

Id4 Expression Induces Apoptosis in Astrocytic Cultures and Is Down-regulated by Activation of the cAMP-Dependent Signal Transduction Pathway

Pedro J. Andres-Barquin, Maria-Clemencia Hernandez, and Mark A. Israel¹

Preuss Laboratory for Molecular Neuro-Oncology, Brain Tumor Research Center, Department of Neurological Surgery, School of Medicine, University of California at San Francisco, San Francisco, California 94143

The Id family of helix-loop-helix transcription factors has been implicated in the regulation of cellular differentiation in several different lineages. We have explored the potential regulatory role of the cyclic AMP-dependent signaling pathway on Id gene expression in astroglial primary cultures. We found that primary cultures of mouse forebrain astrocytes constitutively expressed the four known members of the Id gene family, Id1, Id2, Id3, and Id4. During culture in presence of serum for 4 weeks, the expression of Id4 was up-regulated. In these same cultures, treatment with dibutyryl-cyclic AMP, a cyclic AMP analogue known to promote astrocyte differentiation, dramatically and selectively decreased Id4 gene expression. This effect was detectable after short-term treatment and was maintained during long-term treatment. Forskolin and pentoxifylline, two other agents known to elevate intracellular cyclic AMP through different mechanisms, also potently decreased Id4 gene expression. Furthermore, overexpression of Id4 in an astrocyte-derived cell line induced cells to round up and die by apoptosis. These results indicate that the cyclic AMP pathway acts as an inhibitor of Id4 gene expression in astrocytes, identify a new function for Id4, and suggest that Id4 is strategically positioned in the chain of molecular events regulating astrocyte differentiation and apoptosis. © 1999 Academic Press

Key Words: astrocyte; programmed cell death; differentiation; dominant-negative transcription factor; Id.

INTRODUCTION

Astrocytes are the most abundant cells in the brain and have key roles in normal physiology, development, and pathology of the CNS [1]. These cells adopt morphologies and acquire specialized characteristics and

functions which vary in different locations in the CNS [2, 3]. Excluding radial glia, which are present early during embryogenesis, other astrocyte precursors proliferate and differentiate during perinatal development of the rodent brain. Little is known about the molecular mechanisms that are involved in the regulation of the astrocyte differentiation program. The control of cyclic AMP (cAMP)² synthesis and its regulation of protein kinase A activity are essential for many developmental processes [4–8] and the activation of the cAMP-dependent signaling pathway plays an important role in the regulation of astrocyte cell biology [9]. Both the biochemical and the morphological differentiation of astrocytes are induced in culture by agents which activate the cAMP-dependent pathway [10–12].

Id proteins belong to a class of transcription factors known as helix-loop-helix (HLH) proteins [13, 14]. Members of the HLH family which act positively to enhance transcription typically contain a domain of basic amino acids N-terminal to the HLH motif. Basic HLH (bHLH) transcription factors are known to play an essential role in the regulation of cell determination and differentiation in several cell lineages [15–17]. Following dimerization mediated by their HLH regions, the basic domains of such transcription factors bind to DNA [18]. However, Id proteins lack the basic amino acid domain necessary for DNA binding and inhibit binding to DNA and transcriptional activation by heterodimerization with bHLH factors [13, 19]. This mechanism is considered responsible for the inhibition of cell differentiation attributed to Id proteins in several different tissues [20–24]. Four members of the Id gene family have been identified in mammals: Id1 [13], Id2 [19], Id3 [25], and Id4 [26]. Although all four Id

² Abbreviations used: bHLH, basic HLH; BrdU, bromodeoxyuridine; dB-cAMP, dibutyryl-cAMP; cAMP, cyclic AMP; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HLH, helix-loop-helix; MEM, minimum essential medium; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.

¹ To whom correspondence and reprint requests should be addressed at Preuss Laboratory for Molecular Neuro-Oncology, Brain Tumor Research Center, Department of Neurological Surgery, HSE 722, University of California San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0520. Fax: (415) 476-0388. E-mail: israel@cgl.ucsf.edu.



genes are expressed during CNS development [27–33], very little is known about the biologic activity and regulation of Id gene expression in cells of the nervous system and especially in glial cells. Understanding Id4 may be of particular regard in this respect not only because as the most recently identified member of the family it has been least extensively studied, but also because its expression is the most specific for nervous system tissues [26, 31, 32]. To gain insight into the role of Id genes in astroglial differentiation and into the mechanism of regulation of Id molecules in astrocytes, we investigated the potential regulatory role of the cAMP-dependent signaling pathway on Id gene expression, during the differentiation of primary cultures of mouse forebrain astrocytes.

MATERIALS AND METHODS

Astrocyte culture and transfection. Albino Swiss mice (B & K Universal, Fremont, CA) were euthanized following the UCSF policies and procedures regulating the use of animals. Primary cultures of astrocytes were prepared from the forebrains of 1-day-old neonatal mice as previously described [34]. Briefly, after dissection of the forebrains, the meninges were carefully removed, and tissue was triturated in medium consisting of Eagle's minimum essential medium (MEM) (Life Technologies, Inc., Gaithersburg, MD), 2 mM glutamine, 7 mM glucose, penicillin (100 units/ml), and streptomycin (100 µg/ml) (all from Sigma Chemical Co., St. Louis, MO) supplemented with amino acids and 9% (v/v) fetal bovine serum (FBS) (Life Technologies, Inc.) and filtered through a sterile 80-µm nylon mesh. The cell suspension was incubated at 37°C in 100-mm tissue culture dishes for 1–2 h. Unattached cells were then plated into 60-mm tissue culture dishes in fresh medium and cultured at 37°C in a humidified 5% CO₂/95% air incubator for a maximum of 28 days. The culture medium was changed after 3 days of seeding and then renewed every other day. After 3 weeks of culture the cell population was composed of ~95% glial fibrillary acidic protein (GFAP)-positive cells (astrocytes) and 5% anti-galactocerebroside-positive cells (oligodendrocytes) and anti-Mac1-positive cells (microglia). The astrocyte-derived U373 MG cell line was obtained from the American Type Culture Collection (Rockville, MD; Cat. No. ATCC HTB 17) and cultured at 37°C, 5% CO₂/95% air, in Eagle's MEM with Earle's balanced salt solution (Life Technologies, Inc.) supplemented with nonessential amino acids, 1 mM pyruvate, 50 units/ml penicillin, 50 µg/ml streptomycin, and 10% (v/v) FBS (Life Technologies, Inc.). Where reported, cultures were treated for the indicated periods of time with 1 mM dibutyryl-cAMP (dB-cAMP) (Sigma Chemical Co.), 100 µM forskolin, 100 µM dideoxy-forskolin, or 4 mM pentoxifylline (Research Biochemicals International, Natick, MA). When treatment was longer than 2 days, culture medium was renewed every other day. For transfection, U373 cells were seeded to 70–80% confluence in wells of a six-well tissue culture plate (Falcon, Becton–Dickinson, Franklin Lakes, NJ), incubated 12–24 h, and washed once with phosphate-buffered saline (PBS) and once with OptiMEM medium (Life Technologies, Inc.). A lipofectin–DNA mixture containing 8 µg of pEGFP (Clontech, Palo Alto, CA) and pCMV-Id4 DNAs or pEGFP and pCMV DNAs and 10 µg of lipofectin (Life Technologies, Inc.) diluted in 1 ml of OptiMEM was then added to each well. After incubation at 37°C for 7 h, the medium was replaced with 2 ml of culture medium containing 10% FBS. The cells were examined at different times after transfection.

Isolation of RNA and Northern blot analysis. Total RNA was extracted from cultured cells by the guanidinium thiocyanate–phenol–chloroform extraction procedure [35], and Northern blot analysis was performed as previously described [36]. The hybridization

signal of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe and the methylene blue staining of ribosomal RNAs on the blot were used as an RNA loading and transfer control. Full-length murine Id1 [13], Id2 [19], Id3 (ATCC Cat. No. 63120) [25], and Id4 [26]; human GFAP [37]; and murine GAPDH cDNAs were radiolabeled with [³²P]dCTP by random-priming (Amersham Corp., Arlington Heights, IL). For rehybridization, membranes were stripped for 15 min at 100°C in 0.05× SSPE containing 0.1% SDS and 20 mM EDTA. Id4 films were exposed approximately twice as long as Id1–Id3 films in all experiments.

Immunofluorescence, cell proliferation, and apoptosis assays. For immunofluorescence, primary cultures of astrocytes were cultured as described, fixed with cold methanol for 5 min at –20°C, rinsed with PBS, and incubated for 1 h at room temperature with rabbit anti-cow GFAP (diluted 1:100; Dako Corp., Carpinteria, CA). A normal rabbit immunoglobulin fraction (Dako Corp.) was used in parallel as a control. After washing in PBS, cells were incubated with a 1:100 dilution in PBS of tetramethylrhodamine isothiocyanate (TRITC)-conjugated sheep anti-rabbit IgG for 1 h at room temperature. Cells were finally washed and mounted. Cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation into nuclei following 24 h exposure to BrdU at a final concentration of 10 µM. A cell proliferation kit (Boehringer Mannheim Corp., Indianapolis, IN) was used according to manufacturer specifications to detect BrdU incorporation. For GFAP and BrdU double immunostaining, cells were pulsed with BrdU for the final 24 h of culture. The cells were then fixed in 70% ethanol/50 mM glycine buffer (pH 2.0) for 20 min at –20°C and immunolabeled with an anti-BrdU monoclonal antibody (diluted 1:10) followed by fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (diluted 1:10) (Boehringer Mannheim Corp.). GFAP was immunostained as described above. Cell nuclei were visualized following incubation with 1 µg/ml DAPI (Sigma Chemical Co.) for 5 min at room temperature. To determine the fraction of cells in S phase, approximately 1000 DAPI-positive cells were counted, and the fraction of cells that were also positive for FITC was determined for each sample. Immunofluorescence microscopy was performed using standard epifluorescence optics (Zeiss Axioplan, Oberkochen, Germany).

For green fluorescent protein (GFP) detection and morphological analysis of transfected cells, cells were observed live under fluorescent light in an inverted fluorescence microscope (Zeiss Axiovert 100). A total of 35 fields containing 200–600 GFP-positive cells per sample were counted using 200× magnification. For morphological assessment of chromatin structure, cells were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, washed twice for 5 min each time with PBS, and stained with Hoechst 33342 (Sigma Chemical Co.) (2 µg/ml in PBS) for 10 min at room temperature in the dark. Cells were then washed twice for 5 min in PBS and mounted for photomicrography. DNA fragmentation was detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining using the Apoptag kit (Oncor, Gaithersburg, MD) according to manufacturer specifications. Floating cells were fixed in 7.5% neutral buffered formalin for 10 min at room temperature and 100 µl of the cell suspension was dried on a microscope slide. After Apoptag staining cells were washed in distilled water, counterstained with methyl green, washed in water, and mounted for photomicrography.

RESULTS

Primary cultures of forebrain astrocytes prepared from 1-day-old neonatal mice were analyzed during culture for regulation of Id1, Id2, Id3, and Id4 gene expression by Northern blot analysis. A cell population which contained mostly protoplasmic-like cells and a few fibrous cells grew in the presence of 9% FBS. The cells proliferated rapidly for 2 weeks. These cultures

reached confluence and proliferation was arrested during the third week of culture as estimated by BrdU incorporation (data not shown). As shown in Fig. 1, we detected the transcripts of Id1, Id2, and Id3 and the three characteristic transcripts of Id4 described previously for those genes [19, 25–27]. We determined the steady-state levels of Id1, Id2, Id3, Id4, and GFAP mRNA at 10, 12, 17, 21, 24, and 28 days of culture. While Id1, Id2, and Id3 mRNA steady-state levels did not change significantly over the 4-week culture period (Fig. 1), Id4 mRNA steady-state levels progressively increased approximately threefold from day 10 to day 28 of culture. The pattern of GFAP mRNA expression in these cultures increased during the 2 first weeks of culture and decreased thereafter as has been previously described by others [12] (Fig. 1A).

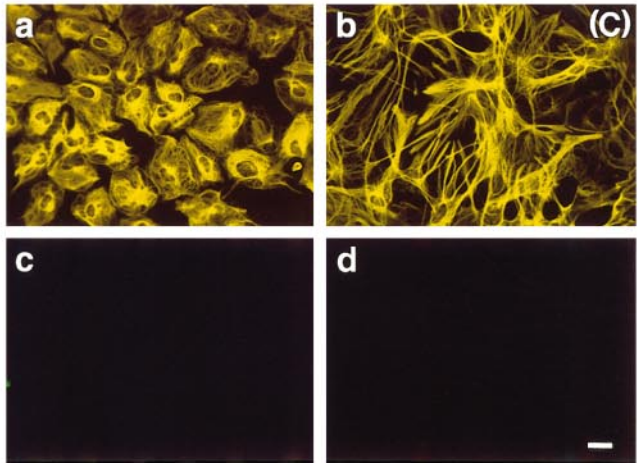
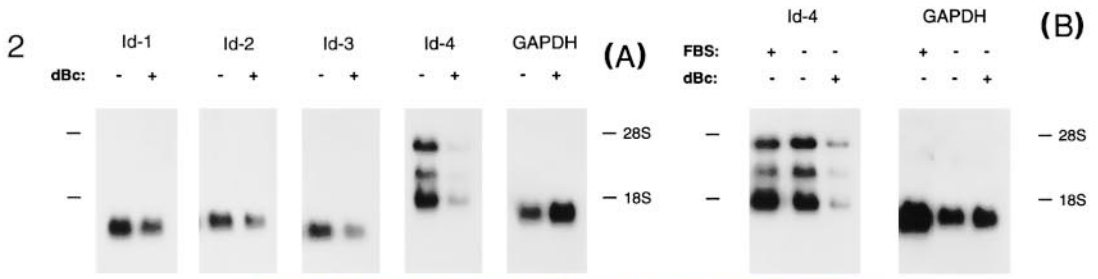
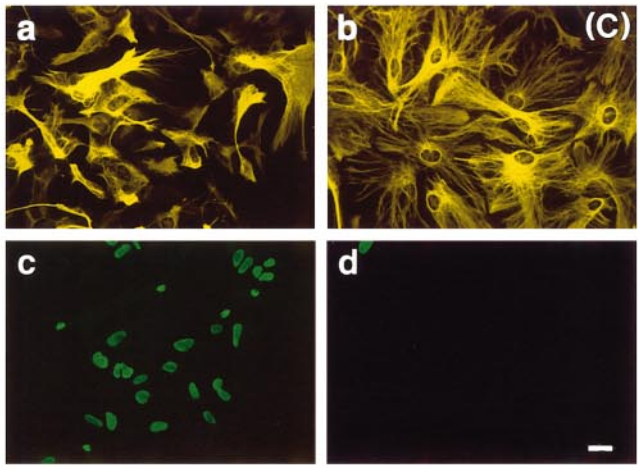
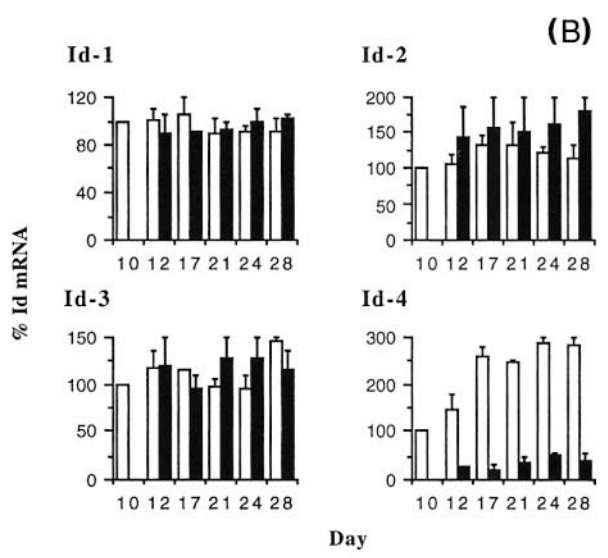
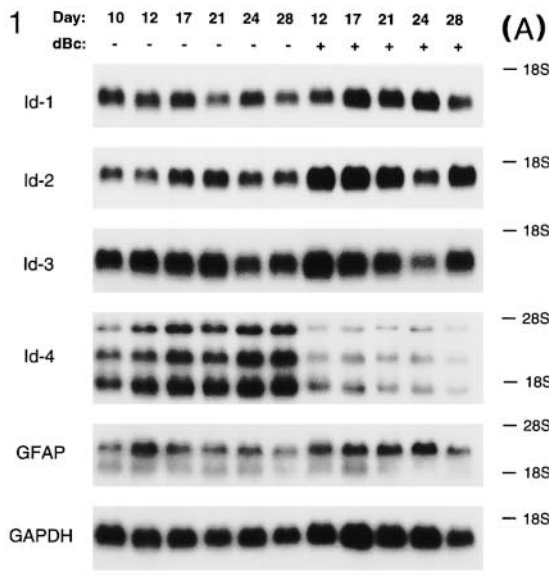
To explore the potential regulatory role of the cAMP pathway on Id gene expression in astroglial primary cultures, dB-cAMP, a cell-permeable analogue of cAMP known to promote astrocyte differentiation, was added to a subset of primary cultures at day 10 of culture. dB-cAMP-treated and mock-treated cultures were examined 2, 7, 11, 14, and 18 days later. We found the Id4 mRNA levels were decreased by approximately 80% in cultures treated with dB-cAMP after a 48-h treatment and that this decrease was maintained through the 28 days of experimentation (Fig. 1B). This effect was accompanied by cell growth arrest and, as has been previously described by others [12], an increase in the number of GFAP-positive cells, an increase in the intensity of GFAP staining, and an increase in the steady-state level of GFAP mRNA together with dramatic changes in the morphology of the cells, which

retract their cytoplasm and extend branched processes (Fig. 1, and data not shown). The steady-state levels of Id1, Id2, and Id3 mRNAs did not change in treated cells in relation to control cells (Fig. 1B). We also examined Id4 gene expression at different times during a 48-h period after dB-cAMP treatment. The steady-state levels of Id4 mRNAs were transiently up-regulated at 8 h of treatment and progressively decreased afterward (data not shown).

To determine whether the decreased levels of Id4 mRNA in dB-cAMP-treated cultures were related to the growth arrest produced by this compound in astrocytes, cell proliferation was arrested by serum deprivation for 2 days and cells were treated with dB-cAMP for 2 additional days under serum-free conditions. As shown in Fig. 2, Id4 mRNA levels did not decrease in the cells that were serum deprived, although a decrease of Id4 gene expression was observed, together with a decrease in the levels of Id1–Id3 transcripts, in the cells that were treated with dB-cAMP under serum-free conditions. Again, this effect correlated with dramatic changes in the morphology of the cells, which displayed hypertrophied, long, branched processes and strong GFAP immunoreactivity (Fig. 2C). To confirm that the changes in Id4 mRNA were due to activation of the cAMP-dependent pathway and to exclude non-specific effects of dB-cAMP, the activity of other compounds known to elevate the level of intracellular cAMP was examined in our primary astrocyte cultures. Forskolin (which activates adenylate cyclase), pentoxifylline (an inhibitor of cell phosphodiesterases), and dB-cAMP all produced the same effect (Fig. 3), which was accompanied by similar phenotypic changes in the

FIG. 1. dB-cAMP selectively suppresses Id4 gene expression in astroglial primary cultures. Cells were cultured for 4 weeks in the presence of 9% FBS. (A) Id1, Id2, Id3, Id4, and GFAP mRNAs were detected by Northern blot analysis. Total RNA was isolated from cells at the indicated days of culture when cultured without any drug or after treatment from day 10 of culture with dB-cAMP (1 mM). 10 micrograms of total RNA were loaded in each gel lane. The hybridization signal of a GAPDH probe used as an RNA loading and transfer control is shown. Id4 film was exposed approximately twice as long as Id1, Id2, and Id3 films. Numbers on the right indicate positions of the 28S and 18S ribosomal RNAs. (B) Id mRNAs were quantified by densitometry, normalized to GAPDH mRNA, and expressed as a percentage of Id mRNA at day 10 of culture in cells that were left untreated. Each point represents the mean of two independent experiments. Bars indicates the range of values obtained. The values obtained for Id4 at day 12 of culture are representative of multiple experiments performed. Values are comparable only within the group of samples hybridized to a single probe. White bars, untreated. Black bars, dB-cAMP treated. (C) The suppression of Id4 message correlates with the differentiation of astroglial primary cultures by dB-cAMP. Fluorescence photomicrographs of cells from the same culture double immunostained at day 12 of culture for GFAP (a and b) and BrdU (c and d) when cultured without any drug (a and c) or after treatment with dB-cAMP (1 mM) for 48 h (b and d). Cells were pulsed with BrdU for the final 24 h of culture. GFAP was detected by indirect immunofluorescence with an anti-GFAP polyclonal antibody followed by TRITC-conjugated sheep anti-rabbit IgG. In the same cells, BrdU was detected with an anti-BrdU monoclonal antibody followed by FITC-conjugated sheep anti-mouse IgG. Photomicrographs were taken from the same field using filters for rhodamine (top) or fluorescein (bottom). Scale bar, 10 μ m.

FIG. 2. dB-cAMP-mediated down-regulation of Id gene expression in astroglial cells is not a consequence of cellular proliferation arrest. Cells were cultured for 10 days in the presence of 9% FBS and growth arrested at that time by serum deprivation. After 2 days in serum-free medium the cells were treated with dB-cAMP (1 mM) and cultured in serum-free medium for 2 additional days. (A) Id1, Id2, Id3, and Id4 mRNAs were detected by Northern blot analysis. Total RNA was isolated at day 14 of culture from dB-cAMP-treated and control cells. 10 micrograms of total RNA were loaded in each gel lane. The hybridization signal of a GAPDH probe used as an RNA loading and transfer control is shown. Id4 film was exposed approximately twice as long as Id1, Id2, and Id3 films. Numbers on the right indicate positions of the 28S and 18S ribosomal RNAs. (B) Comparison of Id4 mRNA levels in cells that were growth arrested by serum deprivation and in cells that grew in the presence of 9% FBS. Id4 mRNAs were detected by Northern blot analysis as in (A). (C) Fluorescence photomicrographs of cells from the same culture double immunostained at day 14 of culture for GFAP (a and b) and BrdU (c and d) when cultured in serum-free medium without any drug (a and c) or after treatment with dB-cAMP (1 mM) for 48 h (b and d). Cells were pulsed with BrdU for 24 h prior to fixation. Photomicrographs were taken from the same field using filters for rhodamine (top) or fluorescein (bottom). Scale bar, 10 μ m.



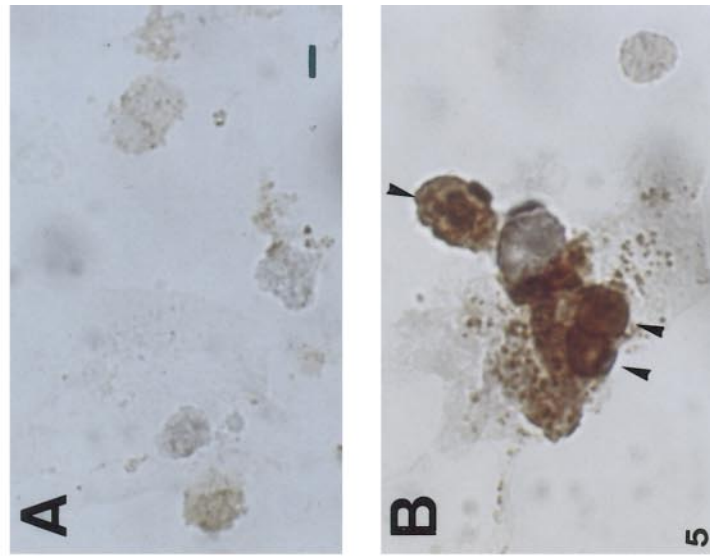
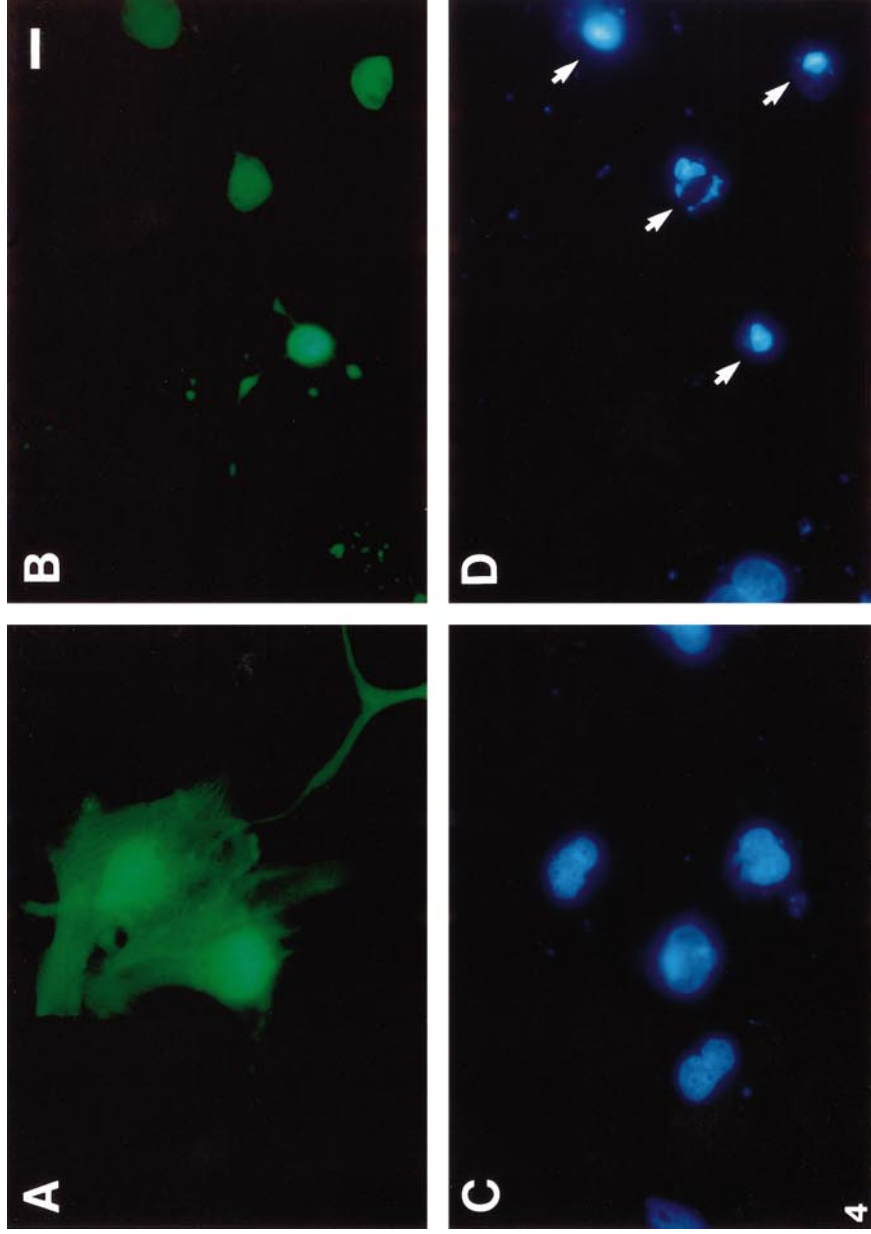


FIG. 4. Effects of Id4 overexpression in U373 astrocytes. Cells were cotransfected by the lipofectin method with pEGFP and pCMV-Id4 plasmid DNAs (B and D) or with pEGFP and pCMV plasmid DNAs as a control (A and C). At 72 h after transfection, the cells were fixed in 4% paraformaldehyde and stained with Hoechst 33342 and the preparations mounted in PBS. Cells were examined under a fluorescence microscope. Photomicrographs were taken from the same field using filters for GFP (top) or Hoechst (bottom). Apoptotic nuclei are indicated by arrows. Scale bar, 5 μ m.

FIG. 5. Apoptotic nuclei in Id4-transfected U373 astrocytes. Cells were transfected by the lipofectin method with pCMV-Id4 plasmid DNA (B) or with pCMV plasmid DNA as a control (A). Twenty four hours after transfection, DNA fragmentation was demonstrated by TUNEL assay. Apoptotic nuclei are indicated by arrowheads. Cells were slightly counterstained with methyl green. Scale bar, 5 μ m.

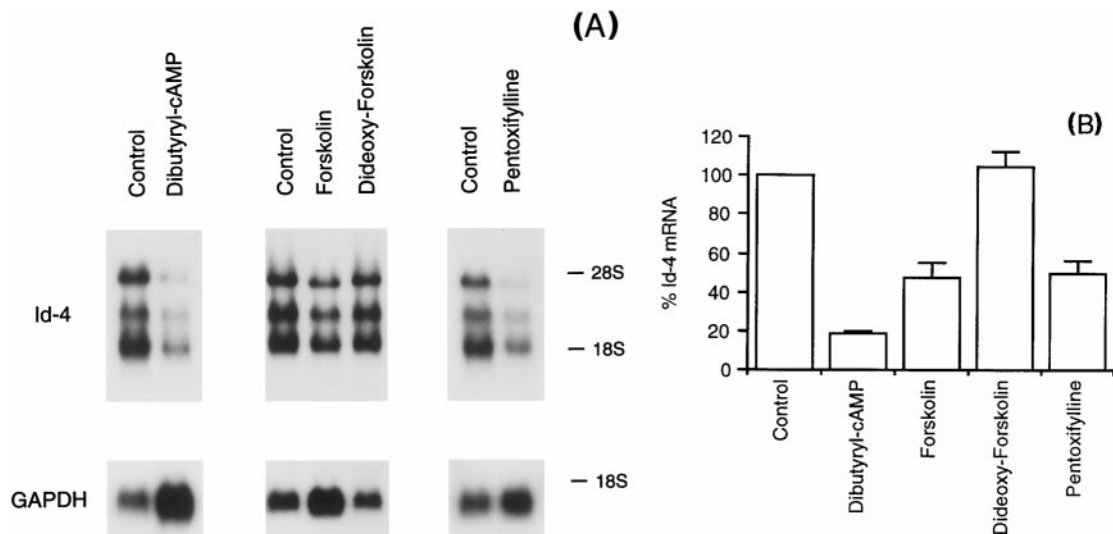


FIG. 3. The expression of the Id4 gene in astroglial primary cultures is decreased by a variety of drugs that stimulate the cAMP-dependent signaling pathway. Cells were cultured for 2 weeks in the presence of 9% FBS and growth arrested at that time by serum deprivation. After 2 days in serum-free medium the cells were treated with dB-cAMP (1 mM), forskolin (100 μ M), dideoxy-forskolin (100 μ M), or pentoxifylline (4 mM) and cultured in serum-free medium for 2 additional days. (A) Northern blot analysis of Id4 mRNAs in treated cells and mock-treated controls. 10 micrograms of total RNA were loaded in each gel lane. (Bottom) The hybridization signal of a GAPDH probe used as an RNA loading and transfer control is shown. Numbers on the right indicate positions of the 28S and 18S ribosomal RNAs. (B) Changes in Id4 mRNA levels detected by Northern blot analysis following treatment. Each point represents the mean of two independent experiments. Bars indicate the range of values obtained. The values obtained for Id4 after dB-cAMP treatment are representative of multiple experiments performed.

cultures (data not shown). Moreover, dideoxy-forskolin (a forskolin analogue which does not stimulate adenylate cyclase) had no effect (Fig. 3).

We examined the effects of Id4 overexpression in U373 astrocytes expecting that high levels of Id4 would block differentiation. U373 cells have been shown to retain morphologic and physiologic properties of primary astrocytes [38]. U373 cells undergo extensive morphological differentiation following cAMP treatment, are very efficiently transfected, and display low levels of Id4 mRNA expression (data not shown). DNAs of pEGFP and pCMV-Id4 expression vectors were co-transfected into the cells in a transient transfection assay, and after 24 h of transfection we studied the cell morphology in GFP-expressing cells at timed intervals. At 24–72 h, 30–50% of the GFP-expressing cells were rounded up (Fig. 4). The round cells showed obvious morphological evidence of cytotoxicity including detachment from the tissue culture surface and contraction of the cytoplasm. Between 72 h and 6 days, there was an increase in the percentage of GFP-expressing cells that had rounded up together with an increase in the number of floating GFP-expressing cells. Furthermore, there was clearly a progressive reduction in the number of GFP-expressing cells attached to the tissue culture surface (by 12 days, almost all the GFP-expressing cells had rounded up and detached), whereas in control cultures transfected with DNA of the CMV expression vector alone, a monolayer of cells containing frequent, normal, GFP-expressing cells was observed.

Staining with Hoechst 33342 for nuclear DNA revealed fragmented nuclei with condensed chromatin characteristic of apoptosis in the round cells expressing GFP which were attached to the tissue culture surface (Fig. 4), as well as in the floating cells which expressed GFP. Flow cytometric analysis of cellular DNA content 48 h after transfection indicated a large proportion of apoptotic cells with subdiploid DNA content (data not shown) and analysis for cell death by the TUNEL assay in the floating cells revealed abundant apoptotic nuclei (Fig. 5). When we attempted to reseed floating cells in tissue culture flasks with fresh culture medium after 48 h transfection, the cells did not attach to the tissue culture surface, and cell viability tests with trypan blue indicated that these cells were dead (data not shown). Finally, to investigate whether Id4 overexpression also induces apoptosis in differentiated cells, we cotransfected DNAs of pEGFP and pCMV-Id4 expression vectors into cells that were differentiated by treatment with 1 mM dB-cAMP for 24 h. The GFP-expressing cells in the differentiated cultures underwent apoptosis to the same extent we observed in undifferentiated cultures (data not shown).

DISCUSSION

This study demonstrates that the level of Id4 mRNA is decreased following activation of the cAMP-dependent signal transduction pathway in primary cultures of mouse forebrain astrocytes. The specificity of this

regulation is notable, since the level of mRNA encoding other members of the Id family is not decreased despite cells appearing to have a fully differentiated phenotype and being growth arrested. This observation contrasts sharply with the long-standing view that high levels of Id mRNA in proliferative and undifferentiated cells decrease as they are induced to differentiate [13, 19, 21, 39], although high levels of Id genes have been found in hematopoietic cells following the induction of differentiation as well [40]. Our findings suggest that different members of the Id family have different tissue specificities in their function to modulate differentiation and that Id molecules may have functions other than inhibition of differentiation [20, 21] or induction of proliferation [41]. Interestingly, it has been reported that Id2 and Id4 mRNAs are expressed in postmitotic neurons of the central and peripheral nervous system [28, 30, 31, 33]. The expression of Id4 in our astroglial cultures after dB-cAMP treatment recapitulates the pattern of expression observed for this gene during astrocytic differentiation of a nervous system precursor cell line [36]. Under serum-free conditions dB-cAMP also down-regulated the expression of Id1, Id2, and Id3 genes, although to a lesser extent than the Id4 gene. It has been reported that the expression of Id1, Id2, and Id3 genes is responsive to serum [13, 25, 42, 43], which could explain the decrease in Id mRNA levels observed under serum-free conditions. An implication of this possibility is that, although strongly GFAP positive, astroglial cultures treated with dB-cAMP require removal of serum to reach complete biochemical differentiation. Alternatively, it is also possible that relatively high levels of Id gene expression in astrocytes are compatible with expression of a differentiated phenotype, as suggested by the finding that significant levels of Id mRNAs are detectable in the brain of adult mouse [25–28, 44].

Cyclic AMP is most commonly considered a positive regulator of transcription. Despite this paradigm, there are a number of examples in which cAMP negatively regulates gene expression. Our astroglial cultures represent a good example of the dual role of cAMP, since activation of the cAMP-dependent pathway up-regulates GFAP mRNA levels, while Id4 gene expression is down-regulated. Inverse levels of expression of Id and genes related to the differentiated phenotype have also been observed in other systems such as vitamin D-mediated differentiation of osteoblastic osteosarcoma cells, in which vitamin D suppressed the expression of Id1 and enhanced osteopontin mRNA levels [39]. In skeletal muscle cells, in which the cAMP pathway inhibits differentiation, cAMP inhibits the activity of the MyoD family of bHLH regulatory factors, which are essential for the activation of muscle-specific genes during myogenesis [45]. bHLH transcription factors play an important role in the regulation of cell differentiation in several tissues, including those of

the nervous system [15, 16, 46–48], and transcriptional regulators belonging to the Id family of proteins efficiently antagonize the function of bHLH proteins [13, 19]. By controlling the synthesis of cAMP, extracellular signals could negatively regulate Id gene expression in astrocytes and in this way trigger differentiation mediated by bHLH differentiation factors. Although the regulation of Id molecules in astrocytes seems to be very complex, a similar pattern of expression is observed for these genes during astrocyte maturation in culture and during mouse brain development [27, 28, 31, 36, and our unpublished results], suggesting that Id gene expression is regulated in astroglial cells *in vivo*.

Data obtained from a variety of developmental systems indicate that programmed cell death is a normal component of development, and specific signaling molecules act to regulate the decision of cells to initiate the cell death process. Glial and neuronal programmed cell death is a fundamental feature of vertebrate nervous system development [49, 50], and it has been reported that most of the dying cells in the postnatal rodent cortex and cerebellum are astrocytes [51, 52]. A provocative aspect of our results is the possibility that Id4 modulates cell death by apoptosis during differentiation. This is likely to be a physiologic function of Id4, since such an activity for Id genes has recently been reported. Id3 expression induces apoptosis in fibroblasts [53] and Id2 expression can enhance apoptosis in myeloid progenitor cells [54]. Differentiation and programmed cell death must be finely tuned during development. Id genes are expressed during CNS development [27–32] and may function not only to regulate cellular differentiation but also may induce apoptosis. Given the proapoptotic function observed for Id4 in astrocytes and the regulation of Id4 by cAMP, it seems possible that during brain development specific signals which modulate cAMP levels mediate astroglial development by decreasing Id4 expression.

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