A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis

Gabriel Gil-Gómez¹, Anton Berns¹ and Hugh J.M.Brady^{2,3,4}

¹Division of Molecular Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, ²Division of Molecular Immunology, The National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA and ³Cancer Biology and Molecular Haematology Units, Camelia Botnar Laboratories, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK

⁴Corresponding author
e-mail: h.brady@ich.ucl.ac.uk

Resting thymocytes undergoing apoptosis in response to specific stimuli degrade the cdk inhibitor p27^{Kip1} and upregulate Cdk2 kinase activity. Inhibition of Cdk2 kinase activity efficiently blocks cell death via certain apoptosis pathways whereas overexpression of Cdk2 accelerates such cell death, suggesting its involvement in the signal transduction pathways activated by certain apoptotic stimuli. We found that Cdk2 activation during thymocyte apoptosis can be regulated by p53, Bax and Bcl-2. The highly elevated Cdk2 kinase activity in the apoptosing thymocytes is not associated with its canonical cyclins, cyclin E and cyclin A, and requires *de novo* synthesis of proteins for activation to take place. We therefore propose Cdk2 activation to be a crucial event in distinct pathways of apoptosis and the point at which the cell cycle and cell death pathways interact.

Keywords: apoptosis/Bax/Bcl-2/Cdk2/cell cycle

Introduction

Apoptosis is a ubiquitous physiological process used to eliminate damaged or unwanted cells in multicellular organisms. For example, apoptosis serves to remove autoreactive or non-functional T cells during their development in the thymus (Suhr and Sprent, 1994). Deregulation of apoptosis is now widely believed to be involved in the pathogenesis of many human diseases (reviewed in Thompson, 1995). The failure of apoptotic cell death has been implicated in various cancers (Lowe *et al.*, 1994; Symonds *et al.*, 1994) as well as autoimmune disorders (Watanabe-Fukunaga *et al.*, 1992; Suda *et al.*, 1993). Conversely, increased apoptosis is associated with a variety of diseases involving cell loss, such as neurodegenerative disorders (Loo *et al.*, 1993; Takashima *et al.*, 1993; Thompson, 1995) and AIDS (Gougeon and Montagnier, 1993). Apoptosis is an active process, regulated by gene products which are conserved from nematodes to mammals and either block or accelerate programmed cell death (reviewed in Penninger and Kroemer, 1998). An understanding of the action of these gene products will provide insights into the process of cell death, and by implication

may have profound consequences for the treatment of many human diseases. Bax and Bcl-2 are two of the gene products that can regulate apoptosis. Bax overexpression has been shown to accelerate cell death in response to certain apoptotic stimuli such as growth factor withdrawal, γ -irradiation or glucocorticoid treatment (Oltvai *et al.*, 1993; Brady *et al.*, 1996a), whereas Bcl-2 can inhibit cell death in such circumstances (Sentman *et al.*, 1991; Strasser *et al.*, 1991). Bax and Bcl-2 can form heterodimers and overexpression of one antagonizes the other's effect (Oltvai *et al.*, 1993). They are part of two classes of Bcl-2-related proteins. One class includes Bcl-2 and Bcl-X_L which can inhibit apoptosis, the other class includes proteins such as Bax, Bak and Bcl-X_S which promote apoptosis (White, 1996).

Like apoptosis, cell division is a fundamental and ubiquitous process in multicellular organisms. The molecules which regulate cell-cycle progression and mitosis, such as the cyclins and cyclin-dependent kinases (cdks) (Norbury and Nurse, 1992; Morgan, 1995), are well characterized. Evidence exists to suggest that the cell cycle and apoptosis may be interconnected (Meikrantz and Schlegel, 1995). For example, expression of the proto-oncogene *c-myc* stimulates cell proliferation and can also predispose cells to apoptosis when growth factors are limiting (Evan *et al.*, 1992). Recent work now indicates that the apoptosis regulatory proteins themselves can directly impinge on the cell-cycle machinery (Brady *et al.*, 1996b; Linette *et al.*, 1996; Mazel *et al.*, 1996; O'Reilly *et al.*, 1996).

We and others (Brady *et al.*, 1996b; Linette *et al.*, 1996; Mazel *et al.*, 1996; O'Reilly *et al.*, 1996) have demonstrated that overexpression of the apoptosis regulatory proteins Bax and Bcl-2 in T cells of transgenic mice leads to perturbations in the dividing population of thymocytes. The effect of the *bax* transgene is to increase the number of cycling thymocytes (Brady *et al.*, 1996b) whereas *bcl-2* has the opposite effect (Brady *et al.*, 1996b; Linette *et al.*, 1996; Mazel *et al.*, 1996; O'Reilly *et al.*, 1996). Furthermore, by studying the interleukin-2 (IL-2)-mediated transition from G₁ to S phase in activated T cells, it was shown that *bcl-2* overexpression delays entry into S phase (Brady *et al.*, 1996b; Linette *et al.*, 1996) whereas *bax* speeds it up (Brady *et al.*, 1996b). This correlates with the levels of p27^{Kip1} in the activated T cells since *bcl-2* overexpression is seen to delay the degradation of p27^{Kip1} whereas *bax* accelerates it. p27^{Kip1} is a negative regulator of cdk activity (Polyak *et al.*, 1994; Totoshima and Hunter, 1994). The cdks in turn are positively regulated by association with cyclins and are required for cell-cycle progression (Norbury and Nurse, 1992; Morgan, 1995; Elledge *et al.*, 1996). Several reports have shown the activation of various Cdks to correlate with apoptosis (Meikrantz *et al.*, 1994; Shi *et al.*, 1994; Li *et al.*, 1995;

Fig. 1. Cdk2 activation during thymocyte apoptosis. (A) Thymocytes from wild-type mice were either treated with 5 Gy of γ -radiation, 2 μ M dexamethasone or untreated then put in culture. Aliquots (5×10^5 cells) were taken at the times indicated and analysed by Western blotting using antibodies against p27^{Kip1}. As a loading control, blots were also probed with a pan-actin antibody. The percentage apoptosis was determined for duplicate aliquots of the thymocytes used in the Western blots. Replicas from the Western blot samples were assayed for Cdk2 kinase activity. (B) Thymocytes from wild-type and *bax* transgenic mice were plated out either without any treatment, after γ -irradiation (5 Gy) or treated with 2 μ M dexamethasone in the presence or absence of 25 μ M roscovitine dissolved in DMSO. Equivalent amounts of DMSO were included in the samples not treated with roscovitine as a control. Aliquots (5×10^5 cells) were taken in duplicate at the times indicated and the percentage of cells in apoptosis determined. The mean values and mean deviation are shown. The experiments were repeated on at least three separate occasions and a representative experiment is shown. (C) Wild-type thymocytes were treated with anti-CD95 antibody (1 μ g/ml) $\pm$ cycloheximide (30 μ g/ml) and plated out in the presence or absence of roscovitine (25 μ M) for an incubation period of 18 h. The experiments were repeated on at least three separate occasions and a representative experiment is shown. (D) Exponentially growing neo clone B1 and Cdk2 clone 6 were rendered quiescent as described in Materials and methods and plated out either with no treatment (Control), after complete IL-3 withdrawal, after treatment with 5 Gy of γ -radiation or in the presence of 50 μ M etoposide. Except in the case of IL-3 withdrawal-induced apoptosis, the BaF3 clones were incubated in starvation medium during the apoptosis time courses. Aliquots (5×10^5 cells) were withdrawn in duplicate from the cultures at the times indicated and apoptosis levels measured as indicated in Materials and methods. Similar results were obtained on at least four separate occasions and a representative single-cell derived BaF3 clones with similar expression levels of the transfected Cdk2 protein behaved similarly to the clone shown (data not shown).

Wei *et al.*, 1997; Zhang *et al.*, 1997; Levkau *et al.*, 1998). It has been shown that dominant negative alleles of *Cdk2* can alleviate tumour necrosis factor- α (TNF α)- and staurosporine-induced apoptosis in HeLa cells (Meikrantz and Schlegel, 1996) and more recently, apoptosis induced by growth factor withdrawal from human umbilical vein endothelial cells (Levkau *et al.*, 1998). It has been suggested that it is the association with cyclin A in these situations which upregulates Cdk2 activity.

Given the data suggesting a connection between Bax/Bcl-2 and the Cdk2 inhibitor p27^{Kip1}, we examined whether Bax and Bcl-2 could modulate Cdk2 activity during

thymocyte apoptosis. We demonstrate a linear relationship between p27^{Kip1} degradation, Cdk2 activation and thymocyte apoptosis which is modulated by p53, Bax and Bcl-2. Furthermore, we show that inhibitors of Cdk2 activity can block various apoptotic pathways although not the pathway regulated by CD95 and, conversely, that Cdk2 overexpression directly accelerates apoptosis in response to specific stimuli. Finally, we also show that the increased Cdk2 kinase activity in dying thymocytes integrates two different signal transduction pathways, does not seem to be mediated by either cyclin E or cyclin A and requires *de novo* protein synthesis. We propose that Cdk2 activation is a crucial

event in distinct pathways of apoptosis and the point at which the cell cycle and cell death pathways interact.

Results

Cdk2 activation is necessary for thymocyte apoptosis

Recent reports have implicated the cell-cycle regulatory protein Cdk2 in apoptosis. In these studies the study of the significance of Cdk2 activation for apoptosis is complicated by the fact that the model cells are either proliferating (Meikrantz and Schlegel, 1996) or undergoing apoptosis as a consequence of growth factor withdrawal (Levkau *et al.*, 1998). In both cases, the cells examined contain large amounts of Cdk2 activity related to normal cell-cycle progression which cannot be distinguished from Cdk2 activity related to apoptosis. Therefore, we have focused on the involvement of Cdk2 in thymocyte apoptosis. Thymocytes from normal mice are a naturally occurring synchronized population since ~90% of them are quiescent, whereas only ~10% are cycling (Huesmann *et al.*, 1991), which makes it possible to separate Cdk2 activity as a regulator of cell-cycle progression from Cdk2 activity involved in apoptosis regulation. Thymocytes were removed from wild-type mice and cultured *in vitro* in medium alone, exposed to γ -irradiation or treated with the glucocorticoid dexamethasone. To determine the level of its activity, Cdk2 was immunoprecipitated from equivalent numbers of thymocytes and then co-incubated with histone H1 and [γ - 32 P]ATP (Kranenburg *et al.*, 1995). Cdk2 phosphorylates histone H1 and the amount of 32 P incorporated into the histone serves as a measure of Cdk2 kinase activity. The level of histone H1 phosphorylation following immunoprecipitation of Cdk2 from the dying thymocytes increased with time when the cells were kept in culture alone. The level of Cdk2 kinase activity increased even more dramatically following an apoptotic stimulus. The percentage of apoptotic cells present during the process, as determined by a flow cytometry-based technique (Nicoletti *et al.*, 1991), correlated with the elevation in Cdk2 kinase activity for all the stimuli tested (Figure 1A). The amount of antibody used in the immunoprecipitations was sufficient to allow the recovery of Cdk2 kinase activities in proliferating cells several fold higher than those measured in apoptotic thymocytes (e.g. see Figure 6) thus showing that the amounts of antibody used in the immunoprecipitations were saturating.

Roscovitin was developed as a competitive analogue of ATP which selectively inhibits cdk kinase activity with specificity for Cdk1 (Cdc2), Cdk2 and Cdk5 (Meijer, 1996; Meijer *et al.*, 1997). We analysed the consequences of incubation with roscovitin for thymocyte apoptosis. Thymocyte suspensions from wild-type and *bax* transgenic mice were treated as above with γ -radiation as well as dexamethasone, in the presence of 25 μ M roscovitin or an equivalent volume of dimethyl sulfoxide (DMSO). As shown in Figure 1B, roscovitin was able to block both p53-independent (dexamethasone) and p53-dependent (γ -radiation and etoposide; data not shown) apoptosis in thymocytes from wild-type mice. Roscovitin was also able to inhibit *bax*-accelerated apoptosis in transgenic thymocytes as well as the residual apoptosis measured in cultures of *bcl-2* transgenic thymocytes triggered to die

with γ -radiation or dexamethasone (data not shown). However, not all apoptotic stimuli were blocked by roscovitin. CD95 (Fas/APO-1)-induced apoptosis in thymocytes was insensitive to the presence of roscovitin, with or without cycloheximide (Figure 1C). Cdk2 kinase activity was unchanged following CD95-induced apoptosis in thymocytes (data not shown). CD95-induced apoptosis does not require protein synthesis and is accentuated by the presence of cycloheximide (Ogasawara *et al.*, 1995). The lack of inhibition by roscovitin on CD95-induced apoptosis is consistent with other data, including our own, that show Bax/Bcl-2-mediated and CD95-induced apoptosis of primary T cells to be separate and distinct pathways (Strasser *et al.*, 1995; Brady *et al.*, 1996b).

Roscovitin can also inhibit Cdk5 and Cdk1 activity (Meijer *et al.*, 1997). However, Cdk5 kinase activity is not present in thymocytes, being restricted to neuronal cells (Tsai *et al.*, 1993). We also assayed Cdk1 activity and found that, as described previously by others (Norbury *et al.*, 1994), its activity declines during apoptosis of thymocytes as the proliferative fraction of thymocytes initially present disappears (data not shown). Therefore, it appears that it is the ability of roscovitin to inhibit Cdk2 activity which permits its use to inhibit certain apoptotic stimuli. We have reproduced these effects on thymocyte apoptosis with other Cdk2 inhibitors (data not shown), such as flavopiridol (de Azevedo *et al.*, 1996), butyrolactone I (Kitagawa *et al.*, 1993) and olomucine (Meijer, 1996), which in the case of flavopiridol and butyrolactone do not share structural homology with roscovitin (Meijer, 1996). Cdk2 inhibitors have been described as apoptosis-inducing drugs in proliferating cell lines (van Engeland *et al.*, 1997; Schutte *et al.*, 1997). Under these conditions, Cdk2 inhibition leads to a cell-cycle block followed by apoptosis. These observations clearly show that the effect of Cdk2 inhibitors on cell death is dependent on the cell-cycle status of the target cell. We have been able to reproduce the apoptosis-inducing properties of Cdk2 inhibitors in the proliferating pro-B cell line BaF3 and recapitulate their apoptosis protecting effects, as seen in thymocytes, after rendering BaF3 cells quiescent by IL-3 deprivation (described below for Figure 1D; data not shown).

In order to prove that Cdk2 activation can lead to apoptosis, we generated stable transfectants expressing Cdk2 wild-type protein in IL-3-dependent BaF3 cells. Since all our observations have been made using thymocytes which are essentially a resting cell population, we looked for conditions suitable to render the BaF3 cells quiescent prior to inducing apoptosis. We titrated down the amount of IL-3 necessary to arrest the BaF3 cells without allowing them to initiate the apoptotic programme. We then found the concentration of IL-3-conditioned medium necessary to have at least 90% of a BaF3 culture in G₀/G₁ but <10% undergoing apoptosis after 15 h in low IL-3. Exponentially growing cultures of control cells and transfected BaF3 clones, as described below, were exhaustively washed and plated out in low IL-3 (starving medium) before triggering them with the apoptotic stimuli.

We generated stably transfected BaF3 clones constitutively expressing wild-type Cdk2 and studied the kinetics of apoptosis induction after IL-3 withdrawal, γ -irradiation and etoposide treatment. The levels of expression of the

Fig. 2. p27^{Kip1} levels during apoptosis. *bax* (A) and *bcl-2* (B) transgenic thymocytes and thymocytes from wild-type littermate controls were treated with 5 Gy of γ -radiation then put in culture. Aliquots (5×10^5 cells) were taken at the times indicated and analysed by Western blotting using antibodies against p27^{Kip1}. As a loading control, blots were also probed with a pan-actin antibody. The percentage apoptosis was determined for duplicate aliquots of the thymocytes used in the Western blots.

wild-type human Cdk2 were comparable to the levels of endogenous mouse Cdk2 present in BaF3 cells (data not shown). As shown in Figure 1D, overexpression of the wild-type Cdk2 leads to a dramatic acceleration in the kinetics of apoptosis appearance in the cultures for both p53-independent (spontaneous apoptosis and IL-3 deprivation) and p53-dependent stimuli (γ -radiation and etoposide treatment). Similar results were obtained with several independent clones expressing comparable levels of Cdk2 (data not shown). The data from the BaF3 transfectants clearly illustrate that elevated Cdk2 activity alone is sufficient to accelerate apoptosis in resting cells.

Bax and Bcl-2 levels influence p27^{Kip1} degradation in thymocytes triggered to die

In activated T cells *bcl-2* overexpression is seen to delay the degradation of p27^{Kip1} whereas *bax* accelerates it (Brady *et al.*, 1996b). Given the apoptosis regulatory role of Bax and Bcl-2, we examined whether they could also regulate p27^{Kip1} levels in a population of cells triggered to die. As thymocytes are virtually all resting, the level of p27^{Kip1} in the total thymocyte population is very high and allowed us to follow the changes in its levels upon triggering of apoptosis. Western blot analysis of wild-type and *bax* transgenic thymocytes following γ -irradiation shows that there is a time-dependent degradation of p27^{Kip1} (Figure 2A). As previously seen with p27^{Kip1} levels during the IL-2-driven G₁→S progression in activated T cells (Brady *et al.*, 1996b), *bax* overexpression in thymocytes accelerates p27^{Kip1} degradation (Figure 2A) while the opposite is seen for *bcl-2* (Figure 2B). This result is surprising since p27^{Kip1} acts as a cell-cycle brake which needs to be degraded to bring cells into S phase, and given the fact that ~90% of the initial population of thymocytes are in the G₀/G₁ state and do not enter S phase, during apoptosis (Pellicciari *et al.*, 1996; data not shown). This suggests that p27^{Kip1} not only functions as a cell-cycle regulator but also controls apoptotic processes.

Cdk2 is activated in apoptotic thymocytes in a Bax- and Bcl-2-regulated manner

p27^{Kip1} is a negative regulator of the activity of cdks (Polyak *et al.*, 1994; Totoshima and Hunter, 1994), notably

Fig. 3. Cdk2 kinase activity during apoptosis. Thymocytes from wild-type mice (5×10^5 cells/time point), *bax* (A) and *bcl-2* (B) transgenic mice were plated out either without any treatment, after γ -irradiation (5 Gy) or in the presence of 2 μ M dexamethasone. The numbers at the bottom of each line indicate the percentage of apoptotic cells at the corresponding time point. As a positive control (BaF), 2×10^5 exponentially growing BaF3 cells were processed in the same way as the thymocyte samples for Cdk2 kinase assay. As a negative control (BaF + Block), a similar amount of cell extract was immunoprecipitated in the presence of an excess of the M2 peptide. All the time courses were repeated on three separate occasions and a representative experiment is shown. The relative intensity of the radioactive bands between two separate treatments can vary slightly due to different autoradiographic exposure times.

Cdk2. We therefore examined whether Bax and Bcl-2 could modulate Cdk2 activity during thymocyte apoptosis. As we have reported previously (Brady *et al.*, 1996a), thymocytes from *bax* transgenic mice are more susceptible to die after triggering with p53-dependent (such as γ -irradiation) or p53-independent (such as dexamethasone) apoptotic stimuli. Measurement of the Cdk2 kinase activity in the conditions described above showed that Bax-accelerated apoptosis correlated with the earlier appearance of Cdk2 kinase activity (Figure 3A). Conversely, overexpression of the survival gene *bcl-2* in transgenic thymocytes led to resistance to apoptotic stimuli and to a delayed rise in Cdk2 kinase activity (Figure 3B). As a positive control (BaF), exponentially growing BaF3 cells were processed in the same way as the thymocyte samples for Cdk2 kinase assay. As a negative control (BaF + Block), a similar amount of cell extract was immunoprecipitated in the presence of an excess of a blocking peptide for the Cdk2 antibody. These results show that Cdk2 activation during apoptosis is a highly regulated process under the control of known apoptosis regulators such as Bax and Bcl-2.

p53 is upstream of Cdk2 activation during apoptosis

In order to establish firmly the correlation between Cdk2 activation and apoptosis, we carried out similar experiments as described above using thymocytes from p53^{+/-} and p53^{-/-} mice. Thymocytes from p53^{-/-} mice are highly resistant to genotoxic agents such as γ -radiation and chemotherapeutic drugs, while remaining fully sensitive to dexamethasone-induced apoptosis (Clarke *et al.*, 1993; Lowe *et al.*, 1993). As in wild-type thymocytes (Figures 1A and 2), there is a time-dependent degradation of p27^{Kip1} and an increase in Cdk2 kinase activity in thymocytes from p53^{+/-} mice triggered to die by γ -radiation or dexamethasone treatment, which correlates with the number of apoptotic cells (Figure 4). As expected, there is no difference in the susceptibility of either p53^{-/-} or p53^{+/-} thymocytes to dexamethasone-induced cell death in terms of cells undergoing apoptosis and this correlates with the kinetics of p27^{Kip1} degradation and Cdk2 induction. Whereas p53^{+/-} thymocytes behave comparably to wild-type thymocytes in γ -radiation-induced apoptosis, p53^{-/-} thymocytes do not show increases in cell death, p27^{Kip1} is not degraded and Cdk2 kinase activity does not rise during the same time course (Figure 4). Cdk2 activity does begin to appear by the 10 h time point of p53^{-/-} γ -irradiated thymocytes as by then even p53^{-/-} thymocytes are beginning to apoptose. From these results we conclude that there is a correlation between the levels of both p53-dependent and p53-independent apoptosis in thymocytes and the activation of Cdk2 kinase activity. These results also prove that Cdk2 activation during apoptosis is downstream of the action of p53, consistent with the current view that p53 is involved in the sensing of DNA damage induced by γ -radiation and in triggering DNA repair mechanisms or apoptosis depending on the severity of the damage (reviewed in Cox and Lane, 1995).

Cdk2 activation during apoptosis results from changes in its specific activity and is not associated with cyclins A or E

Biochemically, the changes in Cdk2 activity measured during apoptosis might be due to an increase in the levels of Cdk2 or an increased specific activity of the protein. In order to distinguish between these two possibilities, we measured the abundance of Cdk2 during the apoptosis time course by Western blot analysis. The amount of Cdk2 protein present did not change appreciably during the apoptosis of γ -irradiated *bax* transgenic thymocytes which

give rise to high levels of Cdk2 kinase activity (Figure 5A). In contrast, Cdk2 kinase activity increased dramatically during the same time course (Figure 3). These results demonstrate that it is the specific activity of Cdk2 rather than its abundance which changes during apoptosis. The relative abundance of the two Cdk2 bands detected by the anti-Cdk2 antibody does not change during the apoptosis time course suggesting that the phosphorylation state of Cdk2 does not contribute to its effect on apoptosis. This is in agreement with the results of Levkau *et al.* (1998) which also detect no change in Cdk phosphorylation status during apoptosis.

Fig. 5. Cdk2 specific activity increases during apoptosis. (A) Cdk2 levels during apoptosis. *bax* transgenic thymocytes and thymocytes from wild-type littermate controls were treated with 5 Gy of γ -radiation then put in culture. Aliquots (5×10^5 cells) were taken at the times indicated and analysed by Western blotting using antibodies against Cdk2. As a loading control blots were also probed with a pan-actin antibody. Exponentially growing BaF3 cells (5×10^5) were used as a positive control. The percentage apoptosis was determined for duplicate aliquots of the thymocytes used in the Western blots. (B) Cell-cycle regulatory proteins during apoptosis. Thymocytes from wild-type mice were either treated with 5 Gy of γ -radiation, 2 μ M dexamethasone or untreated then put in culture. Aliquots (2×10^6 cells) were taken at the times indicated and analysed by Western blotting using antibodies against the indicated proteins. The percentage apoptosis was determined for duplicate aliquots of the thymocytes used in the Western blots. As proliferating cell controls, 10⁶ day 2 concanavalin A-stimulated mouse splenocytes were processed (ConA).

Fig. 4. p53 control of Cdk2 kinase activation during apoptosis. Thymocytes from p53^{+/-} and p53^{-/-} mice (5×10^5 cells/time point) were plated out after γ -irradiation (5 Gy) or in the presence of 2 μ M dexamethasone. Samples were taken at the time points indicated and processed for Western blotting with anti-p27^{Kip1} antibodies or Cdk2 kinase assay as described in Materials and methods. The numbers at the bottom of each line indicate the percentage of apoptotic cells at the corresponding time point. All the time courses were repeated three times and a representative experiment is shown.

During the cell cycle, Cdk2 activity is regulated by association with a cyclin subunit, namely cyclin E, then cyclin A (reviewed in Norbury and Nurse, 1992; Sherr, 1993). We investigated whether the Cdk2 kinase activity present in apoptosing thymocytes was associated with either cyclin. We first measured the levels of different cell-cycle regulators during thymocyte apoptosis. The levels of both cyclin E and cyclin A were low, as expected in a mostly resting cell population, and declined in time (Figure 5B), correlating with the disappearance of the proliferative fraction of thymocytes (data not shown). As shown before, p27^{Kip1} levels decreased as thymocytes underwent apoptosis. Actin levels were measured as loading controls and shown to be constant throughout the time course. D-type cyclins can also bind Cdk2, although only D2-Cdk2 complexes appeared to be active (Ewen *et al.*, 1993). Cyclin D3 levels turned out to be dramatically downregulated quickly after inducing apoptosis in thymocytes (Figure 5B). Similar results were obtained for cyclin D2 (data not shown). These results can be explained by the strict dependence of D-type cyclins on growth factors for expression (reviewed in Sherr, 1995). Thus, once the thymocytes have been removed from their normal thymic environment where the appropriate growth factors are likely to be present, they would immediately downregulate cyclin D expression. If cyclin D2 were responsible for Cdk2 activation during apoptosis then anti-cyclin D2 immunoprecipitates from apoptotic thymocytes should show histone H1 kinase activity. However, in agreement with the expression data, anti-cyclin D2 immunoprecipitates from apoptotic thymocytes did not show measurable histone H1 kinase activity (data not shown), indicating that cyclin D2 is not responsible for Cdk2 activation during apoptosis in thymocytes.

In order to investigate further the putative role of cyclins E- and A-associated kinase activity during apoptosis, the kinetics of the kinase activity measured after immunoprecipitation of cyclin E and A were compared with the corresponding kinetics measured after immunoprecipitation of Cdk2 (Figure 6A). Both wild-type and *bax* transgenic thymocytes (which show higher levels of Cdk2 kinase activity during apoptosis) was processed. Cyclin E immunoprecipitations lead to the recovery of low kinase activities in agreement with the very low levels of cyclin E present (Figure 5B). Cyclin A-associated kinase activities yielded slightly higher values at t_0 that gradually declined to background levels after 6 h. In order to rule out any possible interference of the anti-cyclin A antibody with the kinase activity, the experiment was done with two different antibodies: a polyclonal serum directed against the whole human protein (H-432, Santa Cruz Biotech) and a polyclonal serum directed against a C-terminal epitope (C-19, Santa Cruz Biotech) which both produced identical results. Both antibodies immunoprecipitated high kinase activities from proliferating cells (Figure 6B; data not shown). The differences in the kinetics measured suggest that cyclin E and A are not responsible for Cdk2 activation during the apoptotic process. The cyclin A-associated kinase activity at t_0 is very probably due to the small fraction of proliferating thymocytes present at that time, progressively disappearing as they undergo apoptosis. Quantification of the ^{32}P incorporated into histone H1 showed that the kinase

Fig. 6. Cyclin E- and A-associated kinase activity during apoptosis. (A) Wild-type and *bax* transgenic thymocytes were treated with 5 Gy of γ -radiation. Aliquots (5×10^5 cells) were withdrawn at the times indicated and processed for histone H1 kinase activity after immunoprecipitation of cyclin A or cyclin E. Radioactive bands were excised from the gel and counted by liquid scintillation. These experiments were repeated on several separate occasions and a representative experiment is shown. As controls, 5×10^5 exponentially growing BaF3 cells were processed similarly for cyclin A- and E-associated kinase activity and compared with histone H1 kinase activity measured after Cdk2 immunoprecipitation (B). For comparison, the ^{32}P counts of cyclin A- and E-associated kinase activities in wild-type (WT) and *bax* transgenic (*bax*) thymocytes at t_0 are shown.

activity measured after immunoprecipitation of Cdk2 rose rapidly and was sustained at elevated levels after triggering the thymocytes to die by γ -irradiation. This was in contrast to the low levels of kinase activity recovered following cyclin E or cyclin A immunoprecipitation from replicas of the same lysates. Increasing the amounts of antibodies used for cyclin E and A immunoprecipitations did not increase the yields of kinase activity recovered. Kinase activities as measured after immunoprecipitation of cyclin E, cyclin A or Cdk2 from proliferating cell extracts (BaF3 cells) were used as controls in these experiments, showing consistently high kinase activities in each case (Figure 6B).

p27^{Kip1} degradation alone is not enough to activate Cdk2

Thymocytes from p27^{Kip1}^{-/-} mice do not show perturbations in apoptosis induced by γ -radiation and dexamethasone (Kiyokawa *et al.*, 1996), suggesting that p27^{Kip1} loss does not lead to a constitutive activation of the apoptotic pathway. However, Cdk2 kinase activity is upregulated by apoptotic stimuli in p27^{Kip1}^{-/-} thymocytes

Fig. 7. Protein synthesis dependence of p27^{Kip1} degradation and Cdk2 activation during thymocyte apoptosis. Thymocytes from wild-type mice were plated out either without any treatment (control), after γ -irradiation (5 Gy) or in the presence of 2 μ M dexamethasone, then incubated in the presence or absence of 30 μ g/ml of cycloheximide (CHX). Samples were taken at the indicated time points and processed for Western blotting with anti-p27^{Kip1} and pan-actin antibodies or Cdk2 kinase assay as described in Materials and methods. The numbers at the bottom of each line indicate the percentage of apoptotic cells at the corresponding time point. All the time courses were repeated twice and a representative experiment is shown.

(G.Gil-Gómez, A.Berns and H.J.M.Brady, manuscript in preparation). In order to try to separate p27^{Kip1} degradation from Cdk2 activation, we studied their behaviour following the triggering of apoptosis in the absence of protein synthesis. γ -radiation and dexamethasone-induced apoptosis are absolutely dependent on *de novo* synthesis of proteins (Cohen and Duke, 1984; Sellins and Cohen, 1987). As shown in Figure 7, addition of cycloheximide completely blocks γ -radiation and dexamethasone-induced apoptosis of thymocytes. However, under these conditions, p27^{Kip1} levels decline with the same kinetics as in the presence of *de novo* protein synthesis, showing that its degradation is protein synthesis-independent. The increase in Cdk2 activity is totally abolished. These results show that, in agreement with the phenotype of the knockout mice, p27^{Kip1} loss on its own does not lead to upregulation of Cdk2 activity concomitant with apoptosis. *De novo* synthesized proteins are needed in order to render the Cdk2 complexes active.

Discussion

Apoptosis shares biochemical machinery with cell cycle

Apoptosis has been described as an abortive entry into cell cycle, given the morphological similarities between apoptosis and mitosis (Shi *et al.*, 1994). A number of studies have associated molecules that are clearly linked to cell proliferation, such as Myc (Evan *et al.*, 1992) or E2F-1 (Shan and Lee, 1994), with apoptosis. Other studies have implied that the G₁→S transition of the cell cycle is the most susceptible point for cells to implement a death programme (Meikrantz and Schlegel, 1995). However, it is also clear that resting cells such as thymocytes and post-mitotic cells such as neurons are able to undergo apoptosis very efficiently. We sought to examine the link between cell cycle and apoptosis from the perspective of what happens in the resting cell undergoing programmed cell death. Our data demonstrate that resting thymocytes triggered to die activate biochemical machinery that is shared with the normal cell cycle, i.e. Cdk2 and p27^{Kip1}. However, our data do not support the view of apoptosis being an abortive cell cycle where a highly ordered rise and fall in the level of cyclins (with concomitant changes in cdk activity) takes place. We have not found the distinctive changes in cyclin levels that are indicative for progression from G₀ through G₁ into S phase where a putative 'apoptosis restriction point' has been proposed to

exist (Meikrantz and Schlegel, 1995). Neither is there any indication of DNA synthesis in apoptosing thymocytes as evinced by the appearance of hyperdiploid DNA (data not shown).

Bax and Bcl-2 modulation of Cdk2 activity regulates apoptosis

The fact that Bax and Bcl-2 are able to modulate the levels of p27^{Kip1} and thus Cdk2 activity, identifies both p27^{Kip1} and Cdk2 as downstream components of a common biochemical pathway leading to apoptosis. We have used two independent approaches to verify the requirement for Cdk2 activation in thymocyte apoptosis. In the first approach we inhibited Cdk2 activity by roscovitine. In the second approach, we generated stable transfectants overexpressing wild-type Cdk2. By this means we manipulated Cdk2 activity leading to alterations in the kinetics of cell death. Both approaches led independently to the conclusion that Cdk2 activation is required for thymocyte apoptosis. The concept that Cdk2 kinase activity is involved in apoptosis is in agreement with recently published data on cell death induced by growth factor deprivation in human umbilical vein endothelial cells (Levkau *et al.*, 1998). Our system has the in-built advantage that, given the virtual synchrony of the thymocyte population in the G₀/G₁ phase of the cell cycle, we can unequivocally examine the requirement for proteins normally associated with cell-cycle progression to be involved in apoptosis. Hence, our results suggest that we can rule out the participation of cyclin E and cyclin A in thymocyte apoptosis. In the endothelial cell model, the cells deprived of growth factors show high levels of cyclins A and E, which may be because the cells were not completely quiescent or, more probably, the result of heterogeneity in the cell population (hence cells undergoing apoptosis from different phases of the cell cycle).

The pathway leading to Cdk2 activation is distinct from that utilized by CD95-induced apoptosis. We and others have previously shown that Bax and Bcl-2 do not influence CD95-induced apoptosis of T cells (Strasser *et al.*, 1995; Brady *et al.*, 1996a). Here we show that the Cdk2 inhibitor, roscovitine, can inhibit Bax/Bcl-2-influenced apoptosis such as that induced by dexamethasone or γ -radiation but not by anti-CD95. This correlates with our previous data and implies that Cdk2 activation is not required in the CD95 death pathway in thymocytes. CD95-induced apoptosis signals through Caspase activation (reviewed in Nagata, 1997). Since Cdk2 inhibitors are not able to block

CD95-induced apoptosis, this positions Cdk2 activation upstream of the activation of the executioner caspases.

Role of cyclin-dependent kinase inhibitors in apoptosis regulation

Recent data in an endothelial cell model have also suggested a role for p27^{Kip1} and p21^{Cip1} in apoptosis (Levkau *et al.*, 1998). However, p21^{Cip1} levels are very low in thymocytes and T cells, as they are in normal differentiated adult tissues (MacLeod *et al.*, 1995; Parker *et al.*, 1995; Wu *et al.*, 1996; our results). Furthermore, given the fact that p21^{Cip1} is transcriptionally regulated by p53 (El-Diery *et al.*, 1993), the level of p21^{Cip1} would be predicted to rise during p53-dependent apoptosis rather than decline, resulting in Cdk2 inhibition rather than activation. p21^{Cip1} appears not to have a crucial role in thymocyte apoptosis, though it may well have such a function in other cell types, perhaps synergizing with or replacing p27^{Kip1}.

p53 action is necessary to trigger p27^{Kip1} degradation after γ -radiation (Figure 4). Both γ -radiation and dexamethasone treatment trigger p27^{Kip1} degradation in the presence of cycloheximide (Figure 7) which suggests that protein synthesis is not required for sensing the apoptotic stimuli. It also suggests that p27^{Kip1} degradation is a function of both p53 and the glucocorticoid receptor which is independent of their actions as transcriptional activators. Some of the apoptosis-inducing actions of p53 seem to be independent of its transcription-activating properties (Caelles *et al.*, 1994). In the case of the glucocorticoid receptor, it has been shown that its transrepression function may be even more important than its action as a transcriptional activator for glucocorticoid-induced apoptosis (Helmberg *et al.*, 1995). This transrepression function does not require *de novo* synthesis of proteins and could be involved in the repression of the transcription of a notional short-lived inhibitor of p27^{Kip1} degradation. In the presence of dexamethasone and cycloheximide, the synthesis of this factor would be impaired and p27^{Kip1} degradation would take place normally.

Cdk2 kinase activity may be regulated by a novel apoptosis-related Cdk2 activating subunit

The cycloheximide experiments together with other data from p27^{Kip1}^{-/-} mice indicate that p27^{Kip1} degradation is not enough on its own to activate Cdk2. A newly synthesized factor(s) is necessary to allow Cdk2 to be activated and the apoptotic signal to be propagated. Time-course experiments show that both roscovitine and cycloheximide lose their ability to block apoptosis when added later than 3 h after triggering apoptosis, which is approximately the time at which Cdk2 activity starts to be detected by the kinase assay (data not shown). A Cdk2 inhibitor being unable to block apoptosis when added later than 3 h is in agreement with Cdk2 being a step in the signal transduction pathway of the apoptotic stimuli and thus, once the signal has passed this point, blocking Cdk2 cannot inhibit apoptosis any longer.

The elevation in Cdk2 kinase activity during thymocyte apoptosis does not seem to require the interaction between Cdk2 and its canonical cyclin partners, E and A. According to the phenotype of the knockout mice (Kiyokawa *et al.*, 1996), p27^{Kip1} loss is not enough on its own to lead to full Cdk2 activation; *de novo* synthesis of proteins is also

necessary. Since Cdk2 requires a cyclin partner to have kinase activity (Norbury and Nurse, 1992; Morgan, 1995), our findings could be explained by postulating the presence of a *de novo* synthesized and apoptosis-specific Cdk2 activating subunit in dying thymocytes. This hypothesis would also explain how Cdk2 activation influences the downstream apoptosis machinery. The new regulatory subunit could alter the substrate specificity of Cdk2, as do cyclins E and A, and allow it to act on targets that promote cell death rather than cell-cycle progression (Peeper *et al.*, 1993).

A future task will be to determine what is downstream of the activated Cdk2 in the apoptotic pathway. One possibility to examine is whether Cdk2 kinase activity can cause direct activation of the caspases. Another possibility is that Cdk2 could be part of a feedback mechanism to phosphorylate Bcl-2 or Bax leading to modulation of their function in the mitochondrial membranes regulating cytochrome *c* release to the cytosol (reviewed in Reed, 1997). Cdk2 could turn out to be an attractive target for pharmaceutical intervention to inhibit pathological processes resulting from accelerated apoptosis such as neurodegeneration following trauma leading to the loss of postmitotic neurons or cell death following reperfusion injury (reviewed in Thompson, 1995). The potential is that roscovitine or compounds acting in a similar way could be used as pharmaceutical analogues of Bcl-2 in particular, in tissues where the majority of affected cells are quiescent.

Materials and methods

Apoptosis assay

The assays measuring the percentage of apoptotic thymocytes were performed as described previously (Brady *et al.*, 1996a,b). Thymocytes were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 50 μ M 2-mercaptoethanol. Flow-cytometric quantitation of apoptotic cells was as described in Nicoletti *et al.* (1991). The anti-CD95 antibody was Jo2 (PharMingen). Roscovitine was dissolved in DMSO prior to use and was a gift from Dr Laurent Meijer, CNRS Roscoff, France. BaF3 cells (Palacios and Steinmetz, 1985) were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 μ M 2-mercaptoethanol and 1% X63 IL-3-conditioned medium (gift from A. Strasser, WEHI, Melbourne). Single-cell transfected clones were made quiescent by titrating the amount of X63 IL-3-conditioned medium. A concentration of conditioned medium was used (designated starvation medium) that rendered 90% of the cells in G₀/G₁ phase of the cell cycle after 15 h of culture and showed <10% apoptotic cells.

Immunoblotting

Cell lysates (5 $\times$ 10⁵ cells per lane) were immunoblotted using enhanced chemiluminescence (Amersham) for detection essentially as described previously (Brady *et al.*, 1996b). The anti-p27^{Kip1} (C-19), cyclin E (M20), cyclin A (H-432 and C-19), cyclin D3 (C-16) and Cdk2 (M2) antibodies were purchased from Santa Cruz Biotech and the pan-actin antibody (C4) from Boehringer Mannheim.

Kinase assays

For the determination of kinase activity, an equivalent number of thymocytes (5 $\times$ 10⁵ viable cells at *t*₀) were pelleted and stored at -80°C until assayed. Kinase activity was assayed by immunoprecipitation of the kinase followed by incubation with histone H1, and [γ -³²P]ATP. The immunoprecipitations and histone H1 kinase assays were performed exactly as described (Kranenburg *et al.*, 1995). The M2 antibody (Santa Cruz) was used for Cdk2 and the A17 antibody (PharMingen) for Cdk1 immunoprecipitations. The kinase assays were resolved on a 12% SDS-polyacrylamide gel, stained with Coomassie Blue to visualize the histone H1 bands, dried and exposed for autoradiography. Where required for quantitation, the histone H1 bands were excised and counted on a liquid

scintillation beta counter. Cyclin E- and A-associated kinase activity was assessed following immunoprecipitation with either the H-432 or C-19 antibody for cyclin A and M-20 antibody for cyclin E (all from Santa Cruz Biotech).

Stable transfections

Exponentially growing BaF3 cells were transfected by electroporation as described (Van der Hoff *et al.*, 1992). The expression vector used was pRcCMV (Invitrogen Corporation, CA, USA). G418 selection (1 mg/ml) was started 24 h after electroporation by plating out the transfected cells in selection medium at a concentration of 0.5 G418 resistant clones per well in 96-well plates (assuming an efficiency of one G418 resistant clone per 1000 starting cells) to get single-cell clones. Single-cell clones were picked and expanded for further analysis by Western blotting. Clones expressing the highest levels of transfected protein were chosen for further analysis.

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