Collaboration of G₁ cyclins in the functional inactivation of the retinoblastoma protein

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The retinoblastoma gene product (pRB) constrains cell proliferation by preventing cell-cycle progression from the G₁ to S phase. Its growth-inhibitory effects appear to be reversed by hyperphosphorylation occurring during G₁. This process is thought to involve G₁ cyclins and cyclin-dependent kinases (cdks). Here we report that the cell cycle-dependent phosphorylation of mammalian pRB is faithfully reproduced when it is expressed in *Saccharomyces cerevisiae*. As is the case in mammalian cells, this phosphorylation requires an intact oncoprotein-binding domain and is inhibited by a negative growth factor, in this case a mating pheromone. Expression of pRB in *cln* (–) mutants indicates that specific combinations of endogenous G₁ cyclins, Cln3 and either Cln1 or Cln2 are required for pRB hyperphosphorylation in yeast. Moreover, expression of mammalian G₁ cyclins in *cln* (–) yeast cells indicates that the functions of Cln2 and Cln3 in pRB hyperphosphorylation can be complemented by human cyclin E and cyclin D1, respectively. These observations suggest a functional heterogeneity among G₁ cyclin–cdk complexes and indicate a need for the involvement of multiple G₁ cyclins in promoting pRB hyperphosphorylation and resulting cell-cycle progression.

[Key Words: Cell-cycle; G₁ cyclins; cyclin-dependent kinases; *Saccharomyces cerevisiae*; retinoblastoma protein; phosphorylation]

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Genetic inactivation of the retinoblastoma gene (*RB*) is suspected to play an important role in the development of a variety of human malignancies (for review, see Weinberg 1991; Hollingsworth et al. 1993; Zacksenhaus et al. 1993). *RB* encodes a nuclear phosphoprotein (pRB) that functions as a critical negative regulator of mammalian cell cycle progression (Friend et al. 1987; Lee et al. 1987). The finding that the transforming powers of DNA tumor virus oncoproteins such as adenovirus E1A, simian virus 40 large T antigen, and papilloma virus E7 are dependent, at least in part, on their ability to bind and sequester pRB provides further evidence that pRB acts in normal cells to constrain cell proliferation (DeCaprio et al. 1988; Whyte et al. 1988; Dyson et al. 1989).

The function of pRB appears to be controlled physiologically by cell cycle-dependent phosphorylation (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989, 1992; Mihara et al. 1989). Thus, pRB is found in a hypophosphorylated state in the G₀ and early to mid-G₁ phases of cell cycle but undergoes rapid hyperphosphorylation in late G₁. It remains in this hyperphospho-

rylated state until the end of mitosis. The finding that viral oncoproteins bind preferentially to the hypophosphorylated form of pRB suggests that this form is biologically active in growth regulation (Ludlow et al. 1989; Imai et al. 1991; Templeton et al. 1991). Because the hypophosphorylated form of pRB is observed only during the G₀/G₁ phase, it is assumed that pRB acts primarily in this part of the cell cycle to regulate proliferation; its functional inactivation in mid/late G₁, associated with hyperphosphorylation, is thought to permit progress of the cell into the later phases of the cell cycle.

The cell cycle-dependent phosphorylation of pRB suggests that this process may depend on the actions of cyclin-dependent kinases (cdks) (for review, see Reed 1992). Most of the sites of phosphorylation detectable in pRB isolated from cells can be modified in vitro by incubation of pRB with a cdk, cdc2, and appropriate cyclins (Lees et al. 1991; Lin et al. 1991). However, the details of the phosphorylation mechanism are obscured by the existence of a number of cdks and their regulatory subunits, the cyclins. At present, five different cdks (cdc2, cdk2, cdk3, cdk4, and cdk5) (Lee and Nurse 1987; Elledge and Spottiswood 1991; Ninomiya-Tsuji et al. 1991; Rosenblatt et al. 1992; Tsai et al. 1991; Meyerson et al. 1992) and eight different cyclins (A, B1, B2, C, D1, D2, D3, and

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E) (Pines and Hunter 1989; Wang et al. 1990; Koff et al. 1991; Lew et al. 1991; Matsushime et al. 1991; Motokura et al. 1991; Xiong et al. 1991, 1992a; Inaba et al. 1992; Kiyokawa et al. 1992) have been detected in mammalian cells. Of these, cyclins C, D1, D2, D3, and E are expressed and potentially active in the mid/late portion of the G_1 phase when critical pRB hyperphosphorylation occurs.

Cyclin E seems to be a primary regulator of cdk2 (Dulic et al. 1992; Koff et al. 1991, 1992), whereas D-type cyclins interact with cdk2, cdk4, and cdk5 (Matsushime et al. 1992; Xiong et al. 1992b). Direct evidence for the involvement of cyclin-cdk complexes in the functional inactivation of pRB was provided by the demonstration that ectopic expression of cyclins A and E can overcome pRB-imposed cell growth arrest and induce pRB hyperphosphorylation (Hinds et al. 1992). Expression of cyclin D1, in contrast, failed to induce pRB hyperphosphorylation while partially overriding the pRB growth-suppressing effect. However, another member of D-type cyclins, cyclin D2, seems to induce pRB hyperphosphorylation upon ectopic expression (Ewen et al. 1993). These D-type cyclins have recently been shown to form physical complexes with pRB, suggesting yet another level of functional interaction (Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1993). The respective contributions of the various cyclins in promoting pRB hyperphosphorylation remain unclear.

Lacking genetic tools to dissect further the contributions of these mammalian cyclins to pRB hyperphosphorylation, we turned to yeast cells. Recent studies have shown that very similar molecular mechanisms control cell-cycle progression throughout all eukaryotes, from yeast to mammalian cells (for review, see Norbury and Nurse 1992; Pines 1992; Nasmyth 1993). In the yeast *Saccharomyces cerevisiae*, two major checkpoints control cell cycle advance. One is a late G_1 checkpoint termed Start, which occurs prior to the G_1 -S transition, whereas the other is a G_2 -M checkpoint that controls mitosis. In contrast to mammalian cells, in which various cdks seem to act at distinct cell cycle checkpoints, passage through both checkpoints in yeast depends on the activation of its only known cdk, Cdc28 (Piggott et al. 1982; Reed and Wittenberg 1990; Surana et al. 1991). In these cells, the activity of Cdc28 throughout G_1 is regulated by a distinct set of three G_1 cyclins, termed Cln1, Cln2, and Cln3 (Cross 1988; Nash et al. 1988; Richardson et al. 1989; Tyers et al. 1993). B-type cyclins, in contrast, seem to be specialized for promoting G_2 events leading to the G_2 -M transition (Ghiara et al. 1991; Surana et al. 1991; Fitch et al. 1992; Richardson et al. 1992).

Of the three G_1 cyclins in the yeast *S. cerevisiae*, Cln1 and Cln2 are structurally similar (58% identity) to one other, whereas Cln3 is significantly diverged from Cln1 and Cln2 (23% identity) (Hadwiger et al. 1989). Expression of Cln1 and Cln2 fluctuates periodically during cell cycle, peaking at late G_1 (Wittenberg et al. 1990). Cln3 expression, in contrast, is rather constant throughout the cell cycle (Tyers et al. 1993). This observation suggests

that Cln1/Cln2 and Cln3 may have functionally distinct roles in the regulation of cell-cycle progression.

Interestingly, the expression patterns of Cln1 and Cln2 are reminiscent of mammalian cyclin E, whereas that of Cln3 is similar to mammalian D-type cyclins. Moreover, the amino acid sequence of D1 cyclin aligns better to that of Cln3 than to those of Cln1 or Cln2 (Motokura et al. 1991). The ability of mammalian cyclin genes, including cyclins D and E, to rescue triple *cln1 cln2 cln3* mutants (Koff et al. 1991; Lew et al. 1991; Xiong et al. 1991) indicates a functional conservation of cyclins between yeast and mammalian cells.

To analyze the cell cycle-dependent regulation of pRB, we established a pRB expression system in yeast and have exploited it to demonstrate that pRB phosphorylation mechanisms operating in mammalian cells can be reconstituted in yeast cells. We provide evidence that particular combinations of endogenous G_1 cyclins are required for pRB hyperphosphorylation in yeast. Mammalian G_1 cyclins expressed in yeast can complement the function of yeast G_1 cyclins in pRB hyperphosphorylation. These results imply that multiple G_1 cyclins are involved in the pRB inactivation process in the mammalian cell.

Results

Expression of mammalian retinoblastoma protein in yeast

To express mammalian RB in *S. cerevisiae*, we constructed a high-copy expression vector in which wild-type human RB cDNA or mutant derivatives thereof were placed under the control of the galactose-induced *GAL1,10* promoter. The mutant RB alleles used here, the residue 706 cysteine-to-phenylalanine point mutant (706C-F) and the exon 22 deletion mutant ($\Delta 22$), were isolated originally from human tumors and are known to be functionally inactive in cell growth suppression (Templeton et al. 1991). These various expression plasmids were transformed into the yeast strain L4852 (Leu⁻), and stable transformants (Leu⁺) were selected.

Expression of pRB was induced in the transformants by culturing them in medium containing galactose and was analyzed by immunoprecipitation or immunoblotting with anti-human pRB monoclonal antibodies. In the absence of galactose, no pRB was detected from lysate. However, on galactose induction, pRB was readily detected in the lysates (Fig. 1A,B).

The pRB protein expressed in mammalian cells is known to migrate heterogeneously during gel electrophoresis because of variable levels of phosphorylation (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989). The pRB wild-type protein expressed in yeast also exhibited similar heterogeneity in migration rate during standard 7.5% SDS-PAGE. As anticipated, phosphatase treatment of the pRB protein abolished the slow-migrating species almost completely (Fig. 1A, lane 5), indicating that its retarded mobility was attributable to phosphorylation as is known to be the case in mamma-

Figure 1. Expression of human pRB protein in *S. cerevisiae*. (A) L4852 cells (lanes 1, 2) or transformants containing the human pRB expression vector p2202TRB (lanes 3–6) were labeled with [35 S]methionine in synthetic minimal medium containing 2% glucose (lanes 1, 3) or 2% galactose (lanes 2, 4–6). Lysates were prepared from the labeled cells and immunoprecipitated with the anti-human pRB mAb NM2-21C9 (lanes 2, 4–6) or normal mouse serum (lanes 1, 3). The pRB immunoprecipitates were treated with potato-acid phosphatase in 0.1 M MES (lane 5) or 0.1 M sodium phosphate (lane 6) at 37°C for 30 min. Proteins were resolved on an SDS–7.5% polyacrylamide gel and visualized by autoradiography. Numbers at

left represent the mobility of molecular mass markers. (B) L4852 cells (lane 1) or transformants containing p2202TRB (lanes 2, 3) were grown in synthetic minimal medium containing 2% glucose (lane 2) or 2% galactose (lanes 1, 3). Whole-cell lysates were prepared from the cells, and proteins were resolved on an SDS–7.5% polyacrylamide gel and blotted on a PVDF membrane. The membrane was probed with the anti-human pRB monoclonal antibody G3-245. The bands were visualized by the enhanced chemiluminescence immunodetection system. Numbers at left represent the mobility of molecular mass markers. (C) L4852 cells containing p2202TRB were labeled with [32 P]orthophosphate (32 Pi) in phosphate-depleted medium containing 2% galactose. Lysates prepared from the labeled cells were immunoprecipitated with the anti-human pRB NM2-21C9 (lane 2) or normal mouse serum (lane 1). Proteins were resolved on an SDS–7.5% polyacrylamide gel and visualized by autoradiography. Numbers at left represent the mobility of molecular mass markers.

lian cells. This was confirmed further by immunoprecipitation of pRB from cells metabolically labeled with [32 P]orthophosphate (32 Pi) (Fig. 1C).

The two mutant pRB forms (Δ 22 and 706C-F) expressed in yeast cells migrated as relatively discrete, rapidly moving species (Fig. 2). Immunoblot analysis showed that the expression level of the wild-type pRB was approximately four- to fivefold higher than those of

the mutants on the basis of densitometric analysis. In contrast, incorporation in wild-type pRB of [32 P]orthophosphate was >15-fold higher than by the mutants (Fig. 2C). This clearly indicates that nonfunctional, mutated forms of pRB receive less phosphorylation than wild-type pRB in yeast. The difference precisely parallels their behavior in mammalian cells (Templeton et al. 1991). In mammalian cells, virtually all of the functionally defec-

Figure 2. Expression of mutant pRB proteins in *S. cerevisiae*. (A) L4852 cells containing the expression vectors for wild-type (WT) (p2202TRB) or mutant forms of human pRB (p2202TRB Δ 22, p2202TRB706C-F) were labeled with [35 S]methionine in synthetic minimal medium containing 2% galactose for 3 hr. Lysates from the labeled cells were immunoprecipitated with the anti-human pRB NM2-21C9 (α RB) or normal mouse serum (NMS). Proteins were resolved on an SDS–7.5% polyacrylamide gel and visualized by autoradiography. Because of the small deletion (exon 22 deletion), the molecular size of Δ 22 is slightly smaller than that of

wild-type pRB or the 706C-F mutant. Numbers at left represent the mobility of molecular mass markers. (B) Whole-cell extracts were prepared from L4852 cells containing expression vectors for wild-type (WT) or mutant forms of pRB cultured in synthetic minimal medium containing 2% galactose. Proteins were resolved on an SDS–7.5% polyacrylamide gel and blotted on a PVDF membrane. The membrane was probed with the anti-human pRB mAb G3-245. The bands were visualized by enhanced chemiluminescence immunodetection system. Numbers at left represent the mobility of molecular mass markers. (C) Lysates of [32 P]orthophosphate-labeled L4852 transformant cells containing expression vectors for wild-type (WT) or mutant pRB were immunoprecipitated with the anti-human pRB NM2-21C9. The lysates were prepared from cells grown in phosphate-depleted medium containing 2% galactose. Proteins were resolved on an SDS–7.5% polyacrylamide gel and visualized by autoradiography. Numbers at left represent the mobility of molecular mass markers.

tive forms of pRB are known to escape hyperphosphorylation. This includes mutant pRBs that differ from wild type by only a single amino acid, such as the 706C-F mutant shown here. Thus, the mechanisms governing the specificity of pRB phosphorylation in mammalian cells appear to be operative in yeast cells as well.

In mammalian cells, pRB is known to localize in the nucleus. Upon cell lysis in low salt conditions, the hypophosphorylated form of pRB remains in the nuclear fraction, bound to certain structures that serve to anchor it to the nucleus; the hyperphosphorylated forms, poorly tethered to this anchor, leach out of nuclei under these conditions (Mittnacht and Weinberg 1991). Moreover, functionally defective forms of pRB also have lost the ability to bind tightly to the nucleus. Thus, in mammalian cells there is parallelism between nuclear binding and the growth-suppressing abilities of pRB.

pRB expressed in yeast also localizes in the nucleus as determined by indirect immunofluorescence staining (data not shown). To address whether yeast cells also possess a structure that functions analogously to the nuclear anchor bound by pRB in mammalian cells, we disrupted yeast cells with glass beads in a low salt buffer and extracted pRB protein from their nuclei with buffers containing progressively increasing salt concentrations. In yeast cells expressing wild-type pRB, the hyperphosphorylated forms of pRB were almost completely released from the cell by the low salt buffer, whereas the hypophosphorylated form was retained (Fig. 3A). A significant proportion of the hypophosphorylated form of pRB remained in the nonextractable fraction even after addition of 400 mM NaCl. This observation supports the idea that both yeast and mammalian cells possess analogously functioning nuclear structures that serve to anchor pRB.

Work on pRB mutants in mammalian cells indicates that the wild-type protein associates with its nuclear anchor via its oncoprotein-binding domain, sometimes termed the pRB pocket (Mittnacht and Weinberg 1991). This pocket is also used by a variety of other proteins, including the E2F transcription factor and the D cyclins to bind pRB (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1990, 1991; Bandara and LaThangue 1991; Chellappan et al. 1991; Chittenden et al. 1991; Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1993). As is the case in mammalian cells, the two functionally defective pRB mutants that are mutated in the pocket domain fail to tether tightly to nuclear structures in yeast (Fig. 3B). Accordingly, we suggest that the yeast cell nucleus also contains one or more proteins that act in a specific fashion to bind pRB via its pocket domain.

The growth phenotype of yeast cells expressing human pRB was compared with those of the parental line or transformants expressing mutant pRB. No significant differences in growth rate, cell shape, cell size, or mating pheromone sensitivity were observed among them (data not shown). These observations meant that we could study mechanisms regulating pRB phosphorylation in yeast cells without the confounding effects derived from pRB-mediated suppression of cell growth.

Figure 3. Release of pRB from yeast cells. (A) L4852 cells containing the pRB expression vector p2202TRB were grown to mid-log phase in synthetic minimal medium containing 2% galactose. Cells (1.5×10^8) were broken with glass beads in 500 μ l of ELB buffer without NaCl, and after removing glass beads, the extracts were centrifuged to produce pellet and supernatant fractions. The pellet was sequentially resuspended and extracted in a series of ELB buffers containing different concentrations of NaCl (500 μ l extraction volume) for 10 min on ice. After separating pellet fraction by centrifugation, supernatants were mixed with one-third volume of 4 $\times$ sample buffer. After salt extraction with 400 mM NaCl ELB buffer, pellets were resuspended in 666.7 μ l of 1 $\times$ SDS sample buffer and boiled for 5 min. The lysates (100 μ l) were subject to electrophoresis on SDS-7.5% polyacrylamide gel, and the extractability of pRB from cells was analyzed by immunoblotting with anti-human pRB G3-245. (B) L4852 cells expressing mutant forms of pRB were used to extract proteins by the same procedure described in A.

Effects of mating pheromone on pRB phosphorylation

We wished to determine whether pRB phosphorylation in yeast, as in mammalian cells, is regulated in a cell cycle-dependent fashion. In mammalian cells, pRB hyperphosphorylation is strongly inhibited by the arrest of cells at late G₁ with negative growth factors such as transforming growth factor- β (TGF- β ; Laiho et al. 1990). We therefore examined the effect on pRB hyperphosphorylation of a yeast negative growth factor, the α mating pheromone (α -factor), which also blocks growth in G₁. Treatment of a MATa strain expressing pRB with α -factor caused arrest of cells in late G₁ and inhibited pRB hyperphosphorylation (Fig. 4A). This inhibitory effect

Figure 4. The effect of mating pheromone on pRB hyperphosphorylation. (A) Inhibition of pRB hyperphosphorylation by α -factor. L4852 cells containing p2202TRB were grown to mid-log phase in synthetic minimal medium supplemented with 2% galactose. Cells were then treated with α -factor at a final concentration of 5 μ M. After a 1- or 3-hr treatment with α -factor, cells were collected and whole-cell lysates were prepared from the cells. Proteins were resolved on an SDS-7.5% polyacrylamide gel and blotted on a PVDF membrane. The membrane was probed with the anti-human pRB mAb G3-245. The bands were visualized by enhanced chemiluminescence immunodetection system. Numbers at left represent the mobility of molecular mass markers. (B) Timing of pRB hyperphosphorylation after α -factor release. L4852 cells expressing pRB were arrested in late G₁ by α -factor treatment (5 μ M) for 3 hr at 30°C. Cells were then released from α -factor by vigorous washing to restart the cell cycle in synthetic minimal medium containing 2% galactose. Cell concentration was adjusted to an OD₆₀₀ of 0.75 at the onset of α -factor release, and 5 ml of the suspension was taken from the culture at the times indicated. Lysates were prepared from each 5 ml culture by use of glass beads in ELB buffer. Proteins were resolved on an SDS-7.5% acrylamide gel, and bands were detected by immunoblotting using anti-human pRB G3-245 (top). Aliquots of cell culture from each time points were analyzed for budding index as morphological indicators of the cell cycle progression (bottom).

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was readily detectable within 1 hr of treatment of cells with α -factor and was almost complete by 3 hr of treatment.

Next, we examined the timing of pRB hyperphosphorylation in yeast. To produce a synchronized population, cells were first treated with mating pheromone to arrest them in late G₁ (Tyers et al. 1993). Following release from mating pheromone arrest, cell lysates were prepared at various time points and pRB hyperphosphorylation was determined by immunoblotting (Fig. 4B). pRB hyperphosphorylation, as monitored by shifts in electrophoretic migration, could be seen within 40 min after release and peaked at the 140-min incubation point. In contrast, morphological analysis showed that small budded cells (those entering into S phase) began to appear 60–80 min after the release. These observations indicated that pRB hyperphosphorylation in yeast cells occurs in late G₁ prior to the G₁/S transition. In mammalian cells, this modification occurs at a comparable point in the cell cycle, several hours before S-phase entrance (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989). We note that the amount of total pRB detectable by immunoblotting increased significantly after the release from α -factor arrest. This was likely attributable to an increase of protein synthesis rate in growth-stimulated cells.

Comparison of hypo- and hyperphosphorylated forms of pRB in yeast

The results described above indicated strong similarities in the control of pRB phosphorylation in mammalian

and yeast cells. To extend these parallels, we thought it important to ascertain whether the sites of phosphorylation on pRB in yeast cells are similar or identical to those modified in mammalian cells. We therefore performed phosphopeptide mapping of pRB expressed in yeast. Cells expressing pRB were labeled with ³²P_i, and the labeled proteins were immunoprecipitated with anti-pRB antibody. The pRB polypeptides consisting of hypo- and hyperphosphorylated species were excised, and the eluted proteins were subjected to tryptic phosphopeptide mapping. As seen in Figure 5, the pattern of phosphopeptides closely resembled that seen on analysis of pRB prepared from mammalian cells (Fig. 5A; Lees et al. 1991; Mittnacht et al. 1994). Of the 25 phosphopeptide spots reproducibly associated with pRB isolated from mammalian cells (Lees et al. 1991), 23 comparable spots could be identified in yeast cells (Fig. 5B).

We also analyzed the rapidly migrating, hypophosphorylated pRB obtained by treating yeast cells with α -factor (Fig. 5C). We note the striking observation, also made recently in mammalian cells, that almost all of the phosphopeptides present in hyperphosphorylated pRB were also present in its hypophosphorylated form (Mittnacht et al. 1994). However, it was clear that two spots present in hyperphosphorylated pRB are missing in its hypophosphorylated form, that is, spots 9 and 14 according to the nomenclature of Lees et al. (1991). Two phosphopeptide spots with the same coordinates have also been shown to be missing from hypophosphorylated pRB expressed in mammalian cells (Mittnacht et al. 1994).

As mentioned earlier, we were not able to detect spots 3 and 10 in the hyperphosphorylated form of pRB in

Figure 5. Phosphopeptide mapping of pRB expressed in yeast. (A) Schematic view of phosphopeptide spots obtained from pRB in mammalian cells (Lees et al. 1991). (B) L4852 cells were grown in phosphate-depleted medium containing 2% galactose and metabolically labeled with $^{32}\text{P}_i$ for 90 min. The $^{32}\text{P}_i$ -labeled pRB was then immunoprecipitated, resolved by SDS-PAGE, eluted, and then digested with trypsin. The resulting phosphopeptides were then resolved by electrophoresis and ascending chromatography. (C) The hypophosphorylated form of pRB was obtained by treating cells with α -factor for 3 hr. Cells were labeled with $^{32}\text{P}_i$ for 90 min during α -factor treatment.

yeast cells (Fig. 5B). Of these, phosphopeptide spot 3 of pRB has been reported to increase in intensity on hyperphosphorylation in mammalian cells (DeCaprio et al. 1992). These subtle differences may distinguish the biochemistry of pRB modification in yeast and mammalian cells; all other details of phosphorylation appear to be identical. In addition, we conclude that modification of spot 3 is not essential for the mobility shift seen in

SDS-PAGE associated with hyperphosphorylation of pRB.

Involvement of CDC28 in pRB hyperphosphorylation

To address further whether pRB hyperphosphorylation is regulated by the cell-cycle clocking machinery, we examined pRB phosphorylation in yeast strains with mutant forms of *CDC28*, the only cdk known to be present in these cells. The *cdc28-4* mutation arrests cells at the Start point when shifted to the nonpermissive temperature (Reed and Wittenberg 1990). A second *cdc28* mutation, termed *cdc28-1N*, causes growth arrest at the G_2 -M transition at the nonpermissive temperature (Piggott et al. 1982; Surana et al. 1991).

Cells carrying these *cdc28* mutations were transformed with the pRB expression vector as described previously and were induced to express pRB in galactose media. Cultures of the *cdc28^{ts}*-mutant cells were then shifted to their nonpermissive temperatures and incubated for an additional 3 hr. At the nonpermissive temperatures, >90% of cells arrested at the expected point in the cell cycle as determined by cell morphology (*cdc28-4*, unbudded cells; *cdc28-1N*, cells with large buds).

The pRB protein expressed in late G_1 -arrested *cdc28-4* cells exhibited exclusively the hypophosphorylated (i.e., rapidly migrating) form, whereas that from *cdc28-1N* cells arrested in G_2 -M was predominantly composed of the slowly migrating hyperphosphorylated form (Fig. 6A). To exclude the possibility that the lack of pRB hyperphosphorylation in *cdc28-4* mutant at 36°C was attributable simply to the temperature shift rather than inactivation of *CDC28* function, we expressed the wild-type *CDC28* gene in these temperature-sensitive mutant strains. As shown in Figure 6A, *CDC28* expression in *cdc28-4* strain induced pRB hyperphosphorylation at 36°C. These observations reinforce the previous conclusion that pRB hyperphosphorylation is entrained with progression through the cell cycle and that it occurs in association with transit through the Start point. Moreover, they indicate that this phosphorylation depends directly or indirectly on the function of the Cdc28 kinase.

To explore further the involvement of Cdc28 kinase in pRB phosphorylation, we expressed a mutant form of mouse pRB in which eight cdc2/Cdc28 consensus phosphorylation sites have been replaced by site-directed mutagenesis (Hamel et al. 1992). In mammalian cells, this mutant pRB, termed $\Delta p34$ -HA, is potent in its ability to repress E2F-dependent transcription, an effect that can be explained by its resistance to functional inactivation by cdks and associated cyclins. As is the case in mammalian cells, this pRB mutant did not undergo hyperphosphorylation in yeast as determined by its migration on SDS-PAGE (Fig. 6B). Taken together, these various lines of evidence indicate that in yeast, as in mammalian cells, pRB is phosphorylated in mid/late G_1 . Moreover, they support the notion that this phosphorylation is mediated by cdk, in particular Cdc28.

Figure 6. Involvement of *CDC28* on pRB hyperphosphorylation in yeast. (A) The effect of *cdc28* mutations on pRB hyperphosphorylation. Cells containing either *cdc28-4* or *cdc28-1N* temperature sensitive mutation were transformed with the pRB expression vector p2202TRB. The transformants were grown to mid-log phase in synthetic minimal medium supplemented with 2% galactose at 25°C and then shifted to nonpermissive temperatures. After 3 hr of culture, whole-cell lysates were prepared from cells and were subject to immunoblotting with anti-pRB mAb G3-245. The bands were visualized by enhanced chemiluminescence immunodetection method. To confirm that the temperature-dependent inhibition of pRB

hyperphosphorylation in *cdc28-4* strain is attributable to the temperature-sensitive mutation of *CDC28*, wild-type *CDC28* expression vector pRS314-*CDC28* (a centromeric vector containing the genomic *CDC28* gene and *TRP1* marker; kindly provided by Steve Kron) was introduced into the temperature-sensitive mutant strains. The *CDC28*-expressing transformants were used for the control of the experiments (right four lanes). Numbers at left represent the mobility of molecular mass markers. (B) A phosphorylation-resistant pRB mutant expressed in yeast. An artificial mutant of mouse pRB ($\Delta p34$ -HA) (Hamel et al. 1992) that lacks eight potential *cdc2/Cdc28* phosphorylation sites was inserted into p2202T and was expressed in *S. cerevisiae* by use of the *GAL1,10* promoter for expression. Cultures were grown to mid-log phase in synthetic minimal medium containing 2% galactose at 30°C and labeled with [35 S]methionine for 3 hr in the presence or absence of α -factor. The mutant pRB was immunoprecipitated by anti-human pRB, NM2-21C9, that cross-reacts with mouse pRB. Because G3-245 anti-human pRB does not work on mouse pRB, immunoblot analysis was not done in this experiment.

Involvement of multiple yeast *G₁* cyclins in pRB hyperphosphorylation

The observation that pRB hyperphosphorylation is inhibited by a mating pheromone was most easily explained by the known effects of pheromone on the activities of the yeast *G₁* cyclins regulating Cdc28 kinase. To address the possible role of yeast *G₁* cyclins in causing pRB hyperphosphorylation, we expressed pRB in mutant yeast cells lacking one or more of the complement of the three *G₁* cyclin genes (Fig. 7). Cells with genotypes of *cln1 CLN2 CLN3* or *CLN1 cln2 CLN3* phosphorylated their pRB as efficiently as *CLN1 CLN2 CLN3* cells (Fig. 7A). However, *CLN1 CLN2 cln3* cells showed little, if

any, pRB hyperphosphorylation and instead exhibited a massive accumulation of the hypophosphorylated form. This indicates that *CLN3* plays an essential role for pRB hyperphosphorylation. However, cells expressing only a single *CLN*, including *cln1 cln2 CLN3* cells, also cannot induce pRB hyperphosphorylation (Fig. 7B). We therefore concluded tentatively that a combination of *CLN3* and either *CLN1* or *CLN2* is required for pRB hyperphosphorylation.

It should be noted, however, that the pRB protein expressed in *cln1 cln2 CLN3* cells is seen as a doublet consisting of the fast-migrating hypophosphorylated form and a second form that migrates a bit more slowly. Such a doublet cannot be seen in *CLN1 cln2 cln3* or *cln1*

Figure 7. Effect of mutations in *CLN1*, *CLN2*, and *CLN3* on pRB hyperphosphorylation. The pRB expression vector, p2202TRB or p2206TRB, was introduced into L4852-derived isogenic strains lacking various combinations of endogenous *G₁* cyclins by genetic disruption. Transformants were selected by nutrition markers and were grown to mid-log phase in synthetic minimal medium supplemented with 2% galactose. Whole-cell lysates were prepared from the cells. Proteins were then resolved on an SDS-7.5% polyacrylamide gel and blotted on a PVDF membrane. The membrane was probed with the anti-human pRB mAb antibody G3-245. The bands were visualized by enhanced chemiluminescence immunodetection system. (A) The pRB protein was detected from wild-type and

isogenic mutant strains lacking a single endogenous *CLN* gene. Two independent clones were analyzed for each *cln* (-) mutant. (B) The pRB protein was detected from wild-type and isogenic mutant strains lacking two endogenous *CLN* genes.

CLN2 cln3 cells. This indicates that *cln1 cln2 CLN3* cells have an ability to phosphorylate pRB to a very limited extent, although they are not able to induce the pRB hyperphosphorylation seen in the wild-type as well as the *CLN1 cln2 CLN3*, and *cln1 CLN2 CLN3* cells.

These observed behaviors might have been attributable simply to differences in the doubling times of the various yeast strains. However, strains lacking single *cln* genes doubled at comparable rates. Thus, whereas wild-type cells (*CLN1 CLN2 CLN3*) expressing pRB had a doubling time of 2.38 hr, the doubling times of *cln1 CLN2 CLN3*, *CLN1 cln2 CLN3*, and *CLN1 CLN2 cln3* cells containing pRB were 3.24, 2.43, and 2.60 hr, respectively, in synthetic minimal medium containing 2% galactose.

These results provide strong support for the idea that a combination of at least two different endogenous G_1 cyclins is required for efficient induction of pRB phosphorylation. A combination of either *Cln1*+*Cln3* or *Cln2*+*Cln3* sufficed, whereas *Cln1*+*Cln2* failed to drive pRB hyperphosphorylation.

Complementation of *Cln* function with mammalian G_1 cyclins

Because these data indicated that certain pairs of cyclins may collaborate to promote pRB hyperphosphorylation, we examined whether such a collaboration could also be achieved following introduction of certain mammalian

cyclin genes into yeast cells. The fact that mammalian cyclins can rescue triple *cln1 cln2 cln3* cells indicates that these cyclins can productively interact with yeast Cdc28 to effect transit through Start (Koff et al. 1991; Lew et al. 1991; Xiong et al. 1991). On the basis of the similarities of the mammalian and yeast cyclin expression patterns mentioned earlier, we chose to express human cyclin D1 or cyclin E under control of the *GAL1,10* promoter in strains containing only one active endogenous G_1 cyclin, specifically either the *cln1 CLN2 cln3* or the *cln1 cln2 CLN3* mutant strains.

As seen in Figure 8A, cyclin E was much more efficient than cyclin D1 in inducing pRB hyperphosphorylation in the *cln1 cln2 CLN3* strain. In contrast, cyclin D1 worked better than cyclin E to induce pRB hyperphosphorylation in the *cln1 CLN2 cln3* background. These transformants expressed comparable amounts of the respective human cyclins as determined by immunoblotting (Fig. 8B,C). It should be also noted that heterologous expressions of the human cyclins in these strains did not induce growth stimulation as measured by FACS (data not shown). Rather, cyclin E expression seemed to have negative effect on their growth.

Taken together, these results provide evidence that at least two distinct G_1 cyclins collaborate in the process of pRB hyperphosphorylation in yeast cells. Moreover, the patterns of complementation between mammalian and yeast cyclins suggests that a similar pattern of collaboration may exist in mammalian cells as well. More spe-

Figure 8. Complementation of yeast G_1 cyclin function by human G_1 cyclin in pRB hyperphosphorylation. (A) A *cln1 CLN2 cln3* or a *cln1 cln2 CLN3* strain containing the yeast expression vector for human pRB (p2202TRB) was further transformed with an expression vector for human cyclin D1 or E under the control of the *GAL1,10* promoter (p2208TD1 or p2208TE). The transformants coexpressing the pRB and cyclin cDNAs were grown to mid-log phase in synthetic minimal medium containing 2% galactose, and whole-cell lysates were prepared from the cells. Proteins were then resolved on an SDS-7.5% polyacrylamide gel and blotted on a PVDF membrane. The membrane was probed with the anti-human pRB mAb G3-245. The bands were visualized by enhanced chemiluminescence immunodetection system. In *cln1 CLN2 cln3* cells with vector alone (p2208T), vector with cyclin D1 cDNA (p2208TD1) or the vector with cyclin E cDNA (p2208TE), the ratio of hyper-/hypophosphorylated forms of pRB was 0.03, 0.89, or 0.16, respectively, as determined by densitometric analysis. In *cln1 cln2 CLN3* cells with p2208T, p2208TD1, or p2208TE, the ratio was 0.04, 0.62, or 1.65, respectively. The results were reproducible when other independent transformant clones were used for the analysis. Numbers at left represent the mobility of molecular mass markers. (B) Detection of human cyclin D1 expressed in yeast. Whole-cell lysates prepared from *cln1 CLN2 cln3* cells expressing p2208T (lane 1), *cln1 CLN2 cln3* cells expressing p2208TD1 (lane 2) or *cln1 cln2 CLN3* cells expressing p2208TD1 (lane 3) were run on an SDS-10% polyacrylamide gel, blotted on a PVDF membrane, and probed with rabbit anti-human cyclin D1 antiserum. The arrow indicates the position of cyclin D1. Numbers at left represent the mobility of molecular mass markers. (C) Detection of human cyclin E expressed in yeast. Whole-cell lysates prepared from *cln1 CLN2 cln3* cells expressing p2208T (lane 1), *cln1 CLN2 cln3* cells expressing p2208TE (lane 2) or *cln1 cln2 CLN3* cells expressing p2208TE (lane 3) were run on an SDS-10% polyacrylamide gel, blotted on a PVDF membrane, and probed with anti-human cyclin E monoclonal antibody HE2. The arrow indicates the position of cyclin E. Numbers at left represent the mobility of molecular mass markers.

cifically, the complementation pattern of pRB hyperphosphorylation in yeast suggests that G_1 cyclins can be subclassified into two groups: one consisting of Cln1, Cln2, and cyclin E and the other consisting of Cln3 and cyclin D1. At least one member of each group appears to be required to participate in the processes leading to pRB hyperphosphorylation.

Discussion

The present work indicates that the mechanisms governing pRB phosphorylation are surprisingly similar in mammalian and yeast cells. Human pRB undergoes a hyperphosphorylation in these evolutionary distant organisms that is almost indistinguishable in its biochemical details. As is the case in mammalian cells (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989, 1992; Mihara et al. 1989), hyperphosphorylation of pRB in yeast cells causes a substantial change in its electrophoretic mobility. Moreover, these events occur at similar times in the cell cycle. In mammalian cells, this modification occurs close to or concomitant with passage through a mid/late G_1 gate termed the restriction point; in yeast cells, this modification occurs around a point in G_1 termed Start. These milestones in G_1 are both thought to represent critical events in the commitment of the cell to transit into late G_1 , S, and G_2 . In addition, soluble factors that prevent G_1 passage, TGF- β in the case of mammalian cells and mating pheromone in the case of yeast, work identically to block this modification of pRB.

Subtle differences in pRB structure affect the hyperphosphorylation process identically in mammalian and yeast cells. Thus, alterations of the pRB pocket domain as small as single amino acid substitutions, although they do not affect the cdk-consensus phosphorylation sites, eliminate pRB hyperphosphorylation in both organisms. This indicates the critical involvement of the pocket domain in promoting pRB phosphorylation in both mammalian and yeast cells. Moreover, it suggests a conservation between yeast and mammalian cells of one or more proteins that recognize and associate with the pocket domain.

Although hyperphosphorylation has profound functional effects on pRB in mammalian cells and results in a readily detectable shift in its electrophoretic mobility during SDS-PAGE, this change is achieved in yeast cells through the modification of only a small subset of pRB phosphorylation sites. Similar conclusions have been drawn from the detailed biochemical analysis of the various pRB forms isolated from mammalian cells (Templeton et al. 1991; DeCaprio et al. 1992; Mittnacht et al. 1994). Among the consequences of hyperphosphorylation in mammalian cells is the loss of binding by pRB to other proteins that we have termed operationally as nuclear anchor (Mittnacht and Weinberg 1991). This loss of tight binding and the resulting leaching from the nucleus during cell lysis is also observed in yeast cells.

In sum, these data indicate close parallels in the behavior of pRB in two distantly related eukaryotic cell

types. This similarity in behavior was hardly expected, because neither yeast cells nor those from other non-chordate phyla have been reported to express proteins that are closely related to mammalian pRB. The results suggest the possible existence of one or more proteins in yeast with structural similarities to pRB itself. Moreover, they indicate a cohort of other proteins, including cdk, that interact with pRB in a very similar fashion in both cell types.

A second conclusion from this work is the indication that multiple cyclins are required to effect pRB hyperphosphorylation in yeast cells. Our results obtained by expressing pRB in mutant yeast cells provide strong indication that the activity of a single endogenous yeast G_1 cyclin (Cln1, Cln2, or Cln3) is not sufficient for the induction of pRB hyperphosphorylation. Significantly, any one of these cyclins is able to promote cell cycle progression in yeast (Richardson et al. 1989; Tyers et al. 1993). Thus, pRB phosphorylation can be readily uncoupled from cell-cycle progression in these cells and studied in isolation.

These conditions have allowed us to conclude that combinations of endogenous cyclins are required to drive pRB hyperphosphorylation in yeast cells. We find that Cln1 and Cln3 or Cln2 and Cln3 can collaborate to promote pRB hyperphosphorylation, whereas Cln1 and Cln2 cannot do so. The observation that Cln3 can collaborate with either Cln1 or Cln2 to promote pRB hyperphosphorylation implies a functional heterogeneity among these G_1 cyclins in the activation of Cdc28 kinase. Moreover, it suggests that Cln1 and Cln2, which are structurally very similar (Hadwiger et al. 1989), may function analogously to one another when contributing to pRB modification.

In principle, high levels of G_1 cyclin/Cdc28 kinase activity might be required for pRB hyperphosphorylation simply to compensate for the counteracting effects of yeast phosphatases. Hence, any reduction in kinase activity through loss of one or another G_1 cyclins might lead, in turn, to loss of pRB hyperphosphorylation. Such a model does not fit with our presented observations. Thus, Cln1 and Cln2 are far more potent in activating Cdc28 than is Cln3, as gauged by their respective abilities to cause histone H1 phosphorylation *in vitro* (Tyers et al. 1993). This finding contrasts with the fact that the endogenous expression levels of neither Cln1 nor Cln2 or a combination of the two suffice to cause pRB phosphorylation in cells lacking Cln3 function.

This and other works suggest similarities between various mammalian and yeast cyclins. Although the D cyclins show only distant structural relatedness to Cln3, these two cyclins both show weak histone-modifying activity *in vitro* (Matsushime et al. 1992; Kato et al. 1993; Tyers et al. 1993). On the other hand, the structural similarity of Cln1 and Cln2 has parallels in the structural similarity shared by cyclins A and E of mammalian cells. Like mammalian D cyclins (Lew et al. 1991; Motokura et al. 1991), Cln3 is expressed constantly throughout the cell cycle (Tyers et al. 1993). Cln1 and 2, like cyclins A and E, are expressed in well-defined windows of time as

the cell transits G_1 and S (Richardson et al. 1989; Lew et al. 1991).

Our complementation experiments underscore these analogies further. In yeast cells lacking Cln3, pRB hyperphosphorylation is best restored by cyclin D1; in contrast, Cln2 (and probably Cln1) finds a potent substitute in cyclin E. Although these yeast and mammalian cyclins may not be fully interchangeable, we propose that a similar physiology governs pRB phosphorylation in mammalian cells. More specifically, we suggest that in mammalian cells, D cyclins such as cyclin D1 collaborate with cyclin E in the hyperphosphorylation of pRB in the G_1 phase of the cell cycle.

Cyclin D1 is expressed throughout G_1 in many types of mammalian cells, and therefore cannot be the event that precipitates the hyperphosphorylation of pRB in mid/late G_1 . Instead, this role would seem to be played by cyclin E, expression of which is induced at a time when pRB hyperphosphorylation occurs (Lew et al. 1991). Accordingly, we suggest that cyclin D1 (or its surrogates, cyclins D2 or D3) prepares pRB for subsequent hyperphosphorylation by cyclin E in mid/late G_1 .

Of additional relevance is the fact these D cyclins are able to form physical complexes with pRB and the fact that the formation of these complexes is dependent on an intact pRB pocket domain (Dowdy et al. 1993; Ewen et al. 1993). Mutations that alter this domain prevent cyclin D binding and, at the same time, prevent pRB hyperphosphorylation. For this reason, we suggest that physical complex formation between a D cyclin and the pRB pocket is important for subsequent pRB hyperphosphorylation. Such complex formation, although perhaps necessary, is not sufficient for hyperphosphorylation, as mammalian cells overexpressing cyclin D1 or D3 show little hyperphosphorylated pRB (Hinds et al. 1992).

Our data do not inform us how mammalian D cyclins or yeast Cln3 set the stage for pRB hyperphosphorylation. By forming physical complexes with pRB, the mammalian D cyclins may create a substrate for the subsequent activity of cyclin E/cdk2 complexes. Alternatively, Cln3/cyclin D may cause some type of initial phosphorylation of pRB that is prerequisite for the subsequent changes that we term hyperphosphorylation. The observation of limited, Cln3-specific pRB phosphorylation, as demonstrated in Cln3 single-positive cells,

is consistent with such preparatory phosphorylation. It is also possible that Cln1/Cln2/cyclin E are the only cyclins directly involved in the pRB hyperphosphorylation and Cln3/cyclin D1 regulate the activity of Cln1/Cln2/cyclin E, potentially through the known transcriptional mechanisms (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). In either event, these mechanistic models suggest that the physiologic signals regulating multiple G_1 cyclins converge on the important cell cycle decision that leads to functional inactivation of pRB and the attendant commitment by the cell to pass into the subsequent portions of its life cycle.

Materials and methods

Strain

All strains except L5191 and L5193 used in this experiment are isogenic derivatives of L4852, a *MATa* Gal⁺ haploid strain (Elion et al. 1991). L5191 and L5193 are ascospores derived from *cdc28-1N* and *cdc28-4* strains provided by A. Surana and K. Nasmyth (Research Institute of Molecular Pathology, Vienna, Austria). The relevant genotypes of strains used in this study are listed in Table 1. Yeast cells were grown at 30°C in YEP media (1% yeast extract, 2% peptone) or synthetic minimal media lacking appropriate amino acids for plasmid selection as described (Guthrie and Fink 1991). Either 2% glucose or 2% galactose was added to the culture media. DNA transformations in yeast were performed by the lithium acetate method (Ito et al. 1983).

Vector construction

The expression plasmid p2202T is a 2 μ -based vector that uses the *GAL1,10* promoter to drive expression of the cDNA and contains the *LEU2* gene as a selective marker. The vectors p2206T and p2208T are derived from p2202T, and each contains selectable markers, *URA3* and *HIS3*, respectively. To construct p2202TRB or p2206TRB, an inducible expression vector of human pRB in yeast, a 2.8-kb cDNA fragment that contains the entire human pRB coding sequences was inserted into *Bgl*III–*Hind*III-digested p2202T or p2206T. To construct expression vectors for human cyclins D1 and E, we changed the *Eco*RI site of p2208T to an *Eco*RV site by linker insertion. The cDNAs for human cyclin E (1.5 kb) and human cyclin D1 (1.0 kb) were filled in by T4 DNA polymerase and then ligated into *Eco*RV-cut p2208T.

Table 1. Strain list

Strain	Genotype	Source
L4852	<i>MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100</i>	Elion et al. (1991)
L4874	<i>L4852 cln1::TRP1</i>	Elion et al. (1991)
L4861	<i>L4852 cln2::LEU2</i>	Elion et al. (1991)
L4862	<i>L4852 cln3::URA3</i>	Elion et al. (1991)
L4868	<i>L4852 cln1::TRP1 cln2::LEU2</i>	Elion et al. (1991)
L4869	<i>L4852 cln2::LEU2 cln3::URA3</i>	Elion et al. (1991)
L4873	<i>L4852 cln1::TRP1 cln3::URA3</i>	Elion et al. (1991)
L5191	<i>MATa cdc28-1N ura3 leu2-3,112 trp1-1 his3-11,15 ade2 can1-100</i>	this work
L5193	<i>MATa cdc28-4 ura3 leu2-3,112 trp1-1 his3-11,15 ade2 can1-100</i>	this work

Immunoprecipitation

Overnight cultures grown in synthetic minimal medium containing 2% galactose were diluted to an OD_{600} of 0.75 in methionine-free synthetic minimal medium supplemented with 2% galactose. Cells were cultured for 30 min at 30°C, and [35 S]methionine (TransLabel; ICN) was added to 5 ml of cells at a final concentration of 50 μ Ci/ml. After additional culture for 3 hr at 30°C, the cells were collected and washed once with phosphate-buffered saline. After washing, 1.5×10^8 cells were resuspended in 500 μ l of ELB lysis buffer (0.5% NP-40, 250 mM NaCl, 50 mM HEPES at pH 7.0, 5 mM EDTA, 50 mM NaF, 200 μ M Na_3VO_4 , 2 mM benzamidine, 50 mM β -glycerophosphate, 1 mM PMSF, 10 μ g/ml of aprotinin, 10 μ g/ml of leupeptin, 10 μ g/ml of soybean trypsin inhibitor). Glass beads (0.4 mm) were added to the cell suspension, and the mixture was vortexed for 30 sec and put on ice. The vortex mixing was repeated four times, and then lysates were separated from the beads by centrifugation. The lysates were incubated with 1 μ l of the ascitic form of anti-human pRB monoclonal antibody NM2-21C9 on ice for 1 hr. Anti-mouse Ig(H+L) donkey antiserum (Jackson ImmunoResearch) was added to the lysate and incubated on ice for 30 min. Protein A-agarose beads (10 μ l) were then added to the lysate, and the mixture was rotated at 4°C for 90 min. Suspensions were pelleted and washed four times with 1 ml of lysis buffer. The immunoprecipitates were extracted in 200 μ l of 1 $\times$ SDS sample buffer, and 50 μ l of the sample was loaded on the each lane of the gel. Proteins were separated by 7.5% SDS-PAGE and subjected to fluorography following treatment with 15% PPO/acetic acid. For phosphate labeling, cells were cultured overnight in a phosphate-depleted medium (Guthrie and Fink 1991) containing 2% galactose, pelleted, washed, and resuspended in the same medium at a concentration at OD_{600} of 0.75. Cells were then labeled with [32 P]orthophosphate (1 mCi/ml) for 90 min. Proteins were extracted as described above. For phosphatase treatment, anti-pRB immunoprecipitates prepared as described above were resuspended in 40 μ l of 0.1 M MES (pH 6.0)/1 mM PMSF or 0.1 M sodium phosphate (pH 6.0)/1 mM PMSF. Potato acid phosphatase (0.3 units) (Boehringer Mannheim) was then added to the reaction. After 30 min of incubation at 37°C, 40 μ l of 2 $\times$ SDS sample buffer was added, and the sample was boiled for 5 min before being loaded on the gel.

Immunoblotting

Cells (1.5×10^8) were broken in 500 μ l of ELB buffer by use of glass beads as described before. The cell suspensions were then mixed with 166.7 μ l of 4 $\times$ SDS sample buffer, vortexed twice for 30 sec, and boiled for 5 min. The boiled samples were then centrifuged at 15,000g for 10 min to prepare the whole-cell lysates. One hundred microliters of the whole-cell lysate (corresponding to 2.25×10^7 cells) was loaded on the 7.5% SDS-PAGE gel. Following electrophoresis, proteins were transferred to an Immobilon-P PVDF membrane filter (Millipore). Blots were blocked by incubation with Tris-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin (TBS-T-BSA) for 1 hr at room temperature. The filter was then incubated with anti-human pRB mAb G3-245 (PharMingen) overnight at 4°C with rotation. After primary antibody treatment, blots were washed three times with TBS-T-BSA and were then incubated with goat anti-mouse IgG conjugated to alkaline phosphatase for 1.5 hr. Blots were then developed by the enhanced chemiluminescent detection (ECL) system (Amersham). For the detection of human cyclin D1 or E expressed in yeast, 100 μ l of the whole-cell lysates was run on an SDS-10% acrylamide gel, and separated proteins were blotted onto a PVDF membrane. Cyclin

D1 and E proteins were detected by use of rabbit anti-human cyclin D1 (PharMingen) and anti-human cyclin E monoclonal antibody HE2 (PharMingen) as probes, respectively.

Tryptic phosphopeptide mapping

Tryptic peptide mapping was performed according to the method described previously (Lees et al. 1991). Briefly, anti-pRB immunoprecipitates obtained from 32 P_i-labeled yeast cell lysates were separated by SDS-PAGE. The pRB band was excised, and the labeled protein was eluted from gel after boiling in 50 mM ammonium bicarbonate/0.2% SDS/1% 2-mercaptoethanol. The protein was lyophilized and resuspended in 1 ml of 50 mM ammonium bicarbonate (pH 8.0) with 50 μ g of trypsin (Sigma) treated with tosylamide-phenylethyl-chloromethyl ketone (TPCK). After overnight incubation, the supernatant was lyophilized and resuspended in 10 μ l of electrophoresis buffer [88% formic acid, glacial acetic acid, H₂O (1:3:16)]. Peptides were then separated on a cellulose TLC plate by use of electrophoresis in formic acid, glacial acetic acid, and deionized water (50:56:1794) as the first dimension and chromatography in butanol, pyridine, glacial acetic acid, and deionized water (75:50:15:60) as the second dimension.

Cell-cycle arrest and synchronization

Cells were grown to an A_{600} of 0.75 and treated with synthetic α -factor at a final concentration of 5 μ M for 3 hr at 30°C. Cell synchronization by release from α -factor was carried out by washing α -factor-treated cells and resuspending cells in synthetic minimal medium containing 2% galactose without α -factor as described by Tyers et al. (1993). At 20-min intervals 5 ml of the aliquots was removed, and the lysates were prepared as described above. Percentage budding was assayed by light microscopy of at least 200 cells per time point.

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