The retinoblastoma family proteins pRB, p107, and p130 are phosphorylated and released from E2Fs in the late G1 phase of the cell cycle. This phosphorylation is thought to contribute to the derepression of E2F-responsive genes and to be mediated, in part, by Cdk4 and Cdk6. Evidence that Cdk4/6 activity is inhibited by responsive genes and to be mediated, in part, by Cdk4 and thought to contribute to the derepression of E2F-responsive genes in late G1, it has been suggested that p107 and p130 may be underphosphorylated and remain associated with E2Fs during G1-S progression in cells that lack pRB. To examine this, we evaluated the cell cycle-dependent phosphorylation and E2F binding abilities of p107 and p130 in pRB−, p16(+), and Saos-2 osteosarcoma cells. p130, but not p107, was phosphorylated and released from E2F-4 in late G1 and S phase cells, although p130 phosphorylation differed qualitatively in these and other pRB−, p16(+), and pRB(+), p16(−) cells. p130 phosphorylation occurred in the absence of cyclin D-Cdk4/6 complexes, coincided with cyclin E- and Cdk2-associated kinase activity, and was prevented by expression of dominant negative Cdk2. Moreover, dominant negative Cdk2 prevented the dissociation of endogenous p130-E2F-4 complexes and inhibited E2F-4-dependent transcription. These findings show that p130 can be phosphorylated and functionally inactivated in a Cdk2-dependent process, and they highlight the involvement of distinct Cdks in the regulation of different pRB family proteins.

In mammalian cells, proliferative decisions are frequently made in the late G1 phase of the cell cycle, when under appropriate conditions cells progress through the restriction point and become committed to proceed through the S, G2, and M cell cycle phases (1, 2). G1-S progression is influenced by diverse growth signaling pathways that converge on the control of cyclin-dependent kinases (Cdks), including Cdk4 or Cdk6 in conjunction with D type cyclins and Cdk2 in conjunction with cyclin E (3–8). When active, G1 Cdks are thought to promote the G1-S transition by coordinately phosphorylating specific substrates (for review see Ref. 9).

Among the best characterized substrates of G1 Cdks are the retinoblastoma protein (pRB) and the pRB-related proteins p107 and p130 (10–12). Each of these pRB family members is capable of blocking progression from G1 into S (13–17) and is thought to do so, at least in part, by binding to E2F transcription factors and repressing genes that contribute to S phase entry (15, 17–22). pRB binds preferentially to E2Fs-1, -2, and -3 (23), whereas p107 and p130, which are more highly related to each other than either is related to pRB (24–26), bind preferentially to E2Fs-4 and -5 (17, 20, 27–29). p107 and p130 function redundantly in G1, and they repress E2F-responsive genes that are distinct from those regulated by pRB and that may contribute to cell cycle progression (30–35).

By binding to E2Fs, pRB family proteins inhibit E2F-mediated transactivation and repress E2F-responsive genes (36–42). This transcriptional repression is carried out through the recruitment of histone deacetylases (43–47) and other proteins (48–50) to E2F-responsive promoters and is thought to mediate G1 arrest in response to p16INK4A, transforming growth factor β, and contact inhibition (51, 52). Transcriptional repression is relieved in late G1, when pRB family proteins become highly phosphorylated (53–59) and lose the capacity to bind E2Fs (18, 19, 58–60). Recent studies have shown that different G1 Cdks phosphorylate pRB at distinct residues (61–64) and that cyclin D-Cdk4/6 as well as cyclin E-Cdk2 contribute to pRB hyperphosphorylation and functional inactivation (65–67). As with pRB, full phosphorylation of p107 and p130 requires cyclin D-Cdk4/6 (58, 65), and disruption of p130-E2F complexes requires G1 Cdk activity (68). However, phosphorylation and inactivation of p107 does not require Cdk2 and is not induced by ectopic cyclin E (58, 59).

Whereas pRB phosphorylation is thought to be crucial for G1-S progression as well as for S phase completion in most cell types (69–72), the importance of p107 and p130 phosphorylation has not been established. Because the Cdk4/6-dependent phosphorylation of p107 and p130 coincides with the release of p107 and p130 from E2Fs and with the induction of p107/p130-responsive genes in late G1, it has been suggested that p107 and p130 phosphorylation by Cdk4/6 is required for release from E2F, for induction of p107/p130-responsive genes, and for normal G1-S progression. However, the requirement for such phosphorylation is brought into question in cells that lack functional pRB, because pRB− tumor cells generally express high levels of the Cdk4/6 inhibitor p16INK4A (hereafter referred to as p16) (73–77) and are insensitive to ectopically expressed p16 as well as to other agents that antagonize Cdk4/6 activity (78–84). Accordingly, the growth inhibitory functions of p107 and p130 appear to be circumvented in pRB− cells without p107 and p130 being phosphorylated by the Cdk4/6 kinases. Moreover, the mechanism that is used to circumvent p107-
Cdk2-dependent Phosphorylation of p130 in pRB(−) Cells

Immunoblotting and Immunoprecipitation—Lysates were separated by electrophoresis through SDS-polyacrylamide gels, transferred to nitrocellulose, incubated in 0.05% Tween 20 in PBS (0.05% TPBS) containing 5% nonfat dry milk for 1 h, and washed three times for 10 min each in 0.05% TPBS. Membranes were probed overnight with monoclonal antibodies against human cyclin D3 (NeoMarkers MS-215, 1:100), cyclin E (Upstate Biotechnology 06-134 at 1:1000), monoclonal antibodies against p130 (Transduction Laboratories R27027/L1 at 1:100; used for Fig. 2B, 7A, and 8A), cyclin D1 (NeoMarkers MS-210 at 1:100), cyclin D2 (NeoMarkers MS-214 at 1:100) cyclin D3, (NeoMarkers MS-215 at 1:100), and p16 (NeoMarkers MS-218 at 1:100).

Histone H1 Kinase Assay—Cdk2- and cyclin E-associated histone H1 kinase activities were measured as described (91). Briefly, lysates containing 100 μg of total protein in 200 μl were incubated at 4°C with either 1 μg of rabbit polyclonal anti-Cdk2 (Santa Cruz SC-163) or 2 μg of rabbit polyclonal anti-cyclin E (Upstate Biotechnology 06-134) for 1 h. 10 μl of protein A beads (Zymed Laboratories Inc.) were added and incubated for 1 h. Beads were washed four times with immunoprecipitation buffer (50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 100 mM Na3VO4, pH 7.5). The following antibodies and amounts were used for immunoprecipitation: polyclonal antibodies against p130 (Santa Cruz SC-317, 20 μl for 3 mg lysate) and E2F-4 (Santa Cruz SC-866, 40 μl for 6 mg of lysate (Fig. 4) or 20 μl for 2 mg of lysate (Fig. 8A)); and monoclonal antibodies against human cyclin D3 (NeoMarkers MS-215, 20 μg for 4 mg) and p16 (NeoMarkers MS-218, 20 μg for 4 μg).

Plasmid DNAs—pHook-2 (InVitrogen), pcMV-p16-HA, pcMV-DNcdk2, pcMV-DNcdk4, pcMV-DNcD4, pcMV-CD20 (82), pcMV-E2F-4 (29), pcMV-CDP-D1 (92), pBabe-Puro (95), and pE2-CAT (94) have been described. pHook-2-CC was derived from pHook-2 but has the cytomegaloivirus promoter immediately preceding the Hook gene. It was produced by digesting pHook-2 with EcoRV and SmalI and self-ligating the larger fragment. pHook-2-Cdk2DN and pHook-2-Cdk3DNHA were produced by inserting BamHI DNA fragments containing the indicated Cdk genes (7) into the BamHI site of the pHook-2 polylinker.

Transfection and Selection of Transfected Cells—For Hook selection, 2.2 × 10^6 Saos-2 cells in 10-cm dishes were co-transfected with 2 μg of pHook-2-CC plus 20 μg of expression vector using the calcium phosphate method (95), and serum starved and stimulated as above. Cells were then rinsed with PBS, incubated in 0.06% trypsin (1:4 dilution of 0.25% trypsin in Hanks’s balanced salt solution) at 37°C for 7–10 min, collected by centrifugation, and resuspended in 1 ml of medium containing 1.5 × 10^6 hapten-coated magnetic beads (InVitrogen). Mixtures were stored at 4°C for 1 h, at 0°C for 10 min, and then collected by end-over-end rotation in a magnetic stand for 1 min and washed with Hanks’s balanced salt solution twice while in the stand.

For puromycin selection, 2.2 × 10^6 Saos-2 cells in 10-cm dishes were co-transfected with 2 μg of pBabe-Puro and 15 μg of either pcDNA3 or pcMV-DNcD2 for 14 h, washed twice in PBS, and refed with complete medium for 12 h, serum starved with medium containing 0% serum and 10% FBS and assayed in triplicate, and the means and standard errors of the indicated primary antibodies and then incubated with horseradish peroxidase-conjugated protein A/G (Pierce) at 1:1000 dilution or horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Pharmacia Biotech) at 1:3000 dilution for 1 h, and detected with enhanced chemiluminescence (Kirkgaard and Ferry Laboratories). On occasion, membranes were stripped at 62.5 mM Tris, 0.7% nonfat dry milk, 2% SDS for 20 min at 50°C and reprobed. The following antibodies and dilutions were used in Western analyses: polyclonal antibodies against Cdk2 (Santa Cruz SC-163 at 1:1000), Cdk3 (Santa Cruz SC-826 at 1:1000), Cdk4 (Santa Cruz SC-260 at 1:1000), Cdk6 (Santa Cruz SC-177 at 1:1000), p130 (Santa Cruz SC-317 at 1:1000), used for Figs. 2A, 3, and 4); pRB (Santa Cruz SC-50 at 1:1000), p107 (Santa Cruz SC-318 at 1:1000), E2F (Santa Cruz SC-866 at 1:200), cyclin E (Upstate Biotechnology 06-134 at 1:1000); monoclonal antibodies against p130 (Transduction Laboratories R27027/L1 at 1:100; used for Fig. 2B, 7A, and 8A); cyclin D1 (NeoMarkers MS-210 at 1:100), cyclin D2 (NeoMarkers MS-214 at 1:100) cyclin D3, (NeoMarkers MS-215 at 1:100), and p16 (NeoMarkers MS-218 at 1:100).

At present, mechanisms by which the growth inhibitory effects of p107 and p130 are overcome in pRB(−), p16(+) cells are unknown. As one possibility, p107 and p130 may remain constitutively underphosphorylated, bound to E2Fs, and able to repress p107/p130-responsive genes, with this having little effect on cell proliferation due to the more profound consequences of the loss of pRB. Arguing against this idea, however, is the notion that p107/p130-responsive genes may be important for progression through various stages of the cell cycle and are indeed expressed in pRB(−) cells.2 One means by which transcriptional repression by p107 and p130 might be bypassed is through the exclusion of p107/E2F-4 and p130/E2F-4 complexes from the nucleus in late G1 and S phase cells (86–89), although it is currently unclear whether this can occur in the absence of p107 and p130 phosphorylation. An alternative means by which the growth inhibitory effects of p107 and p130 might be bypassed in pRB(−), p16(+) cells, and which is addressed in the current study is through the release of p107 and p130 from E2Fs, due to their phosphorylation by kinases other than Cdk4 or Cdk6. Here, we show that p130 but not p107 is indeed phosphorylated and released from E2F-4 in a Cdk4/6-independent but Cdk2-dependent process. These findings provide a mechanism by which p130 is inactivated in pRB(−), p16(+) cells and highlight the involvement of distinct Cdns in the regulation of different pRB family proteins.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—Saos-2 cells (clone 2.4) (14) were cultured in Dulbecco’s modified Eagle medium containing 15% fetal bovine serum, 100 unit/ml penicillin + 100 μg/ml streptomycin, and 292 μg/ml glutamine with 5% CO2 at 37°C. HaCaT keratinocytes (90) were grown in α-minimal essential medium with 10% fetal bovine serum and antibiotics and glutamine as above. MDA-MB468, MDA-MB436, BT549, MDA-MB231, MDA-MB4358, RZ75–1, BT483, BT474, and HS57T cells were obtained from American Type Culture Collection and cultured under American Type Culture Collection-supplied conditions.

Cell Cycle Synchronization and Restimulation—Subconfluent Saos-2 and HaCaT cells were trypsinized and plated at 3 × 10^6/100-mm dish and 1.5 × 10^5/100-mm dish, respectively. On the following day, cells were rinsed once with phosphate-buffered saline (PBS) and maintained in serum-free medium for 5 days. Cells were resuspended by addition of complete medium. In some cases in the manuscript, 1 × 10^6 cells were cocultured, [3H]Thymidine Incorporation and Flow Cytometry Analyses—Cells were seeded to 24-well plates at 2.5 × 10^4 cells/well for HaCaT, and restimulated in the presence of 10 μCi/mI [3H]thymidine for 1 h. Labeling was terminated by adding ascorbic acid to 250 mM, cells were washed three times with PBS and once with ice-cold 5% trichloroacetic acid, incubated in 5% trichloroacetic acid on ice for 30 min, washed with H2O, and lysed overnight in 1 M NaOH at 37°C, and [3H]thymidine incorporation was measured by scintillation counting. Samples were assayed in triplicate, and the means and standard errors of the indicated Cdk genes were calculated. Flow cytometry was performed as described previously (15).

Cell Lysates—Cells were washed with ice-cold PBS, incubated in 1 ml of ice-cold cell lysis buffer (50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na3VO4, pH 7.5, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 25 μg/ml benzamidene, 10 μg/ml pepstatin A, and 2 mM phenylmethylsulfonyl fluoride for Fig. 4; and as described (15) for Fig. 8A), removed from plates by scraping, sonicated three times on ice, and subsequently set at 30°C on a Teknor TM50 sonic disruptor, and centrifuged in a microcentrifuge at 14,000 rpm for 10 min at 4°C. The supernatants were collected, and the protein concentrations were determined by a Coomassie Blue binding assay (Bio-Rad).

3 D. Cobrinik, unpublished observations.
RESULTS

Impaired p130 and p107 Phosphorylation and Production of a Novel p130 Phosphoisomer, “Form IIb,” in Late G1, Saos-2 Cells—In this study, we sought to determine whether p107 and p130 are phosphorylated and functionally inactivated during G1-S progression in pRB(−), p16-expressing cells. The Saos-2 osteosarcoma cell subclone 2.4 (14) was used for these analyses. These pRB(−), p16(+) cells (77, 96) can be growth inhibited by serum starvation and induced to enter S phase in a synchronous manner upon serum stimulation. As shown in Fig. 1, [3H]thymidine incorporation increased over basal levels between 15 and 24 h after stimulation and subsequently decreased, indicating that a discrete cohort of Saos-2 cells had been induced to enter and proceed through S. For comparison purposes, p107 and p130 phosphorylation was also examined in pRB(−) HaCaT keratinocytes, which are thought to have active Cdk4 and Cdk6 (66). Fortunately, HaCaT cells entered S phase with kinetics similar to that of Saos-2 (Fig. 1).

The phosphorylation of p130 and p107 in serum-starved and restimulated cells was assessed by immunoblotting for electrophoretically distinct p107 and p130 isoforms. Two p130 species, here termed Forms I and IIa, were detected in serum-starved Saos-2 cells after transfaction for at least 9 h after stimulation (Fig. 2A). Between 9 and 12 h after stimulation, p130 Forms I and IIa declined, and a slightly more slowly migrating p130 species termed Form IIb appeared. This species comprised the majority of p130 from 15 to 24 h after stimulation. Similar to the situation in Saos-2 cells, two p130 isoforms were also present in serum-starved HaCaT cells, and a more slowly migrating species appeared after serum stimulation (Fig. 2B). However, whereas serum-starved Saos-2 and HaCaT cells contained p130 species with identical mobilities, corresponding to Forms I and IIa, the p130 species produced in stimulated HaCaT cells (termed Form III) migrated substantially more slowly than the p130 Form IIb in Saos-2 (Fig. 2C).

In contrast to the situation with p130, no mobility shift of p107 was evident in serum-stimulated Saos-2 cells (Fig. 2, A and C). This behavior differed from that seen in HaCaT cells, where p107 mobility decreased in late G1, coincident with the appearance of hyperphosphorylated p130 and pRB(−), p16-expressing cells. This behavior differed from that seen in HaCaT cells, in which phosphorylation of both p107 and p130 differed in Saos-2 and HaCaT cells may be that the p16 that is expressed in Saos-2 cells may impair Cdk4/6-dependent phosphorylation. If this is the case, then p107 and p130 phosphorylation may be similarly affected in other pRB(−), p16(+) cell types. To evaluate this, the expression of the different p130 phosphoisomers was compared in a series of pRB(−) and pRB(+) breast cancer cell lines. As shown in Fig. 3, the slowly migrating p130 Form III was detected in each of the six pRB(−) cell types examined (lanes 4–9), but only the more rapidly migrating p130 species were detected in three pRB(+) lines (lanes 1–3). As found in many other tumor cell types, p16 was readily detected in each of the pRB(−), but not in the pRB(+)
cells. The failure of pRB(−), p16(+) cells to produce p130 Form III is consistent with p16 inhibiting p130 phosphorylation by cyclin D-Cdk4/6 in these cells.

**Impaired Binding of E2F-4 to p130 Form IIb, but Not to p107, in Late G1 and S Phase Saos-2 Cells**—Earlier studies showed that p107 and p130 were hyperphosphorylated and lost their ability to bind E2F-4 in late G1 in various pRB(+) cell types (58–60, 68, 98). These findings suggested that the release of p107 and p130 from E2F-4 in late G1 might be important for relieving p107- and p130-mediated cell cycle blocks. However, because p107 and p130 were not fully phosphorylated in Saos-2 cells, it was unclear whether they would dissociate from E2F-4 in the late G1 and S cell cycle phases. To address this issue, we evaluated the E2F-4 binding abilities of p107 and p130 in Saos-2 cells that had been serum starved and restimulated for 21 h. In these experiments, hydroxyurea was added to the culture at the time of serum stimulation to prevent asynchronous G1/G1 cells within the culture from obscuring the pattern of E2F association occurring in late G1 and S.

To monitor the association of E2F-4 with p107 and p130, lysates prepared from serum starved and restimulated Saos-2 cells were immunoprecipitated, using either an antibody directed against E2F-4 or an antibody directed against p107 and p130. Levels of p130 Form III as well as hyperphosphorylated pRB were lower in the pRB(-) primary tumor cell lines is shown. p130 Form III was detected in each of the pRB(-), p16(-), but not in the pRB(-), p16(+) cell lines. Levels of p130 Form III as well as hyperphosphorylated pRB were lower in BT483 (lane 7) than in other pRB(+) cell types owing to the decreased growth rate of the culture.

**Absence of Cyclin D-Cdk4/6 Complexes in Late G1 Saos-2 Cells**—Because Saos-2 cells failed to produce p130 Form III, p130 phosphorylation and release from E2F-4 must have come about through a mechanism different from that which operates in pRB(+) cell types. Accordingly, we sought to define the mechanism of p130 Form III phosphorylation by evaluating the involvement of known G1 Cdk's.

We first investigated the role of Cdk4 and Cdk6 in p130 Form IIb phosphorylation. Although Saos-2 cells express p16 (77), this does not preclude their having active Cdk4 or Cdk6, because such kinases may be mutated in a way that prevents binding to p16 and that permits sustained binding to D type

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3 L. Cheng, unpublished data.

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**FIG. 3.** Absence of p130 Form III in pRB(-), p16(+) cells. Western blot analysis of p130, pRB, and p16 in exponentially growing mammary tumor cell lines is shown. p130 Form III was detected in each of the pRB(-), p16(-), but not in the pRB(-), p16(+) cell lines. Levels of p130 Form III as well as hyperphosphorylated pRB were lower in BT483 (lane 7) than in other pRB(+) cell types owing to the decreased growth rate of the culture.

**FIG. 4.** p130 Form IIb fails to bind E2F-4 in late G1, and S phase Saos-2 cells. Saos-2 cells were serum starved and restimulated for 0 h (lanes 1 and 2) or for 21 h in the presence of hydroxyurea (HU; lanes 3 and 4), and lysates were immunoprecipitated (IP) with antibody recognizing both p107 and p130 (using 3 mg of lysate) or with antibody directed against E2F-4 (using 6 mg of lysate), as indicated. The immunoprecipitates were then subjected to Western blotting, and the upper portion of the blot was sequentially probed with anti-p130 and anti-p107 antibodies (top and middle panels), and the lower portion was probed with anti-E2F-4 (bottom panel). The position of the p130 IIb isoform that fails to immunoprecipitate with E2F-4 is indicated.

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**Cdk2-dependent Phosphorylation of p130 in pRB(-) Cells**

**Hours serum stimulation:**

| Time (h) | Lanes |
|---------|-------|
| 0       | 1, 2  |
| 21      | 3, 4  |

**Western:**

- **p130**
  - Lane 1: p130 precipitated with E2F-4 (top panel)
  - Lane 2: E2F-4 precipitated with p130 (middle panel)
  - Lane 3: E2F-4 precipitated with p107 (bottom panel)

- **p107**
  - Lane 4: p107 precipitated with E2F-4 (top panel)
  - Lane 5: E2F-4 precipitated with p107 (middle panel)
  - Lane 6: E2F-4 precipitated with p130 (bottom panel)

**p16**

- Lane 7: p16 precipitated with E2F-4 (top panel)
- Lane 8: E2F-4 precipitated with p16 (bottom panel)
that p130 was phosphorylated in late G1 Saos-2 cells by kinases other than Cdk4 or Cdk6.

Appearance of p130 Form IIb Coincides with Cyclin E-Cdk2 Kinase Activity and Is Impaired by Dominant Negative Cdk2—Previous studies indicated that Cdk2 is needed for S phase entry in Saos-2 cells (7). To assess whether Cdk2 together with cyclin E contributes to the late G1 phosphorylation of p130 in these cells, cyclin E and Cdk2 expression and their associated kinase activities were measured during G1-S progression.

As shown in Fig. 6A, cyclin E levels increased moderately after serum stimulation but were thereafter relatively unchanged. This constitutive expression of cyclin E in Saos-2 cells is similar to that seen in other pRB(−) cell types (31). Cdk2 was also expressed at nearly constant levels in serum-starved and stimulated Saos-2 cells, although a fast migrating Cdk2 species that is thought to represent the active Cdk2 isoform (102) appeared between 9 and 12 h after stimulation (Fig. 6B). Similarly, cyclin E- and Cdk2-associated histone H1 kinase activity appeared between 9 and 12 h and increased progressively thereafter (Fig. 6B, C and D), corresponding to times when p130 Form IIb was being produced (Fig. 2A). These results are consistent with cyclin E-Cdk2 contributing to and being rate-limiting for the production of p130 Form IIb.

To determine whether Cdk2 is required for the late G1 phosphorylation of p130 in Saos-2 cells, we evaluated the effects of dominant negative (DN) Cdk2 on the production of p130 Form IIb. Saos-2 cells were co-transfected with expression vectors encoding DNCdk2 (7) together with a chimeric “Hook” protein composed of the platelet-derived growth factor receptor transmembrane domain and a single chain antibody (103). Transfected cells were then serum starved, restimulated for 21 h, and selected using hapten-coated magnetic beads, and the phosphorylation state of the endogenous p130 protein in the selected cells was assessed by Western analysis.

Selected cells that had been co-transfected with Hook and DNCdk2 vectors produced p130 Form IIb but no detectable Form IIa (Fig. 7A, lane 5). However, selected cells that had been co-transfected with the Hook and DNCdk2 vectors produced p130 Form IIa but no detectable Form IIb (Fig. 7A, lane 2). The mobility difference between p130 Form IIa in DNCdk2-transfected cells and p130 Form IIb in control cells is best appreciated by referring to a consistently migrating protein band that cross reacts with the p130 antibody (labeled with an asterisk in

![Fig. 5.](Image 81x570 to 264x729)

**Fig. 5. Absence of cyclin D-Cdk4 and cyclin D-Cdk6 complexes in late G1 Saos-2 cells.** Saos-2 cells were serum starved and restimulated for 15 h, and lysates were prepared and subjected to three rounds of immunoprecipitation (IP) with anti-p16 (lanes 1–3) followed by three rounds of immunoprecipitation with anti-cyclin D3 (lanes 4–6) antibodies. The immunoprecipitates were then subjected to Western blotting with anti-p16 (top panel), anti-cyclin D3 (middle panel), and a mixture of anti-Cdk4 and anti-Cdk6 antibodies (bottom panel).

![Fig. 6.](Image 81x570 to 264x729)

**Fig. 6. Production of p130 Form IIb coincides with activation of cyclin E-Cdk2.** Saos-2 cells were serum starved and restimulated, and at the indicated times protein lysates were prepared and used for immunoblotting with anti-cyclin E (A) or anti-Cdk2 (B) antibodies or for immunoprecipitation with anti-cyclin E (C) or anti-Cdk2 (D) antibodies followed by assay for histone H1 kinase activity.

![Fig. 7A](Image 81x570 to 264x729)

**Fig. 7A and 7B.** (A) The ability of DNCdk2 to prevent dissociation of p130 from E2F-4 and inhibit E2F-4-dependent transactivation. (B) Production of p130 Form IIb coincides with activation of cyclin E-Cdk2. Saos-2 cells were serum starved and restimulated, and at the indicated times protein lysates were prepared and used for immunoblotting with anti-cyclin E (A) or anti-Cdk2 (B) antibodies or for immunoprecipitation with anti-cyclin E (C) or anti-Cdk2 (D) antibodies followed by assay for histone H1 kinase activity.

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Saos-2 cells were transfected with an E2-CAT reporter construct whose expression depended on the E2F binding sequence (94), together with expression vectors for E2F-4 and DP-1, and CAT activity was determined after 48 h. As shown in Fig. 8B, E2F-4 and DP-1 stimulated E2-CAT expression by -10-fold, whereas transfection of DNCdk2 abolished E2F-4-mediated transactivation. The ability of DNCdk2 to inhibit E2F-4-dependent transactivation is consistent with Cdk2-dependent phosphorylation of p130 being required for E2F-4-mediated transcription in pRB(−), p16(+) cells.

DNCdk2 and DNCdk3 to induce a G1 block was confirmed by co-transfected and selected cells was confirmed by Western blot analysis and DNCdk3 (lane 4) relative to their endogenous counterparts in transfected and selected cells was confirmed by Western blot analysis using a mixture of the corresponding antibodies. The impaired production of Form IIb in DNCdk2-expressing cells (lanes 1–5) or unselected (lanes 6–8) cells was then subjected to Western blotting with anti-p130 antibody. The position of the p130 Form II antibody is indicated, or with the “empty” cDNA3 vector control. At 48 h after transfection, cells were fixed, stained with fluorescein isothiocyanate-labeled anti-CD20 antibody and with propidium iodide, and DNA content of CD20(+) cells was determined by fluorescence-activated cell sorter analysis. The percent increase in G1 cells as compared with cDNA3 vector transfected controls was calculated. Error bars indicate standard deviation for duplicate transfections.

The phosphorylation of p107 and p130 by cyclin D-Cdk4/6 has been thought to contribute to the release of p107 and p130 from E2Fs, to the derepression of E2F-responsive genes, and to G1-S progression (for reviews see Refs. 11, 21, 22, and 105). However, evidence that most pRB(−) cells express high levels of p16 raised the possibility that p107 and p130 might not be phosphorylated or released from E2Fs in such cells. Defining whether p107 and p130 are released from E2Fs, then such release may contribute to the growth of pRB(−) cells has important implications. If p107 and p130 are released from E2Fs, then such release may contribute to the growth of pRB(−) tumors. Alternatively, if p107 or p130 are not released from E2Fs, then other mechanisms may function to overcome the growth inhibitory effects of p107-E2F and p130-E2F complexes and to promote the growth of pRB(−) malignancies.

To address these issues, the current study examined the cell cycle-dependent phosphorylation and E2F-4 binding abilities of p107 and p130 in pRB(−), p16(+) Saos-2 cells. We found that p130 was phosphorylated and released from E2F-4 in late G1, yet this phosphorylation differed from that occurring in numerous pRB(+) cell types. In pRB(+) cells, p130 phosphorylation was accompanied by a substantial electrophoretic mobility shift corresponding to p130 Form III, whereas in pRB(−) Saos-2 cells, phosphorylation was accompanied by a minor mobility shift corresponding to p130 Form IIb. This p130 Form IIb phosphorylation coincided with activation of the cyclin E- and Cdk2-associated kinases and dissociation of p130 from

**DISCUSSION**

The phosphorylation of p107 and p130 by cyclin D-Cdk4/6 has been thought to contribute to the release of p107 and p130 from E2Fs, to the derepression of E2F-responsive genes, and to G1-S progression (for reviews see Refs. 11, 21, 22, and 105). However, evidence that most pRB(−) cells express high levels of p16 raised the possibility that p107 and p130 might not be phosphorylated or released from E2Fs in such cells. Defining whether p107 and p130 are released from E2Fs, then such release may contribute to the growth of pRB(−) tumors. Alternatively, if p107 or p130 are not released from E2Fs, then other mechanisms may function to overcome the growth inhibitory effects of p107-E2F and p130-E2F complexes and to promote the growth of pRB(−) malignancies.

To address these issues, the current study examined the cell cycle-dependent phosphorylation and E2F-4 binding abilities of p107 and p130 in pRB(−), p16(+) Saos-2 cells. We found that p130 was phosphorylated and released from E2F-4 in late G1, yet this phosphorylation differed from that occurring in numerous pRB(+) cell types. In pRB(+) cells, p130 phosphorylation was accompanied by a substantial electrophoretic mobility shift corresponding to p130 Form III, whereas in pRB(−) Saos-2 cells, phosphorylation was accompanied by a minor mobility shift corresponding to p130 Form IIb. This p130 Form IIb phosphorylation coincided with activation of the cyclin E- and Cdk2-associated kinases and dissociation of p130 from
Thus, the different pRB family proteins exhibit consistent with earlier evidence that p107 is phosphorylated requires Cdk4/6 but is unaffected by Cdk2. Finally, as well as Cdk2. In contrast, p107 dissociation from E2F in pRB(−)Saos-2 cells, as seems likely, then it would do so in the absence of prior phosphorylation of p130 by cyclin D-Cdk4/6. This suggests an additional distinction in the regulation of pRB family proteins by Cdks; whereas the Cdk2-dependent phosphorylation of pRB Ser 567 and disruption of the pRB pocket requires prior phosphorylation of the pRB C terminus by Cdk4/6, Cdk2-dependent phosphorylation of the homologous position in p130 and disruption of the p130 pocket may be independent of Cdk4/6 activity.

The association of p107 with E2F-4 in late G1 and S phase Saos-2 cells demonstrates that the persistence of p107-E2F-4 complexes did not prevent S phase entry. One implication of this finding is that p107-mediated repression of E2F-responsive genes may be circumvented without p107 being released from E2Fs. This might occur if p107-E2F-4 complexes are unable to bind to promoter E2F binding sites in late G1, or S, as suggested by in vivo footprint analyses of the cdc-2 and B-myb genes, and is consistent with evidence that p107-E2F-4 complexes localize to the cytoplasm in S phase-enriched cultures.

Interestingly, if p107-E2F-4 complexes localize to the cytoplasm in late G1 Saos-2 cells, then the structurally similar p130-E2F-4 complexes might also localize to the cytoplasm were they not disrupted through p130 phosphorylation. Although Cdk2 activity was required for E2F-4-dependent transcription and S phase entry in Saos-2 cells, it is unclear whether Cdk2-mediated phosphorylation of p130 was required for these processes. Accordingly, it remains possible that Cdk2-mediated p130 phosphorylation and release from E2F-4 is dispensable for overcoming the growth inhibitory effects of p130-E2F-4 complexes. The importance of Cdk2-dependent p130 phosphorylation for the proliferation of pRB(−), p16(+) cells may be resolved by analyzing the effects of p130 phosphorylation site mutants.

If p107 phosphorylation and release from E2F is dispensable for S phase entry in pRB(−) tumor cells, as this study suggests, then this raises the question as to the general importance of p107 and p130 phosphorylation and dissociation from E2Fs in the diverse cell types where it has been observed. As one possibility, phosphorylation of p107 and p130 may be required for S phase entry in pRB(+) cells, whereas phosphorylation may permit the effects of p107-E2F or p130-E2F complexes to be circumvented in pRB(−) cells. Alternatively, p107 and p130 phosphorylation may modulate the expression of E2F-responsive genes without being required for S phase entry in either pRB(+) or pRB(−) cell types. Finally, given that p107 and p130 may be needed to control proliferation in relatively few embryonic or adult tissues, their phosphorylation may contribute to G1-S progression only in certain cell types or at selected times during development, particularly in contexts where other mechanisms for overcoming the effects of p107-E2F and p130-E2F complexes do not exist. To address these issues, it will be of interest to define the requirement for p107 and p130 phosphorylation in diverse biological contexts.

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