Molecular mechanisms of cytokine-mediated proliferation and survival

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Moleculaire mechanismen van cytokine-gemedieerde proliferatie en survival

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Voor mijn vader en moeder

`Would you tell me, please, which way I ought to go from here?'
`That depends a good deal on where you want to get to,' said the Cat.
`I don't much care where--' said Alice.
`Then it doesn't matter which way you go,' said the Cat.
`--so long as I get SOMEWHERE,' Alice added as an explanation.
`Oh, you're sure to do that,' said the Cat, `if you only walk long enough.'

From *"Alice in Wonderland"* by Lewis Carroll

Contents

CHAPTER 1

General Introduction

CONTENTS

Signal transduction

In a multicellular organism a proper balance needs to be established between proliferation, differentiation and cell death in order to maintain tissue homeostasis. To achieve this, a signaling network has evolved by which cells can communicate. To allow communication between cells, in addition to cell-cell contact, mechanisms have evolved that include secretion of growth factors and cytokines which can bind to their cognate receptor on the outside of target cells. Ligand-receptor interaction triggers a cascade of intracellular events, which can ultimately lead to a plethora of responses, including survival or apoptosis (programmed cell death), proliferation, differentiation and modulation of activities of differentiated cells. This cascade of events, which is triggered upon ligand-receptor interaction, is termed signal transduction.

Signal transduction is characterized by the coordinate action of kinases and phosphatases, as well as the release of second messengers. This can result in posttranslational modification, as well as relocalization of proteins, thus altering their activity. The importance for regulation of proliferation, differentiation and apoptosis is illustrated in that dysregulation of this balance is associated with cancer, chronic inflammatory diseases and degenerative diseases as a result of inappropriate cell death. To understand the mechanisms underlying these diseases, ultimately allowing clinical interference, it is important to study signal transduction and how its dysregulation is associated with disease. In this thesis, research investigating mechanisms underlying proliferation and survival by cytokines in inflammatory cells is described. We focused on cytokines of the IL-3, IL-5 and GM-CSF family, which play a crucial role in proliferation, survival and differentiation of cells of the myeloid lineage[1].

The IL-3, IL-5 and GM-CSF family: structure and function

Cytokines of the interleukin (IL)-3, IL-5 and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) are secreted amongst others by activated T cells and mast cells and promote survival, proliferation and differentiation of cells of the hematopoietic lineages. The receptors for IL-3 and GM-CSF are expressed on a broad range of hematopoietic cells, whereas the expression of the IL-5 receptor is more restricted, being present on eosinophils and basophils in man, as well as on B cells in mice[2,3].

Receptors for IL-3, IL-5 and GM-CSF consist of a cytokine-specific alpha chain, IL-3α, IL-5 α and GM-CSFα, glycoproteins of 60-80 kD, and a common beta chain (βc), a 120 kD glycoprotein, which is shared by these receptors. The βc does not bind cytokine itself, but forms a high-affinity receptor with the alpha subunit upon ligand binding[4]. Receptors for IL-3, IL-5 and GM-CSF belong to the cytokine receptor superfamily, which consists of a variety of receptors that are structurally and functionally related[2]. The extracellular domain of the α chains and βc contains structural motifs that are conserved throughout this superfamily. These motifs consist of 4 cysteines and a WSXWS repeat, both of which are involved in ligand binding (Fig. 1). The intracellular domain of α subunits is short (~50 amino acids) and contains a proline-rich motif. In contrast, the intra-

cellular domain of βc is relatively large (436 aa in man and 432 aa in mouse), containing structurally conserved membrane-proximal regions, known as "box 1" and "box 2", and six tyrosine residues in the membrane-distal region. Whereas in man all α subunits bind to βc, in mice an additional β subunit is present, βIL-3, that binds exclusively to IL-3Rα [5]. βIL-3 is highly homologous to βc and has probably arisen by gene duplication[6]. Analysis in knockout mice, however, has revealed no additional function of βIL-3 compared to βc[7]. Six tyrosine residues in βc are conserved from mouse to man, although the murine βc has an additional tyrosine, of which the relevance for signal transduction remains to be established. Ligand binding results in receptor dimerization, triggering subsequent signaling events, transduced through the βc. Although some signal transducing activity has been attributed to the alpha subunit, in particular the proline-rich region, there are conflicting data as to whether this contributes to cell proliferation or survival[8-12]. Given their cytokine-specificity, the alpha subunits might be involved in mediating differentiation of target cells, although this is still largely unconfirmed.

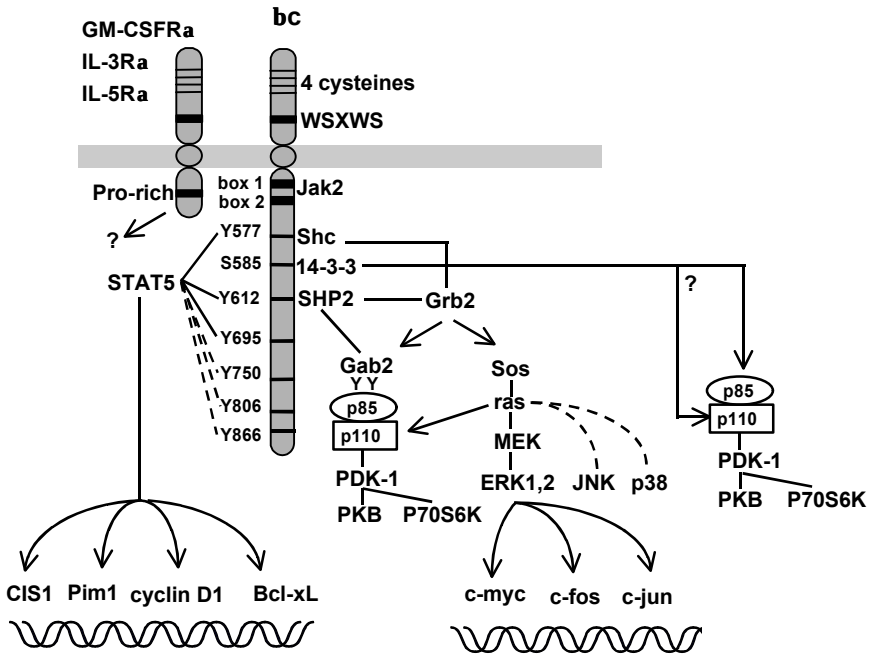

Figure 1. Activation of signaling pathways by the IL-3, IL-5 and GM-CSF receptor family. Binding of IL-3, IL-5 and GM-CSF to their cytokine-specific alpha chain triggers heterodimerization with the βc, resulting in activation of JAK2. JAK2 then phosphorylates tyrosine residues in the βc, which recruits binding of cytoplasmic signaling proteins. These proteins either directly promote transcription (STAT5) or in turn recruit other signaling proteins to the βc, resulting in the activation of multiple signaling pathways.

Intracellular signals elicited by IL-3, IL-5 and GM-CSF

Initial studies on signal transduction of IL-3, IL-5 and GM-CSF revealed that they elicit similar patterns of tyrosine phosphorylation of cytosolic proteins[13,14]. As is true for many cytokine receptors, neither the α chains nor βc possess intrinsic tyrosine kinase activity, but instead a tyrosine kinase, JAK2, is associated with the box 1 region of βc[15,16]. Receptor-ligand-induced dimerization of βc subunits promotes cross-phosphorylation and activation of JAK2, which then phosphorylates tyrosine residues in the cytoplasmic tail of βc (see Fig. 1). Activity of JAK2 is both necessary and sufficient for the initiation of signaling, since mice deficient in JAK2 fail to respond to either IL-3 or GM-CSF[17]. Moreover, constitutive activation of JAK2, by constructing a chimeric receptor (βc /JAK2), consisting of βc extracellular and transmembrane regions fused with JAK2, abrogates the need for cytokines for proliferation or survival[18]. Deletion analysis of βc revealed that signaling through the membrane proximal region induces a transient proliferative response, potentially mediated by induction of the serine threonine kinase pim1 and transcription factor c-myc[19], whereas the membrane distal region is required for cell viability[20,21].

The membrane distal region of βc also contains tyrosine residues that are phosphorylated upon the action of IL-3, IL-5 and GM-CSF[14]. These phospho-tyrosines are capable of recruiting cytoplasmic signaling molecules through their Src-homology 2 (SH2) domain. Subsequently, relocalization of these signaling molecules promotes their activation, triggering subsequent signaling events. Furthermore, the distal region of βc is also important for activation of kinases of the Src family, such as Lyn, Hck and Fyn, which may occur independently of βc tyrosine phosphorylation. Interestingly, this family of kinases has also been proposed to contribute to tyrosine phosphorylation of βc[22-24].

Activation of STAT5 through bc.

One signaling protein that can bind directly to phospho-tyrosines on the βc is the transcription factor STAT5 (Signal Transducer and Activator of Transcription). Binding of STAT5 to the βc results in its subsequent phosphorylation and dimerization that then translocate to the nucleus and promote transcription of target genes which include pim1, CIS1, Bcl-xL and cyclin D1 (reviewed in [25]; Fig. 1). There is some redundancy in βc-mediated activation of STAT5, since STAT5 can bind to multiple phosphorylated tyrosine residues, including Tyr577, Tyr612, Tyr695, and Tyr750 on the human βc[26]. Moreover, Tyr806 and Tyr866 have also been described to promote its activation, but direct binding of STAT5 to these residues has not been demonstrated[27].

Activation of the small GTPase p21ras and its intracellular targets.

Other signaling routes initiated through the distal region of the tyrosine-phosphorylated βc involve the activation of the small GTPase p21ras[28]. Downstream effectors of p21ras activation include the dual specificity kinase MEK and its target ERK (MAPK), resulting in induction of the transcription factors c-fos and c-jun[28,29]. Besides activating MEK-ERK, p21ras can also induce PI3K activity by direct binding to its p110 catalytic subunit[30]

(see below). Finally, signaling through βc can also activate two other members of the MAPK family, p38 and JNK, although their role in transducing a proliferative response and the precise mechanism of their activation still remains to be resolved[31-33].

The activation of p21ras through the βc is complex and requires intermediate proteins. Two proteins that function as adaptor proteins and can bind directly to phosphorylated tyrosine residues on βc are Shc and the protein tyrosine phosphatase SHP2 (also known as PTP-1D, PTP-1C, SH-PTP2 or Syp). Shc binds to phosphorylated Tyr577 of the βc, whereas SHP2 binds Tyr612. Both proteins become tyrosine-phosphorylated upon binding to βc[34-36]. Subsequently, the phosphorylated tyrosine residues on Shc and SHP2 serve as docking sites for the SH2 domain of another adaptor protein, Grb2[37]. In addition to its SH2 domain Grb2 possesses two SH3 domains that bind to the proline-rich region of the GTP-exchange factor Sos. Recruitment of Sos to the plasma membrane subsequently activates p21ras (Fig. 1).

Regulation of phosphatidylinositol 3-kinase activation

Another mechanism by which signals from the distal part of the βc may promote cell survival is through activation of the lipid kinase phosphatidyl inositol 3-kinase (PI3K)[21,38]. The PI3K family consists of several isoforms (reviewed in [39]), of which the classical form consists of two subunits, a p85 regulatory subunit, containing two SH2 domains, and a catalytic subunit, p110. Recruitment of p85 to the βc results in membrane targeting, allowing phosphorylation of the phosphatidylinositol (PI) lipids, phosphatidylinositol-4-phosphate (PI-4P) and phosphatidylinositol-4,5-bisphosphate PI4,5-P_2 on the D3 position (reviewed in [40]). Following this phosphorylation, activation of PI(3,4,5)-trisphosphate dependent kinase (PDK-1) is promoted upon binding to PI(3,4,5)P_3[41]. Downstream targets of PDK-1 include the serine-threonine kinase Protein Kinase B (PKB, also known as Akt)[42], and p70S6K[42-44] (see Fig. 1).

In addition to direct activation by p21ras[30], there are several alternative mechanisms by which PI3K can be activated through βc, which again involve intermediate adaptor proteins. The mechanism of cytokine-mediated PI3K activation has been elusive until the recent discovery of involvement of the adaptor protein p97/ Gab2[45]. Gab2 can be recruited to the βc through association with either tyrosine-phosphorylated SHP2 or Grb2. Following recruitment to βc Gab2 is itself tyrosine-phosphorylated and these tyrosines serve as docking sites for the p85 subunit of PI3K[46] (Fig 1). Interestingly, serine phosphorylation of the βc has also been reported to be critical for PI3K activation[47]. Recently, IL-3 stimulation was demonstrated to result in the phosphorylation of Ser585 in the human βc, and site-directed mutagenesis of this residue specifically impaired the PI3K signaling with a concomitant reduction in cell survival[48]. Ser585 is conserved from mouse to man, and is located in a consensus phosphorylation site for PKA, serving as a docking site for 14-3-3 proteins. 14-3-3 proteins are regulatory molecules that can bind various signaling molecules and might thus serve as adaptor proteins (reviewed in [49]). Binding of 14-3-3 to this site has been suggested to recruit the p85 subunit of PI3K,

either directly or via an intermediate, promoting PI3K activation[47]. This suggests that there are multiple signaling pathways that could contribute to the proliferative and anti-apoptotic signals through βc.

Turning off the signal

Phosphatases

Following IL-3, IL-5 or GM-CSF binding, a transient increase in tyrosine phosphorylation of βc and intracellular proteins is observed. Addition of phosphatase inhibitors both elevates and extends the duration of this tyrosine phosphorylation, resulting in enhanced proliferation and survival[13,14]. This suggests that the action of phosphatases is necessary to regulate a transient signal, and that dysregulation of these phosphatases might be linked to aberrant proliferation or survival. Several phosphatases have been implicated in IL-3, IL-5 or GM-CSF-induced signaling. These include SHP1 (also referred to as PTP-1C, HCP or SH-PTP1), SHP2, SHIP (SH2-containing inositol 5-phosphatase)[36,50,51] and PTEN (Phosphatase and Tensin homolog). SHP1 binds to βc, presumably to Tyr-612, to which it might compete for binding with SHP2[36]. Overexpression of SHP1 decreased IL-3-induced proliferation and was effective in dephosphorylating the βc in vitro[50], suggesting that SHP1 can directly abrogate recruitment of signaling molecules to βc. Little is known regarding the functional significance of SHP2 phosphatase activity in βc-mediated signaling, although it has been proposed to dephosphorylate Gab2[45] and demonstrated to dephosphorylate STAT5 *in vitro*[52]. Another phosphatase involved in inhibiting βc-generated signals is SHIP, an inositol 5' phosphatase that hydrolyzes the PI3K product $PI(3,4,5)P_3$ to $PI(3,4)P_2$[53,54], making it a direct modulator of downstream signaling of PI3K. Indeed, SHIP is important for inhibiting activation of PKB, since SHIP*(-/-)* mice exhibited a prolonged PI3K-dependent $PI(3,4,5)P_3$ accumulation and PKB activation in response to IL-3 and GM-CSF[55]. Moreover, as SHIP binds to tyrosine-phosphorylated SHP2[56] and Shc[57,58], SHIP might interfere with the propagation of βc signals by competing with Grb2 and Gab2 for binding. SHIP-deficient mice exhibit a chronic hyperplasia of myeloid cells which are more resistant to apoptosis[55], suggesting a role for this phosphatase in oncogenesis. The lipid phosphatase PTEN can directly counteract PI3K action by dephosphorylating 3-phosphorylated phosphoinositides on the D3 position[59]. Besides the recent observation that phosphorylation of PTEN at the C-terminus increases its stability to proteasome-mediated degradation[60,61], little is known regarding the regulation of its phosphatase activity. The importance of PTEN in downregulating signals is illustrated in *PTEN (+/-)* mice. These mice were investigated instead of *PTEN (-/-)* mice deletion of both *PTEN* alleles caused early embryonic lethality. *PTEN (+/-)* mice developed a lethal autoimmune disorder accompanied by decreased apoptosis and increased proliferation upon activation in T lymphocytes from these mice[62]. Furthermore, loss of PTEN activity resulting in an elevation of PKB activity has been observed in tumor cells. Reintroduction of PTEN in these cells reduced PKB activity[63] and abrogated their tumorigenicity[64,65].

SOCS proteins

Another mechanism through which cytokine-induced signaling can be abrogated is through downregulating its own signal. The signals that are generated upon cytokine binding to its receptor can promote transcriptional upregulation of proteins that inhibit the further transduction of signals, thus providing a cytokine signaling-induced feedback loop. One family of proteins involved in "turning off" cytokine-induced signals is the SOCS/CIS/SSI (Suppressor Of Cytokine Signaling/ Cytokine Induced SH2 containing protein/ STAT-induced STAT Inhibitor) family (reviewed in [66]). All members of the SOCS/CIS/SSI family contain a conserved domain at their C-terminus, called the SOCS-box, which interacts with components of the ubiquitin system. Thus, SOCS proteins may act as adaptor molecules that target activated cell signaling proteins to the protein degradation pathway[67]. STAT5 transcriptionally upregulates CIS1, which can bind to the tyrosine-phosphorylated βc and thereby reduce the IL-3-induced growth rate upon overexpression[68]. Higher levels of CIS1 inhibit STAT5 activity possibly through "shielding" the βc tyrosines or alternatively, ubiquitin-mediated degradation of the βc by the proteasome[69]. This mechanism of CIS1-induced abrogation of cytokine-induced signaling has been suggested for the erythropoietin receptor[70]. With respect to this it is interesting that treatment of cells with proteasome inhibitors prolonged IL-3-induced activation of JAK/STAT pathway, as well as ERK phosphorylation[71]. Two other SOCS-family members, JAB (Jak Binding protein, also known as SOCS-1 or SSI-1) and SOCS-3 (or Ssi-3, CIS3 OR JAB2) directly bind to JAK2 tyrosine kinase domain, and inhibit its kinase activity[72,73].

A further cytokine-induced inhibitor of proliferation is p56(dok-2), which inhibits phosporylation of Shc through an as yet unidentified mechanism[74]. Altogether, cytokine receptors possess a variety of means to "turn off" cytokine-induced signaling, illustrated in Fig. 2. As discussed above, deletion or mutation of some of these proteins, has recently been demonstrated to play a role in oncogenesis.

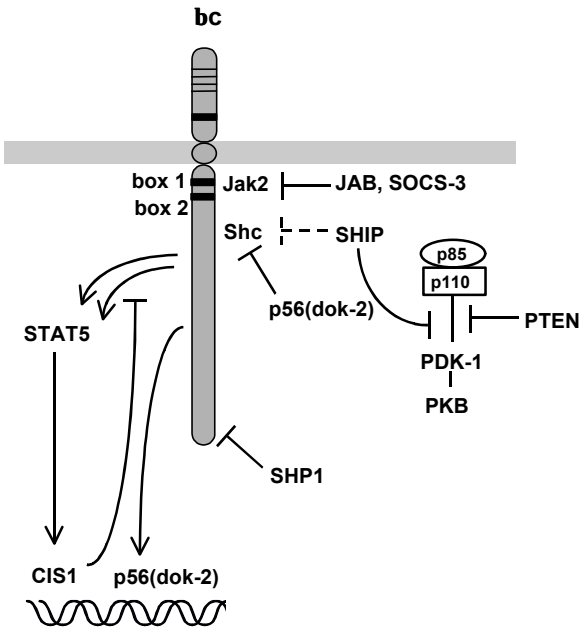

Figure 2. Turning off IL-3, IL-5 and GM-CSF-induced signals. Signals elicited through the βc can be abrogated through the action of phosphatases, SHP1, SHP2, SHIP and PTEN. Alternatively, signal transduction through the βc also promotes transcription of inhibitors, such as CIS or p56(dok-2) that interfere with βc-generated signals.

Mechanisms of growth factor and cytokine-induced proliferation

To get a complete picture as to how cytokines and the signaling pathways that they activate regulate proliferation, one needs to understand the complex process of cell division. Growth factors and cytokines regulate cell proliferation through a network of signaling cascades, regulating assembly and activation of components of the cell cycle machinery, which are described below.

Progression through the cell cycle

The events occurring in the eukaryotic cell cycle include duplication and condensation of the chromosomes, their migration into a spindle network and segregation into two daughter cells (reviewed in [75,76]). With respect to these events, the cell cycle can be divided into four phases: an S (synthesis) phase, in which the chromosomes are duplicated, and M phase (mitosis) separated by two intervals, G_1 (gap 1) the interval between M and S and

G_2, the interval between S and M (Fig. 3). In addition to these phases there is also G_0, representing the quiescent state of cells that are not cycling, which include terminally differentiated cells. Before initiating the cell cycle, controlling systems ensure that the cell only divides when it has attained the proper size and only when it receives positive signals from its environment. In addition, there are regulatory mechanisms to ensure correct chromosome replication and proper alignment before undergoing mitosis. These controlling systems include the so-called "checkpoints", present in G_1 (R, for Restriction point), or G_2 and M (also called spindle formation checkpoint). In this section, focus is on R as passage through this checkpoint is an important means by which growth factors and cytokines are able to override the intracellular controls that block cell cycle progression.

The sequential events of G_1-S-G_2-M are coordinated by so-called "cyclins" and their associated catalytic subunit cyclin-dependent kinases (CDKs). The regulated expression of cyclins and their association with distinct CDKs varies through each phase of the cell cycle (Fig. 3), resulting in an altered CDK substrate specificity[77]. The distinct substrates that are phosphorylated during the different phases of the cell cycle promote the specific events associated with each phase. The cyclin/CDK complexes that are present in G_1 and promote the progression through G_1 and the G_1-S transition are the D-type cyclins, associated with CDK4 and CDK6, and the E-type cyclins, associating with CDK2. The induction of S-phase by cyclin D(1,2,3)/CDK(4,6) and cyclin E/CDK2 is largely dependent on their ability to phosphorylate members of the Rb (retinoblastoma) family, consisting of Rb, p107 and p130[78] (reviewed in [79]). Rb proteins associate with E2F transcription factors and hereby inhibit their activity. Phosphorylation of Rb proteins by G_1 cyclin/CDK complexes results in the release of E2F, allowing transcription of its targets, including cyclin E and cyclin A[80,81], the predominant cyclin in S phase. This allows further progression through G_1 and initiation of S phase (see Fig. 3).

The cyclin-dependent Kinase Inhibitors (CKIs)

In addition to the Rb family, there is another class of proteins that negatively regulates G_1 progression, the Cyclin-dependent Kinase Inhibitors (CKIs). These associate to, and thus inhibit, the activity of cyclin/CDK complexes. CKIs can be divided into two classes, one being the p16/INK4 family, inhibitors of D-type cyclin/CDK4,6, and members of the $p21^{CIP1}$, $p21^{KIP1}$, $p57^{KIP2}$ family (for review, see [82]). The p16/INK4 family will not be discussed further here. The $p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$ family can inhibit cyclin D, as well as cyclin E- associated kinase activities. When present at lower concentrations however, $p21^{CIP}$ and $p21^{KIP1}$ are also involved in the assembly of CDK/ cyclin complexes, without actually inhibiting their activity[83]. $p21^{CIP1}$ promotes DNA-damage induced cell cycle arrest, when its levels are transcriptionally elevated in a p53-dependent manner (reviewed in [84]). In response to growth factors, levels of $p27^{KIP1}$ dramatically decrease[85-87], which appears to be a critical mechanism by which growth factors are capable of inducing cell cycle progression. Moreover, antisense inhibition of $p27^{KIP1}$ expression pre-

vents cell cycle arrest in response to growth factor depletion[85]. This suggests that upregulation of $p27^{KIP1}$ might be a key mechanism by which cell cycle arrest is induced in response to growth factor deprivation. Interestingly, decreased levels of $p27^{KIP1}$ have also been described to be associated with a poor prognosis in cancer[88,89]. An important means by which expression of $p27^{KIP1}$ is regulated is via posttranslational mechanisms; phosphorylation on Thr-187 results in subsequent targeting for degradation by the ubiquitin system[85,90,91]. This phosphorylation is mediated by cyclin E/CDK2[91,92], providing a negative feedback control through cell cycle progression (Fig. 3).

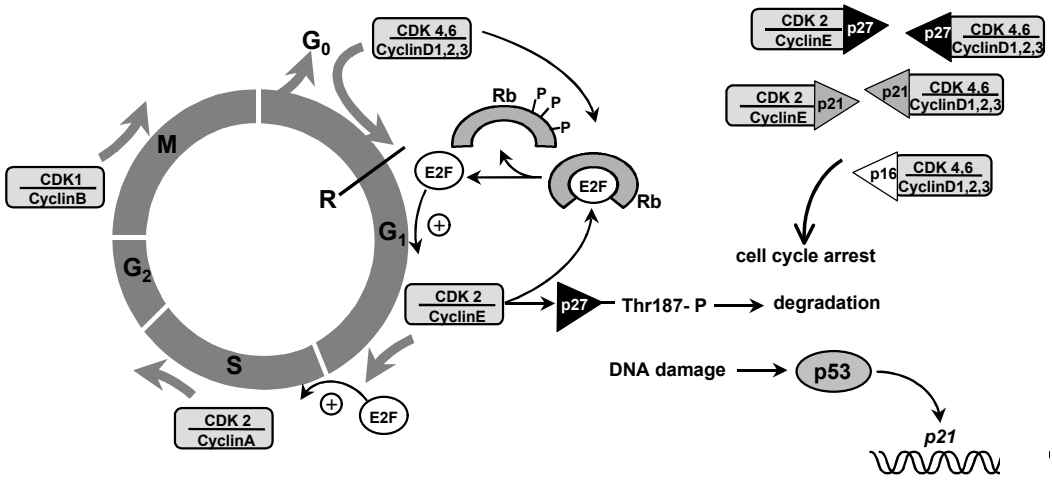

Figure 3. Regulation of the cell cycle. CDK/cyclin complexes promote progression through the different phases of the cell cycle, G_1-S-G_2-M. The initial onset of the cell cycle requires phosphorylation of Rb, thus allowing transcription factor E2F to promote transcription of components of the cell cycle machinery. Activity CDK/cyclin complexes can be inhibited by CKIs. The regulation of their expression includes transcriptional upregulation (p21) or posttranslational modification (p27). Adapted from [1].

Molecular mechanisms regulating the onset of proliferation

Given that cytokines and growth factors can promote proliferation, considerable effort has been invested in elucidating how and which signal transduction pathways are involved in this process. Signaling pathways promoting proliferation generally act in concert and one should keep in mind that there is considerable crosstalk between those pathways. However, it is possible to determine what the relative contribution of each pathway is to inducing proliferation. For this purpose, either pharmacological inhibitors or overexpression of active or dominant-negative forms of components of a signaling pathway are utilized.

Growth factor and cytokine-induced signaling rapidly induces transcription of so-called immediate-early genes, such as c-fos, c-jun and c-myc, all of which have been associated with the induction of a proliferative response[29,93]. One of the earliest events in growth factor-induced proliferation is the synthesis of cyclin D. Higher levels of cyclin D are able to override the inhibitory effects of CKIs, resulting in the activation of associated CDK4,6, cyclin E/CDK2, and the onset of proliferation (reviewed in [79]). Upregulation of cyclin D is cannot be attributed to one specific signaling pathway, since elevation of cyclin D1 has been associated with STAT5[94], p21ras[95], MEK [95,96], as well as PI3K activity[97]. PI3K-promoted entry into S-phase may be mediated through its downstream target p70S6K, which has been demonstrated to be essential for G_1 progression and elevation of cyclin D1[98,99]. The importance for PI3K in proliferation is demonstrated by the observation that proliferation is impaired in B cells that were derived from mice deficient for the p85 subunit of PI3K[100]. PKB has also been implicated in promoting cyclin D expression by enhancing its translation[101], possibly through promoting the phosphorylation and subsequent inhibition of a repressor of mRNA translation, 4E-BP1[102].

Elevation of cyclin D levels might also be linked to the transcription factor c-myc, as growth factor stimulation increases levels of c-myc through transcriptional and posttranscriptional mechanisms involving p21ras[103,104]. Moreover, c-myc has been demonstrated to rapidly induce cyclin E/ CDK2 kinase activity through promoting sequestration of $p27^{KIP1}$ via elevation of cyclin D levels[105,106].

Mechanisms of growth factor and cytokine-induced rescue from apoptosis

Cellular proliferation induced by growth factors or cytokines can only occur in the presence of distinct survival signals. Cells receiving proliferative signals, in the absence of survival signals do not proliferate, but rather die, by a process termed apoptosis. One example illustrating this is that ectopic expression of c-myc alone, which can act as an inducer of proliferation, drives cells into apoptosis in the absence of p21ras and PI3K activity[107-109]. In this way apoptosis may be an important mechanism for the elimination of cells harboring mutations in cell cycle regulatory proteins, which may otherwise create an imbalance in normal cell proliferation. Thus, oncogenesis is promoted by aberrant proliferative signals together with deregulated apoptosis, allowing uncontrolled cell division (reviewed in [110,111]). Programmed cell death is also required during the normal development of a multicellular organism, in the turnover and renewal of cells in the adult body and in the elimination of cells that do not or inappropriately respond to signals from their intracellular environment.

Characteristics of apoptotic cell death

Apoptosis is a process characterized by morphologically distinct features including blebbing and shrinking of the nucleus and the cytoplasm, loss of mitochondrial transmembrane potential, cleavage of intracellular proteins and degradation of chromosomal DNA. DNA is cleaved into fragments of distinct size, called "DNA laddering", a hallmark often used to characterize apoptotic cells[112]. Furthermore, the lipid content on the outside of apoptotic cells changes, resulting in an increase in phosphatidylserine, which can be recognized by macrophages, which phagocytose dying cells. These events are distinct from necrosis, cell death caused by injury, resulting in swelling and bursting of the cell and random degradation of chromosomal DNA.

The specific cleavage of proteins and induction of DNA laddering in cells undergoing apoptosis is carried out by cysteine proteases or "caspases" that cleave specific sites, carboxy-terminal to an aspartate residue (reviewed in [113]. Caspase activity also results in the activation of CAD (caspase-activated deoxyribonuclease), which is responsible for chromosomal DNA degradation during apoptosis[114,115]. Caspases exist as inactive pro-enzymes in the cell and possess an N-terminal prodomain, containing protein-interaction motifs, together with large and small subunits. The prodomain generally targets the caspase for activation, which occurs through proteolytic cleavage, resulting in the release of the prodomain and rearrangement of the large and small subunits to form an active caspase. Initial caspase activity in apoptosis is induced through the recruitment of caspase pro-enzymes to activator molecules, promoting pro-caspase oligomerization and auto-activation. These activated "initiator caspases" then cleave and activate downstream caspases, resulting in cleavage of apoptotic substrates (reviewed in [116,117]).

Molecular events regulating apoptosis or cell survival

An important family of proteins involved in the control of apoptosis is the Bcl-2 family (reviewed in [118]). The first member, Bcl-2, was identified due to its deregulation in B cell lymphomas bearing the t(14;18) translocation, resulting in constitutive Bcl-2 expression[119,120]. Many more Bcl-2 members have been identified and characterized since, based on homology in conserved regions, called Bcl-2 homology (BH 1-4) domains. Bcl-2 proteins are conserved though evolution, and in the nematode worm *Caenorhabditis elegans*, Bcl-2 homolog CED-9 is able to suppress apoptosis by preventing activation of the caspase, CED-3 (reviewed in [121]). Analogous CED-9 members in mammals include Bcl-2, Bcl-xL, A1 and Mcl-1[122]. Further dissection of the apoptotic pathway in *C. elegans* led to the identification of a CED-9 antagonist, EGL-1[123]. EGL-1 binds to and inactivates CED-9, and activates CED-3 through a mechanism involving CED-4, the homolog of mammalian Apaf-1, apoptosis activating factor-1 (Fig. 4).

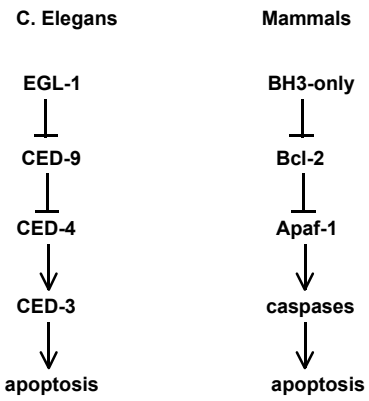

Figure 4. Evolutionary conservation of the apoptotic program. In C. elegans, as well as in mammals, induction of apoptosis requires the activation of caspases. One mechanism of activating caspases can be mediated by Apaf-1/ CED-4 and can be inhibited by the action of Bcl-2/ CED-9. Activity of BH3-only proteins/ EGL-1 can result in induction of apoptosis through the inhibition of Bcl-2/ CED-9.

Interestingly, EGL-1 also belongs to the Bcl-2 family, but of the conserved BH domains in the Bcl-2 family, it only contains the BH3 domain. EGL-1 homologs in mammals have also been identified, including Bad, Bid, Bik, Bim and Hrk (harakiri), which have been termed BH3-only proteins. In addition, mammals also contain other pro-apoptotic Bcl-2 proteins that can induce apoptosis independent of binding to Bcl-2, such as Bax[122]. Interestingly, antiapoptotic Bcl-2 family members were found to inhibit apoptosis by preventing the release from cytochrome c from mitochondria[124]. The mechanism by which cytochrome c could promote activation of caspases has been a mystery until the identification of a cofactor, Apaf-1, which can form a complex with caspase-9 in the presence cytochrome c and dATP, resulting in its activation[125,126] (Fig. 5).

Homology to bacterial toxins with pore-forming activities and experiments in vitro suggested that Bax, but also anti-apoptotic Bcl-2 members form pores in the outer membranes of mitochondria, with Bax promoting release of cytochrome c[127]. Conversely, anti-apoptotic Bcl-2 family proteins prevent mitochondrial changes associated with cytochrome c release[124,128], also through inhibition of Bax channel-forming activity[129].

Maintaining an appropriate balance between pro- and anti-apoptotic Bcl-2 family members is a crucial mechanism by which cellular homeostasis is achieved. Growth factors and cytokines have been demonstrated to be able to promote survival through transcriptional upregulation of anti-apoptotic Bcl-2 family members or inactivation of pro-apoptotic Bcl-2 proteins[130-132]. Interestingly, caspases have also been shown to cleave

Bcl-2, thus inhibiting its anti-apoptotic activity and this way provide a positive feedback loop in executing the apoptotic program[133,134]. With respect to Bcl-2 regulation in lymphocytes an important observation was made in *Bim (-/-)* knockout mice. Lymphocytes derived from these mice failed to undergo apoptosis in response to cytokine withdrawal[135]. This suggests that Bim is indispensable for hematopoietic homeostasis and that there is a unique role for Bim in promoting cytokine withdrawal-induced apoptosis. With respect to this, it is interesting that Bcl-2 deficient mice develop lymphopenia, providing genetic evidence of the importance of maintaining the balance between pro- and anti-apoptotic Bcl-2 family members[136].

Other evolutionarily conserved players in mediating survival are IAPs (Inhibitors of Apoptosis Proteins), that suppress apoptosis by directly binding to and inhibiting the activity of caspases[137], (reviewed in [138]). Although their regulation by growth factors has been suggested in endothelial cells[139], regulation in lymphocytes remains to be demonstrated.

In addition to growth factor deprivation-induced activity of pro-apoptotic members of the Bcl-2 family, there is another mechanism of initiating apoptosis, which occurs through the activation of so-called "death receptors", such as Fas (also known as CD95 or Apo-1) and results in a rapid induction of the apoptotic program. Fas-induced apoptosis has been described as an important mechanism of eradicating activated T cells (reviewed in [140,141]). Ligation of Fas with Fas ligand, FasL, recruits caspase-8 (also known as FLICE) into a multimeric complex via adaptor protein FADD, which triggers its activation (reviewed in [140]; see Fig. 5). Caspase-8 promotes induction of apoptosis though direct cleavage of apoptotic substrates, but can also activate other caspases by cleaving BH3-only protein Bid. Cleaved Bid is then recruited to the mitochondria and inhibits anti-apoptotic Bcl-2 family members, resulting in the activation of further caspases (reviewed in [140,141], also Fig. 4). Cytokines have been reported to interfere with death receptor-induced apoptosis via induction of c-FLIP (FLICE-inhibitory protein), which competes with caspase-8 in binding to FADD[142].

Finally, a third mechanism by which apoptosis can be initiated is through DNA damage. DNA damage results in activation of transcription factor p53, which can trigger apoptosis through transcriptional upregulation of Bax[143], as well as BH3-only protein Noxa[144] (see Fig. 5).

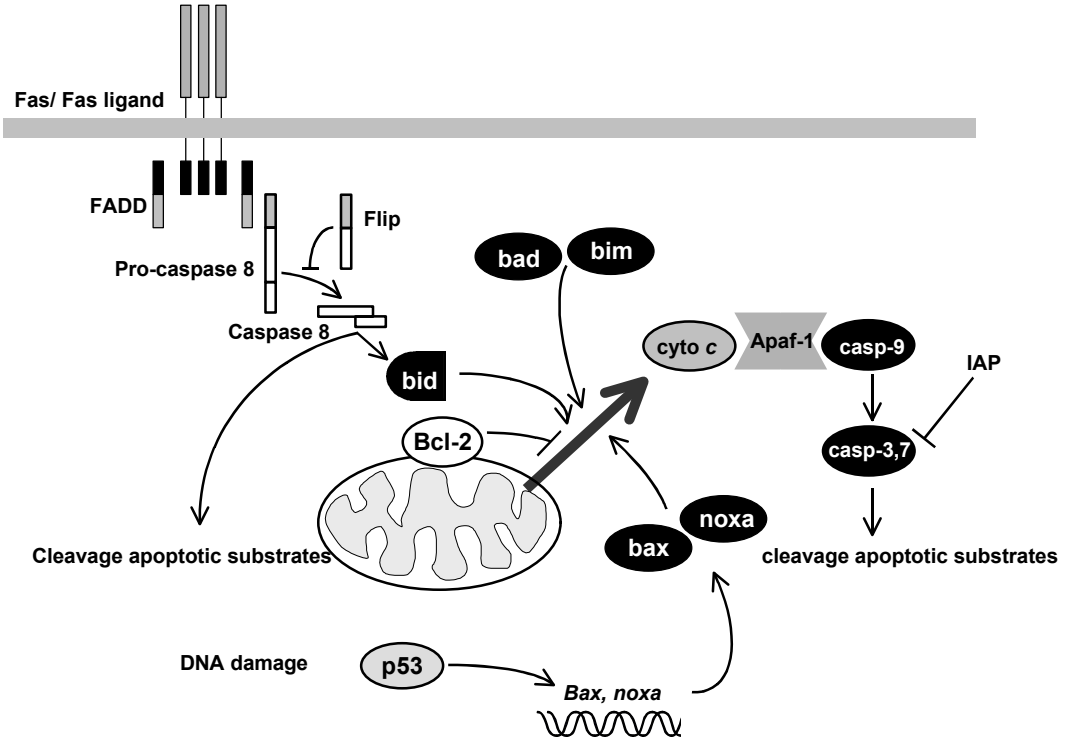

Figure 5. Regulation of apoptosis in higher eukaryotes. Induction of the apoptotic program can be induced through regulation of members of the Bcl-2 family, consisting of anti-apoptotic members, such as Bcl-2 (white) or pro-apoptotic members (black). Enhancement of the activity of the pro-apoptotic Bcl-2 family members results in the release of cytochrome c, which, together with Apaf-1, promotes caspase activation. An alternative way of activating caspases is triggered through Fas/ Fas ligand signaling, resulting in the direct activation of caspase-8. The action of caspase-8 also involves activation of other caspases via cleavage of Bid.

Signal transduction pathways regulating cell survival

As discussed, signaling through growth factors and cytokines can trigger proliferation, which requires simultaneous regulation of components of the cell cycle machinery, as well as inhibiting apoptosis. A predominant mechanism of promoting survival is through enhancing the anti-apoptotic and inhibiting the pro-apoptotic activity of the Bcl-2 family members.

A direct requirement for STAT5-induced Bcl-xL in anti-apoptotic signaling[145] through the EpoR was confirmed in STAT5 deficient mice which had low numbers of erythroid progenitors, showing higher levels of apoptosis[146]. Another anti-apoptotic tar-

get of STAT5 is the serine-threonine kinase pim1[147], which may regulate the expression or activity of pro- and anti-apoptotic members of the bcl-2 family, although this still remains to be demonstrated[148].

Utilizing ectopic expression of an oncogenic p21ras mutant, it was demonstrated that p21ras could also contribute to rescue from apoptosis through upregulation of Bcl-2 and Bcl-xL[149]. This might be mediated by its effector ERK, which can phosphorylate Bcl-2 and increase its anti-apoptotic potential[150,151], possibly by making it more resistant for degradation[152]. Furthermore, p21ras activity has also been linked to the transcription factor Aiolos, described to induce Bcl-2 in T cells[153], and NFIL3 (E4BP4), which can rescue cells from apoptosis[154], although its transcriptional targets remain to be identified.

One protein that has recently received considerable attention as a suppressor of apoptosis is the PI3K effector PKB. Thus far, several mechanisms have been demonstrated by which this serine-threonine kinase mediates cell survival. First, PKB activity can affect the balance of the Bcl-2 family by transcriptionally upregulating anti-apoptotic Bcl-2 family member Mcl-1[155], which is an essential component of the survival response conferred by GM-CSF[156]. Furthermore, phosphorylation of BH3-only protein Bad by PKB inhibits its activity[42,157], however, whether this is a critical target for PKB-mediated rescue from apoptosis is still debatable[158]. Inhibitory phosphorylation of caspase-9 by PKB might also be a way of inhibiting apoptosis in some but not all systems[159,160]. Recently, PKB activity has also been linked to inhibition of death receptor-induced apoptosis through the increase of c-FLIP expression[161]. The importance of PKB in attenuating death receptor-induced apoptosis is supported by the observation that in *PTEN (+/-)* mice Fas-induced apoptosis was impaired[62].

Given that STAT5, p21ras and also PI3K regulate transcription of genes involved in proliferation, as well as rescue from apoptosis, it is no surprise that these signaling molecules or their downstream targets may mediate cellular transformation. Although there is little evidence for a direct oncogenic potential of STAT5, it has been described to contribute to oncogenesis[162,163]. p21ras was initially identified as a molecule that was mutated in certain tumors, including hematological abnormalities, resulting in its constitutive activation, and is one of the most common contributors to induction of malignancies (reviewed in [164]). Increased PI3K activity has also been proposed in lymphoproliferative disorders[165], and inactivation of its inhibitory phosphatase PTEN has been extensively reported in cancer[166,167].

Scope of this thesis

Signal transduction in response to growth factors or cytokines enables cells to appropriately respond to their environment and thus establish a balance between cell proliferation, differentiation and apoptosis in order to maintain homeostasis. Dysregulation of this balance is often correlated with changes in activity of components of the signaling work, which can result in malignancies or inflammation. This study focuses on the mechanisms by which IL-3, IL-5 and GM-CSF mediate proliferation and survival of hematopoietic cells. Utilizing pharmacological inhibitors of signal transduction pathways, we analyzed which signaling pathways are indispensable for cytokine-mediated proliferation and survival. We identified PI3K as the crucial target of IL-3, IL-5 and GM-CSF in mediating proliferation and survival and PKB as the PI3K target involved in mediating the PI3K-induced survival signal. Therefore, we analyzed activation of the PI3K-PKB pathway in response to IL-3, IL-5 and GM-CSF and how this pathway can promote survival and proliferation.

In chapter 2, we analyzed which tyrosine residues of the βc are important for the activation of PKB. Next, we set out to identify novel targets by which PI3K might promote proliferation and survival. This has resulted in the identification of Forkhead transcription factor FKHR-L1, a novel inhibitory target of PI3K-PKB signaling through the βc, whose activity promotes apoptosis, as well as cell cycle arrest (Chapter 3). Upon analyzing how transcriptional activity of FKHR-L1 could result in these events, two FKHR-L1 targets were found (Chapter 3 and 4). Next, we analyzed by which intracellular mechanisms FKHR-L1 could promote apoptosis (Chapter 5). In addition, we provide evidence that cytokine withdrawal-mediated induction of apoptosis is mediated by FKHR-L1. Finally, to identify additional FKHR-L1 targets, we compared gene array filters derived from cells in which FKHR-L1 activity was present or absent (Chapter 6).

Altogether, our findings provide a novel mechanism by which PI3K-PKB can induce rescue from apoptosis, as well as promote induction of proliferation.

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CHAPTER 2

Regulation and Function of Protein Kinase B and MAP Kinase activation by the IL-3/IL-5/GM-CSF Receptor

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Abstract

Interleukin (IL)-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) regulate proliferation, differentiation and apoptosis of target cells. Receptors for these cytokines consist of a cytokine-specific α subunit and a common shared βc subunit. Tyrosine phosphorylation of the βc is thought to play a critical role in mediating signal transduction events. We have examined the effect of mutation of βc tyrosines on the activation of multiple signal transduction pathways. Activation of Protein Kinase B (PKB) required JAK2-binding and was inhibited by dominant-negative phosphatidylinositol 3-kinase (PI3K). Overexpression of JAK2 was sufficient to activate both Protein Kinase B (PKB) and Extracellular Regulated Kinase-1 (ERK1). Tyrosine 577 and 612 were found to be critical for the activation of PKB and ERK1, but not activation of STAT transcription factors. Activation of both PKB and ERK has been implicated in the regulation of proliferation and apoptosis. We generated GM-CSFR stable cell lines expressing receptor mutants to evaluate their effect on these processes. Activation of both PKB and ERK was perturbed, while STAT activation remained unaffected. Tyrosines 577 and 612 were necessary for optimal proliferation; however, mutation of these tyrosine residues did not affect GM-CSF mediated rescue from apoptosis. These data demonstrate that while phosphorylation of βc tyrosine residues 577 and 612 are important for optimal cell proliferation, rescue from apoptosis can be mediated by alternative signaling routes.

Introduction

The cytokine receptor family that consists of the receptors for interleukin (IL)- 3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) have a cytokine-specific alpha chain (IL-3Rα, IL-5Rα and GM-CSFRα) and a common beta chain, βc, which are both required for high affinity ligand binding. The biological effects of these cytokines on hematopoeietic cell lineages and their precursors include induction of proliferation, differentiation, inflammation, cell adhesion, regulation of effector functions and rescue from apoptosis; reviewed in [1-3]. Furthermore, recent data has implicated this family of cytokines in the pathogenesis of various leukemias. A naturally occurring truncated isoform of βc has been identified in a significant proportion of patients with acute leukemia[4], while it has also been demonstrated that inhibition of GM-CSF prevents dissemination and induces remission of myelomonocytic leukemia[5]. Surprisingly, expression of the GM-CSF receptor has also been found in human prostate cancer implying that prostatic tissues may also be responsive to GM-CSF[6].

Signal transduction events elicited by each of these cytokines are thought to be similar, since they all utilize the common βc[2]. Although no specific signaling events have been attributed to the α-chains, intracellular truncation results in aberrant signal transduction[7,8]. The βc does not possess intrinsic tyrosine kinase activity, but associates with a cytoplasmic tyrosine kinase, JAK2 (Janus Kinase 2), via the membrane proximal region[9]. Following cytokine stimulation, the receptor multimerizes, allowing transphosphorylation

of the βc, resulting in the recruitment of signaling molecules to the βc and activation of downstream signaling events[3,9,10]. The precise mechanisms and specific tyrosine residues involved remain to be fully elucidated. The βc contains docking sites for SH2-domain containing proteins such as the cytoplasmic transcription factors STATs (signal transducers and activators of transcription; reviewed in [11]). However, there is a high redundancy for STAT binding sites on the cytoplasmic βc; mutation of a single tyrosine residue does not affect STAT activation[12,13].

Another critical signaling pathway activated by IL-3/IL-5/GM-CSF is initiated by the lipid kinase PI3K, which phosphorylates phosphoinositides on the 3' position[14-18]. Generated phosphatidylinositol-3,4,5-trisphosphate is known to be critical for the activation of several protein kinases including Protein Kinase B/c-Akt (PKB), and the recently identified PtdIns(3,4,5)P_3-Dependent protein Kinase (PDK1)[19,20]. Pathways associated with PI3K have been linked to both proliferation through p70S6K[21], as well as the rescue from apoptosis through PKB[22-24], although the precise mechanism by which PI3K is activated through the βc still needs to be elucidated. Since various candidates regulating βc activation of PI3K have been reported, it is likely that there are multiple pathways from the βc that can trigger activation of PI3K[14,25-27].

In this study, we have investigated which tyrosine residues in the common βc are important for activation of PI3K-mediated signaling events in comparison with activation of the ERK MAP kinases and the STAT transcription factors. We have also assessed the role of βc tyrosine residues in proliferation, as well as in rescue from apoptosis induced by hGM-CSF. We have demonstrated for the first time that activation of PKB by IL-3/IL-5/GM-CSF requires the phosphorylation of specific βc tyrosine residues. Furthermore, PKB appears to play a role in cytokine-mediated proliferation. These data provide novel insights into mechanisms of activation and function of βc-mediated signal transduction.

Materials and Methods

Cells, reagents, antibodies

Rat-1 fibroblasts were cultured in Dulbecco's Modified Eagle's medium (Gibco) supplemented with 8% heat-inactivated FCS; Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3 (mIL-3) produced in COS cells[61]. Human IL-5 (hIL-5) was a kind gift of Dr. D. Fattah (Glaxo Wellcome group research, Stevenage, UK). Recombinant human GM-CSF was obtained from Genzyme (Boston, MA, U.S.A.). The constructs for p21ras N17, Δp85, HA-PKB, HA-ERK1 and JAK2 have been described previously[28,62]. Human IL-5Rα and human βc, as well as βc ΔboxI, βc ΔboxI/II, βc Y577G, βc Y612F and βc Y577G/Y612F mutants were cloned into expression vector pSG513 either with or without the hygromycin gene as described previously[13]. pCDNA3 GM-CSFRα[63] and kinase-dead JAK2[64] were kind gifts from Dr A. Kraft.

Rabbit polyclonal antiserum against the human βc has been described previously[13]. βc mAb and polyclonal ERK1 (C16) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phospho-Ser-473 PKB and phospho-ERK antibodies were from New England Biolabs (Beverly, MA, U.S.A).

Generation of stable transfectants

For the generation of polyclonal transfectants pcDNA3-GMCSFRα containing the neomycin resistance gene was electroporated into Ba/F3 cells (0.28 V; capacitance 960 μFD) together with either empty vector, βc wt, βc Y577G, βc Y612F or βc Y577G/612F cloned into pSG513 containing the hygromycin resistance gene. Cells were cultured in the presence of mIL-3 and selected in 500 μg/ml hygromycin and/or 500 μg/ml G418 (Boehringer Mannheim, Germany).

Western Blotting and Immunoprecipitation

For determining the presence of human βc in the Ba/F3 GM-CSFR stable cell lines $1.5x10^7$ cells were lysed in 20 mM Tris.HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1mM PMSF, 0.1 mM aprotinin and 1 mM sodium orthovanadate. Lysates were cleared by centrifugation at 4°C and incubated with the appropriate antibody on a rotating wheel at 4°C for 1 hr. After that, Protein A beads were added and incubated for another hour. Protein A beads were washed 3x with lysis buffer and boiled in Laemli sample buffer for 5 min at 95°C. Subsequently, samples were run on SDS-polyacrylamide gels and proteins transferred to PolyVinyl DiFluoride (PVDF) membranes. Blots were incubated with appropriate antibodies and developed utilizing Enhanced Chemiluminescence (ECL, Amersham).

In vitro kinase assays

Cells were transfected transiently using calcium phosphate precipitation and the medium refreshed 8 hours later. 36 hours after transfection, the cells were transferred to 0.5% FCS overnight. After a further 12 hours, the cells were stimulated with the appropriate stimulus, washed twice with cold PBS and lysed in a buffer containing 1% Triton X-100, 50mM Tris-HCl pH 7.5, 5 mM EDTA for ERK assays or 1% Triton X-100, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA for PKB assays, both 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. Kinase assays were performed as described previously[28].

CAT assays

Rat-1 cells (6-well plates) were transiently transfected with 3 μg IL-5Rα and 3 μg βc (either wildtype or the tyrosine mutant 577, 612 or 577/612), together with 4 μg 4xIREtkCAT reporter construct[65]. 36 hours after transfection, cells were incubated overnight with IL-5 (10^{-10} M). Cells were washed with PBS and harvested in PBS/EDTA (25 mM) and lysed in 100 μl CAT buffer (250 mM Tris-HCl pH 7.4, 25 mM EDTA). Membranes were spun down and 50 μl of the supernatant was incubated with 150 μl incubation buffer (50 μl CAT buffer, 7.5 μl 50% glycerol, 81.5 μl 250 mM Tris-HCl pH 7.4, 10 μl 6.7 μg/μl butyrylCoA, 1 μl ^{14}C Chloramphenicol 0.025 μCi) for 2 hours at 37°C. Unincorporated ^{14}C Chloramphenicol was separated from butyrylCoA using Xylene-Pristane (1:2) and ^{14}C-ButyrylCoA was counted.

The fold induction was calculated as the amount of counts in the stimulated versus unstimulated cells[65]. Data represent the mean of three independent experiments ± SEM.

Gel retardation assays

Nuclear extracts were prepared from 10^7 stimulated and unstimulated cells as described previously[66]. Synthetic oligonucleotides of the FcγRI GAS[36] were labeled by filling in the cohesive ends with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I. Gel retardation assays were carried out according to published procedures with slight modifications[67]. Briefly, 5 μg nuclear extract were incubated in a final volume of 20 μl, containing 10 mM HEPES pH 7.8, 50 mM KCl, 1 mM EDTA, 5 mM $MgCL_2$, 10% glycerol, 5 mM dithiothreitol, 2 μg poly (dI-dC) and 20 μg bovine serum albumin with 0.1-1.0 ng of ^{32}P-labeled oligonucleotides for 20 min at room temperature. Subsequently, samples were run for 2 hours on a 5% non-denaturing polyacrylamide gel at room temperature, vacuum-dried and exposed to Fuji RX film at -70°C for 1-2 days.

Apoptosis and proliferation assays

For apoptosis assays Ba/F3 cells were counted, washed twice with PBS and seeded in 24 well dishes (0.4x 10^6 cells per well). After two hours cytokines were added and after a further 48 hours cells were harvested, washed twice in PBS and fixed for 2 hours 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 minutes. Next, 750 μl apoptosis buffer (0.1 % Tx100, 10 mM PIPES, 2 mM $MgCl_2$ 40 μg/ml Rnase, 20 μg/ml propidium iodide) was added and incubated for 30 minutes in the dark. The percentage of apoptotic cells was analyzed by FACS as the percentage of cells with a DNA content of <2N. For cell proliferation assays Ba/F3 cells were seeded in 24 well dishes (0.1x 10^6 cells per well) together with hGM-CSF and the number of viable cells was counted every 24 hours by Trypan Blue exclusion.

Results

Activation of PKB and ERK1 requires the βc tyrosine residues 577 and 612.

The mechanisms of PI3K activation by βc are ill-defined[3]. We and others have previously identified PKB as a downstream effector of PI3K activity stimulated by growth factors[28,29] and that PKB is activated by IL-5 and IL-3 in both cell lines and human granulocytes, although the mechanisms have not been defined[18,30]. To analyze the mechanism by which βc can activate PKB we transiently transfected Rat1 cells with IL-5Rα and βc together with epitope-tagged PKB (HA-PKB) and analyzed PKB kinase activity in vitro. These cells have no endogenous βc and demonstrate a relatively strong activation of PI3K pathways by growth factors, thus being a suitable model system[28]. As shown in Fig. 1A, PKB is activated following IL-5 stimulation. This activation is mediated through PI3K, as cotransfection of dominant negative PI3K (Δp85)[31] blocks IL-5 dependent PKB activation. To determine whether IL-5 dependent PKB activation required tyrosine phosphorylation of the βc we cotransfected βc that had a deletion of box I and box II, which prevents the binding of JAK2 and thus prevents receptor phosphorylation[9,32]. We found that box I/II of βc are critical for the activation of PKB, since deletion almost completely abrogated PKB activation (Fig. 1A, left panel). To rule out the possibility that this abrogation is due to a generally non-functional receptor we also transfected kinase-dead JAK2, which has been described to function as a dominant-negative kinase for endogenous JAK2 in interferon-γ signaling[33]. Overexpression of this mutant of JAK2 indeed decreased activation of PKB (Fig. 1A, right panel), indicating the importance of βc phosphorylation for PKB activation. To determine if tyrosine residues 577 or 612 were responsible for mediating activation of PKB, we transfected cells with βc containing either single or double point mutations of these residues. While activation of PKB was mediated predominantly by βc tyrosine residue-577, tyrosine-612 also appears to play a role, since activation is only completely blocked by mutation of both tyrosines (Fig. 1B). Reprobing the same blot revealed equal expression of PKB in all lanes. We also verified whether expression of the various βc constructs was equal (Fig. 1B, left panel). Similar results were obtained by transfecting 293 cells (data not shown).

Previous work has demonstrated a potential role for Shc binding to tyrosine-577 as a mechanism of initiating MAP kinase activation[12,34,35]. To determine if the same tyrosine residues were indeed necessary for activation of the MAP kinase, ERK1, we performed similar cotransfection experiments. ERK1 activation also required an intact box I/II region and was dependent on p21ras (data not shown). Mutation of tyrosine-577 abrogated ERK1 activation (Fig. 1C), suggesting this tyrosine is also critical for activation of ERK1.

To demonstrate that the βc with mutations in these tyrosine residues is still functional in the activation of other signaling pathways we analyzed activation of STAT transcription factors. For this purpose we transiently transfected cells with IL-5Rα and the βc

mutants together with a tkCAT reporter plasmid containing 4XIRE binding sites and analyzed the induction of CAT activity by IL-5. We have previously demonstrated that this is a specific STAT-binding reporter construct[36]. While neither βc tyrosine mutant affected STAT activation, the βc ΔboxI/boxII mutant completely abrogated IL-5 mediated STAT activation (Fig. 1D). Thus, while STAT activation appears to allow redundancy in βc phosphotyrosine residues, p21ras-ERK and PI3K-PKB signaling require tyrosine phosphorylation of specific βc residues.

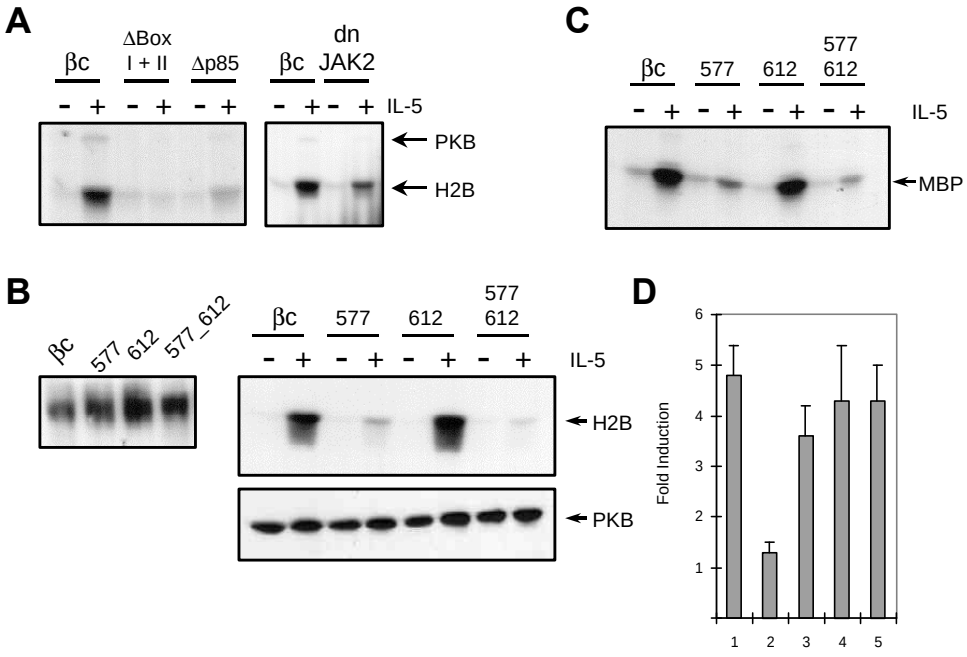

Figure 1. Activation of Multiple Signaling Pathways in response to IL-5.

(A) Rat1 cells were transiently transfected with IL5α (2 μg) and βc (2 μg, lanes 1, 2, 5-10) together with HA-PKB (2 μg) dominant-negative p85 (4 μg; Δp85, lanes 5 and 6), or dn JAK2 (2 μg; lanes 9 and 10) . 48 hours after transfection serum-starved cells were left unstimulated or stimulated with hIL-5 (10^{-10} M) for 7 minutes, HA-PKB was immunoprecipitated and an in vitro kinase assay was performed using Histone 2B (H2B) as a substrate. H2B and autophosphorylated PKB are indicated. (B) Rat1 cells were transfected with βc wildtype or βc tyrosine mutants (10 μg) and the βc was immunoprecipitated, blotted and reprobed with a βc antibody (left panel). For in vitro kinase assays cells were transfected as indicated and stimulated as described above. Equal expression of HA-PKB was determined by 12CA5 Western blotting (lower right panel). (C) Rat1 cells were transfected with HA-ERK1 (2 μg) and βc tyrosine mutants as indicated and stimulated as described above. HA-ERK was immunoprecipitated and an in vitro kinase assay was performed using myelin basic protein (MBP) as a substrate. (D) Rat1 cells were transiently transfected with IL-5Rα and either of the βc mutants together with a tkCAT reporter plasmid (4 μg) containing 4XIRE STAT binding sites (lane 1, wt βc; lane 2, βc ΔboxI/II; lane 3, ΔY577; lane 4, ΔY612; lane 5, ΔY577/612). 36 hours after transfection cells were left unstimulated or hIL-5 was added to the cells overnight and CAT activity was determined the next day as described in Materials and Methods. The fold induction is indicated as the amount of CAT activity of the hIL-5 stimulated cells compared to the unstimulated cells.

Overexpression of JAK2 activates both PKB and ERK1.

As we found that JAK2 binding and phosphorylation of the βc is required for both PKB and ERK activation (Fig. 1), we next addressed whether JAK2 overexpression itself was sufficient to activate these signaling pathways, as has previously been demonstrated for STATs[37]. JAK2 was overexpressed in Rat1 cells and ERK1 or PKB assays were performed. Interestingly, increased JAK2 expression was sufficient to activate both ERK1 (Fig. 2A, compare lane 1 and lane 3) or PKB (Fig. 2B, compare lane 1 and 3), indicating that overexpression of JAK2 is sufficient to activate downstream signaling pathways. Overexpression of JAK2, however, did not result in a 'superinduction' of ERK1 and PKB activity in the presence of IL-5 (Fig. 2A and 2B, compare lanes 2 and 4). This suggests that either the level of βc phosphorylation is playing a limiting role or perhaps more likely, that this activation is independent of βc.

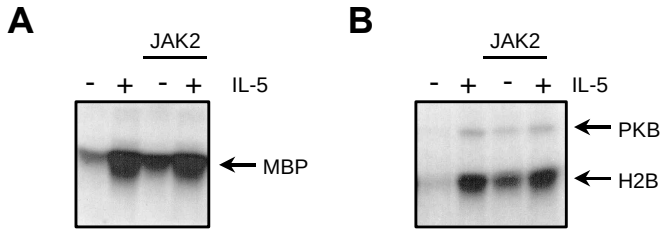

Figure 2. Overexpression of JAK2 activates both ERK1 and PKB.
(A) Rat1 cells were transiently transfected with IL-5Rα (2 μg), βc (2 μg) together with JAK2 (4 μg) and HA-ERK1 (2 μg) and kinase assays were performed as described above. (B) Same as (A), but transfected with HA-PKB (2 μg).

Expression of βc in BaF3 cell lines and activation of signaling pathways.

Activation of both PI3K and MAP Kinase have been proposed to be critical for both proliferative and anti-apoptotic effects of IL-3/IL-5/GM-CSF[30,38-40]. To analyze the potential function of βc tyrosine residues 577 and 612 we utilized Ba/F3 cells, a mouse pre-B cell line that is dependent on murine IL-3 for its growth. As the IL-5Rα subunit was found to interact with the endogenous mouse βc (data not shown) we generated polyclonal stable cell lines with the GM-CSFRα subunit which did not interact with endogenous mouse βc (see Fig. 3A). We transfected either GM-CSFRα alone or together with wildtype human βc (hβc), or hβc in which either tyrosine-577 (Δ577), 612 (Δ612) or both (Δ577/612) had been mutated. Expression of the hβc in the Ba/F3 cell lines was verified by immunoprecipitation with an antibody that specifically recognizes the hβc. Fig. 3A

demonstrates that the expression in βc wt, Δ577, Δ612 and Δ577/612 is comparable. To analyze whether GM-CSF Receptor signaling in these cell lines can be reconstituted, we first analyzed STAT activation following hGM-CSF stimulation, using an electromobility shift assay.
Comparable STAT DNA-binding activity was seen following hGM-CSF stimulation in all stable cell lines except for the Ba/F3 cells expressing only GM-CSFRα, indicating that signaling of these hGM-CSF-R stable cell lines specifically utilizes the human βc (Fig. 3B). Moreover, STAT activity was not diminished in the BaF3 cells containing the hβc with tyrosine mutations. However, when PKB activation was analyzed using activation-specific antibodies against phospho Ser473, which is phosphorylated together with Thr308 following elevation of PtdIns(3,4,5,)P_3, a product of PI3K[41,42]. We found, in agreement with the data for the Rat1 cells, that activation is mediated predominantly through tyrosines 577 and 612, as the double tyrosine mutant was unable to phosphorylate PKB Ser-473 (Fig. 3C). Addition of the PI3K inhibitor

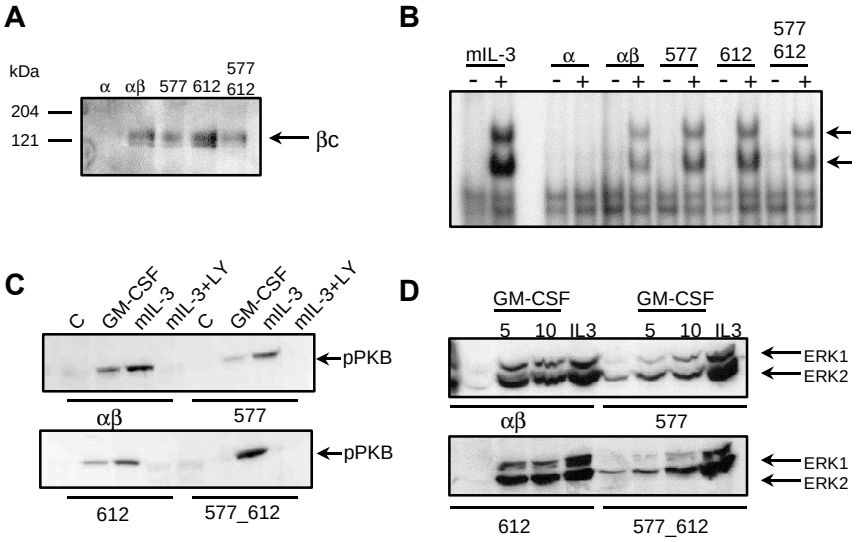

Figure 3. Signaling of hGM-CSFR in Ba/F3 cells.
(A) Ba/F3 cells were stably transfected with hGM-CSFRα together with either empty vector or βc wt, Δ577, Δ612 or Δ577/612. Expression of the human βc was verified by precipitating the human βc from $15x10^6$ cells as described in Materials and Methods. (B) Nuclear extracts were prepared from serum-starved untreated or mIL-3 or hGM-CSF (10^{-10} M) stimulated cells and gel retardation assays using a FcγRI GAS probe were carried out as described in the Materials and Methods. The identity of STAT1 and STAT3 complexes was confirmed by supershift analysis (data not shown). (C) Ba/F3 cells ($0.25x10^6$) were serum-starved for 4 hours and then left untreated stimulated with hGM-CSF (10^{-10} M) or stimulated with mIL-3 with or without pretreatment with 10 μM LY294002 for 20 minutes as indicated. PKB activation was analyzed by phospho-PKB immunoblotting. (D) ERK activation was measured by phospho-ERK immunoblotting as described above.

LY294002 completely abrogated PKB activation following mIL-3 in all GM-CSFR stable cell lines, indicating that phosphorylation of PKB on Ser473 is indeed downstream of PI3K. Activation of ERK1 and ERK2 was also almost completely eliminated by mutation of these residues (Fig. 3D). Additional tyrosine residues may be involved in the activation of ERK1/2, since the Δ577/612 mutant still demonstrated slight ERK activation, although activity was much reduced compared to the wild-type βc. Measuring activity of endogenous ERK2 using a substrate-based assay yielded the same results (data not shown).

Optimal proliferative response to hGM-CSF requires βc tyrosines 577/612.

GM-CSF induces cell proliferation and rescue from apoptosis in a dose-dependent manner. To determine whether mutation of tyrosine-577 and 612 affected proliferation of cells when grown on hGM-CSF, cells were grown for three days with two different concentrations of hGM-CSF and the cell numbers determined every 24 hours. Cells expressing only the GM-CSFRα chain failed to proliferate when challenged with GM-CSF, demonstrating the necessity for interaction with the human βc (Fig. 4). No significant effect was observed by mutating either tyrosine 577 or 612 independently. However, a 2-3-fold decrease in proliferation was seen in Ba/F3 cells containing the βc Δ577/612. A slight, but reproducible, effect was also seen on the proliferation of the βc Δ577 cell line when the cells were grown on a lower concentration (10^{-12} M) hGM-CSF (Fig. 4B). To demonstrate that the effect on proliferation in the Ba/F3 βc Δ577/612 stable cell line is not a clonal artefact we also compared proliferation of this cell line with Ba/F3 βc wt when grown on mIL-3. No difference in proliferation with mIL-3 was found between those cell lines (Fig. 4C), ruling out a clonal difference between those cell lines. Thus it appears that while the ability to proliferate is not completely abrogated by mutation of tyrosine 577 and 612, it is substantially reduced. This suggests that activation of PKB and/or MAP kinase may play a critical role in regulating βc-mediated proliferative responses. Furthermore, since STATs are still activated in this βc mutant (Fig. 3B), it suggests that they are not themselves sufficient to mediate cytokine induced proliferative responses without the cooperation of other signaling pathways.

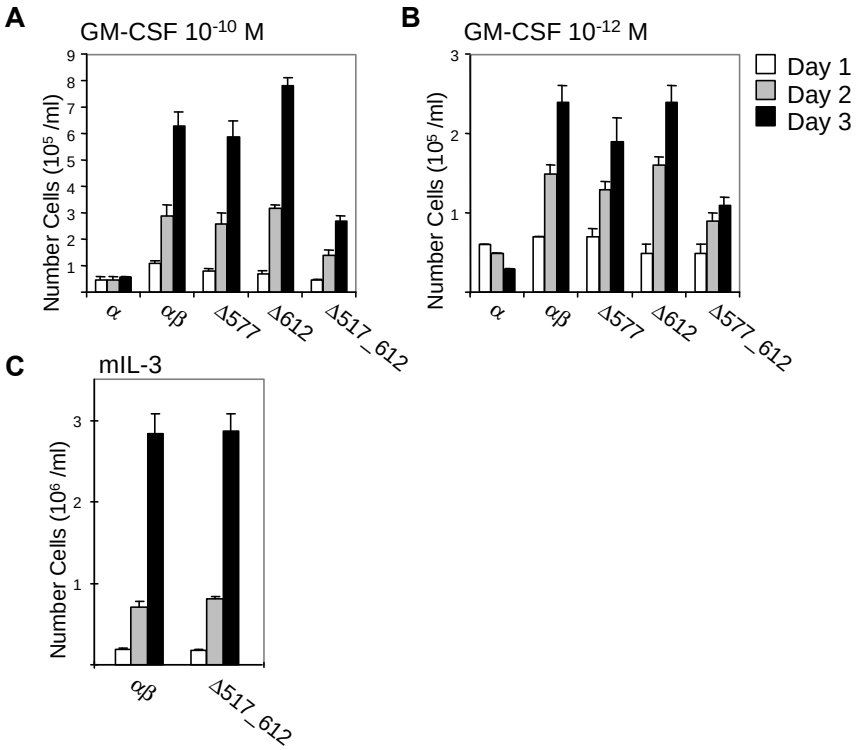

Figure 4. Proliferation of the Ba/F3 hGM-CSFR cell lines.

Ba/F3 cells containing the GM-CSFRα, GM-CSFRα and βc wt, Δ577, Δ612 or Δ577/612 were cultured with hGM-CSF 10^{-10} (A) or 10^{-12} M (B) and the number of cells was counted every 24 hours. (C) Ba/F3 cells containing the GM-CSFRα and βc wt or Δ577/612 were cultured with mIL-3 and the number of cells was counted every 24 hours.

GM-CSF mediated cell survival does not require βc tyrosines 577/612.

In addition to inducing proliferation, agonists such as GM-CSF also prevent apoptosis in responsive cells. To investigate whether the decrease in proliferation that was seen in the Δ577/612 stable cell line was due to increased apoptosis, we determined the percentage of apoptotic cells after 48 hours incubation with or without cytokine. Cells expressing only the GM-CSFRα chain exhibited no GM-CSF mediated rescue from apoptosis, although they could clearly be rescued by incubation with mIL-3 (Fig. 5). We did not observe a significant decrease in the rescue from apoptosis in the single or double Δ577/612 cell lines following incubation with hGM-CSF (10^{-10} M), suggesting that these tyrosine residues are not necessary for mediating apoptotic rescue (Fig. 5). The same results were obtained at lower cytokine concentrations (10^{-12} M hGM-CSF; data not shown). Thus it appears that activation of PKB and, to some extent, ERK are not critical for GM-CSF mediated cell survival. It is possible that the residual ERK

activity may still contribute to the rescue from apoptosis, however, we did not find a difference in the rescue from apoptosis upon addition of the MEK inhibitor PD98059 (see chapter 3).

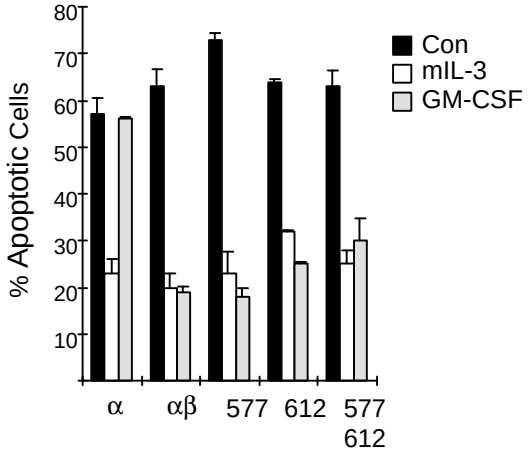

Figure 5. Analysis of apoptosis in the Ba/F3 GM-CSFR stable cell lines.
Ba/F3 cells containing the GM-CSFRα, GM-CSFRα and βc wt, Δ577, Δ612 or Δ577/612 were cultured with hGM-CSF 10^{-12} M for 48 hours and the percentage of apoptotic cells was determined as described in Materials and Methods.

Discussion

Cytokines of the IL-3/IL-5/GM-CSF family are important regulators of hematopoiesis through modulation of proliferation, differentiation and survival of various hematopoietic cell lineages and their precursors[2,3]. Although the receptors for these cytokines do not possess any intrinsic kinase activity, tyrosine phosphorylation of cellular substrates by βc-associated JAK kinases is rapidly observed in stimulated cells. One of these substrates is the βc itself, generating phosphotyrosine docking sites for SH2-containing downstream signaling molecules. In recent reports, as well as in this study, it has been shown that single mutation of any βc tyrosine residue has no effect on STAT activation by IL-3/IL-5/GM-CSF, suggesting a high degree of redundancy[12,13]; Fig. 1D and 3B.

In this paper we report that in various cell lines tyrosine 577 and 612 are important for the activation of both PKB and ERK. One pathway that induces activation of ERK is most likely mediated through the adapter protein Shc, which binds to phosphorylated Y-577 on the βc and is itself tyrosine phosphorylated after IL-3/IL-5/GM-CSF stimulation allowing Grb2-Sos binding and activation of p21ras[34,35,43]. However, an alternative means of ERK activation may be initiated through SHP2, which has been reported to interact through its SH2 domain with Y577 and to be phosphorylated by either Y-577, Y-612, or Y-695[44-47]. Activation of ERK by SHP2 can occur through interaction of phosphorylated SHP2 with Grb2-SOS and subsequent p21ras activation, observations that suggest redundancy in ERK activation[45]. An alternate means of ERK activation may also be provided by Shc binding directly to JAK2, which has been demonstrated for the EPO receptor[48]. This may explain the small residual ERK activity seen in the Ba/F3 βc Δ577/612 stable cell line (Fig. 3D).

Activation of PI3K through βc is complex and likely to be mediated through multiple signaling pathways. While the mechanisms of activation of its downstream effector PKB have not been previously investigated, PI3K activity has been found to be associated with anti-phosphotyrosine immunoprecipitates, but not with anti-βc immunoprecipitates[15]. Studies have reported binding of the regulatory subunit of PI3K to a novel, yet to be identified protein (p80), that may link PI3K to the receptor[26], as well as by associating with Lyn, a Src-like kinase that binds to the βc[14]. Furthermore, SHP2 was also found to coimmunoprecipitate with the p85 subunit of PI3K, potentially linking activation of both PI3K and p21ras pathways[25]. The potential involvement of Lyn in the activation of PI3K is of particular interest, since this kinase has been linked to inhibition of apoptosis in human granulocytes and decreased Lyn activity has been associated with a abrogation of PI3K activity[49-51]. Together, this strongly suggests that there are multiple redundant pathways that promote activation of PI3K. This is in agreement with our finding that both tyrosine-577 and 612 (Fig. 1C) activate its downstream target, PKB. We and others have previously demonstrated that PKB can be activated by cytokines of the IL-3/IL-5/GM-CSF family[18,30]. However, this is the first study to demonstrate the relevance of βc tyrosine residues in PI3K mediated signal transduction and activation of downstream targets such as PKB. Although we have shown that βc-mediated PKB activation requires the p85α subunit of PI3K (Fig. 1A), further studies are needed to determine precisely how PI3K is itself activated after cytokine stimulation.

Interestingly, we found that simply overexpression of JAK2 was sufficient to induce activation of both PKB and ERK1 (Fig. 2). Overexpression of JAK2 in Ba/F3 cells has previously been found to delay apoptosis[52]. In addition, abnormal activation of JAK2 has been implicated in acute lymphoblastic leukemia[53]. Thus overexpression or constitutive activation of JAK2 may lead to an inappropriate activation of p21ras-ERK and PI3K-PKB, resulting in enhanced proliferation or cytokine-independent survival. A direct role for βc itself in leukemogenesis has recently been implied by the recent observation that a truncated βc was found in patients with acute leukemia[4]. This further suggests that inappropriate regulation of βc phosphorylation and subsequent downstream signaling events causes defective proliferative responses in some cells.
We have studied the effect of mutation of βc tyrosines 577 and 612 on proliferation and rescue from apoptosis. Whereas we did not find an effect on cell survival with either of the mutants (Fig. 5), we did observe a decrease in proliferation in the GM-CSFR Δ577/612 cell line (Fig. 4). Interestingly, recent reports have shown that inhibition of STAT activation in Ba/F3 cells, for

example by overexpression of dominant-negative STAT5, significantly repressed IL-3 dependent growth[54]. In contrast, we have demonstrated that repression of proliferation does not have to be linked with STAT activation, since the Δ577/612 cell line effectively activates STATs but has reduced proliferative capacity (Fig. 4). Our approach to determine the effects of the double tyrosine mutant βc has allowed the analysis of effects that may be overlooked with the single mutants. This may explain findings of others suggesting that tyrosine 577 was not necessary for cell viability[12,47]. Since we demonstrated that tyrosines 577 and 612 on the βc were important for activation of both PKB and ERK, we were unable to distinguish their specific role using these tyrosine mutants. Recently, it has been shown that introduction of a dominant-negative MAP kinase kinase (MAPKK) in Ba/F3 cells results in an increase in the level of IL-3 required to stimulate cell proliferation, suggesting a role for MAP kinase activation[39]. A role for MAPK in proliferation may be negligible, since overexpression of dominant negative ras N17 was not found to affect proliferation in Ba/F3 cells[55]. Furthermore, addition of MEK inhibitor PD98059 was not found to affect proliferation in Ba/F3 GM-CSFR stable cells (data not shown). A role for PI3K in proliferation can be further supported by the observation of a profound decrease in proliferation when Ba/F3 GM-CSFR cells were incubated with the immunosuppressant rapamycin, an inhibitor of p70S6K, which is a downstream target of PI3K (data not shown). It has been described previously that blocking mIL-3 induced p70S6K in BaF3 cells with rapamycin partially inhibited mIL-3 dependent 3H-thymidine incorporation, suggesting a role for PI3K signaling in cellular proliferation[21]. However, further work utilizing specific pharmacological inhibitors and interfering mutants of various signaling pathways will be necessary to identify the precise nature of this proliferative mechanism.

The fact that we did not observe a decrease in the rescue from apoptosis by hGM-CSF in the Δ577/612 stable Ba/F3 cell line, which fails to activate PKB may seem in apparent contrast with recently published data[30,56]. Although our data indeed imply the potential for apoptotic rescue independently of PKB, we have recently found that overexpression of an novel effective dominant-negative PKB construct[57] in BaF3 cells abrogated IL-3-mediated rescue from apoptosis (data not shown). Previous studies have relied on the overexpression of constitutively active PKB mutants, demonstrating a cytokine-independent rescue from apoptosis[30]. It is difficult to determine the specificity of these overexpression studies since constitutively active PKB is oncogenic and may induce autocrine or anti-apoptotic effects in these cells and these data should thus be interpreted with caution.

There are several explanations for the apparent discrepancy between survival by hGM-CSF in the Δ577/612 stable Ba/F3 cell line and the observation that overexpression of dominant-negative PKB abrogated IL-3-mediated rescue from apoptosis. First, it might be that there is some residual PKB activity in the Δ577/612 stable Ba/F3 cell line that is not detected by phospho-specific antibodies. Interestingly, after completing these studies, an alternate mechanism of activating PI3K-PKB through βc was provided through phosphorylation of serine-585 on βc[58]. Mutation of this residue impaired IL-3-

mediated survival. An alternate explanation might be that the apoptosis experiments were carried out in the presence of serum, which could contribute to survival mediated by GM-CSF independently of PKB. Indeed, the recent identification of serum and glucocorticoid inducible kinases (SGKs) in the anti-apoptotic response by IL-3[59,60] after completion of this studies supports this. A role for PKB in mediating the anti-apoptotic response is further examined in Chapter 3 and 5.

The studies presented here provide insight not only into the mechanisms of βc-mediated signal transduction but also the potential role of these signaling pathways in maintaining proliferative capacity and viability of cytokine-dependent cells. Activation of PI3K and PKB by IL-3/IL-5/GM-CSF has been previously demonstrated in cell lines and leukocytes[18,30,56]. This is the first study to demonstrate a role for c tyrosine residues in the activation of PKB and suggests specificity between activation of PI3K-PKB, p21ras-ERK and JAK-STAT signaling pathways.

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CHAPTER 3

Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of $p27^{KIP1}$

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Abstract

Interleukin-3 (IL-3), IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) regulate the survival, proliferation and differentiation of hematopoietic lineages. Phosphatidylinositol 3-kinase (PI3K) has been implicated in the regulation of these processes. Here we investigate the molecular mechanism by which PI3K regulates cytokine-mediated proliferation and survival in the murine pre-B cell line Ba/F3. IL-3 was found to repress the expression of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ through activation of PI3K, and this occurs at the level of transcription. This transcriptional regulation occurs through modulation of the forkhead transcription factor FKHR-L1, and IL-3 inhibited FKHR-L1 activity in a PI3K-dependent manner. We have generated Ba/F3 cell lines expressing a tamoxifen-inducible active FKHR-L1 mutant (FKHR-L1(A3):ER*). Tamoxifen-mediated activation of FKHR-L1(A3):ER* resulted in a striking increase in $p27^{KIP1}$ promoter activity and mRNA and protein levels, as well as induction of the apoptotic program. The level of $p27^{KIP1}$ appears to be critical in the regulation of cell survival since mere ectopic expression of $p27^{KIP1}$ was sufficient to induce Ba/F3 apoptosis. Moreover, cell survival was increased in cytokine-starved bone marrow-derived stem cells from $p27^{KIP1}$ null-mutant mice compared to that in cells from wild-type mice. Taken together, these observations indicate that inhibition of $p27^{KIP1}$ transcription through PI3K-induced FKHR-L1 phosphorylation provides a novel mechanism of regulating cytokine-mediated survival and proliferation.

Introduction

Cytokines of the interleukin-3 (IL-3)/IL-5/ granulocyte-macrophage colony-stimulating factor (GM-CSF) family are important regulators of proliferation, differentiation and effector functions of various hematopoietic cell lineages and their precursors[1,2]. IL-3 and GM-CSF regulate the proliferation and survival of multiple hematopoietic lineages, whereas IL-5 has a more restricted role in the differentiation of eosinophils and basophils, as well as of murine B cells[2,3]. Phosphatidylinositol 3-kinase (PI3K), and its downstream target protein kinase B (PKB) have been linked to regulation of proliferation and survival in a variety of hematopoietic systems[4-6]. PI3K activity is negatively regulated by the PTEN (phosphatase and tensin homolog) phosphatase, which specifically dephosphorylates the D3 position of phosphatidylinositol, thus inhibiting the action of PI3K[7-10]. Several mechanisms have been proposed to explain the requirement for PI3K activity in cytokine-mediated cell survival. For example, IL-3 regulates PKB-induced phosphorylation of the pro-apoptotic Bcl-2 family member BAD, inhibiting its pro-apoptotic activity[6]. However, it has recently been shown that this phosphorylation does not correlate well with cell survival[11]. Another target of PKB possibly accounting for its anti-

apoptotic effect is the apoptotic protease caspase-9, which is inactivated upon phosphorylation by PKB[12]. However, this phosphorylation site is not evolutionarily conserved[13], leaving its relevance in vivo to be demonstrated. More recently, PKB was demonstrated to be involved in negatively regulating the activity of the forkhead family of transcription factors which can mediate apoptosis as well as proliferation[14-16].

To identify a potential mechanism by which PI3K could exert its proliferative and anti-apoptotic effects, we focused on cyclin-dependent kinase (CDK) inhibitor p27^{KIP1}. Upregulation of p27^{KIP1} is linked to cell cycle arrest in G_0/G_1 through its interaction with CDK-cyclin complexes[17]. Regulation of p27^{KIP1} levels has been described as occurring predominantly posttranslationally, by cyclin E-CDK2-mediated phosphorylation, which subsequently targets p27^{KIP1} for degradation by the proteasome[17-20]. p27^{KIP1} in turn also inhibits cyclin E-CDK2 complexes, suggesting that the balance of p27^{KIP1} and cyclin E-CDK2 is important for G_1 progression. Mitogens upregulate cyclin D levels, subsequently sequestering away p27^{KIP1} from cyclin E/CDK2 complexes and hereby activating these complexes[21]. Interestingly, p27^{KIP1} has also been implicated in the regulation of immunoglobulin M (IgM)-induced B cell apoptosis, which can be rescued by CD40 ligand engagement[22,23]. The exact mechanism by which cytokines are able to regulate p27^{KIP1} levels and what the importance of this is for mediating its proliferative and anti-apoptotic effects in hematopoietic cells are largely unknown.

Here we show that an important means by which cytokine-mediated proliferation and survival are regulated is through downregulation of p27^{KIP1}. Transcriptional induction of p27^{KIP1} is regulated by the forkhead-related transcription factor FKHR-L1. Activation of FKHR-L1 is sufficient to elevate p27^{KIP1} mRNA and protein levels, as well as to induce apoptosis. Importantly, apoptosis of bone marrow derived hematopoietic stem cells from p27^{KIP1} null-mutant mice is decreased upon cytokine withdrawal compared to that of cells from wild-type mice, demonstrating the importance of regulating p27^{KIP1} levels in vivo for cell survival. Our data provide a novel mechanism by which cytokines can both regulate cell cycle progression and inhibit apoptosis by the PI3K-PKB-mediated downregulation of p27^{KIP1}. We propose that the regulation of p27^{KIP1} transcription by forkhead-related transcription factors may be a general mechanism by which hematopoietic cells can respond appropriately to their environmental conditions, resulting in survival, proliferation, or differentiation.

Materials and Methods

Cell culture

Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3 produced in COS cells[24]. Peripheral blood eosinophils from healthy volunteers obtained from the Blood Bank (Utrecht, The Netherlands) were isolated as described previously[25]. Fetal liver-derived myeloid cultures were prepared from

day-17 mouse embryos by culture of suspension cells in RPMI 1640 supplemented with IL-3, IL-6, and stem cells factor (SCF) as previously described[26]. Bone marrow cells were flushed out of mouse femurs and resuspended in Iscove's modified Eagle Medium containing 20% Myclone Super Plus fetal calf serum and red blood cells were lysed by diluting them 1:1 with acetic acid- phosphate-buffered saline (PBS). Sca1 positive cells were isolated using Sca1 antibody microbeads (Miltenyi, Gladbach, Germany). Cells were cultured for 5 days in medium supplemented with murine IL-3, IL-6, SCF (R&D, Abingdon, United Kingdom) before analyzing apoptosis upon cytokine withdrawal. Twenty-four h after cytokine withdrawal, cells were washed with ice-cold PBS, resuspended in binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin-V for 10 min at room temperature, washed and resuspended in binding buffer containing 1 μg of propidium iodide (PI)/ml and fluorescence was analyzed by fluorescence-activated cell sorter (FACS).

Reagents and antibodies

LY294002, PD098059, and SB203580 were from Alexis (San Diego, CA, U.S.A.). Rapamycin was a kind gift from Dr. N. Lomax from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD, U.S.A.). pRC-$p27^{KIP1}$ (mouse) was a kind gift from R. Bernards (Netherlands Cancer Institute, Amsterdam), and spectrin-linked green fluorescent protein (GFP) was a kind gift from Dr. A. Beavis and T. Sheck (Princeton, USA) and has been described previously[27]. myrPKB:ER* was a kind gift of Dr. A. Klippel (Chiron Corporation, Emeryville, CA, U.S.A.). pSG5-mycPTENcaax was obtained by PCR amplification of PTEN from human neutrophil cDNA and shuttling through pGEM-T_caax[28] before subsequent cloning into pSG5. FKHR-L1 constructs were a kind gift from M.E. Greenberg (Boston, MA, U.S.A.)[14], pCDNA3-FKHR-L1(A3):ER* was generated by cloning FKHR-L1(A3) without the stop codon into a pCDNA3 vector containing the hormone-binding domain of the estrogen receptor (pCDNA3-ER). Constructs for haemagglutinin (HA-PKB, gagPKB, cyclin D1, cyclin D1 promoter, kinase-dead CDK4 and the low-affinity nerve growth factor receptor (LNGFR) have been described previously[29-31]. The pGL2-$p27^{KIP1}$ luciferase promoter construct[32] was a kind gift from Dr. I.P. Touw (Erasmus University, Rotterdam, The Netherlands). Histone H1 and actinomycin D were purchased from Sigma, and protein A agarose was purchased from Boehringer GmbH (Mannheim, Germany). $p27^{KIP1}$ and RACK1 mAb were purchased from Transduction Laboratories (Lexington, Kentucky, U.S.A.), PKB, cyclin E, CDK2, ERK1 and ERK2 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Phospho-Ser473 PKB was from New England Biolabs (Beverly, MA, U.S.A), while FKHR-L1 and phospho-Thr32 FKHR-L1 were from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.).

Western blotting

For the detection of p27^{KIP1}, cells were lysed in Lowry sample buffer and the protein concentration was determined as described previously[33]. Equal amounts of each protein sample were analyzed by 15% SDS-PAGE and Western blotting with p27^{KIP1} antibody. Blots were subsequently probed with RACK1 antibody (or ERK1 and ERK2 in the case of eosinophils) to confirm equal protein loading. For the analysis of CDK2 levels, equal amounts of protein of cells lysed in ELB buffer[33] together with inhibitors (see the description of kinase assays below) and were analyzed in parallel with the cyclin E-associated kinase activity. For detection with phosphospecific antibodies, cells were lysed in ELB buffer together with inhibitors, and equal amounts of protein were run on gel, blotted, and probed with phosphospecific antibodies.

Northern blotting

Ba/F3 cells were cultured with IL-3 and then starved for various times, or were starved for IL-3 overnight and subsequently stimulated with IL-3. In some experiments cells were cultured with IL-3 and 4-hydroxy tamoxifen (4-OHT) was added. Total RNA was isolated as described previously[34]. Twenty micrograms of total RNA was used for Northern blotting and hybridized with a p27^{KIP1} probe consisting of full-length p27^{KIP1} cDNA. Equal RNA loading was verified by stripping and reprobing the blots with a 1.4 kb cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Apoptosis and proliferation assays

For apoptosis assays cells were counted, washed twice with PBS, resuspended in RPMI 1640 containing 8% Hyclone, and seeded in 24-well dishes (0.4 x 10^6 cells per well). After two h inhibitors were added as indicated, and after a further 30 min cytokines were added. After 48 h cells were harvested, washed twice in PBS and fixed for at least 2 h in 300 µl of PBS-700 µl of ethanol. Cells were spun down gently and permeabilized in 200 µl of 0.1% Triton X-100-0.045 M Na_2HPO_4- 0.0025 M sodium citrate at 37°C for 20 min. Next, 750 µl of apoptosis buffer (0.1% Triton X-100, 10 mM PIPES [piperazine-N,N'bis{2-ethanesulfonic acid}], 2 mM $MgCl_2$, 40 µg of Rnase/ml, 20 µg of propidium iodide/ml) was added, and cells were incubated for 30 min in the dark. The percentage of apoptotic cells was analyzed by FACS as the percentage of cells (10,000 cells counted) with a DNA content of <2N. Thresholds were set to gate out cellular debris. For Ba/F3 cells transfected with GFP-spectrin, 5,000 GFP-positive cells were analyzed. Cell cycle profiles were determined using a FACScalibur (Becton Dickson, Mountainview, CA, U.S.A.) and analyzed using Cell Quest and MofFit software. For cell proliferation assays Ba/F3 cells were seeded in 24-well dishes (0.1 x 10^6 cells per well) together with IL-3 with or without inhibitors and the viable cells were counted every 24 h by trypan blue exclusion.

Transient electroporations and generation of stable cell lines

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 24 h after electroporation and analyzed by FACS as described above. For the generation of polyclonal transfectants constructs were electroporated into Ba/F3 cells together with pSG5 conferring neomycin resistance and maintained in 500 μg/ml G418 (Boehringer GmbH) in the presence of IL-3. Monoclonal cell lines were generated by limited dilution, and results shown are representative of at least two separate clones. For the analysis of $p27^{KIP1}$ levels in cells transiently overexpressing $p27^{KIP1}$, cells were electroporated together with LNGFR as a marker[31]. Dead cells were removed, and LNGFR expressing cells were separated using monoclonal LNGFR antibody 20.4[31] and goat anti-mouse microbeads (Miltenyi Biotech). Equal protein concentrations were analyzed by $p27^{KIP1}$ western blotting.

Cyclin E-CDK2 kinase assays

Cyclin E-associated kinase activity was determined as described previously[33], using histone H1 as a substrate. CDK2 levels were determined in parallel by Western blotting.

Luciferase assays

Ba/F3 cells were electroporated with the pGL2-$p27^{KIP1}$ luciferase promoter construct[32], a pGL2 thymidine kinase luciferase construct or a pGL2 control luciferase construct, the internal transfection control (pRL-TK; Promega), and expression plasmids. 24 h after transfection cells were harvested and luciferase activity was measured. Values were corrected for transfection efficiency and growth and represent the means of at least three independent experiments (± standard errors of the means).

Results

Signaling pathways regulating cytokine-mediated proliferation and survival.

Lymphoid and myeloid lineages require cytokines and growth factors to both induce cell division and act as survival factors. The mouse pre-B cell line Ba/F3 requires IL-3 to proliferate as well as to overcome a default apoptotic program. To define signaling pathways critically involved in mediating the proliferative response to IL-3, we analyzed the effect of various pharmacological inhibitors on Ba/F3 cells cultured with IL-3. Cells were cultured for 72 h and the number of trypan blue-excluding cells was determined every 24 h. Proliferation was not affected when the cells were cultured with IL-3 in the presence of mitogen-activated protein kinase (MAPK) kinase inhibitor PD098059[35] or with p38 MAPK inhibitor SB203580[36], indicating that the proliferative response is not affected by inhibition of MAP kinases (Fig. 1A). Activation of ERK and p38 kinases was potently inhibited under these conditions (data not shown). IL-3-dependent proliferation was pro-

foundly inhibited when the cells were cultured in the presence of either PI3K inhibitor LY294002[37] or rapamycin, an inhibitor of the activation of p70S6K, a target of PI3K signaling.

To determine whether the inhibition of proliferation may be due to a decrease in cell survival, we analyzed the effect of pharmacological inhibitors on apoptosis. For this purpose we used FACS analysis of PI-labeled cells and marked cells containing less than 2N DNA content as apoptotic. These results were also confirmed by DNA laddering (data not shown). As expected, addition of PD098059 or SB203580 did not affect cell survival, implying no significant role for MAPKs in the regulation of apoptosis (Fig. 1B). However, IL-3-induced rescue from apoptosis was abrogated when cells were incubated with LY294002. Although rapamycin could efficiently block proliferation, it had no effect on IL-3 mediated rescue from apoptosis, demonstrating that inhibition of cell cycle progression is in itself insufficient to initiate the apoptotic program. Identical results were also found in 32D cells, a murine IL-3-dependent cell line, cultured with IL-3 (data not shown).

To exclude aspecific effects introduced by using pharmacological inhibitors of PI3K we developed a novel inhibitory tool, using the 3-phosphatidylinositol lipid phosphatase PTEN. Although the mechanisms of PTEN regulation are unclear, regulation by membrane localization has been suggested by the recent analysis of its crystal structure[38]. We generated a PTEN construct containing a C-terminal CAAX-box derived from Ki-Ras[28], resulting in constitutive membrane-association (PTENcaax). In contrast to what was found for wild-type PTEN, phosphorylation of PKB was largely abrogated upon expression of this construct, demonstrating that PTENcaax is capable of potently inhibiting PI3K activity (Fig 1C; left, lane 3). To analyze whether PTEN could affect cytokine-mediated rescue from apoptosis, we electroporated cells with PTEN expression vectors. We observed a minor increase in apoptosis in Ba/F3 cells overexpressing wild-type PTEN (data not shown). Ba/F3 cells ectopically expressing PTENcaax exhibited a much higher percentage of apoptosis than control Ba/F3 cells expressing GFP-spectrin alone (Fig. 1C; right). This observation clearly demonstrates the importance of PI3K generated phosphatidylinositol lipids for cell survival.

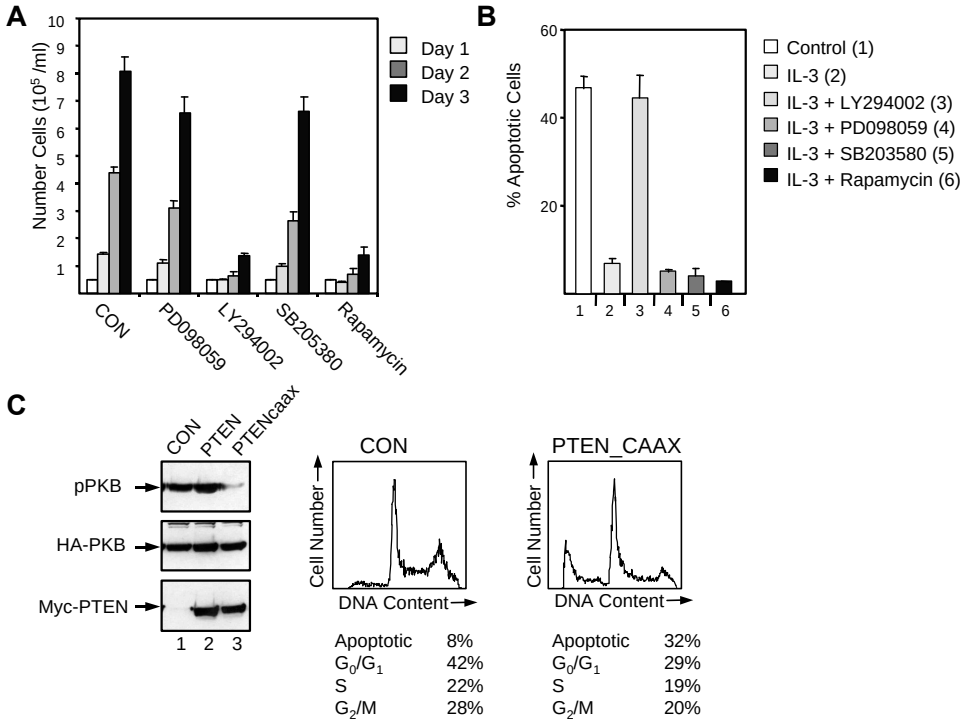

Figure 1. Regulation of IL-3-mediated proliferation and survival.

Ba/F3 cells were cultured in the presence of IL-3 without inhibitors or with PD098059 (50 μM), LY294002 (10 μM), SB203580 (10 μM), or rapamycin (20 ng/ml), and cells were counted every 24 h as indicated. (B) Ba/F3 cells were cultured in the absence of IL-3 (bar 1) or presence of IL-3 either alone (bar 2) or with LY294002 (10 μM; bar 3) PD098059 (50 μM; bar 4), SB203580 (10 μM; bar 5) or rapamycin (20 ng/ml; bar 6) and the percentages of apoptotic cells were determined after 48 h. (C, Left). COS cells were transfected with either 8 μg empty vector, or the myc-PTEN or the myc-PTENcaax vector together with 2 μg of the HA-PKB vector. HA-PKB was immunoprecipitated with an HA antibody (12CA5) and analyzed for activity by immunoblotting with phospho-Ser473 PKB antibody (top). Expression of HA-PKB and mycPTEN was verified by immunoblotting with either 12CA5 (middle) or myc antibody (9E10;bottom). (Right) Ba/F3 cells were electroporated with 2 μg of the spectrin-GFP vector together with either 18 μg of empty vector (pSG5) or 18 μg of the myc-tagged PTENcaax vector. Dead cells were removed 2 h after electroporation by separation through a Ficoll gradient. Twenty-four h after electroporation cells were fixed and stained with PI and the DNA content of 5,000 GFP-positive cells was analyzed by FACS. The data depicted are representative of several independent experiments.

P27^{KIP1} protein levels correlate with induction of apoptosis.

The CDK inhibitor (CKI) p27^{KIP1} is the only CKI whose expression declines upon mitogenic stimulation, as demonstrated for IL-2 and platelet-derived growth factor (PDGF)[39-41]. Upregulation of p27^{KIP1} levels has been correlated not only with a decrease in proliferation, but also with induction of apoptosis, suggesting that PI3K activity might be associated with a decrease in p27^{KIP1} levels.

To determine whether IL-3 can regulate p27^{KIP1} levels, Ba/F3 cells were cultured with or without IL-3 and after 24 h the level of p27^{KIP1} expression was determined by Western blotting. Equal protein loading was confirmed by probing the blot with a RACK1 antibody. Cells cultured without cytokines or with IL-3 in the presence of LY294002 exhibited a significant increase in p27^{KIP1} expression, whereas inhibition of MAPK kinase, p38 MAPK, or p70S6K had no significant effect (Fig. 2A), correlating with a lack of effect of these inhibitors on apoptosis. Expression of another CKI, p21^{CIP1}, was unaffected (data not shown), suggesting that upregulation of p27^{KIP1} upon induction of apoptosis may be specific for this CKI.

Next, we wished to determine the kinetics by which p27^{KIP1} levels changed upon IL-3 withdrawal and the role of transcription therein. Cells were treated with or without the transcription inhibitor actinomycin D, and IL-3 was withdrawn. Levels of p27^{KIP1} increased after IL-3 withdrawal, which precedes induction of the apoptotic program in these cells (data not shown). However, this increase was completely blocked in cells treated with actinomycin D (Fig. 2B), indicating that transcriptional regulation is important for elevating p27^{KIP1} levels following IL-3 withdrawal. Addition of IL-3 to cells that were cytokine-starved overnight resulted in a decrease in p27^{KIP1} levels (Fig. 2C). To determine if cytokine-mediated regulation of p27^{KIP1} levels is a more general phenomenon, we analyzed primary mouse fetal liver cells cultured in the presence or absence of survival factors[26]. Indeed, in cells cultured without cytokines a striking increase in p27^{KIP1} levels also correlated with an induction of apoptosis (Fig. 2D).

These data raise the possibility that repression of p27^{KIP1} levels through cytokine-mediated PI3K activation is required for cell survival. To separate a role for p27^{KIP1} in survival from its role in proliferation, we utilized freshly isolated peripheral blood human eosinophils. Since these terminally differentiated quiescent cells no longer divide, any regulation of p27^{KIP1} will be independent of cellular proliferation. Again, either removal of the cytokine or inhibition of PI3K resulted in both a decrease in cell survival and an induction of p27^{KIP1} (Fig. 2E). We could not detect any expression of the CKI p21CIP1 in these cells (data not shown), suggesting a specific function of p27^{KIP1} distinct from the regulation of cellular proliferation.

Finally, to determine if the increased levels of p27^{KIP1} were indeed functional, we analyzed whether this increase resulted in a decrease in cyclin E-associated kinase activity. In cells cultured without IL-3, little cyclin E-associated CDK2 activity was observed (Fig. 2F, top).

Similarly, addition of LY294002 substantially blocked cyclin E-associated CDK2 activity, correlating with an increase in $p27^{KIP1}$ levels. Together these data demonstrate that PI3K represses the expression of functional $p27^{KIP1}$ and that this strongly correlates with cellular survival.

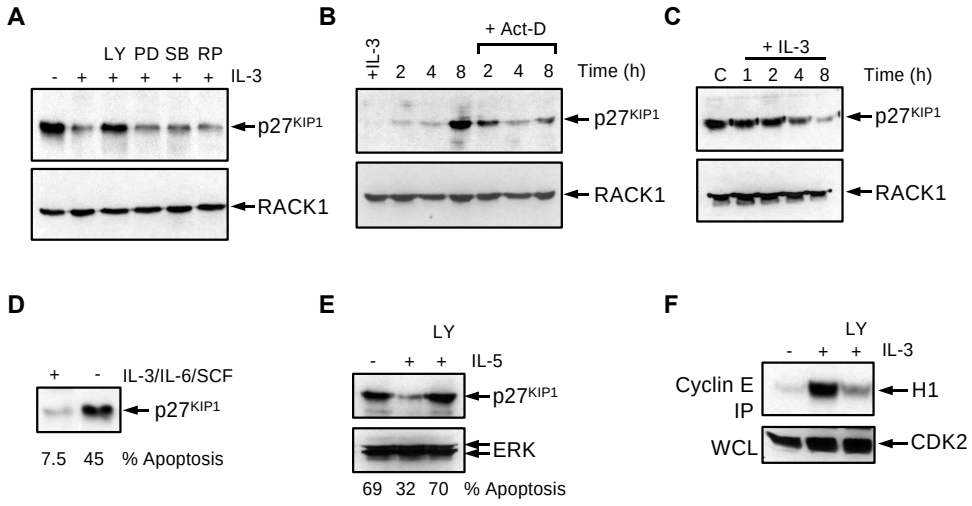

Figure 2. Upregulation of $p27^{KIP1}$ protein levels correlates with apoptosis.
Ba/F3 cells were cultured overnight in the absence or presence of IL-3 without inhibitors or with LY294002 (LY; 10 μM), PD098059 (PD; 50 μM), SB203580 (SB; 10 μM) or rapamycin (rp; 20 ng/ml). Equal amounts of protein were loaded and the levels of $p27^{KIP1}$ (top) and RACK1 (bottom) were determined by immunoblotting as described in Materials and Methods. (B) Ba/F3 cells were cultured overnight with IL-3 and cytokine-starved for the the indicated times in the presence or absence of actinomycin D (5 μg/ml) and $p27^{KIP1}$ levels were analyzed as in for panel (A). (C) Ba/F3 cells were cytokine-starved overnight and were stimulated with IL-3 for the indicated times and levels of $p27^{KIP1}$ were analyzed as for panel (A). (D) Mouse fetal liver cultures were treated with or without cytokines for 24 h, the percentages of apoptotic cells were measured and equal amounts of protein were analyzed for $p27^{KIP1}$ expression. (E) Human peripheral blood eosinophils were cultured without cytokines, with IL-5, or with IL-5 and LY294002 (10 μM). Equal amounts of protein were analyzed for levels of $p27^{KIP1}$ (top) or ERK1 and -2 (bottom) by Western blotting. The percentages of apoptotic cells are shown below. (F) Ba/F3 cells were either cytokine-starved or cultured with IL-3 or IL-3 together with LY294002 (10 μM) overnight, equal amounts of protein were immunoprecipitated (IP) with cyclin E antibody and associated kinase activity was analyzed (top). Equal protein loading was verified by analyzing CDK2 expression (bottom) of whole cell lysate (WCL).

IL-3 downregulates p27^{KIP1} mRNA levels in a PI3K-dependent manner

The regulation of p27^{KIP1} protein expression by phosphorylation, resulting in its degradation by the ubiquitin system, has been extensively studied[42,43]. As upregulation of p27KIP levels upon IL-3 withdrawal was completely abrogated by inhibiting transcription, we investigated whether IL-3 is also capable of regulating p27^{KIP1} mRNA levels. We observed a very rapid upregulation of p27^{KIP1} mRNA upon IL-3 withdrawal, whereas addition of IL-3 rapidly downregulated p27^{KIP1} mRNA (Fig. 3A). To establish a potential role for PI3K in downregulating p27^{KIP1} mRNA, cytokine-starved Ba/F3 cells were either left untreated or preincubated with LY294002 before IL-3 stimulation. In agreement with the findings for p27^{KIP1} protein expression, p27^{KIP1} mRNA expression was also dependent on PI3K activity, since preincubation with LY294002 was found to significantly abrogate downregulation of p27^{KIP1} mRNA expression by IL-3 (Fig. 3B).
In addition to analyzing p27^{KIP1} mRNA, we also examined p27^{KIP1} promoter regulation by IL-3, utilizing a p27^{KIP1} promoter luciferase construct[32]. In agreement with the upregulation of p27^{KIP1} mRNA in cells cultured without IL-3, p27^{KIP1} promoter activity was upregulated in cytokine-starved cells compared to that in cells cultured with IL-3 (Fig. 3C). Addition of LY294002 inhibited IL-3-mediated downregulation of p27^{KIP1} luciferase activity (data not shown). Luciferase activity of control plasmids was unaltered upon IL-3 addition, whereas cyclin D1 promoter activity was upregulated. These data indicate that IL-3 represses p27^{KIP1} transcription in a PI3K-dependent fashion.

FKHR-L1 is inhibited by PI3K-PKB and elevates p27^{KIP1} promoter activity.

The data obtained so far raise the possibility that PI3K activity results in inactivation of a transcription factor responsible for p27^{KIP1} transcription. To identify a possible molecular mechanism by which PI3K could regulate p27^{KIP1} transcription, we focused on the forkhead-related transcription factor FKHR-L1, which has recently been identified as a target of PI3K signaling[14]. The activity of FKHR-L1 is inhibited upon phosphorylation by PKB, resulting in nuclear exclusion[14]. First we analyzed whether IL-3 could regulate the activity of this transcription factor in a PI3K-dependent manner. Indeed, IL-3 stimulation resulted in a rapid transient phosphorylation of endogenous FKHR-L1 (Fig. 4A, left), whereas preincubation of cells with LY294002 completely abrogated this phosphorylation (Fig. 4A, right).

Since PKB has been shown to critically regulate FKHR-L1 we wished to determine whether in Ba/F3 cells FKHR-L1 is phosphorylated in a PKB-dependent fashion. To address this, we constructed a 4-OHT-inducible active-PKB Ba/F3 cell line (myrPKB:ER*)[44]. Concomitant with PKB activation (Fig. 4B), FKHR-L1 phosphorylation was greatly increased upon 4-OHT addition (Fig. 4C). PKB activation was also sufficient to rescue cells from cytokine withdrawal induced apoptosis (data not shown). This demonstrates that ligand-independent activation of PKB alone is sufficient for FKHR-L1 phosphorylation in Ba/F3 cells.

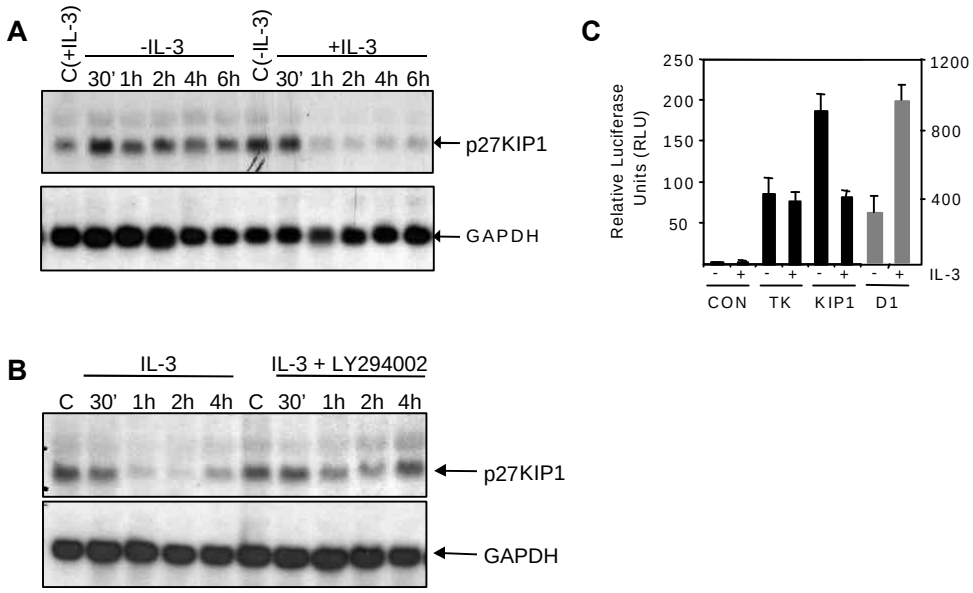

Figure 3. Cytokine-mediated regulation of p27^{KIP1} transcription requires PI3K.
(A) Ba/F3 cells were either IL-3-starved or IL-3-starved overnight and subsequently stimulated with IL-3 for the indicated times. Twenty micrograms of total RNA was used for Northern blotting and hybridized with a p27^{KIP1} probe (top). Equal RNA loading was verified by GAPDH reprobing (bottom). (B) Ba/F3 cells were IL-3-starved overnight and restimulated with IL-3 for the indicated times with or without preincubation with LY294002 (10 μM) and analyzed as for panel (A) (C) Ba/F3 cells were electroporated with 10 μg of either pGL2 (CON), pGL2-TK (TK), pGL2-p27^{KIP1} (KIP1) or cyclin D1 (D1) luciferase constructs together with 500 ng of renilla, cultured with or without IL-3 for 24 h and luciferase activity was analyzed as described in Materials and Methods.

Transcription factor binding site analysis of the p27^{KIP1} promoter sequence revealed consensus forkhead transcription factor binding sites, suggesting that FKHR-L1 may regulate p27^{KIP1} expression. To investigate whether p27^{KIP1} promoter activity could also be enhanced by FKHR-L1, we expressed either wild-type FKHR-L1 or an "active" FKHR-L1 mutant in which all three PKB phosphorylation sites were mutated to alanine [FKHR-L1(A3)][14]. Ectopic expression of FKHR-L1 increased p27^{KIP1} promoter activity, which was further enhanced when FKHR-L1(A3) was expressed (Fig. 4D). To determine whether PKB could regulate FKHR-L1-induced promoter activity, we cotransfected a constitutively active PKB mutant (gagPKB) with FKHR-L1 expression vectors[29]. Cotransfection of gagPKB completely inhibited p27^{KIP1} promoter activity induced by wild-type FKHR-L1, whereas the increase in promoter activity induced by FKHR-L1(A3) was unaffected (Fig. 4D).

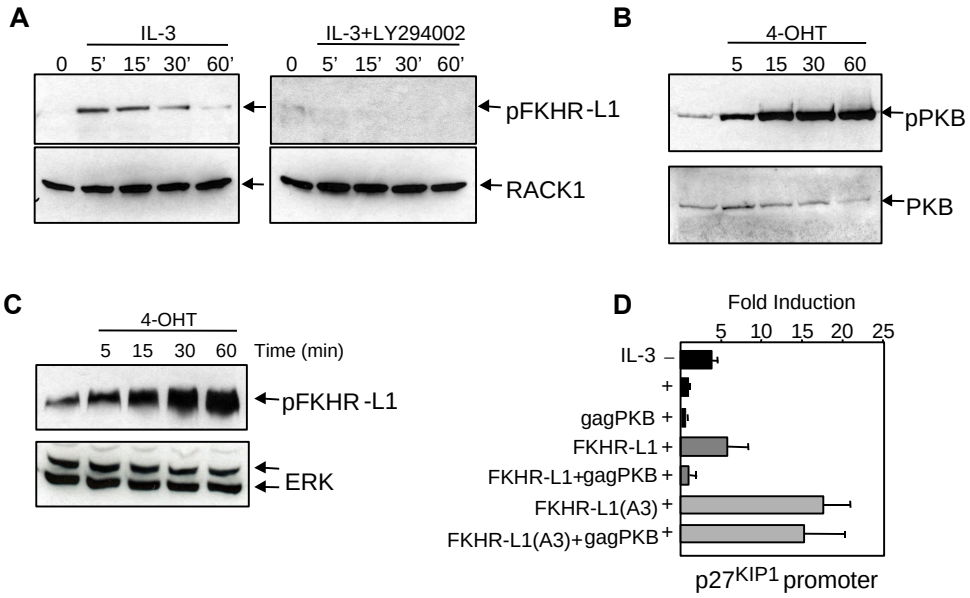

Figure 4. Analysis of FKHR-L1 phosphorylation and activity in Ba/F3 cells.
(A) Ba/F3 cells were cytokine-starved and stimulated with IL-3 for the indicated times (left) or pre-treated with LY294002 (10 μM) for 20 min prior to IL-3 stimulation (right). FKHR-L1 phosphorylation was analyzed using an FKHR-L1(Thr32) specific antibody (top); equal protein loading was verified by RACK1 reprobing (bottom). (B) Ba/F3 cells stably expressing myrPKB:ER* were cytokine-starved overnight and stimulated with 4-OHT (100 nM) for the indicated times. Phosphorylated myrPKB-ER was analyzed using a PKB(Ser473)-specific antibody (top); equal PKB levels were verified by reprobing the blot with a PKB antibody (bottom). (C) Ba/F3 cells stably expressing myrPKB:ER* were cytokine-starved overnight, stimulated with 4-OHT (100 nM) for the indicated times and FKHR-L1 phosphorylation was analyzed using an FKHR-L1(Thr32) specific antibody (top). (D) Ba/F3 cells were electroporated with 12 μg of p27KIP1 luciferase construct together with either 4 μg of pSG5-gagPKB, FKHR-L1(wt), or FKHR-L1(A3) or combinations thereof as indicated. The DNA concentration was adjusted to 20 μg with pSG5. Cells were cultured with IL-3 and luciferase activity was analyzed 24 h later as described in Materials and Methods.

Transcriptional activity of FKHR-L1 directly induces p27^{KIP1} expression.
Previous studies investigating the function of forkhead-related transcription factors have all utilized transient overexpression of these proteins[14,45]. To allow us to specifically analyze the consequence of FKHR-L1 activation in more detail, we generated several monoclonal Ba/F3 cell lines expressing a 4-OHT-inducible FKHR-L1(A3) construct, FKHR-L1(A3):ER*. Expression levels of FKHRL1(A3):ER* in all cell lines were approximately one-third to one-fifth of that of endogenous FKHR-L1 (Fig. 5A). Similar to what was found in the cotransfection experiments (Fig. 4D), p27^{KIP1} promoter activity was upregulated upon 4-OHT addition (Fig. 5B). Furthermore, addition of 4-OHT resulted in a striking upregulation of p27^{KIP1} mRNA within 30 to 60 min (Fig. 5C), providing compelling evidence for direct FKHR-L1 transciptional regulation of p27^{KIP1} expression in vivo. In accordance with induction of p27^{KIP1} mRNA, p27^{KIP1} protein levels were also highly elevated in cells treated with 4-OHT (Fig. 5D). To confirm that upregulation of p27^{KIP1} levels was indeed a result of FKHR-L1 mediated transcription, actinomycin D was added prior to 4-OHT addition. As shown in Fig. 5E this completely abrogated upregulation of p27^{KIP1} protein, as well as mRNA (data not shown). Finally, we analyzed levels of p27^{KIP1} with various concentrations of 4-OHT; the levels were elevated in a dose-dependent fashion (Fig. 5F).

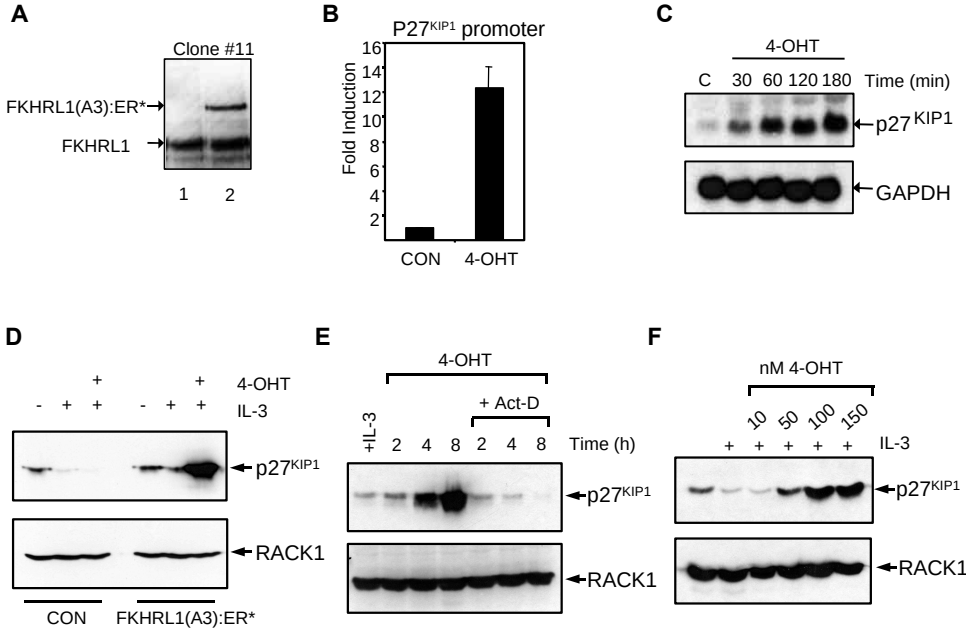

Figure 5. FKHR-L1 directly regulates p27KIP1 transcription.

(A) Expression of FKHR-L1 in Ba/F3 cells or Ba/F3 cells stably expressing FKHR-L1(A3):ER* was verified by immunoblotting with FKHR-L1 antibody. (B) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were electroporated with 12 μg of $p27^{KIP1}$ luciferase construct together with 8 μg of pSG5. Cells were cultured with IL-3 (CON) or with IL-3 and 4-OHT (100 nM), and luciferase activity was analyzed 24 h later as described in Materials and Methods. (C) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were treated with 4-OHT (100 nM) for the indicated times; 20 μg of total RNA was used for Northern blotting and hybridized with a $p27^{KIP1}$ probe (top). Equal RNA loading was verified by GAPDH reprobing (bottom). (D) Ba/F3 cells and Ba/F3 cells stably expressing FKHR-L1(A3):ER* were cytokine-starved overnight and were cultured with IL-3 or with IL-3 and 4-OHT (100 nM). Equal amounts of protein were loaded, and the levels of $p27^{KIP1}$ (upper panel) or RACK1 (lower panel) were determined by immunoblotting as described in Materials and Methods. (E) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were treated with 4-OHT (100 nM) in the absence or presence of actinomycin D (5 μg/ml) for the the indicated times and analyzed as for panel (D). (F) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were cultured in the absence or presence of IL-3 or IL-3 together with various concentrations 4-OHT overnight and were analyzed as for panel (D).

FKHR-L1 function has also been linked with the induction of apoptosis in fibroblasts, cerebellar neurons and T cells[14]. We analyzed the induction of apoptosis upon transient overexpression of either FKHR-L1 or the active mutant FKHR-L1(A3) in Ba/F3 cells. Apoptosis was significantly increased in cells electroporated with FKHR-L1, and was further enhanced when the active mutant FKHR-L1(A3) was overexpressed (Fig. 6C). Next, we analyzed the effect of the addition of increasing 4-OHT concentrations to the FKHR-L1(A3):ER* stable cell lines. 4-OHT addition resulted in the induction of apoptosis in a dose-dependent fashion (Fig. 6D).
Finally, we reasoned that if the elevation of $p27^{KIP1}$ plays a critical role in FKHR-L1 mediated induction of apoptosis, coexpression of cyclin-CDK complexes should be capable of titrating away the induced $p27^{KIP1}$ and thereby rescuing cells from apoptosis[17,45]. Indeed, expression of cyclin D together with a kinase-dead form of CDK4 in 4-OHT treated cells was sufficient to significantly rescue FKHR-L1(A3) induced apoptosis in two independent clones (Fig. 6E). These data confirm that increases in $p27^{KIP1}$ levels play a significant role in FKHR-L1 induced apoptosis.

Regulation of $p27^{KIP1}$ expression is important for maintenance of cell survival

The data described above suggest that repression of $p27^{KIP1}$ levels through PKB-mediated FKHR-L1 phosphorylation may be necessary for cytokine-mediated survival and proliferation. To address whether mere ectopic expression of $p27^{KIP1}$ is sufficient to induce apoptosis, we introduced an expression plasmid for $p27^{KIP1}$ in Ba/F3 cells, together with spectrin-GFP as a marker for transfected cells. Twenty-four h after electroporation, cells were fixed and stained with PI and the DNA content of the spectrin-GFP-expressing cells was analyzed. Cells transfected with both spectrin-GFP and $p27^{KIP1}$ exhibited a significantly higher percentage of apoptotic cells and cells in G_0/G_1 than control cells (Fig. 6A). To exclude the possibility that supra-physiological levels of $p27^{KIP1}$ expression alone cause cells to undergo apoptosis, $p27^{KIP1}$ levels in transfected cells were analyzed. This was performed by coexpressing LNGFR[34], sorting LNGFR expressing cells by magnetic cell sorting (MACS), and analyzing $p27^{KIP1}$ expression levels in corrected protein samples. Levels of $p27^{KIP1}$ inducing apoptosis in transfected cells did not exceed the levels in IL-3-starved cells (Fig. 6B). Thus uncontrolled expression of physiological levels of $p27^{KIP1}$ is sufficient to induce apoptosis in cytokine-dependent cells.

$P27^{KIP1}$ deficiency increases hematopoietic cell survival after cytokine withdrawal.

Finally, to examine the importance of $p27^{KIP1}$ in the regulation of apoptosis in vivo, we utilized hematopoietic stem cells obtained from either wild-type mice, or mice lacking one or both $p27^{KIP1}$ alleles[46]. Bone marrow derived Sca1+ stem cells were cytokine-starved and analyzed 24 h later, using annexin-V staining to label apoptotic cells. Strikingly, stem cells obtained from mice lacking one $p27^{KIP1}$ allele exhibited a moderate protection

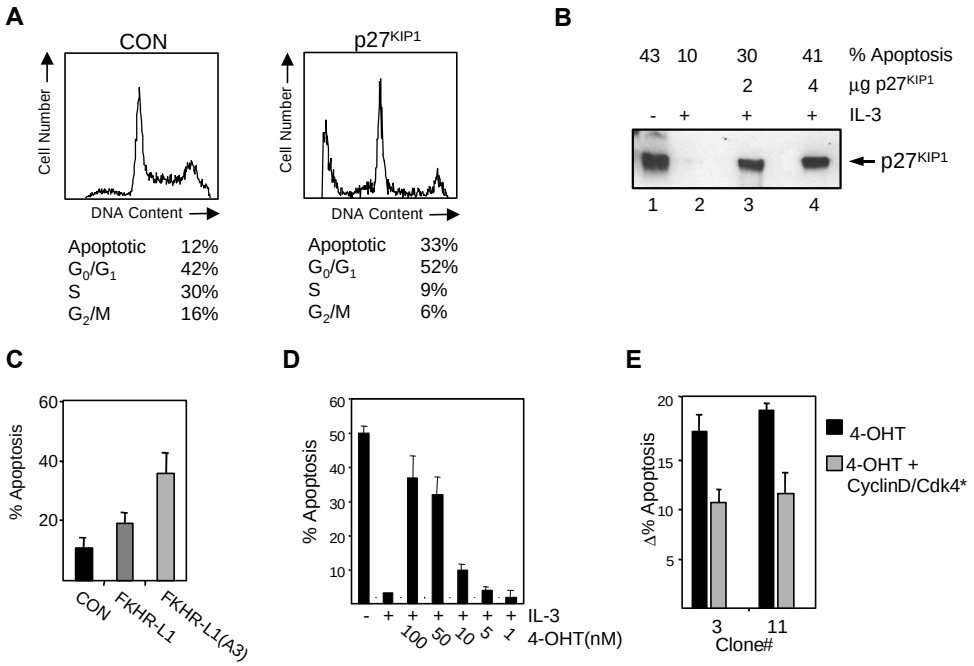

Figure 6. IL-3 mediated survival requires inactivation of FKHR-L1 and downregulation of p27^{KIP1} levels.

(A) Ba/F3 cells were electroporated with 2 µg of spectrin-GFP vector together with either 18 µg of empty vector (pSG5; left) or 18 µg of p27^{KIP1} vector (right). Dead cells were removed by separation through a Ficoll gradient. Twenty-four h after electroporation cells were fixed and stained with PI and the DNA contents of 5,000 GFP-positive cells were analyzed by FACS. The data are representative of several independent experiments. (B) Ba/F3 cells were electroporated with 2 µg of LNGFR DNA, 2 µg LNGR together with 2 or 4 µg of p27^{KIP1} DNA and the total amount of DNA was adjusted to 20 µg with pSG5. Dead cells were removed by separating cells through a Ficoll gradient and LNGFR-expressing cells were analyzed 24 h after transfection as described in Materials and Methods. (C) Ba/F3 cells were electroporated with spectrin-GFP (2 µg) together with either 18 µg pSG5 (CON) vector, FKHR-L1 vector or FKHR-L1(A3) vector and analyzed as for panel (A). The data represent three independent experiments (± standard errors of the means). (D) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were cytokine-starved and cultured with IL-3 or IL-3 together with various concentrations of 4-OHT, and the percentages of apoptotic cells were determined after 48 h by FACS analysis. (E) FKHR-L1(A3):ER*-expressing cells lines were electroporated with either 18 µg of pSG5 and 2 µg of spectrin-GFP (black bars), or 5 µg of kinase-dead CDK4, 5 µg of cyclin D1, 2 µg of spectrin-GFP and 8 µg of pSG5 (grey bars). Dead cells were removed by separation through a Ficoll gradient. Cells were treated with 4-OHT and analyzed 24 h later as for panel (A). The data depicted are representative of several independent experiments.

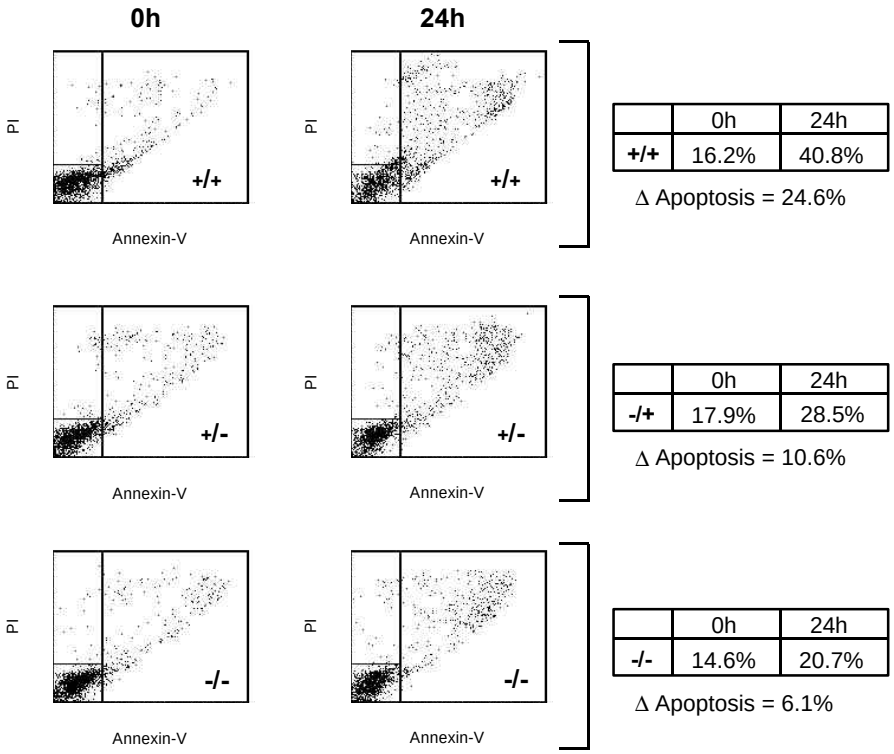

Figure 7 Increased survival of *p27^{KIP1} (-/-)* hematopoietic cells after cytokine withdrawal.

Hematopoietic stem cells were isolated from either wild-type mice *(+/+)* or mice lacking one *(-/+)* or both *(-/-)* alleles of the *p27^{KIP1}* gene and cultured as described in Materials and Methods. Cells were cytokine-starved for 24 h and the percentages of apoptotic cells were analyzed by annexin-V staining. The percentage increase in apoptosis after cytokine withdrawal is shown as Δ(apoptosis). Data are representative of three independent experiments.

against cytokine withdrawal-induced apoptosis compared to those from wild-type mice (Fig. 7). This was significantly enhanced in stem cells from mice lacking both alleles. These data demonstrate the importance of regulating p27^{KIP1} levels in the modulation of hematopoietic cell apoptosis *in vivo*.

Discussion

The control of proliferation and apoptosis by cytokines is critical in the regulation of a variety of hematopoietic lineages[2,47]. Our data demonstrate PI3K signaling to be indispensable in mediating cellular proliferation and survival. The importance of PI3K activity in mediating survival was supported by overexpression the 3-phosphatidylinositol lipid phosphatase PTEN[7], which is a uniquely specific tool for decreasing 3-phosphoinositide levels in cells. Upon overexpression of membrane-localized PTEN, we observed an induction of apoptosis in IL-3-cultured Ba/F3 cells (Fig. 1C). The fact that membrane-targeted PTEN, unlike wild-type PTEN, is potently active (Fig. 1C), suggests that this is a critical aspect of PTEN regulation in vivo. Mutations in the chromosomal region of PTEN resulting in a loss of function of PTEN have been described in a variety of neoplasias, including lymphoid malignancies[48]. These mutations result in the accumulation of PtdIns(3,4,5)P_3 in the absence of cellular stimulation. While inhibition of PTEN activity may have deleterious effects on cell proliferation, resulting in a neoplastic phenotype, our data demonstrate that uncontrolled PTEN activity can result in the induction of an apoptotic program.

In search of a potential mechanism by which PI3K could regulate cytokine-mediated cell survival and proliferation, we focused on the CKI p27^{KIP1}. P27^{KIP1} is an inhibitor of cell cycle progression, exerting its effect through interaction with cyclin-CDK complexes and arresting cells in G_0/G_1[17]. Furthermore, p27^{KIP1} has been implicated in the regulation of apoptosis in immature B cells[22,23]. Cross-linking of surface Ig (IgM) on the WEHI-231 B-cell lymphoma, for example, results in growth arrest and eventually induction of an apoptotic program which can be rescued by CD40 ligand engagement. These IgM-induced changes are correlated with an increase in p27^{KIP1} protein which is inhibited by CD40, although the molecular mechanisms of these observations are unclear[22,23]. A potential role for PI3K in down regulating p27^{KIP1} levels was suggested by the observation that overexpression of PTEN in glioblastoma cells resulted in enhanced p27^{KIP1} levels[49]. We have explored the IL-3-mediated regulation of p27^{KIP1} levels and a possible role for PI3K therein. Survival factor withdrawal resulted in an increase of p27^{KIP1} protein levels in a PI3K-dependent manner (Fig. 2A). In cultures of primary fetal liver cells cytokine withdrawal also resulted in an increase of apoptosis paralleled by upregulation of p27^{KIP1}, suggesting that this may be a common feature of primary lymphocyte lineages (Fig. 2D). Levels of p27^{KIP1} in primary human eosinophils undergoing apoptosis were also analyzed (Fig. 2E). In eosinophils, both cytokine starvation and inhibition of PI3K resulted in significantly higher levels of p27^{KIP1}, correlating with induction of apoptosis (Fig. 1D). Importantly, induction of p27^{KIP1} in these nondividing cells suggests an additional cell cycle independent role for this CKI.

While regulation of p27^{KIP1} levels has been previously considered to occur predominantly posttranslationally[19,50], we found a rapid and dramatic effect of IL-3 on p27^{KIP1} mRNA (Fig. 3A). In addition, IL-3 was also capable of downregulating p27^{KIP1} promot-

er activity in a PI3K-dependent manner (Fig. 3C), prompting us to investigate the role of PI3K-regulated transcription factors in this process. Transcription factors of the AFX/FKHR forkhead family are phosphorylated by the PI3K target PKB, resulting in inhibition of their activity[14,15,51,52]. One member, FKHR-L1, has been linked to induction of apoptosis, possibly by the upregulation of Fas ligand on cells[14]. FKHR-L1 is endogenously expressed in Ba/F3 cells and phosphorylated in a PI3K-PKB-dependent manner (Fig. 4A and C). Furthermore, overexpression of an active FKHR-L1 mutant resulted in induction of apoptosis (Fig. 6C). Since Fas ligand was unable to induce apoptosis in Ba/F3 cells (data not shown), a role for FKHR-L1 in induction of apoptosis must be mediated by an alternative mechanism. The presence of several forkhead transcription factor binding sites in the $p27^{KIP1}$ promoter suggested a possible link between FKHR-L1 and transcription of $p27^{KIP1}$. Indeed, overexpression of FKHR-L1 elevated $p27^{KIP1}$ promoter activity, which could be inhibited by cotransfection of active PKB (Fig. 4D). To specifically analyze the effect of FKHR-L1 on $p27^{KIP1}$ transcription we utilized Ba/F3 cells stably expressing a 4-OHT-inducible active FKHR-L1 construct. Upon FKHR-L1 activation, $p27^{KIP1}$ mRNA was greatly elevated within 30 to 60 min, concomitant with a spectacular elevation of $p27^{KIP1}$ protein levels (Fig. 5C to E). These data clearly demonstrate that activation of FKHR-L1 alone is sufficient to induce rapid upregulation of $p27^{KIP1}$ mRNA in vivo. To determine if $p27^{KIP1}$ is indeed an important target of FKHR-L1-induced apoptosis, we overexpressed cyclin D-CDK4 complexes to titrate away functional $p27^{KIP1}$. Indeed, overexpression of cyclin D-CDK4 complexes was sufficient to significantly reduce FKHR-L1 induced apoptosis, thus suggesting that $p27^{KIP1}$ is an important FKHR-L1 target for the induction of apoptosis. The fact that apoptosis was not completely rescued by overexpression of cyclin D-CDK4, it suggests that there are possibly additional targets accounting for FKHR-L1-induced apoptosis. During the preparation of this paper it was reported that FKHR-L1-related transcription factor AFX was able to induce growth suppression through regulation of $p27^{KIP1}$ expression[45]. However, these overexpression studies were performed with cells normally not expressing AFX. We have now been able to demonstrate that regulation of $p27^{KIP1}$ transcription can be controlled through cytokines and further that this seems to play a role in the regulation of survival.

Here we also provide proof for the importance of $p27^{KIP1}$ in the induction of apoptosis by utilizing mice lacking one or both alleles of the $p27^{KIP1}$ gene[46]. There was a significant decrease in apoptosis upon cytokine withdrawal in mice lacking one $p27^{KIP1}$ gene allele (change in apoptosis [Δapoptosis]=10.6%) compared to that in wild-type mice (Δapoptosis=24.6%), this decrease was even more striking in mice lacking both alleles (Δapoptosis=6.1%). While the role of $p27^{KIP1}$ in regulating growth arrest is fairly well defined, relatively little is known regarding the mechanisms by which this protein may regulate apoptosis. A potential mechanism is suggested by a recent report by Boussiotis et al., who demonstrated that $p27^{KIP1}$ is capable of directly influencing transcription independently of its ability to block cell cycle progression[53]. Increased $p27^{KIP1}$ levels were found to inhibit IL-2 transcription in T cells through the binding, nuclear export, and

subsequent degradation of the Jun transcription factor coactivator JAB1 (Jun activation domain-binding protein-1)[54]. Potentially, inhibition of anti-apoptotic gene expression through p27^{KIP1}-mediated degradation of JAB1 could play a role in the induction of apoptosis. In various malignancies it has been shown that reduced levels of p27^{KIP1} correlate with poor prognosis[55-57]. The levels of p27^{KIP1} do not, however, correlate with the proliferative status of the tumor cells, suggesting that the benefits of p27^{KIP1} reflect an additional function such as increased apoptosis. Indeed, decreased p27^{KIP1} expression in gastric carcinomas correlates with decreased apoptosis and increased aggressiveness of the tumor[58].

The regulation of both proliferation and survival by p27^{KIP1} has parallels with that by the tumor suppressor protein p53. P53 has a major G_1 checkpoint function and can mediate a transient growth arrest in certain situations that favor cell survival, while inducing apoptosis in others[59]. Interestingly, one study has demonstrated that overexpression of Bcl-2 can significantly counteract the apoptotic effects of p27^{KIP1}, preventing caspase activation[60]. This suggests that p27^{KIP1} may either inhibit specific anti-apoptotic Bcl-2 family members or activate pro-apoptotic family members such as Bim that have recently been shown to play a critical role apoptosis induced by cytokine withdrawal[61].

Our findings demonstrate a novel mechanism by which cytokines mediate rescue from apoptosis. This involves the downregulation p27^{KIP1} levels through the PI3K-PKB-regulated inactivation of transcription factors of the AFX/FKHR forkhead family. Exposure of hematopoietic cells to cytokines acts to stimulate both survival and proliferation. The regulation of p27^{KIP1} expression by PI3K allows the modulation of both these processes by altering the levels of a single protein. Our data not only provide insight into the mechanisms of cytokine-mediated signal transduction regulating cell proliferation and survival, but also identify critical components regulating p27^{KIP1} transcription. The mechanism of PI3K-mediated forkhead transcription factor regulation is conserved between the nematode worm *Caenorhabditis elegans*[62] and mammalian cells. Our data implicate the regulation of p27^{KIP1} by this evolutionarily conserved signaling pathway as a general mechanism for controlling cell fate decisions regulating survival and proliferation or differentiation.

Acknowledgements

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CHAPTER 4

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

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Introduction

The regulation of cell death is regulated mainly through an evolutionary conserved form of cell suicide termed apoptosis[1]. Deregulation of this process 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 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 determining cell survival fate[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 to pro-survival Bcl-2 members, 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 Bim is critical for maintenance of the default apoptotic program[4]. Here we show that cytokine withdrawal 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], is sufficient to induce Bim expression. We propose a mechanism by which cytokines promote lymphocyte survival by inhibition of FKHR-L1, preventing Bim expression.

Results and Discussion

While Bim is expressed in many hematopoietic lineages, it is not known how its expression is affected after cytokine withdrawal[1]. 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 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 (Fig. 1A; left panel). To determine whether this observation may represent a more general phenomenon, primary mouse fetal liver cells cultured in the presence or absence of survival factors[6] were analyzed for Bim expression. Indeed, fetal liver cells undergoing apoptosis following cytokine withdrawal exhibited elevated Bim protein levels (Fig 1A; right panel). To determine whether upregulation of Bim expression by IL-3 withdrawal was a result of enhanced transcription, bim mRNA was also analyzed. Bim has three isoforms (BimS, BimL and BimEL) that are generated by alternative splicing[3]. Northern blot analysis has shown several Bim transcripts, although 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 (Fig. 1B).

To determine whether increased expression of Bim is sufficient to induce apoptosis,

we transiently expressed BimS or BimL, together with spectrin-linked GFP in Ba/F3 cells. This approach enables analysis of apoptosis in the transfected (GFP-positive) versus untransfected (GFP-negative). In cells that expressed only spectrin-GFP, cell survival was unaffected relative to control cells (Fig. 1C ; left panel). In cells expressing either BimL or BimS the level of apoptosis in GFP-positive cells was dramatically increased (Fig. 1C; middle and right panels).

Previous work has implicated phosphatidylinositol 3-kinase (PI3K) activity as being critical for cytokine-mediated rescue from apoptosis in lymphocytes[7]. To determine whether changes in Bim expression may be dependent on PI3K activity, Ba/F3 cells were either cytokine-starved, cultured with IL-3 or IL-3 in combination with the specific PI3K inhibitor LY294002. Bim protein levels were elevated in cells undergoing apoptosis induced either by IL-3 withdrawal or by inhibition of PI3K (Fig. 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/Akt), a target of PI3K-activation, has recently been reported to inhibit 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, do 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 (Fig. 2A), which leads to nuclear translocation and activation of this transcription factor[5]. To determine whether activity of FKHR-L1 is linked to upregulation of Bim protein levels we generated a novel inducible FKHRL1(A3) expression construct. FKHR-L1(A3) was fused to the hormone-binding domain of the estrogen receptor[9], resulting in a 4-hydroxy tamoxifen (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 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 (Fig. 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 FKHR-L1(A3):ER* cells 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 FKHRL1-induced apoptosis than GFP-negative control cells (Fig. 2C; lower panel). Finally, to examine whether the enhanced expression of Bim protein by FKHR-L1 activity was a result of transcriptional regulation, bim mRNA was analyzed. Similarly to IL-3 withdrawal (Fig. 1C), 4-OHT mediated FKHR-L1 activation significantly elevated Bim

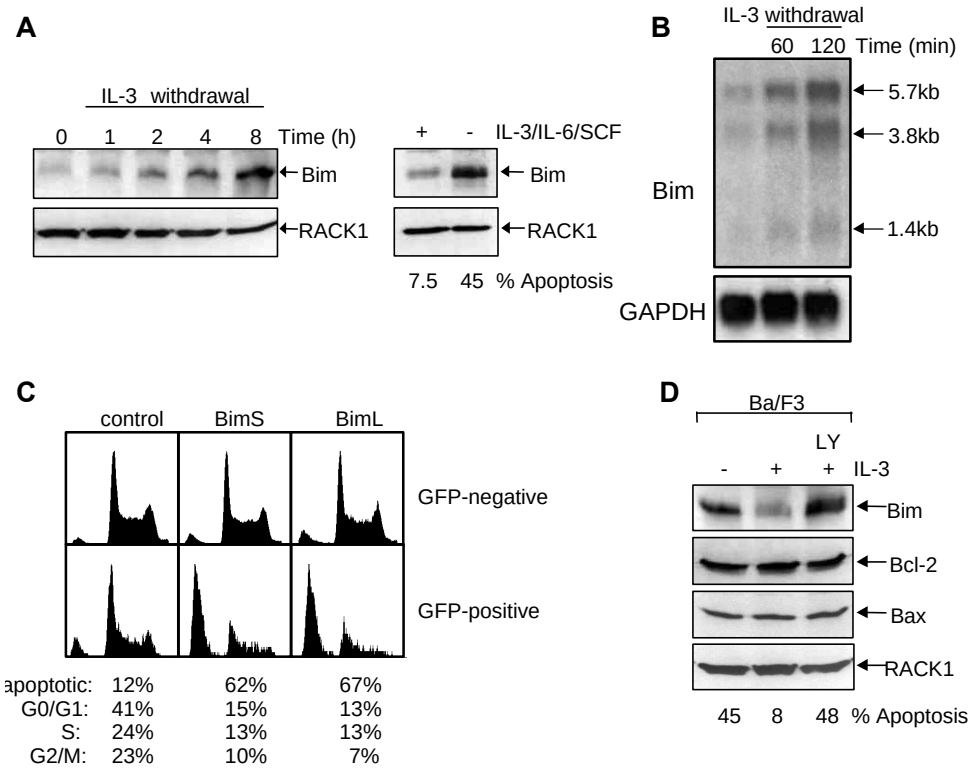

Figure 1 Bim levels are regulated by cytokines and determine cell survival fate.
(A) Left, Ba/F3 cells were IL-3-starved and lysed after the indicated times. Equal amounts of protein were loaded and Bim levels were determined by probing with anti-Bim. The blot was reprobed with anti-RACK1 to confirm equal protein loading. Right, Mouse fetal liver cultures were treated with or without cytokines for 24 hours, and the percentage of apoptotic cells were measured as well as levels of Bim and RACK1. (B) IL-3 withdrawal induces Bim mRNA expression. Ba/F3 cells were IL-3-starved for the indicated time points. PolyA+ RNA was isolated and Bim mRNA levels were analyzed using full length BimL cDNA as a probe. Equal RNA loading was confirmed by reprobing the blot with 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 hours cells were fixed, stained with propidium iodide and the DNA content of 5000 GFP-positive or 20,000 GFP-negative cells was analysed by FACS. The data depicted is representative of several independent experiments. (D) PI3K activity is critical for cytokine-mediated repression of Bim. Ba/F3 cells were either cytokine-starved, cultured overnight with IL-3 without or with LY294002 (10 μM) and levels of Bim, Bcl-2 and Bax were determined. Blots were reprobed with RACK1 to confirm equal protein loading. The percentage of apoptotic cells was also determined by FACS analysis.

transcripts (Fig. 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 indicated timepoints in combination with the protein synthesis inhibitor cycloheximide. An elevation of Bim mRNA was also observed in cycloheximide treated cells (Fig. 2D, right panel), demonstrating that Bim transcription is indeed directly regulated by FKHR-L1.

Relatively little is known regarding 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 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 clearly demonstrate that the regulation of Bim expression by cytokines is very likely 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 since *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 fate 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 lifespan of cytokine-dependent cells.

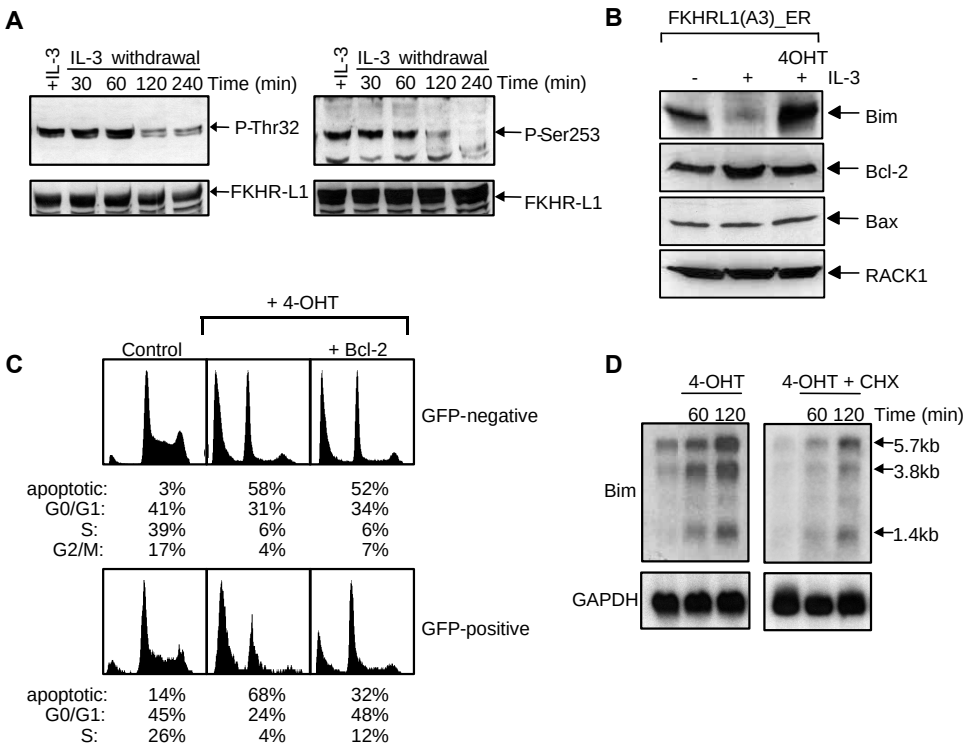

Figure 2. Bim levels are transcriptionally regulated by IL-3 through the forkhead transcription factor FKHR-L1.

(A) FKHR-L1 phosphorylation in Ba/F3 cells. IL-3 Ba/F3 cells were IL-3-starved and lysed at the indicated times. Levels of phosphorylated FKHR-L1 were analyzed by hybridizing with antibodies that detect the Thr32-phosphorylated (P-Thr-32) or Ser253-phosphorylated (P-Ser253) forms of FKHR-L1, and equal protein 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 hours as indicated and levels of Bim, Bcl-2 and Bax were determined. Blots were reprobed with 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 hours was measured as in 1C. (D) Bim mRNA levels are upregulated by FKHR-L1 activity. Left, Ba/F3 cells stably expressing FKHR-L1(A3):ER* were treated with 4-OHT (100 nM) for indicated time points. 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 with GAPDH. Right, same as left panel except that cells were pretreated with cycloheximide (CHX, 10 μg/ml) before addition of 4-OHT.

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. 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 Dr. A. Beavis and T. Sheck (Princeton, U.S.A.) and has been described previously[14]. pSG5-Bcl-2 was a kind gift from Dr. 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 (Golden, CO, U.S.A.), Bcl-2 antibody from Santa Cruz (Santa Cruz, CA, U.S.A.); Bax mAb from BD PharMingen (San Diego, CA, U.S.A.); RACK1 mAb from Transduction Laboratories (Kentucky, U.S.A.); FKHR-L1, phospho-Thr32 FKHR-L1 and phospho-Ser253 FKHR-L1 were from UBI (Lake Placid, NY, U.S.A.).

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 (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 previously described[6].

For transient transfection, Ba/F3 cells were electroporated (0.28 kV; capacitance 960 μFD) and 2 hours after electroporation dead cells were removed by separating through a Ficoll gradient (2500 rpm for 20 minutes). After 24 hours cells were harvested, washed twice in PBS and fixed for at least 2 hours 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 minutes. 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 minutes 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, Mountainview, CA, U.S.A.) 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 (Madison, WI, U.S.A.). Equal RNA loading was verified by reprobing the blots with a 1.4 kb cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

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CHAPTER 5

FKHR-L1 is a critical effector of cell death induced by interleukin-3 withdrawal: PKB-mediated inhibition of FKHR-L1 activity enhances cell survival by maintenance of mitochondrial integrity

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Submitted

Abstract

Survival signals elicited by cytokines include activation of phosphatidyl inositol 3-kinase (PI3K), which in turn promotes activation of Protein Kinase B (PKB). Recently, PKB has been demonstrated to phosphorylate and inactivate Forkhead transcription factor FKHR-L1, which is a potent inducer of apoptosis. To explore the mechanisms underlying induction of apoptosis following cytokine withdrawal or FKHR-L1 activation, we have utilized a cell line in which FKHR-L1 activation can be specifically induced. Both cytokine withdrawal and FKHRL1 activation induce apoptosis as measured by Annexin-V binding and DNA laddering. The onset of apoptosis is 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 FKHR-L1 activation correlates with disruption of mitochondrial membrane integrity and cleavage of effector caspases. This was preceeded by upregulation of the pro-apoptotic Bcl-2 family member Bim, suggesting a critical role for Bim in this process. Apoptosis does not correlate with cleavage of caspase-8, demonstrating that it is a death receptor independent process. Activation of PKB alone was sufficient to promote cell survival through maintenance of mitochondrial integrity and the resultant inhibition of effector caspases. Importantly, these data demonstrate that activation of FKHR-L1 alone is sufficient to induce the complete program of apoptotic events normally associated with survival factor withdrawal, suggesting it is a critical player in this process.

Introduction

In the absence of cytokines, hematopoietic cells stop proliferating and undergo programmed cell death, 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 (reviewed in [1]). The activation of cysteine proteases, caspases, leading to the cleavage of various substrates, including PARP (poly (ADP-ribose) polymerase) and the degradation of chromosomal DNA characterize a crucial step in the induction of apoptosis[2]. Caspases exist as inactive pro-enzymes in the cell, which themselves are activated through proteolytic cleavage upon induction of the apoptotic program (reviewed in [3]).

A well-characterized mechanism of initiating apoptosis is through ligand-mediated activation of cell surface death receptors, such as the TNF receptors and CD95 (APO-1/Fas) (reviewed in [4]). Caspase-8 is indispensable for transducing apoptotic signals initiated by death receptors, demonstrated by the observation that CD95 signaling is abrogated in *caspase-8 (-/-)* mice[5]. Caspase activation can also be triggered via a death receptor-independent mechanism, involving 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 Apaf-1 (apoptosis activating factor 1) promote activation of caspase-9[6,7], which activates downstream caspases, such as caspase-3 and 7[3]. Although loss of mitochondrial integrity can also be induced by death receptors, it is not essential for their induction of apoptosis[8].

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 (reviewed in [9]). One mechanism by which cytokines are believed to promote survival is by inhibiting transcription[10,11] or activity[12,13] of pro-apoptotic members, as well as transcriptionally upregulating anti-apoptotic members[14,15].

The serine and threonine protein kinase, Protein Kinase B (PKB), also known as c-Akt, is involved in cytokine-mediated cell survival[12,16,17]. Anti-apoptotic signals from PKB include upregulation of Mcl-1[18] and inhibitory phosphorylation of Bad[12], although the relevance of Bad phosphorylation for the survival of hematopoietic cells remains unclear[19]. A recently identified mechanism by which PKB can promote rescue from apoptosis is through inhibitory phosphorylation of the Forkhead transcription factor FKHR-L1 (FOXO3a)[10,20]. Activity of this transcription factor has been linked to induction of apoptosis in hematopoietic cells[10,20]. Although PKB is a well-established player in rescue from apoptosis, it is not clear whether PKB exerts its anti-apoptotic effect upstream[21] or downstream[22] of mitochondria. Furthermore, little is known concerning the mechanisms by which FKHR-L1 can lead to activation of caspases, resulting in induction of apoptosis.

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, induces apoptosis through a death receptor-independent pathway. This involves upregulation of the pro-apoptotic Bcl-2 family member Bim, loss of mitochondrial integrity and caspase activation. Thus PKB can protect cells from cytokine withdrawal-induced apoptosis by inhibiting FKHR-L1, resulting in maintenance of mitochondrial integrity. These data shed new light on the mechanisms by which cytokines, through regulation of PKB activity, can modulate the survival of hematopoietic lineages.

Experimental Procedures

Cell culture

Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3 produced in COS cells[23]. Monoclonal Ba/F3 cells stably expressing FKHR-L1(A3):ER* and the polyclonal myrPKB:ER* Ba/F3 cell line have been described previously[10,24] 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 case of the polyclonal

myrPKB:ER* Ba/F3 cell line, cultured for two hours prior to adding 4-hydroxy tamoxifen (4-OHT).

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 (Beverly, MA, U.S.A). Polyclonal caspase-8 antibody (559932) was from BD Pharmingen (San Diego, CA, USA). Bim polyclonal antibody was purchased from Affinity Bioreagents (Golden, CO, U.S.A.). $p27^{KIP1}$ and RACK1 mAb were purchased from Transduction Laboratories (Lexington, Kentucky, U.S.A.). Propidium iodide was from Sigma- Aldrich Chemie (Zwijndrecht, The Netherlands). Annexin V-FITC kit from Alexis (Kordia bv, The Netherlands). Rhodamine-123 was purchased from Molecular Probes (Leiden, The Netherlands). All other chemicals were reagent grade.

DNA laddering

10^7 cells were treated as indicated, lysed on ice for 10 minutes 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 14000 rpm 4°C for 10 minutes. The supernatant was added to an equal volume phenol:chloroform, rocked gently for 10 minutes, centrifuged and the upper phase was added to 1/10th volume sodium acetate (3 M, pH 5.4) and 2.5 volumes ethanol and incubated at -20°C for 15 minutes and subsequently spun down. The pellet was air-dried, resuspended in TE containing 2 μg/ml RNase A, incubated at 37°C for 30 minutes 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)[25]. Protein concentration was measured and equal amounts of protein were analyzed by SDS-PAGE. Blots were incubated overnight at 4°C with the appropriate antibodies (1:1000) and after hybridization with secondary antibodies developed utilizing Enhanced Chemiluminescence (ECL, Amersham).

FACS analysis of apoptosis

Preparation of cells for the analysis of cell cycle profiles has been described previously[10]. For the analysis of apoptosis by Annexin-V staining, cells were washed with ice-cold PBS, resuspended in binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin-V (Bender Medsystems, Vienna, Austria) for 10 min at room temperature, washed and resuspended in binding buffer containing 1 μg of propidium iodide (PI)/ml and fluorescence was analyzed by fluorescence-activated cell sorter (FACS).

Analysis of mitochondrial depolarization

For the analysis of changes in mitochondrial potential, $\Delta\psi_m$ [26], cells were incubated in RPMI together with 10 μg/ml Rhodamine-123 (Rh-123; Molecular Probes, Eugene, OR, U.S.A.) at 37°C for 30 minutes, washed twice with PBS and analyzed by FACS[10]. The percentage of cells falling within the range of Rh-123 fluoresence, indicative of depolarized cells, is shown.

Results

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

Cytokines of the interleukin (IL)-3, IL-5 and GM-CSF (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[27-29]. To examine the mechanisms underlying cytokine withdrawal induced apoptosis we utilized 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, we 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[20,24,30,31]. To specifically analyze the effect of FKHR-L1, we generated cell lines stably overexpressing an inducible form of active FKHR-L1, in which all 3 phosphorylation sites were mutated to alanine, FKHR-L1(A3):ER*. Addition of 4-hydroxy tamoxifen (4-OHT) to these cells results in the rapid induction of FKHR-L1 transcriptional activity, promoting induction of bona fide Forkhead targets[10].

Cells were either cytokine starved or treated with 4-OHT for 24 hours and apoptosis was measured by analyzing binding of Annexin-V-FITC. While cells that are Annexin-V positive represent early apoptotic cells; cells that are stained for both Annexin-V and PI represent cells that have initiated the apoptotic program for a longer period of time. Both cytokine withdrawal, as well as FKHR-L1 activity, induced apoptosis to a similar degree (Fig. 1A).

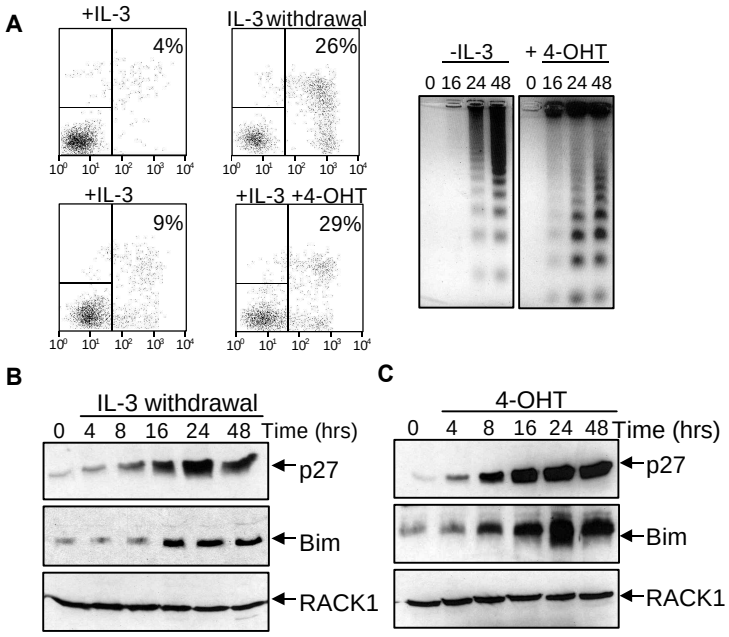

Figure 1. Induction of apoptosis by cytokine withdrawal or FKHR-L1 activity.
(A) Left: Ba/F3 cells were cultured in the presence or absence of cytokines and FKHR-L1(A3):ER* cells were cultured with IL-3 without or with 4-OHT (100 nM) for 24 hours and analyzed for Annexin-V binding and PI-staining as described in Experimental Procedures. Right: 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 Experimental Procedures. (B) Ba/F3 cells were cytokine-starved for the indicated times, lysed and equal amounts of protein were analyzed for levels of $p27^{KIP1}$ (upper panel) or Bim (middle panel). Samples were analyzed with a RACK1 antibody to verify equal protein loading (lower panel). (C) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were treated with 4-OHT for indicated times, lysed and analyzed as above.

Next, we analyzed the kinetics by which apoptosis was induced utilizing DNA laddering, a measure for the final events characterizing apoptosis. Both cytokine withdrawal, as well as FKHR-L1 activity, induced apoptosis within a similar time frame (Fig. 1B). Recently, we have demonstrated that both $p27^{KIP1}$ and Bim (Bcl-2 interacting mediator of cell death) transcriptional targets of FKHR-L1[10,24]. 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. 1B, C). These events occur relatively early and precede the cleavage of DNA observed in Fig. 1B.

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 inhibition of cyclin-CDK complexes[32,33], but has also been described to function in the induction of apoptosis through a yet unidentified mechanism[10,34]. Bim is a potent inducer of apoptosis through binding to and thus inhibiting anti-apoptotic Bcl-2 members[35]. To see whether upregulation of p27^{KIP1} and Bim reflected an altered distribution of cells in the cell cycle, we analyzed the cell cycle profile of cells at various times. Upon cytokine withdrawal, cells stop initiating cell division and accumulate in G_1, within the first 8 hours of starvation (Fig. 2A). After 16 hours of cytokine deprivation cells start to undergo apoptosis, as measured by a DNA content less than 2N chromosomes, the sub-G_1 peak. (Fig. 2A). By 48 hours, a majority of cells has initiated a program of apoptotic cell death. Similar findings were observed in 4-OHT-treated FKHR-L1(A3):ER* cells (Fig. 2B), suggesting that indeed the presence of a G_1 arrest before undergoing apoptosis is related to the initial upregulation of p27^{KIP1} and Bim (Fig . 1B and 1C).

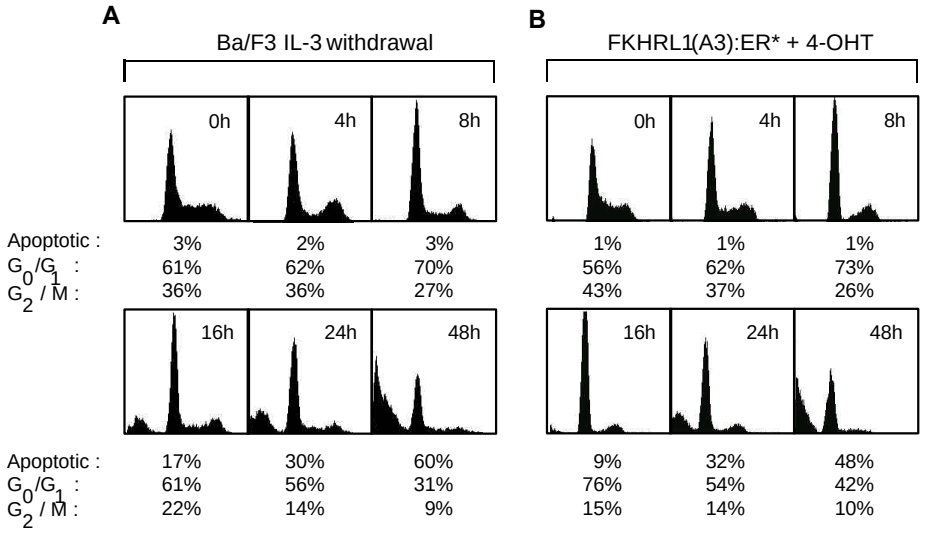

Figure 2. Cell cycle analysis of cytokine-starved cells and cells in which FKHR-L1 activity is induced.

(A) Ba/F3 cells were cytokine-starved for indicated times, fixed, stained with propidium iodide 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.

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

Very little is known of the mechanisms by which cytokine withdrawal promotes caspase activation and cleavage of apoptotic substrates[36,37]. Whether this occurs through a death receptor-dependent or -independent pathway and whether the function of FKHR-L1 is implicated therein remains to be established. We have recently reported that Bim is an important mediator of FKHR-L1-induced apoptosis[10]. Furthermore, Forkhead-mediated upregulation of Fas ligand has been proposed in T cells[20]. This suggests that both death receptor-dependent, as well as death receptor-independent mechanisms may be involved in Forkhead-mediated induction of apoptosis. We sought to investigate this in more detail, examining both cytokine withdrawal and FKHR-L1 activity. Utilizing antibodies specific for cleaved caspase-3 and caspase-7, their activation was analyzed following cytokine withdrawal. Caspase-3 (Fig. 3A, upper panel) and caspase-7 (Fig. 3A, middle panel) were both cleaved after cytokine withdrawal. This occurred approximately 16 hours after removal of cytokine. Analysis of 4-OHT-treated FKHR-L1(A3):ER* cells showed similar kinetics of caspase-3 and caspase-7 cleavage (Fig. 3B, upper and middle panel). A well-characterized caspase substrate that is cleaved when cells undergo apoptosis is PARP (poly (ADP-ribose) polymerase)[38], an enzyme involved in DNA repair (reviewed in [39]). To examine whether PARP is 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. 3C, left), as well as Forkhead activity (Fig. 3C, right) resulted in PARP cleavage with kinetics similar to caspase activation (Fig. 3A,B). These data suggest that similar mechanisms are involved in both cytokine withdrawal and FKHR-L1-induced apoptosis. Furthermore, the activation of caspases proceeds the upregulation of both $p27^{KIP1}$ and Bim (Fig. 1B,C).

Cytokine withdrawal and FKHR-L1 activity promote mitochondrial depolarization.

Mitochondria play a critical role in the initiation of apoptosis, which is accompanied by a loss of mitochondrial transmembrane potential (Ψ_m) and leakage of cytochrome c[40]. This, together with Apaf-1 is required for the onset of the activation of the "caspase-cascade"[6,7] and is indispensable for death receptor-independent induction of apoptosis. To analyze mitochondrial integrity in apoptotic Ba/F3 cells we utilized rhodamine-123 (Rh-123), a dye that binds to mitochondria in a membrane potential-dependent way[26,41]. In cytokine-starved Ba/F3 cells (Fig. 4A), as well as in 4-OHT-treated FKHR-L1(A3):ER* cells (Fig. 4B), loss of Ψm was observed after 16 hours, and increased dramatically over time. The kinetics are similar to those of caspase activation (Fig. 3A and 3B). This suggests that both cytokine withdrawal, as well as FKHR-L1 activation result in loss of mitochondrial transmembrane potential, subsequently resulting in cytochrome c release and activation of caspases.

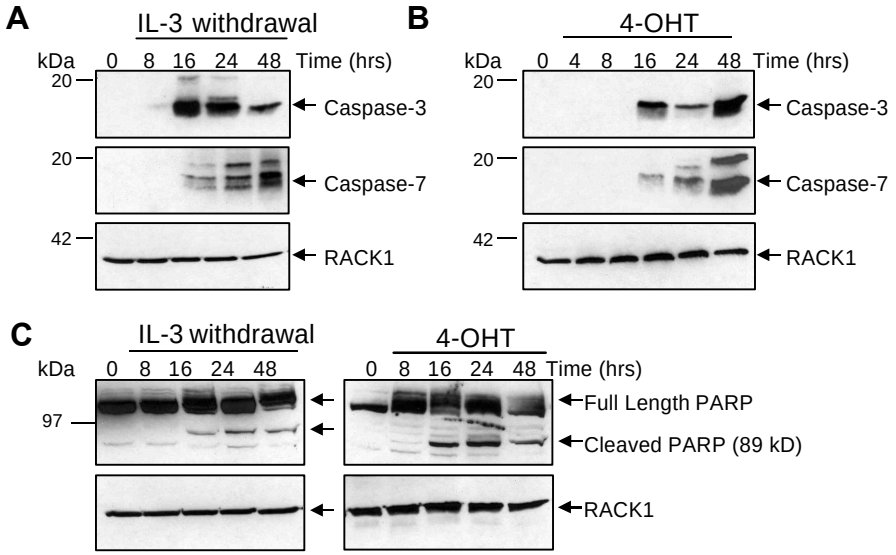

Figure 3. Analysis of caspase activation and PARP cleavage in cytokine-starved cells and 4-OHT-treated FKHR-L1(A3):ER* cells.

(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).

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

PKB is involved in inhibition of apoptosis by cytokines[12,16,17], however, a role for this kinase in the regulation of mitochondrial integrity remains unclear. PKB has been proposed to rescue cells from apoptosis by maintenance of mitochondrial transmembrane potential and preventing cytochrome c release[21]. However, others have observed rescue from apoptosis downstream from cytochrome c release[22]. 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*[10]. Treatment of these cells with 4-OHT resulted in a dramatic phosphorylation of myrPKB:ER* (Fig. 5A, upper panel), allowing us to analyze the effect of PKB activation following cytokine withdrawal. Indeed, activation of PKB in the absence of cytokines was sufficient to rescue cytokine-starved myrPKB:ER* cells from apoptosis (Fig. 5B), as measured by Annexin-V-FITC staining. 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. Ba/F3 cells 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. 5C, upper panel), excluding aspecific effects of 4-OHT. In myrPKB:ER* cells however, addition of 4-OHT substantially decreased the loss of Ψm upon cytokine withdrawal (Fig. 5C, lower panel). This suggests that PKB exerts its anti-apoptotic activity by maintaining mitochondrial integrity. Caspase activity was subsequently analyzed. Activity of both caspase-3 and caspase-7 was substantially reduced in 4-OHT-treated compared to untreated myrPKB:ER* cells. Our findings of a partial rescue from apoptosis (Fig. 5B) and caspase activity (Fig. 5D) 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 maintenance of mitochondrial potential, resulting in a reduction of caspase activity.

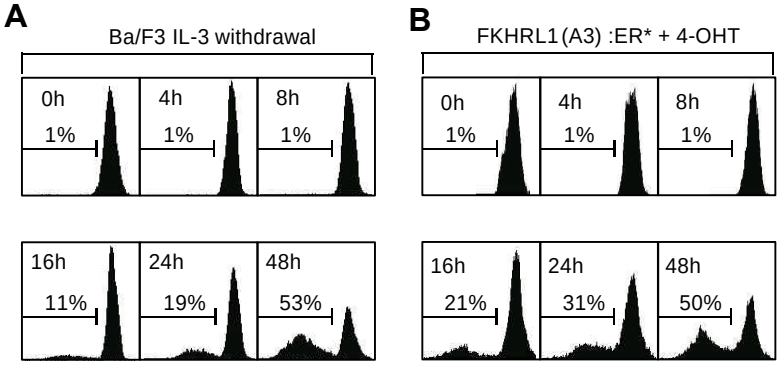

Figure 4. Induction of mitochondrial transmembrane depolarization by cytokine starvation or FKHR-L1 activity.

(A) Ba/F3 cells were IL-3 starved for the times indicated and mitochondrial transmembrane depolarization was measured using rhodamine-123 staining as described in Experimental Procedures. (B) 4-OHT-treated FKHR-L1(A3):ER* cells were analyzed as in (A). Data are representative of several independent experiments.

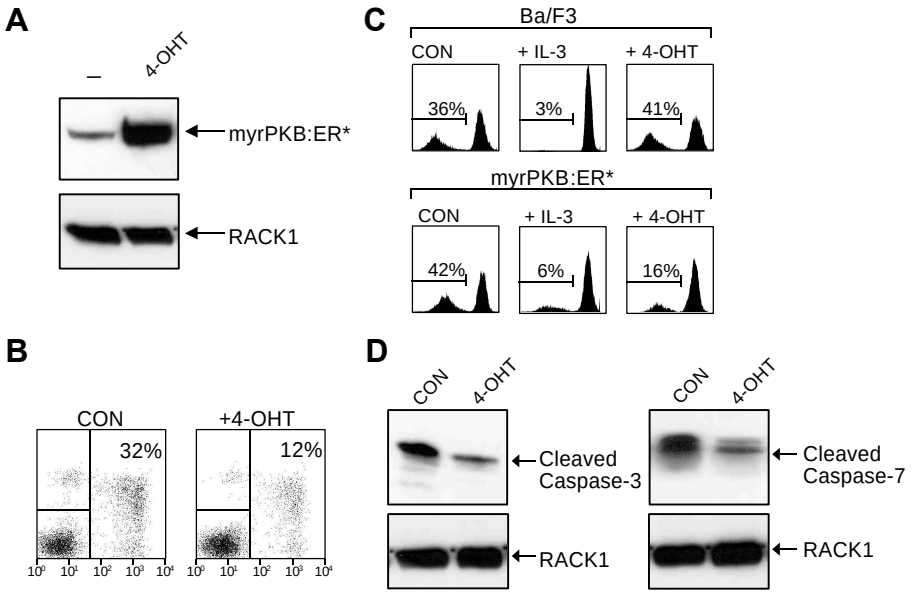

Figure 5. Activation of PKB rescues cells from apoptosis and maintains mitochondrial transmembrane potential.

(A) Ba/F3 cells stably expressing myrPKB:ER* were left untreated or treated with 4-OHT (100 nM) for 36 hours and 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 hours and the percentage of Annexin-V-FITC positive cells was determined as describe in the Experimental Procedures. (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 hours and mitochondrial integrity was examined using rhodamine-123 as described in Experimental Procedures. (D) Caspase-3 (left) and caspase-7 (right) activation was measured in myrPKB:ER* cells that were cytokine-starved for 36 hours in the absence or presence of 4-OHT (100 nM) using activation-specific antibodies and reprobed with RACK1 antibody to verify equal protein loading.

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

FKHR-L1 has been proposed to induce Fas ligand (FasL) in T cells[20], which could contribute to FKHR-L1-mediated induction of apoptosis. Fas/FasL signaling induces cleavage and activation of caspase-8 cleavage which is an indispensable and specific downstream event of death receptor induced apoptosis[5,42]. Analysis of caspase-8 cleavage is thus a means to discriminate between death receptor-dependent or death receptor-inde-

pendent induction of apoptosis. As Ba/F3 cells express Fas, albeit at very low levels[43,44], Forkhead-mediated induction of Fas ligand 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. While Ba/F3 cells expressed caspase-8, neither cytokine withdrawal (Fig. 6A) nor Forkhead activity (Fig. 6B) 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. 6C). This argues against a role for Fas/Fas ligand signaling in FKHR-L1-mediated apoptosis.

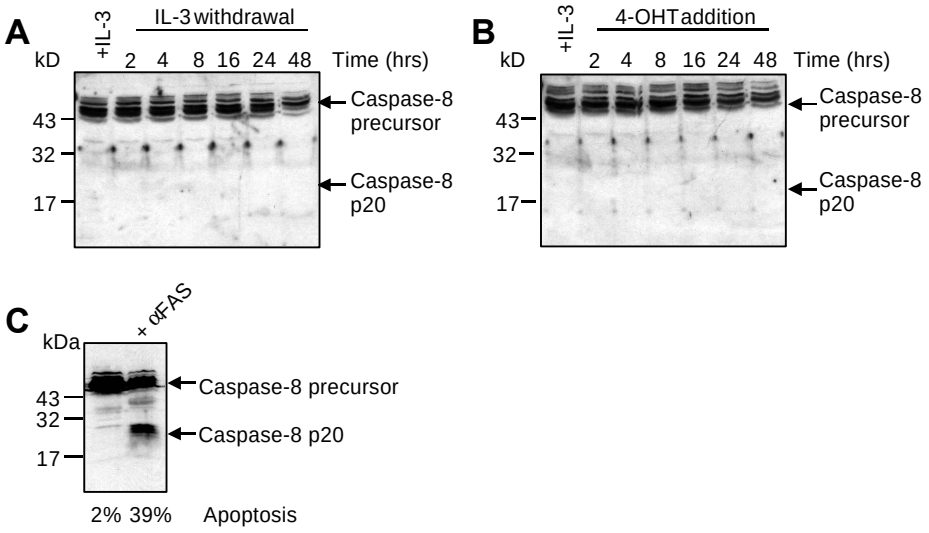

Figure 6. Caspase-8 is not activated in response to cytokine starvation or FKHR-L1 activation. (A) Caspase-8 cleavage was analyzed in Ba/F3 cells that were cytokine-starved for the times indicated. (B) Analysis of capase-8 cleavage in 4-OHT (100 nM) treated FKHR-L1(A3):ER* cells. (C) Jurkat cells were treated with or without αFAS18 (1 μg/ml). After 24 hours samples were harvested and protein concentration measured. Equal amounts of protein were analyzed by SDS-PAGE and anti-caspase-8 western blotting.

Discussion

In this report we analyzed the mechanisms of cytokine withdrawal and FKHR-L1-induced apoptosis, as well as PKB-mediated rescue from apoptosis. Utilizing cells expressing FKHR-L1(A3):ER* allowed us to uniquely analyze the effects of FKHR-L1 activation. Interestingly, no differences between cytokine withdrawal and FKHR-L1-mediated apoptosis were observed. Importantly, our results suggest 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 then underwent apoptosis (Fig. 2). This was accompanied by a sequential upregulation of $p27^{KIP1}$, which is involved in arresting cells in G_1 and the induction of apoptosis[10,32,33], and Bim, which can induce apoptosis by binding to anti-apoptotic members of the Bcl-2 family[35] (Fig. 1B and 1C). Bim appears to be essential for the induction of apoptosis in lymphocytes, since cytokine-deprived lymphocytes from *Bim (-/-)* mice fail to undergo apoptosis[45]. Furthermore, lymphocytes derived from *$p27^{KIP1}$ (-/-)* mice undergo apoptosis at a significantly decreased rate compared to those from wildtype mice[10]. 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.

We also analyzed whether Fas/Fas ligand signaling may be involved in induction of apoptosis upon cytokine withdrawal as previously proposed[20]. Neither cytokine withdrawal nor FKHR-L1 activity resulted in cleavage of caspase-8, an event specific for death receptor signaling[5,42]. This suggests that apoptosis either by cytokine withdrawal 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[46,47] are able to rescue both cytokine withdrawal, as well as FKHR-L1-induced apoptosis[14,24].

PKB has been demonstrated to negatively regulate members of a subfamily of Forkhead transcription factors: AFX, FKHR and FKHR-L1 (reviewed in [48]). Recently, members of the SGK (serum- and glucocorticoid-induced kinases) family, phosphorylating consensus sequences similar to PKB were found to be required for full phosphorylation of FKHR-L1 *in vivo* and IL-3-mediated survival[49,50]. This suggests that both kinases may be required for phosphorylation-mediated inactivation of FKHR-L1. This may explain why PKB was unable to completely inhibit cytokine withdrawal-induced apoptosis (Fig. 5). However, PKB was capable of significantly abrogating cytokine withdrawal induced loss of mitochondrial potential (Fig. 5C). Thus we can conclude that PKB exerts its anti-apoptotic effect at a pre-mitochondrial 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[21], as well as in epithelial cells by detachment from extracellular matrix[51]. However, PKB has also been previously shown to inhibit ceramide-induced apoptosis in

hybrid neuron motor 1 cells downstream of cytochrome c release[22]. These findings may be explained by differences in apoptotic stimuli in different cell types suggesting that PKB has the potential to act at multiple levels. Furthermore, difference in species could be an explanation for the differential contribution of PKB to rescue from apoptosis. PKB promotes rescue from apoptosis by inhibitory phosphorylation of caspase-9 in human cells[52], but not in mouse or rat cells since the PKB phosphorylation site in caspase-9 is not present[53]. PKB has also been linked to the upregulation of anti-apoptotic Bcl-2 member Mcl-1[18], which is essential in cytokine-mediated rescue from apoptosis[14]. 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[54-56], potentially through activation of downstream targets that phosphorylate Bad[55]. Furthermore, MEK initiated signals can result in the phosphorylation of anti-apoptotic members of the Bcl-2 family[56], thereby enhancing their stability[57]. However, utilizing the myrPKB:ER* cell line we have demonstrated that PKB alone is sufficient to protect cells from programmed cell death (Fig. 5B,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[58-60]. 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 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[61-63].

Taken together, our data suggests that cytokine-induced signaling can inhibit cells from apoptosis through activation of PKB (or 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. These events are summarized in a model (Fig. 7).

Our findings provide a greater insight into the mechanisms regulating induction of apoptosis in lymphocytes, and probably other hematopoietic cells, upon cytokine withdrawal. PKB alone is sufficient to inhibit apoptosis through the maintenance of mitochondrial transmembrane potential. This is likely to be due to the inhibition of FKHR-L1, thus preventing transcription of the pro-apoptotic Bcl-2 family member Bim. A greater understanding of the mechanisms by which cytokines regulate cellular survival will help towards the design of novel pharmacological agents for therapeutic intervention in a variety of proliferative and degenerative disorders of the immune system.

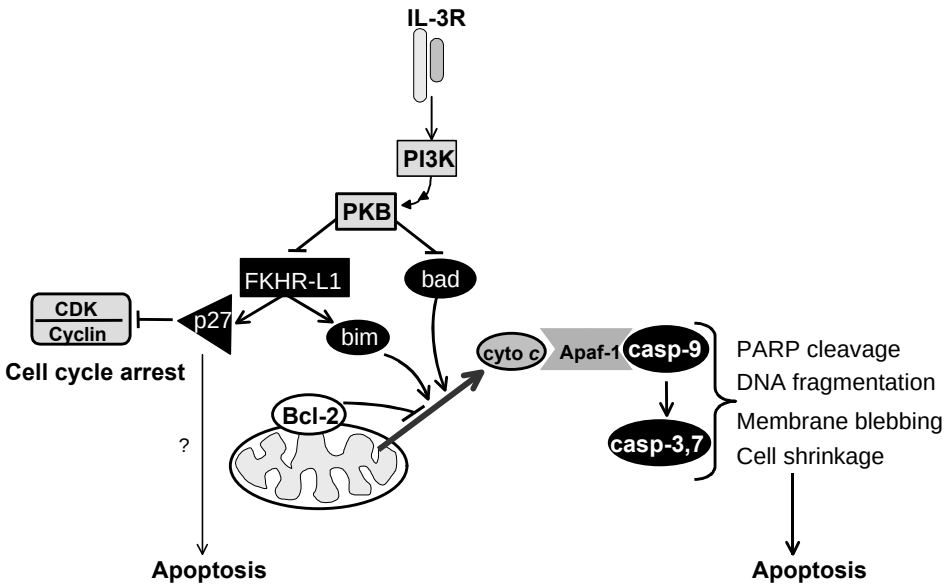

Figure 7. A model for cytokine withdrawal-induced apoptosis.

In the absence of cytokines, PKB and SGK are inactive, resulting in dephosphorylation and subsequent activation of 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 loss of mitochondrial integrity and leakage of cytochrome c. This triggers activation of caspases and subsequent cleavage of downstream targets, resulting in apoptosis.

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CHAPTER 6

Identification of novel targets of the Forkhead transcription factor FKHR-L1 by gene array analysis

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In preparation

Abstract

The Forkhead/ winged helix family of transcription factors shares a structurally related domain, the Forkhead domain, but are highly divergent in their tissue distribution and their contribution to cellular functions. One subclass of this family, consisting of AFX, FKHR and FKHR-L1, have recently been described as targets of Protein Kinase B. Their transcriptional activity, resulting in cell cycle arrest, as well as induction of the apoptotic program, is inhibited by PKB. To obtain further insight into the mechanism of action of this subclass of transcription factors, we performed a gene array screen using cells stably expressing an 4-hydroxy tamoxifen (4-OHT) inducible construct of FKHR-L1. A mouse gene array was probed with cDNA generated from cells treated with or without 4-OHT for three hours. Two members of the AP-1 family of transcription factors, c-Jun and Fra-2, were found to be rapidly and specifically upregulated upon activation of FKHR-L1. Both proteins were also upregulated upon cytokine withdrawal. These findings may help to shed light on the mechanisms by which the AFX, FKHR and FKHR-L1 subclass of transcription factors can regulate proliferation and survival in a variety of cell types.

Introduction

The family of Forkhead/ winged helix transcription factors consists of over 100 members, that vary in their tissue distribution, regulation, and also in their DNA binding sequence specificity and thus also in their transcriptional targets. They all share a conserved 100 amino acid DNA-binding domain, called the Forkhead domain[1]. These transcription factors are involved in a myriad of cellular functions, including embryogenesis, cellular differentiation, mediating cell death, immune homeostasis, maintenance of ovarian follicles[2-6]. Recently, a subclass of Forkhead transcription factors was identified, comprising AFX, FKHR and FKHR-L1, of which chromosomal rearrangement has been linked to oncogenesis[7-10]. These transcription factors are highly homologous in their DNA binding domain which binds to a similar core sequence (TTGTTTAC), but their binding specificity of the surrounding sequences is distinct for each protein[11]. The tissue distribution patterns of AFX, FKHR and FKHR-L1 mRNAs overlaps in the embryo, being high in muscle, adipose tissue and liver. These patterns of expression are similar in the adult, although FKHR-L1 is then ubiquitously expressed[11,12]. These proteins have been identified as targets of PI3K-PKB signaling[6,13-15]. Phosphorylation by PKB results in nuclear exclusion and thus inhibits their activity[6,13-15]. As AFX, FKHR and FKHR-L1 have been linked to both cell cycle arrest and induction of apoptosis[6,14,16,17], chromosomal rearrangement might promote oncogenesis by interfering with their physiological activity. Transcriptional activity of AFX, FKHR and FKHR-L1 can upregulate the cell cycle inhibitor $p27^{KIP1}$(16-18). In addition, transcriptional activity of FKHR-L1 has been shown to upregulate pro-apoptotic protein Bim (Bcl-2 interacting mediator of cell death), which binds to and inactivates anti-apoptotic Bcl-2 members[19]. Furthermore, FKHR-L1

has also been proposed to regulate the expression of Fas ligand[6], which can initiate apoptosis through binding to Fas (CD95, Apo-1) reviewed by Nagata[20]. Recently, signaling by cytokines was also demonstrated to promote survival by inhibiting the activity of Forkhead transcription factor FKHR-L1 through PKB[19,21]. Upon cytokine-withdrawal FKHR-L1 is dephosphorylated[19], which is accompanied by a cell cycle arrest and induction of the apoptotic program, suggesting that these events might be mediated by FKHR-L1. In addition, the activity of this subclass of Forkhead transcription factors might also be involved in the differentiation of cells, as FKHR has recently been described in positive selection of T cells[5].

To identify novel transcriptional targets for AFX, FKHR and FKHR-L1 we made use of cells stably overexpressing an inducible FKHR-L1 mutant, FKHR-L1(A3):ER*, in which all three phosphorylation sites have been mutated. This construct is expressed constitutively in the cell, but is only active when 4-hydroxy tamoxifen (4-OHT) is added. Addition of 4-OHT results in rapid induction of FKHR-L1(A3):ER* activity, and has been proven to be an excellent tool to specifically analyze FKHR-L1 activity[17,19]. To define novel FKHR-L1 target genes we took a gene array approach. ATLAS array filters were screened with probes generated from RNA isolated from FKHR-L1(A3):ER* cells that were treated with or without 4-OHT. Using this strategy, we have identified two potential FKHR-L1 targets, the immediate-early genes c-Jun and Fra-2 (fos-related antigen 2). FKHR-L1 activity, as well as cytokine-withdrawal resulted in an elevation of c-Jun and Fra-2 protein levels. These data have consequences for the regulation of survival, proliferation and differentiation by Forkhead-related transcription factors in a variety of cell systems.

Experimental procedures

Cell culture

Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3 produced in COS cells[22]. Ba/F3 cells stably expressing FKHR-L1(A3):ER* have been described previously[17] and were cultured in the presence of 500 μg/ml G418. For cytokine-withdrawal experiments, cells were washed twice with PBS and resuspendend in RPMI 1640 supplemented with 8% Hyclone.

ATLAS array screen

To perform the ATLAS array screen the Mouse Broad-Coverage ATLAS array #7741-1 was purchased (Clontech, Palo Alto, CA, U.S.A.), which was provided with reagents for all RNA treatments described below. For the isolation of RNA, FKHR-L1(A3):ER* cells (10^8) were cultured in the presence of IL-3 with or without 4-OHT (100 nM) for three hours and RNA was isolated as described previously[23]. Next, 500 μg RNA of each sample was subjected to a DNAse treatment to avoid contamination with genomic DNA. RNA was subsequently analyzed on gel to verify that the RNA was not degraded.

Efficiency of the DNAse treatment was verified by testing whether a PCR (35 cycles) on 2 μg RNA with β-actin primers did not give any visible product. Next, polyA+ RNA was isolated with magnetic beads coated with poly dT using 250 μg of total RNA and a probe of cDNA was generated using specific primers for the genes on the array to specifically generate cDNA of those genes. The reverse transcription of the RNA to generate cDNA was carried out at 54ºC by incubating in the presence of radioactive γ32P-dATP. The duplo array filters were incubated overnight with the probe generated from either untreated or 4-OHT treated cells at 72ºC, washed the next day filter and exposed to X-ray film.

Antibodies and reagents

Constructs for mouse c-Jun[24] and the c-Jun promoter[25] have been described previously. Fra-2 was obtained by PCR amplification from 4-OHT-treated FKHR-L1(A3):ER* cells, cloned into pSG5-myc and verified by sequencing. Polyclonal antibodies against c-Jun (H-79), Jun B (N17), Jun D (329)-G, Fra-2 (Q20), Fra-1 (Q20) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.); p27 and RACK1 mAb were purchased from Transduction Laboratories (Lexington, Kentucky, U.S.A.).

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)[26], protein concentration was measured and equal amounts of protein were loaded. Blots were incubated overnight at 4°C with appropriate antibodies (1:1000) and after hybridization with secondary antibodies developed utilizing Enhanced Chemiluminescence (ECL, Amersham).

Northern blotting

FKHR-L1(A3):ER* cells were cultured in the presence of IL-3 with or without 4-OHT for appropriate times prior to RNA isolation. Total RNA was isolated as described previously[23] and twenty micrograms were used for Northern blotting and hybridized with a Fra-2 probe consisting of full-length Fra-2 cDNA. Equal RNA loading was verified by reprobing the blots with a 1.4 kb cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Luciferase assays

Ba/F3 cells were electroporated (0.28 kV; capacitance 960 μF) with c-Jun-CAT promoter construct together with an internal transfection control (pRL-TK; Promega). Transfections were divided over two wells and 4-OHT was added to one well 2 hours after transfection. 24 hours after transfection, cells were harvested for both CAT assays as described previously[27] to assess c-Jun promoter activity and luciferase activity to correct for transfection efficiency.

Results and discussion

The Forkhead transcription factor FKHR-L1 has previously been shown to be a potent inducer of apoptosis in both T cells, as well in an IL-3-dependent pro B cell line, Ba/F3[6,17]. In T cells, FKHR-L1 has been proposed to promote apoptosis through upregulation of Fas ligand[6]; in Ba/F3 cells, both $p27^{KIP1}$, as well as Bim (Bcl-2 interacting mediator of cell death) have been demonstrated to be upregulated and involved in FKHR-L1-mediated cell cycle arrest, as well as induction of apoptosis[17,19]. As Forkhead transcription factors are also involved in triggering differentiation[5,28,29], the subclass of Forkhead transcription factors AFX, FKHR and FKHR-L1 might also play a role in the differentiation of hematopoietic cells. Indeed, FKHR has recently been described to be involved in the negative selection of thymocytes[5]. To identify novel FKHR-L1 transcriptional targets we used Ba/F3 cells stably expressing FKHR-L1(A3):ER*. This cell line allows us to specifically analyze effects of FKHR-L1 transcriptional activity which is induced upon treatment with 4-OHT. ATLAS array filters (Mouse Broad Coverage #7741) were hybridized with probes generated from either untreated or 4-OHT-treated FKHR-L1(A3):ER* cells (Fig. 1A). $p27^{KIP1}$ was upregulated in 4-OHT-treated cells compared to untreated cells (data not shown), demonstrating that this approach is suitable to identify FKHR-L1 target genes. Of the genes whose expression was regulated on the filter hybridized with the probe from FKHR-L1(A3):ER* cells we focused on two potential FKHR-L1 targets. These proteins are c-Jun and Fra-2 (fos-related antigen 2), which are both members of the transcription factor AP-1 family (activator protein 1, reviewed in [30,31]) (Fig. 1A). AP-1 refers to the assembly of Jun:Jun or Jun:Fos/Fra proteins that bind together to specific regulatory sequences in the promoter of target genes. Jun members include c-Jun, JunB and JunD, whereas Fos members include c-Fos, FosB, Fra-1 and Fra-2. While JunB, JunD and Fos were also present on the ATLAS gene array filter, they were not upregulated following FKHR-L1 activity (data not shown). This suggests that FKHR-L1 activity promotes upregulation of specific AP-1 members.

Although c-Jun[32,33], as well as Fra-2[33] have both been proposed to be involved in proliferation, c-Jun[32,34,35], as well as Fra-2[34] have also been described to be involved in induction of apoptosis as well as differentiation[36-39], thus prompting us to further investigate these proteins. To examine whether the upregulation of c-Jun and Fra-2 was also reflected in an increase in protein levels, FKHR-L1(A3):ER* cells were cultured in the absence or presence of 4-OHT. A dramatic induction of c-Jun, as well as Fra-2 was observed after overnight treatment with 4-OHT (Fig. 1B). The extent of this induction was similar to that of $p27^{KIP1}$, a recently described FKHR-L1 target[16,17].

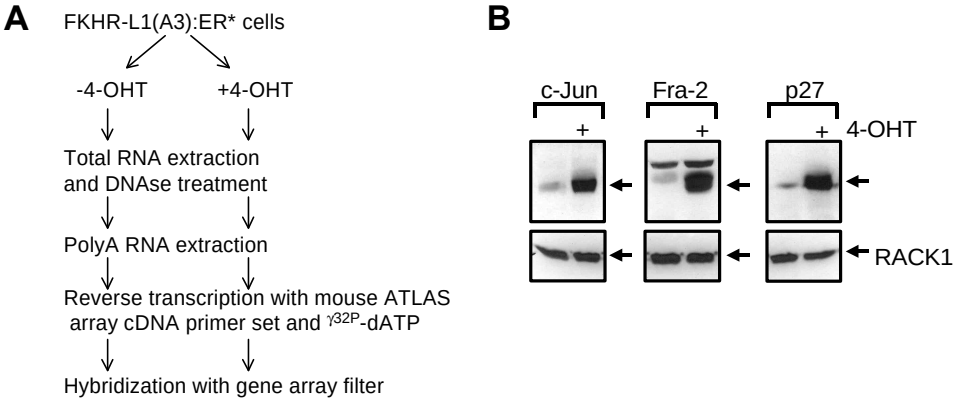

Figure 1 c-Jun, Fra-2 and p27^{KIP1} are upregulated in 4-OHT-treated FKHR-L1(A3):ER* cells.

(A) Schematic representation of the Forkhead array screen (B) FKHR-L1(A3):ER* cells were cultured with IL-3 with or without 4-OHT (100 nM) overnight, lysed and analyzed for levels of c-Jun, Fra-2 or p27^{KIP1}. Blots were reprobed with a RACK1 antibody to verify equal protein loading.

Next, we analyzed the kinetics of FKHR-L1-mediated upregulation of c-Jun and Fra-2 by analyzing FKHR-L1(A3):ER* cells that were treated with 4-OHT for the times indicated (Fig. 2). c-Jun induction was clearly visible as early as 4 hours after induction of FKHR-L1 activity, and increased over time with kinetics similar to those of FKHR-L1 target p27^{KIP1}. Fra-2 protein levels were increased as early as 2 hours after induction of FKHR-L1 activity, but were not further elevated over time (Fig. 2 and data not shown). No elevation in protein levels was observed in JunB, JunD or Fra-1 (data not shown), suggesting that induction of FKHR-L1 activity promotes upregulation of specific AP-1 members.

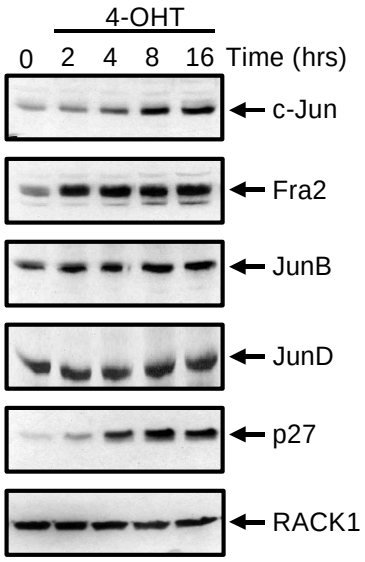

Figure 2 Time course analysis of levels of c-Jun, Fra-2, JunB and p27^{KIP1}.
FKHR-L1(A3):ER* cells were either left untreated or treated with 4-OHT (100 nM) for indicated times, lysed and analyzed for protein levels of c-Jun, Fra-2, JunB, JunD and p27^{KIP1}. Equal protein loading was verified by reprobing the blot with a RACK1 antibody. Data are representative of at least two different clonal FKHR-L1(A3):ER* cell lines.

IL-3 has previously been demonstrated to result in phosphorylation and subsequent inactivation of FKHR-L1[17]. Conversely, withdrawal of IL-3 results in the dephosporylation of FKHR-L1, which is accompanied by upregulation of FKHR-L1 targets p27^{KIP1} and Bim[17,19]. To examine whether IL-3 withdrawal also resulted in an induction of c-Jun and Fra-2, Ba/F3 cells were IL-3-starved for the times indicated and assessed for levels of c-Jun and Fra-2. c-Jun was quite rapidly upregulated following IL-3 withdrawal (Fig. 3A), with levels being highly elevated as early as one hour, which was much faster than the upregulation observed in FKHR-L1(A3):ER* cells (compare with Fig. 1B upper panel). However, levels of c-Jun decreased as early as 4 hours after cytokine withdrawal, with levels returning to basal levels after 16 hours. Similar observations were made for Fra-2 (Fig. 3B). Also in the case of Fra-2, cytokine withdrawal resulted in a much more rapid elevation of Fra-2 than observed in FKHR-L1(A3):ER* cells (Fig. 1B).

The differences in kinetics of upregulation between cytokine withdrawal and 4-OHT-treated FKHR-L1(A3):ER* cells could be explained in that experiments in 4-OHT-treated cells were carried out in the presence of IL-3. IL-3 might act to induce the activity of

a transcriptional repressor of c-Jun and Fra-2, which is rapidly degraded in the absence of IL-3. The kinetics of elevation of c-Jun and Fra-2 protein levels in FKHR-L1(A3):ER* cells might subsequently be delayed as the activity of this repressor needs to be overcome first.

Although activity of FKHR-L1 is probably sustained over time following cytokine withdrawal[17], this is not reflected by an increase in upregulation of c-Jun and Fra-2. An explanation for the sustained c-Jun and Fra-2 levels in 4-OHT-treated cells might be that IL-3 in some way increases their stability. Indeed, MAPK-mediated phosphorylation results in a reduction of ubiquitination and potential degradation of c-Jun[40]. MAPK-mediated stabilization has also been suggested for Fra-2[41].

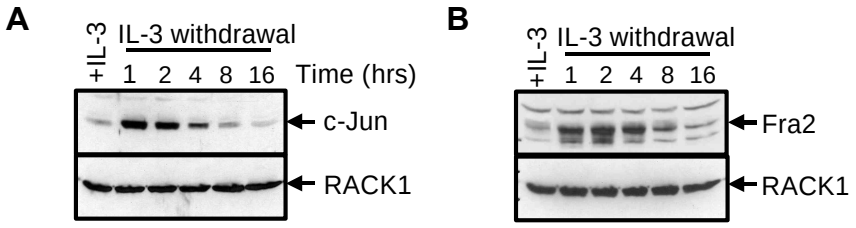

Figure 3 IL-3 withdrawal promotes a transient upregulation of c-Jun, as well as Fra-2.

(A) Ba/F3 cells were IL-3-starved for the indicated times, lysed and assessed for levels of c-Jun (top) or RACK1 (bottom) to verify equal protein loading. (B) As in (A) but probing with Fra-2 instead of c-Jun.

Thus far we only examined the effect of FKHR-L1 transcriptional activity on the elevation of protein levels of c-Jun and Fra-2. To analyze whether indeed upregulation of these protein levels is also reflected by an increase of mRNA levels or promoter activity, FKHR-L1(A3):ER* cells were cultured with or without 4-OHT for the indicated times. Fra-2 mRNA (Fig. 4A) was dramatically elevated upon 4-OHT-treatment, suggesting that indeed transcriptional activity of FKHR-L1 is involved in the elevation Fra-2 protein levels observed. Unfortunately, as pretreatment of cells with protein synthesis inhibitor cycloheximide results in a superinduction of c-Jun[42], as well as Fra-2 (data not shown), we were not able to use this approach to demonstrate that FKHR-L1 directly upregulates c-Jun and Fra-2. However, Forkhead consensus DNA binding sites were found in both the c-Jun[43] and Fra-2[44] promoter, suggesting that FKHR-L1 may indeed directly induce c-Jun or Fra-2 transcription.

Next, we examined whether FKHR-L1 activity could also promote transcriptional activation of the c-Jun promoter. Two clonal Ba/F3 cell lines stably expressing FKHR-L1(A3):ER* cells were analyzed, to exclude artefacts due to clonal selection. In both of the clones examined, 4-OHT treatment of FKHR-L1(A3):ER* cells strikingly elevated c-Jun promoter activity (Fig. 4B, left). This demonstrates FKHR-L1 regulation of both promoter activity, as well as induction of mRNA levels. Furthermore, analysis of c-Jun promoter activity in Ba/F3 cells cultured in the absence or presence of IL-3 also revealed that IL-3 withdrawal resulted in an elevation of c-Jun promoter activity (Fig. 4B, right). Taken together, this suggests that FKHR-L1 activity can result in elevation of c-Jun and Fra-2 protein levels by enhancing their transcription.

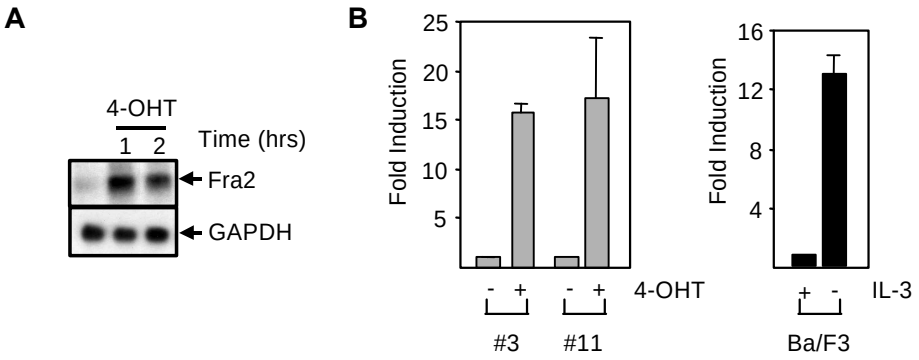

Figure 4 FKHR-L1 induces an increase of Fra-2 mRNA levels, as well as an induction of c-Jun promoter activity.

(A) IL-3-cultured FKHR-L1(A3):ER* cells were either left untreated or treated with 4-OHT (100 nM) for indicated times, 20 μg of total RNA was used for Northern blotting and hybridized with a Fra-2 probe (top). Equal RNA loading was verified by GAPDH reprobing (bottom). (B) Two different clonal Ba/F3 cell lines stably expressing FKHR-L1(A3):ER* (left) or Ba/F3 cells (right) were electroporated with 20 μg of c-Jun-CAT plasmid together with 500 ng of pRL-TK plasmid as an internal transfection control. FKHR-L1(A3):ER* cells were cultured for 24 hours with IL-3 in the absence or presence of 4-OHT (100 nM) and Ba/F3 cells in the absence of presence of IL-3 before analyzing CAT and luciferase activity as described in Materials and Methods. The data are representative of several independent experiments.

If a Forkhead transcriptional target were to be important for regulation of apoptosis or cell cycle arrest, then overexpression of this protein should be sufficient to promote apoptosis or cell cycle arrest. However, overexpression of neither c-Jun nor Fra-2 resulted in Ba/F3 cells resulted in an alteration of cell cycle distribution or decreased survival (data not shown). However, in neuronal cells, c-Jun and Fra-2 have both been demonstrated to promote induction of apoptosis. NGF withdrawal-induced apoptosis is accompanied by elevation of c-Jun mRNA and microinjection of antibodies against c-Jun, as well as Fra-2 protected NGF-deprived neurons from apoptosis[34]. Furthermore, overexpression of dominant negative c-Jun was found to protect sympathetic neurons from apoptosis following NGF-withdrawal[45] and increased c-Jun activity was sufficient to trigger apoptotic cell death in NIH 3T3 fibroblasts[35]. This suggests that activity and function of AP-1 members may differ depending on the cell type and conditions. Thus, in neuronal cells, Forkhead-related transcription factors may play a an important role in the induction of apoptosis through transcriptional induction of AP-1 family members. Importantly, the PI3K-PKB pathway has been shown to be critical in NGF-mediated survival[46].

Fra-2 and c-Jun have also been proposed to function in the differentiation of cells. Ectopic expression of c-Jun is sufficient to induce differentiation of P19 embryonal carcinoma cells[43], as well as murine myelomonocytic WEHI-3B D+ cells[47]. In addition, expression of antisense c-Jun blocked differentiation in a murine erythroleukemia cell line[48]. Fra-2 and JunD heterodimers have also been proposed to be involved in osteoblast differentiation[49] as well as with the transition of proliferating granulosa cells to terminally differentiated, non-dividing luteal cells[39]. These observations suggest that transcriptional upregulation of c-Jun and Fra-2 by FKHR-L1 or related Forkhead family members might be linked to differentiation of cells. It will be interesting to examine this in one of the cell types in which AP-1-mediated differentiation has been proposed.

It should be noted that Fra-2 has a high affinity for JunD and increases the activity of JunD, upon heterodimerization[50]. Thus, upregulation of Fra-2 may promote heterodimerization of Fra-2 to other AP-1 members. c-Jun itself is also involved in the activation of transcription factors other than members of the AP-1 family, such as PU.1[51].

These data demonstrate that FKHR-L1(A3):ER* cells are a powerful tool to obtain further insight into potential targets of Forkhead transcription factors AFX, FKHR and FKHR-L1 and their mechanism of action. While c-Jun and Fra-2 do not appear to have an apparent role in the induction of the apoptotic program in Ba/F3 cells, there may be a role in neuronal cells. A further challenge will be to identify what the consequences of Forkhead-mediated upregulation of c-Jun and Fra-2 is on the transcriptional activity of AP-1 family members and investigate how this is related to their transcriptional regulation of genes in a variety of physiological processes.

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CHAPTER 7

General discussion

General discussion

Maintaining a correct number of cells in the hematopoietic system, as well as having sufficient differentiated cells to carry out specialized tasks and to be able to respond in changes in the extracellular environment requires a highly organized network to coordinate these events. Terminally differentiated cells generally do not divide, but arise from precursors (stem cells), which can either proliferate, giving rise to more precursors, or differentiate, which in general is accompanied with a loss of their proliferative capacity; reviewed in [1,2]. Somewhere in the life of a stem cell the decision is made to proliferate or to undergo differentiation. This decision can be governed by a specific combination of extracellular factors, cytokines, which activate a network of intracellular signaling pathways. Mutations in components of this signaling network can disturb the balance between proliferation and differentiation, as well as abrogate the dependence of the cell for survival factors. This can result in leukemia, characterized by a block in differentiation of hematopoietic precursors, accompanied with inappropriate proliferation and expansion. Thus, an important feature of leukemic cells is that they exhibit a block in differentiation and have gained the capacity for unlimited proliferation, without being restrained by the normal mechanisms that promote apoptosis upon inappropriate division. Generally, in addition to exhibiting unrestrained proliferation, tumor cells also need to have adapted to overcome apoptosis. The differentiation block in leukemias is often a result of chromosomal translocations of genes encoding transcription factors (reviewed in [3,4]). The resulting protein products of these translocations can interfere with the normal growth factor activated signaling network and transcription factors that act in concert to promote differentiation.

Terminal differentiation is in general accompanied with an arrest in the cell cycle and evidence is accumulating that this arrest is mediated by CKIs acting together to induce a G_1 arrest and exit from the cell cycle (known as G_0, reviewed in [5]). In tumor cells, expression of CKIs is often lost or substantially decreased (reviewed in [6,7]). Elevation of the CKI $p27^{KIP1}$ is an adverse prognostic indicator in cancer in that low levels are correlated with a poor prognosis[8-10]. Although it is not clear whether this simply reflects the fact that relatively undifferentiated cells have a higher tumorigenic capacity and lower levels of CKIs, it is worth noticing that induction of differentiation in some leukemias in combination with chemotherapy increases the chance of ablating the leukemia[11]. In addition, lower levels of $p27^{KIP1}$ might also reflect a defect in the pathways regulating $p27^{KIP1}$ expression, which might also include other proteins, whose dysregulation is more directly related to oncogenesis.

To obtain more insight into the mechanisms by which cytokine-activated signaling pathways could promote proliferation and survival and potentially obtain insight into strategies of clinical interference in leukemias or inflammatory diseases, we studied IL-3, IL-5 and GM-CSF-induced signaling. Receptors for IL-3 and GM-CSF are present on a wide variety of hematopoietic cells, whereas expression of the IL-5 receptor is restricted to eosinophils and basophils in man, and in mice also on B cells[12,13]. As eosinophils are

believed to be involved in the pathogenesis of allergic diseases[14,15], potential eradication by interference with signaling pathways involved in IL-5-mediated survival might alleviate the disease symptoms. A useful therapeutic agent might result from the recent development of peptides that bind to and antagonize the IL-5 receptor[16]. However, the requirement for this strategy is that cells are still responsive to IL-5, and have not developed mechanisms that result in cytokine-independent growth or survival.

Upon analyzing which signaling pathways are essential for IL-3, IL-5 and GM-CSF-mediated proliferation utilizing specific pharmacological inhibitors, only inhibition of PI3K abrogated both cytokine-mediated survival and proliferation (Chapter 3, Fig. 1). We have demonstrated that PI3K target PKB can account for the anti-apoptotic effect of PI3K (Chapter 3, Chapter 5, Fig. 5). Previously identified targets of PKB, caspase-9 and Bad, whose activity is inhibited following PKB phosphorylation, have been described in PKB-mediated survival[17,18]. However, phosphorylation of these targets is not required for survival in all cellular systems[19,20]. Therefore we set out to investigate other targets of PKB. This resulted in the observation that PKB-mediated phosphorylation of Forkhead transcription factor FKHR-L1 appears to be required for IL-3, IL-5 and GM-CSF-mediated rescue from apoptosis (Chapter 3 Fig. 4). Phosphorylation of FKHR-L1 by PKB promotes association of FKHR-L1 with 14-3-3 proteins, resulting in cytoplasmic retention, thus inhibiting its transcriptional activity[21]. Upon investigation as to how FKHR-L1 activity promotes cell cycle arrest and induction of apoptosis in cells, two FKHR-L1 targets were identified, $p27^{KIP1}$ and pro-apoptotic Bcl-2 family member Bim (Chapter 3, Chapter 4). These findings are summarized in Fig. 1.

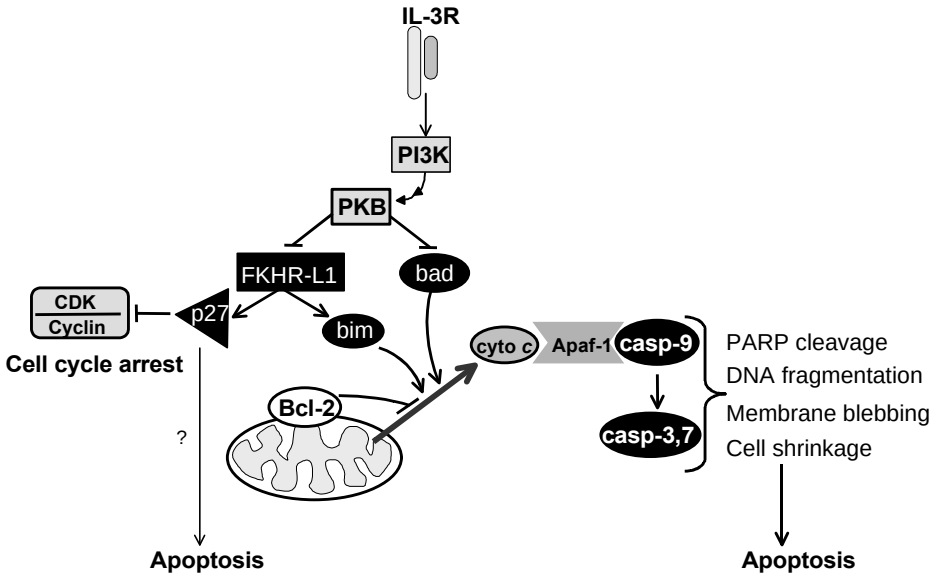

Figure 1 Model.

Cytokine-mediated rescue from apoptosis can be mediated by activation of PKB, which phosphorylates pro-apoptotic Bcl-2 family member Bad and Forkhead transcription factor FKHR-L1, thus inhibiting their activity. In the absence of cytokines, FKHR-L1 is active, promoting transcription of $p27^{KIP1}$ and pro-apoptotic Bcl-2 family member Bim. $P27^{KIP1}$ can promote either cell cycle arrest by inhibiting G_1 CDK-cyclin complexes or inhibit apoptosis through an unidentified mechanism. Bim can promote apoptosis by inhibiting the activity of anti-apoptotic Bcl-2 family members. FKHR-L1 activity results in subsequent activation of caspases, executing the apoptotic program through proteolytic cleavage of substrates, such as PARP.

$p27^{KIP1}$, a critical mediator of proliferation and apoptosis ?

$p27^{KIP1}$ was initially identified as an inhibitor of CDK/ cyclin complexes that are present in G_1 and constitute an important mediator of the "restriction point" (Chapter 1, Fig. 3) which can be overcome by growth factors[22,23]. At the end of G_1, levels of $p27^{KIP1}$ decrease, which is predominantly mediated by phosphorylation by cyclin E/CDK2, resulting in degradation by the ubiquitin system[24-26]. Transcriptional regulation of $p27^{KIP1}$ has, in contrast to our observations in Chapter 3, never been considered an important mechanism of regulation of $p27^{KIP1}$ levels. These differences might be

explained in that many previous studies have been carried out in fibroblasts, in which mRNA levels of $p27^{KIP1}$ do not or only mildly increase in response to growth factor deprivation[23,27]. With respect to this it is also interesting to note that overexpression of $p27^{KIP1}$ in fibroblasts results only in a G_1 arrest, whereas in hematopoietic cells overexpression can in addition promote induction of apoptosis[28,29]. A potential explanation of induction of the apoptotic program by $p27^{KIP1}$ in hematopoietic cells has been provided by Boussiotis and coworkers, who observed that $p27^{KIP1}$ associates with c-Jun co-activator JAB1, resulting in defective AP-1-mediated transcription in T cells[30]. Increases in $p27^{KIP1}$ levels may then result in a decrease in transcription of anti-apoptotic genes, resulting in apoptosis (Fig. 2A). To examine whether this might be an explanation for the induction of apoptosis that we observed upon overexpression of $p27^{KIP1}$, we analyzed the effect of overexpression of $p27^{KIP1}$ on the luciferase activity of an AP1-responsive luciferase construct. Indeed, luciferase activity of cells overexpressing $p27^{KIP1}$ was substantially decreased compared to control cells (Fig. 2B), whereas overexpression of $p27^{KIP1}$ did not alter luciferase activity of an unrelated cyclin D1 luciferase construct.

While this could indicate that displacement of JAB1 from AP-1 complexes is a potential mechanism by which $p27^{KIP1}$ could promote apoptosis in hematopoietic cells, this still awaits further analysis. As an alternative explanation for induction of apoptosis, we cannot exclude that overexpression of $p27^{KIP1}$ in cells which have already passed the restriction point might result in induction of the apoptotic program as a result of simultaneously receiving both proliferative and anti-proliferative signals, as usually levels of $p27^{KIP1}$ decrease towards the end of G_1[22,23]. Further evidence for a function of $p27^{KIP1}$ other than inducing G_1 arrest is provided in that $p27^{KIP1}$ expression is regulated in terminally differentiated eosinophils (Chapter 3 Fig. 2). Although expression of CKIs in terminally differentiated cells has been suggested to be required for the maintenance of their non-proliferative status[2], $p27^{KIP1}$ is upregulated upon IL-5 withdrawal, suggesting that in some cell types $p27^{KIP1}$ might indeed be involved in induction of the apoptotic program.

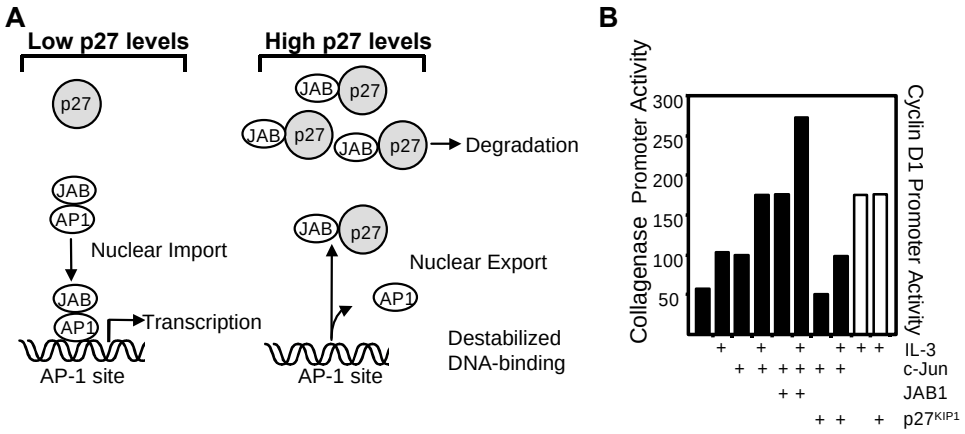

Figure 2 Expression of p27KIP1 decreases collagenase promoter activity.
(A) Model for $p27^{KIP1}$ in induction of apoptosis. When levels of $p27^{KIP1}$ are low, JAB1 translocates to the nucleus and serves as a cofactor for AP-1 complexes. High levels of $p27^{KIP1}$ promote translocation of JAB1 to the cytoplasm, resulting in a destabilization of AP-1 complexes and reduction of AP-1-mediated transcription. (B) Ba/F3 cells were electroporated with either 2 μg of collagenase-luciferase construct (black bars), or cyclin D1 luciferase constructs (white bars) together with 500 ng of renilla as an internal transfection control, together with 8 μg of c-Jun, JAB1 or $p27^{KIP1}$ as indicated. Cells were cultured in the absence or presence IL-3 for 24 hours and luciferase activity was analyzed as described in Chapter 3.

Function of pro-apoptotic Bcl-2 family members in development and potential mechanism of regulation.

A second FKHR-L1 target that we identified was the BH3-only protein Bim (Chapter 4). In addition to Bim there are other BH3-only proteins that interact with anti-apoptotic Bcl-2 family members through their BH3-region and thus inhibit their activity, resulting in induction of the apoptotic program[31]. Interestingly, expression and regulation of Bim was only observed in hematopoietic cells[28], but not in fibroblasts (G. Kops et al., personal communication). Moreover, activity of FKHR-L1 results in a G_1 arrest in fibroblasts, and induction of apoptosis in hematopoietic cells[27,28], suggesting that Bim is indeed a critical mediator of the apoptotic program in hematopoietic cells.

Regulation of activity of BH3-only proteins in mammals can occur posttranslationally, such as for Bad[18,32]. In addition, evidence is accumulating for transcriptional regulation of some of these proteins. DNA damage-induced activation of transcription factor p53 promotes transcription of BH3-only protein Noxa, concomitant with elevation of CKI $p21^{CIP1}$(33; reviewed in [34]). This is similar to our own observations of FKHR-L1-mediated upregulation of a CKI and a BH3-only protein. NGF withdrawal-induced apopto-

sis of neuronal cells, but also survival factor withdrawal of several hematopoietic cell lines and bone marrow-derived CD34(+) cells, induces elevation of the BH3-only protein Harakiri (Hrk, also known as DP5)[35,36]. However, the transcription factor(s) responsible for this upregulation still remains to be elucidated. We have set out to investigate whether FKHR-L1 might be involved in regulation of Hrk, but failed to detect Hrk expression in Ba/F3 cells (data not shown). It is still of course interesting to examine this in the context of other hematopoietic cells.

Could there be a function for BH3-only proteins in apoptosis during development, for example to eliminate unwanted cells during T and B cell selection, where autoreactive cells undergo apoptosis ? The observation that B and T cell development is perturbed in *Bim (-/-)* mice and is accompanied with autoimmune diseases suggests that this might indeed be the case[37]. Here, research on model organisms such as *C. elegans* can shed light on BH3 only proteins in development, as many components of the apoptotic machinery are conserved through evolution[38]. Moreover, the recently elucidated sequence of the human genome will be of help in identifying additional components in human[39]. In *C. elegans*, the BH3-only protein EGL-1 has been demonstrated to be essential for the majority apoptotic cell deaths during development, since loss of function of the *egl-1* gene abrogated apoptosis this process[40]. Additional research suggested that regulation of EGL-1 might be cell-type dependent[41]. In some cells, transcription of *egl-1* can be repressed by the zinc-finger protein CES-1, which in turn is repressed by CES-2, a member of the basic-leucine zipper family of transcription factors[42] (reviewed in [38], see Fig. 3).

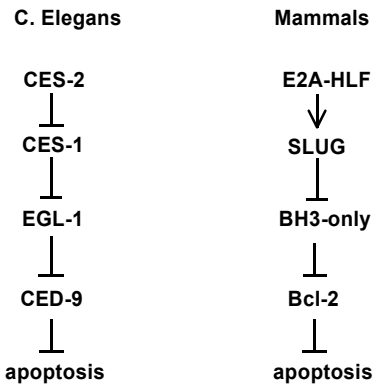

Figure 3 Evolutionary conservation of players of the apoptotic program.
In C. elegans, as well as in mammals, members of the Bcl-2 family play an important role in mediating apoptosis. Upstream regulators of the BH3-only protein EGL-1 in C. elegans have orthologs in mammals, although their function in regulating the expression of BH3-only proteins in mammals still needs to be established.

Upstream regulators of *egl-1* transcription have orthologs in mammals. Recently, a mammalian homolog of CES-1, Slug, was identified, which exhibits anti-apoptotic effects[42]. This suggests that transcriptional repression of pro-apoptotic Bcl-2 members could also be an important means of regulation in mammalian cells. Indeed, IL-3 signaling was recently found to repress protein levels of Bim, which might be due to transcriptional repression[43,44]. Interestingly, chromosomal translocation of a potential mammalian ortholog of CES-2, hepatic leukemia factor (HLF), resulting in the fusion gene E2A-HLF, has been associated with perturbed development of lymphoid cells and leukemia[45-47]. Furthermore, whereas Slug expression appears to be restricted to lymphoid progenitors, expression of E2A-HLF results in sustained expression of Slug, which might be linked to the leukemogenic effects of E2A-HLF[42]. However, the normal activity of HLF or other potential mammalian orthologs of CES-2, as well as regulation of BH3-only proteins by Slug still remains to be demonstrated.

FKHR-L1 and its potential involvement in disease.

In our studies, FKHR-L1 activity promoted G_1 arrest and apoptosis concomitant with an elevation of $p27^{KIP1}$ and Bim[28,43]. The PI3K- PDK-1-PKB- FKHR-L1 pathway is evolutionarily conserved and has orthologs in *C. elegans*. However, a role for the Forkhead ortholog Daf-16 in apoptosis has not yet been demonstrated, although its activity is associated with an increase in life span[48-50].

There are many examples of tumors, and also autoimmune diseases that result from increased activity of PI3K and its downstream effectors[51-54]. In addition to promoting cell division and survival through inhibiting members of the Forkhead family AFX, FKHR and FKHR-L1, rearrangements of FKHR and AFX, have also been demonstrated in certain cancers (reviewed in [55]). However, a potential role for FKHR-L1 in the clearing of inflammatory cells remains to be established. Inflammation in allergic diseases due to enhanced activity, but also enhanced numbers of inflammatory cells, such as neutrophils or eosinophils. Enhanced numbers of these cells might be a result of overproduction or increased mobilization, but also of defective regulation of apoptosis. To discriminate between those possibilities, we analyzed eosinophils of a hypereosinophilic patient having a marked elevation of eosinophil numbers in the peripheral blood (white blood cell count $23x10^9$ cells/l, 95% eosinophils). Cytokine-deprived cells from this patient failed to undergo apoptosis (Fig. 4A, kindly provided by M. Rosas), suggesting that the default apoptotic pathway is affected. Interestingly, whereas in the absence of cytokines activity of FKHR-L1 is induced through dephosphorylation, phosphorylation of FKHR-L1 in eosinophils was sustained even in the absence of cytokines and not altered upon addition of IL-5 (Fig. 4B), in contrast with eosinophils derived from a healthy donor (Fig. 4C). The observation that phosphorylation of ERK decreased in cytokine-starved eosinophils from the patient (Fig. 4A, right) suggests that sustained survival is not simply a result from autocrine survival factor secretion. These data provide evidence that aberrant regulation of FKHR-L1 may not only be related to oncogenesis,

but also to the pathogenesis of inflammatory diseases. We have shown that apoptosis induced by FKHR-L1 is a result from a decrease of mitochondrial transmembrane potential, resulting in release of cytochrome c and activation of caspases (Chapter 5). Although mitochondria are present in low numbers in eosinophils, it has recently been shown that also in these cells mitochondria play a central role in the induction of apoptosis[56]. Indeed, we have recently demonstrated that removal of IL-5 from eosinophils results in decrease of mitochondrial transmembrane potential (data not shown).

Although negative regulation of PI3K and its effectors is mediated by lipid phosphatases[57,58], inhibitory regulation of PKB targets by phosphatases is largely elusive. Recently, the PKB target Bad has been demonstrated to be dephosphorylated by the serine-threonine phosphatases PP1A and PP2A, raising the possibility that these phosphatases could also dephosphorylate FKHR-L1[59,60]. As dysregulation of the activity of these phosphatases has been associated with malignancies (reviewed in [61]), it would be of interest to investigate whether this is associated with constitutive phosphorylation of FKHR-L1.

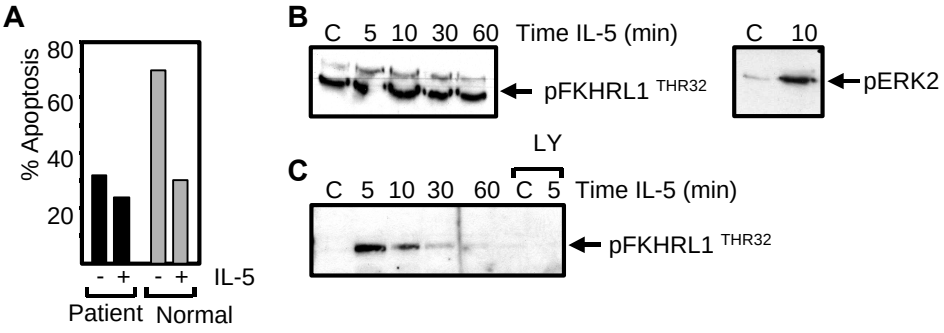

Figure 4 Constitutive phosphorylation of FKHR-L1 correlates with enhanced survival of eosinophils.

(A) Eosinophils from a hypereosinophilic patient (black bars) or a healthy donor (grey bars) were cultured in the absence of presence of IL-5 (10^{-10} nM) for 48 hours and the percentage of apoptotic cells was analyzed as in Chapter 3. (B) Left: cells from a hypereosinophilic patient were stimulated with IL-5 (10^{-10} nM) and the phosphorylation of FKHR-L1 was determined using FKHR-L2-Thr-32 phosphospecific antibody as described in Chapter 3. Right: Cells from the same patient were stimulated with IL-5 (10^{-10} nM) for 10 minutes and ERK2 phosphorylation was analyzed using an ERK2 phospho-specific antibody. (C) Eosinophils from a healthy donor were treated with IL-5 (10^{-10} nM) for indicated times either without or with pretreatment of LY294002 (10 μM) analyzed as in (B).

Induction of apoptosis in response to DNA damage has been attributed to p53 through upregulation of pro-apoptotic Bcl-2 members Bax and Noxa[33,62]. However, mechanisms of transcriptional upregulation of pro-apoptotic proteins in development or in the absence of growth factors is still largely elusive. In addition to FKHR-L1-induced apoptosis in hematopoietic cells, another putative transcription factor, Requiem, might also be involved. Requiem was identified in a screen to identify genes mediating programmed cell death triggered by IL-3-deprivation and encodes a zinc-finger protein, but its mechanism of action still remains to be elucidated[63]. The transcriptional upregulation of pro-apoptotic proteins after cytokine withdrawal might prove to be a common mechanism regulating survival of hematopoietic cells.

Strategies for the development of therapeutic agents.

Cancer cells exhibit unrestricted proliferation independent of environmental regulatory mechanisms having adapted to the apoptotic restraints that are normally be imposed on them. These cells are often more sensitive to irradiation and chemotherapeutic agents than their normal counterparts. Given the side effects of these treatments, it would be desirable to develop a therapy that is more specific and restricted to malignant cells. For many leukemias, this would require a better characterization of the different developmental stages in order to specifically target the therapy. Although this still requires additional research, knowledge of apoptosis is currently being used for clinical interference. For example, adaptive mechanisms in cancer cells often involve the Bcl-2 family, where the activity of anti-apoptotic members is enhanced over pro-apoptotic members (reviewed in [31,64]). Interestingly, treatment of melanoma cells that have elevated levels of Bcl-2 with Bcl-2 antisense oligonucleotides enhanced their susceptibility to chemotherapeutic agents[65]. Studies of the inhibitory effects of BH3-only proteins have resulted in the observation that only a small peptide derived from the BH3 domain, which specifically interacts with and inhibits the activity of anti-apoptotic Bcl-2 family members, is sufficient to trigger apoptosis of cells[66,67]. Recently, research towards developing therapeutic agents to inhibit anti-apoptotic Bcl-2 family members has taken an additional step with the characterization of cell-permeable small molecule inhibitors that compete with the binding of the BH3 domain of BH3-only proteins to anti-apoptotic Bcl-2 family members[68-70]. Addition of these small molecules to cells promotes apoptosis similar to the mechanism of action of BH3-only proteins. Although development of such molecules is promising and has advantage over peptides in that they are more stable, it still requires the development of strategies to be able to specifically target these inhibitors to malignant cells. Taken together, the knowledge generated by fundamental research into the regulation of apoptosis allows the development of more specific means for clinical interference in disease. Further research could contribute to increase the specificity of the therapeutic agent, and limit its effects to malignant cells.

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Nederlandse samenvatting

In het immuunsysteem zorgen witte bloedcellen voor de afweer tegen schadelijke organismen van buitenaf. In laboratorium longziekten te Utrecht wordt onderzoek verricht naar een bepaald type witte bloedcellen, eosinofielen, omdat die een rol spelen bij het ontstaan van allergische astma. Eosinofielen zijn normaliter betrokken bij de afweer tegen bepaalde parasieten, maar die komen in de westerse wereld nog maar weinig voor, dus de fysiologische functie van eosinofielen kan in de westerse wereld wellicht gemist worden. Kortom, je zou dus de aktiviteit van eosinofielen kunnen remmen, zonder dat een astma patient er last van heeft, en alleen een verlichting van zijn ziekte ervaart.

Witte bloedcellen zijn voor hun groei, overleving en specialisatie afhankelijk van groeifactoren. Groeifactoren binden aan de buitenkant van de cel aan een "groeifactor receptor". Er zijn verschillende typen groeifactoren die ieder binden aan hun eigen receptor. Doordat ieder celtype weer andere groeifactor receptoren bezit kunnen verschillende celtypes door specifieke groeifactoren gereguleerd worden. Binding tussen groeifactor en groeifactor receptor resulteert in een cascade van signalen binnenin de cel. Het geheel van deze signaleringscascades, signaaltransductie genaamd, resulteert in stimulatie van witte bloedcellen. Hierdoor kunnen witte bloedcellen aktief zijn in de afweer van schadelijke organismen en gaan delen. Behalve dat groeifactoren ervoor zorgen dat er voldoende cellen zijn doordat de celdeling gestimuleerd wordt, zorgen ze er ook voor dat cellen niet dood gaan. Het idee achter het bestuderen van signaaltransductie in (bijvoorbeeld) eosinofielen is dat als men begrijpt hoe groeifactoren deze cellen stimuleren, vervolgens gekeken kan worden op welke manier deze signalering geremd kan worden. Uiteindelijk zou hierdoor een middel gevonden kunnen worden om (bijvoorbeeld) allergische astma te remmen.

In dit proefschrift wordt beschreven hoe signaaltransductie in een witte bloedcel (zoals een eosinofiel) kan leiden tot celdeling en survival. Er is gekeken naar een bepaalde "groeifactor receptor", de "interleukine-5 receptor", die alleen maar op eosinofielen voorkomt, hetgeen bestudering van de signalering van deze receptor geschikt maakt om uiteindelijk mogelijk specifiek de activiteit van eosinofielen te kunnen remmen.

Behalve dat in dit proefschrift gekeken is hoe groeifactoren kunnen zorgen voor celdeling en survival is ook onderzocht wat er gebeurt met witte bloedcellen in afwezigheid van groeifactoren. Zonder groeifactoren stoppen de meeste witte bloedcellen met delen en ondergaan uiteindelijk "apoptose", ook wel “geprogrammeerde celdood” genoemd. De afhankelijkheid van groeifactoren om te delen en te blijven leven zorgt ervoor dat een cel niet op eigen houtje besluit iets te doen en in zekere zin "in dienst blijft van het organisme". Kortom, enerzijds de vermeerdering van witte bloedcellen in de aanwezigheid van groeifactoren en anderzijds het ondergaan van apoptose in afwezigheid van groeifactoren reguleert het totaal aantal witte bloedcellen in een organisme. Soms kan er echter iets mis zijn met de afhankelijkheid van een cel voor groeifactoren en kan een cel blijven leven of zelfs delen, ook als er geen groeifactoren zijn. Een manier waarop een cel onafhankelijk van groeifactoren kan delen en leven is door mutaties in componenten

van signaaltransductie cascades. Deze mutaties kunnen ertoe leiden dat de cascade ook zonder groeifactoren geaktiveerd is. Als je weet waardoor een cel apoptose ondergaat in afwezigheid van groeifactoren zou het ook wellicht mogelijk zijn om deze kennis te gebruiken om farmacologische drugs te ontwikkelen. Deze farmacologische drugs zouden apoptose kunnen induceren in cellen die blijven leven zonder groeifactoren en zo die ongewenste cellen (met een te grote eigen wil) uit de weg ruimen.

Een cel bezit pro-apoptotische eiwitten die apoptose kunnen induceren en anti-apoptotische eiwitten die apoptose tegengaan. Groeifactor geinduceerde signaal transductie resulteert in verhoging van de aktiviteit van anti-apoptotische eiwitten en remming van de aktiviteit van de pro-apoptotische eiwitten. Vaak kan de aktiviteit van een bepaald type eiwit verhoogd worden simpelweg door ervoor te zorgen dat er meer van dat eiwit (een hogere expressie) in de cel is. Eiwitten die zorgen voor een hogere expressie van een bepaald eiwit in een cel worden "transcriptie factoren" genoemd. Vaak wordt de aktiviteit van transcriptie factoren die zorgen voor meer anti-apoptotische eiwitten in een cel verhoogd door groeifactor signalering.

Of een cel overleeft of apoptose ondergaat is vaak de resultante van een balans tussen pro- en anti-apoptotische eiwitten. Een hele belangrijke balans wordt gevormd door eiwitten van de Bcl-2 (B cell lymphoma) familie. Deze bestaat uit eiwitten die apoptose tegengaan (anti-apoptotische Bcl-2 leden), maar ook eiwitten die apoptose kunnen induceren (pro-apoptotische Bcl-2 leden). De anti-apoptotische Bcl-2 familie leden remmen de aktiviteit van de pro-apoptotische Bcl-2 familie leden en vice versa, waardoor de netto aktiviteit van de Bcl-2 familie belangrijk is voor de cel óf om te overleven óf om apoptose te ondergaan. Signalering van groeifactoren leidt tot verhoging van expressie van anti-apoptotische Bcl-2 en inaktivatie van pro-apoptotische Bcl-2 leden, waardoor survival bewerkstelligd wordt. In de afwezigheid van groeifactoren is de expressie van de anti-apoptotische Bcl-2 familieleden lager, en krijgt de aktiviteit van de pro-apoptotische Bcl-2 familieleden de overhand, waardoor een cel uiteindelijk apoptose ondergaat.

In dit proefschrift is onderzocht welke groeifactor-geinduceerde signaleringsroutes ervoor zorgen dat witte bloedcellen delen en overleven, maar ook waardoor een cel stopt met delen en apoptose ondergaat in afwezigheid van groeifactoren. Een manier waarop groeifactoren kunnen resulteren in celdeling is mogelijkerwijs door verlaging van de expressie van het eiwit $p27^{KIP1}$. In afwezigheid van groeifactoren is er meer $p27^{KIP1}$ aanwezig in de cel (hoofdstuk 3). $p27^{KIP1}$ heeft een belangrijke rol in het stoppen van de celdeling in afwezigheid van groeifactoren. Kortom, verhoging van de expressie van $p27^{KIP1}$ is een manier om celdeling in de afwezigheid van groeifactoren te remmen. Verantwoordelijk voor de verhoging van de expressie van $p27^{KIP1}$ in afwezigheid van groeifactoren bleek de transcriptie factor FKHR-L1. In tegenstelling tot veel andere transcriptie factoren is deze transcriptie factor aktief in afwezigheid van groeifactoren en inaktief in aanwezigheid van groeifactoren. Kortom, door middel van deze transcriptie factor zou bewerkstelligd kunnen worden dat in afwezigheid van groeifactoren de cel niet meer deelt en uiteindelijk apoptose ondergaat. Waarschijnlijk ligt het inderdaad aan de aktivatie van FKHR-L1 dat witte bloedcellen in afwezigheid van groeifactoren stoppen

met delen en apoptose ondergaan (hoofdstuk 5). Aangezien de Bcl-2 familie een belangrijke rol speelt in de balans tussen overleven en apoptose hebben is onderzocht of het mogelijk zou kunnen zijn dat FKHR-L1 aktiviteit leidt tot verhoging van expressie van pro-apoptotische Bcl-2 familieleden. Dit heeft geresulteerd in de bevinding dat FKHR-L1 verantwoordelijk is voor de transcriptionele regulatie van een pro-apoptotische Bcl-2 familielid, Bim (hoofdstuk 4). Dat regulatie van Bim expressie belangrijk is voor de regulatie van de hoeveelheid witte bloedcellen blijkt uit studies in Bim-deficiente muizen. Deze muizen hebben veel te veel witte bloedcellen die geen apoptose ondergaan in afwezigheid van groeifactoren.

Samenvattend hebben de resultaten beschreven in dit proefschrift geleid tot een beter inzicht in groeifactor signalering. De toekomst zal leren in hoeverre kennis van de signaal transductie routes die hier bestudeerd zijn toepasbaar zou kunnen zijn in de ontwikkeling van farmacologische drugs ter bestrijding van bijvoorbeeld allergische astma.

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Pascale

Curriculum Vitae

De schrijfster van dit proefschrift werd op 19 juni 1972 geboren te Veghel. Na het behalen van het diploma op R.K. Gymnasium "Beekvliet" te Sint Michielsgestel werd in 1991 begonnen met de studie Medische Biologie de Universiteit van Utrecht. In agustus 1996 werd het Doctoraal diploma behaald met als hoofdvakken Celbiologie (Dr. P.J. Peters, vakgroep Celbiologie, Universiteit Utrecht en Dr. V.W. Hsu, Department of Rheumatology and Immunology, Brigham and Women's Hospital, Boston, USA) en Fysiologische Chemie (Dr. B.M.T. Burgering, Prof. dr. J.L. Bos, Universiteit Utrecht). Het in dit proefschrift beschreven onderzoek werd uitgevoerd in de periode maart 1997 tot februari 2001 onder begeleiding van Dr. P.J. Coffer, Prof. dr. L. Koenderman en Prof. dr. J-W. J. Lammers in het laboratorium Longziekten te Utrecht.

Vanaf augustus 2001 zal zij werkzaam zijn als post doctoraal onderzoeker, in het lab van Prof. P.H. O'Farrell op het UCSF in San Francisco, USA.