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 p27KIP1 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 cytochrome 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

*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; Rh123, 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; Srinivasa 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 cell 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 p27Kip1 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 late apoptotic cells. Both cytokine withdrawal and FKHR-L1 activity induced apoptosis in a similar degree (Fig. 1 A, top and middle; IL-3 withdrawal: 25% ± 4%; 4-OHT addition: 33% ± 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 p27Kip1 and Bim are transcriptional targets of FKHR-L1 (Dijkers et al., 2000a,b). We examined whether the kinetics of upregulation of p27Kip1 and Bim protein correlated with induction of apoptosis. Both cytokine withdrawal and FKHR-L1 activation resulted in an upregulation of p27Kip1 and Bim (Fig. 1, B and C). These events occurred relatively early and preceded the cleavage of DNA observed in Fig. 1 A.
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 al., 1998). Figure 2 demonstrates that death receptor signaling is not activated in response to cytokine deprivation or FKHR-L1 activation. Caspase-8 cleavage was analyzed in Ba/F3 cells that were cytokine-starved for the indicated times (A). Analysis of caspase-8 cleavage in 4-OHT–treated (100 nM) FKHR-L1(A3):ER* cells demonstrated no activation (B). Jurkat cells were treated with or without a FasL promoter construct (FasL–LUC) or a construct containing three forkhead binding sites from the FasL promoter (FHRE-LUC) (C). After culture with or without IL-3, luciferase activity was measured. Data represent the mean of three independent experiments ± SD. (D) Ba/F3 cells or Jurkat 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, luciferase activity was measured. Data represent the mean of three independent experiments ± SD. (E) Ba/F3 cells or Jurkat cells were treated with 24 h with SUPER Fas Ligand (50 ng/ml) and 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.

Cell cycle analysis of cytokine-starved cells and cells in which FKHR-L1 activity is induced. 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 Fasl signaling in FKHR-L1-mediated apoptosis.

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

p27kip1 is involved in cell cycle arrest in G1 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 p27kip1 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 G1, 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-G1 peak. By 48 h, a majority of cells had initiated a program of apoptosis (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 G1 arrest before the onset apoptosis is probably related to the initial upregulation of p27kip1 (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...
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 p27KIP1 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 \( \Psi_m \) 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-

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**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.

**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.
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 $\Psi_m$ 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...
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 p27kip1 transcription in Ba/F3 cells (Dijkers et al., 2000a), we wished to determine whether inhibition of FKHR-L1 could reduce p27kip1 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 was calculated. As shown in Fig. 8 B, overexpression of FKHRL1-DBD is indeed able to repress the induction of p27kip1 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 FKHR1-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 regulation of Bim expression, 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 G1 and subsequently underwent apoptosis (Fig. 3). This was accompanied by a sequential upregulation of p27KIP1, which is involved in arresting cells in G1, 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 p27KIP1−/− 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 p27KIP1 and Bim may play critical roles in the induction of the apoptotic program initiated by cytokine withdrawal.

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 (Rytmaa 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 p27KIP1 levels. This is supported by the observation that a decrease in p27KIP1 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 Bim 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 p27KIP1 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 p27Kip1. p27Kip1 inhibits cell cycle progression and helps to promote apoptosis in an as yet unidentified manner. Bim, possibly together with Bad, promotes cleavage of cytochrome c and subsequent loss of mitochondrial integrity. This triggers the activation of caspases and subsequent cleavage of downstream targets, resulting in apoptosis.

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 (Caldenhouwen et al., 1995). Monoclonal Ba/F3 cells stably expressing FKHR-L1(3A):ER* and the polyclonal myrPKB:ER* Ba/F3 cell line have been described previously (Dijkers et al., 2000a,b), and were cultured together with L1(A3):ER* and the polyclonal myrPKB:ER* Ba/F3 cell line have been described previously (Dijkers 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 (Amer sham Pharmacia Biotech). PI was from Sigma-Aldrich. The annexin V-FITC kit and monoclonal cytochrome c antibody (clone 7H8.2C12) were from BD Pharmingen. Bim polyclonal antibody was purchased from Affinity BioReagents, Inc. p27Kip1 and RACK1 mAb were purchased from Transduction Laboratories. PI was from Sigma-Aldrich. The annexin V-FITC kit and SUPERFas Ligand was from Alexis Biochemicals Corp. Rh-123 was purchased from Molecular Probes. All other chemicals were reagent grade.

For the analysis of changes in mitochondrial potential, DNA laddering and mitochondrial depolarization, 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, and centrifuged, and the upper phase was added to 1/10 vol sodium acetate (3 M, pH 3.4) 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.

DNA ladder

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 CaCl2). Cells were then incubated with FITC-conjugated annexin V (Bender Medical Systems) 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-phycocerythrin.

Analysis of mitochondrial depolarization

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 CaCl2). Cells were then incubated with FITC-conjugated annexin V (Bender Medical Systems) 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-phycocerythrin.
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 × 10⁷ 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 cytochrome c lysis buffer, and luciferase activity was determined. In some experiments, a luciferase reporter containing the p27Kip1 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 pCEC-HA-FKHR-L1(A3), pSG5-myc-FKHR-L1-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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