MOLECULAR AND CELLULAR BIOLOGY, July 1995, p. 3463–3469 Vol. 15, No. 7
0270-7306/95/$04.00+0

Different Roles for Cyclins D1 and E in Regulation of the G_1-to-S Transition

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Received 30 November 1994/Returned for modification 3 January 1995/Accepted 30 March 1995

Ectopic expression of cyclins D1 and E was previously shown to accelerate the G_1/S-phase transition, indicating that both classes of G_1 cyclin control an event(s) that is rate limiting for entry into S phase. In order to determine whether cyclins D1 and E control the same or two different rate-limiting events, we have created cell lines that express both cyclins in an inducible manner. We show here that ectopic expression of both cyclins E and D1 in the same cell has an additive effect on shortening of the G_1 interval relative to expression of any single cyclin. In order to further explore the molecular basis for G_1 cyclin action, we used cell lines capable of expressing cyclin D1, E, or both prematurely and measured the effect of cyclin expression in early G_1 on phosphorylation of the retinoblastoma susceptibility gene product (pRb). We show here that while premature expression of either cyclin alone advances the G_1/S-phase transition to the same extent, premature expression of cyclin D1 leads to immediate appearance of hyperphosphorylated pRb, while premature expression of cyclin E does not. Ectopic expression of both cyclins E and D1 in the same cell has an additive effect on shortening of the G_1 interval, while the effect on pRb phosphorylation is similar to the effect of cyclin D1 alone. These results suggest that cyclins E and D1 control two different events, both rate limiting for the G_1/S-phase transition, and that pRb phosphorylation might be the rate-limiting event controlled by cyclin D1.

Cyclins are positive regulators of cell cycle progression, initially identified in marine invertebrates as proteins that oscillate dramatically through the cell cycle (20). Cyclins are now known to be positive regulatory subunits of a class of protein kinases termed cyclin-dependent kinases (cdks). These protein kinases have been shown in a number of diverse eukaryotic systems to be the master regulators of major cell cycle transitions (for reviews, see references 39, 44, and 50). This has been demonstrated most clearly in the budding yeast *Saccharomyces cerevisiae*, in which a single cdk, Cdc28, associated with different cyclins is essential for both the G_1-to-S-phase (G_1/S) transition and the G_2-to-M-phase transition (for reviews, see references 28 and 41). In mammalian cells, a higher level of complexity exists than has been found in yeast cells. First, rather than utilizing a single cdk, mammalian cells have been found to have at least six (36, 47, 52). Second, superimposed on this multiplicity of cdk catalytic subunits is a growing list of different structural types of cyclin (28, 47, 57).

The progression from G_1 to S phase in mammalian cells is thought to be regulated by D-type cyclins associated with either cdk4 or cdk6 (4, 34, 37, 58) and by cyclin E associated with cdk2 (10, 24). Three lines of evidence support this notion. First, correlative data from synchronized cells indicate that D- and E-type cyclins are expressed and form active kinase complexes in mid- and late G_1 (35, 52). Second, microinjection of cyclin D1 antisense plasmids or monoclonal antibodies prepared against cyclin D1 prevents entry into S phase (3, 48). Similarly, entry into S phase is blocked by inhibiting cdk2, the functional partner for cyclin E, either by antibody microinjection (46, 54) or by transfection of a dominant negative *cdk2* mutant (55). Third, overexpression of cyclins D and E but not cyclin B1 shortens the G_1 interval in various mammalian cell lines (2, 21, 23, 40, 45, 48, 51, 56), indicating that both classes of G_1 cyclin control a rate-limiting event(s) for the G_1/S transition. However, it is not currently known whether these two classes of G_1 cyclin control the same or two different rate-limiting events (see below).

To understand how cdks promote cell cycle progression, it is important to identify their physiological substrates. Several lines of evidence suggest that the product of the retinoblastoma susceptibility gene (pRb) might be the substrate of G_1 cdks (7, 17, 18, 53). pRb is initially phosphorylated in mid- to late G_1, increases in phosphorylation as a function of cell cycle progression, and is dephosphorylated as cells exit mitosis (5, 6, 8, 31, 38). Hypophosphorylated pRb binds to transcription factors of the E2F family and prevents their transactivation of target genes that promote cell cycle progression. pRb phosphorylation during G_1 has been shown to release E2F from this inhibitory constraint, thereby facilitating entry into S phase (11, 25, 42, 43). In agreement with this model, pRb was shown to block cells in G_1 when introduced into certain *RB*-negative cell lines at high levels that could not be efficiently phosphorylated (12, 13, 16). pRb phosphorylation in G_1 occurs at multiple sites that fit the cdk consensus (26, 29), and a number of different cdks have been shown to be capable of performing this phosphorylation in vitro (1, 12, 19, 22, 26, 29, 34, 35). Cotransfection of genes encoding cyclin A, D2, or E together with pRb into Saos-2, an osteosarcoma-derived cell line, was shown to induce pRb hyperphosphorylation and to rescue the pRb-induced G_1 arrest (12, 16), while cyclin D1, although its associated kinase is capable of phosphorylating pRb in vitro (12, 22, 35, 37), failed to induce pRb phosphorylation in Saos-2 cells (9). Thus, although much circumstantial evidence suggests that G_1 cdks are responsible for pRb phosphorylation, the identity of the kinase(s) responsible for this phosphorylation in vivo remains uncertain.

In order to better understand the roles of cyclins E and D1 in regulation of the G_1/S transition, we have used rat fibroblast cell lines capable of expressing cyclin D1, E, or both in early

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G_1, a time when those cyclins are not normally expressed (51). We have measured the effect of cyclin expression on pRb phosphorylation and on shortening of the G_1 interval. We show here that while expression of either cyclin alone advances the G_1/S transition to a similar extent, expression of cyclin D1 leads to the immediate appearance of hyperphosphorylated pRb, while expression of cyclin E does not. We also show that expression of both cyclins in the same cell line has an additive effect on shortening of the G_1 interval, while the effect on pRb phosphorylation is similar to the effect of cyclin D1 alone. These results suggest that cyclins E and D1 control two different events, both rate limiting for the G_1/S transition, and that pRb phosphorylation is likely to be the rate-limiting event controlled by cyclin D1.

MATERIALS AND METHODS

Cells and culture conditions. Rat-1 clones E2, E19, D3, and D5, capable of expressing cyclin E or D1 in an inducible manner, were described previously (51). Clones DE5 and DE7, capable of expressing both cyclins in the same cell line, were created by cotransfecting tet-cyclin E, tet-cyclin D1, and tk-hygro (51) into R12 Rat-1 cells (51). Selection was performed as previously described (51). Hygromycin-resistant colonies were screened for the ability to express both cyclins upon removal of tetracycline. Two of eight clones were found to be positive. All cell lines were maintained in medium containing 2 μg of tetracycline per ml, 150 μg of hygromycin per ml, and 350 μg of G418 per ml. Other supplements to the medium were as described before (51).

For serum starvation and stimulation experiments, 18×10^5 cells were seeded per 15-cm plate in medium containing 10% fetal calf serum (FCS) and 2 μg of tetracycline per ml. After 24 h, the medium was replaced with starvation medium (Dulbecco's modified Eagle's medium [DMEM], no serum) containing tetracycline. Forty-eight hours later, the medium was replaced again with starvation medium either with (noninduced) or without (induced) tetracycline. After an additional 24 h, the medium was replaced with medium containing 10% FCS, with (noninduced) or without (induced) tetracycline, and samples for cell cycle analysis or preparation of cell lysates were taken at the indicated times.

Immunoprecipitation and immunoblots. Cell lysate (50 μg) prepared as described previously (46) was separated by sodium dodecyl sulfate–8.5% polyacrylamide gel electrophoresis (SDS–8.5% PAGE) and blotted to an Immobilon membrane (Millipore). Western (immunoblot) analysis was performed on different strips of the membrane with anti-pRb monoclonal antibodies (PMG3-245; PharMingen), or anti-cyclin E or D1 polyclonal antibodies (51).

Cell lysate (200 μg) was used to determine cyclin-cdk complex formation following immunoprecipitation with anti-cyclin D1 polyclonal antibodies or anti-cyclin E monoclonal antibodies (HE172 [51]). The immunoprecipitates were washed, heated to 37°C for 10 min, separated by SDS–11% PAGE, and blotted to an Immobilon membrane. Western analysis was performed with anti-cyclin E or D1 polyclonal antibodies, anti-cdk4 polyclonal antibodies (kindly provided by S. Hanks), and anti-cdk2 polyclonal antibodies prepared against a peptide corresponding to the 15 C-terminal amino acids of human cdk2.

In vitro kinase assays. Cell lysate (200 μg) was used to determine cyclin E-associated kinase activity following immunoprecipitation with anti-cyclin E polyclonal antibodies with histone H1 as the substrate, as described before (51).

Cyclin D1-associated kinase activity was determined as described before (35) with slight modifications. Cell lysates were prepared in Tween 20 lysis buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5], 150 mM NaCl, 2.5 mM EGTA [ethylene glycol tetraacetic acid], 1 mM EDTA, 0.1% Tween 20) containing phosphatase and protease inhibitors (1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride, 0.2 U of aprotinin per ml, 10 mM β-glycerolphosphate, 0.1 mM sodium vanadate, 1 mM NaF). Cyclin D1 complexes were immunoprecipitated with DCS11 monoclonal antibodies (33) (kindly provided by J. Lukas and J. Bartek) prebound to protein G beads, after clearing of 250 μg of lysate with protein G beads alone. The immunoprecipitates were washed four times in Tween 20 lysis buffer and twice in kinase assay buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$). The final pellet was resuspended in 30 μl of kinase buffer supplemented with 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerolphosphate, 0.1 mM sodium vanadate, 1 mM NaF, 20 μM ATP, 1 μg of GST-Rb (pRb amino acids 773 to 928 fused to glutathione *S*-transferase [GST]), and 10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol). After incubation for 30 min at 30°C, the reaction was stopped by the addition of Laemmli sample buffer and boiling for 5 min. Phosphorylated pRb was then visualized with a PhosphorImager after separation on SDS-PAGE.

Cell cycle analysis. The cells were labeled for 15 to 30 min with bromodeoxyuridine (BrdU; Amersham), fixed, and stained with fluorescein isothiocyanate-conjugated anti-BrdU (Becton Dickinson) and propidium iodide (Calbiochem) as described before (27). A total of 10,000 stained cells were analyzed in a fluorescence-activated cell sorter (FACScan; Becton Dickinson).

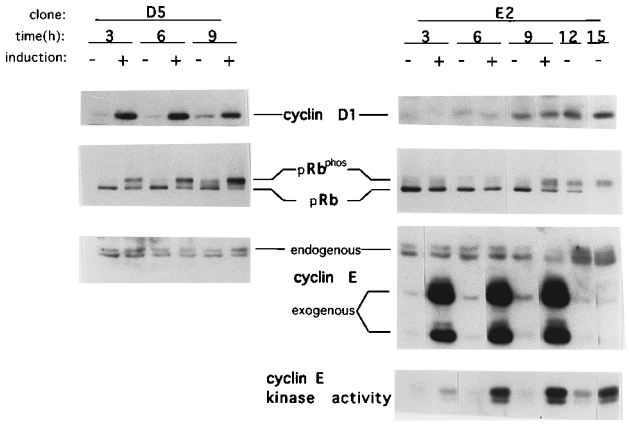

FIG. 1. Premature expression of cyclins E and D1 has different effects on pRb phosphorylation. Clones E2 and D5 (51) were arrested in G_0 by serum starvation and then stimulated to reenter the cell cycle with or without cyclin induction as described in Materials and Methods. Cell lysate (50 μg) prepared at the indicated time points after serum stimulation was analyzed by Western analysis with the indicated antibodies as described in Materials and Methods. Cell lysate (200 μg) from clone E2 was used to determine the cyclin E-associated kinase activity following immunoprecipitation with anti-cyclin E polyclonal antibodies with histone H1 as the substrate, as described before (51). The autoradiogram of ^{32}P-labeled histone H1 is shown.

RESULTS

Different effects of cyclins E and D1 on pRb phosphorylation. We have previously reported the establishment of rat fibroblast cell lines stably transfected with cyclin D1 or E under the control of a tetracycline-repressible promoter and shown that those cell lines can be induced to express the exogenous cyclin upon removal of tetracycline from the medium (51). In the present study, we have used these cell lines to measure the effect of cyclin induction on the phosphorylation state of pRb in a synchronized population. Cell lines capable of inducing cyclin D1 or E were arrested in G_0 by serum starvation under non-induced conditions and then serum stimulated to reenter the cell cycle with or without cyclin induction, as described in Materials and Methods. We have previously shown that, under these conditions, induction of cyclin D1 or E results in accelerated entry into S phase (51). Even though, under this protocol, tetracycline was removed from the medium 24 h before serum addition, expression of the exogenous cyclins was not detected in the absence of serum (data not shown). Western blot analysis indicated that the exogenous cyclins are detectable within 2 h of serum stimulation and are maximally induced by 3 h (data not shown). We therefore monitored the levels of the exogenous cyclins and the phosphorylation state of endogenous pRb at various times in G_1, beginning at 3 h (Fig. 1). Hypophosphorylated pRb, the form that is characteristic of cells in early G_1, has been shown to migrate more rapidly on SDS-polyacrylamide gels then the hyperphosphorylated forms associated with late G_1, S, and G_2 (30, 31). Figure 1 shows that cyclin D1 induction led to the appearance of the slower-migrating form of pRb, which was detected as soon as 3 h after serum stimulation, while induction of cyclin E caused changes in pRb migration only 9 h after serum stimulation (Fig. 1). These relatively late changes might be a direct effect of cyclin E on pRb or, alternatively, a reflection of the cell cycle advance induced by cyclin E through another mechanism (see below, Fig. 5A). The timing of entrance into S phase in this experiment was very similar for the two clones analyzed, 12 and 15 h after serum stimulation for induced and noninduced cells, respectively. The level of the induced cyclin E-associated kinase

activity in early G_1 is comparable to the level of the endogenous cyclin E kinase activity in noninduced cells in late G_1 (Fig. 1, compare induced 3 and 6 h with noninduced 12 and 15 h, respectively). However, hyperphosphorylated pRb is undetectable in induced cells in early G_1 (3 and 6 h), while it is predominant in noninduced cells in late G_1 (12 and 15 h). Comparison between cyclin E protein level and associated kinase activity reveals that while the protein is fully induced 3 h after serum stimulation, its associated kinase activity increases as the cells progress through G_1 (Fig. 1). These results are highly reproducible and suggest the existence of an inhibitory mechanism aimed at preventing premature activation of cyclin E-associated kinase. Figure 1 further shows that induction of cyclin E has no effect on the accumulation of endogenous cyclin D1 protein and that induction of cyclin D1 has no effect on the steady-state levels of endogenous cyclin E protein. The results presented in Fig. 1 clearly demonstrate that expression of active cyclin E by itself cannot promote pRb phosphorylation in early G_1 in vivo, while expression of cyclin D1 can.

The results presented in Fig. 1 strongly suggest that cyclin D1-associated kinase but not cyclin E-associated kinase is responsible in fibroblasts for pRb phosphorylation in G_1 in vivo. However, it remains possible that pRb is not a substrate of cyclin D1-associated kinase and that cyclin D1 expression in early G_1 leads to phosphorylation of pRb indirectly, without creating active kinase complexes. This possibility was further supported by the recent observation that expression of exogenous cyclin D3 and cdk4 in early G_1 is not sufficient for complex formation and creation of active kinase (35). We have therefore studied the complexes between the exogenous cyclins and the endogenous cdks and the activity of cyclin D1-associated kinase in early G_1. Figure 2A shows that exogenous cyclin E is associated with endogenous cdk2, while exogenous cyclin D1 is associated with endogenous cdk4. Thus, these two cyclins, when expressed ectopically in early G_1, are associated with their normal functional partners. Figure 2B further shows that induction of cyclin D1 in early G_1 leads to the creation of active complexes, capable of phosphorylating pRb in vitro. The results presented in Fig. 2 show that cyclin D1-dependent premature phosphorylation of endogenous pRb occurs at a time when active complexes composed of cyclin D1 and cdk4 are present in the cells.

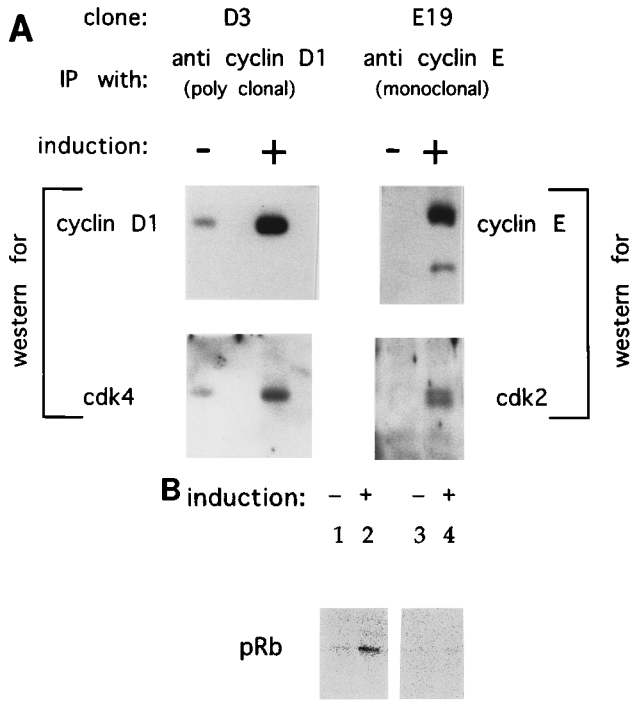

FIG. 2. Induced cyclins D1 and E create active complexes with their normal kinase partner in early G_1. (A) Clones E19 and D3 (51) were arrested in G_0 by serum starvation and then stimulated to reenter the cell cycle with or without cyclin induction as described in Materials and Methods. Cell lysates were prepared 3.5 h after serum stimulation as described before (51). Cell lysate (200 μg) was used for immunoprecipitation (IP) with anti-cyclin D1 polyclonal antibodies for clone D3 and anti-cyclin E monoclonal antibodies (HE172) for clone E19 (51). The immunoprecipitates were washed, heated to 37°C for 10 min, separated by SDS–11% PAGE, and blotted to an Immobilon membrane. Western analysis with cyclin antibodies indicated that the cyclins were induced and the immunoprecipitation was effective (upper panels). The same blots were then reacted with anti-cdk4 or anti-cdk2 antibodies. (B) Cells of clone D5 (51) were arrested in G_0 by serum starvation and then stimulated to reenter the cell cycle with or without cyclin induction as described in Materials and Methods. Cell lysates were prepared 4 h after serum stimulation in Tween 20 lysis buffer as described in Materials and Methods. Cyclin D1-associated kinase activity was determined by using pRb as a substrate as described in Materials and Methods, following immunoprecipitation with DCS-11 monoclonal antibodies (33) prebound to protein G beads (lanes 1 and 2) or protein G beads alone (lanes 3 and 4). Western analysis of the same lysates confirmed that cyclin D1 protein was induced and that endogenous pRb was 60% phosphorylated (not shown).

Inducible expression of cyclins D1 and E in the same cell line. Cyclins D1 and E were previously shown to be rate limiting for the G_1/S transition. However, the relationship between the rate-limiting events controlled by these two cyclins is currently unclear (see Introduction). We have considered two possibilities: either cyclins E and D1 control the same rate-limiting event, or they control independent events, both rate limiting for the G_1/S transition. In the context of the second model, premature expression of a single cyclin accelerates one event, while the other is largely unaffected. Thus, only limited acceleration of the G_1/S transition can be achieved. However, premature expression of both cyclins in the same cell is expected to accelerate both events and to give rise to additive acceleration of the G_1/S transition. In contrast, if both cyclins target the same rate-limiting event, premature expression of both cyclins in the same cell is not expected to accelerate the G_1/S transition more than the expression of either one alone (assuming that the acceleration caused by any one cyclin is saturated; see the Discussion). In order to distinguish between these two models, we used the tetracycline-repressible expression system (14) to create cell lines that express both cyclins in an inducible manner, as described previously (51). Comparison of induced expression levels shows that clone DE5 expresses the same amount of cyclin E as clone E2 and the same amount of cyclin D1 as clone D5 (Fig. 3A). Analysis of the cell cycle distribution in asynchronous cells following cyclin induction has revealed that while expression of cyclin D1 or E alone reduced the duration of G_1 by 32%, expression of both cyclins in the same cells reduced G_1 by 71% (Fig. 3B). It has been reported that expression of either cyclin E or cyclin D1 alone caused a reduction in cell size (21, 45, 48). Although, in our cell lines expressing cyclin E or D1 alone, we could not detect large effects of expression on cell size, a clear reduction in cell size was observed upon expression of both cyclins for 72 h (Fig. 3C). Analysis of the length of G_1 in cells emerging from quiescence showed that G_1 was shortened by 3 h (20%) upon induction of cyclin D1 or E alone and by 9 h (60%) upon induction of both cyclins in the same cells (Fig. 3D). Two independent clones, DE5 and DE7, expressing both cyclins E and D1 to similar levels, were found to shorten the length of G_1 by the same extent, 50 to 60%, in repeated experiments (Table 1).

Taken together, the results presented in Fig. 3 and Table 1

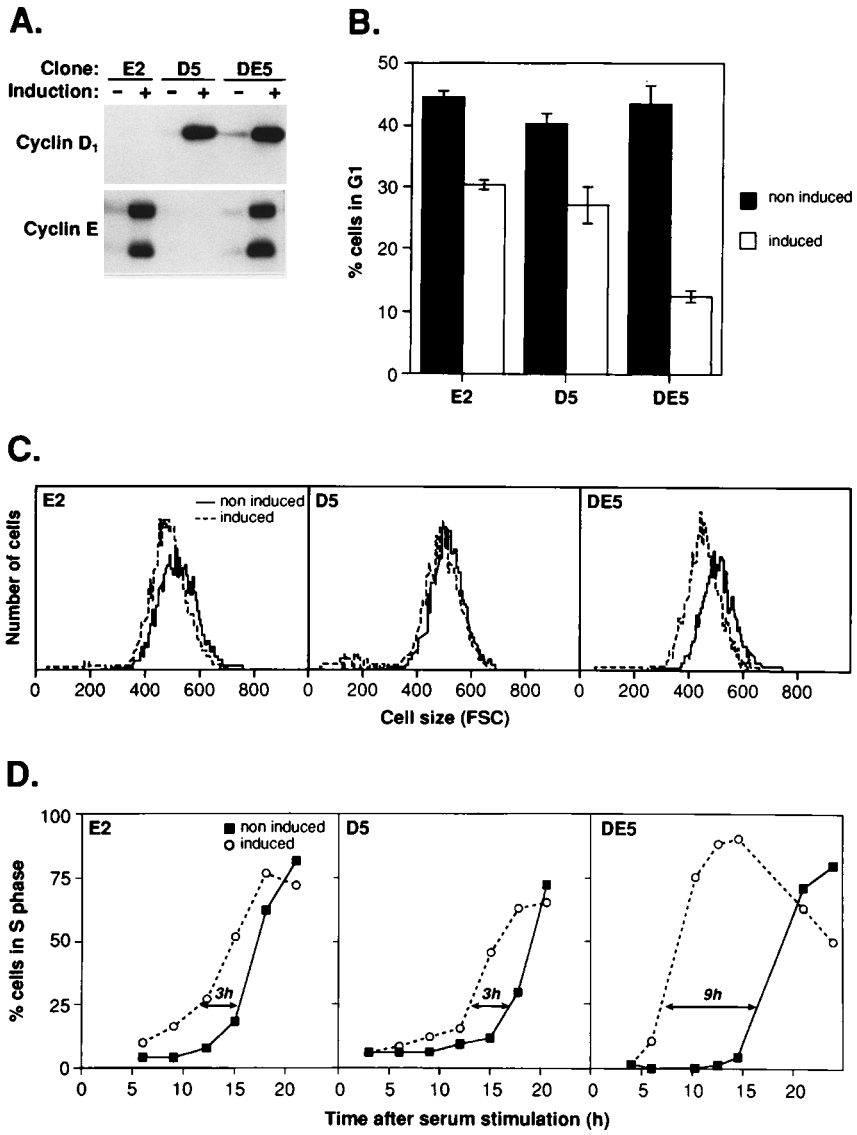

FIG. 3. Additive effects of cyclin expression on the length of G_1 and cell size in a cell line expressing both cyclins E and D1. (A to C) Asynchronous cells of clones E2, D5, and DE5 were seeded with or without tetracycline (noninduced and induced, respectively) in medium containing 10% FCS. (A) Cell lysates were prepared after 48 h and analyzed for cyclin expression as described in the legend to Fig. 1. (B) The percentages of cells in the various cell cycle phases were determined after 48 h by flow cytometric analysis following labeling with BrdU for 30 min, as described in Materials and Methods. Three independent plates were analyzed for each point, and the standard deviations are shown. (C) Cell size was determined after 72 h by forward light scattering as described before (45). (D) Clones E2, D5, and DE5 were arrested in G_0 by serum starvation and then stimulated to reenter the cell cycle with or without cyclin induction as described in the legend to Fig. 1. Cell cycle analysis was performed at the indicated time points as described for panel B.

suggest that expression of both cyclins D1 and E in the same cell line had at least an additive effect on G_1 shortening. In many repeated experiments, the effect was either additive or more than additive, but never less than additive (Fig. 3 and Table 1, and data not shown). The results presented in Fig. 3 and Table 1 are therefore consistent with cyclins D1 and E controlling different rate-limiting events.

The hyperphosphorylated form of pRb appears in late G_1, 3 to 6 h before entry into S phase (7). In order to discriminate between direct effects of cyclin expression on pRb phosphorylation and pRb phosphorylation occurring as a consequence of cell cycle advance, we compared the effects of cyclin expression on cell cycle progression and pRb phosphorylation in the same biological experiment (Fig. 4A). We found that induction of cyclin E caused the same acceleration of S phase and of pRb phosphorylation (3 h; Fig. 4A, top). It is therefore impossible to determine whether the acceleration of pRb phosphorylation is a direct effect of cyclin E induction or a reflection of the cell cycle advance mediated through an as yet unknown mechanism. In contrast, cyclin D1 induction caused a greater acceleration of pRb phosphorylation (6.7 h) than of S phase (3.8 h), which suggests that pRb phosphorylation is a direct result of cyclin D1 induction and might be the event leading to cell cycle advance (Fig. 4A, middle). The same comparison performed in a clone expressing both cyclins revealed that pRb phosphorylation is accelerated to a similar degree as with cyclin D1 expression alone (7 h), but now S phase is accelerated to the same extent (Fig. 4A, bottom). These results suggest that pRb phosphorylation might be the rate-limiting event controlled by cyclin D1 (either directly or through activation of another as

TABLE 1. Effect of cyclin induction on shortening of G_1 in cells expressing both cyclins E and D1[a]

Clone and expt no.	Shortening of G_1 by cyclin induction (h)	Length of G_1 in noninduced cells (h)	% Shortening of G_1 by cyclin induction
D5	3.8	15	25
E2	3.0	15	20
DE5			
1	7.7	12.5	61
2	8	16	50
3	7	13	54
DE7			
1	9	15	60
2	8	16	50

[a] Clones E2, D5, DE5, and DE7 were arrested in G_0 by serum starvation and then stimulated to reenter the cell cycle with or without cyclin induction as described in Materials and Methods. The percentages of cells in the various cell cycle phases were determined every 2 to 3 h for the next 24 h by flow cytometric analysis after labeling with BrdU for 30 min, as described in Materials and Methods. The percentage of cells in S phase versus time was then plotted for induced and noninduced cells (see Fig. 3D). The shortening of G_1 by cyclin induction is represented by the distance between the curves plotted for induced and noninduced cells (see Fig. 3D).

yet unknown kinase), while cyclin E controls another event that is rate limiting for entry into S phase. In a cell line expressing cyclin D1 alone, pRb phosphorylation is accelerated, but entry into S phase is delayed until the cyclin E-controlled event occurs. However, in a cell line expressing both cyclins prematurely, both rate-limiting events are accelerated, and therefore there is no delay between pRb phosphorylation and entry into S phase (see model, Fig. 4B).

DISCUSSION

Overexpression of G_1 cyclins in *S. cerevisiae* causes a dramatic reduction in the length of G_1 (49). In contrast, studies of overexpression of G_1 cyclins in mammalian cells thus far have shown shortening of G_1 by only 20 to 30% (21, 45, 48, 51, 56). These data suggested that in mammalian cells, unlike yeast cells, only a limited window of the G_1 phase is regulated by G_1 cyclins. We show here that expression of two different mammalian G_1 cyclins, cyclins D1 and E, in the same cell line causes a 60 to 70% shortening of the G_1 phase, which is at least an additive effect compared with the shortening of G_1 by expression of each cyclin alone. This is the first demonstration that most of G_1 in mammalian cells is controlled by G_1 cyclins. Furthermore, these results suggest that cyclins E and D1 control different events, both partially rate limiting for the G_1/S transition. Since we cannot be certain that the effect of cyclin E or D1 on G_1 shortening for the single-cyclin-expressing cell lines is saturated, we cannot exclude the possibility that expression of one cyclin alone to a higher degree would have a significant impact. However, the 20 to 30% reduction of G_1 length achieved by ectopic expression of individual cyclins observed in several laboratories with different cell and expression systems (21, 45, 48, 51, 56) suggests that we have at least approached saturation.

We show here that while premature expression of cyclin D1 or E alone advances the G_1/S transition to the same extent, expression of cyclin D1 in early G_1 leads to immediate appearance of the more slowly migrating hyperphosphorylated pRb, while expression of active cyclin E in early G_1 does not. These results are unexpected and in apparent contradiction to earlier published results. Cotransfection of genes encoding cyclin A, D2, or E together with pRb into Saos-2 osteosarcoma cells was shown to induce pRb hyperphosphorylation and rescue the pRb-induced G_1 arrest (12, 16). However, cyclin D1, although capable of rescuing the G_1 arrest, failed to induce pRb phosphorylation in Saos-2 cells (9). Although these results appear to be in conflict with ours, the discrepancy most likely is attributable to differences between the experimental systems. First, Saos-2 cells are transformed, while Rat-1 cells are not. Second, we measured the effects of exogenous stably transfected cyclins on endogenous pRb, while in Saos-2 cells, both cyclin and Rb genes were cotransfected transiently, and therefore the levels of pRb and cyclin expressed are likely to be much higher then the levels reported here. Third, we observed synchronized populations in early G_1, when it was possible to correlate cyclin function with pRb phosphorylation, while the Saos-2 experiments were done on asynchronous populations assayed days after transfection. In the latter case, pRb phosphorylation could be a reflection of cell cycle progression rather than the direct action of the transfected cyclin. It is also possible, however, that cyclin E can cause phosphorylation of pRb later in the cell cycle, but not in early G_1.

D-type cyclins can bind to hypophosphorylated forms of pRb directly, through their Leu-X-Cys-X-Glu sequence, not found in other cyclins but shared with the pRb-binding oncoproteins of DNA tumor viruses (9, 12, 22). Two alternative hypotheses have been suggested to explain the functional role of this interaction. Ewen and Kato and their colleagues have suggested that pRb-cyclin D complex formation physically targets pRb for phosphorylation by cyclin D-dependent kinases (12, 22). In contrast, Dowdy and Hinds and colleagues have proposed that D-type cyclins might be subjected to sequestration by pRb and that pRb phosphorylation by another cdk (possibly cyclin E-dependent kinase) might release D-type cyclins, enabling them to be involved in phosphorylation of downstream targets (9, 15). If indeed cyclin E is responsible for pRb phosphorylation and cyclin D functions downstream, one would predict that expression of cyclin E but not cyclin D would cause premature pRb phosphorylation. Our results show that at least in rat fibroblasts, the opposite is the case. Furthermore, if cyclin D1 is necessary for G_1/S progression through a function downstream of pRb, disruption of cyclin D function is expected to block entrance into S phase whether or not the cells contain functional pRb protein. However, it was recently found that while cyclin D1 is necessary for cell cycle progression in a variety of cell lines (3, 33, 48), it is not necessary in *RB*-deficient cells (32). These results are consistent with a model in which cyclin D1 is necessary for cell cycle progression in normal cells, since it is required to mediate pRb phosphorylation. Our results support this model and suggest further that cyclins E and D1 control two different events, both partially rate limiting for the G_1/S transition. pRb phosphorylation is likely to be the rate-limiting event controlled by cyclin D1, while an as yet unknown critical G_1/S substrate would be the target of cyclin E-cdk2 (see model, Fig. 4B). If the two-event model accurately reflects the regulatory organization of G_1, it might be expected that expression of an individual cyclin would not advance the G_1/S transition at all, since the rate-limiting event controlled by the other cyclin would occur on schedule. The acceleration observed in response to expression of individual cyclins suggests, however, the existence of some communication between the two putative cyclin-controlled pathways, possibly mediated by positive feedback interactions.

As with any model, the proposed mechanism summarized in Fig. 4B should be regarded as a working hypothesis, the objective of which is to stimulate further research. One prediction of this model is that ectopic expression of cyclin D1 in *RB*-deficient cells should have no effect on the length of G_1, while

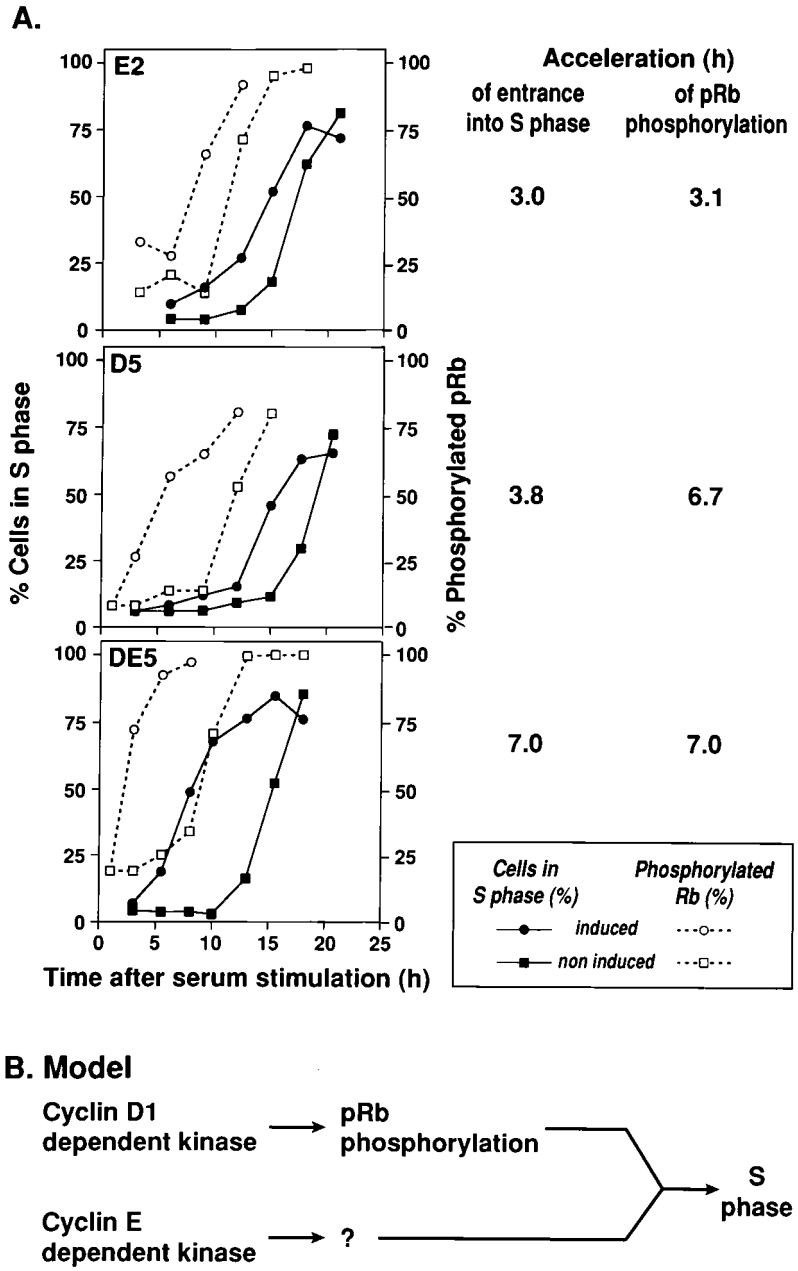

FIG. 4. Cyclins D1 and E may control different rate-limiting events. (A) Clones E2, D5, and DE5 were arrested in G_0 by serum starvation and then stimulated to reenter the cell cycle with or without cyclin induction as described in the legend to Fig. 1. Cell cycle analysis was performed at the indicated time points as described in the legend to 3B. Western analysis with pRb antibody was performed on cell lysates prepared at the different time points, as described in the legend to Fig. 1. The percentage of phosphorylated pRb was determined by laser densitometry of autoradiograms similar to those shown in Fig. 1. (B) Model. We propose two parallel pathways essential for entry into S phase, in which cdks play rate-limiting roles. The function of cyclin D1-associated kinase is likely to be phosphorylation of pRb. The function of cyclin E-associated kinase is unknown.

ectopic expression of cyclin E should cause a decrease in the length of G_1. Indeed, a recent study demonstrated that expression of cyclin E in HeLa cells (in which pRb is inactive because of the presence of the human papillomavirus E7 protein) caused a reduction in the length of G_1 (56). Tests of whether cyclin D1 accelerates the G_1/S transition in such a system are in progress.

ACKNOWLEDGMENTS

We thank S. Hanks, E. Lees, E. Harlow, and V. Dulic for antibodies; J. Lukas and J. Bartek for antibodies and pRb protein; and J. Lukas, J. Bartek, and C. Sherr for suggestions concerning the cyclin D1 kinase assay. We also thank D. Lew and L. Hengst for fruitful discussions and D. Lew, L. Hengst, J. Hanley-Hyde, and J. Bushman for critical reading of the manuscript.

D.R. is supported by a postdoctoral fellowship from the American Cancer Society, California division. This work was supported by U.S. Public Health Service grant GM46006 to S.I.R.

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