The Forkhead Transcription Factor AFX Activates Apoptosis by Induction of the BCL-6 Transcriptional Repressor*

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The activation of the AKT/protein kinase B kinases by mutation of the PTEN lipid phosphatase results in enhanced survival of a variety of tumors. This resistance to apoptosis is partly accomplished by the inhibition of genetic programs induced by a subfamily of forkhead transcription factors, including AFX. Here we describe an AFX-regulated pathway that appears to account for at least part of this apoptotic regulatory system. Cells induced to synthesize an active form of AFX die by activating the apoptotic death pathway. An analysis of genes regulated by AFX demonstrated that BCL-6, a transcriptional repressor, is up-regulated 4–7-fold. An examination of the BCL-6 promoter demonstrated that AFX bound to specific target sites that could activate transcription. BCL-X\(_l\) is an anti-apoptotic protein, contains potential BCL-6 target sites in its promoter. An analysis of endogenous BCL-X\(_l\) levels in AFX-expressing cells revealed enhanced down-regulation of the transcript (~1.3–1.7-fold) and protein, and BCL-6 directly binds to and suppresses the BCL-X\(_l\) promoter. Finally, macrophages isolated from BCL-6–/– mice show enhanced survival in vitro. These results suggest that AFX regulates apoptosis in part by suppressing the levels of anti-apoptotic BCL-XL through the transcriptional repressor BCL-6.

The resistance of tumors to diverse apoptotic stimuli is of major clinical importance. A large number of recent studies has highlighted the significance of hyperactivation of the PI3K pathway to the insensitivity of late stage tumors to chemotherapy and radiation treatments (1–6). The induction of this pathway by growth factor or oncogenic stimuli results in the production of three phosphorylated phosphatidylinositol lipids, which act as binding sites for the pleckstrin homology domains of the downstream kinases, PDK-1 and AKT/PKB (7–13). The membrane colocalization of these kinases results in the phosphorylation of a specific activation site in AKT/PKB by PDK-1 (7, 8, 13–15). Importantly, this pathway is reversed by the lipid phosphatase activity of PTEN, a tumor suppressor that is homozygously deleted in a variety of late stage tumors (2–6). Whereas a number of phosphorylation targets has been described for the AKT/PKB kinases, both biochemical and genetic evidence support a subset of forkhead transcription factors including FKHR, FKHR-L1, and AFX as being important substrates for phosphorylation mediated by these kinases (16–20). Phosphorylation of these forkhead transcription factors by AKT/PKB kinases results in their sequestration to the cytoplasm in which they are unable to activate transcription of their nuclear targets (17–20). This outcome is an important component of cell survival mediated by the PI3K pathway, because nuclear localization of these forkhead family members results in the induction of transcriptional programs that lead to rapid cell death by apoptosis (17–20).

Whereas it is clear that the inhibition of the apoptotic programs activated by these forkhead transcription factors is a critical component of the pro-survival PI3K pathway, the pro-apoptotic genes that are transcribed by these proteins remain largely unknown. The induction of FKHR-mediated transcription results in a profound up-regulation of IGFBP-1 transcript (21) as well as p27\(^{kip}\) protein levels (20, 22, 23), and although it is likely that the induction of p27\(^{kip}\) results in cell cycle arrest, it is not clear how either of these targets could induce the rapid cell death that is observed. Recently, the expression of the pro-apoptotic BH3-only protein, BIM, was found to be induced in lymphocytes by growth factor withdrawal or FKHR-L1. However, although overexpression of this protein induced apoptosis, it was unclear whether BIM alone was sufficient to mediate FKHR-L1-mediated cell death in other cell types (24).

To isolate genes that are potentially involved with AFX-mediated apoptosis, we have produced cell lines that can be induced to synthesize a constitutively active form of AFX, and we have used these cell lines to examine AFX-modulated transcriptional changes. Here we describe an AFX-induced transcriptional program that involves the induction of the transcriptional repressor, BCL-6. An examination of potential targets for BCL-6 repression revealed that the gene encoding the anti-apoptotic BH3 protein, BCL-X\(_l\), contains BCL-6/STAT-binding sites, and we demonstrate that the induction of the AFX-transcriptional program results in the down-regulation of BCL-X\(_l\) transcript and protein levels. Finally, we demonstrate the novel and unexpected finding that macrophages isolated from BCL-6–/– mice die with slower kinetics as compared with wild-type macrophages. These results thus describe a novel pro-apoptotic transcriptional repression program that is activated by nuclear localization of the AFX-type forkhead protein.

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† To whom correspondence should be addressed. Tel.: 415-388-5440; Fax: 650-225-6127; E-mail: larsky@earthlink.net.
‡ The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; AKT/PKB, AKT/protein kinase B; IGFBP-1, insulin-like growth factor-binding protein-1; STAT, signal transducers and activators of transcription; GFP, green fluorescent protein; TM, triple mutant; IRS, insulin-binding protein-1; STAT, signal transducers and activators of transcription; DOX, doxycycline; GST, glutathione S-transferase; mRNA, messenger RNA; pTEN, phosphatase with tensin homology.

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DNA Constructs—The full-length wild-type AFX gene was PCR amplified from human brain Marathon-ReadyTM cDNA (CLONTECH) and subcloned with a FLAG tag at its amino terminus into the pEGFP-N3 vector (CLONTECH). All three AKT/PKB phosphorylation sites in AFX were mutated to alanines by a two-step PCR procedure. The final TM-AFX-GFP sequence was verified by DNA sequencing. This pEGFP-N3 construct was then co-transfected with pEGFP-N3.TM-H152R-AFX from AFX that has an additional mutation in the DNA-binding domain was used to transiently transfected 293E cells as well as BJAB cells for promoter assays. To engineer the HeLa Tet-On™ AFX stable cell lines, the TM-AFX-GFP insert was subsequently subcloned into the pTRE response plasmid (CLONTECH). BCL-6 promoter constructs were made by amplying different length promoter fragments from human genomic DNA (GEX-TECH) using primers shown in Fig. 3A and inserting them upstream of the SV40 promoter in the luciferase pGL3 promoter vector (Promega). The BCL-6 promoter luciferase construct was created using the pGL3 vector (Promega). The pCDNA3.1.BCL-6 plasmid was constructed by subcloning the BCL-6 gene isolated from human brain Marathon-ReadyTM cDNA (CLONTECH) into pCDNA3.1/V5-His TOPO vector (Invitrogen). Plasmids expressing wild-type and mutant AFX-GFP binding domain fused to GST (pGEX.4T.TM-AFXXB and pGEX.4T.TM.H152R-AFXB) were created by inserting the domain sequence (Gly86Ser237) in pGEX.4T-1. Finally, a carboxyl-terminal portion of BCL-6 (Phe510–Cy597), which contains the zinc-finger domain, was fused to GST in pGEX.4T-1 to generate the plasmid pGEX.4T.BCL-6.ZnF.

Cells and Stable Cell Lines—The HeLa Tet-On™ cell line (CLONTECH) was maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, l-glutamine, penicillin-streptomycin, and Geneticin (200 μg/ml, Invitrogen). HeLa Tet-On™-derived AFX stable cell lines maintained in the same medium with an addition of hygromycin B (200 μg/ml, CLONTECH) were engineered by transfecting HeLa Tet-On™ cells with pTRE.TM-AFX and pTRE-Hyg and selecting for hygromycin B-resistant clones.

Differential Gene Expression Analysis—GeneCalling™ software was used to make binary comparison of traces derived from sample sets upon which GeneCalling™ chemistry had been performed and to compare binary comparisons with one another. Sequence information and precise electrophoretic mobility of differentially expressed genes identified by GeneCalling™ software were confirmed as modulated by GeneCalling™ poising (a competitive PCR reaction) or by TaqMan™, quantitative PCR.

Gene Expression—HeLa Tet-On™-derived AFX stable cell lines were induced to synthesize TM-AFX-GFP by doxycycline hydrochloride (2 μg/ml, CLONTECH). For Western blotting, whole-cell extracts were resolved on 4–20% Tris-glycine polyacrylamide SDS gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). Proteins were detected using antibodies directed against BCL-6 (N-3 and/or C-19, 1:200 dilution, Santa Cruz Biotechnology, Inc.), BCL-6 (1:19, 1:200 dilution, Santa Cruz Biotechnology, Inc.), BAX (1:1000 dilution, Pharmingen), β-tubulin (1:2000 dilution, Roche Molecular Biochemicals), P-gp (1:1000 dilution, Pharmingen), p27 KIP1 (1:1000 dilution, PharMingen), cDCX (1:2500 dilution, PharMingen), p18 (1:1000 dilution), p21 (1:1000 dilution), p53 (1:1000 dilution), cdc42 (1:1000 dilution), Ras (1:1000 dilution), Her-2 (1:1000 dilution), cyclin D1 (1:1000 dilution), cyclin A1 (1:1000 dilution), cyclin B1 (1:1000 dilution), cyclin E1 (1:1000 dilution, Santa Cruz Biotechnology, Inc.).

RESULTS

To identify genes that are regulated by the AFX-type forkhead transcription factor, we derived HeLa cell lines that could be induced to generate a mutated form of AFX, which cannot be phosphorylated by the AKT/PKB kinase. Because phosphorylation of this transcription factor regulates its subcellular localization (17–20), a non-phosphorylatable form of the protein would be localized to the nuclear compartment in which it would constitutively activate the transcription of target genes. The AFX cDNA was used to control the expression of the Tet-On™ system, and its expression could thus be regulated by the addition of doxycycline (DOX). In addition, AFX would bind to a carboxyl-terminal fluorescent protein (GFP) at its carboxyl terminus, and we could thus follow the expression of the protein as well as its subcellular localization in real time. Fig. 1A illustrates that cells induced to express the AFX-GFP protein showed complete nuclear localization of the transcription factor. In addition, cells expressing AFX-GFP showed nuclear condensation and cell

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surface blebbing that are characteristic of apoptosis. An analysis of cell viability revealed that cells began to die ~18–24 h after induction of AFX, and ~70% cells were dead at 48 h after induction (Fig. 1B). The 20% apoptosis exhibited by this cell line in the presence of DOX for 18 h was the basal level (Fig. 1D). Further analysis demonstrated that cells expressing AFX

Fig. 1. Characterization of the Tet-On™-inducible AFX stable cell line. A, shown are the phase (left panels) and immunofluorescence (right panels) micrographs of a HeLa cell line engineered to produce a constitutively active non-phosphorylatable mutant of AFX with the green fluorescent protein at its carboxyl terminus (TM-AFX-GFP). TM-AFX-GFP localizes constitutively in the nuclei and induces cell death. No TM-AFX-GFP fusion protein is observed in the HeLa Tet-On™-inducible AFX stable cell line 15-14 in the absence of DOX (top right panel), and the cells appear normal after 24 h (top left panel). In the presence of a 24-h DOX treatment, AFX stable cells produce TM-AFX-GFP, which is detected in the nuclei (bottom right panel). Some cells have undergone apoptosis as indicated by cell surface blebbing (bottom left panel), and there is a noticeable decrease in adherent cell number. B, TM-AFX-GFP induction leads to increasing apoptotic population starting between 18 and 24 h. After 18, 24, and 48 h of incubation in DOX, both floating and adherent cells were harvested and assayed for levels of apoptosis by trypan blue exclusion. Percentage of apoptosis represents the percent of trypan blue-positive cells in the total cell population. Error bars indicate the means ± S.E. p = 0.00097 for 18 versus 24 h, p = 2.31E-06 for 24 versus 48 h. C, DOX induction of AFX results in an increase of p27KIP1 protein levels and poly(ADP-ribose) polymerase cleavage. At the indicated times after the addition of DOX, both floating and adherent cells were harvested and analyzed by Western blotting. TM-AFX-GFP protein detected by an anti-FLAG antibody appears at 3 h after DOX induction and continues to accumulate to significant levels over 24 h. The p27KIP1 protein, a cell cycle inhibitor that is known to be regulated by the AFX-type forkhead transcription factors, begins to accumulate 6–9 h after the addition of DOX. After 12 h of DOX induction, poly(ADP-ribose) polymerase degradation is initiated. D, caspase inhibitor zVAD-fmk prevents cells from AFX-induced apoptosis. AFX stable cells grown for 48 h in the presence or absence of DOX in combination with 0, 25, 50, or 100 μM zVAD-fmk were assayed for levels of apoptosis by trypan blue exclusion. Percentage of apoptosis represents the percent of trypan blue-positive cells in the total cell population. Error bars indicate the means ± S.E. E, IGFBP-1 transcription is activated by AFX induction (21). Inducible AFX stable cells were grown in the presence or absence of DOX for the indicated times. Total RNA was isolated from adherent cells and analyzed by TaqMan™-quantitative PCR. A significant increase in IGFBP-1 RNA level is already detected at 6 h after DOX induction. The RNA levels continue to increase over 18 h of DOX incubation. No induction of IGFBP-1 transcription is observed in the absence of DOX. RNA levels were normalized to GAPDH mRNA levels. Every sample was analyzed in triplicate in each TaqMan™ experiment.
A. GeneCalling™ analysis (25) of transcripts in AFX stable cells induced versus non-induced with DOX for various times reveals several bands that were significantly up-regulated in the induced samples. Subsequent poisoning and TrapPing experiments (25) confirmed that these bands correspond to the BCL-6 gene (data not shown). A representative band, l0c0-124.4, highlighted here by a red vertical line is up-regulated 4.8-fold in cells induced with DOX for 18 h (E118+D) as compared with that in non-induced cells (E118−D). B. BCL-6 transcript levels increase with DOX induction. AFX stable cells were grown in the presence or absence of DOX. At the indicated times after DOX treatment, total RNA was isolated from adherent cells and subjected to TaqMan™-quantitative PCR. Relative BCL-6 RNA levels are depicted after normalizing to GAPDH mRNA levels. Similar results were obtained when RNA levels were normalized to β-actin mRNA levels (data not shown). After 5 h of DOX induction, BCL-6 transcript level is increased ~3-fold over that of a 0-h sample. At 20 h after DOX induction, BCL-6 reaches a maximum induction of ~6.7-fold, similar to that observed by the GeneCalling™ procedure. After 15 h of DOX induction, the level of BCL-6 transcript levels off. Every sample was analyzed in triplicate in each TaqMan™ experiment. Data shown are the averages of three independent experiments. Error bars represent the means ± S.E.C. BCL-6 protein levels increase in the presence of DOX-induced AFX expression. After growing in the presence or absence of DOX for the indicated times, the parental HeLa Tet-On™ cells and the AFX stable cells (cell line 15-14) were lysed and analyzed by Western blotting. Left panel, anti-FLAG antibody reveals that TM-AFX-GFP is not present in HeLa Tet-On™ cells even in the presence of DOX. In the 15-14 AFX stable cells, there is a small but detectable amount of TM-AFX-GFP expression (arrow) in the absence of DOX, although the levels do not increase over time. In contrast, in the presence of DOX, a high level of AFX expression is achieved beginning at 3 h of DOX treatment. Anti-BCL-6 antibody detects a small amount of BCL-6 protein in HeLa Tet-On™ cells at 24 h regardless of DOX treatment (right panel). 15-14 AFX stable cells, which have a slightly leaky expression of AFX, also show small levels of BCL-6 protein in the...
were undergoing death by apoptosis (27). An examination of caspase 3 activation (data not shown) as well as poly(ADP-ribose) polymerase cleavage (Fig. 1C) demonstrated that these two components of the apoptotic death pathway were both induced in response to AFX synthesis. In addition, cell death could be almost completely inhibited by the pan-caspase inhibitor zVAD-fmk (Fig. 1D), consistent with the activation of caspases being responsible for the observed cellular mortality. Finally, two genes that are known to be induced in response to AFX-type forkhead transcription factors, p27kip1 (Fig. 1C) (20, 22, 23) and IGFBP-1 (Fig. 1E) (21), were both up-regulated after AFX induction. Together, these results demonstrated that the induction and nuclear localization of AFX alone were sufficient to activate the apoptotic cell death pathway in this cell system.

The data of Fig. 1 suggest that the activation of a transcription program by AFX is sufficient to induce the apoptotic death pathway through caspase activation. To examine genes that are regulated by AFX, we analyzed the transcripts in cells at various times after AFX induction using the GeneCalling procedure (25). Previously, we and others have utilized (25, 28) this method for the analysis of differentially expressed transcripts in cell lines. An analysis of transcripts in DOX-induced versus non-induced cell lines revealed a large number of AFX-affected mRNAs, and a complete analysis of these changes will be published elsewhere. A potentially interesting gene that appeared to be regulated by AFX encoded BCL-6, a transcriptional repressor that was previously shown to be involved with germinal center formation and apoptosis (29–34). As Fig. 2A illustrates, the GeneCalling procedure revealed that a fragment corresponding to the BCL-6 mRNA was clearly induced ~18 h after AFX synthesis was initiated. To examine this induction in a more quantitative manner, TaqMan-quantitative PCR analysis was performed at various times after AFX induction. Fig. 2B illustrates that BCL-6 transcript was up-regulated in response to AFX synthesis with maximal transcript at ~20 h after AFX induction. In addition, this time course revealed that BCL-6 expression was significantly induced by 5 h after the addition of DOX and that the transcript began to level off after the 15-h time point (Fig. 2B). Whereas these data suggested that the transcript was regulated in response to AFX synthesis, Fig. 2C illustrates that the protein was also induced in response to AFX with a lag time of ~3 h after the appearance of AFX. Importantly, BCL-6 was not induced in the absence of DOX or in cell lines not engineered to express mutant AFX (Fig. 2C). Finally, Fig. 2D illustrates that the BCL-6 transcript is up-regulated by the inhibition of the AKT/PKB pathway with the PI3K inhibitor LY294002, consistent with a role for this survival kinase in the regulation of AFX transcriptional activity. Similar up-regulation of BCL-6 transcript levels was observed in the human embryonic kidney cell line 293E treated with LY294002 for 24 h (data not shown). Together, these data suggested that BCL-6 is a transcriptional target gene for AFX.

An examination of the BCL-6 promoter region (35) revealed that it contains a number of potential consensual AFX-binding sites (5′-(G/C)(A/C)N(G/a)(A/T)(T/c)(G/c-T/g)(T/a)(G/g)−3′) (36). To examine the functional role of these sites, we produced various truncations of the promoter region and analyzed the activation of a downstream luciferase reporter gene in response to cotransfected AFX. As a control, we also cotransfected a form of AFX with a single point mutation (151HR152153) that abolishes DNA binding and transcriptional activation (19). Fig. 3B illustrates that a promoter fragment containing ~1296 nucleotides upstream of the TATA box gave a 6-fold increase in luciferase production in response to transfected wild-type AFX but showed no response to the DNA-binding mutant of AFX. Fragments corresponding to ~600 and ~185 nucleotides upstream of the TATA box also showed a significant although decreased response to AFX, whereas a control plasmid lacking the BCL-6 promoter region showed no enhanced response. These data suggested that multiple sequence elements in the BCL-6 promoter might act as functional AFX-binding sites. To examine these sites for AFX binding, we performed gel shift assays using wild-type and mutant AFX DNA-binding domains. The S6 site bound avidly to the wild type but not to the DNA-binding mutant of AFX (Fig. 3, C and D). Other sites also appeared to bind to the wild-type DNA-binding domain although with apparently less affinity than the S6 site. Furthermore, the S6-binding site can compete specifically for AFX binding with itself as well as with a known forkhead-binding site (irs) derived from the IGFBP-1 promoter (19), whereas the S2 site that did not interact with AFX failed to compete (Fig. 3D). The importance of the S6 target site was further verified by the observation that a single nucleotide mutation of this site that was shown to result in greatly decreased AFX binding (data not shown) resulted in significantly lower AFX activation of the BCL-6 promoter (Fig. 3E), approximately equal to that seen for the truncation mutant lacking the S6-binding site (Fig. 3B). Finally, the examination of the BCL-6 promoter region also revealed a consensus BCL-6-binding site (5′-(T/a/c/g)(T/c)(C/a/t)(C/t/g)/(T/a)/(A/c/g)GAA (A/T)(G/a/c)-3′) (Fig. 3A) (29, 35). Because BCL-6 is a transcriptional repressor, we reasoned that this site might act to regulate negatively AFX-induced BCL-6 expression. Fig. 3F illustrates that increasing levels of AFX expression initially up-regulate and then down-regulate BCL-6 promoter activity. In addition, the mutation of the BCL-6-binding site significantly enhances the response of the promoter to transfected AFX, although the promoter is still ultimately down-regulated. These data suggest that there is a negative feedback effect of AFX on the BCL-6 promoter that is in part probably due to endogenously produced BCL-6 regulating its own promoter negatively. Similar results were observed when luciferase assays were performed in BJAB cells, a lymphoid cell line (Fig. 3G).

Because the PI3K lipid kinase pathway controls the AKT/ PKB protein kinases (1), we searched for survival-regulating genes that might also be regulated by this pathway through the expression of BCL-6. The levels of the critical pro-survival BH3 protein BCL-XL have been found to be regulated by the PI3K pathway in a number of studies, and several reports have suggested that BCL-XL levels can be down-modulated by the overexpression of BCL-6 (19, 33, 37, 38). Therefore, we examined whether BCL-XL levels were modulated in response to AFX expression. An analysis of endogenous transcript levels of BCL-XL demonstrated that although the levels of the transcript appeared to fall because of the depletion of the medium by proliferating cells, the levels in cells expressing AFX fell ~5–6 h earlier than cells not expressing the transcription
factor (Fig. 4A). Importantly, the levels of BCL-X<sub>L</sub> protein were significantly down-regulated with time after AFX induction as compared with β-tubulin and the pro-apoptotic proteins BCL-X<sub>L</sub> and BAX (Fig. 4, B and C). The decline in BCL-X<sub>L</sub> protein level was not detectable until ~24 h after the initiation of DOX treatment. We reasoned that because only a small
percentage of cells had undergone apoptosis at this point (~10% above the basal level), it was difficult to detect any slight change in BCL-XL protein levels by Western blotting of the whole population. Accordingly, we began to observe a more significant reduction in BCL-XL protein levels after 28 h and an almost complete loss of the protein at 48 h at a time when the majority of cells (~70%) had undergone apoptosis. Furthermore, the appearance of cleaved caspase 3, indicative of caspase activation, correlated with the down-regulation of BCL-XL protein levels (Fig. 4C). Fig. 4C also illustrates that the fall in BCL-XL protein levels is not merely because of the activation of caspases, since inclusion of the pan-caspase inhibitor zVAD-fmk modified the rate of BCL-XL down-regulation only slightly. Finally, cotransfection of the triple mutant form of AFX together with a BCL-XL promoter linked to a luciferase reporter revealed down-regulation of this downstream target by AFX (Fig. 4D). Together, these results were consistent with a loss of BCL-XL transcript and protein accompanying the induction of cell death in the AFX-expressing cells.

Previous studies demonstrated that BCL-XL expression is controlled by STAT transcription factor activity (39–41), and the examination of the BCL-XL promoter region revealed the presence of several potential STAT-binding “GAS” sites that are also possible binding sites for BCL-6 (29, 39). To test whether BCL-6 can directly down-regulate the BCL-XL promoter, a cotransfection experiment was performed. A fragment encoding the ~1000-nucleotide promoter region of BCL-XL was placed 5’ to the luciferase reporter, and the expression from this promoter was analyzed in the presence of increasing levels of BCL-6 protein. The expression from the BCL-XL promoter was normalized to that observed for another constitutive promoter to ensure that the observed effects were promoter-specific. Fig. 5A illustrates that the BCL-XL promoter was significantly down-regulated in the presence of increasing levels of
BCL-6. This down-regulation was probably the result of a direct interaction, because gel shift experiments demonstrated that the DNA-binding domain of BCL-6 bound directly to several binding sites within the BCL-XL promoter (Fig. 5B). When GAA was mutated to CCC in these consensus BCL-6-binding sites, the binding of BCL-6 to the BCL-X promoter fragments was abrogated, indicating that the binding was specific (data not shown). An examination of the ability of BCL-6 to induce cell death revealed that although this protein alone was not as effective as AFX at mediating apoptosis, there was still a significant increase of death in cells expressing this protein, which is in agreement with previous studies in other cell lines (33, 34).
The resistance of a diversity of tumors to apoptosis induced by chemotherapy and radiation correlates with the up-regulation of the AKT/PKB kinases through increased PI3K activity (42, 43). It is probable that a significant fraction of this resistance is because of the inhibition of transcriptional programs induced by forkhead transcription factors such as AFX (17–20). The results described here provide for a pro-apoptotic transcriptional mechanism that may be inhibited in tumors endowed with enhanced AKT/PKB kinase activity. Whereas previous data have suggested that the AKT/PKB pathway may regulate the BCL-XL:BAD/BAX pathway by direct phosphorylation of BAD (44), for example, the new results reported here reveal that these two pathways will be regulated by transcriptional modulation (Fig. 7). Our data thus support a transcriptional repression mechanism for the previously observed modulation of BCL-XL.

DISCUSSION

(Fig. 5C). Whereas there are probably to be other mechanisms such as targeted protein degradation involved with the decrease in BCL-XL levels, these data suggest that one of the important anti-apoptotic genes that is regulated by AFX through BCL-6 is BCL-XL. In addition, these data are consistent with in vivo data demonstrating that there are low or undetectable levels of BCL-6 in pre-B cells but high level expression of BCL-XL in this population (31).

Whereas all of these results were consistent with a role for AFX-induced BCL-6 in apoptosis, the use of an in vivo-derived non-transfected cell system would solidify the case dramatically. Therefore, we examined the in vitro survival characteristics of macrophages isolated from BCL-6–/– knockout mice (30). Fig. 6A demonstrates that macrophages from the mutant animals showed enhanced survival in both the presence as well as the absence of the exogenous survival factor macrophage-CSF (M-CSF). A tabulation of the viable cell numbers (Fig. 6B) reveals that the rate of BCL-6–/– macrophage cell death is significantly retarded in the absence of the exogenous survival factor. This effect is even more dramatic in the presence of the survival factor in which at 24 h all of the BCL-6-deficient macrophages are still alive while almost half of the wild-type macrophages have died. These data thus support the contention that BCL-6 is an important mediator of cell death in vivo, although they also suggest that other modulators of apoptosis are also utilized in these cells under these in vitro cell culture conditions.
levels by both the PI3K pathway as well as the BCL-6 transcription factor (19, 33, 37, 38). Because BCL-XL is a major component of cell viability (27), the down-regulation of its expression by BCL-6 would be expected to have negative effects on cell survival. These negative effects on cellular viability would be particularly important under circumstances of limiting growth factors in which the PI3K pathway would be down-regulated (1). These results thus provide a mechanism whereby transcriptional activation by AFX coupled with transcriptional repression by BCL-6 can together induce a cell death pathway, and they are consistent with recent data that demonstrate that an elevated level of BCL-6 expression is a positive prognostic indicator for patients with B-cell lymphoma (45). Whereas it seems clear from our results that the modulation of BCL-6 and BCL-XL levels by AFX is a component of the apoptotic pathway induced by the inhibition of the PI3K pathway, it is probable that this inductive event is substantially more complex. For example, previous data have implicated the induction of the expression of BH3-only proteins such as BIM by the FKHR-L1 forkhead transcription factor (23), although we have been unable to demonstrate an induction of the BIM transcript by the AFX protein in our system (data not shown). In addition, it is improbable that BCL-6 alone is capable of inducing the observed apoptotic response. BCL-6 is expressed in germinal

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**Fig. 6.** BCL-6 plays an anti-survival role in macrophages. Deletion of BCL-6 allows primary mouse macrophages to survive longer in culture. In both the presence and the absence of murine M-CSF, wild-type macrophages with functional BCL-6 exhibit a more accelerated rate of reduction in the number of live adherent cells than macrophages containing no functional BCL-6. A, phase micrographs of wild-type (BCL-6+/+) and BCL-6-deficient (BCL-6−/−) macrophages cultured in the presence or absence of 10 ng/ml M-CSF for the indicated time intervals. B, percentages of cell survival were calculated by dividing the number of live adherent cells at the various time points by the number of live adherent cells at 0 h. p << 0.01 for BCL-6+/+ versus BCL-6−/− at 24, 48, and 72 h.

**Fig. 7.** Transcriptional regulation of apoptosis by AFX. This figure illustrates a model that demonstrates the linkage between the PI3K and BCL-XL:BAD/BAX pathways through the transcriptional regulators AFX and BCL-6. AFX(P), phosphorylated AFX.
center B cells, and BCL-6 null animals are deficient in germinal center formation and suffer from myocarditis and pulmonary vasculitis (30). Interestingly, dysregulation of BCL-6 is correlated with the progression of various lymphomas, and BCL-6 can under some circumstances be anti-apoptotic (32, 46). Therefore, it is likely that the induction of BCL-6 by AFX is only part of the transcriptional program that is involved with the initiation of the apoptotic response. Thus, there appears to be a diversity of other factors that are involved with the AFX-mediated apoptotic cascade. For example, the up-regulation of IGFBP-1, a protein that modulates the activity of the insulin-like growth factor growth and survival factors, is consistent with the hypothesis that inhibition of survival signals upstream of the PI3K pathway is likely to be a component of AFX-induced cell death (19). In addition, the induction of p27kip1, an inhibitor of G1 to S progression, suggests that proteins that modulate the cell cycle are also likely to be involved with forkhead-mediated apoptosis (20, 23). Finally, the transcriptional activation of the forkhead homologue DAF-16 in Caenorhabditis elegans during dauer formation induces a quiescent but not mortal state in these worms, suggesting that forkhead may induce different transcriptional programs under different conditions (16, 47). A further analysis of genes regulated in the system described here will undoubtedly increase our understanding of the mechanisms that are induced by this important transcriptional pathway.

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