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The Forkhead Transcription Factor FoxO Regulates Transcription of p27^{Kip1} and Bim in Response to IL-2[1]

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The cytokine IL-2 plays a very important role in the proliferation and survival of activated T cells. These effects of IL-2 are dependent on signaling through the phosphatidylinositol 3-kinase (PI3K) pathway. We and others have shown that PI3K, through activation of protein kinase B/Akt, inhibits transcriptional activation by a number of forkhead transcription factors (FoxO1, FoxO3, and FoxO4). In this study we have investigated the role of these forkhead transcription factors in the IL-2-induced T cell proliferation and survival. We show that IL-2 regulates phosphorylation of FoxO3 in a PI3K-dependent fashion. Phosphorylation and inactivation of FoxO3 appears to play an important role in IL-2-mediated T cell survival, because mere activation of FoxO3 is sufficient to trigger apoptosis in T cells. Indeed, active FoxO3 can induce expression of IL-2-regulated genes, such as the *cdk* inhibitor p27^{Kip1} and the proapoptotic Bcl-2 family member Bim. Furthermore, we show that IL-2 triggers a rapid, PI3K-dependent, phosphorylation of FoxO1a in primary T cells. Thus, we propose that inactivation of FoxO transcription factors by IL-2 plays a critical role in T cell proliferation and survival. *The Journal of Immunology,* **2002, 168: 5024–5031.**

O ptimal activation of mature resting T lymphocytes requires engagement of the TCR complex, accompanied by a costimulatory signal that can be provided by either CD28 or IL-2 (1, 2). TCR engagement causes the activation of a number of genes, including the high-affinity IL-2Rα chain (or CD25) gene. The expression of CD25 on the cell surface of activated T cells leads to IL-2 responsiveness (3). This is a critical event for the onset of the immune response, because IL-2 is a potent mitogen for the T cells and promotes rapid proliferation of the activated T cells (2–4). Importantly, TCR stimulation also triggers IL-2 gene activation, leading to an autocrine loop of activation (5).

In addition to stimulating T cell proliferation, IL-2 also functions as an important survival factor for T cells. This function of IL-2 has been shown to depend on signaling through the phosphatidylinositol 3-kinase (PI3K)[3] pathway. One important target of PI3K signaling in this respect is protein kinase B (PKB)/Akt (6, 7). PKB activation has been implicated in many metabolic processes and, importantly, in a strong promotion of cell survival (8, 9). Such PKB-dependent cell survival has been proposed to occur through a variety of mechanisms, and most notably through activation of antiapoptotic proteins, such as Bcl-2 (9), and inhibition of proapoptotic proteins, such as Bad (10, 11). Although it is well established that IL-2 promotes T cell survival and proliferation through the activation of PKB, the molecular events downstream of PKB that are involved in these responses remain unclear.

Several lines of evidence have demonstrated that PKB inhibits transcriptional activation of a number of related forkhead transcription factors (FKHR/FKHR-L1/AFX) (12, 13), now referred to as FoxO1, FoxO3, and FoxO4 (14). These forkhead transcription factors recognize a common DNA-binding element (15) that is highly related to insulin response elements (16). Each of these forkhead factors contains conserved phosphorylation sites for PKB, and PKB-mediated phosphorylation was shown to result in translocation of these factors to the cytoplasm (17–19). A conserved pathway is present in the nematode *Caenorhabditis elegans*, where the PI3K and PKB homologs (AGE-1 and AKT, respectively) regulate the activity of a forkhead transcription factor, DAF-16, in a pathway involved in the regulation of survival in response to nutrient starvation (20).

We have recently shown that PKB-regulated forkhead transcription factors are involved in regulation of the *cdk* inhibitor p27^{Kip1} and Bim, a proapoptotic member of the Bcl-2 family (21–23). This indicates that these forkhead factors control expression of genes involved in the regulation of cell cycle progression as well as apoptosis. Therefore, we investigated the role of FoxO proteins in IL-2-dependent T cell proliferation and survival. In this report, we present evidence that points to a role for FoxO3 in IL-2-mediated survival. We found that IL-2 withdrawal leads to activation of FoxO3 and up-regulation of p27^{Kip1} and Bim levels, and that activation of FoxO3 alone is sufficient to mimic the effects of IL-2 withdrawal. Furthermore, in primary T cells, we observed an IL-2-dependent inhibition of the forkhead transcription factors that may potentiate the required down-regulation of p27^{Kip1} for cell cycle reentry.

Materials and Methods

Plasmids and reagents

pCMV-p27^{Kip1} was a kind gift of Dr. R. Bernards (Netherlands Cancer Institute, Amsterdam, The Netherlands), and pCMV-EGFP-spectrin was a kind gift of Dr. A. Beavis (Princeton University, Princeton, NJ). pCMV-FoxO3(A3)ER (or pCMV-FKHR-L1(A3)ER) has been described (23). LY294002 was from Biomol (Plymouth Meeting, PA). Actinomycin D and 4-hydroxy-tamoxifen (4OH-T) were from Sigma-Aldrich (Steinhem, Germany) and Ficoll-Paque was purchased from Amersham Pharmacia Biotech (Little Chalfont, U.K.).

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Cell culture

Murine CTLL-2 T lymphocytes were cultured in RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 10% FCS, 1% penicillin-streptomycin-2-ME, and recombinant human IL-2 (100 IU/ml). CTLL-2-FoxO3(A3)ER stable cell lines were obtained by electroporation of a pCMV-FoxO3(A3)ER construct at 320 V and 960 μF. Stable transfectants were selected in medium containing 500 μg/ml G418/neomycin (Calbiochem, La Jolla, CA), and single-cell clones were obtained by limiting dilution. Primary human lymphocytes were isolated from healthy donor blood on a Ficoll-Paque gradient and cultured in complemented RPMI medium in absence of IL-2. After purification of the T cells, the population was analyzed for CD3 expression by flow cytometry. Cells were only used for further experimentation if the percentage of CD3 positivity was >95%. Cells were activated by addition of recombinant human IL-2 (final concentration, 5 IU/ml) in combination with anti-CD3 mAb (OKT-3 or UHCT-1) as described (24). 4OH-T was diluted in RPMI 1640 and added to the cells at a final concentration of 500 nM. LY294002 was dissolved in DMSO and used at a final concentration of 40 μM.

RNA preparation and Northern blotting

CTLL-2 cells were lysed in a guanidine-isothiocyanate lysis buffer and total RNAs were isolated. Equal amounts were loaded on an agarose-formaldehyde gel, and the blot was probed with $p27^{Kip1}$ or GAPDH cDNA probes.

Abs and Western blotting

$p27^{Kip1}$ and Fas ligand mAb were purchased from Transduction Laboratories (Lexington, KY) and Bim polyclonal Ab were purchased from Affinity BioReagents (Golden, CO). Anti-phospho-Thr^{24}-FoxO1/phospho-Thr^{32}-FoxO3 rabbit Ab (cross-reactive with phospho-Thr^{28}-FoxO4), anti-FoxO3, anti-PKB, and anti-phospho-Ser^{473}-PKB were purchased from Cell Signaling Technology (Beverly, MA). Anti-FoxO4 (N-19) goat Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hemagglutinin Ab was 12CA5 hybridoma supernatant. Cells were lysed in Laemmli buffer (120 mM Tris (pH 6.8), 4% SDS, 20% glycerol) at room temperature, and DNA was sheared by passing through a syringe. The protein content was determined using a Lowry assay, equal amounts of proteins were analyzed by SDS-PAGE, and blots were probed with the appropriate Abs. Proteins were visualized by standard ECL (Amersham Pharmacia Biotech).

FACS analysis

For cell cycle analysis, the harvested cells were washed with PBS and fixed in 70% ethanol for at least 2 h on ice. Cells were spun down for 5 min at $480 \times g$, labeling buffer was added (0.25 mg/ml RNase and 10 μg/ml propidium iodide in PBS), and cells were incubated for 10 min in the dark. The cell population was viewed using a FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed using CellQuest software (BD Biosciences). For determination of $CD3^+$ or $CD25^+$ cells, cells were harvested and incubated for 20 min on ice in the corresponding Ab solution (anti-CD3-FITC or anti-CD25-PE; BD Immunocytometry Systems, San Jose, CA). Cells were washed, resuspended in PBS, and analyzed by flow cytometry.

Results

PI3K/PKB signaling promotes survival of IL-2-dependent T cells

IL-2 induces T cell progression through G_1 into S phase of the cell cycle and controls T cell survival and clonal expansion (2). In addition, many hematopoietic cells have a default program for cell death and require a constant supply of cytokines to promote their survival. The CTLL-2 cell line is dependent on IL-2 for its survival and proliferation and is thus a good model to study IL-2-regulated genes implicated in regulation of survival and proliferation.

When CTLL-2 are cultured in the absence of IL-2, a cell cycle arrest in G_0/G_1 is observed that is most evident after 24 h of IL-2 deprivation (Fig. 1*A*). This initial growth arrest is then followed by apoptosis, so that the majority of the cells in the population have undergone programmed cell death after 48 h of IL-2 deprivation, as indicated by the rise in cells with a sub-G_1 DNA content (Fig. 1*A*).

As mentioned above, cytokine-mediated cell survival was shown to be dependent on PI3K/PKB signaling (8). Activation of PKB correlates with its phosphorylation on certain residues, nota-
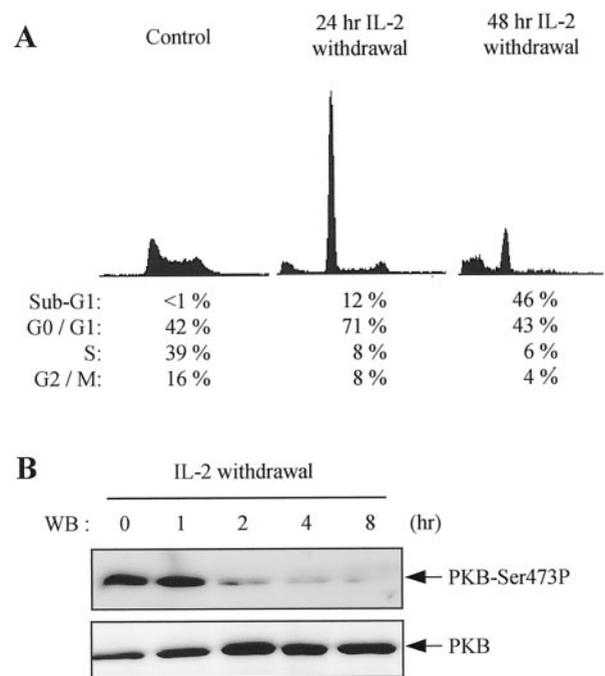

FIGURE 1. IL-2 promotes survival of IL-2-dependent T cells via the PI3K/PKB pathway. *A*, IL-2-dependent CTLL-2 mouse T cells were grown in the absence of IL-2. After 24 and 48 h they were harvested, fixed in ethanol, and labeled with propidium iodide to determine their DNA profiles by flow cytometry. *B*, CTLL-2 were deprived of IL-2 for different periods of time and harvested. They were monitored for PKB expression by Western blotting using a rabbit anti-phospho-Ser^{473}-PKB Ab, recognizing active PKB (*upper panel*), or an Ab directed against total PKB (*lower panel*).

bly on Ser^{473}. Using a phosphospecific Ab that specifically recognizes Ser^{473}-phosphorylated PKB, we observed that PKB was gradually losing its phosphorylation on Ser^{473} in response to IL-2 deprivation of CTLL-2 cells (Fig. 1*B*). Clearly, it takes ~2 h of IL-2 deprivation to see a significant drop in PKB phosphorylation. These kinetics are most likely determined in large part by the time required to turn over the IL-2 bound to the cells, and the subsequent down-modulation of IL-2R signaling. The decrease in Ser^{473} phosphorylation was not due to a reduction in PKB expression levels, as this was constant throughout the course of this experiment (Fig. 1*B*).

To further investigate the requirement for PI3K/PKB signaling in IL-2-mediated survival of CTLL-2 cells, we treated CTLL-2 with LY294002, a potent inhibitor of PI3K. After a 24-h treatment with LY294002, we observed a prominent arrest in G_0/G_1 (Fig. 2*A*). At 48 h after addition of LY294002, a large fraction of the cells had fragmented DNA, as demonstrated by the rise in cells with a sub-G_1 DNA content (Fig. 2*A*), comparable to what was seen after IL-2 withdrawal (Fig. 1*A*). As expected, addition of LY294002 resulted in efficient inhibition of PI3K-mediated signaling, as demonstrated by the rapid reduction in the levels of activated PKB (Fig. 2*B*). Taken together, these data are consistent with the established role for PI3K/PKB signaling in cytokine-dependent cell survival, and show that these CTLL-2 cells critically depend on PI3K activity for their maintenance in culture.

IL-2-mediated cell survival is linked to inactivation of FoxO3

PKB is known to directly phosphorylate a number of forkhead transcription factors belonging to the FoxO subfamily (12, 13, 17). Phosphorylation of these forkhead factors results in their exclusion from the nucleus and a subsequent inhibition in transcriptional
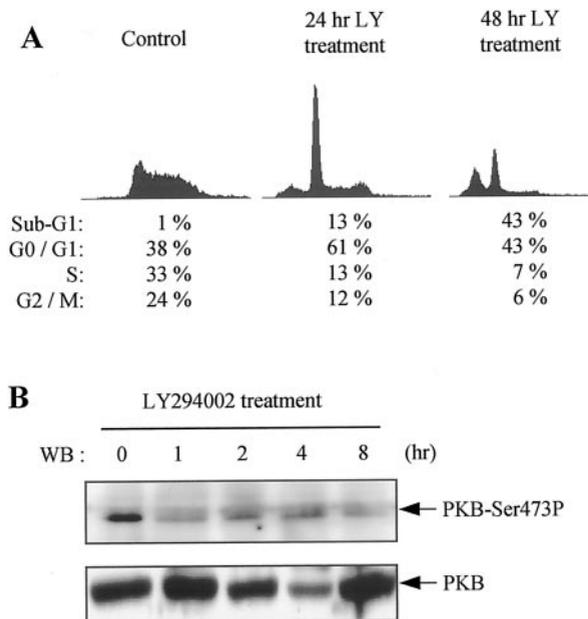
FIGURE 2. Specific interference with the PI3K/PKB pathway mimics IL-2 deprivation. *A*, CTLL-2 mouse T cells were treated with LY294002 for 24 and 48 h in the presence of IL-2. They were harvested, fixed, and labeled as described in Fig. 1*A* for FACS analysis. *B*, CTLL-2 were treated with LY294002 for the corresponding periods of time in presence of IL-2, harvested, and lysed. PKB activity and total PKB were determined using the same Abs as in Fig. 1*B*.

activation of forkhead target genes (18, 19). Moreover, we and others (22, 23, 25) have shown that these forkhead factors can cause apoptosis when expressed at high levels in T and B cell lines. Therefore, we wanted to determine whether the IL-2-mediated survival of CTLL-2 cells was coupled to regulation of FoxO activity. To this end, we analyzed expression of the different PKB-regulated forkhead factors in CTLL-2 cells. Apparently, CTLL-2 cells express relative high levels of FoxO3, while we were unable to detect expression of the related forkhead members FoxO1 and FoxO4 (Fig. 3*A*). We next analyzed PKB-mediated phosphorylation of FoxO3, reflected by its phosphorylation on residue Thr^{32}. As expected, FoxO3 was rapidly dephosphorylated upon IL-2 withdrawal, within 4 h (Fig. 3*B*). Similarly, upon treatment with LY294002, we also observed decreased phosphorylation of FoxO3 (Fig. 3*C*). These results indicate that IL-2 regulates FoxO3 activity through the PI3K/PKB pathway in CTLL-2 cells.

IL-2 mediates p27^{Kip1} and Bim regulation

We have recently shown that FoxO3 controls p27^{Kip1} and Bim levels in a PKB-regulated pathway (22, 23). p27^{Kip1} is a well-known regulator of the G$_1$/S transition through its cyclin-dependent kinase inhibitory activity, which blocks the cell in G$_1$ phase by preventing *cdk*-dependent phosphorylation of pRb (26, 27). Bim is a proapoptotic member of the Bcl-2 family (28, 29), and ectopic expression of Bim is sufficient to trigger apoptosis in a variety of cell types, including CTLL-2 cells (data not shown). To study whether p27^{Kip1} and Bim expression is regulated in an IL-2-dependent fashion in CTLL-2 cells, we analyzed their respective protein levels after IL-2 deprivation or addition of LY294002. We observed an up-regulation of both p27^{Kip1} and Bim protein levels in response to IL-2 starvation (Fig. 4*A*, *upper panels*) as well as after treatment with LY294002 (Fig. 4*A*, *lower panels*). It should be noted that another reported proapoptotic target of FoxO3, Fas ligand (25), does not seem to be an important mediator of cell
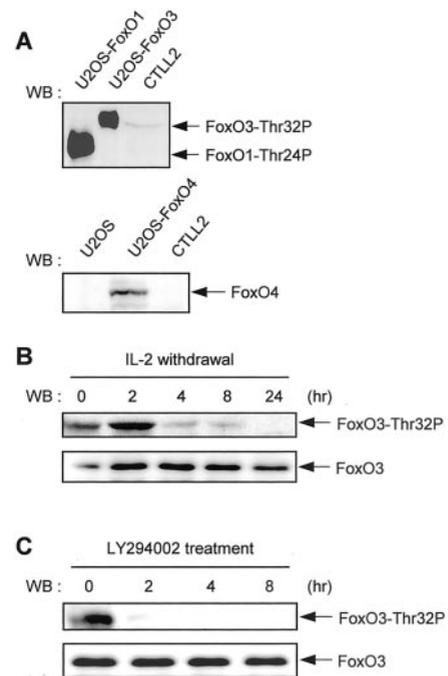

FIGURE 3. IL-2 signaling regulates forkhead transcription factor activity via the PI3K/PKB pathway. *A*, The expression pattern of different FoxO forkhead transcription factors was determined in CTLL-2 mouse T cells. For this purpose, CTLL-2 cells were lysed and analyzed by Western blotting using an anti-phospho-Thr^{24}-FoxO1a/phospho-Thr^{32}-FoxO3 (/phospho-Thr^{28}-FoxO4) Ab (*upper panel*) or an anti-total FoxO4 Ab (*lower panel*). Controls consisting of FoxO4-, FoxO1-, and FoxO3-transfected U2OS cells were loaded in parallel. *B*, CTLL-2 were deprived of IL-2, lysed after different periods of time, and analyzed by Western blotting using a rabbit anti-Thr^{32}-phospho-FoxO3 Ab (*upper panel*) or an Ab recognizing total FoxO3 (*lower panel*). *C*, CTLL-2 were treated for different periods of time with LY294002, lysed, and analyzed by Western blotting using the same Abs as in *B*.

death after cytokine withdrawal, because we could not observe significant changes in the expression of Fas ligand upon IL-2 withdrawal (Fig. 4*B*) or treatment with LY294002 (data not shown).

Because p27^{Kip1} was reported to be a direct target of FoxO3 transactivation (23), we speculated that increased expression of p27^{Kip1} following IL-2 withdrawal is due to transcriptional activation. Indeed, p27^{Kip1} RNA was up-regulated upon IL-2 deprivation in CTLL-2 (Fig. 4*C*). This induction was blocked by addition of actinomycin D, an inhibitor of transcription, indicating that the rise in p27^{Kip1} RNA levels was not due to stabilization of the messenger.

Activation of FoxO3 is sufficient to trigger apoptosis in CTLL-2 cells

The data described above show that IL-2-mediated survival is coupled to FoxO3 inactivation in CTLL-2 cells and that IL-2 withdrawal results in a rapid activation of FoxO3a as determined by its phosphorylation status. However, this does not allow any conclusion as to the importance of FoxO3a activation in the apoptotic program of CTLL-2 cells. To determine whether mere activation of FoxO3 alone is sufficient to mimic the effects of IL-2 deprivation in these cells, we established CTLL-2-derived cell lines expressing a FoxO3-estrogen receptor (ER) fusion protein. In this chimeric protein, the ER module binds heat shock proteins, which preclude translocation to the nucleus. Upon interaction between the ER module and 4OH-T, the heat shock protein mantle is disrupted and the fusion protein is shuttled to the nucleus, where it
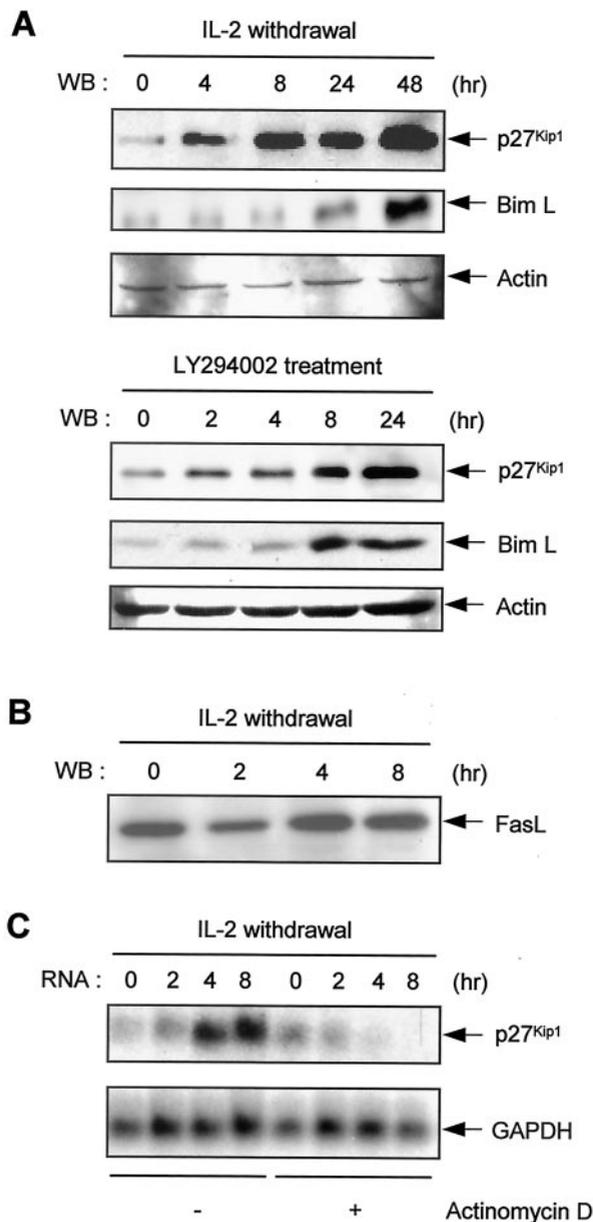
FIGURE 4. FoxO3 directly regulates p27^{Kip1} and Bim expression. *A*, CTLL-2 were either deprived of IL-2 (*upper panels*) or treated with LY294002 (*lower panels*) for different periods of time, harvested, and lysed. p27^{Kip1}, Bim, and actin protein levels were monitored by Western blotting using the appropriate Abs. *B*, CTLL-2 cells were deprived of IL-2 for different periods of time and lysed. Using an anti-Fas ligand Ab, the expression levels of this protein were analyzed by Western blotting to determine whether forkhead transcription factors induce other important proapoptotic target genes in these cells. *C*, CTLL-2 were deprived of IL-2 for the time indicated and treated or not with actinomycin D, a transcription inhibitor. RNAs were extracted at the indicated time points after IL-2 withdrawal and analyzed by Northern blotting using a p27^{Kip1} cDNA probe and a GAPDH cDNA probe as loading control.

can now exert FoxO3 transcriptional activity. To ensure that the fusion protein could not be inactivated by PKB-mediated phosphorylation, the three previously identified PKB phosphorylation sites (Thr^{32}, Ser^{253}, and Ser^{315}) were mutated into alanine residues. This enabled us to trigger FoxO3 activation in the presence of IL-2. Two independent clones (clones 2 and 6) that expressed similar levels of the FoxO3(A3)-ER fusion protein were selected (Fig. 5*A*).
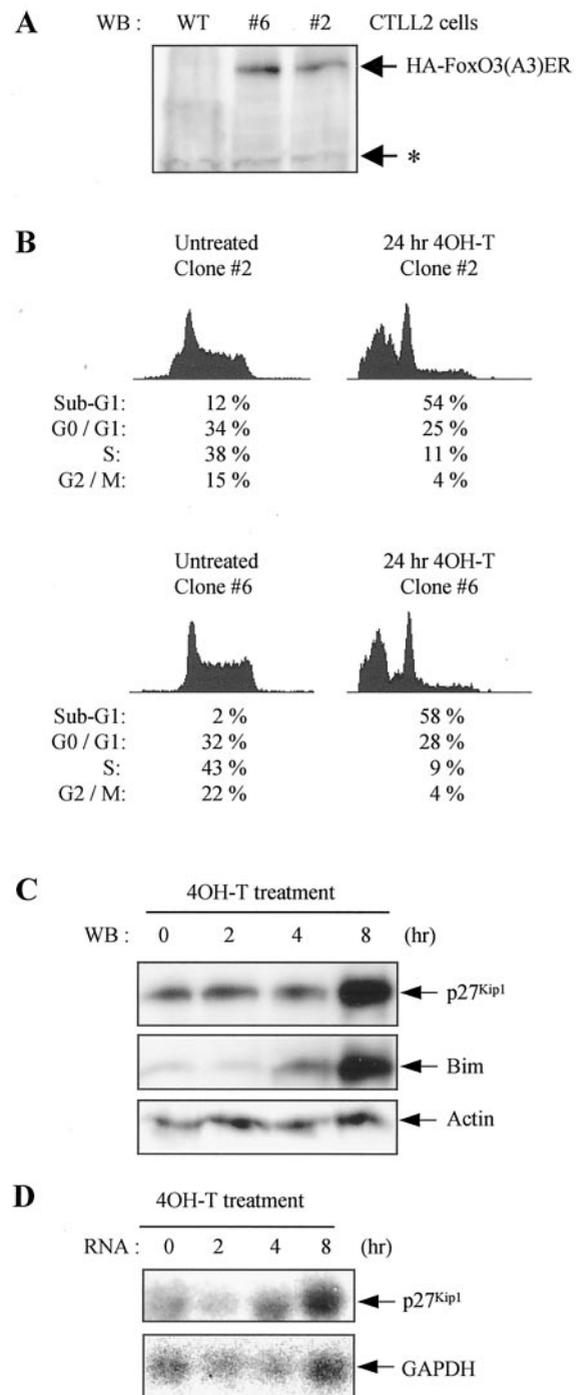
FIGURE 5. PKB-independent activation of FoxO3 mimics IL-2 withdrawal. *A*, CTLL2 cells were electroporated with FoxO3(A3)ER construct. Clonal cell lines expressing this construct in a stable manner were selected on G418. The clones were harvested, lysed, and analyzed by Western blotting using an anti-hemagglutinin mAb. Clones 2 and 6 appeared to express similar levels of FoxO3(A3)ER. *B*, Clones 2 and 6 were treated with 4OH-T for 24 h in presence of IL-2 and analyzed by FACS as described in Fig. 1*A*. *C*, Clone 6 was treated with 4OH-T in the presence of IL-2 for different time lapses and analyzed by Western blotting using the appropriate Abs. *D*, Clone 6 was treated with 4OH-T in presence of IL-2 for the reported time lapses, harvested, and lysed. RNAs were isolated and equal amounts were analyzed by Northern blotting as described in Fig. 4*C*.

Addition of 4OH-T induced a block in cell cycle progression at the G$_1$/S transition, as evidenced by a reduction of cells in S and G$_2$/M in both of these clones (Fig. 5*B*). In addition, a clear increase

in apoptosis was observed at 24 h after addition of 4-OHT (Fig. 5*B*). A certain basal level of apoptosis was observed in clone 2, even in the absence of 4OH-T, suggesting that this clone was somewhat leaky. Indeed, expression of FoxO3(A3)ER was lost over time in clone 2; therefore, clone 6 was used for further experimentation.

We next wanted to study whether activation of FoxO3 alone would result in the induction of the same genes that are up-regulated after withdrawal of IL-2. Indeed, activation of FoxO3 by 4OH-T treatment was able to induce both p27^{Kip1} and Bim protein expression in presence of IL-2 (Fig. 5*C*). Moreover, we were able to confirm that up-regulation of p27^{Kip1} occurred at the transcriptional level, because we could observe a rise in p27^{Kip1} mRNA levels (Fig. 5*D*). These data demonstrate that the sole activation of FoxO3 is sufficient to mimic the effects on p27^{Kip1} and Bim that are normally seen after IL-2 withdrawal, and is sufficient for initiation of programmed cell death.

IL-2 signals rapid inactivation of FoxO in activated T cells

IL-2 signaling is critical throughout T cell development and maturation. The majority of peripheral T cells are quiescent cells but can be stimulated to reenter the cell cycle upon activation of the TCR. However, efficient cell cycle reentry of such resting T cells requires the presence of a costimulatory signal that can be provided by IL-2. Because FoxO3 appears to be such an important target of the IL-2 signaling with respect to T cell survival, we hypothesized that FoxO phosphorylation could be involved during activation of peripheral quiescent T cells upon combined TCR/IL-2 stimulation as well. To address this question, we isolated primary T cells from peripheral blood and activated them with immobilized anti-CD3 mAbs together with IL-2. At 24–48 h after stimulation we could observe that a large proportion of the activated cells had reentered the cell cycle (Fig. 6*A*), but that this cell cycle reentry was efficiently prevented by treatment with
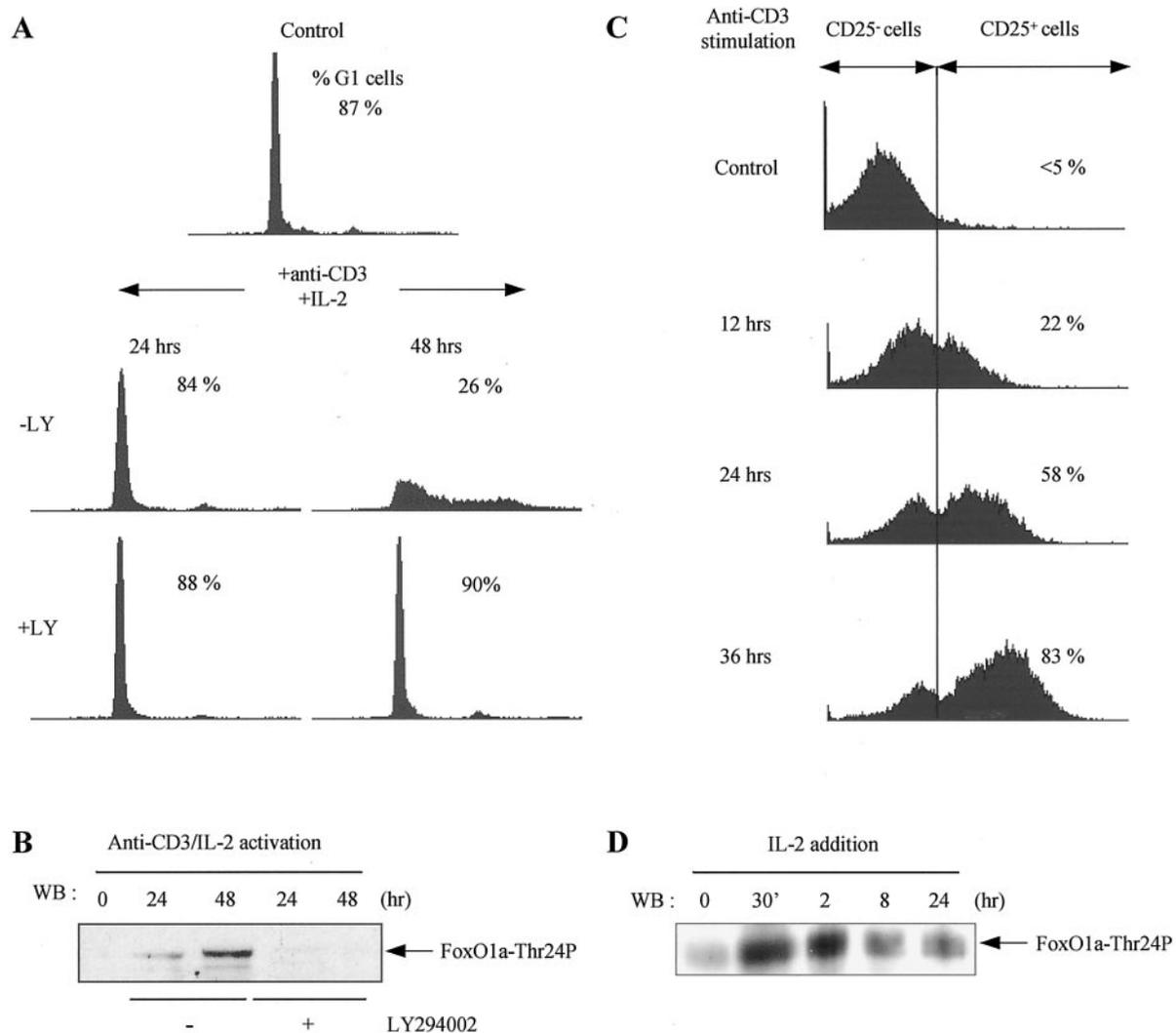

FIGURE 6. Activation of peripheral T lymphocytes is linked with PI3K/PKB-dependent phosphorylation of FoxO1. *A*, Primary T cells were isolated from fresh blood. The cells were stimulated with anti-CD3 mAbs and IL-2 in the presence or absence of LY294002. The cells were harvested after 24 and 48 h, fixed, and stained for FACS analysis as described in Fig. 1*A*. *B*, Protein samples prepared from the cultures shown in *A* were separated on polyacrylamide gels, and FoxO1a-Thr^{24}P levels were detected on Western blot. *C*, Primary T cells were isolated from fresh human blood and stimulated on UCHT-1 anti-CD3-coated dishes for different periods of time. The CD25$^+$ cell population was determined by FACS analysis by incubation with anti-CD25-PE Abs. *D*, Primary T cells were stimulated with anti-CD3 Abs for 24 h, and then with IL-2 for the indicated times. Cells were harvested, lysed, and analyzed by Western blotting with an anti-phospho-Thr^{24}-FoxO1a Ab.

LY294002 (Fig. 6*A*), confirming that activation of resting T cells is dependent on PI3K signaling.

Analysis of the expression of the different FoxO family members showed that these peripheral T cells express relatively high levels of FoxO1a and very low levels of FoxO3a and FoxO4 (data not shown). Phosphorylation of FoxO1a was induced in cells treated with anti-CD3/IL-2 (Fig. 6*B*), indicating that T cell activation correlates with PKB-mediated phosphorylation of FoxO factors. This phosphorylation event was fully dependent on PI3K signaling, because it was prevented when cells were treated with LY294002 in combination with anti-CD3/IL-2. This indicates that PI3K signaling is essential for FoxO phosphorylation in primary T cells. However, because anti-CD3 and IL-2 were added simultaneously in this experiment, and because it takes several hours to induce expression of the IL-2R, we were unable to properly study the kinetics of these events. Therefore, we stimulated the primary T cells with anti-CD3 alone to allow up-regulation of the IL-2R before adding IL-2. As shown in Fig. 6*C*, the high-affinity α-chain of IL-2R (CD25) is not present on resting peripheral T cells but was expressed on 58% of the T cells after 24 h and on ~83% of the cells after 36 h of activation with anti-CD3. To minimize effects of autocrine IL-2 produced by the activated T cells themselves, we used peripheral T cells that had been activated for 24 h only and analyzed the effects of IL-2 addition on FoxO1a phosphorylation. Under these circumstances, FoxO1a was phosphorylated within 30 min, and the extent of phosphorylation peaked at 2 h after stimulation (Fig. 6*D*). At later time points, FoxO1a phosphorylation dropped but was still elevated after 24 h of stimulation with IL-2 compared with the non-IL-2-stimulated population (Fig. 6*D*).

Discussion

IL-2 is a major regulator of T cells throughout their development and differentiation. In this report, we have addressed the molecular mechanism of the IL-2-mediated regulation of T cell proliferation and survival. For this purpose, we have studied IL-2 signaling in the murine T cell line CTLL-2, which is dependent on IL-2 for its proliferation and survival and is therefore a useful model to study IL-2-regulated events. As mentioned before, CTLL-2 cells arrest in the G_1 phase of the cell cycle and undergo apoptosis upon withdrawal of IL-2 from the culture medium. Interestingly, the execution of apoptosis was apparent only at ~24 h subsequent to the onset of a G_1 arrest, suggesting that the onset of apoptosis may require a prolonged arrest in the G_1 phase of the cell cycle to be irreversibly established. In addition, we observed up-regulation of $p27^{Kip1}$ levels upon IL-2 deprivation. $p27^{Kip1}$ is a *cdk* inhibitor that associates with G_1 cyclin/*cdk* complexes and inhibits their enzymatic activity. When $p27^{Kip1}$ protein levels increase, the cyclinE/*cdk2*-associated form of $p27^{Kip1}$ increases and *cdk2* is no longer able to phosphorylate pRb, leading to an arrest in the G_1 phase of the cell cycle (27).

Furthermore, we observed an up-regulation of Bim protein levels upon IL-2 withdrawal from CTLL-2. Bim is a proapoptotic BH3 domain-only member of the Bcl-2 family (28). Interestingly, Bim up-regulation upon IL-2 withdrawal occurred much later than $p27^{Kip1}$ up-regulation, which could explain our findings that the arrest in G_0/G_1 precedes the onset of apoptosis.

The Bcl-2 family consists of pro- and antiapoptotic members that can either homodimerize to promote a common function or heterodimerize to titrate their opposite functions (30, 31). In this model, either up-regulation of proapoptotic member or down-regulation of antiapoptotic member protein levels may lead to the induction of mitochondrial apoptosis. In this respect, it is worth noting that PKB has been described to induce the expression of the antiapoptotic Bcl-2 (9) and to inactivate the proapoptotic Bad by means of phosphorylation (32). Thus, PKB-mediated cell survival and protection from apoptosis occurs notably through interfering with the balance of pro- and antiapoptotic Bcl-2 family members. In this work, we show that proapoptotic Bim is down-regulated in a PI3K-dependent fashion in response to IL-2. This down-regulation appears to require inactivation of FoxO3, because expression of a mutant form of FoxO3 that can no longer be inactivated by the PI3K/PKB signaling pathway is sufficient to induce Bim expression. Bim appears to be an important mediator of the apoptosis seen in response to FoxO3 activation. For one, small changes in the expression of another proapoptotic FoxO3 target gene, namely Fas ligand, are observed upon IL-2 withdrawal or activation of FoxO3 in these cells, consistent with the general idea that apoptosis induced by cytokine withdrawal does not involve Fas signaling (33). Moreover, ectopic expression of Bim very efficiently kills these CTLL-2 cells (data not shown), indicating that the sole up-regulation of Bim is sufficient to drive these cells into apoptosis. Nevertheless, our data do not provide rigorous proof for an involvement of Bim in apoptosis caused by cytokine deprivation, and at present we cannot rule out that other targets of FoxO factors may exist that play a role in this.

Previously, we have reported a role for FoxO3 in IL-3-dependent survival signals in the Ba/F3 pre-B cell line (22). We show in this report that the effect of IL-2 on T cell survival is mediated, at least partly, by FoxO3, indicating that cytokine-mediated cell survival critically depends on the inactivation of FoxO3 or related forkhead factors. Taken together, these observations suggest that there is a conserved pathway from *C. elegans* to mammalians by which PI3K/PKB signaling regulates forkhead transcription factor activity to adapt cell survival and proliferation to the environmental conditions (nutrient, growth factors, and cytokine availability).

When an immune response is initiated, a resting T cell encounters Ag in the context of an APC and subsequent ligation of the TCR together with costimulatory molecules activates the T cell. This leads to expression of a functional IL-2R (through up-regulation of CD25) and secretion of IL-2. Autocrine stimulation by IL-2 now triggers the T cells to proliferate, thereby giving rise to an increased size of the Ag-specific T cell pool. Our results show that in Ag-triggered T cells IL-2 stimulation rapidly activates the PI3K/PKB pathway, which inactivates the forkhead transcription factors, leading to $p27^{Kip1}$ down-regulation (Fig. 7). This down-regulation results in the release of active cyclin-dependent kinase activity, allowing T cells to pass through the G_1 restriction point and to complete a full cell division.

Although a large pool of Ag-primed T cells is necessary to eliminate an invading pathogen, after its eradication the Ag-challenged T cell pool needs to reduce to its normal size to avoid excessive T cell accumulation. Next to a cell death pathway involving the death receptor Fas, the gradual loss of cytokines such as IL-2 has been suggested to be responsible for shutting off an immune response (33). Interestingly, T cell deletion by IL-2 depletion was found to require new gene expression because it can be blocked by actinomycin D and cycloheximide (33). Our data in both primary T cells and the IL-2-dependent CTLL-2 T cell line imply that, in this end stage of an immune response, IL-2 deprivation of the activated T cells leads to inhibition of PKB, activation and nuclear translocation of the forkhead transcription factors, and, finally, expression of $p27^{Kip1}$ and Bim (Fig. 7). The importance of (at least) Bim in immune homeostasis is underscored by the fact that Bim knockout mice accumulate T and B cells and develop signs of autoimmunity later in life (34).
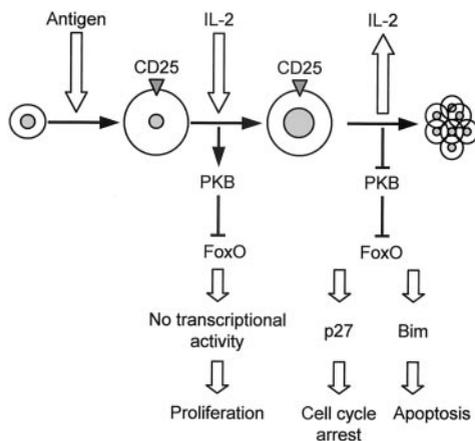
FIGURE 7. The IL-2 downstream pathway bifurcates at the level of the forkhead transcription factors to modulate both cell cycle and apoptosis. Early in an immune response, IL-2 stimulation promotes proliferation of newly Ag-primed CD25$^+$ T lymphocytes through activation of the PI3K/PKB pathway. Activated PKB phosphorylates FoxO members of the forkhead transcription factor family, thereby preventing their translocation to the nucleus and thus transcription of the cell cycle inhibitor p27^{Kip1}, allowing the T cells to proliferate. Later in the immune response, when Ag and IL-2 become limiting, withdrawal of IL-2 shuts down the PI3K/PKB pathway, releasing active FoxO forkhead transcription factors which can in turn activate transcription of target genes, such as p27^{Kip1} and Bim. The Cdk inhibitor p27^{Kip1}, via its brake activity on the cell cycle progression, induces an arrest in G$_1$ phase. Bim, a proapoptotic member of the Bcl-2 family, can induce apoptosis in the activated T cell pool. In conclusion, the IL-2 signaling pathway regulates the FoxO members of the forkhead transcription factor family and bifurcates at that level to exert a dual effect on both cell cycle and cell death via p27^{Kip1} and Bim, respectively.

Interestingly, in contrast to activated T cells, Bim cannot be detected in quiescent primary T cells, although our data suggest that these cells do contain active FoxO1a. Thus, in comparison to cytokine-depleted CTLL-2, transactivation of the Bim promoter by FoxO factors appears to be repressed in resting mature T cells. From a physiological point of view, the reason for such repression is evident, because elevated Bim levels could result in the death of peripheral quiescent T cells, as well as newly Ag-activated T cells. This would severely reduce their potential to generate an adapted immune response to a specific Ag. Nevertheless, the underlying mechanism for the lack of Bim induction is unclear at present. Obviously, the difference could be due to the distinct FoxO factors expressed in these two cell types (FoxO3 in CTLL-2 and FoxO1a in primary T cells), or one could speculate that distinct cofactors must exist that are involved in the activation or repression of the various FoxO target genes.

Taken together, our study indicates that the FoxO transcription factors may play an important role in IL-2-mediated effects on cell cycle progression as well as cell survival. This bifurcation of the IL-2 signaling pathway at the forkhead level enables coupling of G$_1$ progression to apoptosis in T cells. Regulation of the proliferation/death balance in T lymphocytes is critical to insure a fine-tuned immune reaction. Therefore, forkhead transcription factors, being at the crossroad of cell cycle regulation and apoptosis, might be critical modulators of the proliferation/death balance, and interference with their activity might destabilize the proliferation/apoptosis balance and lead to severe pathological disorders.

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