Stress induces depletion of Cdc25p and decreases the cAMP producing capability in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae* the cAMP-dependent protein kinase A pathway antagonizes the cellular response to stress. It is shown here that the cellular content of Cdc25p, the upstream activator of Ras and adenylyl cyclase, decays upon various stresses such as heat shock and oxidative and ethanol shocks, whereas its phosphorylation level and its localization are unaffected. In parallel with the reduction of Cdc25p, the maximal capacity of the cell to accumulate cAMP decreases when its feedback regulation is abolished. A deletion of *CDC25* prevents this decrease. Paradoxically, in wild-type cells, with normal feedback regulation, the level of cAMP, which is much lower, is not reduced but is rather increased upon stress. These observations are consistent with a role of Cdc25p in sensing and transducing stress to downstream targets, either through a cAMP-independent pathway or by large fluctuations in the cAMP content of the cell.

INTRODUCTION

When exposed to sudden environmental changes, the yeast *Saccharomyces cerevisiae* exhibits not only specific adaptive responses but also more general responses which affect the cell cycle, the organization of the cytoskeleton and the expression of a large set of genes (Estruch, 2000). At the level of gene expression, the general stress response system involves two transcriptional activators, Msn2p and Msn4p, which act via stress response elements present in the promoters of stress-responsive genes named the Msn2/4p regulon (Martinez-Pastor *et al.*, 1996; Schmitt & McEntee, 1996). This regulon, which is induced upon various stress conditions as well as during the diauxic transition, has been characterized at the level of protein synthesis (Boy-Marcotte *et al.*, 1998, 1999) and mRNA accumulation (Causton *et al.*, 2001; Gasch *et al.*, 2000). Upon activation, Msn2p and Msn4p translocate to the nucleus (Gorner *et al.*, 1998, 2002) and become hyperphosphorylated (Garreau *et al.*, 2000). A fast rhythmic entry and exit of Msn2p and Msn4p to and from the nucleus is initiated upon stress (Jacquet *et al.*, 2003). These behaviours imply complex regulations which could involve phosphorylation/dephosphorylation processes (Jacquet *et al.*, 2003). The cAMP-protein kinase A (PKA) pathway plays a key role in controlling nucleocytoplasmic shuttling and activity of Msn2p and Msn4p. Unregulated high activity of this pathway prevents nuclear localization, change in phosphorylation state and shuttling, whereas a block or a partial defect in this pathway induces a permanent activation of

Msn2p. Despite a large amount of work devoted to the stress response, the question remains whether or not components of the cAMP pathway contribute to mediation of the cellular response to stress via the Msn2 and Msn4 transcriptional activators.

The cAMP-PKA signalling pathway is tightly regulated. The synthesis of cAMP by adenylyl cyclase requires the activation of at least one of the *RAS1* and *RAS2* gene products (Toda *et al.*, 1985). It is also positively modulated by Gpa2p and Gpr1p in response to glucose addition (Colombo *et al.*, 1998), and negatively regulated by Ira1p and Ira2p (Tanaka *et al.*, 1990). There are two phosphodiesterases of high- and low-affinity, Pde1p and Pde2p (Nikawa *et al.*, 1987a), able to reduce its level, but the most stringent regulation appears to be the feedback exerted by PKA activation upon cAMP accumulation (Nikawa *et al.*, 1987b). Different components of this pathway could be affected by stress and contribute to the control of the cellular response.

We have previously reported that Cdc25p, an upstream element of the cAMP-PKA pathway acting as a guanine nucleotide exchange factor (GEF) for Ras proteins, is able to interact with Ssa (Hsp70 family) and Hsp90 family chaperones (Geymonat *et al.*, 1998). Since these chaperones are recruited to unfold proteins upon stress, their interaction with Cdc25 might be affected. Indeed, it was shown that the amount of Cdc25p was reduced in cells depleted for Ssa chaperones (Geymonat *et al.*, 1998). Therefore, we have chosen to examine if Cdc25p is modified upon stress. We have monitored its cellular content, subcellular distribution and phosphorylation state in response to heat shock

Abbreviation: PKA, protein kinase A.

and other stresses. The results indicated that stress triggers a reduction in the amount of Cdc25p without any change in either phosphorylation state or localization. We have measured the capacity of cells to produce cAMP using mutants in which adenylyl cyclase activity is fully deregulated and we found that this capacity decreases in parallel to the reduction of Cdc25p upon stress. Paradoxically, the low basal level of cAMP measured in wild-type cells increases slightly rather than being reduced. We discuss some hypotheses that could account for such a paradoxical result.

METHODS

Yeast strains. The strains used in this study are listed in Table 1. OL605-2B was obtained from a cross between OL568-3B and S992. OL618-12B was obtained from a cross between RS13-7C-1 and OL566-7. The ORF of *TPK1* and *TPK2* were fully deleted in the diploid cells W303-1B (Rothstein, 1983) with the kanMX2 cassette (Wach *et al.*, 1994), giving rise to G418^R strains OL566-1 and OL566-2, respectively. OL597-1B is a sporulation product of OL566-2. OL566-7 was obtained by sporulation of a heterozygote made with progenies of OL566-1 and OL566-2. The ORF of *CDC25* was deleted and replaced by the *HIS3* gene in the diploid strain FY1679 (Thierry *et al.*, 1995), after insertion of the *RAS2*^{ile152} allele (Camonis & Jacquet, 1988) within the *RAS2* gene promoter using an insertion plasmid containing the *TRP1* gene; the haploid strain OL550-11B results from sporulation of this diploid strain. Strain OL642-13B was selected from a cross between OL597-1B and OL550-11B. Strain OL641-40B was recovered on the basis of its sensitivity to G418 to select for the *tpk2*^w allele and the pattern of

segregation for the other markers, a 2:2 segregation for the Trp⁺ phenotype being the indication of the cosegregation of *tpk3Δ* and *RAS2*^{ile152}.

Yeast growth and stress treatment. For heat shock and ethanol stress, the cells were grown in YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) to mid-exponential phase (OD₇₁₀ = 0.5–1). Heat-shock treatment involved transferring cells from a water bath at 25 °C to 38 °C for the indicated times. Ethanol stress was induced by adding 100 % ethanol to a final concentration of 7 % (v/v) to cells grown at 30 °C. For oxidative stress, early exponential phase cells (OD₇₁₀ = 0.2–0.3) grown at 30 °C in YPD were exposed to H₂O₂ to a final concentration of 0.4 mM.

Cdc25p isolation, immunoblotting and alkaline phosphatase treatment. Cdc25p was tagged at its own locus by three tandem HA epitopes as previously described (Geymonat *et al.* 1998). The tagged version of Cdc25p, Cdc25p-3HA, was fully functional. The Cdc25p protein was extracted as previously described (Garreau *et al.*, 2000). A solution of 1.85 M NaOH plus 1 % 2-mercaptoethanol were added to the cell culture at 1/10 the culture volume to break the cells. The proteins were precipitated by a 50 % TCA solution (5 % final concentration). The precipitated proteins from 4 × 10⁷ cells were dissolved in 500 µl modified Laemmli sample buffer.

Membrane association of Cdc25p was determined as previously described (Garreau *et al.*, 1996) with the following modifications: after centrifugation at 100 000 g for 30 min, the pellet was directly dissolved in Laemmli sample buffer instead of extracting Cdc25p from the membrane by a 2 mM EDTA solution (Gross *et al.*, 1992a, b).

Proteins were separated by 7.5 % SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes by a semi-dry method. Cdc25p-3HA was detected by rat monoclonal anti-HA antibody 3F10

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
W303-1B	<i>MATa/MATx ura3-1/ura3-1 leu2-3, 112/leu2-3, 112 his3-11, 15/his3-11, 15 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100</i>	Rothstein (1983)
FY1679	<i>MATa/MATx ura3-52/ura3-52, his3-Δ200:his3-Δ200, leu2-Δ1/leu2-Δ1, trp1-Δ63:trp1-Δ63</i>	Thierry <i>et al.</i> (1995)
S15-5B	<i>MATa his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2^{w2} tpk3::TRP1</i>	Nikawa <i>et al.</i> (1987b)
RS13-7C-1	<i>MATa his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2^{w2} tpk3::TRP1 bcy1::LEU2</i>	Nikawa <i>et al.</i> (1987b)
S18-1D	<i>MATx his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP</i>	Nikawa <i>et al.</i> (1987b)
RS13-58A-1	<i>MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2</i>	Nikawa <i>et al.</i> (1987b)
S992	<i>MATx ade2-1 ura3-1 his3-11, 15 leu2-3, 12 trp1-1 hsc82::LEU2 hsp82::LEU2 hsp82G170D::HIS3</i>	Nathan & Lindquist (1995)
OL568-3B	<i>MATx ade2-1 ura3-1 his3-11 leu2-3, 112, trp1-1 CDC25:3xHA</i>	Geymonat <i>et al.</i> (1998)
MGL20-4C	<i>MATa ura3 lys2 trp1 ssa1::HIS3 ssa2::LEU2 CDC25:3xHA</i>	Geymonat <i>et al.</i> (1998)
OL566-1	<i>MATa/MATx ade2/ade2 ura3/ura3 leu2/leu2 trp1/trp1 TPK1/tpk1Δ::G418(KanMX2)</i>	This work
OL566-2	<i>MATa/MATx ade2/ade2 ura3/ura3 leu2/leu2 trp1/trp1 TPK2/tpk2Δ::G418^R(KanMX2)</i>	This work
OL566-7	<i>MATx his3 leu2 ura3 trp1 ade2 tpk1::G418 tpk2::G418</i>	This work
OL605-2B	<i>ade2-1 ura3-1 his 3-11, 15 leu2-3, 12 trp1-1 hsc82::LEU2 hsp82::LEU2 hsp82 G170D::HIS3 CDC25:3xHA</i>	This work
OL618-12B	<i>MATx his3 ura3 trp1 ade8 tpk1::G418 tpk2^{w2} tpk3::TRP1 bcy1::LEU2 CDC25:3xHA</i>	This work
OL550-11B	<i>MATa ura3-52, his3Δ, leu2Δ trp1Δ cdc25Δ::HIS3 RAS2::RAS2^{ile152}:TRP1</i>	Jacquet <i>et al.</i> (2003)
OL597-1B	<i>MATx ade2 ura3 leu2 trp1 tpk2Δ::G418^R</i>	This work
OL642-13B	<i>MATa ura3-52, his3Δ, leu2Δ trp1Δ tpk2Δ::G418 cdc25Δ::HIS3 RAS2::RAS2ile152:TRP1</i>	This work
OL641-40B	<i>MATa ura3-52, his3Δ, leu2Δ trp1Δ ade8 tpk1::URA3 tpk2^{w2} tpk3::TRP1 cdc25Δ::HIS3 RAS2::RAS2^{ile152}:TRP1</i>	This work

(Roche Molecular Biochemicals) at a dilution of 1:1000, and anti-rat IgG antibody (alkaline phosphatase conjugate). The band of Cdc25p-3HA was measured by densitometry using Gelscan software (Yvan Zivanovic, Université Paris-Sud).

Alkaline phosphatase treatment was performed as previously described (Garreau *et al.*, 2000) Laemmli extracts (50 μ l) were diluted 10-fold in alkaline phosphatase buffer (100 mM Tris/HCl, pH 8.5, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$), concentrated again to the initial volume by ultrafiltration, and incubated in the presence of 3 μ l calf intestinal alkaline phosphatase (20 U μ l⁻¹; Roche Molecular Biochemicals) for 2 h at 37 °C. The control was incubated under the same conditions without alkaline phosphatase.

RT-PCR measurement of CDC25 mRNA. Total RNA was extracted as described previously (Geymonat *et al.*, 1998), and 5 μ g was used in reverse transcription performed with the Superscript II kit (Gibco-BRL). Specific primers for *CDC25* and *ACT1* were used for PCR amplification with 6 ng cDNA as the template. The PCR product was labelled by [³²P]-dCTP in a 50 μ l reaction volume. As an internal control, *ACT1* primers were added to the *CDC25* PCR reaction after the first 10 cycles and then PCR was continued for another 20 cycles. The [³²P]-labelled PCR products were separated by 6% PAGE. The signal was quantified by a Molecular Dynamics PhosphorImager equipped with ImageQuant software.

cAMP assay. Cell extracts were prepared as previously described by Thevelein *et al.* (1987) with the following modifications: cells were harvested by filtration and cAMP was extracted with 1 ml 15% perchloric acid with three freeze-thaw cycles in liquid nitrogen. After centrifugation, the supernatant was neutralized by 1.5 M potassium carbonate/1 M acetic acid buffer. Potassium perchloride was removed by centrifugation and the supernatant was subjected to the cAMP assay by using the correlate-EIA cyclic AMP kit (Assay Designs, non-acetylated method). The concentration of cAMP was calculated with the Origin 5.0 logistic curve-fitting program (Microsoft).

RESULTS

The cellular content of Cdc25p decreases in response to heat shock

We previously showed that Cdc25p interacts with the Hsp70 family chaperones and that the cellular content of Cdc25p is reduced in a *ssa1Δ*, *ssa2Δ* (encoding members of the Hsp70 family) double-deletion strain (Geymonat *et al.*,

1998). These results raise the possibility that the amount of Cdc25p could also be reduced upon stress, when these chaperones are recruited by unfolded proteins. To address this question, we monitored Cdc25p cellular content and characteristics by immunoblotting. Since Cdc25p is difficult to detect, we constructed a strain containing a HA epitope-tagged copy reinserted at its own locus such that the transcription of *CDC25-3HA* remained under control of the endogenous *CDC25* promoter. The resulting strain, OL568-3B, exhibits the same growth rate at 25, 30 and 37 °C, the same sensitivity to high temperature (50 °C) and the same level of induced thermotolerance as the wild-type strain (data not shown). These results indicate that the properties of tagged Cdc25p were similar to those of the untagged protein, since these criteria are very sensitive to variations in the cAMP-PKA pathway activity.

When cells were transferred from 25 to 38 °C, the amount of Cdc25p, as measured by immunodetection with anti-HA antibodies, decreased significantly and reproducibly within 120 min (Fig. 1a, b). In contrast, the level of *CDC25* mRNA, measured by quantitative RT-PCR, remained stable upon heat shock (Fig. 1c, d). This is consistent with published microarray data that show the *CDC25* mRNA level remains stable under various stress conditions including heat, oxidative, osmotic and acid shock (Causton *et al.*, 2001; Gasch *et al.*, 2000; Jelinsky & Samson, 1999). Therefore, the reduction at the protein level observed in Fig. 1 is due to a post-transcriptional modulation resulting from either reduction of its synthesis or enhanced degradation.

It has been reported that the addition of glucose to glucose-starved cells induces the phosphorylation of Cdc25p and changes its localization from membrane to the cytoplasm (Gross *et al.*, 1992a). In order to determine whether Cdc25p is regulated in the same way in response to heat shock, we checked its phosphorylation state and localization. Under normal growth conditions, Cdc25p migrated as quite a broad, diffuse band in SDS-PAGE, suggesting several poorly resolved species. When the samples were treated with alkaline phosphatase, the Cdc25p band was sharpened and

Fig. 1. Effects of heat shock on the expression of Cdc25p. Wild-type strain OL568-3B (*CDC25:3xHA*) was grown on YPD medium at 25 °C to exponential phase ($OD_{710}=1.0$) and treated at 38 °C for 30, 60 and 120 min. (a) Decrease in Cdc25p quantity, 20 and 40 μ g of crude protein extracts were assessed by Western blotting with the anti-HA 3F10 antibody. Bulk protein was stained by Coomassie blue as a loading control. (b) Quantification of the results. (c) RT-PCR of *CDC25* and *ACT1* mRNA. (d) Quantification of the results, ratio *CDC25:ACT1* (arbitrary values).

Fig. 2. Subcellular distribution and phosphorylation of Cdc25p. Protein extracts from wild-type strain OL568-3B under heat shock were fractionated by centrifugation. Soluble protein (90 µg) and insoluble protein (70 µg) were separated by SDS-PAGE. (a) Western blotting, (b) quantification (arbitrary values). (c) Sample from insoluble fraction, prepared from cells after heat shock at 38 °C for 30 min, was treated with 60 U alkaline phosphatase.

migrated faster (Fig. 2c). These results confirm that Cdc25p is phosphorylated *in vivo*. Moreover, they indicate the existence of several phosphorylated forms. After heat shock, we did not detect any change in migration of the immunoreactive Cdc25p (Fig. 1a) despite various attempts to improve gel resolution by changing the acrylamide/bisacrylamide ratio. We have also investigated whether there is a change in the subcellular distribution of Cdc25p. The protein extract was fractionated into soluble and insoluble fractions at 100 000 g, but no change in Cdc25p distribution was detected after heat shock and the amount of Cdc25p decreased with similar rates in both fractions (Fig. 2a, b).

Taken together, these results led us to conclude that the major effect of stress is to reduce the cellular amount of Cdc25p, while no significant change in either its subcellular distribution or phosphorylation level was detected.

The level of Cdc25p also decreases under oxidative and ethanol stress conditions

To discover whether the reduction of the Cdc25p content of the cell is specific to heat shock or corresponds to a more general stress-related phenomenon we examined the fate of Cdc25p under other classical stress conditions. First, we examined the response to oxidative stress caused by 0.4 mM H₂O₂. In wild-type cells, oxidative stress induced a

more rapid and transient arrest in cell division than that observed under heat-shock conditions. However, Cdc25p showed similar kinetics of decrease as observed in heat-shocked cells (Fig. 3). We also extended this test to a stress induced by 7 % ethanol, which causes cells to stop growing almost immediately. As shown in Fig. 3, ethanol stress also caused the content of Cdc25p to decrease. These results show that the reduction of the cellular content of Cdc25p is a more general response of the cells to stress.

The quantity of Cdc25p is affected by chaperones

We have previously reported that the level of Cdc25p is reduced in the *ssa1Δ*, *ssa2Δ* double-deletion strain (Geymonat *et al.*, 1998). Preliminary results were also suggesting a role of the chaperones of the HSP90 family. We reinvestigated this point by following the stability of Cdc25p in a strain deleted for *HSC82* (encoding hsp90) and carrying a thermosensitive allele for *HSP82* (*hsp82Δ*, *hsc82Δ* double-deletion is lethal). As shown in Fig. 4, at 25 °C, the amount of Cdc25p in these two strains (*ssa1Δ*, *ssa2Δ*) and (*hsc82Δ*, *hsp82ts*) was lower than that in wild-type cells. In response to heat shock, the Cdc25p level in these mutants was further reduced. These results reinforce the possibility that both Hsp70 and Hsp90 play a role in the regulation of the cellular content of Cdc25p.

Fig. 3. Variation of Cdc25p amount in response to hydrogen peroxide and ethanol stress. OL568-3B was grown in YPD medium at 30 °C. Cells in early exponential phase (OD₇₁₀=0.2) were treated with 0.4 mM H₂O₂ (a, b) and with 7 % ethanol (OD₇₁₀=1.0) (c, d). (a, c) Western blotting, (b, d) quantification of the results (arbitrary values).

Fig. 4. Cdc25p content in strains depleted for Ssa and hsp90 chaperones. The strains OL568-3B, MGL20.4C (*ssa1Δ*, *ssa2Δ*) and OL605-2B (*hsc82Δ*, *hsp82Δ*, *hsp82^{G170D}*) were grown and treated as described in Fig. 1. Protein extracts (30 μ g) were separated and tested. (a) Western blotting, (b) quantification of the results (arbitrary values).

The intracellular cAMP level is reduced in *tpk^w* strains under stress conditions

If modulation of the amount of Cdc25p controls the cAMP-PKA pathway, it is expected that upon heat shock

or other stress the production of cAMP would decrease. Unexpectedly, monitoring the intracellular cAMP level during 1 h heat shock showed a two- to threefold increase rather than a decrease (Fig. 5a). Such a result has been previously observed in our laboratory in strain OL3 after a shift from 24 to 36 °C (Camonis *et al.*, 1986).

Since the low level of cAMP measured in the cell population results from a very strong feedback inhibition known to regulate the production of cAMP, we wondered if a possible decrease in cAMP production could be masked by this feedback mechanism. We thus have used *tpk^w* strains with attenuated PKA activity, which have lost the feedback control, to directly assess the capacity of adenylyl cyclase to produce cAMP. In these strains, where two of the three catalytic subunits of PKA are deleted, the third subunit is mutated (*tpk^w*) such that its activity is drastically reduced, leading to loss of negative feedback control on the activity of adenylyl cyclase (Nikawa *et al.*, 1987b). In strain S18-1D (*tpk1^{w1}*, *tpk2Δ*, *tpk3Δ*), the cAMP level was several hundred-fold higher than that in the wild-type (Fig. 5b), as previously reported by Nikawa *et al.* (1987b). Heat shock caused a reproducible decrease in the cAMP level in this strain (Fig. 5b). After 1 h of treatment, the cAMP level was reduced to 30 % of the unstressed level. As also shown in Fig. 5(b), a similar decrease was observed with OL618-12B, a *tpk2^w* strain also deleted for *bcy1*, the regulatory subunit for PKA. This phenomenon was reproduced in other *tpk^w* strains [RS13-58A-1, S15-5B (*tpk1Δ*, *tpk2^{w2}*, *tpk3Δ*) and RS13-7C-1 (*tpk1Δ*, *tpk2^{w2}*, *tpk3Δ*, *bcy1Δ*) (data not shown)]. These results clearly indicate that the capacity of the cell to accumulate cAMP in response to stress decreases steadily after heat shock when the feedback inhibition is abolished.

Fig. 5. cAMP level changes in response to stress. cAMP was measured at different times after applying heat shock (26 °C to 38 °C), 10 mM H₂O₂ or 7 % ethanol (v/v) to exponentially growing cells. (a) cAMP level in wild-type strain OL568-3B. (b) cAMP level in cells without feedback inhibition of adenylyl cyclase, OL618-12B (*tpk1Δ*, *tpk2^{w2}*, *tpk3Δ*, *bcy1Δ*) (Δ); S18-1D (*tpk1^{w1}*, *tpk2Δ*, *tpk3Δ*) ($\blacktriangle$).

We extended this observation to other stresses such as oxidative and ethanol shock in the *tpk2^w* strain (OL618-12B). Oxidative stress induced a decrease in cAMP production in the *tpk2^w* strain which was similar to that observed under heat shock (Fig. 5b). The addition of 7% ethanol, which appears to have a stronger effect on the cell, also leads to a cAMP reduction. Similar to the cells in response to heat shock, the amount of cAMP in the isogenic wild-type strain was much lower and exhibited a small increase upon oxidative and ethanol stress (Fig. 5a). Here again, when the feedback regulation was not operating, the reduction of cAMP accumulation was not limited to heat shock, but was also observed in different types of stress.

Cdc25p is a potential signal transducer in response to stress

To verify that the decrease of cAMP in *tpk2^w* strains correlates with a decrease of Cdc25p in this genetic background, we constructed a *tpk^w* strain in which *CDC25* was tagged with the HA epitope. The Cdc25p amount was reduced in this strain after heat shock with similar kinetics to those of a wild-type strain (Fig. 6). The Cdc25p decrease parallels the reduction of the cAMP level found in a similar genetic background (compare Fig. 6 and Fig. 5b).

We have further established the involvement of Cdc25p in the decay of cAMP accumulated in *tpk^w* strains, by the use of cells lacking Cdc25p. In order to perform this

experiment, we have constructed a strain with a complete deletion of *CDC25* in a *tpk2^w* genetic background. We have used the dominant allele *RAS2^{ile152}* that bypasses the need for a GEF to activate adenylyl cyclase in absence of Cdc25p. The *RAS2^{ile152}* allele encodes a protein that displays a lower affinity for guanine nucleotides, thus allowing spontaneous exchange (Camonis & Jacquet, 1988). The absence of feedback control in this strain leads to a high level of cAMP. As illustrated in Fig. 7, the transfer from 26 to 38 °C did not produce any decrease in cAMP; in fact the opposite occurred, the level of cAMP appears to increase upon increasing temperature. Such an increase, which was reproducible, indicates that other components of the cAMP pathway are sensitive to the rise in temperature.

DISCUSSION

The cAMP-PKA pathway antagonizes the cellular response to stress mediated by the transcriptional regulators Msn2 and Msn4 (Boy-Marcotte *et al.*, 1998, 1999; Gorner *et al.*, 1998, 2002; Estruch, 2000). The contribution of components of this pathway to the transmission of the stress signal has not yet been documented. In a previous report, we have shown the existence of physical and functional interactions between Cdc25p and chaperones of the SSA gene family, suggesting that Cdc25p could be modulated by these chaperones in response to stress. Here we show that the cellular level of Cdc25p is reduced when the cell is under various stresses such as ethanol, oxidative and heat shock.

The decrease of Cdc25p after these stresses is not due to transcriptional repression, as can be deduced from many published microarray data (Causton *et al.*, 2001; Gasch *et al.*, 2000; Jelinsky & Samson, 1999) and confirmed by

Fig. 6. Decreased amount of Cdc25p in the *tpk^w* strain during heat shock. OL618-12B (*tpk1Δ*, *tpk2^{w2}*, *tpk3Δ*, *bcy1Δ*) was grown and treated as described in Fig. 1. Protein extracts (30 and 60 µg) were separated and tested. (a) Western blotting, (b) quantification of the results (arbitrary values).

Fig. 7. Change of cAMP level in a *cdc25Δ*, *RAS2^{ile152}*, *tpk^w* strain in response to heat shock. Strain OL641-40B (*cdc25Δ*, *RAS2^{ile152}*, *tpk^w*) was grown to early exponential phase at 26 °C (OD=0.2) and transferred to 38 °C. Samples were taken at indicated times and cAMP measured. Standard deviation is given for 2–4 independent measurements at each time.

us, using PCR in the strain where Cdc25p decreases. The depletion of Cdc25p in *S. cerevisiae* could then be controlled either by arrest of its production, since it is an unstable protein that decays rapidly (Kaplon & Jacquet, 1995), or by increased degradation. Regulation of the cellular content of the protein has also been reported for a mammalian homologue of Cdc25p, the Ras-GRF2 which contains a CDB motif, also present in Cdc25p, and is controlled by ubiquitination and degradation via the proteasome (de Hoog *et al.*, 2001).

Although we have not addressed the mechanism of depletion of Cdc25p in this report, we have reinforced our previous evidence for the involvement of chaperones in the maintenance of the cellular content of Cdc25p. Indeed, the interaction of Cdc25p with Ssa and Hsp90 chaperones could explain the change in cellular levels following stress, as previously proposed (Geymonat *et al.*, 1998). The unfolding of a large number of proteins upon stress is commonly supposed to recruit these chaperones, which are no longer able to bind their usual partners. Cdc25p could then be destabilized or less accumulated when the availability of these chaperones is reduced by stress. Our finding that the rate of decay of Cdc25p is similar for protein found in both soluble and membrane fractions implies a similar sensitivity to stress or a fast shuttling between both fractions. Our analysis also demonstrated that heat shock did not change the cellular distribution of Cdc25p nor its phosphorylation state.

Cdc25p is present in a limiting amount in the cell. It is known that it could be fully inactivated by sequestration with the product of the dominant negative RAS2^{ala22} mutant, leading to growth arrest (Powers *et al.*, 1984). Its average number has recently been estimated to be approximately 319 per cell compared to the 19 800 molecules of Ras2p (Ghaemmaghami *et al.*, 2003). Therefore, even a small reduction in its level is expected to affect the activity of Ras, and consequently adenylyl cyclase activity. The abrogation of the strong feedback applied on the production of cAMP allows us to show that the accumulation of cAMP is reduced upon stress and correlates with the reduction of Cdc25p. The reduction of the level of accumulated cAMP upon heat shock does not occur when CDC25 is deleted and adenylyl cyclase is activated by the product of RAS2^{ile152}, an allele of Ras2p encoding a constitutively activated protein. This result implies that no other component of the cAMP pathway such as the adenylyl cyclase, Gpr1p, Gpa2p, Ira1p, Ira2p, Pde1p or Pde2p significantly contribute to the reduction of cAMP after heat shock. In contrast, the continuous increase of cAMP observed in this strain suggests that one or several of these components reacts in the opposite way, illustrating the complex dynamic of this system. These results led us to conclude that Cdc25p, which is sensitive to stress, has the potential to contribute to the mediation of stress signals within the cell.

The complexity of the regulation of the cAMP-PKA pathway

was further emphasized by the paradoxical result that the level of cAMP measured in wild-type cells is much lower and increases upon stress. This appears to be in contradiction with the activation of the stress response transcription factors Msn2p and Msn4p, and leads us to propose the existence of more sophisticated regulation than a simple linear pathway. It could be due to an additional regulation acting directly on PKA, as suggested by Thevelein & de Winder (1999). However, it cannot be excluded that Cdc25p, activator of Ras proteins, also controls another Ras-dependent pathway. Supporting this hypothesis, several reports have shown an involvement of Ras in the control of the MAP kinase pathway leading to invasive growth (Cherkasova *et al.*, 2003; Cook *et al.*, 1997). This pathway could converge with the cAMP-PKA pathway upon Msn2p and Msn4p, which are also involved in the control of invasive and filamentous growth (Ho & Bretscher, 2001). In this hypothesis, the reduction of adenylyl cyclase activation would be compensated by the feedback inhibition mechanism and thus remain unnoticed, whereas the other pathway could be more severely affected.

A second, more speculative, hypothesis deserves attention because it stresses the potential of a non-linear phenomenon occurring within cells. It is based upon the consideration that the level of cAMP measured within the cell population does not reflect the real concentration within individual cells but is the average of large fluctuations. The value of cAMP measured in wild-type cells corresponds to a cellular concentration of $\sim 0.5\text{--}2\text{ }\mu\text{M}$. Meanwhile, the K_d for the PKA regulatory subunit is $10\text{ }\mu\text{M}$. Thus if the cAMP content were homogeneous, PKA would never reach its full activity and the ability of adenylyl cyclase to produce several hundred-fold more cAMP would be useless for the cell. Therefore, we think that this value is the average of large variations. The strong feedback loop provides a good device for large amplitude fluctuations. Variations in cAMP level are known to occur upon glucose addition (Thevelein & Beullens, 1985), and have been reported during metabolic oscillations of cellular populations (Satroutdinov *et al.*, 1992; Muller *et al.* 2003). Oscillations in cAMP production might also be involved in the oscillatory shuttling of Msn2p in and out the nucleus (Jacquet *et al.*, 2003). Such oscillations in cAMP accumulation, especially if their amplitude is reduced while their frequency is increased, could explain how the average measured value increases whereas the maximal value decreases. In this case, if the downstream target needs a high threshold of cAMP to be inactivated, then it will respond to the diminution of the amplitude rather than to the average value.

Altogether, the results presented in this paper reveal a new aspect of the stress response in yeast. They show that the cellular level of Cdc25p and the maximal capacity to produce cAMP are reduced. They present a paradoxical result which points to more complex regulation of the signalling pathways in response to stress than previously thought.

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