Journal of Cellular Biochemistry 89:539–549 (2003)

Regulation of p27 in the Process of Neuroblastoma N2A Differentiation

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Abstract Neuronal differentiation implies morphological and biochemical changes to generate a specialized neuron. N2A neuroblastoma cells can be promoted to undergo differentiation associated to neurites outgrowth, a process linked to the arrest of cell division. Using N2A cells as a model, we investigated the detailed molecular aspects on the involvement of p27 in dibutyryl cAMP-induced neuronal differentiation. In the undifferentiated N2A phenotype, an unusually high level of accumulated p27 protein mass was evidenced. Data suggest that in proliferating cells, p27 could be sequestered by direct interaction with cyclin D1, thus preventing its inhibitory action on cell cycle Cdks. Studies also indicate that p27 is functionally active and that its loss of action on Cdks in proliferating cells is due to its strong association with cyclin D1. Therefore, when cell differentiation is triggered, the action of p27 on Cdks seems to depend on both p27 and cyclin D1 degradation during the early steps of differentiation followed by late events of re-synthesis of active p27. In this context, an overexpression of p27 after N2A transfection with a mouse p27 clone induces the outgrowth of neurites associated with a decrease in cyclin D1 expression. On the other hand, treatment of N2A undifferentiated cells with c-myc antisense oligonucleotides led to a decrease in p27 and cyclin D1 levels, similar events as those in early stages of cell differentiation. Studies suggest that blockage in c-myc expression triggers early events in neuronal differentiation. These studies are of the utmost importance to elucidate regulatory mechanisms of molecules that play a critical role in the transition from a proliferating phenotype to differentiated cells. J. Cell. Biochem. 89: 539–549, 2003.
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Key words: p27; cyclin D1; cDNA arrays; protein interactions; cell differentiation; neuroblastoma cells

The arrest of the cell cycle and the consequent induction of the cell differentiation process are cellular events whose regulation appears to be orchestrated by sensitive molecular mechanisms. A set of protein factors such as cyclins and their regulators whose activity is controlled at both spatial and temporal levels promote biochemical and structural changes in cells with an undifferentiated phenotype [Nakagawara, 1998; Anderson, 2001]. Cell division mechanisms involve several protein kinases and their activators, which are necessary for the cell cycle to proceed. The function of these protein kinases is finely modulated by signals that involve changes in the expression patterns of these kinases, post-translational modifications, and protein–protein interactions. On the other hand, the elucidation of the regulatory factors implicated in cell cycle arrest is of the utmost importance in the study of cellular anomalies involved in the pathogenesis of cancer [Kawamata et al., 1996; Li and Sun, 1998; Krimpenfort et al., 2001; Nigg, 2001].

We have focused on determining the precise checkpoints that modulate the transitions between cell proliferation and differentiation in cultured neuroblastoma cells. One of the

Abbreviations: Cdks, cyclin dependent kinases; db-cAMP, dibutyryl-cAMP.

Grant sponsor: Fondecyt; Grant number: 1020155; Grant sponsor: Millennium Science Initiative; Grant number: P99-031F.

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Received 7 January 2003; Accepted 24 February 2003

DOI 10.1002/jcb.10525

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proteins of relevance in cell cycle regulation is p27, a natural inhibitor of Cdks that exerts its action by blocking a wide spectrum of protein complexes between Cdks and cyclins [Nomura et al., 1997; Vidal and Koff, 2000]. Among these, the cdk-2/cyclin E complex is one of the most well studied, since it is involved in the transition from G1 to S phase in the cell cycle [Zetterberg et al., 1995; Hu et al., 1999; Hulleman et al., 1999]. It has been shown that p27 increases its expression in differentiated cells, producing in the meantime a direct effect in arresting the cell cycle [Perez-Juste and Aranda, 1999; Matsuo et al., 2001]. In addition, a relationship between changes in the expression of the oncogene c-myc and p27 has been evidenced [Vlach et al., 1996; Mateyak et al., 1999]. This relationship appears to be of the greatest importance since evidence exists demonstrating that an increase in the expression of c-myc correlates with a concomitant decrease in p27 in tumor cells, even though the precise molecular mechanisms that regulate these processes remain to be elucidated. Cells use mechanisms that involve p27 degradation mediated by SCFskp2 ubiquitin ligase, in which a p27 phosphorylation at threonine 187 occurs at an early stage [Ganoth et al., 2001; Harper, 2001].

Different laboratories have determined that the complexes of Cdks-4 and -6 with cyclin D1 play a non-catalytic role in the progression toward G1 phase of the cell cycle, sequestering the p21 and p27 inhibitors and preventing their action in blocking the cyclin E/cdk-2 complex. Thus, the expression of cyclin D1 in proliferating cells would have a dual function in controlling the action of cdk-4/cdk-6 kinases as well as p27 [Polyak et al., 1994; Soos et al., 1996; Perez-Roger et al., 1999; Sherr and Roberts, 1999; O'Brien et al., 2001].

It has been reported that increased expression of *Ras* oncogene, independently or by a concerted action together with c-myc, causes a significant decrease in the expression of p27 [Leone et al., 1997], while it has been observed that a decrease in the expression of c-myc is concomitant with a significant increase in the expression of p27, in the arrest of the cell cycle [Vlach et al., 1996; Bergmann et al., 2001]. These findings are of relevance due to the direct relationship of these genes to cell proliferation phenomena. Some evidence indicates that c-myc activates proteins that are involved in sequestering p27 and its further degradation [Bouchard et al., 1999; O'Hagan et al., 2000]. Moreover, recent studies have shown that c-myc induces the expression of cdk-4 [Hermeking et al., 2000].

A decrease in p27 expression associated with several tumors and transformed cell lines has been shown. However, in a lower number of cases there is an increase in the levels of p27 associated with the proliferating phenotype, and therefore the loss of p27 found in some tumor cells is not an appropriate marker for cancer. As a matter of fact, a series of reports have revealed an overexpression of p27 in some tumors [Lloyd et al., 1999; Schiffer et al., 1999; Bergmann et al., 2001].

Our present findings show a very peculiar regulation of p27 function in the mouse N2A neuroblastoma induced to differentiate with dibutyryl-cAMP. Interestingly, N2A cells in active proliferation exhibited high levels of p27, a protein whose activity remains controlled as a result of its association with cyclin D1 in the proliferative condition. We also found that both p27 and cyclin D1 degrade upon initiation of cell differentiation. At more advanced stages in the N2A differentiation, the phenotype p27 is re-expressed again while cyclin D1 is absent. Thus, active p27 can exert its inhibitory activity on cell cycle Cdks in differentiated cells.

MATERIALS AND METHODS

Cell Cultures

N2A neuroblastoma cells, which can be induced to undergo differentiation with the outgrow of long neuritic processes after the addition of db-cAMP (Sigma, St. Louis, MO) into the culture medium, were used in these studies. N2A neuroblastoma cells were grown in Petri dishes with Eagles's modified Dulbecco's medium (DMEM) (Gibco-BRL), supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were grown under humidity, with 5% CO_2 and at 37°C. Neurite outgrowth was promoted by changing the cells to DMEM medium plus 0.25% serum, and the addition of 5 mM db-cAMP. After 12-, 24-, and 48-h treatment, cells exhibited a differentiated morphology with long neuritic processes [Muñoz et al., 2000]. For experiments with cycloheximide, N2A cells were treated with 10 ng/ml of cycloheximide in the presence or the absence of db-cAMP.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

To obtain total RNAs from differentiated and undifferentiated N2A cells, these (1×10^6 cells) were detached mechanically using a rubber-ended rod, and the RNA was isolated using the Trizol reagent (Gibco-BRL, Life Technology) method. The amount of RNA obtained was determined spectrophotometrically at 260 nm. The RT-PCR reaction was carried out on 3 μg total RNA, to which 100 ng of the random primer were added in a volume of 12 μl. The samples were incubated for 10 min at 70°C followed by addition of 4 μl of five times transcription buffer, 2 μl 0.1 mM DTT, 1 μl dNTP 10 mM, and 200 U SuperScript II (Gibco-BRL, Life Technology). The mixture was incubated for 50 min at 42°C. The final volume of the reaction was 20 μl. For PCR amplification, different cycle programs and different amounts of synthesized cDNA were analyzed to evaluate the linearity of the reaction. The polymerase reaction of the *p27* gene was carried out in a solution that contained 10× polymerase reaction buffer, 1.5 mM $MgCl_2$, 200 μM of each nucleotide, 10 pmol of the p27 primer, 5 pmol of β-actin primer, and 0.25 U Taq polymerase (Gibco-BRL, Life Technology). The primers were p27 SNS: 5′-TAACCCGGGACTTGGAGAAG-3′ and p27 AS: 5′-GCTTCTTGGGCGTCTGCTC-3′ (450-bp fragment). The amplification for p27 and β-actin were carried out in the same reaction, maintaining ratios of 2:1 for the primer concentrations. β-actin primer was SNS: 5′- TCTACAATGAGCTGCGTGTG- 3′ and β-actin AS: 5′-TACATGGCTGGGGTGTTGAA- 3′ (131-bp fragment). Amplification was carried out by using the following cycle: initial denaturation at 94°C for 3 min, denaturation at 94°C for 40 s, re-association at 57°C for 45 s, extension at 72°C for 5 min. The amplification was carried out for 30 cycles and the same re-association temperature was maintained for p27 and β-actin. The amplification for *c-myc* gene and β-actin was carried out under the same conditions used for p27-β-actin, with the primer c-myc P2 (SNS): 5′-ATGCCCCTCAACGTGAACTTC-3′ and c-myc P1 (AS): 5′-CCTCTTCTCCACAGACACCAC-3′ (810-bp fragment) [Paria et al., 1992]. The number of cycles was 30 and re-association temperature for c-myc and β-actin was 57°C for 45 s. The reaction volume for p27-β-actin and c-myc-β-actin was 50 μl and products were visualized in 1.8% agarose/TAE gels and stained with ethidium bromide (0.5 μg/ml).

cDNA Expression Arrays

Total RNA from differentiated and undifferentiated N2A cells was extracted by the Trizol reagent method (Gibco-BRL, Life Technology). The preparations were treated with DNAse prior to enrichment with Poly A^+, following the description in the Clontech manual. Poli A^+ RNA was used as a template for the synthesis of cDNA probes labeled with ^{32}P. The cDNA probes (2×10^3 cpm/μl) were hybridized to Atlas™ mouse cDNA expression arrays (Clontech Laboratories, Inc.). The arrays were exposed to an autoradiographic film for 3 days. The hybridization signals were analyzed with the Kodak Digital Science 1D 3.0.2 program.

Transfection of N2A Cells

Phosphorothioated oligodeoxynucleotides complementary to c-myc cDNA Antisense (5′-GAAGTTCACGTTGAGGGGCAT-3′) and Sense (5′-ATGCCCCTCAACGTGAACTTC-3′) were used for transfection of N2A cells [Paria et al., 1992]. The mixture of lipofectamine (Gibco-BRL, Life Technology) and oligodeoxynucleotides was added to the medium of cultured N2A cells in the presence of 5% fetal bovine serum at a concentration of 1 μM for 48 h. Under these experimental conditions, the cells remained viable and showed no evidence of toxicity. Medium was changed every 24 h.

Neuroblastoma N2A cells were also transfected with 2 μg of mp27, an expression plasmid for full cDNA of mouse p27 cloning in pcDNA 3.1 (a gift of Dr. K. Miskimins) using a standard lipofectamine protocol (Gibco-BRL, Life Technology). Cells were grown in DMEM 5% fetal bovine serum, and were incubated with the mixture of plasmid and lipofectamine by 48 h. Cells were analyzed by mechanical detachment and protein extraction with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μM leupeptin, and 1 μg/ml pepstatin). Subsequently, an immunodetection analysis was carried out.

Immunoprecipitation

N2A neuroblastoma cells were induced to grow neuritic processes with 5 mM db-cAMP (Sigma, St Louis) for 48 h in DMEM 0.25% fetal bovine serum. Afterward, cells were lysed in

RIPA buffer. Five hundred microgram of total cellular protein was used for immunoprecipitation with an anti-p27 (F8 antibody, Santa Cruz, CA) or anti-Cdk-2 antibodies (M2 antibody, Santa Cruz, CA), used at a final dilution of 1:50. The rinsed agarose beads were washed three times with RIPA buffer. After incubation, the samples were analyzed by SDS–PAGE and Western blot or was used for kinase activity assay.

Protein Kinase Assays

For in vitro kinase assay, the immunoprecipitates were rinsed one time with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, pH 7.5) plus 1 μM cold ATP. The rinsed agarose beads were incubated with kinase buffer containing 2.5 μg of histone H1 plus 5 μCi [γ^{32}P] ATP in a final volume of 50 μl for 30 min at 30°C. After incubation, the samples were analyzed by SDS–PAGE and autoradiography.

Heat treatment experiments were performed as described [O'Brien et al., 2001], the already generated p27 immunoprecipitates were subjected to sonication in 50 mM HEPES, pH 7.5, 150 mM NaCl and clarified by centrifugation (13,000*g* for 10 min at 4°C). Samples were heated at 100°C for 2 min to eliminate thermolabile proteins and Cdks complexes and quenched on ice. Then the treated extracts were added to pre-immunoprecipitated Cdk2 complexes prepared from N2A cells grown under normal conditions. All the immunoprecipitates from heat-treated extracts were clarified by centrifugation (13,000*g* for 5 min at 4°C) and used for kinase assays using histone H1 as substrate, as described above.

Immunofluorescence Studies

For immunofluorescence analysis, N2A cells were fixed in 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 1 min. Immunostaining was done as described by Muñoz et al. [2000], cells was incubated with the polyclonal p27 antibody (Santa Cruz) and a monoclonal antibody against cyclin D1 (Santa Cruz). Cells were then incubated with FITC- or rhodamine-congugated secondary antibodies (Sigma, St Louis, MO). Nuclei were stained with Hoescht. Coverslips were mounted in Prolong Antifade Kit (Molecular Probes).

RESULTS

Levels of p27 Intracellular Pools in Proliferating N2A Cells

It was of great interest to analyze the time course of the changes in the protein expression of p27 in differentiating N2A cells. Determinations by immunodetection of the p27 levels present in undifferentiated N2A cells revealed a significantly higher amount of this protein component in control proliferating cells prior to db-cAMP treatment (Fig. 1A). This was an unexpected observation considering that high proliferating activity of neuroblastoma cells. Interestingly, the protein levels decayed during the first 24 h of differentiation, and then increased again significantly between 24 and 48 h after db-cAMP treatment (Fig. 1A). Densitometric scans after 10 different experiments ($n = 10$) revealed that undifferentiated cells (controls) at zero time had a higher level of p27 protein as compared with differentiated ones ($P \leq 0.01$), and that an increase occurs between 24 and 48 h (Fig. 1B).

In order to further explore the phenomena associated with accumulation of p27 in N2A cells prior to cell differentiation, we analyzed the changes in the cyclin dependent kinases cdk-2, cdk-4, and cdk-6, along with cyclin E. The studies revealed that their expression did not change at all during the course of differentiation; however, the levels of cyclin D1 decayed dramatically upon triggering neuronal differentiation with db-cAMP (Fig. 1C).

It was important to evaluate the turnover of accumulated p27 in proliferating cells. For that purpose, undifferentiated N2A cells were incubated with cycloheximide or cycloheximide plus db-cAMP added at the same time. A gradual decrease in the p27 protein levels was revealed within a period of 12 h in N2A cell treated with db-cAMP and cycloheximide (Fig. 2, lower part). However, in control cell treated only with cycloheximide, p27 protein levels only decreased after 18 h treatment (Fig. 2, upper part). The studies suggest that db-cAMP induces an increase in the turnover of p27, a phenomenon that appears to be similar to the decrease in cyclin D1 as evidenced in Figure 1C.

Dual Control of p27 and Cyclin D1 Expression During Neuronal Differentiation

As an approach, and to ascertain the genes controlling the checkpoints between the arrest

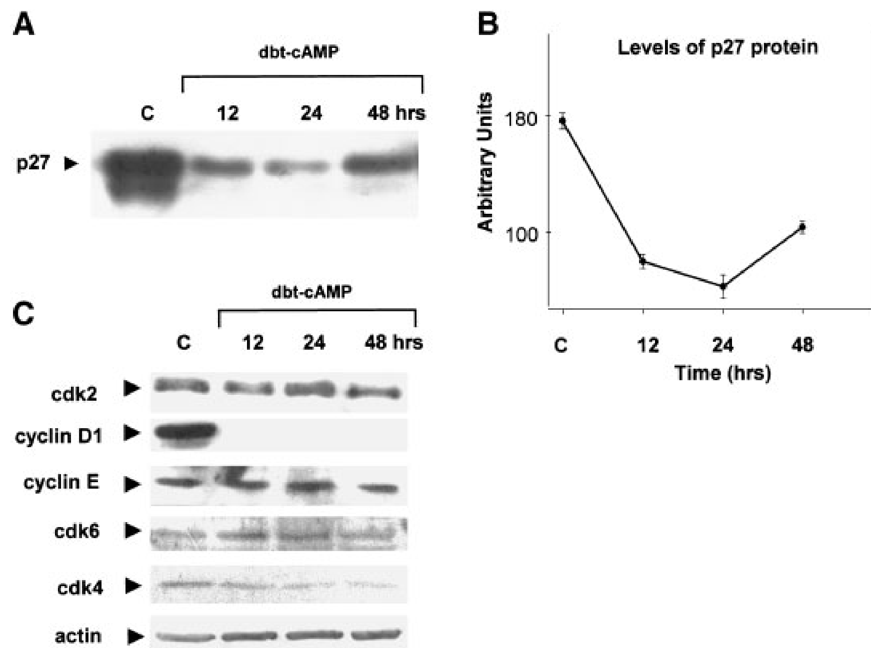

Fig. 1. Western blot analysis of the p27 levels in differentiated and undifferentiated N2A cells. **A**: Immunodetection of the p27 protein in N2A cells after 0, 12, 24, and 48 h following the addition of db-cAMP (5 mM). **B**: Densitometric scans of the p27 protein in N2A cells at 0, 12, 24, and 48 h after addition of db-cAMP (n = 10). Standard deviations are shown. **C**: Immunodetection of the cell cycle-related proteins cdk2, cdk6, cdk4, cyclinD1, and cyclin E in N2A cells after 0, 12, 24, and 48 h following the addition of db-cAMP (5 mM). Actin is shown as internal control.

in cell proliferation and promotion of cell differentiation, DNA array studies were carried out (Fig. 3A). Neuroblastoma N2A cells can be induced to undergo morphological and biochemical differentiation upon incubation in the presence of db-cAMP. The arrest in the N2A cell cycle is associated with the gradual formation of long neuritic processes. Therefore, our

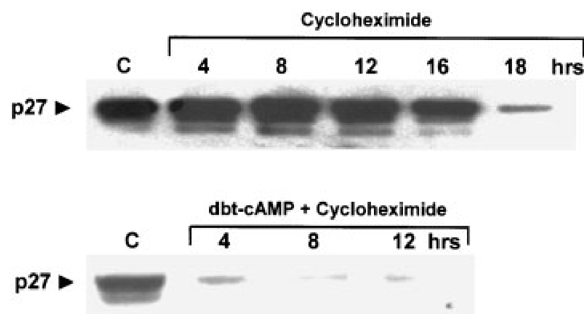

Fig. 2. Differentiation of N2A cells with db-cAMP induces degradation of p27 protein. **Upper**: Undifferentiated N2A cells were treated with cycloheximide for different time intervals and p27 was analyzed by Western blots. **Lower**: Undifferentiated N2A cells were induced to outgrowth neurites with db-cAMP in the presence of cycloheximide for different time intervals, and p27 was analyzed by Western blots. The time-dependent decay in the p27 expression was analyzed by immunodetection of p27 protein in cellular extracts. C, untreated controls.

studies focused on the molecular changes underlying the coordinated control of cell proliferation of these tumor cells and the promotion of neuronal differentiation. A significant decrease in cyclin D1 transcript during the course of cAMP treatment was evidenced after inducing neuronal differentiation of N2A cells, while a concomitant increase in p27 transcript was clearly evidenced after 48 h differentiation (Fig. 3A, left). These results for the transcripts were corroborated by RT-PCR studies using the cDNAs obtained from undifferentiated and 48 h-differentiated neuroblastoma cells. The studies showed a gradual increase in p27 mRNAs during the course of neuronal differentiation (Fig. 3B, right). β-actin was used as a constitutive gene. This approach corroborates the data obtained from cDNA array experiments.

In addition, scanning analysis of the DNA arrays during neuronal differentiation showed a gradual decrease in the transcripts of cyclin D1, H-ras, and B-raf. Cyclin D1 as well as H-ras and B-raf are proteins whose function is also modulated in coordination with the expression of the c-myc/p27 pair in cell proliferation (Fig. 3A, right). The data in Figure 3A represent a relative measurement in arbitrary units of

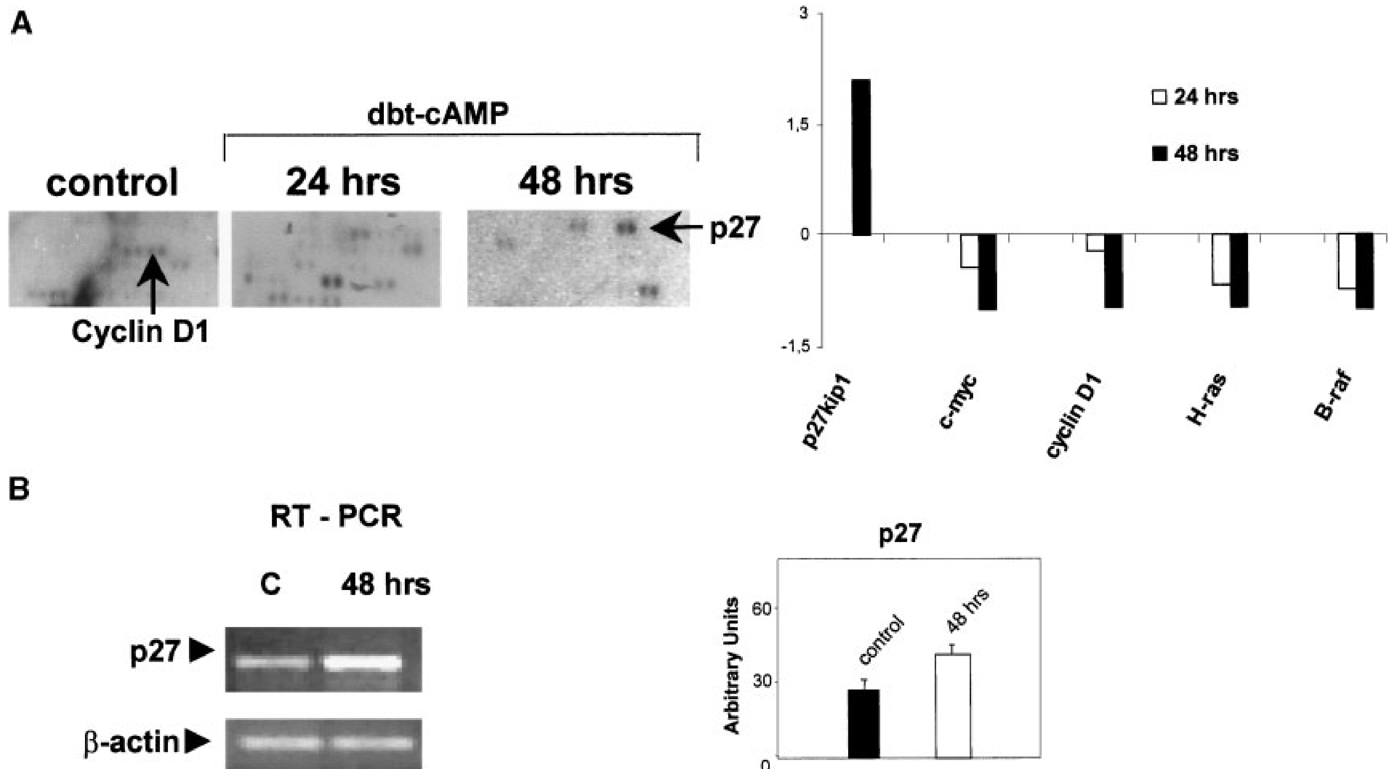

Fig. 3. Changes in p27 expression in N2A cells differentiated with db-cAMP. **A** (**Left**): Changes in the expression of the transcripts for p27 and cyclin D1 as analyzed by cDNA microarray studies. **Right**: Densitometric analysis (in arbitrary units) derived from the cDNA array studies after 24 and 48 h db-cAMP treatment, to evaluate the changes in the expression of the genes involved in the cell proliferation arrest and differentiation of N2A cells. Expression of transcripts was normalized with control cDNA array of undifferentiated N2A cells. **B** (**Left**): RT-PCR analysis of the mRNA levels for p27 in differentiated and undifferentiated N2A cells. A 2% agarose gel of the PCR products for p27 and β-actin obtained for the cDNAs of N2A cells treated with db-cAMP (5 mM) for 48 h and the untreated control cells. Gels were stained with ethidium bromide. Other details are indicated in Materials and Methods. **Right**: Densitometric analysis of RT-PCR showing the error bars of control and 48 h-dbcAMP differentiation.

these transcripts. Expression of transcripts was normalized with control cDNA of undifferentiated N2A cells.

p27 Protein Forms a Complex With Cyclin D1

To examine the mechanisms of accumulation of p27 protein in proliferating N2A cells, studies were focused on its interaction with cyclin D1. Immunofluorescene experiments showed that p27 and cyclin D1 exhibit a similar localization pattern in the undifferentiated N2A morphology (Fig. 4A). Immunodetection experiments have shown that cyclin D1 was also highly expressed under control conditions, that is, neuroblastoma cells with undifferentiated morphology (see Fig. 1C). By using immunoprecipitation with anti-p27 monoclonal antibody, we found that in proliferating cells, most of p27 is associated with cyclin D1 (Fig. 4B), thus explaining the lack of inhibitory activity of p27 on cell cycle Cdks despite its high levels in undifferentiated cells. These data suggest that cyclin D1 could sequester p27, and that as a result of that it regulates p27 activity prior to the induction of cell differentiation.

At this stage it was critical to ascertain whether the loss of p27 function is due to structural alterations such as a possible protein modification [O'Brien et al., 2001], or just directly as a consequence of the counteracting effect of cyclin D1 in undifferentiated N2A cells that may prevent p27 activity. For this purpose we immunoprecipitated p27 with cyclin D1 in proliferative N2A cells, and removed cyclin D1 from complex upon sonication of the neuronal extracts followed by boiling for 2 min based on

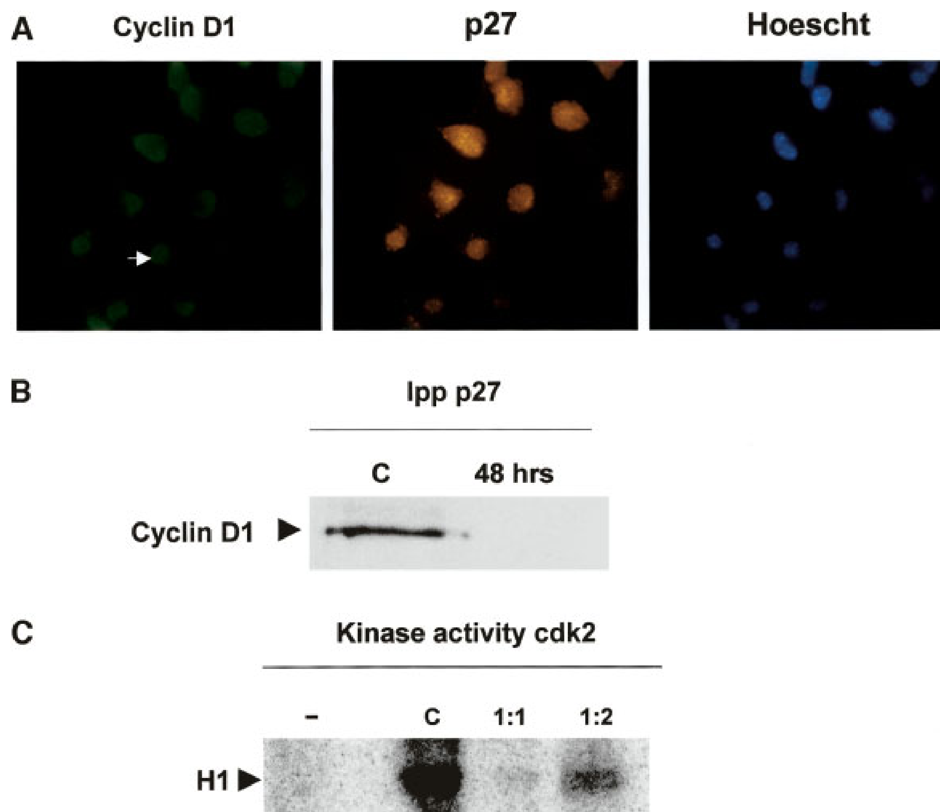

Fig. 4. Interaction of p27 with cyclin D1 in N2A differentiation and effects of p27 in inhibiting Cdks. **A**: Double immunostaning of Cyclin D1 (**left panel**) with p27 (**middle panel**). Arrows indicate the nuclei staining with cyclin D1. The nuclei stained with Hoescht is shown at the **right panel**. **B**: Immunoprecipitation of p27 (Ipp p27) in cell extracts of untreated N2A cells (designated as C), and treated for 48 h with 5 mM db-cAMP to induce differentiation. Immunoprecipitates were analyzed by Western blots using antibodies for cyclin D1. **C**: Protein kinase activity obtained after immunoprecipitation of N2A extract with anti-Cdk2 antibody, following the addition of a fraction of previously N2A immunoprecipitated p27 protein. p27 was precipitated with anti-p27 antibody followed by 2 min boiling. (−), negative control of kinase activity without histone H1 as a susbtrate; (C), positive control of basal total kinase activity of Cdk2 in the undifferentiated N2A cells in the absence of p27; (1:1), Cdk2 kinase activity obtained after mixing (1:1 dilution) immunoprecipitated p27 and the immunoprecipitated Cdk2; and (1:2), Cdk2 kinase activity after mixing (1:2 dilution) the p27 preparation with immunoprecipitated Cdk2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the heat stability property of p27 [O'Brien et al., 2001]. The thermostable p27 protein was capable of inhibit Cdk2 activity using histone H1 as a substrate (Fig. 4C). The data indicate that even though p27 could be sequestered by cyclin D1 it retains its intrinsic activity, and that its functional loss in undifferentiated cells is due to the counteracting effect of cyclin D1.

Overexpression of Exogenous p27 Induces Neuritic Processses

The expression of the p27 transcripts and protein can play a major role in the neuroblastoma cell differentiation, thus exerting its action on the cell cycle Cdks. To further evaluate the effects of p27 expression, undifferentiated neuroblastoma N2A cells were transfected with the full cDNA for mp27 cloned in pcDNA3. We found that a threefold increase in the expression of p27 after transfection, induced cell cycle arrest associated with the growth of N2A neuritic processes (Fig. 5, upper panels). Around 30% of transfected cells contained neurites. Furthermore, an increase in the expression of p27 levels upon cell transfection triggers a decrease in the levels of cyclin D1 (Fig. 5, lower panels).

Inhibition of c-myc Expression Induces Cell Cycle Arrest and N2A Differentiation

Cell differentiation triggers a decay in both the transcript and the protein c-myc, an event that appears to be determinant for N2A differentiation. A progressive decrease in c-myc during the course of neuronal differentiation until 48 h after exposure to db-cAMP was observed by immunodetection and RT-PCR (Fig. 6A–B). In order to evaluate the effects of the c-myc inhibition on N2A proliferation, we used transfection experiments of antisense oligonucleotides to the region involving c-myc mRNA bases 1,396–1,416 in N2A cells. These antisense have been shown to be highly specific [Paria et al., 1992]. The data showed that c-myc inhibition by 40% using the antisense oligonucleotide resulted in an arrest of cell proliferation while inducing outgrowth of N2A neuritic processes (Fig. 6C). On the other hand, cyclin D1 and p27 protein levels decreased upon the antisense treatment, results that are in agreement with that observed in early events of db-cAMP-induced differentiation (Fig. 6D). The data suggest that both c-myc inhibition and the increase in p27 induce cell cycle arrest in the

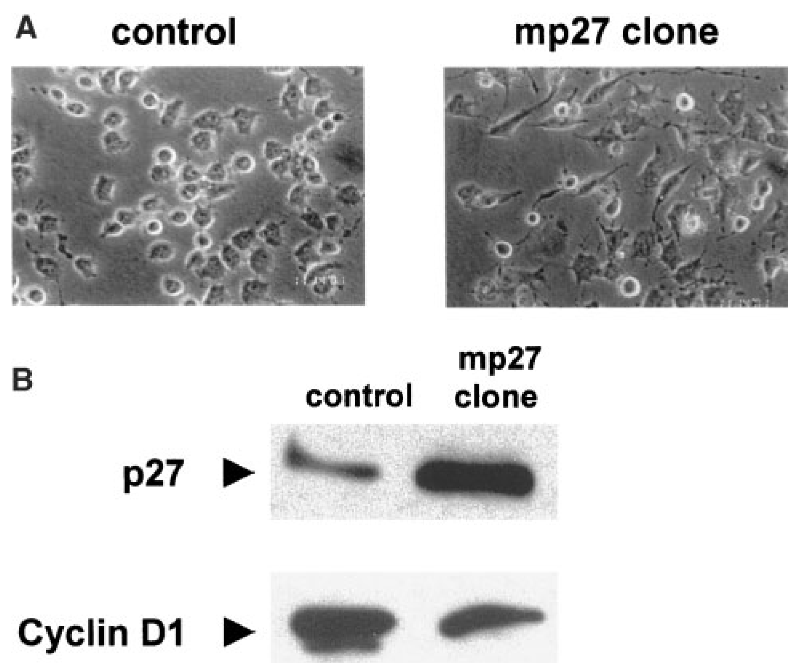

Fig. 5. Overexpression of exogenous p27 induces neuritic outgrowth of N2A cells. **A**: The N2A cells transfected with the pcDNA3 vector without the p27 insert are shown at the **left panel** (control). The N2A cells were transfected with full-length mp27 cDNA cloned in pcDNA3 and analyzed by light microscopy as shown in the **right panel**. Micrographs with a 40× magnification. **B**: Western blots of p27 and cyclin D1 for both the control and mp27 clone-transfected cells.

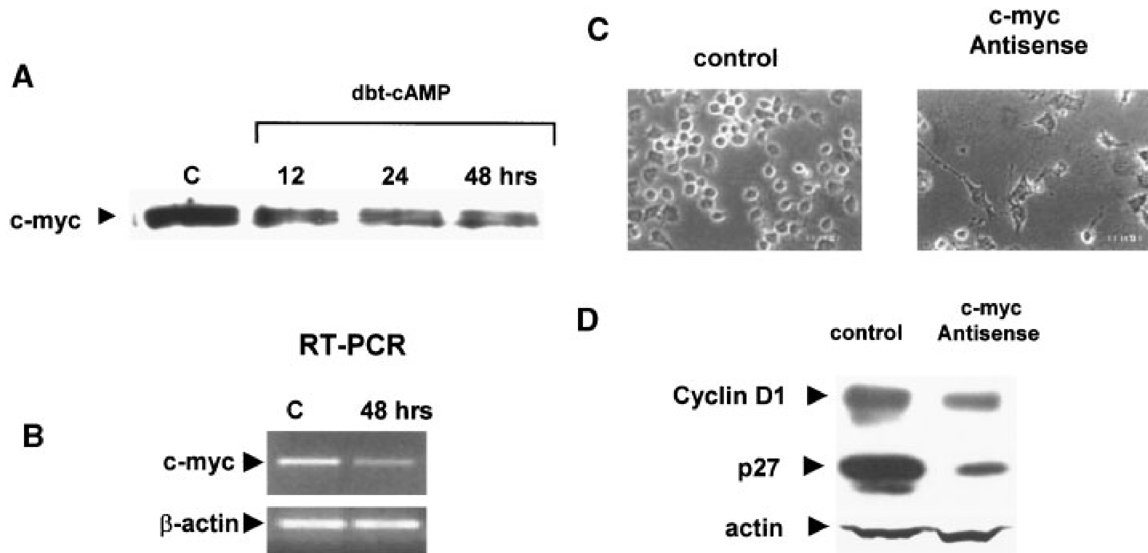

Fig. 6. Inhibition of c-myc induces induces neuritic processses of N2A cells. **A**: Expression of c-myc protein in N2A cells differentiated with 5 mM db-cAMP for 12, 24, 48 h. **B**: RT-PCR of c-myc transcript in undifferentiated and 48 h-differentiated cells. Actin was used as an internal control. **C**: N2A cells were transfected with 1 μM antisense phosphorothioated oligodeoxynucleotides for c-myc (c-myc antisense) for 48 h and analyzed by light microcopy are shown in the **right panel**. Cells treated with the sense oligonucleotide are shown at the **left** (control). **D**: Immunodetection of cyclin D1 and p27 in N2A cells treated with c-myc antisense and the controls treated with the sense oligonucleotides. Actin was used as internal control.

sequence of events that trigger neuronal differentiation, even though the precise mechanisms that coordinate c-myc and p27/cyclin D1 systems in neuronal cells remain to be elucidated.

DISCUSSION

Cell proliferation is regulated by a variety of molecular mechanisms, including gene expression and protein–protein interactions. It was noteworthy to observe a significant level of the p27 protein in proliferating cells, since this protein has been characterized as an inhibitor of cyclin dependent protein kinases such as cdk-2. An explanation for that unusual regulation of p27 in the transition from the undifferentiated to differentiated N2A phenotype is the counteracting effect of cyclin D1, shown to bind p27 in proliferating cells. The role of cyclin D1 in sequestering p27 has been reported in several non-neuronal transformed cells [Vlach et al., 1996; Bouchard et al., 1999; Perez-Roger et al., 1999] as well as its role in counteracting cell differentiation [Spinella et al., 1999] in agreement with the present studies, even though studies were carried out in different cell types. Moreover, the involvement of p27 in cell differentiation has been also claimed in previous reports [Perez-Juste and Aranda, 1999; Baldassarre et al., 2000]. However, the features of p27 found in these neuroblastoma cells are unique. We have found an abnormal accumulation of p27 in highly proliferating neuroblastoma N2A cells, and that this protein is degraded upon triggering differentiation to be re-synthesized in neuronal cells that outgrow long neuritic processes after cAMP treatment. We have also found that the effect of cyclin D1 appears to be the result of its direct interaction with p27 as detected by immunoprecipitation experiments, thus preventing p27 expression of its intrinsic activity in blocking Cdks as a feature of proliferating phenotype.

Previous studies have shown that the factor p27 interacts with the cyclin E/cdk-2 complex, thus originating an arrest in the progression from G1 to S phase during the cell cycle [Mateyak et al., 1999]. The processes that coordinate the regulatory patterns for these proteins have not been elucidated yet, but there is evidence that c-myc can induce the expression of some proteins involved in controlling the accumulation and degradation of p27 [Mateyak et al., 1999]. Interestingly, in the mouse N2A neuroblastoma cells, high amounts of p27 were found in the proliferating undifferentiated cells. As described, the protein mass of p27 decreases gradually to a minimum upon promotion of neuritic outgrowth, and increases again

between 24 and 48 h after exposure of cells to db-cAMP. The observed increase in p27 around 48 h of differentiation is in agreement with experiments on the expression of the transcript, as well as on the functional action of p27 as a cell cycle protein kinase inhibitor in differentiated cells. During this period, de novo synthesis of p27 appears to occur. Similar observations were reported in transformed cells by the E5/E8 protein of bovine papillomavirus type 4, where increments in the mass of p27 were observed. As the p27 protein increased, this was sequestered by binding to the cyclin D1/cdk4 complex [O'Brien et al., 2001].

In the present study, by using DNA microarray and RT-PCR we showed that c-myc and p27 change their expression in a coordinated fashion in the process of differentiation of N2A cells induced to differentiate with db-cAMP. With regard to c-myc, we found a decrease in the mRNA expression associated with neuronal differentiation, as related to cell cycle arrest, and no re-synthesis of the oncogene was observed in the differentiated morphology. The presence of a high levels of p27 in the proliferating phenotype led us to postulate that under these conditions, p27 could not achieve its regulatory role as a result of its interaction and sequestration by the cyclin D1 complex. This phenomenon could lead to a p27 protein more susceptible to degradation. However, it is not clear at all whether degradation may occur in the complex or after p27 dissociates from the cyclin D1 complex. Interestingly, when we determined the cyclin D1 levels during cell differentiation, we found that this cyclin exhibited the highest levels in undifferentiated cells, an observation which is in agreement with previous reports indicating an overexpression of cyclin D1 in tumor cells. Immunoprecipitation experiments were critical to determine that cyclin D1 sequester p27 in actively proliferating neuroblastoma cells. We can speculate that in this way, cyclin D1 can silence p27 activity prior to neuronal differentiation. More precisely, the present studies indicate that even though p27 maintain at least its major activity of controlling Cdks, it cannot exert its action, since it remains sequestered by cyclin D1. The present data show that cyclin D1/p27 complexes are degraded upon differentiation. In this context, a suggested sequence of events could be: cyclin D1/p27 accumulation in proliferating cells, cyclin D1/p27 degradation, p27 synthesis de novo and cdks inhibition, cell growth arrest, and differentiation; even though further studies are required to define the precise relationships of these molecular and cellular events.

An integrated analysis of these results indicates that finely coordinated switches controlling the expression of the transcripts and p27 protein in consonance with c-myc oncogene operate in neuroblastoma cells to control the transition between the proliferating undifferentiated morphology and the differentiated cells. In this context, future directions in this study should address the role of c-myc-dependent p27 mRNA downregulation. On the other hand, treatment of N2A cells with antisense oligonucleotides directed to c-myc promote cell proliferation arrest, while inducing significant neuritic outgrowth. Transfection of a cDNA clone expressing mouse p27 also leads to cell cycle arrest but with only small neuritic processes formation. Similar observations were reported in other neuroblastoma cell lines [Matsuo et al., 2001].

In the present study, we have shown that p27 protein is sequestered by cyclin D1 in N2A proliferating cells, without exerting an effects on the cell cycle Cdks, because its association with the cyclin D1 and with other proteins. Cyclin D1 is highly expressed in proliferated cells. Moreover, these studies indicate that in the process of cell differentiation, this situation changes, the complex is degraded, and p27 is re-expressed while expression of cyclin D1 is inhibited. Therefore, p27 protein produced inhibitory effects on cell cycle Cdks. These studies contribute to shed light on the biochemical and morphological differentiation of a neuronal cell model, and open new paths to investigate these regulations in brain cellular models.

ACKNOWLEDGMENTS

We thank Dr. K. Miskimins for the generous gift of p27 clone. Research was supported by grants from Fondecyt 1020155 and by the Millennium Institute for Advanced Studies (CBB), project P99-031F.

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