Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4633–4637, May 1996
Cell Biology

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected on April 25, 1995.

Regulation of the retinoblastoma protein-related protein p107 by G_1 cyclin-associated kinases

ZHI-XIONG XIAO, DORON GINSBERG, MARK EWEN, AND DAVID M. LIVINGSTON*

Dana–Farber Cancer Institute and Harvard Medical School, 44 Binney Street, Boston, MA 02115

Contributed by David M. Livingston, February 15, 1996

ABSTRACT **p107 is a retinoblastoma protein-related phosphoprotein that, when overproduced, displays a growth inhibitory function. It interacts with and modulates the activity of the transcription factor, E2F-4. In addition, p107 physically associates with cyclin E–CDK2 and cyclin A–CDK2 complexes in late G_1 and at G_1/S, respectively, an indication that cyclin-dependent kinase complexes may regulate, contribute to, and/or benefit from p107 function during the cell cycle. Our results show that p107 phosphorylation begins in mid G_1 and proceeds through late G_1 and S and that cyclin D-associated kinase(s) contributes to this process. In addition, E2F-4 binds selectively to hypophosphorylated p107, and G_1 cyclin-dependent p107 phosphorylation leads to the dissociation of p107–E2F-4 complexes as well as inactivation of p107 G_1 blocking function.**

The retinoblastoma protein (pRB)-related phosphoprotein, p107, is targeted by certain viral oncoproteins, including adenovirus E1A, simian virus 40 large T antigen (T), and human papillomavirus E7 (for review, see ref. 1). p107 shares extensive structural homology in its E1A/T binding domain (the pocket) with other pocket proteins, pRB and p130 (2–5). The pocket domain of all three proteins interacts with a specific set of cellular proteins, and, where studied, these interactions contribute to the regulation of cell growth (1, 2, 6).

E2F is a family of transcription factors, the activity of which contributes to the process of G_1 exit in mammalian cells (7). Among the five known E2F members, pRB binds to and inhibits the transactivation function of E2F-1, E2F-2, E2F-3 (8), and possibly E2F-4 (9). p107 selectively binds to and suppresses the transcription activation functions of E2F-4 *in vivo* (9, 10). p107–E2F complex formation is cell cycle-dependent, as are its interactions with the cyclin E–CDK2 and cyclin A–CDK2 complexes. p107–E2F–cyclin E–CDK2 complexes appear in late G_1; p107–E2F–cyclin A–CDK2 complexes arise at G_1/S, disappearing thereafter (1). The p107 pocket spacer element specifically interacts with cyclins A and E kinases (1), and this interaction appeared to be important for the expression of a p107 growth suppression activity in cells that overproduce the protein (11, 12). Despite this information, it is unclear how p107–E2F complexes normally operate in mammalian cells.

pRB is phosphorylated, at least in part, by cyclin-dependent kinases (CDKs) (for review, see ref. 13). During mid G_1, cyclin D-associated kinases initiate the process, leading to the dissociation of pRB–E2F complexes and to the inactivation of the pRB G_1 exit blocking function (13). In keeping with this view, cyclin D plays an essential role in G_1 exit only when cells synthesize intact pRB (14, 15).

Phosphorylated p107 contains both phosphoserine and phosphothreonine residues (16). Its pocket and C-terminal segment, like the analogous segments of pRB, can bind cyclin D *in vitro* (17). Given that pRB phosphorylation is initiated by cyclin D/kinase (13) and that pRB phosphorylation has profound effects on its function, one wonders whether cyclin D/kinase acts similarly on p107. In this report, we show that p107 is phosphorylated in a cell cycle-dependent manner. The data further suggest that cyclin D/kinase contributes to these phosphorylation events, which in turn lead to dissociation of p107–E2F4 complexes and the inactivation of the p107 growth suppression function. While this manuscript was in preparation, similar results were reported by another laboratory (18).

MATERIALS AND METHODS

Cell Cultures and Antibodies. U-2 OS, Saos-2, and C33A cells were grown in DMEM with 10% Fetal Clone I (HyClone). The mixture of p107 monoclonal antibodies, SD4, SD9, and SD15 (19), was routinely used in anti-p107 immunoprecipitation experiments. Monoclonal antibodies specific for E2F-4 (GG22) (9), cyclin E (HEIII) (20), and E1A (M73) (21) have been described. 12CA5 is a monoclonal antibody specific for an influenza hemagglutinin (HA) epitope (Babco, Richmond, CA). Rabbit polyclonal antiserum to human cyclin D2 was kindly provided by J. Griffin (Dana–Farber Cancer Institute, Boston).

Metabolic Labeling, Immunoprecipitation, Alkaline Phosphatase Treatment, and Western Blot Analysis. Metabolic labeling with [^{35}S]methionine or with [^{32}P]orthophosphate was as described (22). Labeled cells were lysed in EBC buffer (50 mM Tris·HCl, pH 8.0/120 mM NaCl/0.5% Nonidet P-40/50 mM NaF/0.2 mM sodium orthovanadate) containing protease inhibitors (2 μg/ml aprotinin/2 μg/ml leupeptin/100 μg/ml phenylmethylsulfonyl fluoride) and immunoprecipitation was performed as described (23). Phosphatase treatment experiments using alkaline phosphatase (Boehringer Mannheim) were as described (22). Western Blot analyses, using an affinity-purified, rabbit polyclonal antiserum to p107 or with 12CA5 (hybridoma supernatant, diluted 1:50 in TBST containing 1% BSA), were performed as described (22, 23).

Cell Synchronization. Exponentially growing U-2 OS cells were exposed to lovastatin (provided by A. W. Alberts, Merck Sharp and Dohme) at a final concentration of 40 μM for 38 hr or to aphidicolin (5 μg/ml, Sigma) for 16 hr. U-2 OS cells that were blocked in G_1 by lovastatin were released by culturing in DMEM containing 4 mM mevalonic acid (100-fold excess of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: pRB, retinoblastoma protein; CDK, cyclin-dependent kinase; HA, hemagglutinin.

*To whom reprint requests should be addressed.

lovastatin) and 10% fetal calf serum. At each time point after release, cells were labeled for 1 hr with either [^{35}S]methionine or [^{32}P]orthophosphate and lysed as described. Cells were collected by trypsinization at each time point and were subjected to flow cytometric analysis (23).

Transfection and Plasmids. In experiments addressing the role of G_1 cyclins in p107 phosphorylation, C33A cells were transfected by the calcium phosphate procedure (23) with a DNA mixture containing 2 μg of pCMV–HA–p107 (2), 5 μg of pcDNA1–HA–E2F4 (9), and 2 μg of one or more of the indicated expression plasmids: pSG5–cyclin D1, pSG5–cyclin D2, pSG5–cyclin D3 (17), pRC/CMV–cyclin E, pRC/CMV–cyclin A, pCR/CMV–cyclin B1, pCR/CMV–cyclin B2 (24), pRC/CMV–CDK2 and pRC/CMV–CDK4 (25). The total amount of DNA was brought to 25 μg per 100 mm plate by supplementing the mixture with pBSSK-II plasmid DNA (Stratagene). Cells were collected 42 hr after transfection and the cell lysates were immunoprecipitated. Western blot analyses were performed as described.

To analyze the effect of G_1 cyclins on the p107 suppression of E2F-4 transactivation activity, U-2 OS cells were transfected with 2 μg of an E2F reporter plasmid, 3xWT–E2F–Luc (26), alone (mock), or cotransfected with the reporter plasmid and one or more of the indicated plasmids: 0.5 μg of pCMV–E2F4 (9), 0.2 μg of pcDNA1–DP-1 (26), 1 μg of pCMV–p107 (2), 1 μg of pRC/CMV–CDK2 or pRC/CMV–CDK4 (25), and, where indicated, increasing amounts (0.5 μg, 1 μg, and 2.5 μg, respectively) of pSG5–cyclin D1, pSG5–cyclin D2 (17), pRC/CMV–cyclin E, or pRC/CMV–cyclin A (24). Each of the DNA mixtures contained the same amount of pCMV- and pSG5-based vectors by supplementing the final mixture with an appropriate amount of pCMV and pSG5 backbone plasmids. pCMV-β-GAL (1 μg) was used as an internal control in all transfection experiments. The total amount of DNA in the transfection mixture was brought up to 25 μg per 100-mm plate by supplementing with pBSSK-II plasmid DNA (Stratagene). Cells were harvested 42–48 hr after transfection. The luciferase activity was measured, which was then normalized to both an internal control [β-galactosidase (β-GAL) activity] and to the luciferase activity in the mock transfected control, and was presented as fold activation as described (23). The synthesis of transfected cyclins and CDKs was confirmed by immunoprecipitation of [^{35}S]methionine labeled cell proteins.

p107 Growth Suppression Assays. Saos-2 cells (Rb^-/Rb^-) were cotransfected with a DNA mixture containing 8 μg of pCMV–p107, 2 μg of pCMV–CD19, which encodes the CD19 surface marker, and, where indicated, 8 μg of an expression plasmid encoding cyclin D1, cyclin E, cyclin A, CDK4, CDK2, or E2F-4 and DP-1. Each transfection mixture contained an equivalent amount of pCMV and of pSG5-based vector plasmid by appropriately supplementing with pCMV and pSG5 vector DNA, respectively. Cell cycle distribution of $CD19^+$ cells were determined by FACS analysis as described (23).

RESULTS

Phosphorylation of p107 Results in Its Slower Migration in SDS/Polyacrylamide Gels. p107 isolated from asynchronous cultures of U-2 OS cells segregated into faster and slower migrating forms, when electrophoresed in SDS/polyacrylamide gels (Fig. 1 and Fig. 2A). The slower species was overtly phosphorylated as first indicated by its direct ^{32}P-labeling (Fig. 1, lane 5). Moreover, after alkaline phosphatase treatment, it was converted to the faster migrating, ^{35}S-labeled form except in the presence of phosphatase inhibitors. Hence, the observed heterogeneity in p107 mobility is due to differential phosphorylation (Fig. 1, compare lanes 2 and 3). ^{32}P was present in the faster migrating form but at a much lower level than in the slower form (Fig. 1, compare lanes 5 and 6).

Cell Cycle-Dependent Phosphorylation of p107. Given the known, cell cycle dependent phosphorylation of pRB and the structural similarity between pRB and p107, we asked whether p107 phosphorylation is also cell cycle-dependent. U-2 OS cells were synchronized by incubation in lovastatin and aphidicolin. Lovastatin inhibits hydroxymethylglutaryl–CoA reductase and arrests cells in G_1. The block can be reversed by exposure to mevalonic acid (27). Aphidicolin inhibits DNA polymerase α and causes a G_1/S boundary block (28). When U-2 OS cells were arrested with lovastatin, p107 was found to be largely, if not exclusively, hypophosphorylated (Fig. 2*A*). In contrast, p107 was overtly phosphorylated in aphidicolin-treated cells (Fig. 2*A*). Hence, assuming that the drugs do not alter the process significantly, efficient p107 hyperphosphorylation seems to occur during G_1 or at the G_1/S boundary.

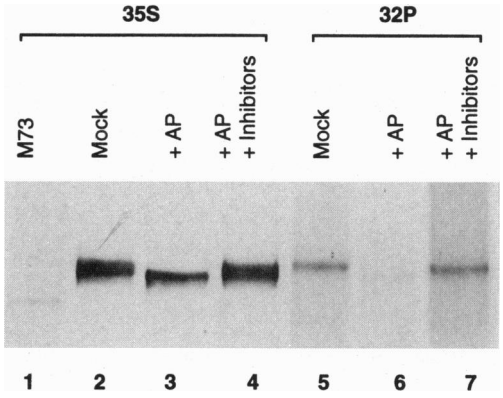

FIG. 1. Phosphorylation of p107 results in its slower migration during SDS/PAGE. U-2 OS cells were labeled with [^{35}S]methionine (lanes 1–4) or [^{32}P]orthophosphate (lanes 5–7). Aliquots of cell extract were immunoprecipitated with p107 monoclonal antibodies or with a control antibody, M73. The immunoprecipitated proteins bound on protein A-Sepharose beads were treated with reaction buffer (Mock), with alkaline phosphatase (AP), or with alkaline phosphatase in the presence of phosphatase inhibitors (AP plus inhibitors). Proteins were then released from the beads, electrophoresed in a SDS/7.5% gel, and visualized by fluorography.

We then asked when p107 phosphorylation occurs in synchronized cells. U-2 OS cells were lovastatin-arrested and then released by incubation in growth medium containing mevalonic acid. As shown in Fig. 2*B*, synthesis of p107 (^{35}S-labeling for 1 hr) and the steady-state level of the protein (Western blotting analysis) were low in early G_1 (data not shown), in keeping with similar observations of others (29). Furthermore, the p107 detected was primarily hypophosphorylated at this time in the cycle. pRB, in contrast, was readily detected and was also hypophosphorylated under these conditions (Fig. 2*B*). At 10 hr, when the vast majority of cells were still in G_1, the p107 level began to rise, and overt phosphorylation of both p107 and pRB was noted (both the mobility shift of and increased ^{32}P uptake by p107 are shown in Fig. 2*B*). These events correlated with the onset of cyclin D synthesis and preceded the first appearance of cyclin E. By 12 hr, most of the detectable p107 and pRB were hyperphosphorylated, and, again, the vast majority of the culture was in G_1. Thus, phosphorylation of p107 and pRB both occurred during mid to late G_1, in keeping with the results developed in lovastatin/aphidicolin treated cells (compare with Fig. 2*A*). Similar results were obtained in serum starved and refed NIH 3T3 cells (data not shown). Hence, cyclical, G_1-dependent p107 phosphorylation is not unique to a given cell line.

Effect of Cyclin D-Associated Kinases on p107 Phosphorylation and E2F Binding. p107 forms stable complexes with E2F-4 *in vivo* (9, 10). Because p107 is differentially phosphorylated, we asked whether the state of p107 phosphorylation affects its binding to this factor. U-2 OS cells were cotransfected with expression plasmids encoding influenza HA-tagged p107 (pCMV–HA–p107) or E2F-4 (pCMV–E2F–4).

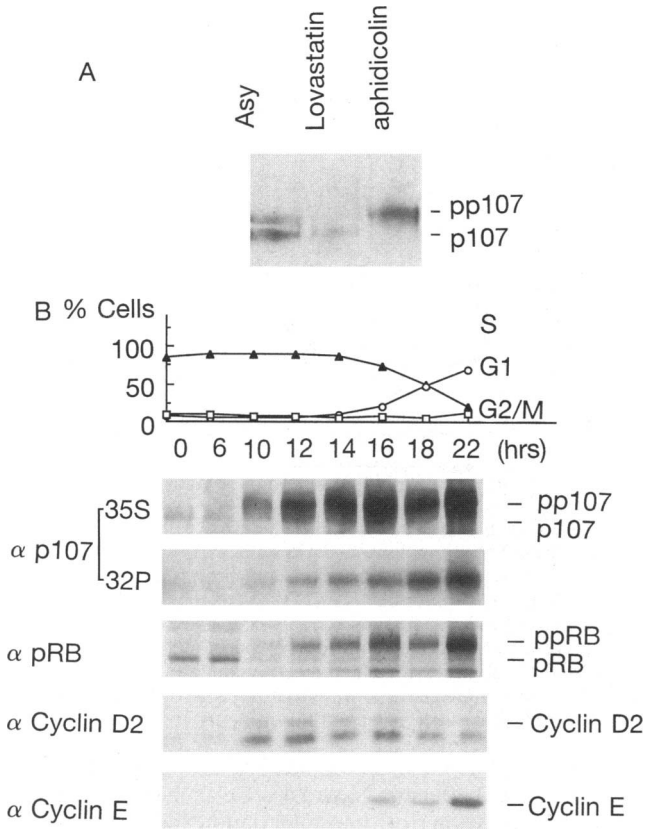

FIG. 2. Cell cycle-dependent phosphorylation of p107. (*A*) p107 is hypophosphorylated in G_1 and hyperphosphorylated at G_1/S. Lysates (1 μg of crude extract protein) of U-2 OS cells, treated with either lovastatin or aphidicolin, were immunoprecipitated (IP) with p107 monoclonal antibodies. The immunoprecipitated proteins were separated in a SDS/6.5% gel and analyzed by Western blotting using an affinity-purified rabbit p107-specific antiserum as probe. (*B*) p107 phosphorylation is synchronous with the phosphorylation of pRB and the appearance of cyclin D. U-2 OS cells were arrested in G_1 with lovastatin and released. ^{35}S-labeled cells or ^{32}P-labeled cells at each time point were immunoprecipitated with antibodies specific for p107, pRB, cyclin D2, or cyclin E, respectively. Immunoprecipitated proteins from this experiment were separated in a 6.5% (p107–IP), a 7.5% (pRB–IP), or a 10% gel (cyclin D–IP and cyclin E–IP) and visualized by autoradiography. The fraction of cells in different cell cycle stages at each time point was determined by flow cytometric analysis.

Using a monoclonal antibody specific for E2F-4 (9) or a mixture of three monoclonal antibodies specific for p107 (19) to immunoprecipitate the protein of interest, the resulting immunoprecipitates were analyzed by Western blotting using, as probe, a monoclonal antibody (12CA5) specific for the HA tag. As shown in Fig. 3*A*, E2F-4 specifically bound hypophosphorylated p107. In contrast, p107 bound the slower migrating (i.e., the hyperphosphorylated) E2F-4 species (the "mock" lane under a p107 immunoprecipitate in Fig. 3*C*, *Left*) (6). Therefore, the un(der)phosphorylated representatives of two related pocket proteins, pRB (1) and p107, bind to their cognate E2F targets.

Because phosphorylation of p107 occurs during G_1, apparently in synchrony with the synthesis of at least one cyclin D, the question of whether the cyclin D–CDK complex(es) was active in this process arose. Therefore, we investigated the effects of overproducing selected CDKs on p107 phosphorylation. Transient transfection of HA-tagged p107 into C33A cells largely resulted in the appearance of the hypophosphorylated form (Fig. 3*B*, Mock lane). In contrast, when C33A cells were cotransfected with p107, cyclin D1, cyclin D2, or cyclin D3 and CDK4, the majority of p107 became hyperphosphorylated, as defined by gel-mobility assay. Cotransfection of cyclin E–CDK2 led to an intermediate gel-shift effect, whereas cyclin A–CDK2 had a yet subtler effect. In contrast, cyclins B1–CDC2 or B2–CDC2 were inactive in this regard (Fig. 3*B*). The same cyclin–CDK cotransfection effects were also observed in Saos-2 and U-2 OS cells (data not shown).

In addition, cotransfection of HA–p107 with p16 or p18, each a specific cyclin D–CDK inhibitor, or p27, another cyclin D–CDK inhibitor (for review, see ref. 30), in U-2 OS cells totally blocked the phosphorylation of HA-p107 (Fig. 3*D*). Cotransfection of a dominant negative CDK4 mutant (CDK4-DN) (25) also blocked the p107 phosphorylation process, presumably by inhibiting endogenous D–CDK activities (data not shown). Taken together, we conclude that the cyclin D-associated kinases can contribute to p107 phosphorylation *in vivo*.

We then asked whether p107 phosphorylation by G_1 cyclins affects the stability of p107–E2F-4 complexes. U-2 OS cells were cotransfected with p107 and HA–E2F-4 expression plasmids. p107–E2F-4 complexes were sought by p107 immunoprecipitation followed by Western blotting, using 12CA5 as probe to detect HA–E2F-4 (Fig. 3*C*, *Left*). In parallel, HA–E2F-4 was directly immunoprecipitated with 12CA5 and Western blotted with anti-HA antibody (Fig. 3*C*, *Right*). While p107 bound a significant amount of the available HA–E2F-4 (Fig. 3*C*, *Left*, compare two "mock" lanes), cotransfection of cyclin D2–CDK4 significantly diminished the abundance of these complexes. In contrast, cotransfection of cyclin E–CDK2 or cyclin A–CDK2 had no such effect (Fig. 3*C*, *Left* and data not shown, respectively). Furthermore, neither the ambient HA–E2F-4 level nor its state of phosphorylation was significantly affected by overproducing cyclin D–CDK4 in these cells (Fig. 3*C*, *Right*). Taken together, these data suggest that p107 phosphorylation by cyclin D-associated kinases leads to the dissociation of p107–E2F-4 complexes.

p107 Suppression of E2F Activity Is Neutralized by Cyclin D-Associated Kinases. E2F-4 activates certain E2F site-containing promoters, and this activity can be specifically suppressed by p107 (9, 10). Because E2F-4 selectively associates with underphosphorylated p107 and phosphorylation of p107 leads to the release of E2F-4 from the p107 pocket, we investigated the effects of G_1 cyclin-associated kinases on p107 suppression of E2F-4 activity.

Cotransfection of expression plasmids encoding E2F-4 and DP-1 into U-2 OS cells led to marked enhancement (57-fold) of E2F transcription reporter activity (3xWT–E2F–Luc), while coexpression of p107 nearly eliminated this effect (Fig. 4). On cotransfecting pCMV–CDK4 and increasing amounts of expression plasmids encoding cyclins D1 or D2, p107 suppression of E2F-4 activity was progressively eliminated (Fig. 4). In contrast, cotransfection of pCMV–CDK2 and increasing amounts of pCMV–cyclin E or pCMV–cyclin A was ineffective in this regard. These differences in stimulation cannot be attributed to differences in abundance of the relevant cyclins and CDKs (data not shown).

Release of p107-Mediated Growth Suppression by G_1 Cyclins. Overproduction of p107 can suppress growth of certain cells, such as Saos-2 and C33A (2, 11). Because ectopically produced p107 was primarily hypophosphorylated in Saos-2 and C33A cells (Fig. 3; data not shown), the question of a link between the state of p107 phosphorylation and the expression of its growth suppressive function arose. To investigate this possibility, we cotransfected a p107 expression plasmid together with various cyclin kinase pairs into Saos-2 cells, and searched for perturbation of p107-growth suppression function. As shown by fluorescence-activated cell sorter analysis of the transfected population (Table 1), overproduction of p107 resulted in a G_1 block, which was overridden by cotransfected E2F-4/DP1. Cotransfection of either cyclin D1–CDK4 or cyclin E–CDK2 effectively overrode this effect, while cyclin

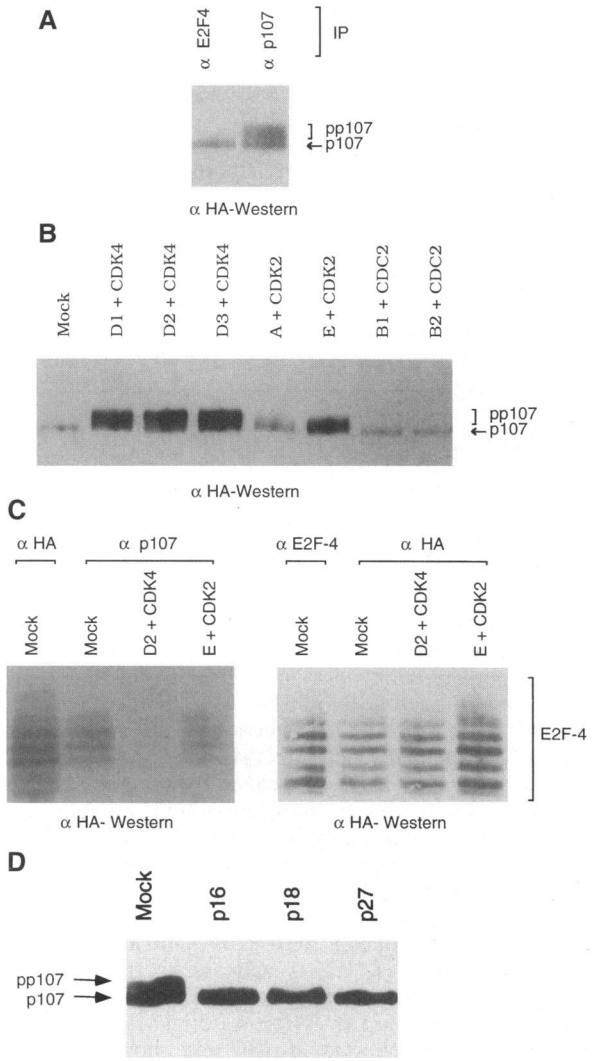

FIG. 3. Cyclin D-dependent phosphorylation of p107 disrupts p107–E2F-4 complexes. (*A*) E2F-4 selectively associates with hypophosphorylated p107. U-2 OS cells were cotransfected with expression plasmids encoding HA-tagged p107 (pCMV–HA–p107) or E2F-4. Cell lysates were immunoprecipitated with monoclonal antibodies specific for E2F-4 or p107. Immunoprecipitates were electrophoresed in a 6.5% gel and analyzed by Western blotting using the monoclonal antibody specific for the HA-tag (12CA5) as probe. (*B*) Cyclin D–CDK4 phosphorylates p107 *in vivo*. C33A cells were transfected with pCMV–HA–p107 alone (Mock) or cotransfected with the indicated plasmids encoding a specific cyclin and a kinase partner (CDK4, CDK2, or CDC2), respectively. Equivalent quantities of the cell lysate were immunoprecipitated with 12CA5 (α HA). Immunoprecipitates were electrophoresed in a SDS/6.5% gel and analyzed by Western blotting using 12CA5 as probe. (*C*) Phosphorylation of p107 by cyclin D–CDK4 disrupts p107–E2F-4 complexes. U-2 OS cells were cotransfected with pCMV–p107 and pCMV–HA–E2F-4 and, where indicated, expression plasmids encoding cyclin D2 and CDK4 or cyclin E and CDK2. Equivalent amounts of each cell lysate were immunoprecipitated with antibodies specific for HA, p107, or E2F-4, respectively. Immunoprecipitates were electrophoresed in a SDS/6.5% gel and analyzed by Western blotting using 12CA5 as probe. (*D*) Endogenous cyclin D-CDK activity is required for p107 phosphorylation. U-2 OS cells were transfected with pCMV–HA–p107 alone (Mock) or cotransfected with an expression plasmid encoding p16, p18, or p27, respectively. Cell lysates were immunoprecipitated with 12CA5, and the immunoprecipitated proteins were electrophoresed in a 6.5% gel and analyzed by Western blotting analysis using 12CA5 as probe.

A–CDK2 had little effect in this assay. Similar results were obtained in C33A cells (data not shown). These data strongly suggest a relationship between p107 phosphorylation and the

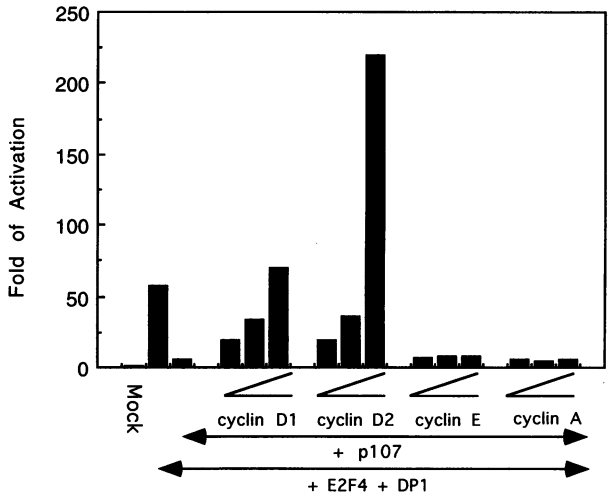

FIG. 4. Cyclin D-associated kinase relieves p107 suppression of E2F-4 transactivation activity. U-2 OS cells were transfected with a E2F reporter (3×WT–E2F–Luc) alone (mock) or cotransfected with indicated expression plasmids. A pCMV–β-GAL plasmid was used as an internal control in all transfections. The luciferase and β-GAL activities in each transfected culture were measured as described (19), normalized to the mock-transfected cell lysate, and reported as fold activation. Data shown here represent an average of the data derived from two independent transfection experiments.

inactivation of its G_1 blocking activity. However, the cyclin E–CDK2 growth effect was noted despite an incomplete effect of cyclin E kinase on p107 phosphorylation and its inability to overcome the p107 suppression of E2F-4 activity. This implies that there is more to the observed p107 growth suppression function than binding to and suppression of the transactivation function of E2F-4 (11).

DISCUSSION

pRB and p107 share certain characteristics, such as binding to and suppression of E2F transactivation function. Both are susceptible to negative regulation by certain G_1 cyclin-associated kinases. Furthermore, because pRB and p107 phosphorylation occurred simultaneously in G_1 in the cell lines tested, because these two events correlated with the first appearance of cyclin D (Fig. 2*B*), and because p16 and p18 blocked the natural p107 phosphorylation process, it seems that cyclin D plays a significant role in p107 phosphorylation.

Results of this study and of another one (18) are both consistent with the view that cyclin D kinase is a physiological participant in p107 phosphorylation and may be a major player in regulating the p107–E2F interaction. However, cyclin E- (and possibly cyclin A-) associated kinase may also contribute to the p107 phosphorylation process, as suggested by the appearance of increasing p107 phosphorylation in late G_1 and S (Fig. 2*B*). In addition, overproduction of cyclin E–CDK2 and cyclin A–CDK2 can lead to an intermediate p107 mobility upshift effect (Fig. 3*B*). However, in agreement with the work of others (18), the p107 phosphorylation effect mediated by overproduction of these two kinases did not lead to dissociation of p107–E2F-4 complexes (see Fig. 3*C* and data not shown), consistent with the finding that p107 suppression of E2F-4 activity was not affected by cyclin E–CDK2 or cyclin A–CDK2 action.

E2F-4 bound selectively to hypophosphorylated p107 (Fig. 3*A*), suggesting that cell cycle-dependent phosphorylation is a key event in the normal modulation of p107-pocket binding activity. Indeed, phosphorylation of p107 following cotransfection of cyclin D kinase closely correlated with the disruption of p107–E2F-4 complexes, alleviation of p107-mediated suppression of E2F transactivation function, and the inactivation

Table 1. Release of p107-mediated growth suppression in Saos-2 cells by G1 cyclins

Expression plasmids	Percent cells		
	G_1	S	G_2/M
Vector	42.9	30.0	27.1
	41.9	30.3	27.7
p107	63.2	20.7	16.1
	62.9	20.1	17.0
p107 plus			
Cyclin D1 + CDK4	48.1	46.0	5.8
	47.4	34.6	18.0
Cyclin E + CDK2	34.6	48.4	17.1
	31.0	50.6	18.4
Cyclin A + CDK2	60.0	29.2	10.7
	61.2	30.6	8.2
E2F4 + DP1	46.4	31.8	21.8
	46.3	31.1	22.6

Saos-2 cells were transfected with the pCMV vector or cotransfected with the indicated expression plasmids. A pCMV–CD19 plasmid encoding a cell surface marker was present in all of the transfection mixtures. Cells were collected at 72 hr after transfection, incubated with a fluorescein isothiocyanate conjugated monoclonal antibody specific for CD19, stained with propidium iodide, and analyzed by flow cytometry. The cell cycle distribution of fluorescein isothiocyanate positive cells from two experiments is presented.

of p107 growth suppression activity in Saos-2 and C33A cells. Hence, loss of p107 growth suppression potential can be linked to its phosphorylation that in turn leads to the release of E2F from the p107 pocket. In keeping with this notion, transfection of cyclin D kinase, which can lead to p107 phosphorylation, resulted in the relief of a p107 G_1 exit block, whereas cyclin A kinase, which had but a subtle effect on p107 phosphorylation, was ineffective in this regard.

However, unlike cyclin A kinase, overproduction of cyclin E kinase also overrode a p107 G_1 exit block, despite its inability to promote release of E2F from p107 (Figs. 3*C* and 4 and Table 1). One interpretation of this finding is that the p107 G_1 exit blocking function is partially linked to its known interaction with cyclin E kinase (11). This would be fully consistent with the observation that the cyclin E-binding domain of p107 contributes to p107-mediated growth arrest (11). In particular, overproduction of p107 may sequester endogenous cyclin E kinase, resulting in its failure to promote G_1 exit.

It is also possible that p107, when overproduced, disrupts cyclin E–cyclin A–CDK–CKI (CDK inhibitor) complexes and releases CDK inhibitors, such as p21, that negatively regulate the G_1/S transition (30). Consistent with this notion is the fact that p107 and p21 share a similar structural domain with which they may compete for binding to cyclins E and A (ref. 12; W. Kaelin, personal communication).

Cyclin E kinase may also operate downstream of E2F, in which case its overproduction does not lead to inactivation of p107 pocket function or to the activation of E2F, but instead leads to an accumulation of enzyme (kinase) activity capable of attacking its physiological target(s). In keeping with this notion, Hofmann and Livingston (F. Hofmann and D.M.L., unpublished data) have shown that E2F can be freed of pocket protein negative regulation and cells will still not exit from G_1 if cyclin E kinase is not activated. This is in agreement with the observation that the cyclin E gene is a key, downstream target of E2F action in *Drosophila* (31).

It now appears that different cyclin-associated kinases have discrete roles in regulating pocket protein functions. For example, cyclin D kinase may initiate pRB (13), p107 (this report; ref. 18) and p130 phosphorylation (32), which leads to the release of E2F during G_1. Cyclin E and cyclin A kinases form specific complexes with p107–E2F during late G_1 and S phase, respectively (for review, see ref. 1). Although the physiological relevance of cyclin E–cyclin A-CDK2-p107-E2F complexes is still unclear, it is conceivable that they may gain certain new, as yet unidentified, functions in late G_1 and S, including certain growth promoting functions.

We thank the members of our division for numerous helpful discussions and Drs. R. Bernards and R. Beijersbergen for sharing their results on p107 phosphorylation before publication. We are grateful to Drs. N. Dyson, L. Zhu, P. Hinds, S. van den Heuvel, Y. Xiong, and W. Krek for kindly providing reagents used in this work. Z.-X.X. was supported by fellowships from the Anna Fuller Fund and the American Cancer Society (Massachusetts Division). D.G. was supported by a fellowship from the Damon Runyon–Walter Winchell Cancer Research Fund and a special fellowship from Leukemia Society of America. This work was funded by grants from the National Institutes of Health and the Dana–Farber Cancer Institute/Sandoz Drug Development Program to D.M.L.

1. Ewen, M. E. (1994) *Cancer Metastasis Rev.* **13,** 45–66.
2. Zhu, L., van der Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N. & Harlow, E. (1993) *Genes Dev.* **7,** 1111–1125.
3. Hannon, G. J., Demetrick, D. & Beach, D. (1993) *Genes Dev.* **7,** 2378–2391.
4. Li, Y., Graham, C., Lacy, S., Duncan, A. M. V. & Whyte, P. (1993) *Genes Dev.* **7,** 2366–2377.
5. Mayol, X., Grana, X., Baldi, A., Sang, N., Hu, Q. & Giordano, A. (1993) *Oncogene* **8,** 2561–2566.
6. Vairo, G., Livingston, D. M. & Ginsberg, D. (1995) *Genes Dev.* **9,** 869–881.
7. Lam, E. W. F. & Thangue, N. (1994) *Curr. Opin. Cell Biol.* **6,** 859–866.
8. Lees, J. A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N. & Helin, K. (1993) *Mol. Cell. Biol.* **13,** 7813–7825.
9. Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z.-X., Xu, G., Wydner, K. L., DeCaprio, J. A., Lawrence, J. B. & Livingston, D. M. (1994) *Genes Dev.* **8,** 2665–2679.
10. Beijersbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlee, L., Voorhoeve, P. M. & Bernards, R. (1994) *Genes Dev.* **8,** 2680–2690.
11. Zhu, L., Enders, G., Lees, J. A., Beijersbergen, R. L., Bernards, R. & Harlow, E. (1995) *EMBO J.* **14,** 1904–1913.
12. Zhu, L., Harlow, E. & Dynlacht, B. D. (1995) *Genes Dev.* **9,** 1740–1752.
13. Weinberg, R. A. (1995) *Cell* **81,** 323–330.
14. Lukas, J., Bartkova, J., Rohde, M., Strauss, M. & Bartek, J. (1995) *Mol. Cell. Biol.* **15,** 2600–2611.
15. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M. & Pagano, M. (1995) *Mol. Cell. Biol.* **15,** 2612–2624.
16. Peeper, D. S., Parker, L. L., Ewen, M. E., Toebes, M., Frederick, F. L., Xu, M., Zantema, A., van der Eb, A. J. & Piwnica-Worms, H. (1993) *EMBO J.* **12,** 1947–1954.
17. Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J.-Y. & Livingston, D. M. (1993) *Cell* **73,** 487–497.
18. Beijersbergen, R. L., Carlee, L., Kerkhoven, R. M. & Bernards, R. (1995) *Genes Dev.* **9,** 1340–1353.
19. Dyson, N., Dembski, M., Fattaey, A., Ngwu, C., Ewen, M. & Helin, K. (1993) *J. Virol.* **67,** 7641–7647.
20. Lees, E., Faha, B., Dulic, V., Reed, S. I. & Harlow, E. (1992) *Genes Dev.* **6,** 1874–1885.
21. Harlow, E., Franza, B. & Schley, C. (1985) *J. Virol.* **55,** 533–546.
22. Qin, X.-Q., Chittenden, T., Livingston, D. M. & Kaelin, W. G. (1992) *Genes Dev.* **6,** 953–964.
23. Xiao, Z.-X., Chen, J., Levine, A., Modjtahedi, N., Xing, J., Seller, W. & Livingston, D. M. (1995) *Nature (London)* **375,** 694–698.
24. Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I. & Weinberg, R. A. (1992) *Cell* **70,** 993-1006.
25. van den Heuvel, S. & Harlow, E. (1993) *Science* **262,** 2050–2053.
26. Krek, W., Livingston, D. M. & Shirodkar, S. (1993) *Science* **262,** 1557–1560.
27. Keyomarshi, K., Sandoval, L., Band, V. & Pardee, A. B. (1991) *Cancer Res.* **51,** 3602–3609.
28. Huberman, J. A. (1981) *Cell* **23,** 647–648.
29. Cobrinik, D., Whyte, P., Peeper, D. S., Jacks, T. & Weinberg, R. A. (1993) *Genes Dev.* **7,** 2392–2404.
30. Sherr, C. J. & Roberts, J. M. (1995) *Genes Dev.* **9,** 1149–1163.
31. Duronio, R. J. & O'Farrell, P. H. (1995) *Genes Dev.* **9,** 1456–1468.
32. Mayol, X., Garriga, J. & Grana, X. (1995) *Oncogene* **11,** 801–808.