p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome *c*-independent apoptosis blocked by Bcl-2

Pei-Feng Li, Rainer Dietz and Rüdiger von Harsdorf[1]

Department of Cardiology, Franz Volhard Klinik, Universitätskliuikum Charité, Humboldt University, Wiltbergstraße 50, 13125 Berlin, Germany

[1]Corresponding author
e-mail: rharsdo@mdc-berlin.de

Downstream mediators of p53 in apoptosis induction remain to be elucidated. We report that p53-induced apoptosis occurred in the absence of cytochrome *c* release into the cytosol. Although Bax was upregulated, it remained largely in the cytosol and there was no detectable translocation to the mitochondria. Bid was not activated as no cleavage could be detected. Thus, the absence of cytochrome *c* release may be due to the lack of Bax translocation to mitochondria and/or Bid inactivation. Nevertheless, p53-induced apoptosis is still caspase dependent because it could be abolished by z-VAD-fmk. To search for alternative downstream targets of p53, we detected production of reactive oxygen species (ROS) as well as mitochondrial membrane potential ($\Delta\psi$). p53 induced ROS generation, which then caused a transient increase of $\Delta\psi$ followed by a decrease. Antioxidants could inhibit the alterations of $\Delta\psi$, thereby preventing apoptosis. z-VAD-fmk was unable to abrogate $\Delta\psi$ elevation but inhibited $\Delta\psi$ decrease, indicating that $\Delta\psi$ elevation and its decrease are two independent events. Bcl-2 may abolish elevation as well as decrease of $\Delta\psi$ without interfering with ROS levels. Thus, the ROS-mediated disruption of $\Delta\psi$ constitutes a pivotal step in the apoptotic pathway of p53, and this pathway does not involve cytochrome *c* release.
Keywords: apoptosis/cytochrome *c*/mitochondrial membrane potential/p53/reactive oxygen species

Introduction

p53 is a transcription factor that can not only induce growth arrest through transactivation of the p21^{CIP1} gene, but can also trigger cell death via apoptosis in certain cell types (Levine, 1997; Agarwal *et al*., 1998). There are several lines of evidence demonstrating the mechanisms by which p53 induces apoptosis. p53 may use transcriptional activation to regulate the gene products of the pro-apoptotic protein Bax (Miyashita and Reed, 1995) or insulin-like growth factor-binding protein-3 (Buckbinder *et al*., 1995). However, p53-induced apoptosis does not necessarily require transcriptional activation, because p53-mediated apoptosis initiated by DNA damage occurs in the presence of actinomycin D or cycloheximide, which block RNA or protein synthesis (Caelles *et al*., 1994).

Inhibition of translation by cycloheximide could not block apoptosis induced by a temperature-sensitive p53 (Wagner *et al*., 1994). Furthermore, inhibitors of protein phosphatases induce p53-dependent apoptosis in the absence of transactivation (Yan *et al*., 1997). In addition, the introduction of a p53 cDNA fragment that encodes amino acid residues 1–214, and that does not bind to DNA or act as a transcription factor, is able to induce apoptosis (Haupt *et al*., 1995). In general, how p53 implements the apoptotic programme remains to be explored in further studies.

Recent studies show that cytochrome *c* participates in activating the programme of cell death. Addition of exogenous cytochrome *c* to cytosol can trigger apoptotic programmes in a cell-free apoptosis system (Liu *et al*., 1996; Kluck *et al*., 1997a), and microinjection of cytochrome *c* to cytosol also results in induction of apoptosis (F.Li *et al*., 1997). Cytochrome *c* may bind to apoptotic protease activating factor-1 (Apaf-1), which then activates caspase-9 (P.Li *et al*., 1997). The cytochrome *c*-required caspase activation pathway is found to be necessary for diverse stimuli to trigger apoptosis and is unlikely to be cell type dependent. Staurosporine and etoposide led to cytochrome *c* release and caspase-3 activation during induction of apoptosis in HL-60 cells (Yang *et al*., 1997). Irradiation induces release of cytochrome *c* in human U-937 cells and in human multiple myeloma cells (Chauhan *et al*., 1997; Kharbanda *et al*., 1997). Tumour necrosis factor α induces cytochrome *c* redistribution in 293 cells (Duckett *et al*., 1998). It is of note that Bax is able to induce cytochrome *c* release (Jurgensmeier *et al*., 1998; Rosse *et al*., 1998). Since Bax has been shown to be a downstream mediator of p53, it is reasonable to hypothesize that cytochrome *c* may be involved in p53-induced apoptosis. However, it is not yet clear whether cytochrome *c* release is a prerequisite for the induction of apoptosis by p53.

Besides releasing cytochrome *c*, mitochondria may participate in apoptosis by opening their membrane permeability transition pores (MPT), an event that is an early step in the signalling cascade of apoptosis (Green and Reed, 1998). Little information exists about whether p53 needs to trigger MPT in order to induce apoptosis.

Bcl-2 and Bcl-X$_L$ may regulate apoptosis by controlling cytochrome *c* release. For example, release of cytochrome *c* in cells undergoing apoptosis could be prevented by overexpression of Bcl-2 or Bcl-X$_L$, thereby abrogating the activation of caspase-3 (Kharbanda *et al*., 1997; Kluck *et al*., 1997b; Yang *et al*., 1997). It has been proposed that Bcl-2 functions as an antioxidant to prevent apoptosis. It may decrease lipid peroxidation and increase the cell resistance to reactive oxygen species (ROS) (Hockenbery *et al*., 1993; Kane *et al*., 1993). On the other hand, Bcl-2 may block ROS production by regulating the opening of MPT (Marzo *et al*., 1998; Zamzami *et al*., 1998). However,

an opposing proposal indicates that Bcl-2 can protect cells from death independently of ROS, because cell death can be rescued by Bcl-2 effectively even in the absence of ROS (Shimizu *et al.*, 1995). It remains enigmatic whether Bcl-2 prevents p53-induced apoptosis by controlling cytochrome *c* release or by interfering with ROS production.

The aim of our present study was to investigate whether p53-induced apoptosis requires cytochrome *c* release and to assess how Bcl-2 can interfere with p53-induced apoptosis.

Results

p53 induces apoptosis independently of cytochrome c release

p53 was overexpressed in HeLa cells, which have been found to contain very low levels of wild-type p53 (Matlashewski *et al.*, 1986). As shown in Figure 1A, p53 was nearly undetectable in adenovirus β-galactosidase (Adβ-gal)-infected HeLa cells, whereas a time-dependent increase of p53 protein levels could be detected in HeLa cells infected with adenovirus p53 (Adp53). Less than 10% of cells were dead in cultures infected with Adβ-gal. However, a time-dependent increase of cell death could be observed in cells infected with Adp53 (Figure 1B). A cell death ELISA was used to assess whether p53-induced cell death occurred by apoptosis in our present study. As depicted in Figure 1C, DNA fragments were noticeably increased in cells infected with Adp53, but not in those infected with Adβ-gal. In order to estimate the significance of p53-induced apoptosis in our model, we used 1.0 mM hydrogen peroxide, which is known to be a potent pro-apoptotic stimulus (Hampton *et al.*, 1998). Treatment with 1.0 mM hydrogen peroxide resulted in a significant increase of DNA fragmentation (Figure 1C). The amount of DNA fragments is in accordance with the percentage of dead cells. Annexin V was next used to assess apoptosis. p53 induces a time-dependent increase in Annexin V-positive cells (Figure 1D). In addition, Adp53-infected cells demonstrated shrinkage and membrane blebbing (Figure 1E). Taken together, these data suggest that over-expression of p53 induces apoptosis in HeLa cells.

In order to detect whether cytochrome *c* release is involved in p53-induced apoptosis, we examined the distribution of cytochrome *c* after Adp53 infection. Subcellular fractions including either cytosol or mitochondria-enriched heavy membranes (HM) were prepared, and cytochrome *c* protein levels were measured by immunoblotting (Figure 2). The majority of cytochrome *c* remained in the mitochondria-enriched HM and there was no increase of cytochrome *c* levels in the cytosolic fractions after Adp53 infection.

p53-induced apoptosis does not involve the subcellular translocation of Bax and cleavage of Bid

To explore the reasons for the lack of cytochrome *c* release in p53-induced apoptosis, we studied factors known to induce cytochrome *c* release in HeLa cells overexpressing p53. p53 is known to transactivate Bax and therefore it is believed that Bax plays an important role in p53-induced apoptosis. Immunoblot analysis demonstrates that there

was a time-dependent increase of Bax protein levels after Adp53 infection (Figure 3A). However, upregulated Bax in Adp53-infected cells remained largely in the cytosolic fraction and there was no detectable translocation to the mitochondria-enriched HM (Figure 3B).

In order to exclude a lack of pro-apoptotic function of Bax in HeLa cells, we transiently overexpressed Bax in these cells. Overexpression of Bax induced cell death in HeLa cells, which was accompanied by a time-dependent release of cytochrome *c* (data not shown). In contrast to cells overexpressing p53, overexpression of Bax itself resulted in an increase of Bax protein not only in the cytosol but also in the mitochondria-enriched HM (Figure 3C).

Bid, a pro-apoptotic factor causing cytochrome *c* release, is known to be activated by its cleavage. As shown by immunoblotting (Figure 3D), Bid was not cleaved in p53-overexpressing cells. In conclusion, these results suggest that the absence of cytochrome *c* release is probably due to the lack of subcellular translocation of p53-upregulated Bax and/or the inactivation of Bid.

p53-induced apoptosis depends on caspases

Released cytochrome *c* has been characterized as binding to Apaf-1 and then activating caspase-9 (P.Li *et al.*, 1997). It would be of interest to detect whether caspases are still required in p53-induced apoptosis that is devoid of cytochrome *c* release (Figure 2). z-VAD-fmk (benzyloxy-carbonyl-Val-Ala-Asp-fluoromethyl ketone) was used to treat the cells after Adp53 infection. z-VAD-fmk could prevent cell death in a dose-dependent manner with 100 μM z-VAD-fmk almost completely blocking cell death (Figure 4A). To assess the potential involvement of caspase-3, activation of caspase-3 was evaluated by detecting its cleavage by immunoblotting. There was no apparent cleavage of caspase-3 proenzyme and no detectable active p17 in Adp53-infected cells (Figure 4B). However, we did observe the existence of caspase-3-like activity in p53-infected cells by assaying DEVD-pNA (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) cleavage (Figure 4C).

The activation of downstream caspases can also be initiated through activation of caspase-8. We therefore detected capase-8 activity by assessing its ability to cleave IETD-pNA (Ile-Glu-Thr-Asp-*p*-nitroanilide). There was no significant caspase-8 activity in p53-infected cells (data not shown). We detected the expression of Fas after Adp53 infection because Fas is able to activate caspase-8 (Li *et al.*, 1998). Fas expression levels remained unaltered in Adp53-infected cells as detected by immunoblotting (data not shown). In addition, the binding of Fas to FADD (Fas-associated death domain protein), as determined by immunoprecipitation, was found to be unchanged (data not shown). Taken together, these data indicate that caspases other than caspase-8 participate in p53-induced apoptosis.

p53 regulates Δψ through reactive oxygen species

The lack of a role for cytochrome *c* implied the search for alternative downstream targets that are causally involved in p53-induced apoptosis. In the following experiments we investigated the role of ROS and Δψ in this context. We detected intracellular ROS production in HeLa cells overexpressing p53 by staining these cells with the ROS-
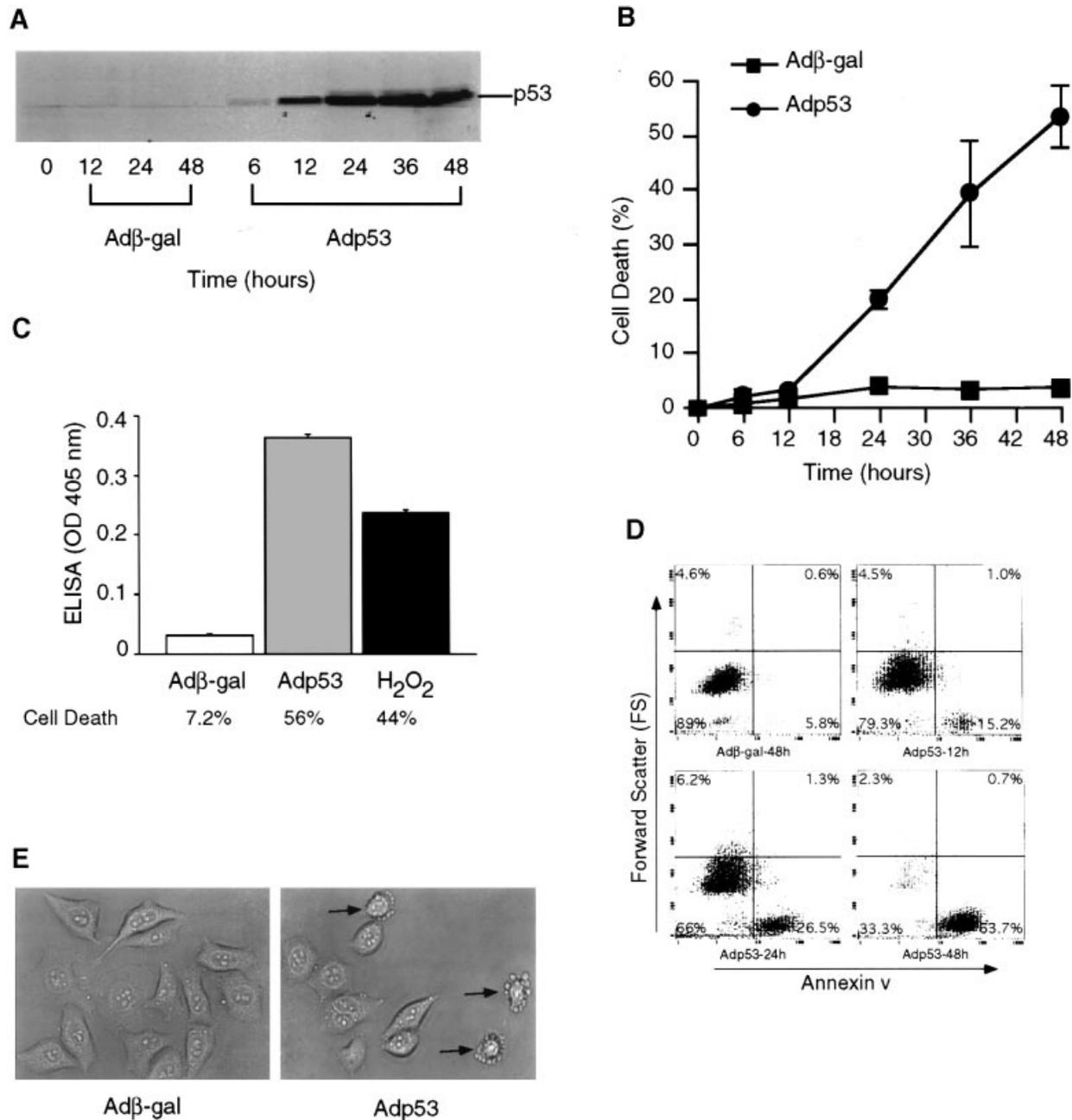
Fig. 1. Overexpression of p53 induces apoptosis. HeLa cells were infected with Adp53 or Adβ-gal at a m.o.i. of 50. (**A**) Detection of p53 expression by immunoblotting. A representative result of three independent experiments is shown. (**B**) Cell death was assessed by Trypan Blue exclusion. Data are expressed as the mean ± SEM of four independent experiments. (**C**) DNA fragmentation analysed by cell death ELISA. Cells treated with 1 mM hydrogen peroxide served as a positive control. Data are expressed as the mean ± SE of one experiment in duplicate, a representative result of three independent experiments. (**D**) Flow cytometry analysis of Annexin V binding. (**E**) Morphological features of cells infected by Adp53. Photographs were taken 24 h after infection. Arrows indicate apoptotic cells. Original magnification 100×.

sensitive fluorescent dyes 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and hydroethidine (HE), respectively. p53 overexpression led to a sharp and transient increase in the intracellular concentration of superoxide peaking at 8 h, as assessed by HE, which was followed by a decrease in the intracellular concentration. Adβ-gal did not induce significant alterations of superoxide concentrations (Figure 5A). In p53-overexpressing cells, hydrogen peroxide, as detected by DCFH-DA, was noticeably elevated at 16 h and reached a peak at 24 h. Adβ-gal led to no

significant alterations of hydrogen peroxide concentrations (Figure 5B). Thus, it seems that the peak in superoxide generation precedes that of hydrogen peroxide generation, although the specificity of DCFH-DA for hydrogen peroxide and that of HE for superoxide is limited. To test whether augmented ROS levels play a role in mediating the death signal of p53, N-acetylcysteine (NAC) and 4,5-dihydroxy-1,3-benzenedisulfonic acid (tiron), two anti-oxidants, were used to treat the cells immediately after Adp53 infection. Both NAC and tiron showed a protective
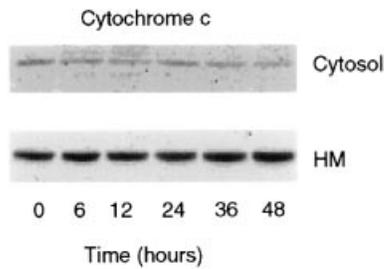
P.-F. Li, R. Dietz and R. von Harsdorf

Cytochrome c

Fig. 2. Overexpression of p53 does not induce cytochrome *c* release. Cytochrome *c* distribution was detected by immunoblotting. Cells were harvested at the time indicated and then subcellular fractions including either cytosol or mitochondria-enriched HM were prepared. A representative result of six independent experiments is shown.
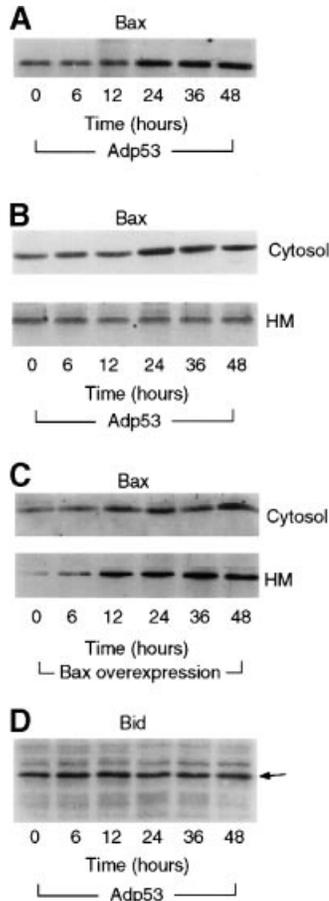

Fig. 3. p53 induces upregulation of Bax. (**A**) Upregulation of Bax by p53. Cells were infected with Adp53 and harvested at the time indicated for the detection of Bax protein levels by immunoblotting. (**B**) Determination of Bax distribution. Subcellular fractions including either cytosol or mitochondria-enriched HM were prepared at the times indicated. Bax protein levels were determined by immunoblotting. Representative results of three independent experiments are shown. (**C**) Overexpression of Bax induces Bax translocation. HeLa cells were transfected with pADBax and subcellular fractions including either cytosol or mitochondria-enriched HM were prepared at the times indicated. Bax was detected by immunoblotting. Representative results of three independent experiments are shown. (**D**) Determination of Bid by immunoblotting.

effect against p53-induced apoptosis in a dose-dependent manner (Figure 5C). These data suggest that the generation of ROS is essential for the induction of apoptosis by p53.

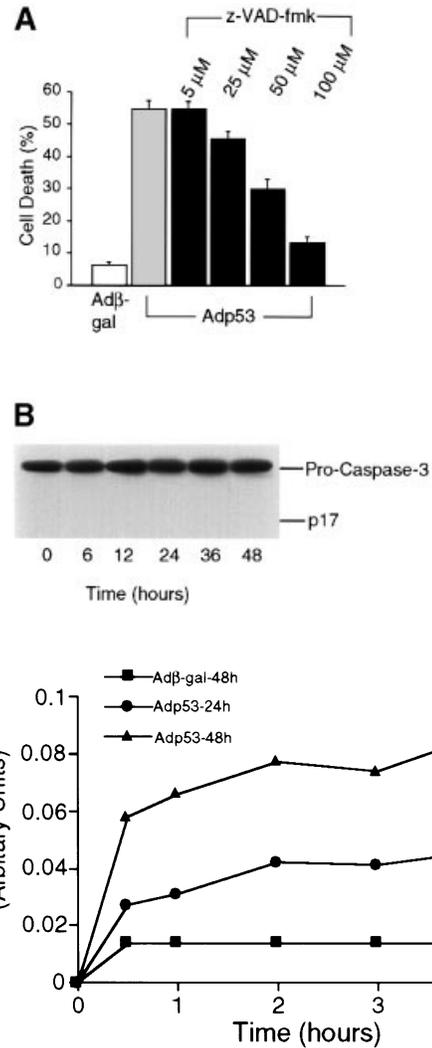We next detected whether p53-induced apoptosis involves alterations of $\Delta\psi$. As shown in Figure 6A, Adβ-

Fig. 4. p53-induced apoptosis is caspase dependent. (**A**) Effect of z-VAD-fmk on cell death. Cell death was assessed by Trypan Blue exclusion. Data are expressed as the mean ± SEM of three independent experiments. (**B**) Analysis of caspase-3 activation by immunoblotting using whole-cell lysates at the time indicated. A representative result of six independent experiments is shown. (**C**) Analysis of caspase-3-like activity by detecting DEVD-pNA cleavage. A representative result of three independent experiments is shown.

gal infection led to no significant change in $\Delta\psi$ detected by 3,3′-dihexyloxacarbocyanine iodide [DiOC$_6$(3)]. However, $\Delta\psi$ began to decrease 24 h after Adp53 infection. Interestingly, there was a transient increase of $\Delta\psi$ 12–24 h after Adp53 infection, preceding the reduction of $\Delta\psi$. Similar results were obtained using tetramethylrhodamine ethyl ester (TMRE) to assess $\Delta\psi$ (data not shown). Administration of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore that dissipates $\Delta\psi$, led to a significant decrease of $\Delta\psi$ either in control cells without adenovirus infection or in cells 16 h after Adp53 infection (Figure 6B), indicating that DiOC$_6$(3) is predominantly located in mitochondria and that the uptake of DiOC$_6$(3) really reflects $\Delta\psi$. To determine whether increased or decreased uptake of DiOC$_6$(3) represents proliferation of mitochondria or originates from a difference in the amount of mitochondria within cells, the cellular content of cytochrome *c* was detected by immunoblotting using
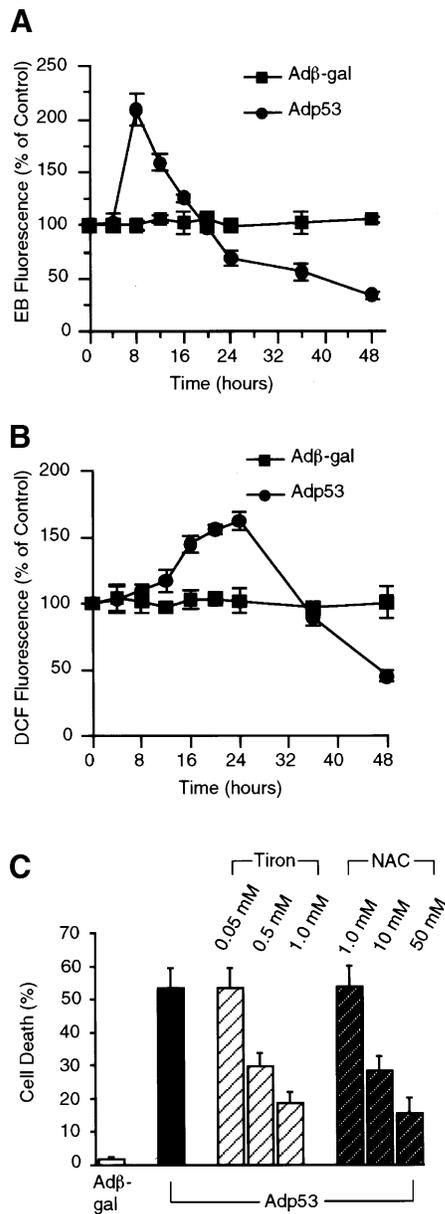
Fig. 5. p53 induces the generation of ROS. ROS were detected with HE (**A**) or DCFH-DA (**B**). The results are expressed as a percentage of the fluorescence intensity compared with uninfected control cells. (**C**) Effects of antioxidants on cell death induced by p53. Cell death was determined by Trypan Blue exclusion 48 h after Adp53 infection. Adβ-gal infection served as a control. Cells were treated with tiron or NAC immediately after Adp53 infection. Data are expressed as the mean ± SEM of four independent experiments.

Fig. 6. p53 induces alterations of $\Delta\psi$ that can be abrogated by antioxidants. (**A**) Analysis of $\Delta\psi$ by $DiOC_6(3)$ at the time indicated after Adβ-gal or Adp53 infection; 1 mM tiron or 50 mM NAC were added immediately after Adp53 infection. The results are expressed as a percentage of the fluorescence intensity compared with uninfected control cells. Data are expressed as the mean ± SEM of three independent experiments. (**B**) Effect of CCCP on $\Delta\psi$. Cells were harvested 16 h after Adp53 infection, then $\Delta\psi$ was detected with $DiOC_6(3)$ in the absence or presence of CCCP. The control cells were treated under the same conditions without Adp53 infection. (**C**) Immunoblotting detection of cytochrome c levels using whole-cell lysates of cells infected with Adp53. A representative result of three independent experiments is shown.

whole-cell lysates. As shown in Figure 6C, cytochrome c levels remained constant after Adp53 infection, indicating that the increased or decreased uptake of $DiOC_6(3)$ is due neither to mitochondrial proliferation nor to an unequal amount of mitochondria.

To determine the relationship between ROS and $\Delta\psi$ in the apoptotic cascade of p53, we used antioxidants to test whether they could influence p53-induced alterations of $\Delta\psi$. The results show that 1 mM tiron or 50 mM NAC, respectively, could inhibit the increase as well as decrease of $\Delta\psi$ (Figure 6A), suggesting that ROS are responsible for the alterations of $\Delta\psi$.
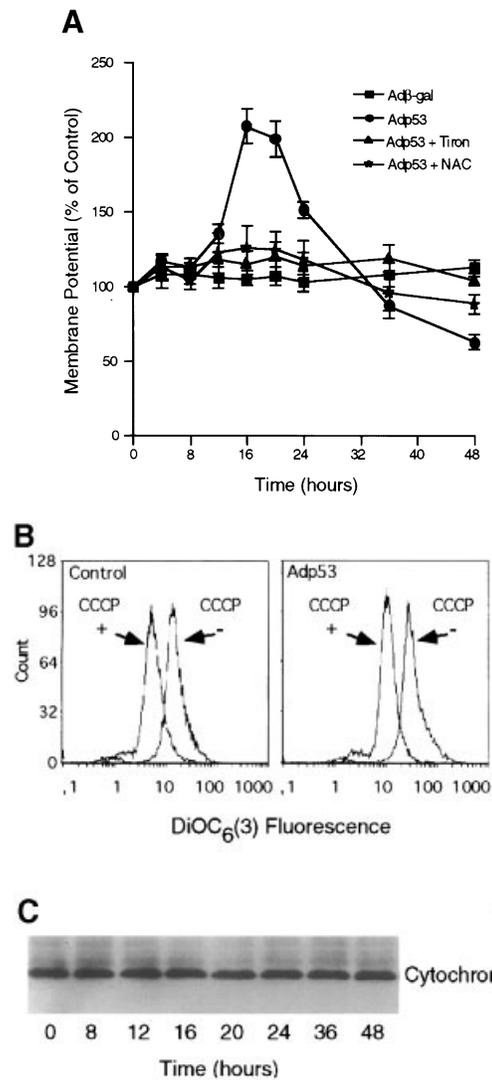
Caspase activation is downstream of ROS production and $\Delta\psi$ elevation but upstream of $\Delta\psi$ decrease

The protective effect of z-VAD-fmk against p53-induced apoptosis led us to test the relationship between caspase activation and ROS production as well as $\Delta\psi$ alterations. z-VAD-fmk at 100 μM, a dose at which cell death could be abolished almost completely (Figure 4A), could prevent neither the production of ROS as detected by HE or DCFH-DA (Figure 7A) nor the increase of $\Delta\psi$ (Figure 7B). However, z-VAD-fmk at 100 μM could abolish the decrease of $\Delta\psi$ (Figure 7C). Also, DNA fragmentation is abrogated completely by 50–100 μM z-VAD-fmk
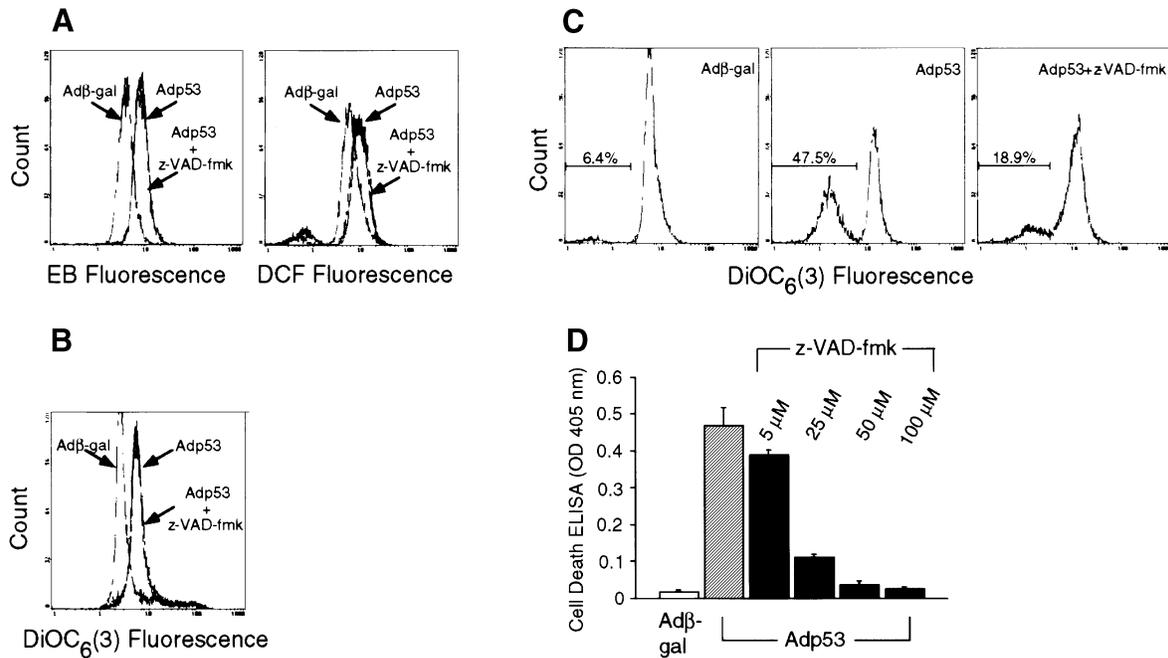
Fig. 7. Effect of z-VAD-fmk on ROS generation and $\Delta\psi$ alterations. (**A**) z-VAD-fmk does not prevent ROS production. ROS were assessed 8 h (HE) or 20 h (DCFH-DA) after Adβ-gal and Adp53 infection. z-VAD-fmk was at 100 µM. (**B**) z-VAD-fmk does not influence $\Delta\psi$ elevation detected by DiOC$_6$(3) 20 h after Adp53 infection. z-VAD-fmk was at 100 µM. (**C**) z-VAD-fmk abolishes the decreases in $\Delta\psi$ detected by DiOC$_6$(3) 48 h after Adp53 infection. z-VAD-fmk was at 100 µM. (**D**) z-VAD-fmk inhibits DNA fragmentation detected by cell death ELISA. Data are expressed as the mean $\pm$ SEM of two independent experiments. Each figure in (A), (B) and (C) is a representative result of three independent experiments.

(Figure 7D). These data suggest that caspases play a role downstream of ROS production and $\Delta\psi$ elevations, and are involved in the decrease of $\Delta\psi$.

Bcl-2 maintains $\Delta\psi$ after ROS generation

To elucidate the effect of Bcl-2 on p53-induced generation of ROS and alterations of $\Delta\psi$, we used HeLa-Bcl-2 cells infected with Adp53. As shown in Figure 8A, Adp53-infected HeLa-Bcl-2 cells underwent apoptosis significantly less than Adp53-infected HeLa-neo cells, suggesting that Bcl-2 could attenuate p53-induced apoptosis. Adβ-gal did not induce significant alterations of $\Delta\psi$ in HeLa-neo cells. Overexpression of p53 in HeLa-neo cells resulted in an increase of $\Delta\psi$ 12–24 h after Adp53 infection and a subsequent decrease of $\Delta\psi$. However, in HeLa-Bcl-2 cells, p53 was unable to cause the elevation and subsequent decrease of $\Delta\psi$ (Figure 8B), suggesting that Bcl-2 may maintain $\Delta\psi$. In addition, in Adp53-infected HeLa-Bcl-2 cells, CCCP could decrease the fluorescence levels of DiOC$_6$(3) significantly, whereas the cellular content of cytochrome c remained unchanged (data not shown). It is thus suggested that the uptake of DiOC$_6$(3) represents $\Delta\psi$ and that the stability of $\Delta\psi$ in HeLa-Bcl-2 cells is not due to the unequal amount of mitochondria.

Finally, we determined whether Bcl-2 regulates $\Delta\psi$ by influencing ROS levels. ROS levels were detected in Adp53-infected and Adβ-gal-infected HeLa-Bcl-2 cells. Adβ-gal infection could not change ethidium bromide (EB) and DCF fluorescence significantly in HeLa-Bcl-2 cells. In contrast, both EB and DCF signals were increased in Adp53-infected HeLa-Bcl-2 cells (Figure 8C and D), and the fluorescence signal was comparable to that obtained in Adp53-infected HeLa cells without Bcl-2 overexpression (Figure 5A and B). These data reveal that

Bcl-2 prevents p53-induced alterations of $\Delta\psi$ downstream of ROS.

Discussion

Our data provide evidence that the regulation of $\Delta\psi$ through the generation of ROS constitutes an important pathway of p53-induced apoptosis. Importantly, this pathway involves neither the subcellular translocation of Bax, which is upregulated by p53, nor the activation of Bid or the release of cytochrome c. Therefore, these results provide a model for cytochrome c-independent induction of apoptosis mediated by p53. Despite the absence of cytochrome c release, caspases are still activated in p53-induced apoptosis and they function downstream of ROS generation and $\Delta\psi$ elevation. Moreover, our observation that Bcl-2 is able to antagonize p53-induced disruption of $\Delta\psi$ downstream of ROS provides a new insight into the anti-apoptotic action of Bcl-2.

Although p53 could upregulate Bax expression, the release of cytochrome c as a consequence of Bax activation could not be detected. In contrast, overexpression of Bax itself resulted in the translocation of cytochrome c from mitochondria to the cytosol. Thus, p53 and Bax may initiate apoptosis using distinct pathways in which cytochrome c is required by Bax, but not p53. Bax in Bax-transfected cells translocated to the mitochondria, whereas Bax in Adp53-infected cells accumulated predominantly in the cytosol. It has been shown that the pro-apoptotic effects of Bax may be elicited through an intrinsic pore-forming activity because it forms channels in synthetic membranes (Antonsson *et al.*, 1997). Such a property of Bax has been shown to regulate the release of cytochrome c. Direct evidence is obtained from an *in vitro* study where addition
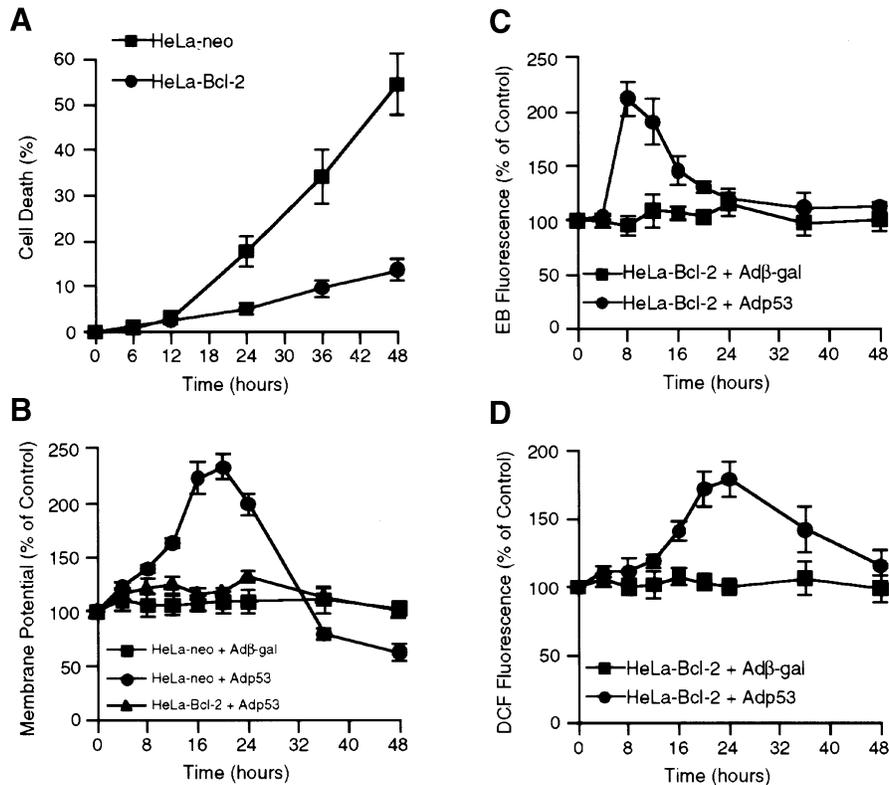
Fig. 8. Effects of Bcl-2 on p53-induced alterations of $\Delta\psi$ and generation of ROS. (**A**) Bcl-2 prevents p53-induced apoptosis. HeLa-neo cells or HeLa-Bcl-2 cells were infected with Adp53 and cell death was assessed by Trypan Blue exclusion at the indicated time. (**B**) Bcl-2 attenuates p53-induced alterations of $\Delta\psi$. $\Delta\psi$ was detected by $DiOC_6(3)$ at the time indicated after Adβ-gal or Adp53 infection. (**C** and **D**) Bcl-2 does not prevent p53-induced generation of ROS detected by HE (C) or DCFH-DA (D). The results are expressed as a percentage of the fluorescence intensity compared with uninfected control cells. Data are expressed as the mean $\pm$ SEM of three independent experiments.

of recombinant Bax protein to isolated mitochondria can induce cytochrome *c* release (Jurgensmeier *et al.*, 1998). An *in vivo* study also indicated that overexpression of Bax results in cytochrome *c* release (Rosse *et al.*, 1998). In addition, Bax has been shown to be located in the cytosol rather than at mitochondrial membranes in cells under physiological conditions. Induction of apoptosis results in the translocation of Bax from the cytosol to mitochondrial membranes, which is a necessary step to trigger apoptosis (Hsu *et al.*, 1997). Thus, the discrepancy between the effects of p53-dependent and -independent expression of Bax may reside in the failure of p53-induced Bax to translocate to the mitochondria, the reason for which remains to be determined. Nevertheless, our data provide an explanation for previous studies reporting Bax-independent apoptosis induced by p53 (Knudson *et al.*, 1995; Brady *et al.*, 1996).

In our study, p53-induced apoptosis involves the activation of caspases. However, the immunoblot analysis demonstrated that caspase-3 was not cleaved, although caspase-3-like activity could be detected. One explanation for such a discrepancy could be that other caspases carrying a catalytic domain similar to caspase-3 may contribute predominantly to the DEVD-pNA cleavage. Downstream caspases are activated not only by the cytochrome *c*–Apaf-1–caspase-9 pathway, but also by the Fas–caspase-8 pathway (Li *et al.*, 1998; Luo *et al.*, 1998). It appears that the Fas–caspase-8 pathway is absent from our model. Although it has been shown that p53 is able to induce the translocation of Fas from the cytosol to the

cell surface, resulting in increases in Fas–FADD binding in vascular smooth muscle cells (Bennett *et al.*, 1998), this is unlikely to occur in our case, since an increase of Fas–FADD binding could not be detected. Furthermore, p53 was unable to upregulate Fas expression in our hands. The lack of activation of caspase-8 and Bid further suggests the absence of a functional Fas pathway, since it would include both the activation of caspase-8 and cleavage of Bid (Li *et al.*, 1998; Luo *et al.*, 1998). It is not surprising that caspase-3-like activity is detectable in the absence of caspase-8 activation and cytochrome *c* release, because caspase-3 could be activated by other caspases such as caspase-6 (Xanthoudakis *et al.*, 1999). Active caspase-3 or caspase-7 create an amplification loop activating upstream caspases (Sun *et al.*, 1999; Xanthoudakis *et al.*, 1999). Future studies should reveal which caspases are activated by p53.

To date, there is no direct evidence indicating that caspase activation requires ROS. Instead, caspases may regulate ROS generation. For example, inhibition of caspases can block ROS production (Kruman *et al.*, 1998; Tan *et al.*, 1998). On the other hand, caspases may function as negative regulators of ROS production because caspase activation can counteract ROS generation (Vercammen *et al.*, 1998). Notably, ROS may even lead to the inactivation of caspases (Hampton and Orrenius, 1997). Our observation that z-VAD-fmk could not prevent ROS production indicates that ROS production is upstream of caspase activation in p53-induced apoptosis. This is consistent with previous work indicating that ROS production

is an early event, whereas the cleavage of poly(ADP-ribose) polymerase is a late event in p53-induced apoptosis (Polyak *et al*., 1997). Our recent work also reveals that ROS-induced apoptosis requires the activation of caspases and that the spectrum of activated caspases is ROS-type dependent (von Harsdorf *et al*., 1999).

Our data suggest the involvement of mitochondria in p53-induced apoptosis with $\Delta\psi$ being the target of p53. p53 induces a transient increase of $\Delta\psi$ followed by a decrease. $\Delta\psi$ is a component of the overall proton motive force that drives ATP production in mitochondria. Its elevation has been shown to have implications for a variety of pathophysiological conditions, in particular for apoptosis. Induction of apoptosis by alkali results in an elevated level of $\Delta\psi$ and overexpression of manganese superoxide dismutase restores $\Delta\psi$, thereby preventing apoptosis (Majima *et al*., 1998). The elevation of $\Delta\psi$ has also been observed in apoptosis triggered by growth factor withdrawal and Bcl-X_L was shown to prevent the increase of $\Delta\psi$, thereby inhibiting apoptosis (Vander Heiden *et al*., 1997). Our results, which indicate that antioxidants prevent the increase of $\Delta\psi$, suggest that ROS may be responsible for the elevation of $\Delta\psi$. This is consistent with a recent report showing that superoxide is able to augment $\Delta\psi$ (Majima *et al*., 1998). The increase of $\Delta\psi$ is an early event in the apoptotic cascade and may be a sign of mitochondrial swelling and disruptions of the outer mitochondrial membrane (Vander Heiden *et al*., 1997). Most recently, the biochemical basis of the increase of $\Delta\psi$ in apoptosis has been demonstrated to be due to a failure to exchange ADP for ATP. Cells with a high $\Delta\psi$ are committed to undergoing apoptosis, whereas those with a low $\Delta\psi$ are capable of exiting the apoptotic pathway (Heiden *et al*., 1999).

Interestingly, z-VAD-fmk is unable to block the increase of $\Delta\psi$, but it can abrogate the decrease of $\Delta\psi$ (Figure 7B and C). These data indicate that $\Delta\psi$ elevation and $\Delta\psi$ decrease are two independent events, or that they are taking place at two different stages of the apoptotic programme in which caspase activation is an intermediate step. Such a phenomenon is not restricted to p53-induced apoptosis, since most recently apoptosis induced by Fas was shown to involve a similiar pattern of alterations of the mitochondrial membrane potential (Banki *et al*., 1999). Here, z-VAD-fmk only prevents the decrease of $\Delta\psi$ and not its elevation. Thus, p53-induced $\Delta\psi$ hyperpolarization is upstream of $\Delta\psi$ decrease. Our present work and that of others (Majima *et al*., 1998; Banki *et al*., 1999) shows that ROS are responsible for $\Delta\psi$ elevation. Superoxide generation reached a peak 8 h after Adp53 infection (Figure 5A), a time at which $\Delta\psi$ started to increase (Figure 6A). The protective effect of tiron against $\Delta\psi$ elevation suggests that superoxide triggers $\Delta\psi$ elevation. In the subsequent cascade, hydrogen peroxide served as a substitute for superoxide to keep $\Delta\psi$ at high levels (Figures 5B and 6A). The ROS production pattern is probably related to the biochemical property of each species with superoxide having a short lifespan but hydrogen peroxide having a long one.

Our results demonstrate for the first time that Bcl-2 may counteract p53-induced apoptosis by maintaining $\Delta\psi$. Bcl-2 has been shown to elevate $\Delta\psi$ from low levels either by enhancing H^+ efflux (Shimizu *et al*., 1998) or

by preventing the opening of MPT (Zamzami *et al*., 1998). Here we found that Bcl-2 stabilizes $\Delta\psi$ by preventing both the increase and decrease of $\Delta\psi$ induced by p53. Since p53 is still able to induce the generation of ROS in Bcl-2-overexpressing cells, Bcl-2 appears to exert its stabilizing effect on $\Delta\psi$ downstream of ROS. This is consistent with previous observations indicating that the anti-apoptotic action of Bcl-2 does not involve the reduction of ROS levels (Satoh *et al*., 1996, 1997). The inability of Bcl-2 to block ROS production may be related to the sources of ROS production. Bcl-2 can prevent ROS production when it is induced by cytochrome *c* release (Cai and Jones, 1998), or by the opening of MPT (Zamzami *et al*., 1998), because Bcl-2 blocks cytochrome *c* release and MPT opening. Thus, Bcl-2 may function at multiple levels and it elicits its anti-apoptotic effect in a manner largely dependent on the stimuli and apoptotic pathways.

Our present work provides evidence that mitochondria participate in p53-induced apoptosis by the alteration of $\Delta\psi$. However, the biochemical mechanisms behind the disruption of $\Delta\psi$ remain to be identified. In this regard, it would be of great interest to understand how ROS lead to the alteration of $\Delta\psi$ and by which means Bcl-2 interacts with this process. Futhermore, the interpretation of our results is limited to the specific model used, which is the overexpression of p53. Therefore, there is a great need for future studies employing different models of p53-dependent apoptosis in order to understand the signalling in p53-induced apoptosis fully.

Materials and methods

Materials
Cell death detection ELISA kit and G418 were purchased from Boehringer Mannheim. Polyclonal anti-Bcl-2 antibody, polyclonal anti-Bax antibody, anti-caspase-3 polyclonal antibody and anti-cytochrome *c* monoclonal antibody were from Pharmingen. Anti-p53 monoconal antibody and z-VAD-fmk were from Calbiochem. Anti-Fas and FADD antibodies were from Transduction Laboratories. Anti-Bid antibody was from Santa Cruz. Caspase-8 assay kit and Annexin V kit were from R&D System. Caspase-3 assay kit was from Biomol. HE and TMRE were from Molecular Probes Inc. CCCP, $DiOC_6(3)$, DCFH-DA, NAC and tiron were from Sigma (St Louis, MO). The pADBax plasmid harbouring the human full-length Bax cDNA driven by the cytomegalovirus (CMV) promoter, the pEBS7-425-Bcl-2 plasmid containing the human full-length Bcl-2 cDNA driven by the CMV promoter and the pCMVneo plasmid were kindly provided by Dr Karsten Brand, Max-Delbrück-Center, Berlin.

Cell culture
HeLa cells (HeLa 229, No. CCL-2.1, American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cell death was determined by Trypan Blue exclusion. Cells were harvested by trypsinization and washed in phosphate-buffered saline (PBS). The numbers of Trypan Blue-positive and -negative cells were counted on a haemocytometer.

Adp53 infection
The adenoviral vector contains deletions of the E1A and E1B region, is replication deficient and harbours human wild-type p53 cDNA under the control of the human CMV promoter (Adp53) (Sandig *et al*., 1997). The identical vector was used to create the adenoviral β-galactosidase construct (Adβ-gal) (Sandig *et al*., 1997). Viruses were amplified in 293 cells and purified on a CsCl gradient. HeLa cells were infected at a multiplicity of infection (m.o.i.) of 50 for 90 min. After washing with PBS, culture medium was added and cells were cultured until the indicated time. For the administration of z-VAD-fmk, cells were treated

immediately after Adp53 infection. After 24 h the inhibitor was added once more. For the treatments with antioxidants, NAC and tiron were added immediately after Adp53 infection.

Transfection with pADBax

Cells that had been passaged the previous day were transfected by the calcium phosphate precipitation method (Sambrook *et al.*, 1989). Plasmid DNA (20 μg) was used for the transfection of 1×10^6 cells. The calcium phosphate–DNA precipitates were left on the cells for 24 h. The control cells were transfected with a control plasmid containing no Bax cDNA using the same method.

Establishment of HeLa cells stably overexpressing Bcl-2

Cells were co-transfected with pEBS7-425-Bcl-2 and pCMVneo at a ratio of 10:1 by the method of calcium phosphate precipitation (Sambrook *et al.*, 1989). Transfected cells were selected in medium containing 1 mg/ml G418 for 30 days and Bcl-2 expression was determined using immunoblotting analysis as described below. The cells stably expressing Bcl-2 are referred to as HeLa-Bcl-2 cells. The cells only transfected with pCMVneo served as a control and are referred to as HeLa-neo cells.

Analysis of DNA fragmentation

DNA fragmentation was detected by cell death detection ELISA, which was performed according to the manufacturer's instructions and as described previously (P.F.Li *et al.*, 1997).

Immunoblotting analysis

Cells were lysed for 1 h at 4°C in a lysis buffer [20 mM Tris pH 7.5, 2 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol (DTT), 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 10 μg/ml each of leupeptin, aprotinin and pepstatin A]. Samples containing 30 μg of protein were subjected to 12% SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Equal protein loading was controlled by Ponceau Red (Sigma) staining of membranes. Blots were probed using primary antibodies, followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG. Antigen–antibody complexes were visualized by enhanced chemiluminescence.

Preparation of subcellular fractions

Cells were washed twice with PBS and the pellet was suspended in 0.5 ml of buffer (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml each of leupeptin, aprotinin and pepstatin A) containing 250 mM sucrose. The cells were homogenized by 10 strokes in a Dounce homogenizer. The homogenates were centrifuged twice at 750 *g* for 5 min at 4°C to collect nuclei and debris. The supernatants were centrifuged at 10 000 *g* for 15 min at 4°C to collect the HM pellet. The resulting supernatants were centrifuged at 100 000 *g* for 1 h at 4°C to yield light membrane pellets (LM). The final supernatants are referred to as cytosolic fractions.

Flow cytometry analysis of cell death

Cells were labelled with Annexin V according to the manufacturer's instructions. Samples (10^4 events) were analysed with a flow cytometer (Coulter Epics) and the distribution of cells was determined using Multicycle Software (Coulter EPICS XL/XL-MCL System II).

Detection of caspase-3 and caspase-8 activity

Caspase-3 and caspase-8 activity were detected using assay kits. The assay procedures were followed according to the kit instructions. Protein concentration was determined using a Bio-Rad protein assay kit.

Detection of ROS and Δψ

HE and DCFH-DA were employed to measure ROS. HE and DCFH-DA have been shown to be relatively specific for superoxide and hydrogen peroxide, respectively (Rothe and Valet, 1990; Narayanan *et al.*, 1997). Superoxide is able to oxidize HE to yield EB and hydrogen peroxide is able to oxidize DCFH to the fluorescent DCF. Cells were harvested and suspended in DMEM medium with 0.2% fetal calf serum. ROS probes were then added and incubated for 30 min at 37°C. The final concentrations of HE and DCF were 10 and 20 μM, respectively. For the detection of Δψ, 40 nM $DiOC_6(3)$ or 100 nM TMRE in the absence or presence of CCCP at 50 μM was added and incubated for 15 min at 37°C. The fluorescence was measured by flow cytometry (Coulter Corp., Miami, FL).

Statistical analysis

The results are expressed as the mean ± SEM of at least three independent experiments, unless stated otherwise. Paired data were evaluated by Student's *t*-test. A one-way ANOVA was used for multiple comparisons. A value of $p < 0.05$ was considered significant.

References

Agarwal,M.L., Taylor,W.R., Chernov,M.V., Chernova,O.B. and Stark,G.R. (1998) The p53 network. *J. Biol. Chem.*, **273**, 1–4.

Antonsson,B. *et al.* (1997) Inhibition of Bax channel-forming activity by Bcl-2. *Science*, **277**, 370–372.

Banki,K., Hutter,E., Gonchoroff,N.J. and Perl,A. (1999) Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling. *J. Immunol.*, **162**, 1466–1479.

Bennett,M., Macdonald,K., Chan,S.W., Luzio,J.P., Simari,R. and Weissberg,P. (1998) Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science*, **282**, 290–293.

Brady,H.J.M., Salomons,G.S., Bobeldijk,R.C. and Berns,A.J.M. (1996) T cells from *bax*α transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53. *EMBO J.*, **15**, 1221–1230.

Buckbinder,L., Talbott,R., Velasco-Miguel,S., Takenaka,I., Faha,B., Seizinger,B.R. and Kley,N. (1995) Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature*, **377**, 646–649.

Caelles,C., Helmberg,A. and Karin,M. (1994) p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature*, **370**, 220–223.

Cai,J. and Jones,D.P. (1998) Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome *c* loss. *J. Biol. Chem.*, **273**, 11401–11404.

Chauhan,D. *et al.* (1997) Cytochrome *c*-dependent and -independent induction of apoptosis in multiple myeloma cells. *J. Biol. Chem.*, **272**, 29995–29997.

Duckett,C.S., Li,F., Wang,Y., Tomaselli,K.J., Thompson,C.B. and Armstrong,R.C. (1998) Human IAP-like protein regulates programmed cell death downstream of Bcl-x$_L$ and cytochrome *c*. *Mol. Cell. Biol.*, **18**, 608–615.

Green,D.R. and Reed,J.C. (1998) Mitochondria and apoptosis. *Science*, **281**, 1309–1312.

Hampton,M.B. and Orrenius,S. (1997) Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. *FEBS Lett.*, **414**, 552–556.

Hampton,M.B., Fadeel,B. and Orrenius,S. (1998) Redox regulation of the caspases during apoptosis. *Ann. NY Acad. Sci.*, **854**, 328–335.

Haupt,Y., Rowan,S., Shaulian,E., Vousden,K.H. and Oren,M. (1995) Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes Dev.*, **9**, 2170–2183.

Heiden,M.G., Chandel,N.S., Schumacker,P.T. and Thompson,C.B. (1999) Bcl-x$_L$ prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol. Cell*, **3**, 159–167.

Hockenbery,D.M., Oltvai,Z.N., Yin,X.M., Milliman,C.L. and Korsmeyer,S.J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, **75**, 241–251.

Hsu,Y.T., Wolter,K.G. and Youle,R.J. (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X$_L$ during apoptosis. *Proc. Natl Acad. Sci. USA*, **94**, 3668–3672.

Jurgensmeier,J.M., Xie,Z., Deveraux,Q., Ellerby,L., Bredesen,D. and Reed,J.C. (1998) Bax directly induces release of cytochrome *c* from isolated mitochondria. *Proc. Natl Acad. Sci. USA*, **95**, 4997–5002.

Kane,D.J., Sarafian,T.A., Anton,R., Hahn,H., Gralla,E.B., Valentine,J.S., Ord,T. and Bredesen,D.E. (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science*, **262**, 1274–1277.

Kharbanda,S. *et al.* (1997) Role for Bcl-x$_L$ as an inhibitor of cytosolic cytochrome *c* accumulation in DNA damage-induced apoptosis. *Proc. Natl Acad. Sci. USA*, **94**, 6939–6942.

Kluck,R.M., Martin,S.J., Hoffman,B.M., Zhou,J.S., Green,D.R. and

Newmeyer,D.D. (1997a) Cytochrome *c* activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system. *EMBO J.*, **16**, 4639–4649.

Kluck,R.M., Bossy-Wetzel,E., Green,D.R. and Newmeyer,D.D. (1997b) The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*, **275**, 1132–1136.

Knudson,C.M., Tung,K.S., Tourtellotte,W.G., Brown,G.A. and Korsmeyer,S.J. (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*, **270**, 96–99.

Kruman,I., Guo,Q. and Mattson,M.P. (1998) Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells. *J. Neurosci. Res.*, **51**, 293–308.

Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.

Li,F., Srinivasan,A., Wang,Y., Armstrong,R.C., Tomaselli,K.J. and Fritz,L.C. (1997) Cell-specific induction of apoptosis by microinjection of cytochrome *c*. Bcl-x$_L$ has activity independent of cytochrome *c* release. *J. Biol. Chem.*, **272**, 30299–30305.

Li,H., Zhu,H., Xu,C.J. and Yuan,J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*, **94**, 491–501.

Li,P., Nijhawan,D., Budihardjo,I., Srinivasula,S.M., Ahmad,M., Alnemri,E.S. and Wang,X. (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, **91**, 479–489.

Li,P.F., Dietz,R. and von Harsdorf,R. (1997) Differential effect of hydrogen peroxide and superoxide anion on apoptosis and proliferation of vascular smooth muscle cells. *Circulation*, **96**, 3602–3609.

Liu,X., Kim,C.N., Yang,J., Jemmerson,R. and Wang,X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell*, **86**, 147–157.

Luo,X., Budihardjo,I., Zou,H., Slaughter,C. and Wang,X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. *Cell*, **94**, 481–490.

Majima,H.J., Oberley,T.D., Furukawa,K., Mattson,M.P., Yen,H.C., Szweda,L.I. and St Clair,D.K. (1998) Prevention of mitochondrial injury by manganese superoxide dismutase reveals a primary mechanism for alkaline-induced cell death. *J. Biol. Chem.*, **273**, 8217–8224.

Marzo,I. *et al.* (1998) The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins. *J. Exp. Med.*, **187**, 1261–1271.

Matlashewski,G., Banks,L., Pim,D. and Crawford,L. (1986) Analysis of human p53 proteins and mRNA levels in normal and transformed cells. *Eur. J. Biochem.*, **154**, 665–672.

Miyashita,T. and Reed,J.C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*, **80**, 293–299.

Narayanan,P.K., Goodwin,E.H. and Lehnert,B.E. (1997) α particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Res.*, **57**, 3963–3971.

Polyak,K., Xia,Y., Zweier,J.L., Kinzler,K.W. and Vogelstein,B. (1997) A model for p53-induced apoptosis. *Nature*, **389**, 300–305.

Rosse,T., Olivier,R., Monney,L., Rager,M., Conus,S., Fellay,I., Jansen,B. and Borner,C. (1998) Bcl-2 prolongs cell survival after Bax-induced release of cytochrome *c*. *Nature*, **391**, 496–499.

Rothe,G. and Valet,G. (1990) Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2′,7′-dichlorofluorescin. *J. Leukoc. Biol.*, **47**, 440–448.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandig,V., Brand,K., Herwig,S., Lukas,J., Bartek,J. and Strauss,M. (1997) Adenovirally transferred p16INK4/CDKN2 and p53 genes cooperate to induce apoptotic tumor cell death. *Nature Med.*, **3**, 313–319.

Satoh,T., Sakai,N., Enokido,Y., Uchiyama,Y. and Hatanaka,H. (1996) Survival factor-insensitive generation of reactive oxygen species induced by serum deprivation in neuronal cells. *Brain Res.*, **733**, 9–14.

Satoh,T., Enokido,Y., Aoshima,H., Uchiyama,Y. and Hatanaka,H. (1997) Changes in mitochondrial membrane potential during oxidative stress-induced apoptosis in PC12 cells. *J. Neurosci. Res.*, **50**, 413–420.

Shimizu,S., Eguchi,Y., Kosaka,H., Kamiike,W., Matsuda,H. and Tsujimoto,Y. (1995) Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-x$_L$. *Nature*, **374**, 811–813.

Shimizu,S., Eguchi,Y., Kamiike,W., Funahashi,Y., Mignon,A., Lacronique,V., Matsuda,H. and Tsujimoto,Y. (1998) Bcl-2 prevents

apoptotic mitochondrial dysfunction by regulating proton flux. *Proc. Natl Acad. Sci. USA*, **95**, 1455–1459.

Sun,X.M., MacFarlane,M., Zhuang,J., Wolf,B.B., Green,D.R. and Cohen,G.M. (1999) Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J. Biol. Chem.*, **274**, 5053–5060.

Tan,S., Sagara,Y., Liu,Y., Maher,P. and Schubert,D. (1998) The regulation of reactive oxygen species production during programmed cell death. *J. Cell Biol.*, **141**, 1423–1432.

Vander Heiden,M.G., Chandel,N.S., Williamson,E.K., Schumacker,P.T. and Thompson,C.B. (1997) Bcl-x$_L$ regulates the membrane potential and volume homeostasis of mitochondria. *Cell*, **91**, 627–637.

Vercammen,D., Beyaert,R., Denecker,G., Goossens,V., Van Loo,G., Declercq,W., Grooten,J., Fiers,W. and Vandenabeele,P. (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J. Exp. Med.*, **187**, 1477–1485.

von Harsdorf,R., Li,P. and Dietz,R. (1999) Signaling pathways in reactive oxygen species-induced cardiomyocyte apoptosis. *Circulation*, **99**, 2934–2941.

Wagner,A.J., Kokontis,J.M. and Hay,N. (1994) Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21$^{waf1/cip1}$. *Genes Dev.*, **8**, 2817–2830.

Xanthoudakis,S. *et al.* (1999) Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. *EMBO J.*, **18**, 2049–2056.

Yan,Y., Shay,J.W., Wright,W.E. and Mumby,M.C. (1997) Inhibition of protein phosphatase activity induces p53-dependent apoptosis in the absence of p53 transactivation. *J. Biol. Chem.*, **272**, 15220–15226.

Yang,J., Liu,X., Bhalla,K., Kim,C.N., Ibrado,A.M., Cai,J., Peng,T.I., Jones,D.P. and Wang,X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science*, **275**, 1129–1132.

Zamzami,N., Marzo,I., Susin,S.A., Brenner,C., Larochette,N., Marchetti,P., Reed,J., Kofler,R. and Kroemer,G. (1998) The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mitochondrial permeability transition. *Oncogene*, **16**, 1055–1063.