Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in Saccharomyces cerevisiae

Dina P. Matheos, Tami J. Kingsbury, U. Salma Ahsan, and Kyle W. Cunningham

Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218 USA

Ca\(^{2+}\) signals regulate gene expression in animal and yeast cells through mechanisms involving calcineurin, a protein phosphatase activated by binding Ca\(^{2+}\) and calmodulin. Tcn1p, also named Crz1p, was identified as a transcription factor in yeast required for the calcineurin-dependent induction of PMC1, PMR1, PMR2A, and FKS2 which confer tolerance to high Ca\(^{2+}\), Mn\(^{2+}\), Na\(^{+}\), and cell wall damage, respectively. Tcn1p was not required for other calcineurin-dependent processes, such as inhibition of a vacuolar H\(^{+}/Ca^{2+}\) exchanger and inhibition of a pheromone-stimulated Ca\(^{2+}\) uptake system, suggesting that Tcn1p functions downstream of calcineurin on a branch of the calcium signaling pathway leading to gene expression. Tcn1p contains three zinc finger motifs at its carboxyl terminus resembling the DNA-binding domains of Zif268, Swi5p, and other transcription factors. When fused to the transcription activation domain of Gal4p, the carboxy terminal domain of Tcn1p directed strong calcineurin-independent expression of PMC1–lacZ and other target genes. The amino-terminal domain of Tcn1p was found to function as a calcineurin-dependent transcription activation domain when fused to the DNA-binding domain of Gal4p. This amino-terminal domain also formed Ca\(^{2+}\)-dependent and FK506-sensitive interactions with calcineurin in the yeast two-hybrid assay. These findings suggest that Tcn1p functions as a calcineurin-dependent transcription factor. Interestingly, induction of Tcn1p-dependent genes was found to be differentially controlled in response to physiological Ca\(^{2+}\) signals generated by treatment with mating pheromone and high salt. We propose that different promoters are sensitive to variations in the strength of Ca\(^{2+}\) signals generated by these stimuli and to effects of other signaling pathways.

[Key Words: Calcineurin; transcription; signal transduction; calcium]
Ca²⁺ influx and calcium signaling promotes cell survival mechanisms through which pheromone signaling stimulates kinases and calcineurin, which independently promote cell survival (Moser et al. 1996). The molecular mechanisms through which pheromone signaling stimulates Ca²⁺ influx and calcium signaling promote cell survival are starting to be elucidated (Iida et al. 1994; Withee et al. 1997).

The calcium signaling pathway of yeast has also been implicated as a regulator of cation homeostasis. Ca²⁺/calmodulin appears to bind and stimulate members of the Pmr2p family of P-type ion pumps (Wieland et al. 1995), which are involved in Na⁺ and Li⁺ efflux (Rudolph et al. 1989; Haro et al. 1991). Additionally, maximal induction of the PMR2A/ENA1 gene in response to high environmental salt requires calcineurin activation by Ca²⁺/calmodulin (Garciadeblas et al. 1993; Cunningham and Fink 1996). Calcineurin may further promote Na⁺ tolerance through other mechanisms (Nakamura et al. 1993; Mendoza et al. 1994; Hirata et al. 1995; Danielsson et al. 1996; Mendoza et al. 1996) and also promotes Na⁺/Ca²⁺ tolerance (Farcasanu et al. 1995; Cunningham and Fink 1996; Pozos et al. 1996). In high Ca²⁺ conditions, activation of calcineurin by Ca²⁺/calmodulin induces the expression of PMC1 and PMR1 (Cunningham and Fink 1996), which respectively encode Ca²⁺-pumping ATPases in the vacuole and Golgi complex (Rudolph et al. 1989; Antebi and Fink 1992; Cunningham and Fink 1994; Sorin et al. 1997). Finally, calcineurin activation appears to strongly inhibit the function of Vcx1p (Cunningham and Fink 1996), a vacuolar H⁺/Ca²⁺ exchanger also known as Hum1p (Pozos et al. 1996). Additional roles of calcineurin have also been detected in mutants deficient in either the vacuolar or plasma membrane H⁺ ATPases (Hemenway et al. 1995; Tanida et al. 1995; Niss et al. 1997). These transcriptional and post-translational effects of calcineurin may be mediated by a number of unidentified factors.

This work aims to identify factors that mediate the calcineurin-dependent induction of PMC1 and to determine their roles in other calcineurin-dependent processes. Through genetic and molecular approaches, we have identified Tcn1p, also called Crz1p (Stathopoulos and Cyert, this issue), as a specific transcription factor required for calcineurin-dependent induction of all the previously reported target genes plus TCN1 itself. We also show that most Tcn1p-dependent genes can be differentially induced based on mechanisms sensitive to both strength of Ca²⁺ signals and other regulatory inputs.
Calcium-dependent gene expression in yeast

Figure 1. Treatments with CaCl$_2$, pheromone, or high salt generate Ca$^{2+}$ signals that differentially induce calcineurin-dependent reporter genes. Wild-type yeast (strain W303-1A) was transformed with either plasmid pKC190 carrying PMC1-lacZ (A), plasmid pKC201 carrying PMR2A-lacZ (B), or plasmid pDM5 containing FKS2-lacZ (C), grown to mid-log phase and treated for 4 hr at 30°C in YPD (pH 5.5) medium with 0.2 µg/ml of FK506 (solid bars) or without FK506 (shaded bars) with the additional supplements of Ca$^{2+}$ (100 mM CaCl$_2$), pheromone (20 µg/ml), Na$^+$ (750 mM NaCl), or combinations thereof as indicated at the base of the plot. Each bar represents the average of three independent determinations of accumulated β-galactosidase activity (± S.D.).

(see Materials and Methods). Thirty-one independent recessive mutants were recovered from this genetic screen and placed into just three complementation groups. All members of the first group (six isolates) behaved like mutants lacking the regulatory B subunit of calcineurin (Cunningham and Fink 1994) and were allelic to cnb1 null mutants. As expected from earlier studies, no members of the second group (11 isolates) or third group (14 isolates) represented mutant alleles of calmodulin or the catalytic A subunit of calcineurin encoded by the yeast CNA1 gene (Cyert et al. 1991).

The predicted 678-amino-acid product of TCN1 contains three C2H2-type zinc finger motifs at the carboxyl terminus that strongly resemble the DNA-binding domains of numerous transcriptional regulators such as Swi5p from yeast and Zif268/early growth response (EGR)-1/Krox-24 from mammals (Fig. 2B). Outside of the zinc finger domain, Tcn1p shows no obvious sequence similarity to any proteins in current databases but contains sequence features found in many other transcriptional regulators, such as three acidic regions (net charges of −7, −23, and −10) separated by a glutamine-rich domain (residues 115–140) and a highly basic region (net charge of +13, residues 398–443) rich in serine and threonine residues (Fig. 2A). These sequence features suggest that Tcn1p may function as a specific transcription activator in the calcium signaling pathway.

Distinct domains of Tcn1p interact specifically with calcineurin

Two functional domains of Tcn1p were defined by analysis of protein fusions constructed with the Gal4p transcription factor. Fusion of the carboxy-terminal zinc finger region of Tcn1p (residues 463–678) with the transcriptional activation domain of Gal4p yielded a functional hybrid protein that strongly induced PMC1-lacZ expression relative to controls in a fashion independent of Ca$^{2+}$ and insensitive to FK506 (Fig. 3A). This constitutive Tcn1(C)::Gal4(AD) hybrid protein did not induce expression of reporter genes that are normally unresponsive to Ca$^{2+}$ signals, such as GAL1-lacZ or CYC1-lacZ but stimulated expression of PMR2A-lacZ and FKS2-lacZ (data not shown). These results show that the carboxy-terminal zinc finger domain of Tcn1p retains the ability to form promoter-specific interactions.

The amino-terminal region of Tcn1p contains a transcriptional activation domain responsive to calcineurin because fusion of residues 11–460 with the DNA-binding domain of Gal4p resulted in a Gal4(DB)::Tcn1(N) hybrid protein that conferred Ca$^{2+}$-stimulated and FK506-sensitive induction to a GAL1-lacZ reporter gene (Fig. 3B). To test whether the amino-terminal domain of Tcn1p interacts directly or indirectly with calcineurin, a two-hybrid experiment was performed using fusions between the amino-terminal domain of Tcn1p and the catalytic subunit of calcineurin encoded by the yeast CNA1 gene (Cyert et al. 1991). Expression of a functional Gal4(AB)::Cna1 hybrid protein in which the DNA-binding domain of Gal4p was fused to calcineurin A failed to induce GAL1-lacZ in standard media or in media supplemented with Ca$^{2+}$ or FK506 (Fig. 3D, right). Similarly, expression of a Tcn1(N)::Gal4(AD) hybrid protein, in which the activation domain of Gal4p was fused to the amino-terminal domain of Tcn1p, also exhibited no ability to induce GAL1-lacZ because this protein lacks the appropriate DNA-binding domain (Fig. 3C, left). Coexpression of both Tcn1(N)::Gal4(AD) and
Gal4(DB)::Cna1 restored induction of GAL1–lacZ by added Ca2+, which was completely inhibited by FK506 (Fig. 3D, left). Similar results were obtained when the carboxy-terminal autoinhibitory domain of calcineurin A was deleted from the hybrid protein (Fig. 3C, right). Overexpression of the hybrid proteins using high-copy plasmid vectors increased the units but did not alter the patterns observed using the low-copy plasmids (data not shown). These results demonstrate that the amino-terminal domain of Tcn1p interacts functionally with activated calcineurin but not with inactive or FK506-inhibited calcineurin. Functional interactions detected using the two-hybrid assay usually reflect direct or indirect physical interactions (Fields and Sternglanz 1994).

Targets of Tcn1p

To determine whether Tcn1p mediates some or all of the effects of calcineurin on gene expression, both expression and function of the four known target genes were examined in a tcn1 null mutant in which the TCN1 coding sequence was deleted and replaced (see Materials and Methods). The tcn1 null mutant grew as well as wild-type strains in standard medium but completely failed to induce PMC1–lacZ in response to growth in high Ca2+ conditions (Table 2, line 1). Furthermore, the normal calcineurin-dependent induction of reporter genes for PMR1, PMR2A, and FKS2 was completely abolished in the tcn1 null mutant (Table 2, lines 2–4), whereas the control CYC1–lacZ reporter was not affected (line 5).

To confirm that PMC1, PMR1, PMR2A, and FKS2 are physiological targets of Tcn1p, the function of each gene was compared in wild-type and tcn1 null mutants. The function of FKS2, for example, was measured qualitatively using a viability assay. Calcineurin-dependent expression of FKS2 is required for viability of cells lacking the homologous gene FKS1 (Parent et al. 1993; Eng et al. 1996).
1994; Garrett-Engele et al. 1995). We found that like calcineurin, Tcn1p is also required for functional expression of FKS2 because all fks1 tcn1 double mutants generated from 18 tetrads of a test cross (strain DMY14 crossed with strain YLIP179) were inviable. The functions of PMR1, PMR2A, and PMC1 in tcn1 mutants were assayed quantitatively using Mn$^{2+}$, Na$^+$, and Ca$^{2+}$ tolerance tests, respectively. tcn1 mutants were significantly less tolerant than wild-type to these ions (Fig. 4). In the presence of FKS06, wild-type strains and tcn1 mutants displayed approximately equal sensitivities to Mn$^{2+}$, Na$^+$, and Ca$^{2+}$, suggesting that Tcn1p retains little or no activity in the absence of calcineurin function and that the two factors function in the same regulatory pathway. The ion tolerance assays shown in Figure 4 also reveal functions of calcineurin that are independent of Tcn1p function. For example, addition of FKS06 to tcn1 mutants and to pmr2 tcn1 double mutants causes a further reduction in Na$^+$ tolerance (Fig. 4B), suggesting that calcineurin affects other Na$^+$ tolerance factors independently of Tcn1p. In tcn1 null mutants, weak effects of calcineurin on PMR2A–lacZ expression were also evident depending on the growth conditions (Table 2, lines 4 and 8). Calcineurin also inhibits the function of Vcx1p in Ca$^{2+}$-tolerance assays by a Tcn1p-independent mechanism because FKS06 addition increased Ca$^{2+}$ tolerance of tcn1 mutants, pmc1 mutants, and pmc1 tcn1 double mutants but only when Vcx1 was present. The finding that both pmc1 tcn1 double mutants and pmr1 tcn1 double mutants (not shown) are viable, whereas pmc1 pmr1 double mutants are inviable (Cunningham and Fink 1994), suggests that Tcn1p-independent basal expression of either Pmc1p or Pmr1p is sufficient for viability in standard media. In summary, the activity of Tcn1p on all known targets required calcineurin function, whereas the activity of calcineurin on at least two additional processes did not require Tcn1p function. These results suggest Tcn1p functions in a branch downstream of calcineurin in the calcium signaling pathway leading to gene expression.

Roles of Tcn1p and calcineurin in response to pheromone

The Ca$^{2+}$ signal generated in response to pheromone induces FKS2 through a calcineurin-dependent mechanism (Mazur et al. 1995). Induction of an FKS2–lacZ reporter gene was also observed after treatment of wild-type MATa cells with pheromone, and this induction was almost completely dependent on calcineurin and Tcn1p (Table 2, line 7). Calcineurin function is required for several additional responses to pheromone treatment, including maintenance of cell viability during prolonged pheromone stimulation (Cyert et al. 1991; Cyert and Thorner 1992; Moser et al. 1996) and for changes in cell morphology (Withee et al. 1997). Using similar methods, we found that tcn1 null mutants were indistinguishable from wild type (data not shown), suggesting that these effects of calcineurin are largely independent of Tcn1p. Another effect of calcineurin during the pheromone response, feedback inhibition of Ca$^{2+}$ uptake, is illustrated in Figure 5. After 4-hr treatment with 20 µM pheromone,
wild-type cells display a small increase in 45Ca2+ accumulation. Inhibition of calcineurin by addition of FK506 greatly potentiated the effect of pheromone but had little or no effect on untreated wild-type cells. In a parallel experiment, a tcn1 null mutant showed a pattern of 45Ca2+ uptake similar to wild type, indicating that Tcn1p is not required for the apparent calcineurin-dependent feedback inhibition of Ca2+ uptake after pheromone treatment. Although Tcn1p becomes activated during the pheromone response and induces genes such as FKS2, it plays no obvious role in several other calcineurin-dependent processes including increased Ca2+ uptake, morphological changes, and cell survival.

Dynamics of the calcium signaling pathway

Complementation tests performed during the characterization of isolated tcn1 mutants, showed that heterozygous tcn1/TCN1 diploids accumulated less than half as much β-galactosidase activity as homozygous TCN1/TCN1 diploids during growth in identical Ca2+ conditions (data not shown). This result implies that Tcn1p abundance may affect responsiveness to Ca2+ signals. Overexpression of Tcn1p by transforming a wild-type strain with the high dosage TCN1 plasmid (pLE66) resulted in higher induction of PMC1-lacZ in response to pheromone treatment to ~50% of maximal levels (Fig. 7A). Additionally, pheromone treatment caused a marked calcineurin-dependent induction of PMC1-lacZ in response to pheromone treatment at ~30% of maximal levels (Fig. 7A). These results suggest pheromone treatment generates a relatively weak Ca2+ signal that is insufficient to induce low-sensitivity genes in vivo. In support of this hypothesis, overexpression of Tcn1p from a high dosage plasmid restored the calcineurin-dependent induction of PMC1-lacZ after pheromone treatment emerging from the above results: The response to pheromone may produce a relatively weak Ca2+ signal that is insufficient to induce low-sensitivity genes. In support of this hypothesis, overexpression of Tcn1p from a high dosage plasmid restored the calcineurin-dependent induction of PMC1-lacZ upon pheromone treatment (Fig. 7A).

Table 2. Expression of reporter genes in wild-type and tcn1 mutants

| Plasmid | Reporter | β-Galactosidase (units) | Induction ratio |
|---------|----------|------------------------|----------------|
|         |          | wild type | tcn1 mutant | wild type | tcn1 mutant |
|         |          | +0 | +Ca | +Ca+FK | +0 | +Ca | +Ca+FK | (+Ca/+Ca+FK) | (+Ca/+Ca+FK) |
| 1. pKC190 | PMC1-lacZ | 0.2 | 19 | 0.1 | 0.1 | 0.1 | 0.1 | 270±90 | 1.0±0.0 |
| 2. pDM5 | FKS2-lacZ | 10 | 232 | 10 | 4.0 | 5.0 | 5.0 | 21±7.0 | 1.1±0.2 |
| 3. pKC199 | PMR1-lacZ | 11 | 28 | 12 | 5.3 | 5.0 | 6.0 | 23.6±0.4 | 0.9±0.1 |
| 4. pKC201 | PMR2A-lacZ | 0.1 | 59 | 4.5 | 0.1 | 0.1 | 0.8 | 14±3.6 | 1.0±0.5 |
| 5. pLG3312 | CYC1-lacZ | 424 | 483 | 466 | 358 | 483 | 415 | 4.2±1.4 | 1.2±1.0 |
| 6. pDM7 | TCN1-lacZ | 4.3 | 21 | 5.3 | 3.1 | 21 | 3.8 | 4.2±1.4 | 1.7±0.4 |
| 7. pDM5 | FKS2-lacZ | 39 | 166 | 50 | 17 | 26 | 24 | 3.4±0.6 | 1.1±0.1 |
| 8. pKC201 | PMR2A-lacZ | 3.3 | 163 | 39 | 1.4 | 53 | 38 | 4.2±0.4 | 1.5±0.2 |

β-Galactosidase activity (units) was measured as described in Methods and Materials after growth in YPD (pH 5.5) medium (lines 1–10) or YPD medium (lines 11, 12) supplemented as indicated with water (+0), 200 mM CaCl2 (+Ca), 20 µM β-Galactosidase activity (units) was measured as described in Methods and Materials after growth in YPD (pH 5.5) medium (lines 1–10) or YPD medium (lines 11, 12) supplemented as indicated with water (+0), 200 mM CaCl2 (+Ca), 20 µM β-galactosidase activity (units) was measured as described in Methods and Materials after growth in YPD (pH 5.5) medium (lines 1–10) or YPD medium (lines 11, 12) supplemented as indicated with water (+0), 200 mM CaCl2 (+Ca), 20 µM m−mating pheromone (+mf), 750 mM NaCl (+Na), or 0.2 µg/ml of FK506 (+FK). The average of three independent transformants is shown in units; induction ratios were calculated separately for each transformant and then averaged (± S.D.). The limit of detection for this experiment is 0.1 unit.

Differential control of Tcn1p-dependent genes involves modulation of Ca2+ signal strength and other promoter-specific factors

One possible explanation for the failure of the calcium signaling pathway to induce PMC1-lacZ after pheromone treatment emerges from the above results: the response to pheromone may produce a relatively weak Ca2+ signal that is insufficient to induce low-sensitivity genes. In support of this hypothesis, overexpression of Tcn1p from a high dosage plasmid restored the calcineurin-dependent induction of PMC1-lacZ after pheromone treatment (Fig. 7A). Additionally, pheromone treatment caused a marked calcineurin-dependent induction of PMC1-lacZ in response to pheromone treatment to ~30% of maximal levels (Fig. 7A). These results suggest pheromone treatment generates a relatively weak Ca2+ signal that is insufficient to induce low-sensitivity genes such as PMC1 and possibly TCN1.

Overexpression of Tcn1p failed to restore calcineurin-dependent induction to PMC1-lacZ by high salt treatment and failed to overcome the inhibitory effect of high salt on PMC1-lacZ induction by high Ca2+ treatment (Fig. 7A). In contrast, treatment with high salt caused
~25% maximal calcineurin-dependent induction of GAL1–lacZ in cells expressing the Gal4(DB)::Tcn1(N) hybrid transcription factor and had only a slight inhibitory effect on induction by high Ca\(^{2+}\) (Fig. 7B). The simplest hypothesis consistent with these results is that the response to high salt includes both the promoter-specific

![Graph A](image)

![Graph B](image)

![Graph C](image)

Figure 4. Mn\(^{2+}\), Na\(^{+}\), and Ca\(^{2+}\) tolerance assays of various yeast mutants showing roles of Tcn1p. All strains were grown to saturation in YPD medium at 30°C and diluted 1000-fold into fresh media containing a range of MnCl\(_2\), NaCl, or CaCl\(_2\) concentrations (with and without 0.2 µg/ml of FK506) and incubated for 1 day at 30°C in flat-bottom 96-well dishes (0.2 ml/well). Optical density at 650 nm was measured for each resuspended culture and plotted directly (A, B) or plotted and used to determine the 50% inhibitory concentration or IC\(_{50}\) (C) as described in Materials and Methods.
blockers of gene expression and the production of relatively weak Ca\textsuperscript{2+} signals that are sensed by calcineurin and the amino-terminal domain of Tcn1p. Based on all these results, we conclude that differential expression of Tcn1p-dependent genes can be accomplished through mechanisms that distinguish both the strength of Ca\textsuperscript{2+} signals and inputs from other signaling pathways.

Discussion

The results reported here and elsewhere (Stathopoulos and Cyert 1997) propose that Tcn1p/Crz1p functions as an important part of a calcineurin-dependent transcription factor in yeast. Tcn1p contains within its amino-terminal region a domain that interacts functionally, and perhaps physically, with activated calcineurin. Genetic analyses of tcn1 null mutants suggest that Tcn1p functions downstream of calcineurin in the calcium signaling pathway on a branch leading to the expression of specific genes. Tcn1p contains three zinc finger motifs in its carboxy-terminal region resembling the DNA-binding domains of numerous transcription regulators and has been shown in vitro to bind a 24-bp element present in the promoter of at least one target gene (Stathopoulos and Cyert 1997). The simplest molecular mechanism consistent with these results would be that calcineurin directly dephosphorylates Tcn1p in response to Ca\textsuperscript{2+} signals and thereby stimulates either nuclear localization or transcriptional activation activity. The available data, however, do not rule out the involvement of unknown intermediary factors or additional steps in the mechanism. Regardless of the molecular mechanism, the analysis of tcn1 null mutants and its gain-of-function variants clarify the roles of specific factors in the calcium signaling pathway during the responses to high Ca\textsuperscript{2+}, high salt, and mating pheromones.

Regulation of calcium transporters

The identification and characterization of Tcn1p confirms our previous model of Ca\textsuperscript{2+} homeostasis in yeast (Cunningham and Fink 1996) and extends our understand-
The analysis of tcn1 mutants also clarifies the role of calcineurin in Mn^{2+} tolerance. Pmr1p contributes strongly to Mn^{2+} tolerance but contributes much less than Pmc1p to Ca^{2+} tolerance (Cunningham and Fink 1994). Strains lacking calcineurin or Tcn1p function fail to induce PMR1-lacZ (Table 2) and are correspondingly less tolerant of added Mn^{2+} (Fig. 4A). This correlation suggests that Pmr1p levels directly determine Mn^{2+} tolerance just as Pmc1p levels directly determine Ca^{2+} tolerance levels. An alternative hypothesis proposed the role of calcineurin in Mn^{2+} tolerance was to limit Mn^{2+} influx by an unknown process (Farcasanu et al. 1995). However, a significant role for calcineurin in Mn^{2+} tolerance can only be detected when both Tcn1p and Pmr1p are functioning (Fig. 4A; Cunningham and Fink 1994). All of these results are consistent with a model in which calcineurin and Tcn1p induce expression of Pmr1p, which increases both Mn^{2+} sequestration in late compartments of the secretory pathway and Mn^{2+} export from the cell. Several other observations support this model. Mutants lacking PMR1 also display numerous secretory defects that can be attributed to insufficient Ca^{2+} and Mn^{2+} accumulation in compartments of the secretory pathway (Rudolph et al. 1998; Antebi and Fink 1992). Sufficient Ca^{2+} and/or Mn^{2+} is required for viability (Loukin and Kung 1995), and pmr1 mutants require much higher levels of these metals than wild type in spite of their increased uptake and sensitivity (Lapinskas et al. 1995). These findings are consistent with a model in which Pmr1p supplies compartments of the secretory pathway with Ca^{2+} and Mn^{2+} during standard conditions and promotes Mn^{2+} tolerance by sequestration and eventual export. Further analysis of tcn1 mutants and Mn^{2+} transport by Pmr1p and other factors (Supek et al. 1996) may resolve this issue.

Because calcineurin-dependent inhibition of the vacuolar H^{+}/Ca^{2+} exchanger Vcx1p is independent of Tcn1p (Fig. 4C), the analysis of tcn1 mutants should give little light on the mechanisms regulating Vcx1p or on the physiological significance of this regulation. Previous work suggested that calcineurin may inhibit Vcx1p post-translationally, although other explanations were not ruled out (Cunningham and Fink 1996). Why yeast cells inhibit Vcx1p when this enzyme can greatly increase Ca^{2+} tolerance also remains unclear. However, analysis of constitutive mutant forms of Vcx1p that resist inhibition by calcineurin revealed inappropriate H^{+}/Ca^{2+} exchange decreases the availability of cytosolic Ca^{2+} for calcium signaling (Cunningham and Fink 1996) and potentially for transport by Pmr1p into the secretory pathway.

Calcium signaling in response to salt stress

Evidence reported here and in previous studies all suggest that the response to high salt includes activation of the calcium signaling pathway, although no change in Ca^{2+} influx or accumulation in the cytosol has been reported. We observed submaximal induction of the Gal1-lacZ reporter by Gal4(DB)::Tcn1(N) after treatment with high salt (Fig. 7), and in tcn1 mutants we observed de-
creases in Na⁺ tolerance and PMR2A expression. These results suggest that high salt may generate a weak Ca²⁺ signal that mildly activates the calcium signaling pathway. In addition, calcineurin contributes to Na⁺ tolerance independently of Tcn1p and the Pmr2p ion pumps (Fig. 4B; Danielsson et al. 1996; Mendoza et al. 1996). Finally, Ca²⁺/calmodulin promotes Na⁺ tolerance by binding and activating Pmr2p ion pumps (Wieland et al. 1995). Together, these findings demonstrate multiple interactions between the calcium signaling pathway and Na⁺ tolerance factors. Another response to high salt appears to be negative regulation of genes such as PMC1 and FKS2 (Figs. 1 and 7). High salt may activate specific repressors or inhibit specific coactivators of transcription such as the MSN5/STE21 gene product, which is important together with Tcn1p for calcineurin-dependent induction of FKS2 and PMC1 (P.M. Alepuz, D.P. Matheos, K.W. Cunningham, and F. Estruch, in prep.).

Calcium signaling during the pheromone response

There is abundant evidence that Ca²⁺ signals are generated in yeast after treatment with high doses of mating pheromones (Ohsumi and Anraku 1985) and that the calcium signaling pathway becomes activated and induces genes such as FKS2 (Mazur et al. 1995). Here we show that induction of FKS2 in response to pheromone treatment also requires Tcn1p. The pattern of FKS2 expression contrasts with other Tcn1p-dependent genes and suggests that the pheromone response generates a relatively weak Ca²⁺ signal that is insufficient for induction of low-sensitivity genes such as PMC1, which required overexpression of Tcn1p before a significant response to pheromone could be observed (Fig. 7). In spite of this clear role for Tcn1p in the response to pheromone, we detected no significant role for Tcn1p in several other calcineurin-responsive phenomena, including feedback inhibition of Ca²⁺ uptake (Fig. 5), changes in cell morphology, or promoting cell survival during pheromone stimulation (not shown). Survival in pheromone was shown previously to depend on Ca²⁺ influx and the functions of calmodulin, calcineurin, and calmodulin-dependent protein kinases (Iida et al. 1990, 1994; Moser et al. 1996; Withee et al. 1997). The targets of the calcium signaling pathway involved in cell survival therefore remain to be identified.

Comparison of Tcn1p regulation with vertebrate systems

The calcineurin-dependent transcriptional activation domain of Tcn1p shows no significant sequence similarity to other proteins in current databases, so extrapolations to specific vertebrate mechanisms are not yet possible. Several parallels are noteworthy, nevertheless. Using the zinc finger domain of Tcn1p to search protein databases, the most similar vertebrate proteins are members of the EGR family of transcription factors. Zif268/EGR-1 is markedly induced by calcium signaling through the serum response factor SRF in many cell types (Cole et al. 1989; Ginty 1997), and induction of EGR-2 in B cells also depends on calcineurin function (Gottschalk et al. 1994). In these cases, the molecular mechanisms controlling EGR expression are not precisely known. We show evidence that Tcn1p may regulate its own expression by a positive feedback mechanism requiring calcineurin and that this autoregulation may affect expression of target genes such as PMC1. Remarkably, expression of vertebrate PMCA genes encoding the plasma membrane Ca²⁺ ATPases homologous to Pmc1p also appears to be regulated in response to Ca²⁺ signals and calcineurin activation in granule cells of the developing rat cerebellum (Carafoli et al. 1996).

An emerging question in understanding signal transduction networks is how cells utilize common signaling modules to generate distinct outputs depending on the type or source of input signal. In neurons, for example, Ca²⁺ signals generated by either activation of the NMDA receptor or activation of the L-type Ca²⁺ channel caused phosphorylation of the critical serine-133 in the CREB transcription factor but only the signal derived from the L-type channel could induce the c-fos gene (Bading et al. 1993). Differences in the spatial or temporal character of these Ca²⁺ signals have been proposed to accomplish this type of differential gene expression (Dolmetsch et al. 1997; Ginty 1997) although differences in Ca²⁺ signal strength or additional regulatory mechanisms analogous to those reported here may also be involved. Although the spatiotemporal characteristics of the Ca²⁺ signals in yeast caused by pheromone, high salt, and high Ca²⁺ are presently unknown, our results indicate that different promoters are sensitive to variations in the strength of Ca²⁺ signals and inputs from other types of signaling pathways. More work is needed to understand this phenomenon and to accurately compare the yeast mechanism with mammalian systems.

Materials and methods

Culture media and isolation/construction of tcn1 mutants

Synthetic complete (SC) and complex (YPD) media were prepared and supplemented with 2% glucose as described previously (Sherman et al. 1986) using reagents from Difco and Sigma Chemical Co. Where indicated, YPD medium was buffered to pH 5.5 by addition of 5 mM succinic acid and supplemented with various salt such as CaCl₂, MnCl₂, NaCl, or G418 sulfate. FK506 was generously provided by Fujisawa Corp. (Tokyo, Japan). The synthetic pheromone -mating factor was obtained from Star Biochemicals.

All yeast strains listed in Table 2 were derived from W303-1A (Wallis et al. 1989) using standard methods of transformation and/or crossing (Sherman et al. 1986), and all strains except Y190 harbored the following genetic markers: MATα ade2-1 can1-100 his3-1 leu2-3,112 trpl-1 ura3-1. The tcp1::kanMX3 null mutation in which the chromosomal TCN1 gene was deleted and replaced was introduced into W303-1A by one-step gene replacement (Rothstein 1991) using a fragment of plasmid pKC287 generated by digestion with BglII plus Xbal. The resulting tcp1 null mutant (strain DMY14) was selected in YPD agar medium supplemented with 0.2 mg/ml of G418 sulfate (GIBCO BRL) and verified by PCR analysis of genomic DNA. Additional
strains containing tcn1::kanMX3 were constructed by crosses between DMY14 and previously described derivatives of W303-1A (Cunningham and Fink 1996). Strains DMY62 and DMY63 were constructed by transformation of strains K473 and K482, respectively, with Apal-digested plasmid pKC217, which integrates a PMC1-lacZ reporter gene at theura3-1 locus. β-Galactosidase accumulation in strains DMY62 and DMY63 was very low during growth in YPD (pH 5.5) medium and very high after growth in YPD (pH 5.5) medium supplemented with CaCl2 as detected using the chromogenic substrates 0-nitrophenyl-β-D-galactopyranoside (ONPG) as described (Guarente 1983) or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, see below).

To identify mutants unable to induce PMC1-lacZ, strains DMY62 and DMY63 were mutagenized to ~30% viability using EMS, spread onto 40 plates of SC minus uracil agar medium at 50°C, and then incubation for 3 days. Colonies were then replica-plated onto paper filters (Whatman No. 3) placed on YPD (pH 5.5) agar plates supplemented with 0.3 mm adenine and 75 mm CaCl2, incubated for 1 day at 30°C, and assayed for β-galactosidase accumulation as follows. Filters were first soaked with 25 mm EGTA (pH 7.5) for 1 min, and finally soaked ~3 hr at room temperature with staining solution containing 0.3 mg/ml of X-gal dissolved in modified Z buffer [100 mm Na phosphate (pH 7.0), 10 mm KCl, 10 mm MgSO4, 0.1% SDS, 0.27% β-mercaptoethanol]. Most colonies stained dark blue after this protocol. All white or light blue colonies were collected, purified, restested, and then subjected to complementation testing where each DMY62 isolate was mated with each DMY63 isolate in all possible combinations and the resulting diploids were selected on SC − Leu-Trp agar medium and tested for β-galactosidase accumulation after calcium treatment as before. All recessive mutations were placed into one of three complementation groups—cnb1, msn5, and tcn1—which were identified by cloning of the functional locus by complementation and/or allelism tests using targeted disruption mutants.

Cloning of TCN1 and recombinant DNA

All recombinant DNA techniques were performed using standard techniques (Sambrook et al. 1989) with reagents supplied by Stratagene and New England Biolabs. To identify the TCN1 gene, a tcn1 mutant isolated in the screen was transformed with a low-copy genomic DNA plasmid library (kind gift of D. Levin, Johns Hopkins University, Baltimore, MD) based on the pRS313 shuttle vector (Sikorski and Hieter 1989) plated on SC − His agar medium, replica plated to filters placed on YPD (pH 5.5) agar medium containing 75 mm CaCl2, and stained with X-gal as described above to identify rare blue clones that regained ability to express PMC1-lacZ. Out of ~10,000 independent transformants, 2 were found to yield plasmids that complemented the tcn1 defect. Restriction mapping and partial sequencing of the insert DNA from both plasmids (pDM1 and pDM2) demonstrated that the two plasmids contain distinct but overlapping inserts spanning two previously uncharacterized open reading frames from chromosome XIV termed YNL026w and YNL027w. Deletion of YNL026w by digestion of pDM1 with SpeI plus XbaI, followed by religation, resulted in plasmid pDM3, which retained the ability to complement tcn1 mutations. Prior to the release of the complete yeast genomic DNA sequence, A. Duesterhoeft and P. Philippsen generously provided the sequence of the TCN1 locus and plasmid p678: lacZ::kanMX3, which was used to construct the tcn1::kanMX3 disruption plasmid pKC287 by digesting with SpeI plus BsrG1 to remove all lacZ sequences, generating blunt ends, and ligating (Philippsen et al. 1997). Null mutants obtained by gene replacement using tcn1::kanMX3 DNA failed to complement each of the tcn1 alleles isolated in the genetic screen, showing that the mutations in this group all resided within the TCN1/YNL027w gene.

Plasmids containing various reporter genes were constructed as follows. An integrating plasmid containing the PMC1-lacZ reporter gene (pKC217) was derived from pKC190 (Cunningham and Fink 1996) after digestion with Sphl and religation to remove the 2u origin of replication. The FKS2-lacZ reporter plasmid (pDM5) containing the DNA segment from position −95 to +6 relative to the initiation codon of FKS2 fused in-frame to lacZ coding sequences was constructed by PCR amplification with oligonucleotides FKS2A (GGAGTCGACAGGGCTACTCAATTAGCT) and FKS2B (GCCCTCTAGGACATACTTATGACAG) followed by digestion with Sall plus XbaI and cloning first into polylinker sites of YEp356R (Myers et al. 1986) and then subcloning into pLG1178 (Guarente and Mason 1983) after first digesting both plasmids with XhoI plus BamHI. A TCN1-lacZ reporter plasmid (pDM7) was constructed by subcloning the DNA segment from −1485 to +42 relative to the TCN1 initiation codon from pDM3 (labeled with Sall plus Sphi) first into Yep356R (digested with Sall plus XbaI) and then subcloning the Sall plus BamHI fragment into pLG1178 digested with XhoI plus BamHI.

All plasmids expressing Gal4 hybrid proteins were constructed from plasmids pC97 and pPc86 (Chevray and Nathans 1992) as follows. The amino-terminal region of Tcn1p corresponding to nucleotides +33 to +1381 was amplified using oligonucleotides DB1 (GCCGCGCGCAATGCGCCGCGCGCAATGCGCCGCGCGCAATGCGCCGCGAGTCGACTAGTAAT) and DB2 (GCCGCGCGCAATGCGCCGCGCGCAATGCGCCGCGAGTCGACTAGTAAT), purified on agarose gels, digested with Sall plus AvrII, and cloned into pC97 digested with Sall plusSpeI to yield pDM15 containing Gal4(DB): Tcn1(N). Plasmid pDM16 expressing Tcn1(C):Gal4(AD) hybrid proteins was constructed by ligating into Sall plus SpeI-digested pcPc86 the Sall plus AvrII-digested PCR product obtained using primers TA1 (GCCGCCCGCGCGAGTCGACTAGTAAT) and TA2 (GCCGCCCGCGCGAGTCGACTAGTAAT), corresponding to nucleotides +1389 to +2033 of TCN1. Plasmids pKC116 and pKC117 containing Gal4(DB): Cna1LC and Gal4(DB): Cna1LC, respectively, were constructed by subcloning the XhoI plus BamHI fragments of pKC73 and pKC74 (Cunningham and Fink 1996) into pC97 digested with Sall plus BamHI. Plasmid pTJK27 containing Tcn1(N):Gal4(AD) was constructed by subcloning a Sall plus Sphi fragment from pDM15 into pPc86 digested with Sall plus Sphi.

β-Galactosidase assays

Strains were grown overnight in SC media lacking uracil to mid-log phase, harvested, and grown for an additional 4 hr in either YPD or YPD (pH 5.5) supplemented with CaCl2, NaCl, α-mating factor, and/or FK506 as indicated in the text. β-Galactosidase activity was assayed at room temperature using chlororm SDS permeabilized cells as described previously (Guarente 1983).

Ion tolerance assay

Ion tolerance assays were performed as described previously (Cunningham and Fink 1996). For CaCl2, yeast strains were grown in YPD (pH 5.5), whereas MnCl2 and NaCl assays used YPD media. Cell density was measured at an OD650 with a Molecular Devices microplate reader. The concentration of cat-
ion resulting in a 50% decrease in cell growth relative to un-supplemented cultures (IC50) was interpolated from linear plots of the ion tolerance data.

**Ca2+ uptake assays**

Yeast cultures were grown to mid-log phase at 30°C in YPD media, harvested, and resuspended to an OD600 of 0.25 in 0.2 ml of YPD and supplemented with -10 μCi/ml of 45Ca2+ (Amer sham Life Science). After incubation for 4 hr at 30°C with occasional mixing, 0.18 ml of culture was filtered through Whatman GFF filters and washed three times with buffer (5 mM Na HEPES, 10 mM CaCl2 at pH 6.5). Filters were dried and radio-activity quantitated using a scintillation counter. 45Ca2+ accumulation per 10^10 cells was calculated using the measured radio-activity retained on filters, the specific activity, and the cell density as determined by culture density using OD600.

**Acknowledgments**

We are indebted to Andreas Duesterhoeft and Peter Philippsen for generously providing DNA sequences prior to publication as well as yeast strains and plasmids. We are very grateful to Paula Alepuz, Trisha Davis, Cameron Douglas, Francisco Estruch, and David Levin for providing reagents and advice and to Fujsawa Corp. for gifts of FK506. This work was supported jointly by the Searle Scholars Program/The Chicago Community Trust, the Basil O'Connor Starter Scholar Research Award (FY96-1131) from the March of Dimes Birth Defects Foundation, and a Research Grant from National Institutes of Health (GM53082).

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Dina P. Matheos, Tami J. Kingsbury, U. Salma Ahsan, et al.

*Genes Dev.* 1997, 11:
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