The tumor suppressor, retinoblastoma (Rb), is involved in both terminal mitosis and neuronal differentiation. We hypothesized that activation of the Rb pathway would induce cell cycle arrest in primary neural precursor cells, independent of the proposed function of cyclin-dependent kinases 4/6 (CDK4/6) to sequester the CIP/KIP CDK inhibitors (CKIs) p21 and p27 of CDK2. We expressed dominant negative adenovirus mutants of CDKs 2, 4, and 6 (dnCDK2, dnCDK4, and dnCDK6) in neural progenitor cells derived from E12.5 wild type and Rb-deficient mouse embryos. In contrast to previous studies, our results demonstrate that in addition to dnCDK2, the dnCDK4/6 mutants can induce growth arrest. Moreover, the dnCDK4/6-mediated inhibition is Rb-dependent. The dnCDK2 partially inhibited cell growth in Rb-deficient cells, suggesting that CDK2 may have additional targets. A previously proposed function of CDK4/6 is CKI sequestration, thereby preventing the resulting inhibition of CDK2, believed to be the key regulator of cell cycle. However, our immunoprecipitation studies revealed that the dominant negative CDK mutants could arrest cell growth despite their interaction with p21 and p27. Taken together, our results demonstrate that both CDK2 and CDK4/6 are crucial for cell cycle regulation. Furthermore, our data underscore the importance of the Rb regulatory pathway in neuronal development and cell cycle regulation, independent of CKI sequestration.

During embryogenesis, cycling neural progenitor cells in the ventricular zones commit to a neuronal fate and as a consequence of that decision undergo terminal mitosis and adopt a neuronal phenotype. A key developmental step in this process is the decision to undergo terminal mitosis. Several lines of evidence indicate that terminal mitosis and terminal differentiation are intimately coupled. First, cells in the mitotic layer of the developing retina induce expression of a retinal ganglion cell marker within minutes of the S phase of terminal mitosis (1, 2). Second, neural precursor cells transplanted throughout the developing cortex exhibit appropriate neuronal identification only if they had undergone terminal mitosis following implantation (3). Third, sensory neurons override the G1 restriction point and undergo S-phase death in the absence of the neurotrophin, NT-3 (4). The importance of terminal mitosis in neuronal development is underscored by the fact that failure to permanently withdraw from the cell cycle results in impaired differentiation and apoptosis. One key regulator of the cell cycle, the tumor suppressor protein pRb, has recently been implicated in terminal mitosis and neuronal differentiation.

Mice nullizygous for Rb1 die by E15.5 from hematopoietic and neurological defects attributed to failed terminal differentiation (5–7). By E12.5 onward, ectopic mitoses and massive cell death are observed throughout the developing nervous system. Studies examining neuronal development have revealed reduced expression of pan-neuronal genes as well as the nerve growth factor receptor TrkA, suggesting that Rb has an important role in differentiation (8). Examination of the expression of a neuron-specific transgene in Rb-deficient embryos revealed impaired neurogenesis in virtually all neuronal cell types examined (9). Furthermore, Rb-deficient neural precursor cells exhibited a dramatic delay in their ability to undergo terminal mitosis (10), indicating that Rb plays an important role in regulating the onset of terminal mitosis in progenitor cells.

While Rb is thought to be a key regulator of terminal mitosis in neural precursor cells (reviewed in Ref. 11), there is little evidence that activation of the Rb pathway is sufficient to induce cell cycle arrest in this cell type. Furthermore, although it is well established that CDK2, CDK4, and CDK6 are all involved in cell cycle regulation, their precise roles and importance are still the subject of controversy. Indeed, previous studies examining the role of cyclin-dependent kinases (CDKs) in cell cycle regulation have indicated that inhibition of CDK4/6, which function through the Rb pathway, is insufficient to induce mitotic arrest in several cell types. Specifically, a dominant negative mutant of CDK2, which sequesters cyclins A and E from endogenous CDKs, could efficiently induce growth arrest, while dominant negative mutants of CDK4/6 which sequester D cyclins, were unable to mediate cell cycle arrest (12). One interpretation of these results is that activation of the Rb pathway alone may not be sufficient to mediate arrest of cell growth. It has recently been suggested that CDK4/6, in its association with D cyclins, may have dual functions. First, these proteins inactivate Rb activity by phosphorylation (13–18). Second, CDK4/6 may sequester the CIP/KIP CDK inhibitors (CKIs) p21 and p27, thus rendering them unavailable to bind and inactivate CDK2 (19). Recently, p21 and p27 have been shown to associate with CDK4/6 in a facilitatory CDK-cyclin interaction without inhibition of kinase activity.
(19–21). In addition, as the INK4 CKIs p15, p16, p18, and p19 become expressed, they displace p21/p27, thereby releasing them to associate with CDK2, an interaction mediating G1 arrest (19, 22, 23). Thus, it is believed that the inhibition of CDK2/cyclin E activity is the crucial step in cell cycle regulation. The notion of CDK2 as the major regulator of cell cycle progression, with CDK4/6 being merely facilitative has become quite pervasive (19). While this model is supported by a number of studies, it should be noted that the experiments using dominant negative mutants were conducted entirely on tumor cells and may not reflect the true mechanism of cell cycle regulation in primary cells.

In view of the importance of Rb, as demonstrated by the severe nervous system defects in transgenic mice carrying null mutations for Rb, we hypothesized that activation of the Rb pathway alone would be sufficient to induce cell cycle arrest in primary neural precursor cells, independent of its function in the sequestration of the p21/p27 proteins from CDK2. To address this question, we have used primary neural progenitor cells derived from E12.5 wild type and Rb-deficient embryos. Dominant negative CDK mutants were introduced into these cells using adeno virus mediated gene delivery. In contrast to studies using cell lines, our results demonstrate that dominant negative mutants of CDK4 and CDK6 can effectively induce mitotic arrest in primary neural precursor cells, independent of the previously proposed binding and sequestration of p21 and p27. The fact that these CDK4/6 mutants are nonfunctional in cells that lack Rb demonstrates that the activation of the Rb pathway in itself is sufficient to cause cell cycle arrest in primary neural precursor cells.

MATERIALS AND METHODS

Transgenic Mice—Rb-deficient transgenic mice, originally generated by Jacks et al. (6), were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on a C57BL6 genetic background. Mice were genotyped by polymerase chain reaction, as described previously (6) using DNA extracted from tails for adults or from remaining embryonic tissue for cell culture studies. For timed pregnancies, mice were bred, and the time of plug identification was counted as day 0.5. For cultures of primary neural precursor cells, embryos were removed at E12.5.

Primary Cultures of Cortical Progenitor Cells, Neurons, and Neural Stem Cells—Neural progenitor cells were prepared as described previously (24), with some modification (9). Cortices were collected from E12.5 mouse embryos, triturated, and aggregates were plated on poly-L-ornithine/laminin-coated dishes. The culture medium consisted of Neurobasal medium (Life Technologies, Inc.), 0.5 mM glutamine, 50 units/ml penicillin-streptomycin, and 1% B27 supplement (Life Technologies, Inc.). To culture Rb-deficient progenitor cells, the same medium, except 1% N2 supplement was now replaced with 2% B27 (Life Technologies, Inc.). To culture Rb-deficient progenitor cells, the same medium, except 1% N2 supplement was now replaced with 2% B27 (Life Technologies, Inc.). To culture Rb-deficient progenitor cells, the same medium, except 1% N2 supplement was now replaced with 2% B27 (Life Technologies, Inc.). To culture Rb-deficient progenitor cells, the same medium, except 1% N2 supplement was now replaced with 2% B27 (Life Technologies, Inc.). To culture Rb-deficient progenitor cells, the same medium, except 1% N2 supplement was now replaced with 2% B27 (Life Technologies, Inc.).

Infection of Primary Neuronal Precursor Cells with Recombinant Adenovirus Vectors—For virus infection, cortical progenitor cells were plated in four-well Nunc tissue culture dishes coated with poly-L-ornithine/laminin in 400 µl of plating medium. After 48 h, another 400 µl of medium containing the appropriate titer of the adenovirus recombinant vector was added to each well. Eighteen hours following infection, a half-medium change was carried out. The multiplicity of infection (m.o.i.) indicates the number of plaque-forming units added per cell. Neural stem cells were infected with adenovirus recombinants 3 days after trituration. The appropriate titer vector was added directly into each dish and incubated for 6 h, after which a complete medium change was carried out. The cells were centrifuged at 1000 × g, and the pellet was resuspended in fresh medium. Samples were harvested 72 h postinfection for immunoprecipitation.

Western Blot Analysis—For Western analysis, protein was harvested in lysis buffer A (50 mM HEPES, pH 7.8, 250 mM KCl, 0.1 mM EDTA, 0.1% Triton X-100, 10% glycerol, 0.1% Nonidet P-40, 1.0 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 2 µg/ml leupeptin, 0.4 mM sodium vanadate) for 20 min on ice, followed by a 10-min micro centrifuge centrifugation. Protein was separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking (5% skim milk), membranes were incubated in the primary antibody for 1–2 h at room temperature. After three 5-min washes in TBPS (100 mM Na2HPO4, 100 mM NaH2PO4, 0.5% NaN3, 0.1% Tween 20), membranes were incubated for 1 h at room temperature in the secondary horseradish peroxidase-conjugated antibody (anti-mouse HRP, Bio-Rad catalog number 170-6515; anti-rabbit HRP, Bio-Rad catalog number 170-6515). Blots were developed with chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Immunoprecipitations—Neural stem cells expressing the dominant negative CDK mutants were harvested 72 h postinfection in lysis buffer B (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1% Triton X-100, 1.0 mM dithiothreitol, 0.5 mM PMSF, 5 µg/ml aprotinin, 2 µg/ml leupeptin, and 0.4 mM sodium vanadate) for 20 min on ice. Cytoplasmic lysates containing 100 µg of protein, as well as a control sample containing beads in lysis buffer only, were rotated overnight at 4 °C with anti-FLAG M2-conjugated agarose beads. The beads were washed in lysis buffer three times and then eluted by boiling for 2 min in SDS-polyacrylamide gel electrophoresis loading buffer. The eluate was then used for Western analysis with antibodies directed against the appropriate cyclins and CKIs as noted in the legends to Figs. 8 and 9.

Antibodies—The following primary antibodies were used: anti-β-galactosidase mouse monoclonal antibody (Promega, Z3789), mouse monoclonal M2 anti-FLAG antibody (Sigma, F3165) and beads (Sigma, A1205), biotinylated M2 anti-FLAG antibody (Sigma, F9291), mouse monoclonal anti-p27 (Transduction Laboratories, R25020), mouse monoclonal anti-BrdUrd (Becton Dickinson, 347580), and mouse monoclonal anti-Rb (PharMingen, 14001A). All other antibodies were purchased from Santa Cruz Biotechnology Inc.: mouse monoclonal anti-p16 (sc-1661), rabbit polyclonal anti-p21 (sc-9397), rabbit polyclonal anti-CDK2 (sc-163), rabbit polyclonal anti-CDK4 (sc-7499), rabbit polyclonal...
anti-CDK5 (sc-173), rabbit polyclonal anti-CDK6 (sc-177), mouse monoclonal anti-cyclin D1 (sc-6281), rabbit polyclonal anti-cyclin D2 (sc-593), rabbit polyclonal anti-cyclin D3 (sc-182), rabbit polyclonal anti-cyclin A (sc-596), and rabbit polyclonal anti-cyclin E (sc-481).

RESULTS

Regulated Expression of CDK, Cyclin, and CKI Proteins in Differentiating Cortical Progenitor Cells—Prior to assessing the role of the Rb pathway in cell cycle regulation of neural precursor cells, Western analysis was conducted to characterize the expression of the cyclin-dependent kinases, cyclins, and cyclin-dependent kinase inhibitors during the time course of differentiation. Stem cells, representing a highly proliferative population, and mature postmitotic neurons (E17) consisting of a quiescent population, were included at either end of time courses as a basis for comparison. First, the expression patterns of the CDKs were examined during the differentiation of cortical progenitor cells. As the population undergoes differentiation in vitro, CDK2 protein gradually becomes down-regulated to very low levels (Fig. 1A). Similarly, CDK4 and CDK6 (Fig. 1, B and D, respectively) are highly expressed in proliferating neural precursor cells and protein levels decrease as neurons develop. In contrast, the expression of CDK5, which is highly expressed in neuronal cells and not believed to function in the regulation of cell cycle (28, 29), is increased during the time course of differentiation, coincident with the appearance of postmitotic neurons in these cultures (Fig. 1C). Thus, in cortical progenitor cells, CDK expression is such that the levels of CDK2, CDK4, and CDK6 become down-regulated to low levels while CDK5 is induced as neurons are generated.

We next examined the expression of cyclins during the time course of neurogenesis (Fig. 2). The D cyclins, including cyclins D1, D2, and D3, are all expressed during neuronal differentiation in vitro; however, distinct differences in their regulation were apparent. Cyclin D1 is present at relatively low levels in proliferating cells but becomes up-regulated as cells undergo differentiation, with highest expression in differentiated, postmitotic neurons (Fig. 2A). In contrast, cyclin D2 is highly expressed in proliferating cells but becomes down-regulated during differentiation, although detectable levels remain in neuronal cultures (Fig. 2B). Cyclin D3 is readily detectable both in differentiated and undifferentiated cells (Fig. 2C). Cyclin E, a CDK2 binding partner, is expressed throughout the time course of differentiation (Fig. 2D). However, cyclin A, similar to the pattern observed with its binding partner CDK2, is expressed at high levels in proliferating cells and becomes down-regulated as neurons are generated (Fig. 2E).

While CDK activity is regulated at several levels including the differential expression of the cyclin binding partners as
Cortical progenitor cells were cultured from wild type E12.5 embryos and allowed to differentiate over 7 days in vitro. Self-renewing neural stem cells (St) and E17 postmitotic cortical neurons (N) cultured for 7 days were included for comparison. After days 0 (initial isolation), 1, 3, 5 and 7, protein was extracted, and 30 μg was loaded into each lane. Western blots were probed with antibodies against p16 (A), p21 (B), and p27 (C).

**Fig. 3.** Cyclin-dependent kinase inhibitor protein expression during neuronal differentiation. Cortical progenitor cells were cultured from wild type E12.5 embryos and allowed to differentiate over 7 days in vitro. Self-renewing neural stem cells (St) and E17 postmitotic cortical neurons (N) cultured for 7 days were included for comparison. After days 0 (initial isolation), 1, 3, 5 and 7, protein was extracted, and 30 μg was loaded into each lane. Western blots were probed with antibodies against p16 (A), p21 (B), and p27 (C).

Well as the expression of the kinase itself, CKIs also play key roles in cell cycle regulation during neural differentiation (30, 31). We therefore examined the expression of the INK4 CKIs p16 as well as the CIP/KIP kinase inhibitors p21 and p27 (Fig. 3). p16 is expressed in neural precursor cells and is up-regulated as cells undergo differentiation (Fig. 3A). p27 exhibits a similar expression pattern, showing low levels in proliferating neural precursor cells that increase as differentiation proceeds (Fig. 3C). In contrast, p21 shows highest levels in rapidly proliferating neural stem cells, which gradually decrease during the time course of differentiation to low or undetectable levels in postmitotic neurons (Fig. 3B). The unexpected pattern of p21 expression, particularly the high levels in neural stem cells, is consistent with the suggestion that p21 has functions beyond that of inhibition of cyclin-dependent kinase activity (20, 21). Western analysis indicated that Rb is present at high levels in proliferating neural precursors and decreases as neurogenesis proceeds (Fig. 4).

**Dominant Inhibitory Mutants of CDK2, CDK4, and CDK6 Induce Growth Arrest in Neural Precursor Cells**—To determine which CDKs are important in the regulation of cell cycle progression in primary neural precursor cells, we constructed recombinant adenovirus vectors carrying FLAG-tagged dominant-negative mutants of CDK2, CDK4, and CDK6 (dnCDK2, dnCDK4, and dnCDK6). These dominant inhibitory mutants have been previously described (12) and contain a point mutation in the kinase region thereby rendering them inactive. These mutants retain the ability to bind cyclins, thus they will inactivate the function of endogenous CDKs by sequestering their required cyclins. To verify that adenovirus recombinants could express the CDK mutant proteins, neural progenitor cells were infected at 50 m.o.i., and Western analysis was carried out 72 h after viral infection. Under these infection conditions, the possibility of viral-mediated effects on cellular function and viability would be minimal (32). Western analysis using antibodies specific for lacZ, CDK2, CDK4, and CDK6 confirmed expression of the appropriate CDK mutants in cortical progenitor cells (Fig. 5). Protein expression was also readily detectable by immunostaining with an anti-FLAG antibody (see below).

Previous studies have shown that the dominant inhibitory mutant of CDK2 was capable of inducing growth arrest in several cell lines, whereas mutants of CDK4/6 were ineffective (12). As these studies were carried out in tumor cell lines, we questioned whether the activities of CDK4/6, which function through the Rb pathway, may be important in cell cycle regulation in primary neural progenitor cells. Wild type neural precursor cells were infected after 2 days in vitro, and BrdUrd incorporation was measured 72 h thereafter. Since viral infections at 50 m.o.i. resulted in less than 20% infection, cells were first stained with anti-FLAG to identify expressing cells followed by immunostaining with anti-BrdUrd (Fig. 6A). The number of FLAG-expressing cells that were BrdUrd-positive were counted, and the rate of BrdUrd incorporation was expressed as a percentage of infected cells. The percent BrdUrd incorporation was then compared with lacZ-expressing controls (Fig. 6B). In contrast to lacZ-expressing cells, which showed a significant rate of BrdUrd incorporation (Fig. 6A), there was very little BrdUrd incorporation in cells expressing dnCDK2 (Fig. 6A). Quantification of these results indicated that 31 ± 3% BrdUrd incorporation was found in lacZ controls, whereas in dnCDK2 expressing cells, only 2 ± 1% were BrdUrd-positive. Consistent with previous studies (12), our results indicate that dnCDK2 can efficiently induce growth arrest in cortical progenitor cells.

We next questioned whether dnCDK4 and dnCDK6 could...
also induce growth arrest in neural precursor cells. Examination of cortical progenitor cells expressing either dnCDK4 or dnCDK6 revealed that the majority of cells transduced were growth arrested and did not enter S phase (Fig. 6). Cell counts revealed that relative to 31 ± 3% in \textit{lacZ}-expressing controls, cells expressing dnCDK4 and dnCDK6 exhibited only 8 ± 2% and 7 ± 2% BrdUrd incorporation, respectively (Fig. 6B). These results indicate that dominant negative mutants, which specifically block the activity of CDK4/6, are sufficient to induce growth arrest in neural precursor cells. While no such inhibition was seen in previous studies using cell lines, our results suggest that this is an important growth regulatory pathway and that CDK4/6 activation is essential for cell cycle progression in primary neural progenitor cells.

Previous studies have shown that cell cycle regulation by CDK4/6 functions primarily through Rb phosphorylation (16–18, 33); therefore, overexpression of such mutants in Rb deficient cells should have no effect. As a control to verify that the dnCDK4/6 mutants are appropriately targeting Rb, we repeated the above experiment in Rb-deficient cortical progenitor cells (Fig. 7A). In the absence of Rb, cells expressing \textit{lacZ} incorporated BrdUrd at a higher rate of 43 ± 6% relative to 31 ± 3% measured in wild type cells (Fig. 7B). Cells expressing dnCDK2 continued to show a decrease in BrdUrd incorporation despite the absence of Rb (Fig. 7B). The ability of dnCDK2 to decrease BrdUrd incorporation relative to control cultures was nevertheless statistically significant. This suggests that in neural precursor cells, CDK2 function is partially Rb-dependent; however there are also other targets. Consistent with Rb as the sole target for CDK4/6, the corresponding dominant negative mutants had no effect on cell growth (Fig. 7). While \textit{lacZ}-expressing cells exhibited 43 ± 6% BrdUrd incorporation, dnCDK4- and dnCDK6-expressing cells showed 40 ± 4% and 46 ± 3% incorporation, respectively (Fig. 7B). These results indicate that recombinant adenovirus vectors carrying the dnCDK mutants are functioning appropriately when overexpressed in neural precursor cells.

**FIG. 6.** Dominant negative mutants of CDK2, CDK4, and CDK6 induce growth arrest in cortical progenitor cells. A, cortical progenitor cells cultured from wild type E12.5 embryos were infected at 50 m.o.i. with Ad\{\textit{lacZ}\} (panels a–c), Ad\{dnCDK2\} (panels d–f), Ad\{dn-CDK4\} (panels g–i), and Ad\{dnCDK6\} (panels j–l) 2 days after plating. BrdUrd was added to the culture medium 18 h prior to fixation. At 72 h following infection, cells were fixed and labeled with anti-\textit{lacZ} (panel a) or anti-FLAG (panels d, g, and j) and anti-BrdUrd (panels b, e, h, and k). Panels displaying \textit{lacZ}/FLAG and BrdUrd labeling were superimposed (panels c, f, i, and l). Arrows point to representative cells. Bar = 50 μm. B, quantification of BrdUrd incorporation. Averages were obtained from eight separate cell cultures (n = 8). Error bars denote S.E.
In view of the biological impact of the dominant inhibitory CDK mutants in cortical progenitor cell cycle regulation, we next sought to verify that the CDK mutants were functioning as predicted and thereby rule out the possibility that overexpression may lead to binding of inappropriate cyclin partners. The dnCDK mutants were expressed in neural stem cells to examine their binding specificities. Stem cells lend themselves readily for immunoprecipitation experiments, because they yield high quantities of tissue and can be readily manipulated with adenovirus vectors such that 60–80% of cells express the exogenous protein. Cells were transduced with lacZ, dnCDK2, dnCDK4, or dnCDK6 at 250 m.o.i. and harvested 72 h after infection. Lysates were prepared for immunoprecipitation with anti-FLAG beads to precipitate the mutant CDK complexes. The immunoprecipitates were examined for CDK-associated proteins by Western analysis. The membranes were subsequently probed for cyclin D3, a physiological binding partner for CDK4/6 and cyclins A and E, which bind CDK2 in vivo, and all of which are endogenously expressed at high levels in neural stem cells (Fig. 2, C, D, and E). Cyclin D3 was detected in immunoprecipitates from stem cells expressing dnCDK4/6, but not in cells expressing dnCDK2 or lacZ (Fig. 8A). Similarly, cyclins A and E were associated with their appropriate partners, the dnCDK2 but not dnCDK4/6 (Fig. 8, B and C). These results demonstrate that despite their overexpression, the dominant negative mutants interact with their appropriate cyclin partners in neural precursor cells.

Although it is known that CDK4/6 function through the regulation of Rb activity by phosphorylation, recently studies suggest that these proteins have an additional function in the
sequestration of the KIP family of CKIs. These CKIs have been shown to have no inhibitory effects on CDK4/6 activity but merely function to stabilize the CDK-cyclin complex (19–21). One possible explanation for the cell cycle inhibition of CDK4/6 is that they are no longer able to bind p21 or p27 efficiently, thereby rendering them available to inhibit the activity of CDK2. We therefore examined whether or not the CDK mutants could interact with the CIP/KIP CKIs, p21 and p27. Immunoprecipitations demonstrated that p21 was present primarily in complexes with dnCDK4/6 but not in complexes with dnCDK2 or lacZ. Similarly, cyclins A and E were detected in complexes with dnCDK2, but not in complexes with dnCDK4/6 or lacZ.

**DISCUSSION**

The onset of pan-neuronal gene expression is closely tied to terminal mitosis (1–4). Understanding the molecules that regulate cell cycle is key to delineating the mechanisms underlying neurogenesis. While studies with RB-deficient mice have demonstrated the importance of this tumor suppressor in nervous system development (5–7), there is little evidence that RB or its upstream regulators can induce cell cycle arrest. In the present study, we demonstrate that the RB pathway is a critical regulator of cell cycle progression in primary neural progenitor cells. In contrast to previous studies (12), we show that dominant negative mutants of CDK4 and CDK6 can induce mitotic arrest in neural precursor cells, despite the binding and sequestration of the KIP CKIs p21 and p27. The activity of these mutants is dependent on the presence of RB, indicating that the activation of the RB pathway is sufficient to cause cell cycle arrest in primary neural precursor cells.

The restriction point of the cell cycle is defined as the time in G1 after which cells no longer require growth factors to enter S phase (36). Studies indicate that CDK2 and CDK4/6 function at two distinct regulatory time points during the G1 phase of the cell cycle. CDK4/6, along with D cyclins, function through the inactivation of Rb early in G1 to regulate the restriction point transition (13–15, 17). The passage through the restriction point and the up-regulation of D cyclins is mitogen-dependent and commits the cell to enter S phase (37, 38). CDK2, with cyclins A and E, regulates the cell cycle later in G1, at which time the cell is committed to undergo the G1/S phase transition (39, 40). Previous studies have shown that dominant negative mutants of CDK2, but not CDK4/6, result in cell cycle arrest in several cell lines (12). These results are consistent with the interpretation that CDK2 activity may be crucial for G1/S phase transition, while CDK4/6 may play a more important role in regulating entry into G1. Indeed it has been suggested that CKIs 4/6 may have dual functions, including the regulation of RB phosphorylation as well as the sequestration of CKIs p21 and p27 to prevent the inhibition of CDK2 activity. It is believed that this latter function may be a crucial one in the regulation of the G1/S transition (19–21).

To determine which CKIs are important cell cycle regulators in neural precursor cells, we delivered dominant negative CDK mutants using adenovirus vectors. Delivery of the dnCDK2 mutant kinase results in a striking growth arrest in neural precursor cells, represented by a 94% decrease in BrdUrd incorporation relative to the lacZ-expressing controls. Unlike previous results with cell lines (12), dnCDK4/6 were also efficient at blocking cell cycle progression, as cells expressing these mutants exhibited a 74% decrease in BrdUrd incorporation. These results indicate that in addition to the established role for CDK2 in regulating cell cycle progression, CDK4/6 activity is also essential for neural precursor cells to enter S phase. To verify that such mutants behave appropriately, we expressed dnCDK4/6 in RB-deficient progenitor cells, and as predicted, our results demonstrated that the cell growth regulation by CDK4/6 activity was entirely dependent on the RB pathway. In contrast, the expression of dnCDK2 in RB null progenitor cells could cause a significant reduction in BrdUrd incorporation relative to lacZ-expressing control cells. This effect, however, did not appear to be as efficient in the absence of RB, suggesting that CDK2 functions partially through the RB pathway as well as additional targets. It is unlikely that cell cycle growth arrest is due to toxic effects resulting from adenovirus infection, because cells expressing Ad[lacZ] exhibited BrdUrd incorporation at a rate typically found in untreated progenitor cells after 5 days in vitro (10), and delivery of dnCDK4/6 to RB-deficient precursor cells had no effect of the rate of BrdUrd incorporation. In addition, the low viral titers and short time courses would minimize the likelihood of viral-mediated effects...
(32). By examining the composition of the CDK complexes we have confirmed that the overexpressed CDK mutants function in the expected manner with respect to retaining their physiological binding specificities to their cyclin partners (Fig. 9). These results suggest that CDK4/6 activity is critical for cell cycle progression in primary neural precursor cells.

Previous studies involving microinjection of cyclin D1 antibodies into primary fibroblasts have demonstrated growth arrest in an Rb-dependent manner (33). Consistent with our results, this suggests that CDK4/6 regulation is critical for cell cycle transition in primary cells. It has been suggested, however, that cyclin D1 antibodies may have also caused the displacement of the CIP/KIP CKIs, thereby leading to CDK2 inhibition, which was the key effector in cell growth arrest. As CDK4/6 have been shown to interact with the CIP/KIP family of CKIs, p21 and p27, one possible mechanism for CDK4/6-mediated growth arrest is the aberrant displacement of p21 and p27, rendering them available to inhibit CDK2. Thus, the observed growth arrest could actually result from the indirect inhibition of CDK2 activity. To address this question in our studies, we performed immunoprecipitations of the dnCDK mutants to examine whether the ability to sequester these CKIs is retained. Our results demonstrate that both p27 and p21 are highly associated with dnCDK4/6 complexes. This indicates that the dnCDK4/6 mutants retain the ability to bind the CIP/KIP CKIs and can arrest cell growth despite this continued association. Clearly, inhibition of the CDK4/6 kinase activity is essential for S phase entry, and Rb is the target for this activity.

The results of the present study demonstrate that CDK4/6 activity, specifically their ability to phosphorylate Rb, is critical for cell cycle transition in primary neural precursor cells. As previous studies were conducted in tumor cells, which pass through the G1 phase of the cell cycle in a mitogen-independent manner, the early G1 phase of the cell cycle may be nonfunctional in this cell type. In primary cells, it appears that both CDK4/6 and CDK2 activity is required to allow the cells to undergo S phase. The requirement of two independent pathways would seem evolutionarily favorable in primary cells for the protection against neoplastic transformation during their normal development.

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