

p27^{Kip1} Enhances Myelin Basic Protein Gene Promoter Activity

Robin Miskimins,^{1*} Rekha Srinivasan,¹ Mireya Marin-Husstege,² W. Keith Miskimins,¹ and Patrizia Casaccia-Bonnel²

¹Division of Basic Biomedical Sciences, University of South Dakota School of Medicine, Vermillion, South Dakota

²Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey

The process of oligodendrocyte differentiation is a complex event that requires cell cycle withdrawal, followed by the activation of a specific transcriptional program responsible for the synthesis of myelin genes. Because growth arrest precedes differentiation, we sought to investigate the role of cell cycle molecules in the activation of myelin gene promoters. We hypothesized that the cell cycle inhibitor p27^{Kip1}, which is primarily responsible for arresting proliferating oligodendrocyte progenitors, may be involved in the transcriptional regulation of myelin genes. In agreement with this hypothesis, overexpression of p27^{Kip1} in the CG4 cell line, but not in 3T3 fibroblasts, enhances the expression of luciferase driven by the myelin basic protein (MBP) promoter. Interestingly, this effect is specific for p27^{Kip1}; overexpression of other cell cycle inhibitors had no effect. Additionally, this effect is independent of halting the cell cycle; treatment with the cell cycle blocker roscovitine did not affect MBP promoter usage. We conclude that p27^{Kip1} contributes to oligodendrocyte differentiation by regulating transcription of the MBP gene. © 2002 Wiley-Liss, Inc.

Key words: p27^{Kip1}; myelin basic protein; oligodendrocyte progenitor

Oligodendrocytes are the myelinating cells of the central nervous system. Mature oligodendrocytes arise from proliferative oligodendrocyte progenitor (OP) cells that pass through a series of developmental stages (Pfeiffer et al., 1993). This progression through the oligodendrocyte lineage is believed to involve both intrinsic timing mechanisms (Noble et al., 1988; Raff et al., 1988) and extrinsic (i.e., growth factors and neuronal signals) mechanisms of regulation (Richardson et al., 1988; McKinnon et al., 1990; McMorris et al., 1993; Canoll et al., 1996). Because terminal differentiation of OP cells is preceded by a withdrawal from the cell cycle (Casaccia-Bonnel, 1997), it is reasonable to propose that cell cycle regulators play an important role in coupling growth arrest to the differentiation process. Cell cycle regulation in mammalian cells is dependent on the activity of cyclin-dependent kinases (CDKs), which are positively regulated by cyclins

and negatively regulated by CDK inhibitors. Two families of mammalian CDK inhibitors have been identified. The members of one family include p21^{Cip1}, p27^{Kip1}, and p57^{Kip2} (Vidal and Koff, 2000). The CDK inhibitor p27^{Kip1} has been shown to be involved in halting cells in G₁ in response to antimitogenic signals (Polyak et al., 1994; Haddad et al., 1999; Rao et al., 1999).

In the oligodendrocyte lineage, progenitors grown in the presence of platelet-derived growth factor (PDGF) accumulate p27^{Kip1}, and its expression is elevated coincidentally with terminal differentiation into mature myelinating cells (Durand, 1997; Friessen, 1997; Tikoo et al., 1997). In addition, ectopic expression of p27^{Kip1} in oligodendrocyte progenitors in the presence of mitogens is sufficient to induce growth arrest (Tikoo et al., 1998; Tang et al., 1999). However, enhanced expression of p27^{Kip1} by itself is not sufficient to trigger the complete process of oligodendrocyte maturation, at least in the absence of an inductive signal, such as thyroid hormone (Tikoo et al., 1998; Tang et al., 1999). Nevertheless, p27^{Kip1} is a key component in growth and development of the oligodendrocyte lineage as indicated by the delayed differentiation process observed *in vitro* in progenitor cells cultured from the brains of p27^{Kip1}-deficient mice (Casaccia-Bonnel, 1997; Durand, 1997).

To address the question of whether the role that p27^{Kip1} may play in oligodendrocyte differentiation is dependent on its function as a cell cycle inhibitor, we asked whether p27^{Kip1} acts on the transcriptional program leading to synthesis of oligodendrocyte differentiation markers. Oligodendrocytes express several gene products that are unique to myelin-forming cells. Among these is the gene-encoding myelin basic protein (MBP). MBP accounts for approximately 30% of central nervous system myelin protein. These basic proteins are found on the

*Correspondence to: Robin Miskimins, Division of Basic Biomedical Sciences, University of South Dakota School of Medicine, 414 E. Clark St., Vermillion, SD 57069. E-mail: rmiskim@usd.edu

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cytoplasmic face of the myelin membrane and, although their function is still largely unknown, are thought to play a role in compaction of the membrane (Givogri et al., 2000). MBP is essential for the formation of myelin, insofar as mutations in the MBP gene that disrupt expression, such as in the shiverer mouse, lead to improper myelin formation (Roach et al., 1983; Kimura et al., 1985). We report here that increased expression of p27^{Kip1} can activate the MBP promoter. This effect is specific to p27^{Kip1} and to MBP and appears to be independent of the role of p27^{Kip1} in halting the cell cycle.

MATERIALS AND METHODS

Cell Culture

CG4 cells were grown at 37°C in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) plus 30 nM Na selenite, 50 ng/ml insulin, 50 µg/ml transferrin, 1 mg/ml bovine serum albumin (BSA), 100 U/ml penicillin, and 100 µg/ml streptomycin. PDGF was added to 5 ng/ml and basic fibroblast growth factor (bFGF) to 10 ng/ml. The cells were maintained in 5% CO₂ and 100% humidity. To induce differentiation, the cells were switched to differentiation medium (DM) consisting of DMEM containing 30 nM Na selenite, 50 µg/ml insulin, 50 µg/ml transferrin, 10 nM biotin, 10 nM hydrocortisone, 30 nM T₃, 20 nM progesterone, 2 mM glutamine, and 1 mg/ml BSA. Dibutyryl cyclic AMP (dbcAMP) was added to a final concentration of 1 mM. NIH3T3 cells were grown at 37°C in DMEM containing 10% calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in 5% CO₂ and 100% humidity.

DNA Constructs

The -1323-luc construct contains MBP gene sequences from -1,323 to +30 upstream of the luciferase coding region in pGL3Basic. The proteolipid protein (PLP) promoter construct (PLPLuc) containing 1.3 kbp of the PLP promoter upstream of the luciferase coding region in pGL2 was a generous gift of Dr. K. Nave. The p27^{Kip1} expression construct (p27) was cloned by polymerase chain reaction (PCR) of the entire cDNA for the mouse p27^{Kip1} and inserted downstream of the CMV promoter in pcDNA3.1(Hygro). The murine p57^{Kip2} construct was kindly provided by Dr. J. Massague.

Transfection and Luciferase Assays

Cells were plated in 35 mm dishes. Transfections were done in GM using GenePorter (Gene Therapy Systems, San Diego, CA) or Effectene transfection reagent (Qiagen, Valencia, CA), according to the manufacturer's protocol. After the transfection solution was left in contact with the cells for 4 hr, the medium was changed to GM containing PDGF and bFGF. The cells that were induced to differentiate had their medium changed to DM plus dbcAMP on the following day. At the indicated time, the cells were rinsed once with phosphate-buffered saline (PBS) and lysed by addition of 1× Reporter lysis buffer (Promega, Madison, WI). The supernatant was used for luciferase assays.

Luciferase assays were performed using Steady-Glo Luciferase assay reagent (Promega) or the Luciferase assay system

(Promega). Activity was measured in a Packard TopCount luminometer and normalized for protein content.

Western Blotting

Cells in 35 mm dishes were rinsed with PBS and lysed by addition of 1× sodium dodecyl sulfate (SDS) sample buffer (2.5 mM Tris-HCl, pH 6.8, 2.5% SDS, 100 mM dithiothreitol, 10% glycerol, 0.025% pyronine Y). Equal protein amounts were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to Immobilon P (Millipore, Bedford, MA) membranes using a Bio-Rad Trans-blot apparatus with a transfer buffer of 48 mM Tris and 39 mM glycine. Transfer blots were processed as described previously (Wang et al., 2000), and detection was carried out using a horseradish peroxidase (HRP)-conjugated secondary antibody and Super Signal chemiluminescent substrate (Pierce, Rockford, IL) or Western blot chemiluminescence reagent (New England Nuclear, Boston, MA). Primary antibodies were used at the following dilutions: anti-p27^{Kip1} (Transduction Laboratories, Lexington, KY) 1:2,500 and anti-p57^{Kip2} (a gift from Dr. E. Harlow) 1:1,000.

BrdU Labeling

CG4 cells were plated in 35 mm dishes and maintained in GM with PDGF and bFGF as described above. Cells were maintained in GM in the presence or absence of roscovitine at a final concentration of 2.5 µM for 24 hr. The cells were labeled with BrdU for 1 hr in GM and processed for detection of BrdU labeling using the BrdU labeling and detection kit II (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol.

RESULTS

Increased Expression of p27^{Kip1} Stimulates MBP Promoter

There is an increasing body of evidence that p27^{Kip1} plays a role in differentiation of cells that is independent of its function in cell cycle control. To determine whether p27^{Kip1} plays a role in transcriptional regulation of oligodendrocyte differentiation markers, the p27^{Kip1} cDNA, under control of the CMV promoter, was cotransfected into CG4 cells with a reporter plasmid containing the luciferase gene under control of the 5' flanking region of the MBP gene. This construct includes MBP sequences extending 1,323 bp upstream of the transcriptional start site, which we have shown to be sufficient to drive high-level, oligodendrocyte-specific expression in transgenic mice (Miskimins et al., 1992). This MBP-luciferase construct drives low-level expression of luciferase in growing CG4 cells (Fig. 1A), an oligodendrocyte precursor cell line (Louis et al., 1992). The level of expression of luciferase from the construct is increased when CG4 cells are switched to differentiation medium, consistent with the increase in MBP gene expression in the cells as they develop into a more mature phenotype. Cotransfection of the MBP-luciferase construct with a construct expressing p27^{Kip1} led to increased luciferase activity (Fig. 1A). This increase was observed in either growing or differentiated cells. Elevated luciferase activity corresponded to en-

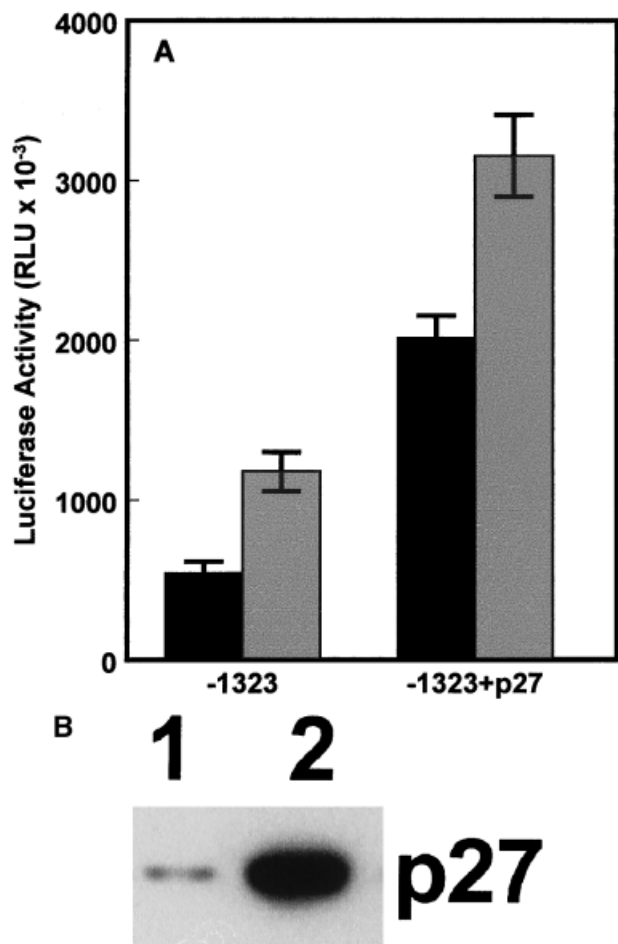


Fig. 1. Increased expression of p27^{Kip1} leads to elevated expression from the MBP promoter. **A:** CG4 cells were transfected with an MBP-luciferase construct and either a control plasmid (-1323) or an expression vector containing the p27^{Kip1} cDNA sequence (-1323 + p27) using GenePorter. Cells were left in growth medium (black bars) or switched to differentiation medium containing 1 mM dibutyryl cAMP (gray bars) and harvested after 3 days. Extracts were assayed for luciferase activity. Results are the mean of a minimum of three replicates \pm SE. The difference between the control (-1323) and the +p27 treatment is statistically significant ($P < 0.001$). **B:** CG4 cells transfected such as in A were harvested in SDS sample buffer and the lysates subjected to western blotting for p27^{Kip1}. Lane 1 is from cells transfected with a control plasmid, and lane 2 is from cells transfected with the p27^{Kip1} expression construct.

hanced p27^{Kip1} expression as determined by western blotting (Fig. 1B). These results indicate that p27^{Kip1} can modulate expression of the MBP gene.

p27^{Kip1} Does Not Affect Transcriptional Activation of Other Myelin Gene Promoters

An important question to address was whether the transcriptional effect observed in CG4 cells overexpressing p27^{Kip1} was specific to the MBP gene or was a general effect that led to increased expression from other myelin

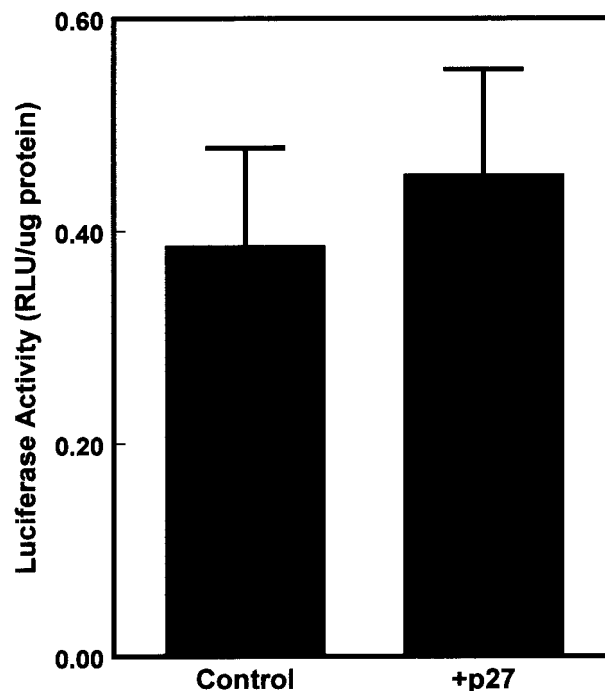


Fig. 2. Elevated expression of p27^{Kip1} does not increase expression from the PLP promoter. CG4 cells were transfected with a PLP-luciferase reporter construct plus either a control plasmid (control) or an expression vector containing the p27^{Kip1} cDNA sequence (+p27) using Effectene. After transfection, cells were maintained in differentiation medium containing dibutyryl cAMP for 3 days and then harvested for luciferase assays. Results are the mean of a minimum of three replicates \pm SE.

gene promoters. To investigate this possibility, CG4 cells were cotransfected with the p27^{Kip1} expression construct together with a plasmid carrying the PLP promoter upstream of luciferase. As indicated by the results shown in Figure 2, there is no response of the PLP promoter to increased p27^{Kip1} levels. This suggests that the effect of p27^{Kip1} is specific to the MBP promoter and does not increase transcriptional activity from all genes encoding myelin-specific products.

Stimulation of MBP Gene Expression Is Specific to p27^{Kip1}

p27^{Kip1} belongs to a family of CDK inhibitor (CKI) proteins that also includes p21^{Cip1} and p57^{Kip2}. When expressed at high levels in the cell, all of these proteins block cell cycle progression by inhibiting CDK2. These CDK inhibitors are closely related within their cyclin/CDK binding domains near the N-terminus of the proteins. However, they are divergent in their C-terminal regions, which may confer unique functions on the proteins. It is possible that the stimulatory effect of p27^{Kip1} on MBP promoter activity is dependent on cell cycle arrest. In this case, all of the CKIs in this family of proteins should have a similar effect. Alternatively, the observed effect

could be mediated through interactions that are specific to p27^{Kip1}. To determine whether expression of other members of this family of CKIs could lead to increased expression from the MBP promoter, the MBP-luciferase construct was cotransfected with p57^{Kip2}. MBP promoter-driven luciferase activity in cells overexpressing p57^{Kip2} was not significantly different from that in cells in which MBP-luciferase was cotransfected with empty vector (Fig. 3A,B). Similar results have been observed using expression vectors encoding p21^{Cip1} and also for a member of a different family of CKIs, p16 (data not shown). Thus p27^{Kip1} is the only CKI protein we have analyzed that stimulates expression from the MBP promoter.

To address whether the effect of p27^{Kip1} on transcription of the MBP gene was dependent on its function as an inhibitor of CDK2 activity, we compared the effect of p27^{Kip1} on MBP promoter activity with that of the pharmacological CDK2 blocker roscovitine. For these experiments, CG4 cells were transfected with the MBP-luciferase construct and then treated with roscovitine. To verify the effect of roscovitine on cell cycle progression, CG4 cells were treated with roscovitine and assayed for bromodeoxyuridine (BrdU) incorporation. In asynchronous cells, about 25% of the population is labeled with BrdU during a 1 hr period (Fig. 3C). Addition of roscovitine decreases the percentage of labeled cells by half (Fig. 3C). If blocking CDK2 were sufficient, we would expect to see increased luciferase activity in cells treated with the inhibitor. In fact, treatment with roscovitine does not lead to increased MBP promoter activity (Fig. 3D). Furthermore, p27^{Kip1} expression activates the MBP promoter even in the presence of roscovitine. This further suggests that the increase in MBP promoter-driven luciferase activity seen when p27^{Kip1} levels are increased is due to a function of p27^{Kip1} that is independent of its role in cell cycle regulation.

Increased MBP Promoter Activity Requires Oligodendrocyte-Specific Factors

Increased expression from the MBP promoter by elevated levels of p27^{Kip1} appears to be a specific effect of p27^{Kip1} on this gene. It is highly unlikely, however, that p27^{Kip1} functions as a DNA-binding protein. This suggests that there are protein-protein interactions that have to occur to effect the increase in MBP promoter usage. Insofar as MBP gene expression is restricted to cells that display a myelinating phenotype, it was of interest to determine whether increased p27^{Kip1} levels would increase MBP promoter usage in cells that were incapable of myelination. For these experiments, the -1323MBP-luciferase construct was cotransfected with the p27^{Kip1} expression construct into NIH 3T3 cells, a fibroblast line. In this case (Fig. 4), there was no increase in the expression of luciferase from the MBP promoter. Rather, a decrease in activity was observed. This indicates that the effect of p27^{Kip1} on the MBP promoter is specific to oligodendrocytes and likely requires oligodendrocyte-specific factors for its function.

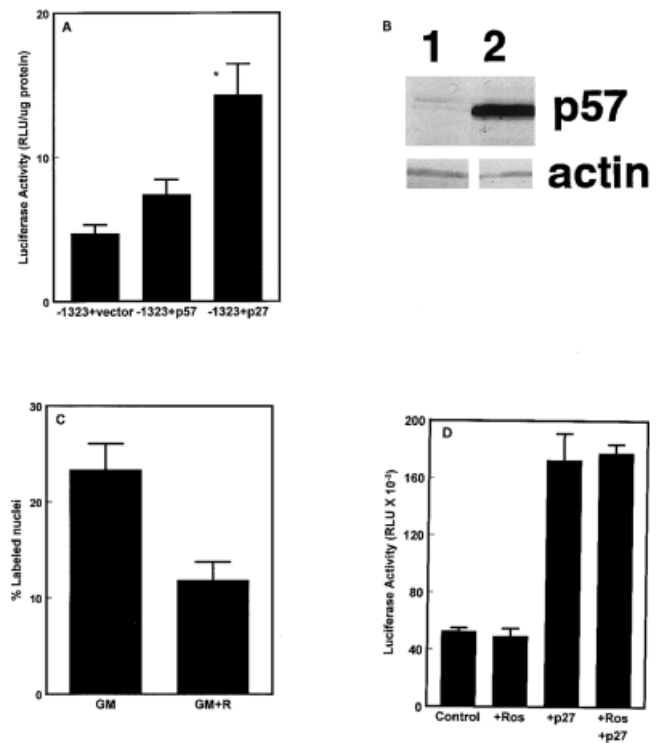


Fig. 3. The effect of p27^{Kip1} on the MBP promoter is specific. **A:** CG4 cells were transfected with the MBP-luc construct and a control plasmid (-1323), a plasmid encoding the p57^{Kip2} cDNA (-1323 + p57), or an expression vector containing the p27^{Kip1} cDNA sequence (-1323 + p27) using Effectene. After transfection, the cells were maintained in differentiation medium plus dibutyryl cAMP for 3 days and harvested for luciferase assays. Results are the mean of a minimum of three replicates \pm SE. *Significantly different from -1323 ($P < 0.01$). **B:** CG4 cells transfected as in A were harvested in SDS sample buffer and the lysates subjected to western blotting for p57^{Kip2} (top). Lane 1 is from cells transfected with a control plasmid, and lane 2 is from cells transfected with the p57^{Kip2} expression construct. The same extracts were blotted for actin (bottom) as a loading control. **C:** CG4 cells were maintained in growth medium plus PDGF and bFGF in the presence (GM + R) or absence (GM) of 2.5 μ M roscovitine for 24 hr. Cells were labeled with BrdU for 1 hr before staining with anti-BrdU antibody. Cells were counted as positively or negatively stained, and the percentage of labeled nuclei was calculated. Results are the average \pm SE. **D:** CG4 cells were transfected with MBP-luc plus either a control plasmid (control) or an expression vector containing the p27^{Kip1} cDNA sequence (+p27) using GenePorter. The cells were grown in the presence (+Ros) or absence of 2.5 μ M roscovitine for 3 days and then harvested for luciferase assays. Results are the mean of a minimum of three replicates \pm SE. Results from cells transfected with p27, with or without roscovitine, were significantly different from those from cells not transfected with p27 ($P < 0.001$).

DISCUSSION

OP cells require withdrawal from the cell cycle to differentiate. The cell cycle inhibitor p27^{Kip1} has been shown to be a key element in OP differentiation, because it is responsible for G₁ arrest by inhibiting cyclin E/CDK2 activity (Casaccia-Bonnel et al., 1997; Tikoo et al., 1998;

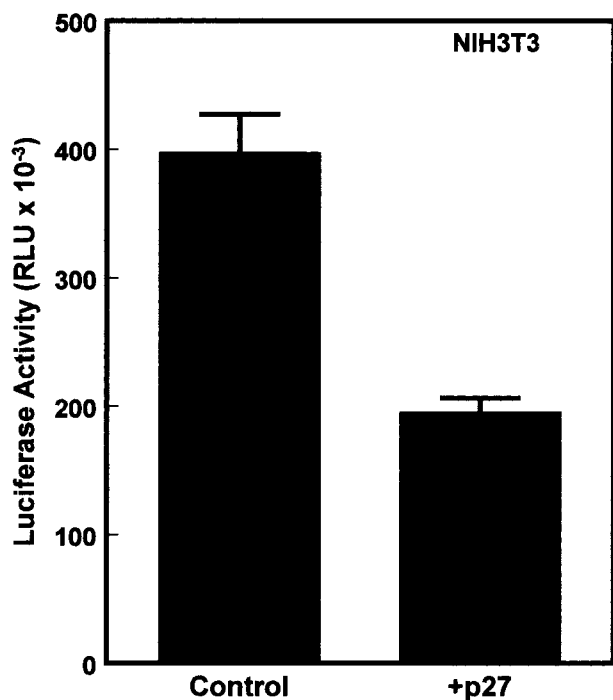


Fig. 4. p27^{Kip1} expression in NIH3T3 cells does not increase MBP promoter usage. NIH3T3 cells were transfected with MBP-luc plus either a control plasmid (control) or an expression vector containing the p27^{Kip1} cDNA sequence (+p27) using GenePorter. Cells were allowed to grow for 1 day after transfection before harvesting for luciferase assays. Results are the mean of a minimum of three replicates \pm SE.

Tang et al., 1999). In agreement with its role in cell cycle regulation, progenitors isolated from p27^{Kip1}^{-/-} mice have a marked deregulation of growth arrest (Casaccia-Bonnelil et al., 1997; Durand et al., 1997), resulting in increased numbers of OPs at specific stages of development in vivo (Casaccia-Bonnelil et al., 1997). These cells have an altered timing of differentiation (Durand, 1998), and only a low percentage acquires the morphology of mature cells (Casaccia-Bonnelil, 1997).

In addition to its role in G₁ arrest in progenitor cells, the presence of high levels of p27^{Kip1} expression in myelinating cells (Durand et al., 1997; Friessen et al., 1997) suggests a role for p27^{Kip1} not only in cell cycle regulation but also in oligodendrocyte differentiation. One possibility is that p27^{Kip1} affects transcription of genes expressed late in differentiation by modulating specific promoters. To test this hypothesis, we used an MBP-luciferase reporter construct that has been shown to be sufficient to drive high levels of oligodendrocyte-specific expression in transgenic mice (Miskimins et al., 1992). When this plasmid is cotransfected with a vector expressing p27^{Kip1} in CG4 cells, we observe a significant increase in luciferase activity. This effect is specific for p27^{Kip1} because other CKIs, such as p57^{Kip2}, p21, and p16^{INK4}, do not produce the same effect on the MBP reporter gene.

In addition, the effect of p27^{Kip1} is a targeted effect that specifically increases MBP expression, not a general

effect on transcription of genes required for oligodendrocyte differentiation. This is demonstrated by the fact that increased levels of p27^{Kip1} do not increase expression from a PLP promoter construct.

A role for p27^{Kip1} during oligodendrocyte differentiation, which is independent of its role in cell cycle regulatory activity, is further suggested by the fact that blocking CDK2 activity with a chemical inhibitor does not lead to increased MBP promoter usage or enhance the effect of overexpression of p27^{Kip1} on the MBP promoter. A role for p27^{Kip1} in differentiation that is independent of its cell cycle regulatory activity has been observed in other systems. For instance, during neuronal differentiation of embryonal carcinoma cells induced by treatment with retinoic acid, p27^{Kip1} levels are increased in a biphasic manner (Sasaki et al., 2000). The early increase is associated with cell cycle arrest, whereas the secondary increase corresponded to the onset of neurite extension. Blocking the secondary increase in p27^{Kip1} in the embryonal carcinoma cells blocked neuronal differentiation, suggesting that p27^{Kip1} plays an integral part in the neuronal differentiation process in these cells.

In addition, other CKI proteins have been shown to have specific transcriptional effects. For example, during muscle cell differentiation, the myogenic transcription factor MyoD is expressed in proliferating myoblasts. Transfection of myoblasts with the CKIs p21 and p16 enhances muscle-specific gene expression (Skapek et al., 1995). In this case, the effect is likely mediated through the action of a cyclin/CDK complex, which, when active, suppresses MyoD function, leading to a decreased level of transcription of muscle-specific genes.

Finally, we demonstrate that the effect of p27^{Kip1} on the MBP promoter is restricted to oligodendrocyte-lineage cells; there is no increase in luciferase activity when the MBP-luciferase construct is expressed in NIH3T3 cells. This implies that p27^{Kip1} requires some oligodendrocyte-specific factor(s) for this function. Although the mechanism by which p27^{Kip1} activates MBP transcription is not known at this time, we can speculate on the mechanism by which this might occur. Insofar as p27^{Kip1} has no known DNA-binding activity, it is likely that the transcriptional effect is indirect. An example of indirect transcriptional control by p27^{Kip1} was recently shown to be operating in control of the P4 promoter of the minute virus of mice (Deleu et al., 1998). In this case, p27^{Kip1} was able to affect the transcription of the P4 promoter by modulating the activity of specific DNA transcription factor binding motifs. Determination of the mechanism by which MBP expression is enhanced by p27^{Kip1} will be of importance in understanding the role played by this protein in oligodendrocyte differentiation.

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