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

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The CDK (cyclin-dependent kinase) family of enzymes is required for the G₁-to-S-phase and G₂-to-M-phase transitions during the cell-division cycle of eukaryotes. We have shown previously that the protein kinase CKII catalyses the phosphorylation of Ser-39 in Cdc2 during the G₁ phase of the HeLa cell-division cycle [Russo, Vandenberg, Yu, Bae, Franza and Marshak (1992) J. Biol. Chem. 267, 20317–20325]. To identify a functional role for this phosphorylation, we have studied the homologous enzymes in the budding yeast *Saccharomyces cerevisiae*. The *S. cerevisiae* homologue of Cdc2, Cdc28, contains a consensus CKII site (Ser-46), which is homologous with that of human Cdc2. Using *in vitro* kinase assays, metabolic labelling, peptide

mapping and phosphoamino acid analysis, we demonstrate that this site is phosphorylated in Cdc28 *in vivo* as well *in vitro*. In addition, *S. cerevisiae* cells in which Ser-46 has been mutated to alanine show a decrease in both cell volume and protein content of 33 %, and this effect is most pronounced in the stationary phase. Because cell size in *S. cerevisiae* is regulated primarily at the G₁ stage, we suggest that CKII contributes to the regulation of the cell cycle in budding yeast by phosphorylation of Cdc28 as a checkpoint for G₁ progression.

Key words: budding yeast, cell division cycle, cyclin-dependent kinase.

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 *cdc2+* 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 G₂-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 G₂-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^{Sic1}, and the synthesis of G₁ 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]. G₁ 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 Clb/Cdc28 kinase activity governs the budding-yeast cell cycle, although evidence for a new, independent G₁ 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 G₁ 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 (α and α'), 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, α ,

Abbreviations used: CDK, cyclin-dependent kinase; CKII, casein kinase II; GST, glutathione S-transferase; S46A, a mutant bearing a Ser-46 → Ala substitution; YNB, yeast nitrogen base.

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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* CKII is required for cell-cycle progression during G₁ phase prior to DNA synthesis, and also at the G₂-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.

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(*MAT α* CDC28::HIS3[WT CDC28-HA] *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*); CY3086(*MAT α* CDC28::HIS3[WT CDC28 on YCp50] *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*); SY129(*MAT α* CDC28::HIS3[WT CDC28 in pRS415] *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*); SY130(*MAT α* 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*-*ClaiI* fragment containing the entire *CDC28* open reading frame [1] was obtained from plasmid CB1503 (*CDC28* in YEpl213), and cloned into the *SmaI* 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 had the following sequence: 5'-ATA AGA CTA GAG GCT GAA GAC GAG GGT G-3'. Both strands of DNA surrounding the Ser-46 $\rightarrow$ Ala mutation (S46A) were sequenced by the dideoxy method using a Sequenase kit (United States Biochemicals 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^{Suc1}-Sephacrose 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 μ g/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 μ l of BSA-Sephacrose beads at 4 °C, with gentle rocking for 4 h. The suspension was centrifuged for 5 min at 13000 g, and 15 μ l of p13^{Suc1}-Sephacrose 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 $\times$ 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')₂ 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 μ l containing 10 μ M [γ -³²P]ATP (20000 c.p.m./pmol); 320 μ M 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 phosphate 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 μ l of Protein G-Sepharose (1:1 suspension; Amersham Pharmacia Biotech), incubated for 1 h at 4 °C after addition of 3 μ g of 12CA5 monoclonal antibody (raised against the epitope peptide) affinity-purified on a Protein G-Sepharose column, following treatment with 40 μ l of Protein G-Sepharose to precipitate the immuno-complex. The immuno-complexes 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 μ l, 10 μ M [γ - 32 P]ATP (20000 c.p.m./pmol) and 2 μ l (46 μ g/ml) of CKII purified to homogeneity from bovine testis [32]. Heparin (Sigma) was added in some experiments to a final concentration of 2 μ g/ml. After incubation for 1 h at 30 °C, reactions were terminated by the addition of 60 μ l of 2 $\times$ 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 [32 P] P_i labelling of proteins, strains SY129 and SY130 were grown in 100 ml of YPD(–) P_i medium (YPD depleted of P_i ; [27]), and absorbance at 600 nm (A_{600}) was measured in a UV-visible spectrophotometer (Amersham Pharmacia Biotech). At an A_{600} of approx. 0.5, the cells from each culture were harvested by centrifugation, resuspended in 15 ml of YPD(–) P_i medium, and divided into two aliquots: one was treated with 5 mCi of [32 P] P_i (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 μ l of p13^{Suc1}-Sepharose beads. Before the addition of 2 $\times$ sample buffer and electrophoresis, beads were treated with 2.5 μ g of RNase and 20 μ g 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 μ l, 10 μ M [γ - 32 P]ATP (20000 c.p.m./pmol) and 1 mM peptide CSH103 (ADAQHATPPKKRKVEDPKDF) 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 1×10^5 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 at Ser-46; the amino acid sequence surrounding this site is homologous with that surrounding the 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 CSH247 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 μ M, 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 vivo*.

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 [³²P]P_i, and the phosphorylation state of Cdc28 and Cdc28-S46A was examined by precipitation with p13^{Suc1}-Sephacrose 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),

Figure 2 *In vitro* phosphorylation of Cdc28 by CKII

(A) Phosphorylation of epitope-tagged Cdc28 (strain SY1061) by CKII enzyme. Cdc28-HA was immunoprecipitated using 12CA5 antibody (lane 2 and 3) or a control serum (lane 1) and phosphorylated *in vitro* by adding pure CKII, as described in the Experimental section. In lane 3, heparin was added to inhibit CKII activity. Arrows indicate the positions of Cdc28-HA. (B) An immunoblot of the experiment shown in (A), indicating the presence of Cdc28-HA protein as a substrate for CKII.

suggesting that the point mutation introduced (Ser-46 to alanine) abolished all serine phosphorylation in Cdc28. After prolonged exposure, a faint spot corresponding to phosphotyrosine 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 *cdc28-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] P_i labelling of Cdc28 and Cdc28-S46A

(A) Autoradiography of phosphorylated wild-type Cdc28 (lane 1) and Cdc28-S46A (lane 2) after precipitation with p13^{Suc1} -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°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 p13^{Suc1} -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.

Substrate	Kinase activity [arbitrary units (c.p.m. $\times 10^{-5}$)]
Cdc28	3.800
Cdc28-S46A	4.000
Cdc28 ctrl	0.067
Cdc28-S46A ctrl	0.036

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 α 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 1×10^5 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, *cka1^{ts}*, arrests, but continues to grow with a cell-polarity defect [40]. Deletion of the *CKB1* gene

elicits a salt-sensitive phenotype [41,42], while deletion of *CKB2* is partly responsible for adaptation in cells bearing a DNA-damage checkpoint [43]. Finally, the temperature-sensitive mutant, *cka2^{ts}*, exhibits defects in both G_1 –S and G_2 –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, several groups demonstrated that Tyr-19 (a homologue of *S. pombe* Tyr-15) is phosphorylated in *S. cerevisiae* [45–47], 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 cAMP 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 G_1 /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 G_1/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^{ts}* arrest within a single cell cycle, exhibiting a dual-arrest phenotype consisting of 50% unbudded and 50% large-

budded cells [22]. The unbudded population of cells showed a clear G_1 arrest either at START, or more likely at the G_1 -S transition. Thus CKII plays multiple roles in cell-size regulation, including a major function at START or G_1 -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 α -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 *cka1^{ts}* in *S. cerevisiae*. Unlike *S. cerevisiae*, *S. pombe* regulates progression through the cell cycle in response to cell size primarily in G_2 , and not in G_1 [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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