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Oxidative stress attenuates Fas-mediated apoptosis in Jurkat T cell line through Bfl-1 induction

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Many types of mammalian cells produce ROS in response to many different stimuli to modulate a number of cellular functions, including apoptosis. However, the correlation between ROS and apoptosis remains controversial, and the mechanisms whereby ROS-induced signals are propagated to critical downstream targets remain largely undefined. Here, we demonstrate that hydrogen peroxide (H_2O_2) upregulates the expression of Bfl-1, an antiapoptotic member of the Bcl-2 family, and that this is responsible for the antiapoptotic activity of ROS. When Jurkat, human leukemic T cells, were pretreated with 100 μM H_2O_2 and then treated with anti-Fas antibody, apoptosis was impaired without change of cell surface Fas expression. An investigation of the expression patterns of Bcl-2 family genes revealed that H_2O_2 treatment induced Bfl-1 gene expression, but left other genes unchanged, and this Bfl-1 expression and H_2O_2-induced antiapoptotic effect was inhibited by antioxidants or NF-κB inhibitor. In addition, an electromobility shift assay revealed that the p65/p50 subunits of NF-κB activated by H_2O_2 bound to a *bfl-1* promoter. Neither the induction of Bfl-1 nor the antiapoptotic effect of H_2O_2 was detected in Bfl-1-knockdown Jurkat cell line containing Bfl-1 antisense (Bfl-1AS). These data indicate that oxidative stress induces the expression of Bfl-1 via NF-κB activation, and this early-response gene protects cells from Fas-mediated apoptosis. This may be a cellular survival mechanism of cells exposed to phagocytes-derived ROS.
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Introduction

Apoptosis is a pivotal physiological process, which occurs during fetal development and in adult tissues. Many proteins including the Bcl-2 family are associated with this process and they play an essential role in the apoptotic machinery that maintains tissue homeostasis (Gross *et al*., 1999). In humans, about 20 members of the Bcl-2 family have been described. Bfl-1, an antiapoptotic member of Bcl-2 family, is an early-response gene and its expression is rapidly and transiently induced by specific stimuli, such as, inflammatory cytokines like TNF-α or IL-β, and by phorbol ester or LPS (Moreb and Schweder, 1997; Busuttil *et al*., 2002).

ROS are a group of reactive oxygen species that include oxygen anions and radicals or milder oxidants, such as H_2O_2. Many types of mammalian cells produce ROS, including H_2O_2, in response to different stimuli. Endogenous H_2O_2 is produced by mitochondria during the reduction of O_2 to H_2O during respiration. Substantial amounts of H_2O_2 are also produced and secreted by activated neutrophils and macrophages via the NADPH oxidase system at sites of inflammation (Baggiolini *et al*., 1993). Both exogenous H_2O_2 and endogenously produced H_2O_2 have been shown to affect a number of cellular functions, including gene activation, proliferation, and apoptosis (Kamata and Hirata, 1999). Moreover, many studies have revealed that the treatment of cells *in vitro* with H_2O_2 causes the activation of caspase, poly (ADP)-ribosylation, DNA strands breaks, the oxidation of proteins, the depletion of cellular energy stores, and apoptosis (Berlett and Stadtman, 1997). In recent years, however, it has been known that sublethal doses of H_2O_2 in the local cellular environment act as a secondary messenger to critically regulate cell functions, and it has an antiapoptotic and mitogenic effects (Reth, 2002; Preston *et al*., 2003). H_2O_2 mediates a number of cell signaling events by affecting protein kinases, phosphatases, and/or transcription factors, and it changes gene expressions (Allen and Tresini, 2000; Xu *et al*., 2002).

In T cells, activated phagocytes-derived H_2O_2 has been shown to decrease the expression of the CD3 ζ chain of the TCR signaling complex, resulting in the impairment of T-cell functions (Otsuji *et al*., 1996). Other studies have suggested that there is a dose window of oxidative stress leading to this T-cell unresponsiveness in the absence of apoptosis (Malmberg *et al*., 2001). In addition, it has been reported that there is a reciprocal relationship between ROS and Bcl-2 levels in Jurkat T cells (Pugazhenthi *et al*., 2003), and Bcl-2 overexpression is

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known to protect cells from ROS-induced cell death (Hildeman *et al*., 2003). Therefore, it is possible that ROS modulate cell viability by changing the levels of antiapoptotic and/or proapoptotic Bcl-2 family members in a concentration-dependent manner. H_2O_2 is continuously produced *in vivo* and achieves a steady state because of its rapid exhaustion. Accordingly, cells exposed to low concentrations of H_2O_2 for a short period can modify their cellular states and change their fates when they encounter with other extracellular signals, including apoptotic stimuli. In this study, we investigated whether oxidative stress can change the susceptibility of cells to an apoptotic stimulus via the transcriptional regulation of Bcl-2 family protein, and we discussed the intracellular mechanism mediating this effect. Here, we demonstrated that low doses of H_2O_2 upregulated antiapoptotic Bfl-1 gene expression in the Jurkat T cell line, and that the expression of this early-response gene prevents cells from Fas-mediated apoptosis.

Results

Attenuation of Fas-mediated apoptosis by H_2O_2 pretreatment via mitochondrial apoptotic pathway inhibition

In order to investigate the effect of H_2O_2 on Fas-mediated apoptosis in Jurkat T cells, we first examined the optimal H_2O_2 concentration and incubation time whereby H_2O_2 itself has little effect on cell viability. Jurkat T cells were treated with various concentrations of H_2O_2 for 6, 12, and 24 h and apoptosis was measured by FACS. As described by others (Finkel and Holbrook, 2000), higher levels of H_2O_2 ($>100\,\mu$M) induced apoptosis, but lower levels of H_2O_2 ($<100\,\mu$M) had little effect on cell viability (Figure 1a). Therefore, the treatment of Jurkat cells with $100\,\mu$M H_2O_2 for 6 h was determined as an optimal condition for the induction of specific signals without inducing considerable cellular

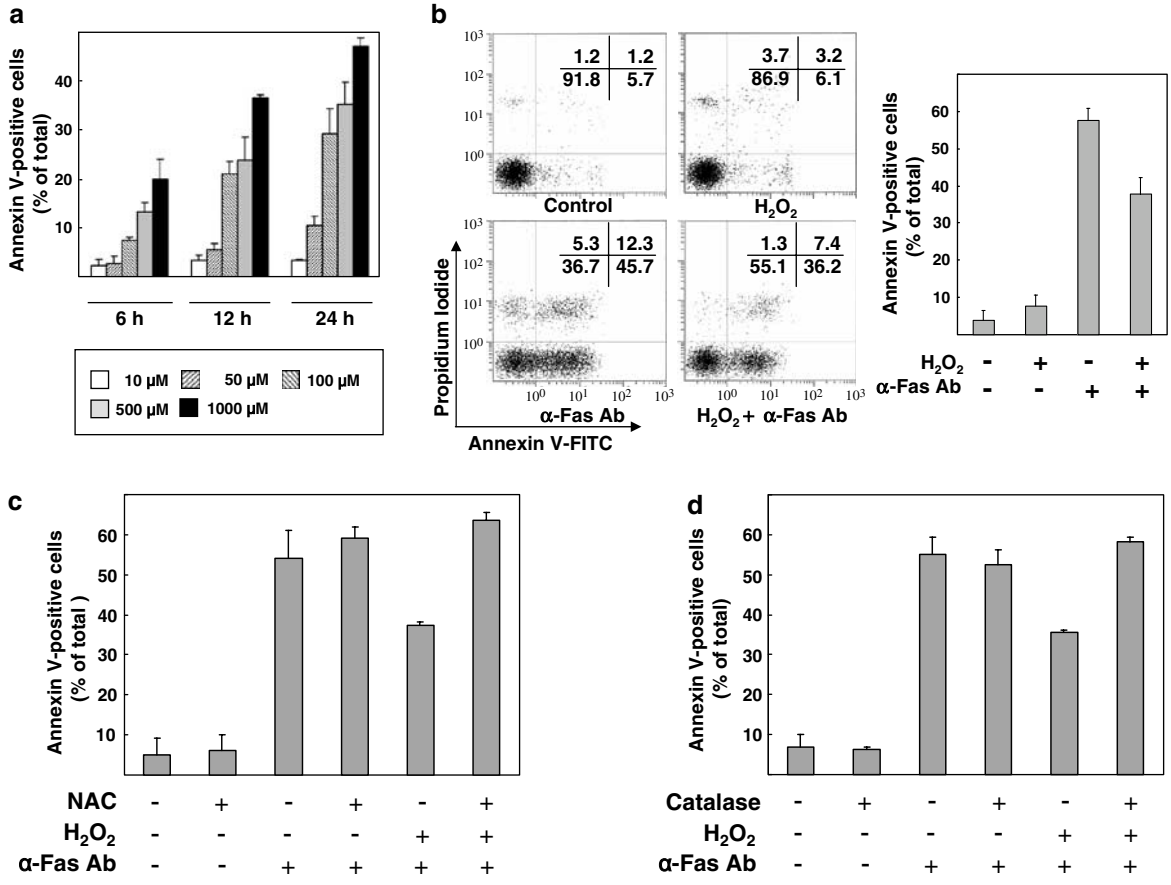

Figure 1 Attenuation of Fas-mediated apoptosis by H_2O_2 pretreatment. (**a**) Effects of H_2O_2 on Jurkat T-cell viability. Jurkat T cells were treated with various concentrations of H_2O_2 for the indicated times and then stained with Annexin V/propidium iodide (PI) and subjected to FACS analysis. The average percentages of Annexin V-positive cells from three independent experiments were presented graphically with s.e. as indicated. (**b**) Effects of H_2O_2 on Fas-mediated apoptosis. Jurkat T cells were pretreated with $100\,\mu$M of H_2O_2 for 6 h. After washing and incubating with 50 ng/ml of anti-Fas antibody for 12 h, cells were harvested and stained with Annexin V/PI, followed by FACS analysis. (**c**) Inhibition of the antiapoptotic effect of H_2O_2 by *N*-acetyl cysteine (NAC). NAC-pretreated cells (5 mM) were incubated with or without H_2O_2, and then stimulated with anti-Fas antibody and analysed by Annexin V/PI-FACS. (**d**) Inhibition of the effect of H_2O_2 on Fas-mediated apoptosis by catalase. Catalase-pretreated cells (1000 U/ml) were treated with or without H_2O_2, and then stimulated with anti-Fas antibody, followed by FACS analysis of Annexin V/PI. Similar results were obtained in three different experiments

damage. To examine whether oxidative stress can influence the death receptor-mediated apoptosis of Jurkat cells, cells were stimulated for 6 h with or without 100 μM H_2O_2. After washing, H_2O_2-pretreated cells were incubated with 50 ng/ml of anti-Fas antibody for 12 h, and then subjected to FACS analysis to determine the level of apoptosis induced by Fas receptor activation. As shown in Figure 1b, Fas receptor activation caused apoptotic cell death in 58%, but H_2O_2 pretreatment significantly attenuated Fas-mediated apoptosis. When Jurkat cells were treated with various concentrations of anti-Fas antibody (> 50 ng/ml) for 24 and 48 h, this antiapoptotic effect was decreased (data not shown). Therefore, the treatment of Jurkat cells with 50 ng/ml for 12 h was determined as a condition for the induction of Fas-mediated apoptosis.

To investigate the association of H_2O_2 with desensitization of Jurkat cells to apoptosis, we examined the effect of an ROS scavenger, NAC, on Fas-mediated apoptosis. When Jurkat cells were pretreated with NAC before exposure to H_2O_2 and then incubated with anti-Fas antibody, the antiapoptotic effect of H_2O_2 was abolished (Figure 1c). To further explore the possibility that pretreatment of H_2O_2 is involved in the insensitivity of Jurkat cells to Fas-mediated cell death, cells were pretreated with another ROS scavenger, catalase, and then treated with H_2O_2 and anti-Fas antibody. In consistent with the results shown in Figure 1c, Fas-mediated cell death was attenuated (Figure 1d), indicating that H_2O_2 induces attenuation of Fas-mediated cell death.

Although it has been shown that low concentrations of H_2O_2 can inhibit apoptosis under specific conditions, the mechanisms that are involved in this process are not well elucidated. It is possible that H_2O_2 could modulate the expression levels of the Fas receptor on the cell surface. To investigate this possibility, we compared the expression levels of Fas receptor by FACS using FITC-conjugated anti-Fas antibody before and after exposing cells to 100 μM H_2O_2. As shown in Figure 2a, Fas expression appeared to be similar in both control and H_2O_2-treated cells, suggesting that this inhibitory effect of H_2O_2 on Fas-mediated apoptosis was not due to a reduction in the surface expression of Fas.

Since mitochondrial apoptotic pathways are known to play a dominant role in the amplification of apoptotic stimuli by forming apoptosome and activating caspase in Fas-mediated apoptosis in Jurkat cells (Scaffidi *et al*., 1998), we next examined whether H_2O_2 treatment can affect the mitochondrial events during Fas-mediated apoptosis. As shown in Figure 2b, anti-Fas antibody treatment resulted in cytochrome *c* release from the mitochondria to the cytoplasm, and this phenomenon was inhibited by H_2O_2 pretreatment. In addition, the antioxidant, *N*-acetyl cysteine (NAC), restored Fas-induced cytochrome *c* release in the H_2O_2-pretreated cells (Figure 2b). Cytochrome *c* levels in cytoplasmic fractions were quantified by densitometer. The released cytochrome *c* is known to form a complex, apoptosome, with Apaf and caspase 9, and this apoptosome activates caspase 3 (Li *et al*., 1997). Therefore, we investigated

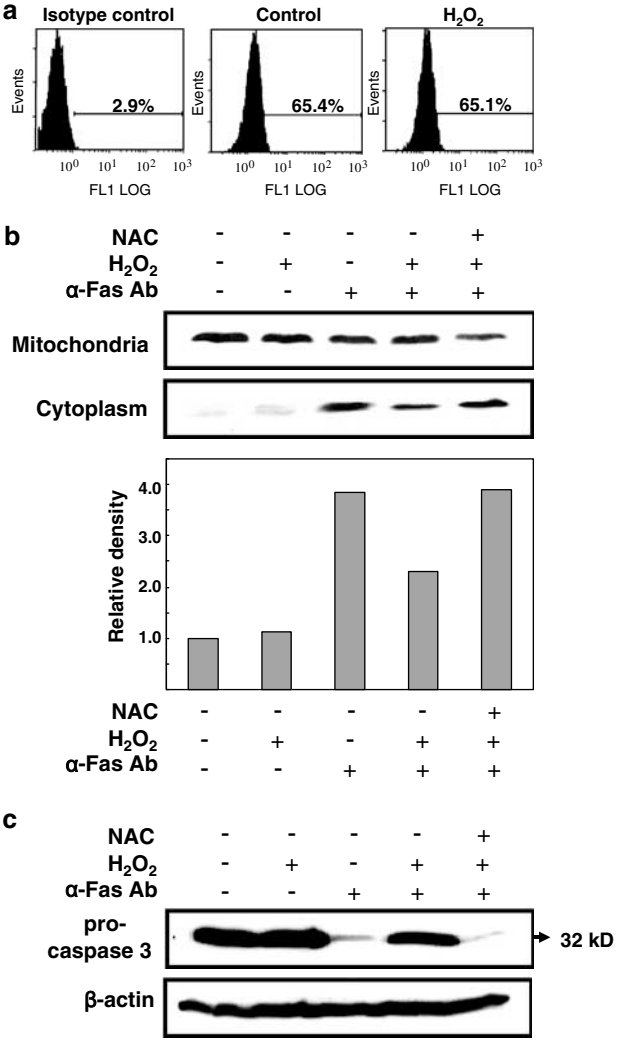

Figure 2 Inhibition of the mitochondrial pathway by H_2O_2-pretreatment in Fas-mediated apoptosis. (**a**) The effect of H_2O_2 on Fas expression. Jurkat T cells were treated with 100 μM of H_2O_2 for 6 h, stained with FITC-conjugated isotype control antibody or FITC-labeled anti-Fas antibody, and analysed by FACS. (**b**) Release of cytochrome *c* from the mitochondria to the cytoplasm. Cells were preincubated with 100 μM of H_2O_2 in the presence or absence of 5 mM NAC for 1 h and then treated with 50 ng/ml anti-Fas antibody for another 12 h. The cells were harvested and fractionated into mitochondria and cytoplasm. Each fraction was subjected to SDS–PAGE and immunoblotted using anti-cytochrome *c* antibody. The densities of bands in cytoplasmic fractions were analysed using Scion-Image software (NIH). (**c**) Activation of caspase 3. Cells were treated with NAC, H_2O_2, and then anti-Fas antibody as described in (**b**). The same amounts of total cellular proteins were subjected to immunoblot analysis using anti-caspase 3 and β-actin antibodies as a loading control. Similar results were also observed in three independent experiments

whether the inhibition of cytochrome *c* release in H_2O_2-pretreated cells is associated with the inhibition of caspase 3 activation. Consistent with the results shown in Figure 2b, Fas-stimulation caused the cleavage of procaspase 3, and H_2O_2 pretreatment blocked this procaspase 3 processing in the Fas-stimulated cells (Figure 2c). The antioxidant, NAC, also reversed the

effect of H_2O_2 on procaspase 3 processing. These findings indicate that the H_2O_2-induced antiapoptotic effect is associated with the inhibition of the mitochondrial apoptotic machinery.

Induction of Bfl-1 expression by H_2O_2

Since ROS are known to regulate gene expression and protein synthesis, we cannot rule out the possibility that H_2O_2 induces the synthesis of some proteins that are responsible for Jurkat cell's insensitivity to Fas-induced cell death. To examine this possibility, Jurkat cells were pretreated with 10 μg/ml cycloheximide (CHX) to inhibit new protein synthesis before exposure to H_2O_2, and then incubated with anti-Fas antibody. Interestingly, CHX abrogated this inhibitory effect of H_2O_2 on Fas-mediated apoptosis (Figure 3a), whereas CHX alone was not significantly cytotoxic. These data indicate that the synthesis of some protein(s) is essential for the antiapoptotic effect of H_2O_2. Furthermore, because several Bcl-2 family members directly regulate the mitochondrial apoptotic machinery, it is possible that H_2O_2 treatment could change the expression levels of these genes in Jurkat cells. To address this possibility, we examined the mRNA levels of the antiapoptotic Bcl-2 family genes, including Bcl-2, Bcl-xL, Mcl-1, and Bfl-1, in cells treated with or without H_2O_2 by RT–PCR. Phorbol ester 12-myristate 13-acetate (PMA)/ionomycin was used as a T cell activator. As shown in Figure 3b, the basal mRNA levels of Bcl-2, Bcl-xL, and Mcl-1 were unchanged by H_2O_2 treatment. Bfl-1 expression was not detectable in untreated cells, but its expression was induced by PMA/ionomycin, which is consistent with a previous report (Karsan *et al*., 1996). Moreover, H_2O_2 induced Bfl-1 gene expression in Jurkat cells, suggesting that Bfl-1 might be involved in antiapoptotic effect of H_2O_2. To further investigate Bfl-1 induction by H_2O_2, we examined the effect of an ROS scavenger, NAC, or catalase. When Jurkat cells were pretreated with different concentrations of NAC or catalase, H_2O_2 no longer induced Bfl-1 gene expression (Figure 3c and d). To determine whether the inability of NAC-pretreated cells to induce Bfl-1 expression by H_2O_2 is associated with a sensitization of the cells to apoptosis, we examined the effect of NAC on Fas-stimulated apoptosis. The pretreatment of Jurkat cells with NAC abolished the antiapoptotic effect of H_2O_2 on Fas-mediated apoptosis. Taken together, these observations demonstrate that Bfl-1 expression is responsive to H_2O_2 treatment in Jurkat T cells, and Bfl-1 induction by H_2O_2

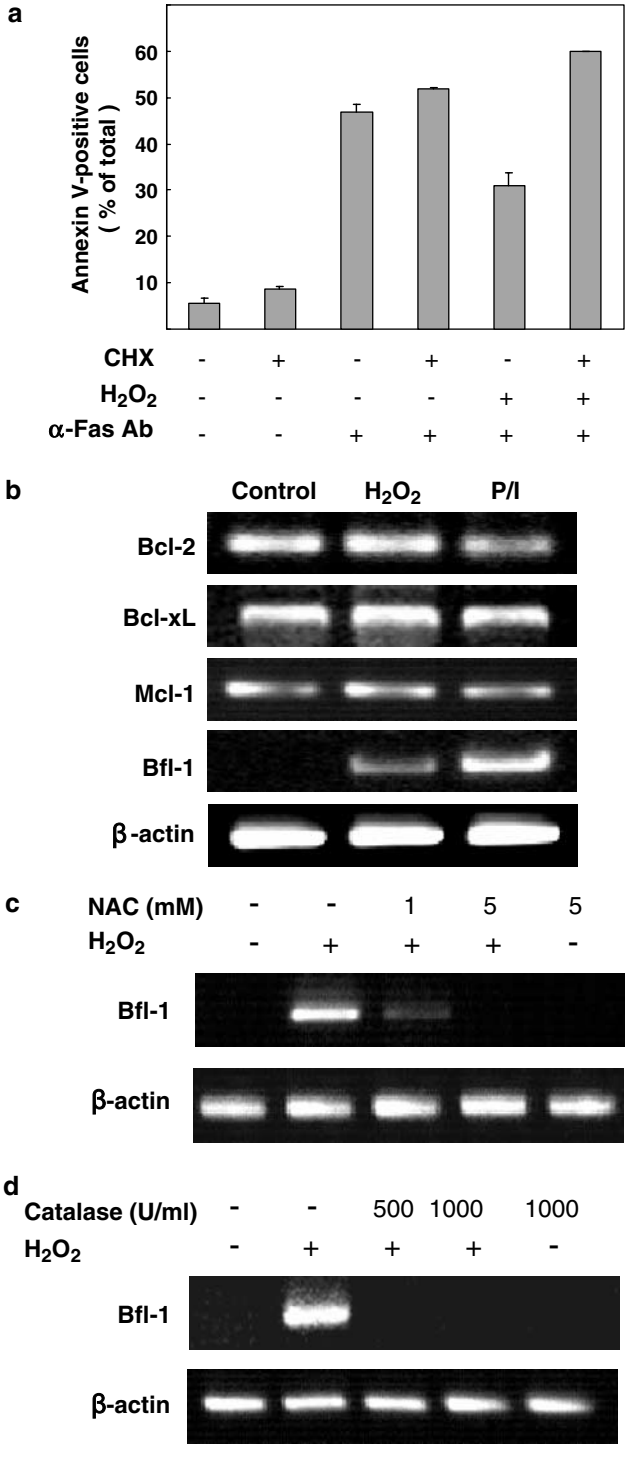

Figure 3 Induction of Bfl-1 gene expression by H_2O_2. (**a**) The effect of a protein synthesis inhibitor on H_2O_2-induced attenuation of Fas-mediated apoptosis. Jurkat T cells were pretreated with 10 μg/ml of cycloheximide (CHX) for 2 h and incubated for another 6 h in the presence or absence of 100 μM H_2O_2. The cells were then treated with anti-Fas antibody (50 ng/ml) for 12 h, and subjected to Annexin V/PI-FACS analysis. (**b**) The gene expression of antiapoptotic Bcl-2 family members by H_2O_2. Jurkat T cells were treated with 100 μM H_2O_2 or 5 μg/ml PMA plus 1 μM ionomycin for 6 h. Total cellular RNAs were prepared and subjected to RT–PCR using appropriate primer sets. (**c**) Inhibition of H_2O_2-induced Bfl-1 expression by NAC. Jurkat T cells were pretreated with the indicated concentrations of NAC and treated with 100 μM H_2O_2. Total cellular RNAs were then prepared and subjected to RT–PCR. (**d**) Inhibition of H_2O_2-induced Bfl-1 expression by catalase. Jurkat T cells were pretreated with the indicated concentrations of catalase and treated with 100 μM H_2O_2. Total cellular RNAs were then prepared and subjected to RT–PCR. All the experiments on RT–PCR were repeated three times and similar results were observed in three independent experiments

is correlated with resistance to Fas-mediated apoptosis as illustrated in Figure 1c and d.

Bfl-1 induction through NF-κB activation in H_2O_2-treated Jurkat cells

By oxidizing proteins, H_2O_2 can modulate many protein functions. These redox-regulated proteins include transcription factors, such as p53, Jun, Fos, and the p50 subunit of NF-κB (Sun and Oberley, 1996). Moreover, H_2O_2 signaling is known to activate IκB kinase by promoting the phosphorylation of serine residues located in its activation loops, thereby activating NF-κB (Kamata *et al*., 2002). Since Bfl-1 is a direct transcriptional target of NF-κB, and it blocks TNF-α-induced apoptosis (Wang *et al*., 1999), it is possible that NF-κB activation is involved in Bfl-1 induction in the H_2O_2-treated cells. To explore this possibility, cells were preincubated with the NF-κB inhibitor SN50, and then treated with H_2O_2 for 6 h. As shown in Figure 4a, inhibition of NF-κB blocked the expression of Bfl-1 gene in the H_2O_2-treated cells. We next investigated the potential involvement of NF-κB activation by H_2O_2 in Fas-mediated apoptosis. Consistent with the result in Figure 4a, NF-κB inhibition reversed the antiapoptotic effect of H_2O_2, indicating that H_2O_2-induced NF-κB activation is associated with this antiapoptotic effect (Figure 4b).

The Rel/NF-κB family of transcription factors is composed of five members (c-Rel, RelA(p65), RelB, NF-κB1(p50), NF-κB2), and the antiapoptotic effect of Rel/NF-κB has been documented (Ghosh and Karin, 2002). Moreover, it has been reported that the c-Rel/p50 heterodimer is the primary regulator of the Bfl-1 gene in PMA/ionomycin-activated Jurkat cells (Edelstein *et al*., 2003). To further determine which NF-κB subunits are activated in H_2O_2-exposed Jurkat cells, we examined the translocations of NF-κB subunits from the cytoplasm to the nucleus by Western blot analysis. Interestingly, H_2O_2 stimulation induced the translocations of both p65 and p50 to the nucleus (Figure 4c), but not of the c-Rel subunit (data not shown). We verified the nuclear and cytosolic fractions by Western blot analysis using anti-

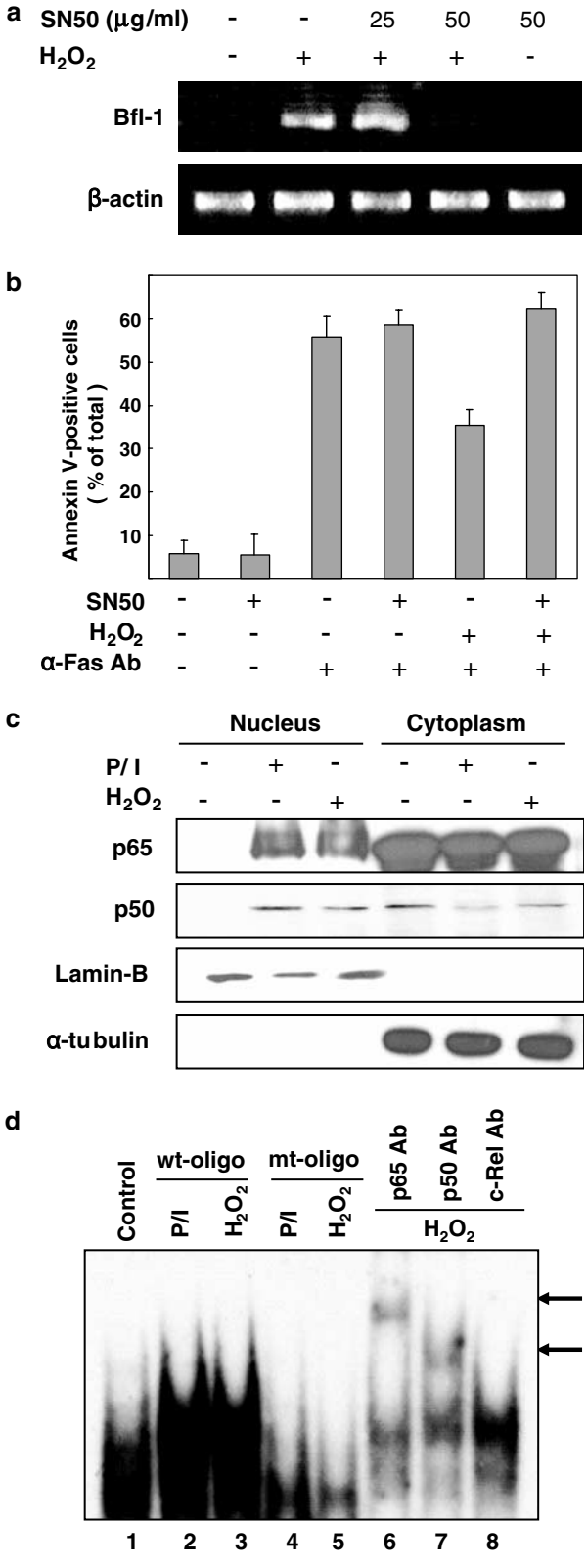

Figure 4 H_2O_2-induced Bfl-1 gene expression through NF-κB activation. (**a**) Inhibition of H_2O_2-induced Bfl-1 gene expression by the NF-κB inhibitor, SN50. Jurkat T cells were pretreated with the indicated concentrations of SN50 for 1 h, and treated with 100 μM H_2O_2. Total cellular RNAs were then prepared and subjected to RT–PCR. (**b**) Effects of SN50 on H_2O_2-induced Fas insensitivity. In total, 50 μg/ml of SN50-pretreated Jurkat T cells were treated with or without H_2O_2, and stimulated with anti-Fas antibody. Apoptosis was determined by Annexin V/PI-FACS. (**c**) Translocation of NF-κB subunits from the cytoplasm to the nucleus by H_2O_2. Nuclear and cytosolic fractions were prepared from Jurkat T cells that had been treated with or without H_2O_2 for 2 h. The presence of NF-κB subunits in each fraction was examined by immunoblotting using anti-p65 and anti-p50 antibodies. PMA and ionomycin were used as a positive control. (**d**) Binding of the p65 and p50 subunits of NF-κB to *bfl-1* promoter fragment in H_2O_2-treated cells. Nuclear extract were prepared from PMA/ionomycin-stimulated or H_2O_2-treated Jurkat cells, and incubated with a ^{32}P-labeled *bfl-1* promoter fragment (wt-oligo) for DNA-binding assay. The NF-κB binding specificity to *bfl-1* promoter was confirmed using *bfl-1* promoter-mutant oligomer (mt-oligo). Supershift assays were performed using NF-κB subunit-specific antibodies. The black arrows point to the supershifted bands. Similar observations were made in three separate experiments

lamin B antibody and anti-α-tubulin antibody, respectively. To obtain evidence that the H_2O_2-stimulated translocation of NF-κB subunits is associated with the binding of NF-κB to the 5′ *bfl-1* regulatory region, thereby inducing Bfl-1 expression, we performed a gel shift assay using a P^{32}-labeled probe derived from the *bfl-1* promoter region and nuclear extracts prepared from Jurkat cells. Shifted bands were observed in the nuclear extract prepared from PMA/ionomycin-stimulated or H_2O_2-treated Jurkat cells (Figure 4d, lanes 2 and 3). Conversely, nonspecific mutant oligomer did not form complexes in the extract from PMA/ionomycin-stimulated or H_2O_2-treated Jurkat cells (Figure 4d, lanes 4 and 5). To determine which subunits of NF-κB bind to the probe, supershift was performed using antibodies against p65, p50, and c-Rel. The shifted bands were found to be supershifted by anti-p65 and anti-p50 antibodies, but not by anti-c-Rel antibody (Figure 4d, lanes 6, 7, and 8), which is consistent with the finding of the translocation of p65 and p50 in the H_2O_2-treated cells. Collectively, these results strongly support the idea that H_2O_2 induces the activation of NF-κB, specifically p65/p50 heterodimer formation, and thereby Bfl-1 induction.

Role of the Bfl-1 gene expression in the antiapoptotic effect of H_2O_2

To further explore the role of Bfl-1 in the antiapoptotic effect of H_2O_2, a Bfl-1-knockdown cell line model was established by transducing Jurkat cells with an antisense retroviral vector of Bfl-1 to inhibit the Bfl-1 gene expression. As shown in Figure 5a, the expression of transduced constructs was confirmed by RT–PCR of the neomycin-resistant (neo^r) mRNA prepared from mock- or Bfl-1AS-transduced cells. When these established cell lines were treated with H_2O_2, only the Bfl-1-knockdown cell line (Bfl-1AS) failed to induce Bfl-1 expression (Figure 5b). To verify the correlation of Bfl-1 expression with antiapoptotic effect of H_2O_2, Fas-mediated apoptosis was induced in Jurkat cells transduced with retroviruses containing Bfl-1AS construct or established Bfl-1-knockdown Jurkat cell line. The antiapoptotic effect of H_2O_2 was abrogated in both of them, indicating that Bfl-1 induction is essential for resistance to Fas-mediated apoptosis in H_2O_2-treated Jurkat cells (Figure 5c and d). These cumulative data demonstrate that oxidative stress induces the expression of Bfl-1 via NF-κB activation, and that this early-response gene protects cells from Fas-mediated apoptosis by inhibiting the mitochondrial apoptotic pathway.

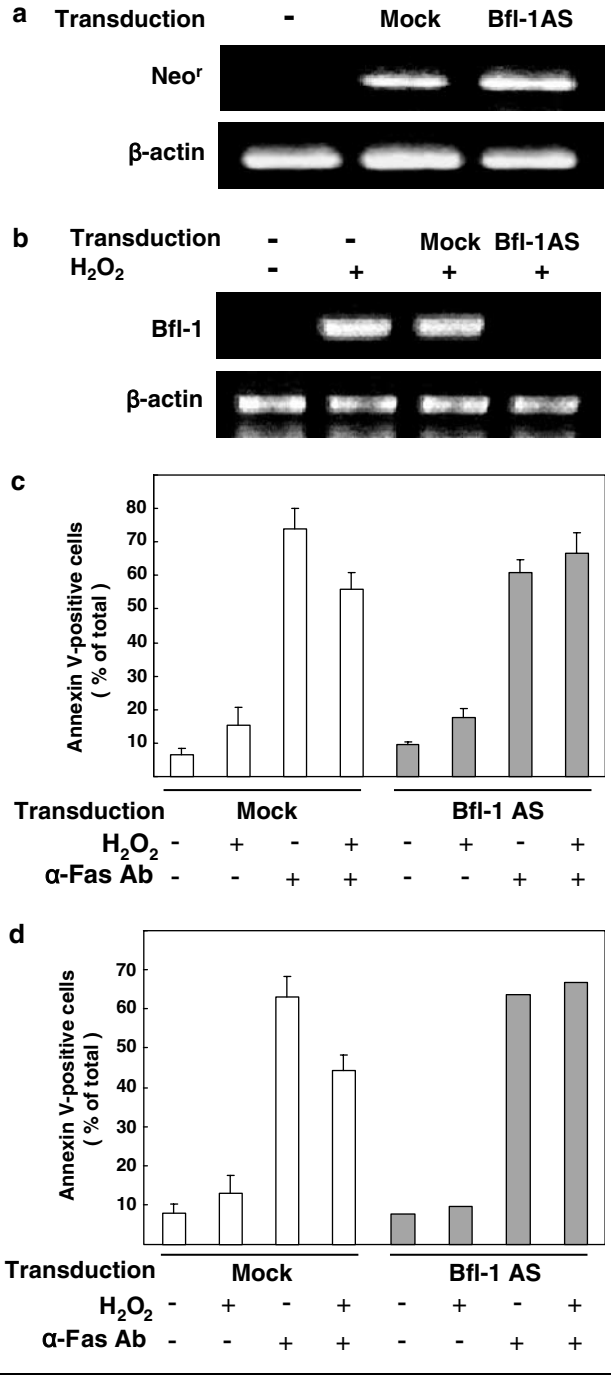

Figure 5 Abrogation of the antiapoptotic effect of H_2O_2 in Bfl-1-knockdown cells. (**a**) Establishment of mock- or Bfl-1AS-transduced Jurkat T cell lines. Jurkat T cells were infected by retroviruses containing either mock or Bfl-1AS constructs, and transduced cells were selected in media containing G418. The presence of constructs was confirmed by RT–PCR with G418 (neomycin)-resistant gene-specific primers. (**b**) Inhibition of H_2O_2-induced Bfl-1 gene expression by Bfl-1AS. Jurkat T cells transduced with either none, mock- or Bfl-1AS retroviruses were incubated with H_2O_2 and Bfl-1 induction was detected by RT–PCR. All the experiments on RT–PCR were repeated three times and similar results were observed in three independent experiments. (**c**) Suppression of the antiapoptotic effect of H_2O_2 by Bfl-1AS. Jurkat T cells were transiently transduced with retroviral supernatants containing mock- or Bfl-1AS construct and treated with H_2O_2 and anti-Fas antibody after 48 h. Apoptosis was analysed by FACS. (**d**) Suppression of the antiapoptotic effect of H_2O_2 in Bfl-1-knockdown cell line. Bfl-1-knockdown cell line was established by transduction with retroviral supernatant and selection with G418. Fas-mediated apoptosis was induced in these modified cells as described in (**c**). These experiments were performed three separate times with comparable results

Discussion

Although it has been demonstrated that ROS are essential in the physiological environment, the precise function of oxidative stress under various conditions remains controversial. Typically, ROS are viewed as detrimental and lead to cell death, ageing, and age-related diseases (Finkel and Holbrook, 2000). In recent years, however, several studies have suggested a role of H_2O_2 as a second messenger in intracellular signaling pathway. For example, TCR signaling of T cells is modified in the presence of H_2O_2 (Hehner *et al*., 2000). In addition, low levels of oxidative stress selectively activate p38 MAPK cascades, leading to inhibition of cell division (Kurata, 2000), and H_2O_2 also activates ERK or Akt/PKB, promoting cell survival (Lee and Esselman, 2001; Martin *et al*., 2002). Therefore, if survived cells from oxidative stress change the cellular state by intracellular modifications, such as gene expressions, it is possible that changes in gene expression can protect cells from encountered apoptotic signals. Here, we report that oxidative stress ($100\,\mu$M H_2O_2) attenuates Fas-mediated apoptosis through Bfl-1 induction in the Jurkat cell line.

It is well known that cells exposed to H_2O_2 undergo apoptotic cell death. Several studies have reported that high doses of H_2O_2 activate intracellular signals, resulting in necrosis rather than apoptosis (Valencia and Moran, 2004). However, the concentrations of H_2O_2 used in many studies supporting this H_2O_2 effect are much higher than concentrations found *in vivo*. Such high doses of H_2O_2 are far from physiological conditions and thus disrupt cell homeostasis by severely oxidizing cell components. Actually, at a site of inflammation, H_2O_2 is produced by activated macrophages at an estimated rate of $2\text{–}6 \times 10^{-14}\,\mu$M/h per cell and T cells may be exposed to 10–100 μM in the physiological microenvironment (Lander *et al*., 1997). Moreover, H_2O_2 at concentrations of 30–100 μM has been shown to induce NF-κB transcription factor activity in one subline of Jurkat cells (Hehner *et al*., 2000). In the present study, we elucidated a novel antiapoptotic function of H_2O_2 through Bfl-1 induction by NF-κB activation.

Even though Bfl-1 was induced at high concentrations of H_2O_2 ($>100\,\mu$M), this Bfl-1 induction did not attenuate Fas-mediated apoptosis (data not shown). This result can be explained by the cytotoxicity of high concentration of H_2O_2. According to a study by Martin *et al*. (2002), the fact that oxidative stress activate contradictory signals of survival and death implies that there must be a sophisticated crosstalk between these opposite signals that dictate cell's fate . In addition, a hypothetical model has been suggested, namely that moderate oxidative stress recruits prosurvival signals to CREB, but that during severe oxidative stress, pro-death signals through JNK and c-jun predominate (Crossthwaite *et al*., 2002). Consistent with this notion, our data also imply that there is a threshold below which H_2O_2-induced Bfl-1 can protect cells from Fas-mediated apoptosis. Taken together, the results on studying the role of ROS are mainly dependent on the experimental conditions such as concentration, exposing time, status and clone or type of cells.

Fas-mediated apoptosis is initiated by interaction with Fas and FasL, resulting in activation of caspase cascade. According to the model of the two Fas signaling pathways, apoptosis is mainly accompanied by activation of the initiator caspase 8 through the death-inducing signaling complex (DISC) in type I cells (Scaffidi *et al*., 1998). In contrast, in type II cells, for example, Jurkat cells, DISC formation is strongly reduced and mitochondrial apoptotic pathways play a dominant role to amplify apoptotic stimulus through formation of apoptosome and activation of caspase. So there are many possibilities to explain this H_2O_2-mediated effect. Since other studies have revealed that H_2O_2 induces upregulation of Fas in human airway epithelial cells via the activation of PARP-p53 pathway (Fujita *et al*., 2002), we examined the modulation of Fas expression with H_2O_2. However, as shown in Figure 2, there was little change of Fas expression in our experimental model, and we have found that this inhibitory effect of H_2O_2 on Fas-mediated apoptosis correlates with mitochondrial apoptotic machinery as a downstream signaling pathway. It has previously been reported that mild oxidative stress downregulates caspase 3 activity, thereby probably leading to resistance to Fas-mediated cell death (Hampton and Orrenius, 1997). In our study, caspase 3 cleavage in H_2O_2 + anti-Fas antibody-treated cells was also reduced versus only anti-Fas antibody-treated cells. However, the observation that CHX abrogated this H_2O_2-mediated antiapoptotic effect led us to speculate that the ability of H_2O_2 to trigger this effect is associated with the expression of novel genes. Moreover, there is a report supporting this notion that ROS trigger several signaling pathways, which leads to the expression of specific genes in T cells (Allen and Tresini, 2000).

As the Bcl-2 family members reside upstream of irreversible cellular damage and focus much of their efforts at mitochondrial level, they act as key regulators of apoptosis (Gross *et al*., 1999). The expression patterns of the antiapoptotic Bcl-2 family in RNA level revealed that $100\,\mu$M of H_2O_2 induces the Bfl-1 gene despite few changes in the expressions of other genes (Figure 3b). Bfl-1 is an early-response gene and its expression is rapidly and transiently induced by specific stimuli (Karsan *et al*., 1996). Many studies have demonstrated the mechanism of the antiapoptotic function of Bfl-1. Bfl-1 interacts strongly with Bax to neutralize its lethality (Zhang *et al*., 2000) and also sequesters truncated Bid to inhibit its collaboration with Bak or Bax (Werner *et al*., 2002). In addition, growth stimulation by ConA or PMA/ionomycin intercepts p53 proapoptotic signaling at the mitochondrial level, probably by upregulating antiapoptotic factors like Bcl-X_L and Bfl-1 (Heinrichs and Deppert, 2003). Therefore, Bfl-1 induced by H_2O_2 might interact with Bax or truncated Bid to inhibit mitochondrial pathway, and thus attenuates Fas-mediated apoptosis.

Interestingly, several reports have demonstrated that the Bfl-1 gene is a direct transcriptional target of NF-κB (Lee *et al*., 1999; Zong *et al*., 1999), and that NF-κB activation is regulated by the ROS level in many cell lines (Li and Karin, 1999). Consistent with these reports, H_2O_2 activated NF-κB, and resulted in Bfl-1 induction in the present study, and pretreatment with antioxidants or NF-κB inhibitor rendered Jurkat cells sensitive to apoptosis (Figures 1c,d and 4a). Many studies have reported that H_2O_2-induced NF-κB activation is highly cell type dependent (Hayakawa *et al*., 2003). The effect of H_2O_2 on NF-κB activation has been mainly reported in Wurzburg Jurkat T cells, subclone JR (Anderson *et al*., 1994; Sen *et al*., 1996). Although some studies have demonstrated that ROS is insufficient to activate NF-κB in E6.1 Jurkat cells, we observed the NF-κB activation induced by H_2O_2 in the same subclone cell line. In addition, other reports have shown the similar results that H_2O_2 can induce NF-κB activation in E6.1 Jurkat cells (Anderson *et al*., 1994; Kazmi *et al*., 1995) and human primary T cells (Gilston *et al*., 2001). Therefore, H_2O_2-mediated Bfl-1 induction through NF-κB activation might be realized *in vivo*.

A recent study provided evidence supporting our data, whereas PMA, a potent inducer of Bfl-1, protects wild-type Jurkat cells from Fas-induced apoptosis, it induces apoptosis in Jurkat variants defective for NF-κB activation (Busuttil *et al*., 2002). Edelstein *et al*. (2003) reported that a c-Rel/p50 heterodimer regulates *bfl-1* expression in PMA/ionomycin-activated Jurkat T cells, but p65 may also contribute to Bfl-1 gene expression in response to other stimuli. In the present study, we showed that p65 and p50 subunits bind the *bfl-1* promoter region to initiate Bfl-1 transcription after H_2O_2 treatment (Figure 4b and c). This fact indicates that different stimuli utilize different combinations of NF-κB heterodimers to induce Bfl-1 gene expression.

To clarify whether this H_2O_2-mediated antiapoptotic effect results from the induction of the Bfl-1 gene, we established a Bfl-1-knockdown cell line model using a viral vector containing Bfl-1 antisense. As shown in Figure 5, the antiapoptotic effect of H_2O_2 appears to depend on Bfl-1 gene induction, and we observed that Bfl-1-overexpressed Jurkat cells are also resistant to Fas-induced apoptosis (data not shown). These data provide novel insights into the mechanism by which the induction of Bfl-1, one of the antiapoptotic Bcl-2 family genes, by H_2O_2 protects cells from apoptosis**.** We tried to detect Bfl-1 protein level changes upon treating with H_2O_2 using many different batches of commercially available antibodies but without success. This difficulty has also been described by others studying Bfl-1 (Moreb and Zucali, 2001). Moreover, although some have detected Bfl-1 at the protein level using antibodies generated in their own laboratories or provided by other researchers, the majority of studies on the role of the Bfl-1 gene have presented mRNA data by RT–PCR, Northern blot analysis, or RPA.

Although ROS are continuously produced *in vivo*, they maintain a steady state because of their rapid metabolic processes. However, activated granulocytes in certain circumstances, such as, at sites of inflammation or those associated with tumors, secrete substantial amounts of H_2O_2, and T cells are exposed to oxidative stress in this microenvironment (Schmielau and Finn, 2001). It has been shown that activated T cells are eventually face activation-induced cell death (AICD) through death receptors such as Fas or TNF receptor (Hildeman *et al*., 2002). Despite overwhelming evidence that oxidative stress induces T cells' hyporesponsiveness without AICD, the precise mechanisms of this event remain controversial. Our findings may provide an important clue to investigations as to how this oxidative stress contributes to the resistance of T cells against AICD in autoimmune processes or tumorigenesis.

Materials and methods

Cell culture, antibodies, and reagents

The Jurkat human T-lymphocytic leukemia cells (E6. 1) were obtained from ATCC (Rockville, MD, USA) and maintained in RPMI 1640 medium (Gibco Life Technologies, New York, NY, USA) containing 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. All cells were grown in a humidified incubator at 37°C and 5% CO_2. Monoclonal antibody against human Fas (clone 7C11) for induction of Fas-mediated apoptosis was purchased from Immunotech (Marseille, France). FITC-labeled mouse anti-human Fas antibody, anti-cytochrome *c* antibody, and anti-caspase 3 antibody were from Pharmingen (San Diego, CA, USA). Antibodies against NF-κB p65, p50, c-Rel, lamin B, and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). H_2O_2, PMA, ionomycin, and NAC were purchased from Sigma (St Louis, MO, USA). CHX was from Bio Vision Inc. (Palo Alto, CA, USA). SN50 and human erythrocyte catalase were obtained from Calbiochem (La Jolla, CA, USA).

Detection of apoptosis by FACS analysis

Apoptosis was measured using an apoptosis detection kit (Annexin V-fluorescein and propidium iodide (PI)) (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Cells were analysed on an EPICS XL™ Flow Cytometer (Beckman Coulter, Fullerton, CA, USA) using EXPO32™ software. Crossover of FITC fluorescence into the PI detection window was electronically compensated.

Total RNA preparation and RT–PCR assay

Total RNA was isolated from cells using TRIzol reagent (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. About 2 μg of total RNA, oligo$(dT)_{18}$ primer, dNTP and Avian Myeloblastosis Virus (AMV) reverse transcriptase XL (Takara, Ohtsu, Japan) were used for the cDNA synthesis reaction. Each cDNA was amplified in a DNA thermal cycler with specific primers (Bcl-2: 5′-GTGT GGAGAGCGTCAACC-3′ (sense) and 5′-GCTGGGGC CGTACAGT TC-3′ (antisense), Bcl-XL: 5′-CCAGAAAGG ATA CAGCTGG-3′ and 5′-CTCCTGGATCCAAGGCTC TA-3′, Mcl-1: 5′-CGGCAGTCGCTGGAGATTAT-3′ and 5′-GTGGTGGTGGTTGGTTA-3′, Bfl-1: 5′-AGCTCAAGA CTTTGCTCTCCACC-3′ and 5′-TGGAGTGTCCTTTCTG GTCAACAG-3′, Neo^r: 5′-TCCATCATGGCTGATGCAAT GCGGC-3′ and 5′-GATAG AAGGCGATGCGCTGCGA

ATCG-3′, β-actin: 5′-GGAAATCGTGCGTGACATTA AGG-3′ and 5′-GGCTTTAGGATGGCAAGGGAC-3′), and human β-actin cDNA was amplified to determine the integrity of RNA and the efficiency of cDNA synthesis. The resulting PCR products were resolved on 1% agarose gel and stained with ethidium bromide.

Western blot analysis

Cellular extracts from cells were prepared using lysis buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 5 mM $MgCl_2$, protease inhibitor). Then, 40 μg of cell lysate was subjected to polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Equal protein loading was verified by staining with Ponceau S reagent. Membranes were blocked with 5% nonfat dry milk in TBST buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature. Immunoblotting with specific antibodies was performed at 4°C overnight, and blots were immunodetected using HRP-conjugated Ig and enhanced chemiluminescence with ECL reagent (Amersham Pharmacia, Buckinghamshire, England).

Electrophoretic mobility shift assay (EMSA)

To prepare nuclear extract, cells were washed in ice-cold PBS and resuspended in Buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.6% Nonidet P-40). After incubation for 20 min on ice, the nuclear pellet was recovered by centrifugation at 1200 *g* and resuspended in Buffer B (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 25% glycerol). Aliquots were then incubated at 4°C for 30 min and supernatants containing nuclear proteins were collected by centrifuging at 21 000 *g*. Labeling of double-stranded oligonucleotide probe containing the NF-κB-binding site of the *bfl-1* promoter region (wild type: 5′-CAGACGGGGATTTACCATATT, mutant: 5′-CAGAC GTTTATTTACCATATT) was performed using T4 polynucleotide kinase (NEB, England, UK), and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham, Les Ulis, France). The binding reaction was carried out in binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, 1 μg poly(dI-dC) for 30 min. Reaction mixtures were loaded onto 4% polyacrylamide gel, and autoradiographed. Supershifts of DNA–protein complexes were detected using anti-p65, anti-p50, or anti-c-Rel antibodies.

Cytochrome c *release assay*

Mitochondrial and cytosolic fractions were purified according to the described protocol with some modifications (Li *et al*., 2001). Cells were washed once in ice-cold PBS, resuspended in extraction buffer (220 mM mannitol, 68 mM sucrose, 50 mM PIPES, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT) supplemented with protease inhibitor mixture, and homogenized using a Dounce homogenizer. Samples were then centrifuged at 800 *g* for 10 min at 4°C to remove the nuclei and unbroken cells. This supernatant was centrifuged at 10 000 *g* for 30 min at 4°C to separate the mitochondria-enriched pellet from the cytosolic supernatant. This pellet was resuspended in RIPA buffer to form mitochondrial fractions, and supernatants were centrifuged at 100 000 *g* for 40 min at 4°C to yield the final cytosolic fractions. These fractions were resolved on a 15% SDS–PAGE and blotted with anti-cytochrome *c* antibody to assess cytochrome *c* release from the mitochondria to the cytoplasm.

Generation of modified Jurkat cell lines

Bfl-1 cDNA was amplified by PCR and cloned into the *Xho*I site of the pLXIN retroviral vector in a reverse orientation for use as an antisense vector. The amphotropic retroviral packaging cell line PA317 was transfected with retroviral vectors (pLXIN or pLXIN-Bfl-1AS) using Lipofectamine reagent (Invitrogen life technologies, Carlsbad, CA, USA). After selection with G418, colony secreting high titer of virus (4×10^6 Neo CFU/ml) was used to infect Jurkat cells. For transient retroviral transduction, Jurkat cells (2×10^5 cells) were infected with viral supernatant (1 ml) by centrifugation at 1000 *g* for 90 min in the presence of polybrene (8 μg/ml, Sigma, St Louis, MO, USA). The spin infection was repeated the next day and analysis was conducted 48 h after infection. The modified Jurkat cell lines stably expressing transduced gene were generated by transduction with viral supernatants and selection with G418 for 4 weeks.

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