Cyclin E-induced S phase without activation of the pRb/E2F pathway

Jiri Lukas,¹ Thomas Herzinger,² Klaus Hansen,¹ Maria Cristina Moroni,³ Dalia Resnitzky,⁴ Kristian Helin,³ Steven I. Reed,² and Jiri Bartek^{1,5}

¹Danish Cancer Society, Division of Cancer Biology, DK-2100 Copenhagen, Denmark; ²Department of Molecular Biology, MB-7, Scripps Research Institute, La Jolla, California 92037 USA; ³Department of Experimental Oncology, European Institute of Oncology, 20141 Milan, Italy; ⁴Department of Molecular Cell Biology, Weizmann Institute of Science, 76100 Rehovot, Israel

In cells of higher eukaryotes, cyclin D-dependent kinases Cdk4 and Cdk6 and, possibly, cyclin E-dependent Cdk2 positively regulate the G₁- to S-phase transition, by phosphorylating the retinoblastoma protein (pRb), thereby releasing E2F transcription factors that control S-phase genes. Here we performed microinjection and transfection experiments using rat R12 fibroblasts, their derivatives conditionally overexpressing cyclins D1 or E, and human U-2-OS cells, to explore the action of G₁ cyclins and the relationship of E2F and cyclin E in S-phase induction. We demonstrate that ectopic expression of cyclin E, but not cyclin D1, can override G₁ arrest imposed by either the p16^{INK4a} Cdk inhibitor specific for Cdk4 and Cdk6 or a novel phosphorylation-deficient mutant pRb. Several complementary approaches to assess E2F activation, including quantitative reporter assays in live cells, showed that the cyclin E-induced S phase and completion of the cell division cycle can occur in the absence of E2F-mediated transactivation. Together with the ability of cyclin E to overcome a G₁ block induced by expression of dominant-negative mutant DP-1, a heterodimeric partner of E2Fs, these results provide evidence for a cyclin E-controlled S phase-promoting event in somatic cells downstream of or parallel to phosphorylation of pRb and independent of E2F activation. They furthermore indicate that a lack of E2F-mediated transactivation can be compensated by hyperactivation of this cyclin E-controlled event.

[Key Words: Cyclin E; cyclin D1; p16; pRb phosphorylation; E2F; S phase]

Received November 11, 1996; revised version accepted April 9, 1997.

Progression from G₁ to S phase of the mammalian cell cycle is regulated by cyclin D-dependent kinases Cdk4 and Cdk6 complexed to D-type cyclins, and by Cdk2 bound to cyclins E or A (Sherr 1993; Peters 1994; Morgan 1995; Pines 1995; Sherr and Roberts 1995; Weinberg 1995; Bartek et al. 1996; Reed 1996). Apart from reflecting cyclin accumulation and binding, Cdk activity is regulated by negative and positive phosphorylation events (Morgan 1995; Pines 1995) and interactions with Cdk inhibitors (Peters 1994; Pines 1995; Sherr and Roberts 1995; Harper and Elledge 1996). Cdk inhibitors of the p16^{INK4} family specifically inhibit Cdk4 and Cdk6, whereas members of the p21^{Cip1}/p27^{Kip1} family inhibit a broader spectrum of Cdks (Peters 1994; Sherr and Roberts 1995; Harper and Elledge 1996). The critical substrates of cyclin D-, E-, and A-associated Cdks remain to be defined, but one important target believed to be phosphorylated in a stepwise manner by some, and possibly all, of these G₁/S cyclin-Cdk complexes is retinoblastoma protein (pRb) (Sherr and Roberts 1995; Weinberg 1995; Bartek et al. 1996).

Phosphorylation of pRb in mid- to late G₁ results in its dissociation from a number of transcription factors, most notably members of the E2F/DP family, that appear to control expression of S-phase genes (Nevins 1992; Helin and Harlow 1993; LaThangue 1994; Beijersbergen and Bernards 1996). Although this scenario accommodates much of the available data, some recent findings indicate that regulation of the G₁/S transition may be more complex, in that cyclins D and E appear to target two distinct and possibly parallel events whose precise molecular nature and mutual coordination are unclear at present (Resnitzky and Reed 1995; Roussel et al. 1995; Reed 1996). Whereas the principal role of cyclin D-associated Cdks depends on functional pRb (Lukas et al. 1994a, 1995a,b; Koh et al. 1995) and most likely entails phosphorylating pRb, thereby releasing E2F and other pRb-bound proteins (Sherr and Roberts 1995; Weinberg 1995; Bartek et al. 1996; Beijersbergen and Bernards 1996), cyclin E remains both rate-limiting and essential for the G₁/S transition in Rb-negative, as well as Rb-positive cells, implying that the cyclin E-associated kinase targets a critical substrate other than pRb (Wimmel et al. 1994; Ohtsubo et al. 1995). In addition, whereas both E2F and cyclin E/Cdk2 appear to be required for

⁵Corresponding author.
E-MAIL bartek@biobase.dk; FAX +45 35 25 77 21.

S-phase entry (van den Heuvel and Harlow 1993; Knoblich et al. 1994; Ohtsubo et al. 1995; Wu et al. 1996), attempts to elucidate the temporal order and/or mutual dependence of the cyclin D-, E2F-, and cyclin E/Cdk2-regulated events at the G₁/S transition have yielded results that, at least in part, are in apparent conflict (DeGregori et al. 1995b; Duronio and O'Farrell 1995; Duronio et al. 1995, 1996; Resnitzky and Reed 1995; Roussel et al. 1995; Hofmann and Livingston 1996; Lukas et al. 1996; Mann and Jones 1996). Taken together, these data raise important questions concerning the role of cyclin E and the extent to which the activity of cyclin D-associated Cdk2, phosphorylation of pRb, and, indeed, the activity of E2F are essential for the promotion of S phase.

In an attempt to understand the G₁/S-controlling pathways regulated by cyclin D and cyclin E, and to elucidate the interdependence of cyclin E and E2F, we examined the respective abilities of cyclins D1 or E to overcome a G₁ arrest imposed by either p16 or a phosphorylation-deficient mutant pRb. Ectopic expression of cyclin E, but not cyclin D1, was able to rescue the G₁ block in either case. Cyclin E-driven progression into S phase and through the rest of the cell division cycle occurred even under conditions where the transactivating potential of E2F was suppressed by the nonphosphorylatable mutant pRb or dominant-negative mutant DP-1. We suggest that cyclin E may play multiple roles in promoting S phase, and that at least one of the critical events controlled by cyclin E is independent on the pRb/E2F pathway and sufficient, when hyperactivated, to bypass the requirement for E2F activity.

Results

Cyclin E, but not cyclin D1, can overcome a p16-mediated G₁ arrest

G₁ cyclin-dependent kinases commonly have been regarded as upstream activators of E2F, acting via phosphorylation of pRb and the other E2F-binding pocket proteins, p107 and p130 (Sherr and Roberts 1995; Weinberg 1995; Beijersbergen and Bernards 1996). Cyclin D-associated kinase may initiate the process by phosphorylating some of the serine and/or threonine residues in mid-to late G₁, and Cdk2 complexed to cyclins E and/or A may contribute additional pRb phosphorylation events at the G₁/S transition (Weinberg 1995). To investigate the roles of cyclin D1 and E in promoting S-phase entry, we first asked whether either of these G₁ cyclins could overcome a G₁ block imposed by inhibition of cyclin D-associated Cdk2. Cultured rat fibroblasts (R12) arrested in G₀ by serum starvation were microinjected with a plasmid expressing the human Cdk4/6 inhibitor p16 (Serrano et al. 1993), either alone or in combination with plasmids expressing cyclin D1 or cyclin E, and the effects on G₁/S progression were examined by monitoring DNA synthesis. As expected, ectopic expression of p16 prevented incorporation of 5-bromodeoxyuridine

(BrdU) into newly synthesized DNA of serum-stimulated R12 cells. Coexpression of cyclin D1 did not influence the G₁ arrest caused by p16, whereas expression of cyclin E significantly counteracted the p16-mediated G₁ block (Fig. 1A,B). The effect of cyclin E was dependent on its kinase partner, because coexpression of a dominant-negative mutant Cdk2 (van den Heuvel and Harlow 1993) abolished the stimulation of S-phase entry (not shown). We next employed a related model system, microinjecting the p16 plasmid into R12-derived clones R12/D5 and R12/E2 in which tetracycline-repressible expression of exogenous cyclins D1 or E, respectively, had been established (Resnitzky et al. 1994). Upon removal of tetracycline from the medium, the p16-injected R12/D5 cells expressed cyclin D1 yet remained in G₁ phase. In contrast, in p16-injected R12/E2 cells induction of cyclin E stimulated entry into S phase in a significant proportion of the injected cell population (Fig. 1B). The observed lower percentage of R12/E2 cells induced to enter S phase in this experiment relative to the experiment employing direct coinjection of p16 and cyclin E expression plasmids (Fig. 1B) is consistent with variable expression of cyclin E in individual R12/E2 cells upon tetracycline removal (Resnitzky et al. 1994; Resnitzky and Reed 1995). Finally, the ability of cyclin E to rescue a G₁ arrest imposed by p16 was independently confirmed by cotransfecting p16 and either cyclin D1 or cyclin E expression plasmids into exponentially growing human U-2-OS osteosarcoma cells. Flow cytometric analyses of U-2-OS cells productively transfected with p16 and control DNA, p16 and cyclin D1, or p16 and cyclin E expression plasmids demonstrated that only the cyclin E-transfected cells were able to enter S phase as efficiently as control cells (Table 1). Thus, cyclin E can rescue the G₁ block conferred by expression of p16, whereas cyclin D1 cannot.

Cyclin E can rescue a G₁ blockade imposed by phosphorylation-deficient mutant pRb

These results indicate that expression of cyclin E can overcome the blockade of cyclin D-Cdk activity mediated by p16, and raise the question of whether or not cyclin E can induce S phase in the presence of hypophosphorylated pRb, which also normally blocks the G₁/S transition. To test this possibility, we employed site-directed mutagenesis to further modify the mouse *RB1* gene mutant (Δ p34) constructed by Hamel et al. (1992) that showed a variable growth inhibitory effect (data not shown). Our new mutant (designated Δ cdk) eliminates 10 Cdk target sites including the 8 mutations present in Δ p34 (in the serine and threonine residues controlling E2F and c-Abl binding; Wang et al. 1994; Knudsen and Wang 1996), and, additionally, the Thr-814 and Thr-819, equivalent to Thr-821 and Thr-826 of human pRb, recently identified as critical for regulation of pRb binding to LXCXE-containing proteins (Knudsen and Wang 1996). To verify that the additional threonine to alanine substitutions made in the Δ cdk mutant influence the

Figure 1. Cyclin E but not cyclin D1 rescues the G_1 block imposed by p16. (A) Immunofluorescence detection of cyclin D1 or E in R12 fibroblasts 24 hr after microinjection of expression plasmids, using antibodies DCS-6 and HE12, respectively (top). R12 cells expressing p16 are stimulated to enter S phase by coinjected cyclin E (bottom) but not cyclin D1 (middle) expression plasmids. Quiescent R12 cells were injected directly into nuclei with indicated expression plasmids and stimulated to reenter the cell cycle by medium containing 10% FCS. Twenty-four hours later, productively injected cells expressing p16 were detected by immunostaining with antibody DCS-50 and scored for simultaneous incorporation of BrdU. Matched cells are marked by arrowheads. (B) Quantitation of the relative ability of cyclins E or D1 to overcome p16-elicited G_1 arrest assayed by coinjection of the expression plasmids in R12 cells (left) or by expression of p16 in cyclin E (E2) or D1 (D5) tetracycline-repressible clones at 24 or 30 hr after injection or cyclin induction, respectively (right). Data are presented as a mean $\pm$ S.D. from three to five independent experiments.

Table 1. Effect of cyclin D1 and cyclin E on G_1 arrest imposed by 16 or pRb in U-2-OS cells

Expression plasmids	ΔG_1 (%) ^a	
	experiment 1	experiment 2
p16 + control DNA	34	26
p16 + cyclin D1	47	41
p16 + cyclin E	2	2
pRb($\Delta B/X$) + control DNA	9	5
pRb(Δcdk) + control DNA	31	27
pRb(Δcdk) + cyclin D1	32	25
pRb(Δcdk) + cyclin E	1	7

^aThe data are presented as ΔG_1 , the percent change in the proportion of cells in G_1 relative to the vector control sample.

phosphorylation of pRb in vivo, plasmids expressing hemagglutinin (HA)-tagged wild-type ($\Delta B/X$) or mutant (either $\Delta p34$ or Δcdk) pRb were transfected into U-2-OS cells and incubated with [^{32}P]orthophosphate. Wild-type and mutant pRb immunoprecipitated with anti-HA antibody from cell extracts was then subjected to two-dimensional phosphoamino acid and phosphopeptide analyses. A considerably higher level of incorporation was detected in the wild-type pRb than in either mutant (Fig. 2). More important, consistent with a loss of two additional potential phosphothreonines, the phosphothreonine-to-phosphoserine ratio in the residual signal detected in the Δcdk mutant was reproducibly about threefold lower than in the $\Delta p34$ mutant pRb (Fig. 2). Furthermore, two phosphopeptides were selectively missing in tryptic digests of the Δcdk mutant, consistent with the substitutions of the two additional threonine residues in the pRb(Δcdk) (Fig. 2). The identity of these two tryptic phosphopeptides with those predicted to contain residues Thr-814 and Thr-819, respectively, was confirmed by Edman sequencing of phosphopeptides isolated from two-dimensional tryptic maps (data not shown).

Injection of an HA-tagged wild-type Rb($\Delta B/X$) expression plasmid into serum-starved R12 cells led to moderate inhibition of entry into S phase upon serum stimulation. This effect was reversed and S-phase onset even accelerated by coinjection of either cyclin D1 or cyclin E expression plasmids (Fig. 3A,B). By contrast, R12 cells injected with the mutant Rb(Δcdk) lacking 10 Cdk target sites were efficiently arrested in G_1 , and although this block could be rescued fully by coinjection of the cyclin E expression plasmid, it was influenced only marginally by the cyclin D1 expression plasmid (Fig. 3A,B). Parallel experiments with coinjections of plasmids expressing catalytically inactive Cdk2 and Cdk4 (van den Heuvel and Harlow 1993), respectively, showed that the dramatic effect of cyclin E was dependent on the activity of its Cdk partner, whereas the small effect of cyclin D1 was not (not shown). In addition, cyclin E was able to stimulate S-phase entry retarded by wild-type pRb($\Delta B/X$) or prevented by the phosphorylation-deficient mutant

Figure 2. Reduced in vivo phosphorylation of the pRb(Δcdk) mutant. (*Top*) PhosphorImage analysis of gel-separated pRb proteins immunoprecipitated from transfected U-2-OS cells. (*Middle*) Phosphoamino acid analysis of pRb(Δp34) and pRb(Δcdk), with the calculated phosphothreonine-to-phosphoserine ratios indicated under the corresponding images; arrowheads point to a nonspecific signal distinct from phosphotyrosine. (*Bottom*) Tryptic phosphopeptide mapping. Closed arrowheads mark the identical phosphopeptide preserved in both pRb mutants; open arrowheads indicate positions of two phosphopeptides selectively absent in pRb(Δcdk).

pRb(Δcdk) in both R12/E2 cells upon removal of tetracycline (Fig. 3B), and in U-2-OS cells cotransfected with the Rb and cyclin E expression plasmids (Table 1). Ectopic cyclin D1 expression could reverse the effects of wild-type pRb, but had little impact on the cell cycle block conferred by the Δcdk mutant pRb in either R12/D5 (Fig. 3B) or U-2-OS cells (Table 1). Furthermore, to examine whether cells arrested in G₁ by the mutant pRb(Δcdk), and rescued by coexpression of cyclin E can traverse the rest of the cell division cycle, R12 fibroblasts presynchronized by serum starvation and coinjected as above with the appropriate combinations of expression plasmids (see Material and Methods for details) were followed for 40 hr after serum stimulation, and divisions of the individual microinjected cells recorded as a function of time. This analysis revealed that all cells rescued by cyclin E can complete all phases of the cell cycle, including mitosis and cell division, despite continuous presence of active pRb(Δcdk), thereby excluding the possibility that such cells enter S phase but then remain arrested in S and/or G₂ phases, or initiate a cell death program, never completing the cell cycle.

Cyclin E can induce S phase without activation of E2F

Taken together, the above results demonstrate that ectopic cyclin E can induce DNA replication even in cells whose G₁/S transition is otherwise prevented by a phosphorylation-deficient mutant pRb. Moreover, the fact that such cells rescued by cyclin E can complete the cell cycle including undergoing cell division raised the possibility that cyclin E-triggered cell cycle progression can occur in the absence of free S-phase transcription factors such as E2F. To assess the status of E2F under the conditions used, we employed both qualitative and quantitative in situ reporter assays, using luciferase constructs controlled by either of the following three promoters: an artificial promoter containing six consensus E2F sites in front of the TATA box (6xE2F-luc kindly provided by Ali Fattaey, ONYX Pharmaceuticals, Richmond, CA), and endogenous promoters of genes known to be either activated (DHFR promoter; Means et al. 1992) or repressed (B-myb promoter; Lam and Watson 1993) by E2F. The use of the 6xE2F-luc reporter plasmid was validated by cotransfection and/or microinjection experiments in human and rodent cells, which showed that the 6xE2F promoter can be activated effectively by any of the known members of the E2F family, either alone or in combination with DP-1, and that it is significantly more sensitive in the luciferase reporter assays than the constructs driven by endogenous promoters of E2F-target genes such as dihydrofolate reductase (DHFR) (K. Helin and J. Lukas, unpubl.).

In the first set of single-cell reporter experiments designed to address the status of E2F in cells rescued by cyclin E, serum-starved R12 cells were microinjected with combinations of the cyclin E expression plasmid in conjunction with the 6xE2F-luc reporter plasmid and either the wild-type Rb(ΔB/X) or mutant Rb(Δcdk) plasmids. Expression from the reporter gene was examined by double-immunofluorescence to concurrently visualize the luciferase protein and the ectopic HA-tagged pRb forms, at 24 hr after serum stimulation, a time at which most cyclin E-expressing cells were shown to be in S phase under any of the conditions used. The luciferase protein was clearly induced in cells coinjected with wild-type Rb and cyclin E, but undetectable in R12 cells injected with mutant Rb(Δcdk) and cyclin E (Fig. 4A,B). Very low luciferase levels seen in experiments with a control TATA box-driven expression plasmid lacking the 6 E2F sites verified the specificity of these results (not shown). To complement the qualitative data from the above high-throughput assay of scoring luciferase-positive versus -negative cells (Fig. 4A,B), we next quantitated the luciferase-specific immunofluorescence signals in analogous microinjection experiments, using the Olympus computer-assisted image analysis system. As can be seen in Figure 4C, the phosphorylation-deficient mutant pRb(Δcdk) prevented expression of the luciferase from the 6xE2F-luc reporter plasmid in microinjected R12 cells. More significant, even upon coexpression of cyclin E sufficient to induce S phase (see above), the luciferase-specific fluorescence remained indistinguish-

Figure 3. Cyclin E overcomes G_1 arrest imposed by a phosphorylation-deficient mutant pRb(Δcdk). **(A)** Examples of original data on S-phase induction in pRb(Δcdk)-arrested R12 cells by ectopic cyclin E but not cyclin D1. Cells expressing the HA-tagged pRb forms were identified by anti-HA staining and evaluated for BrdU incorporation 24 hr after injection/serum stimulation. Arrowheads mark identical cells in matched fields. Cyclin D1 induces S phase when coexpressed with wild-type (*top*), but not with the mutant (*middle*) pRb. In contrast, cells arrested by pRb(Δcdk) resume DNA replication when coinjected with cyclin E expression plasmid (*bottom*). **(B)** Quantitation of the S phase-inducing effects of cyclin E

or D1 in cells arrested by mutant pRb(Δcdk), assayed in directly coinjected R12 cells (*left*), presented as a mean $\pm$ s.d. from two to five experiments; or in cells with tetracycline-repressible cyclin E (clone E2) or D1 (clone D5) (*right*) (data from two experiments are shown).

able from the background signal detected in the noninjected cells, equal to $\leq 1\%$ of the integrated optical density in control microinjected cells or those coexpressing wild-type pRb (Fig. 4C).

The next set of in situ reporter experiments, based on plasmids in which luciferase expression was controlled by either the DHFR or the B-myb gene promoters, yielded results consistent with those obtained with the artificial 6x-E2F-luc reporter. Thus, luciferase-specific immunofluorescence signals generated from either reporter in synchronized R12 cells were suppressed by the mutant pRb(Δcdk) to the low levels seen in serum-deprived cells, and coexpression of cyclin E failed to induce any detectable increase of the luciferase signal quantitated by image analysis (see Fig. 4C for examples of the DHFR-luc reporter data). Compared with the values obtained with the 6x-E2F-luc reporter (Fig. 4C), both the DHFR and B-myb promoters gave somewhat lower signals when activated/derepressed in the R12 cells (Fig. 4C; data not shown), and they both showed low yet reproducibly detectable expression of luciferase even in the presence of mutant pRb(Δcdk), which, however, never exceeded the signal detected in serum-starved cells (Fig. 4C). The lower induced signals produced by the natural promoters indicate that the 6x-E2F promoter is more sensitive, and the higher basal signals likely reflect the presence of other, non-E2F regulatory elements in the more complex natural promoters (Means et al. 1992;

Lam and Watson 1993). Furthermore, these observations are consistent with data from in situ reporter assays using a mutant version of the DHFR-luc plasmid lacking the E2F site, whose luciferase expression was even slightly increased compared with the wild-type promoter, and which could no longer be influenced by the mutant pRb(Δcdk) (data not shown).

Thus, both qualitative estimation and image analysis of in situ luciferase reporter assays strongly indicate that mutant pRb(Δcdk) is able to prevent any E2F-dependent promoter activation or derepression in R12 cells, and that cyclin E-induced S phase and completion of the cell division cycle can occur in the absence of E2F-mediated transactivation. Additional microinjection experiments revealed that the mutant pRb(Δcdk) can sequester not only the available endogenous E2Fs but also extra ectopic E2F-1 and E2F-2 expressed at levels shown previously (Lukas et al. 1996) to be sufficient to drive quiescent cells into S phase (data not shown).

Noninvasive imaging of reporter luciferase activity in living cells confirms that E2F is dispensable for the cyclin E-mediated cell cycle progression

All approaches to study cell cycle-related transcription employed to date, and including our reporter assays described above, suffer from the necessity to either fix or lyse the cells under investigation, thereby disrupting the

Figure 4. S-phase induction by cyclin E without concomitant stimulation of endogenous E2F-dependent transactivation. (A) Examples of in situ luciferase immunodetection in R12 cells coinjected with Rb plus cyclin E expression plasmids, together with the 6xE2F-luc reporter plasmid. Induced luciferase protein was detected with affinity-purified antibody at 22–24 hr after injection/serum stimulation. Although cyclin E induces S phase in either scenario, note that the reporter construct was activated selectively in cells coinjected with wild-type Rb($\Delta B/X$) (top) but not with mutant pRb(Δcdk) expression plasmids (bottom). (B) Graph summary of the qualitative in situ immunodetection of luciferase in the reporter assay exemplified in A, derived from five to six experiments corresponding to several hundred injected cells (mean values $\pm$ S.D.). (C) Quantitation of luciferase-associated fluorescence obtained from a representative assay identical to the experiments described in A and B, using the 6xE2F-luc plasmid or DHFR-luc reporter plasmid, and evaluated by computer-assisted image analysis (mean values $\pm$ S.D.).

dynamic homeostasis of the cells and potentially creating artefacts. To avoid this fundamental limitation of current technologies, and in view of the key importance of accurate quantitation of E2F activity, we have used noninvasive real-time measurement of gene expression to monitor E2F-dependent transcription in living cells.

The microinjection into synchronized R12 cells of Rb and cyclin E expression plasmids together with the luciferase reporter plasmids was performed as described above, but instead of fixing the cells and immunostaining, the enzymatic activity of luciferase was measured directly in living cells upon addition of luciferin to the culture medium, using an image-intensified charged-coupled device camera (ICCD). The attached photon-counting device collects light emission from cells in a low-power microscopic field virtually without background (Rutter et al. 1995), allowing quantitation of the luciferase activity over the recorded interval. An example of the data obtained from a 10-min recording in R12 cells is shown in Figure 5A. Whereas easily detectable specific light-emission signals were recorded in cells expressing HA-tagged wild-type pRb with cyclin E and the 6xE2F-luc reporter, the signal was totally abolished when the phosphorylation-deficient mutant Rb(Δcdk) was expressed (Fig. 5A–C). Analogous results were obtained when using the DHFR-luc reporter, except that the overall signal generated in control cells with wild-type pRb was lower than that from the stronger syn-

thetic 6xE2F promoter, and very low light emission comparable to values in G_0 cells was detected in the presence of pRb(Δcdk) (Fig. 5C).

The graphs shown in Figure 5C summarize data from four independent photon-counting reporter experiments recorded at 24 hr postinjection/serum stimulation, corresponding to a time when most cells expressing either Rb form had entered S phase (see above, Fig. 3A,B). To exclude the unlikely possibility that cyclin E could liberate some E2F activity despite the presence of mutant pRb(Δcdk), which might have been missed because of the use of a single time point, we performed the photon-counting reporter assay in a time course, recording the luciferase activity of the same group of microinjected live cells in real time at 18, 22, 24, and 30 hr, corresponding to late G_1 , early S, and late S/ G_2 phases of the cell cycle. No measurable light signals were recorded in the R12 cells at any of the time points (data not shown), which considering the protein half-life of luciferase (2.5–3 hr; Rutter et al. 1995), and the likely cell-to-cell variability of S-phase entry, excluded inappropriate timing of the recording period as a potential source of a false-negative result.

In addition, analogous comicroinjection experiments of Rb and cyclin E expression plasmids with the 6xE2F-luc reporter, followed by quantitative imaging of luciferase activity in living cells, were also performed with exponentially growing U-2-OS. These experiments inde-

Figure 5. Cyclin E-mediated rescue of G_1 block imposed by pRb(Δ cdk) does not restore E2F-mediated promoter transactivation, as detected by quantitative photon estimation. (A) Examples of single-cell photon estimation assayed directly in live R12 cells microinjected with 6x E2F-luc reporter together with the indicated Rb and cyclin E expression plasmids. Luciferase activity was recorded by an ultrasensitive ICCD camera; each image represents a 10-min data collection period from a field containing 16–20 productively injected cells (as verified by subsequent immunostaining). Note the total absence of released photons when the reporter plasmid was coinjected with pRb(Δ cdk) and cyclin E. (B) Diagram summarizing distribution of specific luminescence intensities among cells productively coinjected with cyclin E, wild-type (wt) or mutant (mut) Rb, and 6x E2F-luc plasmids as in A. (C) Summary of quantitative single-cell luminescence imaging in live R12 cells using 6x E2F-luc or DHFR-luc reporter, as indicated. The data represent summary from four independent experiments, in which fields with 10–20 productively injected cells were recorded and quantified.

pendently confirmed that ectopic cyclin E did not restore the E2F-mediated transactivation potential suppressed by the phosphorylation-deficient mutant pRb(Δ cdk) (data not shown).

Taken together, the data obtained using a noninvasive

and quantitative in situ reporter assay demonstrate that the cells coexpressing cyclin E and the mutant pRb(Δ cdk) are able to traverse the cell cycle with an E2F-dependent transactivation potential that is two to three orders of magnitude below that normally generated in these cells during the G_1 /S transition.

Cyclin E-associated kinase activity does not promote the accumulation of free E2F

If cyclin E can indeed induce S phase in the absence of E2F-mediated transactivation, some important and experimentally testable predictions can be made. First, premature expression of cyclin E early during progression from G_0 to S phase, known to shorten G_1 (Resnitzky et al. 1995), would be expected to accelerate the G_1 /S transition in the presence of endogenous wild-type pRb without concurrently activating/derepressing endogenous E2Fs.

To determine directly whether activation of cyclin E-Cdk2 kinase to levels that accelerate the G_1 /S transition is sufficient to promote an increase in free E2F, an E2F electrophoretic mobility shift experiment was performed on G_1 -phase R12/E2 cells induced to express cyclin E by removal of tetracycline. After serum stimulation, ectopic expression of cyclin E was detected in the induced cells at 3 hr (not shown; Resnitzky and Reed 1995; Resnitzky et al. 1995) followed by near maximal levels of cyclin E-dependent kinase activity at 11 hr (Fig. 6B). At this time, there was no detectable increase in free E2F levels, as determined by observing the band shift associated with free E2F (Fig. 6B). Conversely, there was no decrease observed in the levels of E2F complexed to pRb and the related pocket protein p130 (E2F G_0 / G_1 ; Fig. 6B). The identity of relevant bands in these displays was determined by competition and supershift experiments (Fig. 6A). An increase in free E2F as well as a decrease in E2F-pocket protein complexes (E2F G_0 / G_1) was, however, observed simultaneously for both induced and uninduced cells (Fig. 6B), even though cells expressing cyclin E entered S phase 3 hr before their uninduced counterparts (not shown; Resnitzky and Reed 1995; Resnitzky et al. 1995). These data indicate that the timing of disruption of E2F-pocket protein complexes and the accumulation of free E2F does not correlate at all with the activation of cyclin E-dependent kinase. In contrast, in a parallel experiment involving ectopic expression of cyclin D1, there was a strong correlation between activation of cyclin D1-dependent kinase and a shift from E2F complexes to free E2F (Fig. 6C). At 6 hr after serum stimulation, where cyclin D1-dependent kinase is already at maximal levels in the induced cells, which are at least 6 hr away from the G_1 /S phase boundary (not shown; Resnitzky and Reed 1995; Resnitzky et al. 1995), E2F-pRb and E2F-p130 complexes are no longer apparent in the band shifts, whereas there is a significant increase in free E2F, as compared with uninduced controls (Fig. 6C). The increase in a slower-migrating E2F-containing complex observed in the induced lane (Fig. 6C) most likely corresponds to an E2F-p107 complex, con-

Figure 6. Activation of cyclin E-dependent kinase does not lead to immediate increase of free E2F/DNA complexes. (A) Characterization of E2F complexes in early G_1 R12E2 cells. Cells were synchronized by serum starvation, and stimulated with 10% serum for 6 hr in the absence of tetracycline (cyclin E-induced). Nuclear extracts were prepared and analyzed by gel retardation assay. Binding reactions included a 100-fold molar excess of unlabeled oligonucleotide corresponding to the *c-myc* promoter E2F site (lane 2) or a point mutant E2F site (lane 3), or antibodies against pRb, p130, E2F-1, or E2F-4 (lanes 4–7, respectively). (B) Activation of cyclin E kinase does not lead to a concomitant increase in free E2F. Cyclin E-inducible R12E2 cells were synchronized by serum starvation, and stimulated with 10% serum in either the absence (induced) or presence (noninduced) of tetracycline. Cell lysates from the indicated time points after serum stimulation were analyzed by immunoblotting with the HE12 antibody against human cyclin E (top). Two hundred micrograms of cell lysate was used to determine the cyclin E-associated kinase activity following immunoprecipitation with anti-human cyclin E monoclonal antibody (HE172) as described (middle). A gel retardation assay was performed in parallel (bottom). (C) Induction of cyclin D1 kinase activity in R12D5 cells leads to early increase in free E2F. R12D5 cells were harvested 6 hr after emergence from quiescence in the absence or presence of tetracycline. Whole-cell extracts were assayed for cyclin D1 protein (top) and kinase activity (middle). Nuclear extracts were assayed for E2F/DNA complexes (bottom).

sistent with p107 being an E2F-induced protein (Zhu et al. 1995). These data confirm that whereas cyclin D1-associated kinase has a profound effect on E2F metabolism independent of cell cycle progression, cyclin E-associated kinase does not, even when sufficiently active to alter the kinetics of S-phase entry.

Cyclin E can rescue G_1 arrest conferred by dominant-negative mutant DP-1

The second prediction based on the outcome of the quantitative reporter assays is that cyclin E should be able to overcome a G_1 blockade imposed by dominant-negative mutant DP-1 (Wu et al. 1996), a heterodimeric partner of the E2F transcription factors. To test this possibility, R12 cells synchronized by serum starvation–stimulation were microinjected with expression plasmids coding for either wild-type or mutant DP-1, and the effects of their expression upon progression into S phase was monitored by BrdU incorporation. Consistent with the original report by Wu et al. (1996), expression of dominant-negative DP-1 prevented a majority of the productively injected cells from initiating the DNA synthesis (Fig. 7A). More important, coexpression of cyclin E with the dominant-negative mutant DP-1 restored the rate of S-phase entry to the level seen in cells treated with control DNA or expressing the wild-type DP-1 (Fig. 7A). Similar data were also obtained with the tetracycline-regulatable

model of R12/E2 cells, in which cyclin E induction resulted in restoration of S-phase entry despite the continuous presence of the dominant-negative mutant DP-1 expressed from the preinjected expression plasmid (data not shown). Finally, the ability of the dominant-negative mutant DP-1 to repress the activity of endogenous E2F transcription factors was verified by both qualitative and quantitative image analysis of the luciferase-specific immunofluorescence, by use of the sensitive 6x E2F-luc reporter in R12 cells with or without ectopic expression of cyclin E. The summary graphs shown in Figure 7B demonstrate that the level of ectopic mutant DP-1 was sufficient to prevent any detectable expression of luciferase, and that coexpression of cyclin E capable of rescuing the G_1 block did not significantly restore the E2F activity sequestered by the mutant DP-1. These data fully support our conclusion that the activity of E2F is dispensable for G_0 - to S-phase traverse and completion of the cell cycle in the presence of serum growth factors and sufficient levels of cyclin E.

Discussion

The results of this study demonstrate that mammalian cyclin D1 and cyclin E, respectively, promote the G_1 /S transition by different molecular mechanisms. We show, using a variety of techniques and complementary cellular models, that, whereas the activities associated with

Figure 7. Cyclin E can rescue G_1 block imposed by dominant-negative DP-1. (A) Serum-starved R12 cells microinjected with expression plasmid coding for dominant-negative allele of DP-1 remain largely arrested in G_1 when assayed 24 hr after serum stimulation in the presence of BrdU. Coexpression of cyclin E restores their ability to enter S phase to control levels seen in the presence of wild-type DP-1 (also identical to mock-treated or backbone DNA-injected cells). (B) Cyclin E-mediated rescue of cells blocked by dominant-negative DP-1 does not restore E2F-mediated transactivation. Quiescent R12 cells microinjected with 6x-E2F-luc reporter together with indicated expression plasmids were stimulated with serum and 24 hr later analyzed by an in situ luciferase assay described in Fig. 4. The graphs represent mean values from three experiments, $\pm$ S.D.

D-type cyclins target the pRb/E2F pathway, as has been suggested by other studies, cyclin E-associated kinase stimulates entry into S phase by mechanisms that are distinct from induction of E2F activity. In addition, our finding that cyclin E-mediated S phase and completion of the cell division cycle can occur in the absence of E2F-mediated transactivation is unexpected in view of the contemporary thinking about cell cycle control, and contributes new insights into the temporal order and mutual dependence of E2F- and cyclin E-controlled regulatory events.

Following some initial controversy as to the temporal sequence of G_1 -regulatory events controlled by cyclin D and E, respectively, a consensus view of D-type cyclins operating upstream of pRb has been reached and supported experimentally (Sherr and Roberts 1995; Weinberg 1995; Bartek et al. 1996). In particular, studies demonstrating that both cyclin D1 (Tam et al. 1994; Lukas et al. 1994a, 1995a) and the activity of cyclin D-dependent kinases Cdk4 and Cdk6 (Koh et al. 1995; Lukas et al. 1995a,b; Medema et al. 1995; Parry et al. 1995) are dispensable in cells lacking functional pRb, were consistent with pRb being a key substrate whose phosphorylation by cyclin D-associated kinases is necessary for traverse through G_1 . On the other hand, accumulating recent evidence suggests that cyclin D-dependent kinases may target additional substrates, potentially including the pRb-

related p107 and p130 E2F-binding proteins (Beijersbergen et al. 1995; Mayol et al. 1995), the E2F transcription factors (Fagan et al. 1994), and DMP1 (Hirai and Sherr 1996). Although the existence of multiple substrates indicates the possibility that cyclin D-associated kinases may regulate gene expression also in a pRb-independent manner, our present demonstration that ectopic expression of cyclin D1 did not overcome a G_1 block imposed by phosphorylation-deficient mutant pRb corroborates the notion of the pRb/E2F pathway as a critical target of the cell cycle-promoting function of D-type cyclins.

Similar to cyclin D, cyclin E has been regarded as a candidate for an upstream activator of E2F transcription factors. In such a scenario, cyclin E/Cdk2 kinase, which becomes activated closer to the G_1 /S transition than cyclin D-dependent kinases, presumably acts via phosphorylating pRb and/or the other pocket proteins that have been partially phosphorylated by cyclin D/Cdks during G_1 (Sherr and Roberts 1995; Weinberg 1995). The E2F-activating role of cyclin E has been documented in the unique model of rapidly cycling cells of the central nervous system in developing *Drosophila* embryos (Duronio and O'Farrell 1995), and more indirectly supported by the findings that pRb can serve as an in vitro substrate for cyclin E/Cdk2 and that ectopic expression of cyclin E could rescue a cell cycle block conferred by ectopic expression of pRb (Hinds et al. 1992). Consistent with this concept, we found that cyclin E expression in R12 or U-2-OS cells, blocked in G_1 by the p16 inhibitor of Cdk4/6, resulted in partial restoration of E2F-mediated transactivation (J. Lukas and J. Bartek, unpubl.). However, the evidence for an E2F-activating role of cyclin E in mammalian cells should be interpreted cautiously as the observed effects might be rather indirect. For instance, cyclin E-dependent kinase might help activate another G_1 /S kinase, such as cyclin A-dependent kinase, which in turn could be responsible for phosphorylation of pRb (Resnitzky and Reed 1995; Resnitzky et al. 1995). One way cyclin E-dependent kinase might achieve such an effect is via phosphorylating, and thereby targeting for rapid destruction by the ubiquitin/proteasome pathway one or more of the Cdk inhibitors such as p27 (Pagano et al. 1995). This mechanism would be analogous to G_1 /S control in the yeast *Saccharomyces cerevisiae*, where G_1 cyclins contribute to regulation of protein turnover of at least one Cdk inhibitor operating at the G_1 /S transition (Schwob et al. 1994; Dirick et al. 1995). The demonstration that activation of cyclin E-dependent kinase in G_1 did not lead to an increase in free E2F or a dissociation of E2F from pRb and other pocket proteins is consistent with an indirect role for cyclin E in the activation of E2F in cycling cells. In conclusion, although cyclin E appears capable of activating E2F in higher eukaryotes, the underlying molecular mechanisms remain to be elucidated.

The most significant result of our present study is the currently most direct demonstration in mammalian cells of a role played by cyclin E-dependent kinase in promoting DNA replication independently of activating the pRb/E2F pathway. Our data imply that cyclin E-associated kinase targets one or more proteins controlling

the onset of DNA replication. These putative targets function downstream from or on a parallel pathway relative to pRb, and are most likely distinct from E2F-binding pocket proteins because overexpressed pRb can sequester all known E2F isoforms, including E2F-4 and E2F-5 (Ikeda et al. 1996; Moberg et al. 1996), and because no significant E2F-dependent transactivation was detected during cyclin E-induced S phase in the presence of mutant pRb(Δ cdk). The ability of cyclin E to bypass the G₁ block imposed by p16 or phosphorylation-deficient, constitutively active mutant pRb is unexpected and in some respects analogous and complementary to the ability of E2F-1, E2F-2, or E2F-3 overexpression to stimulate S-phase entry in cells arrested by p16 (DeGregori et al. 1995b; Lukas et al. 1996; Mann and Jones 1996). Although expression of cyclin E itself is controlled by E2F (Ohtani et al. 1995; Botz et al. 1996; Geng et al. 1996), a simple linear pathway in which cyclin E mediates all the S phase-inducing effects of E2F appears unlikely, as E2F isoforms have been shown to regulate many other S-phase genes (Nevins 1992; Helin and Harlow 1993; LaThangue 1994; DeGregori et al. 1995a; Beijersbergen and Bernards 1996). On the other hand, transcription of the E2F-regulated genes that encode enzymes involved in DNA replication is controlled by multiple transcription factors, and expression of these genes is also controlled by several post-transcriptional mechanisms (Johnson 1992; Slansky and Farnham 1996). This complex mode of regulation, together with the slow turnover of the replication proteins, can help reconcile the reported requirement of E2F activity for proliferation (Wu et al. 1996) with our present finding that cyclin E can bypass the sequestration of E2F proteins by nonphosphorylatable mutant pRb or dominant-negative DP-1. We suggest that the critical S phase-promoting event controlled by cyclin E occurs downstream of E2F (upon up-regulation of cyclin E transcription by E2F), at least in most cell types during the G₀- to S-phase progression. The lack of E2F is compatible with at least one complete cell division cycle, provided that, in addition to sufficient cyclin E, some limited pool of the replication proteins is available because of their stability and/or E2F-independent regulation of expression (Johnson 1992; Slansky and Farnham 1996). Because cyclin E is a labile protein and controls a critical S-phase promoting event other than activation of E2F (this study; Duronio et al. 1996), it represents a key rate-limiting factor regulating G₁/S transition. This powerful stimulation of S-phase entry by cyclin E can also help reconcile the findings that even relatively low residual activity of cyclin E/Cdk2 detected in cells treated with γ -irradiation or Cdk inhibitors (DeGregori et al. 1995b) was sufficient to cooperate with overexpression of E2F-1 to promote progression through an apparently complete round of cell division cycle. It is possible that the critical targets of cyclin E-dependent kinase activity are also targets of E2F-mediated transactivation, thus accounting for the ability of excess kinase activity to compensate for a shortfall in E2F-dependent gene expression and vice versa.

Our demonstration of the E2F-independent function of

cyclin E is consistent with recent evidence obtained in both mammalian cells (Ohtsubo et al. 1995; Resnitzky and Reed 1995; Hofmann and Livingston 1996) and *Drosophila* (Duronio and O'Farrell 1995). Furthermore, genetic evidence for requirement of cyclin E in E2F-induced S phase was published after completion of the present study, independently confirming one of our conclusions yet using a reverse approach based on inactivation of cyclin E, and the phylogenetically simpler model of *Drosophila* (Duronio et al. 1996). Taken together with our present data based on a phosphorylation-deficient mutant Rb, and dominant-negative mutant DP-1, these results support a role for cyclin E downstream or independent of the pRb/E2F pathway, sufficiently powerful to drive cells into S phase and through the rest of the cell division cycle, and even able to compensate for a lack of E2F-mediated transactivation. It remains to be determined whether elevated expression of cyclin E can compensate for lack of E2F-mediated transactivation over the long term. Nevertheless, one can conclude that the E2F-mediated and cyclin E-mediated S phase-promoting pathways complement and potentiate each other during normal somatic cell cycles in possibly all metazoa (Resnitzky and Reed 1995; Duronio et al. 1996; this study). Furthermore, this powerful positive impact of cyclin E upon cell cycle progression may also explain the emerging role of this G₁ cyclin as a proto-oncogene. Cyclin E can become overexpressed as a result of amplification of the gene and/or other mechanisms including loss of *Rb* (Dou et al. 1996; Hall and Peters 1996; Herrera et al. 1996), thereby potentially exceeding the threshold level compatible with normal G₁/S control mechanism.

Finally, the quantitative imaging of the luciferase reporter assays employed here provides, to our knowledge, the first example of the noninvasive measurement of cell cycle-dependent gene expression in living cells. The method is highly sensitive, quantitative, and nondisruptive, and allows E2F-regulated gene expression to be monitored repeatedly in the same cells in real time. This combination of desirable features is unique among the currently available technologies. Thus, this approach should find wide application to future analyses of cell cycle-related transcriptional control mechanisms that underlie normal cell growth and development and, in addition, should help elucidate deregulation of these processes in hypoproliferative and hyperproliferative diseases, including cancer.

Materials and methods

Plasmids and mutagenesis

The expression plasmids pX^{myc}-p16 (Lukas et al. 1995b), pCMV-CD20 (Lukas et al. 1995b), pRcCMV-cyclin D1 (Hinds et al. 1992), and pRcCMV-cyclin E (Hinds et al. 1992), and murine wild-type (pECE- Δ B/X-HA) and mutant (pECE- Δ p34-HA) pRb (Hamel et al. 1992) were described. The pGL3-TATA-6xE2F (referred to as 6xE2F-luc) and control pGL3-TATA-basic luciferase reporter plasmids were kindly donated by Ali Fattaey; the pGL2(-536) and pGL2(-536)mut reporter plasmids contain the

luciferase gene under control of wild-type B-myb promoter and the same promoter with a mutation in the E2F DNA-binding site, respectively (Lam and Watson 1993); and the DHFR-luc and DHFRmut-luc reporter constructs contain the fragments -270 to +20 of the DHFR promoter in the pGL2 basic vector (Means et al. 1992; Slansky et al. 1993). The plasmids pCMV-HA-DP-1 wild-type and pCMV-HA-DP-1 dominant-negative mutant (Δ 103-126) were described in Wu et al. (1996). The pECE- Δ cdk-HA, in which Thr 814 and Thr 819 of mouse Rb were changed to Ala, was derived from pECE- Δ p34-HA (Hamel et al. 1992) by use of a Chameleon Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions and with the following mutagenic primer: 5'-GGTCTGCCAG-CACCCACAAAAATGGCTCCGAGATC-3'. Clones containing the desired Thr to Ala substitutions were verified by sequencing.

Cell culture, microinjection, and transfection

Rat embryonic fibroblasts (clone R12; Resnitzky et al. 1994) were rendered quiescent by growing for 48 hr in serum-free medium. Expression plasmids for p16, HA-tagged wild-type (Δ B/X) or mutant (Δ cdk) pRb, cyclins E or D1 (25 μ g/ml of DNA each), wild-type or dominant-negative DP-1 (150 μ g/ml of DNA) were injected in desired combinations directly into cell nuclei using the Zeiss AIS microinjection system (Lukas et al. 1996), and the cells were subsequently stimulated to reenter the cell cycle by medium containing 10% fetal calf serum (FCS) and 100 μ M BrdU. Productively injected cells were located and assayed for BrdU incorporation by double immunostaining as described (Lukas et al. 1996). Unless indicated otherwise, all samples were evaluated 24 hr after microinjection/restimulation from quiescence.

Cyclin E (R12/E2) and cyclin D1 (R12/D5) inducible clones were synchronized in G_0 by growth in serum-free medium supplemented with 2 μ g/ml of tetracycline (Resnitzky and Reed 1995), and microinjected with the desired expression plasmids as described above, in serum- and tetracycline-free medium. Cyclin induction and reentry into the cell cycle were stimulated with tetracycline-free medium containing 10% serum and BrdU (100 μ g/ml). At the indicated time points, the cells were fixed and BrdU incorporation scored in productively injected cells as compared with closely neighboring noninjected cells, and the data obtained in multiple experiments were expressed as a mean $\pm$ S.D..

Calcium phosphate transfections into exponentially growing U-2-OS cells were performed as described (Lukas et al. 1995b), using the expression plasmids for p16 (4 μ g), wild-type (Δ B/X) or mutant (Δ cdk) pRb (4 μ g), cyclins E or D1 (16 μ g), and CD-20 (2 μ g), in combinations specified in the text. After 36 hr, the CD20-positive cells were sorted by flow cytometry (Coulter Epics XL) and analyzed for DNA profile using Multicycle software (Lukas et al. 1995b).

Immunoprecipitation and phosphoamino acid analysis

U-2-OS cells were transfected with either the control DNA or the indicated pRb expression plasmids (10 μ g per 80 cm² culture flask) by the calcium phosphate method (Lukas et al. 1995b). After 48 hr, the cells were labeled for 3 hr with 6 mCi/ml of [³²P]orthophosphate and lysed in 50 mM HEPES at pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 5 μ g/ml leupeptin, 1 mM PMSE, 1% (vol/vol) aprotinin, 1 mM DTT, 50 mM NaF, 10 mM β -glycerophosphate, and 400 μ M orthovanadate; the ectopically expressed pRb was immunoprecipitated with the anti-HA anti-

body 12CA5, and the phosphorylation status of pRb evaluated by SDS-gel electrophoresis and PhosphorImage analysis of proteins transferred to a nitrocellulose membrane. The bands corresponding to pRb were cut out and processed for tryptic digestion and phosphoamino acid analysis according to Boyle et al. (1991), and selected phosphopeptides isolated from thin layer chromatography (TLC) plates were subject to Edman sequencing.

Cell division assay

Quiescent R12 fibroblasts were microinjected with expression plasmids for wild-type or Δ cdk mutant Rb, in combination with cyclin E or D1 plasmids or control backbone DNA (all DNAs at 25 μ g/ml). Expression plasmid coding for green fluorescent protein (pGFP-N1; Clontech) was also coinjected (50 μ g/ml). Following short recovery, the injected cells were stimulated with 10% FCS and incubated undisturbed for 22 hr, when the starting numbers of productively injected cells were recorded (typically 50 productively injected cells per experiment) based on green fluorescence detected during a brief exposure of live cells to light within the fluorescein isothiocyanate (FITC) spectrum. Cells were then returned to the incubator and cell divisions monitored by counting the green fluorescence-positive cells at 3-hr intervals up to 40 hr after mitogen stimulation.

Luciferase immunodetection and quantitation in situ

Serum-starved R12 cells were coinjected with Rb (25 μ g/ml) or DP-1 (150 μ g/ml) plus cyclin E (25 μ g/ml) expression plasmids together with the luciferase reporter constructs containing 6x E2F, DHFR, or B-myb promoter fragments (250 μ g/ml) and subsequently stimulated with 10% FCS or left in G_0 as specified in figure legends. Induced luciferase was detected by double immunofluorescence with an affinity-purified rabbit antibody against luciferase (Promega), combined with mouse monoclonal antibody to HA epitope (12CA5) to identify the productively injected cells expressing pRb or DP-1 (incubation with the mixture of primary antibodies proceeded for 1 hr at room temperature). After washing with PBS (3 $\times$ 5 min), the cells were incubated with FITC-coupled anti-rabbit antibody and biotinylated anti-mouse antibody at room temperature for 30 min, followed by Texas red-coupled streptavidin (all secondary reagents from Amersham). Luciferase-associated fluorescence was recorded and quantitated by a computer-assisted image analysis system (analySIS; Soft-Imaging GmbH, Germany) attached to Olympus BX50 microscope. Briefly, defined objects of identical areas were introduced into multiple sites of a flat part of the cytoplasmic compartments of the productively injected cells (measurement reproducibility was facilitated by the fact that at the time of evaluation, the cells were highly synchronous and had very similar morphology). The relative fluorescence, expressed as mean gray value per defined area, was determined and subtracted with the background signal detected in closely neighboring noninjected cells. Data from multiple fields containing productively injected cells were exported to Microsoft Excel software and processed for statistical evaluation.

Noninvasive cell luminescence imaging

Luminescence produced by live cells microinjected with luciferase reporter plasmids (250 μ g/ml) and expression plasmids in combinations specified in legend to Figure 5 was measured using the Zeiss Axiovert 135M microscope with a Fluor 10 $\times$ objective and ICCD camera (ICCD-576-S/RB EM detector at-

tached to a ST-138S controller, Princeton Instruments, Inc., NJ). This highly sensitive, interactive photon-estimation system can discriminate single-photon events. Twenty-four hours after serum stimulation of the injected cells, the medium was supplied with 1 mM beetle luciferin (Promega); the area with the injected cells was found using coordinates on grid coverslips (Eppendorf); and the emitted photoelectrons were acquired in complete dark. The total acquisition time was 10 min per field containing minimum of 10 productively injected cells as verified by subsequent fixation and immunostaining. Quantitation of the luminescence images was performed after dark charge subtraction and corrections of distortions across the field following the manufacturer's instructions. (Further technical details are available upon request from J. Lukas and J. Bartek).

Histone H1 and pRb kinase assays

For H1 kinase assays, cells were lysed in 50 mM Tris-HCl at pH 7.5, 1% deoxycholate (DOC), 1% Triton X-100, 0.1% SDS, 150 mM NaCl, containing 10 mM PMSF, 10 µg of leupeptin per ml, 10 µg/ml of pepstatin, 20 µg/ml of aprotinin, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, and 10 mM sodium pyrophosphate (PPi). Two hundred micrograms of protein was used per immunoprecipitation with monoclonal anti-cyclin E antibody HE172. Immunoprecipitates were washed twice in lysis buffer, once in 50 mM Tris-HCl at pH 7.5, 1% DOC, 1% Triton X-100, once in kinase assay buffer (40 mM Tris-HCl, 15 mM MgCl₂), and incubated in 5 µl of kinase assay buffer with 0.01 mCi of [γ -³²P]ATP (4500 Ci/mmol; ICN) and 2 µg of histone H1 for 30 min at 37°C. Reaction products were separated by SDS-PAGE, and the gel was stained with Coomassie blue, dried, and exposed to X-ray film. Rb kinase assays were performed essentially as described previously (Lukas et al. 1995a) by use of the DCS-11 monoclonal anti-cyclin D1 antibody (Lukas et al. 1994b) for immunoprecipitation and 1 µg of recombinant full-length Rb protein (QED Bioscience, Inc.) as substrate. Phosphorylated pRb was visualized with a PhosphorImager.

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was performed with 1 µg of nuclear extracts (Schreiber et al. 1989) in a 10-µl reaction volume containing 10 mM HEPES (pH 7.9), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 4% Ficoll, 0.4 mM spermine, 500 ng of sonicated salmon sperm DNA, and 0.5 ng of end-labeled oligonucleotide encompassing the E2F-DNA-binding site of the *c-myc* promoter. DNA-protein complexes were allowed to form for 20 min at room temperature and separated on a 4.5% polyacrylamide gel in 0.25× Tris-borate EDTA (TBE) at 280 V at 4°C for 2.5 hr. Antibodies used for supershift experiments were from Santa Cruz, CA.

Acknowledgments

We thank L. Rönstrand for phosphopeptide sequence information, P. Hamel, A. Fattaey, R. Watson, P. Farnham, C.-L. Wu, and G. Peters for some of the reagents, and acknowledge grant support from the Danish Cancer Society (J.L., K. Hansen, J.B.), the Nordic Cancer Union (J.B.), the National Institutes of Health (GM46006, to S.I.R.), Human Frontier Science Programme (RG-557/96, to J.B. and K. Helin), and Associazione Italiana per la Ricerca sul Cancro (AIRC) (K. Helin, M.C.M.). T.H. is a fellow of the Deutsche Forschungsgemeinschaft.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Bartek, J., J. Bartkova, and J. Lukas. 1996. The retinoblastoma protein pathway and the restriction point. *Curr. Opin. Cell Biol.* **8**: 805–814.
- Beijersbergen, R.L. and R. Bernards. 1996. Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. *Biochim. Biophys. Acta* **1287**: 103–120.
- Beijersbergen, R.L., L. Carlee, R.M. Kerkhoven, and R. Bernards. 1995. Regulation of the retinoblastoma protein-related p107 by G₁ cyclin complexes. *Genes & Dev.* **9**: 1340–1353.
- Botz, J., K. Zerfass-Thome, D. Spitkovski, H. Delius, B. Vogt, M. Eilers, A. Hatzigeorgiou, and P. Jansen-Dürr. 1996. Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. *Mol. Cell. Biol.* **16**: 3401–3409.
- Boyle, W.J., P. Van der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* **201**: 110–149.
- DeGregori, J., T. Kowalik, and J.R. Nevins. 1995a. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis and G1/S-regulatory genes. *Mol. Cell. Biol.* **15**: 4215–4224.
- DeGregori, J., G. Leone, K. Ohtani, A. Miron, and J.R. Nevins. 1995b. E2F-1 accumulation bypasses a G₁ arrest resulting from the inhibition of G₁ cyclin-dependent kinase activity. *Genes & Dev.* **9**: 2873–2887.
- Dirick, L., T. Bohm, and K. Nasmyth. 1995. Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. *EMBO J.* **14**: 4803–4813.
- Dou, Q.P., A.B. Pardee, and K. Keyomarsi. 1996. Cyclin E—A better prognostic marker for breast cancer than cyclin D? *Nature Med.* **2**: 254.
- Duronio, R.J. and P.H. O'Farrell. 1995. Developmental control of the G₁ to S transition in *Drosophila*: Cyclin E is a limiting downstream target of E2F. *Genes & Dev.* **9**: 1456–1468.
- Duronio, R.J., P.H. O'Farrell, J.E. Xie, A. Brook, and N. Dyson. 1995. The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. *Genes & Dev.* **9**: 1445–1455.
- Duronio, R.J., A. Brook, N. Dyson, and P.H. O'Farrell. 1996. E2F-induced S phase requires cyclin E. *Genes & Dev.* **10**: 2505–2513.
- Fagan, R., K.J. Flint, and N. Jones. 1994. Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene product and the adenoviral E4 19 kDa protein. *Cell* **78**: 799–811.
- Geng, Y., E. Ng Eaton, M. Picón, J.M. Roberts, A.S. Lundberg, A. Gifford, C. Sardet, and R.A. Weinberg. 1996. Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene* **12**: 1173–1180.
- Hall, M. and G. Peters. 1996. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. *Adv. Cancer Res.* **68**: 67–108.
- Hamel, P.A., R.M. Gill, R.A. Phillips, and B.L. Gallie. 1992. Regions controlling hyperphosphorylation and conformation of the retinoblastoma gene product are independent of domains required for transcriptional repression. *Oncogene* **7**: 693–701.

- Harper, J.W. and S.J. Elledge. 1996. Cdk inhibitors in development and cancer. *Curr. Opin. Genet. Dev.* **6**: 56–64.
- Helin, K. and E. Harlow. 1993. The retinoblastoma protein as a transcriptional repressor. *Trends Cell Biol.* **3**: 43–46.
- Herrera, R.E., V.P. Sah, B.O. Williams, T.P. Mäkelä, R.A. Weinberg, and T. Jacks. 1996. Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts. *Mol. Cell. Biol.* **16**: 2402–2407.
- Hinds, P.W., S. Mittnacht, V. Dulic, A. Arnold, S.I. Reed, and R.I. Weinberg. 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70**: 993–1006.
- Hirai, H. and C.J. Sherr. 1996. Interaction of D-type cyclins with a novel myb-like transcription factor, DMP1. *Mol. Cell. Biol.* **16**: 6457–6467.
- Hofmann, F. and D.M. Livingston. 1996. Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G₁ exit. *Genes & Dev.* **10**: 851–861.
- Ikeda, M.-A., L. Jakoi, and J.R. Nevins. 1996. A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. *Proc. Natl. Acad. Sci.* **93**: 3215–3220.
- Johnson, L.F. 1992. G1 events and the regulation of genes for S-phase enzymes. *Curr. Opin. Cell Biol.* **4**: 149–154.
- Knoblich, J.A., K. Sauer, L. Jones, H. Richardson, R. Saint, and C.F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for arrest of cell proliferation. *Cell* **77**: 107–120.
- Knudsen, E.S. and J.Y.J. Wang. 1996. Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. *J. Biol. Chem.* **271**: 8313–8320.
- Koh, J., G.H. Enders, B.D. Dynlacht, and E. Harlow. 1995. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* **375**: 506–510.
- Lam, E.W.-F. and R.J. Watson. 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. *EMBO J.* **12**: 2705–2713.
- LaThangue, N.B. 1994. DRTF1/E2F: An expanding family of heterodimeric transcription factors implicated in cell-cycle control. *Trends Biochem. Sci.* **19**: 108–114.
- Lukas, J., H. Müller, J. Bartkova, D. Spitkovsky, A.A. Kjerulff, P. Jansen-Dürr, M. Strauss, and J. Bartek. 1994a. DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1. *J. Cell Biol.* **125**: 625–638.
- Lukas, J., M. Pagano, Z. Staskova, G. Draetta, and J. Bartek. 1994b. Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumour cell lines. *Oncogene* **9**: 707–718.
- Lukas, J., J. Bartkova, M. Rohde, M. Strauss, and J. Bartek. 1995a. Cyclin D1 is dispensable for G1 control in retinoblastoma gene-deficient cells independently of cdk4 activity. *Mol. Cell. Biol.* **15**: 2600–2611.
- Lukas, J., D. Parry, L. Aagaard, D.J. Mann, J. Bartkova, M. Strauss, G. Peters, and J. Bartek. 1995b. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature* **375**: 503–506.
- Lukas, J., B.O. Petersen, K. Holm, J. Bartek, and K. Helin. 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes p16^{INK4}-mediated growth suppression. *Mol. Cell. Biol.* **16**: 1047–1057.
- Mayol, X., J. Garriga, and X. Grana. 1995. Cell cycle-dependent phosphorylation of the retinoblastoma-related protein p130. *Oncogene* **11**: 801–808.
- Mann, D.J. and N.C. Jones. 1996. E2F-1 but not E2F-4 can overcome p16-induced G1 cell-cycle arrest. *Curr. Biol.* **6**: 474–483.
- Means, A.L., J.E. Slansky, S.L. McMahon, M.W. Knuth, and P.J. Farnham. 1992. The HIP1 binding site is required for growth regulation of the dihydrofolate reductase gene promoter. *Mol. Cell. Biol.* **12**: 1054–1063.
- Medema, R.H., R.E. Herrera, F. Lam, and R.A. Weinberg. 1995. Growth suppression by p16^{ink4} requires functional retinoblastoma protein. *Proc. Natl. Acad. Sci.* **92**: 6289–6293.
- Moberg, K., M.A. Starz, and J.A. Lees. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Mol. Cell. Biol.* **16**: 1436–1449.
- Morgan, D.O. 1995. Principles of CDK regulation. *Nature* **374**: 131–134.
- Nevins, J.R. 1992. E2F: A link between the Rb tumor suppressor protein and viral oncogenesis. *Science* **258**: 424–429.
- Ohtani, K., J. DeGregori, and J.R. Nevins. 1995. Regulation of the cyclin E gene by transcription factor E2F1. *Proc. Natl. Acad. Sci.* **92**: 12146–12150.
- Ohtsubo, M., A.M. Theodoras, J. Schumacher, J.M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol. Cell. Biol.* **15**: 2612–2624.
- Pagano, M., S.W. Tam, A.M. Theodoras, P. Beer-Romero, G. Del Sal, V. Chau, P.R. Yew, G.F. Draetta, and M. Rolfe. 1995. Role of the ubiquitin-proteasome pathway in regulating amounts of the cyclin-dependent kinase inhibitor p27. *Science* **269**: 682–685.
- Parry, D., S. Bates, D.J. Mann, and G. Peters. 1995. Lack of cyclin D-cdk complexes in RB-negative cells correlates with high levels of p16^{INK4/MTS1} tumour suppressor gene product. *EMBO J.* **14**: 503–511.
- Peters, G. 1994. Cell cycle: Stifled by inhibitions. *Nature* **371**: 204–205.
- Pines, J. 1995. Cyclins and cyclin-dependent kinases: A biochemical view. *Biochem. J.* **308**: 697–711.
- Reed, S.I. 1996. Cyclin E: In mid-cycle. *Biochim. Biophys. Acta* **1287**: 151–153.
- Resnitzky, D. and S.I. Reed. 1995. Different roles for cyclins D1 and E in regulation of the G1-to-S transition. *Mol. Cell. Biol.* **15**: 3463–3469.
- Resnitzky, D., M. Gossen, H. Bujard, and S.I. Reed. 1994. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with inducible system. *Mol. Cell. Biol.* **14**: 1669–1679.
- Resnitzky, D., L. Hengst, and S.I. Reed. 1995. Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G1 by p27^{Kip1}. *Mol. Cell. Biol.* **15**: 4347–4352.
- Roussel, M.F., A.M. Theodoras, M. Pagano, and C.J. Sherr. 1995. Rescue of defective mitogenic signaling by D-type cyclins. *Proc. Natl. Acad. Sci.* **92**: 6837–6841.
- Rutter, G.A., M.R.H. White, and J.M. Tavaré. 1995. Involvement of MAP kinase in insulin signalling revealed by non-invasive imaging of luciferase gene expression in single living cells. *Curr. Biol.* **5**: 890–899.
- Schreiber, E., P. Matthias, M.M. Müller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with “mini-extracts,” prepared from a small number of cells. *Nucleic Acids Res.* **17**: 6419.
- Schwob, E., T. Böhm, M.D. Mendenhall, and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor p40^{SIC1} controls the G1 to S transition in *S. cerevisiae*. *Cell* **79**: 233–244.
- Serrano, M., G.J. Hannon, and D. Beach. 1993. A regulatory motif in cell-cycle control causing specific inhibition of cyclin D/cdk4. *Nature* **366**: 704–707.
- Sherr, C.J. 1993. Mammalian G1 cyclins. *Cell* **73**: 1059–1065.
- Sherr, C.J. and J.M. Roberts. 1995. Inhibitors of mammalian G₁

- cyclin-dependent kinases. *Genes & Dev.* **9**: 1149–1163.
- Slansky, J.E. and P.J. Farnham. 1996. Introduction to the E2F family: Protein structure and gene regulation. *Curr. Top. Microbiol. Immunol.* **208**: 1–30.
- Slansky, J.E., Y. Li, W.G. Kaelin, and P.J. Farnham. 1993. A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. *Mol. Cell. Biol.* **13**: 1610–1618.
- Tam, S.W., A.M. Theodoras, J.W. Shay, G.F. Draetta, and M. Pagano. 1994. Differential expression and regulation of cyclin D1 protein in normal and tumor human cells: Association with cdk4 is required for cyclin D1 function in G1 progression. *Oncogene* **9**: 2663–2674.
- van den Heuvel, S. and E. Harlow. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262**: 2050–2054.
- Wang, J.Y.J., E.S. Knudsen, and P.J. Welch. 1994. The retinoblastoma tumor suppressor protein. *Adv. Cancer Res.* **64**: 25–85.
- Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell* **81**: 323–330.
- Wimmel, A., F.C. Lucibello, A. Sewing, S. Adolph, and R. Müller. 1994. Inducible acceleration of G1 progression through tetracycline-regulated expression of human cyclin E. *Oncogene* **9**: 995–997.
- Wu, C.-L., M. Classon, N. Dyson, and E. Harlow. 1996. Expression of dominant-negative mutant DP-1 blocks cell cycle progression in G1. *Mol. Cell. Biol.* **16**: 3698–3706.
- Zhu, L., L. Zhu, E. Xie, and L.S. Chang. 1995. Differential roles of two tandem E2F sites in repression of the human p107 promoter by retinoblastoma and p107 proteins. *Mol. Cell. Biol.* **15**: 3552–3562.

Cyclin E-induced S phase without activation of the pRb/E2F pathway.

J Lukas, T Herzinger, K Hansen, et al.

Genes Dev. 1997, **11**:

Access the most recent version at doi:[10.1101/gad.11.11.1479](https://doi.org/10.1101/gad.11.11.1479)

References

This article cites 67 articles, 32 of which can be accessed free at:
<http://genesdev.cshlp.org/content/11/11/1479.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).