Phosphorylation of Cdc28 and regulation of cell size by the protein kinase CKII in Saccharomyces cerevisiae

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INTRODUCTION

Cyclin-dependent kinases (CDKs) are critical protein kinases regulating mitosis in eukaryotic cells. In the budding yeast, Saccharomyces cerevisiae, the major CDK involved in cell division cycle regulation is encoded by the CDC28 gene [1], whereas its homologue cdc-2 is present in fission yeast Schizosaccharomyces pombe [2]. In yeasts, the Cdc28/Cdc2 protein kinase is required at two crucial points: at START, when the cells become committed to entry into S phase, and at the G1–M transition [3–6]. A family of Cdc28/Cdc2 kinases exists in mammalian cells (CDKs) to control cell-cycle progression, differentiation and transformation [7–9].

Genetic analysis of the cell-cycle regulation in S. cerevisiae and S. pombe has revealed that the two species differ in several aspects of cell-cycle control. As in mammalian cells, S. cerevisiae regulates entry into the cell cycle at G1, and it is during this phase that S. cerevisiae monitors its cell size and/or environmental conditions, such as levels of nutrients. During nutritional deprivation, the G1 phase is elongated [4,6]. In contrast, in rapidly growing S. pombe, the G1 phase is very short, and information regarding cell size and nutrition is mainly regulated at the G1–M transition [5]. In S. cerevisiae, START arrests have been classified according to whether arrested cells continue active growth (e.g. in the presence of mating pheromones: START-I) or become quiescent, as in the case of nutrient starvation (START-II) [4]. Many START-II arrests are dependent on low levels of cAMP, which reduce both cAMP-dependent phosphorylation of Cdc28 and its binding to p40<sup>ppi</sup>, and the synthesis of G<sub>1</sub> cyclins [4,10–13].

The activity of Cdc28/Cdc2 is regulated by cell-cycle-specific association with regulatory molecules called cyclins, and by phosphorylation [14]. G<sub>1</sub> cyclins (encoded by the CLN1, CLN2 and CLN3 genes) are essential for progression through START, while B-type cyclins (encoded by genes CLB1 to CLB6) appear to be necessary for entry into S phase and for mitosis [15]. The oscillation between low and high Cib/Cdc28 kinase activity governs the budding-yeast cell cycle, although evidence for a new, independent G<sub>1</sub> oscillator has been presented recently [16]. These two transitions are linked by regulated ubiquitin-dependent proteolysis of key molecules, such as the Cdc28 inhibitor, Sic1, and some regulators of the APC (anaphase-promoting factors complex) [15,17].

Previously, we have identified and characterized a novel Cdc2 phosphorylation site at residue Ser-39, which is specifically phosphorylated in vivo during the G<sub>1</sub> phase of the HeLa cell cycle by CKII (formerly known as casein kinase II) [18]. Protein kinase CKII is a well-characterized kinase that is present ubiquitously in all tissues and cell lines investigated to date [19,20]. The enzyme exists as a tetramer composed of two catalytic subunits (z and z<sup>′</sup>), and two regulatory subunits (β) differently assembled among them [21].

Data from a large number of studies suggest an involvement of CKII in signal transduction, and its activity changes following stimulation by a variety of extracellular signals [19]. In S. cerevisiae, CKII is composed of at least four different subunits, z,
α’, β and β’, which are encoded by four distinct genes, CKA1, CKA2, CKB1 and CKB2 respectively (reviewed in [22]). Glover and colleagues [22] showed that deletion of the gene for either of the catalytic subunits alone has little effect, but deletion of both subunits results in a loss of viability that is characterized by a substantially increased size prior to growth arrest [23]. Using temperature-sensitive alleles of the CKA2 gene, Hanna et al. [24] demonstrated that S. cerevisiae CKI is required for cell-cycle progression during G1 phase prior to DNA synthesis, and also at the G1-M phase, providing further evidence for CKII involvement in growth control.

Analogous to human Cdc2, S. cerevisiae Cdc28 contains a serine residue (Ser-46) embedded in a consensus sequence for CKII phosphorylation. An 18-amino-acid sequence of Cdc28 containing this serine is nearly identical with the human Cdc2 sequence: the only difference is a conservative amino acid exchange of glutamate for aspartate at residue 48 in Cdc28. Ser-46 is a target for CKII in growth control. In the present paper, we show that Cdc28 is phosphorylated at Ser-46 in vitro by CKII, and that this site is also phosphorylated in vivo. The mutant CDC28-S46A, in which the serine is replaced by alanine, exhibited a histone H1 kinase activity (Cdc28), and a growth rate similar to that of wild-type cells. However, the S46A mutation resulted in a decrease of 33% in cell size. This result demonstrates a functional role for the phosphorylation of Cdc28 by CKII in S. cerevisiae cell-size control.

**EXPERIMENTAL**

**Strains and media**

The relevant genotypes of strains used in the present study are as follows: SY1061 (MATa CDC28::HIS3[WT CDC28-HA] ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100); CY3086 (MATa CDC28::HIS3[WT CDC28 on YCp50] ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100); SY129 (MATa CDC28::HIS3[WT CDC28 in pRS415] ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100); SY130 (MATa CDC28::HIS3[CDC28-S46A in pRS415] ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100).

Transformations were made using the lithium acetate procedure [25]. YPD medium consisted of 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) d-glucose. Standard genetic methods for strain construction in S. cerevisiae were as reported previously [25].

**Site-specific mutagenesis and construction of the CDC28-S46A allele**

To mutate residue Ser-46 of Cdc28 to alanine, a 2.4 kb PvuII-ClaI fragment containing the entire CDC28 open reading frame [1] was obtained from plasmid CB1503 (CDC28 in YEp213), and cloned into the Smal site of plasmid pRS415, provided by Dr J. Kuret (Ohio State University, Columbus, OH, U.S.A.) according to standard techniques [26]. Site-directed mutagenesis of the resulting clone (pRS-CDC28) was performed by the method of Zoller and Smith [26] using uracil-containing single-stranded DNA. The oligonucleotide used for mutagenesis of Ser-46 to alanine was DNA. The oligonucleotide used for mutagenesis of Ser-46 to alanine was sequenced by the dideoxy method using a Sequenase kit (United States Biochemical Corp., Cleveland, OH, U.S.A.).

The wild-type (pRS-CDC28) and mutagenized (pRS-CDC28-S46A) plasmids were transformed into strain CY3086. Loss of the YCp50 plasmid containing wild-type CDC28 was selected for by plating transformants on to 5-fluoro-orotic acid [25]. The resulting strains, SY129 and SY130, contained wild-type CDC28 and CDC28-S46A on pRS415 respectively.

**Preparation of cell extracts, p13-Sepharose bead precipitation and immunoblotting**

Exponentially growing cells were harvested and lysed as reported previously [27] using buffer B [100 mM Tris/HCl (pH 7.5)/200 mM NaCl/1 mM EDTA/5% (v/v) glycerol/0.5 mM dithiothreitol/1 mM PMSF/1 µg/ml each of chymostatin, leupeptin, antipain and pepstatin/1 mM sodium pyrophosphate/1 mM sodium vanadate/10 mM sodium fluoride/20 mM p-nitrophenyl phosphate], and the protein concentration of the extracts was determined. To pre-clear cellular extracts, 0.25 mg of total proteins in a final vol. of 1 ml was incubated with 40 µl of BSA–Sepharose beads at 4 °C, with gentle rocking for 4 h. The suspension was centrifuged for 5 min at 13000 g, and 15 µl of p13-Sepharose–Sepharose beads [28] was added to each supernatant, followed by a 4 h incubation at 4 °C. Finally, beads were washed three times in buffer B, and once in 20 mM Tris/HCl, pH 8. An equal volume of 2 x gel-sample buffer was added to each sample; these were then heated for 3 min at 95 °C. The supernatants were subjected to electrophoresis in SDS/PAGE (12% gels). The separated proteins were analysed by immunoblotting [29]. Anti-phosphotyrosine polyclonal antibody (B2; a gift from Dr A. Rossomando, Bayer Pharmaceutical, West Haven, CT, U.S.A.) was used at a dilution of 1:1500, and an enhanced chemiluminescence system (ECL*, Amersham Pharmacia Biotech, Uppsala, Sweden) was used for immunoblot detection according to the manufacturer’s instructions. Cdc28 was evidenced using a specific antiserum (antibody Ab182; generously given by Dr B. Futcher, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.) at a dilution of 1:1000. Specific bands were detected using sheep anti-rabbit immunoglobulin F(ab’), conjugated to alkaline phosphatase (Sigma, St Louis, MO, U.S.A.) as a secondary antibody (at the dilution recommended by the manufacturer), or by the ECL* system.

**In vitro kinase assays**

Phosphorylation of peptide CSH247 (KKIRLESEEEGVPST- AIR; residues shown by the single-letter amino acid code, spanning Lys-33 to Arg-50 of human Cdc2) by CKII from strain SY129 was tested as follows: cells were grown in 50 ml of YPD medium to mid-exponential phase, harvested, washed once in ice-cold distilled water, once in 10 ml of lysis buffer A [30 mM Tris/HCl (pH 8.0)/350 mM NaCl/0.5 mM EDTA/0.5 mM dithiothreitol/10% (v/v) glycerol/0.2 mM PMSF]), broken by vortex-mixing using glass beads, and then CKII was partially purified as described previously [30]. The enzymic activity was performed using peptide CSH247 as the substrate. Increasing amounts of enzyme were added to a reaction mixture of 50 µl containing 10 µM [γ-32P]ATP (20000 c.p.m./pmol); 320 µM peptide in CKII reaction buffer [50 mM Tris/HCl (pH 8)/150 mM NaCl/10 mM MgCl2]. The reaction was incubated for 30 min at 30 °C, and the incorporation of phosphorus was determined by adsorption of aliquots of the reaction mixture on phosphocellulose paper (P81; Whatman International Ltd, Maidstone, Kent, U.K.) according to procedures described previously [31]. To map CKII phosphorylation site(s) on CSH247, free ATP was eliminated from the reaction mixture, and the radioactive sequence of phosphopeptide was obtained as described previously [18].
In vitro phosphorylation of Cdc28 was performed using strain SY1061 carrying Cdc28 fused to an epitope tag (Cdc28-HA) derived from the HA protein of influenza virus. Yeast cell extracts (0.450 mg) were prepared as described above. They were diluted to 0.85 ml with buffer B, and, after a pre-clearing step of 1 h in the presence of 30 μl of Protein G-Sepharose (1:1 suspension; Amersham Pharmacia Biotech), incubated for 1 h at 4°C after addition of 3 μg of 12CA5 monoclonal antibody (raised against the epitope peptide) affinity-purified on a Protein G-Sepharose column, following treatment with 40 μl of Protein G-Sepharose to precipitate the immunocomplex. The immunocomplexes obtained after removal of supernatants were washed three times in buffer B and three times in CKII reaction buffer, as described above. The reaction mixture contained, in a total volume of 50 μl, 10 μM [γ-32P]ATP (20000 c.p.m./pmol) and 2 μl (46 μg/ml) of CKII purified to homogeneity from bovine testis [32]. Heparin (Sigma) was added in some experiments to a final concentration of 2 μg/ml. After incubation for 1 h at 30°C, reactions were terminated by the addition of 60 μl of 2× sample buffer to the beads after removal of the reaction mixtures. Finally, the samples were heated for 5 min at 95°C, electrophoresed on SDS/12% polyacrylamide gels, and the dried gel was then subjected to autoradiography. In some experiments, the gel was subjected to immunoblotting in order to localize the band phosphorylated by CKII and corresponding to Cdc28-HA.

In vivo labelling, peptide mapping and phosphoamino acid analysis

For [32P]P, labelling of proteins, strains SY129 and SY130 were grown in 100 ml of YPD(−) medium (YPD depleted of Pi; [27]), and absorbance at 600 nm (A600) was measured in a UV–visible spectrophotometer (Amersham Pharmacia Biotech). At an A600 of approx. 0.5, the cells from each culture were harvested by centrifugation, resuspended in 15 ml of YPD(−) medium, and divided into two aliquots: one was treated with 5 mCi of [32P]P, (NEX-053; New England Nuclear, Boston, MA, U.S.A.), whereas the other represented the unlabelled control. After labelling for 70–80 min at 30°C, cell extracts were prepared as described above, and Cdc28 was precipitated with 60 μl of p13-Sepharose beads. Before the addition of 2× sample buffer and electrophoresis, beads were treated with 2.5 μg of RNase and 20 μg of DNase, followed by extensive washes in buffer B.

To recover the phospholabelled Cdc28 and Cdc28-S46A after electrophoresis, the wet gel was exposed to X-ray film for several hours at room temperature. Cdc28 was identified by alignment with the autoradiogram, and by comparison with immunoblotting of unlabelled samples. The Cdc28-containing band was excised and eluted as described previously [33]. Unlabelled samples were treated exactly as labelled samples, and Cdc28 was identified by immunoblotting. Cdc28 was subjected to tryptic peptide mapping and phosphoamino acid analysis, as reported previously [18,33].

Cdc28 kinase assay

Cdc28 was precipitated as described above and the catalytic activity associated with the beads was measured as reported previously [34]. Briefly, assays contained, in a final volume of 30 μl, 10 μM [γ-32P]ATP (20000 c.p.m./pmol) and 1 mM peptide CSH103 (ADAQHAATPPKKKKRVEDPKDF) representing a sequence of simian-virus-40 large T antigen, modified as described previously [34]. The reaction was incubated for 30 min at 30°C, and the incorporation of phosphate was determined using P81 paper, as reported above for CKII kinase assay.

Measurement of cell volumes and FACS analysis

Cells were grown in yeast nitrogen base (YNB)–glucose medium at a start-up density of 1×10^6 cells/ml, in a roller at 30°C to mid-exponential phase. Cultures were then placed on ice and briefly sonicated. The proliferation rate was monitored as the increase in cell number, determined with a Coulter Counter ZBI (Coulter Electronics Inc., Hialeah, FL, U.S.A.). The mean cell volume was estimated from the cell-volume distribution obtained by a Coulter Channalyzer C-1000, as described before [35]. The percentage of budded cells was determined by microscopy counting on at least 400 cells that were fixed in 4% (v/v) formalin and mildly sonicated. Flow-cytometric analysis of the yeast population was usually performed on a Coulter Epics 752 apparatus, after staining with FITC for protein content, as described previously [36]. Protein content distribution was obtained with a FACS IV cytofluorimeter (Becton–Dickinson, Franklin Lakes, NJ, U.S.A.) as described before [36].

Digital figures

Original data presented below in Figures 2 and 3 were digitally generated after being scanned in (HP ScanJet IIcx; resolution 300 d.p.i.) and processed using the following software: Microsoft PowerPoint 97 and Adobe Photoshop 4.0.

RESULTS

In vitro phosphorylation of Cdc28 by CKII

Residues 45–49 of Cdc28 contain a canonical CKII phosphorylation site on human Cdc2 (Figure 1A; [18]). To investigate whether CKII from S. cerevisiae is able to phosphorylate residue Ser-46 of Cdc28, we tested the ability of CKII from S. cerevisiae to phosphorylate a peptide (CSH247) that contains the relevant sequence. CKII was purified from strain SY129 containing wild-type CDC28, and its activity was measured using peptide CSH1247 as a substrate, as described in the Experimental section. The yeast CKII [30] was able to phosphorylate peptide CSH247, exhibiting a K_m value of 900 μM, identical with that calculated for the human enzyme [18].

Mapping of the phosphorylation site(s) on CSH247 identified the serine residue at position 7, corresponding to Ser-46 in S. cerevisiae, as the phosphorylated residue (Figure 1B).

To verify further that CKII was able to catalyse phosphorylation of Cdc28 in vitro, we immunoprecipitated Cdc28-HA from a cell lysate of S. cerevisiae, and phosphorylated the precipitated protein using exogenous CKII enzyme purified from a bovine source[32]. The autoradiogram in Figure 2(A) shows that Cdc28-HA is phosphorylated in the presence of CKII (lane 2), and not phosphorylated when heparin, an inhibitor of CKII activity [37], was added to the reaction (lane 3). To confirm that the band phosphorylated by CKII corresponded to Cdc28-HA, a control immunoblotting experiment was performed on the same samples (Figure 2B).

We have shown that a peptide containing a sequence from human Cdc2 can be phosphorylated by S. cerevisiae CKII at a residue corresponding to Ser-46 in Cdc28; in addition, Cdc28 is a substrate of bovine CKII in vitro. Our data in vitro suggest that residue Ser-46 of S. cerevisiae Cdc28 is a potential phosphorylation site in vitro.
Figure 1 CKII phosphorylation site on Cdc28

(A) Homology between human Cdc2 and S. cerevisiae Cdc28 in the region containing the CKII phosphorylation site. (B) Radioactive sequence analysis of peptide CSH247 phosphorylated in vitro using CKII purified from strain SY129. Ser-46 on Cdc28 is embedded in a sequence highly homologous with that surrounding Ser-39 of human Cdc2, and a peptide containing this sequence is phosphorylated by S. cerevisiae CKII in vitro. Abbreviation: A, alanine.

In vivo phosphorylation of Cdc28

To investigate if residue Ser-46 was also phosphorylated in vivo, strains SY129 and SY130 were labelled in vivo with \[^{32}P\]P\(_i\), and the phosphorylation state of Cdc28 and Cdc28-S46A was examined by precipitation with p13\(^{32}P\)–Sepharose beads (Figure 3A). The two-dimensional phosphopeptide maps obtained (Figures 3B and 3C) show very similar patterns. However, two phosphopeptides (indicated by arrowheads in Figure 3B) are present in wild-type Cdc28, but not in the Cdc28-S46A mutant. These spots are likely to correspond to peptides phosphorylated on Ser-46. The presence of two spots is probably derived from the partial proteolytic degradation of the protein; in fact, the region of Cdc28 spanning from residues 40 to 57 (including Ser-46) contains four possible sites for trypsin digestion (Lys-40, Lys-41, Arg-43 and Arg-57), increasing the probability of partial degradation. Partial tryptic digestion was described for the homologous region in human Cdc2, yielding spots in a similar relative position [18].

Using phosphoamino acid analysis of Cdc28 isolated from strains SY129 and SY130, we detected no phosphoserine in strain SY130 carrying the \(CDC28-S46A\) allele (Figure 3D), suggesting that the point mutation introduced (Ser-46 to alanine) abolished all serine phosphorylation in Cdc28. After prolonged exposure, a faint spot corresponding to phosphothreonine was detected in both wild-type Cdc28 and Cdc28-S46A (results not shown).

Characterization of Cdc28 and Cdc28-S46A

To investigate the effect of the \(cdu28-S46A\) mutation, we compared the kinase activities, the protein expression levels, the levels of tyrosine phosphorylation and the cyclin A-binding activity of Cdc28 and Cdc28-S46A in strains SY129 and SY130 respectively. First, we examined whether the mutation had an effect on Cdc28 kinase activity, and no significant difference in kinase activity was measured comparing strains SY129 and SY130, using the peptide as substrate (Table 1). Similar results were obtained employing histone H1 as substrate (results not shown). Secondly, wild-type Cdc28 and Cdc28-S46A were precipitated and subjected to immunoblotting analysis, as described in the Experimental section. Nitrocellulose membrane was probed with anti-Cdc28 polyclonal antibody, and bands of similar intensity were detected for both Cdc28 and Cdc28-S46A, corresponding to an apparent \(M_r\) of 34000 (results not shown), indicating similar levels of protein. Thirdly, to test if the point mutation introduced affected tyrosine phosphorylation levels on Cdc28, the membrane was probed with anti-phosphotyrosine antibodies. For both Cdc28 and Cdc28-S46A, bands of similar intensity were detected (results not shown), indicating similar tyrosine phosphorylation levels for the wild-type and mutant. This observation suggests that the \(CDC28-S46A\) mutation does not affect Tyr-19 phosphorylation. Finally, we used glutathione S-transferase (GST)–cyclin A cross-linked to Sepharose to pre-
Figure 3  In vivo $^{32}$P labeling of Cdc28 and Cdc28-S46A

(A) Autoradiography of phosphorylated wild-type Cdc28 (lane 1) and Cdc28-S46A (lane 2) after precipitation with p13Suc1-Sepharose beads. Lanes 3 and 4, immunoblots of control unlabelled experiments for Cdc28 and Cdc28-S46A respectively. (B) and (C) Tryptic phosphopeptide maps of Cdc28 and Cdc28-S46A respectively. For both experiments, 600 c.p.m. (Cerenkov) were loaded on cellulose plates. The exposure time was 10 days at $-70^\circ$C. (D) Phosphoamino acid analysis of Cdc28 (lane 1) and Cdc28-S46A (lane 2). The signal for phosphotyrosine was visible only after prolonged exposure (results not shown). No phosphoserine was detected in Cdc28-S46A.

Table 1  Catalytic activity of Cdc28 and Cdc28-S46A

Cdc28 and Cdc28-S46A exhibit similar catalytic activities. Cdc28 and Cdc28-S46A were precipitated from strains SY129 and SY130 respectively using p13Suc1-Sepharose beads, or BSA-Sepharose as a control (ctrl). The complexes obtained were tested for kinase activity towards CSH103 peptide. The values shown are the means for two duplicates in a representative experiment (values differed by less than 10%). This experiment was performed three times with similar results.

<table>
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<tr>
<th>Substrate</th>
<th>Kinase activity [arbitrary units (c.p.m. $\times 10^{-5}$)]</th>
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<tr>
<td>Cdc28</td>
<td>3.800</td>
</tr>
<tr>
<td>Cdc28-S46A</td>
<td>4.000</td>
</tr>
<tr>
<td>Cdc28 ctrl</td>
<td>0.067</td>
</tr>
<tr>
<td>Cdc28-S46A ctrl</td>
<td>0.036</td>
</tr>
</tbody>
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To precipitate Cdc28 from strains SY129 and SY130, and compared the histone H1 kinase activity of wild-type Cdc28 with that of the mutant. In fact, the amino acid cluster surrounding Ser-39 in human Cdc2 is essential for cyclin A binding and kinase activity [38,39]. The kinase activity of the Cdc28-S46A mutant was similar to that of the wild-type (results not shown).

In summary, the Cdc28-S46A mutant is therefore similar to the wild-type protein with regard to the total amount of protein expressed, its catalytic activity, the level of tyrosine phosphorylation and its effect on cyclin binding.

Effect of the CDC28-S46A mutation on cell size

To determine the functional relevance of Ser-46 phosphorylation in Cdc28, we looked for phenotypical differences between SY129 (wild-type) and SY130 (S46A mutant) strains. We found no difference in sensitivity to heat shock, UV irradiation, or to the mating pheromone $a$ factor. In addition, we did not observe any changes in bud morphology in strain SY130 compared with wild-type (results not shown). However, we did find a decrease in cell size for the mutant strain, SY130, as compared with the wild-type. Whereas the effect was modest for exponentially growing cells (Figure 4A), it was pronounced in cells grown to stationary phase (Figure 4B). Growth of strains SY129 and SY130 was monitored at different times, in terms of both cell number and the percentage of budded cells. No significant differences were reported. However, the cell size analysis indicated that, at stationary phase, the mutant cells (Cdc28-S46A in Figure 4B) were greatly reduced in cell size (33 %) compared with those of the control strain (Cdc28, Figure 4B). The difference was found to apply to both small and big cells (compare the left- and right-hand parts of the graph in Figure 4B). This phenotype resembles that of cells with a defect in the Ras/cAMP signalling pathway [11,35,36]; however, in those mutants an altered cell size can be readily observed too during the exponential phase of growth. To assess whether the significant difference in size was due to...
Figure 4  Growth parameters of asynchronously growing SY129 (CDC28) and SY130 (CDC28-S46A) strains

Strains were inoculated in YNB-glucose at a starting density of $1 \times 10^5$ cells/ml at 30 °C. Samples were taken at the indicated time points, and processed for cell-volume distributions and protein-content measurements (FACS). (A) Cell-volume distributions during exponential-phase growth: Cdc28 (—), Cdc28-S46A (⋯⋯⋯). (B) Cell-volume distributions in stationary-phase growth: Cdc28 (—), Cdc28-S46A (⋯⋯⋯). (C) Protein flow-cytometric analysis of Cdc28 and Cdc28-S46A grown to mid-exponential phase, and stained with FITC. (D) Protein flow-cytometric analysis of Cdc28 and Cdc28-S46A in the stationary phase.

changes in biomass, protein content of the cells was directly measured by flow cytometry. Cell-protein distributions during the exponential-growth stage and the stationary phase are shown in Figures 4(C) and 4(D) respectively. For the first case, no significant differences were observed, whereas starved wild-type cells (Cdc28, in Figure 4D) were about 25%, larger than those of CDC28-S46A. This result indicates that, towards the end of the exponential growth phase (3–5 h), the CDC28-S46A mutant shows the same difference observed in cell-size measurement (Figure 4B). In addition, we did not detect any differences in DNA distribution between the mutant strain and wild-type in either growth condition, i.e. the exponential growth phase or the stationary one (results not shown).

DISCUSSION

The role of CKII in the cell division cycle and normal cell physiology has been intensely studied, and S. cerevisiae has been a useful model to this end. The holoenzyme is composed of four subunits encoded by four different genes: CKA1, CKA2, CKB1 and CKB2 (reviewed in [22]). At the restrictive temperature, the temperature-sensitive mutant, cka2ts, exhibits defects in both G1–S and G2–M transitions [24]. These observations, together with our previous finding that the cell-cycle-regulated kinase Cdc2 is phosphorylated by CKII in mammalian cells [18], led us to examine the role of CKII phosphorylation in yeast cell-size control.

We present evidence that Cdc28 from S. cerevisiae is phosphorylated in vivo at residue Ser-46, the same site that is phosphorylated in vitro by CKII. The mutation of this residue to alanine resulted in a significant decrease in cell volume and protein distribution, both in cells at stationary phase and in the late stage of exponential growth. This is the first demonstration of a cellular phenotype associated with phosphorylation of a serine on a cyclin-dependent kinase.

The observation that Cdc28 is a substrate for CKII in vitro was obtained by two separate methods. First, a synthetic peptide encompassing the region of Cdc28 containing the putative CKII consensus site was phosphorylated by CKII purified from S. cerevisiae. Secondly, epitope-tagged Cdc28 (Cdc28-HA) was immunoprecipitated from yeast cell (strain SY106) lysates, and was found to be a substrate of pure bovine CKII in vitro.
Phosphoamino acid analysis of Cdc28 isolated from yeast indicates that the only serine residue phosphorylated in vivo is Ser-46. This is the first demonstration of phosphorylation on a specific serine residue in S. cerevisiae Cdc28. The data on in vitro and in vivo phosphorylation of Cdc28 confirm the results we obtained previously for Cdc2 in HeLa cells [18]. In both cases, the canonical CKII consensus site, Ser-46 for Cdc28 and Ser-39 for Cdc2, was phosphorylated in vivo. Previous studies have shown that Cdc28 is a phosphoprotein containing phosphoserine, -tyrosine and -threonine residues [44]. Subsequently, shown that Cdc28 is a phosphoprotein containing phospho-specific serine residue in Ser-46. This is the first demonstration of phosphorylation on a the canonical CKII consensus site, Ser-46 for Cdc28 and Ser-39 and Thr-18 is also phosphorylated in exponentially growing cells [45]. Thus four amino acid residues have been shown to be phosphorylated in vivo in S. cerevisiae; that is, Tyr-19, Thr-18, Ser-46 and Thr-169. However, the tryptic phosphopeptide maps of wild-type Cdc28 show an extremely complex pattern ([45]; also see Figure 3) compared with that reported for fission yeast Cdc2 [48] or vertebrate Cdc2 [18,49,50], suggesting that the regulation of Cdc28 by phosphorylation might have further complexity.

The small-cell-size phenotype associated with the Ser-46 mutation to alanine, reported here, is the first demonstration of a functional relationship between CKII and Cdc28. During exponential growth, the mutant strain SY130 showed a slight decrease in cell size of $\approx 10\%$ with respect to the wild-type strain. This difference reached 33 $\%$ between the mutant and wild-type when the size of cells grown to stationary phase was measured. FACS analysis of protein distribution in strains SY130 and SY129 confirmed the cell-size observations. The agreement between cell-size measurement and protein-distribution analysis is consistent with an actual decrease in cell size, and excludes the possibility of interfering phenomena, such as differential vacuole formation.

The phenotype of CDC28-S46A is similar to that of cdc25-1, a mutant with a partially defective AMP pathway [35,36]. In the stationary phase, the deprivation of nutrients induces cell-cycle arrest at a point known as START [4,5], which involves the Ras/cAMP signalling pathway [11,12,35]. The cdc25-1 mutant, which is temperature-sensitive but "leaky" at the permissive temperature, shows a longer G1/unbudded phase of the cell cycle and has a smaller critical size required for budding without changing the growth rate [35]. We observed the same phenotype in the CDC28-S46A mutant.

To explain the role of Ser-46 phosphorylation in controlling S. cerevisiae cell size, we propose the following model. Phosphorylation of Ser-46 might have a negative effect on the activation of Cdc28 by cyclin, albeit not necessarily altering cyclin binding in itself. The Ala-46 mutant, which remains unphosphorylated at this site, would enable more, and/or an earlier appearance of, sufficient quantities of active Cdc28 kinase to trigger G1/S progression prior to the cell reaching normal size. We cannot exclude the possibility that, additionally, the CKII and cAMP pathway could interact to account for the data, although no direct correlation has yet been found between CKII kinase and cAMP-dependent events.

It is interesting that the loss of the CKII phosphorylation site on Cdc28 yields small cells, whereas deletion of both CKII catalytic subunits is lethal, and the cells arrested display a greatly enlarged morphology [23]. Because the CDC28-S46A mutant is defective in one specific site on a single substrate (Cdc28) of CKII, these results together indicate that there are other substrates of CKII involved in the regulation of cell size in budding yeast. In fact, recent work has demonstrated that conditional alleles of cka2 + arrest within a single cell cycle, exhibiting a dual-arrest phenotype consisting of 50 $\%$ un budded and 50 $\%$ large-budded cells [22]. The unbudded population of cells showed a clear G1 arrest either at START, or more likely at the G1-S transition. Thus CKII plays multiple roles in cell-size regulation, including a major function at START or G1-S, probably involving Cdc28.

In S. pombe, Cdc2 lacks a CKII phosphorylation site and has only one gene for the CKII catalytic subunit. Mutations in the S. pombe CKII a-subunit gene (orb-5) lead to an alteration in cell polarity without any clear effect on cell growth [51], similar to the situation found with cka2 + in S. cerevisiae. Unlike S. cerevisiae, S. pombe regulates progression through the cell cycle in response to cell size primarily in G1, and not in G0 [5], so it might not require phosphorylation of Cdc2 by CKII.

In summary, the studies described here contribute to the concept of a multi-functional role for CKII in budding yeast. In S. cerevisiae, CKII appears to regulate cell-cycle progression and cell size by catalysing the phosphorylation of Cdc28.

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