The N-terminal region of the *Saccharomyces cerevisiae* RasGEF Cdc25 is required for nutrient-dependent cell-size regulation

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In the yeast *Saccharomyces cerevisiae*, the Cdc25/Ras/cAMP/protein kinase A (PKA) pathway plays a major role in the control of metabolism, stress resistance and proliferation, in relation to the available nutrients and conditions. The budding yeast RasGEF Cdc25 was the first RasGEF to be identified in any organism, but very little is known about its activity regulation. Recently, it was suggested that the dispensable N-terminal domain of Cdc25 could negatively control the catalytic activity of the protein. In order to investigate the role of this domain, strains were constructed that produced two different versions of the C-terminal domain of Cdc25 (aa 907–1589 and 1147–1589). The carbon-source-dependent cell size control mechanism present in the wild type was found in the first of these mutants, but was lost in the second mutant, for which the cell size, determined as protein content, was the same during exponential growth in both ethanol- and glucose-containing media. A biparametric analysis demonstrated that this effect was essentially due to the inability of the mutant producing the shorter sequence to modify its protein content at budding. A similar phenotype was observed in strains that lacked CDC25, but which possessed a mammalian GEF catalytic domain. Taken together, these results suggest that Cdc25 is involved in the regulation of cell size in the presence of different carbon sources. Moreover, production of the aa 876–1100 fragment increased heat-stress resistance in the wild-type strain, and rescued heat-stress sensitivity in the *ira1Δ* background. Further work will aim to clarify the role of this region in Cdc25 activity and Ras/cAMP pathway regulation.

INTRODUCTION

In the budding yeast *Saccharomyces cerevisiae*, the Cdc25/Ras/cAMP signal transduction pathway regulates many cellular and physiological processes, such as growth, resting state and sporulation (reviewed by Thevelein & de Winde, 1999; Thevelein *et al.*, 2000), carbohydrate and nitrogen metabolism, stress tolerance, and cell wall resistance to lyticase digestion (Martegani *et al.*, 1998; Plesset *et al.*, 1987; Van Dijck *et al.*, 1995). The cAMP pathway is involved in the control of cell cycle progression at G₀/G₁ and/or G₂ phases (Drebot *et al.*, 1990; Thevelein, 1992, Anghileri *et al.*, 1999), Cln3 G₁ cyclin translation (Barbet *et al.*, 1996; Hall *et al.*, 1998; Polymenis & Schmidt, 1997), and nutritional modulation of the critical cell size required for entry into the S phase (Baroni *et al.*, 1989; Mitsuzawa, 1994).

In yeast, cyclic AMP is synthesized by adenylyl cyclase, which is encoded by the *CYR1/CDC35* gene, and degraded by Pde1 and Pde2 phosphodiesterases (Ma *et al.*, 1999). Yeast adenylate cyclase is activated by two different systems: the G-protein-coupled receptor system, acting through the G-protein Gpa2 (Nakafuku *et al.*, 1988; Colombo *et al.*, 1998), and the Cdc25/Ras system (Toda *et al.*, 1985). The Gpa2 protein is closely related to the extracellular glucose receptor Gpr1, and it seems to be mainly involved in producing the rapid increase of cAMP levels that occurs upon addition of high glucose concentrations (100 mM) to glucose-derepressed cells. Cdc25 and Ras are also required for the glucose- (and fructose-) induced cAMP increase (Rolland *et al.*, 2000); they are essential for cell viability, and play a central role in adenylate cyclase activity regulation during yeast growth (Tamanoi, 1988).

Activity of Ras proteins is modulated by their ability to switch between an inactive state, when bound to GDP, and an active state, when associated with GTP. The ratio of GDP/GTP on Ras proteins is controlled by the guanine nucleotide exchange factors (GEFs) Cdc25 (Jones *et al.*, 1991) and Sdc25 (Damak *et al.*, 1991; Boy-Marcotte *et al.*, 1996), which stimulate the GDP–GTP exchange on Ras, and by the Ira1 and Ira2 proteins, which promote intrinsic Ras GTPase activity (Tanaka *et al.*, 1989, 1990a, b).

Abbreviations: BI, budding index; CDB, cyclin destruction box; FACS, fluorescence-activated cell sorting; GEF, guanine nucleotide exchange factor; PI, propidium iodide; PKA, protein kinase A; P见解, total protein content.
The \textit{S. cerevisiae} Cdc25 protein was the first RasGEF to be identified (Camonis et al., 1986; Martegani et al., 1986). Cdc25 is responsible for Ras1 and Ras2 activation, and is required for the glucose-induced rapid increase of Ras2-GTP levels (Rudoni et al., 2001; Colombo et al., 2004), and the activation of adenylate cyclase (Engelberg et al., 1990); however, the molecular basis of the mechanism that regulates the exchange activity of Cdc25 in response to nutrients is yet to be elucidated.

The yeast \textit{CDC25} gene encodes a 1589 aa protein that is produced as a polypeptide of about 180 kDa (Vanoni et al., 1990; Jones et al., 1991; Gross et al., 1992). The C-terminal fragment (aa 1256–1589), containing the GEF domain, is essential for normal growth and viability of \textit{cdc25A} and \textit{cdc25 ts} mutants (Lai et al., 1993; Coccetti et al., 1995).

In the large N-terminal region of Cdc25 (aa residues 1–1101), there is an SH3 motif (aa 60–130) that binds adenylate cyclase, and seems to enhance its responsiveness to activation by Ras \textit{in vitro} (Mintzer & Field, 1995). Next to the SH3 motif, there is a cyclin destruction box (CDB) motif, and the Cdc25 protein content in the yeast cell is controlled by a ubiquitin-dependent degradation process specifically driven by this CDB motif (Kaplon & Jacquet, 1995).

A possible biological function for the large Cdc25 N-terminus has been suggested as a result of the finding that upon glucose stimulation in yeast, some residues within aa 114–348 become phosphorylated, leading to decreased association of Cdc25 with the membranes and accessibility to Ras (Gross et al., 1992). This phosphorylation is believed to be part of a negative-feedback loop resulting from the action of cAMP-dependent protein kinase A (PKA).

The Cdc25 N-terminus has been suggested to have weak dominant-negative properties, which inhibit the function of the whole Cdc25 protein \textit{in vivo}, possibly by interacting with the complete endogenous Cdc25, thus interfering with its ability to activate Ras (Chen et al., 2000).

To better characterize the function of the N-terminal region of the RasGEF protein, two yeast mutants that lacked most of the Cdc25 N-terminal domain were constructed. In addition, we made two other mutants in which the whole \textit{CDC25} gene was replaced by a catalytic domain from a heterologous mammalian RasGEF: the mouse RasGRF1/\textit{Cdc25}\textsuperscript{Mm} or the human Sos1 (Martegani et al., 1992; Gross et al., 1999). Since the homology between \textit{S. cerevisiae} Cdc25 and mammalian GEFs is restricted to the Ras-exchange domain, and is not very high (about 30 \% similarity), it was thought that the latter two mutants would help to clarify whether some regions close to or inside the Cdc25 exchange domain are also responsible for some regulation. Finally, the effect of overproduction of different fragments of the Cdc25 N-terminus was tested in the wild-type strain, and in different mutant backgrounds.

Here, we show that lack of the N-terminal region of Cdc25, or the presence of an unregulated heterologous GEF activity, causes a defect in the nutrient modulation of cell size and cell cycle regulation.

**METHODS**

**Strains, plasmids and growth conditions.** \textit{S. cerevisiae} haploid strains used in this work are described in Table 1. Deletion mutants were generated in the diploid W303 strain (Thomas & Rothstein, 1989), using plasmid-derived gene replacement cassettes. The cassettes were prepared in a pGEM3z (with the \textit{Sall} site disrupted) vector (Promega) by insertion of the \textit{URA3} marker and the \textit{CDC25} gene flanking regions, which were extracted from plasmid pPCDC25(LEU2)-2 (Broek et al., 1987). Briefly, first, the \textit{Smal}–\textit{Ndel} (filled-in) fragment from plasmid pVT-102U (Vernet et al., 1987) was inserted into the \textit{BamHI} (filled-in)–\textit{HincII} sites; then, the \textit{BamHI} (filled-in)–\textit{HincII} fragment, spanning the 5' \textit{CDC25} flanking region (bp positions +837 to −314), was inserted in the \textit{HindIII} (filled-in) site, upstream of the \textit{URA3} marker; finally, the \textit{SphI}-excised fragment, spanning the 3' \textit{CDC25} flanking region (bp positions +4653 to +5230), was inserted into the \textit{Smal} site, downstream of the marker. The cassettes used to generate the \textit{WACdc25}\textsuperscript{Mm} and \textit{WAhsos1} strains were excised by \textit{SphI} cutting from the pVTU-Cdc25\textsuperscript{Mm} (Coccetti et al., 1995) and pVTU-hSos1 (M. Vanoni, 1989).

![Table 1. Strains used in this study](image-url)
University plasmids, respectively, and inserted into the SphI site, between the 5' CDC25 flanking region and the URA3 marker. To generate the N-terminal deleted strains, deleted ORFs were prepared by PCR using the oligonucleotides GTGCGGGATCCAGATTCGAG, and either GAGATCTCTGATAACATCTGAAATCCAC (for the 1–2628 deletion, leading to the generation of a new ORF, starting from the first following ATG, and encoding aa 907–1589) or GTATCCATGGGATACACTATCCATATCCAC (for the 1–3304 deletion, creating a new ORF encoding aa 1147–1589), to amplify the CDC25 promoter, and then to substitute the SstII or the NcoI CDC25 gene fragment, respectively. The newly generated ORF was then inserted instead of the 5’ flanking region in the pGEM3z gene replacement cassette. The cassettes were finally excised by restriction, or amplified by PCR to transform the diploid W303 strain. Gene replacement was verified by PCR in the heterozygote diploids, and haploid mutant strains were recovered by sporulation.

Plasmid pYX212-CDC25(aa 1–875) was prepared by inserting into the BamHI site of pYX212 (Novagen) the 3000 bp fragment obtained by BamHI–BglII partial digestion (containing nt 1–2628 of CDC25 ORF) of the HindIII fragment of the CDC25 gene, previously subcloned in the HindIII site of the same plasmid. Plasmid pYX212-CDC25(aa 1–1100) was obtained by inserting into the NcoI site of pYX212 the 3700 bp fragment obtained by NcoI digestion (spanning nt 1–3304 of CDC25 ORF) from the same CDC25-HindIII-fragment-containing plasmid. Plasmid pYX212-CDC25(aa 353–1100) and plasmid pYX212-CDC25(aa 876–1100) were prepared by subcloning into the BamHI site of pYX212 either the 2200 bp fragment obtained by BamHI–BglII partial digestion from pYX212-CDC25(aa 1–1100) or the 680 bp fragment obtained by BglII–BamHI digestion from pYX212-CDC25(aa 1–1100). Plasmid pYX212-CDC25(aa 353–875) was obtained by deleting the BamHI–BglII fragment in pYX212-CDC25(aa 1–875). Plasmid pYX212-CDC25(aa 1–352) was prepared by inserting the 1400 bp fragment obtained by BamHI–BglII digestion from pYX212-CDC25(aa 1–1100).

Rich (YPE) medium contained 1% yeast extract and 2% peptone (Bioline), and was supplemented with 2% glucose (YPD), 2% ethanol (YEPE), 2% raffinose, 2% potassium acetate or 2% glycerol as the carbon source. Synthetic minimal medium contained 6% (YPE), 2% raffinose, 2% potassium acetate or 2% glycerol as the carbon source. Synthetic minimal medium contained 6% ammonium sulfate and 5 mg l⁻¹ of the appropriate auxotrophic requirements, and was supplemented with a carbon source, as for YEP medium. Nitrogen starvation experiments were performed using synthetic medium containing 1% yeast extract and 2% peptone (BioLife), and was supplemented with 2% glucose (YPYD), 2% ethanol (YEP), 2% raffinose, 2% potassium acetate or 2% glycerol as the carbon source. Synthetic minimal medium contained 6% YNB (Biolife), and was supplemented with 2% glucose (YPYD), 2% ethanol (YPD), 2% ethanol (YPD), 2% ethanol (YPD). For the heat-shock sensitivity assay, on ir1A strains, cells were grown on selective medium for 2 days, then exposed to 51°C for 17 min, replica plated on fresh medium, and incubated at 30°C for 2 days.

Different mutants were created by modifying the CDC25 locus, as described in Methods. The N-terminal domain was deleted from the CDC25 locus in two different mutants, one with a shorter (aa 1–906) deletion, and the other with a longer (aa 1–1146) deletion. In other mutants, the entire CDC25 ORF was deleted, and substituted with an expression cassette containing the Ras-exchange domain of mammalian GEF RasGRF1/Cdc25Sm or human Sos1; the production of either of these mammalian RasGEFs domains is able to rescue the growth defect of a temperature-sensitive cdc25 mutant (Martegani et al., 1992; Gross et al., 1999), and the lethality caused by CDC25 deletion. The modified alleles were introduced in the diploid W303 strain, and the mutant haploid strains were recovered after tetrad dissection.
The dissections of the tetrads from all of the heterozygous diploid strains containing a mutated \( cdc25 \) allele presented a segregation pattern \( 2:2 \) (large:small colonies), and the mutant genotype, determined by PCR analysis of the colonies, always co-segregated with the small colony size (Fig. 1a). The small size of the mutant colonies was not due to a growth defect: all the mutant strains grew well, with doubling times comparable with that of the wild-type strain, not only in YPD medium (Fig. 1b), but in all the conditions tested (Table 2), including synthetic minimal medium supplemented with acetate, glycerol or ethanol as the carbon source (data not shown). Rather, it was thought that the small colony size was due to a delay in spore germination. However, all the mutant strains, except, surprisingly, \( \text{WN}2 \), showed only a short delay in recovery of growth from stationary phase (Fig. 2), and a little longer in recovery from nitrogen starvation (data not shown). Therefore, it is probable that normally regulated Cdc25 activity is critical in the transition from the \( G_0 \) phase to entry into the cell cycle. All the mutant strains were able to enter stationary phase normally, at least in YPD medium, with a high fraction of unbudded cells (up to 90%), as shown in Fig. 2.

Different \( cdc25 \) mutants show defects in nutrient-dependent cell size modulation

As shown above, mutant strains had similar growth rates to the wild-type (Table 2), and they were able to arrest normally, mostly as unbudded cells, when grown to saturation on YPD medium, or starved of nitrogen (data not shown). Nonetheless, they showed some abnormal features during exponential growth on different carbon sources: compared with the wild type, the \( \text{WN}1 \) strain always had a larger cell volume (Fig. 3), and a higher percentage of budded cells. In contrast, \( \text{WN}2 \) (Fig. 3), \( \text{WACdc25}^{\text{MM}} \) and \( \text{WAhSos1} \) (data not shown) showed a smaller cell volume than the wild type; these mutant strains also showed a reduction in Bl (Table 2). To better characterize the alterations in cell size and cell cycle regulation, we used flow cytometry to analyse the protein and DNA distributions in the wild-type and mutant strains during the exponential growth, using glucose and ethanol as carbon sources.

Protein distribution was determined (Baroni et al., 1989), and it was compared with the glucose-grown wild-type strain W303 (Fig. 4a). The mean total protein content (\( P_0 \)), calculated from the protein distribution, was different in the mutant strains compared with the wild-type, in all the conditions tested; compared with the wild type, \( \text{WN}1 \)

**Table 2. Growth parameters of \( cdc25 \) mutant strains at 24 °C**

<table>
<thead>
<tr>
<th>Medium</th>
<th>( t_d )</th>
<th>BI</th>
<th>( t_d )</th>
<th>BI</th>
<th>( t_d )</th>
<th>BI</th>
<th>( t_d )</th>
<th>BI</th>
<th>( t_d )</th>
<th>BI</th>
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<tbody>
<tr>
<td>YPD</td>
<td>2 ± 0.2</td>
<td>66 ± 4</td>
<td>2 ± 0.2</td>
<td>66 ± 6</td>
<td>2 ± 0.2</td>
<td>57 ± 1</td>
<td>2 ± 0.2</td>
<td>57 ± 4</td>
<td>2 ± 0.2</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>SM</td>
<td>2 ± 0.2</td>
<td>66 ± 4</td>
<td>2 ± 0.2</td>
<td>66 ± 6</td>
<td>2 ± 0.2</td>
<td>57 ± 1</td>
<td>2 ± 0.2</td>
<td>57 ± 4</td>
<td>2 ± 0.2</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>YPE</td>
<td>4 ± 0.2</td>
<td>56 ± 4</td>
<td>4 ± 0.2</td>
<td>56 ± 4</td>
<td>4 ± 0.2</td>
<td>56 ± 4</td>
<td>4 ± 0.2</td>
<td>56 ± 4</td>
<td>4 ± 0.2</td>
<td>56 ± 4</td>
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\( t_d \), duplication time (h); BI, budding index (%). SM, synthetic minimal medium supplemented with glucose.

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**Fig. 1.** Tetrad analysis and growth kinetics of the mutant strains. (a) Tetrads obtained from the dissection of mutant diploid strains showed a segregation ratio of 2:2, small versus large colonies. Tetrads were dissected, and incubated on YPD agar for 3 days. The figure shows representative tetrads obtained from the heterozygote strains indicated. (b) The mutant strains grew in YPD medium at a rate comparable to that of the wild-type strain. Cells were inoculated in YPD medium at 24 °C, and grown to early exponential phase. Cell density was plotted against time. Strains: W303 (●), W\( \text{WN}1 \) (■), W\( \text{WN}2 \) (▲), W\( \text{ACdc25}^{\text{MM}} \) (○), W\( \text{AhSos1} \) (□).

**Fig. 2.** Protein distribution was determined (Baroni et al., 1989), and it was compared with the glucose-grown wild-type strain W303 (Fig. 4a). The mean total protein content (\( P_0 \)), calculated from the protein distribution, was different in the mutant strains compared with the wild-type, in all the conditions tested; compared with the wild type, \( \text{WN}1 \)
showed higher $P_t$ values, while the other mutants had smaller $P_t$ values (Fig. 4b). Moreover, of the mutant strains, only WΔN1 was like the wild type in being able to change its mean $P_t$ in response to the nutritional conditions: the mutant strain showed a decrease of about 30% in its $P_t$ in ethanol medium compared with glucose medium. In contrast, WΔN2, WΔCdc25Mm and WΔhSos1 showed smaller $P_t$ values than the wild-type strain, and the values obtained for each strain were almost identical in the two conditions tested.

A possible cause for the different regulation of cell size and cellular protein content during exponential growth in different cultural conditions could be a defect in setting the $P_s$ according to the environmental conditions. In fact, the critical size for entry into the S-phase constitutes the main nutrient-dependent control on the cell size, and is also dependent on the Ras/cAMP pathway (Baroni et al., 1989). $P_t$ values were experimentally determined in exponentially growing populations of the different strains by using a biparametric FACS analysis of the mean $P_t$ of gated early S-phase cells (Fig. 5), according to Coccetti et al. (2004). The results confirmed the hypothesis that the mutant strains WΔN2, WΔCdc25Mm and WΔhSos1 were defective in the nutrient-dependent regulation of the $P_t$ threshold (Table 3).

Since the fraction of budded cells in the mutant strains was different from that of the wild-type, the cell distribution in the cell cycle phases was also expected to be different. FACS analysis of the DNA content distribution of the mutant strains during exponential growth on glucose (Fig. 6a) revealed that the WΔN2, WΔCdc25Mm and WΔhSos1 strains had a higher percentage of cells in G1 phase than the wild-type strain. Moreover, while the number of G1-phase
cells in the wild-type strain almost doubled in ethanol medium compared with glucose medium, the mutant strains were completely unable to perform normal carbon source-dependent regulation of the cell cycle (Fig. 6a, b). **WDN1** was the only strain to show a decrease in the fraction of cells in G1 phase in comparison with the wild-type strain.

The defects in cell size modulation and growth recovery from G0 phase suggest that, in mutants, unregulated RasGEF activity could allow cells to grow, but some defects in transitory situations are to be expected. We analysed the behaviour of the mutant strains during a growth transition, namely a nutritional shift-up, where cells growing in ethanol medium were supplemented with glucose. In the wild-type strain, a transient increase in the length of G1 phase occurs in this transition, in order for the strain to adapt to the new cultural conditions that require a higher Ps value than that required during growth on ethanol (Alberghina et al., 1998). This transition step can be monitored as a transient drop in BI, followed by recovery and adaptation to the typical BI obtained in glucose-containing medium.

**Fig. 4.** Mutant strains show a defect in nutrient-dependent cell size regulation. (a) FACS analyses of the protein content distribution of strains growing in YPD medium (filled curve) and YPE medium (open curve). (b) Samples were taken from several cultures of each strain during exponential growth in YPD medium (black bars) and YPE medium (white bars), and these were analysed by FACS in order to obtain the protein content distribution. The Pt was calculated, and the mean values from at least four independent analyses were plotted. The error bars indicate SD. A, W303; B, WDN1; C, WDN2; D, WΔCdc25Mm; E, WΔhSos1.

**Fig. 5.** Mutant strains show defects in nutrient-dependent modulation of the critical Ps. Cells were collected during exponential growth in YPD or YPE medium, and analysed by FACS with biparametric staining. FITC fluorescence was plotted versus PI fluorescence as a density plot. The scales are in arbitrary units. The arrow indicates an example of a gated region used to evaluate the Ps.
WDN1 showed normal control of this transition step, and the pattern of the BI during the nutritional shift-up was similar to that of the wild type (Fig. 7). In WDN2, WΔCdc25Mm and WΔhSos1, the BI did not show a decrease, but it steadily increased, leading to the values typical for growth on glucose.

cdc25 mutants exhibit alterations in PKA activity

An expected consequence of reduced or unregulated activity of the Cdc25 protein would be alteration of PKA activity. In order to verify this hypothesis, other phenotypic properties usually related to PKA activity were considered. The most immediate effect of abnormal PKA activity is sensitivity to heat shock: high activity correlates with high sensitivity to heat shock, and vice versa. Thus, heat-shock sensitivity of exponentially growing cells of the mutant strains was compared with the wild-type: WΔN1 showed a high sensitivity to heat shock, whilst WDN2, WΔCdc25Mm and WΔhSos1 were very resistant to heat-shock treatment (Fig. 8a).

In addition, an assay was performed to determine trehalase activity. The trehalase activity in S. cerevisiae is directly controlled by PKA phosphorylation (Wera et al., 1999). Consistent with the observations reported above, trehalase activity was higher in the WΔN1 strain, and lower in the WΔN2, WΔCdc25Mm and WΔhSos1 strains, when compared with the wild type (Fig. 8b).

This led us to propose the hypothesis that in WΔN1, the deletion of the N-terminal CDB motif could have resulted in greater accumulation of the GEF protein and/or an increase in GEF activity, leading to hyperactivation of the Ras/cAMP pathway. On the other hand, the heterologous GEFs are probably less efficient on yeast Ras. For WΔN2, the phenotype observed was similar to the heterologous GEF-producing strains; the deletion in the CDC25 locus in the WΔN2 strain resulted in a catalytic domain lacking a complete RasGEFN motif, which probably led to a basal activity of GEF that was reduced and not able to adapt to different conditions.

**Table 3. P<sub>s</sub> values during exponential growth**

P<sub>s</sub> values were calculated as mean protein content of gated early S-phase cells on the density plots obtained by biparametric FACS analysis, and expressed as relative units with respect to the value for the wild-type in YPD medium.

<table>
<thead>
<tr>
<th>Strain</th>
<th>YPD</th>
<th>YPE</th>
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<tr>
<td>W303</td>
<td>1.00</td>
<td>0.66</td>
</tr>
<tr>
<td>WΔN1</td>
<td>1.64</td>
<td>0.95</td>
</tr>
<tr>
<td>WΔN2</td>
<td>0.70</td>
<td>0.72</td>
</tr>
<tr>
<td>WΔCdc25Mm</td>
<td>0.72</td>
<td>0.50</td>
</tr>
<tr>
<td>WΔhSos1</td>
<td>0.61</td>
<td>0.62</td>
</tr>
</tbody>
</table>

WΔN1 showed normal control of this transition step, and the pattern of the BI during the nutritional shift-up was similar to that of the wild type (Fig. 7). In WΔN2, WΔCdc25Mm and WΔhSos1, the BI did not show a decrease, but it steadily increased, leading to the values typical for growth on glucose.

Overproduction of the SH3-containing N-terminal fragment of Cdc25 rescues ira1Δ strain growth and heat-stress sensitivity defects

To further characterize the role played by the large N-terminal region of Cdc25, we made several constructs that overproduced different fragments of this region (Fig. 9a). Since it has been reported that overproduction of the entire N-terminal fragment is able to rescue the heat-shock sensitivity defect of the ira1Δ strain (Chen et al., 2000), we tested for this in constructs. The two larger fragments (encoding aa 1–1100 and 1–875) were able to rescue the heat-shock sensitivity of ira1Δ cells during exponential growth (data not shown) and in stationary phase (Fig. 9b).
Moreover, overproduction of a smaller fragment (aa 876–1100) was also able to rescue the heat-shock sensitivity of \textit{ira1} \textit{D}. Interestingly, the two larger fragments, containing the SH3 domain, not only conferred normal heat-shock resistance (Fig. 9b) to the \textit{ira1} \textit{D} strain, but also higher viability to the mutant cells. In fact, the \textit{ira1} \textit{D} strain producing either of the large fragments resumed growth quickly after being spotted from an exponentially growing population, and did not show any delay in recovery from stationary phase (data not shown). Overproduction of the smaller fragment (aa 876–1100) rescued the heat-shock sensitivity defect only; the cells still required a long incubation time to recover growth.

**Overproduction of the central region of Cdc25 confers heat-shock resistance to the wild-type strain**

Since it has been suggested that the ability of the N-terminal region to rescue \textit{ira1} \textit{A} strain defects probably relies on an inhibitory effect of this domain on the Cdc25 protein itself (Chen \textit{et al.}, 2000), we looked for a similar effect in the wild-type strain when different fragments of this region were overproduced. In this background, none of the fragments tested affected the growth parameters for the wild-type strain during exponential growth on different carbon sources (data not shown). The two larger fragments (encoding aa 1–1100 and 1–875) had no effect on heat-shock sensitivity. However, the central fragments, encoding aa 353–1100 and 876–1100, conferred a slightly stronger resistance to heat shock (data not shown), indicating that these fragments are able to interfere with PKA activity, and maybe with Cdc25 activity.

**DISCUSSION**

The Cdc25 protein is an exchange factor for Ras, and its activity is essential for growth of yeast cells. Despite the fact that Cdc25 was the first Ras GEF to be identified and characterized, it remains unclear whether its activity is regulated or not, and how the Cdc25/Ras/cAMP pathway transduces the signal originating from nutrients. Cdc25 is a large protein, and the essential exchange domain is restricted to the last 300 aa, but the presence of regulatory regions has never been clearly demonstrated. However, there is increasing evidence that the Cdc25 protein could dimerize, and possibly be part of a large complex with other elements of the pathway, i.e. adenylate cyclase and Ira proteins (Mintzer & Field, 1999); in addition, Cdc25 has been found to interact with the heat-shock protein Ssa1 (Geymonat \textit{et al.}, 1998), and to be associated to

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**Fig. 7.** \textit{W}Δ\textit{N}2, \textit{W}Δ\textit{Cdc25} \textit{Mm} and \textit{W}Δ\textit{hSos1} mutant strains show a rapid increase in Bi during a nutritional shift-up. Glucose was added to a final concentration of 2% (w/v) to cultures growing exponentially in YPE medium. The Bi was monitored as a function of time. (a) \textit{W}303 (■) and \textit{W}Δ\textit{N}1 mutant (△), (b) \textit{W}303 (■) and \textit{W}Δ\textit{N}2 mutant (△), (c) \textit{W}303 (■) and \textit{W}Δ\textit{Cdc25} \textit{Mm} mutant (△), (d) \textit{W}303 (■) and \textit{W}Δ\textit{hSos1} (△).
Cdc25 is required for cell size regulation

Fig. 8. Analyses of PKA-activity-related phenotypes in the mutant strains. (a) Heat-shock resistance in exponentially growing cells. Cells were incubated in YPD medium to exponential phase, and then exposed to heat shock at 51 °C for 0, 1.5, 2 and 3 min. Approximately 10⁴ cells were spotted on YPD agar, and incubated at 30 °C for 3 days. (b) CDC25 mutants have different trehalase activity levels during the exponential growth phase. Samples of cells growing in YEP medium supplemented with 2% glycerol, at 30 °C, were assayed for trehalase activity. The results shown are the means of at least three experiments. The error bars indicate SD. A, W303; B, WN1; C, WN2; D, WΔCdc25Mm; E, WΔhSos1.

several different large protein complexes (Ho et al., 2002; Archambault et al., 2004).

Following several contradictory reports stating either the necessity (Munder & Küntzel, 1989; Van Aelst et al., 1990; Schomero et al., 1990; Van Aelst et al., 1991) or the dispensability (Goldberg et al., 1994) of Cdc25 for Ras activation upon glucose stimulation, Cdc25 has recently been identified as necessary for Ras2 GTP loading after glucose addition (Colombo et al., 2004). Furthermore, the N-terminal domain of Cdc25 was initially proposed to be involved (Munder & Küntzel, 1989; Schomero et al., 1990) and not involved (Van Aelst et al., 1990) in the glucose-induced cAMP response; however, recently, a negative regulatory role has been suggested for this region (Chen et al., 2000).

In order to investigate the role played by the putative regulatory domains of Cdc25 in the nutrient-sensing mechanism, we constructed several strains in which (1) Cdc25 was completely substituted by heterologous GEF domains, which were probably insensitive to any yeast regulatory mechanisms, but able to rescue the severe phenotype of CDC25 deletion; and (2) the N-terminal portion of Cdc25 was deleted, up to either the 906 aa position or the 1146 aa position.

All the mutant strains showed growth parameters that were almost normal in both glucose- and ethanol-containing media (Fig. 1b, Table 2), and the strains were able to arrest in stationary phase upon nitrogen starvation, producing a very low percentage of budded cells. In addition, all the mutant strains were able to grow in minimal medium containing acetate, glycerol or ethanol, showing that the N-terminal portion of Cdc25 was not required for growth on either glucose or non-fermentable carbon sources; these findings contradict a report by Munder et al. (1988). Moreover, our results indicate that the CDC25 requirement for growth can be substituted by RasGEF activity. In fact, our strains with a full deletion of the CDC25 gene, and producing the GEF domain of RasGRF1/Cdc25Mm or human Sos1, grew as well as the wild-type, and were able to enter stationary phase as normal at the end of exponential growth.

However, all the mutants presented a delay in spore germination (Fig. 1a); this could not be explained by a growth defect, since the duplication time was not significantly different in the mutants strains, or by the slight delay observed in growth recovery from stationary phase (Fig. 2) or nitrogen starvation (data not shown). These findings clearly indicate that a complete Cdc25 protein is required for normal spore germination, and that it may also play a role in re-entry of the cell into the cell cycle.

Although all the mutant strains grew as well as the wild-type strain in all the conditions tested, nevertheless they presented some peculiar characteristics during exponential growth. Flow cytometry analysis demonstrated that WN2, WΔCdc25Mm and WΔhSos1 were not able to modulate cell size and P₁ in different nutrients. In fact, each of these strains had the same P₁ in glucose and ethanol, whereas a wild-type strain normally modulates its P₁ in a nutrient-dependent manner (Table 3). In the mutants, this defect in P₁ modulation was accompanied by abnormal regulation of the cell cycle, as indicated by the increase of G₁-phase cells (Fig. 6b). The inability to regulate cell size in response to different nutrients is also in agreement with the behaviour of these mutant strains during a nutritional shift-up (Fig. 7). These defects have also been shown to be accompanied by phenotypic traits that are typical of reduced PKA activity (small cell volume, heat-shock resistance, and low trehalase activity) (Baroni et al., 1989; Mitsuzawa, 1994; Martegani...
et al., 1984; Wera et al., 1999). WN1 was able to maintain the ability to modulate cell size and P, in response to nutrients, but, in all the conditions tested, it presented an increase in protein content compared with the wild-type, and showed several phenotypic traits consistent with a hyperactivated Ras/cAMP pathway (large volume, heat-shock sensitivity and high trehalase activity).

These results indicate that the Cdc25 N-terminal region exerts a regulatory role, and is required for a normal glucose-sensing mechanism, at least for the nutrient-dependent cell size regulation typical of normal yeast cells. In addition to this, the marked difference between WN2 and WN1 strains suggests that the region between aa 907 and 1146 could be important for exchange activity and nutrient sensing.

To further characterize the role played by the large N-terminal region of Cdc25, several constructs were prepared that overproduced different fragments of this region. Overproduction of the fragments caused no visible alterations to growth, cell size and cell cycle regulation. A possible explanation for the weakness of the phenotypes generated by the overproduction of the N-terminal regions of Cdc25 could be that these domains need to be linked to an active RasGEF domain in order to exert their regulatory functions. Alternatively, perhaps the overproduced fragments could not properly localize in the specific cellular region (membranes?) where the whole Cdc25 protein is localized, and therefore were not able to interact properly with the endogenous Cdc25 GEF domain. The existence of an organized complex containing Cdc25 could easily explain the weak dominant-negative effect of the large N-terminal fragment spanning aa 1–875, which improved heat-shock resistance and survival of ira1D mutants; this fragment spans important protein–protein interaction sites (SH3 domain), and could destabilize the complex by competing for their binding sites. The aa 875–1100 region conferred an increase in heat-shock resistance in wild-type cells; this region does not contain any known domain, but it appears to be as well conserved as the catalytic domain in several fungal RasGEF proteins (data not shown). This finding reinforces the hypothesis that the aa 875–1100 region could play an important role in the regulation of the GEF activity, as suggested by our results.

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REFERENCES


