BJ www.biochemj.org

BJ Signal

Biochem. J. (2010) **427**, 275–287 (Printed in Great Britain) doi:10.1042/BJ20091334

online data

Calcineurin/Crz1 destabilizes Msn2 and Msn4 in the nucleus in response to Ca^{2+} in *Saccharomyces cerevisiae*

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Although methylglyoxal is derived from glycolysis, it has adverse effects on cellular function. Hence, the intrinsic role of methylglyoxal *in vivo* remains to be determined. Glyoxalase 1 is a pivotal enzyme in the metabolism of methylglyoxal in all types of organisms. To learn about the physiological roles of methylglyoxal, we have screened conditions that alter the expression of the gene encoding glyoxalase 1, *GLO1*, in *Saccharomyces cerevisiae*. We show that the expression of *GLO1* is induced following treatment with Ca^{2+} and is dependent on the MAPK (mitogen-activated protein kinase) Hog1 protein and the Msn2/Msn4 transcription factors. Intriguingly, the Ca^{2+}-induced expression of *GLO1* was enhanced in the presence of FK506, a potent inhibitor of calcineurin. Consequently, the Ca^{2+}-induced expression of *GLO1* in a mutant that is defective in calcineurin or Crz1, the sole transcription factor downstream of calcineurin, was much greater than that in the wild-type strain even without FK506. This phenomenon was dependent upon a *cis*-element, the STRE (stress-response element), in the promoter that is able to mediate the response to Ca^{2+} signalling together with Hog1 and Msn2/Msn4. The level of Ca^{2+}-induced expression of *GLO1* reached a maximum in cells overexpressing *MSN2* even when FK506 was not present, whereas in cells overexpressing *CRZ1* the level was greatly reduced and increased markedly when FK506 was present. We also found that the levels of Msn2 and Msn4 proteins in Ca^{2+}-treated cells decreased gradually and that FK506 blocked the degradation of Msn2/Msn4. We propose that Crz1 destabilizes Msn2/Msn4 in the nuclei of cells in response to Ca^{2+} signalling.

Key words: calcineurin, calcium, Crz1, FK506, methylglyoxal, Msn2/Msn4.

INTRODUCTION

MG (methylglyoxal) is a typical 2-oxoaldehyde that is synthesized during glycolysis, a ubiquitous energy-generating pathway [1,2]. Although MG is a natural metabolite it can inhibit the growth of cells [1–3] and in some cases induce apoptosis or necrosis, with the mode of cell death depending upon the cell line examined [4–6]. MG was once believed to be a major intermediate of glycolysis, but that hypothesis has been disproved; nevertheless, MG and its metabolic enzymes have received considerable attention, because MG is involved in diseases such as diabetes and its complications, cancers, Alzheimer's disease and autism [7–12]. Hence, there must be an ingenious system to avoid any metabolic disorder involving MG in cells, thereby reducing the risk of suffering such diseases. However, the molecular mechanisms by which MG causes such diseases are not well understood.

To find a clue as to the physiological role of MG, we searched for conditions that alter intracellular levels of MG, as well as the activities of MG-metabolizing enzymes in the yeast *Saccharomyces cerevisiae*. We have reported previously that the expression of *GLO1* is specifically induced by osmotic stress via the MAPK (mitogen-activated protein kinase) Hog1 [13] and that expression of *GLO1* is crucial for the metabolism of MG [14]. The *GLO1* promoter contains a characteristic *cis*-acting element, the STRE (stress-response element). Genes possessing the STRE usually respond to a wide variety of stress stimuli, such as oxidative stress, heat shock stress and osmotic stress [15]. *MSN2* and *MSN4*, originally cloned as multicopy suppressors of the *SNF1* mutation, encode C_2H_2-type zinc finger transcription factors that have a high degree of functional redundancy; they have been shown to translocate into the nucleus, and bind to the STRE under environmental stress [15a]. Even though the *GLO1* promoter possesses two STREs, intriguingly, *GLO1* did not respond to any stress other than osmotic stress [13]. *S. cerevisiae* produces glycerol as a compatible osmolyte under high-osmotic stress [16]. We found that the uptake of glucose, and subsequently, the flux of glucose into glycolysis, was enhanced when cells were exposed to high-osmotic stress in order to facilitate glycerol production [13]. Consequently, intracellular MG increased [13], because the major source of MG is glycolysis. Therefore we have proposed previously that the physiological significance of the specific expression of *GLO1* under high-osmotic stress is probably due to the efficient metabolism of MG, which increases during the response to osmotic stress [2,13].

On the other hand, we have studied the effect of MG on cellular function. As a result, we revealed that MG functions as a signal initiator in yeasts [17–19]. For example, we found that the AP-1 (activator protein 1)-like bZIP (basic leucine zipper) transcription factor Yap1 is constitutively activated in *glo1*Δ cells. This occurred via MG-mediated modification of cysteine residues and these residues are crucial for the determination of its nucleocytoplasmic localization [17]. Yap1 also plays crucial

Abbreviations used: AP-1, activator protein 1; CDMD, Crz1-dependent Msn2/4 degradation; CDRE, calcineurin-dependent response element; MAPK, mitogen-activated protein kinase; MG, methylglyoxal; SD, synthetic dextrose; STRE, stress-response element; YPD medium, 1% (w/v) yeast extract/2% (w/v) peptone/2% (w/v) glucose medium.

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roles in the response to oxidative stress, as well as drug stress in *S. cerevisiae* (for a review see [20]). Recently, we found that MG attenuates overall protein synthesis through phosphorylation of the translation initiation factor eIF2α in a TOR (target of rapamycin)-independent manner [21], and another AP-1-like transcription factor Gcn4 plays a role in the adaptive response to MG stress [22]. These results show that cellular levels of MG must be controlled adequately to warrant normal cellular functions.

In the present study we show that Ca^{2+} induces the expression of *GLO1*, and that the induction is strictly dependent upon Hog1, Msn2/Msn4 and the two STREs in the *GLO1* promoter. Notably, the Ca^{2+}-induced expression of *GLO1* was further augmented by FK506, a potent inhibitor of calcineurin [23]. Calcineurin is a protein phosphatase whose activity is regulated by Ca^{2+}/calmodulin. Full induction of the expression of *GLO1* following treatment with Ca^{2+} was observed in a mutant defective in either calcineurin or Crz1, which encodes the sole transcription factor functioning under the control of calcineurin [24–27]. We also provide evidence that Crz1 negatively affects the functions of Msn2/Msn4 in the nuclei of cells treated with Ca^{2+} by facilitating the degradation of Msn2 and Msn4. Consequently, a further increase in the Ca^{2+}-induced expression of *GLO1* was seen when either FK506 was present, or calcineurin or Crz1 was disrupted. Our results suggest that Crz1 destabilizes Msn2 and Msn4 proteins in the nucleus when cells are treated with Ca^{2+}.

EXPERIMENTAL

Strain, plasmids and medium

Unless otherwise stated, yeast strains used in the present study have the YPH250 background (*MAT*a *trp1-Δ1 his3-Δ200 leu2-Δ1 lys2–801 ade2–101 ura3–52*). Construction of the *hog1*Δ, *pbs2*Δ, *ssk1*Δ, *sho1*Δ, *ssk1*Δ*sho1*Δ, *cnb1*Δ, *crz1*Δ, *msn2*Δ and *msn2*Δ*msn4*Δ mutants in YPH250 has been described previously [13,18]. The *erg6*Δ (deficient in ergosterol biosynthesis) mutant has the BY4741 background (*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Cells were cultured in YPD medium [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] or SD (synthetic dextrose) minimal medium [2 % (w/v) glucose and 0.67 % yeast nitrogen base without amino acids] with appropriate amino acids and bases at 28 °C with reciprocal shaking.

Enzyme assay

The preparation of cell extracts and measurement of the activities of Glo1 and β-galactosidase were as described previously [13,14]. One unit of the activity of Glo1 was defined as the amount of enzyme forming 1 μmol of *S*-D-lactoylglutathione per min using a millimolar absorption coefficient of 3.37 $mM^{-1}\cdot cm^{-1}$ [14]. One unit of β-galactosidase activity was defined as the amount of enzyme that increases the A_{420} by 1000 per h [13]. Protein concentrations were determined by the Bradford method [28].

Northern blotting

Cells were cultured in YPD medium until the D_{610} was 1.0, and 300 mM $CaCl_2$ and/or 1 μg/ml FK506 were added. After 30 min of incubation, total RNA was prepared according to the method described by Schmitt et al. [29]. The DNA probe, labelled with [α-^{32}P]dCTP was prepared as described previously [13].

Detection of Hog1 phosphorylation

Cells were cultured in YPD medium until the D_{610} was 1.0, and 300 mM $CaCl_2$ and/or 1 μg/ml FK506 were added. After 60 min of incubation, cell lysates were prepared as described by Bell et al. [30]. Yeast cellular proteins were separated by SDS/PAGE (10 % gels) and transferred on to a PVDF membrane. Anti-phospho-p38 monoclonal antibody (Sigma–Aldrich) was used as the primary antibody and anti-(rabbit IgG) antibody conjugated with horseradish peroxidase (New England Biolabs) was used as the secondary antibody. To measure the level of Hog1 protein, an anti-Hog1 antibody (Santa Cruz Biotechnology) was used as a primary antibody.

Detection of Msn2 and Msn4

To determine the levels of Msn2 and Msn4 proteins, cells carrying pRS316+Msn2-GFP or pGR247 (pAdh1-Msn4-GFP) [31] were cultured in YPD medium until they were growing exponentially and they were then treated with 300 mM $CaCl_2$. Cells were collected (2000 ***g*** centrifugation for 15 s) at the indicated prescribed time, and disrupted with glass beads in 100 mM Tris/HCl buffer, pH 7.0, containing 5 mM $MgCl_2$, 10 % (v/v) glycerol, 0.1 % Triton X-100, 50 mM NaCl, 1 mM dithiothreitol and protease inhibitor cocktail (Nacalai Tesque). Cell extracts (20 μg of protein) were subjected to SDS/PAGE (7.5 % gels). Anti-GFP (green fluorescent protein) antibody (Santa Cruz Biotechnology) was used to detect Msn2–GFP and Msn4–GFP. Detection of Cdc28 was used as a loading control [the membrane was treated in 0.2 M glycine (pH 2) for 5 min and reprobed with anti-Cdc2 antibody (Santa Cruz Biotechnology)].

Construction of *GLO1–lacZ* reporter gene

The *GLO1–lacZ* cassette in pRSGlac415 [13] was cloned into the SalI site of YIp5 (to give YIp5+GLO1-lacZ), and the cassette was then amplified by PCR with primers 5TO358F (5′-AGGGCATCGGTCGACGGATCCGGGTAATTC-3′) and 5TO358R (5′-TAAAACGACGGAATTCCCGGGTTTCTCAAT-3′) using pYIp5+GLO1-lacZ as a template. SalI and EcoRI sites were added to 5TO358F and 5TO358R respectively (underlined nucleotides). The PCR fragment was digested with SalI and EcoRI, and cloned into the SalI/EcoRI site of YIp358R. The resultant plasmid (YIp358R+GLO1-lacZ) was digested with NcoI and the linearized fragment was integrated into the *ura3* locus of YPH250.

To insert a point mutation into the STREs (5′-AGGGG-3′→5′-AGATG-3′), an overlap extension PCR was conducted [32]. For the site-directed mutagenesis, four internal primers (GSTRE1, 5′-AATAGGTAAAGAGATGGGTGGGGGTGG-3′; GSTRE1R, 5′-CCACCCCCACCCATCTCTTTACCTATT-3′; GSTRE2, 5′-CTGAATAAACAAGATGCTTTACGATGG-3′; and GSTRE2R, 5′-CCATCGTAAAGCATCTTGTTTATTCAG-3′) were designed. To create the mutation in STRE1 (from −432 to −428 relative to the initiation ATG codon), the first PCR was performed with 5TO358F plus GSTRE1R, and 5TO358R plus GSTRE1. The second PCR was performed with 5TO358F and 5TO358R as the primers and a mixture of the products of each first PCR as the template. The PCR product was digested with SalI and EcoRI and the resultant fragment was inserted into the SalI/EcoRI site of YIp358R to yield YIp358R + PMS1. Similarly, to create the mutation in STRE2 (from −229 to −225), the first PCR was performed with 5TO358F plus GSTRE2R and 5TO358R plus GSTRE2. The second PCR was performed with 5TO358F and 5TO358R and a mixture of the products of each first PCR as the template. The PCR product was cloned into YIp358R as described above (YIp358R + PMS2). To construct the *GLO1–lacZ* reporter gene carrying both mutated STRE1 and STRE2 (PMS12), the first PCR was performed with

5TO358F plus GSTRE2R and 5TO358R plus GSTRE2 using YIp358R + PMS1 as a template. The second PCR was performed using 5TO358F and 5TO358R and a mixture of the products of each first PCR as the template. The PCR product was digested with SalI and EcoRI, and the resultant fragment was inserted into the SalI/EcoRI site of YIp358R to yield YIp358R + PMS12. The correct introduction of the mutation was verified by DNA sequencing.

Construction of glutathione peroxidase reporter gene *GPX1–lacZ*

To amplify the region between −365 and +8 of *GPX1* (relative to the translation initiation ATG codon), PCR was performed with primer GPX1–lacZ-F (5′-GGAGTCGAC-GGACTTGATAGAATCCACCTT-3′) and GPX1-lacZ-R (5′-GGTGAAAAAGAATGAATTCCTTGCATCGTT-3′) using the genomic DNA of YPH250 as a template. SalI and EcoRI sites were designed in GPX1–lacZ-F and GPX1-lacZ-R respectively (underlined nucleotides). The PCR fragment was digested with SalI and EcoRI, and cloned into the SalI/EcoRI site of YIp358R. The resultant plasmid (YIp358R-GPX1-lacZ) was digested with NcoI, and the linearized fragment was integrated into the *ura3* locus of YPH250.

Construction of *HOG1*-overexpression plasmid

The *HOG1* gene was amplified by PCR with primers HOG1S (5′-GTTGTTAGGAAAGCATGCTTTATCTCCAAG-3′) and HOG1R (5′-CCTTTTATGGGATCCTAATTTCTTAAGGAG-3′) using the genomic DNA of YPH250 as a template. SphI and BamHI sites were designed in HOG1S and HOG1R respectively (underlined nucleotides). The PCR fragment was digested with SphI and BamHI, and cloned into the SphI/BamHI sites of plasmid pRS423 to yield pRS423+HOG1.

Fluorescence microscopy

Cells expressing GFP-tagged proteins were cultured in YPD medium to a D_{610} of 0.5, and 300 mM $CaCl_2$ was added in the presence or absence of 1 μg/ml FK506. After incubation for the prescribed time at 28 °C, the intracellular localization of each GFP-tagged protein was observed using a fluorescence microscope (Olympus BX51). Plasmids carrying Hog1–GFP, Msn2–GFP and Crz1–GFP were pRS4-Hog1-GFP [18], pAMG (pAdh1-Msn2-GFP) [33] and pASM463 [34] respectively.

RESULTS

Expression of *GLO1* is induced by Ca^{2+} in a Hog1 MAPK cascade-dependent manner

We first searched for conditions under which either the cellular concentration of MG is changed or the expression of *GLO1*, which encodes an enzyme crucial for the metabolism of MG [2,13], is altered. We found that levels of *GLO1* mRNA were increased following treatment with $CaCl_2$ (Figure 1A). We therefore constructed a *GLO1–lacZ* reporter gene to verify the Ca^{2+}-induced expression of *GLO1* and found that the activity of β-galactosidase driven by *GLO1–lacZ* was increased following treatment with $CaCl_2$ (see Figure 5B). The activity of Glo1 was also increased in cells treated with $CaCl_2$ (Figure 1B). Therefore we concluded that Ca^{2+} induces the expression of *GLO1*.

Hog1 is one of the MAPKs in *S. cerevisiae* and plays a crucial role in the response to osmotic stress (for reviews see [35,36]).

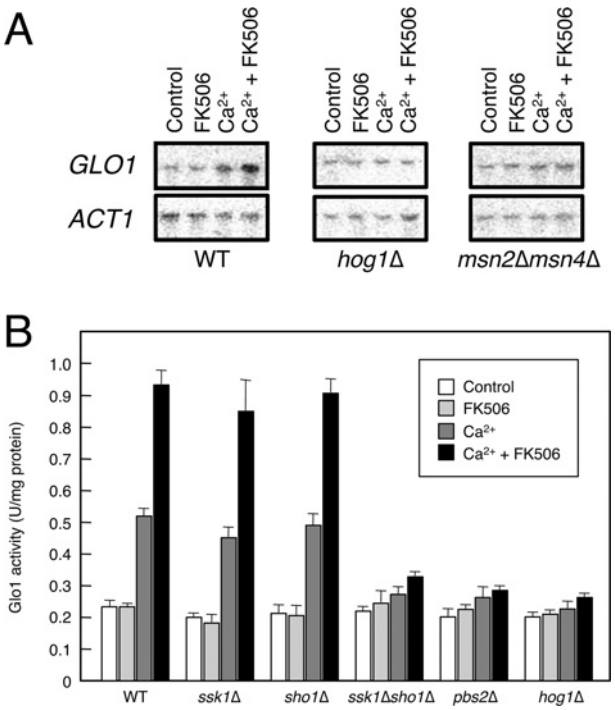

Figure 1 Effects of Ca^{2+} and FK506 on the expression of *GLO1*

(**A**) Wild-type (WT), *hog1*Δ and *msn2*Δ*msn4*Δ cells were cultured in YPD medium until an exponential phase of growth and were then treated or not with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 30 min of incubation, total RNA was prepared and a Northern blot analysis for *GLO1* was conducted. *ACT1* (actin) was used as a control. (**B**) Wild-type (WT) and mutant cells were treated with $CaCl_2$ and/or FK506 as described in (**A**). After 60 min of incubation, cell extracts were prepared, and Glo1 activity was measured. U, unit.

Therefore we conducted a Northern blot analysis to determine the *GLO1* mRNA level in a *hog1*Δ mutant. As shown in Figure 1(A), the level of *GLO1* mRNA was not increased in *hog1*Δ cells following the treatment with $CaCl_2$. Induction of Glo1 activity by $CaCl_2$ also did not occur in the *hog1*Δ nor the *pbs2*Δ mutant, which is defective in a MAPK kinase responsible for the activation of Hog1 (Figure 1B).

Besides Hog1, we have reported previously that the Msn2 and Msn4 C_2H_2 zinc finger transcription factors are necessary for the up-regulation of *GLO1* in response to osmotic stress [13]. To determine whether these transcription factors are also involved in the Ca^{2+}-induced expression of *GLO1*, we conducted Northern blotting in an *msn2*Δ*msn4*Δ double mutant. As shown in Figure 1(A), the levels of *GLO1* mRNA in *msn2*Δ*msn4*Δ cells did not increase following the treatment with $CaCl_2$, indicating that Msn2 and Msn4 are necessary for the response of *GLO1* to Ca^{2+}.

Next, we determined the roles of the osmosensors functioning upstream of the Hog1 MAPK cascade. In *S. cerevisiae*, two such osmosensors, Sln1 and Sho1, have been thus far identified. The Sln1 branch consists of the phospho-relay protein Ypd1 and the response regulator Ssk1, constituting a two-component system [37]. Two redundant MAPK kinase kinases (Ssk2 and Ssk22) function downstream of the Sln1 branch. In the Sho1 branch, Sho1 physically interacts with Pbs2 through the SH3 (Src homology 3) domain [38,39]. To determine whether these two osmosensors are involved in the Ca^{2+}-induced expression of *GLO1*, we measured the Glo1 activity in cells defective in these branches. As *SLN1* is an essential gene, we disrupted *SSK1* and/or *SHO1* to inactivate the osmotic-stress signalling pathways upstream of Hog1. As shown

in Figure 1(B), the induction of *GLO1* expression was observed in mutants defective in either *SSK1* or *SHO1*, although it was repressed in the *ssk1*Δ*sho1*Δ double mutant.

Response of *GLO1* to Ca^{2+} and FK506

Although we have shown that the Hog1 MAPK cascade is necessary for the Ca^{2+}-induced expression of *GLO1*, we also set out to determine whether the Ca^{2+}/calmodulin-dependent calcineurin system was also involved in this response, because the system is a well-known pathway mediating Ca^{2+} signalling in eukaryotic cells, including in *S. cerevisiae* [40]. In the Ca^{2+}/calcineurin system, extracellular Ca^{2+} enters the cell and binds calmodulin to activate a protein phosphatase calcineurin, which in turn dephosphorylates Crz1, a transcription factor functioning downstream of calcineurin [24,25]. As FK506 is a potent inhibitor of calcineurin [23], the expression of the Ca^{2+}-induced genes regulated by the Ca^{2+}/calcineurin system, e.g. *GSC2* (glucan synthase of cerevisiae, also known as *FKS2*) and *GPX2* [41,42] is usually markedly repressed in its presence. To address whether the calcineurin system is involved in the Ca^{2+}-induced expression of *GLO1*, we determined the effect of FK506. Surprisingly, and rather bizarrely, the Ca^{2+}-induced expression of *GLO1* mRNA was further increased when FK506 was present (Figure 1A). Similarly, the activities of β-galactosidase, driven by the *GLO1–lacZ* reporter, as well as Glo1 were further increased by the simultaneous treatment of cells with $CaCl_2$ and FK506 (Figures 1B and 5B). This was observed in the *ssk1*Δ and *sho1*Δ mutants, but not in the *ssk1*Δ*sho1*Δ mutant (Figure 1B). These results show that two osmosensors function as the upstream modules for the transduction of the Ca^{2+} signal to Hog1, and subsequently, that there was a bizarre response, whereby Glo1 is induced by Ca^{2+} and FK506.

Calcineurin/Crz1 pathway is involved in the bizarre response of *GLO1*

We confirmed that the bizarre response of *GLO1* to Ca^{2+} and FK506 as described above was also seen when employing much lower concentrations (50 mM) of $CaCl_2$ (results not shown). However, as the maximal induction of *GLO1* is brought about by 200–300 mM $CaCl_2$, we subsequently analysed this response in the presence of 300 mM $CaCl_2$.

To verify whether the bizarre response caused by FK506 in terms of the Ca^{2+}-induced expression of *GLO1* was brought about by a certain action of FK506 on the machinery involved in Ca^{2+} signalling or whether it was dependent upon the calcineurin system we conducted a genetic analysis. We disrupted both the regulatory subunit of calcineurin (*CNB1*), to abolish the phosphatase activity [43], and also Crz1. As shown in Figure 2(A), the expression of *GLO1* in both *cnb1*Δ and *crz1*Δ mutants reached a maximum level on treatment with $CaCl_2$ alone, and the treatment of these mutants with $CaCl_2$ and FK506 simultaneously did not enhance the expression of *GLO1* any further. Thereby we confirmed that the effect of FK506 on the expression of *GLO1* with respect to the response to Ca^{2+} was exerted through the calcineurin/Crz1 pathway.

This positive effect of FK506 was not observed for the NaCl-induced expression of Glo1 (Figure 2B). This was also the case for the KCl- and sorbitol-induced expression of *GLO1* mRNA (results not shown). Furthermore, basal levels of *GLO1* mRNA, as well as Glo1 activity, did not increase in the *cnb1*Δ and *crz1*Δ mutants (Figures 2A and 2B), therefore the calcineurin/Crz1 pathway does not act as a negative regulator of the expression of *GLO1* under

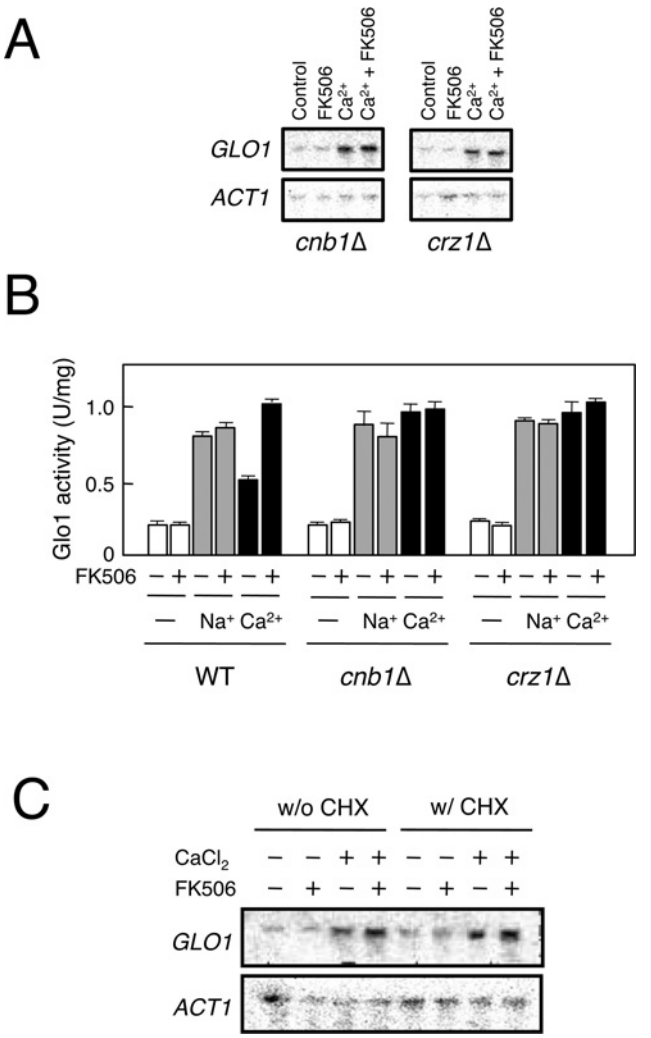

Figure 2 Calcineurin/Crz1 pathway is involved in the Ca^{2+}-induced expression of *GLO1*

(**A**) *cnb1*Δ or *crz1*Δ cells were cultured in YPD medium until an exponential phase of growth and were then treated or not with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 30 min of incubation, total RNA was prepared and a Northern blot analysis was conducted. *ACT1* (actin) was used as a control. (**B**) Wild-type (WT), *cnb1*Δ or *crz1*Δ cells were treated or not with 300 mM $CaCl_2$ or 500 mM NaCl and/or 1 μg/ml FK506 as indicated. After 60 min of incubation, cell extracts were prepared, and Glo1 activity was measured. (**C**) Wild-type cells (w/o CHX) and wild-type cells that had been pre-treated with 10 μg/ml cycloheximide (w/ CHX) for 10 min were treated with 300 mM $CaCl_2$ and/or 1 μg/ml FK506 and analysed as in (**A**). U, unit.

normal conditions. It is likely that the effect of FK506 on the Hog1-dependent induction of the expression of *GLO1* occurs only when Ca^{2+} is present, and FK506 does not enhance the response to osmotic stress in general. Therefore we temporally referred to this phenomenon as the 'bizarre response', because Crz1 is a transcription factor that essentially has a positive effect on its target gene, but nevertheless, Crz1 acted as a negative regulator for the Ca^{2+}-induced expression of *GLO1*.

One possible explanation for the bizarre response is that some newly synthesized protein(s), the synthesis of which is regulated by the calcineurin/Crz1 pathway, function as a negative regulator of the expression of *GLO1* in response to Ca^{2+}. To address this possibility, we treated yeast cells with $CaCl_2$ and FK506 in the presence of cycloheximide. As shown in Figure 2(C), the bizarre response was observed even under conditions where

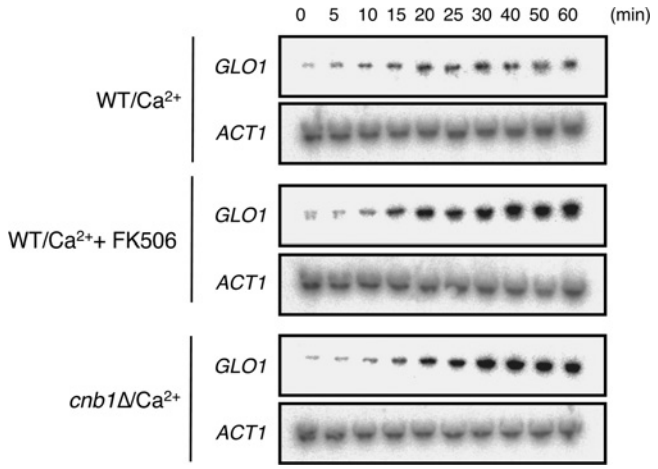

Figure 3 Time course of the expression of *GLO1* in response to Ca^{2+}

Wild-type (WT) or *cnb1*Δ cells were cultured in YPD medium until growing exponentially, and 300 mM $CaCl_2$ or 300 mM $CaCl_2$ plus 1 μg/ml FK506 was added (time 0 min). Total RNA was prepared periodically as indicated and Northern blotting for *GLO1* was conducted. *ACT1* (actin) was used as a control.

protein synthesis is blocked. Hence, the bizarre response is likely to be caused by the pre-existing machinery in yeast cells.

Time course of the expression of *GLO1*

To gain further insights into the role of the calcineurin pathway in the Ca^{2+}-induced expression of *GLO1*, we monitored the level of *GLO1* mRNA in cells treated with $CaCl_2$. As shown in Figure 3, the amount of *GLO1* mRNA in wild-type cells gradually increased upon the addition of $CaCl_2$, reaching a maximum after 30–40 min of incubation. The timing of the increase in the level of *GLO1* mRNA in cells treated with $CaCl_2$ plus FK506 was almost the same as that in cells treated with $CaCl_2$ alone, although the amount of *GLO1* mRNA was significantly higher. The pattern of the changes in the level of *GLO1* mRNA in *cnb1*Δ cells treated with $CaCl_2$ was substantially the same as that in wild-type cells treated with $CaCl_2$ plus FK506. The expression profile of *GLO1* in *crz1*Δ cells in response to Ca^{2+} was essentially similar to that in *cnb1*Δ cells (results not shown). Therefore the calcineurin pathway seems to negatively influence the maximal level of *GLO1* mRNA in response to Ca^{2+}.

Phosphorylation and nucleocytoplasmic localization of Hog1

Hog1 is phosphorylated by Pbs2, and then translocates into the nucleus in response to high osmotic stress [33,44,45]. As the Ca^{2+}-induced expression of *GLO1* was strictly dependent upon Hog1, we determined the level of Hog1 phosphorylation following treatment with $CaCl_2$. As shown in Figure 4(A), Hog1 was phosphorylated in cells treated with $CaCl_2$; however, the simultaneous treatment of cells with $CaCl_2$ and FK506 did not enhance the phosphorylation of Hog1. Similarly, the level of phosphorylated Hog1 in *cnb1*Δ, as well as *crz1*Δ cells treated with $CaCl_2$, was the same as that in wild-type cells and the addition of FK506 did not give rise to any further increase in Hog1 phosphorylation.

Next, we determined the nucleocytoplasmic localization of Hog1. As shown in Figure 4(B), Hog1 was concentrated in the nucleus upon $CaCl_2$ treatment and the intensity of the fluorescence derived from Hog1–GFP in the nuclei in cells treated with $CaCl_2$ was the same as that observed in cells treated with $CaCl_2$ and FK506 simultaneously.

We also determined the time course of the nuclear localization of Hog1 following the treatment with $CaCl_2$ and FK506 (Figure 4C). In wild-type cells, Hog1 was seen to immediately accumulate in the nucleus upon the treatment with $CaCl_2$ and redistributed to the cytoplasm after 30–45 min of incubation in the presence of $CaCl_2$. No distinct difference was observed in the timing when FK506 was present (Figure 4C). This was also the case in *cnb1*Δ and *crz1*Δ cells (Figure 4C). Therefore although Hog1 is necessary for *GLO1* to respond to extracellular Ca^{2+}, neither the level of phosphorylation nor the nucleocytoplasmic localization of Hog1 is likely to be a cause of the bizarre response.

Roles of STREs in the *GLO1* promoter in the bizarre response

The *GLO1* promoter contains two STREs (STRE1 at −432 to −428 and STRE2 at −229 to −225) [13]. As the Ca^{2+}-induced expression of *GLO1* was dependent upon the Msn2/Msn4 transcription factors (Figure 1A), and it has been reported that Msn2 and Msn4 bind to the STRE [33], we next determined whether the STREs are involved in the bizarre response. A point mutation, known to render the STRE non-functional [46], was introduced into either or both of the STREs (5′-AGGGG-3′→5′-AGATG-3′) (Figure 5A). The Ca^{2+}-induced expression of the *GLO1–lacZ* reporter was reduced by approx. 45 % by introduction of a mutation into either STRE1 or STRE2, and greatly repressed by the simultaneous introduction into both STREs (Figure 5B). Importantly, the bizarre response was repressed by the mutation in either STRE1 or STRE2, although STRE2 seemed to have a more important role in the response.

Next, we determined the response of *GLO1–lacZ* reporters with various STRE constructs in an *msn2*Δ mutant. As Msn2 is the primary transcription factor for the STRE-dependent gene, the Ca^{2+}-induced expression of *GLO1–lacZ* was impaired in *msn2*Δ cells when compared with wild-type cells (Supplementary Figure S1 available at http://www.BiochemJ.org/bj/427/bj4270275add.htm). Although the induction rate was quite small, the Ca^{2+}-induced expression of *GLO1* did occur in *msn2*Δ cells and this may be attributable to activation by Msn4; however, only a limited increase in β-galactosidase activity was observed in the presence of FK506 (Supplementary Figure S1). As the Ca^{2+}-induced expression of PMS1 was comparable with that of the wild-type STRE construct in *msn2*Δ cells, Msn4 may bind to STRE2 in response to Ca^{2+}. No induction was observed in *msn2*Δ*msn4*Δ cells (Figure 1A).

As we have demonstrated that Hog1 plays a crucial role in the bizarre response, we further determined the effect of Hog1-deficiency on the expression of the *GLO1–lacZ* reporters with various STRE constructs. As shown in Figure 5(C), as Hog1 is crucial, the level of Ca^{2+}-induced expression of *GLO1* was markedly reduced in a *hog1*Δ mutant (note that the vertical scale of Figure 5B is 50-fold that of Figure 5C) and the bizarre response was not clearly observed in the presence of FK506 together with $CaCl_2$, even in the *GLO1–lacZ* reporter with wild-type STREs. We also determined the response of *GLO1–lacZ* in Hog1-overexpressing cells. Intriguingly, as shown in Figure 5(D), the bizarre response was not observed.

STREs regulated by both Mns2/Msn4 and Hog1 exhibit the bizarre response

To verify whether the STRE is sufficient for the bizarre response, we used the $STRE_{CTT1}$–*lacZ* reporter gene; this contained two

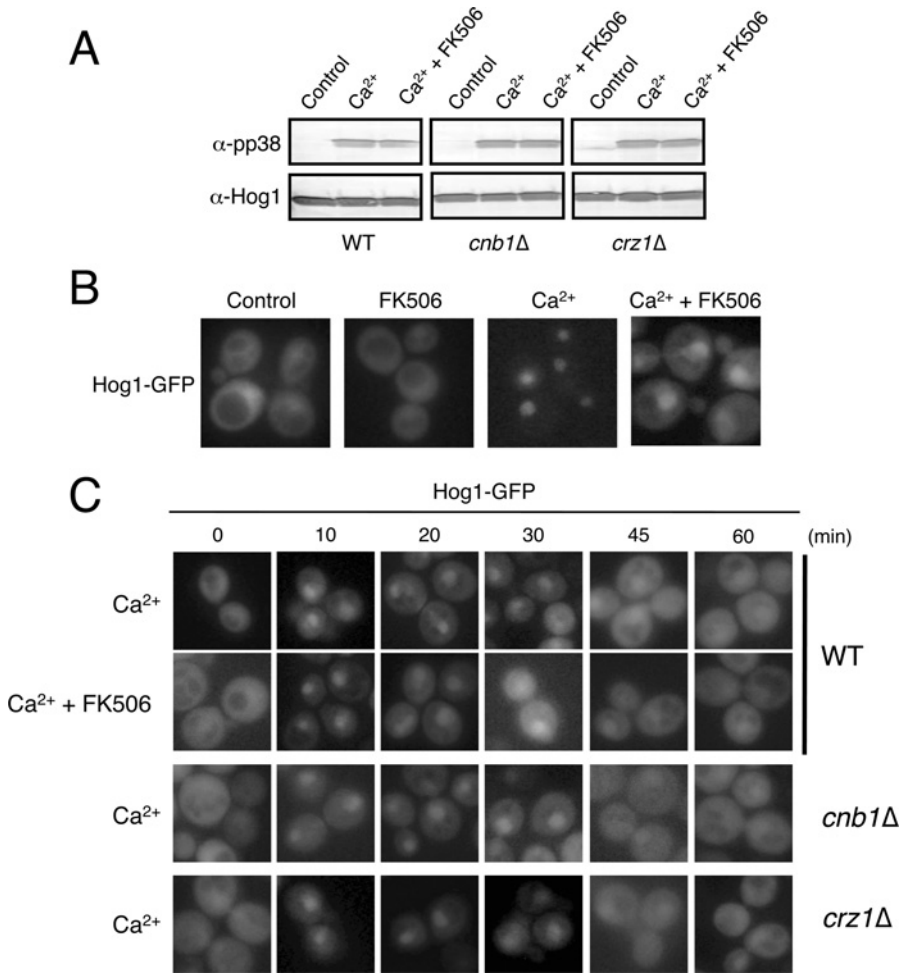

Figure 4 Effects of Ca^{2+} and FK506 on the phosphorylation and nucleocytoplasmic localization of Hog1

(**A**) Wild-type (WT), *cnb1*Δ or *crz1*Δ cells were cultured in YPD medium until growing exponentially, and were then treated or not with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 10 min of incubation, the level of phosphorylation of Hog1 and the level of Hog1 protein was determined using an anti-phospho-p38 antibody (α-pp38) and an anti-Hog1 antibody (α-Hog1) respectively. (**B**) *hog1*Δ cells expressing Hog1–GFP (pRS4-Hog1-GFP; [18]) were cultured in YPD medium until growing exponentially, and were then treated or not with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506 for 10 min. The nucleocytoplasmic localization of Hog1–GFP was then determined using fluorescence microscopy. (**C**) Wild-type (WT), *cnb1*Δ or *crz1*Δ cells expressing Hog1–GFP were cultured in YPD medium until growing exponentially. Then 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506 was added, and the nucleocytoplasmic localization of Hog1–GFP was determined periodically.

STREs from the promoter of the catalase-T-encoding gene *CTT1* (5′-TTCAAGGGGATCACCGGTAAGGGGCCAAG-3′; the STREs are underlined) followed by the TATA box of *CYC1* [47]. As shown in Figure 6(A), the bizarre response was observed with this reporter gene. As the *CTT1* gene encodes a cytoplasmic catalase [48], we measured the catalase activity. The Ca^{2+}-induced increase in catalase activity was further increased by addition of FK506 (Figure 6B). In addition, as observed in Glo1, catalase activity was markedly increased in *cnb1*Δ, as well as *crz1*Δ, cells following treatment with $CaCl_2$ alone, but did not increase any further when FK506 was present (Figure 6B).

Very recently, we found that the expression of *GPX1*, encoding an *S. cerevisiae* homologue of mammalian glutathione peroxidase [49], is induced following treatment with $CaCl_2$ in an Msn2/Msn4-dependent manner (Figure 6C) and that the *GPX1* promoter contains two functional STREs [50]; nevertheless, *GPX1* did not exhibit the bizarre response (Figure 6C). We therefore have found that Hog1 is not necessary for the Ca^{2+}-mediated induction of *GPX1* [50], whereas, the Ca^{2+}-induced response for both catalase, as well as $STRE_{CTT1}$–*lacZ*, is dependent upon Hog1 (Figures 6A and 6B). Collectively, these results suggest that STREs regulated by both Hog1 and Msn2/Msn4 mediate the bizarre response.

Effect of Crz1 on the function of Msn2

The results presented so far imply two possibilities: (i) that Crz1 could compete with Msn2 to bind to the STRE in the *GLO1* promoter to reduce the effect of Msn2 on the Ca^{2+}-induced expression of *GLO1*; or (ii) that Crz1 inhibits the activity of Msn2 towards the STRE. It is known that Crz1 binds to a distinct DNA sequence, the CDRE (calcineurin-dependent response element) [24], the consensus sequence of which (5′-GAGGCTG-3′) does not resemble the STRE. In addition, the *GLO1* promoter does not contain a sequence exactly coinciding with that of the CDRE near the STREs, so the first scenario is not likely. To address the possibility of the latter mechansim, the response of *GLO1* to Ca^{2+} was determined in cells overexpressing *MSN2* or *CRZ1*. As shown in Figure 7(A), the activity of Glo1 in Msn2-overexpressing cells following treatment with $CaCl_2$ was increased to a much

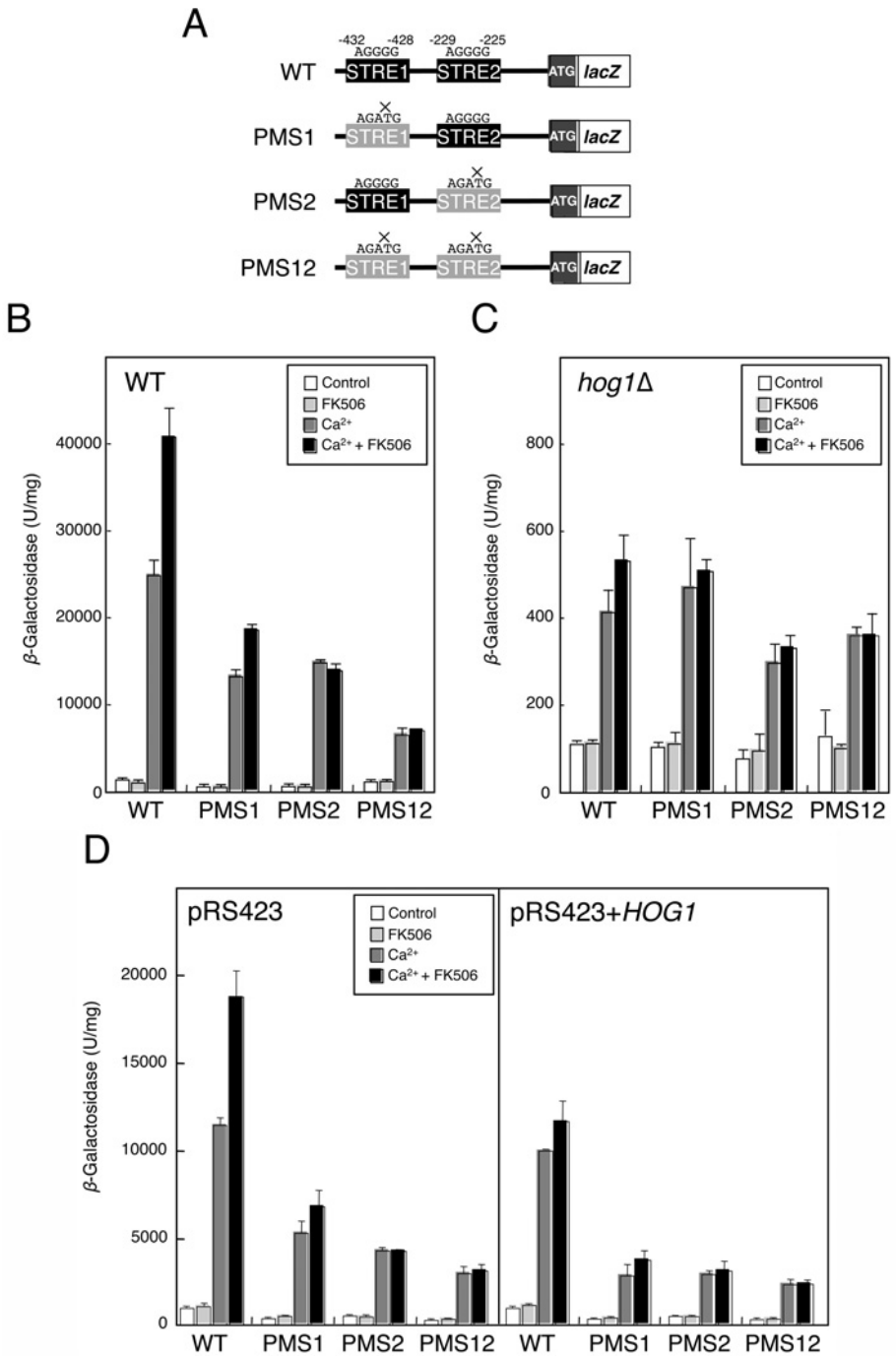

Figure 5 Role of STRE in the bizarre response

(**A**) Schematic diagram of the STRE-mutant *GLO1–lacZ* reporter genes used in the present study. (**B**) Wild-type (WT) or (**C**) *hog1*Δ-mutant cells each carrying the *GLO1–lacZ* reporter gene were cultured in YPD medium until growing exponentially. They were then treated or not with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 60 min of incubation, β-galactosidase activity was determined. (**D**) Wild-type cells carrying pRS423 or pRS423 + *HOG1* (to overexpress Hog1) were cultured in SD medium until growing exponentially. Cells were collected by centrifugation and were suspended in fresh YPD medium, containing or not 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 60 min of incubation, β-galactosidase activity was determined. U, units.

greater extent than that in cells carrying the vector alone, and the addition of FK506 did not bring about a further increase in the activity. On the other hand, the induction of Glo1 activity by $CaCl_2$ in *CRZ1*-overexpressing cells was repressed, but the activity increased to a maximal level when $CaCl_2$ and FK506 were added simultaneously.

We have reported previously that Msn2 is accumulated in the nucleus following treatment with $CaCl_2$ [42]. The time course of Msn2 accumulation is shown in Figure 7(B); Msn2 was found in the nucleus immediately upon the treatment of cells with $CaCl_2$ where it resided for up to 30 min and redistribution in the cytoplasm was observed after 45–60 min of incubation. The

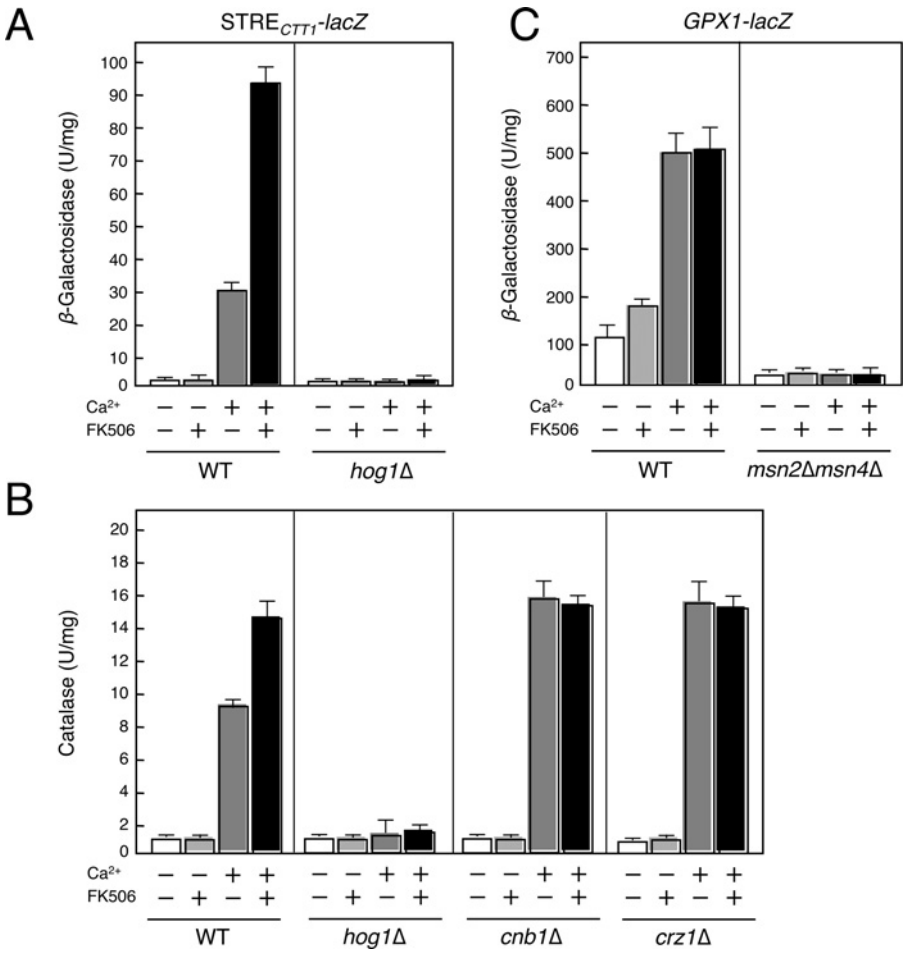

Figure 6 STRE is involved in the bizarre response

(**A**) Wild-type (WT) and *hog1*Δ cells carrying the STRE$_{CTT1}$–*lacZ* reporter gene were cultured in YPD medium until growing exponentially, and were then treated or not with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 60 min of incubation, β-galactosidase activity was determined. (**B**) Wild-type (WT), *hog1*Δ, *cnb1*Δ or *crz1*Δ cells with an additional mutation *cta1*Δ (defective in peroxisomal catalase) in the YPH250 background was cultured to a exponential phase of growth, and treated or not with $CaCl_2$ in the presence or absence of FK506 as described in (**A**). After 60 min of incubation, cell extracts were prepared and catalase activity was measured. (**C**) Cells (YPH250 background) carrying a *GPX1–lacZ* reporter in the *ura3* locus in wild-type (WT) and an *msn2*Δ*msn4*Δ (*msn2*Δ::*KanMX4 msn4*Δ::*TRP1*) strain were cultured in YPD medium until growing exponentially, and were treated or not with $CaCl_2$ in the presence or absence of FK506 as described in (**A**) after which β-galactosidase activity was determined. U, units.

behaviour of Msn2 in terms of its nucleocytoplasmic localization, i.e. the timing of the beginning of the nuclear accumulation and the retention period in the nucleus, was not affected by the presence of FK506 (Figure 7B). Similarly, the behaviour of Msn2 in *cnb1*Δ cells was the same as that in wild-type cells (Figure 7B). The nucleocytoplasmic localization of Msn2 in response to Ca^{2+} and FK506 in *crz1*Δ cells was substantially the same as that in wild-type and *cnb1*Δ cells (results not shown). Furthermore, Crz1 also accumulates in the nucleus upon treatment with $CaCl_2$ [51], but we found that the accumulation was blocked by FK506 (Figure 7C). The nuclear accumulation of Crz1 did not occur following treatment with NaCl (Figure 7C). Therefore the Ca^{2+}-induced Crz1 nuclear accumulation seems to cause this transcription factor to function as a negative regulator of Msn2 in the nucleus.

Crz1 affects the stability of Msn2 in the nucleus in response to Ca^{2+}

Durchschlag et al. [52] have reported that Msn2 is degraded in the nucleus. Hence, one feasible explanation for the bizarre response is that the nuclear Crz1 affects the stability of Msn2 in the nucleus when Ca^{2+} is present. To address this possibility, we determined the level of Msn2 protein following treatment with $CaCl_2$ with or without FK506. As shown in Figure 8(A), the level of Msn2 protein in wild-type cells gradually decreased in the presence of $CaCl_2$, but the reduction was blocked when FK506 was present. This is in contrast with *crz1*Δ cells, where the reduction in the level of Msn2 protein following the treatment with $CaCl_2$ was repressed even in the absence of FK506 (Figure 8A). Substantially, the same results in terms of the stability of Msn2 were obtained in *cnb1*Δ cells (results not shown). Additionally, the same results were obtained with respect to the stability of Msn4 (Figure 8B). Taken together, Crz1 seems to destabilize Msn2 in response to Ca^{2+}.

As the bizarre response was observed in genes where the expression is dependent upon Hog1, we determined the stability of Msn2 in *hog1*Δ cells and Hog1-overexpressing cells. The Ca^{2+}-induced degradation of Msn2 was observed in *hog1*Δ cells and was blocked by the addition of FK506 (Figure 8C). In contrast, in Hog1-overexpressing cells, the Ca^{2+}-induced degradation of Msn2 was not blocked by the presence of FK506 (Figure 8D); this is consistent with the results presented above whereby further increases in the expression of *GLO1–lacZ* reporter in the presence

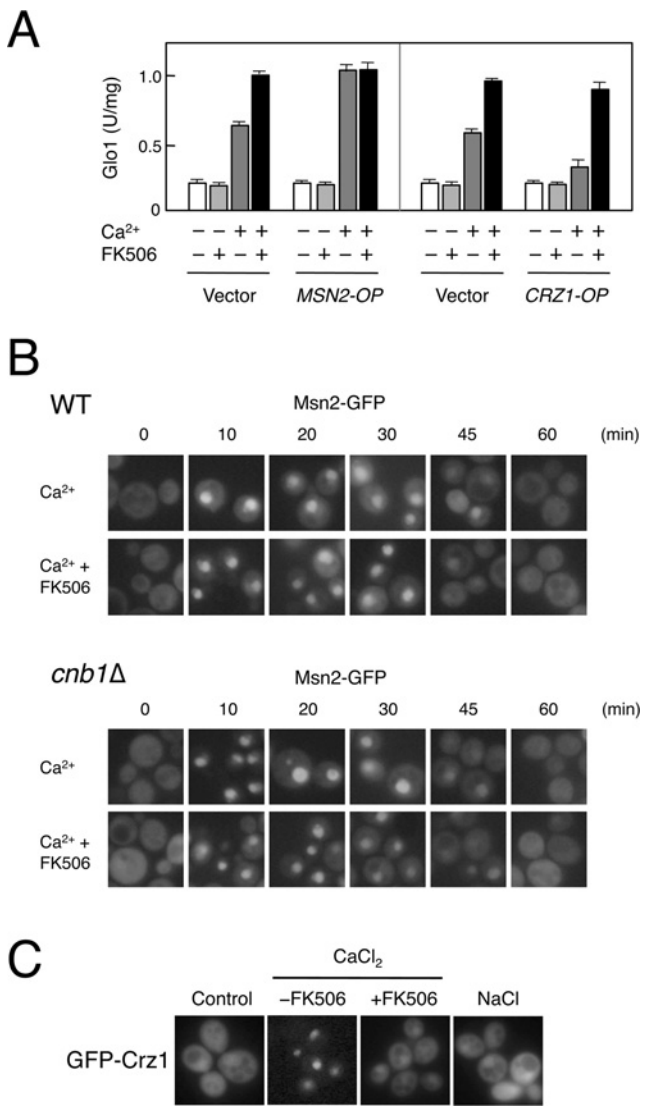

Figure 7 Negative effect of Crz1 on Msn2

(**A**) Cells carrying the *MSN2*-overexpression plasmid (pAMG; *MSN2-OP*) [33] or its control vector (YCplac111; Vector), and *CRZ1*-overexpression plasmid (pAMS453, *CRZ1-OP*) [24] or its control vector (YEp351; Vector) were cultured in YPD medium until growing exponentially, and were treated or not with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 60 min of incubation, Glo1 activity was determined. (**B**) Cells carrying pAdh1-Msn2-GFP were cultured in YPD medium until growing exponentially, and treated with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506, after which the nucleocytoplasmic localization of Msn2–GFP was determined periodically. (**C**) Cells carrying a plasmid expressing GFP–Crz1 (pAMS463) [34] were cultured in YPD medium until growing exponetially, and were treated or not with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 10 min of incubation, the nucleocytoplasmic localization of GFP–Crz1 was determined. The distribution of GFP-tagged Crz1 in cells treated with 500 mM NaCl for 10 min was also determined. U, unit.

of $CaCl_2$ and FK506 (i.e. the bizarre response) were not observed in Hog1-overexpressing cells (Figure 5D).

DISCUSSION

Negative effect of Crz1 on Msn2

To learn about the physiological role of MG in yeast cells, we searched for conditions that alter the expression of *GLO1*, and revealed that *GLO1* is expressed following treatment with $CaCl_2$. During the course of the present study, intriguingly, we found that the Ca^{2+}-induced expression of *GLO1* was further enhanced if FK506 was present, which we referred to as the bizarre response.

In the present study, we found that Crz1 negatively influences the function of Msn2 on the STRE in the *GLO1* promoter in cells treated with $CaCl_2$. Therefore the bizarre response occurred only with a combination of Ca^{2+} and FK506, and not with a combination of FK506 and other ions (Na^+ and K^+) or sorbitol, at concentrations that provoke the nuclear accumulation of Msn2. This is presumably because such chemicals do not activate the calcineurin system, and therefore Crz1 does not accumulate in the nucleus (Figure 7C) preventing interaction with Msn2. However, although several genes have expression that is regulated by Msn2 and the STRE, we found that only Hog1-dependent genes exhibited the bizarre response (Figure 5B). In other words, a gene whose expression is dependent upon these three factors will exhibit the bizarre response (e.g. *CTT1*, but not *GPX1*). As calcineurin is a protein phosphatase, a simple model explaining the role of Hog1 in the bizarre response is that calcineurin dephosphorylates Hog1, thereby preventing the Ca^{2+} signal from reaching the target gene. However, as shown in Figure 4(A), disruption of *CNB1* or *CRZ1* did not affect the level of phosphorylation or the nuclear accumulation of Hog1. These results support our conclusion that Crz1 affects the function of Msn2/Msn4 and not Hog1.

There are several explanations as to the mechanism whereby Crz1 acts negatively on the expression of genes regulated by Hog1, Msn2/Msn4 and STREs. For example, Crz1 could bind directly to Msn2/Msn4 to reduce its function as a transcription factor. To address this possibility, we tried to detect any direct interaction between Crz1 and Msn2 by conducting a GST (glutathione transferase) pull-down and co-immunoprecipitation assay, however, we were unable to obtain evidence of physical interactions (results not shown).

Another possibility is that Crz1 influences the machinery involved in the nuclear localization of Msn2. However, as shown in Figure 7(B), the behaviour of Msn2, in terms of its nucleocytoplasmic localization in response to Ca^{2+}, was virtually unaffected by FK506. In addition, Durchschlag et al. [52] have reported that Msn2 is constitutively concentrated in the nucleus in *msn5*Δ cells because Msn5 is an exportin for Msn2 [34,53]; nevertheless, the basal level of *CTT1* mRNA and the stress response of *CTT1* expression in the *msn5*Δ mutant were normal [52]. This is accounted for by a decrease in the total amount of Msn2 protein in *msn5*Δ cells (see below). The expression of *CTT1* under environmental stress is regulated by Hog1, Msn2/Msn4 and the STRE [48], and consequently the *CTT1* promoter shows the bizarre response (Figures 6A and 6B). Although Msn2 is a positive regulator for the expression of *GLO1*, the basal activity of Glo1 did not increase in the *msn5*Δ mutant (results not shown), as observed in the case of *CTT1*, despite the constitutive nuclear accumulation of Msn2. Therefore the cause of the bizarre response is not likely to be the nuclear retention period of Msn2.

Next, we examined the possibility that Crz1 titrates Hog1 away from Msn2, thereby reducing the activity as transcription factor in the presence of $CaCl_2$. Although a direct physical interaction between Crz1 and Hog1 has not been reported so far, if the nuclear Crz1 titrates Hog1 away from Msn2, further increases in the expression of *GLO1–lacZ* would be expected to occur in the presence of FK506, which blocks the nuclear accumulation of Crz1; however, this was not the case (Figure 5D). Furthermore, in contrast with in Msn2-overexpressing cells (Figure 7A), full activation of Msn2, in terms of the expression of *GLO1–lacZ*, was not observed in Hog1-overexpressing cells (Figure 5D). We propose that this is because the proportion of Msn2 that is not bound to STRE in the *GLO1* promoter is increased. It has been reported

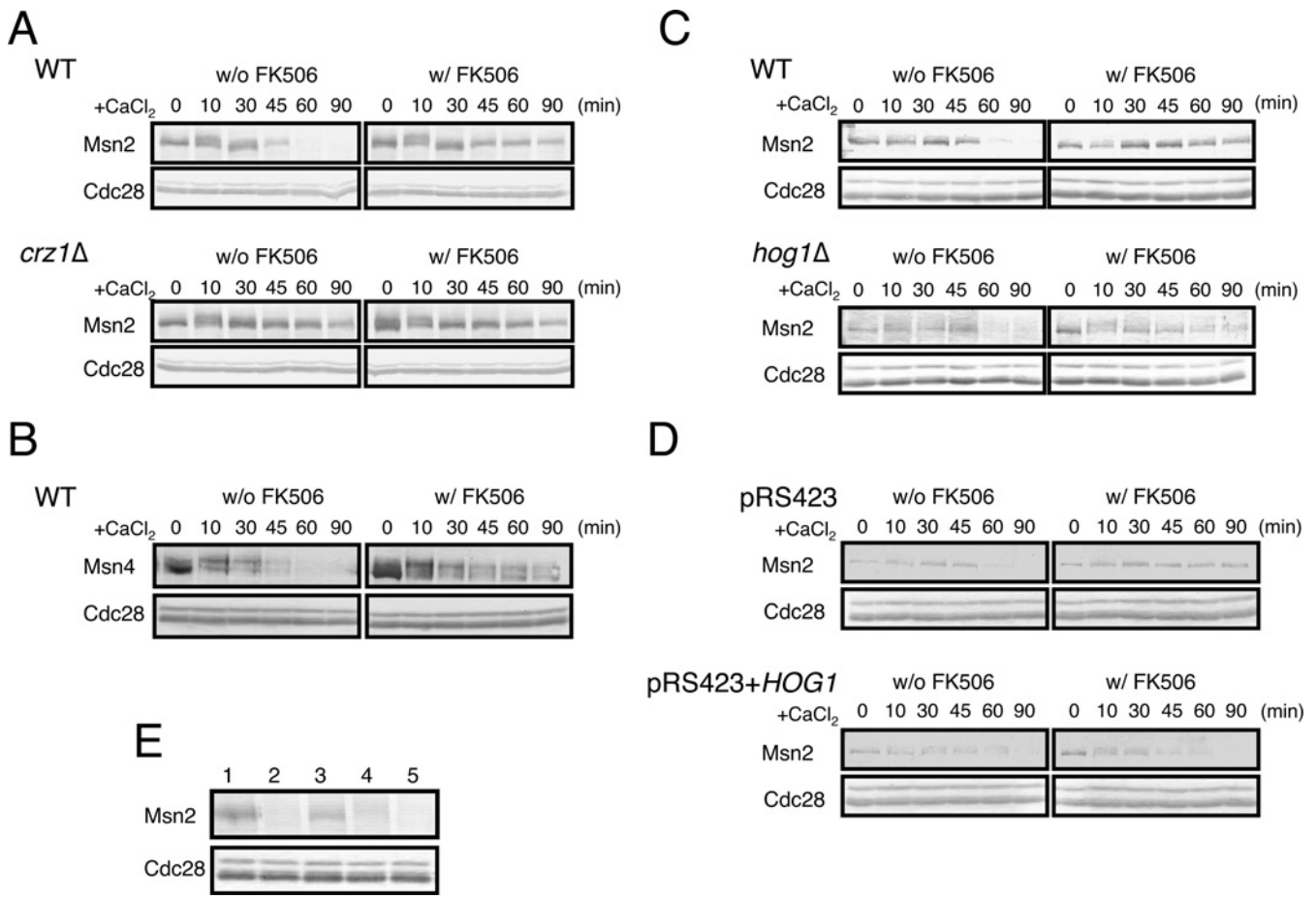

Figure 8 Effect of Ca^{2+} on degradation of Msn2

(**A**) Wild-type (WT) or *crz1*Δ cells expressing Msn2–GFP were cultured in YPD medium until growing exponentially, and were treated with 300 mM $CaCl_2$ in the presence (w/ FK506) or absence (w/o FK506) of 1 μg/ml FK506. At the indicated time, the level of Msn2 protein was determined using an anti-GFP antibody. The level of Cdc28 protein is shown as a loading control. (**B**) Wild-type (WT) cells expressing Msn4–GFP (pGR247; [31]) were treated as described in (**A**). (**C**) Wild-type (WT) or *hog1*Δ cells expressing Msn2–GFP were treated as described in (**A**). (**D**) Wild-type (WT) cells expressing Msn2–GFP and a control vector (pRS423) or the Hog1-overexpression vector (pRS423+*HOG1*) were cultured in SD medium until growing exponetially. Cells were collected by centrifugation, and suspended in a fresh YPD medium containing 300 mM $CaCl_2$, in the presence (w/ FK506) or absence (w/o FK506) of 1 μg/ml FK506. At the indicated time, the level of Msn2 protein was determined. (**E**) *erg6*Δ::*KanMX4* cells in the BY4741 background carrying pRS316+Msn2-GFP were treated with $CaCl_2$ as described in (**A**) for 90 min. Lane 1, control (YPD); lane 2, 300 mM $CaCl_2$; lane 3, 300 mM $CaCl_2$ plus 1 μg/ml FK506; lane 4, 300 mM $CaCl_2$ plus 50 μM MG132; and lane 5, 300 mM $CaCl_2$ plus 1 % DMSO (the solvent used to dissolve MG132).

that Hog1 forms a complex with Msn2, although Hog1 in itself is not a transcription factor. Therefore overexpression of Hog1 may increase the proportion of Hog1–Msn2 complex that is not bound to the STRE thereby removing Msn2 away from the STRE target sequence; this reduces the opportunity of Msn2 to function as transcription factor on the promoter. Subsequently, full activation is not observed following treatment with $CaCl_2$. Additionally, if Crz1 was titrated by Hog1, the induction of the expression of Crz1-dependent genes by Ca^{2+} would be impaired in Hog1-overexpressing cells. So, we determined the response of *GPX2* to Ca^{2+}, the expression of which is regulated by the calcineurin/Crz1 pathway [42]. However, *GPX2* responded normally to Ca^{2+} even in Hog1-overexpressing cells (Supplementary Figure S2A available at http://www.BiochemJ.org/bj/427/bj4270275add.htm). Taken together, we concluded that Hog1 was not titrated by Crz1 in the presence of Ca^{2+}.

Genome-wide search for the genes showing the bizarre response

Although a typical Crz1-binding consensus sequence (5′-GAGGCTG-3′) has not been found in the *GLO1* promoter, Ruiz et al. [54] have recently reported that a 5′-GGGGCTG-3′ sequence in the promoter of the hexose transporter *HXT2* is functional as a CDRE. A similar sequence (5′-GGGGCTT-3′; −228 to −222) overlaps the STRE2 (5′-AGGGG-3′; −229 to −225) in the *GLO1* promoter. So, to address the possibility that the bizarre response is mediated by the co-existence or the proximity of STRE- and Crz1-binding sites, we searched for the CDRE-like sequences [5′-GGGGCT(G/T)-3′] in the promoters that exhibit the bizarre response using the DNA microarray data set produced by Yoshimoto et al. [26].

First, we looked for the genes whose expression was induced by more than 2-fold when cells were treated with $CaCl_2$ for 30 min and among those, we then looked for genes whose expression was induced further when FK506 is present together with $CaCl_2$. Next, we calculated the ratio of the fold induction between the Ca^{2+}-induced expression, [Ca^{2+}], and the Ca^{2+} plus FK506-induced expression, [Ca^{2+}+FK506]. We set the threshold value for [Ca^{2+}+FK506]/[Ca^{2+}] at >1.67, because the value of *GLO1* is 1.67 when calculated based on the database. Consequently, 22 genes, including *CTT1* (ranking number 4), were found. Finally, we searched for the STRE

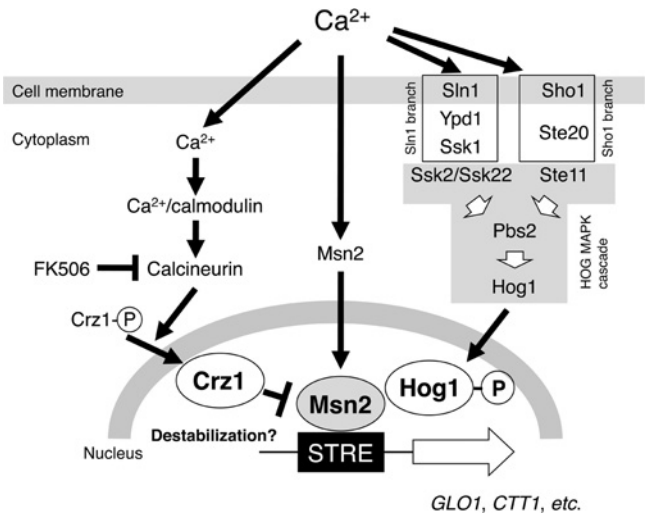

Figure 9 A tentative model for the CDMD

Extracellular Ca^{2+} activates the Hog1 MAPK cascade, and subsequently, phosphorylated Hog1 is translocated into the nucleus. Extracellular Ca^{2+} induces the nuclear accumulation of Msn2. Meanwhile, extracellular Ca^{2+} enters the cell, and activates calcineurin in a Ca^{2+}/calmodulin-dependent manner. Calcineurin dephosphorylates Crz1, thereby allowing its nuclear accumulation. Crz1 in the nucleus, in turn, enhances the degradation of Msn2, which leads to the insufficient induction of genes whose expression is dependent upon both STRE and Msn2–Hog1. Hence, if calcineurin is inhibited by FK506, or inactivated by disruption of its regulatory subunit (*cnb1*Δ) to block the nuclear accumulation of Crz1, or in *crz1*Δ cells, the degradation of Msn2 is suppressed, which leads to full activation of Msn2-dependent genes.

and CDRE-like sequences in the promoter region (to approx. −500 bp relative to the initiation codon) of the genes. As a result, we found that all of the genes have one or more STREs, whereas only six genes contained the typical CDRE or CDRE-like sequence (Supplementary Table S1 available at http://www.BiochemJ.org/bj/427/bj4270275add.htm). Therefore the co-existence or the proximity of STREs and CDREs seems unnecessary for the bizarre response. We believe that the function of such gene products found in this experiment are not likely to be involved in a certain common biological process; however, some of them are involved in the energy metabolism and we are now investigating the physiological relevance of the bizarre response using these results.

Crz1 destabilizes Msn2/Msn4 in the nucleus in response to Ca^{2+}

A striking feature of the negative effect of Crz1 on Msn2 is that nuclear Crz1 affects the stability of Msn2 and Msn4 in the nucleus when Ca^{2+} is present (Figure 8A). We call this phenomenon CDMD (Crz1-dependent Msn2/4 degradation). Taking into account the overall results of the present study, we conclude the following mechanism for the CDMD 'bizarre response' (Figure 9): Crz1 accumulates in the nucleus upon Ca^{2+}-treatment via calmodulin and calcineurin; in the nucleus it can interact with Msn2/Msn4 and reduce their stability by a yet unknown mechanism. The addition of FK506, inhibiting calcineurin, prevents the simultaneous accumulation of Crz1 and Msn2/Msn4 thereby allowing a further increase in the Ca^{2+}-induced expression of *GLO1*. Hence, direct disruption of expression of genes encoding calcineurin or Crz1, thereby inhibiting the degradation of Msn2/Msn4, also increases the level of Ca^{2+}-induced expression of *GLO1*.

Msn2 under conditions of chronic stress or low PKA (protein kinase A) activity is known to be degraded by the 26S proteasome in the nucleus [52]. To examine whether Crz1 enhances the activity of this protein degradation pathway, we treated yeast cells with the proteasome inhibitor MG132. In this experiment, we used an *erg6*Δ mutant because MG132 take-up is minimal in wild-type cells [55]. As shown in Figure 8(E), Msn2 was degraded in *erg6*Δ cells following treatment with $CaCl_2$, and this was blocked by FK506. We confirmed that the bizarre response also occurred in the *erg6*Δ mutant (results not shown). However, the addition of MG132 did not repress the degradation of Msn2 in the presence of $CaCl_2$.

Importantly, the CDMD was not seen in genes where the response to Ca^{2+} was independent of Hog1, even though the Ca^{2+}-induced expression is still dependent upon Msn2/Msn4 and the STRE (e.g. for *GPX1*). This means a reduction in the level of Msn2 protein might decrease the integrity of a Hog1–Msn2 complex, which may limit the efficacy of the expression of its STRE-possessing target gene. As far as we could determine, the behaviour of Msn2, with respect to its nuclear localization in response to Ca^{2+}, closely resembled that of Hog1 (Figures 4C and 7C). Hence, the Ca^{2+}-induced expression of *GLO1* may be limited by the degradation of Msn2 in addition to the nuclear export of Msn2 and Hog1. The genetic disruption of Crz1, or the inhibition of the nuclear accumulation of Crz1 by inhibiting calcineurin with FK506, block the breakdown of Msn2, thereby stabilizing a Hog1–Msn2 complex to warrant a full response to Ca^{2+}. However, intriguingly, we found that FK506 did not block the degradation of Msn2 in the presence of Ca^{2+} in Hog1-overexpressing cells (Figure 5D). One clue which explains this result is that we found Msn2 resided in the nucleus for longer periods in Hog1-overexpressing cells in the presence of Ca^{2+} and FK506; i.e. Crz1 was concentrated in the nucleus following treatment with Ca^{2+} and redistributed in the cytoplasm after 30–45 min in cells carrying the control vector, but Crz1 resided in the nucleus for 60 min during treatment with $CaCl_2$ in Hog1-overexpressing cells (Supplementary Figure 2B). Durchschlag et al. [52] have reported that longer residence of Msn2 in the nucleus causes the degradation of Msn2 in a 26S proteasome-dependent manner. Thus in this case we propose that Msn2 might have been degraded in Hog1-overexpressing cells due to the longer retention in the nucleus.

Our findings suggest that an indirect interaction between transcription factors, affecting their stability, may be a regulatory mechanism in the response to stress in addition to other mechanisms (such as the nucleocytoplasmic dynamics, changes to the affinity for their target sequences through post-translational modifications, chromatin remodelling and interactions with RNA polymerase). For example, Williams and Cyert [56] have reported that the oxidative stress-responsive transcription factor Skn7 regulates the turnover of Crz1; however, the protease involved has not been identified. A network system regulating the stability of transcription factors might exist in yeast cells to warrant an appropriate and distinct stress response to a wide variety of environmental stimuli that partially overlap. This would be the case for a high concentration of Ca^{2+}, which provokes both an osmotic stress response and a Ca^{2+} signalling response simultaneously. One possible physiological interpretation of the CDMD is that it minimizes unnecessary Hog1–Msn2/Msn4-dependent osmotic responses induced by Ca^{2+} by destabilizing Msn2/Msn4 through Crz1.

AUTHOR CONTRIBUTION

Yoshifumi Takatsume and Takumi Ohdate contributed equally; Yoshifumi Takatsume undertook most of the experimentation shown in Figures 1–7; Takumi Ohdate undertook most of the experimentation shown in Figures 5D, 8 and the Supplementary material and discovered the Crz1-dependent degradation of Msn2/Msn4 in the presence of Ca^{2+}.

Kazuhiro Maeta, Wataru Nomura and Shingo Izawa contributed to valuable discussion about the work. Yoshiharu Inoue initiated the study, after discovering the fundamental phenomenon, and wrote the paper.

ACKNOWLEDGEMENTS

We thank Dr Y. Kikuchi (Institute for Biomolecular Science, Gakushuin University, Tokyo, Japan) for pRS316+Msn2-GFP, Dr C. Schüller (Department of Biochemistry and Cell Biology, University of Vienna, Vienna, Austria) for pAMG (pAdh1-Msn2-GFP), Dr A. Goldbeter (Faculté des Sciences, Université Libre de Bruxelles, Brussels, Belgium) for pGR247 (pAdh1-Msn4-GFP) and Dr M. Cyert (Department of Biological Sciences, Stanford University, Palo Alto, U.S.A.) for pAMS435 (YEp351+CRZ1) and pAMS463 (GFP–Crz1). We are grateful to Astellas Pharma (formerly Fujisawa Pharmaceutical) for their gift of FK506.

FUNDING

This research was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan [grant number 20380187].

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Received 26 August 2009/4 January 2010; accepted 2 February 2010
Published as BJ Immediate Publication 2 February 2010, doi:10.1042/BJ20091334

Biochem. J. (2010) **427**, 275–287 (Printed in Great Britain) doi:10.1042/BJ20091334

online data

SUPPLEMENTARY ONLINE DATA

Calcineurin/Crz1 destabilizes Msn2 and Msn4 in the nucleus in response to Ca^{2+} in *Saccharomyces cerevisiae*

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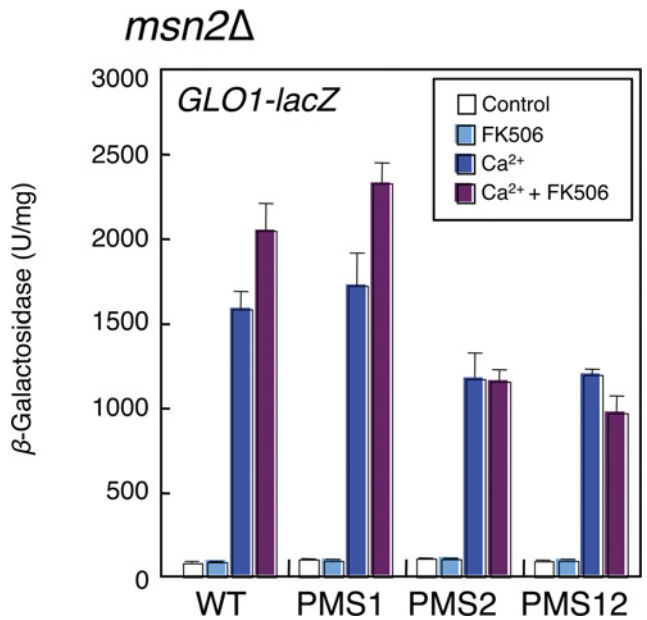

Figure S1 Role of the STRE in the bizarre response in *msn2*Δ cells

*msn2*Δ cells each carrying a *GLO1–lacZ* reporter gene were cultured in YPD medium until growing exponentially, and were treated with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 60 min of incubation, β-galactosidase activity was determined. U, units.

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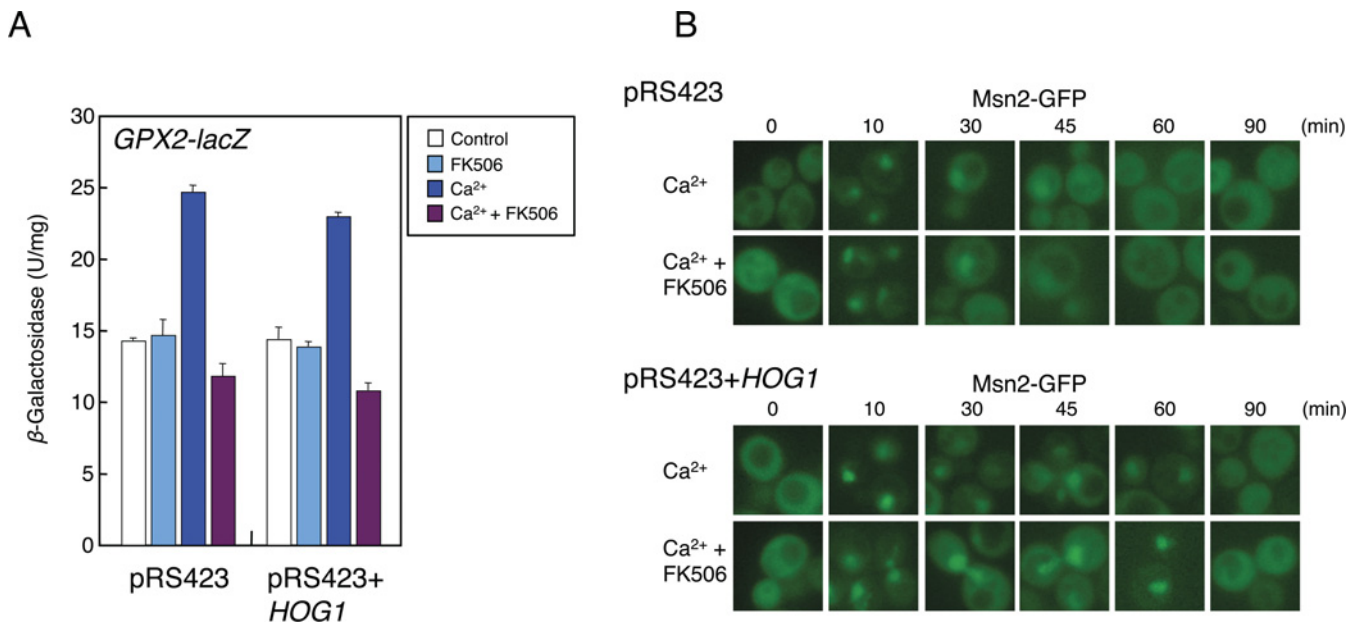

Figure S2 Effect of Hog1 overexpression on the function of Crz1

(**A**) Cells carrying the *GPX2–lacZ* reporter gene [1] were cultured in YPD medium until growing exponentially, and treated with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 60 min of incubation, β-galactosidase activity was determined. (**B**) Cells expressing GFP–Crz1 (via the plasmid pAMS463 [2]) and a control (pRS423) or the Hog1-overexpressing (pRS423 + *HOG1*) plasmid were cultured in SD medium until growing exponentially, and were then treated with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. The nucleocytoplasmic localization of GFP–Crz1 was determined using fluorescence microscopy at the indicated time.

Table S1 Genome-wide search for genes showing the bizarre response

A DNA microarray approach was used to find genes whose expression was induced more than 2-fold when cells were treated with $CaCl_2$ for 30 min and whose expression was induced further when FK506 is present together with $CaCl_2$. The genes where the $[Ca^{2+} + FK506]/[Ca^{2+}]$ ratio was >1.67 are presented. The position of the STRE is given relative to the translation initiation codon. ER, endoplasmic reticulum; –, not present. The function/descriptions are taken from http://www.yeastgenome.org/.

Open-reading frame	Gene name	$[Ca^{2+}+FK506]/[Ca^{2+}]$ ratio	Sequence (5′→3′) and position of the CDRE-like sequence	Position(s) of the STRE	Localization	Function/Description
YMR175W	*SIP18*	2.53	–	−388; −369; −158	Unknown	Phospholipid-binding protein; expression is induced by osmotic stress
YKL035W	*UGP1*	2.41	GGGGCGCA; −533	−483; −445; −272; −260	Cytoplasm; plasma-membrane-enriched fraction	UGPase (UDP-glucose pyrophosphorylase), catalyses the reversible formation of UDP-glucose from glucose 1-phosphate and UTP, involved in a wide variety of metabolic pathways, expression modulated by phosphate metabolism Pho4–Pho85 proteins
YBR117C	*TKL2*	2.39	–	−77	Cytoplasm; nucleus	Transketolase, similar to Tkl1; catalyses conversion of xylulose 5-phosphate and ribose 5-phosphate into sedoheptulose 7-phosphate and glyceraldehyde-3-phosphate in the pentose phosphate pathway; needed for synthesis of aromatic amino acids
YGR088W	*CTT1*	2.31	–	−378; −363; −133	Cytoplasm	Cytosolic catalase T, has a role in protection from oxidative damage by hydrogen peroxide
YPL186C	*UIP4*	2.31	–	−301; −219; −95	ER; nuclear envelope; mitochondrion	Protein that interacts with Ulp1, a Ubl (ubiquitin-like protein)-specific protease for Smt3p protein conjugates; detected in a phosphorylated state in the mitochondrial outer membrane; also detected in ER and nuclear envelope
YFL014W	*HSP12*	2.06	GGGGCTG;−234	−435; −414; −377; −232; −190	Plasma membrane; cytoplasm; nucleus	Plasma-membrane-localized protein that protects membranes from desiccation; induced by heat shock, oxidative stress, osmostress, stationary phase entry, glucose depletion, oleate and alcohol; regulated by the HOG and Ras/PKA pathways
YOR173W	*DCS2*	2.03	GGGGCTG ;−190	−160; −148	Cytoplasm	Non-essential, stress induced regulatory protein containing a HIT (histidine triad) motif; modulates m^7G−oligoribonucleotide metabolism; inhibits Dcs1; regulated by Msn2/4, and the Ras/cAMP/cAPK (cAMP-dependent protein kinase) signalling pathway, similar to Dcs1.
YGR256W	*GND2*	2.00	–	−299; −163	Cytoplasm	6-Phosphogluconate dehydrogenase (decarboxylating), catalyses an NADPH-regenerating reaction in the pentose phosphate pathway; required for growth on D-glucono-Δ-lactone
YDR516C	*EMI2*	2.00	–	−355; −173	Cytoplasm	Non-essential protein of unknown function required for transcriptional induction of the early meiotic-specific transcription factor IME1; required for sporulation; expression is regulated by glucose-repression transcription factors Mig1/2
YMR297W	*PRC1*	1.99	–	−194; −163	Cytoplasm; ER; vacuole	Vacuolar carboxypeptidase Y (proteinase C), broad-specificity C-terminal exopeptidase involved in non-specific protein degradation in the vacuole; member of the serine carboxypeptidase family
YNL160W	*YGP1*	1.97	–	−434	Extracellular region	Cell-wall-related secretory glycoprotein; induced by nutrient deprivation-associated growth arrest and upon entry into stationary phase; may be involved in adaptation prior to stationary phase entry; has similarity to Sps100

Table S1 Continued

Open-reading frame	Gene name	$[Ca^{2+}+FK506]/[Ca^{2+}]$ ratio	Sequence (5′→3′) and position of the CDRE-like sequence	Position(s) of the STRE	Localization	Function/Description
YOR382W	*FIT2*	1.93	GGGGCTT ;−420	−242	Cell wall	Mannoprotein that is incorporated into the cell wall via a GPI (glycosylphosphatidylinositol) anchor, involved in the retention of siderophore−iron in the cell wall
YCL042W		1.92	GAGGCTG ;−441	−449	Cytoplasm	Putative protein of unknown function; epitope-tagged protein localizes to the cytoplasm
YBR183W	*YPC1*	1.89	–	−361; −270; −83	ER	Alkaline ceramidase that also has reverse (CoA-independent) ceramide synthase activity, catalyses both breakdown and synthesis of phytoceramide; overexpression confers fumonisin B1 resistance
YPL004C	*LSP1*	1.83	–	−215; −193; −182	Cytoplasm; mitochondrion	Primary component of eisosomes, which are large immobile patch structures at the cell cortex associated with endocytosis, along with Pil1 and Sur7; null mutants show activation of Pkc1/Ypk1 stress resistance pathways
YGR052W	*FMP48*	1.82	–	−338	Mitochondrion	Putative protein of unknown function; the authentic, untagged protein is detected in highly purified mitochondria in high-throughput studies; induced by treatment with 8-methoxypsoralen and UV-A irradiation
YNR001C	*CIT1*	1.81	–	−159; −134	Mitochondrion	Citrate synthase, catalyses the condensation of acetyl-CoA and oxaloacetate to form citrate; the rate-limiting enzyme of the tricarboxylic acid cycle cycle; nuclear-encoded mitochondrial protein
YOL052C-A	*DDR2*	1.77	–	−472; −248; −203; −175	Cytoplasm; vacuole	Multistress response protein, expression is activated by a variety of xenobiotic agents and environmental or physiological stresses
YBR105C	*VID24*	1.76	–	−356	Cytoplasmic membrane−bounded vesicle (extrinsic to membrane); GID complex	Peripheral membrane protein located at Vid (vacuole import and degradation) vesicles; regulates FBPase (fructose-1,6-bisphosphatase) targeting to the vacuole; promotes proteasome-dependent catabolite degradation of FBPase
YKL150W	*MCR1*	1.71	–	−308; −268	Mitochondrion	Mitochondrial NADH-cytochrome *b*5 reductase, involved in ergosterol biosynthesis
YDR070C	*FMP16*	1.68	–	−201	Mitochondrion	Putative protein of unknown function; proposed to be involved in responding to conditions of stress; the authentic, untagged protein is detected in highly purified mitochondria in high-throughput studies
YML004C	*GLO1*	1.67	GGGGCTT ;–228	–432; –229	Cytoplasm; nucleus	Monomeric glyoxalase 1, catalyses the detoxification of MG via condensation with glutathione to produce *S*-D-actoylglutathione; expression regulated by MG levels and osmotic stress

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Received 26 August 2009/4 January 2010; accepted 2 February 2010
Published as BJ Immediate Publication 2 February 2010, doi:10.1042/BJ20091334