Journal of Cellular Biochemistry 74:313–322 (1999)

FAST TRACKS

Induction of Apoptosis and Differentiation in Neuroblastoma and Astrocytoma Cells by the Overexpression of Bin1, a Novel Myc Interacting Protein

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Abstract Bin1 is a novel protein that specifically binds Myc and inhibits, at least in part, Myc transactivation. Bin1 seems to play a role in cell cycle control, acting as a tumor suppressor gene. Since MYC family genes play a regulatory role in the proliferation, differentiation, and apoptosis of the nervous system, we studied the effects of the overexpression of the Myc-interacting protein, Bin1, in neuroblastoma and astrocytoma cell lines, which were chosen as neural cell system models. The major effects of BIN1 overexpression observed in undifferentiated neuroblastoma and astrocytoma cells were a significant reduction of cell growth, an increase in the G_0/G_1 cell population and the induction of apoptosis. The trigger of programmed cell death by Bin1 is described for the first time. Bin1 overexpression in undifferentiated cells did not induce any maturation process as neither neuronal nor astrocyte differentiation markers were upregulated in neuroblastoma and astrocytoma cells, respectively. On the other side, the effects of Bin1 overproduction in neuroblastoma and astrocytoma cells committed towards neuronal and astrocyte differentiation, respectively, were different from those observed in undifferentiated cells. Although we did not evidence any triggering of programmed cell death, we did notice a further induction towards more differentiated phenotypes. Our studies suggest that Bin1 overexpression in neuroblastoma and astrocytoma cells can result in one of the following pathways: (1) suppressed cell proliferation, (2) induced differentiation, or (3) apoptosis. Thus, it appears that Bin1 operates through different pathways that involve activation of different genes: the chosen pathway however will depend on the proliferating or differentiated state of the cell. J. Cell. Biochem. 74:313–322, 1999.

Key words: neuroblastoma; astrocytoma; proliferation; apoptosis; differentiation

The study of tumor suppressor genes and oncogenes involved in neural proliferation, differentiation, and apoptosis has received great attention over the past decade. During development, neuronal cell death clearly plays a role in the sculpturing and organisation of neuronal circuitry. Furthermore, neuronal loss also is a prominent feature in aging and in neurodegenerative diseases [Ross, 1996; Birge et al., 1998].

Under physiological conditions, MYC family proto-oncogenes encode for transcription factors that regulate cell growth, differentiation, and the apoptotic process [Packham and Cleveland, 1995; Evan and Littlewood, 1998]. Bin1 is a novel protein that binds the functional critical Myc box regions at the N terminus of the Myc oncoprotein [Sakamuro et al., 1996; Schmidt, 1996; Wechsler-Riva et al., 1997]. The primary structure of Bin1 suggests a putative role as a tumor suppressor gene. In fact, Bin1 seems to inhibit Myc transactivation, at least in part. In addition, Bin1 blocks malignant cell transformation by the Myc and the E1A adenovirus proteins and is poorly expressed in many cancer cell lines [Sakamuro et al., 1996; Schmidt, 1996; Wechsler-Riva et al., 1997]. Bin1 is related to amphiphysin, a neuronal protein which plays a crucial role in the endocytosis of synaptic vesicles. The meaning of the amphiphy-

Grant sponsor: MURST; Grant sponsor: C.N.R. P. F. Beni Culturali; Grant number: 97.00618.36.

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Received 30 March 1999; Accepted 6 April 1999

sin-Bin1 relationship is unclear, although both proteins have a Src homology 3 (SH3) domain, typically present in signal transduction proteins [Sakamuro et al., 1996; Butler et al., 1997]. These Bin1 properties motivated us to study its biological role in neuroblastoma and astrocytoma cell lines.

When established in culture, neuroblastoma cells maintain the differentiation potential of embryonic neuroblasts and can be induced to differentiate toward mature neurons [Galderisi et al., 1999]. However, astrocytomas are tumors composed of cells showing varying degrees of differentiation toward fully developed astrocytes [Ellsworth and Cheng-Mei, 1995]. Thus, neuroblastoma and astrocytoma cells represent a good homogeneous cellular context for investigating the Bin1 role in the neural cell system.

MATERIALS AND METHODS

Cell Cultures

SKNBE(2)C human neuroblastoma and ADF astrocytoma cell lines grown in monolayer were maintained at 37°C, 5% carbon dioxide in RPMI containing 10% fetal calf serum, (FCS) 2 mM L-glutamine, 50 U/ml penicillin, and 100 µg/ml streptomycin. To induce neuronal differentiation, SKNBE(2)C cells were treated with 10 µM retinoic acid and added to the culture medium [Galderisi et al., 1999]. The treatment was prolonged for 6 days. The media were replaced every 3–4 days. To induce astrocyte differentiation, ADF cells were treated with 3 mM phenyl acetate for 6–10 days. The cells were stable transfected with either BIN1 sense or BIN1 antisense or pCDNA3 plasmids by FUGENE 6 transfection reagent (Boehringer Mannheim) according to the manufacturer's protocol.

Proliferation Assay

Neuroblastoma and astrocytoma cells were seeded at 3,000/well in 96 multi-well plates. After 24, 48, and 72 h, cellular proliferation assays were performed by measuring the metabolic activity of cellular enzymes by tetrazolium dyes (Promega, Madison, WI). Each assay was repeated three times.

Cytofluorimetric Analysis

Cells were examined in a Becton Dickinson cytofluorograph (FACSscan) using the 488-nm emission of an argon laser. For each stained cell sample, the forward scatter (related to cell size), right-angle scatter (related to granularity), green fluorescence (FITC), and red fluorescence (propidium iodide) values were measured for 20,000 cells.

Live cells were those without bright propidium iodide staining and with sufficient forward scatter to exclude the remaining cellular debris. The percentages of fluorescence-positive live cells and the mean fluorescence and scatter values of the fluorescence-positive cells were determined using Lysis II software.

RNA Extraction and RT-PCR

Total RNA was extracted from cell cultures using an RNAzol reagent (Biotec Lab.) according to the manufacturer's protocol. The mRNA levels of the genes under analysis were measured by reverse transcription-polymerase chain reaction (RT-PCR) amplification, as previously reported [Melone et al., 1998]. Sequences for the human Bin1, N-Myc, c-Myc, p53, p21, GADD45, Bcl-2, Bax, Bak, Naip, Rb, Rb2/p130 HPRT, neuron-specific enolase (ENO), and Ret mRNAs from GeneBank (DNASTAR Company) were used to design primer pairs for RT-PCR experiments (OLIGO 4.05 software). Primers were 20/22 nucleotides long and contained 50–60% G/C. Appropriate regions of HPRT cDNAs were used as controls. The amplifications carried out for 28–30 cycles were as follows: 94°C for 1′, 57°C for 1′ and 72°C for 1′. Each RT-PCR experiment was repeated at least three times. Amplification products were electrophoresed on 2% agarose gel in a 1× TAE buffer. A semiquantitative analysis of mRNA levels was carried out by the GEL DOC 1,000-UV Fluorescent Gel Documentation System (BioRad).

Western Blotting

ADF cell aliquots were lysed in a buffer containing 0.1% Triton for 30 min at 4°C. The lysates then were centrifuged for 10 min at 10,000*g* at 4°C. After centrifugation, 20 µg of each sample was loaded, electrophoresed in a 10% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Primary antibody incubation was carried out with GFAP polyclonal antibody (Sigma Chemical Co., St. Louis, MO). The blots then were incubated with horseradish peroxidase (HPO) secondary antibodies and developed with an ECL system (Amersham). Equal protein loading of the proteic fractions of ADF cells was checked with monoclonal antibody anti-Actin (Sigma) and the rela-

tive amounts of GFAP and actin bands were determined by densitometric analysis.

TUNEL Assays and Determination of Apoptic Index

The cells for TUNEL assays were grown on glass coverslips. They were fixed for 15′ with 4% paraformaldehyde, and the TUNEL reaction was performed according to the manufacturer's instructions (Boehringer Mannheim). The cells were observed under light optical microscope. The apoptotic index was calculated by the number of positive TUNEL cells out of 1,000 cells in 4/5 different microscope fields.

RESULTS

Growth Suppression Cell Cycle Arrest and Apoptosis in Undifferentiated Cells

The SKNE(2)C neuroblastoma and the ADF astrocytoma cells were stable transfected with plasmids containing the entire coding sequence of BIN1 inserted into the pCDNA3 vector in both sense and antisense orientiation. We also transfected cells with an empty pCDNA3 vector. The RT-PCR assay demonstrated a two- to threefold increased expression of BIN1 in sense transfected cells compared with the antisense and the empty vector (Fig. 1A,B). Because the cell lines bearing the BIN1 antisense plasmid and those carrying the empty vector showed the same level of BIN1 mRNA, we only used the antisense BIN1 plasmid transfected cells as control in the later experiments.

In comparison with the untransfected cells, none of the transfected cells showed morphological changes. However, the BIN1 overexpression produced a significant reduction of cell proliferation compared with the antisense transfected cells (data not shown).

Figure 2 depicts the cell cycle distribution of neuroblastoma and astrocytoma cells overexpressing Bin1 mRNA compared with controls. Both cell lines showed a significant increase in G_0/G_1 cell population, indicating that Bin1 overproduction can reduce the G_1/S progression. In several flow cytometry experiments, the DNA histograms of Bin1 overexpressing cells showed a subdiploid peak, which is one of the hallmarks of apoptosis, but this peak was not clearly reliable. However, several other investigators have not observed subdiploid peaks in cells, which have undergone apoptosis by means of other criteria. Furthermore, certain subdiploid peaks

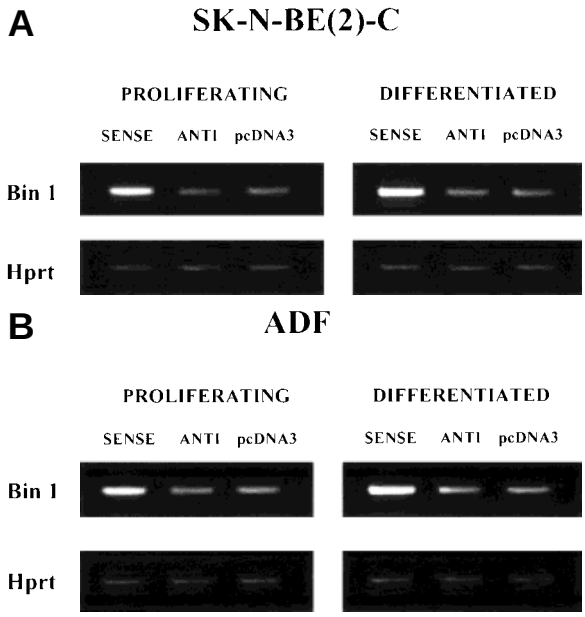

Fig. 1. A,B: Agarose gel electrophoresis analysis of reverse transcription-polymerase chain reaction (RT-PCR) products of Bin1 and Hprt mRNA in neuroblastoma and astrocytoma cells transfected with either BIN1 sense, or BIN1 antisense or empty pcDNA3 (inset). Experiments were performed on proliferating and differentiated cells.

may include debris and not apoptotic bodies [Elstein et al., 1995]. To clarify whether BIN1 could induce programmed cell death in certain cell types, we evaluated the number of apoptotic cells by employing the in situ cell death detection technique (TUNEL reaction). Several cells with evident apoptosis features were identified in the BIN1 sense transfected cell lines (Fig. 3A,B). A significant increase ($P < 0.01$) of apoptotic index was observed in neuroblastoma and astrocytoma cells that overexpressed Bin1 mRNA compared with the controls.

Effects of Elevated BIN1 Activity on the Gene Expression of Undifferentiated Neuroblastoma Cells

Bin1 appears to regulate MYC gene family activity [Sakamuro et al., 1996; Schmidt, 1996] and affects cell growth and apoptosis. For these reasons, we evaluated the effects of Bin1 elevated expression on the N-Myc mRNA level. In addition, we examined the level of p53, a key gene in the regulation of DNA repair, cell cycle arrest and apoptosis, and the expression of p53 downstream genes such as p21 and GADD45 involved in G1 arrest and DNA repair [Levine, 1997; Giacca et al., 1998]. Bin1 overproduction did not greatly affect the N-Myc mRNA level,

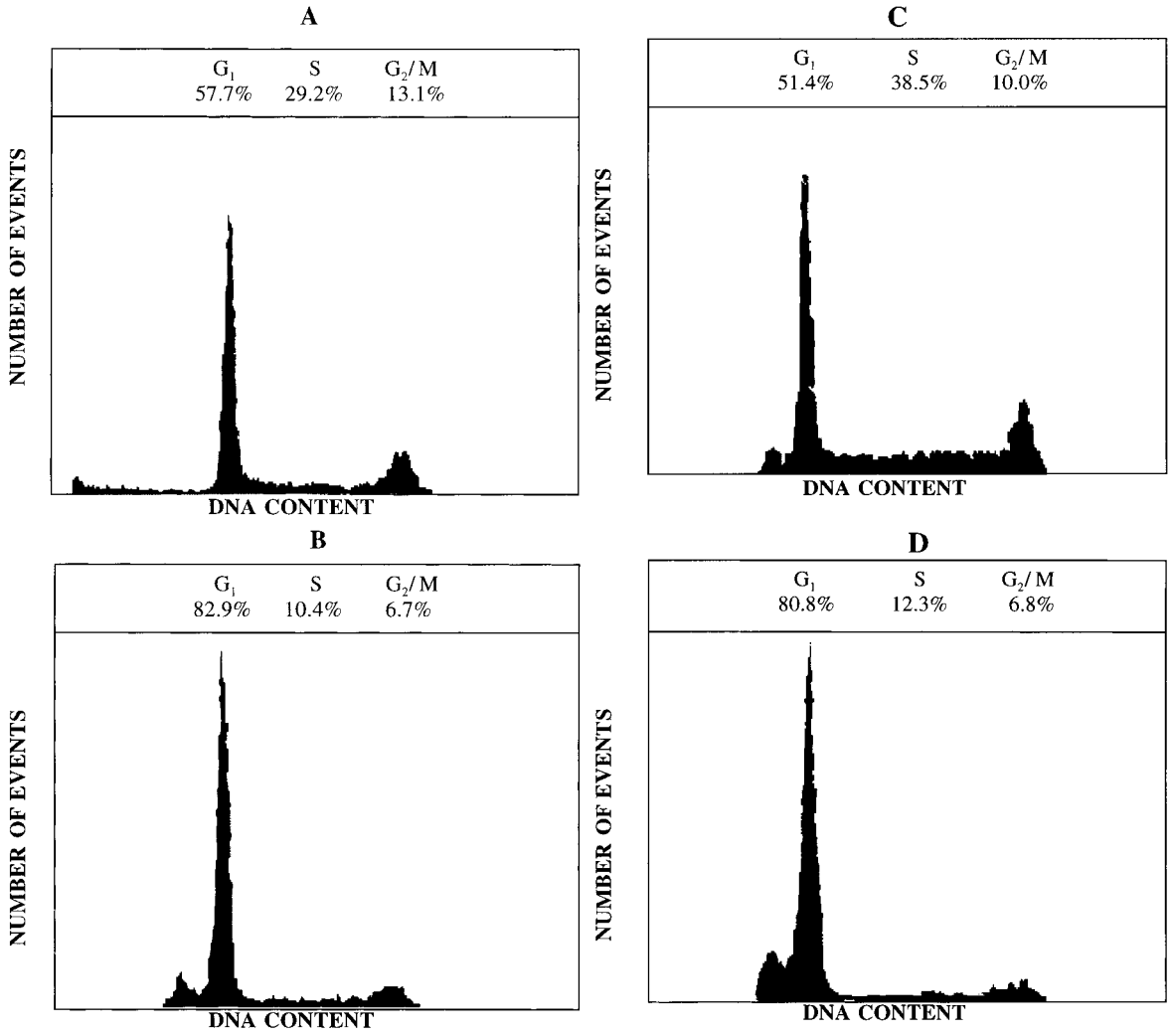

Fig. 2. FACS profile of neuroblastoma and astrocytoma nuclei treated with propidium iodide. Histograms of DNA content versus cell number are shown. Proliferating neuroblastoma cells transfected with either BIN1 antisense **(A)** or BIN1 sense **(B)**. Proliferating astrocytoma cells transfected with either BIN1 antisense **(C)** or BIN1 sense **(D)**.

but we did observe a substantial increase of p53, p21, and GADD45 gene expression (Fig. 4A and Table I). These results appear to be in agreement with the cell cycle arrest detected in cells with high Bin1 activity.

RB and RB2/p130 genes control cell cycle arrest, differentiation and apoptosis and regulate the activity of important cellular transcription factors, such as the E2F family [Paggi et al., 1996; Stiegler et al., 1998].

We examined whether RB and RB2/p130 gene expressions were affected by BIN1 increased activity. A significant reduction in the Rb mRNA level was observed in BIN1 sense transfected cells, while no modification in the Rb2 mRNA level was detected (Fig. 5A and Table I).

The Bcl-2 family comprises death-inducing and death-inhibitory members, which differ in tissue and activation expression patterns. Among these genes, Bcl-2 with antiapoptic activity, and Bax or Bak, which have proapoptic features, play a central role in almost all cell types. The ratio of death agonist to death antagonist genes can determine cell fate [Reed, 1997; Adams, 1998].

Another gene encoding a neuronal apoptosis inhibitor protein (NAIP) has recently been isolated [Liston et al., 1996]. This gene appears to play a fundamental role in the development of the nervous system, and it is partly deleted in individuals with neurodegenerative disorders [Liston et al., 1996]. In order to confirm that

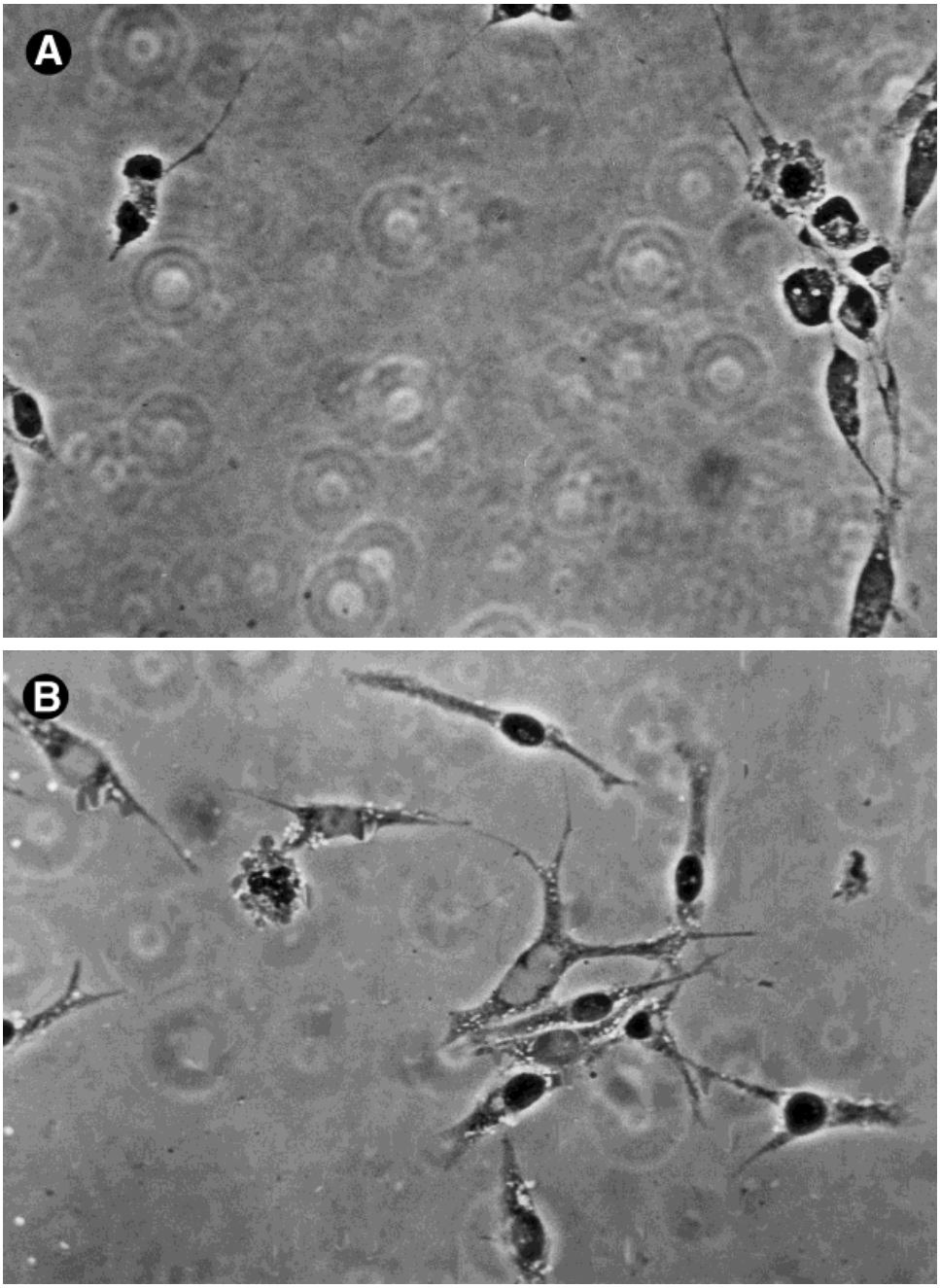

Fig. 3. TUNEL reaction perfomed on undifferentiated neuroblastoma **(A)** and astrocytoma **(B)**-type cells grown on glass coverslips. The apoptic cells, showing condensed chromatin mainly at nuclear margins, were stained by the Tunel dye. ×1,200.

Bin-1 overexpression could act to induce programmed cell death in certain cell types, the expression of the above described genes was evaluated in neuroblastoma cell cultures transfected either with BIN1 sense or BIN1antisense plasmids.

The upregulation of Bin1 activity produced a clear increase in pro-apoptotic BAX gene expression, while Bcl2 and Naip mRNA levels did not change (Fig. 6A and Table I). This is in agreement with the increase in the apoptotic index we observed in BIN1 sense transfected cells. The Bin1 ectopic expression did not seem to induce a neuroblastoma cell differentiation because the mRNA level of Neuronal specific enolase and Ret, which are neuronal markers [Gal-

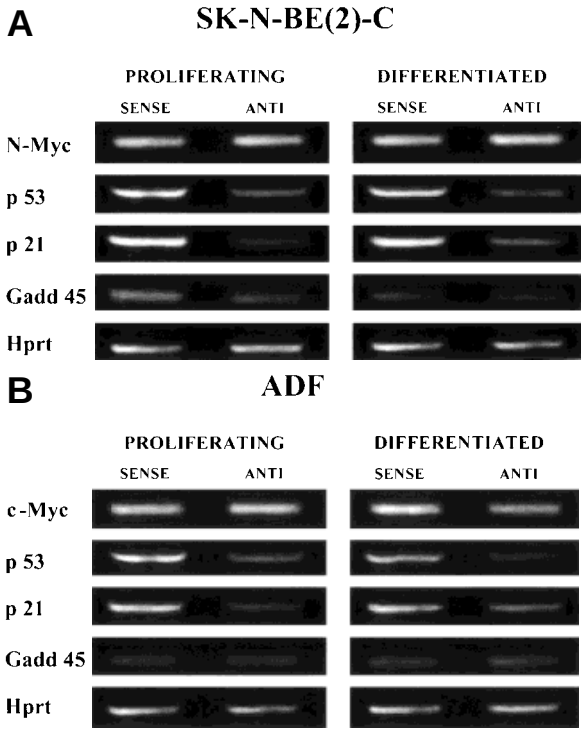

Fig. 4. Agarose gel electrophoresis analysis of c-Myc, N-Myc, p53, p21, GADD45, and Hprt of reverse transcription-polymerase chain reaction (RT-PCR) products in proliferating and differentiated neuroblastoma **(A)** and astrocytoma cells **(B)**, transfected with either BIN1 sense or antisense plasmids (inset).

TABLE I. RT-PCR Determination of Gene Expression

	SK	SK + RA	ADF	ADF + PA
BIN 1	+	+++	+++	+++
P53	++	+	++	+
BAX	+	=	+	=
BCL-2	=	+	++	=
BAK	=	+	++	+
NAIP	=	+	+	=
Rb	−	−	−−	=
Rb2	=	+	=	=
N-myc	+	=	//	//
c-myc	//	//	=	+
p21	+++	+	++	+
GADD45	+	+	=	=
ENO	=	+	//	//
RET	=	+	//	//

mRNA expression levels of the indicated genes in proliferating and differentiated neuroblastoma and astrocytoma cells, transfected with either BIN1 sense or antisense plasmids, as shown in the insert. The mRNA levels were measured by a GELDOC instrument and normalised with respect to Hprt mRNA, which was chosen as the RT-PCR control. Each value is the mean of at least 3 different experiments. The variations in the gene expression in BIN1 sense transfected cells compared with the control are indicated as follows: (+) 40–70%, (++) 70–100%, (+++) >100% increased expression; (=) no variation; (−) 40–70%, (−−) >100% decreased expression; (//) not detected. SK and SK + RA correspond to proliferating and differentiated neuroblastoma cells, respectively. ADF and ADF + PA correspond to proliferating and differentiated astrocytoma cells, respectively.

derisi et al., 1999], did not show an increase in BIN1 sense transfected cells compared to the controls (Fig. 5A and Table I).

Effects of Elevated Bin1 Activity on the Gene Expression of Undifferentiated Astrocytoma Cells

N-Myc was the only Myc family member whose activity we detected by RT-PCR in neuroblastoma cells, however, we observed only c-Myc expression in astrocytoma cells. Bin1 overproduction did not modify the c-Myc mRNA level in astrocytoma undifferentiated cells (Fig. 4B and Table I).

We also noticed an upregulation of p53 and p21 expressions in BIN1 sense transfected cells (Fig. 4B and Table I). Bin1 overproduction induced an Rb downregulation but did not affect Rb2/p130 mRNA expression (Fig. 5B and Table I). The mRNA level of all the death-inducing and death-inhibiting genes we detected was greater in Bin1 overexpressing cells. However, the equilibrium among anti- and pro-apoptotic gene expression may have shifted toward apoptosis, because we observed an increase in the apoptotic index in Bin1 sense transfected cells (Fig. 6B and Table I).

No differentiation process was induced by Bin1 because the GFAP protein level, a marker associated with astrocyte differentiation, was not upregulated in BIN1 sense transfected cells (Fig. 5B and Table I).

Effects of Bin1 Overexpression in Neuroblastoma Cells Induced Toward Neuronal Differentiation

A number of studies have shown that the mitogenic and pro-apoptotic properties of Myc family members depend on "cell state" and on "cell type" [Packham and Cleveland, 1995; Evan and Littlewood, 1998; Galderisi et al., 1999]. For these reasons we evaluated the effects of Bin1 overexpression in neuroblastoma cells induced toward neuronal differentiation. For this purpose, neuroblastoma transfected cells were induced toward differentiated ganglion-like neurons by adding retinoic acid to the cell culture medium. The differentiation process proved not

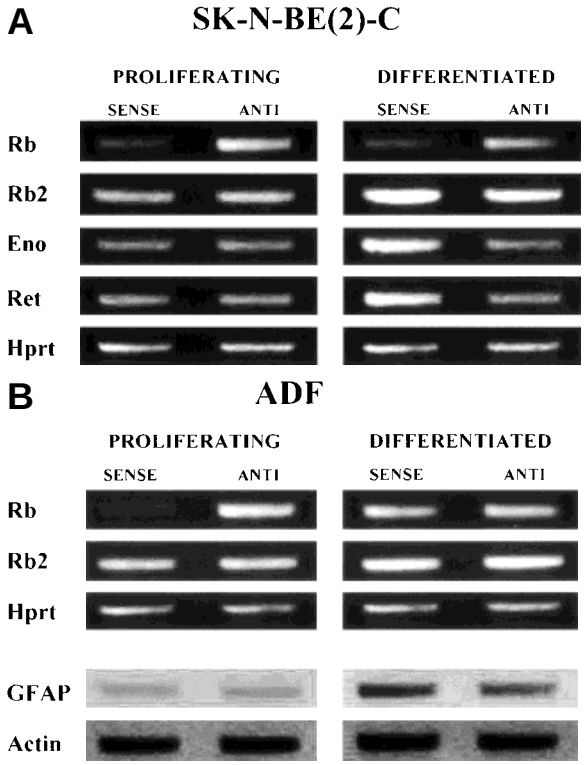

Fig. 5. **A:** Agarose gel electrophoresis analysis of Rb, Rb2/p130, Neuron-specific enolase, Ret, and Hprt of reverse transcription-polymerase chain reaction (RT-PCR) products in proliferating and differentiated neuroblastoma transfected with either BIN1 sense or antisense plasmids (inset). **B:** Agarose gel electrophoresis analysis of Rb, Rb2/p130, and Hprt RT-PCR products in proliferating and differentiated astrocytoma cells transfected with either BIN1 sense or antisense plasmids (inset). Immunoblot of proteins detected with antibody to the protein GFAP and Actin in proliferating and differentiated astrocytoma cells transfected with either BIN1 sense or antisense plasmids (inset).

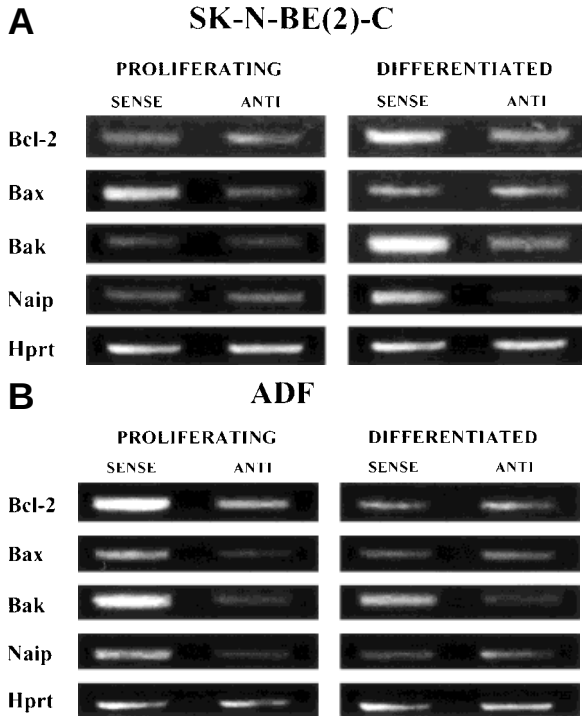

Fig. 6. Agarose gel electrophoresis analysis of Bcl-2, Bax, Bak, Naip, and Hprt of reverse transcription-polymerase chain reaction (RT-PCR) products in proliferating and differentiated neuroblastoma **(A)** and astrocytoma cells **(B)**, transfected with either BIN1 sense or antisense plasmids (inset).

to affect the activity of BIN1 sense plasmids as the Bin1 mRNA level was three times higher in sense transfected cells than in antisense transfected cells (Fig. 1A).

Because neuroblastoma differentiated cells exit the cell cycle, showing almost exclusively a G_0/G_1 cell population, we were unable to observe a further block in G_1/S progression induced by Bin1 overexpression. Bin1 overexpression did not change the N-Myc mRNA level, but it did induce a clear upregulation of p53 and p21 gene expression (Fig. 4A and Table I). BIN1 sense transfected cells showed a decrease in the Rb mRNA level along with a Rb2/p130 upregulation (Fig. 5A and Table I). This Rb family expression pattern is in agreement with a further induction toward neuronal phenotype [Raschellà et al., 1997]. Moreover, we noticed an upregulation of the neuronal differentiation markers Ret and neuronal specific enolase in BIN1 sense transfected cells (Fig. 5A and Table I).

While ectopic Bin1 activity induced an upregulation of the pro-apoptotic gene BAX in proliferating cells, Bin1 did not modify the BAX expression in differentiated cells and induced the upregulation of the anti-apoptotic genes BCL-2 and NAIP counteracted by an increased expression of the pro-apoptotic gene BAK (Fig. 6A and Table I). This expression pattern is in agreement with a non-induction of the apoptosis process as confirmed by the TUNEL technique (Fig. 3A).

Effects of Bin1 Overexpression in Astrocytoma Cells Induced Toward Astrocyte Differentiation

To assess whether Bin1 overexpression also could have an effect on the astrocyte differentiation process, BIN1 sense and antisense transfected astrocytoma cells were induced to differentiate toward a mature astrocyte phenotype by adding phenyl acetate to cell cultures. As observed in the neuroblastoma cell line, the

differentiation process did not shut off the activity of BIN1 sense plasmids (Fig. 1B).

Astrocytoma cells treated with phenyl acetate exit from the cell cycle so we could not evaluate the consequence of Bin1 overexpression on cell cycle progression. In proliferating astrocytoma cells, Bin1 overproduction triggered an apoptosis process as determined by the TUNEL reaction, but we did not see such a phenomenon in differentiated cells.

No modification in c-Myc mRNA level was noted in BIN1 sense transfected cells, although Bin1 overproduction determined a p53 and a p21 upregualtion (Fig. 4B and Table I). BIN1 sense transfected cells did not show a modification of RB and p130/RB2 gene expression (Fig. 5B and Table I). When overexpressing Bin1 in proliferating cells, we observed a clear upregulation of all the anti- and pro-apoptotic genes investigated, while in differentiated cells the Bin1 ectopic expression induced only the upregulation of the pro-apoptotic gene BAK (Fig. 6B and Table I). However, we did not observe any induction of apoptosis by the TUNEL reaction. The Bin1 ectopic expression in phenyl acetate treated cells appeared to induce a further differentiation toward a mature astrocyte phenotype, and in fact we observed an upregulation of GFAP expression, which is a marker of astrocyte differentiation (Fig. 5B).

DISCUSSION

Bin1 is a novel protein that specifically binds Myc and inhibits, at least in part, Myc transactivation. Bin1 seems to play a role in cell cycle control, acting as a tumor suppressor gene [Sakamuro et al., 1996; Schmidt, 1996; Wechsler-Riva et al., 1997]. Because MYC family genes play a regulatory role in proliferation, differentiation and apoptosis of the nervous system [Packham and Cleveland, 1995; Zindy et al., 1998; Galderisi et al. 1999], we studied the effects of the overexpression of the Myc-related protein Bin1 in neuroblastoma and astrocytoma cell lines, which were chosen as neural cell system models.

In undifferentiated neuroblastoma and astrocytoma cells, BIN1 overexpression resulted in a significant reduction of cell growth. The decrease in proliferation rate can be explained by the fact that Bin1 overproduction produced an increase in the G_0/G_1 cell population as well as an increase in apoptotic cell number (Figs. 2, 3).

We did not observe a huge modification in the Myc mRNA level in BIN1 transfected cells compared with controls. This is in accordance with the proposed Bin1 function. In fact, Bin1 should regulate Myc activity at the protein level [Sakamuro et al., 1996].

p53 induction and the resultant p21 upregulation in BIN1 overexpressing cells are in agreement with the cell cycle arrest we detected. It should be pointed out that, generally speaking, the predominant regulation of p53 occurs at the protein level [Donehower and Bradley, 1993; Giacca et al., 1998]. Thus, Bin1 overexpression seems to be one of a few situations in which p53 activity is controlled by altering the level of p53 mRNA. The decreased level in Rb mRNA observed in undifferentiated cells overproducing Bin1 was in all likelihood the consequence of cell cycle arrest. In fact, the Rb protein level slightly increases during G_1/S progression because the RB promoter is responsive to E2F transcription factors, whose activity is reduced in quiescent cells [Paggi et al., 1996. Nevins, 1998]. The major control of the Rb family proteins is the phosphorylation state of the proteins. Given that we have no data on Rb proteins, our analysis can not be complete.

A striking feature of Bin1 overproduction in undifferentiated cells was the triggering of apoptosis. In neuroblastoma cells the programmed cell death process clearly appeared linked to the p53 pathway. Indeed, the pro-apoptotic and p53 dependent gene BAX was the only activated gene among all the pro- and anti-apoptotic genes we detected. In undifferentiated astrocytoma cells, the Bin1 ectopic expression triggered apoptosis along with the induction of both the pro- and anti-apoptotic genes we studied. Pro- and anti-apoptotic family members can titrate one another's function, acting as a rheostat for the suicide program [Reed, 1997; Adams, 1998]. Thus, in undifferentiated astrocytoma cells overexpressing Bin1, the effects of pro-apoptotic protein upregulation appeared to exceed that of anti-apoptotic proteins.

The role that Bin1 seems to play in apoptosis may depend on a partial inhibition of Myc activity as it appears able to block the mitogenic and not the apoptotic activity of Myc proteins. Indeed, there is evidence to support this hypothesis:

1. In one of our previous studies performed on neuroblastoma cells, we drastically reduced N-MYC activity by decreasing its mRNA

translation with antisense technology [Galderisi et al, 1999]. When N-Myc downregulation was performed in undifferentiated neuroblastoma cells, we observed a decrease in cell growth but it did not trigger apoptosis. Antisense molecules preventing N-Myc protein production inhibited both mitogenic and apoptotic activities.

2. Bin1 does not appear to affect transactivation from minimal promoters containing multimerized Myc binding sites [Sakamuro et al., 1996].
3. p53 showed an increased expression in Bin1 overproducing cells, although the p53 gene has a Myc consensus sequence in its promoter and is induced in cells overexpressing c-Myc [Donehower and Bradley, 1993; Zindy et al., 1998].

However, Bin1 also may have an Myc independent activity because it has a Src homology domain typically present in signal transduction proteins [Sakamuro et al., 1996]. We have no data to support or exclude this hypothesis.

Bin1 overproduction in itself could induce apoptosis in neural proliferating cells. Several proteins associated with cell proliferation have been demonstrated to induce apoptosis when overexpressed [Packham and Cleveland, 1993]. However, this does not seem to be the case because Bin1 ectopic expression in neural differentiated cells did not promote programmed cell death.

Bin1 overexpression in undifferentiated cells did not induce any maturation process as neither neuronal nor astrocyte differentiation markers were upregulated in neuroblastoma and astrocytoma cells, respectively. This observation is in agreement with the role played by Myc in the differentiative process. During differentiation, c-Myc and N-myc mRNA levels progressively decrease. However, several studies indicate that Myc downregulation is not the key event in neural cell differentiation. In fact, when N-myc activity is downregulated before neuroblastoma cells are committed to differentiate, the result is only cell cycle arrest. In addition, N-Myc downregulation performed in cells already induced to differentiate promote further cell maturation [Packham and Cleveland, 1993; Galderisi et al., 1999].

The effects of Bin1 overproduction in neuroblastoma and astrocytoma cells committed toward neuronal and astrocyte differentiation, respectively, were different from those observed in undifferentiated cells. We did not see evidence of any triggering of programmed cell death, although we did notice a further induction toward more differentiated phenotypes. In neuroblastoma cells overexpressing Bin1 and treated with retinoic acid, we observed an upregulation of p53 and p21. Interestingly, we observed an induction of the anti-apoptotic genes BCL-2 and NAIP that could counteract the increased activity of the pro-apoptotic gene BAK. These results are in agreement with the absence of cell death increase we observed by TUNEL reaction.

In astrocytoma cells overproducing BIN1 and committed toward astrocyte phenotype by phenyl acetate treatment, p53 and p21 induction persisted. While in the proliferating astrocytoma cells we analyzed BIN1 overexpression determined a strong induction of all the "apoptotic machinery," we detected only a slight upregulation of the pro-apoptotic gene BAK in cells induced toward mature phenotype. Because the TUNEL reaction did not show any triggering of the apoptosis process in these BIN1 overexpressing cells, we hypothesize that BAX upregulation might be counteracted by anti-apoptotic genes not included in our studies, or that BAK in itself is not capable of inducing apoptosis in this cellular context.

BIN1 ectopic expression in cells committed to neuronal or astrocyte phenotypes gave rise to a further induction in the direction of a more differentiated phenotype. During the differentiation process, a progressive reduction of c-Myc and N-Myc activity occurs [Packham and Cleveland, 1993; Galderisi et al. 1999]. Thus, the overexpression of BIN1 in cells already committed to differentiating could promote a further differentiation by reducing Myc protein activity to a greater extent. In addition, BIN1 ectopic expression in cells committed to differentiating still induced p53, which plays a regulatory role in the maturation and differentiation of neural cells [Eizenberg et al., 1996].

In conclusion, the data presented indicate that BIN1 overexpression in neuroblastoma and astrocytoma cells can result in one of the following pathways: (1) suppressed cell proliferation, (2) induced differentiation, or (3) apoptosis. Thus, it seems that Bin1 operates through different pathways that involve activation of different genes; however, the chosen pathway will

depend on the proliferating or differentiated state of the cell.

ACKNOWLEDGMENTS

We are indebted to Professor G. Prendergast of the University of Pennsylvania, Philadelphia, PA, for providing us with BIN1 sense and BIN1 antisense plasmids. We are grateful to the P.I.T.A.G.O.R.A. Institute for stimulating cultural support. The research was partially financed by funds from MURST 40% (to A.C.) and C.N.R. P. F. Beni Culturali 97.00618.36 (to M.C.).

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