The transcription factor early growth response factor-1 (EGR-1) promotes apoptosis of neuroblastoma cells

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Early growth response factor-1 (EGR-1) is an immediate early gene, which is rapidly activated in quiescent cells by mitogens or in postmitotic neurons after depolarization. EGR-1 has been involved in diverse biological functions such as cell growth, differentiation and apoptosis. Here we report that enforced expression of the EGR-1 gene induces apoptosis, as determined by flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick-end labelling (TUNEL) analysis, in murine Neuro2A cells. In accordance with this role of EGR-1 in cell death, antisense oligonucleotides increase cell viability in cells cultured in the absence of serum. This apoptotic activity

of the EGR-1 appears to be mediated by p73, a member of the p53 family of proteins, since an increase in the amount of p73 is observed in clones stably expressing the EGR-1 protein. We also observed an increase in the transcriptional activity of the mdm2 promoter in cells overexpressing EGR-1, which is paralleled by a marked decrease in the levels of p53 protein, therefore excluding a role of this protein in mediating EGR-1-induced apoptosis. Our results suggest that EGR-1 is an important factor involved in neuronal apoptosis.

Key words: apoptosis, cell death, early genes, mdm2, p73.

INTRODUCTION

Regulation of cell death is essential for normal development and is an important defence against viral infection, cancer and degenerative diseases [1]. Cells of the central nervous system leave the mitotic cycle and differentiate early in development [2]. Later, in maturation, a massive-scale death of neurons and oligodendrocytes occurs as part of the normal physiological process of development [3,4]. The affected cells are thought to be neurons that have failed to form connections with a target and thus lack target-derived support, such as specific neurotrophic factors [4], and oligodendrocytes that have failed to obtain the growth factors, cytokines and neurotrophic factors necessary for their survival [5,6]. This neuronal cell death has been shown to occur via apoptosis [7]. Neuronal cell death can occur abnormally by its timing, its causes or its extent. This is obviously of particular importance when the human nervous system is concerned, where extensive neuronal cell death is observed in neurodegenerative diseases and after stroke and trauma [8–10].

The protein products of numerous genes exert activating or inhibitory effects on apoptosis. Therefore the identification and characterization of the function of these genes will help to define the process of cell death at the biochemical level and suggest sites of therapeutic intervention. The initial nuclear events that trigger the apoptotic programme in developing neurons are still unknown although a number of immediate early genes, including members of the *fos* and *jun* families, have been implicated. Previous studies observed a significant induction in the level of *c-jun* and *c-fos* in cultured sympathetic neurons that were destined to die [11,12], and an augmented expression of early growth response factor-1 (EGR-1) has been recently associated with cerebellar granule cell death in culture [13].

EGR-1 (also known as NGFI-A, krox24, zif 268 and tis 8) is an immediate early gene that was identified simultaneously in different cell systems [14,15]. EGR-1 is a nuclear DNA-binding protein which specifically recognizes the sequence: 5'-GCGKG-GGCG-3' and can transactivate promoters containing the appropriate cognate site [16]. Promoters containing active binding/transcription sites for EGR-1 include those of the thymidine kinase, tumour necrosis factor, both chains of platelet-derived growth factor, synapsins [17,18], acetylcholinesterase and *N*-methyl-D-aspartate receptor 1 (NMDAR-1) [19,20]. Several studies have suggested a role for the EGR-1 protein in the regulation of cell division, differentiation and apoptosis. It has been shown that expression of EGR-1 is induced during differentiation of myeloid cells [21,22], and more recently we have demonstrated an involvement of this protein in the differentiation of neuronal cells [23]. Accumulated evidence suggests that the EGR-1 gene may play an important role in the nervous system. First it is expressed widely in the peripheral and central nervous systems in adult and developing animals [24,25]. Second, EGR-1 gene expression is highly responsive to neuronal stimulation and the levels of EGR-1 mRNA in the brain increases dramatically in animals that have had a seizure [26]. We have also reported an up-regulation of EGR-1 gene expression by thyroid hormone [25,27], receptors for which are likely to play a role in events such as neuronal differentiation, establishment of interneuronal connections and cell death [28,29].

In this study we have used the Neuro2A cells (N2A cells) to investigate the role of the transcription factor EGR-1 on programmed cell death. Our results show a significant increase in apoptosis in N2A cells stably transfected with an expression vector for EGR-1, as well as a reduction in cells treated with EGR-1 antisense oligonucleotides. Furthermore, EGR-1-induced

Abbreviations used: EGR-1, early growth response factor-1; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP-fluorescein nick-end labelling; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PI, propidium iodide.

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apoptosis appears to be independent of the cell-cycle checkpoint gene p53, but dependent on the p73 protein, since a simultaneous decrease in p53 and increase in p73 protein was observed in cells overexpressing EGR-1.

MATERIALS AND METHODS

Cell culture and transfection

Mouse N2A and clones NG9 and NG10 [23], overexpressing the EGR-1 protein, were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10 % fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY, U.S.A.), 2 mM glutamine and 0.01 mg/ml sodium pyruvate. For all experiments, cells were seeded at a density of 20 000/cm² and grown for 24 h in complete medium before any treatment was started. In the absence of serum, N2A cells first differentiate (12–24 h) and later they start to die (24–32 h) [23]. Antisense or sense oligonucleotides were added to the culture medium at a concentration of 40 μ M at the same time of serum withdrawal. For transient transfection experiments (EGR-1, mdm2 and pG13), semiconfluent cells were transfected by the calcium phosphate precipitation technique in 24-well plates. After 8 h of exposure to the Ca²⁺–DNA mixture, cells were washed with TD buffer (20 mM Tris/HCl, pH 7.4, 1 mM Na₂HPO₄, 140 mM NaCl and 5 mM KCl), incubated for another 24 h in complete medium, and harvested for determination of luciferase activity as described previously [30]. Each transient transfection experiment was repeated at least three times in triplicate. For the study of EGR-1 transcriptional activity, we used a construct containing two EGR-1 consensus-binding sites upstream of a prolactin minimal promoter [31].

The pSUPER vector directing the synthesis of a 19-bp double-stranded p53 target sequence (pSUPER/p53) was used to suppress the endogenous p53 gene [32]. N2A parental cells and clones NG9 and NG10 were transfected either with the anti-p53 construct or with the empty vector (pSUPER) by the calcium phosphate precipitation technique, as indicated above. With this method we obtained more than 90 % transfection efficiency. After transfection (50 h) the amount of p53 protein and the percentage of apoptotic cells were determined.

Oligonucleotides

EGR-1 antisense and sense oligodeoxyribonucleotides with the sequences (antisense) 5'-GCG GGG TGC AGG GGC ACA CT-3' and (sense) 5'-AGT GTG CCC CTG CAC CCC GC-3' were synthesized with an Applied Biosystems 391 DNA synthesizer and purified on acrylamide gels. The antisense oligonucleotide is complementary to a sequence located 120–101 bp upstream of the AUG initiation codon. A 15-mer antisense oligodeoxyribonucleotide with the sequence 5'-GGG GTA GTT GTC CAT-3', complementary to a sequence starting at the AUG initiation codon, was also used with the same results. The oligonucleotides used to amplify a 400-bp fragment of the mouse P2 mdm2 internal promoter [33] were as follows: antisense, 5'-GGG CCC GCT CCG GGT CGC GCT-3', and sense, 5'-ATG CAT TTA CGA AGG AGA-3'. The oligonucleotides used to construct the pSUPER/p53 vector were as follows: forward primer, 5'-GAT CCC CGA CTC CAG TGG GAA CCT TCT TCA AGA GAG AAG GTT CCC ACT GGA GTC TTT TTG GAA A-3', and reverse primer, 5'-AGC TTT TCC AAA GAC TCC AGT GGT AAT CTA TCT CTT GAA GAA GGT TCC CAC TGG AGT CGG G-3'.

Western immunoblot analysis

N2A cells were lysed in 500 μ l of PBS containing 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS and protease inhibitors, centrifuged at 12 000 g for 20 min and the supernatants removed and stored at –70 °C until analysis. Equal amounts of protein (70 μ g) were electrophoresed in SDS/10 %-acrylamide gels, and blotted on to nitrocellulose membranes. Transferred membranes were blocked with 5 % non-fat milk in TBST buffer (20 mM Tris/HCl, pH 7.6, 130 mM NaCl and 0.1 % Tween 20) for 1 h at room temperature and incubated with EGR-1-, p53-, mdm2- or p73-specific antibodies (Santa Cruz Biotechnology) used all at 1:1000 dilution, in TBST with 4 % non-fat milk for 2 h. The anti- α -tubulin antibody was obtained from Sigma and used at a 1:4000 dilution. After several washes with TBST, the membranes were incubated with the corresponding horseradish peroxidase-coupled secondary antibodies in the same buffer. After washing, immunoreactive bands were visualized using Amersham's ECL detection kit according to the manufacturer's instructions.

Cell viability and apoptosis assays

To assess cell viability, aliquots of cells were mixed with Trypan Blue (1:1) and loaded on to a haemocytometer, and the percentage of dead cells per sample was calculated. Survival assays were carried out independently at least three times. To calculate the percentage of dead cells, 1×10^6 cells were cultured in 10-cm-diameter tissue-culture plates and grown in DMEM for 24 h before switching to DMEM without FBS for a further 32 h. Apoptosis was assayed using propidium iodide (PI) staining and FACS analysis, and a minimum of 10 000 events were collected for each sample. Data are presented using the dot density plot function. Regions R3 plus R4 correspond to the apoptotic population. Cells in panel R2 correspond to dead cells that have altered the membrane permeability. The data were analysed using the CellQuest software package (Becton Dickinson). For microscopy analysis, cells were grown on coverslips for 32 h in normal or serum-free medium, stained with 0.01 % PI and examined with a Zeiss Axiophot microscope.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-fluorescein nick-end labelling (TUNEL) assay

Apoptosis was also measured by the TUNEL method. Genomic DNA strand breaks characteristic of apoptosis were labelled *in situ* by TdT using an *in situ* cell-death-detection kit (Kamiya Biomedical Com., Seattle, WA, U.S.A.) according to the manufacturer's instructions. Briefly, N2A cells were grown in chamber slides (Becton Dickinson) for 24 h, changed to serum-free media and maintained for 32 h. Cells were fixed with 5 % paraformaldehyde solution in PBS for 30 min at room temperature and then permeabilized with 0.1 % Triton X-100 in PBS for 10 min. The cells were then exposed to the TUNEL reaction mixture containing the TdT enzyme and fluorescein-labelled nucleotides, washed and photographed under a fluorescence Zeiss Axiophot microscope.

Statistical analysis

The data were analysed using the StatWorks software package. Significant effects were determined using Student's *t* test. A statistically significant difference was considered to be present at *P* $\leq$ 0.05.

Figure 1 Levels of EGR-1 protein and transcriptional activity

(A) Representative Western blot of EGR-1 protein in clones NG9 and NG10 grown in the absence or presence of FBS. (B) N2A cells and clones NG9 and NG10 were transfected with 0.5 μ g of an EGR-1 reporter construct, and 24 h after transfection luciferase activity of cell lysates was determined. Values represent the means $\pm$ S.D. from three different experiments; * $P \leq 0.05$, ** $P \leq 0.01$.

RESULTS

Effects of EGR-1 on N2A survival

First of all, we analysed the transcriptional activity of the EGR-1 protein in N2A parental cells and in the clones overexpressing this protein (NG9 and NG10). For that purpose, we transfected parental N2A cells and clones NG9 and NG10 with a vector containing two EGR-1 consensus binding sites cloned in front of the prolactin minimal promoter. As shown in Figure 1(B) the transcriptional activity of this construct, in the presence as well as in the absence of serum, is 3-fold higher in NG9 and NG10 clones, compared with the activity observed in parental N2A cells. These data suggest that the transcriptional activity of EGR-1 is increased in cells overexpressing this protein. In addition, as can be observed in Figure 1(A), the levels of EGR-1 protein do not change significantly in cells growing in serum-free medium compared with cells growing in regular conditions (medium plus FBS).

To determine whether overexpression of EGR-1 directly affected the survival of N2A cells, we tested control cells and clones NG9 and NG10, overexpressing EGR-1, for their ability to exclude a vital dye such as Trypan Blue. As shown in Figure 2, EGR-1 overexpression caused a significant increase in the number of Trypan Blue-positive cells. This increment was observed in the presence as well as in the absence of serum, although the differences in survival between controls and cells overexpressing EGR-1 were higher in the cells growing in the presence of serum. Our results show 3- and 2-fold (NG9 and NG10, respectively) increases in the number of Trypan Blue-positive cells, compared with parental N2A and vector-alone-expressing cells (pLNCX), in cells growing in the presence of serum, whereas this increase was only 1.5- and 1.4-fold (NG9 and NG10, respectively) in cells cultured in serum-free medium.

To analyse the effect of EGR-1 on apoptosis, N2A control cells and clones NG9 and NG10 were treated with 0.01 % PI, visualized

Figure 2 Percentage of Trypan Blue-positive cells in different clones overexpressing the EGR-1 protein

Cells were grown in DMEM plus 10 % FBS for 24 h and then some of the plates were switched to medium without serum. Afterwards all the cells were incubated for a further 32 h. pLNCX represents N2A cells stably transfected with an empty vector. Values represent the means $\pm$ S.D. from three different experiments; * $P \leq 0.05$, ** $P \leq 0.01$.

by fluorescence microscopy and quantified by flow cytometry. Morphological examinations by fluorescence microscopy showed that the amount of apoptotic nuclei, in cells growing in the presence or absence of serum, was higher in transfectant compared with control cells (Figure 3A). Quantification of apoptosis by flow cytometry (Figures 3B and 3C) showed that, in the presence of serum, up to 12.5 and 18.2% of the NG9 and NG10 cells, respectively, displayed a typical pattern of apoptosis (R3 + R4), compared with 6% in the control cells. In the absence of serum, the EGR-1-expressing cells showed also a significant enhancement of the apoptotic population (15.4 and 18.5% in NG9 and NG10 respectively) in comparison with control cells (10.5%). Cells expressing the vector alone gave the same results, as did the wild-type N2A cells (not shown). The total percentages of necrotic + apoptotic population (R2 + R3 + R4), measured by flow cytometry, were essentially identical to those obtained by Trypan Blue exclusion (Figure 2).

A typical molecular characteristic of apoptosis is genomic DNA cleavage into internucleosomal segments attributed to the specific activation of an endogenous endonuclease. This apoptosis-specific genomic DNA cleavage was also assessed by the TUNEL assay. Using this technique we demonstrated further that EGR-1-induced cell death was due to an apoptosis-like mechanism. N2A control and NG9 and NG10 clones were cultured in the absence of serum and assayed for DNA strand breakage. Control (N2A) cells showed very limited fluorescent labelling, whereas NG9 and NG10 clones showed numerous positive cells (Figure 4). These results indicate that death is due to an apoptotic process.

Once we established that EGR-1 overexpression induces apoptosis in N2A cells, we next studied whether treatment with EGR-1 antisense oligodeoxyribonucleotide could result in a diminution of the apoptotic population induced by serum deprivation. For that purpose, parental N2A cells were grown in serum-deprived media in the presence or absence of EGR-1 antisense for 32 h and stained with PI. As shown in Figure 5 the addition of EGR-1 antisense oligonucleotides to the culture medium produces a dramatic decrease in the levels of EGR-1 protein (Figure 5A) and

Figure 3 Apoptosis induction by the overexpression of the EGR-1 gene

(A) Two different clones were cultured for 32 h in normal or serum-free medium and apoptotic cells were detected by PI staining. Phase-contrast (right-hand images) and fluorescence (left-hand images) photomicrographs were taken; magnification, $\times 100$; scale bars, $30 \mu\text{m}$. (B) Representative dot density plot of freshly prepared N2A clones, cultured in the same conditions as (A) and examined by flow cytometry for PI staining. R1, live cells; R2, necrotic cells; R3 + R4, apoptotic cells. fsc, forward scatter. (C) Quantitative determination of apoptotic and necrotic cells determined by PI staining and flow cytometry. Values represent the means $\pm$ S.D. from at least three different experiments; * $P \leq 0.05$, ** $P \leq 0.01$.

Figure 4 Detection of N2A cells undergoing apoptosis using the TUNEL technique

Cells were seeded and grown in medium containing 10% FBS for 24 h. Then, some of the cultures were induced to die by growing in the absence of serum for 32 h. Afterwards the TUNEL assay was performed by labelling the apoptosis-specific DNA strand breaks with fluorescent label as described in the Materials and methods section.

Figure 5 Effect of EGR-1 antisense oligonucleotides on PI staining of N2A cells

Cells were seeded and grown for 24 h in medium containing 10% FBS. Then the cultures were grown for 32 and 48 h in normal or serum-deprived medium with or without 40 μ M EGR-1 sense or antisense oligodeoxynucleotides. (A) Representative Western blot showing the effects of antisense oligodeoxynucleotides on EGR-1 protein levels. (B) Fluorescent photomicrographs of cultures stained with PI, as indicated in the Materials and methods section. S, sense; AS, antisense. Magnification, $\times 50$; scale bars, 40 μ m.

the number of PI-positive cells (5B), suggesting an implication of the endogenous EGR-1 gene in the apoptosis induced by serum withdrawal.

Figure 6 Effect of EGR-1 on p53 expression

(A) The protein levels of p53 in parental N2A cells and clones NG9 and NG10 were determined by Western-blot analysis. Equal protein amounts (20 μ g) of each sample were subjected to SDS/PAGE, transferred to nitrocellulose and blots incubated with specific anti-p53 as described in the Materials and methods section. (B) Parental N2A cells were transiently transfected with a luciferase reporter plasmid containing 400 bp of the P2 mdm2 promoter and 0.3 μ g of an EGR-1 expression vector. Data represent the means $\pm$ S.D. of luciferase activity determined in triplicate in at least two independent experiments, and are expressed relative to the basal value obtained with only the reporter construct. (C) Parental and EGR-1-overexpressing cells were transiently transfected with the same luciferase reporter plasmid as in (B), and luciferase activity was measured. Data represent the means $\pm$ S.D. of luciferase activity determined in triplicate in at least two independent experiments, and are expressed relative to the value obtained with the parental N2A cells; ** $P \leq 0.01$, *** $P \leq 0.001$.

Regulation of p53 family proteins by EGR-1

Since p53 protein has been implicated in EGR-1-mediated apoptosis in melanoma cells [34], we further tested the ability of EGR-1 to regulate the endogenous p53 gene in N2A cells. Therefore, we measured the levels of p53 protein in parental N2A cells and clones NG9 and NG10. Surprisingly, these two clones showed significantly lower levels of p53 protein than did the wild-type N2A cells (Figure 6A). These findings suggest that p53 may not be required for EGR-1-induced apoptosis in these cells.

It has been shown that stability of the p53 protein is reduced significantly by the mdm2 protein; therefore we studied whether the decreased levels in p53 protein observed in the clones NG9 and NG10 could be due to the regulation of the mdm2 gene by EGR-1. To this end we performed Western-blot analysis, using a specific anti-mdm2 antibody, and transient co-transfections of parental N2A cells with a reporter construct that contains 400 bp of the mdm2 promoter region (P2) and an EGR-1 expression plasmid. As shown in Figure 6(A), the content of mdm2 protein is undetectable in parental N2A cells and its levels are clearly induced in the NG9 and NG10 clones. The activity of the reporter construct was also significantly increased (2.2-fold) by co-transfection with EGR-1 (Figure 6B). We next tested mdm2 promoter activity in parental N2A cells and cells overexpressing EGR-1 (Figure 6C). In these experiments, we also noted a strong increase in luciferase reporter activity in cells stably expressing the EGR-1 protein: 3.2- and 6.3-fold (NG9 and NG10 respectively) increases over basal values (i.e. N2A cells), which is in agreement with the higher levels of mdm2 protein observed in these clones. These findings suggested that an induction of mdm2 could be responsible for the observed decrease in p53 and also allow us to add the mdm2 gene to the growing list of genes regulated by EGR-1.

Figure 7 Effect of p53 suppression on EGR-1-induced apoptosis

Parental N2A cells and clones NG9 and NG10 were grown in 60-mm plates and transfected with 2 μ g of pSUPER/p53 or pSUPER vectors, as indicated in the Materials and methods section, and analysed 50 h later. (A) The protein levels of p53 in parental N2A cells and clones NG9 and NG10 were determined by Western-blot analysis. Equal protein amounts (20 μ g) of each sample were subjected to SDS/PAGE, transferred to nitrocellulose and blots were incubated with specific anti-p53 as described in the Materials and methods section. (B) Quantitative determination of apoptotic cells determined by PI staining and flow cytometry. Values represent the means $\pm$ S.D. from at least three different experiments; ** $P \leq 0.01$, *** $P \leq 0.001$.

To further confirm that the EGR-1-induced apoptosis in N2A cells does not require the p53 gene, we designed a construct to knockdown p53 protein. As shown by Brummelkamp et al. [32], appropriate constructions with the pSUPER vector are able to direct the synthesis of small-interfering-RNA- ('siRNA')-like transcripts causing efficient and specific down-regulation of gene expression resulting in functional inactivation of the target gene. In Figure 7(A), we show that clones NG9 and NG10, and parental N2A cells transfected with the pSUPER/p53 construct, presented diminished levels of p53 protein compared with the same cells transfected with pSUPER empty vector. This marked decrease of p53 protein had no effect on apoptosis induced by overexpression of EGR-1 (Figure 7B), suggesting again that the EGR-1-induced apoptosis of N2A cells is independent of p53.

Although p53 plays an essential proapoptotic role, this function is shared with other family members such as the p73 protein. Therefore, to investigate whether the p73 protein could mediate the enhanced apoptosis observed in the clones overexpressing the EGR-1 protein, we first performed Western-blot analysis with a specific anti-p73 antibody. As shown in Figure 8(A) there is a significant increase in p73 in clones NG9 and NG10 as compared with parental N2A cells. Several reports have shown that p73 binds to p53-binding sites and, consequently, activates promoters containing these sequences. We next performed transient transfections experiments using the reporter construct pG13-luc, which contains multiple p53-binding sites linked to a basal promoter [35]. As can be seen in Figure 8(B), when the p53 reporter construct pG13-luc was transfected into N2A cells and clones

Figure 8 Effect of EGR-1 on p73 expression

(A) The protein levels of p73 in parental N2A cells and clones NG9 and NG10 were determined by Western-blot analysis. Equal protein amounts (20 μ g) of each sample were subjected to SDS/PAGE, transferred to nitrocellulose and blots were incubated with specific anti-p73 as described in the Materials and methods section. (B) N2A cells and clones NG9 and NG10 were transfected with 0.5 μ g of pG13-luc vector, and 24 h after transfection, luciferase activity of cell lysates was determined. The histogram shows the means $\pm$ S.D. from at least three separate experiments in triplicate; *** $P \leq 0.001$.

NG9 and NG10, a clear induction of luciferase activity was observed in the two clones. These data suggest a possible involvement of p73 protein in the EGR-1-induced apoptosis in N2A cells.

DISCUSSION

A hallmark of the nervous system is the extensive histogenetic cell death that takes place relatively late in maturation and results in the loss of 20–70% of all neurons [36]. This cell death occurs by apoptosis, a genetically controlled response for cells to commit suicide, a process in which many genes seem to be involved. In this study we have shown that the EGR-1 gene is involved in the apoptosis induced by serum withdrawal in neuroblastoma cells. Furthermore, the enforced expression of EGR-1 in these cells is able to induce apoptosis even in the presence of serum, suggesting that this gene could participate in the decision to commit to the death pathway directly, rather than having an impact on the manifestation of the death process once the pathway has been activated. The apoptotic effect of EGR-1 could be mediated by the p73 protein and seems to be independent of p53 since an increase in p73 protein, paralleled by a decrease in p53, was observed in cells overexpressing EGR-1.

Since the discovery of the EGR-1 gene as an 'early growth response gene', research was directed towards a function of this gene in growth and proliferation. However, EGR-1 expression has also been linked to cell death [37]. In the nervous system, an enhanced expression of EGR-1 has been linked with neuronal apoptosis of both primary cultures of cerebellar granule cells and neuroblastoma cell lines [38–40]. Cerebellar granule cell death induced by withdrawal of depolarizing concentrations of K^+

is accompanied by an induction of endogenous EGR-1 levels. However, although a dominant-negative inhibitor construct blocks granule-cell apoptosis, increased expression of EGR-1 was not sufficient to activate the apoptotic process [41]. Also, it has been reported that an impairment of EGR-1 biosynthesis has no effect upon apoptosis induced by anti-depressants in HT22 immortalized hippocampal neurons [40]. Our results clearly show that, in N2A cells, EGR-1 is required for apoptosis induced by serum deprivation. Furthermore, overexpression of the EGR-1 protein can efficiently promote the apoptotic programme of neuroblastoma cells.

Previous reports have shown that EGR-1 plays a role in thapsigargin or ionizing radiation-inducible apoptosis in human melanoma cells A375-C6 and prostate cells [34,42]. In human melanoma cells, wild-type p53 was required for EGR-1-dependent apoptosis caused by thapsigargin. Nair et al. [34] observed two EGR-1-binding sites in the p53 promoter and one of the sites tested was found to bind EGR-1; consistent with this finding, EGR-1 caused transcriptional up-regulation of the p53 gene promoter and increased p53 transcript and protein levels. However, the possibility that EGR-1 may utilize a p53-independent pathway, in the case of ionizing radiation-inducible apoptosis, has been suggested by the finding that a dominant-negative mutant of EGR-1 abrogated radiation-inducible apoptosis despite induction of wild-type p53 [43]. This hypothesis was confirmed by studies in prostate cancer PC-3 cells that lack p53 protein [44]. In our system, overexpression of EGR-1 results in a decrease of p53 protein levels, which is in contrast with the previous observation by Nair et al. [34] in human melanoma cells, commented on above. This could be the consequence of a different cell background since it has been shown that the activity of EGR-1 is dependent on multiple factors [45]. The effect of EGR-1 on p53 protein levels, shown in this paper, suggests that this protein does not mediate EGR-1-induced apoptosis of N2A cells and, therefore, that p53 is not required for all EGR-1 signalling pathways leading to apoptosis. This conclusion is further supported by the results showing that, in NG9 and NG10 clones transfected with the pSUPER/p53 construct and presenting even lower levels of p53 protein, the percentage of apoptotic cells doesn't change.

Two genes, p63 and p73 [46], have been found to encode several proteins whose structure and functions are similar, but not identical, to those of p53. It has been shown that p73 is able to induce apoptosis when overexpressed [47]. p73 apoptotic functions are also recruited in response to DNA damage, through a p53-independent pathway that requires the activation of the nuclear tyrosine kinase c-Abl [48,49]. Our data suggest that the apoptosis induced by EGR-1 in N2A cells could be mediated by an increase in p73 protein levels and the subsequent enhancement in its transactivation activity. The effect of EGR-1 protein on p73 gene expression could be a direct one since it has been described in the presence of EGR-1 binding sites in the promoter of this gene [50]. Therefore cell-type-specific mechanisms may determine the mechanism of action of EGR-1 upon programmed cell death. Differential regulation by EGR-1 has been described for other genes, like the platelet-derived growth factor A-chain that is dependent on cell type [51,52]. It is interesting to point out that a suppression in p53 protein levels [53] and an overexpression of p73 [54] have also been associated with an increase in neurite outgrowth and expression of neuronal differentiation markers. In agreement with this, we have previously found that the ectopic expression of EGR-1 in N2A cells induces neuronal differentiation [23].

The p53 protein is significantly destabilized by the overexpression of mdm2 [55,56], leading to the postulation that mdm2

may play a role in maintaining low levels of p53 in normal cells as well as in removing p53 during recovery from stress-induced arrest through a feedback-regulatory loop. The mdm2 protein is able to form a protein-protein interaction with p53, which inactivates the transactivation function of p53 [57,58] and overexpression of mdm2 leads to a significant destabilization of the p53 protein inhibiting the ability of p53 to evoke cell-cycle arrest or induce apoptosis [55,56,59]. Our results clearly show an up-regulation of the mdm2 promoter and protein content by the EGR-1 protein, which probably plays an important role in the reduction of p53 protein levels observed in clones overexpressing the EGR-1 protein. This observation is particularly important since it implicates the EGR-1 gene as a regulator of a gene involved in crucial cellular processes such as apoptosis and cell-cycle regulation.

The studies described herein, together with previous reports regarding the regulation of EGR-1 gene expression and its possible role in neuronal tissues [25,60,61] suggest that EGR-1 levels may play a significant role in neuronal cell survival during development of the central nervous system. Our results also suggest an implication of the p73 protein in the effect of EGR-1 on N2A cell death through a mechanism independent of p53. Analysis of the downstream signal transduction of the EGR-1-induced apoptosis might be useful for understanding the general mechanism of neuronal cell death.

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