S-phase entry upon ectopic expression of G1 cyclin-dependent kinases in the absence of retinoblastoma protein phosphorylation

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In mammalian cells, the retinoblastoma protein (Rb) is thought to negatively regulate progression through the G1 phase of the cell cycle by its association with the transcription factor E2F [1–3]. Rb–E2F complexes suppress transcription of genes required for DNA synthesis ([4], reviewed in [3,5]), and the prevailing view is that phosphorylation of Rb by complexes of cyclin-dependent kinases (Cdks) and their regulatory cyclin subunits, and the subsequent release of active E2F, is required for S-phase entry [1–3]. This view is based, in part, on the fact that ectopic expression of cyclin–Cdks leads to Rb phosphorylation and that this modification correlates with S-phase entry [6–8]. In *Drosophila*, however, cyclin E expression can bypass a requirement for E2F, suggesting that cyclins may activate replication independently of the Rb/E2F pathway [9]. We sought to examine whether Rb phosphorylation is a prerequisite for S-phase entry in Rb-deficient SAOS-2 osteosarcoma cells, using a commonly used cotransfection assay [6–8,10]. We find that a G1 arrest in SAOS-2 cells mediated by an Rb mutant lacking all 14 consensus Cdk phosphorylation sites is bypassed by coexpressing G1-specific E-type or D-type cyclin–Cdk complexes, and that injection of purified cyclin–Cdks during G1 accelerates S-phase entry. Our results indicate that Rb phosphorylation is not essential for S-phase entry when G1 cyclin–Cdks are overexpressed, and that other substrates of these kinases can be rate-limiting for the G1 to S-phase transition. These data also reveal that the SAOS-2 cotransfection assay is complicated by Rb-independent effects of the coexpressed Cdks.

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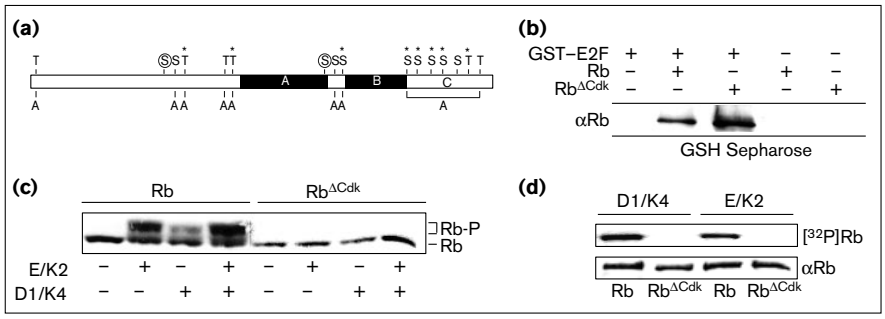
Results and discussion

An Rb mutant ($Rb^{\Delta Cdk}$) was generated containing serine and threonine to alanine substitutions at all 14 consensus Cdk phosphorylation sites (Figure 1a). Like wild-type Rb, $Rb^{\Delta Cdk}$ associates with E2F when coexpressed in insect cells (Figure 1b) and binds avidly to a peptide affinity column in which the peptide, derived from the papilloma E7 oncoprotein, contains the motif Leu–X–Cys–X–Glu, in which X is any amino acid (Figure 1d), indicating that both the E2F-binding domain and the large pocket of Rb are functional in this mutant. This mutant would, therefore, be expected to interact with cellular targets, thereby blocking S-phase entry, but would not be expected to be regulated by Cdk-mediated phosphorylation. When co-expressed with cyclin E–Cdk2 or cyclin D1–Cdk4, $Rb^{\Delta Cdk}$ comigrated with the hypophosphorylated form of Rb, whereas wild-type Rb underwent a characteristic decrease in electrophoretic mobility associated with its phosphorylation (Figure 1c). Consistent with the inability of $Rb^{\Delta Cdk}$ to be phosphorylated by Cdks, neither cyclin E–Cdk2 nor cyclin D1–Cdk4 phosphorylated $Rb^{\Delta Cdk}$ *in vitro* under conditions in which Rb is extensively phosphorylated on a subset of *in vivo* phosphorylation sites (Figure 1d and [11]).

We then tested $Rb^{\Delta Cdk}$ in a SAOS-2 cotransfection assay that has previously been used to establish the roles of Rb and Cdks in cell cycle entry [6–8,10]. As expected, expression of $Rb^{\Delta Cdk}$ in SAOS-2 cells led to a G1 arrest, as assessed by flow cytometry (Figure 2), and also produced the 'flat cell' phenotype characteristic of Rb expression in this cell line (data not shown). Surprisingly, however, cotransfection of $Rb^{\Delta Cdk}$ with plasmids expressing cyclin E–Cdk2, cyclin D1–Cdk4, or cyclin E alone resulted in a substantial increase in the S-phase population at the expense of G1 cells (Figure 2 and data not shown). For example, cotransfection of $Rb^{\Delta Cdk}$ with cyclin E–Cdk2 led to a 21–41% increase in the fraction of S-phase cells in three independent experiments, similar to the 25–44% increase observed with Rb (Figure 2). Although cyclin D1–Cdk4 was somewhat less effective at overriding Rb in this assay, the increase in S-phase cells was comparable for both Rb and $Rb^{\Delta Cdk}$ (10–26%). Expression of Cdk complexes alone also increased the percentage of S-phase cells compared to cells transfected with empty vector (Figure 2).

Similar results were obtained in a pulse bromodeoxyuridine (BrdU) incorporation assay that examines DNA synthesis directly in Rb-expressing cells (Figure 3). As expected, less than 1% of cells positive for Rb or $Rb^{\Delta Cdk}$ became labeled with BrdU in the absence of cyclin expression. Over 80% of Rb-positive cells were BrdU-positive, however, when Rb was coexpressed with cyclin E–Cdk2 or a mixture of cyclin E–Cdk2 and cyclin D1–Cdk4, regardless of whether

Figure 1



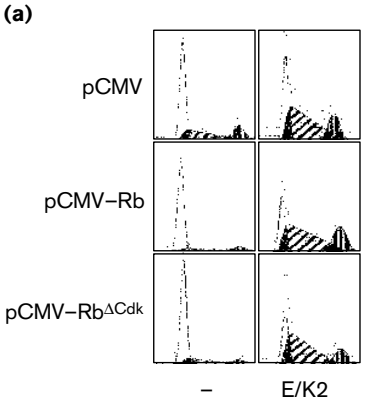
(a) Rb structure showing consensus Cdk phosphorylation sites (S/T), sites of phosphorylation by E-type and D-type cyclin–Cdks *in vitro* [11] (asterisks), and phosphorylation sites mutated to alanine (A) [11] in $Rb^{\Delta Cdk}$. Encircled serines are within Ser–Pro sequences that do not fit the Cdk consensus (Ser–Pro–X–Arg/Lys, in which X is any amino acid). **(b)** $Rb^{\Delta Cdk}$ associates with E2F in insect cells. Insect cells infected with the indicated baculovirus combinations were lysed as described [11]. Complexes of Rb and a fusion protein of glutathione-S-transferase and E2F (GST–E2F) were purified using glutathione (GSH) Sepharose, and proteins were separated by gel electrophoresis before immunoblotting with anti-Rb antibodies (Santa Cruz SC-050). **(c)** Rb, but not $Rb^{\Delta Cdk}$, is converted to slower migrating phosphorylated forms (Rb-P) upon coexpression with cyclin–Cdks in SAOS-2 cells (see Figure 2 legend for transfection methods). Cell lysates were immunoblotted using anti-Rb antibodies (Pharmingen 10041A). **(d)** $Rb^{\Delta Cdk}$ is not phosphorylated *in vitro* by cyclin–Cdks. Rb and $Rb^{\Delta Cdk}$ were expressed in insect cells and purified by affinity chromatography on a resin of Sepharose covalently joined to E7 peptide (Thr–Asp–Leu–Tyr–Cys–Tyr–Glu–Gln–Leu–Asn) and eluted with unbuffered 50 mM Na_2CO_3 containing 150 mM NaCl and 1 mM DTT before neutralization with 1 M HEPES, pH 7.0 [19]. For Rb phosphorylation, Rb proteins (500 nM) were treated with 20 nM cyclin D1–Cdk4 (D1/K4) or cyclin E–Cdk2 (E/K2), as described [11]. Rb was then purified from reaction mixtures by immunoprecipitation prior to gel electrophoresis. Replica gels were either immunoblotted to verify Rb levels (αRb) or subjected to autoradiography to visualize phosphorylation ($[^{32}P]$Rb).

or not the Rb they expressed had Cdk phosphorylation sites (Figure 3). Cyclin D1–Cdk4 (Figure 3b) and cyclin D3–Cdk4 (data not shown) were also capable of inducing S phase in cells expressing $Rb^{\Delta Cdk}$, albeit with a lower efficiency than cyclin E–Cdk2. However, neither cyclin D1 alone nor cyclin D1 together with either a kinase-inactive Cdk4 mutant (D158N) [10] or Cdk2 effectively overcame arrest by Rb or $Rb^{\Delta Cdk}$ in this assay (Figure 3 and data not shown). Cyclin D1 does not efficiently activate Cdk2 as an Rb kinase [8].

These data suggested that expression of cyclin–Cdks can effect replication independently of Rb phosphorylation. Whereas both cyclin E and cyclin D1 have been shown to accelerate G1 progression in Rb-positive fibroblasts [12–14], and cyclin E–Cdk2 is required for DNA replication *in vitro* [15], the question of whether these cyclins can accelerate S-phase entry independently of Rb has not been addressed. To examine this question, active cyclin–Cdk complexes were injected into SAOS-2 cells during early or mid G1, and BrdU incorporation was used

Figure 2

(a)

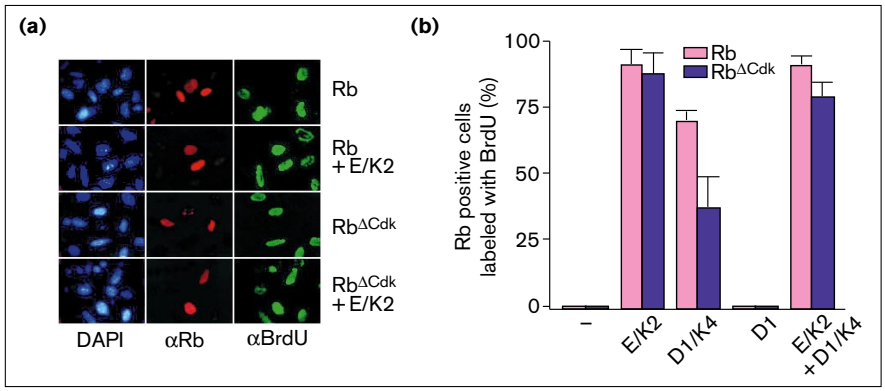


(b)

		Experiment 1			Experiment 2			Experiment 3		
		G1	S	G2/M	G1	S	G2/M	G1	S	G2/M
pCMV	–	54	27	19	57	26	17	54	26	20
	E/K2	40	42	18	30	51	19	34	39	27
	D1/K4	43	34	23	37	37	26	39	34	27
	E/K2+D1/K4	37	46	17	20	52	28	35	39	26
pCMV–Rb	–	76	13	11	81	10	9	72	14	14
	E/K2	44	43	13	23	54	23	36	39	25
	D1/K4	50	27	23	40	31	29	48	32	20
	E/K2+D1/K4	32	43	25	19	64	17	40	43	17
pCMV–$Rb^{\Delta Cdk}$	–	66	16	18	76	17	7	76	16	8
	E/K2	36	42	22	26	58	16	36	51	13
	D1/K4	51	26	23	48	34	18	50	26	24
	E/K2+D1/K4	21	56	23	25	57	18	35	45	20

(a) Cyclin–Cdks override G1 arrest by $Rb^{\Delta Cdk}$. SAOS-2 cells were transfected using calcium phosphate and 1 μg each of either empty vector (pCMV) or vector encoding Rb (pCMV–Rb) or $Rb^{\Delta Cdk}$ (pCMV–$Rb^{\Delta Cdk}$), and vectors encoding cyclin E and Cdk2 (E/K2) and/or cyclin D1 and Cdk4 (D1/K4), as well as 0.5 μg of pCMV–CD20, which encodes CD20, a cell surface marker used to identify transfected cells [10], in a total of 10 μg DNA. All proteins were expressed under the control of the cytomegalovirus (CMV) promoter and constant levels of CMV-promoter-containing DNA were maintained. The DNA content of 1000–3000 CD20-positive cells was determined by flow cytometry 48 h later, as described [10]. Of three experiments performed, **(a)** histograms from the second experiment and **(b)** the DNA content of transfected cells from all three experiments are shown.

Figure 3

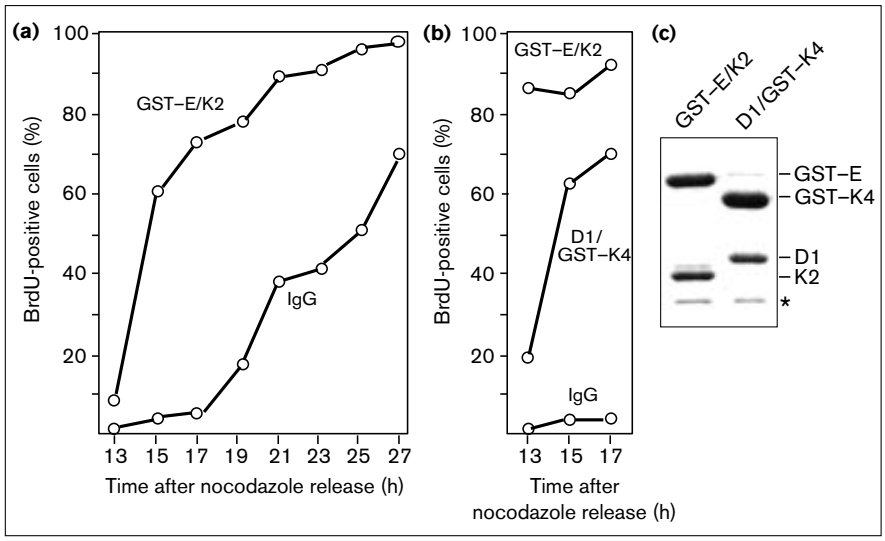


(a) Cells cotransfected to express the indicated proteins, as described in Figure 2, were incubated for 6 h with 10 μM BrdU to label S-phase cells before immunofluorescence. BrdU incorporation was visualized using anti-BrdU antibodies (Becton-Dickinson; green) and Rb expression using anti-Rb antibodies (red). Nuclei were identified using 4,6-diamidino-2-phenylindole (DAPI; blue). **(b)** Summary of three experiments depicting the proportion of Rb-expressing cells that incorporated BrdU. For each experiment, about 100 Rb-positive cells were counted.

to measure the fraction of cells entering S phase over time (Figure 4). When injected with cyclin E–Cdk2 10 h after release from mitosis, 60% of the cells were in S phase 15 h after release, compared with less than 10% in mock-injected cells (Figure 4a). Mock-injected cells reached 60% BrdU incorporation 25–27 h after release, indicating a dramatic 10–12 h acceleration of S-phase entry by cyclin E–Cdk2. When injected with cyclin E–Cdk2 4 h after release, over 85% of the cells were BrdU-positive at 13 h, intimating that the earlier cyclin E–Cdk2 is present in G1, the earlier cells will initiate replication (Figure 4b). Interestingly, cyclin D1–Cdk4 can also accelerate S-phase entry, albeit less efficiently than cyclin E–Cdk2; 17 h after release from mitosis, less than 2% of mock-injected cells were BrdU positive, whereas over 60% of the cyclin D1–Cdk4-injected cells had initiated replication (Figure 4b). Thus, G1 cyclin–Cdk complexes can affect the timing of and/or commitment to S phase, independent of Rb.

Consistent with our studies, Lukas *et al.* [16] recently reported that cyclin E can bypass an Rb mutant lacking Cdk phosphorylation sites. Importantly, their studies also showed that transcription from an E2F promoter–reporter plasmid is still repressed by the Rb mutant while cells are entering S phase as a result of cyclin E expression. Taken together with our work, these results indicate that the prevailing view of how Cdks regulate the G1 to S phase transition is incomplete. Although Rb phosphorylation and release of active E2F are induced by cyclin–Cdk cotransfection ([6–8,12] and Figure 1), these kinases can simultaneously promote cell cycle progression independently of Rb/E2F, presumably by acting upon downstream targets that are rate-limiting for S phase. Further studies are required to determine whether the ability of ectopic cyclin E to bypass the Rb–E2F pathway simply reflects the fact that Rb normally represses cyclin E expression while not significantly affecting other E2F-regulated genes [17,18].

Figure 4



Injection of active cyclin–Cdk complexes accelerates S-phase entry. SAOS-2 cells (about 100 per time point) were injected with either biotinylated rabbit immunoglobulin G (IgG) or a mixture of IgG and the indicated kinase (at 10 μM) **(a)** 10 h or **(b)** 4 h after release from a nocodazole block [11], and 10 μM BrdU was added. As a kinase source, a fusion protein of glutathione-S-transferase (GST) and cyclin E, complexed to Cdk2 (GST–E/K2), and a GST–Cdk4 fusion protein complexed to cyclin D1 (D1/GST–K4) were purified from insect cells [11] and exchanged into injection buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl] using a Centricon-30. Cells were processed at the indicated times for immunofluorescence to detect BrdU-positive, IgG-positive cells [11]. **(c)** Gel electrophoresis of the kinases used for injection; insect cell GST protein is marked with an asterisk.

Our results also indicate that the Rb/cyclin SAOS-2 cotransfection assay is confounded by Rb-independent effects of cyclin–Cdks on DNA replication. We have recently presented an alternative assay for examining Rb regulation in which Rb modified *in vitro* by Cdks is assayed for growth suppression by microinjection in the absence of exogenous cyclin–Cdks [11]. In this assay, mutation of a single critical cyclin D1–Cdk4 phosphorylation site in Rb (Ser795→Ala) blocks the ability of this kinase to inactivate Rb's growth suppressive function upon phosphorylation of Rb *in vitro*, indicating that this Rb mutant acts in a dominant fashion when Cdks are not introduced into the cell [11]. In contrast, we have shown here that when cyclin D1–Cdk4 is introduced into cells, growth arrest mediated by Rb lacking all its cyclin D1–Cdk4 phosphorylation sites is bypassed (Figures 2 and 3). We also found previously that hyperphosphorylation of Rb *in vitro* by cyclin E–Cdk2 is not sufficient to inactivate Rb's G1 arrest activity [11] but, as for cotransfection, introduction of Rb and cyclin E–Cdk2 into cells by coinjection leads to S-phase entry (L.C-C., J.W.H. and D.G., unpublished observations). Thus, introduction of cyclin–Cdks into cells can affect DNA replication independently of upstream regulatory pathways.

Surprisingly, we found that cyclin D1–Cdk4, but not cyclin D1 alone, can also bypass a G1 arrest mediated by $Rb^{\Delta Cdk}$. Moreover, we also found that cyclin D1–Cdk4 can accelerate S-phase entry in the absence of Rb, indicating that this kinase can affect replication independently of its ability to phosphorylate Rb. Lukas *et al*. [16] have also shown that cyclin D1 alone is not sufficient to bypass $Rb^{\Delta Cdk}$ but did not address whether cyclin D1–Cdk4 can bypass this 'non-phosphorylatable' Rb mutant. It is unlikely that the ability of cyclin D1–Cdk4 to bypass $Rb^{\Delta Cdk}$ depends upon a physical association of cyclin D1 with Rb [7,8], as neither cyclin D1 alone nor cyclin D1 associated with kinase-defective Cdk4 can efficiently bypass Rb-mediated growth arrest. The latter finding also suggests that Cdk4 activity is required, which would argue against Cdk-inhibitor sequestration by cyclin D1–Cdk4 being involved in $Rb^{\Delta Cdk}$ bypass, but does not absolutely rule it out. Whereas cyclin D1 is not required for S-phase entry in cells lacking Rb (reviewed in [1,2]), it is possible that cyclin D1–Cdk4 shares substrates with cyclin E–Cdk2, making cyclin D1–Cdk4 expendable for S-phase entry in Rb-deficient cells. In this case, cyclin D1–Cdk4 may induce S phase using pathways that are normally operative in the cell, but which are redundant in the presence of active cyclin E–Cdk2. Alternatively, the effects of overexpressed cyclin D1–Cdk4 may reflect a non-physiological loss of specificity of the kinase activity when expressed at high levels. The identification of downstream targets for these kinases is required to resolve this issue. Nevertheless, our results suggest the possibility that overexpression of cyclin D may contribute to cell cycle progression and transformation [2] through both Rb-dependent and Rb-independent pathways.

Acknowledgements

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