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Ssa1p chaperone interacts with the guanine nucleotide exchange factor of ras Cdc25p and controls the cAMP pathway in *Saccharomyces cerevisiae*

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Summary

We have found that the guanine nucleotide exchange factor for ras, Cdc25p, interacts with Ssa1p in *Saccharomyces cerevisiae*. This interaction was observed with GST-fused Cdc25p polypeptides and confirmed by co-immunoprecipitation with the endogenous Cdc25p. Hsp82 appeared also to be co-immunoprecipitated with Cdc25p, albeit to a lower level than Hsp70. In a strain deleted for *SSA1* and *SSA2*, we observed a reduced cellular content of Cdc25p. Consistent with a reduced activity of the cAMP-dependent PKA pathway, the rate of accumulation of both trehalose and glycogen was stimulated in the *ssa*-deleted strain. Expression of *SSA1* reversed these effects, whereas co-expression of *SSA1* and *PDE2* restored high accumulation. The expression of genes repressed by cAMP, *GAC1* and *TPS1*, fused to β-galactosidase, was also stimulated by deletion of *SSA* genes. The effect of *ssa* deletion on glycogen accumulation was lost in a strain deleted for *CDC25* rescued by the *RAS2^{ile152}* allele. Altogether, these results lead to the conclusion that Ssa1p positively controls the cAMP pathway through Cdc25p. We propose that this connection plays a critical role in the adaptation of cells to stress conditions.

Introduction

Ras proteins are molecular switches involved in signal transduction pathways. They are activated by guanine nucleotide exchange factors (GEF) that transduce various signals. In cells of higher eukaryotes, tyrosine kinase receptors, when phosphorylated in response to extracellular signals, recruit the GEF of the SOS family linked to Grb2, or grb2-like, adapters (Schlessinger, 1994). In unicellular lower eukaryotes such as yeast, in the absence of tyrosine kinase receptors and grb2-like adapters, the mode of control of GEF is poorly understood. In *Saccharomyces cerevisiae*, Cdc25p, the GEF for ras proteins, is essential for cAMP production and PKA activation. As it is an unstable protein containing a cyclin destruction box (Kaplon and Jacquet, 1995), its cellular content is likely to be a limiting factor potentially used for its regulation. This protein is tightly associated with the membrane (Garreau *et al*., 1996), and the control of the ras pathway by its recruitment to the membrane (Gross *et al*., 1992a) remains to be further substantiated. This protein belongs to a multimolecular complex, it interacts with itself and potentially Sdc25p *in vitro* and forms a dimer when expressed in *Escherichia coli* (Camus *et al*., 1997) and it contains an SH3 domain that has been reported to interact with the adenylate cyclase (Freeman *et al*., 1996). Therefore, the assembly process of a multimolecular structure at the membrane level could also be part of a control of ras by its GEF.

In the search for interacting proteins expected to be involved in the control of the Cdc25p function, we have found Ssa1p, a chaperone of the HSP70 superfamily. The *SSA* subfamily, is composed in yeast of four members *(SSA1–4)*. During growth, only *SSA1* and *SSA2* are expressed at high level, whereas *SSA3 and SSA4* are not. After heat shock or during a stress response, Ssa1p is overexpressed and *SSA3* and *4* are induced (Werner-Washburne *et al*., 1987). These chaperones have multiple effects and control different functions in the cell. Ssa1/2p have been shown, in addition to their role in protein translocation *in vivo* (for review see Craig *et al*., 1994), to participate *in vitro* in the prevention of protein aggregation and in the refolding of denatured proteins (Bush and Meyer, 1996). Hsp70 are often found in association with Hsp90 chaperones that are encoded in yeast by two genes, *HSP82* and *HSC82* (Chang and Lindquist, 1994).

In this report we describe the identification of Ssa1p as a protein tightly bound to Cdc25p. We present evidence for a role of Ssa in the control of the Cdc25–Ras–cAMP pathway. We then conclude with the proposal that, in response to stress, the recruitment of chaperones to unfolded proteins

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reduces the amount of functional Cdc25p and then the activity of the PKA pathway.

Results

Ssa1p *co-purifies with a GST–Cdc25 fusion protein*

We have previously demonstrated that a Cdc25p region of about 100 aa located in the C-terminal part of the protein is responsible for the membrane localization of Cdc25p (Garreau *et al*., 1996). To search for protein(s) that could interact with this region, we have constructed a plasmid in which the coding sequence of the C-terminal fragment of Cdc25p (from amino acids 1441–1589) has been fused to that of GST. The fused gene expressed in yeast produces the GST–Cdc25Cter chimera with the predicted molecular weight. It was localized in the same insoluble fraction as the complete Cdc25p or the C-terminal domain alone. The GST–Cdc25Cter chimera was solubilized by 2 mM EDTA pH 12, which is the only relatively mild treatment able to solubilize Cdc25p (Gross *et al*., 1992b). Indeed this treatment allows the detection of Cdc25–Ras interaction (Gross *et al*., 1992a) and Cdc25p dimerization (Camus *et al*., 1997). As shown in Fig. 1A a major band of 70 kDa was co-purified with the GST–Cdc25Cter chimera but not with the GST alone used as a control. Although the relative amount of this protein was lower than that of the GST chimera, as judged from staining, this ratio was highly reproducible, indicating a specific interaction. Furthermore, treatments with ATP (up to 400 mM) or high salt concentration (up to 1 M KCl) were not able to release the 70 kDa associated protein (data not shown).

The eluted 70 kDa material was analysed by microsequencing of peptides produced with endoprotease Lys C (Boehringer). Although the different members of the Hsp70 family in yeast, namely Ssa1p, Ssa2p, Ssa3p and Ssa4p, present strong sequence similarities, the sequences of five peptides obtained in stoichiometric amounts correspond to the product of *SSA1* (Fig. 1B). In addition the N-terminus of the 70 kDa protein is blocked as reported for the Ssa subfamily of Hsp70 proteins in yeast.

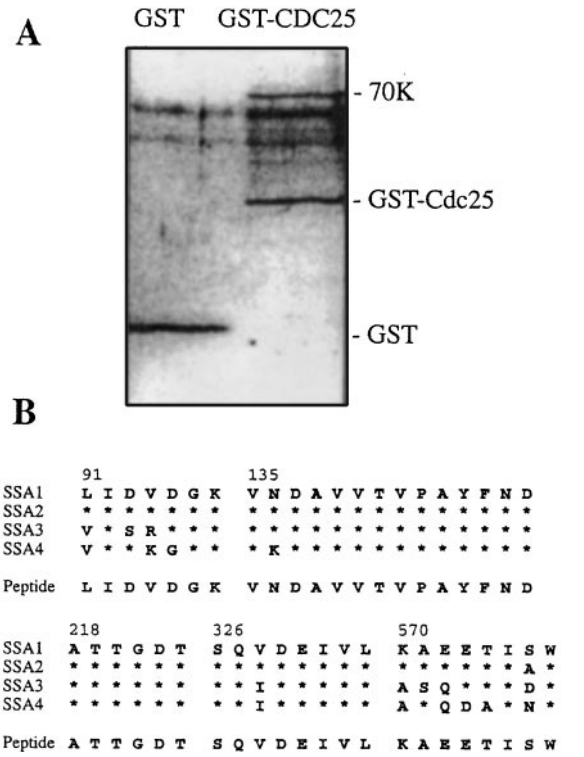

Fig. 1. Ssa1p associates with a Cdc25p C-terminal fragment fused with GST.
A. C13ABYS86 yeast cells transformed with pEG(KG) (GST alone) or pEG-FS4 (GST–Cter–Cdc25 fusion) were grown in a galactose-based medium. Glutathione–agarose-bound proteins derived from cells harbouring pEG(KG) (GST) and pEG-FS4 (GST–Cter–Cdc25 fusion) were eluted, subjected to SDS–PAGE and stained with Coomassie blue. The 70 kDa specifically co-purified polypeptide is indicated as well as GST and GST-Cdc25 polypeptides.
B. The sequences of the analysed peptides are compared with the sequences of the corresponding regions of Ssa1p, Ssa2p, Ssa3p and Ssa4p. Asterisks represent conserved amino acids (SWISSPROT references: P10591, P10592, P09435, P22202).

Hsp70 and possibly Hsp82 are co-immunoprecipitated with endogenous Cdc25p

To characterize further the interaction between Cdc25p and Ssa1p, we have performed immunoprecipitation experiments. In a first experiment we have used epitope-tagged (HA) full-length Cdc25 protein, tagged at the C-terminus (pTK3). To assess the presence of Ssa1p in the immunoprecipitate, we have taken advantage of the polyclonal antibody Ab119 raised against the last 13 aa of the human Hsp90β (Lees-Miller and Anderson, 1989), which recognizes α- and β-Hsp90 vertebrate forms and cross-reacts with vertebrate Hsp70 (Jerome *et al*., 1993). Figure 2A shows that Ab119 recognizes the co-purified Ssa1p previously characterized (lanes a and b) as well as the yeast Hsp82 (lanes c and d). The recognition of the Hsp82 was confirmed by the use of a strain deleted for *HSP82* and *HSC82* complemented either by the yeast *HSP82* (lane c) or by chicken HSP90α (lane d). The difference in migration of the immunodetected bands corresponds to the difference between the sizes of chicken HSP90α and yeast Hsp82p. The recognition of Hsp70p and Hsp82 in yeast is likely to be the result of the conservation of the last four C-terminal aa between Hsp90 family and Hsp70 of the Ssa subfamily. In Fig. 2B the epitope-tagged Cdc25p is co-immunoprecipitated with Hsp70. The interaction of the endogenous Cdc25p with Hsp was also tested by immunoprecipitation using the polyclonal antibody A323 directed against the C-terminal portion of Cdc25p (Garreau *et al*., 1996). As shown in Fig. 2C, Hsp70 was also

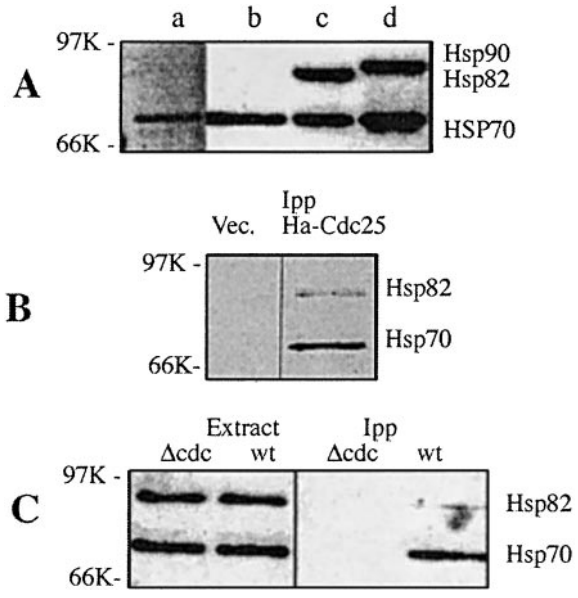

Fig. 2. Co-immunoprecipitation of Cdc25p and chaperones.
A. Specificity of the polyclonal antibody Ab119. Western blot analysis of proteins extracted from C13ABYS86 transformed with pEG-FS4 (GST–Cter–Cdc25 fusion) bound to gluthatione–agarose beads (lanes a and b) and of membrane proteins extracted from ΔECU82a (*hsp82*, *hsc82*) transformed either with pKAT6, expressing the yeast *HSP82* (lane c), or with pTGpd90α, expressing the chicken HSP90α (lane d). Lane a was stained with Ponceau S, and lanes b, c and d were labelled with Ab119.
B. Immunoprecipitation of a Cdc25-tagged protein. Membrane proteins immunoprecipitated with 12CA5 from C13ABYS86 transformed with the empty vector (vec) or expressing the complete tagged Cdc25p in pKT3 (Ha-Cdc25) were blotted onto nitrocellulose and labelled with Ab119.
C. Immunoprecipitation of the endogenous Cdc25p. Crude membrane extract (Extract) or proteins immunoprecipitated with A323 (anti-Cdc25p) (Ipp) from OL550–11A (Δcdc) or from C13ABYS86 (wt) were blotted onto nitrocellulose and labelled with Ab119. Size markers are on the left.

co-immunoprecipitated from a membrane extract of a *CDC25* wild type but not from a strain deleted for *CDC25* rescued by *RAS2*^{Ile152}, a *CDC25*-independent ras allele (Camonis and Jacquet, 1988), although Hsp70 and Hsp82 were present at the same level in the two strains (Fig. 2C, left). It is interesting to note that a small amount of Hsp82 was co-immunoprecipitated together with the Cdc25–Ssa1p complex in the case of both the HA-tagged version and the endogenous Cdc25p. This result was reproducible and could suggest the existence of a complex with a member of the Hsp82 family.

To determine whether the interaction between Cdc25p and the Hsp70 is specific for Ssa1p or can also occur with the related Hsp70 encoded by *SSA2*, *SSA3* and *SSA4*, similar immunoprecipitation experiments were performed in strains deleted for *ssa1*, and for *ssa1* and *ssa2*, both of them transformed with pTK3. In Fig. 3, it can be seen that Ab119 recognizes a 70 kDa protein in both strains. This protein could be Ssa2p in the *Δssa1* strain because *SSA3* and *SSA4* are not expressed at 26°C in this strain and Ssa3/4p in the *Δssa1*, *Δssa2* mutant, because Ssa3/4p migrate at a slightly slower rate than Ssa1/2p in SDS–PAGE and are overexpressed in this strain (see Fig. 2) (Werner-Washburne *et al*., 1987). From this result it can be concluded that the interaction between Cdc25p and Ssa1p can be extended to Ssa2p, Ssa3p and/or Ssa4p, at least in the absence of Ssa1p.

The relative abundance of Cdc25p is reduced in a Δssa1, Δssa2 *mutant*

The biochemical function of chaperones is thought to assist protein folding, thus it can be hypothesized that the interaction with Cdc25p plays a role in the correct folding of this protein or in its assembly with membrane or other protein components. If this is the case, a defect in chaperones could lead to a lower amount of functional Cdc25p. To test this hypothesis, we have compared the amount of Cdc25p between a *Δssa1*, *Δssa2* mutant and the isogenic strain complemented with *SSA1*. As the amount of Cdc25p is very low and barely detectable with Cdc25 antibodies, we have replaced the *CDC25* gene at its locus with a three HA epitope tagged version. The tagged Cdc25p (T-Cdc25p) is functional, allowing normal growth. As seen in Fig. 4, T-Cdc25p migrates in gel electrophoresis at the expected position from its size. In contrast to other cellular proteins, the amount of T-Cdc25p found in the *Δssa1*, *Δssa2* mutant (V) was lower than in the *SSA1* complemented strain (SSA1). To establish further that the reduction of Cdc25p was due to an effect at the protein level rather than on its expression, we have measured the relative amount of mRNA in the two strains. We have used a quantitative RT-PCR assay using *GPH1* and *BCY1* as internal controls. From the experiment illustrated in Fig. 4B, no significant variation was observed at the mRNA level contrasting with the reduction seen at the protein level in the absence of Ssa1p. Therefore the limitation of Hsp70 because of the double deletion, although partially compensated by overexpression of *SSA3* and *SSA4*, reduces the cellular content of Cdc25p but not of its mRNA relative to other cellular proteins.

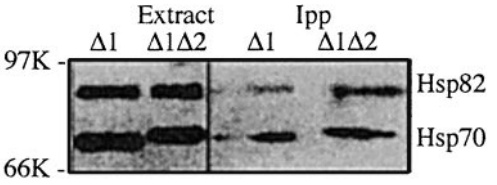

Fig. 3. Cdc25p also interacts with Ssa2p and Ssa3/4p. Crude membrane extract (Extract) or proteins immunoprecipitated with 12CA5 (Ipp) from T141 lacking *SSA1*(*Δ1*) or from OL565 lacking *SSA1* and *SSA2*(*Δ1*,*Δ2*) transformed with pTK3 were blotted onto nitrocellulose and labelled with Ab119. Size markers are on the left.

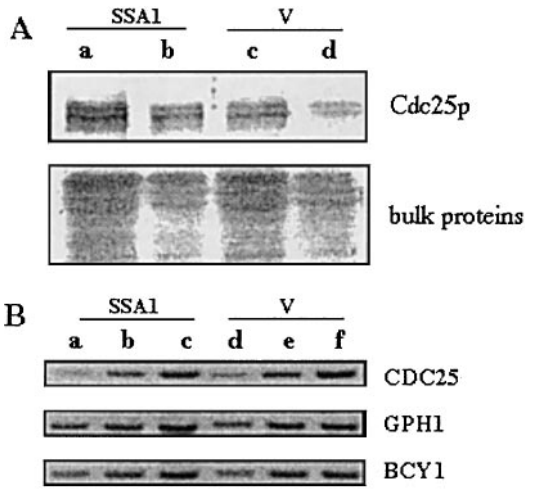

Fig. 4. Relative abundance of the Cdc25 protein and its mRNA in *ssa1* and *ssa2* mutant. The *ssa1*, *ssa2*, mutant strain (-4C) transformed either by the control plasmid pRS314 (V) or by pGRSSSA1 (SSA1) was grown at 30°C in minimal medium and harvested in log growth phase ($OD_{700} = 0.5$) for proteins and RNA measurements.
A. Protein content. A 70 μg sample (a and c) and 35 μg sample (b and d) of total proteins from each culture were separated on 7.5% acrylamide gels as previously described (Garreau *et al.*, 1996). After transfer, the upper part of the nitrocellulose sheet was processed for immunostaining of T-Cdc25p, the lower part was stained with Ponceau S. Densitometric analysis showed a threefold reduction in Cdc25p content in the *ssa1*, *ssa2* mutant, when compared with the *SSA1*-complemented strain.
B. mRNA content. A 5 μg sample of total RNA from each culture (SSA1, and V) was used in reverse transcription performed with the Superscript II kit (Gibco-BRL). Then PCR was primed with oligonucleotides specific for *CDC25*, *GPH1* and *BCY1*. The reaction was run for 23 (a, d), 26 (b, e) and 29 (c, f) cycles. Densitometric quantification as well as independent radioactive measurements during kinetic gave a ratio of *CDC25* mRNA found between the *SSA1*-containing cell and the double mutant in the range of 0.8 after normalization with either *GPH1* or *BCY1*.

Ssa chaperones affect the cAMP-dependent rate of production of trehalose, glycogen and gene expression

If Hsp70 proteins control the cellular content of Cdc25p, responses to cAMP are expected to be modified by deletion of *SSA1* and *SSA2*. To test this hypothesis, we have compared the behaviour of the *Δssa1*, *Δssa2* mutant containing or not the *SSA1* gene on a plasmid. We have followed the rate of production of glycogen and trehalose as markers of the efficiency of the cAMP pathway. The cellular content in glycogen is controlled by the cAMP pathway at the enzymatic level on both glycogen synthetase and glycogen phosphorylase (Rothman-Denes and Cabib, 1970; Huang and Cabib, 1972) and at the level of gene expression (François *et al.*, 1992). Several mutants defective in glycogen accumulation were found to be affected on genes of the cAMP pathway [*glc1* = *ira1*, *glc4* = *ira2*, *glc5* = *ras2* (Cannon *et al.*, 1994)]. Trehalase activation and trehalose degradation are also mediated by cAMP-dependent PKA (van Solingen and van der Plaat, 1975).

Glycogen is not accumulated constitutively during the culture but appears later before glucose exhaustion. As the cellular contents of the cell vary during the culture, we used the 'Monod plot' to compare the different strains. This representation (specific compound plotted versus total protein) allows the rate of accumulation (slope of the curve) to be followed and to be compared under different situations and even at different rates of growth. We found that the *Δssa1*, *Δssa2* mutant complemented by *SSA1* on a plasmid, which does not exhibit an altered phenotype, has a normal rate of glycogen accumulation (Fig. 5A). In contrast, in the absence of *SSA1* and *SSA2* the rate of

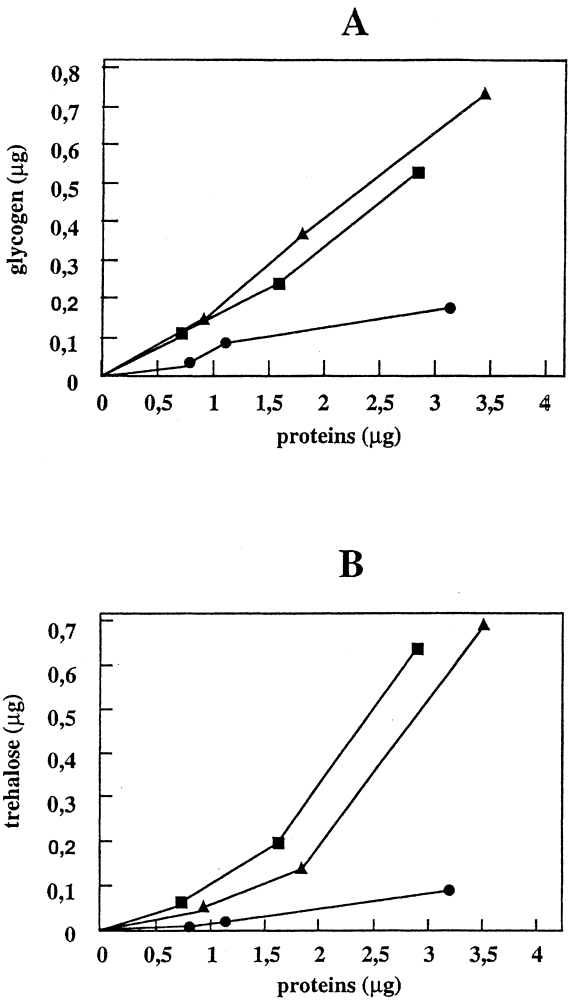

Fig. 5. Accumulation of intracellular glycogen and trehalose depends on the presence of the *SSA1* gene product. Cells grown on minimal medium (YNB) were harvested during exponential growth between OD_{700} 0.4 and 1.6. The amount of glycogen (A) or trehalose (B) is plotted versus. the amount of proteins (Monod plot) measured in a 1 ml culture. OL565 (*ssa1*, *ssa2*) transformed with pRS314 and YEp352 (■), pGRSSA1 (*SSA1*) and YEp352 (●) or pGRSSA1 and pGR103 (*PDE2*) (▲).

accumulation of glycogen was dramatically increased. A similar increase was seen when the high-affinity cAMP phosphodiesterase, encoded by the *PDE2* gene, was overexpressed from a plasmid in the strain containing *SSA1*. We have checked in the *SSA1*-containing strain that the rate of glycogen accumulation relative to protein was independent from the growth rate. Indeed we have obtained superimposable curves at different temperatures for different growth rates (data not shown).

We have also measured trehalose accumulation in the *Δssa1*, *Δssa2* mutant (Fig. 5B). Trehalose is accumulated after the glycogen during batch cultures on glucose, most likely as the result of a different regulatory circuit but also as a function of the cAMP pathway. In contrast to the situation found for glycogen, the overexpression of phosphodiesterase did not result in a linear relationship between trehalose and protein, but a variation in the differential rate of accumulation was still observed, suggesting the existence of a cAMP-independent control on trehalose accumulation. Interestingly, the curve obtained in the non-complemented double mutant is very close to that obtained with the complemented mutant harbouring the *PDE2* plasmid. The differential rate of trehalose accumulation was increased earlier in the non-complemented mutant and reached higher values, as in cells containing low cAMP levels.

It is also known that the cAMP pathway negatively regulates genes that contain stress-responsive elements (STRE) in their promoter (Ruis and Schüller, 1995). In the *Δssa1*, *Δssa2* mutant, we have analysed the expression of β-galactosidase controlled by the promoters of two genes repressed by cAMP and containing STRE sequences in their promoter region, *GAC1* (François *et al*., 1992) and *TPS1* (Winderickx *et al*., 1996). In the early exponential growth phase, the transcription of *GAC1* and *TPS1* was four-fold higher in a strain deleted for both *SSA1* and *SSA2* than in the same strain complemented with *SSA1* (Table 1). By contrast, the level of transcription of the *CYC1–LacZ* fusion, used as a control, was independent from the presence of Ssa1/2p. Therefore, the loss of *SSA1* and *SSA2* mimics a low intracellular level of cAMP for glycogen and trehalose accumulation as well as cAMP-dependent gene expression.

A CDC25 *deletion rescued by* RAS2^{ile152} *is epistatic on* SSA *deletions for the control of glycogen accumulation*

The experiments presented above illustrate the effect of ssa chaperones on cAMP targets. To analyse further whether or not these effects are mediated by the cAMP pathway and more specifically Cdc25p we have used a cell in which *CDC25* was completely deleted. As *CDC25* is an essential gene, this experiment was performed in a strain rescued by the *RAS2^{ile152}* allele (Camonis and Jacquet, 1988). We chose this suppressor rather than others such as *RAS2^{val19}* to avoid any bias due to overactivity of the pathway. Therefore we have constructed the strain OL576-3C containing four mutations: (i) the deletion of the two genes *ssa1* and *ssa2* inherited from the strain MW123; and (ii) the complete deletion of the *CDC25* open reading frame (ORF) with the integrated copy of *RAS2^{ile152}*. This strain still exhibits cAMP-independent phenotypes for the defect of chaperones, reduced growth and thermosensitivity. As shown in Fig. 6, the relative rate of glycogen accumulation in the strain containing the four mutations was almost identical to that of the strain with the *Δcdc25 RAS2^{ile152}* mutations, whereas it was lower than the *ssa*-deleted strain. This difference was also observed in synthetic medium rather than the rich medium used here. Thus, it can be concluded that the lack of *CDC25* and the activity of the product of *RAS2^{ile152}* is epistatic upon the deletion of *ssa1* and *ssa2* on the stimulation of glycogen accumulation.

Table 1. *GAC1* and *TPS1* promoters are regulated by *SSA1* gene product.

Strain and plasmids used	OL565 with pSSA1 β-gal units per protein	OL565 with pRS314 β-gal units per protein	Stimulation factor
GAC1–lacZ	0.105	0.426	4.06
TPS1–lacZ	0.173	0.596	3.45
CYC1–lacZ	3.491	5.466	1.59

The specific activity of the β-galactosidase (arbitrary units) was measured in early exponential phase ($OD_{700} = 0.3$) of OL565 (*ssa1*, *ssa2*) co-transformed with pJF169 (*GAC1*–LacZ), pTPS1 (*TPS1*–LacZ) or pLG669Z (*CYC1*–LacZ) and pGRSSA1or pRS314.

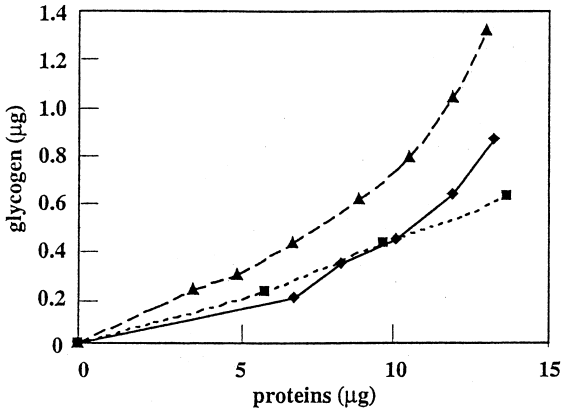

Fig. 6. Epistatic effect of *Δcdc25*, *RAS2^{ile152}* on *Δssa1*, *Δssa2* for glycogen accumulation. Cells grown in rich medium (YEPD) were harvested during exponential growth between OD_{700} 1.5 and OD_{700} 5.5. The amount of glycogen is plotted versus the amount of proteins measured in 1 ml of culture for the strain OL565 (*ssa1*, *ssa2* mutant) (▲), OL550–11A (*Δcdc25*,*RAS2^{ile152}*) (■) and OL576–3C (*ssa1*, *ssa2*, *cdc25*, *RAS2^{ile152}*) (♦). The standard deviation on glycogen measurement is less than 10%.

Discussion

Cdc25p interacts with chaperones

Searching for proteins interacting with the *S. cerevisiae* Ras GEFCdc25p, we have found that the *SSA1* gene product co-purified with a GST–Cdc25p fusion protein containing a C-terminal fragment of Cdc25p. The reproducibility of this interaction has been assessed by immunoprecipitation experiments of either an HA-tagged Cdc25 polypeptide or the endogenous Cdc25p. In addition, using an antibody directed against Hsp70p of the Ssa subfamily and Hsp82, we have shown that both the HA-tagged and the endogenous Cdc25p were co-immunoprecipitated with Hsp70p chaperones. Hsp82 was also found associated with Cdc25p in immunoprecipitation experiments. As this interaction was weaker than with HSP70, we have not investigated it further in this report, although it might be important because interactions between members of the Hsp70 and Hsp80 families have already been described in yeast (Chang and Lindquist, 1994).

Hsp70 and other members of the cellular chaperones family have a general capability to bind unfolded proteins (Palleros *et al*., 1991). Overexpressed proteins are potential targets for such interactions, nevertheless we have shown that the interaction also occurs using the functional endogenous Cdc25p. It could also be argued that the solubilization procedure of Cdc25p is responsible for the binding to chaperone, but if this were the case the more abundant chaperones such as the Ssb proteins (Nelson *et al*., 1992) or even Ssa2p, which is more abundant than Ssa1p in growing cells (Boucherie *et al*., 1996), would have been preferentially found. Indeed, Cdc25p is able to bind to other Ssa proteins such as Ssa2p in the *ssa1* mutant, but Ssa1p was the only Hsp70 protein identified in microsequencing analysis, suggesting a higher affinity for this chaperone, which does not preclude a partially redundant role of the other members of the family. As discussed below, genetic and physiological interactions between the cAMP pathway and *SSA1* further establish the relevance of this interaction *in vivo*.

Ssa chaperones control the cAMP signalling pathway

The biological significance of this interaction was first assessed by comparison of the level of Cdc25p between the *Δssa1*, *Δssa2* mutant and the *SSA1*-complemented strain. The amount of Cdc25p relative to other proteins was significantly reduced in this mutant. This reduction is not the result of a lower expression because the level of mRNA was found to be quite similar between the two strains. This result suggests a direct effect of the chaperones on Cdc25p. As Cdc25p, an unstable protein, is present in very low amounts in the cell (Kaplon and Jacquet, 1995), a regulation on its cellular content will be readily transmitted to the signalling pathway. Indeed, the activity of the cAMP pathway depends upon the amount of Cdc25p as deduced from the slow growth observed in cells where the GEF domain of *CDC25* is expressed from a poor endogenous promoter (M. Jacquet, unpublished; Petitjean *et al*., 1990). Therefore, a reduction in activity of the Ras–cAMP–PKA pathway can be predicted in cells having a reduced amount of Ssa protein such as in the double *ssa*-deleted strain. Members of the *SSA* family are essential for growth and partially redundant; the double deletion of *SSA1* and *SSA2* is still viable because of the overexpression of *SSA3* and *SSA4*, but this is not sufficient to maintain a normal growth rate. Among the pleiotropic phenotypes associated with the defect of these chaperones, several can be explained by the activation of Hsf1p (Craig and Jacobsen, 1984; Werner-Washburne *et al*., 1987; Boorstein and Craig, 1990) and are not suppressed by suppressors of the cAMP pathway, but some could be related to the cAMP pathway. Constitutive thermotolerance, which is a paradoxical phenotype for a defect of chaperone, could be related to the cAMP pathway because it was also found associated with mutations reducing the activity of the cAMP pathway (De Virgilio *et al*., 1993; Shin *et al*., 1987). In addition, in a strain depleted for Ssa chaperones, an increased level of trehalose after heat shock was found to result from a decrease in trehalase activity that is directly controlled by PKA (Hottiger *et al*., 1992). In the present report we have confirmed this observation on trehalose and further extended the conclusion to other targets of the cAMP pathway. We have shown that the rate of accumulation of trehalose depends upon the presence of *SSA1* when *SSA2* is lacking. The *SSA1* effect requires the cAMP pathway function because it is lost when the pathway is blocked by reducing the level of cAMP with excess phosphodiesterase. The control of the glycogen content of the cell by *SSA1* is a newly described phenotype for these chaperones. As for trehalose, the limitation of cellular chaperones mimics a reduced activity of the cAMP pathway; a modulation of the cAMP pathway by overexpression of the phosphodiesterase is again epistatic on the effect of *SSA1* on glycogen accumulation. In addition, looking at transcriptional targets of the cAMP pathway, we have extended the effect of the Ssa chaperones to the expression of *TPS1* and *GAC1* promoters. The promoters of both *TPS1* and *GAC1* lacking canonical HSE, contain STRE sequences that mediate negative response to the cAMP/PKA pathway; they have been shown to respond to cAMP (D. Tadi and E. Boy Marcotte, unpublished). Therefore, their Ssap-dependent expression is not likely to be mediated by *HSF* but rather by the cAMP pathway, most likely through STRE.

The diversity of the cAMP-dependent phenotypes controlled by Ssa strongly argues for an effect of these chaperones on the control of the cAMP pathway. The evidence

that this control acts upstream of the cascade comes from our last experiment, in which we have analysed the effect of the *SSA* deletions in a strain with a normal cAMP pathway and a strain where this pathway was defective in *CDC25* and bypassed at the level of ras. Although the difference between the curves is small because Ras2p^{ile152} is less effective in activating the cAMP pathway than a normal protein, this difference was reproducible and leads us to conclude that Ssa proteins should control the pathway upstream of ras. Altogether, the result of physical interaction of Cdc25p with the Ssa proteins and the physiological effect of Ssap on the cAMP–PKA pathway led us to propose a model in which the interaction of Hsp with the GEF of ras: Cdc25p, is one of the regulatory systems acting on the cAMP signalling pathway.

Why are chaperones modulating the cAMP signalling pathway?

A lowered activity of the PKA pathway appears to be required for an adaptation of the cell to stress conditions, as deduced from the inhibitory effect of a high PKA activity (Ruis and Schüller, 1995) (Boy-Marcotte *et al*., 1998), or from the thermotolerance acquisition due to deregulating mutations affecting this pathway (Shin *et al*., 1987). During heat shock, several genes containing STRE elements in their promoter are also induced (Lahskari *et al*., 1997). This element is controlled by Msn2p and Msn4p, which have been shown to have their nuclear localization controlled by the cAMP pathway (Görner *et al*., 1998). The interaction between Cdc25p and Hsp70 and Hsp90 could then play a critical role in this model to connect the activity of the cAMP pathway to the state of the cell. Hsp70 has been proposed to play the role of a cellular thermometer, and it would be recruited by unfolded proteins produced after heat shock and thus would be less available to repress Hsfp (Craig and Gross, 1991) (Shi *et al*., 1998). A similar model could be proposed for the control of the cAMP pathway: a transient accumulation of unfolded proteins after a stress would reduce the availability of Hsp70 for Cdc25p as for Hsf1p and then reduce the activity of the cAMP pathway. This reduction would allow the expression of the regulon controlled by STRE that is induced in response to several conditions of stress (Ruis and Schüller, 1995) or metabolic changes (Boy-Marcotte *et al*., 1998) when many proteins have to be removed from the cell.

Experimental procedures

Yeast strains

The previously described strains we used are W303-1B, W3031B/D (Nasmyth, 1985), C13ABYS86 (Heinemeyer *et al*., 1991), T141 (*MATa*, *ura3-52*, *his3-11,15*, *leu2-3,112*, *lys2*, *ssa1*::*LEU2*), MW123 (*MAT a*, *ura3*, *his3*, *leu2*, *lys2*, *Δtrp1*, *ssa1*::*HIS3*, *ssa2*::*LEU2* (Werner-Washburne *et al*., 1987), ΔECU82a (*MATa*, *ade2-1*, *ura3-1*, *his3-11,15*, *leu2-3,112*, *trp1-1*, *hsc82*::*LEU2*, *hsp82*::*LEU2*).

The following strains have been constructed for the present work. OL565 (*MATa*, *ura3*, *his3*, *leu2*, *lys2*, *trp1*, *ssa1*::*HIS3*, *ssa2*::*LEU2*) was derived from a cross between MW123 and W303-1B/D. To construct the *CDC25* deleted strains: (i) the *RAS2^{ile152}* allele (Camonis and Jacquet, 1988) contained within a *Cla*1–*Bam*H1 fragment was cloned into the integration plasmid pRS304 (Sikorski and Hieter, 1989) to produce pGR113, this plasmid linearized with *Pst1* was introduced into the diploid yeast strain FY1679 (Baudin *et al*., 1993) to give the trp$^+$ OL549 strain; (ii) the *CDC25* gene was replaced by the *HIS3* gene amplified with two oligonucleotides corresponding to the 35 nucleotides upstream of the *CDC25* ATG (at −26) and downstream of the stop codon (+48), respectively, extended by the *HIS3* primers (Baudin *et al*., 1993); (iii) the resulting strain OL550 was sporulated and asci dissected. Among the progeny His$^+$,Trp$^+$ cells were recovered and analysed by PCR to confirm gene replacement. Two of these strains were used in this report: OL550–11A (*MATα*, *ura3-52*, *his3-Δ200*, *leu2-Δ1*, *trp1-Δ63*, *cdc25*::*HIS3*, *RAS2-RAS2^{ile152}*::*TRP1*) and OL550-11B (*MATa*, *ura3-52*, *his3-Δ200*, *leu2-Δ1*, *trp1-Δ63*, *cdc25*::*HIS3 RAS2-RAS2^{ile152}*::*TRP1*).

The *Δssa1*, *Δssa2* mutant containing the *cdc25*::*HIS3*, *RAS2^{ile152}* was obtained from a cross between the strain OL550-11B and OL565. One strain among the progeny, OL576-3C, was found to contain (i) the double *ssa1* and *ssa2* deletion, as judged from *HIS3* and *LEU2* segregation in the tetrad and the associated phenotypes: slow growth and thermosensitivity; (ii) the *CDC25*::*HIS3* replacement from PCR analysis; and (iii) the presence of *RAS2^{ile152}* from *TRP1* segregation and growth rescue of the *cdc25* deletion.

The OL568-1C strain was derived from the diploid strain W303–1B by insertion of three tandem repeats encoding the influenza virus haemagglutinin epitope (HA) at the 5′ end of the CDC25 gene, between codons 4 and 5, using the method of Schneider *et al*. (1995). The 3xHA-*URA3*-3xHA cassette of the pMPy-3xHA plasmid was amplified by PCR, using ol25NHA up and ol25NHA down and was transferred to the W303-1B strain. Ura$^+$ transformants with the *CDC25* gene replaced were checked by PCR and plated onto 5-fluoro-orotic acid to select ura$^-$ cells. After sporulation and tetrad analysis, the haploid OL568-1C strain (*ade2-1*, *ura3-1*, *his3-11*, *leu2-3*, *112*, *trp1-1*, *CDC25:3xHA*) was recovered. As checked by PCR, it contained three tandem repeats of the HA epitope. The MGL20-4C strain (*ura3*, *lys2*, *trp1*, *ssa1*::*HIS3*, *ssa2*::*LEU2*, *CDC25*:*x3HA*) was obtained from a cross between OL568-1C and OL565.

Plasmid constructions

Plasmids used in this study are listed in Table 2. pTK3 was constructed by insertion of a three HA tag coding sequence within the Nhe1 site of pTK2, allowing expression of the full *CDC25* gene under the *GAL1-10* promoter (Kaplon and Jacquet, 1995). pEG-FS4 was obtained by insertion of a PCR fragment containing the last 148 codons of *CDC25* in the *Sma*I–*Sal*I sites of the pEG(KG) (Mitchell *et al*., 1993); this fragment was amplified with olYF1 and olYF2 on the plasmid

Table 2. Plasmids and oligonucleotides used.

Plasmids	Inserts/comments	Source/references
pTK3	Cdc25p, tagged at C-terminal, under *GAL10* promoter, *URA3*, 2μ	This work
pYFS	Codons 937–1589 of *CDC25* in pYeF1	Garreau *et al.* (1996)
pYFS4	Codons 1441–1589 of *CDC25* in pYeF1	This work
pEG-FS4	Codons 1441–1589 of *CDC25* tagged at N-terminal in pEG(KG)	This work
pKAT6	Yeast HSP82 in a YEp24 vector	Gift from S. Lindquist
pTGpd90a	Chicken HSP90α in a pRS314 derivative vector	Gift from M. G. Catelli
pGRSSA1	*SSA1* in pRS314	This work
pGR103	*PDE2* in YEp352	This work
Oligonucleotides		
Sequence		
ol25NHA up	TGGATATTGGATAGTTGTATCATGTCCGATACTAACACGTCTATTAGGGAACAAAAGCTGG	
ol25NHA down	AGTTTGTGAAGCATTGCCTGCCTCCCTTGCAGAACTTGTATTGGGCTGTAGGGCGAATTGG	
ol1690	GGGGCGGCCGCTGTGAAGTATAGAGAG	
ol2218	GGAGATCCCCCGCGAATT	
olYF1	TTCCCGGGGTACCCATACGACGTCCCA	
olYF2	TGAATTCTTCGAGTGTAGCAATCAA	
ol600	TGGCTCGAGGCTCGGTTGTCGACAAATTGTT	
ol1053	TTATACCCGGGAAACGAAACTACAACTCAAT	
ol3155	CGTTTCCCGGGTATAATGAGGAATTTTCCGA	
ol3657	CCGGGCTAGCCCTCACACTGTACATGATAA	

pYFS4 with ol1690 and ol2218 and inserted into YeF1 (Cullin and Minvielle-Sebastia, 1994) by *Not*1 and *Eco*R1.

The *SSA1* gene was cloned by gap repair into the plasmid pGRSSA1 using the intergenic flip-flop procedure (Mallet and Jacquet, 1996). The *SSA1* promoter sequence (from −579 to −108 of ATG) and terminator sequence (from +50 to +585 of the stop codon) were amplified using oligonucleotides ol600, ol1053 and ol3155, ol3657 respectively. The second PCR has been primed with ol600 and ol3657 and the two amplified fragments. The resulting fragment was cut with *Xho*I, and *Nhe*I was cloned into *Xho*I–*Spe*I sites of pRS314 (Sikorski and Hieter, 1989). The *Sma1* linearized plasmid was transformed into W303 on medium lacking tryptophan. The plasmid pGRSSA1 complements the thermosensitivity and the reduced rate of growth of a *Δssa1*, *Δssa2* mutant.

pGR103, expressing the *PDE2* gene, was obtained by cloning a 3 kb *Bam*HI–Bgl*II* fragment obtained from pW4 (Wilson and Tatchell, 1988) into the Bam*HI* site of YEp352 (Hill *et al.*, 1986).

Whole-cell protein extract, subcellular fractionation, GST fusion proteins purification and immunoprecipitation

Crude cell extracts and membrane fractions were prepared as previously described (Garreau *et al.*, 1990) with the addition of 20 mM sodium molybdate in the lysis buffer. Solubilization of complete Cdc25p or fused protein was performed as described by Gross *et al.* (1992b) with 2 mM EDTA pH 12. GST fusion proteins were further purified by incubation of the neutralized supernatant with glutathione–agarose beads for 1 h at 4°C. After extensive washing in washing buffer (PBS, 0.5% Triton X-100, 0.25 M KCl), bound proteins were recovered by boiling the beads in 2× Laemmli sample buffer and subjected to SDS–PAGE. For GST control, the supernatant of the first centrifugation was directly incubated with glutathione–agarose beads.

Immunoprecipitation was performed by incubation of the neutralized supernatant from 50 ml of culture, at 4°C, 1 h with the antibody and then 1 h in the presence of protein A–Sepharose. Bound proteins were recovered after washing by boiling in 2× Laemmli sample buffer and subjected to SDS–PAGE. Immunoreactive bands were detected by goat anti-rabbit or anti-mouse IgG serum, alkaline phosphatase conjugate (ProMega) or peroxidase conjugate (Amersham).

Glycogen, trehalose, β-galactosidase and protein determinations

Glycogen and trehalose were measured as described (Parrou and François, 1997). β-Galactosidase was measured from cell extracts with the Ozyme Galacto-Light plus chemiluminescent reporter assay kit as recommended by the manufacturer. Protein concentrations of extracts were measured according to the method of Bradford.

Peptide analysis

Digestion and separation of peptides were performed by the Laboratoire de Microséquençage des Protéines of the Institut Pasteur (Paris) as described in Kawasaki and Suzuki (1990) with slight modifications: proteins separated on SDS polyacrylamide gel were stained with 0.03% Amido Black; the buffer used for digestion was 0.1 M Tris-HCl pH = 8.8, 0.03% SDS, an Aquapore AX300 (Brownlee) column was used to remove SDS and the peptides were separated on a C_{18} Vydac 218TP52 column. Amino acid sequences were determined with an Applied Biosystems Procise ABI 498 (PROCISE-HT).

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