Domains of E1A that Bind p105Rb, p130, and p300 are Required to Block Nerve Growth Factor-Induced Neurite Growth in PC12 Cells

Daniel Kalman,^{*†} Katherine Whittaker,[‡] J. Michael Bishop,^{*} and Paul H. O'Lague[‡]

[‡]Department of Biology, University of California at Los Angeles, Los Angeles, California 90024; and ^{*}The G.W. Hooper Foundation and The Department of Microbiology and Immunology, University of California at San Francisco, San Francisco, California 94143

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Nerve growth factor (NGF) causes PC12 cells to cease division and undergo sympathetic neuron-like differentiation, including neurite outgrowth. We have tested whether differentiation and division share overlapping control mechanisms in these cells. To do this, we have perturbed the activity of proteins known to participate in cell-cycle regulation by introducing the E1A oncogene or its mutant forms via microinjection into PC12 cells. The E1A protein binds to several putative cell cycle control proteins, including p105Rb (the product of the retinoblastoma susceptibility gene), as well as others of unknown function such as p130, p107, and p300. Similar to previous results, we find that wild-type E1A abrogates NGF-induced neurite extension. However, NGF does cause neurite outgrowth in the presence of E1A mutants known to have greatly reduced binding to either p105Rb and p130 or p300. Our experiments suggest that p105Rb, p130, and p300 may participate either in E1A-mediated inhibition of differentiation or in the NGF signal transduction pathway. We also report here that NGF affects phosphorylation of p105Rb, suggesting that Rb mediates at least some of NGF's effects. Our results raise the possibility that putative cell-cycle control proteins may participate not only in NGF-induced cessation of division but also in differentiation.

INTRODUCTION

The decision by neuroblasts to exit mitosis and differentiate plays a central role in the formation and plasticity of the nervous system. Factors that trigger neuronal development have been described extensively and include extracellular substrates and growth factors (Halegoua *et al.*, 1991; Reichardt and Tomaselli, 1991). However, information on the molecular mechanisms and signal transduction pathways by which such triggering factors inhibit mitosis and initiate differentiation remains more limited.

PC12 cells, a clonal line isolated from a rat adrenal chromaffin tumor (Greene and Tischler, 1976, 1982), have proven useful as a model system for studying the mechanisms of action of the neurotrophic molecule

nerve growth factor (NGF) (Greene and Tischler, 1976, 1982). When cultured in the presence of NGF, PC12 cells exhibit hallmarks of sympathetic neuron differentiation that include slowing or cessation of cell division (Ignatius *et al.*, 1985; Rudkin *et al.*, 1989), acquisition of excitable properties (e.g., Dichter *et al.*, 1977; Kalman *et al.*, 1990), activation of neurotransmitter biosynthetic pathways (McTigue *et al.*, 1985), and neurite growth (Greene and Tischler, 1976).

NGF initiates the differentiation program in PC12 cells by binding to a specific high affinity receptor (p140^{trk}) (Klein *et al.*, 1991) and possibly a low affinity receptor (p75) (Schechter and Bothwell, 1981; Berg *et al.*, 1991). Activation of p140^{trk} by NGF results in stimulation of its intrinsic tyrosine kinase activity (Kaplan *et al.*, 1991). This in turn leads to activation of several molecules, including serine/threonine kinases (McTigue *et al.*, 1985; Gomez and Cohen, 1991), tyrosine kinases

[†] Corresponding author.

(e.g., pp60^{src}) (Halegoua *et al.*, 1991; Kremer *et al.*, 1991), GTP-binding proteins (e.g., p21^{ras}) (Halegoua *et al.*, 1991; Kremer *et al.*, 1991), and phospholipases (e.g., Vetter *et al.*, 1991). Transcription factors or other nuclear proteins have been proposed to mediate NGF's effects on gene expression (e.g., Bartel *et al.*, 1989) and may play a key role in initiating the developmental program. We have undertaken experiments to identify nuclear proteins involved in inhibiting mitosis and initiating differentiation. Our approach relies on the use of the adenovirus transforming protein E1A (Flint and Shenk, 1989) as an inhibitor of differentiation in PC12 cells (Maruyama *et al.*, 1987).

E1A is a potent oncogene that can transform established cells on its own and primary cells in cooperation with other oncogenes (Montell *et al.*, 1984; Moran *et al.*, 1986; Moran and Zerler, 1988; Quinlan and Douglas, 1992). E1A can transactivate gene transcription (Lillie and Green, 1989) and bind nuclear proteins (Harlow *et al.*, 1986; Whyte *et al.*, 1988), some of which have been associated with mitotic control (Whyte *et al.*, 1988; Giordano *et al.*, 1989; Goodrich *et al.*, 1991). Studies with mutants of E1A suggest that the transactivation and binding activities reside within different domains of the protein (Whyte *et al.*, 1989; Lee *et al.*, 1991). Transactivation maps to a domain (called conserved region 3 [CR3]; Figure 1) that is conserved among several adenoviral serotypes (Berk, 1986; Flint and Shenk, 1989; Lillie and Green, 1989). Other conserved regions of E1A mediate its binding to cellular proteins (Whyte *et al.*, 1989) (Figure 1). Conserved region 2 (CR2) contributes to the binding of the product of the retinoblastoma susceptibility gene (p105Rb) (Lee *et al.*, 1988; Whyte *et al.*, 1988; Hollingsworth and Lee, 1991; Weinberg, 1991), a cell cycle control molecule p60 cyclin A (Giordano *et al.*, 1989), and several other proteins of unknown function, including p107 (Harlow *et al.*, 1986; Ewen *et al.*, 1991) and p130 (Harlow *et al.*, 1986). Another domain, conserved region 1 (CR1), contains binding sites for both p105Rb and a 300-kDa phosphoprotein of unknown function (Whyte *et al.*, 1989; Yaciuk and Moran, 1991). p300 also binds to an amino terminal domain of E1A (Moran and Zerler, 1988).

It has been reported that E1A inhibits NGF-induced neurite growth in PC12 cells and that the transactivation domain (CR3) is not required (Maruyama *et al.*, 1987). This observation prompted us to ask which of the remaining E1A domains might be responsible for inhibition. This strategy might allow identification of cellular proteins involved in NGF-induced differentiation. To do this, we set out to express in PC12 cells either wild-type E1A proteins or mutant forms that show impaired binding to various subsets of cellular proteins (Figure 1). We then determined whether NGF could induce neurite growth. We report here that inhibition of differentiation by E1A requires domains binding p300, p130, and p105Rb. We also provide more direct evi-

dence that p105Rb participates in the NGF signal transduction pathway. Heasley *et al.* (1991) have used a similar strategy but arrived at conclusions somewhat different from ours.

MATERIALS AND METHODS

Cell Culture

PC12 cells were obtained from L. Greene (Cornell University, Ithaca, NY) and grown and passaged as described previously (Kalman *et al.*, 1990). For microinjection experiments, cells were plated on coverslips prepared as follows. Five-millimeter square pieces were cut from large (45 × 72 mm) polystyrene planning slides (Miles Laboratories, Naperville, IL). Coverslips were sterilized with ultraviolet light for 30 min and coated with laminin (Sigma, St. Louis, MO; dissolved in RPMI 1640 without serum) at 0.4 mg/coverslip. Approximately 100 cells in 10-ml aliquots were transferred to the center of these coated coverslips and allowed to adhere for 45 min at 37°C in a moist chamber. Coverslips with attached cells were then transferred into a 50-mm tissue culture dish containing 5 ml of medium and returned to the incubator. During microinjection of DNA into cells, coverslips were bathed in a modified Hanks' balanced salt solution consisting of 151 mM NaCl, 5.4 mM KCl, 0.5 mM KH₂PO₄, 1.6 mM Na₂HPO₄, 1 mM CaCl₂, 11 mM MgCl₂, 5.5 mM glucose, and 6 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Sigma). Phenol red (0.01 mg/ml; Sigma) was added and the pH adjusted with NaOH to 7.2. Bovine serum albumin (5%) was then added, and the solution was sterilized by filtration.

For immunoprecipitation experiments, cells were grown in 150-cm² tissue culture flasks (Corning Glass, Corning, NY) at a density of 2 × 10⁵ per cm². In some cases, flasks were coated with laminin (Sigma) at 100–200 mg/flask. In addition to the normal culture medium, the following agents were included depending on the growth experiment: NGF (2.5S; Boehringer Mannheim, Indianapolis, IN) at 500 ng/ml; epidermal growth factor (Boehringer Mannheim) at 100 ng/ml. MgCl₂ (10 mM) was routinely added to the culture medium in experiments where NGF was used. This addition resulted in an ~20% increase in the rate of neurite growth in the presence of NGF but had no distinguishable effect on control cultures.

For microinjection experiments, cells were fused as described previously (O'Lague and Huttner, 1980). One day after fusion, cells were gently removed from flasks by rinsing with phosphate-buffered saline (PBS), and the resulting cell suspension then passed through a 26-mm Nitex filter (TETKO, Elmsford, NY). The cells remaining on the filter were gently rinsed off and transferred onto coverslips as described above.

Microinjection

Plasmid DNA was purified using Qiagen columns (Qiagen, Chatsworth, CA) followed by phenol-chloroform extraction and ethanol precipitation. DNA was then resuspended in TE buffer (0.01 M tris(hydroxymethyl)aminomethane [Tris], 0.001 M EDTA, pH 7.4; Sigma). Plasmids pE1A.12Swt, pE1A.2-36, pE1A.Cxd1, pE1A.928, and pE1A.Psdl were provided by J. Pietenpol (Johns Hopkins University, Baltimore, MD). Plasmids E1A.Psdl and E1A.Cxd1 were provided by E. Moran (Cold Spring Harbor Laboratories, NY). pE1A.13Swt was a gift from Arnold Berk (University of California, Los Angeles, CA). All DNA solutions were centrifuged (13 000 × g for 1 min) to eliminate particulate material before use. For all plasmid injections, ~1 ml of DNA at a concentration of 0.01 mg/ml was transferred using a polyethylene needle into the tip region of an acid-washed fiber-filled glass micropipette (TW150F-6, WPI, New Haven, CT). Before being transferred to a Leitz manipulator (Max Erb Instruments, Burbank, CA) for positioning over the cells, filled micropipettes were placed in a humid chamber for a few minutes to allow the tip region to become free of bubbles.

Under constant N₂ pressure, the DNA solutions were injected into the nuclei of cells on coverslips previously transferred into a dish

Figure 1. Characteristics of wild-type and mutant E1A proteins. (Left) Schematic diagram of wild-type and mutant E1A proteins. Open boxes represent E1A proteins. Numbers above the boxes refer to amino acid residues. CR1, CR2, and CR3 refer to conserved regions 1, 2, and 3, respectively. Darkened boxes in E1A.12S and mutants refer to in-frame deletions or mutations as described in the text. (Right) Capacity of E1A proteins to associate with cellular proteins or to transactivate gene expression. Proteins found to associate with wild-type or mutant E1A proteins in other cell types are shown above the table. "Rb-like" refers to several proteins binding to the E1A CR2 domain including p107 (Harlow *et al.*, 1986). The ability of E1A alleles to bind cellular proteins (+ or -) has been determined in previous coimmunoprecipitation studies (Harlow *et al.*, 1986; Whyte *et al.*, 1989). Note that the capacity to transactivate gene expression ("X-activation") requires the presence of an intact CR3 domain.

containing Hanks' medium (see above) and placed onto the stage of an inverted Zeiss phase contrast microscope. Cells were viewed at 400 $\times$ magnification. All injections were done at room temperature (20–22°C). We considered an injection successful if, after insertion of the pipette, the nucleus swelled slightly and became pale. We did not attempt to determine the injection volume. Blunt-tipped pipettes proved an extremely useful means of delivering DNA into the nucleus because they did not clog and did not damage the cells.

Detection of Proteins Expressed from Injected Plasmids

Cells on coverslips previously injected with plasmids encoding E1A proteins were fixed for 5 min in PBS supplemented with 2 g/100 ml fresh paraformaldehyde (Aldrich Chemical, Milwaukee, IL; pH adjusted to 7.2 with NaOH). Cells on coverslips were washed extensively in PBS, then permeabilized for 5 min in PBS supplemented with 1% Triton X-100 (Sigma), and again washed extensively in PBS. Coverslips were then incubated for 45 min at 37°C in a moist chamber with two anti-E1A monoclonal antibodies, M58 (a gift of E. Harlow, Harvard University, Charlestown, MA) and M73 (Oncogene Science, Manhasset, NY), each diluted 1:50 in PBS. Coverslips were washed in PBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse monoclonal antibodies (Cappel, Cochranville, PA) diluted 1:100 in PBS. After extensive washing the cells were exposed to 500 mg/ml diaminobenzidine dissolved in water and 0.2% H₂O₂. In some experiments, a plasmid encoding the β -galactosidase gene was injected into cells. To determine if a protein product was made, cells on coverslips were fixed in PBS containing 2% glutaraldehyde and 2% formaldehyde. The coverslips were washed in PBS and incubated for 24 h in PBS supplemented with 5 mM ferrocyanide, 5 mM ferricyanide, and 2 mg/ml X-Gal. After 1–2 h a blue precipitate was evident in some cells, indicating the presence of β -galactosidase activity. The precipitate darkened over a 24-h period. It was never observed in identically treated cultures of uninjected cells.

Immunoprecipitation

p105Rb was immunoprecipitated from PC12 cells using procedures adapted from Huang *et al.* (1990). PC12 cells, grown in 150-cm² tissue culture flasks to a density of 2×10^5 per cm², were washed in PBS and lysed for 1 h in a solution of 250 mM NaCl, 50 mM Tris (pH 7.4), 5 mM

EDTA, 0.1% NP-40 (Pierce Chemical, Rockford, IL), 0.1% Triton-X-100 (Sigma), 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim), 50 mM leupeptin (Boehringer Mannheim), and 0.05 mg/ml aprotinin. The suspension was then centrifuged (10 000 $\times$ g for 10 min at 4°C), and the supernatant removed and incubated for 1 h at 4°C with 15 ml of the anti-Rb monoclonal antibody PMG245 (O.D. 0.2), generously provided by Phang Lang Chen and Wen-Hwa Lee (University of Texas Health Science Center, Dallas, TX). Protein A sepharose beads (0.125 g/25 ml; Pharmacia, Piscataway, NJ), previously suspended and washed in lysis buffer, were added to the lysate and incubated at 4°C for 1 h. The mixture was centrifuged for 15 s at 10 000 $\times$ g, the supernatant removed, and the beads washed five times with fresh lysis buffer. In most experiments, the beads were resuspended in running buffer consisting of 4% β -mercaptoethanol, 62 mM Tris (pH 7.4), 11.5% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, and 10% glycerol. In some experiments, the beads were incubated with 0.5 units of potato acid phosphatase (Boehringer Mannheim) at 37°C for 15 min before addition of the running buffer. The running buffer-bead mixture was boiled for 10 min to dissociate the protein/bead complexes, centrifuged at 10 000 $\times$ g for 30 s, and the supernatant run on a 7.5% SDS-polyacrylamide discontinuous buffer system. The separated proteins were electroblotted onto an Immobilon-P membrane (Millipore, Bedford MA) for 1.5 h at 150 V. The membrane was blocked for 1 h at 22°C with a buffer containing 125 mM NaCl, 25 mM Tris (pH 7.4), 0.1% Tween-20, and 4% Nonfat milk solids (Carnation, Los Angeles, CA) and then incubated for an additional hour at 22°C with PMG-245 antibody diluted 1:200 in blocking buffer. After extensive washing, the membrane was incubated for 1 h at 22°C with an HRP-conjugated goat anti-mouse monoclonal antibody (Cappel) diluted 1:2000 in blocking buffer. Immunoreactivity was resolved using an enhanced chemiluminescence system (ECL, Amersham, Arlington Heights, IL) according to the manufacturers specifications. The membrane was then exposed to x-ray film for 1–5 min.

RESULTS

Effects of Wild-Type E1A Proteins on NGF-Induced Neurite Growth

To extend the findings of Maruyama *et al.* (1987) and determine which of the protein-binding domains of E1A

were required for inhibition of differentiation, we microinjected plasmid expression vectors into PC12 cells (Capecci, 1980; Kalman *et al.*, 1990) and measured the ability of NGF to cause neurite growth. For these studies, cells were injected with control vectors or vectors encoding either wild-type or various mutant E1A alleles. The cells were then cultured in the presence of NGF for 3–5 d. Although most experiments were carried out with fused cells because of their accessibility to microinjection, identical results were obtained using unfused cells. Under our conditions, 30–80% of the injected cells expressed the protein encoded by an introduced plasmid when either the Rous sarcoma virus LTR or E1A promoter was used. Proteins expressed from injected plasmids did not appear to nonspecifically obstruct or alter neurite growth. For example, cells expressing β -galactosidase from an injected LacZ construct grew neurites in response to NGF as well as uninjected cells (Figures 2, A and B and 3). In our experiments, an NGF-treated cell was considered to grow neurites if the neurite length exceeded twice the cell diameter.

We tested the effects of expressing, under the control of the E1A promoter, either wild-type E1A protein (called 13S) or a smaller form encoded by a spliced

mRNA (called 12S) (Flint and Shenk, 1989) that lacked the CR3 transactivation domain. Expression of E1A proteins from injected plasmids was assessed by immunohistochemical staining with the anti-E1A monoclonal antibodies M73 and M58. Both antibodies recognize the products of all E1A alleles used in this study (Whyte *et al.*, 1989). Staining was generally detected in the nucleus and was never observed in uninjected cells treated identically or in injected cells in which the primary antibody was omitted during staining. The percentage of cells extending neurites decreased from 96% in control cells ($n = 5$, 200 total cells) to 23% in 13S expressing cells ($n = 8$, 225 total cells) and to 24% in cells expressing the 12S construct ($n = 10$, 200 total cells; Figures 2, C and D and 3). The standard error of the mean ranged from 1 to 4%, depending on the injected construct. Similar percentages were obtained in injections of unfused cells (e.g., 93% in control cells [$n = 30$]; 21% in E1A12S expressing cells [$n = 24$]).

We also tested the ability of the 12S E1A protein to reverse the effects of NGF. Cells were cultured in NGF for 4 d, injected with the 12S expression vector, cultured for an additional 3 d in the presence of NGF, and stained for E1A. We found that many of the stained cells had

Figure 2. Effects of wild-type or mutant E1A plasmids on NGF-induced neurite growth in PC12 cells. Representative photographs of fused PC12 cells microinjected with no DNA (A), plasmid expression vector encoding the β -galactosidase (Lac-Z) gene under control of the Rous sarcoma virus LTR (B), or vectors expressing wild-type E1A.12S (C,D) or the E1A mutants E1A.2-36 (E) and E1A.928 (F) under control of the E1A promoter. Cells were treated with NGF for 3–4 d and then fixed and stained for either β -galactosidase (B) or E1A (A, C–F). The presence of E1A is indicated by the presence of the orange precipitate in the nuclei of some cells. Scale bar is 120 μ m for A–C and 60 μ m for D–F.

Figure 3. E1A alleles that cannot bind p300 or p105Rb/p130 do not inhibit NGF-induced neurite growth. Measurement of NGF-induced neurite growth in uninjected PC12 cells or PC12 cells expressing β -galactosidase, wild-type E1A, or mutant E1A alleles. Uninjected cells, or cells injected with the plasmids indicated on the abscissa, were grown for 3–4 d in the presence of NGF after injection. If a cell grew neurites greater than two cell diameters, it was scored as positive. Among injected cells, only those with detectable expression (e.g., a blue or orange precipitate) were scored. Each bar represents ≥ 200 cells from at least five experiments. The bar height represents the cumulative percentage of cells with neurites from all experiments. The error bar amplitude is the standard error of the mean calculated from the means of each experiment.

stunted or decayed neurites, an effect similar to that observed when NGF is removed from cultures of PC12 cells. Together these results suggested that the E1A protein both inhibited and reversed NGF-induced neurite growth and that the transactivation domain (CR3) was not required.

Effects of Mutant E1A Proteins

To determine which domains of the 12S protein were required for inhibition of neurite growth, we tested the effects of E1A plasmids lacking both the transactivation domain and regions required for binding to cellular proteins. The protein encoded by the mutant E1A allele Psdl has deletions that remove the CR3 transactivation domain and the CR1 domain. In immunoprecipitation studies, the Psdl protein has severely impaired binding to p105Rb and p300 compared with parental E1A (Moran and Zerler, 1988). PC12 cells expressing Psdl responded normally to NGF with respect to the percentage of cells growing neurites (Figure 3). The results with Psdl eliminate the possibility that the E1A promoter itself inhibited neurite growth in cells expressing the wild-type E1A protein. In addition, the results suggest that the regions of E1A that bind either p300, p105Rb, or both appear to be required to inhibit neurite growth.

To more clearly distinguish the region(s) of the E1A protein responsible for inhibition of neurite growth,

we tested the effects of plasmids expressing mutant 12S E1A proteins that bind to either p300 or p105Rb. The plasmid E1A.2-36 encodes a protein with a deletion of 34 amino acids in the amino terminus (Stein *et al.*, 1990). The 2-36 protein has no detectable binding to p300 in immunoprecipitation studies, although it retains the capacity to bind p105Rb, p130, p107, p60 cyclin A, and other proteins that associate with the CR2 domain (Whyte *et al.*, 1989; Stein *et al.*, 1990). We also tested the effects of the plasmid Cxd1 that encodes a protein lacking the CR2 domain (Moran *et al.*, 1986). This deletion disrupts binding of all the proteins associated with CR2 but does not affect p300 binding (Stein *et al.*, 1990). Finally, we tested the effects of plasmid E1A.928 that encodes a protein with a mutation at codon 928 that results in a glycine instead of a cysteine at amino acid 124 within CR2 (Moran *et al.*, 1986). Like Cxd1, the E1A.928 protein retains normal binding to p300. However, the mutation in CR2 is sufficient to disrupt binding of only two CR2 associated proteins: p105Rb and p130 (Whyte *et al.*, 1989).

PC12 cells microinjected with plasmids encoding E1A.2-36, Cxd1, or E1A.928 all exhibited marked neurite growth in response to NGF treatment (e.g., Figures 2F and 3). For all these plasmids, the percentage of cells with neurites was slightly reduced with respect to uninjected or lacZ controls or to Psdl but was still threefold greater than with wild-type E1A.

Proteins encoded by E1A.2-36 and E1A.928 are mutually complementary such that together they might be expected to bind the same spectrum of cellular proteins as the wild-type. In accordance with this idea, we found that coinjection of E1A.2-36 with E1A.928 resulted in a substantial reduction in the percentage of cells extending neurites in NGF, though not to the same level as achieved with wild-type E1A (Figure 3). Because the monoclonal antibodies used here detect both mutant proteins, we were unable to discriminate between protein products from the two plasmids. However, when we carried out experiments with two vectors that encode independently detectable markers in PC12 cells, such as lacZ and mutated c-Ha-ras (Kalman *et al.*, 1990; Halegoua *et al.*, 1991) or lacZ and E1A, we found that 94% of the cells expressed both ($n = 323$ cells). We conclude that the combination of the two proteins was able to inhibit neurite growth but not as effectively as wild-type E1A. A similar conclusion was reached by Moran and Zerler (1988), who found that the same E1A alleles were less efficient than wild-type E1A in cooperating with *ras* to transform primary BRK cells. Our results using these mutant E1A plasmids suggest that inhibition of NGF-induced neurite growth was relieved by disrupting E1A domains involved in the binding of either p105Rb/p130 or p300.

Effects of NGF on the Electrophoretic Mobility of p105Rb

In search of evidence to implicate p105Rb in the NGF signal transduction pathway, we next determined whether NGF affected the electrophoretic mobility of p105Rb. Previous work has established that Rb migrates at an apparent molecular mass of 105 kDa when isolated from cells in the G₀/G₁ phase of the cell cycle and at an apparent molecular mass of 110–112 kDa at other times. The 110-kDa form results from multiple phosphorylations of the 105-kDa species during the G₁/S transition. Given that NGF causes unsynchronized cells to accumulate in G₁ after 3 d (Rudkin *et al.*, 1989), we asked whether NGF might lead to the accumulation of the 105-kDa form of Rb. Rb was immunoprecipitated from untreated and NGF-treated PC12 cells (Figure 4A). Two forms of Rb of apparent molecular masses of 105 and 110 kDa were evident in lysates from untreated cells and from cells treated with NGF for up to 3 d. After 4 d in NGF, only the 105-kDa species was detectable. The 105-kDa species persisted for up to 3 d after NGF treatment, the longest time measured. This effect on the electrophoretic mobility of Rb was specific to NGF treatment because epidermal growth factor (EGF), a mitogen for PC12 cells, did not change the relative proportions of the 105- and 110-kDa forms (Figure 4B), even after extended incubations of up to 7 d. Similar to results in other cell types, p110Rb from untreated PC12 cells also appeared to be phosphorylated. Incubation of lysates from untreated PC12 cells with potato acid phosphatase resulted in Rb migrating solely as a 105-kDa species (Figure 4C). Thus, our results provide evidence that NGF leads to the accumulation of a dephosphorylated or hypophosphorylated form of Rb.

DISCUSSION

Several lines of evidence suggest that a variety of molecules participate in the NGF-signal transduction pathway including GTP-binding proteins, protein tyrosine and serine/threonine kinases, and transcription factors (reviewed by Halegoua *et al.*, 1991). Much less is known about how such signal transduction pathways stop the cells from dividing and initiate differentiation. In an effort to identify nuclear proteins that might be targets of regulation by NGF, we carried out experiments to determine how the E1A protein inhibits NGF-induced neurite growth (Maruyama *et al.*, 1987).

What Domains of E1A are Required to Inhibit Differentiation?

We find that inhibition does not require the presence of CR3, a domain of E1A involved in transactivation (Lillie and Green, 1989; Lee *et al.*, 1991), in general agreement with previously published reports (Maru-

Figure 4. NGF but not EGF causes accumulation of hypophosphorylated p105Rb. (A) Western blot of p105Rb immunoprecipitated from untreated PC12 cells (lanes 2, 4, 7, 8) or PC12 cells treated with NGF for 3 h (lane 1), 2 d (lane 3), or 4.5 d (lanes 5 and 6). The lower mobility band is ~110 kDa and the higher mobility band is ~105 kDa. The times depicted above the lanes indicate the duration of NGF treatment. Rb protein was isolated from a separate flask of untreated cells at each time point. Data at the 4.5-d time point was acquired in duplicate. (B) Western blot of Rb protein immunoprecipitated from untreated PC12 cells (lane 1) or PC12 cells treated with EGF for 4.5 d (lane 2). (C) Western blot of Rb protein immunoprecipitated from untreated PC12 cells. Before loading the gel, the immunoprecipitated protein was incubated with (lane 1) or without (lane 2) potato acid phosphatase.

yama *et al.*, 1987). Our experiments with E1A proteins lacking both the transactivation domain and other regions suggest that at least three proteins that bind to E1A may be involved in inhibiting differentiation. These include p300 (a DNA-binding phosphoprotein of unknown function), p105Rb, and p130 (an uncharacterized protein). We find that mutations in E1A that disrupt binding of either p300 alone, or p105Rb and p130 together, are sufficient to abrogate inhibition of NGF activity in PC12 cells by E1A. At this time, we cannot dissociate the roles of p105Rb and p130. Our results with Psdl, a mutant that fails to inhibit differentiation, are not informative in this regard, even though Psdl binds p130 but not p105Rb (Moran *et al.*, 1986; Stein *et al.*, 1990). Psdl also fails to bind p300, and our results with E1A.2-36 show that disruption of p300 binding alone is sufficient to relieve inhibition of differentiation. Moreover, our results raise the possibility that other proteins bound by the CR2 domain, such as p60 cyclin A and p107, might also play a role in E1A-mediated inhibition of differentiation.

Using a stable transfection assay, Heasley *et al.* (1991) have come to similar conclusions as to the potential role of p300 in the inhibition of NGF activity in PC12 cells. However, our results differ from theirs regarding involvement of p105Rb and p130. In our assay system,

alleles containing CR2 deletions or mutations (e.g., Cxdl or E1A.928) do not inhibit neurite growth. We consider it unlikely that our results are due to low expression levels of Cxdl or 928 proteins relative to the wild-type for two reasons. First, we can readily detect expression with immunohistochemistry, the least sensitive of protein detection methods. Second, the 928 protein retains some measureable activity because injection of pE1A.928 together with pE1A.2-36 inhibits differentiation to a significantly greater extent than either plasmid alone. In corroboration of our results with E1A.928 and Cxdl, we have achieved similar results in studies with the adenovirus strain 12S-Ctdl-930 (Whyte *et al.*, 1989) that synthesizes a mutated E1A protein unable to bind Rb and p130 or to transactivate genes (D. Kalman and P.H. O'Lague, unpublished results). In contrast, Heasley *et al.* find that Cxdl does inhibit neurite growth. We surmise that this discrepancy arose because transient expression of E1A in our study may have different effects than constitutive expression in clonal lines (the protocol used by Heasley *et al.*). In this regard, it is possible that the 928 or Cxdl proteins exert slower or weaker effects on differentiation than wild-type E1A and that such a difference was detected in our transient assay but not in the cell lines generated by Heasley *et al.* Alternatively, the discrepancy may be ascribed to clonal differences in the PC12 lines used in the two studies. The possibility exists that genetic alterations (e.g., p53 mutations) may lead to different responses to E1A mutants.

How does E1A Contribute to Immortalization, Transformation, and Inhibition of Growth Factor Action?

Mutational analysis of E1A has been used by others to study the mechanisms by which E1A immortalizes primary cells (Quinlan and Douglas, 1992), transforms cells in cooperation with other oncogenes (Ruley, 1983; Montell *et al.*, 1984; Moran *et al.*, 1986; Moran and Zerler, 1988; Stein *et al.*, 1990), or inhibits growth factor activity. Immortalization of primary BRK cells by E1A or transformation in cooperation with the activated *ras* oncogene requires intact p300 and p105Rb binding sites (Moran *et al.*, 1986; Moran and Zerler, 1988; Stein *et al.*, 1990), the same sites required to inhibit differentiation of PC12 cells in our experiments. In all of these experimental systems, each of the p105Rb or p300 binding sites is necessary but not sufficient to exert the measured effect. In contrast, Pietenpol *et al.* (1990a) showed that E1A inhibited the TGF- β -induced down regulation of *c-myc* expression and growth arrest in primary keratinocytes (see also Moses *et al.*, 1990; Pietenpol *et al.*, 1990b). In this case, disruption of the p105Rb/p130 binding domain of E1A is sufficient to abrogate the observed inhibition (see also Laiho *et al.*, 1990).

The E1A protein associates in several cell types with nuclear proteins that control entry into or exit from the

cell cycle (e.g., Whyte *et al.*, 1988). E1A binds p105Rb (Whyte *et al.*, 1988), a well-characterized tumor suppressor gene product (Weinberg, 1991) that, in its hypophosphorylated form, inhibits the G₁/S transition (Goodrich *et al.*, 1991) and counteracts neoplastic phenotypes (Huang *et al.*, 1988; Takahashi *et al.*, 1991). It has been proposed that, like phosphorylation, binding of p105Rb to DNA tumor virus proteins such as SV40 large T antigen, papilloma virus E7, or E1A abrogates the inhibitory activity of p105Rb and allows the cell to enter S phase (Cooper and Whyte, 1989; Ludlow *et al.*, 1989; Hu *et al.*, 1990; Huang *et al.*, 1990; Ludlow *et al.*, 1990; Templeton *et al.*, 1991).

Inactivation of p105Rb by binding to tumor virus proteins is consistent with reports that the CR2 domain of E1A is required to stimulate quiescent cells to transit the cell cycle during immortalization by E1A (Quinlan and Douglas, 1992) and with the CR2-dependent inhibition of TGF- β . This hypothesis is also consistent with our results that the p105Rb binding domain is required for inhibition of NGF activity. Whereas the inhibition of growth arrest may be restricted to the p105Rb binding domain of E1A, as in the case of TGF- β , E1A-mediated inhibition of NGF activity, immortalization, or transformation may require more complex changes in cellular physiology, thereby necessitating use of additional E1A domains (see Quinlan and Douglas, 1990). A precise role in E1A-mediated processes for proteins binding to other E1A domains (e.g., p300) or of the additional proteins bound in the p105Rb binding domain (e.g., p130) awaits further characterization of these proteins.

Does Rb Play a Role in NGF-Induced Cessation of Division and/or Differentiation?

Several lines of evidence suggest that NGF arrests PC12 cells in G₀/G₁ either as a prelude to or coincident with differentiation. One possible role for p105Rb in NGF-treated PC12 cells might be to arrest cell division. In support of this hypothesis, results presented here suggest that in response to NGF, Rb protein accumulates in a hypophosphorylated form with the same kinetics as the cells reportedly accumulate in G₁ (Figure 4A) (Rudkin *et al.*, 1989). Our results suggest a role for p105Rb in NGF action and make the prediction that some NGF-triggered signal transduction pathways may serve to cause dephosphorylation of the Rb protein either by inhibiting the activity of a kinase or by activating a phosphatase. Such biochemical evidence for the involvement of p105Rb in the NGF signal transduction pathway suggests that E1A can inhibit differentiation by affecting molecules that participate in, rather than modulate, the pathway. Our results also show that accumulation of hypophosphorylated p105Rb occurs with the same time course as cessation of division and neurite extension suggesting that NGF causes PC12 cells to ex-

press neuronal phenotypes only after they reach G₁ (see also Rudkin *et al.*, 1989). Recent work with transgenic mice lacking functional copies of the Rb gene suggests a role for p105Rb in neuronal and hematopoietic development (Lee *et al.*, 1992; Jacks *et al.*, 1992; Clarke *et al.*, 1992). It remains to be determined whether the presence of hypophosphorylated Rb protein in PC12 cells contributes to maintenance of or entry into the differentiation program in addition to cell-cycle arrest.

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