Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in Saccharomyces cerevisiae

Hervé Garreau,1 Rukhsana Nilofer Hasan,1 Georges Renault,1 Francisco Estruch,2 Emmanuelle Boy-Marcotte1 and Michel Jacquet1

Author for correspondence: Hervé Garreau. Tel: +33 1 69 15 46 30. Fax: +33 1 69 15 72 96.
E-mail: garreau@igmors.u-psud.fr

In response to various stresses, as well as during the diauxic transition, the Msn2p and Msn4p transcription factors of Saccharomyces cerevisiae are activated and induce a large set of genes. This activation is inhibited by the Ras/cAMP/PKA (cAMP-dependent protein kinase) pathway. Here we show by immunoblotting experiments that Msn2p and Msn4p are phosphorylated in vivo during growth on glucose, and become hyperphosphorylated at the diauxic transition and upon heat shock. This hyperphosphorylation is correlated with activation of Msn2/4p-dependent transcription. An increased level of cAMP prevents and reverses these hyperphosphorylations, indicating that kinases other than PKA are involved. These results suggest that PKA and stress-activated kinases control Msn2/4p activity by antagonistic phosphorylation. It was also noted that Msn4p is transiently increased at the diauxic transition. Msn2p and Msn4p present different hyperphosphorylation patterns in response to different stresses.

Keywords: Saccharomyces cerevisiae, stress response, transcription factor, cAMP-dependent protein kinase

INTRODUCTION

All organisms have developed mechanisms of adaptation to environmental challenges. In the yeast Saccharomyces cerevisiae, the cellular responses involve a transcriptional activation of numerous genes, some of which promote a specific adaptation to the new environmental conditions, whereas others appear to be less specific, since they are induced under a wide variety of changes. The so-called stress-response system that involves the STRE (stress-response element) motif CCCCT and two transcriptional activators, Msn2p and Msn4p (Estruch & Carlson, 1993; Mager & De Kruijff, 1995; Marchler-Bauer et al., 1998), is an instance of such a broad-specificity response. As an example, heat shock induces the expression of both the Hsf1p regulon, which controls a specific subset of chaperones involved in the protection of proteins, and the Msn2/4p regulon (Boy-Marcotte et al., 1999). Similarly, oxidative stress triggers the activation of the Yap1p and Skn7p transcription factors, which induce genes playing a crucial role in maintaining the redox potential of the cell, as well as the Msn2/4p regulon (Godon et al., 1998; Lee et al., 1999). The Msn2/4p regulon is also activated in response to nutritional limitation. It is involved in the diauxic shift, the reprogramming of gene expression which occurs upon glucose exhaustion and ethanol-sustained growth (Boy-Marcotte et al., 1998). In these cases, the signal transduction pathway(s) from extracellular stress to gene induction is still largely unravelled. Whether or not Msn2p and/or Msn4p receive the same signals as the specific transcription factors (such as Hsf1p, Yap1p and Skn7p) remains unknown. It is also possible that the cAMP-PKA (cAMP-dependent protein kinase) pathway, which controls the nuclear localization of Msn2p and Msn4p, could mediate the general-stress response (Görner et al., 1998). Indeed most, if not all, of the genes found to be regulated by Msn2/4p during the diauxic shift, heat shock or oxidative stress are also repressed by cAMP (Boy-Marcotte et al., 1999, 1998). To explain how Msn2/4p could be activated in response to different stresses and to evaluate the role of the cAMP pathway in this control, we have examined the state of

Abbreviations: PKA, cAMP-dependent protein kinase; STRE, stress-response element.
phosphorylation of both Msn2p and Msn4p by their differential migration on gel electrophoresis at the diauxic transition and in response to heat shock. We have shown that Msn2/4p become hyperphosphorylated in vivo during the diauxic shift and upon heat shock. Msn2/4p, capable of relaying many different stresses, could be the targets of different signal-transducing kinases. This activating mechanism is antagonized by cAMP. Thus the cAMP–PKA pathway cannot be the transducer of the stress signal to these trancitivators but acts in addition to them, to counteract the adaptive response of the Msn2/4p regulon.

**METHODS**

**Plasmids and strains**

**Plasmids.** pGEX-3X-MSN2B contains the EcoRV–EcoRI fragment from the MSN2 gene (Estruch & Carlson, 1993) inserted into the Smal and EcoRI sites in pGEX-3X (Pharmacia). It contains codons 44 to 302 of the MSN2 ORF. pGEX-3X-MSN4B contains the NclI–NruI fragment from the MSN4 gene (Estruch & Carlson, 1993) inserted into the EcoRI site of pGEX-3X, after filling the 5′ protruding NclI and EcoRI ends. It contains codons 34 to 256 of the MSN4 ORF. pEL-32/45 was derived from pEL32 by replacing the PvuII–PvuII fragment (not including the PvuII site) with the EcoRI fragment (containing the MSN4 gene) from pEL45 (Estruch & Carlson, 1993). It contains both MSN2 and MSN4 genes under the control of their own promoters inserted into the Yep24 vector (Carlson & Botstein, 1982).

pAdh1-Msn2-GFP, encoding a Msn2–GFP fusion under the control of the strong constitutive ADH1 promoter, was a generous gift from C. Schüller (Görner et al., 1998).

**Yeast strains.** The following strains were used: W303-1A (MATa ade2 his3 leu2 trp1 ura3 ) (Estruch & Carlson, 1993) and the diploid OL556 (MATa/MATα cdc25-5/cdc25-5 his3/his3 leu2/leu2 trp1/Trp1 rca1/rca1 ura3/ura3) (Boy-Marcotte et al., 1996). The construction of the OL556-STRE strain containing the reporter gene lacZ under the control of four STRE motifs has been already described (Boy-Marcotte et al., 1998).

**Antibodies.** Rabbit anti-Msn2p and anti-Msn4p antibodies were raised against the N-terminal parts of Msn2p and Msn4p (expressed in E. coli from pGEX-3X-MSN2B and pGEX-3X-MSN4B, respectively) and showed no cross-reactivity. They were purified by immunoblotting (Harlow & Lane, 1988) with the GST-fused peptides expressed in E. coli from pGEX-3X-MSN2 and pGEX-3X-MSN4B. For immunoblotting, these antibodies were used at 1/20 to 1/50 dilutions.

**Yeast growth, protein extracts, immunoblotting and alkaline phosphatase treatment.** For the diauxic transition, yeast cells were grown in YES medium with 2% glucose (Boy-Marcotte et al., 1996). Glucose concentration in the culture medium was monitored enzymically with glucose oxidase using Sigma Diagnostic Glucose reagent kit no 510-A. Glucose concentration in the culture medium was measured by the glucose oxidase method at the diauxic transition and in response to heat shock. We have shown that Msn2/4p become hyperphosphorylated in vivo during the diauxic shift and upon heat shock. Msn2/4p, capable of relaying many different stresses, could be the targets of different signal-transducing kinases. This activating mechanism is antagonized by cAMP. Thus the cAMP–PKA pathway cannot be the transducer of the stress signal to these trancitivators but acts in addition to them, to counteract the adaptive response of the Msn2/4p regulon.

**RESULTS**

**cAMP prevents hyperphosphorylation of Msn2p and Msn4p at the diauxic transition**

We have previously reported that Msn2p and/or Msn4p were essential for full induction of a set of genes that occurs at the diauxic transition, and that cAMP inhibited this transition (Boy-Marcotte et al., 1996). Therefore we looked at the phosphorylation state of Msn2p and Msn4p throughout the growth curve, in the presence and in the absence of cAMP (Fig. 1a). We used the OL556 strain, carrying the rca1 mutation in the PDE2 gene, which allows one to increase the intracellular level of cAMP by adding cAMP to the medium (Boy-Marcotte et al., 1996; Wilson et al., 1993). During the exponential-growth phase, two immunoreactive bands of Msn2p were detected (apparent molecular mass around 100 kDa): their electrophoretic mobilities with or without cAMP was the same (Fig. 1b, + /− cAMP, lanes 1). Msn2p was already phosphorylated, as shown by alkaline phosphatase treatment (Fig. 1c, sample 1). As the glucose concentration decreased, but before complete exhaustion, the fast-mobility band almost completely disappeared and new species with lower mobility were detected (Fig. 1b, −cAMP, lanes 2–4). This mobility shift was due to hyperphosphorylation (Fig. 1c, sample 4). With cAMP in the medium, no mobility shift was detected without fixation essentially as described by Görner et al. (1998).

**β-Galactosidase assay.** Determination of β-galactosidase activity was performed as previously described (Boy-Marcotte et al., 1998).
Msn2p phosphorylation, stress and cAMP

**Fig. 1.** cAMP prevents hyperphosphorylation of Msn2p at the diauxic transition. (a) Two cultures of the OL556 strain were grown in YNBS at 26°C, one in the absence (white symbols) and the other one in the presence of 3 mM cAMP (black symbols). At the times indicated by the arrows, samples were withdrawn from the cultures for glucose assay in the medium, immunotransfer and RNA analysis. OD700, glucose concentration in the culture medium. (b) Samples (40 ml) were withdrawn at the times indicated in (a) and proteins extracted. For each sample, 5 and 10 µl were loaded on polyacrylamide gel (7.5% acrylamide). After electrophoresis and transfer, the upper part of the PVDF sheets were probed for immunodetection of Msn2p using specific antibodies (upper panel), the lower part was used for Coomassie blue staining of bulk proteins (lower panel). (c) Alkaline phosphatase treatment of protein extracts of samples number 1 and number 4 in the absence of cAMP. Aliquots were incubated with or without alkaline phosphatase and Msn2p was detected by immunoblotting. s, starting material; m, mock incubation in alkaline phosphatase buffer; p, incubation in the presence of alkaline phosphatase. (d) Northern analysis of HSP12 mRNA in RNA extracts from samples taken from OL556 cultures with or without cAMP. ACT1 mRNA was analysed as the loading control.

was observed during the glucose-concentration decrease (Fig. 1b, +cAMP, lanes 1–4). A similar phenomenon was also observed for the closely related Msn4p. Indeed, during the exponential-growth phase, Msn4p was detected as two species having an apparent molecular mass around 95 kDa, the faster mobility form being predominant (Fig. 2b, –cAMP, lane 1). These forms were already phosphorylated, as shown by alkaline phosphatase treatment (Fig. 2c). After alkaline phosphatase treatment, two faster migrating bands were observed for Msn4p, probably as a result of either incomplete dephosphorylation or some proteolysis (Fig. 2c). A partial mobility shift occurred before complete exhaustion of glucose; several poorly resolved species of lower mobility were detected in addition to the faster-mobility forms present in exponential phase (Fig. 2b, –cAMP, lanes 2 and 3). The amount of Msn4p increased before complete exhaustion of glucose and Msn4p became barely detectable when the glucose was completely consumed (Fig. 2b, –cAMP, lanes 4 and 5). This was not due to a lower protein content of the sample, as checked by protein staining in the lower part of the transfer. Nevertheless, the faint band detected in lane 4 has a slower mobility than the major fast-mobility form detected in the exponential-growth phase, indicating hyperphosphorylation. In the presence of cAMP, during exponential growth, one major band was detected that had the same mobility as the low-mobility form detected in the exponential-growth phase in the absence of cAMP. No isoform with lower mobility was observed when glucose became limiting and no significant variation in the amount of Msn4p was observed (Fig. 2b, +cAMP, lanes 1 to 5).

The same type of experiment was performed on strain W303-1A. Here again, as described for the OL556 strain, both Msn2p and Msn4p were already phosphorylated during the exponential-growth phase and became hyperphosphorylated as glucose was consumed. A transient increase in the level of Msn4p was also observed prior to the end of exponential growth, reaching a peak before complete glucose exhaustion (data not shown).

As a reporter gene of the transcriptional activity of Msn2/4p (Martinez-Pastor et al., 1996), the accumu-
H. GARREAU and OTHERS

Fig. 2. cAMP prevents hyperphosphorylation of Msn4p at the diauxic transition. (a) Strain OL556 was grown in YNBS at 26 °C with or without 3 mM cAMP. At the times indicated by the arrows, samples were withdrawn from the culture for glucose assay in the medium and immunotransfer, as described in Fig. 1. The symbols are the same as in Fig. 1(a). (b) Immunotransfer of Msn4p. The same protocol as for Msn2p was applied. (c) To assess the phosphorylation state of Msn4p during exponential growth, we used strain OL556 carrying the multicopy plasmid pEL-32/45. A sample was withdrawn from a culture, grown in YNBS at 26 °C, at OD710 ≈ 0.8 and the protein extract was treated with alkaline phosphatase. An electrophoresis and transfer, Msn4p was detected by immunostaining. s, starting material; m, mock incubation in alkaline phosphatase buffer; p, incubation in the presence of alkaline phosphatase.

Fig. 3. Hyperphosphorylation of Msn2p and Msn4p upon heat shock and effect of cAMP. (a) Strain OL556 grown at 26 °C in YNBS medium (OD710 0) was split into two and 3 mM cAMP was added to one part. After 30 min, both cultures were shifted to 38 °C. Samples (40 ml) were taken at the times indicated (numbered 1 to 6). (b) Protein extracts were prepared from these samples and processed as described in Fig. 1. Top panels, immunostaining of Msn2p and Msn4p; bottom panels, Coomassie blue staining.

The phosphorylation state of Msn2p and Msn4p was examined when a heat shock from 26 °C to 38 °C was applied in the absence or in the presence of cAMP (Fig. 3a). At 26 °C, Msn2p was detected as two immunoreactive bands (Fig. 3b, left, lane 1). After heat shock in the absence of cAMP, the faster-mobility band dis-
The hyperphosphorylation promoted by heat shock was reversed by the addition of cAMP. Indeed, when cAMP was added 15 min after heat shock (Fig. 4a), the faster-mobility form of Msn2p again became visible and predominant (Fig. 4b). A similar reversion of the heat-shock-induced mobility shift was also observed with Msn4p (data not shown).

The transcriptional activity of Msn2/4p during heat shock in the presence and in the absence of cAMP was assessed by monitoring the expression of β-galactosidase as a reporter gene under control of Msn2/4 binding sites (STRE motifs). As shown in Fig. 5, specific activity was increased 18-fold after heat shock in the absence of cAMP. In contrast, in the presence of cAMP, the specific activity remained constant.

Different hyperphosphorylation forms are produced upon different stresses

The pattern of heat shock-induced hyperphosphorylated forms of Msn2p (Figs 3 and 4) was different from the one obtained during the diauxic shift (Fig. 1), suggesting that different stresses could lead to different states of phosphorylation. To confirm this hypothesis and further extend this notion, we have compared the pattern of phosphorylation obtained in cells either submitted to heat shock or grown on 2% ethanol. As shown in Fig. 6(a), Msn2p presented different phosphorylation patterns under the two conditions: several low-mobility bands were detected in ethanol-grown cells compared to the unique low-mobility band observed after heat shock, all of them migrating still more slowly than heat-shock-induced species. After alkaline phosphatase treatment of the protein extracts from heat-shocked cells, as described in Methods, the electrophoretic pattern of Msn2p was split into two and 3 mM cAMP was added to one part (+). Samples were withdrawn from the two cultures at the times indicated by the arrows. (b) Protein extracts were prepared from these samples and processed as described in Fig. 1. Top panel, immunostaining of Msn2p; bottom panel, Coomassie blue staining.

Fig. 4. Hyperphosphorylation of Msn2p induced by heat shock is reversed by cAMP. (a) Strain OL556 grown in YNBS medium (OD710 0.8) was submitted to a heat shock from 26°C to 38°C in the absence of cAMP (–). After 15 min heat shock, the culture was split into two and 3 mM cAMP was added to one part (+). Samples were withdrawn from the two cultures at the times indicated by the arrows. (b) Protein extracts were prepared from these samples and processed as described in Fig. 1. Top panel, immunostaining of Msn2p; bottom panel, Coomassie blue staining.
DISCUSSION

Msn2/4p are hyperphosphorylated and activated by stress-signalling kinases

Msn2p and Msn4p induce transcription of a set of genes in response to various stresses via the STRE motif. The negative regulation of STRE-containing genes by the cAMP–PKA pathway has focused attention on a direct effect of this pathway on Msn2p and Msn4p. This hypothesis was further substantiated by the existence of several sites of PKA phosphorylation in each of these factors. Indeed, Msn2p has been reported to be phosphorylated in vitro by PKA (O’Brien et al., 1995; H. Garreau, J. d’Alayet and M. Jacquet, unpublished); mutation of these sites in Msn2p has been shown to abolish cAMP-promoted nuclear export and cytoplasmic localization in non-stressed cells (Görner et al., 1998). These results led to the suggestion that inactivation of PKA and/or dephosphorylation of the PKA sites would be sufficient to fully activate Msn2/4p–STRE transcription. To test this hypothesis, we analysed by immunoblotting the in vivo phosphorylation state and how it could be modified under stress or during the diauxic transition. Our data clearly show that under two different conditions that have been previously shown to activate the STRE-Msn2/4p system, heat shock and the diauxic transition (Boy-Marcotte et al., 1999, 1998), hyperphosphorylation rather than dephosphorylation was observed for both of these transcription factors. This definitely eliminates the simplistic model in which the cAMP pathway would have been the unique integration system for stresses. It points to the existence of other protein kinases transducing stress signals by catalysing hyperphosphorylations.

In both the diauxic transition and heat shock, hyperphosphorylation of Msn2/4p was correlated with induced transcriptional activity of Msn2/4p. Moreover, the correlation between activation and hyperphosphorylation could be extended to the subcellular localization of these factors. We have confirmed the results previously obtained (Görner et al., 1998): the nuclear translocation of Msn2p induced by heat shock was inhibited and reversed by cAMP (data not shown). Therefore, it appears that hyperphosphorylation could be required for activation of Msn2p and Msn4p. The question therefore arises whether hyperphosphorylation is required for stress-induced nuclear localization or if it occurs only after migration to the nucleus.

cAMP–PKA pathway antagonizes both hyperphosphorylation and activation of Msn2/4p

We observed that high cAMP levels prevent stress-induced hyperphosphorylation of Msn2/4p and induction of HSP12 transcription. Thus, dephosphorylation of PKA sites could be a prerequisite for stress-promoted hyperphosphorylation and activation. In addition, the fact that added cAMP reversed hyperphosphorylation suggests that the PKA-phosphorylated sites have been dephosphorylated during stress-induced activation in order to restore responsiveness to cAMP. As illustrated in this report, Msn2p and Msn4p were already dephosphorylated during exponential growth on glucose, when the intracellular cAMP level remains higher than in the absence of glucose (Russell et al., 1993). This basal phosphorylation could be due to PKA activity. The decrease in cAMP that occurs upon glucose exhaustion could lead to dephosphorylation of the PKA sites, allowing phosphorylation by stress-activated protein kinases. In this respect, it was recently reported how the TOR pathway leads to Msn2/4p sequestration in the cytoplasm by association with Bmh2p, a member of the 14-3-3 protein family. Inhibition of the TOR signalling pathway by rapamycin led to release of Msn2p from Bmh2p and to nuclear translocation of Msn2p (Beck & Hall, 1999). The fact that these 14-3-3 proteins are anchoring partners for serine-phosphorylated proteins (Muslin et al., 1996) strongly argues for such phosphorylation of Msn2/4p during exponential growth.
growth, to keep them present in the cytoplasm in the absence of stress.

These results suggest the existence of a switch between active and inactive forms controlled by two types of antagonistic phosphorylation. A similar phosphorylation switch has been observed at the diauxic transition on Not5p, a subunit of the Not complex, a negative regulator of TATA-less promoters. Not5p is phosphorylated upon glucose starvation and this phosphorylation is inhibited by high PKA activity (M. Collart, personal communication). Multiple phosphorylations with different effects on the proteins appear to be used by the cell for the fine tuning of regulation such as in the case of Pho4p, which is phosphorylated at several sites, each of them being involved at a different level of control (Komeili & O'Shea, 1999). Msn2p and Msn4p are other examples of such complex regulation of transcription factors by multiple phosphorylations.

**Different stress signals are transduced by different pathways**

In this report we show that different conditions (the diauxic transition, heat shock and growth on ethanol) give different patterns of hyperphosphorylation of Msn2p and Msn4p. That could be due to differences in the antagonistic activity of PKA, higher in glucose medium, even after heat shock, than in ethanol. Indeed, the intracellular cAMP level has been shown to be lower in ethanol-grown cells than in glucose-grown cells (François et al., 1987). Another explanation could be the involvement of different protein kinases, according to different stresses. Several stress-signalling pathways have been identified that involve specific kinases (Gustin et al., 1998). Among them, the HOG pathway has been shown to activate Msn2/4p/STRE-driven transcription in response to osmotic shock (Inoue et al., 1998; Martinez-Pastor et al., 1996; Rep et al., 1999). Another candidate could be the Snf1p protein kinase, which is activated during the diauxic transition (Wilson et al., 1996). Although protein kinase C mediates a response to heat-shock-induced cell-wall damage, this pathway does not seem to be involved in Msn2/4p activating phosphorylation, since STRE/Msn2/4p-driven transcription is independent of this pathway (Kamada et al., 1995).

**Msn2p and Msn4p seem to be differentially regulated**

We have observed some differences between Msn2p and Msn4p. Their cellular content appeared to be differentially regulated during diauxic transition: the amount of Msn4p transiently increased at the end of the exponential-growth phase, whereas Msn2p remained constant. This result is consistent with the data obtained by micro-array analysis (De Risi et al., 1997): MSN4 mRNA increases at the diauxic transition and MSN2 expression is repressed by Tup1p, whereas MSN2 mRNA decreases at the diauxic transition. The rapid decrease of Msn4p when growth is arrested due to glucose exhaustion suggests an instability of this protein, which was not observed for Msn2p. The ratios of these two factors could vary between growth conditions. Some subtle differences in the phosphorylation patterns between Msn2p and Msn4p after different stresses were also observed. This suggests differential susceptibility to stress-induced phosphorylation and to PKA. As with many other partially redundant genes, MSN2 and MSN4 could play partially overlapping functions with some biological specificity. The amount of the active forms induced by different stresses could be different for each of the two genes. That could lead to a differential induction of two partially overlapping subsets of genes among the Msn2/4p regulon, each depending on its own transcription factor. Indeed, as reported by Treger et al. (1998), the relative contribution of each factor varies among different Msn2/4p-regulated genes, indicating some specificity for their functions.

**ACKNOWLEDGEMENTS**

We are grateful to D. Zickler and F. Jammes for their help and advice in fluorescence microscopy and to W. Heidman for critical reading of the manuscript. We thank C. Schüller and H. Ruis for sending us plasmids encoding GFP–MSN2 fusions and M. Collart for communicating unpublished results. This work was supported by the Centre National de la Recherche Scientifique, the Ministère de l’Education Nationale, de la Recherche et de la Technologie and by a grant from l’Association pour la Recherche sur le Cancer. R.H. was supported by a fellowship from l’Association pour la Recherche sur le Cancer.

**REFERENCES**


Received 22 March 2000; revised 12 June 2000; accepted 15 June 2000.