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Ca^{2+}-Calmodulin Promotes Survival of Pheromone-Induced Growth Arrest by Activation of Calcineurin and Ca^{2+}-Calmodulin-Dependent Protein Kinase

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The *cmd1-6* allele contains three mutations that block Ca^{2+} binding to calmodulin from *Saccharomyces cerevisiae*. We find that strains containing *cmd1-6* lose viability during cell cycle arrest induced by the mating pheromone α-factor. The 50% lethal dose (LD_{50}) of α-factor for the calmodulin mutant is almost fivefold below the LD_{50} for a wild-type strain. The calmodulin mutants are not more sensitive to α-factor, as measured by activation of a pheromone-responsive reporter gene. Two observations indicate that activation of the Ca^{2+}-calmodulin-dependent protein phosphatase calcineurin contributes to survival of pheromone-induced arrest. First, deletion of the gene encoding the calcineurin regulatory B subunit, *CNB1*, from a wild-type strain decreases the LD_{50} of α-factor but has no further effect on a *cmd1-6* strain. Second, a dominant constitutive calcineurin mutant partially restores the ability of the *cmd1-6* strain to survive exposure to α-factor. Activation of the Ca^{2+}-calmodulin-dependent protein kinase (CaMK) also contributes to survival, thus revealing a new function for this enzyme. Deletion of the *CMK1* and *CMK2* genes, which encode CaMK, decreases the LD_{50} of pheromone compared with that for a wild-type strain but again has no effect in a *cmd1-6* strain. Furthermore, the LD_{50} of α-factor for a mutant in which the calcineurin and CaMK genes have been deleted is the same as that for the calmodulin mutant. Finally, the CaMK and calcineurin pathways appear to be independent since the ability of constitutive calcineurin to rescue a *cmd1-6* strain is not blocked by deletion of the CaMK genes.

The mating pheromone response pathway in *Saccharomyces cerevisiae* is one of the best-understood signal transduction pathways today. Genetics and biochemistry have led to elucidation of the major components required for transduction of a signal from the cell surface to the nucleus. Secreted peptide pheromone binds to a G-protein-coupled receptor on the surface of a cell of the opposite mating type. Pheromone binding to receptor stimulates dissociation of the G-protein α subunit from the β and γ subunits. This dissociation activates a mitogen-activated protein (MAP) kinase cascade, resulting in the activation of two MAP kinases. MAP kinase activation results in transcriptional and morphological changes, including inactivation of a complex containing G_1 cyclins and the yeast Cdc28 protein kinase. Inactivation of this complex results in a reversible arrest at the G_1 phase of the cell cycle (for reviews, see references 1 and 23).

While the cascade required to induce cell cycle arrest has been worked out in detail, escape from pheromone-induced cell cycle arrest is less well understood. Resumption of cell division following arrest is a function of at least four processes: sensitivity, recovery, adaptation, and survival. Sensitivity to pheromone is the level of cellular response elicited by a given amount of α-factor. Mutations in the *SST2* gene, which encodes a protein of unknown function, make *S. cerevisiae* supersensitive to pheromone (6, 8). Mutations in the third intracellular loop of the α-factor receptor, Ste2p, decrease sensitivity to pheromone (44).

Recovery is the ability of cells to quickly resume growth when the pheromone signal has been removed. Wild-type yeast cells of the **a** mating type resume growth simultaneously following a transient treatment with α-factor. This property has been widely exploited as the method of choice for isolation of populations of yeast cells proceeding through synchronous cycles of cell division. There are at least four known mechanisms for recovery. Bar1p, a secreted protease that specifically degrades α-factor, is required for recovery (6, 8, 26). A second pathway is mediated by the C-terminal cytoplasmic tail of the α-factor receptor, Ste2p (22, 38), which acts in conjunction with Afr1p (21). This pathway may be modulated by phosphorylation by analogy with the arrestin–β-adrenergic receptor system of desensitization in mammalian cells (24, 38). A third pathway involves the third intracellular loop of Ste2p, which may act through Sst2p (7, 44). Finally, mutations that prevent phosphorylation of Ste4p, the G protein β subunit, confer a defect in recovery independently of Sst2p or the C terminus of Ste2p (9).

Adaptation, or desensitization, is a process that allows cells treated continuously with a pheromone to eventually ignore the transduced signal elicited by pheromone binding at the cell surface. Thus, yeast cells exposed to a constant level of α-factor will become resistant to pheromone and will resume growth even in the presence of peptide (31). Although adaptation occurs in the presence of pheromone, some of the same components may be required to reverse the cell cycle block both in the presence and in the absence of pheromone. For example, both adaptation and recovery require the *SST2* gene product (2, 7).

Survival of exposure to pheromone differs from the ability to recover from arrest. Arrested cells are alive, will produce a shmoo, and will continue to increase in volume. Thus, if a cell is arrested but is somehow defective in recovery or adaptation, it will continue metabolism and growth even if it cannot prop-

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TABLE 1. Yeast strains

Strain name	Genotype	Source or reference
BCY66	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cnb1Δ1::LEU2*	This study
CRY1	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100*	Robert Fuller
JGY149	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmd1-6*	This study
MMY9	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cna1::LEU2 cna2::URA3*	This study
MMY18-3C	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmd1-6 cnb1Δ1::LEU2*	This study
MMY22	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 FUS1:lacZ:TRP1*	This study
MMY23	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cnb1Δ1::LEU2 FUS1:lacZ:TRP1*	This study
MMY25	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmd1-6 FUS1:lacZ:TRP1*	This study
MMY34-12D	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmd1-6 cna1::LEU2 cna2::URA3*	This study
MMY41-10B	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 CNA2ΔC*	This study
MMY44-6C	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmd1-6 CNA2ΔC*	This study
MMY71	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmk1Δ1::HIS3 cmk2::TRP1*	This study
MMY72	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmk1Δ1::HIS3 cmk2::TRP1 cnb1Δ1::LEU2*	This study
MMY75	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmd1-6 cmk1Δ1::HIS3 cmk2::TRP1*	This study
MMY76	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmd1-6 cmk1Δ1::HIS3 cmk2::TRP1 cnb1Δ1::LEU2*	This study
MMY77	*MAT***a** *ade2-1^{oc} leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 cmd1-6 cmk1Δ1::HIS3 cmk2::TRP1 CNA2ΔC*	This study
MPY62	*MAT***a** *ade2-101 his3-Δ300 leu2-Δ1 lys2-801a trp1-Δ63 ura3-52 cmk1Δ1::HIS3 cmk2Δ2::TRP1*	36

erly reenter the cell cycle. Cells that lose viability after exposure to pheromone are incapable of any growth or metabolism. Ca^{2+} is one factor required for survival of pheromone-induced arrest. Thirty to forty minutes after treatment with α-factor the cytoplasmic concentration of Ca^{2+} increases. External Ca^{2+} is required for the increased cytoplasmic Ca^{2+} and for survival because cells treated with pheromone in Ca^{2+}-depleted medium show no increase and lose viability (20). Two recently identified genes, *MID1* and *MID2*, also appear to be required for cells to survive exposure to α-factor and may be a part of the pathway involving Ca^{2+} influx (18, 35).

The *S. cerevisiae* homologs of the mammalian Ca^{2+}-calmodulin-dependent phosphatase calcineurin are required for some aspect of survival of or recovery from pheromone-induced arrest. In a halo assay, the halos that are formed on strains deficient in calcineurin do not fill in as well as those formed on wild-type strains (12, 13). The same result is observed if calcineurin is inhibited with either FK506 or cyclosporin (14). Therefore, we expected that a Ca^{2+}-binding-site mutant of yeast calmodulin should not be able to activate yeast calcineurin and thus would confer a defect in survival of or recovery from exposure to pheromone. Here, we show that while Ca^{2+}-calmodulin is required to activate calcineurin, it is also required to activate the Ca^{2+}-calmodulin-dependent protein kinase (CaMK). Furthermore, we found that Ca^{2+}-calmodulin and its targets do not contribute to pheromone sensitivity but do contribute to the maintenance of cell viability in the continuous presence of pheromone.

MATERIALS AND METHODS

Media and reagents. Cultures of *Escherichia coli* were grown in Luria-Bertani medium (30) containing 50 μg of ampicillin per ml. Cultures of *S. cerevisiae* were grown in yeast-peptone-dextrose (YPD) or synthetic dextrose (SD) medium (40) supplemented as described previously (17). Synthetic α-factor was quantified by using an extinction coefficient of 12,240 M^{-1} cm^{-1} at 280 nm.

Plasmids. Plasmid pJG81, which was used to replace *CMD1* with *cmd1-6*, which encodes calmodulin carrying mutations D20A, D56A, and D93A, was constructed by inserting the *Bam*HI-*Sal*I fragment from pJG26 (17) containing *cmd1-6* into pTD53 (17) digested with *Bam*HI and *Sal*I.

A DNA fragment containing the *CNA2ΔC* gene, encoding Cna2p with C-terminal residues 501 to 604 (comprising the calmodulin and autoinhibitory domains) removed, was synthesized by PCR and ligated into the pGEM-5Zf(+) T vector (Promega), creating pMM112. A 5′ primer (GGTCATGACTTCAGA CGCTATAAG) containing a *Bsp*HI site at the initiating ATG codon and a 3′ primer (GAAGTACGTACGGTGGAAATAGGAGCTTTCTCTAATGTTCG TCATCTAAAATGGAAGC) converting a *Bsr*GI site to a *Bsi*WI site were used for amplification. A 252-bp *Bsr*GI-*Sph*I fragment from plasmid pHT3 (25) containing the *CNA2* 3′ noncoding region was joined to the *CNA2ΔC* fragment in pMM112 cut with *Bsi*WI and *Sph*I, creating pMM119. Plasmid pMM122, which was used to replace *CNA2* with *CNA2ΔC*, was made by inserting the 2.0-kb *Spe*I-to-*Sac*I fragment of pMM119 containing *CNA2ΔC* into pRS306 (41) cut with *Spe*I and *Sac*I.

Strains. The strains used in this study are listed in Table 1. BCY66 was made by integrating the *cnb1Δ1::LEU2* allele, encoded on the 3.8-kb *Bam*HI-*Pst*I fragment of pCNB1::LEU2 (13), into CRY1. Proper integration of *cnb1Δ1::LEU2* was confirmed by Southern blot analysis. JGY149 was made by two-step gene replacement (3) by using strain CRY1 and plasmid pJG81 cut with *Sph*I. Proper integration of *cmd1-6* was confirmed by Southern blotting, PCR, and DNA sequence analysis. MMY9 was made by integrating the 4.8-kb *Bam*HI-*Hin*dIII fragment of pYL3 containing *cna1::LEU2* and the 2.3-kb *Eco*RI fragment of pHT3 containing *cna2::URA3* (25) into CRY1 (W303 background). Proper integration of *cna1::LEU2* and *cna2::URA3* was confirmed by Southern blot analysis. MMY18-3C was isolated as a *MAT***a** Leu^+ non-Ts segregant of diploid MMY18 constructed by mating JGY149 to TDY123-7C (*cmd1-3 cnbΔ1::LEU2*). Plasmid pFL-TRPa2 (gift of P. Pryciak), containing a *FUS1-lacZ* fusion and *TRP1*, was cut with *Sph*I and integrated at *FUS1* in strains CRY1, BCY66, and JGY149, creating strains MMY22, MMY23, and MMY25, respectively. MMY34-12D, containing *cmd1-6*, *cna1::LEU2*, and *cna2::URA3*, was isolated as a *MAT***a** Leu^+ Ura^+ non-Ts spore from diploid MMY34 constructed by mating JGY149 to TDY124-5C (*cmd1-3 cna1::LEU2 cna2::URA3*). The wild-type *CNA2* gene was replaced with a calmodulin-independent allele of calcineurin, *CNA2ΔC*, in strain MMY39 by a two-step gene replacement (3) in which strain EHY2 (*kex2::LEU2 cmd1-3*) was transformed with pMM122 cut with *Xba*I. Proper integration of *CNA2ΔC* was confirmed by Southern blot analysis. MMY39 was mated to TDY124-9A (*CNA1 cna2::URA3*), creating diploid MMY41. A *MAT***a** Leu^- Ura^- non-Ts spore containing *CNA2ΔC* was isolated and called MMY41-10B. A Leu^- Ura^- Ts spore was also isolated; this spore, called MMY41-7A, contained *cmd1-3* and *CNA2ΔC*. MMY41-7A was mated to MMY34-12D, creating diploid MMY44. A *MAT***a** Leu^- Ura^- non-Ts spore containing *CNA2ΔC* and *cmd1-6* was isolated and called MMY44-6C.

Strains MMY71, MMY72, MMY75, MMY76, and MMY77, containing *cmk1Δ1::HIS3* and *cmk2::TRP1*, were made as follows. The *cmk1Δ1::HIS3* allele was amplified by PCR from strain MPY62 (36) with primers GCCATGGACG ACAAAGTTTCAG and CTCTTACAGGCTGCATTTCG. A 3.5-kb *Bam*HI-*Sph*I fragment was excised from plasmid pJOY213-22T; this plasmid carried the *cmk2::TRP1* allele, which contained *TRP1* inserted at the *Nsi*I site of *CMK2* (gift of Y. Ohya). These two DNA fragments were used together to transform CRY1, BCY66, JGY149, MMY18-3C, and MMY44-6C to histidine and tryptophan prototrophy. Proper integration of *cmk1Δ1::HIS3* and *cmk2::TRP1* was confirmed by both PCR and Southern blot analysis.

α-Factor halo assay. *MAT***a** cells from logarithmic-phase cultures in YPD at 30°C were diluted to 2.5×10^6 cells per ml in YPD top agar. Synthetic pheromone was added to sterile filter discs placed on the surface of the agar. Halos were allowed to develop at 21°C and were photographed after 2 or 3 days.

LD_{50} determination for α-factor. Cultures of *MAT***a** yeast strains were grown until they reached a density of 2.5×10^7 cells per ml in YPD at 30°C as determined by cytometry with a Multisizer II (Coulter). Aliquots were sonicated and then plated in triplicate at a 10^{-5} dilution onto freshly prepared 10-ml YPD plates containing various amounts of synthetic α-factor. The fraction of viable cells (in CFU per milliliter) at each concentration of pheromone was calculated by normalizing the mean viable count from the pheromone-containing plates to the mean viable count from six YPD plates containing no pheromone. Plates

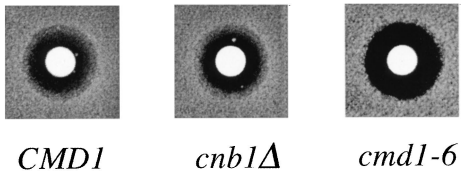

FIG. 1. Mating pheromone halo assay. *MAT***a** strains of the genotypes shown were exposed to 5 nmol of synthetic α-factor at 21°C in YPD top agar. Halos were photographed after 2 days of incubation. Strains corresponding to the genotypes are as follows: *CMD1*, CRY1; *cnb1*Δ, BCY66; and *cmd1-6*, JGY149.

were incubated at 30°C for 3 days. Longer incubations did not result in any additional colonies. A 50% lethal dose (LD_{50}) was calculated by determining the equation of a line from the nearest datum point greater than 50% viability to the nearest datum point less than 50% viability. If one datum point fell extremely close to 50% viability, the three datum points nearest to 50% viability were used in a linear regression to determine the equation of a line spanning 50% viability. The mean LD_{50}s and standard deviations of experiments performed in at least triplicate are reported.

CFU of cultures in liquid media containing α-factor. Cells from logarithmic-phase cultures grown in YPD at 30°C were diluted from 2.5×10^7 to 2.5×10^2 cells per ml in 30°C YPD containing 10, 40, or 160 nM synthetic α-factor. Initially and after 10 h, 250 μl of cells was withdrawn, sonicated, and then plated for viability in quadruplicate on YPD plates. Plates were incubated at 30°C for 3 days.

Viability of cells after exposure to pheromone. Cells from logarithmic-phase cultures grown in YPD at 30°C were diluted from 10^7 to 10^5 cells per ml in 30°C YPD containing 500 nM synthetic α-factor. Increased cell density necessary for cytometry favors Bar1p activity and required a higher concentration of pheromone to maintain arrest of wild-type cells. Initially and after 5 h, cells were washed in SD complete medium and then stained in SD complete medium containing 100 μg of methylene blue per ml. The number of cells stained with methylene blue was determined by examination with phase optics on a BH-2 microscope (Olympus) at a magnification of ×400. Aliquots of cells were fixed with formaldehyde for cell size determination by cytometry with a Multisizer II (Coulter). The median log diameter of a sphere with a volume equivalent to that of a measured cell was reported. Viability was also determined by plating a 5×10^2 dilution on YPD plates grown at 30°C.

RESULTS

Halo assay. The *cmd1-6* allele encodes an alanine substituted for the aspartate in the first position of the three functional Ca^{2+}-binding sites of yeast calmodulin (17). A strain carrying *cmd1-6* had a severe defect in recovery as determined by a mating-pheromone halo assay. The *cmd1-6* cells arrested in response to α-factor and halos formed. However, the halos did not fill in and the edges stayed sharp and well defined even after several weeks (Fig. 1). In contrast, the halos that formed on the wild-type strain began to fill in almost immediately. The halo assay gave more subtle results for the *cnb1*Δ mutant, in which the *CNB1* gene, encoding the regulatory subunit of calcineurin, is deleted. Under our conditions (rich medium with cells in the logarithmic phase of growth), the defect conferred by the loss of *CNB1* was difficult to detect and never appeared as severe as that conferred by *cmd1-6* (Fig. 1).

Plating survival assay. Because of the inherent difficulty in quantifying how quickly a halo fills in, we developed a plating assay to measure the ability of a single cell to survive and adapt to a uniform concentration of pheromone. Cells were spread on plates made with medium containing increasing concentrations of α-factor. Cells that survive and adapt to pheromone can escape growth arrest and form a colony. The calmodulin mutant carrying *cmd1-6* formed colonies only at concentrations of α-factor up to 80 nM. In contrast, a strain carrying *cnb1*Δ formed colonies at up to 200 nM α-factor, while a wild-type strain formed colonies at up to 900 nM α-factor (Fig. 2).

The trends observed with the pheromone plating assay are very reproducible, as demonstrated by determination of the

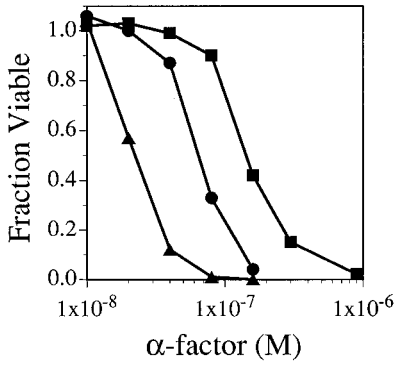

FIG. 2. Plate assay for survival of exposure to pheromone. Cells from logarithmic-phase cultures were plated on solid YPD media containing different concentrations of synthetic α-factor. The CFU at each concentration of pheromone was determined and normalized to the CFU on medium containing no pheromone. ■, wild-type *CMD1* strain CRY1; ●, strain BCY66, containing the *cnb1*Δ::*LEU2* mutation; ▲, *cmd1-6* mutant strain JGY149.

LD_{50} of α-factor for various strains by plating assays performed in at least triplicate (Fig. 3). The α-factor LD_{50} for the calmodulin mutant carrying *cmd1-6* is 31 ± 3 nM, the LD_{50} for a *cnb1*Δ strain is 58 ± 6 nM, and that for a wild-type strain is 139 ± 22 nM (Fig. 3A).

One possible explanation of the more severe defect conferred by *cmd1-6* is that the deletion of the calcineurin regulatory subunit gene does not completely inactivate calcineurin, whereas the calmodulin mutant does. However, the LD_{50} for a strain containing deletions of both catalytic subunit genes, *CNA1* and *CNA2*, is 51 ± 1 nM, a value similar within the error of the experiment to that conferred by deletion of *CNB1* (Fig. 3A). Thus, as previously described (10, 13), the *cnb1*Δ mutation inactivates calcineurin.

The more severe phenotype of the *cmd1-6* mutant suggests that calmodulin performs either a function exclusive of that of calcineurin or an additional function besides that of calcineurin. If calmodulin has a function different from that of calcineurin, then the defects conferred by the calmodulin mutant and the calcineurin mutant should be additive. Instead, deletion of *CNB1* or deletion of *CNA1* and *CNA2* from a strain containing *cmd1-6* does not further exacerbate the defect conferred by the *cmd1-6* mutation alone (Fig. 3A). The LD_{50}s of α-factor for strains containing either *cmd1-6* and *cnb1*Δ or *cmd1-6* and *cna1*Δ *cna2*Δ are 29 ± 2 nM and 28 ± 2 nM, respectively (Fig. 3A), values that are no different from the LD_{50} observed for the *cmd1-6* mutant alone. Thus, Ca^{2+}-calmodulin is required to activate calcineurin but calmodulin has additional Ca^{2+}-dependent functions required for survival in the presence of pheromone.

Another known Ca^{2+}-dependent calmodulin target in yeast cells is CaMK. Deletion of the genes *CMK1* and *CMK2*, encoding yeast CaMK, is not known to confer any defect in pheromone recovery (34, 36), although their deletion interferes with heat-induced thermotolerance (19). If calcineurin and CaMK both function in survival of exposure to pheromone, the loss of either target alone would have an intermediate effect. Furthermore, the combined loss of both Ca^{2+}-calmodulin-dependent protein phosphatase and kinase activity would result in a phenotype for survival resembling that of a Ca^{2+}-binding-site mutant of calmodulin. As a test of this hypothesis, the LD_{50}s of pheromone for a series of strains containing deletions of the *CMK1* and *CMK2* genes were determined (Fig. 3B). The LD_{50} for a *cmk1*Δ *cmk2*Δ strain was 99 ±

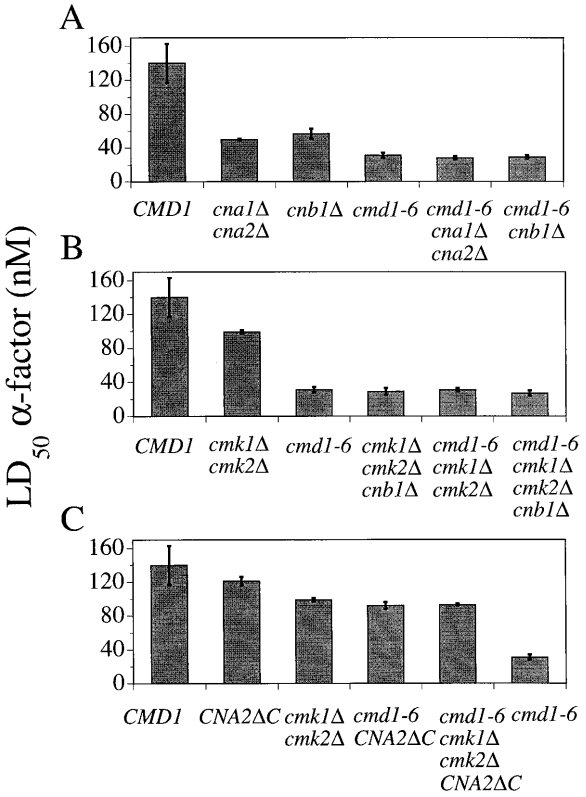

FIG. 3. Determination of LD_{50} of α-factor. Survival plating assays as shown in Fig. 2 were performed in at least triplicate, and the LD_{50}s of α-factor for strains corresponding to the listed genotypes were determined as described in Materials and Methods. Values are means ± standard deviations. The strain and number of trials corresponding to each genotype are as follows. (A) *CMD1*, CRY1, 11; *cna1*Δ *cna2*Δ, MMY9, 3; *cnb1*Δ, BCY66, 8; *cmd1-6*, JGY149, 11; *cmd1-6 cna1*Δ *cna2*Δ, MMY34-12D, 3; *cmd1-6 cnb1*Δ, MMY18-3C, 3. (B) *CMD1*, CRY1, 11; *cmk1*Δ *cmk2*Δ, MMY71, 3; *cmd1-6*, JGY149, 11; *cmk1*Δ *cmk2*Δ *cnb1*Δ, MMY72, 4; *cmd1-6 cmk1*Δ *cmk2*Δ, MMY75, 3; *cmd1-6 cmk1*Δ *cmk2*Δ *cnb1*Δ, MMY76, 3. (C) *CMD1*, CRY1, 11; *CNA2*Δ*C*, MMY41-10B, 3; *cmk1*Δ *cmk2*Δ, MMY71, 3; *cmd1-6 CNA2*Δ*C*, MMY44-6C, 3; *cmd1-6 cmk1*Δ *cmk2*Δ *CNA2*Δ*C*, MMY77, 3; *cmd1-6*, JGY149, 11.

2 nM, less than that for a wild-type strain, indicating a new function for CaMK in survival of exposure to pheromone. The LD_{50} of pheromone for a *cnb1*Δ *cmk1*Δ *cmk2*Δ mutant strain was 29 ± 4 nM, which is statistically identical to the value for the *cmd1-6* mutant, 31 ± 3 nM. Thus, the loss of both calcineurin and CaMK confers the same phenotype for survival of exposure to pheromone caused by a lack of Ca^{2+}-calmodulin. The LD_{50}s of α-factor for a *cmd1-6 cmk1*Δ *cmk2*Δ strain and a *cmd1-6 cnb1*Δ *cmk1*Δ *cmk2*Δ strain were measured and found to be 30 ± 3 nM and 27 ± 2 nM, respectively. The LD_{50}s for these strains do not differ from that of the *cmd1-6* mutant alone, indicating that neither CaMK nor calcineurin functions independently of Ca^{2+}-calmodulin. Thus, Ca^{2+}-calmodulin functions in survival of exposure to pheromone by activating both CaMK and calcineurin.

A constitutive calcineurin mutant was used to examine the relationship between CaMK and calcineurin in survival of exposure to pheromone and to confirm the contribution of calcineurin to maintaining viability (Fig. 3C). The *CNA2* gene, encoding a calcineurin catalytic A subunit, was mutated to encode a C-terminal truncation, *CNA2*Δ*C*. Removal of the C terminus of the A subunit, containing the autoinhibitory and calmodulin-binding domains, is predicted to result in a constitutive Ca^{2+}-calmodulin-independent calcineurin phosphatase (27). The LD_{50} for a strain in which a single copy of *CNA2*Δ*C* precisely replaces the *CNA2* gene is 121 ± 5 nM, a value similar within the error of the experiment to the LD_{50} for a wild-type strain. Thus, introduction of the truncation allele *CNA2*Δ*C* has no obvious effect on an otherwise-wild-type strain. However, the LD_{50} of pheromone for a *cmd1-6 CNA2*Δ*C* strain was found to be 92 ± 4 nM, a value that is threefold higher than that for a *cmd1-6* strain. Thus, as previously suggested by plasmid overexpression (10), constitutive calcineurin can reverse the phenotype conferred by mutations in the Ca^{2+}-binding sites of calmodulin. However, the effect of constitutive calcineurin is partial, allowing only *cmd1-6* cells to survive exposure to higher concentrations of pheromone up to the level for a *cmk1*Δ *cmk2*Δ strain. Furthermore, the LD_{50} for a *cmd1-6 CNA2*Δ*C cmk1*Δ *cmk2*Δ strain is 94 ± 2 nM, identical to the LD_{50} for a strain containing only *cmd1-6* and *CNA2*Δ*C*. Therefore, the ability of the constitutively active calcineurin to rescue the *cmd1-6* strain is independent of the *CMK1* and *CMK2* genes, demonstrating that the yeast Ca^{2+}-calmodulin-dependent kinase and phosphatase perform in independent yet functionally overlapping pathways that help yeast cells survive exposure to pheromone.

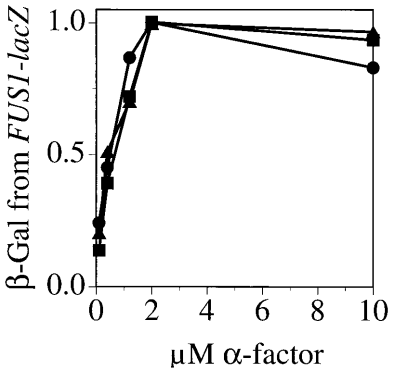

FIG. 4. β-Galactosidase (β-Gal) induction from *FUS1-lacZ* by α-factor. The sensitivities of strains to pheromone were measured with a *TRP1*-marked reporter construct. The construct contained *E. coli lacZ* fused to the *S. cerevisiae* pheromone-responsive gene *FUS1* and was integrated at *FUS1* into either *CMD1*, *cnb1*Δ, or *cmd1-6*, resulting in strains MMY22, MMY23, and MMY25, respectively. Cells in the logarithmic phase of growth in YPD at 30°C were treated with synthetic α-factor for 60 min and then assayed for β-galactosidase activity (46). Activity levels from at least two trials were normalized and then averaged. ■, wild-type *CMD1* strain MMY22; ●, strain MMY23, containing the *cnb1*Δ mutation; ▲, *cmd1-6* mutant strain MMY25.

Sensitivity to, recovery from exposure to, and adaptation to α-factor. The inability of calmodulin mutant strains to form colonies on medium containing α-factor is not caused by an increased sensitivity to pheromone. Sensitivity to α-factor was assayed by measuring β-galactosidase activity expressed from an integrated reporter gene consisting of the pheromone-responsive *FUS1* gene fused to *lacZ*. No difference in sensitivity was observed (Fig. 4).

The processes of adaptation and recovery seem to be intact in the mutant strains. Adaptation was measured by exposing wild-type and calcineurin and calmodulin mutant strains to continuous levels of pheromone in liquid medium and monitoring the percentage of unbudded cells at different times. Experiments were performed at extremely low cell densities shown to eliminate any effects from the secreted α-factor-specific protease Bar1p (31). The cells initially responded to pheromone by accumulating as unbudded cells and then were released from the block by desensitization to pheromone (Fig.

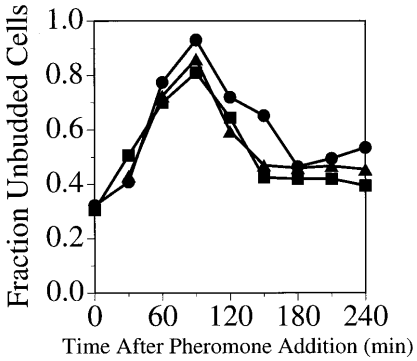

FIG. 5. Adaptation to a continuous concentration of pheromone. An adaptation assay was performed as described previously (31), with the following modifications. Strains in the logarithmic phase of growth in YPD were treated with 2 nM synthetic α-factor at 30°C at a density of 2×10^2 cells per ml. At 30-min intervals, cells were withdrawn and fixed. After concentration by filtration, cells were visually scored for the presence or absence of a bud. ■, wild-type *CMD1* strain CRY1; ●, strain BCY66, containing the *cnb1*Δ mutation; ▲, *cmd1-6* mutant strain JGY149.

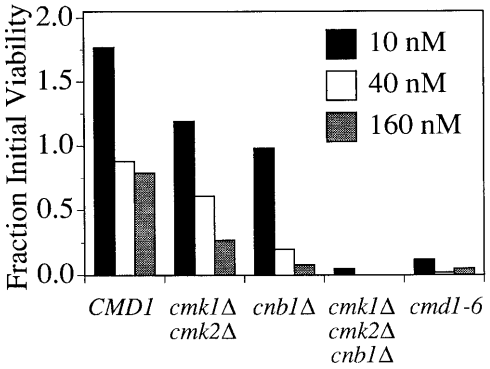

FIG. 7. Viability during continuous exposure to pheromone. Strains in the logarithmic phase of growth in YPD were treated with 10, 40, and 160 nM synthetic α-factor at 30°C at a density of 2.5×10^2 cells per ml. The titers of cells were determined for viability initially and after 10 h. The CFU after 10 h of pheromone treatment was normalized to the CFU immediately following pheromone addition. For the strains corresponding to the listed genotypes, see the legend for Fig. 3.

5). The calcineurin and calmodulin mutant strains adapted to a low concentration of pheromone as well as a wild-type strain. Recovery was assayed by monitoring the increase in CFU after pheromone withdrawal. When the data were normalized to the number of CFU present immediately following pheromone withdrawal, the *cmd1-6* strain and the strain containing the *cnb1*Δ mutation were found to have recovered with the same kinetics as a wild-type strain (Fig. 6).

Viability in α-factor. The LD_{50} of α-factor for the Ca^{2+}-binding-site calmodulin mutant carrying *cmd1-6* was fivefold-lower than that for a wild-type strain. Yet, this strain does not differ from wild-type strains in sensitivity to pheromone or in ability to adapt to the continuous presence of pheromone. The *cmd1-6* strain also appeared to recover from arrest as well as a wild-type strain. However, we observed that the calmodulin mutant culture lost viability during the pheromone treatment. Therefore, the abilities of the calmodulin, calcineurin, and CaMK mutants to survive continuous exposure to pheromone in liquid media were carefully measured and compared with that of a wild-type strain. Experiments were performed at low culture densities shown to eliminate the effects of Bar1p. Cells were exposed to 10, 40, or 160 nM pheromone for 10 h (Fig. 7). At 10 nM α-factor, wild-type cells stayed viable. Higher concentrations of pheromone had a deleterious effect on wild-type cells, resulting in lower viability at 40 and 160 nM α-factor. Strains defective in either CaMK or calcineurin survive 10 nM α-factor, but not as well as wild-type strains. At 40 and 160 nM pheromone, the CaMK and the calcineurin mutants lost viability. When mutations in CaMK and calcineurin were combined, a cumulative effect was seen. In 10 nM α-factor, only slight viability was observed, and at higher concentrations no viable cells were detected. Similar to the CaMK-and-calcineurin mutant, the calmodulin mutant had a greatly reduced viability at all concentrations of pheromone. Thus, Ca^{2+}-calmodulin functions to activate both calcineurin and CaMK to maintain yeast viability in the continuous presence of α-factor.

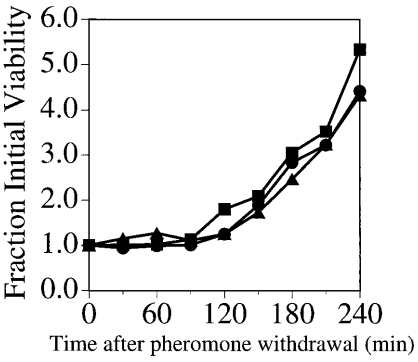

FIG. 6. Recovery from pheromone-induced arrest. Cells from logarithmic-phase cultures grown in YPD at 30°C were diluted from 10^7 to 10^5 cells per ml in 30°C YPD containing 500 nM synthetic α-factor. After 2 h the cells were collected by filtration and washed with 6 volumes of 30°C YPD. The cells were resuspended in fresh 30°C YPD, sonicated to disperse cell aggregates, and then cultured at 30°C. Initially and at 30-min intervals, cells were withdrawn and sonicated, and their titers were determined in triplicate for viability on YPD plates incubated at 30°C. The viable count (in CFU per milliliter) at each time point was normalized to the value measured immediately following pheromone withdrawal. ■, wild-type *CMD1* strain CRY1; ●, strain BCY66, containing the *cnb1*Δ mutation; ▲, *cmd1-6* mutant strain JGY149.

The observed number of CFU for the calmodulin mutant could decrease after α-factor treatment either because the cells become metabolically inactive or because the cells are permanently arrested but still metabolically active. The metabolic activity of cells in culture was determined by using the vital stain methylene blue. Methylene blue can be reduced to the colorless compound leukomethylene blue only by cells that are metabolically active (39). After 5 h of treatment with pheromone, wild-type cells maintained the ability to reduce methylene blue but *cmd1-6* cells lost the ability to reduce the dye (Table 2). A second criterion of cell viability is the ability to continue growth even when cell division is blocked. The median cell diameter of wild-type and *cmd1-6* cells was determined by cytometry. The wild-type cells continued to grow while arrested, increasing in size to almost twice their initial cell diameter, but the calmodulin mutant cell diameter increased only slightly (Table 2). Thus, the calmodulin mutant lost viability, becoming metabolically inactive.

DISCUSSION

We have developed an assay to quantify the ability of yeast cells to resume growth after treatment with mating pheromone by measuring their ability to form colonies on solid media containing different concentrations of α-factor. Using this assay, we demonstrated that Ca^{2+}-calmodulin is required to re-

TABLE 2. Viability of cells after exposure to pheromone

Genotype[a]	Without α-factor		With α-factor[b]		
	Fraction unstained[c]	Median cell diam (μm)[d]	Fraction unstained[c]	Median cell diam (μm)[d]	Fraction initial viability[e]
CMD1	0.99	3.5	0.92	6.0	1.28
cmd1-6	0.99	3.5	0.31	4.4	0.04

[a] Strains corresponding to the listed genotypes: *CMD1*, CRY1; *cmd1-6*, JGY149.

[b] Cells grown in YPD liquid at 30°C at a density of 10^5 cells per ml were treated with 500 nM synthetic α-factor for 5 h.

[c] A minimum of 200 cells stained with 100 μg of methylene blue per ml in SD complete medium were visually scored. Only dark blue cells were scored as stained; faint blue and clear cells were excluded.

[d] Cells were fixed with formaldehyde, and the median log cell diameter was determined by cytometry.

[e] Viability after pheromone treatment was determined for comparison by calculating cell titers on YPD medium.

sume growth. The LD_{50} of α-factor for mutant cells relying on calmodulin with inactivated Ca^{2+}-binding sites is almost fivefold lower than that for a wild-type strain. The low LD_{50} for the calmodulin mutant cells does not reflect an increased sensitivity to α-factor, as measured by induction of a pheromone-responsive reporter gene. Instead, the ability to resume growth after a given signal is defective. An examination of mutant cells incubated in liquid medium with α-factor revealed that the cells become metabolically inactive. The mutant cells begin to form shmoos but stay small and cannot reduce methylene blue. Under similar conditions, wild-type cells stay metabolically active because they form shmoos, continue to increase in size, and reduce methylene blue.

Determination of the contribution of Ca^{2+}-calmodulin to adaptation and recovery from pheromone-induced arrest is difficult since these conditions cause cell death. However, the calmodulin mutant is able to adapt to the continuous presence of a low concentration of pheromone (2 nM). Thus, the basic process of adaptation is intact. Furthermore, the mutant cells that do survive a 2-h treatment with pheromone recover at the same rate as a wild-type strain when the pheromone is removed. These observations lead us to conclude that calmodulin is not likely to be involved in recovery or adaptation but instead is required to survive prolonged exposure to mating pheromone.

The Ca^{2+}-calmodulin-dependent phosphatase calcineurin is required for a normal response in a mating pheromone halo assay (12, 13) and also allows cells to survive pheromone exposure in low-pH medium (45). Several results presented here suggest that calcineurin is activated by Ca^{2+}-calmodulin in order for cells to survive pheromone treatment. First, although deletion of the gene encoding the calcineurin regulatory subunit or deletion of the genes encoding the catalytic subunits decreases the LD_{50} of α-factor for a strain with wild-type calmodulin, the same deletions have no effect on a strain already carrying mutations in the Ca^{2+}-binding sites of calmodulin. Second, a constitutively activated calmodulin-independent calcineurin increases the LD_{50} of pheromone for a Ca^{2+}-binding-site calmodulin mutant. Finally, the *cnb1*Δ mutant loses viability after prolonged exposure to pheromone in liquid medium, albeit to a lesser extent than does the *cmd1-6* mutant.

However, calcineurin is not the only Ca^{2+}-calmodulin-dependent enzyme required for survival of pheromone treatment. The LD_{50} of α-factor for the calmodulin mutant is lower than that for a strain deficient in calcineurin, and expression of a calmodulin-independent calcineurin only partially rescues a calmodulin mutant. We show here that the other Ca^{2+}-calmodulin-dependent target required for survival is CaMK, thus identifying a new function for this enzyme. The LD_{50} of α-factor for a mutant lacking CaMK is lower than that for a wild-type strain. Furthermore, the LD_{50} of pheromone for a mutant lacking both calcineurin and CaMK is the same as that for a *cmd1-6* mutant. The *cmk1 cmk2* mutant also loses viability after exposure to pheromone in liquid media, although the phenotype is not as severe as it is for the *cnb1* mutant.

The two pathways for survival of exposure to pheromone defined here appear to be separate but additive. Deletion of either calcineurin or CaMK alone results in a phenotype that is less severe than that of a Ca^{2+}-binding-site calmodulin mutant. A strain deficient in both calcineurin and CaMK has a phenotype that is very similar to that of the *cmd1-6* mutant. The two pathways appear to be separate since a constitutive calcineurin mutant can partially rescue the defect in survival of exposure to pheromone in a *cmd1-6* strain, even though CaMK is presumably not active. Further confirmation of the separate nature of the CaMK and calcineurin pathways comes from the demonstration that the deletion of *CMK1* and *CMK2* has no effect on the ability of *CNA2ΔC* to suppress *cmd1-6*.

The cellular event that requires Ca^{2+}-calmodulin for survival remains to be defined. Cell cycle arrest is not sufficient to cause a loss of viability because at the low concentration of α-factor used in the adaptation assay (2 nM), the mutant cells arrest but survive. Similarly, formation of a mating projection or shmoo is not sufficient to cause loss of viability. Instead, some event that occurs only during prolonged arrest caused by exposure to higher concentrations of α-factor results in death. Our survival assay indicates that 31 nM α-factor causes 50% of the *cmd1-6* cells to lose viability on solid medium.

Iida and coworkers (20) demonstrated a requirement for external Ca^{2+} in order for cells to survive pheromone arrest. They measured an LD_{50} of α-factor of 3 μM for cultures grown in medium with a low concentration of Ca^{2+}. In contrast, we measured an LD_{50} for the *cmd1-6* mutant that was 2 orders of magnitude lower. A likely reason for the difference may be that Iida and coworkers performed their assays with dense liquid cultures that maximize the effects of the Bar1p protease. Our assays were performed at a much lower cell density on solid medium. When Iida and coworkers measured the LD_{50} for a strain lacking the Bar1p protease, they obtained a value of 100 nM, which is still higher than the 31 nM we measured for the calmodulin mutant. Perhaps the concentration of Ca^{2+} in their low-Ca^{2+} medium (240 nM) is sufficient for activation of enough calmodulin to partially protect cells against α-factor.

Calmodulin has at least two functions required during the mitotic cell cycle. Binding to the spindle pole component Spc110p is required for proper assembly of spindle pole components (42). Also, calmodulin interacts with an unconventional myosin during bud growth (5). The *cmd1-6* allele used in this study confers no defects in either spindle function or bud growth, and Cmd1-6p interacts well with both Myo2p and Spc110p (4, 5, 16, 42). Furthermore, the survival defect conferred by *cmd1-6* is mimicked by deletion of the genes encoding calcineurin and CaMK. Therefore, the Ca^{2+}-dependent function of calmodulin in surviving exposure to pheromone is independent of its two previously identified functions during the mitotic cell cycle.

The identities of targets downstream of calcineurin and CaMK are not known. The *FKS2* gene, encoding a glucan synthase, is induced by pheromone, and this induction requires calcineurin (28). However, deletion of *FKS2* does not confer pheromone-induced death (28). Furthermore, constitutive expression of *FKS2* (28) fails to rescue the defect of the *cmd1-6*

mutant in survival of exposure to pheromone (unpublished observation). Although induction of new cell wall formation is likely to be important for both shmoo and zygote formation, defects in glucan synthesis do not appear to cause cell death during pheromone-induced arrest.

Calcineurin plays an integral role in regulation of ion homeostasis. The loss of calcineurin or Ca^{2+}-calmodulin allows strains lacking *PMC1*, which encodes a plasma membrane Ca^{2+}-ATPase, to survive in medium containing high levels of Ca^{2+} (10). This may occur because calcineurin represses a vacuolar H^+/Ca^{2+} exchanger, encoded by the *VCX1*/*HUM1* gene, by a posttranslational mechanism (11, 37). Furthermore, calcineurin induces expression of the *PMC1* gene (11). Strains lacking calcineurin display decreased tolerance for Na^+, Li^+, Mn^{2+}, and OH^- (15, 29, 32, 43). Pheromone treatment causes an increase in the intracellular concentration of Ca^{2+} (20). Therefore, Ca^{2+}-calmodulin, calcineurin, and potentially CaMK may act to regulate the levels of Ca^{2+} and other ions after pheromone treatment. Perhaps a loss of normal ion homeostasis in the calmodulin mutant during prolonged incubation with uniform amounts of α-factor leads to cell death.

The *cmd1-6* mutant does not display obvious defects in mating. Normally, mating occurs within a few hours. When mating is efficient, the cell death pathway examined here may not have sufficient time to function. Also, rather than being exposed to a uniform concentration of pheromone in all directions, haploids in a mating mixture are exposed to a gradient of pheromone. Furthermore, the product of a normal mating is a diploid zygote which may have mechanisms to survive pheromone exposure different from those of a haploid cell.

In conclusion, Ca^{2+}-calmodulin is required to activate two enzymes that allow yeast cells to survive exposure to the mating pheromone α-factor. Surprisingly, one target protein is the yeast homolog of the mammalian calcineurin phosphatase, while the other is the yeast CaMK. In contrast to their roles in T-lymphocyte activation (33), in budding yeast cells calcineurin and CaMK appear to function in parallel rather than in opposing signal transduction pathways. Despite the fact that they have opposing enzymatic activities, either target alone will help promote survival during prolonged exposure to pheromone. Our results combined with those of others define a pathway whereby pheromone induces an increase in internal Ca^{2+} (20), perhaps via the *MID1* gene product (18). Increased intracellular Ca^{2+} activates calmodulin, which in turn activates both calcineurin and CaMK to promote survival.

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