in most PMCA isoforms and is involved in calmodulin binding and autoinhibition is absent in Pmc1p and replaced by a much smaller COOH-terminal domain lacking significant similarity to other proteins in current databases. Despite these structural differences, *PMC1* appears to retain all conserved features of P-type ATPases and is the first reported fungal homolog of PMCA.

The product of *PMC1* is expressed during exponential growth as judged by immunoblotting (Fig. 3) and immunofluorescence microscopy (Fig. 4). For these experi-

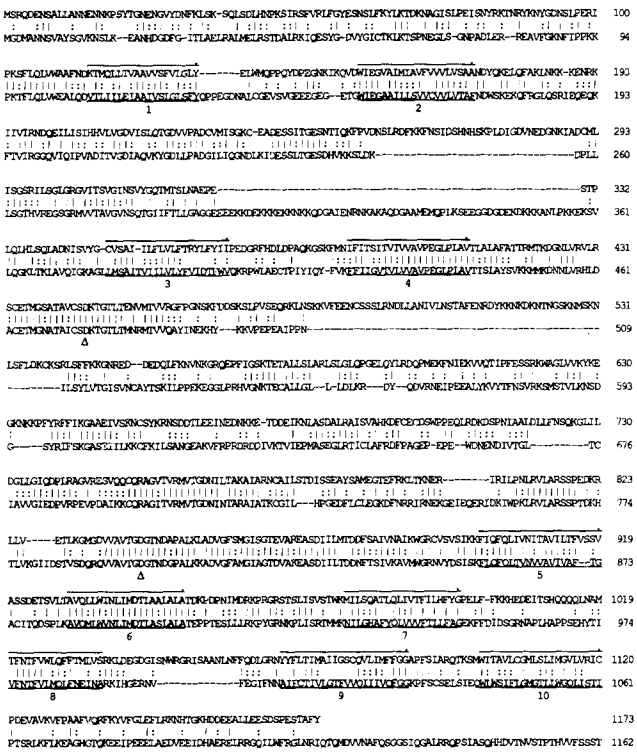


Figure 2. Alignment of the predicted *PMC1* gene product (*top sequence*) with rat PMCA1a (*bottom sequence*, [Shull and Greeb, 1988]). Gaps (−) were introduced to maximize homology (Lipman and Pearson, 1985). Predicted transmembrane domains 1–10 are indicated by underline and overline. Arrowheads (Δ) mark the highly conserved catalytic autophosphorylation and ATP-binding domains employed in the design of degenerate oligonucleotides for PCR analysis.

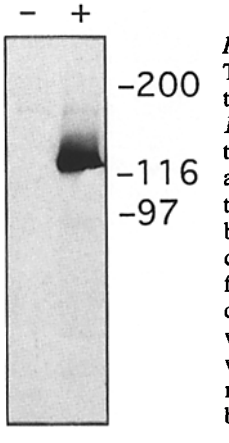


Figure 3. Immunoblot analysis of Pmc1p. Total cell lysates prepared from exponentially grown strains W303-1A *PMC1* (lane *1*) or K470 *PMC1::HA* (lane 2) were fractionated by SDS-PAGE on 7.5% gels and analyzed by immunoblot using a 1:1,000 dilution of 12CA5 monoclonal antibody which binds specifically to HA epitopes. Immune complexes were visualized on radiographic film (Kodak) using the ECL chemilluminescence detection kit. Protein molecular weight markers loaded in adjacent wells were marked after Ponceau S staining of the nitrocellulose blot prior to treatment with blocking agents.

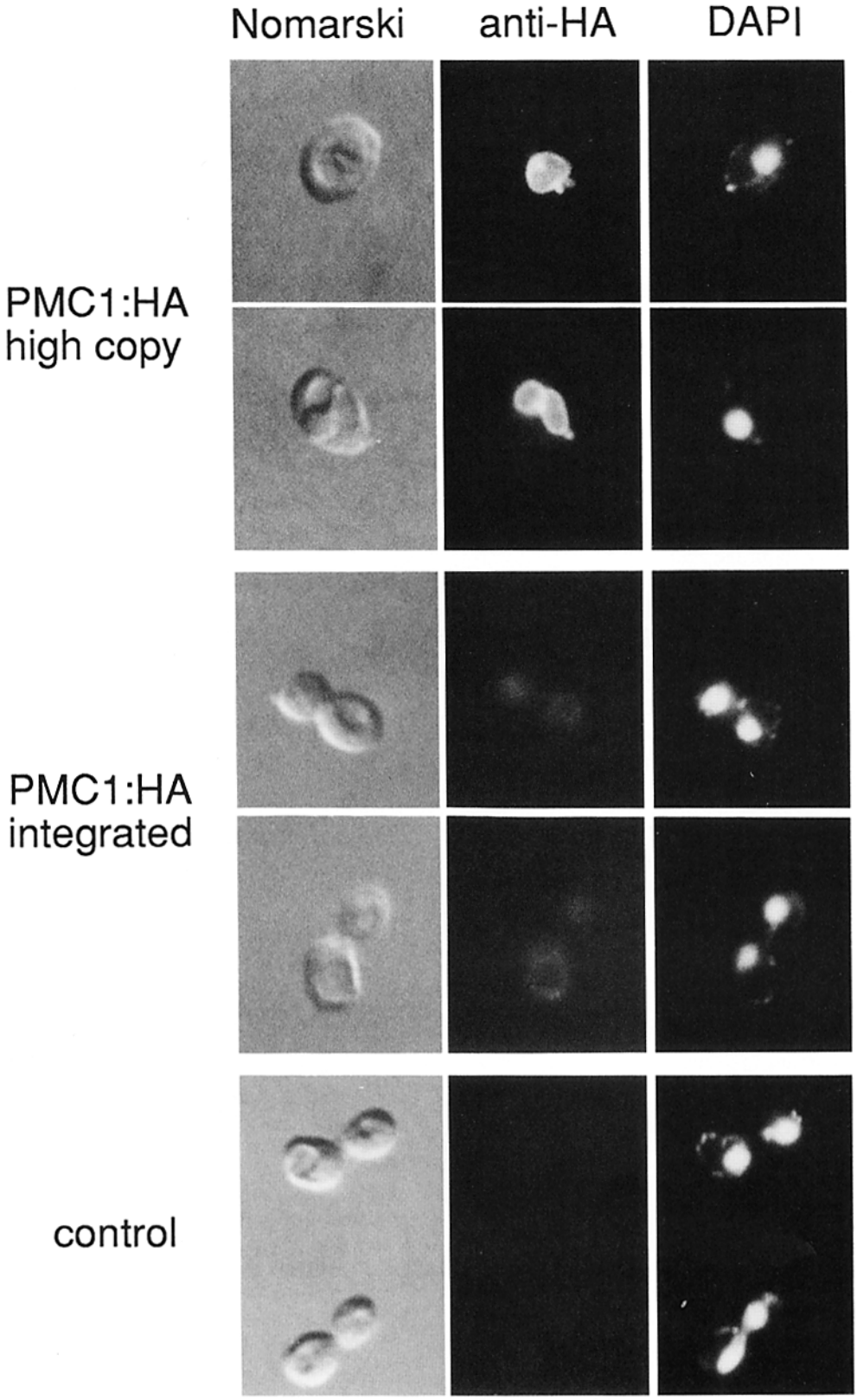


Figure 4. Immunofluorescence localization of Pmclp to vacuole membranes. W303-1A strains transformed with the high copy plasmid pKC60 (*top*), the integrating plasmid pKC55 (*center*), or the control plasmid B2205 (*bottom*) were grown to early log phase, fixed, and processed for immunofluorescence microscopy using the 12CA5 monoclonal antibody. The *PMC1::HA* product is visible at the vacuole periphery in overexpressing strain (*top*) and the single copy strain (*center*), but only nonspecific background staining is visible in similar exposures of the strain lacking the epitope. Nomarski microscopy and DAPI staining identify the vacuoles and nucleus, respectively.

ments, the *PMC1::HA* gene product was visualized using the 12CA5 monoclonal antibody, which recognizes a 34-amino acid insertion at codon +2 in the *PMC1* open reading frame (Fig. 1 *C*). This epitope-tagged version of *PMC1* is functional in vivo when integrated into its chromosomal locus or when expressed from high copy plasmids (see below). Immunoblotting of total cell lysates revealed a major immunoreactive polypeptide at ~130 kD, in good agreement with the predicted molecular mass of 131 kD (Fig. 3, lane 2). Only minor cross-reacting species were observed in

lysates prepared from strains lacking the epitope (Fig. 3, lane *1*).

Analysis by immunofluorescence microscopy revealed that the epitope-tagged *PMC1::HA* gene product is localized predominantly to vacuole membranes in log-phase cells (Fig. 4). Cells producing the *PMC1::HA* product from its chromosomal locus displayed staining of one to three large vesicular structures corresponding to vacuoles by Nomarski optics. Expression of *PMC1::HA* from a multi-copy plasmid greatly increased the staining of the vacuole periphery. No staining of the nucleus, endoplasmic reticulum, mitochondria, or plasma membrane was detected in these experiments. These results suggest that the *PMC1* product might function to transport Ca^{2+} ions into the vacuole from the cytosol. High affinity Ca^{2+} ATPase or Ca^{2+} transport activities have not yet been detected in vacuole membrane vesicles (Ohsumi and Anraku, 1983).

Pmc1p Sequesters Ca^{2+} In Vivo

The effects of mutating the chromosomal *PMC1* gene were examined to infer its function in yeast. The *PMC1* gene is not essential for viability since deleting 97% of the *PMC1* open reading frame (Fig. 1 *A*) had no significant effect on growth rate in rich or synthetic media at a variety of temperatures. Additionally, disruption of *PMC1* does not noticeably affect mating, sporulation, starvation, or a variety of other responses. These observations show that the function of *PMC1* is not required for the execution of many processes under standard conditions.

The effect of deleting *PMC1* on steady state Ca^{2+} pools suggests that Pmc1p functions in Ca^{2+} sequestration in vivo. The yeast vacuole accumulates over 95% of the total cell-associated calcium (Eilam et al., 1985), the majority of which is nonexchangeable in pulse/chase experiments (Eilam, 1982*a*,*b*). Accordingly, the exchangeable and nonexchangeable Ca^{2+} pools were measured in exponentially growing *PMC1* and *pmc1* cells. The exchangeable Ca^{2+} pool was increased only slightly (12%) in *pmc1* cells relative to wild-type cells. However, the nonexchangeable Ca^{2+} pool in *pmc1* cells was over fivefold lower than that of isogenic wild-type cells (Fig. 5). This difference reflects a large reduction in the vacuole Ca^{2+} pool in *pmc1* mutants, and suggests that Pmc1p is necessary for efficient Ca^{2+} sequestration in vivo.

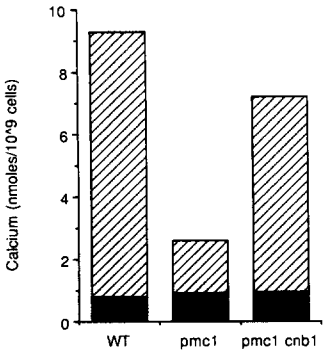
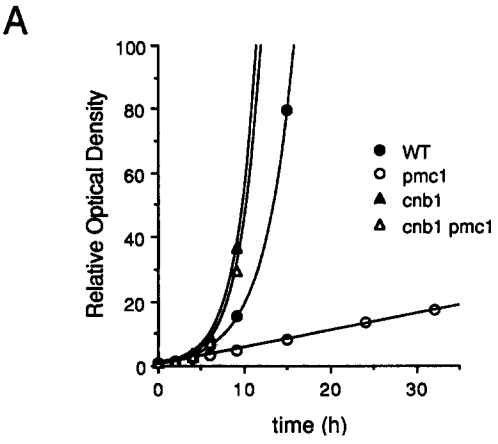


Figure 5. Ca^{2+} compartmentalization in wild-type, *pmc1*, and *pmc1 cnb1* strains. The exchangeable (*black*) and nonexchangeable (*striped*) pools of cells associated Ca^{2+} were measured in strains W303-1A (*WT*), K605 (*pmc1*), and K607 (*pmc1 cnb1*) using cells uniformly labeled with $^{45}Ca^{2+}$ (41 cpm/pmol) as described in Materials and Methods. The values shown are the average of duplicate experiments. (SEM less than 5% of the total for each determination).



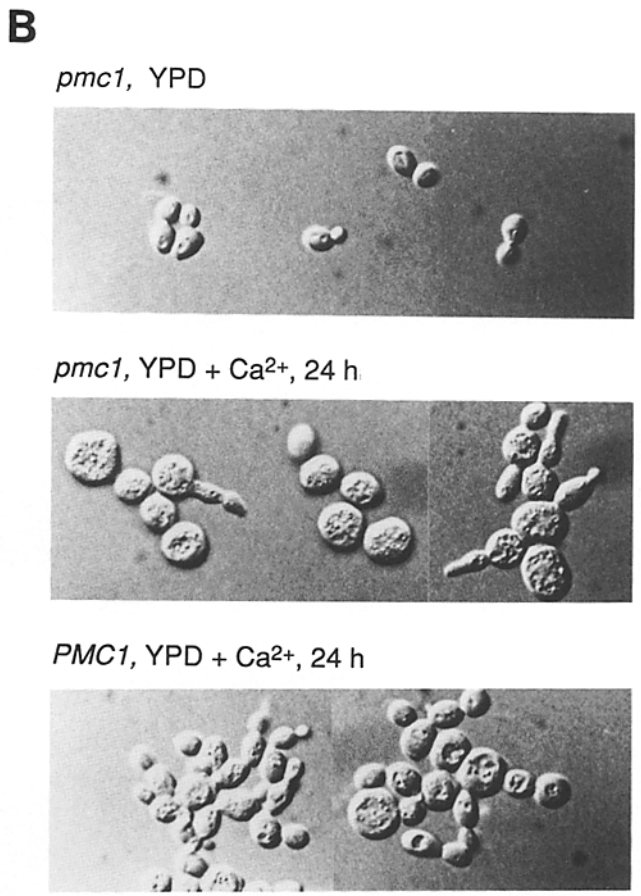


Figure 6. Growth and morphology of *pmc1* mutants in high Ca^{2+} media. Cultures of W303-1A (*WT*), K603 (*cnb1*), K605 (*pmc1*), and K607 (*pmc1 cnb1*) growing exponentially in YPD, pH 5.5, medium were collected by centrifugation and suspended in YPD, pH 5.5, medium supplemented with 200 mM $CaCl_2$ and shaken at 30°C. (*A*) The relative optical density at 600 nm (normalized to the initial level) was determined for each culture at the indicated time intervals. To maintain low cell densities, serial dilutions into fresh prewarmed media were made as necessary and factored into the calculation of relative optical density. (*B*) Nomarski micrographs of *pmc1* mutants growing for 24 h in YPD medium (*top*) and in YPD+200 mM Ca^{2+} (*center*) and of W303-1A growing for 24 h in YPD+200 mM Ca^{2+} (*bottom*).

PMC1 Is Required for Ca^{2+} Tolerance

As *pmc1* mutants have decreased ability to sequester Ca^{2+} in the vacuole, we tested their ability to tolerate very high or very low Ca^{2+} concentrations in the growth medium. Wild-type and *pmc1* null mutants are indistinguishable during growth in synthetic media containing <1 μM Ca^{2+} (Rudolph et al., 1989) or in complex YPD media containing varying amounts of EGTA (not shown). However, *pmc1* null mutants grow very poorly in YPD medium, pH 5.5, supplemented with 200 mM $CaCl_2$ (Figs. 6 and 7). The growth defect of *pmc1* mutants was not observed in YPD, pH 5.5, supplemented with 200 mM $MgCl_2$ (Fig. 7). Additionally Ca^{2+} sensitivity was not observed in strains expressing only the epitope-tagged *PMC1::HA* hybrid from its chromosomal locus or from high copy plasmids (not shown). These observations demonstrate that *PMC1* is necessary for tolerance to high Ca^{2+} and confirm the importance of the vacuole in yeast Ca^{2+} homeostasis (Klionsky et al., 1990; Anraku et al., 1991).

In liquid growth medium supplemented with 200 mM Ca^{2+}, wild-type strains grew exponentially after a brief lag (Fig. 6 *A*). In contrast, *pmc1* mutants shifted to high Ca^{2+} media increased culture density at a slow linear rate that continued over at least 32 h. After 24 h of incubation, the optical density of the *pmc1* culture increased 10-fold and the number of viable cells increased approximately 5-fold, as determined by colony forming units on agar YPD, pH 5.5, medium supplemented with 200 mM $MgCl_2$. Though the morphology of *pmc1* cells changed somewhat during incubation in high Ca^{2+} (Fig. 6 *B*), they did not accumulate with a uniform morphology. Thus, the growth inhibition of *pmc1* mutants by high Ca^{2+} is reversible and apparently not a consequence of a specific block in the cell division cycle.

Calcineurin Mediates Growth Inhibition of pmc1 Mutants in High Ca^{2+} Medium

The mechanism by which external Ca^{2+} inhibits the growth of *pmc1* mutants was investigated by characterizing spontaneous revertants carrying secondary mutations that restore the ability of *pmc1* null mutants to proliferate in high Ca^{2+} media (see Materials and Methods). Recessive mutations in three complementation groups were found to reverse the Ca^{2+} sensitivity of *pmc1* mutants. The first group of revertants (termed *crm1* for Ca^{2+} resistant mutation) was found to define the *CNB1* gene which encodes the Ca^{2+} binding regulatory subunit of calcineurin (Kuno et al., 1991; Cyert and Thorner, 1992). The *crm1* alleles displayed a defect in recovery from pheromone arrest similar to *cnb1* null mutants (Cyert and Thorner, 1992), were genetically linked to the *CNB1* locus (44:0:0, parental ditype/non-parental ditype/tetratype), and were complemented by plasmids carrying the wild-type *CNB1* gene. Additionally, disruption of *CNB1* by gene replacement restores growth of *pmc1* mutants (Fig. 7). Growth of *pmc1* mutants is also restored by simultaneous deletion of both genes encoding the catalytic subunit of calcineurin, *CNA1* and *CNA2* (Fig. 7). Deletion of either *CNA1* alone or *CNA2* alone is not sufficient to suppress the Ca^{2+} sensitivity of *pmc1*. By this assay, the *CNA1* and *CNA2* genes are functionally redundant, which explains why no spontaneous *cna1* or *cna2* mutants were recovered in the initial

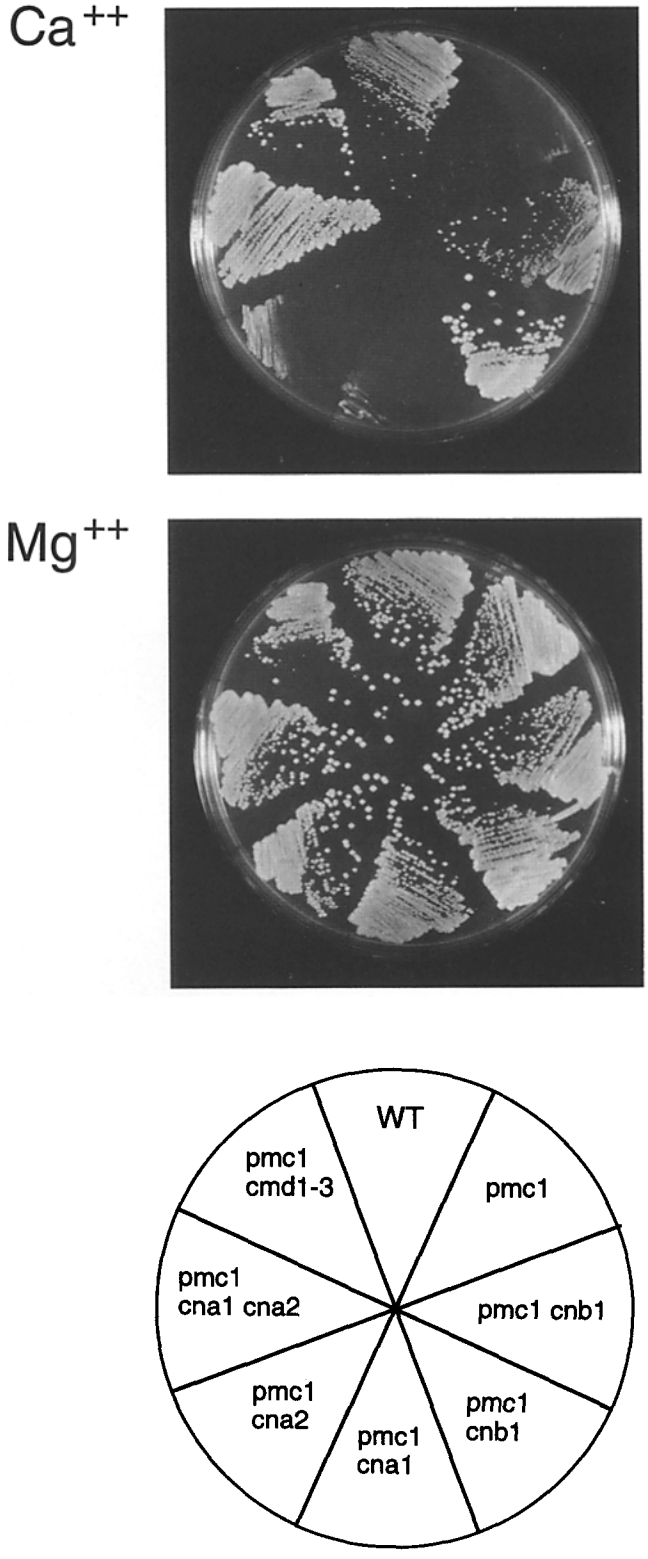


Figure 7. Growth inhibition of *pmc1* by high Ca^{2+} is prevented by mutations in calmodulin and calcineurin. Yeast strains were spread onto the surface of YPD agar medium, pH 5.5, supplemented with either 200 mM $CaCl_2$ (*A*) or 200 mM $MgCl_2$ (*B*) and incubated at 30°C for 3 d. Strains clockwise from top: W303-1A, K473, K473-a7, K607, K541, K557, K559, and K510 with relevant genotypes (*C*).

revertant screen. Loss of calcineurin function did not affect growth of *pmc1* mutants in standard media or in media supplemented with 200 mM $MgCl_2$. Thus, growth inhibition of *pmc1* mutants by high Ca^{2+} requires the function of both the catalytic and regulatory subunits of calcineurin.

In high Ca^{2+} liquid media (Fig. 6 *A*), *cnb1* and *pmc1 cnb1* strains grow at faster rates (1.6 h doubling time) than an isogenic wild-type strain (2.5 h doubling time). This result suggests that calcineurin activity limits the rate of yeast growth in high Ca^{2+} conditions, and is consistent with earlier observations that the *CNA1*, *CNA2*, and *CNB1* genes are not required for vegetative growth in standard media (Cyert et al., 1991; Liu et al., 1991*b*; Cyert and Thorner, 1992). Interestingly, *pmc1 cnb1* mutants have a larger pool of nonexchangeable Ca^{2+} than *pmc1* mutants (Fig. 5). If Ca^{2+} sequestration into the vacuole is improved by inactivation of calcineurin, this might explain the enhanced growth rate of *pmc1 cnb1* (and *cnb1*) strains in high Ca^{2+} media.

Activation of Calcineurin by Ca^{2+}/Calmodulin Is Necessary for Inhibition of pmc1 Mutants

The protein phosphatase activity of mammalian calcineurin in vitro is greatly stimulated by binding of Ca^{2+} and calmodulin (Stewart et al., 1982). Similarly, calmodulin binds to the *CNA1* and *CNA2* gene products only in the presence of Ca^{2+} (Cyert et al., 1991; Liu et al., 1991*b*). Mutant forms of calmodulin that are unable to bind Ca^{2+} fail to activate target enzymes in vitro (Hurwitz et al., 1988), but still provide functions necessary for yeast viability (Geiser et al., 1991). Using *cmd1-3* strains, which express a calmodulin mutant containing two amino acid substitutions in each of

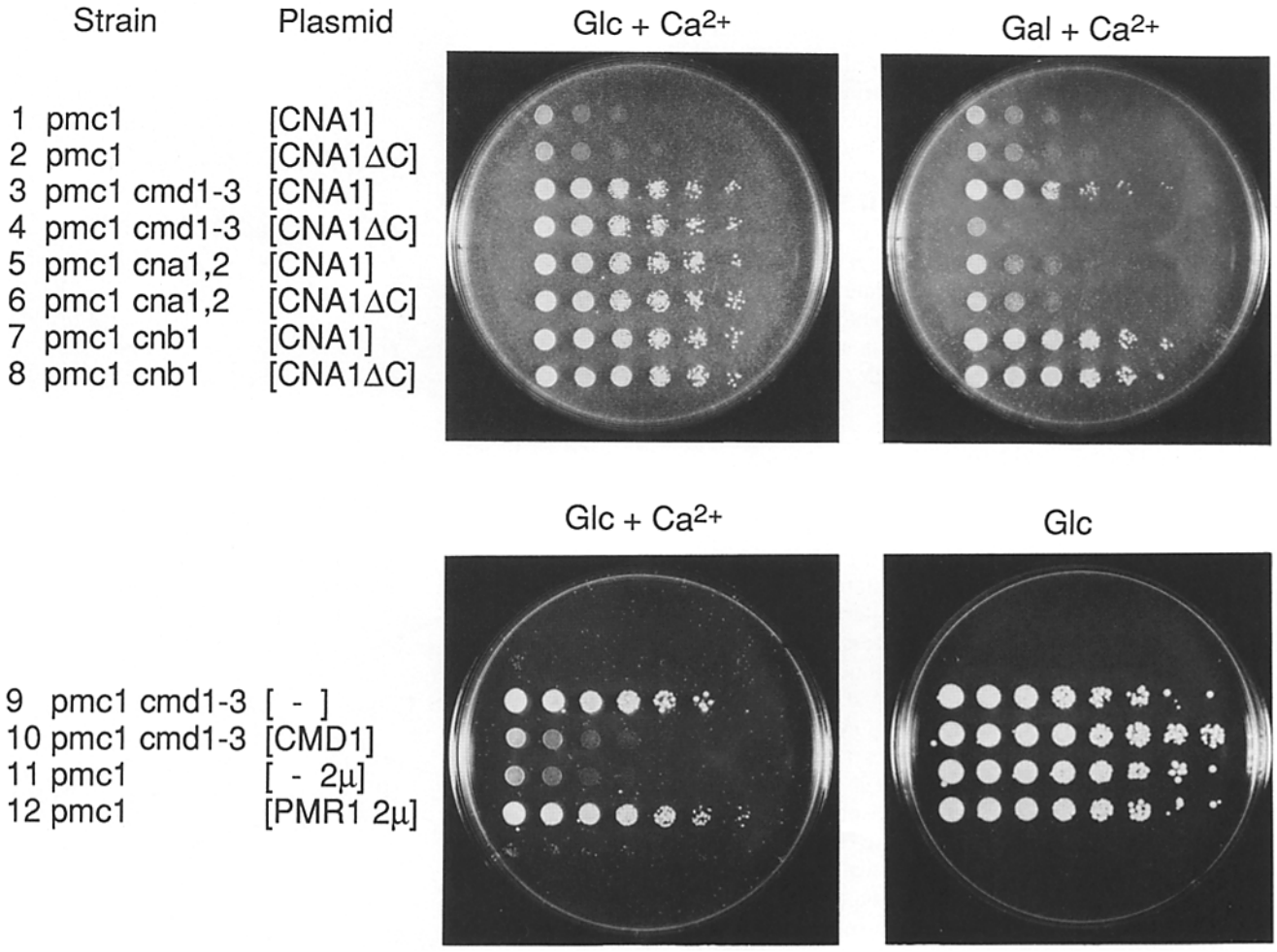


Figure 8. Expression of COOH-terminal truncated *CNA1* (*CNA1ΔC*) bypasses *cmd1-3* in *pmc1* mutants. Strains K473 (lines *1* and *2*), K510 (lines *3* and *4*), K559 (lines *5* and *6*), and K607 (lines *7* and *8*) were transformed with low copy plasmids containing derivatives of *CNA1* (pKC74, lines *1*, *3*, *5*, and *7*) or *CNA1ΔC* (pKC73, lines *2*, *4*, *6*, and *8*) under control of the galactose-inducible promoter of *GAL1*. Strain K510 (lines *9* and *10*) was also transformed with a control plasmid pUN50, or pTD59 containing the *CMD1* gene (Geiser et al., 1991). Strain K473 was also transformed with the high copy control plasmids B2205 or pKC11 containing the *PMR1* gene. Serial fivefold dilutions of saturated cultures were spotted onto complete medium lacking uracil (to maintain selection for the plasmids) supplemented with the 2% glucose or galactose and 400 mM $CaCl_2$ as indicated. The plates were photographed after 3 d incubation at 30°C. High Ca^{2+} inhibits growth of *pmc1 cmd1-3* and *pmc1 cna1 cna2* but not *pmc1 cnb1* mutants expressing *CNA1ΔC*. High Ca^{2+} does not inhibit growth of *pmc1 cmd1-3* or *pmc1 cnb1* mutants expressing *CNA1*.

the three high affinity Ca^{2+}-binding domains, it is possible to test whether Ca^{2+} binding to calmodulin is necessary for growth inhibition of *pmc1* mutants by high Ca^{2+}.

In contrast to *pmc1* mutants, a *pmc1 cmd1-3* double mutant grows well on high Ca^{2+} media (Fig. 7). Introduction of a low copy plasmid carrying the wild-type *CMD1* gene into the *pmc1 cmd1-3* strain restored Ca^{2+} sensitivity (Fig. 8, lines *9* and *10*), indicating that the mutant calmodulin has not gained a function (dominant over wild type), but rather has specifically lost a function required for Ca^{2+}-induced growth inhibition. Identical results were obtained with a *pmc1::HIS3 cmd1-6* strain (K445), which contains only three point mutations in the *CMD1* gene (data not shown). Thus, a mutant with amino acid substitutions in the high affinity Ca^{2+} binding sites of calmodulin has the same phenotype as a mutant that has lost calcineurin function.

Ca^{2+}/calmodulin activates calcineurin in vitro by binding to a COOH-terminal domain of the catalytic subunit and relieving autoinhibition by this domain (Stewart et al., 1982; Hashimoto et al., 1990). Proteolytic removal of the autoinhibitory domain causes calmodulin-independent stimulation of protein phosphatase activity (Manalan and Klee, 1983). These biochemical data predict that a *CNA1* truncation mutant lacking the autoinhibitory domain would become activated independent of Ca^{2+}/calmodulin and would prevent growth of *pmc1 cmd1-3* strains in high Ca^{2+} media. As predicted, a *pmc1 cmd1-3* strain expressing the truncated *CNA1ΔC* allele from an inducible promoter (*GAL1*) fails to grow in high Ca^{2+} medium (Fig. 8, line *4*). In contrast, expression of wild-type *CNA1* from this promoter does not inhibit growth of the *pmc1 cmd1-3* strain in high Ca^{2+} medium (Fig. 8, line *3*). The *GAL1*- induced expression of *CNA1* and *CNA1ΔC* does not inhibit growth of yeast strains in the absence of added Ca^{2+} (not shown). Furthermore, expression of either *CNA1* or *CNA1ΔC* inhibits growth of a *pmc1 cna1 cna2* strain (Fig. 8, lines *5* and *6*) but not a *pmc1 cnb1* strain (lines *7* and *8*) in high Ca^{2+} media. Thus, removing the COOH-terminal autoinhibitory domain of calcineurin A specifically bypasses the requirement for Ca^{2+}/calmodulin to promote growth arrest, though calcineurin B and Ca^{2+} are still required for this arrest. These data suggest that the major role of Ca^{2+}/calmodulin in growth inhibition by Ca^{2+} is to activate calcineurin via its COOH-terminal region. The *cmd1-3* mutant calmodulin is unable to perform this function.

FK506 and Cyclosporin A Inhibit Yeast Calcineurin In Vivo

The immunosuppressant drug FK506 binds tightly to a complex of proteins present in wild-type yeast strains and absent in extracts made from strains lacking calcineurin A, calcineurin B, or the yeast homolog of FKBP-12 encoded by *FPR1* (Foor et al., 1992). A similar complex formed in mammalian cells results in the complete inhibition of calcineurin activity towards phosphopeptide substrates (Liu et al., 1991*a*). These considerations prompted us to test whether FK506 would restore growth of *pmc1* mutants in high Ca^{2+} media by inhibiting yeast calcineurin.

Addition of FK506 dramatically restores growth of *pmc1* mutants in high Ca^{2+} medium, but has no effect on *pmc1 fpr1* strains lacking FKBP-12 (Fig. 9 *A*). In this assay, the

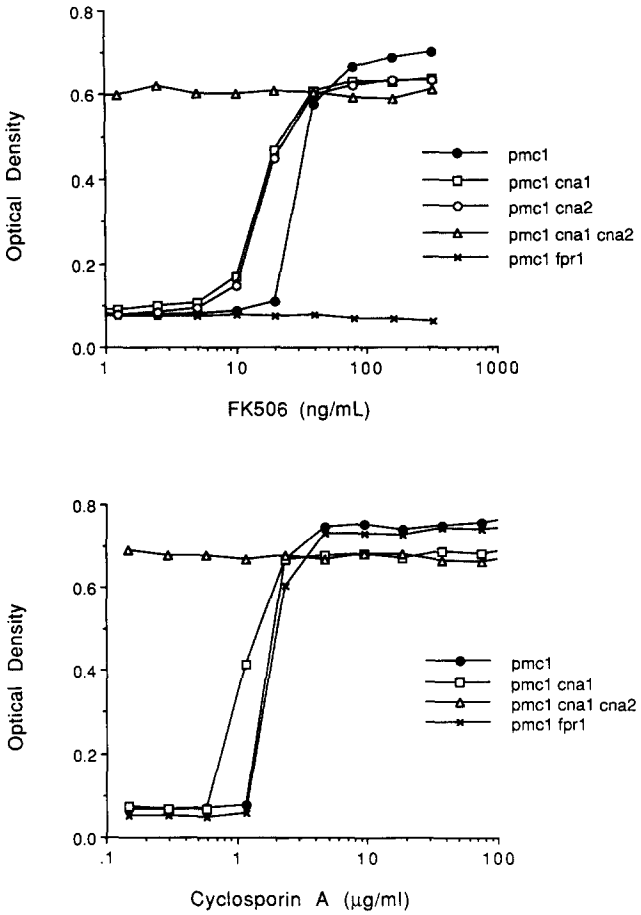


Figure 9. FK506 (*A*) and cyclosporin A (*B*) suppress the Ca^{2+} sensitivity of *pmc1* mutants. Yeast strains (Table I) were inoculated at low density into YPD medium, pH 5.5, supplemented with 200 mM $CaCl_2$ and the indicated concentrations of FK506 (*A*) or cyclosporin A (*B*). After 24 h incubation at 30°C in flat bottom microtiter dishes, the optical density of each culture was measured at 570 nm (GR600 Microplate Reader; Dynatech Labs. Inc., Chantilly, VA). The effect of FK506 but not cyclosporin A requires functional *FPR1*. The effective dosages of both drugs correlate with the gene dosage of *CNA*.

50% effective dosage (ED_{50}) of FK506 was estimated at 25 ng/ml which is over 1,000 times lower than the 50% lethal dosage (LD_{50}) of ~50 μg/ml (Heitman et al., 1991). There is also a direct correlation between the effectiveness of the drug and the dosage of calcineurin A genes. *pmc1 cna1* and *pmc1 cna2* double mutants each required approximately half as much FK506 (12 ng/ml) when compared to the *pmc1* strain. These results fully support the hypothesis that a direct target of FK506/FKBP-12 in yeast is activated calcineurin.

Cyclosporin A, a structurally distinct inhibitor of calcineurin acting through its own binding protein (Liu et al., 1991*a*), also restores growth of *pmc1* mutants in high Ca^{2+} media (Fig. 9 *B*). The ED_{50} of cyclosporin A in this assay (1–2 μg/ml) is much lower than its LD_{50} (~200 μg/ml), is directly proportional to *CNA* gene dosage, and is independent of FKBP-12. The effectiveness of both drugs can be explained by a model where a growth inhibitory complex of Ca^{2+}/calmodulin/calcineurin forms in *pmc1* mutants during

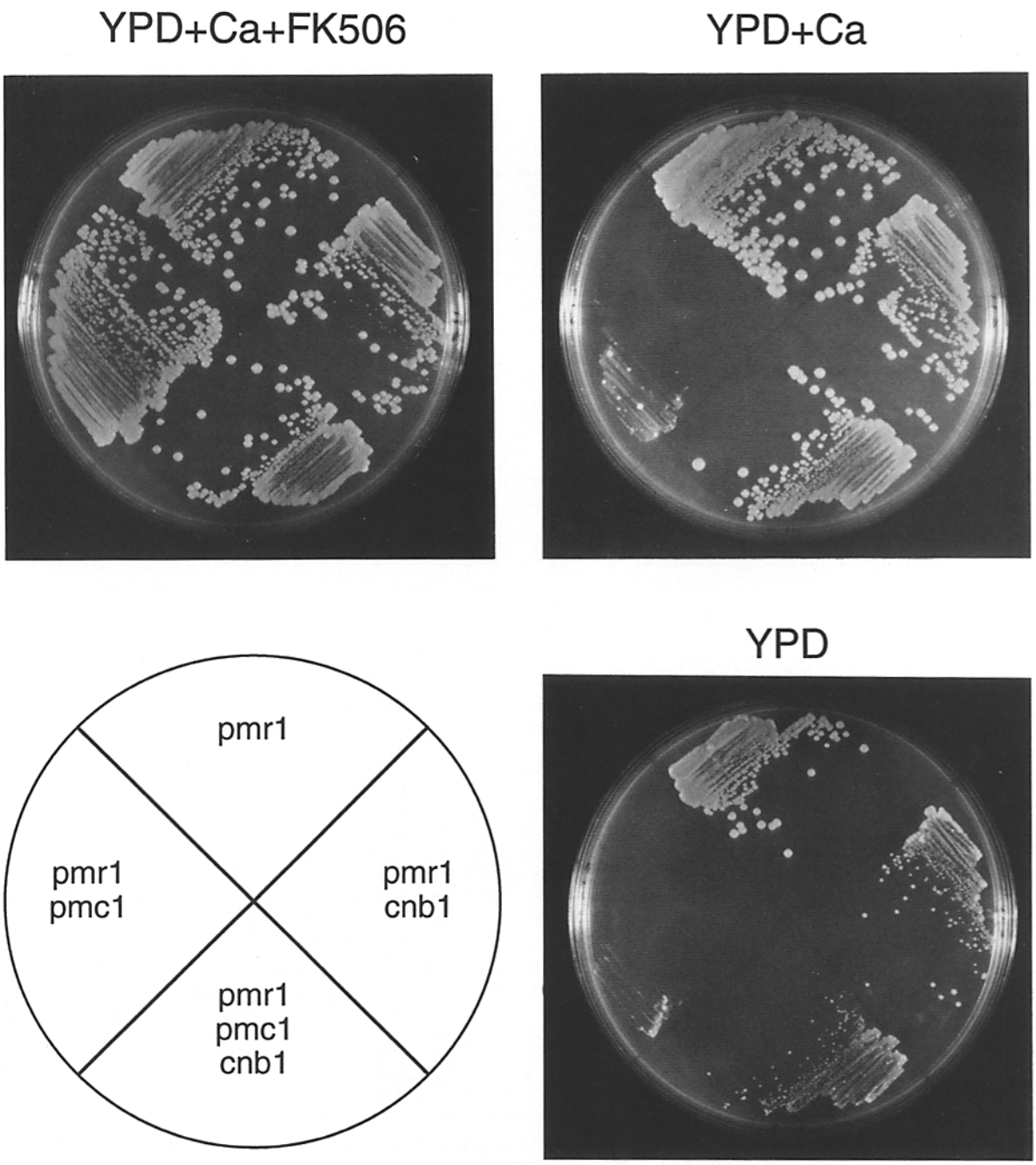


Figure 10. The synthetic lethality of *pmc1 pmr1* double mutants is reversed by a *cnb1* mutation or by addition of FK506. Strains K610 (*pmr1*), K612 (*pmr1 cnb1*), K614 (*pmr1 pmc1*), and K616 (*pmr1 pmc1 cnb1*) were streaked onto YPD agar medium supplemented with FK506 (0.4 μg/ml) and/or $CaCl_2$ (20 mM) as indicated. The strains were initially recovered by germinating ascopores produced from a triply heterozygous diploid on YPD+FK506+$CaCl_2$ medium where all genotypes are viable. The colonies were photographed after 4 d of incubation at 30°C. The *pmr1 pmc1* double mutants did not form visible colonies on YPD or YPD plus 20 mM $CaCl_2$.

growth in high Ca^{2+} media, and that this complex is inhibited by the stoichiometric binding of FK506/FKBP-12 or cyclosporin A/cyclophilin (Foor et al., 1992). This model implies that $[Ca^{2+}]c$ is elevated in *pmc1* mutants during incubation in high Ca^{2+} media, in agreement with the genetic data suggesting that Ca^{2+}/calmodulin but not apo-calmodulin (as produced by *cmd1-3*) is required for the inhibitory effect of calcineurin.

The Inviability of a pmr1 pmc1 Double Mutant Is Suppressed by Inactivation of Calcineurin

The role of *PMC1* in Ca^{2+} tolerance may be shared by *PMR1* (Rudolph et al., 1989; Antebi and Fink, 1992), a putative Ca^{2+} ATPase in the Golgi complex related to Ca^{2+} ATPases of the mammalian SERCA. This possibility was verified by the observation that overexpression of *PMR1* in

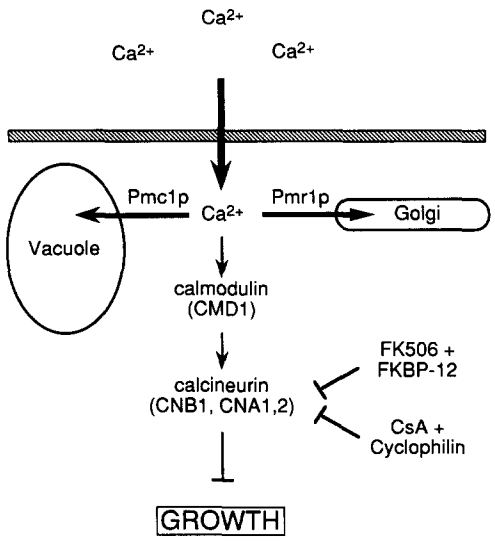


Figure 11. A working model for Ca^{2+} transport in yeast and Ca^{2+}/calcineurin dependent inhibition of growth. The cytoplasmic pool of Ca^{2+} is increased by influx of external Ca^{2+} and decreased by sequestration activities of Pmclp and Pmrlp which directly pump Ca^{2+} into the vacuole and Golgi complex, respectively. Other Ca^{2+} transporters may also contribute to control of cytoplasmic Ca^{2+}. Elevation of the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]c$, activates calcineurin by increasing the binding of Ca^{2+} and calmodulin. Inappropriate activation of calcineurin, attained by increased Ca^{2+} influx and/or decreased sequestration, dephosphorylates protein substrates whose phosphorylation is necessary for growth during these conditions. The downstream targets of calcineurin and their roles in growth control are unknown. It is possible that calcineurin negatively regulates a Ca^{2+}-sequestering mechanism distinct from Pmclp (Fig. 5).

pmcl mutants restores growth in high Ca^{2+} media (Fig. 8, lines *11* and *12*). Moreover, simultaneous deletion of both *PMC1* and *PMR1* causes a phenotype more severe than either mutation alone. Strains containing both *pmr1 pmc1* null mutations are inviable on all media regardless of the external Ca^{2+} levels (from 0.2 to 200 mM). However, the lethality of *pmr1 pmc1* strains is prevented by inactivation of calcineurin with either FK506 (400 ng/ml) or the *cnb1* null mutation (Fig. 10). Thus, *PMC1* and *PMR1* are redundant for an essential function only when calcineurin can be activated.

The simplest model consistent with these findings (see Fig. 11) is that both *PMC1* and *PMR1* gene products transport Ca^{2+} from the cytosol into internal compartments. Adequate Ca^{2+} ATPase activity is essential for yeast growth in order to prevent the inhibitory activation of calcineurin by calmodulin in response to elevated $[Ca^{2+}]c$. Calcineurin activation may inhibit growth of *pmc1* mutants by a variety of mechanisms, such as decreasing the activity of unknown factors necessary for growth or Ca^{2+} tolerance.

Discussion

PMC1 Encodes a Vacuolar Ca^{2+} ATPase Involved in Ca^{2+} Sequestration and Regulation of $[Ca^{2+}]c$

Several lines of evidence indicate that Pmclp transports Ca^{2+} from the cytoplasm into the vacuole. First, the predicted product of *PMC1* contains all the conserved features of P-type ion pumps and is highly homologous to mammalian PMCAs (41% identical through 81% of its sequence). Second, the phenotypes of *pmc1* mutants are consistent with a direct role for Pmclp in Ca^{2+} transport. Although *pmc1* null mutants show no obvious growth or morphological phenotypes on standard media, they accumulate significantly less Ca^{2+} in the vacuole and are intolerant of high Ca^{2+} in the growth medium. Third, the behavior of *pmc1* mutants is dramatically affected by modifications of a Ca^{2+}-sensitive regulatory pathway consisting of calmodulin and calcineurin. Finally, *PMC1* shares an essential function with *PMR1*, a member of SERCA-type Ca^{2+} pumps (Serrano, 1991). All these properties are consistent with a general model of Ca^{2+} transport and signaling thought to exist in most eukaryotic cells (Fig. 11). However, the study of Ca^{2+} metabolism in yeast has revealed some noteworthy distinctions.

Ca^{2+} uptake into the yeast vacuole in vitro is predominantly due to low affinity H^+/Ca^{2+} antiport activity (Ohsumi and Anraku, 1983). However, during growth in 0.18 mM Ca^{2+} YPD medium (Ohya et al., 1984), the majority of Ca^{2+} sequestered in the vacuole requires Pmclp (Fig. 5). Thermodynamic considerations also argue that Ca^{2+} ATPases are necessary to account for the high levels of Ca^{2+} sequestered in fungal vacuoles (Miller et al., 1990). In the absence of Pmclp, the residual Ca^{2+} sequestration can be attributed to the vacuolar antiporter and/or Pmrlp in the Golgi complex. *PMR1* is required for viability of *pmc1* mutants and overexpression of *PMR1* restores Ca^{2+} tolerance to *pmc1* mutants. The simplest interpretation of these findings is that Pmclp and Pmrlp both transport Ca^{2+} from the cytoplasm into internal compartments and therefore function redundantly in controlling cytoplasmic Ca^{2+} levels. This effect is not unlike the roles of PMCA and SERCA in returning $[Ca^{2+}]c$ to submicromolar levels after Ca^{2+} influx is induced by external stimuli (Carafoli, 1987). The major distinction is that yeast utilize Pmclp for Ca^{2+} sequestration into the vacuole whereas mammalian cells utilize PMCA for Ca^{2+} efflux through the plasma membrane.

Calcineurin Activation by Ca^{2+}/Calmodulin Is Growth Inhibitory in pmc1 Mutants

Our results show that the inviability of *pmc1* strains in high Ca^{2+} media requires calcineurin activation by Ca^{2+}/calmodulin. The growth defects of *pmc1* strains in high Ca^{2+} media are completely reversed in strains lacking either the catalytic or regulatory subunits of calcineurin or in strains containing mutant forms of calmodulin with defective Ca^{2+} binding sites. Expression of a truncated calcineurin A lacking the autoinhibitory domain bypasses the requirement for Ca^{2+}/calmodulin, preventing growth of *pmc1 cmd1-3* double mutants in high Ca^{2+} medium. These in vivo data are consistent with the mechanism of calcineurin activation in vitro where maximal stimulation of phosphopeptide phosphatase activity requires binding of Ca^{2+}/calmodulin to relieve autoinhibition by the COOH-terminal domain of the catalytic A subunit.

The clear effect of inactivating calcineurin in *pmc1* mutants was exploited here in a bioassay for potential antagonists of yeast calcineurin. The immunosuppressant drugs FK506 and cyclosporin A, together with their respective immunophilin binding proteins, form inactive complexes with yeast and

mammalian calcineurin (Liu et al., 1991*a*; Foor et al., 1992). As demonstrated here, both of these compounds mimic the effect of calcineurin mutations, restoring growth to *pmcl* mutants in high Ca^{2+} media. Moreover, the effective dosages of these drugs decreased about twofold when the gene dosage of calcineurin A was reduced by half. These findings provide strong evidence that calcineurin is the direct target of FK506 and cyclosporin A in yeast cells. Based on these results, we propose that calcineurin activation occurs during incubation of *pmcl* mutants in high Ca^{2+} media.

Calcineurin activation also occurs in standard media in *pmcl pmrl* double mutants, and therefore is not restricted to conditions of high external Ca^{2+}. Calcineurin-dependent growth inhibition can be observed in *pmcl pmrl* strains in media containing 0.18–200 mM Ca^{2+}. Because Pmclp and Pmrlp function in Ca^{2+} sequestration, their shared essential function (in >0.18 mM Ca^{2+}) is most likely to maintain sufficiently low $[Ca^{2+}]c$ to prevent activation of calcineurin. Though growth inhibition by calcineurin activation was most evident in *pmcl pmrl* and *pmcl* strains, it is possible that calcineurin inhibits growth of wild-type strains in response to high Ca^{2+} environments (Fig. 6) or in response to natural Ca^{2+} signals. Transient Ca^{2+} signals might be generated during some situations by the physiological modulation of Pmclp and Pmrlp activities or other Ca^{2+} transporters.

Calcineurin-dependent Signaling Pathways

The results reported here suggest that calcineurin activation can have a net negative effect on growth, which itself might obscure other positive effects. A positive (growth enhancing) role for calcineurin in yeast has been observed in response to prolonged cell cycle arrest by mating pheromones (Cyert et al., 1991; Cyert and Thorner, 1992). The process of recovery from pheromone arrest is inefficient in calcineurin mutants and in strains treated with FK506 or cyclosporin A (Foor et al., 1992). Thus, calcineurin activation can affect yeast proliferation in a positive or negative way depending on the circumstances. The net effect of activated calcineurin may be a consequence of differences in the activity or availability of phosphoprotein substrates, in the nature of the cytosolic Ca^{2+} signal, or possibly the input of additional regulatory signals.

Calcineurin-dependent signaling in human cells can also have a net positive or negative effect depending upon the cell status. Cyclosporin A and FK506 can prevent activation and proliferation of mature T cells, whereas these compounds block the induced suicide of immature T cells (Bierer et al., 1991). The calcineurin-dependent inhibition of yeast growth reported here is reversible, occurs at many stages of the cell cycle, and is therefore distinct from the type of cell death observed in immature T cells. A more complete understanding of the different responses will require the identification of distinguishing calcineurin targets.

Our results show that in *pmcl* mutants, calcineurin function decreases tolerance to external Ca^{2+} (Fig. 6) and decreases sequestration of Ca^{2+} into the nonexchangeable (vacuolar) pool (Fig. 5). It is unlikely that the latter effect is due to increased influx or decreased efflux of Ca^{2+} since both these models would predict decreased tolerance to external Ca^{2+}. An alternative explanation is that another enzyme involved in Ca^{2+} transport into the vacuole, such as the H^+/Ca^{2+} antiporter (Ohsumi and Anraku, 1983), is negatively regulated by calcineurin. This explanation would also account for the calcineurin-dependent growth inhibition of *pmcl* mutants in high Ca^{2+} conditions.

Given the remarkable interspecies conservation of the mechanism by which calcineurin is activated and inhibited, it is plausible that the physiological roles of calcineurin are similar in human and yeast cells. The bioassays reported here will be useful to identify the downstream targets and functions of yeast calcineurin as a framework for understanding Ca^{2+}-signaling mechanisms and responses in human cells.

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