Inhibition of Cyclin-dependent Kinase Activity Triggers Neuronal Differentiation of Mouse Neuroblastoma Cells

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Abstract. Studies on the molecular mechanisms underlying neuronal differentiation are frequently performed using cell lines established from neuroblastomas. In this study we have used mouse N1E-115 neuroblastoma cells that undergo neuronal differentiation in response to DMSO. During differentiation, cyclin-dependent kinase (cdk) activities decline and phosphorylation of the retinoblastoma gene product (pRb) is lost, leading to the appearance of a pRb-containing E2F DNA-binding complex. The loss of cdk2 activity is due to a decrease in cdk2 abundance whereas loss of cdk4 activity is caused by strong association with the cdk inhibitor (CKI) p27^{KIP1} and concurrent loss of cdk4 phosphorylation. Moreover, neuronal differentiation can be induced by overexpression of p27^{KIP1} or pRb, suggesting that inhibition of cdk activity leading to loss of pRb phosphorylation, is the major determinant for neuronal differentiation.

THE retinoblastoma susceptibility gene, *RB1*, is essential for murine neurogenesis (Clarke et al., 1992; Jacks et al., 1992; Lee et al. 1992) and is highly expressed in cell types undergoing neuronal differentiation in vivo and in vitro (Slack et al., 1993; Kranenburg et al. 1994; Lee et al., 1994). In *RB1*-deficient mouse embryos both the central and peripheral nervous systems are grossly abnormal. In regions where only postmitotic cells are found in wild-type (wt)[1] animals, many cells attempt to divide and subsequently die in Rb$^{-/-}$ mice (Lee et al., 1992, 1994), suggesting that these cells require retinoblastoma gene product (pRb) for cell cycle arrest and survival. In addition, loss of *RB1* affects the ability of neuronal cells to differentiate properly. These results demonstrate that pRb not only suppresses cell growth, but may also help to establish neuronal differentiation.

The molecular mechanism by which pRb inhibits cell proliferation is becoming increasingly clear. pRb inhibits the activity of proteins that function as inducers of DNA synthesis. Among these proteins are members of the E2F family of transcription factors (Hamel et al., 1992; Flemington et al., 1993; Helin et al., 1993), and the tyrosine kinase c-Abl (Welch and Wang, 1993). Thus, pRb constrains cell growth by preventing S-phase entry (for review see Farnham et al., 1993; LaThangue, 1994).

The molecular mechanism(s) underlying pRb-mediated induction of terminal differentiation is less clear. During

myogenic differentiation pRb stimulates the formation of active heterodimeric transcription factors that drive muscle-specific gene expression (Gu et al., 1993). However, additional differentiation pathways are likely to be regulated by pRb, since loss of *RB1* affects the differentiation state of neurons, lens fibre cells, retina cells and cells from the cerebellar cortex (Lee et al., 1994; Morgenbesser et al., 1994; Robanus-Maandag et al., 1994; Williams et al., 1994). Furthermore, the fact that in an Rb$^{-/-}$ background, p107 substitutes for pRb in the regulation of myogenesis, suggests that pRb may regulate differentiation pathways that remain unaffected in Rb$^{-/-}$ mice.

The activity of pRb both as a suppressor of cell growth and as an inducer of (myogenic) differentiation is impaired by phosphorylation (reviewed by Sherr, 1994; Gu et al., 1993). During terminal differentiation of a number of cell types in vitro, underphosphorylated pRb accumulates and the hyperphosphorylated forms of pRb are lost (Chen et al., 1989; Mihara et al., 1989). The activity of pRb kinases is therefore likely to be impaired during terminal differentiation. Cyclin D- and E-dependent kinases are thought to cooperate in the hyperphosphorylation of pRb in cycling cells (Sherr, 1994). Indeed, during various differentiation pathways the expression levels of cyclins or cyclin-dependent kinase's (cdk) decline, resulting in loss of cdk activity (Kato and Sherr, 1993; Kiyokawa et al., 1994; Rao et al., 1994; Kiess et al., 1995). The importance of cdk-inactivation during terminal differentiation is illustrated by the fact that artificially sustained cdk activity through overexpression of cyclins or cdk's is sufficient to prevent differentiation (Kato and Sherr, 1993; Kiyokawa et al., 1994; Rao et al., 1994). In the present study we have used the mouse N1E-115 neuroblastoma cell line as an in vitro model system for neuronal differentiation. N1E-115 cells

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1. *Abbreviations used in this paper*: bHLH, basic helix-loop-helix; cdk, cyclin-dependent kinase; PAA, polyacrylamide; PCNA, proliferating cell nuclear antigen; pRb, retinoblastoma gene product; wt, wild-type.

proliferate in normal culture medium but undergo neuronal differentiation in response to DMSO, cAMP, or serum withdrawal (Kimhy et al., 1976; reviewed by De Laat and Van der Saag, 1982; Reagan et al., 1990; Larcher et al., 1992). Upon induction of differentiation, proliferation of N1E-115 cells ceases, extensive neurite outgrowth is observed and the membranes become highly excitable (Kimhy et al., 1976; De Laat and Van der Saag, 1982). Thus, N1E-115 cell differentiation is an excellent in vitro model system for studying neuronal differentiation. We show that both cdk2 and cdk4 activities strongly decline during neuronal differentiation albeit via different mechanisms: the abundance of cdk2 drops to undetectable levels whereas cdk4 remains present but is inactivated due to association with p27^{KIP1}. Moreover, overproduction of p27^{KIP1} or pRb efficiently induces differentiation in neuronal precursor cells, implying that down-regulation of cdk activity or overproduction of pRb is sufficient to switch from proliferation to differentiation.

Materials and Methods

Cells, Cell Culture, Transfection, and Differentiation Protocols

N1E-115 mouse neuroblastoma cells (kindly provided by Dr. R. P. de Groot) were cultured in DMEM containing 2% FCS, supplemented with antibiotics. Transient transfections were performed by overnight exposure of the cells to calcium phosphate-DNA precipitates as described (van der Eb and Graham, 1980). The cells were then washed three times with PBS and received fresh medium. Transfection efficiencies of approximately 50% are routinely obtained using this procedure on N1E-115 cells. Expression vectors for pRb (pRcCMV-Rb) and p27^{KIP1} (pCMVX-p27^{KIP1}) were kindly provided by Drs. R. Bernards (Cancer Institute, Netherlands) and T. Hunter (The Salk Institute, La Jolla, CA), respectively.

For differentiation, cells were cultured in DMEM/FCS (2%) supplemented with 1.25% DMSO. Cell cultures were then analyzed for four consecutive days.

Western Blot Analysis

Cells were washed twice with PBS, pelleted, and lysed in ice-cold lysis buffer (50 mM Tris pH 7.4, 50 mM NaCl, 0.5% NP-40, 0.1% SDS supplemented with 1 mM phenylmethylsulfonylfluoride, 0.1 mg/ml trypsin inhibitor 0.1 mg/ml NaF, 0.1 mg/ml β-glycerophosphate, 0.1 mg/ml Na$_3$VO$_4$ and 1 mg/ml Na$_4$P$_2$O$_7$). After 30-min incubation on ice, lysates were centrifuged and protein concentrations were determined with the Bio-Rad assay kit. 50 µg of the cell extracts were boiled for 5 min in sample buffer and were subsequently run out on 12 or 8% SDS–polyacrylamide (PAA) gels. The proteins were blotted onto Immobilon-I (Millipore).

First antibodies were anti-cyclin D1/D2 (polyclonal rabbit IgG; UBI, Lake Placid, NY), anti-cyclin D3 (18B6-10; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclin E (C19; Santa Cruz), anti-cdk2 (rabbit polyclonal antiserum; Kranenburg et al., 1995), anti-cdk4 (C22; Santa Cruz), anti-cdc2 (C17; Santa Cruz), and anti-hRb (G3-245; PharMingen, San Diego, CA). The second antibodies were horseradish peroxidase-conjugated anti-mouse, -rabbit, or -rat IgG (Jackson ImmunoResearch Labs., Inc., West Grove, PA). Signals were visualized with the Amersham ECL detection system according to the manufacturer's protocol.

Metabolic Cell Labeling

Cell cultures were rinsed in either PBS or phosphate-free medium and were labeled for 3 h with 150 µCi [^{35}S]methionine or 1 mCi [^{32}P]orthophosphate (Amersham Corp., Arlington Heights, IL). Label medium was aspirated and the cells were washed once with ice-cold PBS. Cells were then scraped in ice-cold i.p. buffer (Western lysis buffer without SDS) and left on ice for 30 min. The lysates were then cleared by centrifugation and the incorporated label was determined by liquid scintillation counting. Equal amounts of extract were then used for immunoprecipitation as described below.

Immunoprecipitation

Cells were washed with ice-cold PBS, pelleted, and lysed in ice-cold i.p. buffer. After a 30-min incubation on ice, lysates were centrifuged and protein concentrations were determined with the Bio-Rad assay kit. Labeled cell extracts were quantified by scintillation counting. 200 µg of cell extracts were incubated with antibody-coated protein A–Sepharose beads under constant rotation. Immunocomplexes were collected by centrifugation and were washed four times with lysis buffer. Immunocomplexes, after boiling in sample buffer, were run out on SDS-PAA gels. The [^{35}S] gels were incubated in PPO-DMSO and were subsequently dried. Proteins were visualized by autoradiography. When unlabeled cell extracts were used, the presence of associating proteins was analyzed by Western blotting.

Immunokinase Assays

Cdk2 was immunoprecipitated from 50 µg cell extracts as described above. The complexes were assayed for kinase activity towards histone H1 as follows. Immunocomplexes were washed three times with lysis buffer and once with kinase buffer (20 mM Tris, pH 7.4, 10 mM MgCl$_2$, 1 mM DTT). Subsequently, the beads were resuspended in 50 µl kinase buffer containing 2 µg histone H1, 10 µCi [γ-^{32}P]ATP and 20 µM ATP. The reaction mixtures were incubated at 30°C for 30 min. Reaction mixtures were boiled in sample buffer and run on SDS-PAA gels. Gels were stained with Coomassie blue, dried, and autoradiographed. Quantitation of the signals was performed by phosphorimaging. Cdk4 was immunoprecipitated from 200 µg cell extracts, prepared as described (Matsushime et al., 1994). The immunocomplexes were assayed for kinase activity towards soluble GST-Rb that was purified from bacteria. The beads were washed three times with lysis buffer and once with kinase buffer (50 mM Hepes, pH 7.4, 10 mM MgCl$_2$, 1 mM DTT). Subsequently, the beads were resuspended in 50 µl kinase buffer containing 0.5 µg GST-Rb, 10 µCi [γ-^{32}P]ATP and 20 µM ATP. Reaction mixtures were then incubated at 30°C for 1 h. Gels were stained with Coomassie blue, dried, and autoradiographed.

E2F Gel Shift

Cells were scraped in ice-cold PBS, centrifuged, and taken up in two packed cell volumes of lysis buffer (400 mM KCl, 20 mM Hepes, pH 7.6, 1.5 mM MgCl$_2$, 0.5 mM NaF, 1 mM DTT, 1 mM PMSF, 0.1 mg/ml trypsin inhibitor, and 1 mg/ml NaVO$_4$). Cell suspensions were frozen and subsequently thawed on ice. After centrifugation for 20 min at 13,000 rpm, the concentrations of the cell extracts were determined and 10 µg of extract was assayed for binding activity to a ^{32}P-labeled double-stranded oligonucleotide (5'-ACTAGTTTCGCGCCCTTTA-3') harboring an E2F binding site. The binding reaction was performed for 30 min at room temperature in 20 µl buffer containing 50 mM KCl, 20 mM Hepes, pH 7.6, 10 mM MgCl$_2$, 10% glycerol, 0.5 mM DTT, 0.1% NP40, 2 µg sonicated salmon sperm DNA, 1 ng of labeled oligo, supplemented with 1 mM PMSF, and 0.1 mg/ml trypsin inhibitor. DNA-protein complexes were separated on a 4% nondenaturing PAA gel and visualized by autoradiography. For supershifts the extracts were incubated for 20 min at room temperature with 1 µg of purified IgG (anti-Rb, Ab2, or anti-PCNA, PC10; Oncogene Science, Inc., Manhasset, NY) before the binding reaction.

V8 Proteolytic Digestion

Radiolabeled proteins were excised from dried SDS-PAA gels. The slices were incubated for 10 min in the wells of the stacking gel in a buffer containing 0.1% SDS, 1 mM EDTA, 2.5 mM DTT, 0.125 M Tris, pH 6.8. The slice was subsequently overlayed with the same buffer containing 20% glycerol and 200 ng V8 protease. The gel was run for 30 min after which the power was shut off. After a 30-min incubation period the power was applied again. Normal procedures were then followed to visualize the degradation pattern.

Results

Neuronal Differentiation Is Accompanied by Changes in Cyclin and cdk Abundances, Loss of cdk2 Activity and Loss of pRb Phosphorylation

When cultured in the presence of DMSO, mouse N1E-115

neuroblastoma cells undergo neuronal differentiation. In the absence of DMSO these cells proliferate with a doubling time of ~16 h and have a relatively small and rounded appearance (Fig. 1 A). When cultured in the presence of DMSO, cell proliferation ceases (Fig. 1 B), cells flatten, enlarge, and extensive outgrowth of neurites is observed (Fig. 1 A). As neuronal differentiation requires a pRb-mediated cell cycle arrest and pRb function is inhibited by phosphorylation, down-regulation of pRb kinase activity is likely to be of major importance in the proliferation-differentiation switch. Therefore we analyzed the regulation of cyclins, cdks, and pRb during differentiation. During differentiation, the steady-state protein level of the proliferating cell nuclear antigen (PCNA) strongly declines, confirming cessation of cell proliferation (Figs. 1 B and 2). Similarly, the abundance of cyclin D1 declines, whereas cyclin D2 is undetectable and the level of cyclin D3 strongly increases (Fig. 2). The steady-state protein level of the kinase partner for D-type cyclins, cdk4, remains constant throughout the differentiation process (Fig. 2). In addition, the level of cyclin E remains constant, but the level of its kinase partner, cdk2, strongly declines (Fig. 2). Concomitant with the decline in cdk2 abundance, phosphorylation of pRb is lost: in exponentially growing cells the underphosphorylated, rapidly migrating species of pRb (Fig. 2, *lower arrow*) is hardly detectable, whereas

two days after induction of differentiation this form of pRb is, and remains, predominant. The level of pRb does not change significantly (Fig. 2). These results are in agreement with previous studies showing the accumulation of underphosphorylated pRb and the decrease in cdc2 and cdk2 abundance in cells undergoing neuronal differentiation in vivo and in vitro (Gaetano et al., 1991; Slack et al., 1993; Lee et al., 1994; Buchkovic and Ziff, 1994; Yan and Ziff, 1995).

Loss of pRb Phosphorylation Leads to the Appearance of a pRb/E2F Complex

Suppression of cell growth by pRb is mediated (in part) through complex formation with E2F transcription factors, leading to their inactivation (Farnham et al., 1993; LaThangue, 1993). We analyzed whether the loss of pRb phosphorylation in response to the differentiation signal (Fig. 2) would lead to the appearance of E2F complexes containing pRb. To this end we prepared extracts of differentiating cells and assayed these extracts for the presence of complexes that could bind to an E2F consensus site. As shown in Fig. 3, one predominant complex is detected in extracts prepared from exponentially growing cells. However, simultaneously with the appearance of underphosphorylated pRb (Fig. 2, day 1), a second, more slowly migrating, E2F complex is observed. To confirm the presence of pRb in the slower migrating complex we incubated the extracts with anti-pRb or control antibodies prior to the binding reaction. Fig. 3 shows that the anti-pRb antibody, but not the control antibody (anti-PCNA), supershifts the slower migrating complex, demonstrating the presence of pRb in this complex. Antibodies directed against cyclin D3 did not affect the migration pattern of the E2F complexes (not shown). These results show that loss of pRb phosphorylation leads to complex formation between pRb and E2Fs, presumably contributing to growth arrest.
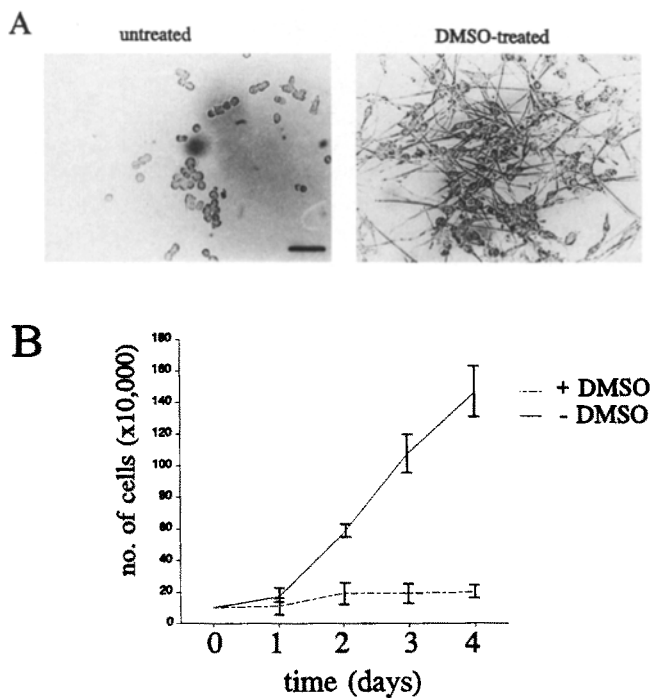

Figure 1. Morphology and growth characteristics of N1E-115 cells. *(A)* Untreated N1E-115 cells are relatively small and have a rounded appearance. After four days of culturing in the presence of 1.25% DMSO the cells have enlarged, flattened, and extensive outgrowth of neurites is observed. *(B)* Proliferation of N1E-115 cells in normal and differentiation-inducing medium. Cells (100,000) were seeded in 5-cm dishes and the cells were counted for four consecutive days, using a Burker chamber. The values are means and standard deviations of three independent experiments. Bar, 100 μm.
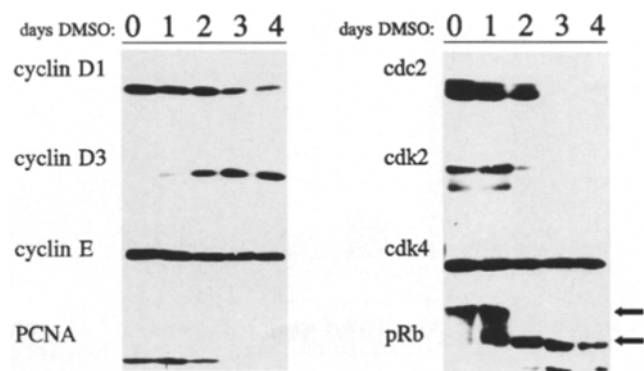

Figure 2. Western blot analysis of cyclins, cdks and pRb. Terminal differentiation of N1E-115 cells was induced by addition of DMSO and cells were harvested with 1-d intervals for four consecutive days. The abundance of the indicated proteins was analyzed using specific antibodies (see Materials and Methods). Note the difference in electrophoretic mobility between hyperphosphorylated pRb (*upper arrow*) and underphosphorylated pRb (*lower arrow*). The loss of pRb phosphorylation is concomitant with the decline in cdk2 abundance.
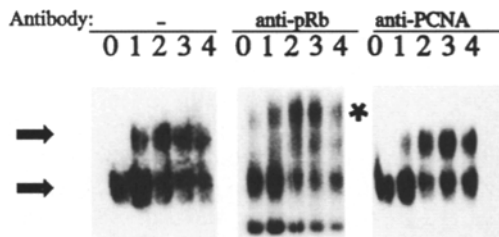
Figure 3. E2F DNA-binding complexes during terminal neuronal differentiation. Extracts from differentiating N1E-115 cells were analyzed for the presence of complexes that can bind to the consensus E2F sequence. Untreated cycling cells contain one predominant complex (*bottom arrow*). Concomitant with the appearance of underphosphorylated pRb, a second more slowly migrating complex is detected (*top arrow*). Pre-incubation of the extracts with an antibody directed against pRb, but not antibodies directed against PCNA, or cyclins A and D3 (not shown) supershifts the upper complex (*), demonstrating the presence of pRb in the upper complex.

Inhibition of cdk2 and cdk4 Activities via Different Mechanisms

As hyperphosphorylation of pRb occurs during a burst of cyclin E-dependent cdk2 activity late in G1, cdk2 activity may be the major determinant of the pRb phosphorylation state. We therefore assayed the cdk2 kinase activity during differentiation and found that concomitant with the disappearance of hyperphosphorylated pRb and of cdk2, cdk2 activity is lost (Fig. 4).

Although the loss of cdk2 activity may suffice in explaining the loss of pRb phosphorylation, cyclin D-dependent kinases have also been shown to induce phosphorylation of pRb (Sherr, 1994). As shown in Fig. 2, the cyclin D-dependent kinase cdk4 persists, and D-type cyclins (first D1, later D3) are available throughout the differentiation pro-
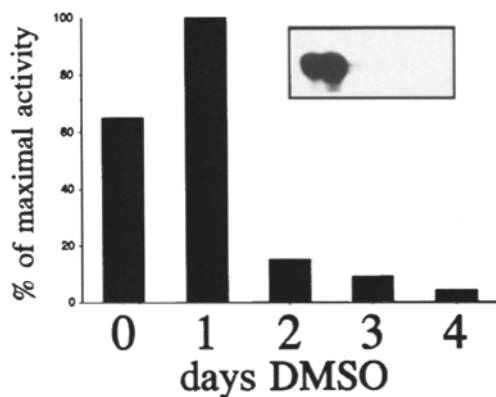
cess. Both cyclin D1 and cyclin D3 are capable of activating cdk4 towards pRb in in vitro kinase assays and when coexpressed in the baculovirus/insect cell overexpression system (Kato et al., 1993; Matsushime et al., 1994). Therefore we assessed whether the D-type cyclins complex with and activate cdk4 during neuronal differentiation. Cdk4 and cyclin D1 were immunoprecipitated from extracts prepared from differentiating cells and the immunocomplexes were subsequently analyzed for coprecipitating partners by Western blotting. As shown in Fig. 5, decreasing amounts of cdk4 coprecipitate with cyclin D1 and vice versa, demonstrating a decline in the amount of cyclin D1-cdk4 complexes. Furthermore, increasing amounts of cyclin D3 coprecipitate with cdk4 during differentiation, reflecting the increase in cyclin D3 abundance (Figs. 2 and 5). These results demonstrate that cdk4 is complexed to a D-type cyclin throughout the differentiation process. Apart from association to a cyclin partner, the activity of cdk4 is also determined by its phosphorylation state. Phosphorylation of cdk4 by the cdk-activating kinase (CAK) is a prerequisite for its activity (Kato et al., 1994a). We therefore analyzed the phosphorylation state of cdk4 during differentiation by immunoprecipitating the protein from extracts prepared from ^{32}P-labeled cells. As is shown in Fig. 6, phosphorylation of cdk4 is strongly diminished during differentiation (*top*), whereas the total amount of immunoprecipitated cdk4 does not change, as determined by subsequent Western blot analysis of the same gel (*bottom*). The observation that CAK-mediated cdk activation is inhibited by binding of the cdk inhibitor (CKI) p27[KIP1] to cyclin-cdk complexes (Kato et al. 1994a,b) prompted us to investigate whether the loss of cdk4 phosphorylation during differentiation is accompanied by increased binding of p27[KIP1]. Cdk4 was immunoprecipitated from extracts prepared from ^{35}S-labeled cells. As shown in figure 7 A, a 27-kD protein associates with cdk4 in differentiated, but not in undifferentiated cells. To confirm the identity of p27 we transfected undifferentiated cells with an expression vector encoding p27[KIP1] and found that the trans-
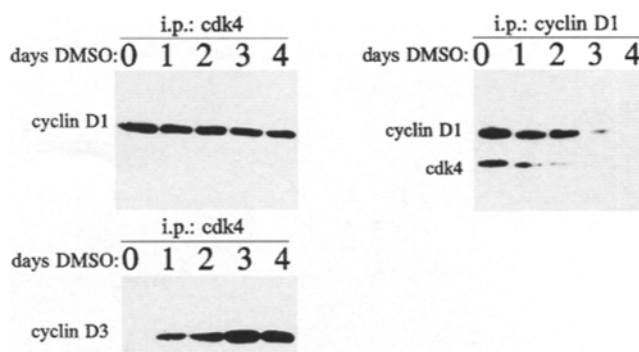

Figure 4. Cdk2 kinase activity during terminal neuronal differentiation. Cdk2 immunoprecipitates isolated from extracts prepared from differentiating cells, were tested for kinase activity towards histone H1. The reaction mixtures were separated on an SDS-PAA gel and the amount of incorporated label into histone H1 was subsequently analyzed by phosphorimaging. The data are represented as % of maximal activity. An autoradiogram of a representative experiment is shown in the inset. Note that the loss of activity is concomitant with the loss of cdk2 abundance and the loss of pRb phosphorylation (see Fig. 2).

Figure 5. Analysis of cdk4-containing complexes during differentiation. Cdk4 and cyclin D1 were immunoprecipitated from extracts prepared from differentiating cells. The immunocomplexes were then analyzed for the presence of associating proteins by Western blotting as indicated. The blot containing the cyclin D1 immunocomplexes was probed for the presence of both cyclin D1 and cdk4. The amount of cyclin D1-cdk4 complexes is clearly decreased, whereas the amount of cyclin D3-cdk4 complexes is strongly increased during differentiation.
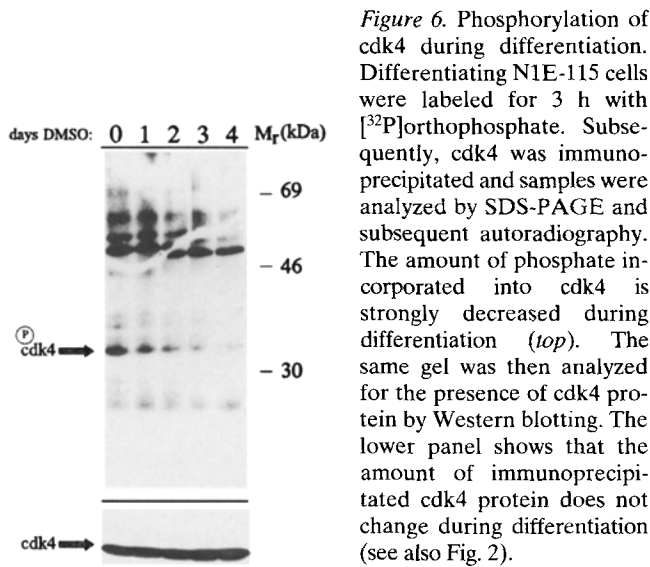
Figure 6. Phosphorylation of cdk4 during differentiation. Differentiating N1E-115 cells were labeled for 3 h with [^{32}P]orthophosphate. Subsequently, cdk4 was immunoprecipitated and samples were analyzed by SDS-PAGE and subsequent autoradiography. The amount of phosphate incorporated into cdk4 is strongly decreased during differentiation *(top)*. The same gel was then analyzed for the presence of cdk4 protein by Western blotting. The lower panel shows that the amount of immunoprecipitated cdk4 protein does not change during differentiation (see also Fig. 2).

fected protein precisely comigrated with p27 from differentiated cells, when analyzed by coimmunoprecipitation with cdk4 (Fig. 7 *A*). Furthermore, proteolytic digestion of the two proteins with V8 protease yields indistinguishable
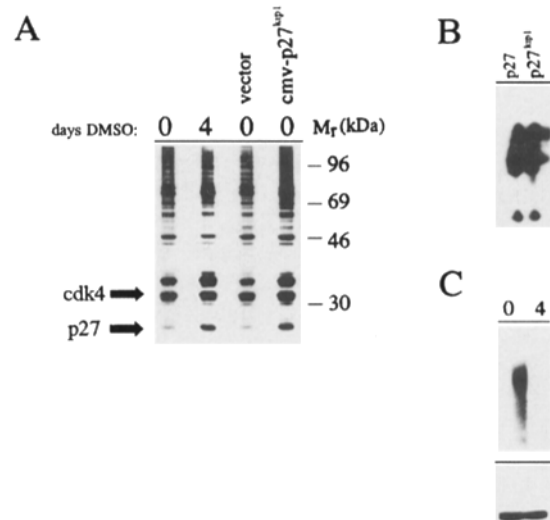

Figure 7. Regulation of cdk4 activity by p27^{KIP1}. *(A)* Cdk4 was immunoprecipitated from extracts prepared from differentiated neurons and untreated cycling cells labeled for 4 h with [^{35}S]methionine. A 27 kD protein associates to cdk4 in terminally differentiated neurons (4 d DMSO), but not in precursor cells (0 d DMSO). This 27-kD protein comigrates with p27^{KIP1} transfected into precursor cells and subsequently analyzed by coprecipitation with cdk4. The p27-kD band is not observed in cells transfected with the control vector. *(B)* The 27-kD protein isolated from neurons was subsequently compared with p27^{KIP1} by subjecting the two excised bands to proteolytic digestion with 200 ng V8 protease. The degradation patterns were then analyzed by SDS-PAGE and autoradiography. *(C)* Cdk4 was immunoprecipitated from terminally differentiated neurons (4 d DMSO) and from untreated precursor cells (0 d DMSO). The immunocomplexes were subsequently analyzed for kinase activity towards GST-Rb *(top)* in an in vitro kinase assay, and for the amount of cdk4 immunoprecipitated by Western blotting *(bottom)*. The experiment shows that although cdk4 is present in both extracts, it is only active when isolated from undifferentiated precursor cells.

degradation patterns, confirming that p27 is p27^{KIP1} (Fig. 7 *B*). The fact that cdk4 phosphorylation is lost and p27^{KIP1} strongly associates to cdk4 in differentiated neurons suggests that its activity may be inhibited, despite association to cyclin D3. We therefore tested the activity of cdk4 in undifferentiated and differentiated cells in an in vitro kinase assay. Fig. 7 *C (top)* shows that cdk4 immunocomplexes isolated from cycling precursor cells, but not from terminally differentiated neurons, efficiently phosphorylate GST-Rb, even though the amount of immunoprecipitated cdk4 was similar, as judged by Western blotting *(bottom)*. From these experiments we conclude that during neuronal differentiation, the activity of cdk4 is lost, despite association to cyclin D3. Furthermore, the decline in activity is most likely due to p27^{KIP1}-mediated inhibition of cdk4 phosphorylation.

Inhibition of cdk Activity Is Sufficient to Induce Neuronal Differentiation

The above mentioned experiments show that cdk2 and cdk4 activities strongly decline and that pRb is activated when neuronal differentiation is induced. These data, together with experiments demonstrating that loss of *RB1* impairs neuronal differentiation (Lee et al., 1994; Williams et al., 1994), suggest that the neuronal proliferation-differentiation switch requires activation of pRb, presumably resulting from loss of cdk2 and cdk4 activities. This raises the question whether overproduction of pRb, or inhibition of cdk activity, would be sufficient to induce neuronal differentiation. We chose to address this question by transfecting neuronal precursor cells with expression vectors for pRb or the cdk2/cdk4-inhibitor p27^{KIP1} (Toyoshima and Hunter, 1994), together with a lacZ expression vector as transfection marker. Analysis of DNA synthesis in the transfected (blue) cells showed that pCMV-Rb and pCMV-p27, but not the control vector, efficiently inhibited N1E-115 cell cycle progression (not shown). Four days after transfection, cells were stained for β-gal activity and the morphology of transfected (blue) cells was examined microscopically. As shown in Fig. 1 *A*, differentiated cells are easily distinguishable from undifferentiated cells. Fig. 8 *A* shows that transfection of neuronal precursor cells with expression vectors for pRb or p27^{KIP1}, but not with the control vector, efficiently stimulated differentiation. Up to 48% of the transfected cells showed a fully differentiated phenotype as characterized by cell flattening, enlargement, and extensive outgrowth of neurites (Fig. 8, *A* and *B*). Expression of pRb was determined by Western blot analysis (Fig. 8 *C*) and expression of p27^{KIP1} was confirmed by coimmunoprecipitation with cdk4 (not shown, see also Fig. 7 *A*). The anti-pRb Western also shows that the underphosphorylated forms of pRb are readily detectable in cells transfected with pRb or p27^{KIP1}, but not in cells transfected with the control vector, demonstrating that overproduction of either pRb or p27^{KIP1} yields active pRb in these cell populations (Fig. 8 *C*). Moreover, transfection of pRb or p27^{KIP1} expression plasmids into N1E-115 cells greatly enhanced the expression of cyclin D3, which is, as shown in Fig. 2, confined to differentiated neurons (Fig. 8 *C*). These results demonstrate that overproduction of pRb, or inhibition of cdk activity by over-
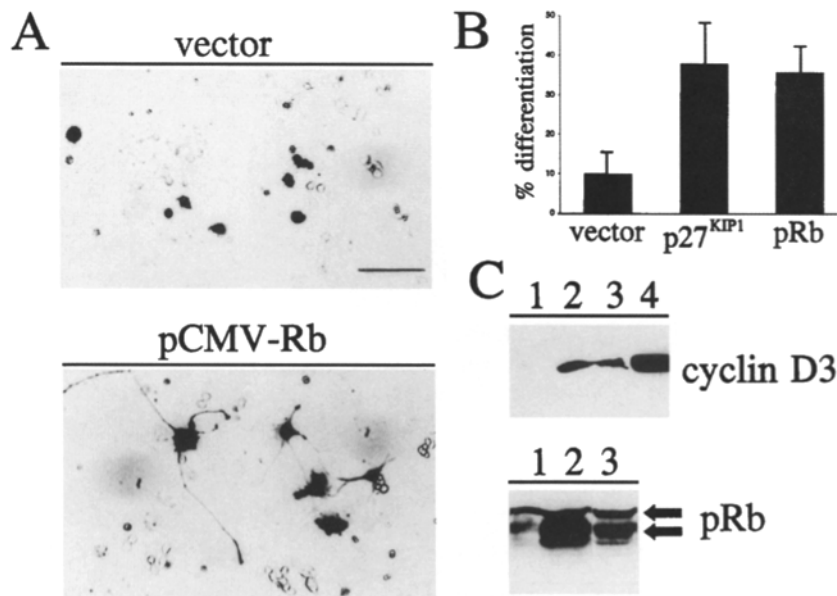
A vector

pCMV-Rb

B % differentiation

vector   p27^KIP1   pRb

C   1 2 3 4

cyclin D3

1 2 3

pRb

Figure 8. Induction of terminal neuronal differentiation by overexpression of pRb or inhibition of cdk activity. *(A)* Untreated cycling precursor cells were transfected with expression vectors for pRb or p27^KIP1 in five-fold excess over a lacZ expression vector. In total, 12 µg (10+2) of plasmid DNA was transfected. After transfection, the cells were cultured for 4 d in normal growth medium. The cells were then fixed on the dishes, stained for β-gal activity and photographed. Cells transfected with the control vector retain an undifferentiated morphology *(top)*, whereas up to 50% (see below) of the cells transfected with pCMV-Rb *(bottom)* or pCMV-p27^KIP1 (not shown) are differentiated. *(B)* Microscopic analysis of percentage of differentiation in response to pRb or p27^KIP1 overproduction. Cells were scored "differentiated" if the length of neurite extensions was at least the diameter of one cell body. The depicted values are means and standard deviations of four independent experiments. *(C)* The abundance of cyclin D3 was tested as a marker for terminal differentiation in cell cultures transiently transfected with the control vector (lane *1*), pCMV-pRb (lane *2*), or pCMV-p27^KIP1 (lane *3*). The cyclin D3 levels in a culture of terminally differentiated neurons is shown as a reference (lane *4*). Western blot analysis for pRb *(bottom)* shows that underphosphorylated, active pRb *(bottom arrow)* accumulates in cells transfected with either pCMV-Rb (lane *2*) or pCMV-p27^KIP1 (lane *3*) expression vectors but not in cells transfected with the control vector (lane *1*). The presence of p27^KIP1 was confirmed by coimmunoprecipitation with cdk4 (not shown, see also Fig. 7 *A*). Bar, 100 µm.

production of p27^KIP1, is sufficient to induce neuronal differentiation.

Discussion

The close correlation between cell growth and the phosphorylation state of pRb was noted several years ago (Buchkovic et al., 1989; Chen et al., 1989; Mihara et al., 1989). The loss of pRb phosphorylation in non-proliferating cells correlates with loss of activity of the pRb kinases cdk2 and cdk4 (Dulic et al., 1993; Ewen et al., 1993; Koff et al., 1993; Kato et al., 1994b; Kiyokawa et al., 1994; Polyak et al., 1994b; Rao et al., 1994; Kiess et al., 1995). In the present study we have demonstrated that during neuronal differentiation both cdk2 and cdk4 activities decline. However, inactivation of these kinases was found to occur through different mechanisms. Cdk2 activity is lost simply because of a strong decline in steady-state levels of cdk2 protein, whereas cdk4 activity is regulated in a more complex way. Phosphorylation of cdk4 is lost, possibly mediated by p27^KIP1. It has recently been reported that, during a cAMP-induced growth arrest of murine macrophages, cdk4 activation by CAK is inhibited by association of p27^KIP1 to the kinase complex (Kato et al., 1994b). Interestingly, N1E-115 cells differentiate in response to elevated cAMP levels and it has been shown that cAMP levels rise in response to DMSO treatment of these cells (Reagan et al., 1990). Thus, during DMSO-induced differentiation cAMP may mediate the induction of p27^KIP1-binding to cdk4, leading to loss of cdk4 phosphorylation and activity. p27^KIP1 may also directly inhibit the activity of CAK-phosphorylated cdk4 (Polyak et al. 1994a,b). In addition to an increase in intracellular cAMP levels DMSO

treatment also causes an increase in intracellular Ca^{2+} levels (Reagan et al., 1990) and an activation of membrane-bound tyrosine kinase activity (Rubin and Earp, 1983; Srivastava, 1984; Barnekow and Gessler, 1986). These responses may well contribute to the induction of differentiation by DMSO. First, a rise in intracellular Ca^{2+} stimulates neuroblastoma cell differentiation (Reboulleau, 1985). Second, activation of the receptors for neurotrophins and fibroblast growth factors results in enhanced tyrosine kinase activity (reviewed by Marshall, 1995). In response to sustained activation of these kinases, PC12 pheochromocytoma cells undergo neuronal differentiation (Marshall, 1995). Since the signal transduction pathways underlying NGF-induced (tyrosine kinase-dependent) and cAMP-induced differentiation function independently (Szeberenyi et al., 1990), DMSO may induce differentiation by activating distinct signalling pathways. The cAMP-dependent pathway may lead to activation of p27^KIP1, whereas the tyrosine kinase-dependent pathway may generate a signal that is similar to that evoked by NGF or FGF in PC12 cells. Yet another differentiation-inducing signal for neuroblastoma cells including N1E-115 is serum withdrawal, implying that serum contains differentiation-inhibiting signalling molecules (De Laat and Van der Saag, 1982). However, serum withdrawal also leads to massive neuronal apoptotic cell death (O. Kranenburg, unpublished observation; reviewed by Johnson and Deckwerth, 1993).

Thus far, all reports describing the regulation of cdk activity during terminal differentiation pathways show that these activities decline (Gaetano et al., 1991; Buchkovic and Ziff, 1994; Kato et al. 1994b; Kiyokawa et al., 1994; Rao et al., 1994; Kiess et al., 1995; Yan and Ziff, 1995).

The importance of this decline in cdk activity is illustrated by several studies in which forced overexpression of cyclin or cdk subunits inhibits the differentiation process (Kato and Sherr, 1993; Kiyokawa et al., 1994; Rao et al., 1994). Sustained cdk activity would prevent activation of pRb in response to a differentiation signal, although other targets for cdk-mediated phosphorylation may also contribute to the observed effect (see below). Activation of pRb-function may be regarded as a critical event during the neuronal proliferation-differentiation switch. First, overexpression of pRb is sufficient to induce neuronal differentiation in neuronal precursor cells (this study). Second, loss of pRb leads to deregulated neuronal cell proliferation in vivo and is thus essential in mediating cell cycle arrest prior to terminal differentiation (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1994). Third, surviving $Rb^{-/-}$ neurons are less differentiated than their wild-type counterparts, demonstrating that pRb helps to establish the genetic program that leads to terminal differentiation (Lee et al., 1994). It has been suggested that the dual role of pRb during myogenic differentiation may also apply to other terminal differentiation processes (Gu et al., 1993). If this is the case, a master-class of transcription factors analogous to the basic helix-loop-helix (bHLH) MyoD/myogenin class may function to drive neuronal differentiation together with pRb. Indeed, neuronal-specific bHLH transcription factors have been cloned (Guillemot et al., 1993; Turner and Weintraub, 1994; Lee et al., 1995), but it is not yet known whether these transcription factors function in conjunction with pRb to drive neuronal-specific gene expression.

Neuronal differentiation was not only induced by overproduction of pRb, but also by overproduction of $p27^{KIP1}$. Similar results were obtained with overproduction of the $p27^{KIP1}$-like CKI $p21^{WAF1}$, or the cyclin D-dependent kinase inhibitor $p16^{INK4A}$ (not shown). Most likely, $p27^{KIP1}$ overexpression leads to inactivation of cdk2 and cdk4 activities (Polyak et al., 1994a,b; Toyoshima and Hunter, 1994), resulting in activation of endogenous pRb (Fig. 8 C). Since a cell cycle arrest sec potentiates but is not sufficient for the induction of morphological differentiation in neuroblastoma cells (Larcher et al., 1992; LoPresti et al., 1992), overproduced pRb and $p27^{KIP1}$ most likely perform additional functions. One such possible function could be the activation of neuronal-specific transcription factors. In analogy with myoD (Skapek et al., 1995), these transcription factors may be prone to negative regulation by cyclin D-dependent kinases. Thus, $p27^{KIP1}$ may activate neuronal-specific transcription by releasing neuronal-specific transcription factors from repression by cdk4.

In summary, we have shown that neuronal differentiation of mouse N1E-115 cells into postmitotic neurons is accompanied by decreased cdk2 and cdk4 activities and loss of pRb phosphorylation. In the absence of an exogenous differentiation signal, suppression of cdk activities by $p27^{KIP1}$ overproduction or overproduction of pRb is sufficient to trigger neuronal differentiation. Thus, loss of cdk-activity and loss of pRb phosphorylation is not only essential but is also sufficient for the induction of neuronal differentiation.

References

Barnekow, A., and M. Gessler. 1986. Activation of the pp60^c-src protein kinase during differentiation of monomyelocytic cells in vitro. EMBO J. 5:701–705.

Buchkovic, K. J., and E. B. Ziff. 1994. Nerve growth factor regulates the expression and activity of p33^cdk2 and p34^cdc2 kinases in PC12 pheochromocytoma cells. Mol. Biol. Cell. 5:1225–1241.

Buchkovic, K., L. A. Duffy, and E. Harlow. 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell. 58:1097–1105.

Chen, P.-.L., P. Scully, J.-Y. Shew, J. Y. J. Wang, and W.-H. Lee. 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell. 58:1193–1198.

Clarke, A. R., E. R. Maandag, M. van Roon, N. M. T. van der Lugt, M. van der Valk, M. L. Hooper, A. Berns, and H. te Riele. 1992. Requirement for a functional Rb-1 gene in murine development. Nature (Lond.). 359:328–330.

De Laat, S. L., and P. T. van der Saag. 1982. The plasma membrane as a regulatory site in growth and differentiation of neuroblastoma cells. Int. Rev. Cyt. 74:1–53.

Dulic, V., L. F. Drullinger, E. Lees, S. Reed, and G. Stein. 1993. Altered regulation of G1 cyclins in senescent human diploid fibroblasts: Accumulation of inactive cyclin E- cdk2 and cyclin D1-cdk2 complexes. Proc. Natl. Acad. Sci. USA. 90:11034–11038.

Ewen, M., H. K. Sluss, L. L. Whitehouse, and D.M. Livingston. 1993. TGFβ inhibition of cdk4 synthesis is linked to cell cycle arrest. Cell. 74:1009–1020.

Farnham, P., J. E. Slansky, and R. Kollmar. 1993. The role of E2F in the mammalian cell cycle. Biochem. Biophys. Acta. 1155:125–131.

Flemington, E. K., S. H. Speck, and W. Kaelin. 1993. E2F1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc. Natl. Acad. Sci. USA. 90:6914–6918.

Gaetano, C., K. Matsumoto, and C. J. Thiele. 1991. Retinoic acid negatively regulates p34^cdc2 expression during human neuroblastoma cell differentiation. Cell Growth & Differ. 2:487–493.

Goodrich, D. W., N. P. Wang, Y.-W. Qian, E. Y.-H. P. Lee, and W.-H. Lee. 1991. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell. 67:293–302.

Gu, W., J. W. Schneider, G. Condorelli, S. Kaushai, V. Mahdavi, and B. Nadal-Ginard. 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell. 72:309–324.

Guillemot, F., L.-C. Lo, J. E. Johnson, A. Auerbach, D. J. Anderson, and A. L. Joyner. 1993. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell. 75:463–476.

Hamel, P., R. Montgomery Gill, R. A. Phillips, and B. Gallie. 1992. Transcriptional repression of the E2-containing promoters EIIaE, c-myc, and RB1 by the product of the RB1 gene. Mol. Cell. Biol. 12:3431–3438.

Helin, K., E. Harlow, and A. Fattaey. 1993. Inhibition of E2F1 transactivation by direct binding of the retinoblastoma protein. Mol. Cell. Biol. 13:6501–6508.

Jacks, T., A. Fazelli, E. M. Schmitt, R. T. Bronson, M. A. Goodell, and R. Weinberg. 1992. Effects of an Rb mutation in the mouse. Nature (Lond.). 358:295–300.

Johnson, E. M. J., and T. L. Deckwerth. 1993. Molecular mechanisms of developmental neuronal death. Annu. Rev. Neurosci. 16:31–46.

Kato, J.-Y., and C. J. Sherr. 1993. Inhibition of granulocyte differentiation by G1 cyclins D2 and D3 but not D1. Proc. Natl. Acad. Sci. USA. 90:11513–11517.

Kato, J.-Y. H. Matsushime, S. W. Hiebert, M. Ewen, and C. J. Sherr. 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase cdk4. Genes & Dev. 7: 331–342.

Kato, J.-Y., M. Matsuoka, D. K. Strom, and C. Sherr. 1994a. Regulation of cyclin D-dependent kinase 4 by cdk4-activating kinase. Mol. Cell. Biol. 14: 2713–2721.

Kato, J.-Y., M. Matsuoka, K. Polyak, J. Massagué, and C. J. Sherr. 1994b. Cyclic AMP- induced G1 phase arrest mediated by an inhibitor (p27^kip1) of cyclin-dependent kinase 4 activation. Cell. 79:487–496.

Kiess, M., R. Montgomery Gill, and P. Hamel. 1995. Expression of the positive regulator of cell cycle progression, cyclin D3, is induced during differentiation of myoblasts into quiescent myotubes. Oncogene. 10:159–166.

Kimhy, Y., I. Spector, Y. Barak, and U. Z. Littauer. 1976. Maturation of neuroblastoma cells in the presence of dimethylsulfoxide. Proc. Natl. Acad. Sci. USA. 73:462–466.

Kiyokawa, H., V. M. Richon, R. A. Rifkind, and P. A. Marks. 1994. Suppression of cyclin-dependent kinase 4 during induced differentiation of erythroleukemia cells. Mol. Cell. Biol. 14:7195–7203.

Koff, A., M. Ohtsuki, K. Polyak, J. Roberts, and J. Massagué. 1993. Negative regulation of G1 in mammalian cells: Inhibition of cyclin E-dependent kinase by TGFβ. Science (Wash. DC). 260:536–539.

Kranenburg, O., R. P. De Groot, A. J. Van der Eb, and A. Zantema. 1995. Differentiation of P19 EC cells leads to differential regulation of cyclin-dependent kinase activities and to changes in the cell cycle profile. Oncogene. 10: 87–95.

Larcher, J. C., M. Basseville, J. L. Vaysierre, L. Cordeau-Lossouarn, B. Croizat,

and F. Gros. 1992. Growth inhibition of N1E-115 mouse neuroblastome cells by c-myc or N-myc antisense oligodeoxynucleotides causes limited differentiation but is not coupled to neurite formation. *Biochem. Biophys. Res. Commun.* 185:915–924.

La Thangue, N. B. 1994. DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. *Trends Biochem. Sci.* 19:108–114.

Lee, E.Y.-H., C.-Y. Chang, N. Hu, Y.-C. J. Wang, C.-C. Lai, K. Herrup, W.-H. Lee, and A. Bradley. 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoieseis. *Nature (Lond.).* 359:288–294.

Lee, E. Y.-H., N. Hu, S.-S. F. Yuan, L. A. Cox, A. Bradley, W.-H. Lee, and K. Herrup. 1994. Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes & Dev.* 8:2008–2021.

Lee, J. E., S. M. Hollenberg, L. Snider, D. L. Turner, N. Lipnick, and H. Weintraub. 1995. Conversion of Xenopus ectoderm into neurons by NeuroD, a basic Helix-Loop-Helix protein. *Science (Wash. DC).* 268:836–844.

Lopresti, P., W. Poluha, D. K. Poluha, E. Drinkwater, and A. H. Ross. 1992. Neuronal differentiation triggered by blocking cell proliferation. *Cell Growth & Differ.* 3:627–635.

Marshall, C. J. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell.* 80: 179–185.

Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and J.-Y. Kato. 1994. D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* 14:2066–2076.

Mihara, K., X.-R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A. T'Ang, and Y.-K.T. Fung. 1989. Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. *Science (Wash. DC).* 246:1300–1303.

Morgenbesser, S. D., B. O. Williams, T. Jacks, and R. A. DePinho. 1994. p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature (Lond.).* 371:72–74.

Polyak, K., J.-Y. Kato, M. Solomon, C. J. Sherr, J. Massague, J. Roberts, and A. Koff. 1994a. p27^{KIP1}, a cyclin-cdk inhibitor links transforming growth factor-β and contact inhibition to cell cycle arrest. *Genes & Dev.* 8:9–22.

Polyak, K., M.-H. Lee, H. Erdjument-Bromage, A. Koff, J. M. Roberts, P. Tempst, and J. Massagué. 1994b. Cloning of p27^{kip1}, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell.* 78:59–66.

Rao, S. S., C. Chu, and S. K. Kohtz. 1994. Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators. *Mol. Cell. Biol.* 14:5259–5267.

Reagan, L. P., X. Ye, R. Mir, L. R. DePalo, and S. J. Fluharty. 1990. Up-regulation of angiotensin II receptors by in vitro differentiation of murine N1E-115 neuroblastoma cells. *Mol. Pharmacol.* 38:878–886.

Reboulleau, C. P. 1986. Extracellular calcium-induced neuroblastoma cell differentiation: involvement of phosphatidylinositol turnover. *J. Neurochem.* 46:920–930.

Robanus-Maandag, E. C., M. van der Valk, M. Vlaar, C. Feltkamp, J. O'Brien, M. van Roon, N. van der Lugt, A. Berns, and H. te Riele. 1994. Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. *EMBO J.* 13:4260–4268.

Rubin, R. A., and H.S. Earp. 1982. Dimethyl sulfoxide stimulates tyrosine residue phosphorylation of rat liver epidermal growth factor receptor. *Science (Wash. DC).* 219:60–63.

Sherr, C. J. 1994. G1 phase progression: cycling on cue. *Cell.* 79:551–555.

Skapek, S. X., J. Rhee, D. B. Spicer, and J. B. Lassar. 1995. Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science (Wash. DC).* 267:1022–1024.

Slack, R., P. A. Hamel, T. S. Bladon, R. Montgomery-Gill, and M. McBurney. 1993. Regulated expression of the retinoblastoma gene in differentiating embryonal carcinoma cells. *Oncogene.* 8:1585–1591.

Srivastava, A. K. 1985. Stimulation of tyrosine protein kinase activity by demethyl sulfoxide. *Biochem. Biophys. Res. Commun.* 126:1042–1047.

Szeberenyi, J., H. Cai, and G. M. Cooper. 1990. Effect of a dominant inhibitory Ha-ras mutant on neuronal differentiation of PC12 cells. *Mol. Cell. Biol.* 10: 5324–5332.

Toyoshima, H., and T. Hunter. 1994. p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity is related to p21. *Cell.* 78:67–74.

Turner, D. L., and H. Weintraub. 1994. Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes & Dev.* 8:1434–1447.

Van der Eb, A. J., and F. L. Graham. 1980. Assay of transforming activity of tumor virus DNA. *Method Enzymol.* 65:826–839.

Welch, P. J., and J. Y. J. Wang. 1993. A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. *Cell.* 75:779–790.

Williams, B. O., E. M. Schmitt, L. Remington, R. T. Bronson, D. M. Albert, R. A. Weinberg, and T. Jacks. 1994. Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. *EMBO J.* 13:4251–4259.

Yan, G.-Z., and E. B. Ziff. NGF regulates the PC12 cell cycle machinery through specific inhibition of the cdk kinases and induction of cyclin D1. *J. Neurosci.* In press.