Identification of 24p3 as a Direct Target of Foxo3a Regulated by Interleukin-3 through the Phosphoinositide 3-Kinase/Akt Pathway

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Pro-apoptotic protein 24p3, a member of lipocalin family, is induced upon interleukin-3 (IL-3) deprivation and plays a pivotal role in induction of apoptosis in hematopoietic cells. However, the molecular mechanism by which IL-3 regulates 24p3 expression remains largely unknown. Here, we show that 24p3 is a direct target of Foxo3a and that phosphoinositide 3-kinase (PI3K)/Akt mediates IL-3-repressed 24p3 through regulation of Foxo3a. Inhibition of the PI3K/Akt (but not MAPK) pathway induced 24p3 expression and programmed cell death in FL5.12 cells. Furthermore, constitutively active Akt largely attenuated 24p3 expression and apoptosis in response to IL-3 withdrawal. Foxo3a directly bound to the 24p3 promoter and induced promoter activity. Akt abrogated wild-type Foxo3a-induced (but not Akt-non-phosphorylatable Foxo3aΔA)-induced 24p3 expression and promoter activity. Therefore, these data indicate for the first time that 24p3 is a Foxo3a target gene and that PI3K/Akt (but not MAPK) mediates IL-3-regulated 24p3 expression in hematopoietic cells.

Apoptosis is a genetically controlled cell suicide program that has been implicated in diverse biological processes such as the development and homeostasis of multicellular organisms and the defense mechanism against pathogens (1–3). Apoptosis in hematopoietic cells can be induced by withdrawal of cytokines such as interleukin-3 (IL-3)2 (4). IL-3 exerts its survival and proliferative functions through a common signaling subunit, the β2 receptor. Signal transduction events that regulate survival by IL-3 are mediated in part by cytosolic tyrosine kinases (5). In the absence of IL-3, IL-3-dependent progenitor cells undergo apoptosis (6, 7).

Another signaling molecule that is activated by IL-3 is phosphoinositide 3-kinase (PI3K) (8, 9). PI3K has been implicated in the regulation of survival in various cell types (10). Akt is a downstream target of PI3K and is able to support the survival of a number of cell types after survival factor deprivation (3, 11, 12). A previous study also examined the ability of activated forms of Akt to protect cells from death induced by withdrawal of IL-2 in BAF/3 cells (13). Akt activity is induced rapidly by IL-3, and the activation of Akt by IL-3 depends upon the activity of PI3K. Previous studies demonstrated that Akt protects IL-3-dependent FL5.12 cells from IL-3 depletion-induced apoptosis through phosphorylation of BAD (14, 15).

Pro-apoptotic protein 24p3 has been shown to be significantly elevated at the mRNA and protein levels in FL5.12 cells after IL-3 withdrawal, which was demonstrated to be essential for IL-3 deprivation-induced apoptosis (16). The conditioned medium from IL-3-deprived cells, which contains secreted 24p3, induces apoptosis in naïve cells, even when IL-3 is present. 24p3 also induces apoptosis in a wide variety of leukocytes, indicating that IL-3 deprivation enhances 24p3 transcription and leads to synthesis and secretion of 24p3, which induces apoptosis in an autocrine manner (16). In addition to murine FL5.12 pro-B cells, many other cell types are susceptible to 24p3-mediated apoptosis, including murine primary thymocytes, murine primary splenocytes, murine primary bone marrow cells, human primary neutrophils, and human peripheral blood lymphocytes. In contrast, human primary macrophages, HeLa cells, and Jurkat cells are not susceptible to 24p3-mediated apoptosis (16). In addition, the 24p3 receptor was recently identified in FL5.12 cells and found to bind to iron-bound and iron-free forms of 24p3 (17). However, their effects on cell survival are different. Iron-bound 24p3 increases intracellular iron concentration without promoting apoptosis; iron-free 24p3 decreases intracellular iron levels, which induces expression of the pro-apoptotic protein Bim, resulting in apoptosis. Recent studies also showed that the BCR-ABL oncoprotein activates expression of 24p3 but inhibits 24p3 receptor transcription in BCR-ABL-positive cells. Secreted 24p3 leads neighboring cells to apoptosis (17, 18). By inhibiting BCR-ABL, imatinib/Gleevec inhibits 24p3 transcription and induces 24p3 receptor expression. As a result, BCR-ABL-positive chronic myeloid leukemia cells undergo programmed cell death (17, 18).

Members of the forkhead transcription factor family play a pro-apoptotic role in neurons or hematopoietic cells. Akt antagonizes FOXO pro-apoptotic function through phosphorylation of serine/threonine residues, leading to FOXO translocation from the nucleus to the cytoplasm (19, 20). In this work, we show that Foxo3a, a major member of the FOXO
family, directly binds to the 24p3 promoter and induces 24p3 transcription in response to IL-3 withdrawal. Inhibition of the PI3K/Akt (but not MAPK) pathway antagonizes IL-3 down-regulation of 24p3. Activation of Akt inhibits 24p3 expression and apoptosis induced by IL-3 deprivation in FL5.12 cells through phosphorylation of Foxo3a.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Reagents, and Transfection**—IL-3-dependent FL5.12 murine pro-B cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% WEHI-3B-conditioned medium (a source of IL-3) (21). PD98059, wortmannin, and LY294002 were obtained from Calbiochem. Human recombinant insulin-like growth factor-1 was purchased from Invitrogen. Transfection of FL5.12 cells was carried out by electroporation. Cells were electroporated at 1 × 10^6 cells/400 µl at 275 V and 960 microfarads with 25 µg of plasmid DNA. Stable clonal cell lines were established after selection with G418 (600 µg/ml).

**Plasmids**—The human 24p3 gene was amplified by nested PCR using a human myeloid leukemia cDNA library (Stratagene). The primers used were 5'-1(5'-AGCAGCCACACAGGGGGGTGGAAG-3'), 5'-2 (5'-CTAGGTCTCCTGTG-3'), 3'-1 (5'-TCAATGTTGCTGCGGCTGGTGTG-3'), and 3'-2 (5'-TGCTCTAGACTCGAGGCCCGTCACTGTCGATTG-3'). Amplified DNA products were subcloned into the mammalian expression vector pcDNA3.5-GW-FLAG and the gluthathione S-transferase-fused vector pGEX-4T-1. For cloning the human 24p3 promoter, a 1.3-kb DNA fragment upstream transcription start site was amplified from human genomic DNA using nested PCR and primers 5-1(5'-AAGACGAATATCGCTCCTGCCCTAGGTCTCCTGTG-3'), 5-2, (5'-AGGAATTCCGATCCATGCTG-3'), 3-1 (5'-CTAGGTCTCCTGCGGCTGGCAGTGAAG-3'), and 3-2 (5'-CACCAGTTAGGAGTCCGCAGTGAAG-3'). Amplified DNA fragments were subcloned into the pGL3-Luc reporter vector (Promega). The integrity of all constructs was confirmed by DNA sequencing. Hemagglutinin-Foxo3a-Luc reporter vector (Promega). The integrity of all constructs was confirmed by DNA sequencing.

**Northern and Western Blot Analyses**—Northern blot analysis was performed as described previously (22). Briefly, the cells were lysed in lysis buffer, separated by SDS-PAGE, and immunoblotted with the appropriate antibodies as indicated in the figure legends.

**Cell Viability**—Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium dye reduction assay measuring mitochondrial respiratory function (23). FL5.12 cells were plated in 96-well plates (10^4 cells/well) and treated with conditioned media and inhibitors as indicated in the figure legends. Cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium dye (20 µl/well) for 2 h and solubilized with 10% SDS (25 µl/well) at room temperature for 4 h. Absorbance was determined in a Titertek plate reader at 490 nm.

**Chromatin Immunoprecipitation Assay**—Chromatin immunoprecipitation assay was performed essentially as described previously (24). Briefly, solubilized chromatin was prepared from a total of 2 × 10^7 asynchronously growing FL5.12 cells that were electroporated with hemagglutinin-Foxo3a. The chromatin solution was diluted 10-fold with chromatin immunoprecipitation dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1, 0.01% SDS, and protease inhibitors) and precleared with protein A beads. The precleared chromatin solution was utilized for immunoprecipitation assay with either Foxo3a (Upstate) or anti-actin antibody. Following washing, the antibody-protein-DNA complex was eluted from the beads by resuspending the pellets in 1% SDS and 0.1 M NaHCO_3 at room temperature for 20 min. After a reverse cross-link, protein and RNA were removed by incubation with 10 µg of proteinase K and 10 µg of RNase A at 42 °C for 3 h. Purified DNA was subjected to PCR with primers specific for three putative Foxo3a-binding sites in the 24p3 promoter. The PCR primers used were as follows: region 1, 5'-CAGAGACAGCAACAGTGTTTCAATGTGCCC-3' (forward) and 5'-CATCCCTCTGTGCTGCGCCATACACC-3' (reverse); region 2, 5'-TACCTTTGGAAGGCGCCAACGGGCGTG-3' (forward) and 5'-AACAGACCGTGTTTCCGACGTG-3' (reverse); and region 3, 5'-TGCTCTAACCAGTCAGTGTCGTTG-3' (forward) and 5'-GGCCATGCTTCCACACAGCTACATGTGTCGTT-3' (reverse). Amplified PCR products were resolved on 1.2% agarose gel and visualized by BioImage.

**RESULTS**

**PI3K/Akt (but not MAPK) Pathway Inhibits 24p3 Expression Induced by IL-3 Deprivation and Mediates IL-3 Survival Signal in FL5.12 Cells**—Previous studies have demonstrated that IL-3 activates Akt, which results in the phosphorylation of BAD and cell survival in IL-3-dependent FL5.12 cells. Deprivation of IL-3 reduces BAD phosphorylation, leading to programmed cell death (14, 15). Transcriptional induction of 24p3 has been shown, however, to play a pivotal role in induction of apoptosis...
We further examined the role of the PI3K/Akt and MAPK pathways in IL-3-dependent cell survival. FL5.12 cells were treated with or without LY294002 or PD98059 in the presence and absence of IL-3. As shown in Fig. 1 (B and C), inhibition of PI3K induced greater cell death compared with blockade of the MAPK pathway. Moreover, ectopic expression of constitutively active Akt inhibited cell death induced by IL-3 deprivation (Fig. 1, D and E), whereas expression of dominant-negative Akt largely abrogated IL-3-protected apoptosis (Fig. 1D). However, neither constitutively active nor dominant-negative Akt had a significant effect on FL5.12 cell proliferation (data not shown). These data indicate that the PI3K/Akt pathway mediates primarily IL-3 action in 24p3 expression and cell survival.

**PI3K/Akt Reduces 24p3 mRNA Expression**—Having observed the PI3K/Akt pathway to mediate IL-3-regulated 24p3 protein expression, we next investigated whether PI3K/Akt regulates 24p3 at the transcriptional level. Following culture of FL5.12 cells in the absence and presence of IL-3 and treatment with LY294002 or PD98059, Northern blot analysis showed that the mRNA level of 24p3 was elevated upon IL-3 withdrawal. The addition of IL-3 repressed 24p3 mRNA expression. Inhibition of PI3K (but not MEK) reduced the effect of IL-3 on 24p3 mRNA expression (Fig. 2A). Furthermore, expression of constitutively active Akt inhibited IL-3 withdrawal-induced 24p3, whereas dominant-negative Akt partially abrogated IL-3-repressed 24p3 mRNA expression (Fig. 2, A and B).

**Isolation of the Human 24p3 Promoter and IL-3 Inhibition of 24p3 Promoter Activity through the PI3K/Akt Pathway**—To further analyze the transcriptional regulation of 24p3 by Akt, we defined the transcription start site using 5'-rapid amplification of cDNA ends and cloned the 1.3-kb DNA upstream transcription start site of the human 24p3 gene (Fig. 3A and supplemental Fig. 1). Sequence analysis revealed three putative binding sites (TTGTTTAC) for Foxo3a (20, 24, 25). Because the 24p3 promoter contains three putative Foxo3a-binding sites and because Foxo3a is a major target of Akt (20), we investigated whether the 24p3 pro-

**FIGURE 1.** PI3K/Akt (but not MAPK) pathway mediates IL-3 regulation of 24p3 and cell survival. A, FL5.12 cells were transfected with the indicated plasmids, treated with 50 μM PD98059 or 10 μM LY294002 for 12 h, and then immunoblotted with the indicated antibodies. HA, hemagglutinin. B and C, FL5.12 cells were treated with the indicated agents as described for A. Cell death was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and DNA fragmentation. D and E, FL5.12 cells were stably transfected with constitutively active Akt or dominant-negative (DN) Akt and treated with 50 μM PD98059 or 10 μM LY294002 for 12 h. For cell survival, the experiments were done three times with triplicate samples for each treatment. Cell death was quantified with Student’s t test. The error bars represent S.D. (n = three independent experiments).
moter is regulated by Foxo3a. HEK293 cells were transfected with pGL3-24p3-Luc, β-galactosidase, and increasing amounts of Foxo3a. Fig. 4A shows that 24p3 promoter activity was induced by Foxo3a in a dose-dependent manner. Coexpression of constitutively active Akt repressed the promoter activity induced by Foxo3a but not by Foxo3aA3, a mutant form that cannot be phosphorylated by Akt (Fig. 4B). Moreover, Foxo3a-induced 24p3 promoter activity was largely inhibited by IL-3 treatment in FL5.12 cells. In contrast, IL-3 treatment had no effect on Foxo3aA3-induced 24p3 promoter activity (Fig. 4C).

To further demonstrate that IL-3 regulates 24p3 through the Akt/Foxo3a axis, we examined the phosphorylation level of Foxo3a and Akt expression in response to IL-3 treatment. FL5.12 cells were transfected with constitutively active Akt [myristoylated Akt (Myr-Akt)] or pcDNA3 vector and then treated with and without IL-3. Immunoblot analysis showed that IL-3 deprivation reduced the phosphorylation levels of Foxo3a and Akt and increased 24p3 expression in pcDNA3-transfected cells (Fig. 5A, second lane) but not in Myr-Akt-transfected cells (fourth lane). Furthermore, expression of Akt-non-phosphorylatable Foxo3aA3 abrogated IL-3-inhibited
24p3 expression at the protein (Fig. 5B) and mRNA (Fig. 5C) levels. In addition, knockdown of Foxo3a largely attenuated 24p3 levels induced by IL-3 withdrawal (Fig. 5D). In contrast, inhibition of Akt by either Akt inhibitor API-2 or Akt short hairpin RNA induced 24p3 expression (Fig. 5, E and F). Taken collectively, these data indicate that 24p3 is a direct downstream target of Foxo3a and that Foxo3a-regulated 24p3 is controlled by the IL-3/Akt pathway.

Defining the Foxo3a Response Element(s) in the 24p3 Promoter—The 24p3 promoter contains three putative Foxo3a-binding sites, and thus, we next defined which binding site(s) is required for the promoter to respond to Foxo3a. A series of deletion mutants of the 24p3 promoter were created and used for the luciferase reporter assay. As shown in Fig. 6A, the deletion mutant promoter (P3) created by removal of region 1/10-1002/340 largely induced by Foxo3a, whereas the mutant promoters (P4 and P5) with deletion of the region(s) containing the third and/or the second Foxo3a-binding sites largely reduced the ability to respond to Foxo3a, suggesting that major Foxo3a response elements in the promoter are located in the last two Foxo3a-binding sites and that a repression element(s) may reside in the region between bp 304 and 18. Furthermore, chromatin immunoprecipitation assay revealed that Foxo3a bound to the second and third sites in vivo (Fig. 6B). In addition, luciferase reporter assay using point mutation of each Foxo3a-binding site demonstrated that the last two binding sites are indeed required for the 24p3 promoter to respond to Foxo3a (Fig. 6C).
**IL-3 Regulation of 24p3 via the PI3K/Akt/FOXO Pathway**

**A**

Conditioned Medium from Myr-Akt-FL5.12 Cells Fails to Induce Apoptosis, and Myr-Akt-FL5.12 Cells Resist Apoptosis Induced by Conditioned Medium Expressing 24p3—Previous studies have shown that conditioned medium from IL-3-deprived FL5.12 cells expresses 24p3 and induces apoptosis in a number of hematopoietic cell lines (16). Because Akt/Foxo3a mediates IL-3-regulated 24p3 expression, we further examined the cell viability of naïve FL5.12 cells following treatment with different conditioned media. As shown in Fig. 7A, the conditioned media from pcDNA3-transfected and parental FL5.12 cells induced naïve FL5.12 cell death, whereas the conditioned medium from constitutively active Akt-transfected FL5.12 cells contained very low levels of 24p3 and had no significant effect on cell viability. In contrast, all conditioned media failed to induce cell death in constitutively active Akt-transfected FL5.12 cells (Fig. 7B). These results further support our findings that 24p3 is regulated by IL-3 through the PI3K/Akt/FOXO pathway and that activation of Akt not only inhibits 24p3 expression induced by IL-3 deprivation but also reduces 24p3-induced cell death (Fig. 7C).

**DISCUSSION**

Previous studies have shown that Akt mediates the IL-3 cell survival signal in hematopoietic cells through phosphorylation of BAD, which leads to the switching of BAD from the Bcl-2 and Bcl-xL complex into 14-3-3 and the loss of its pro-apoptotic function (14). 24p3 has recently been identified as a key molecule for IL-3 withdrawal-induced apoptosis in IL-3-dependent cells (16). Neutralization of 24p3 using anti-24p3 antibody largely reduces cell death induced by IL-3 deprivation (16). 24p3 is transcriptionally regulated by IL-3 signaling, i.e. withdrawal of IL-3 induces and addition of IL-3 represses 24p3 expression at both the protein and mRNA levels. However, the underlying molecular mechanism is unknown. We have demonstrated in this study that IL-3 regulation of 24p3 is mediated by the Akt/Foxo3a axis. Transcription factor Foxo3a directly binds to and transactivates the 24p3 promoter. The regulation of 24p3 by Foxo3a is controlled by IL-3 through the PI3K/Akt (but not MAPK) pathway. IL-3 activates Akt, which leads to phosphorylation and inhibition of Foxo3a function for 24p3. IL-3 deprivation results in dephosphorylation and activation of Foxo3a and increases the expression of 24p3. Moreover, expression of constitutively active Akt inhibits FL5.12 cell death after deprivation of IL-3, which is due to suppression of 24p3 transcription through phosphorylation of Foxo3a.

FOXO transcription factors are crucial regulators of cell fate. It has been shown that FOXO transcription factors control cell survival by regulation of its downstream target genes. The common FOXO target genes that are involved in apoptosis include BNIP3 and bim (24, 25). In addition to direct targets, FOXO also indirectly down-regulates the expression of pro-survival Bcl-xL by inducing the expression of transcriptional repressor Bcl-6 (26). In neurons, Foxo3a triggers cell death circuitously by inducing the expression of Fas ligand, which activates programmed cell death through the death receptor pathway (20). In hematopoietic cells, we have shown in this study that 24p3 is a direct target of Foxo3a and provokes cell death after IL-3 deprivation in naïve FL5.12 cells. Foxo3a up-regulates 24p3 transcription through directly binding to the 24p3 promoter.

In addition, a previous study demonstrated that 24p3 induces apoptosis in FL5.12 cells even in culture medium containing IL-3 (16). However, our data show that constitutively active Akt-transfected FL5.12 cells resist apoptosis induced by conditioned medium expressing 24p3 (Fig. 7A). Because IL-3 induces Akt activation, it is difficult to understand how constitutively active Akt can override 24p3-induced cell death. A possible mechanism is that constitutively active Akt might regulate the 24p3 receptor cascade and other pro-apoptotic molecules prior to 24p3 treatment. Further investigations into the underlying mechanism are required.
ings indicate that 24p3 is a direct target of Foxo3a and that the PI3K/Akt (but not MAPK) pathway mediates IL-3 function in 24p3 in hematopoietic cells.

Acknowledgments—We are grateful to the DNA Sequence and Flow Cytometry Facilities at the H. Lee Moffitt Cancer Center.

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In summary, we have demonstrated that Foxo3a directly regulates 24p3 expression and mediates IL-3 signaling in hematopoietic cells. Depreservation of IL-3 decreases Akt phosphorylation of Foxo3a, which leads to the induction of 24p3 transcription and cell death. Inhibition of PI3K/Akt (but not MAPK) increases 24p3 expression, whereas activation of Akt reduces 24p3 and inhibits 24p3-induced apoptosis.