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Mg^{2+} Deprivation Elicits Rapid Ca^{2+} Uptake and Activates Ca^{2+}/Calcineurin Signaling in *Saccharomyces cerevisiae*▽

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To learn about the cellular processes involved in Mg^{2+} homeostasis and the mechanisms allowing cells to cope with low Mg^{2+} availability, we performed RNA expression-profiling experiments and followed changes in gene activity upon Mg^{2+} depletion on a genome-wide scale. A striking portion of genes up-regulated under Mg^{2+} depletion are also induced by high Ca^{2+} and/or alkalinization. Among the genes significantly up-regulated by Mg^{2+} starvation, Ca^{2+} stress, and alkalinization are *ENA1* (encoding a P-type ATPase sodium pump) and *PHO89* (encoding a sodium/phosphate cotransporter). We show that up-regulation of these genes is dependent on the calcineurin/Crz1p (calcineurin-responsive zinc finger protein) signaling pathway. Similarly to Ca^{2+} stress, Mg^{2+} starvation induces translocation of the transcription factor Crz1p from the cytoplasm into the nucleus. The up-regulation of *ENA1* and *PHO89* upon Mg^{2+} starvation depends on extracellular Ca^{2+}. Using fluorescence resonance energy transfer microscopy, we demonstrate that removal of Mg^{2+} results in an immediate increase in free cytoplasmic Ca^{2+}. This effect is dependent on external Ca^{2+}. The results presented indicate that Mg^{2+} depletion in yeast cells leads to enhanced cellular Ca^{2+} concentrations, which activate the Crz1p/calcineurin pathway. We provide evidence that calcineurin/Crz1p signaling is crucial for yeast cells to cope with Mg^{2+} depletion stress.

Mg^{2+} is the most abundant divalent cation in cells, where the ion predominantly serves as a counterion for solutes, particularly ATP and other nucleotides, RNA and DNA. By binding to RNAs and many proteins, Mg^{2+} also contributes to establishing and maintaining physiological structures and acts as an important cofactor in catalytic processes. Mg^{2+} also stabilizes membranes and active conformations of macromolecules (reviewed in references 18, 37, and 38). Cellular Mg^{2+} concentrations are in the millimolar range (~15 to 20 mM), some 3 orders of magnitude higher than those of Ca^{2+} (100 to 200 nM) (4, 5, 17, 20, 38). The vast majority of Mg^{2+} is bound to ligands, leaving a small fraction of up to 5% in a free ionized state (38, 40). Cellular Mg^{2+} homeostasis involves systems facilitating influx and others that mediate extrusion of the ion. Mg^{2+} influx is an electrogenic process driven by the inside negative membrane potential and mediated by channels in the plasma membrane, either by TRPM6 and TRPM7 proteins in mammals (42, 43) or by members of the heterogeneous CorA/Mrs2/Alr1 protein family in prokaryotes, organelles, lower eukaryotes, and plants (15, 17, 23, 24, 53, 54). These high-affinity Mg^{2+} uptake systems allow cells to grow even in the presence of very low external Mg^{2+} concentrations. In mutants lacking these systems, cells survive only when provided with high external Mg^{2+} concentrations. Extrusion of Mg^{2+} occurs against the electrochemical gradient and is mediated by exchange against Na^+, H^+, or other ions, making use of their inside-directed gradients to drive the process (10, 40).

Although Ca^{2+} concentrations are several orders of magnitude lower than those of Mg^{2+}, the two ions appear to affect each other in a mostly antiparallel fashion. In yeast, vacuolar Ca^{2+} accumulation is blocked by increased Mg^{2+} in the medium, and *alr1*Δ mutants having lower Mg^{2+} exhibit elevated Ca^{2+} (5, 17). In pancreatic acinar cells, an increase in intracellular Mg^{2+} results in a decrease of Ca^{2+} influx, whereas intracellular Ca^{2+} mobilization is associated with a reduction in Mg^{2+} (31). Moreover, extracellular Mg^{2+} is known to regulate K^+ and Ca^{2+} channels in the plasma membrane (6, 29, 44). Intracellular Mg^{2+} concentrations in mammalian cells have been reported to change in response to hormonal stimuli, albeit much more slowly than do Ca^{2+} concentrations (10, 31, 40). In some cases, these mutual modulations may simply reflect a replacement of one divalent cation by the other, but Mg^{2+} effects on Ca^{2+} signaling have frequently been observed (31).

Mg^{2+} starvation of rats has been reported to elicit significant up-regulation of expression of genes involved in oxygen stress in thymocytes (35). These effects result from long-lasting Mg^{2+} starvation conditions (2 days) and may include immediate responses of cells to Mg^{2+} withdrawal as well as secondary effects reflecting induction of stress phenomena.

In an attempt to understand the direct effects of Mg^{2+} starvation, we followed a whole-genome approach in *Saccharomyces cerevisiae*. We set out to analyze short-term responses to Mg^{2+} withdrawal in yeast cells by transcriptomal analysis. A relatively confined set (<2%) of the total of 6,300 genes responded with a significant, at least twofold, increase in tran-

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script levels. Most of them were found to be similarly up-regulated by other treatments that elicited a Ca^{2+} peak. In fact, we observed an increase of cytoplasmic Ca^{2+} immediately after cells were transferred to low-Mg^{2+} medium, and up-regulation of the calcineurin/Crz1p (calcineurin-responsive zinc finger protein) signaling pathway.

MATERIALS AND METHODS

Yeast strains, plasmids, and media. *S. cerevisiae* strains used in this study are Y00000 (BY4741), Y05353 (BY4741; *crz1*::*kanMX4*) and Y05040 (BY4741; *cnb1*::*kanMX4*) from the EUROSCARF collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/). The presence of the deletions was confirmed by qualitative PCR using two specific primer pairs for wild-type and deletion mutant strains (data not shown). Wild-type strain JS034-4C is described by Stadler and Schweyen (47). The Crz1-green fluorescent protein (GFP) fusion construct (pRSP97) is described by Polizotto and Cyert (36). Synthetic medium was prepared according to Sherman (46): for standard medium, 1 mM $MgCl_2$ was added, and for Mg^{2+} free medium, the $MgCl_2$ was omitted. EGTA (10 mM, pH 8.0) was added where indicated. Medium containing high Ca^{2+} was buffered with 50 mM MES (morpholineethanesulfonic acid [pH 6.0]).

Mg^{2+} starvation and RNA isolation. For each experiment, two identical yeast cultures were grown in synthetic medium containing 1 mM $MgCl_2$ to an optical density at 600 nm (OD_{600}) of 0.5. The cultures were centrifuged, washed twice with prewarmed synthetic medium containing either 1 mM $MgCl_2$ ($+Mg^{2+}$) or no $MgCl_2$ ($-Mg^{2+}$), and resuspended in the same medium. For time course experiments, aliquots were removed at the indicated time points; for all other experiments, cells were harvested 70 min after the first wash. For the FK506 experiment, FK506 (Fujisawa GmbH, Munich) in dimethyl sulfoxide was added to a final concentration of 1 μg/ml (1.25 μM) 10 min before centrifugation and for all subsequent steps. Total RNA was isolated using the hot acidic phenol method (1). For microarray experiments, three chloroform extractions were performed instead of one.

DNA microarray analyses. Yeast cDNA arrays were obtained from the Ontario Cancer Institute Microarray Centre. Reverse transcription, probe cleanup, and microarray hybridization were performed according to the manufacturer's protocol. Two individual experiments including dye swap were performed. Microarrays were read using an axon GenePix 4000B laser scanner (Axon Instruments) and analyzed with the GenePix Pro 3.0 software. The Saccharomyces Genome Database (http://www.yeastgenome.org/) was used to extract the information on the genes regulated by Mg^{2+} starvation. The geneXplorer 2.0: Megayeast site from Stanford University (http://genome-www.stanford.edu/cgi-bin/yeast_stress/gx?n = megayeast&rx = 5&ry) was used to search for genes induced under general stress conditions.

Northern blot experiments. Twenty-five-microgram samples of total RNA were subjected to gel electrophoresis and blotted to nitrocellulose membranes (27). ^{32}P-labeled probes for hybridization were generated by either random primed labeling (Roche) from PCR-synthesized DNAs or hot PCR on genomic DNA using the following oligonucleotide primers: ENA1F (5′-TTATCGCGGTCAATGTGCTC), ENA1R (5′-ATCAAACTCACGTTGCCCTC), PHO89F (5′-TGCTTTACTGCTGGTTGGTG), and PHO89R (5′-AGCGTTGGCAACGTCATTAG). For quantification of RNA levels, the blots were rehybridized to an actin probe generated using the primers ACT1F (5′-ACCAAGAGAGGTATCTTGACTTTACG) and ACT1R (5′-GACATCGACATCACACTTCATGATGG). Documentation and analyses of the Northern blots were performed using the Amersham Biosciences Typhoon 8600 phosphorimaging system and the Molecular Dynamics Image Quant software.

Fluorescence microscopy. For determination of the subcellular location of Crz1p, plasmid pRSP97 (a GFP-*CRZ1* fusion construct) (36) was transformed into BY4741. The transformants were grown in synthetic complete medium lacking uracil (SC −Ura [containing 1 mM $MgCl_2$ and 10 mg/liter methionine]) to an OD_{600} of 1 to 1.5. One-milliliter aliquots were spun in a tabletop centrifuge (30 s at 7,000 rpm), washed twice in SC −Ura medium (prewarmed to 30°C, with 1 mM Mg^{2+}, 0 mM Mg^{2+}, or 200 mM $CaCl_2$, respectively), resuspended in the same medium, and incubated for 10 min at 30°C. Prior to microscopy, the cells were briefly spun in a Qualtron microcentrifuge and resuspended in a small volume of the supernatant.

Determination of cytoplasmic Ca^{2+} concentrations. To express YC2-12 in yeast, a 2.5-kb BamHI-XhoI fragment from YC2.12 in pCS2 (30) was cloned into the yeast expression plasmid pVT-U (51). The resulting plasmid (pGW845) was transformed into BY4741. Transformants were grown in selective medium containing 1 mM Mg^{2+} to an OD_{600} of 0.5 to 1.5 and concentrated to 1/100 volume in a Qualtron microcentrifuge. Cytosolic Ca^{2+} concentration was measured as previously described (14, 26). Briefly, yeast cells stably expressing the sensor in the cytosol were immobilized on glass coverslips with concanavalin A (Sigma-Aldrich) and placed into an experimental chamber that allowed continuous perfusion and fast buffer switch. The microscope consists of a Nikon inverted microscope (Eclipse 300TE, Nikon, Vienna, Austria) equipped with CFI Plan Fluor ×40 oil immersion objective (NA 1.3; Nikon, Vienna, Austria), an epifluorescence system (150 W XBO; Optiquip, Highland Mills, NY), and a liquid-cooled charge-coupled device camera (−30°C; Quantix KAF 1400G2, Roper Scientific, Acton, MA). All devices were controlled by Metafluor 4.0 (Visitron Systems, Puchheim, Germany). To monitor the cytosolic free Ca^{2+} concentration, the cells were illuminated at 440 nm (Cameleon; 440AF21; Omega Optical, Brattleboro, VT). An optical beam splitter (Dual-View Micro-Imager; Optical Insights, Visitron Systems) was used in order to allow simultaneous emission rationing at 480 nm (480AF30; Omega Optical) and 535 nm (535AF26 with dichroic 455DRVP; Omega Optical).

Microarray data accession number. Microarray data from this study are available at the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE6687.

RESULTS

Genome-wide analysis of gene expression in response to Mg^{2+} starvation. To determine how cells cope with deprivation of the essential metal ion Mg^{2+} and to learn about the cellular processes involved in Mg^{2+} transport, we performed whole-genome microarray experiments. Cells were grown in synthetic medium containing standard concentrations of Mg^{2+} (1 mM $MgCl_2$) and then shifted to nominally Mg^{2+}-free medium or to fresh medium containing standard concentrations of Mg^{2+}. The expression profile of cells shifted to Mg^{2+}-free medium was compared to that of cells grown in 1 mM Mg^{2+}. Ninety minutes after the shift from 1 mM to nominally Mg^{2+}-free medium, we found genes belonging to particular functional clusters to be up-regulated (Table 1). Among the genes most significantly induced were several genes encoding proteins involved in Na^+, phosphate, and energy homeostasis: i.e., Na^+ pump P-type ATPase genes and their stabilizing factors (*ENA1*, *ENA2*, *ENA5*, and *STF2* and its homologue, *YLR327c*) and *PHO89*, required for sodium-dependent phosphate uptake. In contrast, genes encoding acid phosphatases (*PHO3*, *PHO11*, and *PHO12*) were found to be down-regulated. Other genes found to be up-regulated upon Mg^{2+} deprivation are involved in cytoskeleton organization (*ABP1*, *MTI1*, *RVS167*, *SAC6*, and *SRV2*) and membrane synthesis (*ARE2*, *ERG26*, and *PLB3*). The proteins encoded by these genes might stabilize the cytoskeleton and membranes to compensate for the lack of Mg^{2+}, as Mg^{2+} is known to be crucial in stabilizing the cell shape and for membrane integrity. Moreover, typical stress response genes were induced as well as genes for carbohydrate and amino acid metabolism, vacuolar protein degradation, and a large set of genes with known or unknown function, which do not form obvious functional clusters (see Table 1 for details).

A striking number of genes up-regulated upon Mg^{2+} starvation are also up-regulated under one ore more of the following conditions: Ca^{2+} stress, Na^+ stress (57), or alkalization of the growth medium (45, 52) (see Table 1). In particular, of the 112 genes significantly up-regulated (± standard deviation of ≥2) 42 (38%) are known to be up-regulated by Ca^{2+} and 13 (12%) by high Na^+, of which 11 are also induced by Ca^{2+}, giving a total of 44 out of 112 genes (39%) up-regulated by temporal Mg^{2+} deprivation and also by short-term Ca^{2+} and/or Na^+ stress. Furthermore, many of the genes induced/

TABLE 1. Gene expression upon magnesium starvation

Name and function	Identification no.	Induction or reduction	Coregulation[a]
Induction			
Na, P, and energy homeostasis			
ENA1	YDR040C	4.1 ± 1.5	C A
ENA2	YDR039C	3.5 ± 1.3	C
ENA5	YDR038C	3.1 ± 0.7	C A
PMC1	YGL006W	5.5 ± 0.5	C
PHO89	YBR296C	7.9 ± 2.5	C A S
STF2	YGR008C	4.9 ± 2.0	S
C metabolism			
ARA1	YBR149W	2.9 ± 0.6	
ATH1	YPR026W	2.7 ± 0.4	S
GLK1	YCL040W	4.6 ± 1.2	S
GPD1	YDL022W	3.2 ± 1.2	S
MDH2	YOL126C	2.9 ± 0.6	
TPS2	YDR074W	3.4 ± 0.7	S
TSL1	YML100W	8.2 ± 3.1	S
Amino acid metabolism			
APE2	YKL158W	2.6 ± 0.3	
ARO9	YHR137W	3.9 ± 1.5	S
BNA2	YJR078W	3.9 ± 1.5	C
GTT1	YIR038C	4.9 ± 1.9	S
MET14	YKL001C	3.8 ± 1.7	C A
MET3	YJR010W	3.9 ± 1.6	C
Vacuolar protein degradation			
ATG19	YOL082W	2.9 ± 0.5	C S
PBI2	YNL015W	4.0 ± 1.7	S
PEP12	YOR036W	4.5 ± 0.8	C S
PEP4	YPL154C	4.5 ± 1.7	A S
PRB1	YEL060C	9.3 ± 4.0	C N S
Cytoskeleton organization			
ABP1	YCR088W	3.1 ± 0.8	
MTI1	YJL020C	3.5 ± 1.3	
Membrane synthesis			
ERG26	YGL001C	3.1 ± 0.9	C
OSH6	YKR003W	3.0 ± 0.8	
PLB3	YOL011W	3.6 ± 1.4	N
SLI1	YGR212W	3.5 ± 1.2	C S
Transporters			
ENB1	YOL158C	2.9 ± 0.8	C
MEP1	YGR121C	3.7 ± 0.6	C N S
SUL1	YBR294W	2.8 ± 0.4	
Stress response			
DDR2	YOL052C-A	4.0 ± 1.9	
DDR48	YMR173W	4.8 ± 1.6	A S
GRX1	YCL035C	2.8 ± 0.8	
HSP104	YLL026W	4.5 ± 1.3	S
HSP42	YDR171W	4.0 ± 1.0	S
SGE1	YPR198W	4.4 ± 1.7	C
Others of known function			
AKL1	YBR059C	2.2 ± 0.2	
ATG5	YPL149W	3.8 ± 1.3	C
CPS1	YJL172W	3.5 ± 1.2	C N S
DCS1	YLR270W	2.7 ± 0.6	S
DCS2	YOR173W	3.0 ± 0.5	S
DIA1	YMR316W	9.3 ± 3.5	C N S
ECM13	YBL043W	2.7 ± 0.2	
ETR1	YBR026C	2.5 ± 0.2	
FMS1	YMR020W	2.6 ± 0.5	C
GPI18	YBR004C	3.2 ± 0.7	C A
HAL5	YJL165C	3.3 ± 0.7	N a
MRP8	YKL142W	3.4 ± 1.1	S
PNC1	YGL037C	4.6 ± 1.2	S
PTP2	YOR208W	2.9 ± 0.8	
RCR1	YBR005W	6.3 ± 1.8	C N S
REX3	YLR107W	3.7 ± 1.3	
RPN7	YPR108W	2.8 ± 0.8	
SOL4	YGR248W	2.7 ± 0.4	S
TIS11	YLR136C	5.8 ± 2.0	C A S
URA1	YKL216W	3.7 ± 1.3	S
WTM1	YOR230W	4.2 ± 1.4	a S
YPK1	YKL126W	3.4 ± 1.1	C
	YDL124W	7.4 ± 2.9	S
Others of unknown function			
FMP12	YHL021C	4.2 ± 1.3	A a S
FMP46	YKR049C	2.6 ± 0.5	
HOR7	YMR251W-A	7.4 ± 3.0	A S
HUA1	YGR268C	4.1 ± 1.5	C S
IML2	YJL082W	2.7 ± 0.4	
MSC1	YML128C	2.6 ± 0.3	A
ORM2	YLR350W	3.6 ± 1.5	C
PIN3	YPR154W	4.1 ± 1.4	S
PRM8	YGL053W	2.8 ± 0.7	C
RTA1	YGR213C	6.6 ± 2.0	C A
SRF4	YDL023C	2.9 ± 0.5	
UBX6	YJL048C	2.7 ± 0.6	
UIP3	YAR027W	3.3 ± 0.5	C S
YSW1	YBR148W	2.9 ± 0.6	
ZSP1	YBR287W	4.9 ± 2.0	C A S
	YLR327C	5.1 ± 2.4	C A S
	YDL010W	3.2 ± 0.7	C
	YDR391C	4.3 ± 1.5	S
	YEL074W	4.9 ± 1.3	
	YHR087W	6.2 ± 2.9	A
	YHR097C	6.0 ± 0.6	C N S
	YJL171C	5.3 ± 2.3	C N A S
	YKL151C	3.1 ± 0.7	S
	YLR414C	11.0 ± 4.5	C N S
	YNL208W	7.7 ± 2.7	C A S
	YOR220W	16.3 ± 1.5	C N S
	YOR289W	2.9 ± 0.7	S
	YOR385W	3.1 ± 0.8	C N S
	YCL042W	4.5 ± 0.3	S
	YDL011C	2.7 ± 0.3	
	YGL165C	2.5 ± 0.2	C N
	YGR110W	2.6 ± 0.4	C
	YIL055C	3.1 ± 0.5	
	YJL015C	4.2 ± 0.4	
	YJL016W	4.3 ± 0.9	C
	YJL144W	3.6 ± 1.4	S
	YJL152W	3.0 ± 0.9	
	YLR162W	3.6 ± 0.5	
	YLR194C	11.5 ± 3.0	C N
	YMR007W	4.2 ± 1.0	C
	YMR102C	2.6 ± 0.4	
	YMR304C-A	3.3 ± 1.0	C A s
	YNL092W	4.3 ± 1.3	C
	YNL115C	3.9 ± 1.5	
	YNL134C	3.5 ± 0.8	A S
	YOL048C	2.9 ± 0.8	
	YOR152C	2.3 ± 0.2	
	YPR197C	3.0 ± 0.5	
Reduction			
P homeostasis			
PHO3	YBR092C	−3.2 ± 1.2	
PHO5	YBR093C	−2.9 ± 0.9	s
PHO12	YHR215W	−3.4 ± 1.4	A s
Others			
YHB1	YGR234W	−4.5 ± 1.4	
	YLR413W	−2.8 ± 0.7	s

[a] Induction is shown by uppercase letters as follows: C, Ca^{2+}; N, Na^{+}; A, alkalinization; S, stress. Reduction is shown by lowercase letters as follows: a, alkalinization; s, stress (16, 52, 57).

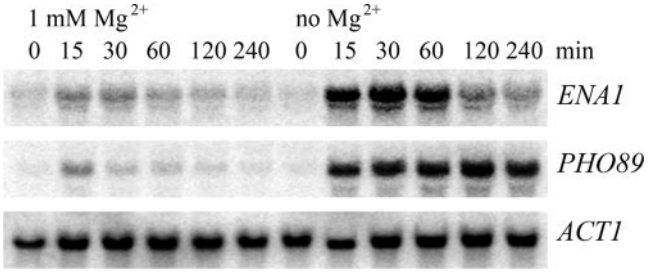

FIG. 1. *ENA1* and *PHO89* transcripts are induced upon Mg^{2+} starvation. Strain JS034-4C was grown in synthetic medium containing 1 mM Mg^{2+} to an OD_{600} of 0.5, washed twice with SD medium containing either 1 mM Mg^{2+} or lacking Mg^{2+}, and then incubated in the same medium. Samples were drawn at the indicated time points, and total RNA was prepared. Twenty-five micrograms of total RNA was loaded per lane, and Northern blot analysis was performed using radiolabeled probes specific for *ENA1*, *PHO89*, and *ACT1* as a loading control.

repressed by Mg^{2+} starvation seem to respond to general stress conditions as they are similarly regulated under various conditions of stress (16) (Table 1).

***ENA1* and *PHO89* transcripts are rapidly induced upon Mg^{2+} starvation.** To confirm that induction of transcripts is due to depletion of Mg^{2+} and to investigate the kinetics of transcriptional induction by Mg^{2+} starvation, we performed Northern blot analyses of two genes highly induced in the microarray experiment, *ENA1*, which encodes a P-type ATPase (19), and *PHO89*, a gene required for phosphate uptake (28). For this experiment, two cultures were grown under identical conditions. One culture was washed and resuspended in medium lacking Mg^{2+} as described in Materials and Methods, and the other was mock treated with medium containing 1 mM Mg^{2+}. As shown in Fig. 1, both *ENA1* and *PHO89* were indeed highly induced within 15 min of Mg^{2+} starvation (lanes 8 to 12), whereas only a very small transient increase of *ENA1* and *PHO89* transcript levels can be observed in cultures treated the same way with the regular growth medium (1 mM Mg^{2+}). This small response in the control samples is probably due to the treatment of the cells (centrifugation and supply of fresh medium). While *PHO89* mRNA steady-state levels remained high for at least 4 h after Mg^{2+} depletion, *ENA1* induction appeared more temporal as the gene expression was again down-regulated after 2 h. Thus, at least for certain genes, this induction by Mg^{2+} depletion appears transient.

Induction of *ENA1* and *PHO89* transcription by low Mg^{2+} is mediated by the transcription factor Crz1p and requires calcineurin signaling. Many of the genes induced by Mg^{2+} starvation have previously been shown to be under the control of the calcineurin-dependent transcription factor Crz1p: i.e., the calcineurin/Crz1p-dependent signaling pathway (9, 57). Thus, we investigated whether transcriptional induction upon Mg^{2+} starvation is dependent on the transcription factor Crz1p. For this purpose, Mg^{2+} starvation and subsequent Northern blot analysis were performed on wild-type and *crz1*Δ strains. As seen in the Northern blot (Fig. 2A, lanes 1 and 2), both the *ENA1* and *PHO89* transcripts were increased by Mg^{2+} starvation. No such induction was observed in the *crz1*Δ cells (lanes 3 and 4). Moreover, steady state levels of *ENA1* and *PHO89* were slightly lower in *crz1*Δ cells grown in regular medium compared to the isogenic wild type (compare lanes 1 and 3).

The activity of Crz1p is regulated by the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin. Dephosphorylation of the transcription factor Crz1p results in its translocation to the nucleus, where it becomes active (36, 49). To determine whether Mg^{2+} starvation also promotes nuclear localization of Crz1p, we followed the localization of GFP-tagged Crz1p (36) under standard and Mg^{2+} starvation conditions, respectively. GFP-Crz1p is located in the cytoplasm under normal growth conditions (Fig. 2B, a and d). Yet, when the cells were shifted to Mg^{2+}-free medium, nuclear accumulation of GFP-tagged Crz1p was observed within 10 min (Fig. 2B, b and e). The same was true when cells were challenged with 200 mM Ca^{2+} (Fig. 2B, c and f), indicating that under both circumstances Crz1p is targeted to the nucleus to induce its target genes. However, it has to be noted that more GFP-Crz1p stays in the cytoplasm upon Mg^{2+} depletion than under Ca^{2+} stress. This observation is reminiscent of Crz1p nuclear translocation upon Na^+ (800 mM) or mild Ca^{2+} ($\leq$150 mM) stress, where only partial translocation to the nucleus had been seen (49). When cells were mock treated (centrifuged and washed) with fresh medium containing 1 mM $MgCl_2$, GFP-Crz1p stayed in the cyto-

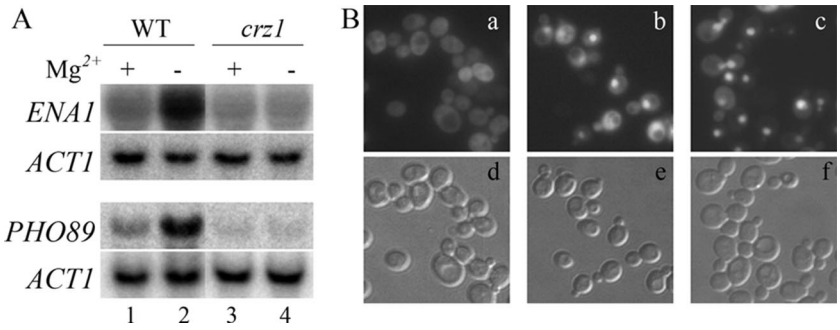

FIG. 2. The transcription factor Crz1p is required for the induction of *ENA1* and *PHO89* and translocated to the nucleus upon Mg^{2+} starvation. (A) Levels of expression of *ENA1* and *PHO89* in wild-type (WT [BY4741]) (lanes 1 and 2) and *crz1*Δ (lanes 3 and 4) cells were compared under standard conditions (1 mM Mg^{2+}, lanes 1 and 3) or 70 min of Mg^{2+} starvation (lanes 2 and 4). Northern blot analysis is shown. (B) Strain BY4741-*CRZ1*-GFP was grown at 28°C to log-phase in SD −Ura medium containing 1 mM $MgCl_2$ (a and d) and then washed and incubated for 10 min at 28°C in SD −Ura medium containing either no $MgCl_2$ (b and e) or 1 mM $MgCl_2$–200 mM $CaCl_2$–50 mM MES (pH 6) (c and f) before cells were harvested for microscopy and analyzed.

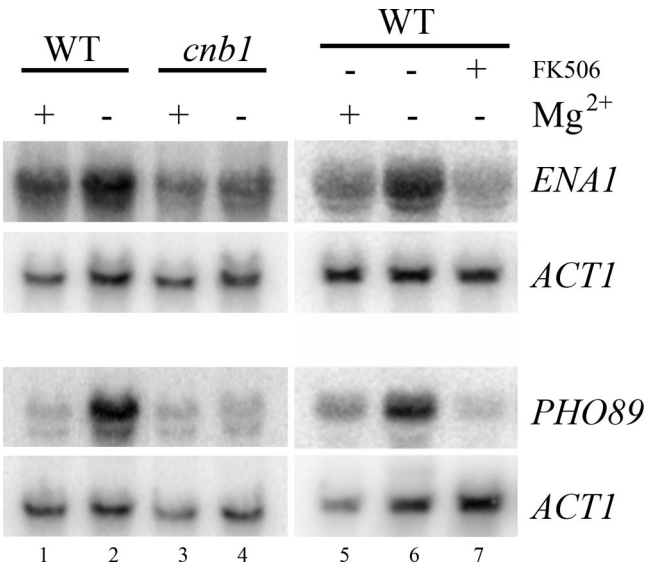

FIG. 3. The transcriptional response to Mg^{2+} starvation is dependent on calcineurin. Expression of *ENA1* and *PHO89* upon Mg^{2+} starvation (70 min) was followed in *cnb1*Δ mutant cells and in wild-type (WT) cells in the presence or absence of the calcineurin inhibitor FK506 (1.25 μM), which was added to the culture 10 min prior to the washes. Northern blot analysis is shown.

plasm (data not shown). Neither GFP alone, nor an Msn2-GFP fusion protein was translocated to the nucleus upon Mg^{2+} depletion (data not shown). Msn2, a transcriptional activator related to Msn4p, is activated under stress conditions, resulting in its translocation from the cytoplasm to the nucleus, where it binds DNA at stress response elements of responsive genes, inducing gene expression (http://www.yeastgenome.org).

To ascertain whether transcriptional induction of *ENA1* and *PHO89* is also dependent on calcineurin, the phosphatase responsible for dephosphorylation of Crz1p, we performed Mg^{2+} starvation experiments in the calcineurin mutant (*cnb1*Δ) and in wild-type cells in the presence of the calcineurin inhibitor FK506. As shown in Fig. 3, transcriptional activation of *ENA1* and *PHO89* was indeed dependent on calcineurin function: while the wild-type cells starved for Mg^{2+} showed normal induction of both transcripts (Fig. 3, lanes 1, 2, 5, and 6), *cnb1*Δ cells (Fig. 3, lanes 3 and 4) or cells pretreated with FK506 did not induce the two transcripts upon Mg^{2+} depletion (Fig. 3, lane 7). Treatment of the cells with only dimethyl sulfoxide (the solvent for FK506) did not abolish the transcriptional response to Mg^{2+} depletion (data not shown). Similarly, addition of FK506 to the control experiment (1 mM Mg^{2+}) did not affect expression (data not shown). Taken together, our results show that removal of Mg^{2+} from the growth medium results in activation of the calcineurin/Crz1p signaling pathway.

Cells lacking calcineurin/Crz1p signaling are sensitive to low Mg^{2+}. We have shown that yeast cells react to Mg^{2+} deprivation by activating a number of genes via the calcineurin/Crz1p pathway. To investigate whether this response is necessary for cells to cope with low-Mg^{2+} stress, we analyzed the growth on Mg^{2+}-depleted medium of mutants defective for calcineurin signaling. As shown in Fig. 4 (left panel), growth of cells lacking either Crz1p or Cnb1p (calcineurin B), the regulatory subunit of calcineurin, is indistinguishable from that of wild-type cells on synthetic medium containing standard magnesium concentrations. In contrast, when Mg^{2+} is omitted from the medium *crz1*Δ or *cnb1*Δ mutants exhibit clearly reduced growth compared to the wild type (Fig. 4, right panel), indicating that the induction of the calcineurin pathway is important for cells to cope with Mg^{2+} depletion.

External Ca^{2+} is required for the induction of *ENA1* and *PHO89* in response to low Mg^{2+}. Next, we investigated the role of Ca^{2+} in the calcineurin/Crz1p dependent up-regulation of *ENA1* and *PHO89* upon Mg^{2+} depletion. While in animal cells, the endoplasmic reticulum is the major site of intracellular Ca^{2+} storage and release, this does not seem to be the case for yeast (50). The two major sources for calcium in yeast are the vacuole and the medium (12, 41). Therefore, we asked whether the source of the calcium signal is outside the cell. We compared the *ENA1* and *PHO89* induction from cells grown in

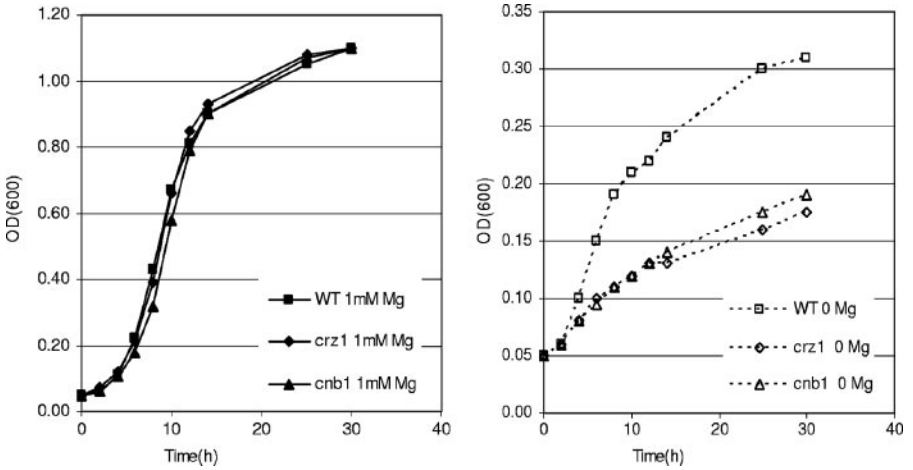

FIG. 4. *crz1*Δ and *cnb1*Δ cells are sensitive to low Mg^{2+}. BY4741 wild-type (WT) and *crz1*Δ and *cnb1*Δ mutant cells were cultured in synthetic SD medium containing 1 mM Mg^{2+} overnight, washed three times in distilled H_2O, and then inoculated (OD_{600} of 0.05) into synthetic SD medium containing 1 mM Mg^{2+} (left panel) or no Mg^{2+} (right panel). Cells were incubated at 28°C with shaking, and growth was followed by measuring the OD_{600}.

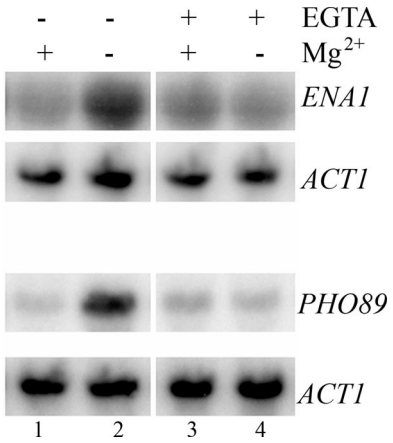

FIG. 5. External Ca^{2+} is required for the induction of *ENA1* and *PHO89* upon Mg^{2+} starvation. Expression of *ENA1* and *PHO89* upon Mg^{2+} starvation (70 min) was followed in the presence or absence of the Ca^{2+} chelator EGTA (10 mM). Northern blot analysis is shown.

medium containing standard concentrations of Ca^{2+} with those grown in medium containing the Ca^{2+} chelator EGTA (Fig. 5). When 10 mM EGTA was added to the medium before and after the shift to Mg^{2+}-free medium, the induction was completely abolished (Fig. 5, lanes 3 and 4), as compared to standard Ca^{2+} conditions (Fig. 5, lanes 1 and 2). Thus, we conclude that external calcium contributes to the transcriptional induction by Mg^{2+} depletion and that external calcium can indeed become limiting for the response, as the signal is completely abolished when EGTA is added to the media.

Cytoplasmic Ca^{2+} levels increase upon Mg^{2+} depletion with dependence on external Ca^{2+}. Since removal of Mg^{2+} from the medium resulted in activation of the calcineurin/Crz1p pathway and was dependent on external Ca^{2+}, we wanted to know if this was caused by an increase in cytosolic Ca^{2+} concentration upon Mg^{2+} removal. A number of external stimuli, such as Ca^{2+} or Na^{+} stress, alkalinization of the growth medium, alpha factor, and hyper- and hypo-osmotic stress have so far been shown to trigger a rise in cytoplasmic Ca^{2+} (3, 11, 32, 52, 57). To determine cytoplasmic Ca^{2+} concentrations, we expressed the cytosol-targeted Ca^{2+}-sensing protein YC2-12 (30) in yeast under the strong constitutive *ADH1* promoter. This sensor is a member of the "cameleon family" of Ca^{2+} indicators and consists of tandem fusions of a cyan-emitting fluorescent protein, calmodulin, the calmodulin-binding peptide M13, and a yellow-emitting fluorescent protein. Upon binding of Ca^{2+} to calmodulin, this protein binds to the M13 domain, resulting in an increase in the fluorescence resonance energy transfer between the flanking fluorophores. To show that the sensor is working in yeast, we challenged cells with 200 mM external Ca^{2+}. This treatment resulted in a cytoplasmic Ca^{2+} peak (Fig. 6, right panel). Next, we investigated cytosolic Ca^{2+} upon Mg^{2+} depletion. As shown in Fig. 6 (left panel), removal of extracellular Mg^{2+} resulted in a rapid increase of the cytosolic Ca^{2+} concentration in the presence of 2 mM external Ca^{2+}. This signal was reversible and normalized upon readdition of Mg^{2+} into the external solution. In contrast, in the absence of extracellular Ca^{2+} (i.e., Ca^{2+}-free buffer containing 10 mM EGTA), no cytosolic Ca^{2+} elevation was obtained in response to Mg^{2+} removal. These data indicate that the cyto-

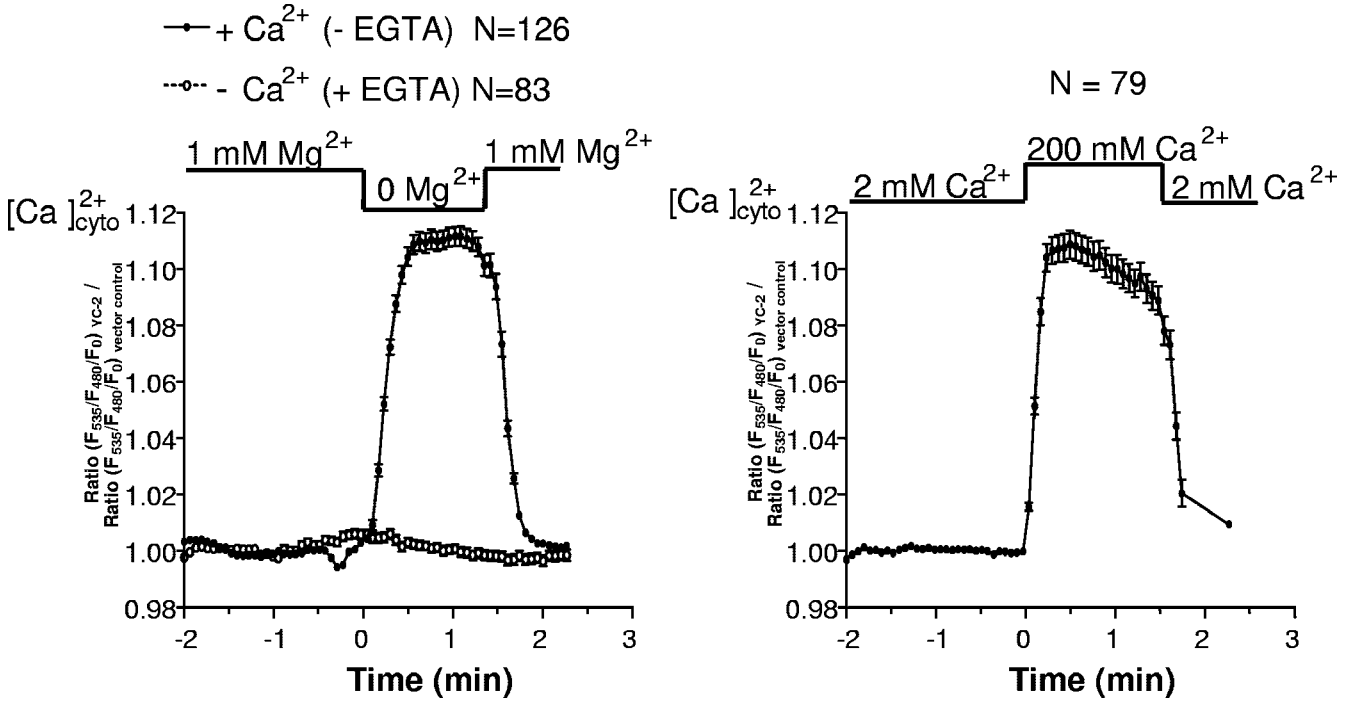

FIG. 6. Cytoplasmic Ca^{2+} levels increase upon Mg^{2+} depletion in dependence of external Ca^{2+}. Cytosolic free Ca^{2+} concentrations were analyzed upon Mg^{2+} depletion (left panel) or upon addition of 200 mM Ca^{2+} (right panel) using fluorescence resonance energy transfer microscopy.

plasmic elevation of Ca^{2+} in response to the removal of external Mg^{2+} essentially depends on the presence of extracellular Ca^{2+}, strongly indicating that an influx of Ca^{2+} is occurring under these conditions. The cytoplasmic Ca^{2+} elevation upon removal of extracellular Mg^{2+} closely matched the peak obtained in response to 200 mM external Ca^{2+}.

DISCUSSION

Interactions of Ca^{2+} and Mg^{2+}. Cellular Ca^{2+} and Mg^{2+} levels appear linked in many circumstances, such that high Mg^{2+} results in low Ca^{2+} and vice versa. Thus, internal Mg^{2+} or Ca^{2+} concentrations can be reciprocally modulated by altering medium concentrations of Mg^{2+} or Ca^{2+}, by hormone stimulation, or by mutations of ion transporters (5, 17, 31). There are Ca^{2+}-dependent Mg^{2+} transporters, possibly antiporters, present in hepatocytes (13, 39), as well as a Ca^{2+}/Mg^{2+} exchanger in the apical rat liver plasma membrane (7). Also, Mg^{2+} acts at an extracellular site on L-type Ca^{2+} channels to regulate Ca^{2+} influx (2); the same is found on T-type Ca^{2+} channels (44). There is evidence that this modulatory effect of Mg^{2+} involves the EF-hand motif of the COOH-terminus of Ca^{2+} channels (6). In the present study, we found that the transcriptomal response of Mg^{2+} depletion is very similar to the Ca^{2+} stress response: i.e., yeast cells respond to Mg^{2+} withdrawal from growth medium with an immediate upshift of intracellular Ca^{2+} concentrations; activation of the transcription factor Crz1p via calcineurin, a Crz1p phosphatase; and the upregulation of a gene set known to be under the control of the calcineurin/Crz1p pathway. Mg^{2+} depletion thus elicits a response which is widely similar to that of Ca^{2+} stress, high Na^+, or alkalinization.

Induction of the calcineurin/Crz1p pathway by low-Mg^{2+} stress. This study commenced with the observation that short-term Mg^{2+} depletion in the yeast *S. cerevisiae* induces a number of genes known to be calcineurin/Crz1p regulated (45, 52, 57). This induction is immediate and transient. Crz1p is the vital transcription factor for Ca^{2+} signaling in yeast. Crz1p contains a zinc finger motif for DNA binding and binds specifically to the calcineurin-dependent response element, a 24-bp DNA sequence both necessary and sufficient for Ca^{2+}-induced, calcineurin-dependent gene expression (48). The conserved Ca^{2+}/calmodulin-regulated protein phosphatase calcineurin dephosphorylates and thereby activates Crz1p (49). Calcineurin is inhibited by the immunosuppressive drugs FK506 and cyclosporine and is essential for the antigen-dependent activation of T lymphocytes in higher eukaryotes (8, 25, 34). When dephosphorylated by calcineurin, Crz1p translocates from the cytoplasm to the nucleus, where it binds its target DNA in order to activate downstream genes (49; reviewed in reference 9). Similarly, we found that Crz1p shifted to the nucleus also upon Mg^{2+} depletion, as in the case of Ca^{2+} or Na^+ stress (57), and Crz1p target genes were upregulated. Since this effect was missing in the calcineurin mutant and was inhibited by the calcineurin inhibitor FK506, dephosphorylation of Crz1p by calcineurin is a prerequisite. Taken together, short-term Mg^{2+} depletion results in the induction of the calcineurin/Crz1p pathway and requires both calcineurin and Crz1p function. This response is necessary for yeast cells to function properly under conditions of Mg^{2+} depletion, as calcineurin/Crz1p pathway mutants displayed reduced growth in Mg^{2+}-depleted medium. The parallel induction of Crz1p by Mg^{2+} shortage and Ca^{2+} stress is reminiscent of the regulation of the transcription factor Aft1p, which, upon iron shortage as well as Co^{2+} stress, moves to the nucleus and induces its target genes (47, 55). In both instances, shortage of the more abundant ions (iron/Mg^{2+}) mimics the effects induced by imbalances in the respective ions at lower physiological concentrations (Co^{2+}/Ca^{2+}). Also in both cases, these distinct responses confer resistance to the stress induced by metal ion shortage, or overexposure, respectively.

Mg^{2+} depletion induces rapid Ca^{2+} influx. Depletion of external Mg^{2+} concentrations has no obvious short-term effect on intracellular Mg^{2+} concentrations (4, 17). Accordingly, the signal for switching on the calcineurin/Crz1 pathway is likely to arise from effects of low Mg^{2+} at the cell surface and to be mediated through the cell membrane. K^+ and Ca^{2+} channels have been reported to open when Mg^{2+} falls below threshold concentrations (2, 33, 44, 56), and an effect of Mg^{2+} withdrawal on the opening of Ca^{2+} channels and influx of Ca^{2+} thus appears to be likely. In fact, we have demonstrated here that external Ca^{2+} is essential for the induction of Crz1p target genes by Mg^{2+} depletion. Moreover, upon Mg^{2+} depletion external Ca^{2+} is rapidly internalized, leading to high cytoplasmic Ca^{2+} similar to that observed in the presence of high Ca^{2+} concentrations in the medium.

High intracellular Ca^{2+} may also be helpful for the release of internal Mg^{2+} stores, perhaps maintaining the availability of free Mg^{2+} upon medium Mg^{2+} depletion.

Ca^{2+}/calcineurin signaling is conserved from yeast to mammals. Activation of Crz1p by calcineurin is reminiscent of the regulation of the mammalian NFAT1 to -4 transcription factor proteins by Ca^{2+}/calcineurin-dependent signaling. Similar to the situation in yeast, a rise in intracellular Ca^{2+} activates calcineurin in mammals, which in turn dephosphorylates all four NFAT proteins, leading to their rapid nuclear import (8, 22). Depending upon which binding partners are involved Ca^{2+}/calcineurin-NFAT-mediated signaling pathways regulate gene expression either positively or negatively. Binding partners can be AP-1 (composed of Fos and Jun proteins), MEF2, GATA proteins, and histone deacetylases (21). Especially with respect to diseases associated with altered serum Mg^{2+} levels (hypomagnesemia and hypermagnesemia), it will be interesting to learn whether changes in the serum Mg^{2+} availability also influence Ca^{2+} signaling in higher eukaryotes.

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