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T cells from *baxα* transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53

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Baxα was isolated due to its interaction with Bcl-2. Baxα overexpression in an interleukin (IL)-3 dependent cell line accelerates apoptosis upon removal of the cytokine. The ratio of Baxα to Bcl-2 appears to be crucial for the effect. To study the action of the *bax* gene product *in vivo*, we have generated transgenic mice overexpressing Baxα specifically in T cells. Such T cells show accelerated apoptosis in response to γ-radiation, dexamethasone and etoposide. By crossing *baxα* mice with *bcl-2* transgenics we show that the critical nature of the Baxα:Bcl-2 ratio holds in primary T cells and that it can be manipulated to elicit a strong response to previously resisted stimuli. p53 has a role in the regulation of apoptosis in response to DNA-damaging agents. p53 directly activates transcription of the *bax* gene. The presence of the *baxα* transgene accelerated apoptosis in thymocytes from both p53–/– and p53+/– mice in response to dexamethasone. Thymocytes from p53–/– mice with the *baxα* transgene showed similar resistance to apoptosis by DNA-damaging agents as did p53–/– mice without the transgene. Baxα overexpression alone cannot restore the DNA damage apoptosis pathway, suggesting that p53 is required to induce or activate other factor(s) to reconstitute the response fully.

Keywords: apoptosis/Bax/Bcl-2/p53/transgenic

Introduction

The homeostatic regulation of cell populations is an essential requirement of the developing embryo as well as of adult tissues. This regulation is controlled by the balance between proliferation, growth arrest and programmed cell death (apoptosis) (Williams, 1991; Evan *et al.*, 1992). During apoptosis, cells are eliminated in a highly characteristic fashion. The cells display a marked shrinkage, membrane blebbing, chromatin condensation and eventual internucleosomal DNA cleavage (Wyllie, 1980). Lymphocytes, in particular, are highly susceptible to apoptosis and this is very evident during early stages of their development (Cohen *et al.*, 1992; Krammer *et al.*, 1994). The reason for the particular susceptibility of lymphocytes is unknown, but it has been suggested that since they have a high intrinsic potential for mutation and clonal expansion, it may be advantageous to the organism to kill off lymphocytes that incur such damage (Strasser *et al.*, 1994). Apoptosis of immature T cells and T cell hybridomas can be induced readily by DNA-damaging agents such as ionizing radiation (Umansky, 1991) and etoposide (Clarke *et al.*, 1993) as well as glucocorticoids (Wyllie, 1980) and various agents that induce activation (Green and Scott, 1994). Much effort is now being expended to elucidate the genetic and biochemical mechanisms of apoptosis. Prominent among the genes already discovered to be involved is the *bcl-2* oncogene.

bcl-2 was first isolated by virtue of its translocation into the immunoglubolin gene locus in some follicular B cell lymphomas (Cleary *et al.*, 1986; Tsujimoto and Croce, 1986). Bcl-2 overexpression was subsequently shown to block cell death in an interleukin (IL)-3-dependent cell line upon withdrawal of the cytokine (Vaux *et al.*, 1988). Transgenic mice overexpressing Bcl-2 in thymocytes conferred resistance to the induction of apoptosis by glucocorticoid, γ-radiation and anti-CD3 treatments (Sentman *et al.*, 1991; Strasser *et al.*, 1991). However, Bcl-2 overexpression in these studies could not prevent the deletion of self-reactive T cells by negative selection, suggesting that not all forms of programmed cell death are inhibited by Bcl-2. Recently, Bcl-2-related gene products which also have roles in apoptosis have been isolated (Boise *et al.*, 1993; Oltvai *et al.*, 1993). One of these gene products, Bax, has been shown to form homodimers as well as heterodimers with Bcl-2 (Oltvai *et al.* 1993). Alternative splicing within the *bax* gene produces three classes of transcript of which the α mRNA encodes the 21 kDa protein that can heterodimerize with Bcl-2 (Oltvai *et al.*, 1993). In this same study, the overexpression of Baxα in an IL-3-dependent cell line is seen to accelerate apoptosis upon removal of the cytokine. Furthermore, the ratio of Baxα to Bcl-2 appears to be critical since a predominance of Baxα accelerates apoptosis in response to factor withdrawal, whereas overexpressed Bcl-2 greatly diminishes apoptosis.

As mentioned above, DNA-damaging agents can exact an apoptosis response in many cell types, particularly lymphocytes. A critical regulator of the cellular response to DNA damage is the transcription factor encoded by the p53 tumour suppressor gene (Cox and Lane, 1995; Selivanova and Wiman, 1995). Low levels of p53 protein are found in normal cells but its level rises rapidly after irradiation or exposure to other DNA-damaging agents (Maltzman and Czyzyk, 1984; Kuerbitz *et al.*, 1992; Lowe *et al.*, 1993). When non-irradiated cells are forced to express high levels of p53 they undergo either growth arrest (Michalovitz *et al.*, 1990) or apoptosis (Yonish-Rouach *et al.*, 1991). Analysis of mice in which both p53 alleles have been inactivated (p53–/–) by gene targeting has demonstrated that p53 is required for the induction of apoptosis in the thymocytes by γ-radiation and by some

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DNA-damaging drugs (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993). Irradiated fibroblasts from p53–/– mice fail to arrest in G_1, strongly suggesting that p53 regulates the G_1 radiation checkpoint (Livingstone *et al.*, 1992; Yin *et al.*, 1992). This requirement for p53 in apoptosis seems to be very important for its action as an *in vivo* suppressor of tumour formation. A study of transgenic mice expressing SV40 large T antigen found that tumour development associated with the loss of p53 resulted in decreased rates of cell death rather than increased rates of cell proliferation (Symonds *et al.*, 1994). The way in which p53 induces apoptosis is not clear. However, evidence has been obtained recently that implicates Bax/Bcl-2. Restoration of p53 in a murine leukaemia cell leads to increases in *bax* mRNA and protein levels accompanied by lower steady-state levels of *bcl-2* mRNA and protein (Miyashita *et al.*, 1994; Selvakumaran *et al.*, 1994). Moreover, p53 has been shown to activate transcription of the *bax* gene directly (Miyashita and Reed, 1995). Additional evidence that Bax/Bcl-2 is downstream of p53 comes from data on Bcl-2 overexpression in a cell line with inducible p53 wherein Bcl-2 blocks p53-induced apoptosis (Wang *et al.*, 1993; Chiou *et al.*, 1994; Strasser *et al.*, 1994).

To gain an insight into the action of the *bax* gene *in vivo*, we have generated transgenic mice specifically overexpressing Baxα in the T cell compartment. Thymocytes from the *baxα* transgenics show accelerated apoptosis in response to multiple stimuli. Baxα overexpression above a certain level can overcome the protective effects of high Bcl-2 levels in primary T cells. However, while *baxα* transgenic thymocytes in a p53–/– background still show accelerated apoptosis in response to glucocorticoid treatment they do not exhibit a restored response to DNA damage-induced apoptosis.

Results

Baxα transgenic mice express HABaxα protein in their T cells

Several transgenic lines of mice were produced carrying a construct containing the human CD2 promoter and locus control region (LCR) element as well as the mouse *baxα* cDNA with a haemagglutinin (HA) epitope attached. The HA epitope was added to the N-terminus of murine *baxα* using PCR so that a consensus Kozak sequence (Kozak, 1987) preceded the 11 amino acid tag containing a well characterized epitope of the influenza virus HA protein (Kolodziej and Young, 1991) followed directly by the cDNA coding for mouse *baxα*. The HA epitope used is recognized specifically by the monoclonal antibody (mAb) 12CA5 (Kolodziej and Young, 1991) so that HABaxα protein produced by the transgene can be distinguished from endogenous Baxα.

The HA*baxα* insert was generated by PCR such that it had *Eco*RI sites at each end for ease of cloning into the *Eco*RI site of the human CD2 expression cassette. The CD2 HA*baxα* transgene is described in Figure 1A. Both HA*baxα* and CD2 sequences are transcribed, but only functional HABaxα protein is produced since a frameshift mutation engineered in the unique *Eco*RV site of the coding region of the human CD2 gene ensures that functional CD2 protein is not translated. The human CD2

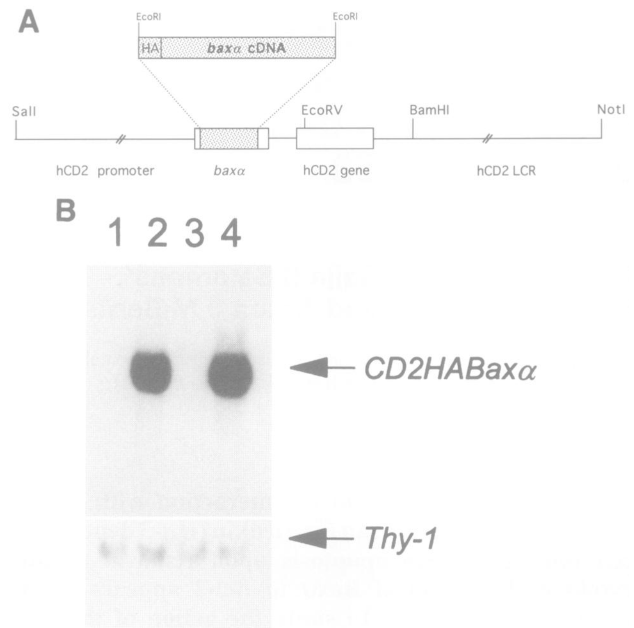

Fig. 1. *Baxα* transgene. (**A**) *Baxα* transgene construction. The HA-tagged *baxα* insert flanked by *Eco*RI sites was generated by PCR from the murine *baxα* cDNA. The insert was cloned into the *Eco*RI site in exon 1 of the human CD2 expression cassette containing the CD2 promoter, shortened CD2 coding region and 3′ LCR. The *Sal*I–*Not*I fragment was isolated for injection into fertilized oocytes. (**B**) Southern blot of transgene. To check the integrity and copy number of the *baxα* mouse transgene, genomic DNA was cut by *Hin*dIII and probed with human CD2 transgene- and *Thy-1*-specific probes. Track 1 is from a non-transgenic and track 2 from a transgenic littermate of the Bax 18 line. Track 3 is from a non-transgenic and track 4 from a transgenic littermate of the Bax 25 line.

gene and LCR are known to direct expression of inserted genes to the T cell lineage beginning early in differentation and to continue expression in double positive and single positive cells in the thymus as well as in peripheral T cells (Lang *et al.*, 1988; Owen *et al.*, 1988).

DNA from the transgenic lines generated was analysed by Southern blot analysis, and subsequent studies focused on the two lines with the highest copy numbers of transgene. These lines were designated CD2HABax 18 (Bax 18) and CD2 HABax 25 (Bax 25). Figure 1B shows a Southern blot on DNA from Bax 18 (tracks 1 and 2) and Bax 25 (tracks 3 and 4) transgenic mice as well as non-transgenic littermates. *Thy-1* was used as a control because it is a single copy gene. The transgene is seen to be intact in each line, and the copy number, as determined by phosphorimaging, was ~25 for the Bax 18 line and 40 for the Bax 25 line.

Western blot analysis was used to examine the level of HABaxα expression in the T cells of the transgenic mice. Thymus was isolated from a transgenic and non-transgenic littermate from both lines. Cell extracts were prepared from single cell suspensions of each thymus. One hundred micrograms of each extract were then separated on a denaturing polyacrylamide gel and transferred to a PVDF membrane. Baxα expression was then detected using a rabbit polyclonal antibody against Baxα and the 12CA5 mouse mAb against the HA epitope. The left side of Figure 2A shows the respective transgenic and non-transgenic littermate thymus lysates probed with the rabbit

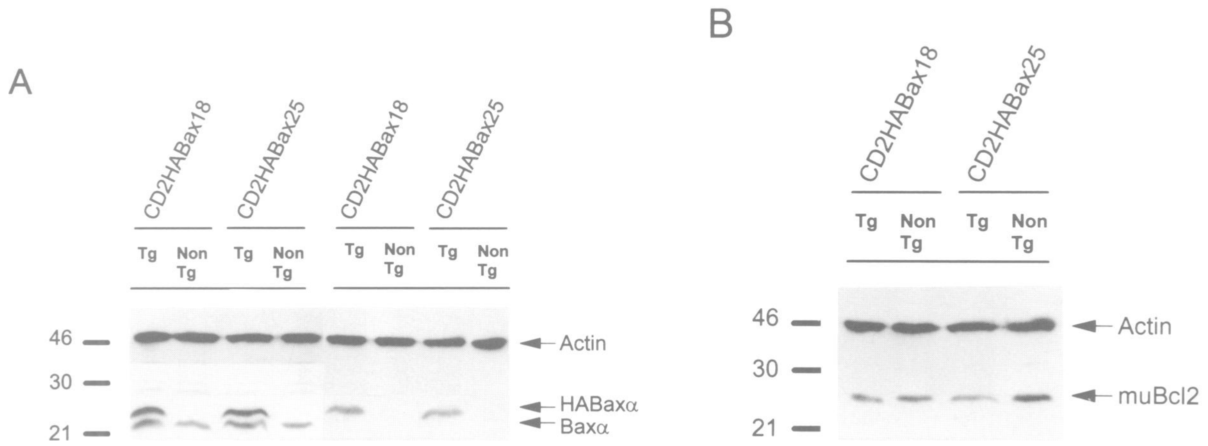

Fig. 2. Western blot analysis of thymus lysates. (**A**) Lysates were separated on a denaturing polyacrylamide gel and transferred to a PVDF membrane. The left side of the figure shows the respective transgene (Tg) and non-transgene (Non Tg) thymocyte lysates probed with a rabbit polyclonal against Baxα. The right side shows replica tracks probed with the mAb to 12CA5. The top part of the filters was also probed with an mAb against actin as a loading control. Visualization of the bands followed incubation with an appropriate ^{125}I-linked secondary antibody and autoradiography. Band intensity was measured on a phosphorimager, normalizing against the actin levels. (**B**) Replica samples to the four shown in (A) were probed with mAbs against muBcl-2 and actin followed by a ^{125}I-linked anti-mouse or anti-rat Ig Ab.

polyclonal against Baxα. Two bands are visible in the transgenic lanes and only one in the non-transgenic lanes. The lower band corresponds to the endogenous Baxα protein which runs close to the 21 kDa molecular weight marker (Oltvai *et al.*, 1993). The upper band on the left side is the only band present on the right side where four replica tracks have been probed with the 12CA5 mAb specific for the HA epitope. The addition of the 11 amino acid HA epitope means that the HABaxα protein is larger than the endogenous Baxα. The ratio of HABaxα to endogenous Baxα was determined by phosphorimaging using the actin control to normalize each track. The ratio HABaxα:Baxα was 145% for CD2 HABax18 and 155% for CD2 HABax25. Lysates from the spleens of transgenic and non-transgenic littermates from both lines were tested, and similarly high levels of expression of the HA*baxα* transgene were found in the transgenic samples (data not shown). To assess whether overexpression of Baxα did not perturb endogenous Bcl-2 levels, the Bax 18 and Bax 25 thymocyte lysates were probed with a mAb specific for murine (mu) Bcl-2 (Figure 2B). The endogenous Bcl-2 level is seen to be unchanged regardless of the Baxα overexpression. No differences in total thymocyte numbers were observed (data not shown).

T cells from baxα transgenics show accelerated apoptosis in response to some apoptotic stimuli

Since it has been shown previously that Baxα overexpression in an IL-3-dependent cell line can accelerate cell death (Oltvai *et al.*, 1993), we investigated the effect of various apoptotic stimuli on T cells from our CD2HABaxα lines 18 and 25. We tested a variety of stimuli known to cause cell death by apoptosis and, in particular, dexamethasone, radiation and etoposide, which are known to be inhibited by Bcl-2. Assays were performed by removing the thymus and making a single cell suspension of thymocytes in RPMI medium supplemented by 10% fetal calf serum (FCS) and 50 μM 2-mercaptoethanol. After the cells received the apoptotic stimulus, they were incubated at 37°C in 5% CO_2 and harvested at various time points. Rather than assessing apoptosis by estimating cell viability using trypan blue, we used a flow cytometry-based technique which measures propidium iodide staining of cellular DNA in a hypotonic buffer (Nicoletti *et al.*, 1991). Using this method, apoptotic nuclei appear as a broad hypodiploid DNA peak easily discriminable from the narrow peak of thymocyte nuclei with a normal diploid DNA content, allowing the percentage of apoptotic cells to be calculated.

The glucocorticoid dexamethasone has been shown to induce apoptosis in thymocytes (Wyllie, 1980). The result of dexamethasone treatment of thymocytes from the Bax 18 and 25 transgenic as well as non-transgenic littermates is shown in Figure 3A and B respectively. The cells were harvested after 4–5 h incubation with 2 μM dexamethasone. More than 60% of the Bax 18 transgenic thymocytes are in apoptosis whereas only 30% or so of those from non-transgenic thymocytes are apoptotic. Similarly, ~60% of the Bax 25 transgenic thymocytes are apoptotic as opposed to <30% for the non-transgenic thymocytes. Thymocytes in which HABaxα is overexpressed are therefore significantly more sensitive to glucorticoid-induced apoptosis than are wild-type thymocytes.

The *baxα* transgenic thymocytes were assessed further for their sensitivity to apoptosis induced by DNA damage. Certain cell types, particularly lymphocytes, are known to be highly sensitive to DNA damage-induced apoptosis caused by γ-radiation or cytotoxic drugs, as used in chemotherapy, such as etoposide, a DNA topoisomerase II inhibitor (Cohen *et al.*, 1992). This sensitivity is known to be reduced greatly by Bcl-2 overexpression (Sentman *et al.*, 1991; Strasser *et al.*, 1991). Therefore, it seemed likely that Baxα overexpression would have the opposite effect.

Low doses of γ-radiation were administered to the Bax 18 and Bax 25 thymocytes. The doses were in the range 50–200 cGy. As shown in Figure 4, for both lines the transgenic thymocytes are substantially more sensitive to γ-radiation than are those of non-transgenic littermates. After 8 h, >30% apoptosis is found in Bax 18 mice irradiated with only 100 cGy (Figure 4A). A

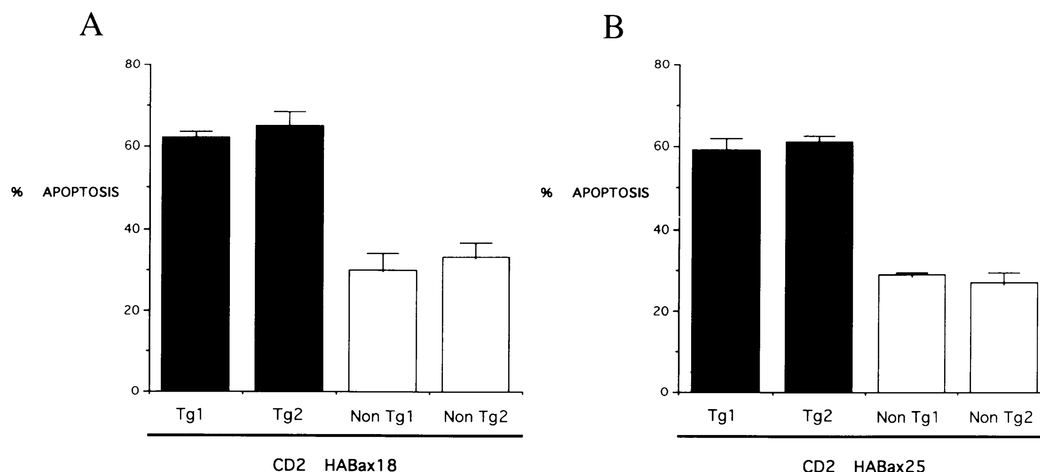

Fig. 3. Percentage of apoptosis in *baxα* transgenic thymocytes treated with 2 μM dexamethasone. (**A**) Thymocytes were isolated from transgenic (Tg1,2) and non-transgenic littermates (Non Tg1,2) of the CD2 HABax 18 line and treated with 2 μM dexamethasone. 4.5 h after treatment, the cells were harvested and prepared for FACS to measure the DNA content of the nuclei by propidium iodide staining, as described in Materials and methods. FACS analysis allowed apoptotic nuclei to be identified as a broad hypodiploid DNA peak easily separable from the narrow peak of thymocyte nuclei with a normal diploid DNA content. The percentage of apoptotic cells shown is normalized to the amount of apoptotic cells found in untreated cultures for the same animal at the same time point. Similar results to those shown were observed in three independent experiments. Each value represents the mean ± range of duplicate determinations of apoptosis in 10 000 cells each. (**B**) Thymocytes were isolated from transgenic (Tg1,2) and non-transgenic littermates (Non Tg1,2) of the CD2 HABax 25 line and treated with 2 μM dexamethasone for 4.5 h. Cells were harvested and analysed as described in (A). The percentage of apoptotic cells is shown.

similar situation is found in the Bax 25 mice, and in both lines there is a very clear differential between transgenic and non-transgenic thymocytes at each dose (Figure 4C). As shown for Bax 18 mice, the differential is still clearly seen at 20 h after irradiation where the transgenic thymocytes have between 2- and 3-fold more apoptotic cells than the non-transgenic thymocytes (Figure 4B).

Further analysis of the apoptotic response to DNA damage was assayed by treating thymocytes with 50 μM etoposide. Etoposide is believed to stabilize the topoisomerase–DNA complex during its cleavage–religation cycle, so generating double strand DNA breaks even in non-replicating cells (Roy *et al.*, 1992). Etoposide has been shown to initiate apoptosis in various cell types including thymocytes (Clarke *et al.*, 1993). Figure 4D shows that the level of apoptotic cells in the Baxα-overexpressing transgenic thymocytes is virtually double that in non-transgenic cells; therefore, Baxα overexpression clearly sensitizes the thymocytes to etoposide-induced apoptosis.

Baxα overexpression can rescue the anti-apoptotic effect of Bcl-2 in response to apoptotic stimuli on primary T cells

As described above, Baxα was identified on the basis of its ability to interact with Bcl-2 (Oltvai *et al.*, 1993). This same study also shows that the ratio of transfected *bcl-2* and transfected *baxα* in an IL-3-dependent cell line can determine the rate of cell death following removal of IL-3. We sought to demonstrate this in primary T cells by crossing *baxα* transgenic mice with Eμ*bcl-2* transgenic mice (McDonnell *et al.*, 1989). The thymocytes of these *bcl-2* mice, which overexpress human Bcl-2 (huBcl-2), have been shown to be very resistant to multiple apoptotic stimuli (Sentman *et al.*, 1991; Strasser *et al.*, 1991).

Initially, we crossed heterozygous transgenic mice of both Bax 18 and 25 with heterozygote Eμ*bcl-2*. Analysis of thymocytes from mice heterozygous for Bax 18 or 25 and Eμ*bcl-2* for response to apoptotic stimuli showed an almost non-transgenic wild-type phenotype, suggesting insufficient HABaxα to completely reverse the *bcl-2* phenotype to that found for *baxα* transgenics. We then took CD2 HABaxα/Eμ*bcl-2* mice and crossed them again to CD2 HABaxα mice. This produced mice with the array of genotypes as described in Figure 5. Litters were obtained containing mice with a non-transgenic genotype, mice heterozygous for either Bax 18 (Bax 18 +/–) or *bcl-2* (EμBcl-2 +/–), mice homozygous for Bax 18 (Bax 18 +/+) and, finally, mice homozygous for Bax 18 but heterozygous for *bcl-2* (Bax 18 +/+, EμBcl-2 +/–). Thymocytes from these mice were then assayed for their response to dexamethasone (shown in Figure 5A) and radiation (shown in Figure 5B and C). In response to dexamethasone, the Eμ*bcl-2* heterozygote was more resistant to apoptosis than were wild-type mice. Mice heterozygous and homozygous for Bax 18 both showed greatly increased thymocyte apoptosis in comparison with those from the non-transgenic littermate. Thymocytes heterozygous for both Bax 18 and Bcl-2 have approximately the same level of apoptosis as wild-type, indicating that the level of HABaxα produced is insufficient to overcome the high level of transgenic Bcl-2 completely. However, thymocytes from mice homozygous for the Bax 18 transgene, while heterozygous for Eμ*bcl-2*, have levels of dexamethasone-induced apoptosis similar to heterozygous Bax 18-only mice. Therefore, the level of transgenic HABaxα in these mice is sufficient to reverse completely the protective effect of Bcl-2 and restore the *baxα* transgenic phenotype. The response of the thymocytes to γ-radiation again reflects the relative amounts of HABaxα and huBcl-2 as for the dexamethasone response (Figure 5B

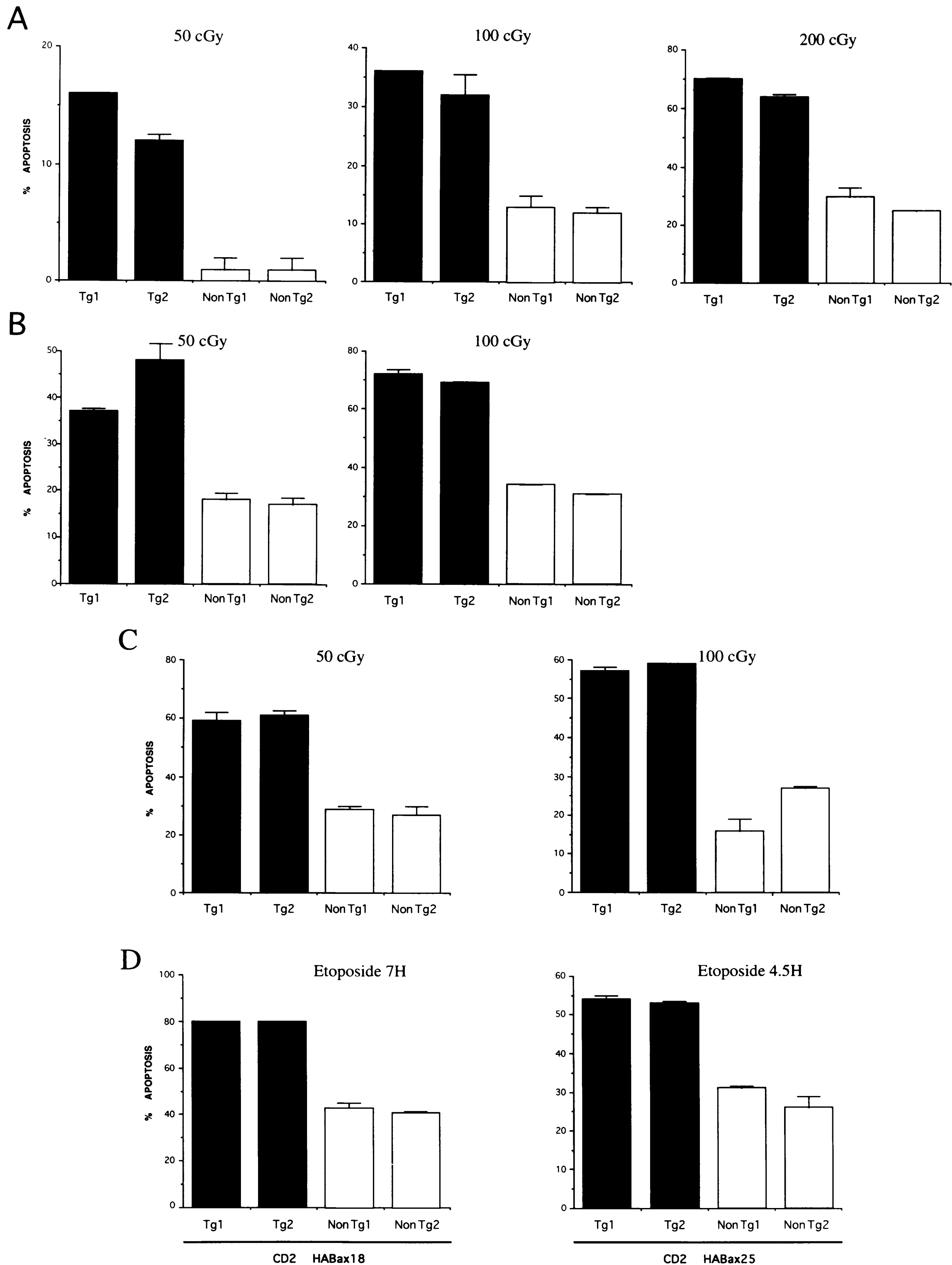

Fig. 4. Percentage of apoptosis in *baxα* treated with γ-radiation and 50 μM etoposide. (**A**) Thymocytes were isolated from transgenic (Tg1,2) and non-transgenic littermates (Non Tg1,2) of the Bax 18 line and irradiated with 50, 100 or 200 cGy. The cells were cultured for 8 h, harvested and prepared for DNA content measurement. Percentage apoptosis was determined as described in Figure 3. (**B**) Thymocytes were isolated as in (A) except, following 50 cGy and 100 cGy of γ-radiation, they were cultured for 20 h before harvesting and analysis. (**C**) Thymocytes were isolated from transgenic (Tg1,2) and non-transgenic (Non Tg1,2) littermates of the Bax 25 line and treated with 50 or 100 cGy of γ-radiation. The cells were cultured for 8 h before harvesting and analysis. (**D**) Thymocytes were isolated from transgenic (Tg1,2) and non-transgenic littermates (Non Tg1,2) of the Bax 18 line (as indicated) and treated with 50 μM etoposide for 7 h. Similarly, thymocytes were isolated from the Bax 25 line (as indicated) and treated with 50 μM etoposide for 4.5 h before harvesting and analysis. Similar results to those shown were observed in three independent experiments. Each value represents the mean ± range of duplicate determinations of apoptosis in 10 000 cells each.

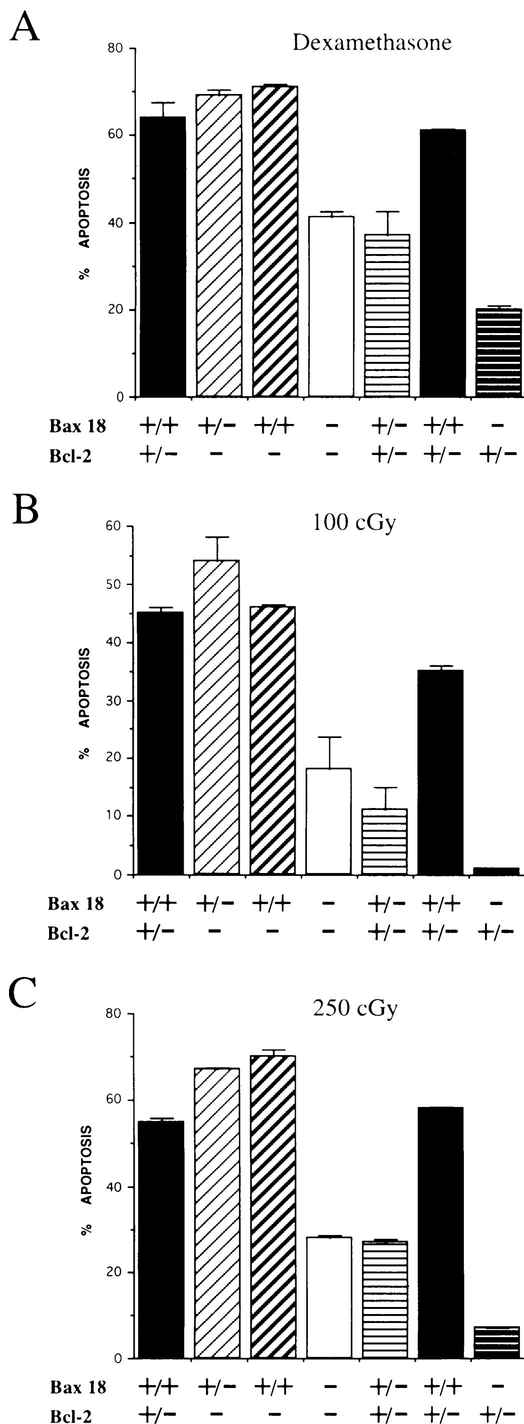

Fig. 5. Percentage of apoptosis in *baxα/bcl-2* transgenic thymocytes treated with dexamethasone and γ-radiation. Thymocytes were isolated from mice produced by the cross of Bax 18 and Eμ*bcl-2* mice. The genotype of these mice is indicated. +/+ represents a mouse homozygous for a transgene, +/– is a mouse heterozygous for the transgene and – indicates no transgene present. (**A**) The cells were treated with 2 μM dexamethasone for 5 h and then harvested and prepared for DNA content measurement. The percentage apoptosis was calculated as described in Figure 3A. (**B**) Thymocytes were isolated as in (A) and subjected to 100 cGy of γ-radiation or (**C**) 250 cGy of γ-radiation and then harvested after 9 h in culture. The percentage of apoptotic cells is shown. Similar results to those shown were observed in three independent experiments. Each value represents the mean ± range of duplicate determinations of apoptosis in 10 000 cells each.

and C). This is illustrated further in Figure 6. The level of HABaxα is shown to be 2.0 times that (as measured by phosphorimaging) in the thymocytes homozygous for the transgene, as compared with that seen in the heterozygous thymocytes (Figure 6A) and is able to overcome the high level of huBcl-2 expressed by the transgene (Figure 6C). The level of endogenous muBcl-2 remains constant regardless of the alterations in the level of transgene products (Figure 6B).

HABaxα overexpression in p53 null T cells still accelerates glucocorticoid-induced apoptosis but does not rescue DNA damage-induced apoptosis

The CD2 HABaxα mice were also crossed so as to produce mice homozygous for the p53 null mutation (Donehower *et al.*, 1992) while being *baxα* transgenic. Thymocytes from p53 null mice have been shown to be resistant to apoptosis induced by DNA-damaging agents such as γ-radiation or etoposide (Clarke *et al.*, 1993; Lowe *et al.*, 1993). It has been shown that *baxα* expression is activated directly by p53 (Miyashita and Reed, 1995). These authors suggest that since p53 activity is increased by DNA-damaging agents this will lower the resistance of cells to apoptosis through p53-mediated effects on *baxα* expression and hence altering the Bcl-2:Baxα ratio. We sought to examine the effect of Baxα overexpression in p53–/– thymocytes on the response to apoptotic stimuli. Thymocytes were analysed from mice heterozygous and homozygous for the p53 null allele and then plus or minus the *baxα* transgene. The result of dexamethasone treatment is shown in Figure 7A. High levels of apoptosis were found in the mice transgenic for CD2 HABaxα regardless of p53 status. Much lower levels of apoptosis are seen in thymocytes from mice heterozygous or homozygous for the p53 null allele without *baxα*.

A different situation exists in thymocytes treated with etoposide (Figure 7B) or γ-radiation (Figure 7C and D). High levels of etoposide-induced apoptosis are seen in p53 heterozygous thymocytes, and somewhat higher levels in those also expressing HABaxα. There are only very low levels seen in p53 null homozygotes, regardless of the presence or absence of the *baxα* transgene. The same situation applies to thymocytes irradiated with 100 cGy (Figure 7C) or 500 cGy (Figure 7D) of γ-radiation. Irradiated p53 null thymocytes showed a slightly increased level of apoptosis with *baxα* than without, but we do not consider this significant outside of inherent experimental variation. Therefore, it is clear that Baxα overexpression accelerates apoptosis due to glucocorticoid treatment in a p53-independent manner. However, while Baxα overexpression can accelerate apoptosis due to DNA damage, this process is dependent on p53 status since it is not effective in the absence of p53.

Discussion

We have demonstrated that the overexpression of Baxα in primary T cells can accelerate apoptosis in these cells in response to different stimuli. Comparing protein expression, we find that thymocytes from both Bax 18 and Bax 25 have transgenic HA Baxα levels of ~150% that of endogenous Baxα. This level of HABaxα is sufficient to accelerate apoptosis substantially in thymo-

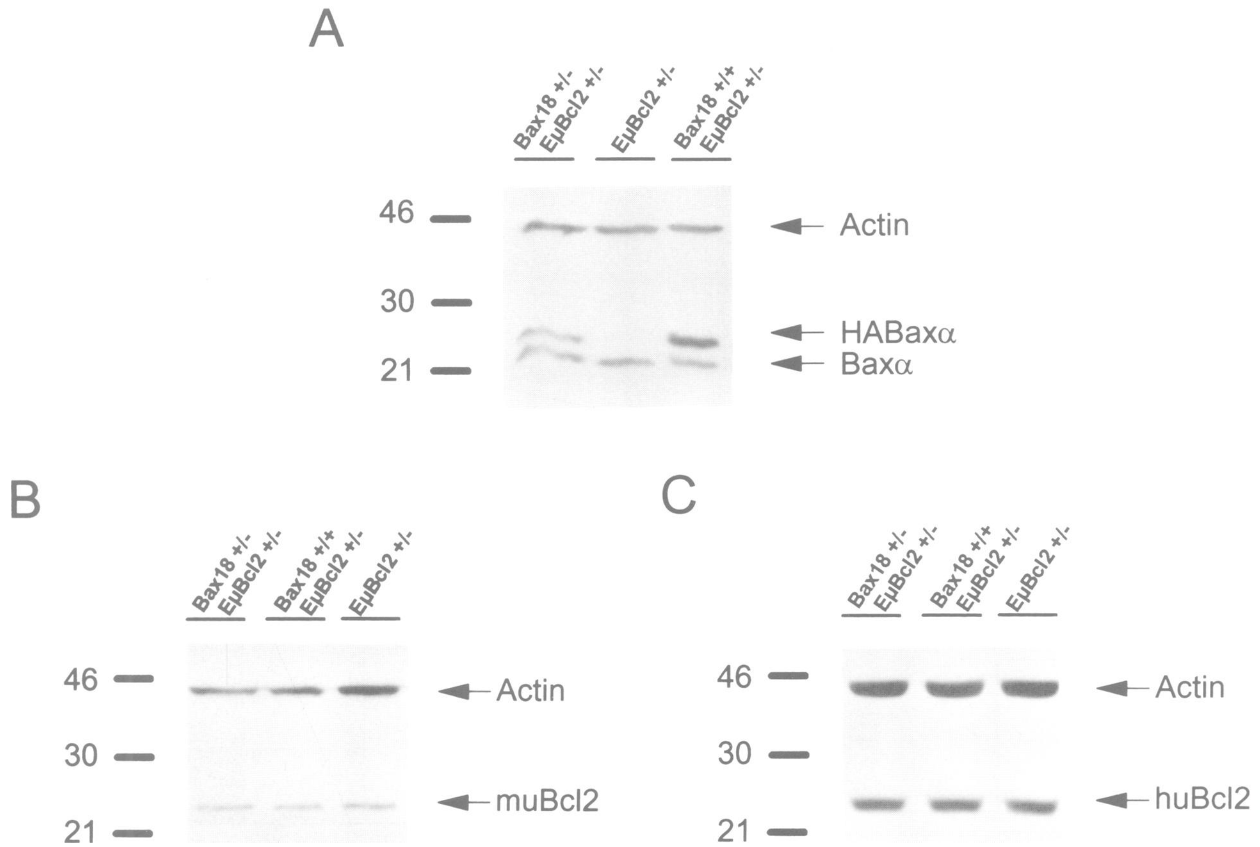

Fig. 6. Western blot analysis of thymus lysates from *baxα/bcl-2* transgenic mice. (**A**) Lysates were isolated on a denaturing polyacrylamide gel and transferred to a PVDF membrane. The filters were probed with rabbit polyclonal antibody against Baxα followed by a ^{125}I-linked anti-rabbit Ig Ab and autoradiography. The filters were also probed with an mAb against actin as a loading control. Band intensity was calculated following phosphor-imaging and normalization against actin levels. (**B**) Replica tracks to those shown in (A) were probed with mAbs against muBcl-2 and actin followed by ^{125}I-linked anti-mouse or anti-rat Ig Ab and autoradiography. (**C**) Replica tracks to those shown in (A) were probed with an mAb against huBcl-2 and actin and visualized as above.

cytes in response to low doses of γ-radiation, dexamethasone and etoposide. This is in direct contrast to thymocytes from *bcl-2* transgenic mice which become more resistant to these apoptotic stimuli (Sentman *et al.*, 1991; Strasser *et al.*, 1991). Our data and the previous work of others point to a situation in T cells where the ratio of Baxα to Bcl-2 is very important for determining response to apoptotic stimuli.

To probe this point further, we crossed *bcl-2* transgenics with our *baxα* transgenics to see whether we could reverse the protective effect of Bcl-2 in primary T cells. We found that the level of HABaxα in *baxα* heterozygotes was insufficient to reverse completely the effect of high huBcl-2 in mice also heterozygous for *bcl-2*. In this case, it was only sufficient to allow a virtual wild-type response to various apoptotic stimuli, so that while the effect of the *bcl-2* transgene was counteracted the *baxα* transgenic phenotype was not apparent. However, thymocytes from Eμ*bcl-2* mice homozygous for the *baxα* transgene, and hence having double the amount of HABaxα in the heterozygote, show a clear and substantial acceleration to cell death in response to dexamethasone, etoposide and low doses of γ-radiation. The effect is almost the same as that seen in *baxα*-only heterozygote mice. No significant difference is seen in the sensitivity of homozygous *baxα*-only thymocytes compared with heterozygous *baxα*-only thymocytes in response to dexamethasone or irradiation. This may well reflect a situation in which, once the Baxα:Bcl-2 ratio is tilted in favour of Baxα predominance beyond a certain threshold, further increasing the amount of Baxα present has no effect (Oltvai and Korsmeyer, 1994). Therefore, although in our system we cannot measure the stoichiometry of the Baxα:Bcl-2 ratio precisely, we can see that it operates in primary T cells and that it can be manipulated to elicit a strong response to stimuli which previously were resisted. We also see that while the levels of transgene products are being manipulated the steady-state levels of endogenous Baxα and muBcl-2 in primary cells are not affected, which reinforces the view that susceptibility to apoptosis is a cell autonomous property (Oltvai and Korsmeyer, 1994). This also implies that proposed therapeutic approaches based on the manipulation of apoptosis-related gene products in primary cells are possible without altering the endogenous levels of those gene products (Fisher, 1994; McDonnell *et al.*, 1995).

Clearly, p53 has a central role in the regulation of apoptosis in response to DNA-damaging agents (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993). In addition, p53 suppresses tumour formation *in vivo*, at least in part through this ability to facilitate cell death (Symonds *et al.*, 1994). p53 also directly activates tran-

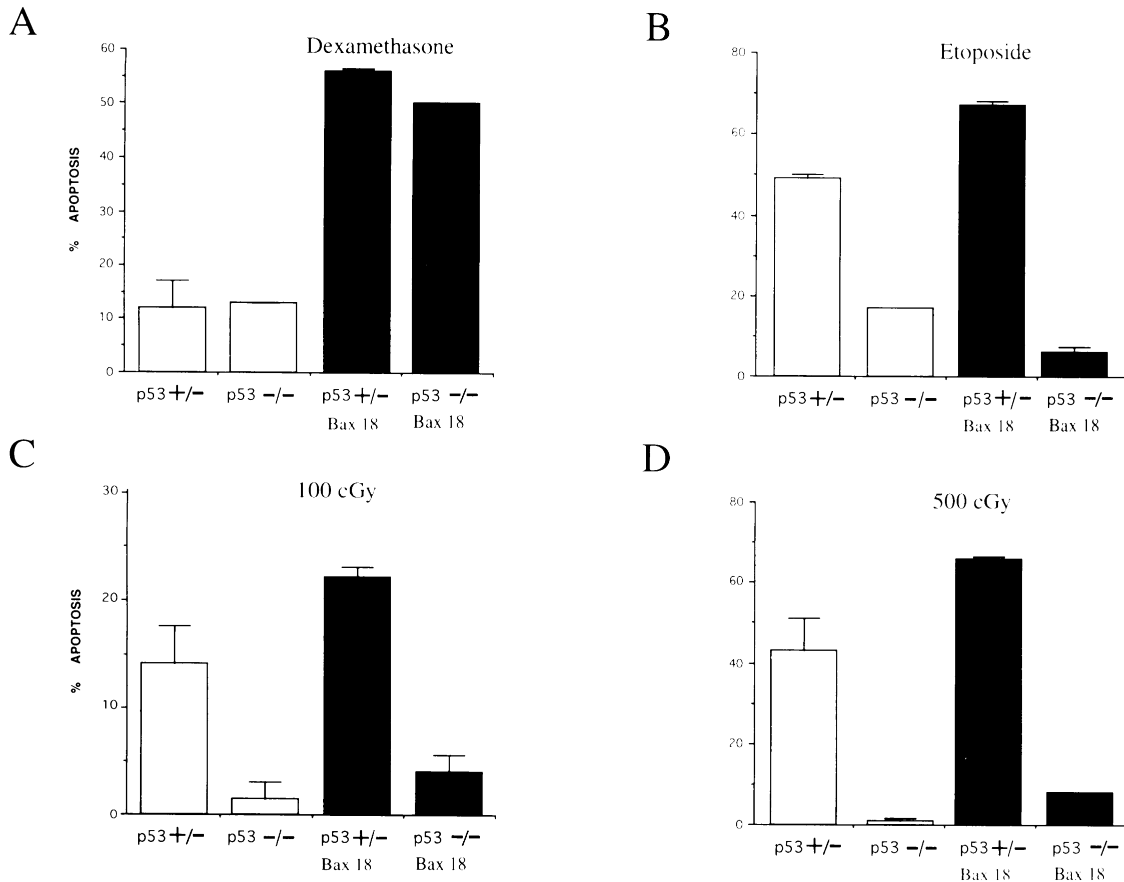

Fig. 7. Percentage apoptosis in thymocytes from mice with or without p53 and/or the *bax*α transgene following treatment with dexamethasone, etoposide and γ-radiation. (**A**) Thymocytes were isolated from mice heterozygous for a p53 null allele (p53+/–) or homozygous for p53 null alleles (p53–/–) and either containing the *bax*α transgene from the Bax 18 line or not (as indicated). The cells were treated with 2 μM dexamethasone for 4.5 h, harvested and prepared for DNA content measurement. Percentage apoptosis was determined as in Figure 3. Thymocytes isolated as for (A) were treated with 50 μM etoposide for 7 h (**B**), cultured for 8 h following 100 cGy of γ-radiation (**C**) or cultured for 8 h following 500 cGy of γ-radiation (**D**), before harvesting and analysis. The percentage of apoptotic cells is shown. Similar results to those shown were observed in four independent experiments, including experiments where the Bax 25 line was used instead of the Bax 18 line. Each value represents the mean ± range of duplicate determinations of apoptosis in 10 000 cells each.

scription of the *bax* gene (Miyashita and Reed, 1995). Taken together, these findings suggested that Baxα may act downstream of p53 in a p53-dependent apoptosis pathway. To investigate this, we performed serial crosses between *bax*α transgenics and p53–/– mice (Donehower *et al.*, 1992) to generate p53–/– and p53+/– animals with or without a *bax*α transgene. The presence of the *bax*α transgene accelerated apoptosis in thymocytes from both p53–/– and p53+/– mice in response to dexamethasone. Therefore, glucocorticoid-mediated apoptosis clearly is p53 independent and Baxα's role in this is also independent of the p53 status of the cell. This is consistent with the previous finding that p53 levels do not increase detectably in p53+/+ thymocytes after exposure to dexamethasone (Lowe *et al.*, 1993).

In contrast to the response to dexamethasone, the presence of the *bax*α transgene accelerated apoptosis only in thymocytes from p53+/– mice after exposure to γ-radiation and etoposide. Thymocytes from p53–/– mice with the *bax*α transgene showed similar resistance to apoptosis by these DNA-damaging agents as did p53–/– mice without the *bax*α transgene. Therefore, the suggestion that p53 acts to induce apoptosis following DNA damage by altering the Baxα:Bcl-2 ratio in favour of Baxα to accelerate cell death (Miyashita *et al.*, 1994; Cox and Lane, 1995; Miyashita and Reed, 1995) is a part of but clearly not the complete picture. We can see that the *bax*α transgene is active in accelerating apoptosis in a p53–/– background in response to dexamethasone as well as in response to DNA damage in a p53+/– background. Therefore, this suggests that, since it is not effective in p53–/– cells after DNA damage, the presence of p53 is required to induce other necessary factor(s) to hasten cell death or perhaps directly activate such factor(s) by a protein–protein interaction. DNA damage clearly leads to the activation of p53 and this in turn leads to the transcriptional activation of several genes including $p21^{CIP/WAF1}$, *gadd* 45 and Mdm2 (reviewed in Cox and Lane, 1995). Of these genes, p21 has been suggested as being the most likely to control the cell cycle in G_1 (Harper *et al.*, 1993). Recently, experiments on fibroblasts from p21–/– mice have shown them to be defective in G_1 arrest in response to DNA damage (Deng *et al.*, 1995). However, thymocytes from the same mice show no defect in response to DNA damage-induced apoptosis, in marked contrast to p53–/– thymocytes. Therefore, any additional factor required to complement Baxα overexpression in re-establishing the apoptotic response to DNA damage is probably not p21.

Previous work examining mice transgenic for *bcl-2* and

p53–/– has shown no significant additive effect of Bcl-2 in resting thymocytes on resistance to DNA damage-induced apoptosis as compared with p53–/– only (Strasser *et al.*, 1994). However, interpretation of this is somewhat ambiguous since p53–/– thymocytes are already so resistant. Our data unequivocally show that although Bcl-2 and Baxα may lie downstream of p53 in DNA damage-induced apoptosis, their action is wholly dependent on p53. This is further confirmation of p53's pivotal role as 'guardian of the genome' (Lane, 1992), since the presence of large amounts of a downstream effector of p53-mediated apoptosis, i.e. Baxα, is not sufficient to precipitate apoptosis in the absence of the sentry, p53.

Clearly, the role of p53 in apoptosis remains to be elucidated, as does the mechanism of Baxα action. Our experiments show the profound effect of Baxα in accelerating apoptosis and its abilty to antagonize Bcl-2 in primary T cells. They also show the inability of Baxα to overcome the absence of p53 in DNA damage-induced apoptosis, confirming the centrality of p53 to the process. They also highlight the need to identify other p53-induced factors(s) that may interact with *baxα* to accelerate cell death after DNA damage. Future studies with the mice described here will examine the interaction of Baxα with other apoptotic effector molecules *in vivo*. In addition, a study of the T cell lymphoma incidence of *baxα*/p53–/– mice is planned. T cell lymphomas arising from such will be studied for their response to DNA damage and other stimuli via p53-independent mechanisms (Strasser *et al.*, 1994).

Materials and methods

Generation of transgenic mice and DNA analysis

The HA*baxα* transgene insert was generated by PCR using murine *baxα* cDNA (Oltvai *et al.*, 1993). The 11 amino acid tag containing the well characterized epitope of the HA protein of influenza virus (Kolodziej and Young, 1991) was attached to the murine *baxα* cDNA (gift of S.J.Korsmeyer) by a single-step PCR method. The 5′ end of the *baxα* cDNA was extended to contain the HA epitope preceded by a Kozak consensus translation start site (Kozak, 1986) and this was preceded by an *Eco*RI site. The 3′ end was extended to incorporate an *Eco*RI site directly after the translation stop codon. The 5′ primer used was (5′ to 3′) CCGGAATTCACCATGGCTTACCCATACGACGTCCCAGACTA-CGCTAGCGACGGGTCCGGGGAGCAGCTT and the 3′ primer (5′ to 3′) CCGGAATTCTCAGCCCATCTTCTTCCAGATG. PCR amplification was carried out using *Pfu* polymerase (Stratagene) and the manufacturer's incubation buffer for 35 cycles as follows: 10 min denaturation at 94°C as first step, followed by 35 cycles of denaturation at 94°C for 1 min; annealing 55°C for 1.5 min; extension, 72°C for 2 min (last cycle 10 min). The PCR product was purified, digested with *Eco*RI and then cloned into the *Eco*RI site of the p2629 CD2 expression plasmid (gift from D.Kioussis) essentially as described previously (Brady *et al.*, 1993). A 5.5 kb *Bam*HI–*Not*I fragment containing the 3′ CD2 LCR from p2694 (gift from D.Kioussis) was then ligated into the unique *Bam*HI–*Not*I sites in p2629 HA *baxα* to give pCD2HA *baxα*. The transgene insert was completely sequenced by dideoxy sequencing to confirm that no mutations were introduced following PCR amplification. The *Sal*I–*Not*I fragment of pCD2 HA*baxα* was separated from the vector sequence by gel electrophoresis, and microinjected as described previously (van Lohuizen *et al.*, 1989).

The DNA was injected into fertilized oocytes from FVB mice, and founder mice were bred to FVB mice to maintain the line. The presence of the *baxα* transgene was determined by Southern blot analysis of tail DNA (10 μg). The DNA was digested with *Hin*dIII and probed with a randomly primed 2.0 kb *Nde*I fragment from pCD2 2629 or *baxα* cDNA. A 1.2 kb *Thy-1.2* fragment was used as a loading control probe. Quantitation for copy number was performed by phosphorimaging.

The Eμ*bcl-2* mice used were a gift of T.McDonnell and S.Korsmeyer (McDonnell *et al.*, 1989). They were maintained on a C57BL/6J background before crossing. The p53–/– mice used were a gift of L.Donehower and A.Bradley (Donehower *et al.*, 1992). Prior to the experiments described here, the p53+/– mice were backcrossed with Balb/c mice for five generations and then crossed for two generations to FVB before crossing to generate p53–/– mice. *baxα*/p53+/– mice arising from p53–/– mice crossed with the *baxα* transgenics were crossed to p53–/– mice to generate the mice used in the studies described. Wild-type and targeted p53 alleles in offspring were identified by PCR essentially as described in Donehower *et al.* (1992).

Although some genetic backgrounds may partially influence the sensitivity of thymocytes to treatments such as irradiation, we obtained highly consistent results from all animals with a given transgenic genotype, as previously described by others, e.g. Lowe *et al.* (1993).

Transgene expression analysis

Single cell suspensions were prepared from thymus (and occasionally spleen) of transgenic and non-transgenic mice. Erythrocytes were removed by lysis in Tris-buffered ammonium chloride. Protein extracts were prepared by lysing cells at 4°C for 20 min in 5 vol of lysis buffer (1% NP-40, 20 mM Tris pH 8.0, 137 mM NaCl and 10% glycerol) supplemented with protease inhibitors. The lysate was centrifuged at 4°C for 10 min at 14 000 r.p.m. and the supernatant retained. Protein concentration was measured using the Bradford assay reagent (Bio-Rad). One hundred μg of protein was used per track, adjusted to 1× Laemmli sample buffer, boiled for 5 min and then loaded on a 15% SDS–polyacrylamide gel. After resolution, the gel was transferred to an Immobilon-P, PVDF membrane (Millipore) by electroblotting. All antibody incubations were performed in blocking buffer [0.15% bovine serum albumin, 0.5 mM EDTA, 0.5% Tween in phosphate-buffered saline (PBS)] at 4°C. The primary antibodies used were a mouse mAb to the HA epitope, 12CA5 (Kolodziej and Young, 1991), a rabbit polyclonal antibody to Baxα (SanverTech), a mouse mAb to human Bcl-2 (clone 124, DAKO) and a rat mAb to muBcl-2 (SanverTech). The secondary antibodies were ^{125}I-labelled sheep anti-mouse and ^{125}I-labelled donkey anti-rabbit (Amersham). Protein bands were detected by autoradiography and levels quantitated on a phosphorimager.

Apoptosis assays and FACS analysis

For the assays measuring response to apoptotic stimuli, single cell suspensions were prepared from the thymuses of mice aged between 4 and 6 weeks and erythrocytes removed as above. The cells were then washed twice in medium (RPMI 1640 supplemented with 10% FCS and 50 μM 2-mercaptoethanol) and cell viability assessed by trypan blue exclusion and counting in a haemocytometer. The thymocytes were resuspended at 1×10^6 cells/ml and plated in 24-well plates in either 2 μM dexamethasone (Sigma) or 50 μM etoposide (Sigma), or after exposure to varying doses of γ-radiation from a ^{137}Cs source (Von Gahler Nederland B.V.). Duplicate samples were taken of the thymocytes from each mouse at each time point. To estimate the percentage undergoing apoptosis, the cells were processed essentially as described in Nicoletti *et al.* (1991). The 1×10^6 cells per sample were washed once in PBS, pelleted gently, then resuspended in 300 ml of a hypotonic fluorochrome solution (propidium iodide 50 mg/ml in 0.1% sodium citrate plus 0.1% Triton X-100) and placed in the dark overnight before FACS analysis. Flow cytometric quantitation of apoptotic cells was performed as indicated in Nicoletti *et al.* (1991) and debris was excluded on the basis of forward and side light-scattering properties. The percentage apoptosis in the treated thymocytes was normalized to that found in untreated cultures derived from the same animal.

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