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Cyclin D1 is an essential mediator of apoptotic neuronal cell death

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Many neurons in the developing nervous system undergo programmed cell death, or apoptosis. However, the molecular mechanism underlying this phenomenon is largely unknown. In the present report, we present evidence that the cell cycle regulator cyclin D1 is involved in the regulation of neuronal cell death. During neuronal apoptosis, cyclin D1-dependent kinase activity is stimulated, due to an increase in cyclin D1 levels. Moreover, artificial elevation of cyclin D1 levels is sufficient to induce apoptosis, even in non-neural cell types. Cyclin D1-induced apoptosis, like neuronal apoptosis, can be inhibited by 21 kDa E1B, Bcl2 and pRb, but not by 55 kDa E1B. Most importantly, however, overexpression of the cyclin D-dependent kinase inhibitor $p16^{INK4}$ protects neurons from apoptotic cell death, demonstrating that activation of endogenous cyclin D1-dependent kinases is essential during neuronal apoptosis. These data support a model in which neuronal apoptosis results from an aborted attempt to activate the cell cycle in terminally differentiated neurons.

Keywords: apoptosis/cdk4/cell cycle/cyclin D1/neuronal cell death

Introduction

During the development of the nervous system, approximately half of the generated neurons die by apoptosis (reviewed by Oppenheim, 1991), a process that is characterized by cytoplasmic blebbing, cell shrinking, neurite degeneration, fragmentation of the nucleus and fragmentation of the genomic DNA. Neurotrophic factors suppress apoptosis and are presumably the limiting survival factors for neuronal cell types *in vivo* (Oppenheim, 1991). The fact that neuronal cell death does not occur in the presence of inhibitors of mRNA and protein synthesis indicates that the process requires activation of a specific genetic programme (Martin *et al.*, 1988).

As yet, little is known about the genetic programme driving neuronal apoptosis. Recent evidence suggests that the events that trigger neuronal apoptosis may be similar to the events that trigger transformation: activation or overexpression of (proto-) oncogenes may lead to transformation of proliferation-competent cells, but may lead to apoptosis in quiescent or terminally differentiated cells (Evan *et al.*, 1992; Heintz, 1993; Lowe and Ruley, 1993; Hoang *et al.*, 1994). Therefore, it has been hypothesized that the response of a given cell type to oncogene activation (i.e. transformation or apoptosis) may be determined by the proliferation/differentiation state of the challenged cell (Heintz, 1993).

Among the (proto-) oncogenes with apoptosis-inducing properties are the genes encoding c-Myc, cyclin A, E2F1 and adenovirus E1A (Evan *et al.*, 1992; Debbas and White, 1993; Lowe and Ruley, 1993; Hoang *et al.*, 1994; Wu and Levine, 1994). Furthermore, during neuronal apoptosis there is an induction of the proto-oncogenes encoding c-Jun and cyclin D1 (Estus *et al.*, 1994; Freeman *et al.*, 1994). Cyclin D1 regulates progression through the G_1 phase of the cell cycle by stimulating the activity of the cyclin D-dependent kinases cdk4 or cdk6 (Matsushime *et al.*, 1994; Meyerson and Harlow, 1994; reviewed by Sherr, 1994). An important substrate for these kinases is the product of the retinoblastoma susceptibility gene, pRb (Ewen *et al.*, 1993; Kato *et al.*, 1993). In G_1, underphosphorylated pRb suppresses initiation of S phase (Goodrich *et al.*, 1991) but, during mid-to-late G_1, pRb is progressively phosphorylated by G_1 cdks and thereby loses its growth suppressive effect (Sherr, 1994). The fact that cyclin D-dependent kinase activity is only required for cell cycle progression in cells expressing functional pRb (Lukas *et al.*, 1994, 1995a,b; Guan *et al.*, 1995) is consistent with the view that pRb is a key substrate for cyclin D-dependent kinases. Overexpression of cyclin D1 causes a reduction in cell size, reduces the requirement for growth factors and shortens the G_1 cell cycle phase (Jiang *et al.*, 1993; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994). In addition, overexpression of cyclin D1 contributes to the oncogenic transformation of cells *in vitro* and *in vivo* (Jiang *et al.*, 1993; Bodrug *et al.*, 1994; Hinds *et al.*, 1994; Lovec *et al.* 1994a,b; Wang *et al.*, 1994).

Given the correlation between cell transformation and apoptotic cell death, we investigated a possible function for cyclin D1 in the induction of apoptosis. We found that high levels of cyclin D1 indeed induce apoptotic cell death in a variety of cell types. Upon characterization of cyclin D1-induced apoptosis, we found a remarkable similarity between the gene products that inhibit cyclin D1-induced apoptosis and those that are known to inhibit neuronal apoptosis. The relevance of this observation is demonstrated by experiments showing that the activity of the cyclin D-dependent kinase cdk4 is greatly stimulated during neuronal apoptotic cell death, due to increased abundance of cyclin D1. Moreover, by using a specific inhibitor of cyclin D-dependent kinase activity, we show that the induction of this activity is an essential event during neuronal apoptosis.

The results presented here show that the stimulation of G_1 cdk activity is essential for neuronal cell death. A model in which neuronal apoptosis results from an aborted

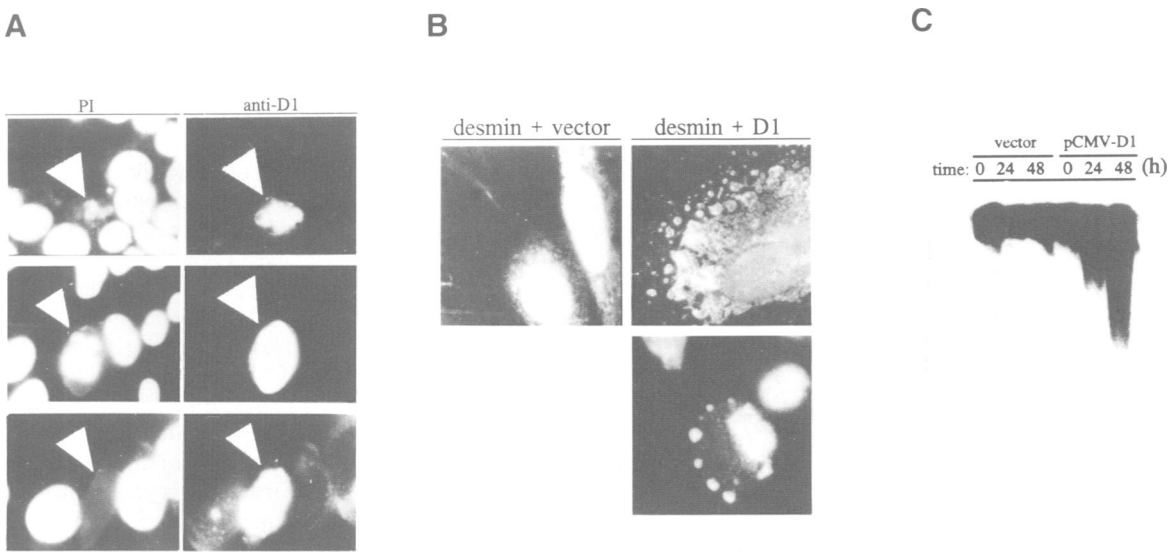

Fig. 1. Overexpression of cyclin D1 induces apoptosis. (**A**) Immunofluorescence analysis of transiently transfected 3T3 (upper panel), R970B (middle panel) and CV-1 (lower panel) cells shows that cyclin D1-positive cells (indicated by arrowheads) contain nuclei with apoptotic features like nuclear fragmentation and loss of PI staining. In addition, many cyclin D1-positive cells show highly condensed chromatin (not shown). (**B**) Immunofluorescence analysis shows that co-transfection of pCMV-desmin with pCMV-D1 but not with pCMV-neo leads to cytoplasmic blebbing. The upper photograph shows a cell with extensively blebbed cytoplasm. The lower photograph shows a cell in which the process has just been initiated. The nuclei were counterstained with PI. (**C**) Southern blot analysis of total genomic DNA isolated 24, 48 and 72 h after transfection of R970B cells with either pCMV-D1 or pCMV-neo. Fragmentation of genomic DNA is detected readily in cell cultures transfected with pCMV-D1, but not with pCMV-neo.

attempt to start the cell division cycle in post-mitotic neurons is therefore strongly supported.

Results

Overexpression of cyclin D1 induces apoptosis

A number of studies have established that overexpression of cyclin D1 may lead to oncogenic transformation (Jiang *et al.*, 1993; Bodrug *et al.*, 1994; Hinds *et al.*, 1994; Lovec *et al.*, 1994a,b; Wang *et al.*, 1994). Since transformation and apoptosis may be related processes (Heintz, 1993), we initiated these studies by analysing whether overexpression of cyclin D1 would lead to apoptotic cell death. To this end, an expression vector driving cyclin D1 expression from the cytomegalovirus (CMV) promoter (pCMV-D1) was transfected into NIH 3T3 ($p53^{+/+}$), R970B ($p53^{-/-}$) or CV-1 (monkey kidney) cells. Three days after transfection, the cyclin D1-positive cells were analysed for apoptotic properties. Fluorescence microscopic analysis showed that the nuclei of cyclin D1-positive cells exhibit apoptotic features such as fragmentation of the nucleus and loss of DNA staining by propidium iodide (PI; Figure 1A).

Moreover, staining of the cytoplasm by immunofluorescence for co-transfected desmin indicates that the cytoplasm of many cyclin D1-expressing cells showed extensive blebbing, which was not observed in cells transfected with desmin and the control vector (Figure 1B).

In addition to changes in the nuclear and cytoplasmic morphologies, apoptotic cell death is characterized by fragmentation of the genomic DNA. To assess whether cyclin D1 overexpression would induce this phenomenon, R970B cells were transfected with pCMV-D1 or pCMV-neo and the genomic DNA was isolated 24, 48 and 72 h after transfection. Analysis of the genomic DNA by Southern blotting shows that DNA fragmentation is readily detected in cyclin D1-expressing cell cultures but not in cell cultures transfected with the control vector (Figure 1C; see also Figure 3B).

Thus, cells overexpressing cyclin D1 show at least four hallmarks of apoptotic cell death: aberrant nuclear morphology, loss of nuclear staining by propidium iodide, blebbing of the cytoplasm and fragmentation of the genomic DNA.

Cyclin D1-induced apoptosis does not require p53 and is only marginally inhibited by serum factors

The tumour suppressor protein p53 regulates some, but not all, pathways that lead to apoptosis (reviewed by Oren, 1994). To assess the dependency of cyclin D1-induced apoptosis on p53, the fluorescence assay was used to determine the kinetics of apoptosis in the presence or absence of p53. NIH 3T3 ($p53^{+/+}$) and R970B ($p53^{-/-}$) mouse fibroblasts were transfected with pCMV-D1 and the percentage of cyclin D1-positive cells with apoptotic features was determined at the indicated times, as described in Materials and methods. Figure 2A shows that the kinetics of cyclin D1-induced apoptosis are similar in $p53^{+/+}$ and $p53^{-/-}$ mouse fibroblasts. From this experiment, we conclude that p53 is not required for cyclin D1-induced apoptosis.

Some apoptotic processes are inhibited by serum factors (Evan *et al.*, 1992; Lowe and Ruley, 1993; Hoang *et al.*, 1994). To assess whether serum factors could suppress cyclin D1-induced apoptosis, R970B cells were transfected with pCMV-D1 and were subsequently cultured in 0.5 or 8% fetal calf serum (FCS), for the indicated times. The kinetics of apoptosis were then determined. Figure 2B shows that cyclin D1-induced apoptosis is only marginally suppressed by serum factors.

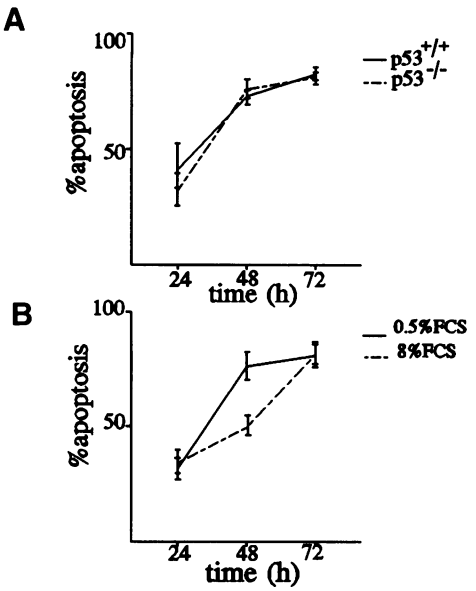

Fig. 2. Cyclin D1-induced apoptosis is independent of p53 and hardly inhibited by serum factors. (**A**) Immunofluorescence analysis was used to determine the percentage of cyclin D1-positive nuclei with apoptotic features at the indicated times after transfection of NIH 3T3 ($p53^{+/+}$) and R970B ($p53^{-/-}$) cells with pCMV-D1, as described in Materials and methods. The kinetics of apoptosis induction are similar in $p53^{+/+}$ and $p53^{-/-}$ cells. (**B**) Cyclin D1-induced apoptosis in R970B cells is only marginally suppressed in the presence of 8% FCS.

Cyclin D1-induced apoptosis depends on cdk activation and can be inhibited by adenovirus 21 kDa E1B, Bcl2 and pRb, but not by PCNA or 55 kDa E1B

Since cyclin D1 is a regulator of cdk activity, apoptosis may result from the aberrant activation of cdk4/6. To test whether kinase activation would be required for cyclin D1-induced apoptosis, we made use of an expression vector producing a mutant form of cyclin D1(KE) that cannot bind to cdks (Hinds *et al.*, 1994). Figure 3A (left upper panel) shows that this mutant cyclin D1 protein is largely ineffective in inducing apoptosis. The background level of cell death induced by transfection of pCMV-D1(KE) into R970B cells is similar to that induced by transfection of the control vector pCMV-lacZ.

In another approach to study the dependency of cyclin D1-induced apoptosis on kinase activation, we made use of the specific inhibitor of cyclin D-dependent kinases, $p16^{INK4A}$ (Serrano *et al.*, 1993). R970B cells were transfected with expression plasmids for cyclin D1 and $p16^{INK4A}$, and the kinetics of apoptosis were determined subsequently by the fluorescence assay. Co-production of $p16^{INK4A}$ with cyclin D1 largely abrogates cyclin D1-induced apoptosis (Figure 3A, left lower panel). However, these experiments do not rule out the possibility that cyclin D1-induced apoptosis occurs as an indirect result of titration of cdk inhibitors (CKIs) and subsequent activation of other cyclin–cdk complexes. Therefore, we used a mutant form of cdk4(dn) (van den Heuvel and Harlow, 1993). Cdk4(dn) binds to cyclin D1 but is catalytically inactive and acts in a dominant-negative fashion, presumably by displacing wild-type cdk4 from cyclin D1. Cyclin D1–cdk4(dn) complexes can still titrate CKIs but, as shown in Figure 3 (left lower panel), co-expression of cdk4(dn) with cyclin D1 abrogates cyclin D1-induced apoptosis. From these experiments, we conclude that induction of cyclin D1-dependent kinase activity is a prerequisite for the induction of apoptosis by cyclin D1.

Co-production of pRb, a substrate for cyclin D1-dependent kinases (Kato *et al.*, 1993), with cyclin D1 also efficiently inhibits apoptosis (Figure 3A, left lower panel). In a similar way, we analysed whether the anti-apoptotic proteins Bcl2, adenovirus (type 5) 21 kDa E1B or 55 kDa E1B, could inhibit cyclin D1-induced apoptosis. Adenovirus 21 kDa E1B is an anti-apoptotic protein that is a functional homologue of the cellular Bcl2 protein and prevents premature host cell death during lytic adenovirus infections. The E1B gene also encodes a larger (55 kDa) protein that inhibits the function of the cellular tumour suppressor protein p53. Since p53 is essential for various apoptotic pathways (Oren, 1994), 55 kDa E1B may function as an inhibitor of cell death. It is shown in Figure 3A (right panel) that co-expression of either Bcl2 or 21 kDa E1B, but not 55 kDa E1B, with cyclin D1 efficiently inhibits cyclin D1-induced apoptosis. The fact that 55 kDa E1B does not inhibit cyclin D1-induced apoptosis was expected, since p53 is not expressed in R970B cells and is not required for cyclin D1-induced apoptosis (Figure 2). The inhibitory actions of Bcl2, 21 kDa E1B, pRb, p16 and cdk4(dn) only become clearly apparent at 48 and 72 h after transfection. At 24 h after transfection, the level of background cell death is only marginally lower than the level of cyclin D1-induced apoptosis (Figure 3A, left upper panel) and may therefore mask the inhibitory actions of these proteins. Furthermore, the fact that co-transfection of plasmids will not be complete may also contribute to the observed lack of inhibition at 24 h.

In contrast to the inhibitory effects of Bcl2, 21 kDa E1B, $p16^{INK4}$ or pRb, co-expression of proliferating cell nuclear antigen (PCNA) did not affect the kinetics of cyclin D1-induced apoptosis (Figure 3A, right panel), suggesting that the functional interaction between cyclin D1 and PCNA (Pagano *et al.*, 1994) is not essential for the induction of apoptosis. The production of the proteins encoded by the transfected expression plasmids (21 kDa E1B, 55 kDa E1B, Bcl2, PCNA, $p16^{INK4A}$, pRb) was confirmed by Western blotting, immunoprecipitation or immunofluorescence (not shown).

Analysis of genomic DNA isolated from the transfected cell cultures (adherent and floating cells) confirmed the above-mentioned fluorescence data, since fragmentation of genomic DNA is detected readily in cells expressing cyclin D1 and is largely inhibited by co-expression of $p16^{INK4}$, Bcl2, 21 kDa E1B and pRb, but not by 55 kDa E1B (Figure 3B). In addition, transfection of cells with the control vector or with mutant cyclin D1(KE) did not induce DNA fragmentation (Figure 3B).

Cyclin D1-dependent kinase activity is induced during neuronal apoptosis

The fact that overexpression of cyclin D1 induces apoptosis raises the question whether cyclin D1 is a physiological regulator of apoptotic cell death. The features of cyclin D1-induced apoptosis as described above show a remarkable similarity to those reported for neuronal apoptosis: both

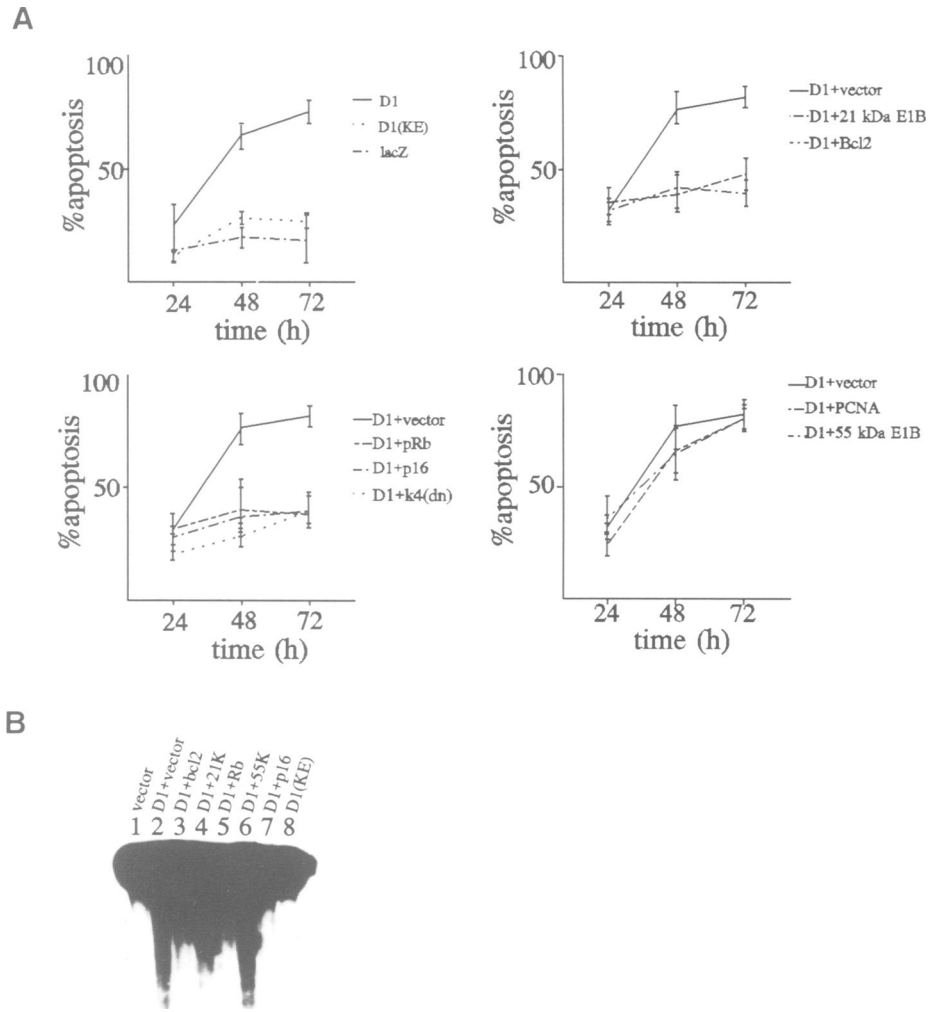

Fig. 3. Identification of factors that inhibit cyclin D1-induced apoptosis. (**A**) Immunofluorescence analysis (as in Figure 2) of transfected R970B cells. R970B cells were transfected with 1 μg of pCMV-D1, pCMV-D1(KE) or pCMV-lacZ. The cyclin D1 or β-gal-positive nuclei were then analysed for apoptotic properties as described in Materials and methods. R970B cells were subsequently co-transfected with 1 μg of pCMV-D1 and 5 μg of either pCMV-neo, pCMV-bcl2, pCMV-21KE1B, pCMV-$p16^{INK4}$, pCMV-cdk4(dn), pCMV-Rb, pCMV-PCNA or pCMV-55KE1B and the kinetics of apoptosis were determined. Co-expression of 21 kDa E1B, Bcl2, $p16^{INK4}$, cdk4(dn) or pRb suppresses cyclin D1-induced apoptosis, whereas PCNA and 55 kDa E1B do not interfere with this process. (**B**) Southern blot analysis of total genomic DNA isolated from the cell populations transfected as in (A). pCMV-D1, but neither pCMV-neo nor pCMV-D1(KE), induces DNA fragmentation. In accordance with the immunofluorescence data, Bcl2, 21 kDa E1B, $p16^{INK4}$ and pRb inhibit DNA fragmentation, whereas 55 kDa E1B does not.

apoptotic processes are inhibited by Bcl2, 21 kDa E1B and pRb, but not by 55 kDa E1B (Figure 3; Allsop *et al.*, 1993; Lee *et al.*, 1994; Martinou *et al.*, 1995). In addition, it was found recently that cyclin D1 mRNA levels increase during neuronal apoptosis (Freeman *et al.*, 1994).

The possible involvement of cyclin D1-dependent kinases in the regulation of neuronal apoptotic cell death was then studied using cultures of post-mitotic neurons prepared from the mouse N1E-115 neuroblastoma cell line. To correlate our findings with those made by Freeman *et al.* (1994), we analysed cyclin D1 protein levels in terminally differentiated neurons induced to undergo apoptosis (by serum withdrawal). Double fluorescence staining for DNA (PI) and cyclin D1 shows that in apoptotic nuclei (loss of PI staining) the level of endogenous cyclin D1 protein is increased enormously (Figure 4). Thus, the induction of cyclin D1 mRNA during neuronal apoptosis (Freeman *et al.*, 1994) results in an increased abundance of cyclin D1 protein.

Transient transfection of cyclin D1 into N1E-115 cells leads to loss of nuclear PI staining (Figure 5A), as was observed in the cell types shown in Figure 1A. The specificity of apoptosis induction by cyclin D1 was tested in a time course experiment similar to that shown in Figure 3. As is shown in Figure 5B, the induction of apoptosis in N1E-115 cells is not due to aspecific consequences of the transfection procedure, as only low levels of apoptosis are detected following transfection with the control vector pCMV-lacZ.

The N1E-115-derived neurons have been studied extensively with respect to morphological, biochemical and electrophysiological differentiation, and are an excellent *in vitro* model system for studying the behaviour of terminally differentiated neurons (reviewed by de Laat

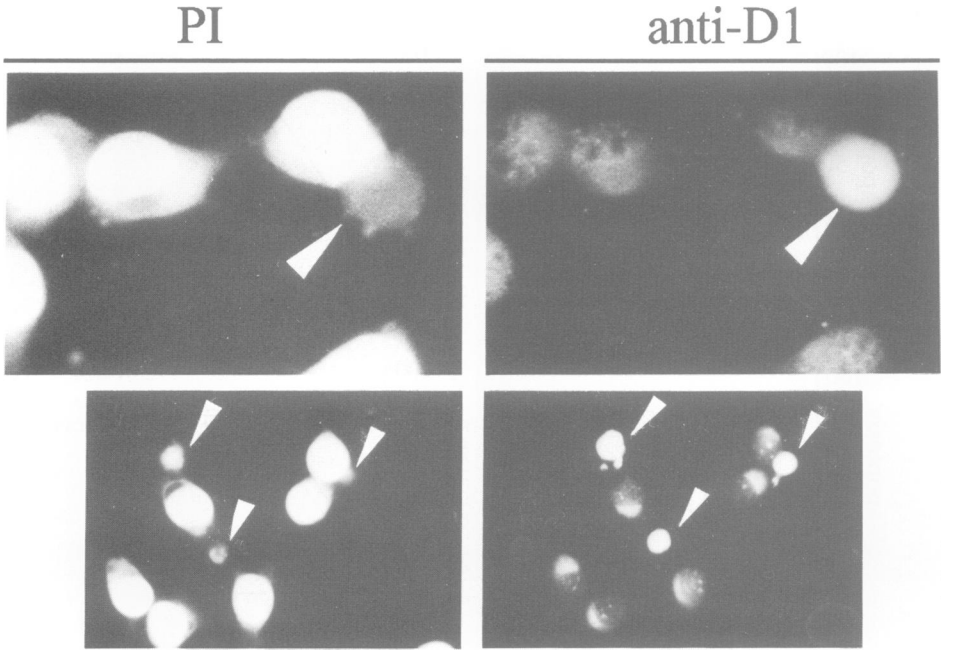

Fig. 4. Accumulation of cyclin D1 in apoptotic neurons. N1E-115 cells were grown on glass coverslips and were differentiated into post-mitotic neurons. Serum was then withdrawn from the neurons and, after 6 h, cells were fixed and processed for anti-cyclin D1 immunofluorescence. The nuclei were counterstained with PI. In the PI-negative (apoptotic) neurons (indicated by arrowheads), high levels of cyclin D1 are detected, whereas in the surrounding non-apoptotic neurons cyclin D1 is hardly detectable. Two different fields are shown, photographed at 1000× and 400× magnification respectively.

and Van der Saag, 1982, Jalink *et al.*, 1994). Upon serum withdrawal from these neurons, apoptosis is rapidly induced as analysed by DNA fragmentation (Figure 6A). During this process, cyclin D1 protein levels increase whereas the levels of cdk4 protein remain constant (Figure 6B). With the increase of cyclin D1 protein, the amount of cyclin D1–cdk4 complexes increases (Figure 6C) and the activity of cdk4 is greatly stimulated (Figure 6D). Thus, neuronal apoptosis is accompanied by a strong induction of cyclin D1–cdk4 kinase activity due to increased cyclin D1 protein levels.

Activation of cyclin D1-dependent kinases is essential for neuronal apoptosis

The experiments presented above indicate that cyclin D1-dependent kinase activity may regulate neuronal apoptosis. The importance of the observed induction of this activity was then studied by using $p16^{INK4A}$. This kinase inhibitor specifically inhibits cyclin D-dependent kinases (Serrano *et al.*, 1993) and, as demonstrated in Figure 3, can inhibit cyclin D1-induced apoptosis efficiently. The expression vector for $p16^{INK4A}$ was introduced into N1E-115 neuronal precursor cells, in excess of a reporter gene (either pRSV-CAT or pCMV-lacZ). After transfection, the cells were differentiated into post-mitotic neurons and subsequently were induced to undergo apotosis (by serum withdrawal). At different times after the induction of apoptosis, adherent cells were harvested and tested for expression of the reporter gene. This scheme is outlined in Figure 7A. Expression of $p16^{INK4A}$ was confirmed by co-immunoprecipitation with cdk4 from ^{35}S-labelled cell extracts (Figure 7B).

Co-transfection of the control vector together with the reporter (pRSV-CAT or pCMV-lacZ) into neuronal

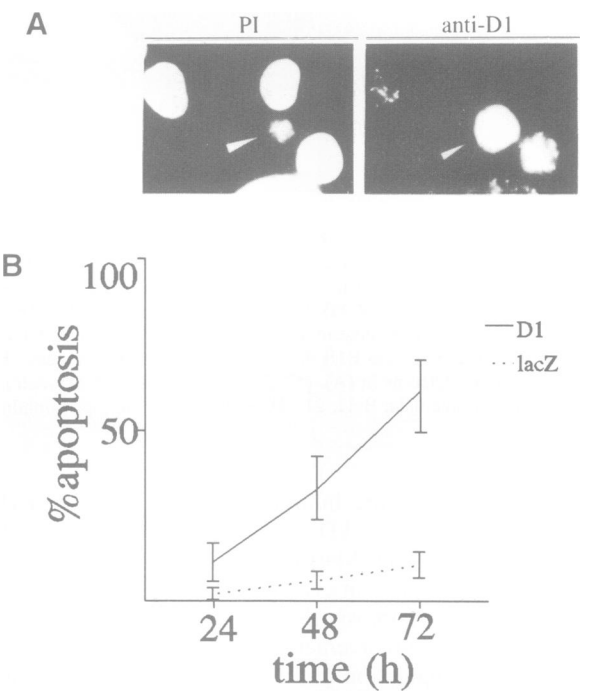

Fig. 5. Induction of apoptosis in N1E-115 neuronal precursor cells by cyclin D1. N1E-115 cells were grown on glass coverslips and were transfected with pCMV-D1. (**A**) Double fluorescence analysis for cyclin D1 and DNA shows that the cyclin D1-positive nucleus (indicated by arrowheads) is negative for PI staining.
(**B**) Quantification of cyclin D1-induced apoptosis in N1E-115 cells. Twenty-four, 48 and 72 h after transfection with either pCMV-D1 or pCMV-lacZ, the cells were processed for immunofluorescence (anti-D1 or anti-β-gal) and apoptosis was quantified as described in Materials and methods. As is shown in the graphs, cyclin D1, but not β-gal, induces apoptosis in this cell line.

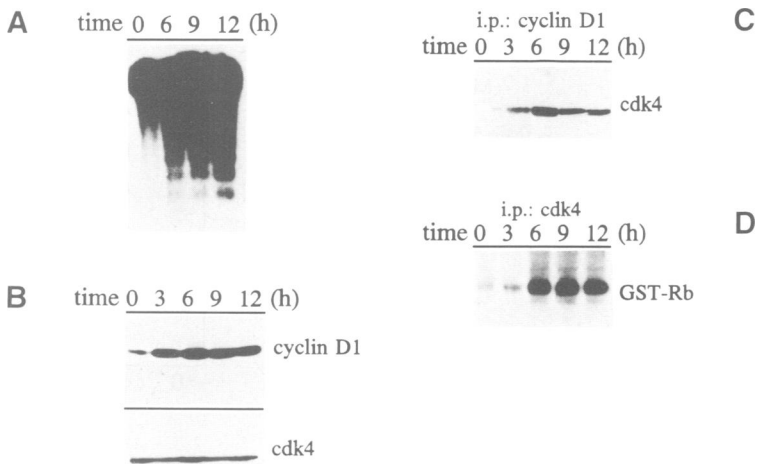

Fig. 6. Activation of cyclin D1-dependent kinases during apoptotic neuronal cell death. Serum was withdrawn from terminally differentiated neurons and the cells were harvested 0, 6, 9 and 12 h after serum withdrawal. (**A**) Apoptosis was monitored by Southern blot analysis of total genomic DNA. (**B**) Western blot analysis of cyclin D1 and cdk4 protein levels during the induction of apoptosis. (**C**) Complex formation between cyclin D1 and cdk4. Cyclin D1 was immunoprecipitated from cell extracts and the amount of co-precipitating cdk4 was determined subsequently by Western blotting. Increasing amounts of cdk4 co-precipitate with cyclin D1 upon induction of apoptosis. (**D**) cdk4 was immunoprecipitated and tested for kinase activity towards GST–Rb. cdk4 is activated during neuronal apoptosis in parallel with the increased abundance of cyclin D1–cdk4 complexes.

precursor cells does not protect the neurons from cell death: 48 h after the induction of apoptosis, expression of the reporter gene is lost from the adherent neuron population (Figure 7C and D). However, in neurons expressing $p16^{INK4A}$, expression of the reporter gene is preserved (Figure 7C and D). The increase in CAT activity in the $p16^{INK4A}$-transfected cell cultures is due to the accumulation of (stable) CAT enzyme, as it is also observed in control–vector-transfected cells that are not induced to undergo apoptosis (not shown). Similarly, 48 h after the induction of apoptosis, many β-gal-positive neurons are observed in the population of neurons expressing $p16^{INK4A}$, but not in the control cell culture (Figure 7D). From these experiments, we conclude that $p16^{INK4A}$ protects neurons from apoptotic cell death, and thus that the observed induction of cyclin D-dependent kinase activity during neuronal apoptosis (Figure 6) is essential.

Discussion

Unscheduled expression of the genes encoding c-Myc, E2Fs and adenovirus E1A may cause cell transformation, but may also cause apoptotic cell death (Houweling *et al.*, 1980; Luscher and Eisenman, 1990; Evan *et al.*, 1992; Debbas and White, 1993; Lowe and Ruley, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Singh *et al.*, 1994; Wu and Levine, 1994; Xu *et al.*, 1995). Therefore, transformation and apoptosis may be regarded as related phenomena. In this study we have shown that cyclin D1, known as a transforming gene product, can also cause apoptotic cell death when overexpressed. The induction of apoptosis by overproduced cyclin D1 provides an explanation for the extremely low efficiency with which stable cyclin D1-overexpressing cell lines are generated (Quelle *et al.*, 1993). How then, can these findings be related to the fact that overexpression of cyclin D1 contributes to tumorigenesis? Many tumour cell lines with gross cyclin D1 mRNA overexpression only moderately overproduce the protein (E.Schuuring, personal communication). This suggests that there is a selection against extremely high levels of cyclin D1. In line with this, we have found that the level of cyclin D1 in transiently transfected cells largely exceeds the level of cyclin D1 in tumour cells with cyclin D1 amplification. Moreover, transient overexpression of cyclin D1 also induces apoptosis in these cell lines (not shown). Thus, the absolute level of cyclin D1 in a given cell type determines the cellular response to overexpression: moderate overexpression results in growth stimulation, whereas high overexpression results in apoptotic cell death.

Cyclin D1-induced apoptosis is inhibited by pRb (Figure 3), a substrate for cyclin D-dependent kinases (Kato *et al.*, 1993). Possibly, the apoptosis-inhibitory effect of pRb (Haas-Kogan *et al.*, 1995) is antagonized by overexpression of cyclin D1. Co-overexpression of pRb itself may then compensate for pRb inactivation by overexpressed cyclin D1. It was shown recently that loss of pRb sensitizes mouse fibroblasts to apoptosis induced by methotrexate (Almasan *et al.*, 1995). In addition, loss of pRb leads to the apoptotic cell death of neuronal cell types (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992, 1994), a process that is not inhibited by 55 kDa E1B and thus, like cyclin D1-induced apoptosis, occurs independently of p53 (Figure 2; Martinou *et al.*, 1995). Conversely, loss of pRb in lens fibre cells leads to p53-dependent apoptosis (Morgenbesser *et al.*, 1994). Therefore, it remains unclear presently to what extent the inactivation of pRb (or pRb-related proteins) contributes to cyclin D1-induced apoptosis.

An alternative mechanism for cyclin D1-induced apoptosis could involve the cdk4/6-mediated phosphorylation of substrates that would normally only be phosphorylated by related cdk enzymes. For instance, premature activation of cdk1 (cdc2) leads to, and is essential for,

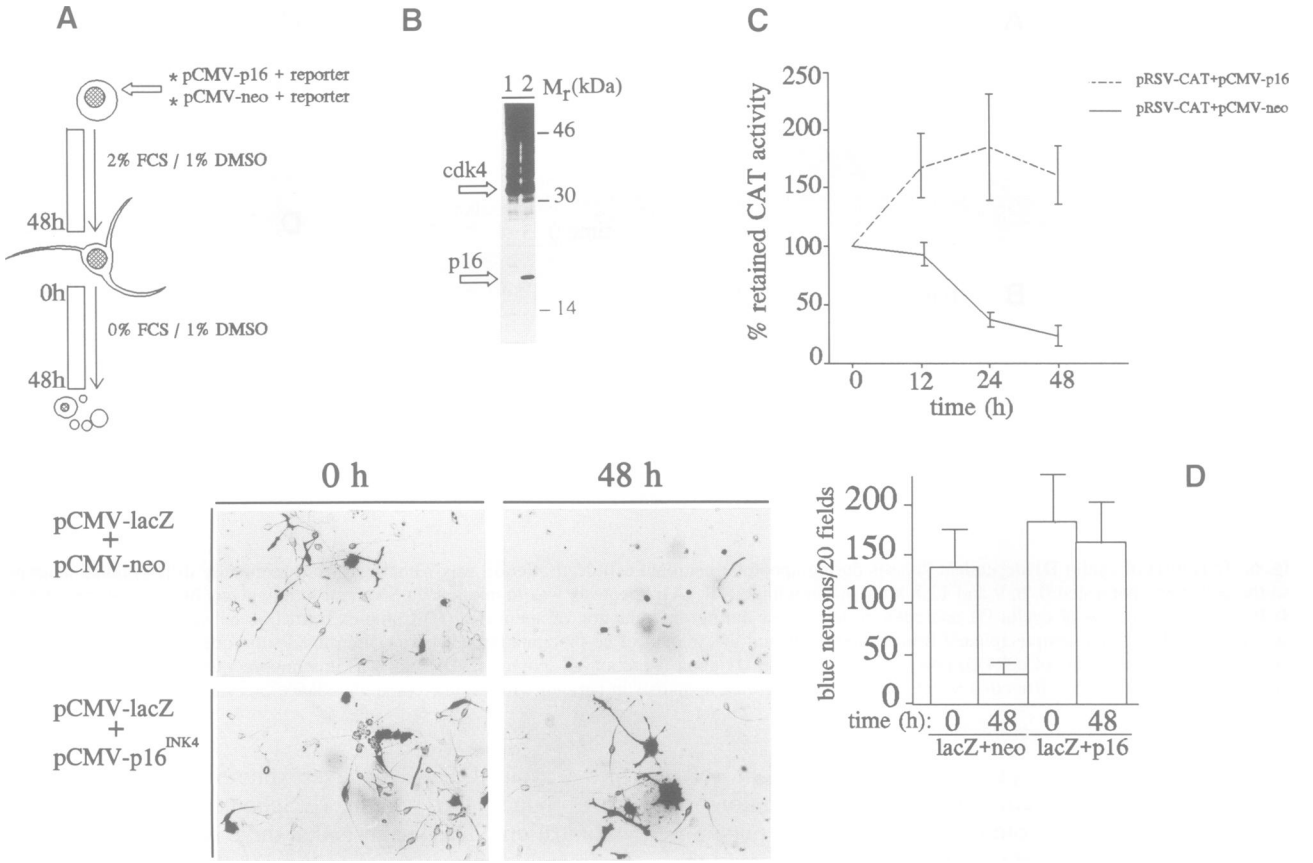

Fig. 7. Apoptotic neuronal cell death requires activation of cyclin D1-dependent kinases. (**A**) Schematic outline of the experiment. N1E-115 cells were transfected with either pCMV-neo or pCMV-p16^{INK4} together with a reporter plasmid, and differentiation was induced for 48 h. Subsequently, apoptosis was induced and, at the indicated times, the adherent cell population was tested for reporter gene expression. (**B**) The production of p16^{INK4} was analysed in cell populations transfected with pCMV-neo (lane 1) or with pCMV-p16^{INK4} (lane 2), by co-immunoprecipitation with cdk4 from ^{35}S-labelled extracts. (**C**) CAT activity is rapidly lost from control-transfected cells, but is retained in cells transfected with pCMV-p16^{INK4}. Means and standard deviations of four independent transfection experiments were calculated and are presented in the graph as percentages of control cell (time 0) activity. (**D**) The experiment was performed as described in (A) but pCMV-lacZ was used as a reporter instead of pRSV-CAT. Forty-eight hours after the induction of apoptosis, β-gal activity is lost from the adherent cell population in control-transfected cells, but the neurons derived from pCMV-p16^{INK4}-transfected cells survive and β-gal activity is retained. These results were quantified by counting the number of blue neurons in 20 microscopic fields (40× magnification) prior to and 48 h after the induction of apoptosis. The means and standard deviations of four separately transfected dishes are shown.

apoptosis (Shi *et al.*, 1994). Thus, phosphorylation of cdk1 substrates by aberrantly activated cdk4 may result in apoptosis.

Although it may be argued that the induction of apoptosis by overexpressed cyclin D1 is not a physiological process, we have also demonstrated that (endogenous) cyclin D1 regulates a physiological apoptotic process. When the experiments in Figure 3 were performed, we noted that the gene products that could inhibit cyclin D1-induced apoptosis were similar to those that had been described to inhibit neuronal apoptosis (Allsop *et al.*, 1993; Lee *et al.*, 1994; Martinou *et al.*, 1995). Furthermore, it was found recently that cyclin D1 mRNA is induced during this process (Freeman *et al.*, 1994). We have shown by immunofluorescence that apoptotic (but not surviving) neurons stain strongly for cyclin D1 protein (Figure 4). In agreement with this, the abundance of cyclin D1 protein in a culture of terminally differentiated neurons is induced strongly upon the induction of apoptosis (Figures 5). These findings may be related to the fact that the activities of Ras and c-Jun are essential during neuronal apoptosis (Estus *et al.*, 1994; Ferrari and Greene, 1994), since both Ras and c-Jun can induce cyclin D1 expression (Filmus *et al.*, 1994; Herber *et al.*, 1994). Thus, activation of Ras and c-Jun may be responsible for the induction of cyclin D1 expression during neuronal cell death.

During emergence from quiescence, the cyclin D-dependent kinases are the first cdks (known) to be activated during mid-to-late G_1 (Sherr, 1994). Interference with this activity leads to a G_1 cell cycle arrest, indicating that activation of cyclin D-dependent kinases is essential for G_1 progression (Baldin *et al.*, 1993). The key substrate for cyclin D1-dependent kinases is pRb, as in pRb$^{-/-}$ cells cyclin D1 function is not required (Lukas *et al.*, 1994, 1995a). In addition, the specific cyclin D-dependent kinase inhibitors p16^{INK4A} and p18^{INK4C} only arrest the cell cycle in cells expressing functional pRb (Guan *et al.*, 1995; Hirai *et al.*, 1995; Lukas *et al.*, 1995b). Interestingly, in pRb$^{-/-}$ mice, many neuronal cell types undergo ectopic cell divisions, do not mature properly and undergo apoptotic cell death (Clarke *et al.*, 1993; Jacks *et al.*, 1993; Lee *et al.*, 1993, 1994). Thus, pRb is essential for

establishing cell cycle arrest, for terminal differentiation and for survival of neuronal cell types. We have shown here that the activity of the pRb-inactivating kinase cdk4 is strongly induced during neuronal cell death (Figure 6). Thus, the first cyclin–cdk complex that drives the cell cycle and initiates pRb phosphorylation (inactivation) is activated during neuronal apoptosis. Moreover, we have shown in Figure 7 that specific inhibition of this activity by overexpression of $p16^{INK4A}$ prevents neuronal apoptosis, thereby strongly supporting a model in which activation of the cell cycle in terminally differentiated neurons is essential for the induction of apoptosis.

Materials and methods

Plasmids, cell lines and transfection

pCMV-D1, pCMV-PCNA and pCMV-bcl2 were created by ligating the respective cDNAs into pCMV-neo (Baker *et al.*, 1990). The cDNAs were kindly provided by Drs E.Schuuring (cyclin D1), R.Bravo (PCNA) and S.Korsmeyer (bcl2). pCMV-55K encoding the 55 kDa E1B protein of adenovirus type 5 was described previously (van den Heuvel *et al.*, 1993). pCMV-21K, encoding only the 21 kDa E1B protein of adenovirus type 5, is described by Steegenga *et al.* (1995). pCMV-D1(KE), pCMV-Rb, pCMV-$p16^{INK4}$ and pCMV-cdk4(dn) were kindly provided by Drs S.Dowdy, R.Bernards, D.Beach and S.van den Heuvel respectively. The R970B cell line was provided by Dr W.Steegenga and was established from $p53^{-/-}$ mouse embryo fibroblasts that were provided by Dr T.Jacks. Cultures of post-mitotic neurons were prepared by culturing N1E-115 neuronal precursor cells (de Laat and Van der Saag, 1982) in the presence of 1% dimethyl sulfoxide (DMSO) and 2% FCS for 4 days, after which 80–100% of the cells has undergone morphological differentiation. All transfections were performed essentially as described (van der Eb and Graham, 1980).

Immunofluorescence

Immunofluorescence analysis was performed as described in Buchou *et al.* (1993). Briefly, the cells were grown on glass coverslips and were transfected. At the indicated times, cells were fixed and processed for immunofluorescence. First antibodies were anti-cyclin D (UBI; 06–137), anti-desmin (Boehringer Mannheim) and anti-β-gal (Sigma). Second antibodies were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or rabbit anti-mouse IgG (Jackson Immunoresearch Inc.). Nuclei were stained with PI (1 μg/ml).

Detection of apoptosis by analysis of genomic DNA

Both the adherent and floating cell populations were harvested. Apoptosis was detected by analysis of the integrity of total genomic DNA as described in Lee *et al.* (1994), except that the Southern blots were probed with randomly labelled total mouse genomic DNA.

Detection of apoptosis by combined DNA/cyclin D1 fluorescence

The cells were grown on glass coverslips and were fixed 24, 48 and 72 h after transfection. Anti-cyclin D1 or anti-β-gal antibodies were used as first antibodies and were followed by FITC-conjugated second antibodies to obtain green fluorescence for the transfected proteins. The nuclei were counterstained with PI (1 μg/μl), giving red fluorescence. The nuclei of cyclin D1-positive cells were scored apoptotic if PI staining was lost or if nuclear fragmentation was apparent (see e.g. also Meikrantz *et al.*, 1994; Sgonc and Wick, 1994; Brunner *et al.*, 1995; Dhein *et al.*, 1995). At least 100 cyclin D1-positive nuclei were analysed for each time point. The graphs in Figures 2, 3 and 5 show the means and standard deviations of 3–5 independent experiments.

Detection of cytoplasmic blebbing

Cells were co-transfected with 0.5 μg of pCMV-desmin and 2.5 μg of either pCMV-neo (vector) or pCMV-D1. Twenty-four hours after transfection, the cells were fixed and processed for anti-desmin immunofluorescence. The nuclei were counterstained with PI.

Induction of neuronal apoptosis

Cultures of differentiated neurons were derived from the mouse N1E-115 neuroblastoma cell line as described above. Apoptosis was induced subsequently by serum withdrawal.

Western blotting, immunoprecipitation and cdk4 kinase assay

Western blotting and immunoprecipitation were performed as described in Kranenburg *et al.* (1995). The antibodies used were anti-cyclin D (UBI) and anti-cdk4 (Santa Cruz, C22). The cdk4 kinase assay was performed as described in Matsushime *et al.* (1994).

CAT assays and β-gal assays

Standard procedures were followed to assay CAT and β-gal activities. For CAT assays, 1% of the cell extracts was used. CAT activities at 12, 24 and 48 h after the induction of apoptosis were compared with the activity at time 0. The percentage of conversion in each reaction was determined by phosphorimaging.

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