

Regulation of the retinoblastoma protein-related p107 by G₁ cyclin complexes

Roderick L. Beijersbergen, Leone Carlée, Ron M. Kerkhoven, and René Bernards¹

Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands

The orderly progression through the cell cycle is mediated by the sequential activation of several cyclin/cyclin-dependent kinase (cdk) complexes. These kinases phosphorylate a number of cellular substrates, among which is the product of the retinoblastoma gene, pRb. Phosphorylation of pRb in late G₁ causes the release of the transcription factor E2F from pRb, resulting in the transcriptional activation of E2F-responsive genes. We show here that phosphorylation of the pRb-related p107 is also cell cycle regulated. p107 is first phosphorylated at 8 hr following serum stimulation of quiescent fibroblasts, which coincides with an increase in cyclin D1 protein levels. Consistent with this, we show that a cyclin D1/cdk4 complex, but not a cyclin E/cdk2 complex, can phosphorylate p107 in vivo. Furthermore, phosphorylation of p107 can be abolished by the overexpression of a dominant-negative form of cdk4. Phosphorylation of p107 results in the loss of the ability to associate with E2F-4, a transcription factor with growth-promoting and oncogenic activity. A p107-induced cell cycle block can be released by cyclin D1/cdk4 but not by cyclin E/cdk2. These data indicate that the activity of p107 is regulated by phosphorylation through D-type cyclins.

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The cell division cycle in mammalian cells is regulated by the cyclical activation of a number of kinases whose activity depends on their association with a cyclin subunit (Sherr 1993). In the G₁ phase of the cell cycle, cyclins D and E are expressed. D-type cyclins are encoded by a family of three closely related genes, cyclin D1, D2, and D3, whereas only a single gene for the E-type cyclin has been isolated [Koff et al. 1992; Matsushime et al. 1991]. Several lines of evidence indicate that D- and E-type cyclins are rate-limiting for passage through G₁. Antibodies to cyclin D1 block S-phase entry in several cell types [Baldin et al. 1993; Lukas et al. 1994] and overexpression of both cyclin D1 and E shortens G₁ [Ohtsubo and Roberts 1993; Quelle et al. 1993; Resnitzky et al. 1994; Wimmel et al. 1994]. Because induction of cyclin D1 or E expression in serum-starved cells does not result in S-phase entry, it appears that the expression of these cyclins is necessary but not sufficient to progress through G₁ into S phase [Resnitzky et al. 1994].

An important difference between cyclins D and E is that D-type cyclins are implicated as a causal agent in cancer; cyclin D1 gene amplification has been found in breast cancer, esophageal carcinoma, and squamous cell carcinoma [Lammie et al. 1991; Jiang et al. 1992; Schuurin et al. 1992a,b]. Furthermore, cyclin D1 is translocated in parathyroid adenomas and in centrocytic lymphomas [Motokura et al. 1991; Rosenberg et al. 1991; Withers et al. 1991; Seto et al. 1992]. More recently, the

product of the *mts-1* tumor suppressor gene was identified as p16, a strong inhibitor of cyclin D1-associated kinase activity [Serrano et al. 1993; Kamb et al. 1994]. Finally, in vitro, cyclin D1 can cooperate with other oncogenes to transform fibroblasts [Hinds et al. 1994; Lovet et al. 1994], and in transgenic mice overexpression of cyclin D1 in breast epithelium results in breast cancer [Wang et al. 1994].

Because of the involvement of D-type cyclins, and not E-type cyclins, in the genesis of several types of human cancer, important functional differences are likely to exist between these two G₁ cyclins. One such difference may be the kinetics of induction of D- versus E-type cyclins. Cyclin E is a generic cyclin in that its expression is induced in a cyclical fashion in the cell cycle, reaching maximal levels toward the end of G₁, and cyclin E-associated kinase activity has been shown to peak at the G₁/S transition [Dulic et al. 1992; Koff et al. 1992]. In contrast, cyclin D1 appears to be induced most strongly following mitogen stimulation of quiescent cells [Matsushime et al. 1991 1994; Won et al. 1992; Ajchenbaum et al. 1993; Sewing et al. 1993]. Whether D1 cyclin is also expressed in a cyclical fashion in exponentially growing cells is controversial. Some have shown invariant expression of cyclin D1 during the cell cycle, whereas others have seen cell cycle-dependent variation in cyclin D1 levels [Matsushime et al. 1991; Baldin et al. 1993; Sewing et al. 1993; Lukas et al. 1994]. Because several strong inhibitors of cyclin D1-associated kinase activity exist, an important question that has not been addressed so far

¹Corresponding author.

is whether the cyclin D1-associated kinase activity varies during the cell cycle.

The product of the retinoblastoma gene, pRb, is a substrate for G₁ cyclin/cyclin-dependant kinase (cdk) complexes. Extracts from insect cells infected with recombinant baculoviruses that express either cyclin E and cdk2 or D-type cyclins and cdk4 efficiently phosphorylate pRb in vitro (Ewen et al. 1993; Kato et al. 1993). In human osteosarcoma cells, expression of cyclin E, D2, and D3, but not D1, resulted in hyperphosphorylation of pRb (Hinds et al. 1992; Ewen et al. 1993). Significantly, a pRb-induced cell cycle block could be released by ectopic expression of cyclin E, D2, and D3, but not effectively by cyclin D1 expression (Hinds et al. 1992; Ewen et al. 1993).

The retinoblastoma protein and the related p107 and p130 interact with several cellular polypeptides including E2F, a transcription factor that controls gene expression during the cell cycle (Cao et al. 1992; Cobrinik et al. 1993). E2F DNA-binding activity consists of a heterodimeric complex containing an E2F component complexed to a DP component (Bandara et al. 1993; Helin et al. 1993; Krek et al. 1993). The E2F component of the heterodimer is encoded by at least five closely related polypeptides. E2F-1, E2F-2, and E2F-3 associate in vivo only with pRb and not with the related p107 (Lees et al. 1993). E2F-4, in contrast, interacts with p107 and p130 but not with pRb (Beijersbergen et al. 1994b; Ginsberg et al. 1994; Vairo et al. 1995; R.L. Beijersbergen and R. Bernards, unpubl.), whereas E2F-5 interacts preferentially with p130 (Hijmans et al. 1995). E2F binds preferentially to hypophosphorylated pRb, suggesting that complex formation between pRb and E2F is regulated by phosphorylation of pRb by cyclin/cdk complexes (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Chellappan et al. 1991). Hypophosphorylated pRb is found mostly in the G₁ phase of the cell cycle, whereas the hyperphosphorylated form of pRb is first observed at the G₁ to S transition (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989).

The complexes between E2F and p107 show a more complex pattern of appearance during the cell cycle. In late G₁, DNA-binding complexes have been observed that contain E2F, p107, cyclin E, and cdk2. In S phase cyclin E is no longer found in these complexes; instead, E2F is found associated with p107, cyclin A, and cdk2 (Lees et al. 1992; Shirodkar et al. 1992). In spite of the presence of cyclin/cdk complexes in association with p107, very little is known about the regulation of p107 by phosphorylation. In contrast to pRb, cell cycle-regulated phosphorylation of p107 has not been observed. We show here that the growth inhibitory activity of p107 is subject to regulation by phosphorylation by cyclin/cdk complexes.

Results

Effect of cyclin E on p107

In late G₁ phase of the cell cycle, p107 is found in a multiprotein complex that contains E2F DNA-binding

activity, cyclin E, and cdk2 (Lees et al. 1992). To study the effect of cyclin E and its associated kinase cdk2 on the E2F/p107 complex, we used a transient transfection assay with E2F-4. We have shown recently that E2F-4 associates in vivo specifically with p107 and not with pRb. As a control, we studied the effect of cyclin E/cdk2 on the pRb/E2F-1 complex. We cotransfected a chloramphenicol acetyltransferase (CAT) reporter gene harboring upstream E2F sites with either E2F-4 and DP-1 expression vectors or with E2F-1 and DP-1 expression vectors. Figure 1, A and B, shows that both the E2F-4/DP-1 and the E2F-1/DP-1 heterodimers efficiently activated the CAT reporter gene (tracks 3). As expected, cotransfection of p107 inhibited E2F-4 *trans*-activation and pRb expression suppressed E2F-1 *trans*-activation (tracks 5). When cyclin E/cdk2 and pRb expression vectors were cotransfected, a release of E2F-1 inhibition by pRb was observed, presumably as a result of phosphorylation of pRb by cyclin E/cdk2 (Fig. 1A, track 6) (Hinds et al. 1992; Ewen et al. 1993). Surprisingly, cotransfection of cyclin E/cdk2 with p107 failed to relieve p107 inhibition of E2F-4 (Fig. 1B, track 6). We conclude that cyclin E/cdk2 acts differently on pRb and p107 in that cyclin E/cdk2 releases pRb, but not p107 inhibition of E2F.

Effect of D-type cyclins on p107

Next, we studied the effect of cyclin D1 on p107. Figure 2 shows that in contrast to cyclin E/cdk2, cyclin D1, together with its major catalytic partner cdk4, can release p107 inhibition of E2F-4 and pRb inhibition of E2F-1 (Fig. 2A,B, cf. tracks 4 and 7). The inactivation of p107 as an inhibitor of E2F-4 activity could be caused by several mechanisms. First, cyclin D1/cdk4 could phosphorylate p107, thereby releasing active E2F-4. An alternative mechanism of cyclin D1 action could be the direct binding of cyclin D1 to the pocket region of p107, as cyclin D1 shares a motif (LXCXE) with a number of viral transforming proteins that bind avidly to p107 and pRb through this motif (Dowdy et al. 1993). However, neither cyclin D1 alone nor cdk4 alone was able to release p107 and pRb inhibition of E2F activity (Fig. 2A,B). This suggests that a cyclin D/cdk complex is required to release p107 inhibition of E2F-4.

To study the kinase requirement of D-type cyclins to inactivate p107, we cotransfected E2F-4 and p107 with other combinations of D-type cyclins and cdks. To address the specificity of the kinase, we expressed cyclin D3, instead of cyclin D1, in combination with either cdk2 or cdk4. Both cdk2 and cdk4, when bound to cyclin D3, have significant kinase activity toward pRb, whereas cyclin D1 only forms an active complex with cdk4 (Ewen et al. 1993). Figure 3 shows that although cyclin D1/cdk4 and cyclin D3/cdk4 effectively rescued E2F-4 inhibition by p107, no release of inhibition was observed with cyclin D3/cdk2 (Fig. 3, cf. tracks 6, 8, and 9). These data indicate that not only the cyclin, but also the associated kinase contributes to the activity of the cyclin/cdk complex toward p107.

The data shown above are consistent with a model in

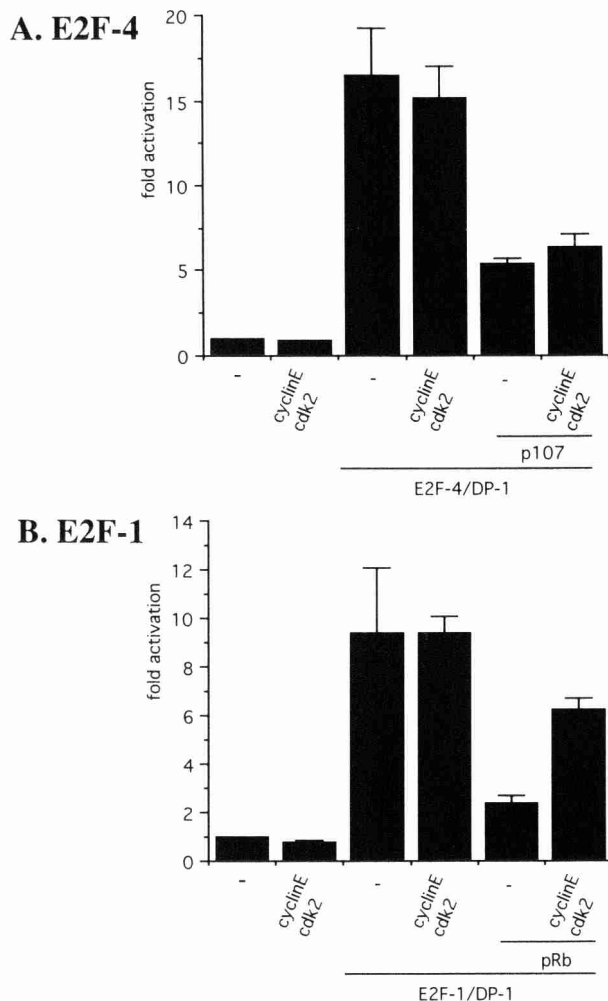


Figure 1. Effect of cyclin E/cdk2 on p107-E2F and pRb-E2F interactions. (A) C33A cells were transfected with 100 ng of pCMVE2F-4 and 100 ng of pCMVDP-1 in combination with 200 ng of pCMVp107 and with 2.0 μ g of pCMVcyclin E and 2.0 μ g of pCMV cdk2 as indicated. Together with the expression plasmids, the cells were transfected with 2 μ g of E2F₄-CAT and 0.2 μ g of pRSV luciferase. CAT activity was normalized to the luciferase internal control. Fold activation was calculated relative to the basal level of E2F₄-CAT, which was set to 1 unit. The experiments were performed in duplicate, and activity was also assayed in duplicate. Data are representative for at least three independent experiments. (B) C33A cells were transfected with 100 ng of pCMVE2F-1 and 100 ng of pCMVDP-1 in combination with 200 ng of pCMVpRb and with 2.0 μ g of pCMV cyclin E and 2.0 μ g of pCMV cdk2 as indicated. Fold activation was determined as described in A.

which some, but not all, cyclin D/cdk complexes can phosphorylate and functionally inactivate p107. To further study the molecular mechanism by which cyclin D1 acts on p107, we used two mutants of cyclin D1. The first mutant, cyclin D1-GH carries a mutation in the LXCXE motif that is required for high affinity binding of cyclin D1 to pocket proteins. As a result, the GH mutant of cyclin D1 is defective for binding to pocket proteins

(Dowdy et al. 1993; Hinds et al. 1994). The second mutant, cyclin D1-KE carries a mutation in the cyclin box, a domain that mediates association with cdk4. The KE mutant is therefore unable to form a stable complex with cdk4 (Hinds et al. 1994). Figure 4 shows that the cyclin D1 GH mutant retained the ability to release E2F-4 from p107 inhibition, whereas the KE mutant was inactive in this assay. These data are consistent with the notion that cyclin D1/cdk4 phosphorylates p107 and do not support the hypothesis that cyclin D1 competes with E2F-4 for binding to the p107 pocket.

To further substantiate that phosphorylation is essen-

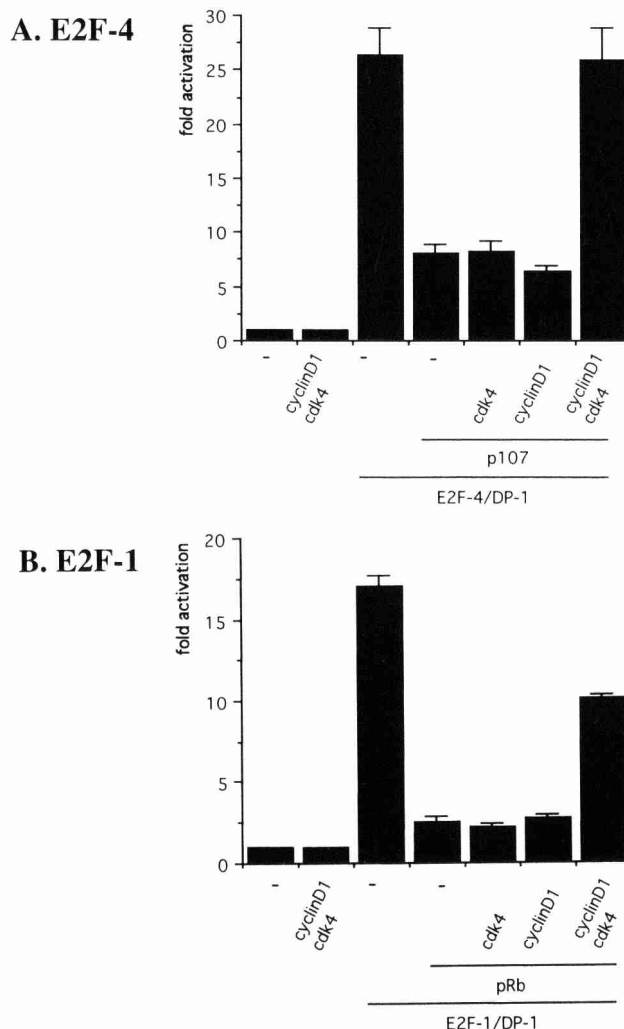


Figure 2. Effect of cyclin D1 and cdk4 on p107 and pRb. (A) C33A cells were transfected with 100 ng of pCMVE2F-4 and 100 ng of pCMVDP-1 in combination with 200 ng of pCMVp107 and 2.0 μ g of pCMV cyclin D1 and 2.0 μ g of pCMVcdk4 as indicated. Together with the expression plasmids, the cells were transfected with 2 μ g of E2F₄-CAT and 0.2 μ g of pRSV luciferase. (B) C33A cells were transfected with 100 ng of pCMVE2F-1 and 100 ng of pCMVDP-1 in combination with 200 ng of pCMVpRb and 2.0 μ g of pCMV cyclin D1 and 2.0 μ g of pCMV cdk4 as indicated. Experiments were performed as described in Fig. 1A; fold activation was calculated as described in Fig. 1A.

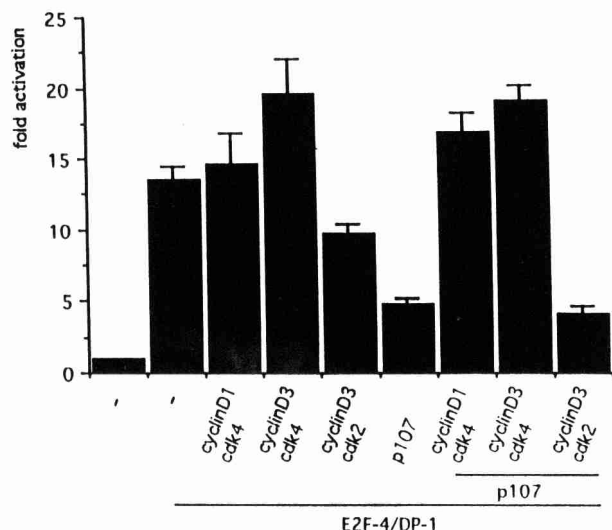


Figure 3. Cyclin D1 and D3 in combination with cdk4 can release p107 inhibition. Cells were transfected as described in Fig. 2. Cyclin D1, cyclin D3, cdk2, or cdk4 (2.0 μ g) was cotransfected as indicated.

tial for the inactivation of p107, we used a dominant-negative mutant of cdk4 (van den Heuvel and Harlow 1993). Figure 5A shows that this kinase-inactive cdk4, in combination with cyclin D1, was not able to release the p107 inhibition of E2F-4, indicating that kinase activity of the cyclin D/cdk4 complex is essential for the release of the p107 mediated inhibition. Recently, a strong in-

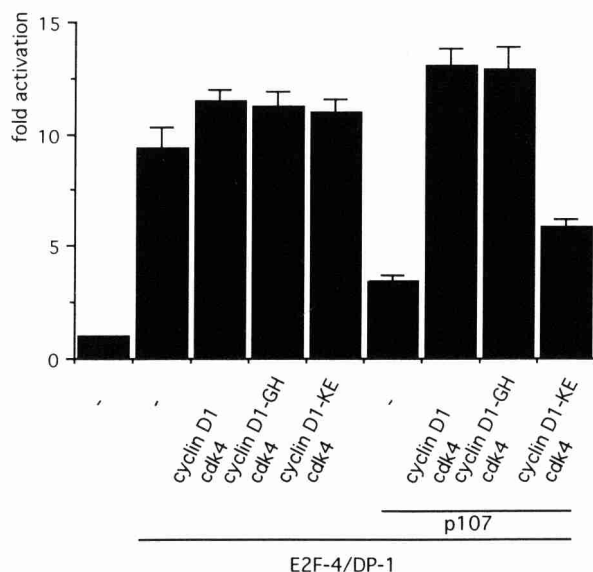


Figure 4. Effect of Cyclin D1 mutants on p107/E2F-4. C33A cells were transfected as described in Fig. 3. pCMV cyclin D1-KH (2.0 μ g), mutant cyclin D1 that carries a mutation in the LXCXE domain, or pCMV cyclin D1-KE (2.0 μ g) mutant cyclin D1 that lacks the cdk4 interaction site, was transfected as indicated.

hibitor of cyclin D1/cdk4 kinase activity, p27^{kip1}, was isolated (Polyak et al. 1994; Toyoshima and Hunter 1994). We also studied the effect of p27^{kip1} expression on cyclin D1/cdk4 in the assay described above. Figure 5B shows that whereas cyclin D1/cdk4 effectively released p107 inhibition of E2F-4, cotransfection of a p27^{kip1} expression vector completely blocked the release by cyclin D1/cdk4. Thus, inhibition of kinase activity of cyclin D1/cdk4 by either mutation of cdk4 or coexpression of p27^{kip1} prevented the release of p107 inhibition. These data lend further support to the notion that cyclin D1/

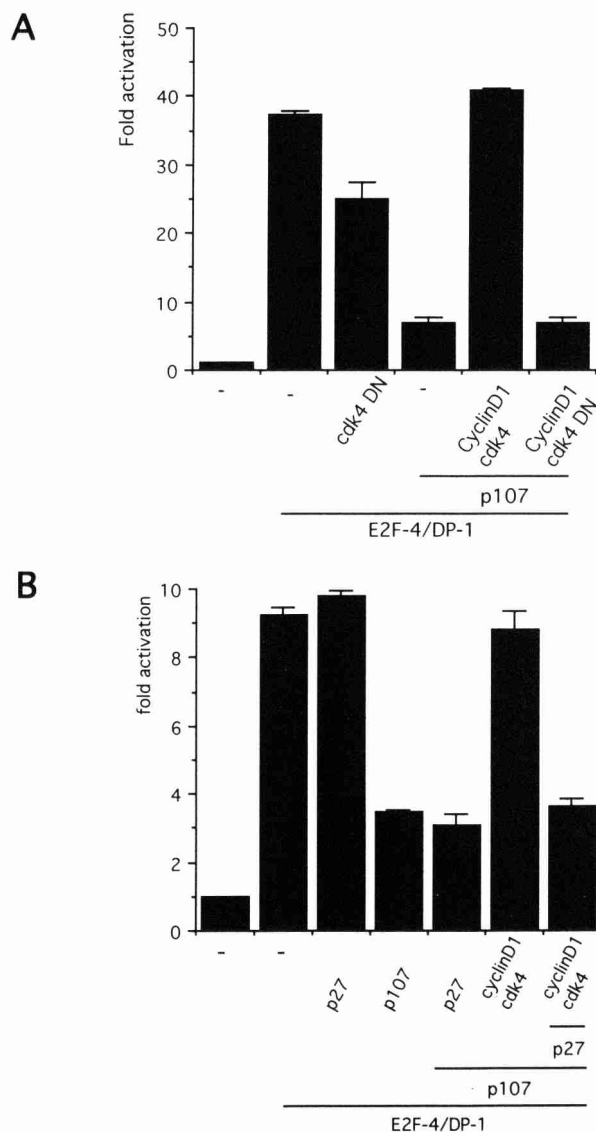


Figure 5. (A) Cyclin D1 in combination with a dominant-negative cdk4 cannot release p107 inhibition. C33A cells were transfected as described in Fig. 2; 2 μ g of cdk4 dominant-negative (cdk4 DN) was transfected as indicated. (B) Release of p107 inhibition by cyclin D1/cdk4 is blocked by p27^{kip1}. C33A cells were transfected as described in Fig. 2; 2.0 μ g of p27^{kip1} was transfected as indicated.

cdk4 kinase activity is required to release p107 inhibition of E2F-4.

Cyclin D/cdk4 complexes phosphorylate p107

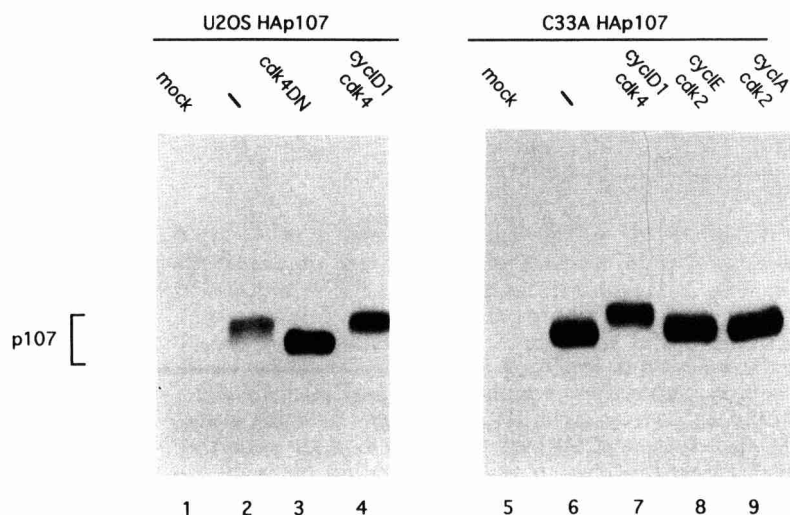
The effect of cyclin D1/cdk4 could be the result of phosphorylation of the E2F-4 protein, the p107 protein, or both. Although we have shown recently that E2F-4 is a phosphoprotein, we did not see any alterations in E2F-4 phosphorylation upon expression of cyclin D1/cdk4 either in the absence or presence of p107 (Beijersbergen et al. 1994b; data not shown). We therefore focused on the effect of cyclin D1/cdk4 on p107. The cell cycle-regulated phosphorylation of pRb can be readily detected because of the increased apparent molecular weight of hyperphosphorylated pRb in SDS-acrylamide gels (Buchkovich et al. 1989; Ludlow et al. 1989). To study the effect of cyclin D1/cdk4 on p107 phosphorylation, we transfected the osteosarcoma cell line U2-OS with either an epitope-tagged p107 expression vector alone, or one cotransfected with the dominant-negative form of cdk4 or cyclin D1/cdk4. Two days after transfection, cells were lysed and whole cell extracts were subjected to 7.5% SDS-PAGE, and the p107 protein was detected by Western blot analysis with monoclonal antibody 12CA5 directed against HA-tagged p107. Figure 6 shows that p107 migrates as a doublet when expressed in U2-OS cells (lane 2). This doublet is reduced to a single band that migrates with the lower apparent molecular weight species when the dominant-negative form of cdk4 is coexpressed (lane 3). This result suggests that the p107 protein is phosphorylated upon expression in U2-OS cells and that this phosphorylation can be abolished by the coexpression of a dominant-negative form of cdk4. Cotransfection of cyclin D1/cdk4 resulted in an increase of p107 species with higher apparent molecular weight (lane 4), most likely as a result of phosphorylation of p107 by cyclin D/cdk4. U2-OS cells express multiple p107 species (Fig. 6, lane 2) probably as a result of endogenous kinase activity present in these cells. We searched

for additional cell lines that lack significant p107 kinase activity. Figure 6, lane 6, shows that C33A cervical carcinoma cells only express a single species of p107, which comigrates with the fastest migrating species of p107 in U2-OS cells (cf. lanes 2 and 6). As expected, cotransfection of cyclin D1/cdk4 resulted in a slower migrating species of p107 (lane 7). In contrast, cotransfection of p107 with cyclin E/cdk2, or cyclin A/cdk2 did not result in a significant increase in p107 mobility (Fig. 6, cf. lanes 6, 8, and 9). This lack of phosphorylation of p107 by cyclin E/cdk2 in C33A cells correlates with the absence of the release of the p107-mediated inhibition of E2F-4 *trans*-activation in the same cells (Fig. 1A). U2-OS cells that were labeled with [³²P]orthophosphate after transfection with HA-tagged p107, alone or together with the different cyclin/cdk combinations, showed an increase in the amount of ³²P-labeled p107 only in the presence of cyclin D1/cdk4 and not cyclin E/cdk2 or cyclin A/cdk2 (data not shown). To show that the increase in apparent molecular weight of p107 is the result of phosphorylation, we treated p107 immunoprecipitates with λ phosphatase. p107 immunoprecipitated from U2-OS cells transfected with HA-tagged p107, displays different p107 molecular weight species (Fig. 7, lane 1). Lambda phosphatase treatment of p107 immunoprecipitates resulted in the appearance of a single band that comigrates with the faster migrating p107 species (Fig. 7, lane 3). A p107 immunoprecipitate treated with λ phosphatase in the presence of phosphatase inhibitors contained both the slower and faster migrating species of p107 protein (Fig. 7, lane 2). We conclude that p107 is phosphorylated *in vivo*, which is most likely attributable to cyclin D-associated kinase activity and that expression of cyclin D1/cdk4 leads to hyperphosphorylation of p107.

Phosphorylation of p107 prevents interaction with E2F-4

To address whether p107 hyperphosphorylation results in a loss of association with the E2F-4/DP-1 het-

Figure 6. Effect of cdk4 dominant-negative and cyclins on the phosphorylation of p107. U2-OS cells (lanes 1–4) were transfected with HA-tagged p107 alone (lane 2) or in combination with dominant-negative cdk4 (lane 3) or cyclin D1/cdk4 (lane 4). C33A cells (lanes 5–9) were transfected with HA-tagged p107 alone (lane 6) or in combination with cyclin D1/cdk4 (lane 7), cyclin E/cdk2 (lane 8), or cyclin A/cdk2 (lane 9). Forty hours after transfection, the cells were lysed and cell extracts were subjected to low percentage SDS-PAGE. The separated proteins were transferred to nitrocellulose, and HA-tagged p107 protein was detected by Western analysis with mAb 12CA5.



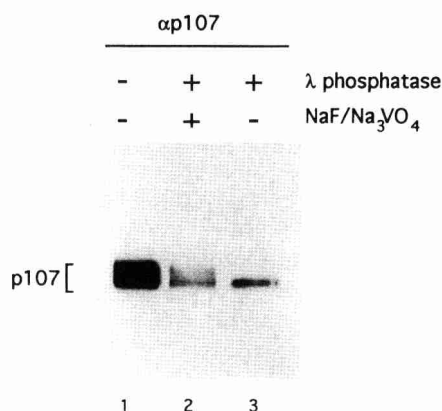


Figure 7. Electrophoretic heterogeneity of p107 is attributable to differential phosphorylation. U2-OS cells were transfected with HA-tagged p107. Forty hours after transfection, the cells were lysed and the lysate was split in three. HA-tagged p107 protein was immunoprecipitated and resuspended in SDS-containing sample buffer (lane 1) or treated with λ -phosphatase in the presence (lane 2) or absence of phosphatase inhibitors (lane 3), as described in Material and methods. The immunoprecipitates were subjected to low-percentage polyacrylamide SDS-PAGE and transferred to nitrocellulose. HA-tagged p107 protein was detected by Western analysis using mAb 12CA5.

erodimeric complex, we performed the following experiment. An epitope tagged DP-1 expression vector was cotransfected with E2F-4 and p107 expression vectors in the presence or absence of cyclin/cdk expression vectors. Two days after transfection, cells were metabolically labeled with [³⁵S]methionine and lysed in nonionic detergent. One-half of each lysate was immunoprecipitated with p107 antibodies, and the other half with mAb 12CA5 directed against HA-tagged DP-1. Immunoprecipitated proteins were resolved on a low-percentage SDS-polyacrylamide gel. Figure 8, lane 4, shows that in the absence of cotransfected cyclin/cdks the DP-1/E2F-4 heterodimer is bound to p107. Expression of cyclin D1/cdk4 completely abolished this interaction, whereas expression of cyclin E/cdk2 did not cause a release of p107 from E2F-4 (Fig. 8, lanes 5,6). Thus, cyclin D1/cdk4-mediated phosphorylation of p107 prevents an interaction between p107 and the E2F-4/DP-1 heterodimer, whereas the expression of cyclin E/cdk2 does not affect the complex.

U2-OS cells contain both hypo- and hyperphosphorylated species of p107 protein. The experiments described above indicate that phosphorylation of p107 prevents association with E2F-4; therefore, p107 species that are associated with E2F-4 should be in the hypophosphorylated state. To address the phosphorylation status of p107 protein that is bound to E2F-4, we transfected U2-OS cells with HA-tagged E2F-4 alone or together with DP-1. Cells were lysed and E2F-4 protein was immunoprecipitated with mAb 12CA5 antibody directed against HA-E2F-4. As a control for the p107 species present in cells transfected with both HA-E2F-4 and DP-1, p107 protein was immunoprecipitated with the p107 antibody

SD4. The immunoprecipitated proteins were separated on a low-percentage SDS-polyacrylamide gel and p107 protein was detected by Western blot with a p107 antibody. As can be seen in Figure 9, lane 1, both species of p107 were again present in these cells. In contrast, p107 that was coimmunoprecipitated with HA-E2F-4 only consisted of the faster migrating species (Fig. 9, lanes 3,4). These results indicate that E2F-4 preferentially associates with the hypophosphorylated p107 species. We can conclude, then, that p107 is phosphorylated, most likely by cyclin D/cdk4 kinase complexes, and that phosphorylation of p107 prevents the association between E2F-4 and p107.

p107 phosphorylation is cell cycle regulated

To study whether p107 is phosphorylated in a cell cycle-dependent fashion, we studied the phosphorylation status of p107 in untransfected NIH-3T3 fibroblasts. NIH-

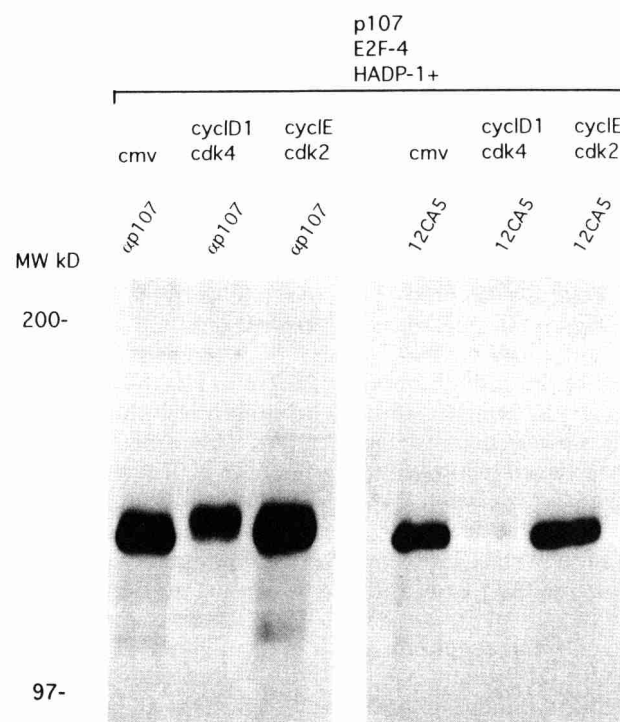


Figure 8. Cyclin D1/cdk4 phosphorylates p107 and prevents association of E2F-4 with p107. U2-OS cells were transfected with pCMVE2F-4 and pCMVDP-1, together with pCMVp107 in combination with control vector pCMV (lanes 1,4) or pCMV cyclin D1 and pCMV cdk4 (lanes 2,5) or pCMV cyclin E and pCMV cdk2 (lanes 3,6). The cells were labeled with [³⁵S]methionine 40 hr after transfection. Cell lysates were split, and one-half was immunoprecipitated with p107 antibodies (lanes 1-3); the other half with 12CA5 directed against HA-tagged DP-1 (lanes 4-6). Immunocomplexes were separated on a 7.5% SDS-polyacrylamide gel, dried, and subjected to fluorography. DP-1 was added in these transfections because the association between E2F-4 and p107 depends on its presence [Beijersbergen et al. 1994b].

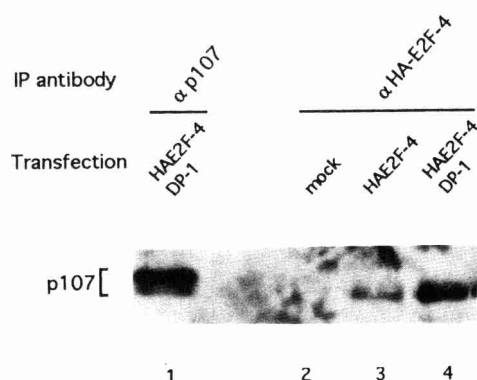


Figure 9. E2F-4 associates preferentially with the underphosphorylated species of p107. U2-OS cells were transfected with pCMV HA-E2F-4 alone or together with pCMVDP-1. Forty hours after transfection the cells were lysed and 90% of the lysate was immunoprecipitated with mAb 12CA5 directed against HA-E2F-4. As a control 10% of the lysate from cells transfected with both HA-E2F-4 and DP-1 was immunoprecipitated with p107 antibody SD-4. Immunoprecipitated proteins were separated by electrophoresis in 7.5% SDS-polyacrylamide gels and p107 species were detected by Western blotting with p107 antibody C18.

3T3 cells were made quiescent by culturing for 40 hr in low serum (0.2%). After this period the cells were restimulated with 10% serum and serum-stimulated cells were harvested at different time points. Cell extracts were subjected to low-percentage SDS-PAGE, and p107 protein was detected by Western blot analysis. As shown in Figure 10, quiescent cells express only the fastest migrating species of p107. After 8 hr of serum stimulation, part of the p107 protein has shifted to a higher apparent molecular weight. After 10 hr, most p107 protein is of the slower migrating species (Fig. 10). In addition, we observed an increase in the amount of p107 after 10 hr of serum stimulation. At this stage, we do not know whether this is the result of increased protein synthesis or protein stabilization as a result of phosphorylation. To examine the role of the D-type cyclins in the phosphorylation of p107, we analyzed the same extracts for cyclin D1 expression. As can be seen in Figure 10, middle, cyclin D1 protein expression is induced at 6–8 hr after serum stimulation and remains constant at later time points. As a control we also analyzed the same extracts for the expression of cyclin E. Cyclin E is not induced until 12–14 hr after serum stimulation (Fig. 10, bottom). These results indicate that phosphorylation of p107 is regulated in a cell cycle-dependent manner and that this phosphorylation takes place in the cell cycle shortly after cyclin D1 induction and well before cyclin E induction, suggesting that cyclin D is the most likely cyclin candidate to control p107 phosphorylation. To investigate how these differences in p107 phosphorylation affect E2F-p107 complex formation during the cell cycle, we analyzed E2F DNA-binding complexes in the same NIH-3T3 cells after serum stimulation. The most predominant complex in G_0 cells is E2F in complex with

p130 [Cobrinik et al. 1993]. In our experiments the complexes did not change until 8 hr after serum stimulation (Fig. 11A, lanes 1–4) and contained predominantly p130 as confirmed by p130 antibody-dependent supershifts (data not shown; Cobrinik et al. 1993). A slower migrating complex was first detected at 10–12 hr after serum stimulation (lanes 5–7). Only after time points of >18–24 hr (lane 8), as cells begin to pass from G_1 into S phase of the cell cycle (as determined by flow cytometry; data not shown) the slowest migrating complex became prominent. To determine whether p107 was present in this slowly migrating complex, the same extracts were analyzed in the presence of a p107 antibody. Between 10 and 16 hr after serum stimulation, very small amounts of the supershifted complexes were detectable and remained constant (Fig. 11B, lanes 1–4). At 24 hr after serum stimulation, the majority of the higher order E2F complexes was supershifted by p107 antibody. When the presence of E2F complexes containing p107 is compared to the presence of p107 protein in these cells (cf. Figs. 10 and 11), one can see that although there is a strong increase in the expression of phosphorylated p107 protein at time points 10–14 hr after stimulation this does not result in the increase in E2F/p107 complexes. The in-

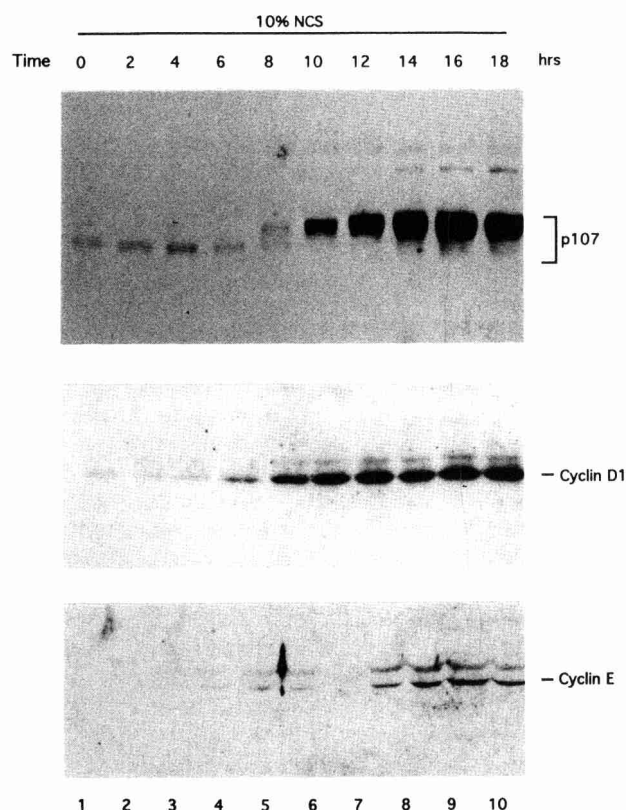


Figure 10. p107 is phosphorylated in a cell-cycle dependent manner. Extracts of equal numbers of NIH-3T3 cells were prepared after serum starvation (lane 1) or after serum stimulation at different time points as indicated (lanes 2–10) and subjected to gel electrophoresis followed by Western blot detection of p107 (top), cyclin D1 (middle), or cyclin E (bottom).

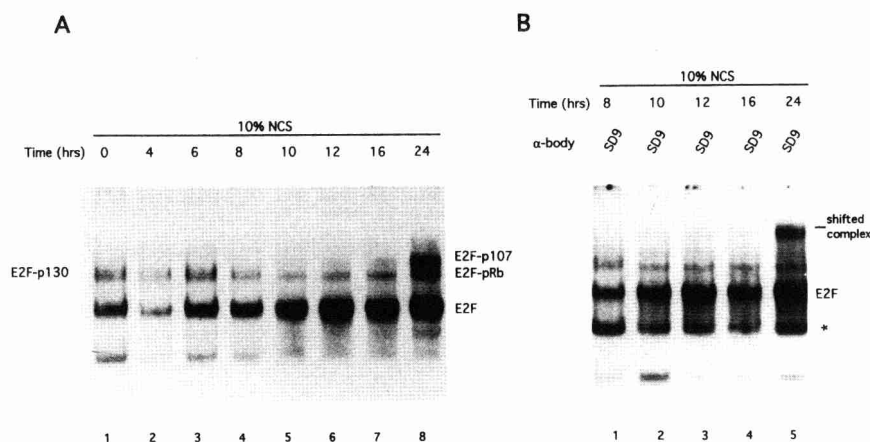


Figure 11. Analysis of E2F DNA-binding activity in G_0 and serum-stimulated 3T3 fibroblasts. (A) Extracts were prepared from NIH-3T3 fibroblasts after serum starvation (lane 1) or at different time points after serum stimulation (lanes 2–8) and subjected to mobility shift analysis as described in Materials and methods. The position of free E2F and the different E2F complexes, E2F–p130, E2F–p107, and E2F–pRb are indicated. (B) The same extracts that were used in A for the time points 8, 10, 12, 16, and 24 hr after serum stimulation (lanes 1–5) were subjected to mobility shift analysis in the presence of p107 antibody SD9. The position of free E2F and the p107 antibody-dependent supershift complexes are indicated. (*) A nonspecific background band.

crease in E2F complexes containing p107 at later time points could indicate that newly synthesized p107 protein is no longer phosphorylated at these timepoints and can therefore associate with E2F and form the E2F/p107 complex that is detected in S-phase cells.

Although p107 is phosphorylated when cells progress from G_1 into S phase of the cell cycle, E2F activity can be found associated with p107 in S-phase cells and asynchronously growing cells. We therefore asked whether hypophosphorylated p107, capable of forming a complex with E2F, is present in asynchronously growing cells. Both quiescent and asynchronously growing 3T3 cells were analyzed for the presence of the different p107 species. As can be seen in Figure 12, the level of p107 is much lower in G_0 cells and only consists of the hypophosphorylated form. In asynchronously growing cells, much higher levels of p107 were detected, and these cells contain both hypo- and hyperphosphorylated forms of p107. These results indicate that although p107 is phosphorylated in the G_1 phase of the cell cycle after serum stimulation, a detectable amount of p107 remains underphosphorylated in the later stages of the cell cycle. This hypophosphorylated p107 is able to associate with E2Fs, as shown in Figure 9, and is likely to be responsible for the p107-associated E2F activity detected in Sphase and in asynchronous cells.

Cyclin D1/cdk4 can rescue a p107-induced cell cycle block

E2F transcription factors play an important role in the regulation of the G_1 - to S-phase transition. When transiently transfected, both pRb and p107 have the ability to arrest SAOS-2 cells in the G_1 phase of the cell cycle (Zhu et al. 1993). The pRb-induced cell cycle-block can be rescued by overexpression of cyclin A, cyclin E, and cyclin D2 or D3, resulting in hyperphosphorylation of pRb (Hinds et al. 1992; Ewen et al. 1993). In contrast, a p107-induced cell cycle block cannot be rescued effectively by either cyclin A or cyclin E expression (Zhu et al. 1993).

The data described above are in agreement with this, in that they show that p107 cannot be inactivated by ectopic expression of cyclin E. Because p107 inhibition of E2F-4 can be released by cyclin D1/cdk4, we asked whether expression of cyclin D1/cdk4 was able to release SAOS-2 cells from a p107-induced cell cycle block. SAOS-2 cells were transfected with the cell-surface marker CD20 alone, with CD20 and p107 or with CD20, p107, and cyclin/cdk expression vectors. Three days after transfection, cells were selected for CD20 expression by FACS analysis, and the cell cycle distribution of transfected ($CD20^+$) cells was determined by propidium iodide staining.

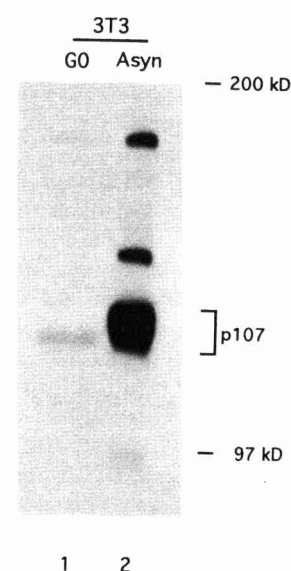


Figure 12. Hypo- and hyperphosphorylated p107 species are present in asynchronous 3T3 cells. Total protein (80 μ g) from quiescent or asynchronously growing NIH-3T3 cells was subjected to electrophoresis through a 7.5% SDS–polyacrylamide gel. p107 protein was detected by Western analysis.

Table 1 shows that expression of p107 in SAOS-2 cells resulted in a significant accumulation of CD20-positive cells in G₁. Expression of cyclin D1/cdk4 completely released the p107-induced cell cycle block, decreasing the G₁ population and increasing both S- and G₂/M-phase cells. Consistent with the results of Zhu et al. (1993), we observed only a partial release of the p107 cell cycle block by cyclin E/cdk2 (Table 1). Additional controls with cyclin D3 alone or in combination with cdk2 or cdk4 showed that as expected, only the cyclin D3/cdk4 complex was able to release the p107 cell cycle block. Taken together, our data indicate that p107 growth inhibitory activity in the cell cycle is regulated by D-type cyclins in association with cdk4 but not effectively by cyclin E and its associated kinase.

Discussion

In this paper we present several lines of evidence to indicate that p107 is negatively regulated as a result of phosphorylation. The p107 protein is found to be phosphorylated differently, and this phosphorylation changes dramatically during the G₁ to S phase of the cell cycle in 3T3 fibroblasts (Fig. 11).

p107 is a pocket protein that can form a complex with the cellular transcription factor E2F-4 *in vivo*. E2F-4 plays a role in the regulation of cell cycle progression (Beijersbergen et al. 1994b; Ginsberg et al. 1994). The activity of the transcription factor E2F-4 can be suppressed by the expression of p107. Overexpression of p107 results in G₁ cell cycle arrest in certain cell types (Zhu et al. 1993). We show here that both the ability of p107 to interact with E2F-4 and the ability to induce a G₁ cell cycle arrest are both abolished upon phosphorylation

of p107. Furthermore, our experiments indicate that p107 is a target for cyclin D/cdk4 complexes.

In a first set of experiments, we studied the effects of cyclin/cdk complexes on the interaction between p107 and its E2F partner, E2F-4 (Beijersbergen et al. 1994b, Ginsberg et al. 1994). *Trans*-activation by E2F-4 can be suppressed by expression of p107. Importantly, p107 suppression of E2F-4 could be released by coexpression of both cyclin D1 and cdk4 but not by either protein alone. This activity of cyclin D1/cdk4 was abolished completely when a dominant-negative form of cdk4 was used or by coexpression of p27^{kip1}, a strong inhibitor of the cyclin D1/cdk4 kinase complex. Both these results indicate that cyclin D1-associated kinase activity is required to release p107 inhibition of E2F-4. Expression of cyclin D1/cdk4 caused hyperphosphorylation of p107 and resulted in a loss of association with E2F-4. In correlation with a role for cyclin D1 in phosphorylating p107 is the finding that p107 can be phosphorylated *in vitro* by cyclin D1/cdk4. Li et al. (1993) showed that p107 is found in complex with D-type cyclins *in vivo*, and *in vitro* kinase reactions with these complexes show that p107 can be phosphorylated by D-type cyclin associated kinase activity. The binding of p107 and cyclin D, by analogy to cyclin D and pRb, suggests an alternative mechanism of D-type cyclin action in that D-type cyclins might interfere with both p107 and pRb function through direct binding to the pocket (Dowdy et al. 1993; Ewen et al. 1993; Hinds et al. 1992), thereby functionally inactivating p107 or pRb in the absence of phosphorylation. As discussed above, our data support a model in which p107 is inactivated through phosphorylation by the cyclin D/cdk4 complex and do not support the hypothesis that cyclin D1 acts by competing with E2F for p107 pocket binding.

Further evidence that phosphorylation of p107 is mediated by cyclin D complexes was obtained when we serum-stimulated quiescent fibroblasts: Phosphorylation of p107 occurs shortly after cyclin D1 is induced but well before cyclin E induction (Fig. 11). Also, overexpression of a dominant-negative form of cdk4 resulted in the disappearance of the hyperphosphorylated forms of p107 in U2-OS cells, strongly suggesting that D-type cyclin-associated kinase activity is responsible for this modification. Together, these data provide strong evidence that p107 is an *in vivo* substrate for cyclin D/cdk4. In agreement with the hypothesis that p107 is a target for cyclin D-associated kinase activity is the finding that p107 phosphorylation as observed in U2-OS cells is absent in both SAOS-2 and C33A cells (Fig. 6; data not shown). The absence of p107 phosphorylation in C33A and SAOS-2 cells, which have only very little cyclin D-associated kinase activity (Bates et al. 1994; Tam et al. 1994), shows a correlation between the presence of cyclin D-associated kinase activity and the phosphorylation status of p107 (R.L. Beijersbergen and R. Bernards, unpubl.).

Recent data show that cyclin D1 can cooperate with a mutant form of adenovirus E1A and an activated *ras* oncogene in the transformation of primary fibroblasts. Importantly, the GH mutant of cyclin D1 that had retained

Table 1. Rescue of p107-induced cell cycle arrest in SAOS2 cells by G₁ cyclins

Rescue	Vector			p107		
	G ₁	S	G ₂ /M	G ₁	S	G ₂ /M
	47	12	41	73	6	21
	42	15	43	67	9	24
	44	14	42	61	10	29
Cyclins						
+pCMV cyclin D1/cdk4	35	22	42	38	24	38
				37	16	47
+pCMV cyclin E/cdk2	39	22	39	58	19	23
				54	15	31
+pCMV cyclin D3				54	14	31
+pCMV cyclin D3/cdk4				34	18	48
+pCMV cyclin D3/cdk2				56	13	31

SAOS-2 cells were transfected with pCMV CD20 (2 µg) expression vector in combination with expression vectors pCMV or pCMVp107 (5 µg), together with different cyclins and cdks (10 µg each) as indicated. Transfected cells were analyzed by FACS, and the cell cycle profile of CD20-positive cells was determined. The percentage of cells in G₀/G₁, S, and G₂/M phases of the cell cycle are depicted.

the ability to transform in this assay was also able to release p107 inhibition of E2F-4 (Fig. 5; Hinds et al. 1994). Conversely, the KE mutant of cyclin D1, which is unable to form a stable complex with cdk4, did not transform and did not release p107 inhibition of E2F-4; thus, the ability of cyclin D1 mutants to transform segregates with the ability to inactivate p107. This suggests that cyclin D1 derives its oncogenic activity, at least in part, by releasing p107-associated proteins from their inactive, p107-bound state. We and others have shown recently that p107 interacts with at least two cellular proteins, E2F-4 and c-Myc, both of which are endowed with oncogenic activity (Beijersbergen et al. 1994a,b; Ginsberg et al. 1994; Gu et al. 1994).

To our surprise, the cyclin E/cdk2 complex was unable to affect the complex between E2F-4 and p107. This result was unexpected because p107 forms a stable complex with cyclin E *in vivo*. In contrast to pRb, p107 contains within its pocket region a spacer that allows the formation of higher order complexes with cyclins and cdk. These complexes contain, apart from p107 and E2F (which interacts with the pocket region), cyclin A or cyclin E, together with their associated kinase cdk2. In spite of this, cyclin E/cdk2-mediated phosphorylation of p107 does not appear to occur in the E2F-containing complex *in vivo*, at least not with the result that p107 is released from E2F. The role of the cyclin E/cdk2 complex in the p107/E2F complex therefore remains to be elucidated. It is possible that cyclin E/cdk2 is present in the p107/E2F complex to phosphorylate the E2F and/or the DP-1 component. Alternatively, cyclin E could be bound and inactivated by p107. In this scenario, p107 would act as a molecular sink to bind and inactivate cyclin E. Active cyclin E/cdk2 could then only occur above a certain threshold level of cyclin E. A similar threshold-setting role has been proposed recently for cdk inhibitory molecules (Peters 1994), and we have recently obtained evidence to support a role for p107 in down-modulating cyclin A and E activity (Zhu et al. 1995).

In S phase, p107 is found in complex with E2F and cyclin A (Lees et al. 1992). Cyclin A can also interact directly with E2F-1, resulting in the phosphorylation of at least one member of the E2F/DP complex (Krek et al. 1994; Dynlacht et al. 1994). This phosphorylation results in the down-modulation of E2F-1 activity, presumably through loss of DNA binding of the E2F-1/DP-1 complex. We did not find a release of p107 inhibition by cyclin A/cdk2 overexpression (data not shown). This indicates that like cyclin E/cdk2, cyclin A/cdk2 is also unable to inactivate p107 by phosphorylation.

Although hypophosphorylated p107 is present in G₀, E2F complexes containing p107 are not readily detected. Instead, E2F is found in complex with p130. Only after disappearance of the E2F-p130 complex (Fig. 11, lane 5), E2F/p107 complexes can be detected, albeit in very low amounts. This could indicate that p130 can effectively compete with hypophosphorylated p107, which is present at this stage of the cell cycle, for binding to E2F-4. That p130 can bind E2F-4 was shown recently by us and others (Vairo et al. 1995; R.L. Beijersbergen and R.

Bernards, in prep.). The observation that p107 is phosphorylated at 8 hr after serum stimulation would suggest that at this moment newly synthesized E2F-4 would not be inactivated through the association with p107. At the same moment E2F/p130 complexes disappear. This would suggest a scenario in which E2Fs that can be inactivated by p130 and p107 would be free to activate transcription. On the other hand, an increase in p107-containing E2F complexes is observed at the G₁/S transition and throughout S phase of the cell cycle, in our experiments at 18 hr postserum stimulation (Fig. 11). The result of this regulation is that the p130 and p107-associated E2Fs are only free to activate transcription during a very small time frame from 8 to 18 hr following serum stimulation, before and after which the higher order complexes are present. The formation of these higher order complexes may play an important role in the active repression of genes when cells progress to the later stages of the cell cycle.

A possible explanation for the reoccurrence of E2F/p107 complexes at S phase is that there is a strong increase in p107 protein expression. The majority of this newly synthesized protein is phosphorylated, but as a result of a decrease in cyclin D-associated kinase activity, a significant amount of p107 remains hypophosphorylated in S phase. This newly synthesized hypophosphorylated p107 can form a complex with E2F-4.

Overexpression of p107 in SAOS-2 cells results in a growth arrest in the G₁ phase of the cell cycle (Zhu et al. 1993). This p107-induced growth arrest could be rescued by cotransfection of cyclin D1/cdk4 or cyclin D3/cdk4 (Table 1). Thus, phosphorylation of p107 by cyclin D1/cdk4 not only leads to the release of E2F-4 but also inactivates almost completely the growth-suppressive activity of p107. In contrast, cyclin E/cdk2 only resulted in a partial release of the p107 cell cycle block. Thus, the different ability of cyclins D and E to release E2F-4 from p107 is correlated with their ability to inactivate the growth inhibitory activity of p107. Cellular targets of p107 may therefore play an important role in the progression of G₁ to S phase. Consistent with this, both E2F-4 and c-Myc can stimulate S-phase entry (Eilers et al. 1991; Beijersbergen et al. 1994b).

Our data reveal significant differences in substrate specificity between the two most prominent G₁ cyclin/cdk complexes: p107 can be inactivated by D-type cyclins and not by cyclin E, whereas pRb that can be inactivated by cyclin D2, D3, and E. Because cyclin D1 is induced prior to cyclin E in the cell cycle, our data also suggest that the p107-associated E2Fs are activated at an early point in G₁, when cyclin D-associated kinase activity is induced. That p107 E2Fs can function early in G₁ is also supported by the recent findings that, in contrast to E2F-1, E2F-4 mRNA is already present in quiescent cells (Ginsberg et al. 1994; Johnson et al. 1994). Furthermore, E2F-4 is transcriptionally induced prior to E2F-1 when cells progress through G₁ into S (R.L. Beijersbergen and R. Bernards unpubl.).

Even though cyclin E/cdk2 and cyclin D1/cdk4 complexes share many functional properties (Ewen et al.

1993; Resnitzky et al. 1994), a striking difference is that cyclin D1 is frequently involved in the genesis of human cancer, whereas little evidence for deregulated cyclin E expression in cancer exists to date. Our present data show that cyclin D1, when in complex with cdk4, can functionally inactivate p107, whereas cyclin E/cdk2 was inactive toward p107. It is possible that the higher oncogenic activity of cyclin D1 stems from its ability to inactivate the growth inhibitory activity of both pRb and p107, whereas overexpression of cyclin E would leave the p107-mediated growth inhibitory circuit intact.

Materials and methods

Cell lines

Human C33A, U2-OS, and SAOS-2 were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum. NIH-3T3 cells were cultured in DMEM with 10% newborn calf serum. Transfections were performed using the calcium phosphate precipitation technique (Graham and Van der Eb 1973).

Synchronization of cells

NIH-3T3 cells were allowed to grow 24 hr and were then incubated with DMEM containing 0.2% serum for 40 hr. Restimulation of quiescent cells was performed by incubation with DMEM containing 10% serum for the indicated times.

Plasmids

pCMVp27 was generated by the polymerase chain reaction (PCR) using p27-specific primers on DNA isolated from a BALB/c brain cDNA library. DNA sequencing showed that the amplified p27 cDNA was identical to the published sequence (Toyoshima and Hunter 1994). pCMVHA E2F-4 was generated by cloning the hemagglutinin epitope tag at the amino terminus of E2F-4 in pCMV. The plasmids pCMVpRb, pCMVp107, pCMVp107DE, pCMVCD20, pCMV-E2F-4, pCMV-E2F-1, pCMV-DP-1, pCMV cyclin E, cyclin A, cyclin D1, cyclin D2, cdk2, cdk4, cdk4 dominant-negative, cyclin D1-GH, and cyclin D1-KE have all been described elsewhere (Hinds et al. 1992, 1994; van den Heuvel and Harlow 1993; Zhu et al. 1993; Beijsbergen et al. 1994b).

Immunological reagents

For the experiments described here we used mAb RK13 directed against E2F-4. Antibodies against p107 (SD2, SD4, SD9, SD6, and SD15) and against the HA epitope (12CA5) have been described previously (Field et al. 1988; Zhu et al. 1993). The antibody used for the detection of endogenous p107 in Western, C-18, and cyclin E, C-19, were obtained from Santa Cruz. Cyclin D1 was detected by DSC-6 antibody from Sanbio.

CAT assays

C33A cells were transiently transfected with the expression vectors as indicated together with 2 μ g E2F₄CAT (Helin et al. 1993), 0.2 μ g RSV luciferase. pRc/CMV was added to a total of 20 μ g of DNA per 10-cm plate. Cells were harvested 40 hr after transfection. Cells were collected and resuspended in 100 μ l of 0.1 M Tris-HCl (pH 8.0). Cells were freeze/thawed three times and centrifuged at 15,000g at 4°C for 10 min. Supernatants were assayed for luciferase activity (Promega, luciferase system) and

CAT activity using the phase extraction assay (Seed and Sheen 1988). In all experiments, CAT activity was normalized to luciferase activity.

Phosphatase treatment

Immunoprecipitates were washed twice in phosphatase buffer (50 mM Tris-HCl at pH 7.8 and 5 mM DTT) and resuspended in 60 μ l of phosphatase buffer. Lambda phosphatase (NEB, 400 units) was added, where indicated, and incubated for 60 min at 30°C. Phosphatase inhibitors NaF (5 mM) and Na₃VO₄ (5 mM) were added, where indicated.

Metabolic labeling and immunoprecipitations

Cells were incubated for 1 hr in methionine-free DMEM in the presence of 10% fetal calf serum, followed by incubation in methionine-free DMEM supplemented with 250 μ Ci of Tran-³⁵S-label for 4 hr. After labeling, cells were lysed in ELB⁺ buffer [ELB buffer containing 5 mM EDTA, 1 mM DTT, 10 mM NaF, 10 mM sodium orthovanadate, 0.2 mM sodium pyrophosphate, 1 μ g/ml of chymostatin and aprotinin, and 1 mM phenylmethylsulfonylfluoride (PMSF)] and immunoprecipitated as described previously (Beijsbergen et al. 1994a).

Western blot analysis

Cells were lysed in SDS-containing sample buffer and sonicated for 10 sec. The cell extracts were then separated on SDS-polyacrylamide gels and transferred to nitrocellulose. The membrane was blocked in TBST (15 mM NaCl, 10 mM Tris-HCl at pH 8.0, 0.05% Tween 20) containing 5% dried milk for 1 hr at room temperature and C18 antibody diluted 1:2000 in TBST containing 1% dried milk for 16 hr at 4°C. The membrane was then washed, and the antibody was detected using horseradish peroxidase-linked goat anti-rabbit IgG and enhanced chemiluminescence (Amersham). HA-tagged p107 was detected with mAb 12CA5 at 1:1000 dilution, cyclin D1 with DSC-6 in 1:250 dilution, and cyclin E with C-19 in 1:500 dilution in TBST containing 1% dried milk.

Mobility shift assays

For the preparation of whole cell extracts of quiescent or serum-stimulated 3T3 cells, 10-cm tissue culture dishes were washed with cold PBS, and cells were collected and resuspended in 100 μ l of lysis buffer (20 mM HEPES at pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10 mM DTT, 1 mM PMSF 25% glycerol). The cells were kept on ice for 20 min and lysed by two freeze/thaw cycles (−80°C and 0°C). After centrifugation for 10 min at 15,000 rpm and 4°C, the supernatant was stored at −80°C. Gel shifts were performed using 10 μ g of cell extract in a 20- μ l reaction volume containing 10 mM HEPES (pH 7.9), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 4% Ficoll, and 1 μ g of sonicated and boiled salmon sperm DNA. Where indicated, SD9 antibody (Santa Cruz) was added to the reaction mixture. Reactions were incubated for 10 min at room temperature after which 0.5 ng of ³²P-labeled oligonucleotide containing the consensus E2F DNA-binding site (Santa Cruz) was added and the reaction was incubated for an additional 20 min at room temperature. The reaction products were separated on a 3.5% polyacrylamide gel in 0.25× TBE at 100 V for 3 hr at room temperature. The gel was then dried and subjected to autoradiography.

SAOS-2 cell cycle block rescue

SAOS-2 cells were transfected using calcium phosphate precipitates containing 30 µg of plasmid DNA for each 15-cm dish. After 16 hr the cells were washed twice with DMEM/10% FCS and incubated with fresh medium at 37°C. Forty-eight hours later the cells were collected in PBS containing 3 mM EDTA, spun down, and incubated with 20 µl of FITC-conjugated anti-CD20 monoclonal antibody (Becton-Dickinson) for 20 min on ice. The cells were washed and fixed in 80% ethanol at 4°C. Before FACS analysis the cells were washed with PBS supplemented with 0.1% serum and incubated with 20 µg/ml of propidium iodide and 200 µg/ml of RNase A. The FACS analysis was performed on a Becton-Dickinson FACScan apparatus. A gate was set to select CD20-positive cells with a fluorescence intensity of >20-fold the control population. The propidium iodide signal was used to determine the cell cycle distribution of the selected cells.

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