FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B–enhanced cell survival through maintenance of mitochondrial integrity

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Survival signals elicited by cytokines include the activation of phosphatidylinositol 3-kinase (PI3K), which in turn promotes the activation of protein kinase B (PKB). Recently, PKB has been demonstrated to phosphorylate and inactivate forkhead transcription factor FKHR-L1, a potent inducer of apoptosis. To explore the mechanisms underlying the induction of apoptosis after cytokine withdrawal or FKHR-L1 activation, we used a cell line in which FKHR-L1 activity could be specifically induced. Both cytokine withdrawal and FKHR-L1 activation induced apoptosis, which was preceded by an upregulation in $p27^{KIP1}$ and a concomitant decrease in cells entering the cell cycle. Induction of apoptosis by both cytokine withdrawal and activation of FKHR-L1 correlated with the disruption of mitochondrial membrane integrity and cytochrome c release. This was preceded by upregulation of the pro-apoptotic Bcl-2 family member Bim. Ectopic expression of an inhibitory mutant of FKHR-L1 substantially reduced the levels of apoptosis observed after cytokine withdrawal. Activation of PKB alone was sufficient to promote cell survival, as measured by maintenance of mitochondrial integrity and the resultant inhibition of effector caspases. Furthermore, hematopoietic stem cells isolated from $Bim^{-/-}$ mice exhibited reduced levels of apoptosis upon inhibition of PI3K/PKB signaling.

These data demonstrate that activation of FKHR-L1 alone can recapitulate all known elements of the apoptotic program normally induced by cytokine withdrawal. Thus PI3K/PKB–mediated inhibition of this transcription factor likely provides an important mechanism by which survival factors act to prevent programmed cell death.

Introduction

In the absence of cytokines, hematopoietic cells stop proliferating and undergo programmed cell death, a process also known as apoptosis. This dependence on cytokines is necessary to maintain homeostasis in the immune system, and dysregulation of this process has been associated with autoimmune diseases, as well as malignancies (for review see Rinkenberger and Korsmeyer, 1997). The activation of cysteine proteases (caspases), which leads to the cleavage of various substrates, including poly(ADP-ribose) polymerase (PARP),* and degradation of chromosomal DNA, characterizes a crucial step in the induction of apoptosis (Cryns and Yuan, 1998). Caspases exist as inactive pro-enzymes in the cell that are activated through proteolytic cleavage upon induction of the apoptotic program (for review see Budihardjo et al., 1999).

A well-characterized mechanism of initiating apoptosis is through ligand-mediated activation of cell surface death receptors, such as the tumor necrosis factor receptors and

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*Abbreviations used in this paper: 4-OHT, 4-hydroxy tamoxifen; DBD, DNA-binding domain; FasL, Fas ligand; GFP, green fluorescence protein; IL, interleukin; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; Rh-123, rhodamine-123; SGK, serum and glucocorticoid-induced kinases.

CD95 (APO-1/Fas) (for review see Nagata, 1999). Caspase-8 is indispensable for transducing apoptotic signals initiated by death receptors, as demonstrated by the observation that CD95 signaling is abrogated in caspase-$8^{-/-}$ mice (Varfolomeev et al., 1998). Caspase activation can also be triggered via a death receptor–independent mechanism, involving the regulation of mitochondrial membrane permeability. Central to this "intrinsic" means of mitochondrial-initiated caspase activation is the release of cytochrome c from the intermembrane space of mitochondria into the cytosol. Cytochrome c, together with apoptosis activating factor 1 (Apaf-1), promotes activation of caspase-9 (Li et al., 1997; Srinivasula et al., 1998), which then activates downstream caspases, such as caspase-3 and -7 (Budihardjo et al., 1999). Although loss of mitochondrial integrity can also be induced by death receptors, it is not essential for their induction of apoptosis (Strasser et al., 1995).

Indispensable for the regulation of mitochondrial integrity are proteins of the Bcl-2 family. These consist of anti-apoptotic members, such as Bcl-2, Mcl-1, and Bcl-XL and pro-apoptotic members, such as Bad, Bim, and Bid (for review see Adams and Cory, 1998). One mechanism by which cytokines are believed to promote survival is by inhibiting transcription (Dijkers et al., 2000a; Shinjyo et al., 2001) or activity (del Peso et al., 1997; Songyang et al., 1997) of pro-apoptotic members, as well as transcriptionally upregulating anti-apoptotic members (Chao et al., 1998; Kuribara et al., 1999).

Protein kinase B (PKB), also known as c-akt, is regulated by agonist-induced phosphatidylinositol 3-kinase (PI3K) activation, and has been proposed to regulate cytokine-mediated cell survival (Ahmed et al., 1997; Songyang et al., 1997; Eves et al., 1998). Anti-apoptotic signals from PKB include upregulation of Mcl-1 (Wang et al., 1999) and inhibitory phosphorylation of Bad (Songyang et al., 1997), although the relevance of Bad phosphorylation for the survival of hematopoietic cells remains unclear (Scheid and Duronio, 1998). A recently identified mechanism by which PKB can promote rescue from apoptosis is through inhibitory phosphorylation of the forkhead transcription factor FKHR-L1 (FOXO3a) (Brunet et al., 1999; Dijkers et al., 2000a). Activity of this transcription factor has been linked to the induction of apoptosis in hematopoietic cells (Brunet et al., 1999; Dijkers et al., 2000a,b). Although it has been demonstrated in several systems that PKB can mediate rescue from apoptosis, it is not clear whether PKB exerts its anti-apoptotic effect upstream (Kennedy et al., 1999) or downstream (Zhou et al., 2000) of mitochondria. Furthermore, little is known concerning the mechanisms by which activation of FKHR-L1 can lead to induction of the apoptotic program.

Here, we investigate the mechanisms of cytokine withdrawal– and forkhead-induced apoptosis and the role of PKB in rescue from apoptosis in cytokine-deprived cells. Our data demonstrate that FKHR-L1, as well as cytokine withdrawal, induce apoptosis through a death receptor–independent pathway. This involves transcriptional upregulation of the pro-apoptotic Bcl-2 family member Bim, loss of mitochondrial integrity, cytochrome c release, and caspase activation. Thus, PKB can protect cells from cytokine withdrawal–induced apoptosis by inhibiting FKHR-L1, resulting in the maintenance of mitochondrial integrity. These data shed new light on the mechanisms by which cytokines, through regulation of PI3K activity, can modulate the survival of hematopoietic lineages.

Results

Induction of apoptosis correlates with upregulation of $p27^{KIP1}$ and Bim

Cytokines of the interleukin (IL)-3, IL-5, and granulocyte macrophage colony–stimulating factor family have a well-established function in transducing a proliferative and anti-apoptotic response in hematopoietic target cells and their precursors (de Groot et al., 1998; Guthridge et al., 1998; Reddy et al., 2000). To examine the mechanisms underlying cytokine withdrawal–induced apoptosis, we used the mouse pre-B cell line, Ba/F3, which requires IL-3 both for proliferation as well as to overcome the default apoptotic program. Previously, ourselves and others have shown that one mechanism by which cytokine-mediated rescue from apoptosis may be achieved is through inhibitory phosphorylation of the forkhead transcription factor FKHR-L1 by PKB (Brunet et al., 1999; Dijkers et al., 2000a; Kashii et al., 2000; Uddin et al., 2000). To specifically analyze the effect of FKHR-L1 activity, we generated cell lines stably overexpressing an inducible form of active FKHR-L1, in which all three inhibitory phosphorylation sites were mutated to alanine, FKHR-L1(A3):ER* (Dijkers et al., 2000a). Addition of 4-hydroxy tamoxifen (4-OHT) to these cells results in the rapid induction of FKHR-L1 transcriptional activity, promoting the induction of bona fide forkhead targets (Dijkers et al., 2000a,b). This occurs within a similar time frame as FKHR-L1 dephosphorylation and activation after cytokine deprivation. Importantly addition of 4-OHT to Ba/F3 cells not expressing FKHR-L1(A3):ER* has no effect on apoptosis.

Cells were either cytokine-starved or treated with 4-OHT for 24 h and apoptosis was measured by analyzing binding of annexin V-FITC. Cells that are annexin V positive represent early apoptotic cells, whereas cells that are stained for both annexin V and propidium iodide (PI) represent cells that have initiated the apoptotic program for a longer period of time. Cells that are dead through necrosis are PI positive and annexin V negative. Both cytokine withdrawal and FKHR-L1 activity induced apoptosis to a similar degree (Fig. 1 A, top and middle; IL-3 withdrawal: 25% $\pm$ 4%; 4-OHT addition: 33% $\pm$ 4%). Next, we analyzed the kinetics by which apoptosis was induced using DNA laddering, a measure for the final events characterizing apoptosis. Both cytokine withdrawal and FKHR-L1 activity induced apoptosis within a similar time frame (Fig. 1 A, bottom). Recently, we have demonstrated that both $p27^{KIP1}$ and Bim are transcriptional targets of FKHR-L1 (Dijkers et al., 2000a,b). We examined whether the kinetics of upregulation of $p27^{KIP1}$ and Bim protein correlated with induction of apoptosis. Both cytokine withdrawal and FKHR-L1 activation resulted in an upregulation of $p27^{KIP1}$ and Bim (Fig. 1, B and C). These events occurred relatively early and preceded the cleavage of DNA observed in Fig. 1 A.

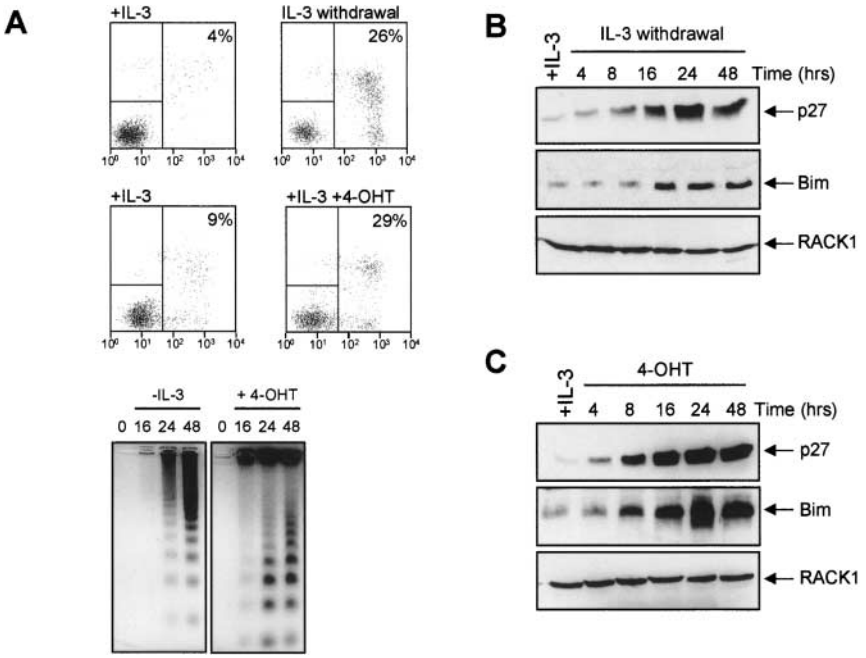

Figure 1. **Induction of apoptosis by cytokine withdrawal or FKHR-L1 activity.** (A, top) Ba/F3 cells were cultured in the presence or absence of cytokines. FKHR-L1(A3):ER* cells were cultured with IL-3 without or with 4-OHT (100 nM) for 24 h and analyzed for annexin V binding and PI staining as described in the Materials and methods. (Bottom) Ba/F3 cells were IL-3 starved (left) or FKHR-L1(A3):ER*–expressing cells were treated with 4-OHT (100 nM, right) for the times indicated. DNA laddering was analyzed as described in the Materials and methods. (B) Ba/F3 cells were cytokine-starved for the indicated times, lysed, and equal amounts of protein were analyzed for levels of $p27^{KIP1}$ (top) or Bim (middle). Samples were analyzed with a RACK1 antibody to verify equal protein loading (bottom). (C) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were treated with 4-OHT for the times indicated, lysed, and analyzed as above. Data depicted are representative of at least three independent experiments.

Cytokine withdrawal and FKHR-L1 activity induce apoptosis independently of death receptor activation

FKHR-L1 has been proposed to induce Fas ligand (FasL) in T cells (Brunet et al., 1999), which could contribute to FKHR-L1–mediated induction of apoptosis. Fas/FasL signaling induces cleavage and activation of caspase-8, which is an indispensable and specific downstream event of death receptor–induced apoptosis (Juo et al., 1998; Varfolomeev et

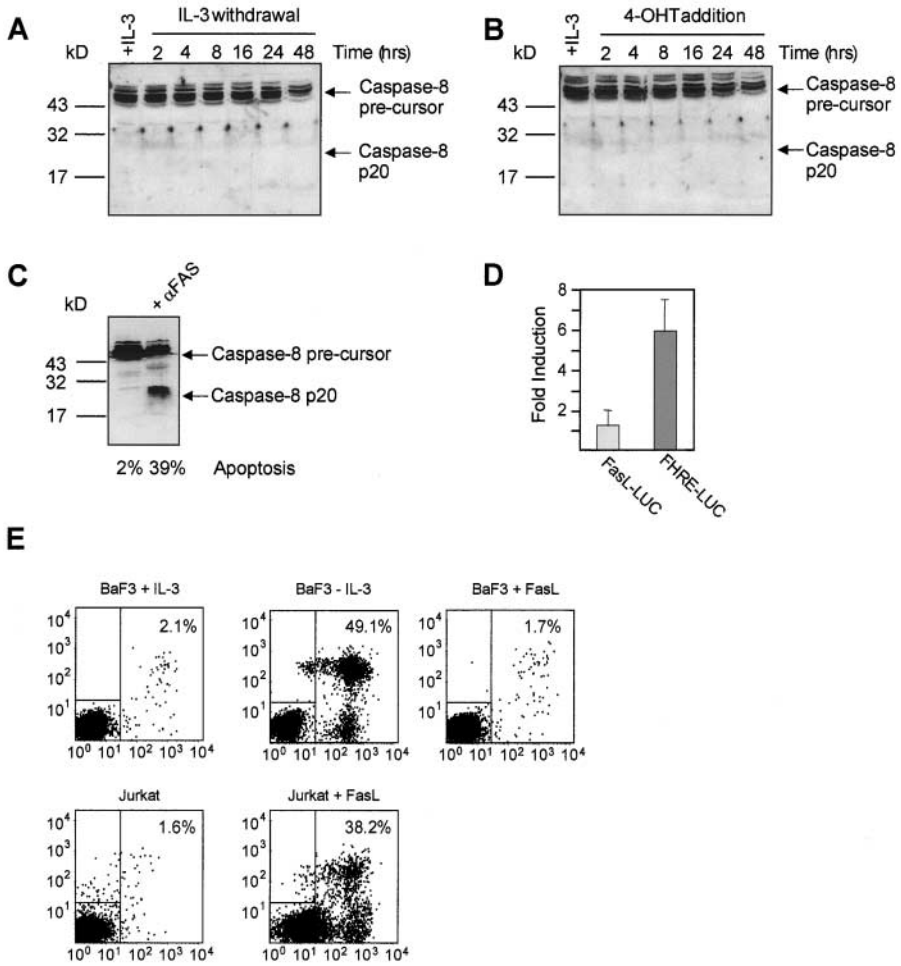

Figure 2. **Death receptor signaling is not activated in response to cytokine deprivation or FKHR-L1 activation.** (A) Caspase-8 cleavage was analyzed in Ba/F3 cells that were cytokine starved for the times indicated. (B) Analysis of caspase-8 cleavage in 4-OHT–treated (100 nM) FKHR-L1(A3):ER* cells. (C) Jurkat cells were treated with or without αFAS18 (1 μg/ml). After 24 h, samples were harvested and protein concentration was measured. An equal amount of protein was analyzed by SDS-PAGE and anti–caspase-8 Western blotting. Data depicted are representative of at least three independent experiments. (D) Ba/F3 cells were electroporated with 1 μg renilla luciferase and either 20 μg of a FasL promoter construct (FasL–LUC) or a construct containing three forkhead binding sites from the FasL promoter (FHRE-LUC). After culture for 18 h with or without IL-3, cells were lysed and analyzed for the amount of produced luciferase protein. Data represent the mean of three independent experiments ± SD. (E) Ba/F3 cells or Jurkat cells were treated for 24 h with SUPER-Fas Ligand (50 ng/ml) and analyzed for annexin V binding and PI staining as described in the Materials and methods.

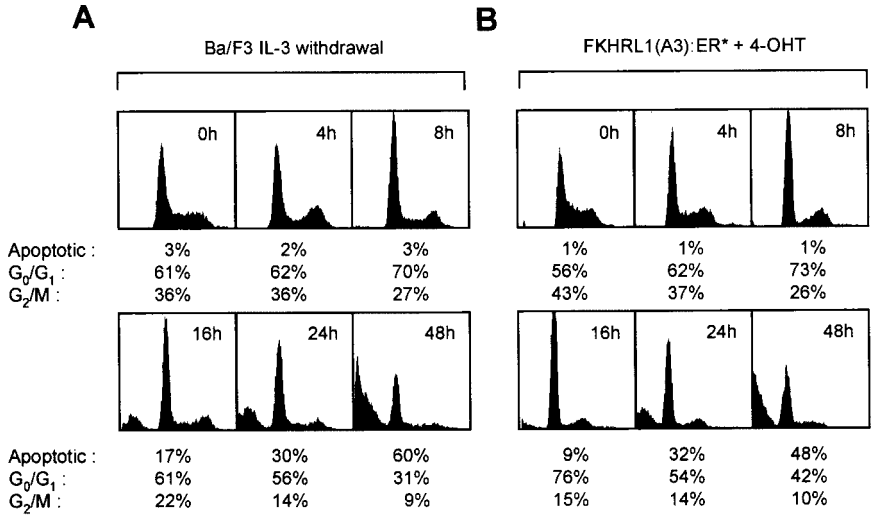

Figure 3. **Cell cycle analysis of cytokine-starved cells and cells in which FKHR-L1 activity is induced.** (A) Ba/F3 cells were cytokine starved for the times indicated, fixed, stained with PI, and analyzed by FACS®. (B) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were treated with 4-OHT (100 nM) for the times indicated and processed as in A. Data depicted are representative of at least three independent experiments.

al., 1998). Analysis of caspase-8 cleavage is thus a means to discriminate between death receptor–dependent and –independent induction of apoptosis. As Ba/F3 cells express Fas, albeit at very low levels (Marshall et al., 2000; Santos-Beneit and Mollinedo, 2000), forkhead-mediated induction of FasL could potentially trigger apoptosis by inducing caspase-8 activation. We analyzed a potential role for Fas/FasL signaling in the induction of apoptosis by cytokine withdrawal or forkhead activation by measuring caspase-8 cleavage. Although Ba/F3 cells expressed caspase-8, neither cytokine withdrawal (Fig. 2 A) nor forkhead activity (Fig. 2 B) resulted in caspase-8 cleavage. As a positive control, Jurkat T cells were treated with a cross-linking anti-Fas antibody, which clearly induced cleavage and activation of caspase-8 (Fig. 2 C).

In addition, to examine the effect of cytokine withdrawal on FasL induction, Ba/F3 cells were transiently transfected with a FasL promoter reporter construct (Holtz-Heppelmann et al., 1998; Brunet et al., 1999). Cytokine withdrawal had no effect on FasL promoter activity (Fig. 2 D). In contrast, a reporter construct under the control of the portion of the FasL promoter that contains three forkhead binding sites (FHRE-LUC) was strongly induced upon cytokine withdrawal. This suggests that although in an artificial context these FasL promoter forkhead binding sites can be regulated, this is not the case in Ba/F3 cells in the context of the intact FasL promoter itself. Furthermore, we were unable to detect an upregulation of FasL protein by Western blotting (unpublished data). In accordance with these results, treatment of Ba/F3 cells for 24 h with recombinant, species cross-reactive, active FasL had no effect on the percentage of apoptotic cells, whereas in Jurkat cells FasL at this concentration strongly induced apoptosis (Fig. 2 E). Taken together, these data demonstrate that Ba/F3 cells are not susceptible to FasL-mediated killing and argue against a role for Fas/FasL signaling in FKHR-L1–mediated apoptosis.

Cytokine withdrawal and FKHR-L1 activity induce cell cycle arrest followed by apoptosis

$p27^{KIP1}$ is involved in cell cycle arrest in G_1 through the inhibition of cyclin–CDK complexes (Polyak et al., 1994; Toyoshima and Hunter, 1994), but has also been described to function in the induction of apoptosis through a yet unidentified mechanism (Wang et al., 1997; Dijkers et al., 2000a). To see whether upregulation of $p27^{KIP1}$ reflected an altered distribution of cells in the cell cycle, we analyzed the cell cycle profile of cells at various time points. Upon cytokine withdrawal, cells stopped initiating cell division and accumulated in G_1, within the first 8 h of starvation (Fig. 3 A). After 16 h of cytokine deprivation cells started to undergo apoptosis, as measured by cells having a DNA content $<2n$ chromosomes, the sub-G_1 peak. By 48 h, a majority of cells had initiated a program of apoptotis (58% ± 5%). Similar findings were observed in 4-OHT–treated FKHR-L1(A3):ER* cells (Fig. 3 B; 48 h, 49% ± 3%), suggesting that the presence of a G_1 arrest before the onset apoptosis is probably related to the initial upregulation of $p27^{KIP1}$ (Fig. 1, B and C).

Caspase activation and PARP cleavage follow cytokine withdrawal and FKHR-L1 activation

Very little is known regarding mechanisms by which cytokine withdrawal promotes caspase activation and cleavage of apoptotic substrates (Hieronymus et al., 2000; Khaled et al., 2001), and the function of FKHR-L1 therein remains to be established. We sought to investigate this in more detail, examining both cytokine withdrawal and FKHR-L1 activity. Using antibodies specific for cleaved caspase-3 and -7, their activation was analyzed after cytokine withdrawal. Caspase-3 (Fig. 4 A, top) and -7 (Fig. 4 A, middle) were both cleaved after cytokine withdrawal. This occurred ~16 h after cytokine removal. Analysis of 4-OHT–treated FKHR-L1(A3):ER* cells also showed similar kinetics of caspase-3 and -7 cleavage (Fig. 4 B, top and middle). A well-characterized caspase substrate that is cleaved when cells undergo apoptosis is PARP (Lazebnik et al., 1994), an enzyme involved in DNA repair (for review see D'Amours et al., 1999). To examine whether PARP is also cleaved in either cytokine withdrawal– or forkhead-induced apoptosis, lysates of cytokine-starved cells or 4-OHT–treated FKHR-L1(A3):ER* cells were again analyzed. Both IL-3 withdrawal (Fig. 4 C, left) and forkhead activity (Fig. 4 C, right) resulted in PARP

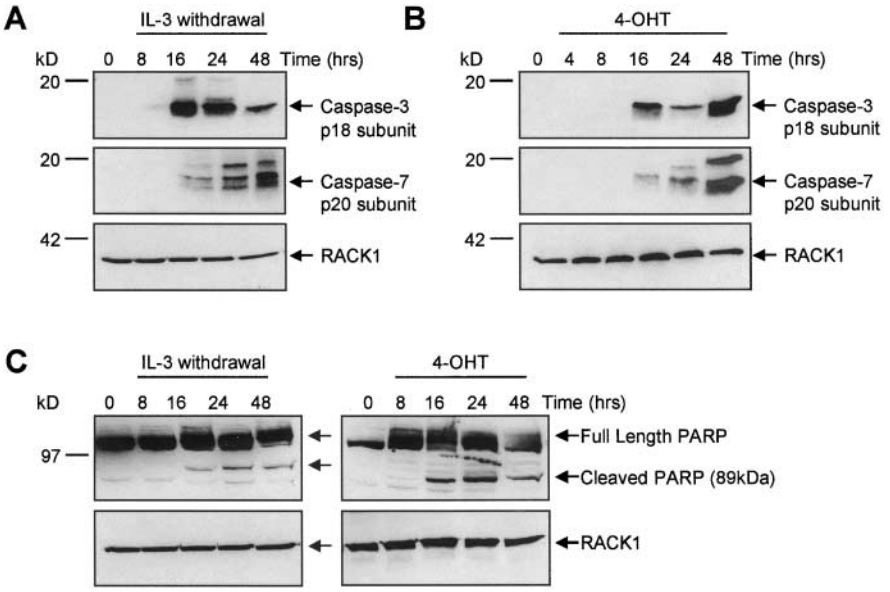

Figure 4. **Analysis of caspase activation and PARP cleavage in cytokine-starved cells and 4-OHT–treated FKHR-L1(A3):ER* cells.** (A) Cells were IL-3 starved for the times indicated and caspase-3 and -7 activation was analyzed using cleavage-specific antibodies. Equal protein loading was verified by analyzing samples for RACK1 expression. (B) 4-OHT–treated FKHR-L1(A3):ER* cells were analyzed as in A. (C) PARP cleavage was examined in cytokine-starved cells (left) or 4-OHT–treated FKHR-L1(A3):ER* cells (right). Data depicted are representative of at least three independent experiments.

cleavage with kinetics similar to caspase activation (Fig. 4, A and B). These data suggest that similar mechanisms are involved in both cytokine withdrawal– and FKHR-L1–induced apoptosis. Furthermore, upregulation of both $p27^{KIP1}$ and Bim precedes the activation of caspases (Fig. 1, B and C). The kinetics of cell cycle arrest, caspase activation, and cell death are remarkably similar between 4-OHT–induced FKHR-L1 activation and IL-3 withdrawal. These observations can be reconciled, as 4-OHT addition results in FKHR-L1 activation within 30 min, and IL-3 withdrawal results in FKHR-L1 dephosphorylation after 60 min (Dijkers et al., 2000a,b).

Cytokine withdrawal and FKHR-L1 activity promote mitochondrial depolarization

Mitochondria can play a critical role in the initiation of apoptosis, which is accompanied by a loss of mitochondrial transmembrane potential and leakage of cytochrome c (Liu et al., 1996). This, together with apoptosis activating factor 1 (Apaf-1), is required for activation of the "caspase cascade" (Li et al., 1997; Srinivasula et al., 1998) and is indispensable for death receptor–independent induction of apoptosis. To analyze mitochondrial integrity in apoptotic Ba/F3 cells, we used rhodamine-123 (Rh-123), a dye that binds to mitochondria in a membrane potential–dependent way (Ferlini et al., 1996; Scaduto and Grotyohann, 1999). In cytokine-starved Ba/F3 cells (Fig. 5 A), as well as in 4-OHT–treated FKHR-L1(A3):ER* cells (Fig. 5 B), loss of Ψ_m was observed after 16 h, and mitochondrial transmembrane depolarization increased dramatically over time (48 h: IL-3 withdrawal 50% ± 5%; 4-OHT addition 49% ± 3%). The kinetics were similar to those of caspase activation (Fig. 4, A and B). This suggests that both cytokine withdrawal and FKHR-L1 activation result in a loss of mitochondrial transmembrane potential, subsequently resulting in cytochrome c release and activation of caspases.

Inhibition of PI3K or direct activation of FKHR-L1 results in cytochrome c release from mitochondria

Next, we examined whether the loss of mitochondrial transmembrane potential coincided with cytochrome c release into the cytosol. Therefore, Ba/F3 cells were deprived of cytokines and release of cytochrome c was measured as described in the Materials and methods. As shown in Fig. 6, Ba/F3 cells cultured in the presence of IL-3 showed little cytosolic cytochrome c, whereas cytokine withdrawal strongly induced cytochrome c release from the mitochondria. This release appears to coincide with the mitochondrial mem-

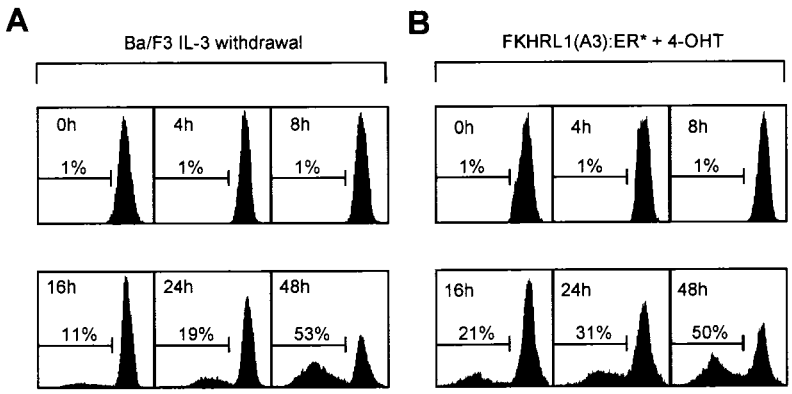

Figure 5. **Induction of mitochondrial transmembrane depolarization by cytokine starvation or FKHR-L1 activity.** (A) Ba/F3 cells were IL-3 starved for the times indicated and mitochondrial transmembrane depolarization was measured using Rh-123 staining as described in the Materials and methods. (B) 4-OHT–treated FKHR-L1(A3):ER* cells were analyzed as in A. Data depicted are representative of at least three independent experiments.

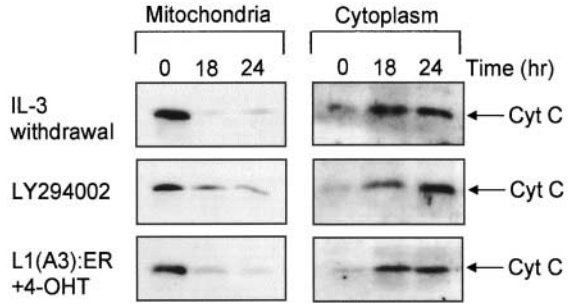

Figure 6. **Inhibition of PI3K or direct activation of FKHR-L1 results in cytochrome c release from mitochondria.** Ba/F3 cells were IL-3 starved or treated with LY294002, and FKHR-L1(A3):ER cells were treated with 4-OHT for the times indicated (in the presence of IL-3). Mitochondrial and cytosolic fractions were prepared as described in the Materials and methods. Protein concentrations were measured, and an equal amount of protein from the different fractions was analyzed by SDS-PAGE and cytochrome c Western blotting.

brane depolarization measured in Fig. 5. To investigate the involvement of the PI3K/PKB pathway in cytochrome c release, Ba/F3 cells were treated with the PI3K inhibitor LY294002. Again, in control cells, cytochrome c was localized in the mitochondria, whereas in the presence of LY294002, cytochrome c was released into the cytosol. Similarly, treatment with 4-OHT strongly induced cytochrome c release into the cytosol in FKHR-L1(A3):ER* Ba/F3 cells. These data demonstrate that inhibition of PI3K or activation of FKHR-L1 alone is sufficient to promote cytochrome c release from the mitochondria into the cytosol.

PKB-mediated rescue from apoptosis correlates with maintenance of mitochondrial integrity

Although PKB has been shown to be involved in the inhibition of apoptosis by survival factors (Ahmed et al., 1997; Songyang et al., 1997; Eves et al., 1998), a role for this kinase in the regulation of mitochondrial integrity remains unclear. PKB has been proposed to rescue cells from apoptosis by maintaining mitochondrial transmembrane potential and preventing cytochrome c release (Kennedy et al., 1999). However, others have observed rescue from apoptosis downstream of cytochrome c release (Zhou et al., 2000). To investigate the role of PKB in maintenance of mitochondrial integrity in more detail, we made use of a polyclonal Ba/F3 cell line stably expressing a 4-OHT–inducible, active form of PKB, myrPKB:ER* (Dijkers et al., 2000a). Treatment of these cells with 4-OHT resulted in a dramatic phosphorylation of myrPKB:ER* (Fig. 7 A, top), allowing us to analyze the effect of PKB activation after cytokine withdrawal. Indeed, activation of PKB in the absence of cytokines was sufficient to rescue cytokine-starved myrPKB:ER* cells from apoptosis, as measured by annexin V-FITC staining (Fig. 7 B). To further elucidate a role for PKB upstream or downstream of mitochondria, we analyzed whether PKB was capable of abrogating cytokine withdrawal–induced loss of mitochondrial transmembrane potential. For this purpose, Ba/F3 and myrPKB:ER* cells were cultured with or without IL-3 in the presence or absence of 4-OHT. In Ba/F3 cells, loss of mitochondrial transmembrane potential in the absence of cytokines could not be rescued by 4-OHT (Fig. 7 C, top), excluding aspecific effects of 4-OHT. In myrPKB:ER* cells, however, addition of 4-OHT substantially decreased the loss of Ψ_m upon cytokine withdrawal (Fig. 7 C, bottom). Subsequently, caspase activity was analyzed. Activity of both caspase-3 and -7 was substantially reduced in 4-OHT–treated compared with untreated myrPKB:ER* cells. Our findings of a partial rescue from apoptosis (Fig. 7 B) and caspase activity (Fig. 7 D) may be explained by the fact that the myrPKB:ER* cell line is a polyclonal cell line, potentially expressing heterogeneous levels of myrPKB:ER*, the lower levels being insufficient to rescue cells from cytokine withdrawal–induced apoptosis. Taken together, these findings

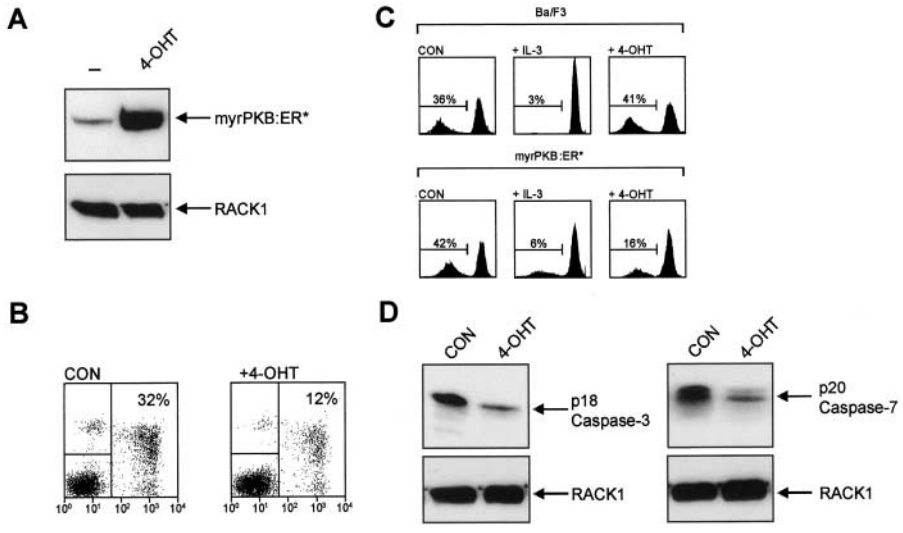

Figure 7. **Activation of PKB rescues cells from apoptosis and maintains mitochondrial transmembrane potential.** (A) Ba/F3 cells stably expressing myrPKB:ER* were left untreated or were treated with 4-OHT (100 nM) for 36 h. PKB phosphorylation was measured using a PKB phospho ser-473–specific antibody. (B) myrPKB:ER* cells were cytokine starved in the absence (left) or presence (right) of 4-OHT (100 nM) for 36 h, and the percentage of annexin V-FITC–positive cells was determined as described in the Materials and methods. (C) Ba/F3 cells or Ba/F3 cells stably expressing myrPKB:ER* were cytokine starved in the absence or presence of 4-OHT (100 nM) or cultured with IL-3 for 36 h. Mitochondrial integrity was examined using Rh-123 as described in the Materials and methods. (D) Caspase-3 (left) and -7 (right) activation was measured in myrPKB:ER* cells that were cytokine starved for 36 h in the absence or presence of 4-OHT (100 nM) using activation-specific antibodies. These cells were then reprobed with RACK1 antibody to verify equal protein loading. All data depicted are representative of at least three independent experiments.

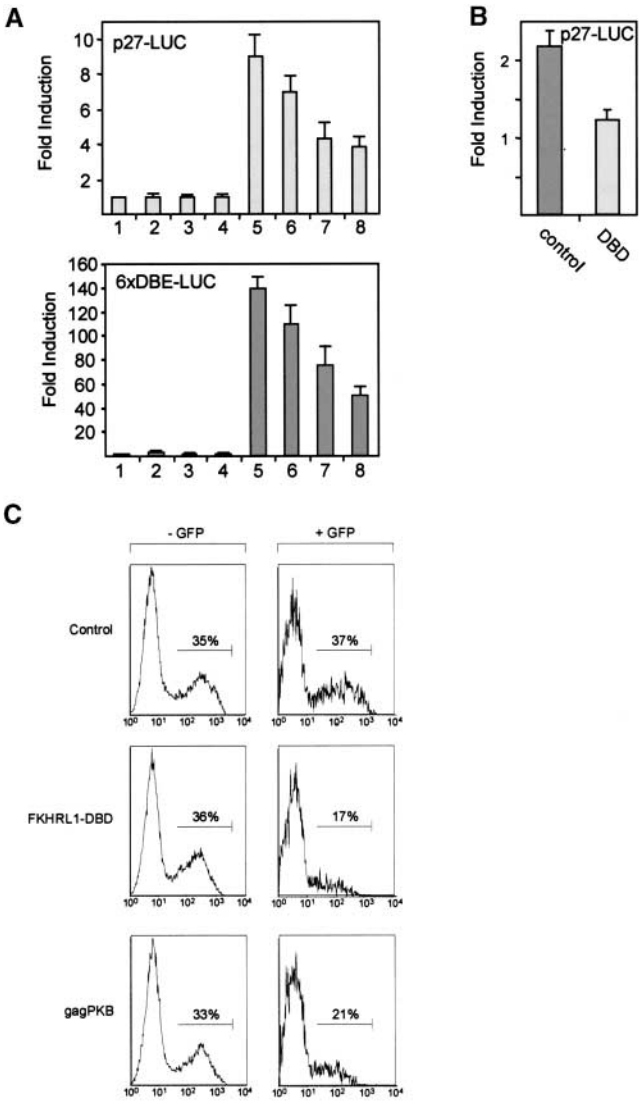

Figure 8. **Inhibition of FKHR-L1 transcriptional activity protects cells from apoptosis induced by cytokine withdrawal.** (A) COS cells (6-cm wells) were transfected with 50 ng of a renilla luciferase construct together with 2 μg of the pGL2-p27kip luciferase promoter construct (top) or 2 μg of a pGL2–6xDBE luciferase construct (bottom). Cells were cotransfected either with an empty vector (lane 1) with 1, 2, or 4 μg of pSG5–mycFKHRL1-DBD (lanes 2–4, respectively), or with 2 μg pECE-HA-FKHR-L1(A3) (lane 5) together with 1, 2, or 4 μg of pSG5–mycFKHRL1-DBD (lanes 6–8, respectively). After 24 h, cells were lysed and analyzed for luciferase activity. Data represent the mean of three independent experiments ± SEM. (B) Ba/F3 cells (10^7) were electroporated with 10 μg pGL2-p27kip luciferase promoter construct together with 10 μg of either a control plasmid (pSG5) or pSG5–mycFKHRL1-DBD. After 24 h, cells were lysed and analyzed for luciferase activity. Data represent the mean of three independent experiments ± SD. (C) Ba/F3 cells (10^7) were electroporated with 2 μg spectrin–GFP and either 18 μg control vector (top), FKHRL1-DBD (middle), or gagPKB (bottom). After electroporation, dead cells were removed as described in the Materials and methods. 48 h later GFP-positive (right panels) and GFP-negative (left panels) cells were analyzed by FACS® for apoptosis by annexin V–phycoerythrin binding. The percentage of annexin V–positive cells is shown. Data depicted are representative of at least three independent experiments.

demonstrate that PKB-mediated rescue from apoptosis correlates with mitochondrial potential maintenance and a reduction of caspase activity.

FKHR-L1 transcriptional activity is important for induction of apoptosis after cytokine withdrawal

As demonstrated above, induction of FKHR-L1 transcriptional activity recapitulates all known elements of the apoptotic program normally induced by cytokine withdrawal. However, the question remains whether FKHR-L1 activity is also necessary for induction of apoptosis after removal of survival factors. To attempt to answer this question, we generated a novel inhibitory mutant of FKHR-L1 (see Materials and methods). This deletion mutant, FKHRL1-DBD, contains only the DNA-binding domain (DBD) of FKHR-L1, allowing it to bind DNA but not activate transcription. We reasoned that ectopic expression of this protein results in competitive inhibition at the level of FKHR-L1–mediated transcription. A similar mutant for the related transcription factor AFX has been previously described (Medema et al., 2000). It should, however, be noted that although this mutant should inhibit FKHR-L1–induced transcription, it has the potential to also interfere with both FKHR- and AFX-mediated transcription as well.

To determine whether FKHRL1-DBD could indeed inhibit FKHR-L1–mediated transcription, COS cells were transiently transfected with the pGL2-p27kip luciferase promoter construct together with a constitutively active FKHR-L1(A3) mutant and increasing concentrations of the inhibitory mutant FKHRL1-DBD. As shown in Fig. 8 A, FKHR-L1(A3) strongly induced pGL2-p27kip promoter activity, which was inhibited by FKHRL1-DBD in a concentration-dependent manner. Similar results were shown for the pGL2–6xDBE construct, which contains six consecutive FKHR-L1 binding sites. Again, FKHRL1(A3) strongly induced promoter activity, which was inhibited by increasing concentrations of FKHRL1-DBD.

Because we have also demonstrated that FKHR-L1 is sufficient to induce $p27^{KIP1}$ transcription in Ba/F3 cells (Dijkers et al., 2000a), we wished to determine whether inhibition of FKHR-L1 could reduce $p27^{KIP1}$ transcription in response to cytokine withdrawal. Ba/F3 cells were electroporated with the pGL2-p27kip luciferase reporter construct together with FKHRL1-DBD or a control plasmid. Luciferase activity was measured in the presence or absence of IL-3, and the fold induction of promoter activity after cytokine withdrawal was calculated. As shown in Fig. 8 B, overexpression of FKHRL1-DBD is indeed able to repress the induction of $p27^{KIP1}$ transcription normally associated with cytokine withdrawal in these cells.

To determine then whether inhibition of forkhead-related transcription factors (FKHR-L1, FKHR, and AFX) could protect cells from cytokine withdrawal–induced apoptosis, Ba/F3 cells were transfected with spectrin–green fluorescence protein (GFP) plus a control vector, FKHRL1-DBD, or constitutively active PKB (gagPKB). Cells were cultured without IL-3 and levels of apoptosis were measured by annexin V binding, as described in the Materials and methods. Cells transfected with the control vector exhibited the same

level of apoptosis as untransfected cells (Fig. 8 C, top). However, cotransfection of FKHRL1-DBD (Fig. 8 C, middle) dramatically reduced the level of apoptosis compared with untransfected cells (17% vs. 36%, respectively). This rescue from apoptosis was comparable with that observed in cells transfected with gag-PKB (Fig. 8 C, bottom). Taken together with our previous findings (Dijkers et al., 2000a,b), these data demonstrate that FKHR-L1 is indeed an important component in regulating the initiation of the apoptotic program.

Inhibition of Bim expression is important for PI3K-mediated rescue from apoptosis

To examine the relevance of inhibition of Bim expression for PI3K/PKB–mediated rescue from apoptosis in vivo, we used hematopoietic stem cells isolated from either wild-type mice or mice lacking both Bim alleles ($Bim^{-/-}$; Bouillet et al., 1999). Bone marrow Sca1+ stem cells were cultured either with cytokines, without cytokines, or with cytokines as well as the PI3K inhibitor LY294002. Cells were subsequently analyzed after 24 h by annexin V staining, or after 48 h by Rh-123 binding (Fig. 9). In cells cultured with cytokines, the level of apoptosis, as measured by annexin V or Rh-123, is low and exhibits little difference between mice. Upon removal of survival factors, cells isolated from the $Bim^{-/-}$ mice have a significantly enhanced survival advantage, demonstrating that Bim is indeed critical in the regulation of hematopoietic stem cell survival. This is particularly apparent as measured by Rh-123 after 48 h. To determine if inhibition of Bim levels is a critical component of the mechanism by which PI3K/PKB regulates cell survival, stem cells were treated with LY294002. Treatment of cells isolated from wild-type mice with this PI3K inhibitor induced apoptosis to a level at least equal to that observed upon cytokine withdrawal (Fig. 9). However, in $Bim^{-/-}$ mice, the percentage of apoptotic cells observed after LY294002 treatment was significantly reduced compared with wild-type, particularly when mitochondrial integrity was analyzed after 48 h (Fig. 9 B). These data suggest that Bim is a critical downstream target of PI3K action.

Taken together, our data indicate that inhibition of FKHR-L1 activity by PKB-mediated phosphorylation, with the resultant effect of inhibiting Bim expression, is possibly one of the critical events by which survival factors prevent hematopoietic cells from initiating their intrinsic apoptotic program.

Discussion

In this report, we analyze the mechanisms of cytokine withdrawal– and FKHR-L1–induced apoptosis, as well as PKB-mediated rescue from apoptosis. Using cells expressing FKHR-L1(A3):ER* allowed us to uniquely analyze the effects of FKHR-L1 activation. Interestingly, no measurable differences between cytokine withdrawal– and FKHR-L1–mediated induction of apoptosis were observed. This suggests that FKHR-L1 alone could account for induction of the apoptotic program triggered by cytokine withdrawal. In both cases, cells were first arrested in G_1 and subsequently underwent apoptosis (Fig. 3). This was accompanied by a sequential upregulation of $p27^{KIP1}$, which is involved in arresting cells in G_1 and the induction of apoptosis (Polyak et al., 1994; Toyoshima and Hunter, 1994; Dijkers et al., 2000a), and Bim, which can induce apoptosis by binding to anti-apoptotic members of the Bcl-2 family (O'Connor et al., 1998) (Fig. 1, B and C). Bim appears to be essential for the induction of apoptosis in lymphocytes, because cytokine-deprived lymphocytes from $Bim^{-/-}$ mice fail to undergo apoptosis (Bouillet et al., 1999). Furthermore, lymphocytes derived from $p27^{KIP1-/-}$ mice undergo apoptosis at a significantly decreased rate compared with those from wild-type mice (Dijkers et al., 2000a). This suggests that the upregulation of both $p27^{KIP1}$ and Bim may play critical roles in the induction of the apoptotic program initiated by cytokine withdrawal.

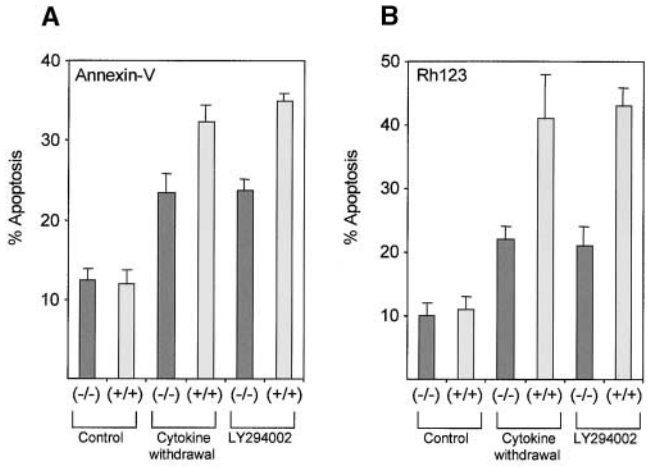

Figure 9. **Regulation of Bim expression is critical for PI3K-mediated rescue from apoptosis.** Hematopoietic stem cells were isolated from either wild-type mice or mice lacking both (−/−) alleles of the *BIM* gene as described in the Materials and methods. Cells were treated with cytokines, with or without incubation with 10 μM LY294002, or were cytokine starved. (A) Annexin V binding was analyzed after 24 h. (B) The integrity of the mitochondrial transmembrane potential was measured by FACS® analysis of Rh-123 binding after 48 h. Data represent the mean of four independent experiments ± SEM.

We also analyzed whether Fas/FasL signaling may be involved in the induction of apoptosis upon cytokine withdrawal as previously proposed (Brunet et al., 1999). Neither cytokine withdrawal nor FKHR-L1 activity resulted in caspase-8 cleavage, an event specific for death receptor signaling (Juo et al., 1998; Varfolomeev et al., 1998). In addition, cytokine withdrawal had no effect on FasL promoter activity (Fig. 2 D) or FasL protein expression (unpublished data). Importantly, treatment of Ba/F3 cells with FasL did not induce apoptosis, suggesting a lack of a functional Fas/FasL death receptor signaling pathway. This suggests that apoptosis induced either by cytokine withdrawal, PI3K inhibition, or FKHR-L1 activity is initiated through a death receptor–independent mechanism. In support of this, overexpression of anti-apoptotic Bcl-2 members, which rescue death receptor–independent apoptosis, but not death receptor–dependent apoptosis in lymphocytes (Itoh et al., 1993; Scaffidi et al., 1998), are able to rescue both cytokine withdrawal– as

well as FKHR-L1–induced apoptosis (Chao et al., 1998; Dijkers et al., 2000b). Interestingly, although the FasL promoter was not activated by cytokine withdrawal, a small region of this promoter previously shown to contain three forkhead binding sites was (Brunet et al., 1999; Fig. 2 D). This suggests that in the context of the intact FasL promoter, secondary factors are responsible for mediating promoter activity. Possibly, there are critical forkhead cofactors absent in Ba/F3 cells, or other factors are expressed that actively repress FasL promoter activity. This might explain the differences between our data and that previously described by Brunet et al. (1999) in T cells.

PKB has been demonstrated to negatively regulate members of a subfamily of forkhead transcription factors: AFX, FKHR, and FKHR-L1 (for review see Datta et al., 1999). Recently, members of the SGK (serum and glucocorticoid-induced kinases) family, which phosphorylate consensus sequences similar to PKB, were found to be required for full phosphorylation of FKHR-L1 in vivo and in IL-3–mediated survival (Liu et al., 2000; Brunet et al., 2001). This suggests that both kinases may be required for phosphorylation-mediated inactivation of FKHR-L1, and may explain why PKB was unable to completely inhibit cytokine withdrawal–induced apoptosis (Fig. 7). However, PKB was capable of significantly abrogating cytokine withdrawal–induced loss of mitochondrial potential (Fig. 7 C). Thus, we can conclude that PKB exerts its anti-apoptotic effect at a premitochondrial level, preventing intracellular release of cytochrome c. A potential role for PKB in rescue from apoptosis and prevention of cytochrome c leakage has also been proposed in apoptosis induced in Rat1 fibroblasts by UV irradiation (Kennedy et al., 1999), as well as in apoptosis induced in epithelial cells by detachment from the extracellular matrix (Rytomaa et al., 2000). However, PKB has also been previously shown to inhibit ceramide-induced apoptosis in hybrid neuron motor 1 cells downstream of cytochrome c release (Zhou et al., 2000). These findings may be explained by differences in apoptotic stimuli in different cell types, and indicates that PKB has the potential to act at multiple levels. Furthermore, difference in species could be an explanation for the differential contribution of PKB in rescue from apoptosis. PKB has been suggested to promote rescue from apoptosis by inhibitory phosphorylation of caspase-9 in human cells (Cardone et al., 1998), but not in mouse or rat cells because the PKB phosphorylation site in caspase-9 is not present (Fujita et al., 1999). PKB has also been linked to the upregulation of the anti-apoptotic Bcl-2 member Mcl-1 (Wang et al., 1999), which is essential in cytokine-mediated rescue from apoptosis (Chao et al., 1998). This regulation of an anti-apoptotic Bcl-2 member, involved in the maintenance of mitochondrial integrity, also supports a role for PKB upstream of cytochrome c leakage in cytokine-mediated rescue from apoptosis.

The p21ras-activated protein kinase MEK has also been proposed to rescue cells from apoptosis (Perkins et al., 1996; Shimamura et al., 2000), potentially through activation of downstream targets that phosphorylate Bad (Shimamura et al., 2000). Furthermore, MEK-initiated signals can result in the phosphorylation of anti-apoptotic members of the Bcl-2 family, thereby enhancing their stability (Breitschopf et al., 2000). However, using the myrPKB:ER* cell line, we have demonstrated that PKB alone is sufficient to protect cells from apoptosis (Fig. 6, B and C). Our data do not, however, rule out the possibility that MEK plays a role in these events.

Increased PKB activity can result in cellular transformation (Bellacosa et al., 1991; Cheng et al., 1992; Haas-Kogan et al., 1998), although the exact mechanisms by which PKB is capable of promoting oncogenesis remains to be established. Inhibitory phosphorylation of FKHR-L1 could very well contribute to this process, leading to a decrease in both Bim and $p27^{KIP1}$ levels. This is supported by the observation that a decrease in $p27^{KIP1}$ levels is associated with a poor prognosis in cancer (Catzavelos et al., 1997; Loda et al., 1997; Ohtani et al., 1999).

Two critical experiments have demonstrated that FKHR-L1 is a critical effector of cell death induced by cytokine withdrawal, and that Bim is an important downstream target of PI3K/PKB action. First, in Fig. 8 we demonstrate that ectopic expression of an inhibitory FKHR-L1 significantly reduces the levels of apoptosis observed after cytokine withdrawal. This is similar to the effect of expressing a constitutively active mutant of PKB. Although inhibition of apoptosis is dramatic, it is not complete. This could either be due to additional pro-apoptotic pathways, or simply that the levels of expression of these transfected proteins is relatively low in Ba/F3 cells. Using Bim knockout mice, we demonstrate that hematopoietic stem cells isolated from these animals have much reduced levels of apoptosis compared with wild-type mice upon cytokine withdrawal. This is in agreement with observations previously made in leukocytes isolated from these mice (Bouillet et al., 1999). If inhibition of Bim transcriptional levels is indeed critical for PI3K/PKB–mediated cell survival, it follows that inhibition of PI3K activity would have a significantly reduced effect on apoptosis in cells isolated from Bim-deficient mice. Indeed our data demonstrate this to be the case (Fig. 9). Thus, it appears that in cytokine-dependent cells, repression of Bim expression may be one of the major mechanisms by which PI3K/PKB signaling results in enhanced cell survival in vivo. It should, however, be noted that the cytoprotective effects of FKHRL1-DBD and Bim knockout are not complete. This suggests the possibility that there are other targets and mechanisms contributing to the induction of apoptosis upon cytokine withdrawal. Similarly, it has not been shown that FKHR-L1 is critical for the induction of Bim upon cytokine withdrawal.

Taken together, our data suggest that cytokine-induced signaling can inhibit cells from apoptosis through activation of PKB (or possibly SGK), which inhibits FKHR-L1 and Bad through phosphorylation and transcriptionally upregulates Mcl-1. In the absence of cytokines, PKB is inactive, resulting in dephosphorylation and activation of Bad and transcription of FKHR-L1 targets $p27^{KIP1}$ and Bim. This results in induction of the apoptotic program through loss of mitochondrial integrity, leakage of cytochrome c, subsequent activation of caspases, and cleavage of substrates. The events mediating cytokine withdrawal–induced apoptosis, as well as, cytokine-mediated rescue from apoptosis are summarized in Fig. 10.

Our findings provide greater insight into the mechanisms regulating induction of apoptosis in lymphocytes, and probably other hematopoietic lineages, upon cytokine with-

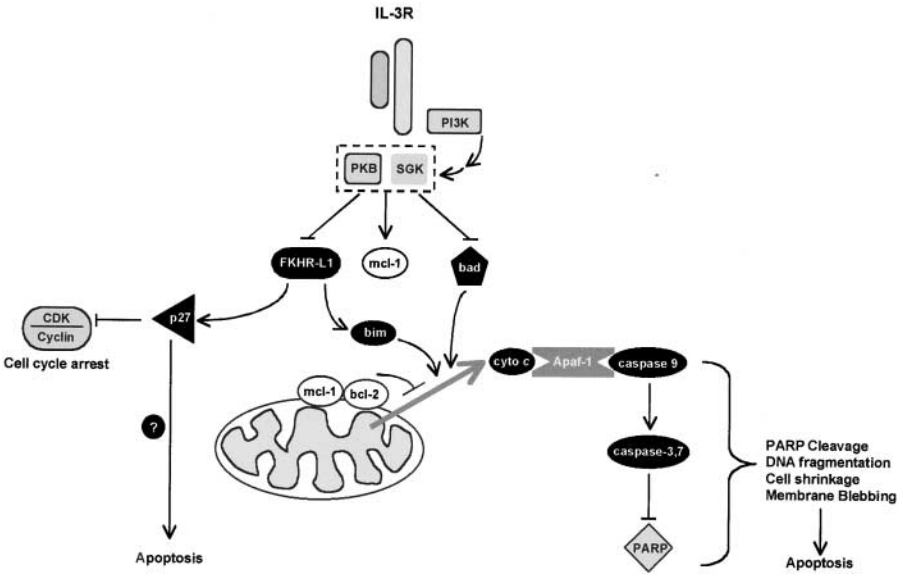

Figure 10. **A model for cytokine withdrawal–induced apoptosis.** In the absence of cytokines, PKB and SGK are inactive, preventing elevation of the anti-apoptotic Bcl-2 member Mcl-1, and resulting in dephosphorylation and subsequent activation of the pro-apoptotic Bcl-2 member Bad and the FKHR-L1 transcription factor. Transcriptional activity of FKHR-L1 elevates levels of Bim and $p27^{KIP1}$. $p27^{KIP1}$ inhibits cell cycle progression and helps to promote apoptosis in an as yet unidentified manner. Bim, possibly together with Bad, promotes leakage of cytochrome c and subsequent loss of mitochondrial integrity. This triggers the activation of caspases and subsequent cleavage of downstream targets, resulting in apoptosis.

drawal. PKB alone is sufficient to inhibit apoptosis through the maintenance of mitochondrial transmembrane potential. This is likely due to the inhibition of FKHR-L1, which prevents transcription of the pro-apoptotic Bcl-2 family member Bim. A greater understanding of the mechanisms by which cytokines regulate cellular survival will help toward the design of novel pharmacological agents for therapeutic intervention in a variety of proliferative and degenerative disorders of the immune system.

Materials and methods

Cell culture

Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (GIBCO BRL) and recombinant mouse IL-3 produced in COS cells (Caldenhoven et al., 1995). Monoclonal Ba/F3 cells stably expressing FKHR-L1(A3):ER* and the polyclonal myrPKB:ER* Ba/F3 cell line have been described previously (Dijkers et al., 2000a,b), and were cultured together with 500 μg/ml G418. For cytokine withdrawal experiments, cells were washed twice with PBS and resuspended in RPMI 1640 supplemented with 8% Hyclone serum and, in the case of the polyclonal myrPKB:ER* Ba/F3 cell line, cultured for 2 h without cytokines before adding 4-OHT. COS cells were cultured in DME (GIBCO BRL) supplemented with 8% FCS.

Mice deficient for Bim have been described previously (Bouillet et al., 1999). Each $Bim^{-/-}$ mouse used in this study was produced by crossing (C57bl.6J background) and genotyped by PCR analysis. All $Bim^{-/-}$ mice were compared with age-matched wild-type littermates (3–4 mo old). After sacrifice, the femurs were removed and bone marrow was flushed out with Iscove's modified Dulbecco's medium (IMDM) containing 20% (vol/vol) FCS, penicillin, and streptomycin through a MACS prefilter (Miltenyi Biotec). An aliquot was removed, and nucleated cells were counted with an improved Neubauer chamber after lysing the red cells in 1% (vol/vol) acetic acid. The remaining cells were pelleted and incubated for 15 min at 4°C with 200 ml Sca1 MultiSort microbeads per 10^8 cells. Sca1+ cells were isolated through two columns, either manually (MACS-MS columns) or with an AutoMACS machine, according to the manufacturer's protocol. The Sca1+ cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) containing 20% (vol/vol) FCS, penicillin, and streptomycin at 10^6 cells/ml, and their purity was determined as around 95% by flow cytometry. The Sca1+ cells were isolated for 6–7 d in the presence of IL-3 (10 ng/ml), IL-6 (10 ng/ml), and stem cell factor (100 ng/ml), and fresh medium containing cytokines was added as necessary (Kurata et al., 1998).

Antibodies and reagents

Polyclonal antibodies against cleaved caspase-3 (#9661), cleaved caspase-7 (#9491S), and PARP (#9542) and phospho-Ser473 PKB (#9271S) were from New England Biolabs, Inc. Polyclonal caspase-8 antibody (559932) and monoclonal cytochrome c antibody (clone 7H8.2C12) were from BD PharMingen. Bim polyclonal antibody was purchased from Affinity BioReagents, Inc. $p27^{KIP1}$ and RACK1 mAb were purchased from Transduction Laboratories. PI was from Sigma-Aldrich. The annexin V-FITC kit and SUPERFas Ligand were from Alexis Biochemicals Corp. Rh-123 was purchased from Molecular Probes. All other chemicals were reagent grade. pSG5–mycFKHRL1(DBD) was made by PCR cloning nucleotides corresponding to amino acids 141–268 from FKHR-L1(A3) in-frame into pSG5–myc. FasL promoter and 6×DBE luciferase reporter constructs were provided by Anne Brunet (Children's Hospital, Boston, MA) and Boudewijn (University Medical Center, Utrecht, Netherlands), respectively.

DNA laddering

10^7 cells were treated as indicated, lysed on ice for 10 min in buffer A (10 mM Tris-Cl, pH 7.4, 10 mM EDTA, 0.2% TX-100, supplemented with 1 mM PMSF, 0.1 mM aprotinin, and 1 mM leupeptin), and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was added to an equal volume phenol/chloroform, rocked gently for 10 min, centrifuged, and the upper phase was added to 1/10 vol sodium acetate (3 M, pH 5.4) and 2.5 vol ethanol, incubated at −20°C for 15 min, and subsequently spun down. The pellet was air dried, resuspended in TE containing 2 μg/ml RNase A, incubated at 37°C for 30 min, and run on a 2% agarose gel.

Western blotting

For the detection of all proteins, cells were lysed in ELB buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM EDTA together with inhibitors) (Medema et al., 1998). Protein concentration was measured and equal amounts of protein were analyzed by SDS-PAGE. Blots were incubated overnight at 4°C with appropriate antibodies (1:1,000), and after hybridization with secondary antibodies, they were developed using ECL (Amersham Pharmacia Biotech).

FACS® analysis of apoptosis

Preparation of cells for the analysis of cell cycle profiles has been described previously (Dijkers et al., 2000a). For the analysis of apoptosis by annexin V staining, cells were washed with ice cold PBS and resuspended in binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were then incubated with FITC-conjugated annexin V (Bender Medsystems) for 10 min at room temperature, washed, and resuspended in binding buffer containing 1 μg of PI/ml. Fluorescence was analyzed by FACS®.

For transient transfections, Ba/F3 cells were electroporated (0.28 kV; capacitance, 960 μF), and 2 h after electroporation, dead cells were removed by separation through a Ficoll gradient (2,500 rpm for 20 min). Cells were harvested 48 h after electroporation and analyzed by FACS® as described above, but using annexin V–phycoerythrin.

Analysis of mitochondrial depolarization

For the analysis of changes in mitochondrial potential, $\Delta\psi_m$ (Ferlini et al., 1996), cells were incubated in RPMI together with 10 μg/ml Rh-123 (Mo-

lecular Probes) at 37°C for 30 min, washed twice with PBS, and analyzed by FACS® (Dijkers et al., 2000b). The percentage of cells falling within the range of Rh-123 fluorescence, indicative of depolarized cells, is shown.

Analysis of intracellular cytochrome c release from mitochondria

Mitochondria and cytosol fractions were obtained using the mitochondria/cytosol fractionation kit (Kordia) according to the manufacturer's protocol. In brief, 5×10^7 cells were treated as indicated, washed once in PBS, resuspended in 500 μl cytosol extraction buffer, and incubated on ice for 10 min. Subsequently, cells were homogenized in an ice cold tissue grinder. The homogenate was centrifuged at 700 *g* for 10 min at 4°C. The remaining supernatant was then centrifuged at 10,000 *g* for 30 min at 4°C. Subsequently, the supernatant was collected as the cytosolic fraction. The pellet was resuspended in 100 μl mitochondrial extraction buffer and vortexed for 10 s (mitochondrial fraction). Protein concentration was measured and equal amounts of protein were analyzed by SDS-PAGE. The amount of cytochrome c in the different fractions was determined by Western blotting using a monoclonal antibody against cytochrome c.

Luciferase assays

For transient transfections, Ba/F3 cells were electroporated (0.28 kV; capacitance, 960 μF) with 20 μg of a luciferase reporter plasmid containing the FasL enhancer region (Holtz-Heppelmann et al., 1998) or a luciferase reporter containing the portion of the FasL promoter that contains three forkhead binding sites (FHRE-LUC reporter) (Brunet et al., 1999). Cells were cotransfected with 50 ng of a renilla luciferase plasmid (pRL-TK; Promega) to normalize for transfection efficiency. After transfection, cells were cultured with or without IL-3 for 18 h. Cells were then harvested, lysed in commercially available luciferase lysis buffer, and luciferase activity was determined. In some experiments, a luciferase reporter containing the $p27^{KIP1}$ promoter was used.

COS cells were transiently transfected with the pGL2-p27kip luciferase promoter construct or a pGL2–6xDBE luciferase construct (containing six FKHR-L1 binding sequence), together with pECE-HA-FKHR-L1(A3), pSG5-myc-FKHRL1-DBD expression, or control vectors and the internal transfection control (pRL-TK) by calcium phosphate precipitation. Values were corrected for transfection efficiency and represent the mean of at least three independent experiments (± SEM).

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