Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity

Wolfram Görner,1 Erich Durchschlag,1 Maria Teresa Martinez-Pastor,2 Francisco Estruch,2 Gustav Ammerer,1 Barbara Hamilton,1 Helmut Ruis,1 and Christoph Schüller1,3

1Vienna Biocenter, Institut für Biochemie und Molekulare Zellbiologie der Universität Wien und Ludwig Boltzmann-Forschungsstelle für Biochemie, A-1030 Wien, Austria; 2Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universitat de València and Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), 46100 Burjassot, Spain

Msn2p and the partially redundant factor Msn4p are key regulators of stress-responsive gene expression in Saccharomyces cerevisiae. They are required for the transcription of a number of genes coding for proteins with stress-protective functions. Both Msn2p and Msn4p are Cys2His2 zinc finger proteins and bind to the stress response element (STRE). In vivo footprinting studies show that the occupation of STREs is enhanced in stressed cells and dependent on the presence of Msn2p and Msn4p. Both factors accumulate in the nucleus under stress conditions, such as heat shock, osmotic stress, carbon-source starvation, and in the presence of ethanol or sorbate. Stress-induced nuclear localization was found to be rapid, reversible, and independent of protein synthesis. Nuclear localization of Msn2p and Msn4p was shown to be correlated inversely to cAMP levels and protein kinase A (PKA) activity. A region with significant homologies shared between Msn2p and Msn4p is sufficient to confer stress-regulated localization to a SV40–NLS–GFP fusion protein. Serine to alanine or aspartate substitutions in a conserved PKA consensus site abolished cAMP-driven nuclear export and cytoplasmic localization in unstressed cells. We propose stress and cAMP-regulated intracellular localization of Msn2p to be a key step in STRE-dependent transcription and in the general stress response.

[Key Words: Stress; yeast; STRE; nuclear localization; protein kinase A; Msn2p; Msn4p]

Received June 25, 1997; a revised version accepted December 9, 1997.
tions is mediated by the stress response element (STRE; core consensus 5′-WAGGGG-3′) (Ruis and Schüller 1995; Siderius and Mager 1997). STRE-driven transcription is regulated by the high-osmolarity glycerol (HOG) pathway (Schüller et al. 1994), a MAP kinase pathway of the yeast S. cerevisiae involved in protection of cells against increases in external osmolarity (Brewster et al. 1993; Estruch and Carlson 1993; Martinez-Pastor et al. 1996; Schmitt and McEntee 1996). The Cys2His2 Zn-finger DNA-binding domains of these proteins are almost identical and both bind specifically to STRE sequences. These proteins appear to be functionally redundant, as double but not single mutants exhibit pleiotropic stress sensitivity. Msn2p seems to have a more pronounced role, as mutants lacking only MSN2 exhibit an already distinct decrease in STRE-mediated transcription (C. Schüller and G. Marchler, unpubl.). Full stress-induced expression of STRE-regulated genes is dependent on the presence of both Msn2p and Msn4p.

A central issue for our understanding of the general stress-response system is the question of how the response to multiple stresses and to PKA activity can be mediated by only one type of transcription factor. We found that Msn2p and Msn4p are translocated reversibly to the nucleus in a stress-dependent manner. This suggests that Msn2p and Msn4p translocation is the key regulatory event in stress induction of transcription. We show that high PKA reverses the nuclear localization of Msn2p under stress conditions. Our results provide a model of how stress and nutrient controls might be integrated in S. cerevisiae.

Results

Msn2p binds to STREs in stressed cells

Msn2p and Msn4p have been shown previously to bind to STREs in vitro (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996). In initial experiments, we observed no significant changes of the STRE-binding activity of myc9 epitope for indirect immunolocalization (Piatti et al. 1996) or, for examination of living cells, a green fluorescent protein (GFP) tag. To verify the functionality of the carboxy-terminally tagged Msn2p derivatives, we assayed the expression of a STRE-driven gene (CTT1) in their presence. In msn2 msn4 mutant cells transformed with empty control vector, only low levels of catalase expression were observed with or without stress (Fig. 2D). Catalase activity was restored to normal and inducible levels by plasmids containing a wild-type MSN2 gene or genes encoding either the myc9-tagged version or the Msn2p–GFP fusion. In all of these cases, expression of Msn2p derivatives was driven by the native promoter demonstrating that physiological levels of tagged versions of Msn2p are functional.

For initial indirect immunofluorescence studies, we used epitope-tagged versions of Msn2p driven by the authentic MSN2 promoter. Although a specific signal is barely detectable under normal growth conditions, a distinct nuclear signal appears after stress treatment. We therefore increased the expression level by replacing the native promoter through the ADH1 promoter. Overexpression of untagged and tagged genes (pAdh1–Msn2 derivatives) resulted in an ~20-fold increase of both basal and stress-induced expression of the CTT1 gene (Fig. 2D).
Indirect immune fluorescence data using monoclonal anti-myc antibody (9E10) are shown in Figure 2A. In unstressed cells, the Msn2p–myc9 fusion protein is distributed diffusely throughout the cytoplasm and is partly excluded from the nucleus. Immunofluorescence of the myc-tagged Msn2p co-stains with DAPI in cells treated for 20 min by either heat shock, ethanol, sorbate, or osmostress (0.4 M NaCl or 0.9 M sorbitol; the latter not shown). Together with the observation that stress causes STRE protection, these data suggest that Msn2p localization controls promoter occupancy.

To confirm this finding in living cells, we used a Msn2p–GFP fusion protein. Logarithmic cells carrying the Msn2p–myc9 fusion protein is distributed diffusely throughout the cytoplasm and is partly excluded from the nucleus. Immunofluorescence of the myc-tagged Msn2p co-stains with DAPI in cells treated for 20 min by either heat shock, ethanol, sorbate, or osmostress (0.4 M NaCl or 0.9 M sorbitol; the latter not shown). Together with the observation that stress causes STRE protection, these data suggest that Msn2p localization controls promoter occupancy.

To confirm this finding in living cells, we used a Msn2p–GFP fusion protein. Logarithmic cells carrying the Msn2p–GFP fusion showed uniform cytoplasmic staining in the absence of stress (Fig. 2B). After stress treatment, we observed that the nuclei of almost all cells were stained within a few minutes. In addition to the stress conditions documented in Figure 2B, we found a similar nuclear concentration of Msn2p–GFP in cells exposed to sudden glucose starvation (see Fig. 3), low pH (2.8) or methanol (10%) (data not shown). Msn2p–GFP fluorescence colocalizes with DAPI-stained DNA in stressed cells. No influence of a hog1 mutation on Msn2p translocation by osmostress (Fig. 2B) or by other stresses (data not shown) was observed. Western control experiments demonstrated that the levels of Msn2p–GFP remain unchanged under these conditions (Fig. 2C).

Msn2p nuclear import and export is fast and protein synthesis-independent

To study the kinetics of Msn2p translocation (Fig. 3), logarithmic W303 msn2 msn4 cells transformed with a Msn2p–GFP fusion plasmid were treated with 7.5% ethanol and fluorescence of a group of cells was recorded after 30 and 300 sec, when nuclear import was completed (Fig. 3A). De novo protein synthesis was not required for stress-induced nuclear import, as preincubation with 100 µg cycloheximide/ml did not prevent translocation (data not shown). These data demonstrate that stress-induced translocation of Msn2p is a direct and rapid process.

Because the activation of stress genes is normally transient and tightly controlled, one can assume that the relief of stress might, by analogy, cause a rapid shutdown of stress gene transcription. In the case of nuclear-localized Msn2p, the shutdown could either be achieved by modification, degradation, or export of the factor. Western blotting shows that the levels of Msn2p do not exhibit large variations before stress, after stress, or after stress relief (Fig. 3D). When import was induced by a short heat shock (36°C), relocation of Msn2p–GFP to the cytoplasm was observed at room temperature within several minutes (data not shown). A consecutive heat shock again caused nuclear concentration of the GFP-tagged protein.

Figure 1. In vivo footprint analysis on the CTT1 (A) and HSP104 (B) promoters during heat shock. DNA of unstressed (25°C) and heat-shocked (37°C) W303 (lanes 1,3) and W303 msn2 msn4 (lanes 2,4) cells was prepared after methylation in vivo with DMS. Primer extension reactions were run on a sequencing gel and autoradiographed. Gels were quantitated with a PhosphorImager. The aligned curves are shown comparing the relative extent of methylation of DNA from mutant (continuous lines) and wild-type (broken lines) strain under nonstress and stress conditions. Bands were identified by a conventional sequencing reaction. The STRE core sequence is indicated by arrows or underscoring. (C) In vivo footprint analysis of the CTT1 and HSP104 promoter under osmotic (0.4 M NaCl) and weak organic acid (10 mM sorbic acid) stress. W303 (lanes 1–3) and W303 msn2 msn4 (lanes 4–6) were grown to logarithmic phase and stress-treated for 20 min. Primer extension reactions were run on a sequencing gel and autoradiographed.
Similarly, export of Msn2p–GFP from the nucleus was observed when stress agents (sorbic acid, ethanol, NaCl) were removed by transferring the cells to fresh medium. Activation of transcription by glucose starvation has been shown to be mediated by Msn2p and Msn4p (Martinez-Pastor et al. 1996). Carbon-source starvation is achieved by transferring the cells to medium lacking glucose and results in rapid nuclear concentration of the Msn2p–GFP fusion protein. Relief of stress can be accomplished by adding glucose to cells in carbon-source starvation medium. Figure 3B shows glucose-starved cells to which 1% glucose was added. Immediately after addition of glucose (30 sec), Msn2p–GFP is still concentrated in the nuclei. Within 300 sec, nuclear fluorescence faded with concomitant increase of cytoplasmic staining. The constancy of the overall cellular concentration of Msn2p–GFP under these conditions was demonstrated by a control Western blot (Fig. 3D), suggesting that no significant degradation occurred. Therefore, Msn2p–GFP is exported from the nucleus to the cytoplasm on stress relief. Analogous to stress-induced import, no significant dependence of the export on de novo protein synthesis was evident. To demonstrate the reversibility of Msn2p import and export on individual cells, stress relief and stress agent were applied simultaneously. Carbon-starved cells were treated with 1% glucose and 10 mM sorbate. The time course of Msn2p–GFP localization under these conditions is shown in Figure 3C (30, 300, and 600 sec). Apparently, glucose refeeding (relief of stress) and sorbate stress compete under such conditions so that Msn2p–GFP, which is concentrated initially in the nucleus at time point 30 sec, is exported after 300 sec, but partly re-imported into nuclei after 600 sec. This implies that Msn2p localization is dynamically con-

**Msn2p stress-regulated nuclear translocation**

**Figure 2.** Subcellular localization of Msn2p–myc9 and Msn2p–GFP. (A) Indirect immunofluorescence. W303 msn2 msn4 cells transformed with pAdh1-Msn2–myc9 were grown to logarithmic phase, treated with stress for 20 min and fixed. Indirect immunodetection was performed using monoclonal anti-myc (9E10) antibody and rabbit anti-mouse IgG CY3-conjugated secondary antibody (Sigma). DNA was stained with DAPI. (B) Distribution of Msn2p–GFP fluorescence. Stressed and unstressed W303 msn2 msn4 and W303 hog1 cells transformed with pAdh1-Msn2–GFP were grown to logarithmic phase in synthetic medium. DAPI was added to the cultures 10 min before they were subjected to the indicated stress treatment for 5 min. In living cells, DAPI stains mitochondrial DNA brightly, whereas chromosomal DNA appears as a diffuse spot. (BF) Bright field microscopy. (C) Western blot using a polyclonal anti-Msn2 antiserum showing amounts of Msn2p–GFP under conditions used for fluorescence microscopy; (control) cells without Msn2–GFP plasmid. (D) Biological activity of Msn2p and Msn2p derivatives in W303 msn2 msn4 cells. Mutant cells were transformed with vector (YCplac111), pMsn2, pMsn2–myc9, pMsn2–GFP, pAdh1–Msn2, pAdh1–Msn2–myc9, and pAdh1–Msn2–GFP constructs under control of the native MNS2 or the ADH1 promoter. Cells were grown to logarithmic phase (open bars), one aliquot was treated with sorbic acid (10 mM, 45 min; stippled bars). Catalase activity of extracts was assayed from at least three independent cultures (standard deviation <20%).
PKA regulates Msn2p localization

The PKA pathway is known to negatively regulate STRE-dependent transcription (Marchler et al. 1993). To determine how Msn2p function is affected by PKA, we studied the localization of Msn2p in cells with different levels of kinase activity. Constitutively low PKA activity is exhibited in strain RS58-5A-1 (bcy1 tpk1w tpk2 tpk3) lacking the gene for the regulatory subunit (BCY1) and two of the three genes for catalytic subunits of PKA (TPK2, TPK3), while carrying a functionally impaired allele of the third (tpk1w) (Cameron et al. 1988). Such mutants have abnormally high levels of STRE-regulated transcripts. Consistent with a major role for Msn2p, deletion of MSN2 in this strain abolished the high level of catalase T produced in this genetic background in the absence of stress (Fig. 4B). In vivo footprinting analysis shows enhanced protection of STREs (indicated by arrows) in the CTT1 promoter in unstressed logarithmic RS58-5A-1 cells in comparison with the isogenic wild-type strain SP1 (Fig. 4A). Consistently, Msn2p–GFP displayed constitutive nuclear localization in RS58-5A-1 cells, in contrast to cytoplasmic staining observed in wild-type cells (Fig. 4C). Notably, levels of Msn2p–GFP are significantly lower in strain RS58-5A-1 compared with wild type (data not shown). This is probably an indirect effect attributable to the mutant background as Msn2p–GFP was found to be stable when PKA activity was impaired in a cdc25-1 strain (see below). These results implied that PKA is negatively regulating STRE-dependent transcription through control of Msn2p localization.

PKA activity can also be manipulated by exogenous cAMP in cells lacking the high-affinity phosphodiesterase gene (pde2). Strain OL556 carries a temperature-sensitive allele of the CDC25 gene (cdc25-5) and a pde2 mutation that allows uptake of externally supplied cAMP (Boy-Marcotte et al. 1996). At the nonpermissive temperature (37°C) endogenous cAMP production ceases, attributable to the cdc25-5 allele whose product fails to activate the yeast Ras proteins and thereby prevents activation of adenylate cyclase. At the permissive
temperature (26°C) we observed no difference in the regulation and levels of expression of CTT1 and of a STRE-LacZ fusion in this strain compared with wild-type strains (data not shown). To assay the effect of cAMP on Msn2p, OL556 cells were grown to logarithmic phase at 26°C and cAMP was added (3 mM). After 1 hr, the culture was shifted to the nonpermissive temperature of 37°C. To recover cells from heat shock, the culture was kept at 37°C for at least 1 hr. During this time span, heat shock-induced CTT1 mRNA levels decrease again to the basal level in wild-type cells (Martinez-Pastor et al. 1996). In the OL556 strain, the heat shock induction of catalase activity was suppressed by 3 mM cAMP indicating inhibition of stress-induced transcription by high PKA activity (data not shown). The culture was divided and cAMP was removed by washing one aliquot in 37°C medium. The cultures were then incubated further at 37°C. CTT1 mRNA rose to a detectable level within 15 min in the absence of cAMP, but not in its presence, in a cycloheximide-insensitive manner (Fig. 5B). OL556 cells treated in the same way were subjected to in vivo footprinting analysis. Protection of CTT1 STREs was increased significantly in the cAMP-free cells compared with cAMP-containing cells (Fig. 5A). In absence of cAMP, but not its presence, Msn2p–GFP accumulated in the nucleus at the nonpermissive temperature (Fig. 5C). To investigate whether cAMP depletion is sufficient to trigger nuclear accumulation of Msn2p at room temperature, we used a strain (TC41-1; Hubler et al. 1993) that is unable to synthesize cAMP because of a deletion of the adenylate cyclase gene (CDC35). TC41-1 cells expressing Msn2p–GFP were grown in the presence of cAMP (2 mM). cAMP was removed by washing the cells in medium and nuclear localization of Msn2p–GFP was observed within 20 min (data not shown). Taken together, these data suggest that high PKA activity inactivates transcription of STRE-regulated genes by lowering the nuclear localization of Msn2p. To establish whether an increase of PKA activity causes nuclear export, we followed the fate of already nuclear localized Msn2p–GFP. Nuclear localization of Msn2p–GFP was triggered by treating OL556 cells with 10 mM sorbate at the permissive temperature (Fig. 5D). Activation of PKA by subsequent addition of cAMP (25 mM) caused fading of the nuclear GFP signal with a concomitant increase of fluorescence activity in the cytoplasm within 10 min (Fig. 5D). According to a control Western blot, no significant degradation of Msn2p–GFP occurs during this type of experiment (Fig. 5E). These results demonstrate that translocation of Msn2p to nuclei, even under sustained stress conditions, is reversed by cAMP, a factor activating PKA activity.

Stress and cAMP influence the localization of the Msn2p homolog Msn4p

Msn4p has been reported to bind to STREs and to have functions overlapping with Msn2p (Martinez-Pastor et al. 1996). We investigated the intracellular localization of Msn4p using a GFP fusion protein (Fig. 6). Msn4p–GFP is distributed diffusely in the cytoplasm of unstressed cells, whereas, very much like Msn2p, it is rapidly concentrated in nuclei on sorbate stress. Other stresses had comparable effects (data not shown). cAMP responsive-

Figure 5. cAMP levels influence Msn2p localization. (A) In vivo footprint analysis of the CTT1 STREs. OL556 (cdc25-5 pde2) cells were grown and shifted to nonpermissive temperature (37°C) in the presence of 3 mM cAMP and released by dividing the culture and removing cAMP from one aliquot. STRE protection in the CTT1 promoter was determined after 20 min in cells with (+cAMP) and without cAMP (−cAMP). (B) CTT1 mRNA levels decrease (+cAMP) and levels of expression of Msn2–GFP in extracts from cells grown under conditions described in D.

GENES & DEVELOPMENT 591
ness was tested in strain OL556. The protein was found to be nuclear in the absence of cAMP, but localized in the cytoplasm in its presence. Therefore, Msn2p and Msn4p may share common control mechanisms regulating intracellular localization by stress and cAMP.

Analysis of Msn2p regions required for regulation of localization

To identify the domains involved in regulation of Msn2p, we carried out a deletion and mutagenesis analysis (Fig. 7A). Whereas a truncated protein lacking the DNA-binding domain behaved like wild type, regulation was lost with deletion of the carboxy-terminal 136 amino acids (data not shown). The carboxy-terminal region of the protein (pAMG3) is sufficient for nuclear localization of a GFP fusion and may therefore contain a NLS that can be replaced by the SV40–NLS (pASMG1). Deletion of the amino-terminal 264 amino acids (pAMG4) did not impair regulated localization. Further deletion to position 287 (pAMG2) resulted in constitutive nuclear localization. To determine whether potential PKA modification sites (RRXS) are important for Msn2p regulation, we created a series of serine to alanine and aspartate substitutions. S288A and S288D mutations resulted in a GFP fusion protein (pAMG5/pAMG6) with nuclear localization under nonstress conditions, which, however, translocated to the cytoplasm in presence of high exogenous cAMP. Export was abolished completely in combination with the exchange of S620, S625, and S633 to aspartate (pAMG7) or S582, S620, S625, and S633 to alanine (pAMG8). Therefore, the PKA sites are important but partially redundant for nuclear export driven by high exogenous cAMP.

To determine the specific function of S288, a fragment of Msn2p (264 to 568; pAMG9) in which all carboxy-terminal PKA sites are eliminated was fused to the SV40–NLS. The fusion protein (pASMG1) showed wild type-like behavior in response to stress and cAMP. Mutagenesis of S288 abolished both stress responsiveness and cAMP-driven nuclear export (pASMG2, pASMG3). Further carboxy-terminal truncation of pASMG1 destroyed any kind of regulation (pASMG4, amino acids 264–363). In summary, nuclear export requires the region containing the PKA consensus site around S288 and

Figure 7. Functional regions of Msn2p. Deletion constructs were assayed for stress-induced import and cAMP-driven export in OL556 cells. Microcolonies of transformants were suspended in fresh medium (−stress), treated with 10 mM sorbate (+stress), and subsequently incubated with 25 mM cAMP (+high cAMP). The effects of stress and cAMP treatment were scored after 10 min (cyt: cytoplasmic localization due to indeterminable nuclear staining; nuc: clearly identifiable nuclear staining). Serine to alanine or aspartate substitutions are indicated. All SV40–NLS-directed constructs exhibit a certain background of cytoplasmic fluorescence. (HD I) Homology domain I; (HD II) homology domain II; (Zn) zinc finger. (B) Alignment of HD I and HD II (pileup algorithm, Wisconsin GCG package).
the region between amino acids 363 and 568. Within this region (264–568), two stretches with high similarity to the Msn4p sequence (Fig. 7B) are apparent, both of which are included in the domains necessary for Msn2p nuclear export. These homology domains are indicated in Figure 7A as HD I and HD II. The PKA consensus site in HD I (containing S288 of Msn2p) is conserved between both proteins (Fig. 7B, arrow). These data suggest that functional PKA consensus sites are important for the regulation of the intracellular localization of Msn proteins.

Discussion

In the budding yeast S. cerevisiae, many different stress situations activate a common response system whose central parts are two transcription factors, Msn2p and Msn4p. These two factors have partly overlapping functions—being able to bind and activate STRE dependent promoters. Double mutants (msn2 msn4) are normally unable to survive severe stress probably because they are defective in mounting proper countermeasures. Any attempt to explain the generality of the stress response to various individual challenges might have to deal with the question of how these transcription factors function. The results presented here strongly support the notion that diverse stress signals converge on mechanisms that determine the nuclear concentration of Msn2p. Nuclear transport phenomena might therefore be the key to understanding how different stress conditions are integrated into one cellular response.

Nuclear localization of Msn2p is a general response to stress

Our conclusion that Msn2p and Msn4p localization must be at the heart of the system is mostly based on cytological observations. Both in situ immunodetection of an epitope-tagged Msn2p as well as GFP fusions reveal the same situation. Under favorable environmental conditions, the amount of nuclear Msn2p/Msn4p appears low in most cells. After cells encounter any stressful condition, however, the pool of existing proteins is quickly redistributed toward high nuclear concentrations. This effect can be seen in virtually all cells. A causal relationship between the translocation event and gene activity can be inferred by the fact that an increase in nuclear Msn2p concentration correlates well with the occupation of STREs, as suggested by in vivo footprinting experiments. STRE-dependent transcriptional induction is normally occurring rapidly, that is, within minutes after exposing cells to a variety of stress conditions. According to the observed kinetics of this process, Msn2p/Msn4p nuclear translocation appears to be the rate-determining step in the activation process.

In many STRE containing promoters, transcriptional induction occurs after sudden temperature upshift, lowering of the pH, osmotic shock, glucose starvation, and treatment with alcohols or weak organic acids (sorbic acid). In almost every case, the induction is dependent completely on the presence of Msn2p and Msn4p (Martínez-Pastor et al. 1996). One notable exception is the response to osmotic stress, which is still discernible in the msn2 msn4 double mutant even if the absolute levels of transcription are affected severely. In this case, the wild-type response requires a functional Hog1p MAP kinase whose activity is induced specifically by high external osmolarity (Brewster et al. 1993). Our observations on Msn2p nuclear import correlate very well with the STRE expression data. In all stress situations, including osmotic challenge, the nuclear levels of Msn2p become elevated. This increase in nuclear concentration can be observed in hog1 null mutants under osmotic stress. The data suggest that a separate, specialized HOG pathway-dependent mechanism must enhance the osmotic response (Schüller et al. 1994; Martínez-Pastor et al. 1996). After initial perception, the osmostress signal may bifurcate at one point to converge again on the level of the STRE employing different and perhaps interacting transcription factors with Msn2p being one of them. Another explanation could be that high osmolarity normally causes enough collateral damage to induce the general stress response system. Whatever the primary cause of Msn2p and Msn4p translocation, we suggest that this translocation is an integrating response to activate a large set of stress-related genes. Therefore, Msn2p localization may be regarded as a measure of a yeast cell’s well-being.

How might nuclear localization of Msn2p be regulated?

Two different modes of regulation could impinge on Msn2p to achieve the observed differences in spatial distribution—regulated nuclear import and regulated export. With regard to import control, two major themes have emerged over the last few years. One involves the obscuring of a nuclear import signal by phosphorylation. The other mechanism involves the retention of a transcription factor by a cytoplasmic anchor. Our dissection of Msn2p clearly indicates that position and nature of the NLS is apparently not so important for regulated translocation. Instead, the integrity of a 300-amino-acid domain seems crucial to cause cytoplasmic accumulation under nonstress conditions. This observation may point to a retention mechanism that depends on a cytoplasmic partner. The interaction of NF-κB with IκB is perhaps the prototypical example in this regard. Curiously, like Msn2p in yeast, NF-κB seems to guide a large variety of stress responses in higher eukaryotic systems. Here, stress signal-activated kinases seem to influence the phosphorylation-induced instability of IκB. Unfortunately, there is no structural similarity between the two transcription factors to allow any meaningful extrapolation from NFκB on Msn2p control. Compared with these two factors, a more specialized situation has been studied in the case of the S. cerevisiae transcription factor Yap1p (Kuge et al. 1997). Localization of Yap1p involves a carboxy-terminal cystein-rich domain sufficient to cause cytoplasmic retention in unstressed cells. As some of these cystein residues are required for proper regula-
tion they have been proposed to act as sensors for oxidative damaging conditions. The mechanism of retention, however, is not yet understood but might also require a cytoplasmic anchor. In any case, translocation of this factor appears to be controlled specifically by oxidative stress, whereas a broad variety of stresses affect cellular localization in the case of Msn2p.

What is the role of PKA in the regulation of Msn2p?

With the exception of the HOG pathway, which seems to bypass Msn2p, little is known about signal generation and transduction for any of the Msn2p-activating stresses. Nevertheless, one might ask whether modification of Msn2p could be important for releasing or maintaining cytoplasmic anchorage. For example, Pho4p, a factor inducing the response to low phosphate, was shown to be modified by a cyclin-CDK complex, and this phosphorylation may provide the information for anchorage-dependent retention (O’Neill et al. 1996). Glucose-regulated Mig1p nuclear localization is correlated dynamically and reversibly with its phosphorylation status, which is probably regulated by the Snf1 protein kinase. Again phosphorylation may prevent nuclear import (DevIt et al. 1997). We presented here an intriguing connection between Msn2p localization and the activity of protein kinase A. Similar to Pho4p, there is an inverse correlation between PKA activity and nuclear localization. The finding that substitutions at the consensus PKA sites, particularly at position 288, lead to constitutive nuclear residence of Msn2p is highly suggestive for a role of direct modification.

Despite this evidence, we have no clear indication where and how PKA interferes mechanistically with Msn2p and Msn4p distribution. For example, one cannot completely disregard the possibility that PKA regulates the activity of stress signal transduction systems before they converge on Msn proteins. Similarly, one cannot yet exclude that stress might regulate the intracellular distribution of the transcription factors through modulating PKA activity. What we have made clear, however, is that the fact that stress conditions generally lead to higher nuclear concentration, whereas increases in PKA activity induce cytoplasmic accumulation. Changes in the distribution between nucleus and cytoplasm happen rapidly and without noticable changes in the overall protein concentration. Therefore we find considerable attraction in a model in which stress-induced import and PKA induced export are mediated by independent mechanisms.

In a dynamic equilibrium, either stress factors or PKA activity might dominate leading to a steady-state situation where the major part of Msn2p and Msn4p is located either in the nucleus or in the cytoplasm depending on the extent of stress as well on PKA activity.

What is the physiological role of dynamic Msn2p localization?

The influence of PKA activity on Msn2p provides a model explaining how growth conditions determine stress resistance in yeast. Previous observations indicated that strains with low PKA activity are more stress resistant than cells with high PKA activity (Shin et al. 1987). We show here that in mutants with constitutive low PKA activity (bcy1 tpk1w tpk2 tpk3), Msn2p is localized predominantly in the nucleus, which causes high levels of expression of stress-inducible genes and increased stress resistance (Schüller et al. 1994; Ruis 1997). In contrast, high PKA activity would shut down expression of stress genes by preventing nuclear accumulation of Msn2p. This would explain why mutants with high PKA activity (e.g., bcy1) are stress-sensitive (Thevelein 1994; de Winde et al. 1997). Our results suggest that PKA sets the threshold by preventing the nuclear accumulation of Msn2p/Msn4p for how much stress signal is required to mount the stress defense program.

For yeast, it might be advantageous to adjust stress protein synthesis according to growth conditions, thereby avoiding any unnecessary burden detrimental to optimal growth. A high level of expression of Msn2p or Msn4p reduces the growth rate, perhaps by the constitutive activation of STRE-regulated genes (Estruch and Carlson 1993; Martinez-Pastor et al. 1996; C. Schüller, unpubl.). In a competitive environment, a return to fast growth after stress relief and adaptation could be of vital importance.

Materials and methods

Yeast strains, media, and growth conditions

In strain W303-STRE-lac2 (MATaura3-1 leu2-3 his3-11 ura3, trp1-1 ade2-1 cam1-1000 URA3::STRE–lacZ) the MSN2 and MSN4 genes were disrupted using the msn2::HIS3 and msn4::TRP1 (Estruch and Carlson 1993); HOG1 was deleted with TRP1 (Brewster et al. 1993). OL556 (MATa [cdc25-5/cdc25-5 pde2/pde2 his3 his3 leu2 leu2 ura3 ura3::STRE–lacZ; Boy-Marcotte et al. 1996) was obtained from E. Boy-Marcotte. TC41-1 (MATa leu2-3 his3-112 his3 his4 ura3-cdc35::ura3 cam) was provided by W. Heidemann. SP1 (MATa leu2 his3 trp1 ade8 can1 ura3) and the derivative RS58-5A-1 (MATa leu2 his3 trpl ade8 can1 ura3 tpk1-1 tpk2-1 HIS3 tpk3::TRP1 bcy1::LEU2) have been described previously (Cameron et al. 1988). RS58-5A-1 msn2::URA3 was created by transformation. Cells were generally grown to logarithmic growth phase from OD ∼ 0.2 to OD ∼ 1 by dilution of overnight precultures in rich medium (YPD) or in synthetic medium. Logarithmic cultures were used to visualize GFP fusions. GFP fusions were also visualized in medium suspensions of microcolonies (diameter up to ~1 mm). Stress was caused by addition of concentrated solutions to logarithmic cultures or suspensions to the desired final concentration followed by incubation with shaking for the indicated times. For heat stress treatment cultures were grown at least four generations at 26°C before the growth temperature was raised to 37°C within 3–4 min. Glucose starvation was achieved by washing cells two times in glucose free medium.

Plasmids

Plasmid pMsn2::Ura3 was created from pt32-deltaXB::His3 (Estruch and Carlson 1993) by replacing the HIS3 sequences with URA3. Plasmid pMsn2 was generated by cloning a 3.5-kb KspI-HindIII fragment containing the MSN2 gene into a modified
EcoRI site (changed to a KspI site with oligonucleotide 5'-AAT-
TGCCTGGGCGGC-3') of Ycplac111 centromere vector (Gietz and 
Sugino 1988). A NotI site was introduced by creating a linker into 
the Sall site (position +7) of the MSN2 gene. A NotI site at the 3' 
end of MSN2 was introduced before the stop codon by PCR 
using 5' oligonucleotide 5'-TCGAGCTCTTGCC- 
GATCTCTTGGACTCTTACCAC-3' and 3' oligonucleo-
tide 5'-TTTAAGCTTTAAAAATTGGGCCCACATGTC-
ATCATGTTTTATGAGT-3' (NotI site, italicized; stop 
codon, underscored; HindIII site, bold). The 0.3-kb Ndel–HindIII 
fragment of pMS2 was replaced by the Ndel–HindIII-cut PCR 
fragment generating pMS2–NotI. pMS2–myc9 was generated by 
ligating a myc9 fragment into pMS2–NotI sites, italicized; vector pEGFP–N3 
(Clonetech) served as template. Plasmid pMS2–GFP was generated 
by ligation of a GFP NotI cassette into pMS2–NotI. 

pAdh1–Msn2–GFP (Sikorski and Hieter 1989). Expression of fusion 
proteins was verified by Western blotting using monoclonal antibody to the 
Myc epitope (9E10) or polyclonal rabbit antiserum against 
Msn2p stress-regulated nuclear translocation

The 3' oligonucleotide 5'-GCGGCCGC- 
ACCATGGTCGACGTTATAACAAAC-3' (NotI site, italicized; stop 
codon, underscored; HindIII site, bold). The 3' end of MSN2 was introduced before the stop codon by PCR 
using 5' oligonucleotide 5'-TCGACTCGCTTACCA-
GATCTCTTGGACTCTTACCAC-3' and 3' oligonucleo-
tide 5'-TTTAAGCTTTAAAAATTGGGCCCACATGTC-
ATCATGTTTTATGAGT-3' (NotI site, italicized; stop 
codon, underscored; HindIII site, bold). The 0.3-kb Ndel–HindIII 
fragment of pMS2 was replaced by the Ndel–HindIII-cut PCR 
fragment generating pMS2–NotI. pMS2–myc9 was generated by 
ligating a myc9 fragment into pMS2–NotI sites, italicized; vector pEGFP–N3 
(Clonetech) served as template. Plasmid pMS2–GFP was generated 
by ligation of a GFP NotI cassette into pMS2–NotI. 

pAdh1–Msn2–GFP (Sikorski and Hieter 1989). Expression of fusion 
proteins was verified by Western blotting using monoclonal antibody to the 
Myc epitope (9E10) or polyclonal rabbit antiserum against 
Msn2p.
Extract preparation for EMSA and Western blot analysis

Extracts for EMSA were prepared from cells grown at 30°C at OD₆₀₀ 1–2. Pellets were suspended in cold buffer A [50 mM HEPES, pH 8.0, 0.4 M (NH₄)₂SO₄, 1 mM EDTA, and 5% glycerol], broken with glass beads, and clarified by centrifugation. Extracts were precipitated with (NH₄)₂SO₄ to 50% saturation and resuspended in buffer B (75 mM KCl, 25 mM HEPES, pH 8.0, 1 mM EDTA, and 12% glycerol).

For Western analysis, cells from OD₆₀₀ = 1.0 cultures were washed once, resuspended in buffer A, and then broken up by vortexing with glass beads for 3 × 4 min. Glass beads and debris were spun out by two 20-min centrifugation steps. Extracts (100 μl) were boiled with 50 μl 4× sample buffer. For Western blot analysis, 50 μg of protein were loaded on 8% SDS-PAGE and separated using the Mini Protean system (Biorad). Proteins were transferred to Nitrocellulose membranes (Schleicher & Schuell) by Semi-dry Blotting Device (Biorad). Immunodetection of proteins was carried out as described in the ECL manual (Amersham) using primary polyclonal affinity-purified αMsn2p and αCdc28p antibodies. Complete protease inhibitor mix tablets (Boehringer) were used in all buffers.

Electrophoretic mobility shift assays, Northern analysis, and enzyme assays

EMSA was carried out as described by Martinez-Pastor et al. (1996). CTT1-20 and CTT1-23 are described by Marchler et al. (1993). A supershift was performed by addition of 9E10 monoclonal antibody to the binding mix 2 hr before the addition of the labeled oligonucleotide. Northern analysis and measurement of β-galactosidase and catalase enzyme activities were carried out as described by Martinez-Pastor et al. (1996). To test whether the processes studied require protein synthesis, cycloheximide (100 μg/ml) was added 30 min before harvesting.

Acknowledgments

We thank W. Zachariae and T. Dechat for help with immunofluorescence; E. Waigmann for providing her microscope; E. Boy-Marcotte, W. Heidemann, and J. Aitchison for materials; A. Marchler, G. Marchler-Bauer, S. Kuge, S. Nomoto, and A. Nomoto. 1997. Regulation of yAP-1 stress conditions.

1996. Nuclear translocation of the Mgl1 glucose repressor. Mol. Biol. Cell 8: 1603–1618.

DeWitte, M.J., J.A. Waddle, and M. Johnston. 1997. Regulated nuclear localization in response to oxidative stress. EMBO J. 16: 7119–7120.

Marchler, M.T., G. Schüller, G. Adam, and H. Ruis. 1993. A Saccharomyces cerevisiae UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J. 12: 1997–2003.

Martinez-Pastor, M.T., G. Marchler, C. Schüller, A. Marchler-Bauer, H. Ruis, and F. Estruch. 1996. The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J. 15: 2277–2283.

Nasmyth, K., G. Adolf, D. Lydall, and A. Seddon. 1990. The identification of a second cell cycle control on the HO promoter in yeast: cell cycle regulation of SWI5 nuclear entry. Cell 62: 631–647.

References

Baldwin, A.S., Jr. 1996. The NF-kappa B and I kappa B proteins: New discoveries and insights. Annu. Rev. Immunol. 14: 649–683.

Baroni, M.D., P. Monti, G. Marconi, and L. Alberghina. 1992. cAMP-mediated increase in the critical cell size required for the G1 to S transition in Saccharomyces cerevisiae. Exp. Cell Res. 201: 299–306.

Baroni, M.D., P. Monti, and L. Alberghina. 1994. Repression of growth-regulated G1 cyclin expression by cyclic AMP in budding yeast. Nature 371: 339–342.

Beals, C.R., C.M. Sheridan, C.W. Turner, P. Gardner, and G.R. Crabtree. 1997. Nuclear export of N-F-AF-Tc enhanced by glycolgen synthase kinase-3. Science 275: 1930–1933.

Brewster, J.L., T. de Valoir, N.D. Dwyer, E. Winter, and M.C. Gustin. 1993. An osmosensing signal transduction pathway in yeast. Science 259: 1760–1763.

Boy-Marcotte, E., D. Tadi, M. Perrot, H. Boucherie, and M. Jacquet. 1996. High cAMP levels antagonize the reprogramming of gene expression that occurs at the diauxic shift in Saccharomyces cerevisiae. Microbiology 142: 459–467.

Cameron, S., L. Levin, M. Zoller, and M. Wigler. 1988. cAMP-independent control of sporulation, glycolgen metabolism, and heat shock resistance in S. cerevisiae. Cell 53: 555–566.

Chen, R.-H., C. Samiecki, and J. Blenis. 1992. Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Mol. Cell. Biol. 12: 915–927.

DeWitte, M.J., J.A. Waddle, and M. Johnston. 1997. Regulated nuclear translocation of the Mgl1 glucose repressor. Mol. Biol. Cell 8: 1603–1618.

De Winde, J.H., J.M. Thevelein, and J. Winderickx. 1997. From feast to famine: Adaptation to nutrient depletion in yeast. In Yeast stress responses (ed. S. Hohmann and W.H. Mager ), pp. 7–52. Landes, Austin, TX.

Estruch, F. and M. Carlson. 1993. Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of Saccharomyces cerevisiae. Mol. Cell. Biol. 13: 3872–3881.

Gietz, R.D. and A. Sugino. 1988. New yeast—Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74: 527–534.

Görlich, D. and I.W. Mattaj. 1996. Nucleocytoplasmic transport. Science 271: 1513–1518.

Hubler, L., J. Bradshaw-Rouse, and W. Heidemann. 1993. Connections between the Ras-cyclic AMP pathway and G1 cyclin expression in the budding yeast Saccharomyces cerevisiae. Mol. Cell. Biol. 13: 6274–6282.

Jans, D.A. 1995. The regulation of protein transport to the nucleus by phosphorylation. Biochem. J. 311: 705–716.

Koch, C., A. Schleffer, G. Ammerer, and K. Nasmyth. 1996. Switching transcription on and off during the yeast cell cycle Cin/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at start, whereas Cib/Cdc28 kinases displace it from the promoter in G2. Genes & Dev. 10: 129–141.

Kuge, S., N. Jones, and A. Nomoto. 1997. Regulation of yAP-1 nuclear localization in response to oxidative stress. EMBO J. 16: 2670–2680.

Marchler, M.T., G. Schüller, G. Adam, and H. Ruis. 1993. A Saccharomyces cerevisiae UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J. 12: 1997–2003.

Martinez-Pastor, M.T., G. Marchler, C. Schüller, A. Marchler-Bauer, H. Ruis, and F. Estruch. 1996. The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J. 15: 2277–2283.

Nasmyth, K., G. Adolf, D. Lydall, and A. Seddon. 1990. The identification of a second cell cycle control on the HO promoter in yeast: cell cycle regulation of SWI5 nuclear entry. Cell 62: 631–647.
Nigg, E.A. 1997. Nucleocytoplasmic transport: Signals, mechanisms and regulation. Nature 386: 779–783.

O’Neill, E.M., A. Kaffman, R.J. Jolly, and E.K. O’Shea. 1996. Regulation of PHO4 nuclear localization by the PHO80-PHO85 cyclin-CDK complex. Science 271: 209–212.

Piatti, S., T. Böhm, J.H. Cocker, J.F.X. Diffley, and K. Nasmyth. 1996. Activation of S-phase-promoting CDKs in late G1 defines a “point of no return” after which Cdc6 synthesis cannot promote DNA replication in yeast. Genes & Dev. 10: 1516–1531.

Ruis, H. 1997. Yeast stress responses: Achievements, goals and a look beyond yeast. In Yeast stress responses (ed. S. Hohmann and W.H. Mager), pp. 231–247. Landes, Austin, TX.

Ruis, H. and C. Schüller. 1995. Stress signaling in yeast. BioEssays 17: 959–965.

Schmitt, A.P. and K. McEntee. 1996. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. 93: 5777–5782.

Schüller, C., J.L. Brewster, M.R. Alexander, M.C. Gustin, and H. Ruis. 1994. The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the Saccharomyces cerevisiae CTT1 gene. EMBO J. 13: 4382–4389.

Shin, D.-Y., K. Matsumoto, H. Iida, I. Uno, and T. Ishikawa. 1987. Heat shock response of Saccharomyces cerevisiae mutants altered in cAMP-dependent protein phosphorylation. Mol. Cell. Biol. 7: 244–250.

Siebenlist, U., K. Brown, and G. Franzoso. 1995. NF-κB: A mediator of pathogen and stress responses. In Inducible gene expression, Vol.1, Environmental stresses and nutrients (ed. P. Baeuerle), pp. 93–142. Birkhäuser, Boston, MA.

Siderius, M. and W.H. Mager. 1997. General stress response: In search of a common denominator. In Yeast stress responses (ed. S. Hohmann and W.H. Mager), pp. 213–230. Landes, Austin, TX.

Sikorski, R.S. and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122: 19–27.

Thevelein, J.M. 1994. Signal transduction in yeast. Yeast 10: 1753–1790.

Tokiwa, G., M. Tyers, T. Volpe, and B. Futcher. 1994. Inhibition of G1 cyclin activity by the Ras/cAMP pathway in yeast. Nature 371: 342–345.
Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity

Wolfram Görner, Erich Durchschlag, Maria Teresa Martinez-Pastor, et al.

Genes Dev. 1998, 12:

References

This article cites 28 articles, 14 of which can be accessed free at:
http://genesdev.cshlp.org/content/12/4/586.full.html#ref-list-1

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.