Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1

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Cell death is regulated mainly through an evolutionarily conserved form of cell suicide termed apoptosis [1]. Deregulation of apoptosis has been associated with cancer, autoimmune diseases and degenerative disorders. Many cells, particularly those of the hematopoietic system, have a default program of cell death and survival that is dependent on the constant supply of survival signals. The Bcl-2 family, which has both pro- and anti-apoptotic members, plays a critical role in regulating cell survival [2]. One family member, the Bcl-2 interacting mediator of cell death (Bim), contains only a protein-interaction motif known as the BH3 domain, allowing it to bind pro-survival Bcl-2 molecules, neutralizing their function [3]. Disruption of the *bim* gene results in resistance to apoptosis following cytokine withdrawal in leukocytes, indicating that regulation of the pro-apoptotic activity of Bim is critical for maintenance of the default apoptotic program [4]. Here, we report that withdrawal of cytokine results in upregulation of Bim expression concomitant with induction of the apoptotic program in lymphocytes. Activation of the forkhead transcription factor FKHR-L1, previously implicated in regulation of apoptosis in T lymphocytes [5], was sufficient to induce Bim expression. We propose a mechanism by which cytokines promote lymphocyte survival by inhibition of FKHR-L1, preventing Bim expression.

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Results and discussion

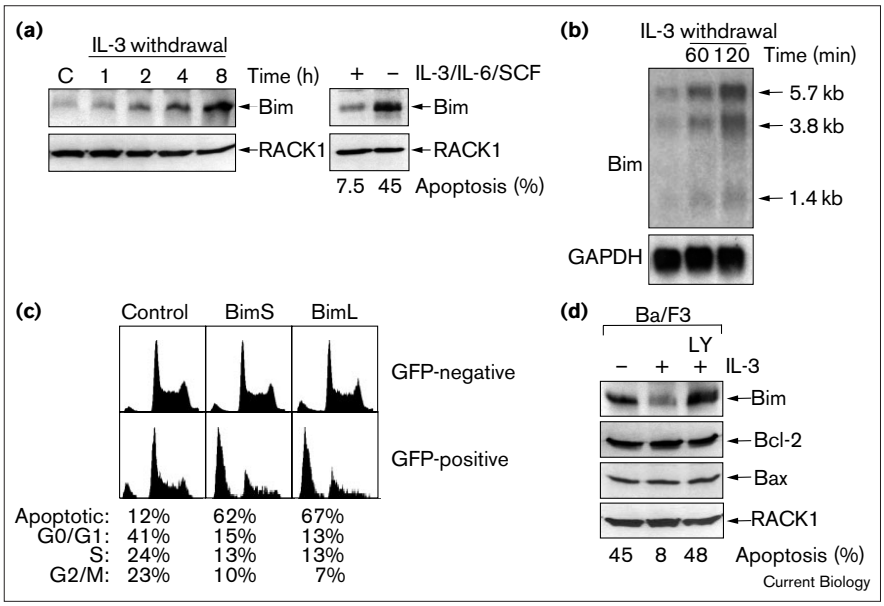
Although Bim is expressed in many hematopoietic lineages, it is not known how its expression is affected by cytokine withdrawal [3]. Here, we determined whether changes in the levels of Bim expression could be responsible for regulation of cell survival. The mouse pro-B cell line Ba/F3 has a default apoptotic pathway, which is repressed upon the addition of interleukin-3 (IL-3). We examined Bim protein levels in these cells following cytokine withdrawal. Interestingly, Bim expression increased steadily after IL-3 deprivation, correlating with induction of the apoptotic program (Figure 1a; left panel). To determine whether this observation might represent a more general phenomenon, primary mouse fetal liver cells cultured in the presence or absence of survival factors [6] were analysed for Bim expression. Indeed, fetal liver cells undergoing apoptosis following cytokine withdrawal exhibited elevated Bim protein levels (Figure 1a; right panel). To determine whether upregulation of Bim expression by IL-3 withdrawal was a result of enhanced transcription, *bim* mRNA was also analysed. Bim has three isoforms (BimS, BimL and BimEL) that are generated by alternative splicing [3]. Northern blot analysis has shown several Bim transcripts, but the specific relationship between these transcripts and the three Bim isoforms is unclear [3]. We detected several transcripts as described previously and, importantly, expression of these mRNAs was significantly elevated upon IL-3 withdrawal (Figure 1b).

To determine whether increased expression of Bim is sufficient to induce apoptosis, we transiently expressed BimS or BimL, together with spectrin-linked green fluorescent protein (GFP) in Ba/F3 cells. This approach enables analysis of apoptosis in the transfected (GFP-positive) versus untransfected (GFP-negative) cells. In cells that expressed only spectrin–GFP, cell survival was unaffected relative to control cells (Figure 1c; left panels). In cells expressing either BimL or BimS, the level of apoptosis in GFP-positive cells was dramatically increased (Figure 1c; middle and right panels).

Previous work has implicated phosphatidylinositol (PI) 3-kinase activity as being critical for cytokine-mediated rescue from apoptosis in lymphocytes [7]. To determine whether changes in Bim expression are dependent on PI 3-kinase activity, Ba/F3 cells were either starved of cytokine, or cultured with IL-3 or IL-3 in combination with the specific PI 3-kinase inhibitor LY294002. Bim protein levels were elevated in cells undergoing apoptosis induced either by IL-3 withdrawal or by inhibition of PI 3-kinase (Figure 1d). As there was no change in Bax levels, it appears that a general increase in pro-apoptotic Bcl-2 family members is not in itself a feature of cell death.

Protein kinase B (PKB, also known as Akt), a target of PI 3-kinase activation, has recently been reported to inhibit

Figure 1

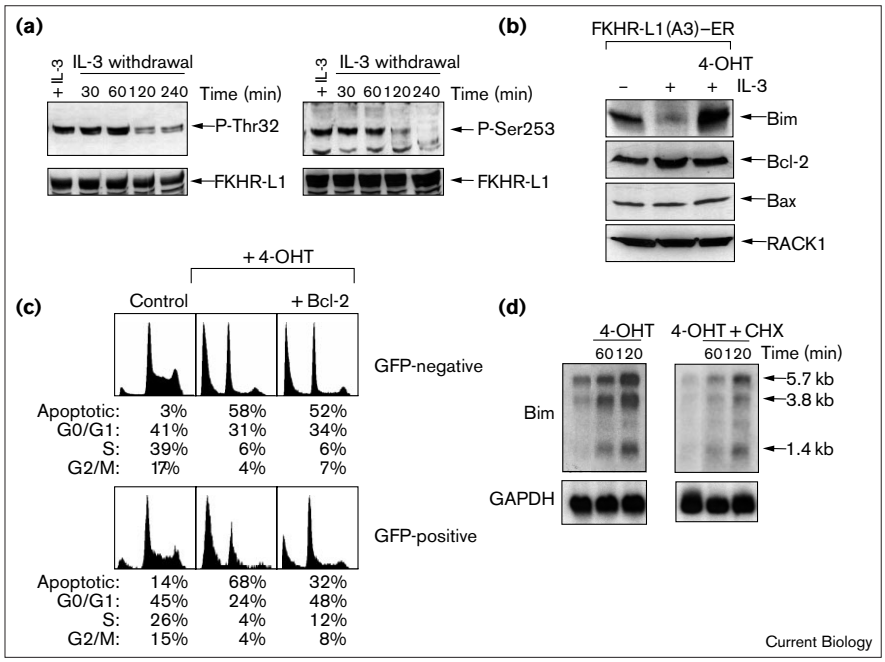


Bim levels are regulated by cytokines and determine cell-survival fate. **(a)** Left, Ba/F3 cells were starved of IL-3 and lysed after the indicated times. Equal amounts of protein were loaded and Bim levels were determined by probing with anti-Bim antibody. The blot was reprobed with anti-RACK1 antibody to confirm equal protein loading. Lane C corresponds to Ba/F3 cells cultured with IL-3. Right, mouse fetal liver cultures were treated with or without cytokines for 24 h, and the percentage of apoptotic cells was measured as well as levels of Bim and RACK1. SCF, stem cell factor. **(b)** IL-3 withdrawal induces *bim* mRNA expression. Ba/F3 cells were starved of IL-3 for the indicated times. PolyA+ RNA was isolated and *bim* mRNA levels were analysed using full-length BimL cDNA as a probe. Equal RNA loading was confirmed by reprobing the blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **(c)** Bim expression is sufficient to induce cell death. Ba/F3 cells were electroporated with 18 μg empty vector, BimS or BimL, together with 2 μg spectrin–GFP. After 24 h, the cells were fixed, stained with propidium iodide and the DNA content of 5000 GFP-positive or 20,000 GFP-negative cells was analysed by fluorescence-activated cell sorting (FACS). The data depicted is representative of several independent experiments. **(d)** PI 3-kinase activity is critical for cytokine-mediated repression of Bim. Ba/F3 cells were either starved of cytokine, or cultured overnight in the presence of IL-3 with or without LY294002 (LY, 10 μM). The levels of Bim, Bcl-2 and Bax were determined. Blots were reprobed for RACK1 to confirm equal protein loading. The percentage of apoptotic cells was also determined by FACS analysis.

transcriptional activity of a subfamily of forkhead transcription factors, which include FKHR-L1, AFX and FKHR [8]. FKHR-L1 activity, for example, is inhibited by PKB phosphorylation on three sites, resulting in an inability to translocate to the nucleus [5]. Mutation of these phosphorylation sites results in the generation of a constitutively active transcription factor and such a mutant, FKHR-L1(A3), has recently been shown to induce apoptosis in T cells through induction of Fas-L [5]. Ba/F3 cells, however, did not appear to be susceptible to Fas-L-induced apoptosis (P.J.C. and P.F.D., unpublished observations). IL-3 withdrawal resulted in dephosphorylation of FKHR-L1 (Figure 2a), which leads to nuclear translocation and activation of this transcription factor [5].

To determine whether the activity of FKHR-L1 is linked to upregulation of Bim protein levels, we generated a novel inducible FKHR-L1(A3) expression construct. FKHR-L1(A3) was fused to the hormone-binding domain of the estrogen receptor [9], resulting in a 4-hydroxytamoxifen (4-OHT)-inducible protein, FKHR-L1(A3)–ER. We generated several stable clonal Ba/F3 cell lines in which expression levels of FKHR-L1(A3)–ER were approximately 3–5 times lower than that of endogenous FKHR-L1 (data not shown). Addition of 4-OHT in the presence of IL-3 resulted in a dramatic induction of apoptosis, coinciding with an elevation of Bim protein levels (Figure 2b,c). Levels of Bcl-2 and Bax were unaffected, demonstrating that the FKHR-L1 effects appear to be specific for Bim. Elevation of Bcl-2 levels, however, has previously been shown to counteract the pro-apoptotic activity of Bim [3]. To determine whether the increase in Bim levels may be a critical mechanism by which FKHR-L1 is able to induce apoptosis, Ba/F3 cells expressing FKHR-L1(A3)–ER were electroporated with or without Bcl-2, together with spectrin–GFP as a marker for transfected cells. GFP-positive cells transiently expressing Bcl-2 were considerably more resistant to FKHR-L1-induced apoptosis than GFP-negative control cells (Figure 2c). Finally, to examine whether the enhanced expression of Bim protein by FKHR-L1 activity was a result of transcriptional regulation, *bim* mRNA was analysed. Similarly to IL-3 withdrawal (Figure 1c), 4-OHT-mediated FKHR-L1 activation significantly elevated Bim transcripts (Figure 2d; left panel). To demonstrate that upregulation of Bim levels through FKHR-L1 occurs directly and does not require *de novo* protein synthesis, Ba/F3 FKHR-L1(A3)–ER cells were treated with 4-OHT for the indicated times in combination with the protein synthesis inhibitor cycloheximide. An elevation of *bim* mRNA was also observed in cycloheximide-treated cells (Figure 2d, right panel), demonstrating that Bim transcription is indeed directly regulated by FKHR-L1.

Figure 2



Bim levels are transcriptionally regulated by IL-3 through the forkhead transcription factor FKHR-L1. **(a)** FKHR-L1 phosphorylation in Ba/F3 cells. Ba/F3 cells were starved of IL-3 and lysed at the indicated time points. Levels of phosphorylated FKHR-L1 were analysed by hybridizing with antibodies that detect the Thr32-phosphorylated (P-Thr32) or Ser253-phosphorylated (P-Ser253) forms of FKHR-L1. Equal loading was verified by reprobing with FKHR-L1 antibody. **(b)** Activation of FKHR-L1 induces Bim expression. Ba/F3 cells stably expressing FKHR-L1(A3)–ER were treated with 4-OHT (100 nM) for 24 h as indicated and the levels of Bim, Bcl-2 and Bax were determined. Blots were reprobed for RACK1 to confirm equal protein loading. **(c)** Ba/F3 cells expressing FKHR-L1(A3)–ER were electroporated with 2 μg spectrin–GFP together with either 18 μg empty vector (control) or Bcl-2, and the percentage of apoptotic cells upon treatment with 4-OHT (100 nM) for 24 h was measured as in Figure 1c. **(d)** FKHR-L1 activity upregulates *bim* mRNA levels. Left, Ba/F3 cells stably expressing FKHR-L1(A3)–ER were treated with 4-OHT (100 nM) for the indicated times. PolyA+ RNA was isolated and *bim* mRNA levels were analysed using full-length BimL cDNA as a probe. Equal RNA loading was confirmed by reprobing the blot for GAPDH. Right, same as left panel except that cells were pre-treated with cycloheximide (CHX, 10 μg/ml) before addition of 4-OHT.

Relatively little is known about transcriptional regulation of pro-apoptotic proteins in cells undergoing apoptosis. Our data identify cytokine-mediated inhibition of Bim expression as a novel mechanism of apoptotic regulation. In *Caenorhabditis elegans*, transcriptional repression of Egl-1, a pro-apoptotic protein related to Bim, is critical for regulating developmental cell death [10]. Recently, expression of the pro-apoptotic protein Hrk in hematopoietic progenitor cells was also found to be rapidly upregulated upon growth factor withdrawal [11]. Bim, like other BH3-domain proteins, exerts its pro-apoptotic activity through heterodimerization with anti-apoptotic Bcl-2 members [3]. Previously, regulation of the pro-apoptotic activity of Bim has been reported to occur through its re-localization [12]. In this model, Bim is sequestered to the microtubular motor complex by binding to dynein light chain (LC8). Pro-apoptotic stimuli release LC8 together with Bim into the cytoplasm, allowing interaction of Bim with anti-apoptotic Bcl-2 members [12]. While this may indeed modulate the activity of Bim isoforms, we have established that the regulation of Bim expression by cytokines is very likely to be a contributory factor, defining the balance between cell survival and apoptosis. Moreover, the identification of Bim as a novel target of FKHR-L1 highlights the functional importance of this recently identified subfamily of forkhead transcription factors.

Bim levels are critical in regulating apoptosis as $Bim^{-/-}$ lymphocytes have an increased resistance to cell death induced by cytokine withdrawal, surviving 10–30 times better than wild-type cells [4]. These results suggest that, at least in lymphocytes, Bim is the dominant transducer of death signals. By controlling the level of Bim through cytokine-mediated regulation of forkhead transcription factors, cell-survival decisions can be rapidly made in response to changes in the local lymphocyte environment. This is analogous to the regulation of DAF-16, a *C. elegans* forkhead transcription factor, which controls longevity in response to changes in environmental nutrient content [13]. Our data suggest that this evolutionary conserved signaling pathway has been exploited in mammals to regulate the life span of cytokine-dependent cells.

Materials and methods

Plasmids

BimS and BimL constructs were generated by PCR from Ba/F3 cDNA isolated from IL-3-deprived cells, cloned into pSG5-MYC and verified by sequencing. Plasmid pCDNA3-FKHR-L1(A3)–ER was generated by cloning FKHR-L1(A3) without the stop codon into pCDNA3 containing the hormone-binding domain of the estrogen receptor (pCDNA3-ER). Spectrin-linked GFP was a kind gift from A. Beavis and T. Sheck (Princeton) and has been described previously [14]. Plasmid pSG5-Bcl-2 was a kind gift from R.P. de Groot.

Western blotting

For determining protein levels, cells were lysed in a buffer containing (0.1% NP-40, 20 mM HEPES pH 7.5, 5 mM EDTA, 150 mM NaCl,

supplemented with 10 μg/ml aprotinin, 1 mM leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 40 mM β-glycerophosphate and 50 mM NaF). Protein content was determined and equal amounts of protein were analysed by SDS–PAGE and blots were probed with the appropriate antibodies. Bim polyclonal antibody was purchased from Affinity Bioreagents, Bcl-2 antibody from Santa Cruz, Bax monoclonal antibody from BD PharMingen, and RACK1 monoclonal antibody from Transduction Laboratories. FKHR-L1, phospho-Thr32 FKHR-L1 and phospho-Ser253 FKHR-L1 antibodies were from UBI.

Cell culture, transient electroporation and FACS analysis

Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3 produced in COS cells. Fetal-liver-derived myeloid cultures were prepared from day 17 mouse embryos by culture of suspension cells in RPMI supplemented with IL-3, IL-6 and SCF as described previously [6]. For transient transfection, Ba/F3 cells were electroporated (0.28 kV; capacitance 960 μFD) and, 2 h after electroporation, dead cells were removed by separating through a Ficoll gradient (2500 rpm for 20 min). After 24 h, cells were harvested, washed twice in PBS and fixed for at least 2 h in 300 μl PBS and 700 μl ethanol. Cells were spun down gently and permeabilized in 200 μl 0.1% Triton X-100, 0.045 M Na_2HPO_4 and 0.0025 M sodium citrate at 37°C for 20 min. Next, 750 μl apoptosis buffer (0.1% Triton X-100, 10 mM PIPES, 2 mM $MgCl_2$, 40 μg/ml RNase, 20 μg/ml propidium iodide) was added and incubated for 30 min in the dark. The percentage of apoptotic cells was analysed by FACS as the percentage of cells with a DNA content of $< 2N$, counting 5,000 cells. Thresholds were set to gate out cellular debris. Cell-cycle profiles were determined using a FACS calibur (Becton and Dickson) and analysed using Cell Quest and MofFit software.

Northern blotting

Total RNA was isolated from Ba/F3 and 500 μg was used for the isolation of polyA+ RNA using polyA Tract mRNA isolation kit from Promega. Equal RNA loading was verified by reprobing the blots with a 1.4 kb cDNA fragment of the human GAPDH gene.

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