Calcineurin-dependent Growth Control in *Saccharomyces cerevisiae*
Mutants Lacking *PMCl*, a Homolog of Plasma Membrane Ca\(^{2+}\) ATPases
Kyle W. Cunningham and Gerald R. Fink
Whitehead Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142

Abstract. Ca\(^{2+}\) ATPases deplete the cytosol of Ca\(^{2+}\) ions and are crucial to cellular Ca\(^{2+}\) homeostasis. The *PMCl* 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 *PMCl* grow well in standard media, but sequester Ca\(^{2+}\) into the vacuole at 20% of the wild-type levels. *pmcl* null mutants fail to grow in media containing high levels of Ca\(^{2+}\), suggesting a role of *PMCl* 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 *pmcl* 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 *PMRI* gene acts together with *PMCl* 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\(^{2+}\)]\(_{c}\), 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, cyclophillin and FKBP-12, as potent inhibitors of calcineurin (Liu et al., 1991a). 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
wild-type strains by addition of cyclosporin A or FK506 (Poo 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., 1991b; 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, \(\sim 0.1-0.3 \mu M\) (Halachmi and Eilam, 1989; Ohyaa 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 (Har 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 (Carafoi, 1992).

This study reports the identification of the PMCI gene, which encodes a homolog of mammalian PMCAs. Genetic analysis suggests that the product of PMCI (Pmclp) transports Ca\(^{2+}\) into the vacuole and participates in the control of [Ca\(^{2+}\)]\(_{c}\) together with Pmrlp. High external Ca\(^{2+}\) inhibits the growth of pmcl 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 PMCI 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 DH5\(\alpha\) grown in Luria Broth medium with appropriate antibiotics (Maniatis et al., 1982). Protocols used for polymerase chain reaction (Boehhringer-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 PMCI gene was identified by PCR amplification of genomic DNA from the pmrl::LEU2 pmr2::URA3 strain LA420 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 (CCCGATCGTATNTGGWSVGAYAARACNGGNA) and B1 (CGGAAATGSGRTTNNRNYCRCRTCNCCG) produced a novel 1.2-kb product distinct from the 0.8-kb product from PMCl and PMA2 (Serrano et al., 1986; Schlessner 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 PMCI locus near the PMAI gene on chromosome VII (see Fig. 1) by hybridization to an ordered library of genomic DNA (Riles et al., 1993). The entire PMCI locus was subcloned from plasmid B157 containing PMAI (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 ExoI H/ExoVII nuclease digestion according to manufacturer’s specifications (GIBCO-BRL, Gaithersburg, MD).

The integrating plasmid pKC55, used to create a chromosomal PMCI:: HA epitope-tagged allele, was constructed by transferring a 1.7-kb fragment of pKC45 (containing the 5’ end of PMCI) 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

---

The Journal of Cell Biology, Volume 124, 1994 352
DNA sequencing, linearized by digestion with CiaI, and integrated into the chromosomal PMCl locus leaving full-length PMCl::HA adjacent to a truncated pmcl::URA3 allele. Plasmid pKC60 contains the complete epitope-tagged PMCl::HA gene (from HindIII to BamHI 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 PMCl::HA alleles produced immunoreactive polypeptides and functionally complemented pmcl mutations for growth in high Ca2+ media. The plasmid pKCl1 contains the PMRI locus (4.4-kb PvuII to SpeI fragment) from pL149 (Rudolph et al., 1989) inserted into B2205 (2 μm URA3) digested with SmaI plus XbaI.

The plasmids pKC52 and pKCl9 used to replace the chromosomal PMCl gene with pmcl::LEU2 and pmcl::TRPl, 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 pKC58 allele. Plasmid pKC60 contains the complete epitope fusion protein. The pKC74 allele was restored in pKC74 by replacing the 3' segment from SalI to HindIII in the 3' noncoding region. Both pKC73 and pKC74 complemented cnal cn2 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 CaCl2. 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 CaCl2 or SC-Leu-Trp plus 400 mM CaCl2. 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 cmdl, defines the CNBl gene (Cytet and Thorner, 1992). The cmd2 and cmd3 groups (11 and 3 members, respectively) are not complemented by pTD59 containing the CMDI gene (Geiser et al., 1991) and will be described in future reports.

Table 1. List of Yeast Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1A</td>
<td>MATa</td>
<td>Wallis et al., 1989</td>
</tr>
<tr>
<td>W303-1B</td>
<td>MATa</td>
<td>Wallis et al., 1989</td>
</tr>
<tr>
<td>JGY41</td>
<td>MATa cmdl-3</td>
<td>Geiser et al., 1991</td>
</tr>
<tr>
<td>JGY48</td>
<td>MATa cmdl-6</td>
<td>Geiser et al., 1991</td>
</tr>
<tr>
<td>K444</td>
<td>MATa pmcl::His3</td>
<td>W303-1A</td>
</tr>
<tr>
<td>K445</td>
<td>MATa cmdl-6 pmcl::His3</td>
<td>JGY148</td>
</tr>
<tr>
<td>K470</td>
<td>MATa PMCl::HA (pmcl::URA3)</td>
<td>W303-1A</td>
</tr>
<tr>
<td>K473</td>
<td>MATa pmcl::LEU2</td>
<td>W303-1A</td>
</tr>
<tr>
<td>K473-a7</td>
<td>MATa pmcl::LEU2 cnal-1</td>
<td>K473</td>
</tr>
<tr>
<td>K482</td>
<td>MATa pmcl::TRP1</td>
<td>W303-1B</td>
</tr>
<tr>
<td>K484</td>
<td>MATa pmcl::LEU2 fprl::URA3</td>
<td>K473</td>
</tr>
<tr>
<td>K510</td>
<td>MATa pmcl::LEU2 cmdl-3</td>
<td></td>
</tr>
<tr>
<td>K541</td>
<td>MATa pmcl::LEU2 cnal::His3</td>
<td></td>
</tr>
<tr>
<td>K547</td>
<td>MATa pmcl::LEU2 cnal::G</td>
<td></td>
</tr>
<tr>
<td>K559</td>
<td>MATa pmcl::LEU2 cnal::HisG cnal::His3</td>
<td></td>
</tr>
<tr>
<td>K603</td>
<td>MATa cnal::LEU2</td>
<td></td>
</tr>
<tr>
<td>K605</td>
<td>MATa pmcl::TRP1</td>
<td></td>
</tr>
<tr>
<td>K607</td>
<td>MATa pmcl::TRP1 cnal::LEU2</td>
<td></td>
</tr>
<tr>
<td>K610</td>
<td>MATa pmcl::His3</td>
<td></td>
</tr>
<tr>
<td>K612</td>
<td>MATa pmcl::His3 cnal::LEU2</td>
<td></td>
</tr>
<tr>
<td>K614</td>
<td>MATa pmcl::LEU2 pmcl::TRP1</td>
<td></td>
</tr>
<tr>
<td>K616</td>
<td>MATa pmcl::His3 pmcl::TRP1 cnal::LEU2</td>
<td></td>
</tr>
</tbody>
</table>

* All strains harbor the following additional mutations: ade2-1 can1-100 his3-11,15 leu2-3,112 trpl-1 ura3-1.
‡ Strains K444 through K484 were constructed by transformation of the source strain. Strains K510 through K616 were produced by crosses involving derivatives of W303-1A, W303-1B, and JGY41 which contained mutations in cnal, cn2, 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 (Johnston 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 4\(^{Ca}\)Cl\(_2\) (Amersham Corp., Arlington Heights, IL) were used to supplement growth medium. FK506 was a generous gift from Vertex Pharmaceuticals.

**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\(^{2+}\) transmembrane 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 PMCl 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 Pmclp and replaced by a much smaller COOH-terminal domain lacking significant similarity to other proteins in current databases. Despite these structural differences, PMCl appears to retain all conserved features of P-type ATPases and is the first reported fungal homolog of PMCA.

The product of PMCl is expressed during exponential growth as judged by immunoblotting (Fig. 3) and immunofluorescence microscopy (Fig. 4). For these experi-
Figure 4. Immunofluorescence localization of Pmc1p 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 PMCI::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 PMCI::HA gene product was visualized using the 12CA5 monoclonal antibody, which recognizes a 34-amino acid insertion at codon +2 in the PMCI open reading frame (Fig. 1 C). This epitope-tagged version of PMCI 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 PMCI::HA gene product is localized predominantly to vacuole membranes in log-phase cells (Fig. 4). Cells producing the PMCI::HA product from its chromosomal locus displayed staining of one to three large vesicular structures corresponding to vacuoles by Nomarski optics. Expression of PMCI::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 PMCI 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).

**Pmclp Sequesters Ca$^{2+}$ In Vivo**

The effects of mutating the chromosomal PMCI gene were examined to infer its function in yeast. The PMCI gene is not essential for viability since deleting 97% of the PMCI 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 PMCI does not noticeably affect mating, sporulation, starvation, or a variety of other responses. These observations show that the function of PMCI is not required for the execution of many processes under standard conditions.

The effect of deleting PMCI on steady state Ca$^{2+}$ pools suggests that Pmclp 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, 1982a,b). Accordingly, the exchangeable and nonexchangeable Ca$^{2+}$ pools were measured in exponentially growing PMCI and pmcl cells. The exchangeable Ca$^{2+}$ pool was increased only slightly (12%) in pmcl cells relative to wild-type cells. However, the nonexchangeable Ca$^{2+}$ pool in pmcl 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 pmcl mutants, and suggests that Pmclp is necessary for efficient Ca$^{2+}$ sequestration in vivo.

**Figure 5**. Ca$^{2+}$ compartmentalization in wild-type, pmcl, and pmcl cnbl strains. The exchangeable (black) and nonexchangeable (striped) pools of cells associated Ca$^{2+}$ were measured in strains W303-IA (WT), K605 (pmc/), and K607 (pmcl cnbl) 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 pmcl mutants in high Ca$^{2+}$ media. Cultures of W303-IA (WT), K603 (cnbl), K605 (pmc/), and K607 (pmcl cnbl) 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 pmcl mutants growing for 24 h in YPD medium (top) and in YPD+200 mM Ca$^{2+}$ (center) and of W303-IA growing for 24 h in YPD+200 mM Ca$^{2+}$ (bottom).
**PMCl Is Required for Ca²⁺ Tolerance**

As pmcl mutants have decreased ability to sequester Ca²⁺ in the vacuole, we tested their ability to tolerate very high or very low Ca²⁺ concentrations in the growth medium. Wild-type and pmcl null mutants are indistinguishable during growth in synthetic media containing <1 µM Ca²⁺ (Rudolph et al., 1989) or in complex YPD media containing varying amounts of EGTA (not shown). However, pmcl null mutants grow very poorly in YPD medium, pH 5.5, supplemented with 200 mM CaCl₂ (Figs. 6 and 7). The growth defect of pmcl mutants was not observed in YPD, pH 5.5, supplemented with 200 mM MgCl₂ (Fig. 7). Additionally, Ca²⁺ sensitivity was not observed in strains expressing only the epitope-tagged PMCl::HA hybrid from its chromosomal locus or from high copy plasmids (not shown). These observations demonstrate that PMCl is necessary for tolerance to high Ca²⁺ and confirm the importance of the vacuole in yeast Ca²⁺ homeostasis (Klionsky et al., 1990; Anraku et al., 1991).

In liquid growth medium supplemented with 200 mM Ca²⁺, wild-type strains grew exponentially after a brief lag (Fig. 6 A). In contrast, pmcl mutants shifted to high Ca²⁺ 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 pmcl 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 MgCl₂. Though the morphology of pmcl cells changed somewhat during incubation in high Ca²⁺ (Fig. 6 B), they did not accumulate with a uniform morphology. Thus, the growth inhibition of pmcl mutants by high Ca²⁺ is reversible and apparently not a consequence of a specific block in the cell division cycle.

**Calcineurin Mediates Growth Inhibition of pmcl Mutants in High Ca²⁺ Medium**

The mechanism by which external Ca²⁺ inhibits the growth of pmcl mutants was investigated by characterizing spontaneous revertants carrying secondary mutations that restore the ability of pmcl null mutants to proliferate in high Ca²⁺ media (see Materials and Methods). Recessive mutations in three complementation groups were found to reverse the Ca²⁺ sensitivity of pmcl mutants. The first group of revertants (termed crml for Ca²⁺ resistant mutation) was found to define the CNBI gene which encodes the Ca²⁺ binding regulatory subunit of calcineurin (Kuno et al., 1991; Cyert and Thorner, 1992). The crml alleles displayed a defect in recovery from pheromone arrest similar to cnbl null mutants (Cyert and Thorner, 1992), were genetically linked to the CNBI locus (44:0:0, parental ditype/non-parental ditype/tetraplotype), and were complemented by plasmids carrying the wild-type CNBI gene. Additionally, disruption of CNBI by gene replacement restores growth of pmcl mutants (Fig. 7). Growth of pmcl 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 Ca²⁺ sensitivity of pmcl. By this assay, the CNA1 and CNA2 genes are functionally redundant, which explains why spontaneous cna1 or cna2 mutants were recovered in the initial

**Figure 7. Growth inhibition of pmcl by high Ca²⁺ 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 CaCl₂ (A) or 200 mM MgCl₂ (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 \textit{pmcl} mutants in standard media or in media supplemented with 200 mM MgCl\textsubscript{2}. Thus, growth inhibition of \textit{pmcl} mutants by high Ca\textsuperscript{2+} requires the function of both the catalytic and regulatory subunits of calcineurin.

In high Ca\textsuperscript{2+} liquid media (Fig. 6A), \textit{cnbl} and \textit{pmcl cnbl} strains grow at faster rates (1.6 h doubling time) than an isogenic wild-type strain (2.3 h doubling time). This result suggests that calcineurin activity limits the rate of yeast growth in high Ca\textsuperscript{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., 1991b; Cyert and Thorner, 1992). Interestingly, \textit{pmcl cnbl} mutants have a larger pool of nonexchangeable Ca\textsuperscript{2+} than \textit{pmcl} mutants (Fig. 5). If Ca\textsuperscript{2+} sequestration into the vacuole is improved by inactivation of calcineurin, this might explain the enhanced growth rate of \textit{pmcl cnbl} (and \textit{cnbl}) strains in high Ca\textsuperscript{2+} media.

### Activation of Calcineurin by Ca\textsuperscript{2+}/Calmodulin Is Necessary for Inhibition of \textit{pmcl} Mutants

The protein phosphatase activity of mammalian calcineurin in vitro is greatly stimulated by binding of Ca\textsuperscript{2+} and calmodulin (Stewart et al., 1982). Similarly, calmodulin binds to the CNA1 and CNA2 gene products only in the presence of Ca\textsuperscript{2+} (Cyert et al., 1991; Liu et al., 1991b). Mutant forms of calmodulin that are unable to bind Ca\textsuperscript{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 \textit{cmdl-3} strains, which express a calmodulin mutant containing two amino acid substitutions in each of

![Figure 8](https://jcb.rupress.org/)

**Figure 8.** Expression of COOH-terminal truncated CNA1 (CNA1ΔC) bypasses cmdl-3 in pmcl mutants. Strains K473 (lines 1 and 2), K510 (lines 3 and 4), K539 (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 \textit{GAL1}. Strain K510 (lines 9 and 10) was also transformed with a control plasmid pUN50, or pTD59 containing the \textit{CMD1} gene (Geiser et al., 1991). Strain K473 was also transformed with the high copy control plasmids B2205 or pKC1 containing the PMRI 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\textsubscript{2} as indicated. The plates were photographed after 3 d incubation at 30°C. High Ca\textsuperscript{2+} inhibits growth of \textit{pmcl cmdl-3} and \textit{pmcl cnal cna2} but not \textit{pmcl cnbl} mutants expressing CNA1ΔC. High Ca\textsuperscript{2+} does not inhibit growth of \textit{pmcl cmdl-3} or \textit{pmcl cnbl} mutants expressing CNA1.
the three high affinity Ca\textsuperscript{2+}-binding domains, it is possible to test whether Ca\textsuperscript{2+} binding to calmodulin is necessary for growth inhibition of pmcl mutants by high Ca\textsuperscript{2+}.

In contrast to pmcl mutants, a pmcl cmdl-3 double mutant grows well on high Ca\textsuperscript{2+} medium (Fig. 7). Introduction of a low copy plasmid carrying the wild-type CMDI gene into the pmcl cmdl-3 strain restored Ca\textsuperscript{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\textsuperscript{2+}-induced growth inhibition. Identical results were obtained with a pmcl::HIS3 cmdl-6 strain (K445), which contains only three point mutations in the CMDI gene (data not shown). Thus, a mutant with amino acid substitutions in the high affinity Ca\textsuperscript{2+} binding sites of calmodulin has the same phenotype as a mutant that has lost calcineurin function.

Ca\textsuperscript{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 CNA\textsubscript{1} truncation mutant lacking the autoinhibitory domain would become activated independent of Ca\textsuperscript{2+}/calmodulin and would prevent growth of pmcl cmdl-3 strains in high Ca\textsuperscript{2+} media. As predicted, a pmcl cmdl-3 strain expressing the truncated CNA\textsubscript{1}\Delta C allele from an inducible promoter (GAL\textsubscript{1}) fails to grow in high Ca\textsuperscript{2+} medium (Fig. 8, line 4). In contrast, expression of wild-type CNA\textsubscript{1} from this promoter does not inhibit growth of the pmcl cmdl-3 strain in high Ca\textsuperscript{2+} medium (Fig. 8, line 3). The GAL\textsubscript{1}-induced expression of CNA\textsubscript{1} and CNA\textsubscript{1}\Delta C does not inhibit growth of yeast strains in the absence of added Ca\textsuperscript{2+} (not shown). Furthermore, expression of either CNA\textsubscript{1} or CNA\textsubscript{1}\Delta C inhibits growth of a pmcl cnal cna2 strain (Fig. 8, lines 5 and 6) but not a pmcl cnbl strain (lines 7 and 8) in high Ca\textsuperscript{2+} media. Thus, removing the COOH-terminal autoinhibitory domain of calcineurin A specifically bypasses the requirement for Ca\textsuperscript{2+}/calmodulin to promote growth arrest, though calcineurin B and Ca\textsuperscript{2+} are still required for this arrest. These data suggest that the major role of Ca\textsuperscript{2+}/calmodulin in growth inhibition by Ca\textsuperscript{2+} is to activate calcineurin via its COOH-terminal region. The cmdl-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 (Poore et al., 1992). A similar complex formed in mammalian cells results in the complete inhibition of calcineurin activity towards phosphopeptide substrates (Liu et al., 1991a). These considerations prompted us to test whether FK506 would restore growth of pmcl mutants in high Ca\textsuperscript{2+} media by inhibiting yeast calcineurin.

Addition of FK506 dramatically restores growth of pmcl mutants in high Ca\textsuperscript{2+} medium, but has no effect on pmcl fprl strains lacking FKBP-12 (Fig. 9 A). In this assay, the 50% effective dosage (ED\textsubscript{50}) of FK506 was estimated at 25 ng/ml which is over 1,000 times lower than the 50% lethal dosage (LD\textsubscript{50}) of ~50 \(\mu\)g/ml (Heitman et al., 1991). There is also a direct correlation between the effectiveness of the drug and the dosage of calcineurin A genes. pmcl cnal and pmcl cna2 double mutants each required approximately half as much FK506 (12 ng/ml) when compared to the pmcl 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., 1991a), also restores growth of pmcl mutants in high Ca\textsuperscript{2+} media (Fig. 9 B). The ED\textsubscript{50} of cyclosporin A in this assay (1-2 \(\mu\)g/ml) is much lower than its LD\textsubscript{50} (~200 \(\mu\)g/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\textsuperscript{2+}/calmodulin/calcineurin forms in pmcl mutants during
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 pmcl 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 cmdl-3) is required for the inhibitory effect of calcineurin.

The Inviability of a pmrl pmcl Double Mutant Is Suppressed by Inactivation of Calcineurin

The role of PMCl in Ca\(^{2+}\) tolerance may be shared by PMRI (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 PMRI in
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 pmcl 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 pmcl strains is prevented by inactivation of calcineurin with either FK506 (400 ng/ml) or the cnbl 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 pmcl mutants by a variety of mechanisms, such as decreasing the activity of unknown factors necessary for growth or Ca\(^{2+}\) tolerance.

**Discussion**

**PMCI Encodes a Vacuolar Ca\(^{2+}\) ATPase Involved in Ca\(^{2+}\) Sequestration and Regulation of [Ca\(^{2+}\)]c**

Several lines of evidence indicate that Pmc1p transports Ca\(^{2+}\) from the cytoplasm into the vacuole. First, the predicted product of PMCI contains all the conserved features of P-type ion pumps and is highly homologous to mammalian PMCA (41% identical through 81% of its sequence). Second, the phenotypes of pmcl mutants are consistent with a direct role for Pmc1p in Ca\(^{2+}\) transport. Although pmcl 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 pmcl mutants is dramatically affected by modifications of a Ca\(^{2+}\)-sensitive regulatory pathway consisting of calmodulin and calcineurin. Finally, PMCI shares an essential function with PMRI, 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 Pmc1p (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 Pmc1p, the residual Ca\(^{2+}\) sequestration can be attributed to the vacuolar antiporter and/or Pmr1p in the Golgi complex. PMRI is required for viability of pmcl mutants and overexpression of PMRI restores Ca\(^{2+}\) tolerance to pmcl mutants. The simplest interpretation of these findings is that Pmc1p and Pmr1p 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 Pmc1p 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 pmcl Mutants**

Our results show that the inviability of pmcl strains in high Ca\(^{2+}\) media requires calcineurin activation by Ca\(^{2+}\)/calmodulin. The growth defects of pmcl 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 pmcl cmdl-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 autoinhibitory activity by the COOH-terminal domain of the catalytic A subunit.

The clear effect of inactivating calcineurin in pmcl 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., 1991a; Foor et al.,
1992). As demonstrated here, both of these compounds mimic the effect of calcineurin mutations, restoring growth to pmcl mutants in high Ca²⁺ 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 Ca²⁺ media.

Calcineurin activation also occurs in standard media in pmcl pmrl double mutants, and therefore is not restricted to conditions of high external Ca²⁺. Calcineurin-dependent growth inhibition can be observed in pmcl pmrl strains in media containing 0.18–200 mM Ca²⁺. Because Pmclp and Pmrp function in Ca²⁺ sequestration, their shared essential function (in >0.18 mM Ca²⁺) is most likely to maintain sufficiently low [Ca²⁺]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 Ca²⁺ environments (Fig. 6) or in response to natural Ca²⁺ signals. Transient Ca²⁺ signals might be generated during some situations by the physiological modulation of Pmclp and Pmrp activities or other Ca²⁺ 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 (Cryer et al., 1991; Cryer 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 Ca²⁺ 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 Ca²⁺ (Fig. 6) and decreases sequestration of Ca²⁺ into the nonexchangeable (vacuolar) pool (Fig. 5). It is unlikely that the latter effect is due to increased influx or decreased efflux of Ca²⁺ since both these models would predict decreased tolerance to external Ca²⁺. An alternative explanation is that another enzyme involved in Ca²⁺ transport into the vacuole, such as the H⁺/Ca²⁺ 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 Ca²⁺ 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 Ca²⁺-signaling mechanisms and responses in human cells.

We would like to acknowledge all members of the lab for excellent advice and discussions, especially Adam Antebi, Hans Rudolph, Steven Kron, and Amy Chang. We thank Per Ljungdahl, David Pellman, and Judy Bender for critically reading the manuscript. We are grateful to Trisha Davis, Martha Cyert, and Joe Heitman for generously providing plasmids and/or yeast strains that were important to this study and to Harvey Lodish and Vertex Pharmaceuticals, Inc., for supplying samples of cyclosporin A and FK506, respectively.

K. W. Cunningham was supported by grant PF-3472 from the American Cancer Society. G. R. Fink is American Cancer Society Professor of Genetics. This work was supported by National Institutes of Health grant GM-40266.

Received for publication 23 September 1993 and in revised form 8 November 1993.

References


