Increased Expression of Mcl-1 Is Required for Protection against Serum Starvation in Phosphatase and Tensin Homologue on Chromosome 10 Null Mouse Embryonic Fibroblasts, but Repression of Bim Is Favored in Human Glioblastomas*

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Inactivating mutations in the tumor suppressor gene phosphatase and tensin homologue on chromosome 10 (PTEN) result in elevated levels of phosphatidylinositol (3,4,5)-trisphosphate, activation of protein kinase B (PKB), and protection against apoptotic insults such as withdrawal of survival factors. Protection may arise through the inhibition of the pro-apoptotic protein Bim, which is normally repressed by a PKB-dependent mechanism. Here we show that PTEN−/− immortalized mouse embryonic fibroblasts (MEFs) exhibit elevated PKB phosphorylation and are resistant to serum withdrawal-induced death, but exhibit normal Bim expression following withdrawal of serum. In contrast, expression of Mcl-1, a pro-survival member of the Bcl-2 family, was elevated in PTEN−/− MEFs. Transient or stable overexpression of Mcl-1 in PTEN−/− MEFs conferred resistance to serum withdrawal, whereas ablatively expressing expression of Mcl-1 in PTEN−/− MEFs, using RNA interference, abolished their resistance to serum withdrawal-induced apoptosis. To determine if Mcl-1 is selected for overexpression in human tumors we examined human glioblastoma cell lines but found that loss of PTEN had no effect on Mcl-1 expression. In contrast, two of three PTEN−/− glioblastoma cell lines exhibited low expression of Bim, which was refractory to serum withdrawal. These results indicate that the resistance of PTEN−/− MEFs to serum withdrawal is largely due to the up-regulation of Mcl-1 but that loss of PTEN in tumor cell lines is more complex and may favor de-regulation of different apoptotic regulators such as Bim.

A large number of early and advanced human cancers contain mutations in the tumor suppressor Phosphatase and TENsin homologue on chromosome 10 (PTEN). Loss of heterozygosity (LOH) at the human PTEN locus (10q23) is associated with many high grade glioblastomas and endometrial tumors as well as some melanoma, prostate, and breast carcinomas (1). Germ line mutations of PTEN are also found in rare autosomal dominant syndromes such as Cowden disease, which are characterized by benign hyperplastic outgrowths (2). Re-introduction or overexpression of wild type PTEN in human carcinomas results in suppression of tumor growth, apoptosis, or both (3–6). Complete loss of PTEN in mice is embryonic lethal, whereas PTEN+/− mice are viable but develop various neoplasms by adulthood (7).

The principal role of PTEN is to negatively regulate the phosphoinositide 3-kinase (PI3K) pathway via its in vivo lipid phosphatase activity (8). PTEN converts phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) to phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) such that resting cells harboring PTEN mutations often have elevated levels of PtdIns(3,4,5)P3 (9). One effect of elevated PtdIns(3,4,5)P3 levels is the activation of the serine/threonine protein kinase B (PKB/Akt) that in turn confers a strong survival signal (10). Increased PKB activity has been observed in PTEN-deficient tumors (9, 11) and is associated with increased resistance to cytotoxic drugs, UV irradiation, matrix detachment, and withdrawal of survival factors (12–14).

Resistance to apoptosis can be achieved via the up-regulation and/or down-regulation of anti-apoptotic or pro-apoptotic Bcl-2 family members, respectively. Anti-apoptotic family members share four defined regions of homology, BH1–4, and include Bcl-2, Bcl-XL, A1, and Mcl-1. Pro-apoptotic members are divided into two subfamilies: those that contain domains BH1–3 (Bak and Bax) and those that contain only the BH-3 domain such as Bim, Bad, and Bmf (15). Recent studies have drawn attention to Bim as a target for PKB-dependent survival signals. For example, cytokine withdrawal in mouse Ba/F3 cells results in de novo expression of Bim and apoptosis (16). Bim expression is induced by the transcription factor FOXO3A that is normally inhibited by PKB-dependent phosphorylation (17). Indeed, inhibition of the PI3K pathway is sufficient to induce Bim expression in a variety of cells (16, 18, 19).

In this study we sought to address whether Bim is repressed following PTEN LOH. We found that PTEN−/− immortalized mouse embryonic fibroblasts (MEFs) are resistant to serum withdrawal-induced apoptosis, but this protection was not associated with suppression of Bim but rather with elevated levels of Mcl-1. Indeed, the increase in expression of Mcl-1 was required for PTEN−/− MEFs to resist serum starvation-induced apoptosis. In contrast, loss of PTEN in human glioblastomas (3,4,5)-trisphosphate; PtdIns(4,5)P3, phosphatidylinositol (4,5)-bisphosphate; RT, reverse transcription; shRNA, small hairpin ribonucleic acid; LOH, loss of heterozygosity.
Protection by Loss of PTEN Requires Mcl-1

Real-time RT-PCR—Preparation of total RNA was performed as previously described (18). (Q)RT-PCR was performed according to the protocol supplied with the TaqMan® reverse transcription reagents (Applied Biosystems) as described previously (18). For mouse Mcl-1 we used 5'-TGTAGGACCAAGAGCCGACT-3' as the forward primer and 5'-AAAGCCAGCAATCATTTCT-3' as the reverse primer. For mouse glyceraldehyde-3-phosphate dehydrogenase we used 5'-TCAAGCGACCTTCTTGCACGA-3' as the forward primer and 5'-GTTCCTGTCAACTCCATAT-3' as the reverse primer. For human β-Actin we used 5'-TGGTTGAGACCCCTTCAACC-3' as the forward primer and 5'-TGGAGACCAGACGATTAAT-3' as the reverse primer.

RESULTS

PKB Phosphorylation Is Elevated in Immortalized PTEN−/− Mouse Embryonic Fibroblasts—We sought to characterize the effect of serum withdrawal on immortalized MEF cell lines derived from mice heterozygous or homozygous null for PTEN (13). In the first instance, we confirmed by immunoblotting that PTEN was expressed in the PTEN+/− cells but not in the PTEN−/− cells (Fig. 1). PTEN LOH resulted in elevated PKB phosphorylation in cycling cells compared with PTEN+/− cells (9, 13), whereas total PKB and total Bax protein levels did not differ and served as a loading control.

The difference in PKB phosphorylation was more pronounced following serum starvation (Fig. 2A). In PTEN+/− MEFs PKB phosphorylation declined rapidly following withdrawal of serum and remained low. In contrast, in PTEN−/− MEFs, the decline was less pronounced, and the levels of PKB phosphorylation recovered from 3 h onwards. ERK1/2 phosphorylation also declined rapidly following serum starvation in the PTEN+/− cells and only started to recover after 24 h; however, we did observe in some experiments that this recovery was more rapid in PTEN−/− cells, perhaps suggesting that loss of PTEN may impact on the ERK pathway in some instances (21). No differences in total ERK1/2, PKB, or Bax levels were observed (Figs. 1 and 2A).

PTEN−/− MEFs Are Resistant to Serum Withdrawal-induced Apoptosis—Apoptosis following serum withdrawal was initially assessed by immunoblotting total cell lysates with an antibody that recognizes the cleaved (17 kDa) active form of caspase-3. Following withdrawal of serum from cycling PTEN+/− cells, levels of cleaved caspase-3 protein increased rapidly, being apparent by 1 h and maximal by 3 h (Fig. 2A). Although serum withdrawal did promote an increase in cleavage of caspase-3 in PTEN−/− cells, levels were substantially reduced when compared with the corresponding PTEN+/− samples. To further quantify the reduction in caspase activation we compared the ability of whole cell extracts to cleave the synthetic caspase substrate Ac-DEVD-AMC (a DEVDase assay). In PTEN+/− cells DEVDase activity was clearly elevated 4 h after serum withdrawal and was maximal by 24 h, whereas

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Invitrogen. The following antibodies were used throughout this study: PTEN (6H2-1) was from Cascade Bioscience; ERK1/2 and Bcl-XL were from BD Biosciences Pharmingen; Bim was from Chemicon; Bmf was from Calbiochem; Actin, ERK2, Mcl-1, Bcl-2, Bak, and Bax (N-20) were from Santa Cruz Biotechnology; phospho-ERK1/2 (Thr-202/Tyr-204), cleaved caspase-3 PKB, phospho-PKB (Thr-308), and phospho-PKB (Ser-473) were from Cell Signaling Technologies. Horseradish peroxidase-conjugated antibodies were from Bio-Rad. Hygromycin B was from Roche Applied Science; Zeocin was from Invitrogen. All other chemicals were purchased from Sigma and were of the highest grade available.

Cell Culture—PTEN+/− and PTEN−/− immortalized MEFs were obtained from V. Stambolic (Amgen Institute, Toronto). U251-MG cells were obtained from D. Deen (Brain Tumor Research Centre, University of California San Francisco). U87-MG, LN229, and LN18 cells were from N. Rainov (University of Liverpool, UK). U373-MG cells were from E. Chastre (Faculte de Medecine X., Paris, France). PTEN+/− MEFs overexpressing HA-Mcl-1 were derived by transfection (FuGENE 6, Roche Applied Science). Zeocin selection (300 μg/ml) and ring clonning after limiting dilution. PTEN+/− MEFs expressing shRNA against Mcl-1 were derived by transfection (FuGENE 6, Roche Applied Science). Hygromycin B selection (500 μg/ml) and ring cloning after limiting dilution. All cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 4.5 mg/ml glucose, 100 units/ml penicillin, 10 μg/ml streptomycin, 2 mM glutamine, 10% (v/v) FBS. For serum starvation, cells judged to be 50–60% confluent were washed free medium for the times indicated in the figure legends.

Transient Overexpression of Mcl-1—HA-tagged human Mcl-1 cDNA (Hugh Brady, Institute of Child Health, London) was subcloned into pcDNA3.1/Zeo (Invitrogen). PTEN+/− cells were transfected with 10 μg of pcDNAHA:Mcl-1 or empty vector and 2 μg of pcDNA:EGFP-spectrin (Paul Coffer, University of Utrecht), using a calcium phosphate precipitation protocol to ensure that EGFP-expressing cells were also expressing HA-Mcl-1. 20 h later the cells were subjected to a 24-h serum starvation. The difference in PKB phosphorylation was more pronounced following serum starvation (Fig. 2A). In PTEN+/− MEFs PKB phosphorylation declined rapidly following withdrawal of serum and remained low. In contrast, in PTEN−/− MEFs, the decline was less pronounced, and the levels of PKB phosphorylation recovered from 3 h onwards. ERK1/2 phosphorylation also declined rapidly following serum starvation in the PTEN+/− cells and only started to recover after 24 h; however, we did observe in some experiments that this recovery was more rapid in PTEN−/− cells, perhaps suggesting that loss of PTEN may impact on the ERK pathway in some instances (21). No differences in total ERK1/2, PKB, or Bax levels were observed (Figs. 1 and 2A).

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Protection by Loss of PTEN Requires Mcl-1

FIGURE 2. PTEN+/− MEFs are resistant to serum withdrawal-induced apoptosis. Cycling PTEN+/− and PTEN−/− MEFs were maintained in complete medium (C, 10% FBS) or switched to serum-free (SF) medium for the indicated time points. A, normalized cell lysates were immunoblotted with antibodies for cleaved caspase-3, phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2, phospho-PKB (Ser473), total PKB and Bax. B, DEVDase activity, expressed as relative fluorescent units (FU) per microgram total protein, was assayed from normalized detergent extracts prepared from triplicate cell samples. Data (mean ± S.D.) is taken from a single experiment that is representative of three independent experiments. C, PTEN+/− and PTEN−/− MEFs were maintained in complete medium (C, 10% FBS), or switched to SF medium for 12, 24, or 36 h. Cells were then stained with propidium iodide before analyzing their cell cycle profile by flow cytometry. D, PTEN+/− and PTEN−/− MEFs were maintained in complete medium (C, 10% FBS), switched to SF medium for 24 h, or maintained in complete medium supplemented with etoposide (30 μM) for 24 h. Data represent the mean ± S.D. sub-G1 content of three replicate cell populations and is representative of two independent experiments. An asterisk indicates values for PTEN−/− cells are significantly different from corresponding PTEN+/− values by Student’s t test; *, p < 0.05.

samples derived from serum-starved PTEN−/− cells exhibited significantly reduced DEVDase activity (p < 0.05) such that it barely increased above that at time zero (Fig. 2B). In addition, PTEN−/− cell cultures contained significantly fewer cells with sub-G1 DNA than the equivalent PTEN+/− population following 12 and 24 h (p < 0.05) of serum withdrawal (Fig. 2, C and D). However, despite the strong inhibition of caspase activation, we did observe that protection conferred by loss of PTEN was progressively lost after 24 h. This may indicate that cells can also undergo cell death by a parallel PTEN-independent pathway at later time points, perhaps reflecting a later necrotic response. A much smaller, but still significant (p < 0.05), reduction in sub-G1 DNA content was also observed in PTEN−/− cells following a 24-h treatment with the DNA-damaging agent etoposide (Fig. 2D). These data clearly show that PTEN LOH in MEFs prevents the activation of caspases and the appearance of downstream markers of apoptosis following serum withdrawal.

Serum Withdrawal-induced Apoptosis in PTEN+/− MEFs Occurs in the Absence of de Novo Bim Expression—The up-regulation or activation of pro-apoptotic BH3-only proteins serves to link cellular stress to initiation of the cell death response (15). Bim, in particular, is strongly implicated in promoting cell death following withdrawal of survival factors (16, 18, 19, 22–26). In viable cells Bim expression is repressed, in part, by PKB-dependent phosphorylation and inactivation of FOXO transcription factors (17, 19), so we investigated whether Bim expression was down-regulated in PTEN−/− cells. Western blot analysis revealed that Bim expression did increase following serum withdrawal (Figs. 3A and 4A). However, there was no difference in the magnitude of Bim expression between PTEN+/− and PTEN−/− cells.

Death following withdrawal of survival factors is blocked by cycloheximide in many cell types, indicating a requirement for de novo synthesis of a pro-apoptotic protein, such as Bim (18, 23). Indeed, the increase in expression of Bim following serum withdrawal was blocked by cycloheximide (Fig. 3A). However, cycloheximide did not block serum withdrawal-induced caspase activation in PTEN+/− cells (Fig. 3B); PTEN−/− cells again failed to activate caspases. Thus, even though
Bim expression increases following serum withdrawal, this is not required for cell death in these immortalized MEFs. Rather, serum withdrawal-induced death proceeds through pre-existing components, and the ability of PTEN status to determine this response is unlikely to be due to differences in de novo Bim expression.

Mcl-1 mRNA and Protein Levels Are Elevated in PTEN−/− MEFs—Because changes in Bim expression were unlikely to be the PTEN-dependent changes in cell death in MEFs, we examined the expression of other Bcl-2 family members (Fig. 4A). Following serum withdrawal from PTEN+/− and PTEN−/− MEFs, we observed no difference between the cell lines with respect to the expression of Bim and Bmf (BH3-only subfamily) or Bax and Bak (Bax sub-family) (Fig. 4A). Levels of the anti-apoptotic protein Bcl-2 steadily decreased upon serum starvation in both cell lines, although absolute levels were higher in PTEN+/− cells. Bcl-XL protein levels remained largely unchanged throughout the experiment, although again, absolute levels were higher in PTEN+/− cells. However, we did observe changes in the expression of Mcl-1 that were consistent with it conferring protection from apoptosis. First, we observed that the basal expression of Mcl-1 in PTEN−/− cells was typically 2.5-fold greater than that observed in PTEN+/− cells (Fig. 4A and B). Second, the level of Mcl-1 declined following serum withdrawal in PTEN+/− cells but was maintained in serum-starved PTEN−/− cells (Fig. 4A).

Quantitative real-time RT-PCR was used to determine levels of Mcl-1 mRNA throughout a time course of serum withdrawal (Fig. 4C). Mcl-1 mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels and then plotted as -fold change over basal levels (time = 0) in PTEN+/− cells. The basal level of Mcl-1 mRNA in PTEN−/− cells was 2-fold higher than that observed in PTEN+/− cells and increased still further following serum starvation. In contrast, Mcl-1 mRNA levels remained unchanged in PTEN+/− cells so that from 9 h Mcl-1 mRNA levels were typically 3.5-fold higher in PTEN−/− cells versus PTEN+/− cells.

Overexpression of Mcl-1 Protects PTEN+/− Cells from Serum Withdrawal—We deemed that elevated expression of Mcl-1 was a plausible mechanism to account for the observed protection against serum withdrawal in PTEN−/− cells. To test this directly, we transiently transfected PTEN+/− cells with plasmids expressing HA:Mcl-1 and an EGFP:spectrin fusion protein, before serum starving for 24 h (Fig. 5A, A and B). Cells transfected with empty vector and EGFP:spectrin served as a negative control. Immunoblots confirmed expression of Mcl-1 and EGFP:spectrin served as a negative control. Immunoblots confirmed expression of Mcl-1 and EGFP:spectrin (Fig. 5A). When EGFP-positive cells were analyzed for sub-G1 DNA content we observed that transient expression of Mcl-1 caused a significant (3-fold) reduction in serum withdrawal-induced cell death (Fig. 5B). Immunoblotting confirming that this Mcl-1-dependent protection did not block the serum starvation-induced increase in Bim expression (Fig. 5A).

In addition to these transient expression studies, we derived PTEN+/− clonal cell lines (+/−19 and +/−23) overexpressing HA:Mcl-1 (Fig. 6A) and compared their sensitivity to serum withdrawal with that of parental PTEN+/− and PTEN−/− cells. These results revealed that stable overexpression of HA-Mcl-1 strongly protected PTEN−/− cells as illustrated by a 81% (+/−19) and 78% (+/−23) reduction in the serum starvation-induced increase in DEVDase activity (Fig. 6B). Upon serum starvation, HA-Mcl-1 overexpressing clone +/19 also displayed a significant reduction (p < 0.05) in the proportion of cells with sub-G1 DNA (Fig. 6C). These results show that transient overexpression (Fig. 5) or a stable and more modest overexpression (Fig. 6) of Mcl-1 alone is sufficient to confer resistance to serum withdrawal-induced apoptosis in immortalized PTEN+/− MEFs.
Protection by Loss of PTEN Requires Mcl-1

Mcl-1 Is Required for Resistance to Serum Withdrawal in PTEN\(^{-/-}\) MEFs—To test whether the increase in Mcl-1 expression in PTEN\(^{-/-}\) MEFs was required for their protection against serum withdrawal, we used a small hairpin RNA interference strategy to reduce Mcl-1 expression in PTEN\(^{-/-}\) MEFs. Two independent PTEN\(^{-/-}\) clonal cell lines (−/−1 and −/−8) were derived from the parental PTEN\(^{-/-}\) MEFs. Western blot analysis showed that endogenous Mcl-1 protein levels were reduced in both clones, relative to that of the parental PTEN\(^{-/-}\) cell line (Fig. 7A). PTEN\(^{-/-}\) clones 1 and 8, in which Mcl-1 expression was reduced, were sensitized to serum withdrawal-induced apoptosis relative to parental PTEN\(^{-/-}\) cells, as judged by an increase in DEVDase activity and an increase in sub-G\(_1\) DNA content (Fig. 7B and C). Furthermore, the cells that displayed the most effective knockdown of Mcl-1 (−/−8) exhibited the greatest sensitivity to serum starvation. Thus, Mcl-1 is necessary for most, if not all, of the resistance to serum withdrawal-induced apoptosis observed in immortalized PTEN\(^{-/-}\) MEFs.

Deletion of PTEN in Human Glioblastoma Cells Does Not Affect Mcl-1 Expression but Does Inhibit Bim Expression—Prompted by our results in MEFs, we wished to address whether Mcl-1 expression was increased in PTEN\(^{-/-}\) human GBMs (1, 27). In the first instance we compared expression of Mcl-1 in the two MEF cell lines with that in three GBM cell lines, including those that were wild type (LN18 and LN229) or homozygous null (U87-MG) for PTEN (Fig. 8A). Once again, Mcl-1 levels were elevated in PTEN\(^{-/-}\) MEFs compared with PTEN\(^{+/+}\) MEFs. The PTEN\(^{-/-}\) GBM cell line U87-MG exhibited elevated levels of PKB phosphorylation in comparison to the two PTEN\(^{+/+}\) GBM cell lines (LN18 and LN229), whereas total PKB levels remained unchanged. Side-by-side analysis of the MEFs and the human GBMs revealed that human Mcl-1 resolved at a slightly higher molecular weight than murine Mcl-1 on SDS-PAGE gels. More importantly it revealed that human Mcl-1 content by flow cytometry. Each data point represents the mean ± S.D. of triplicate samples. Values marked with an asterisk indicate that expression of Mcl-1 in clone ± 19 caused a significant reduction in cell death compared with cells transfected with pcDNA3.1, by Student’s t test (p < 0.05).

FIGURE 5. Transient overexpression of HA-Mcl-1 in PTEN\(^{-/-}\) MEFs confers resistance to serum withdrawal-induced apoptosis. Cycling PTEN\(^{-/-}\) MEFs maintained in complete medium (C, 10% FBS) were transfected with pCMV/EGFP:spectrin and either pcDNA3.1:HA-Mcl-1 or pcDNA3.1. A, HA-Mcl-1 and EGFP:spectrin expression was confirmed by immunoblotting total lysates with antibodies for Mcl-1 and GFP. Total lysates were blotted with Bax antibodies to confirm equal loading and Bim antibodies to confirm induction upon serum withdrawal. B, twenty hours after transfection, cells were treated with serum free (SF) medium for 24 h then fixed and stained with propidium iodide. The sub-G\(_1\) DNA content of EGFP-positive cells was determined by flow cytometry. Each data point represents the mean ± S.D. of triplicate samples. Data are mean ± S.D. taken from a single experiment, representative of three. An asterisk indicates that expression of HA-Mcl-1 caused a significant reduction in cell death compared with cells transfected with pcDNA3.1, by Student’s t test (p < 0.05).

FIGURE 6. Stable overexpression of HA-Mcl-1 protects PTEN\(^{-/-}\) MEFs from serum withdrawal-induced apoptosis. A, two independent PTEN\(^{-/-}\) clones (clones 19 and 23) express HA-Mcl-1 at low and high levels of HA-Mcl-1, respectively. Whole cell lysates from cycling cells were blotted with antibodies to Mcl-1, Bcl-x\(_L\), and Bax. In the Mcl-1 blot the upper band represents HA-Mcl-1 protein, whereas the lower band represents endogenous Mcl-1 protein. B, PTEN\(^{-/-}\) and PTEN\(^{+/+}\) MEFs, and PTEN\(^{-/-}\) clones 19 and 23 were maintained in complete (C) medium or switched to serum-free (SF) medium for 5 h. DEVDase activity was assayed from normalized protein extracts prepared from triplicate cell samples. Data are mean ± S.D. taken from a single experiment, representative of three. An asterisk indicates the reduction in DEVDase activity in clone ± 19 and clone ± 23 was significantly different from the corresponding PTEN\(^{-/-}\) SF control value by Student’s t test (p < 0.05). C, PTEN\(^{-/-}\) MEFs and Clone ± 19 were serum-starved for 12, 24, or 36 h. Cells were stained with propidium iodide before analyzing their sub-G\(_1\) DNA content by flow cytometry. Each data point represents the mean ± S.D. of triplicate samples. Values marked with an asterisk indicate that expression of Mcl-1 in clone ± 19 caused a significant reduction in cell death by Student’s t test (p < 0.05).
In the course of this analysis, however, we did observe substantial differences in the expression of Bim that correlated well with PTEN status (Fig. 8B). Serum starvation caused a clear increase in Bim expression in both PTEN+/− and PTEN−/− MEFs and two independent PTEN−/− clones expressing a Mcl-1-specific, U6 promoter-driven, shRNA construct (+/− 1 and −/− 8) were immunoblotted with antibodies for Mcl-1 and Bax. B, PTEN+/− and PTEN−/− MEFs and the Mcl-1 silenced clones −/− 1 and −/− 8 were maintained in complete medium or switched to serum-free medium (SF) for 8 h. DEVDase activity, expressed as relative fluorescent units (FU) per microgram of total protein, was assayed from normalized protein extracts prepared from triplicate cell samples. C, PTEN−/− MEFs and the Mcl-1 silenced clones −/− 1 and −/− 8 were maintained in complete medium or switched to serum-free medium (SF) for 24 h before staining with propidium iodide. Following cell cycle analysis by flow cytometry the mean ± S.D. sub-G0 content of three replicate cell populations was determined. An asterisk indicates values for clones −/− 1 and −/− 8 were significantly different from the corresponding PTEN−/− SF value by Student’s t test, (p < 0.05).

DISCUSSION

Increased Expression of Mcl-1 Is Required for Resistance to Serum Starvation in PTEN−/− MEFs—Deletion of PTEN is associated with increased resistance to multiple death stimuli, orchestrated primarily through activation of the key survival kinase PKB and its downstream targets (9, 13, 28–30). PKB can regulate cell survival indirectly through phosphorylation of the pro-apoptotic transcription factors FOXO3A (31) and YAP (32), the JNK scaffold proteins JIP1 and POSH (33, 34), and the p53-binding protein MDM2 (35). The relative importance of these many PKB targets may vary in different cell types. Furthermore, some PKB targets may be influenced by other pathways making interpretation more difficult; for example, Bim is regulated by the PKB, ERK (18, 35), and JNK (24) pathways.

To simplify interpretation we examined serum withdrawal-induced apoptosis, because (a) this is a relatively benign form of cell stress that doesn't involve DNA damage, (b) survival signals would be expected to determine in part the activation state of the PKB pathway, and (c) loss of growth factor dependence for survival is a hallmark of cancer cells (36). Our results confirm that PTEN LOH in MEFs causes constitutive activation of PKB and confers considerable protection against serum starvation (Figs. 1 and 2) (9). Analysis of pre- and antiapoptotic Bcl-2 family members showed that Mcl-1 protein and mRNA levels were elevated in PTEN−/− cells. Furthermore, RNA interference-mediated knockdown showed that this increase in Mcl-1 expression in PTEN−/− MEFs was required for resistance to serum withdrawal, whereas transient or stable overexpression of Mcl-1 alone was sufficient to protect the otherwise sensitive PTEN−/− MEFs. Thus, of all the changes that could result from loss of PTEN, increased expression of Mcl-1 alone is necessary and sufficient to account for the resistance of PTEN−/− MEFs to serum starvation.

Mcl-1 differs from the majority of Bcl-2 proteins in that it does not possess a BH-4 domain, has a short half-life, and is degraded by the proteasome (37). Indeed, the PI3K/PKB pathway has previously been implicated in increasing Mcl-1 protein translation (38), whereas glycolytic macrophage-colony stimulating factor has been shown to increase Mcl-1 protein stability via PKB and ERK-dependent phosphorylation (39). However, we observed no difference in the half-life of either Mcl-1 protein or mRNA in PTEN+/− and PTEN−/− cells, suggesting that loss of PTEN in MEFs increases Mcl-1 protein levels primarily through increasing de novo Mcl-1 gene expression. The PI3K/PKB pathway has previously been implicated in up-regulating Mcl-1 gene expression (40–43), but the majority of these studies have been in hematopoietic cells. For example, Mcl-1 gene expression is regulated by the PKB pathway in the interleukin-3-dependent Ba/F3 pro-B cell line (40) and in human macrophages (43). However, PI3K-dependent up-regulation of Mcl-1 expression was also seen in Hep3B cells during interleukin-6-mediated protection (42).

* M. Austin and S. Cook, unpublished observations.

Protection by Loss of PTEN Requires Mcl-1

Dase activity or sub-G0 DNA content. However, when we examined the activation of Bax, using the appearance of the conformationally active N-20 epitope (18), we observed that the PTEN null U87-MG cells exhibited no activation of Bax following serum withdrawal, whereas the LN229 cells exhibited clear activation of Bax. The total, activable Bax was the same in the two cell lines as judged by the ability of triton X-100 to fully expose the N-20 epitope.

Taken together, these data suggest that in at least two independent human GBM cell lines loss of PTEN results not in the up-regulation of Mcl-1 but rather the down-regulation of Bim mRNA and protein. Furthermore, the reduction in Bim expression in a PTEN−/− GBM correlates with decreased Bax activation following serum starvation.
Protection by Loss of PTEN Requires Mcl-1

Increased Mcl-1 expression certainly has the capacity to confer a malignant phenotype (44) and is often associated with increased resistance to apoptosis (45–47). In addition, Taniai and co-workers (48) have recently shown that RNA interference-mediated down-regulation of Mcl-1, but not Bcl-2 or Bcl-XL, was sufficient to confer sensitivity to tumor necrosis factor-related apoptosis-inducing ligand-induced death in cholangiocarcinoma cells. Our results suggest that increased expression of Mcl-1 alone is necessary and sufficient to account for the resistance of PTEN−/− MEFs to serum starvation, and we believe it represents the first demonstration of a clear genetic link between PTEN LOH and an increase in Mcl-1 expression.

Loss of PTEN Favors Bim Repression in Glioblastomas but Not MEFs—The BH3-only protein Bim is implicated in cell death following withdrawal of survival factors (16, 18, 23–26). Although Bim protein levels may be determined by ERK-dependent degradation (25, 49), Bim mRNA levels increase when FOXO3A is activated following cytokine withdrawal and/or inactivation of PKB (16, 19). Indeed, inhibition of PI3K is sufficient to induce Bim expression in a variety of cell types (16, 18, 19).

Prompted by these observations we anticipated that loss of PTEN in MEFs would protect cells from serum withdrawal by blocking the increase in Bim expression. However, two observations argue against this. First, there was no difference between PTEN+/− and PTEN−/− MEFs in the magnitude or kinetics of Bim expression following serum withdrawal, indicating that de-regulation of the PKB pathway following loss of PTEN does not impair Bim expression in MEFs. Second, although serum starvation did promote an increase in Bim expression, this was not required for cell death in PTEN+/− cells, because cycloheximide blocked the increase in Bim expression but did not inhibit cell death. This is in striking contrast to the situation in CCl39 fibroblasts (18), sympathetic neurons (50), and lymphocytes (16), where death requires de novo protein synthesis. Indeed, it is notable that following serum withdrawal the appearance of cleaved, activated caspase-3 was very rapid in MEFs, being apparent within 1 h (Fig. 2), whereas in CCl39 fibroblasts caspase activation is not apparent until 5–6 h after serum withdrawal and can be blocked by cycloheximide (18). Thus, in contrast to many cell types, serum withdrawal-induced cell death must proceed through the post-translational modification of pre-existing components in MEFs. This doesn’t rule out a role for Bim in cell death following serum starvation in these MEFs, but it does argue that Bim would need to be regulated by some post-translational mechanism under these circumstances (25, 51, 52).

PTEN mutation and/or LOH in human GBMs is well documented (1, 27, 53, 54). Despite our data showing that PTEN status was a clear...
Protection of PTEN Loss Requires Mcl-1

Bressieux, J.-M., Cabarrot-Moreau, A., Chompret, A., Demange, L., Eles, R. A., Yabanda, A. M., Fearon, E. R., Fricker, J.-P., Gorlin, R. J., Hodgson, S. V., Husson, S., Lacombe, D., LePrat, F., Odent, S., Toulouse, C., Olopade, O. L., Sobol, H., Tishler, S., Woods, C. G., Robinson, B. G., Weber, H. C., Parsons, R., Peacocke, M., Longy, M., and Eng, C. (1998) *Hum. Mol. Genet.* 7, 507–515

Li, J., Simpson, L., Takashahi, M., Miliareis, C., Myers, M. P., Tonks, N., and Parsons R. (1998) *Cancer Res.* 58, 5667–5672

Lu, Y., Lin, Y.-Z., LuPusinh, R., Cuevas, B., Fang, X., Yu, S. X., Davies, M. A., Khan, H., Furui, T., Mao, M., Zinner, R., Hung, M.-C., Steck, P., Simonovitch, K., and Mills, G. B. (1999) *Oncogene* 18, 7034–7045

Weng, L.-P., Gimmi, O., Kum, J. B., Smith, W. M., Zhou, X.-P., Wynford-Thomas, D., Leone, G., and Eng, C. (2001) *Hum. Mol. Genet.* 10, 251–258

Zhao, H., Dupont, J., Yakar, S., Karas, M., and LeRoith, D. (2004) *Oncogene* 23, 786–794

Wu, H., Goel, V., and Halaska, F. G. (2003) *Oncogene* 22, 3113–3122

Myers, M. P., Pass, L., Batty, I. H., van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P., and Tonks, N. K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 13513–13518

Sun, H., Lesche, R., Li, D. M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Muller, B., Liu, X., and Wu, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 6199–6204

Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) *Genes and Dev.* 13, 2905–2927

Haas-Kogan, D., Shalev, N., Wong, M., Mills, G., Yount, G., and Stokoe, D. (1998) *Carr. Biol.* 8, 1195–1198

Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) *Science* 276, 661–665

Stambolic, V., Suzuki, A., de la Pompa, J. L., Murot, C., Maito, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Maka, T. W. (1998) *Cell* 95, 29–39

Yamada, K. M., and Araki, M. (2001) *J. Cell Sci.* 114, 2375–2382

Cory, S., Huang, D. C. S., and Adams, J. M. (2003) *Oncogene* 22, 8590 – 8607

Dijiksters, P. F., Medema, R. H., Lammers, J. W. L., Koenderman, L., and Coffier, P. J. (2000) *Carr. Biol.* 10, 1201–1204

Dijiksters, P. F., Birkenkamp, K. U., Lam, W. F.-M., Thomas, N. S. B., Lammers, J. W. I., Koenderman, L., and Coffier, P. J. (2002) *Cell Mol. Biol.* 156, 531–542

Weston, C. R., Balmanno, K., Hadfield, K., Miliaresis, C., Myers, M. P., Tonks, N., and Parsons, R. (2001) *Cell Mol. Biol.* 47, 26915–26921

Stiles, B., Gilman, V., Khanzenzon, N., Lesche, R., Liu, X., and Wu, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 6199–6204

Gilley, J., Coffier, P. J., and Ham, J. (2003) *J. Cell Biol.* 162, 613–622

Garner, A. P., Weston, C. R., Todd, D. E., Balmanno, K., and Cook, S. J. (2002) *Oncogene* 21, 8089–8104

Suzuki, A., Yamaguchi, M. T., Otobe, T., Sasaki, T., Karish, K., Kimura, Y., Yoshida, R., Wakeham, A., Ito, H., Fukumoto, M., Tsutahara, T., Ohashi, P. S., Koyasu, S., Penninger, J. M., Nakano, T., and Mak, T. W. (2001) *Immunity* 14, 523–534

Shinjyo, T., Kuribara, R., Inui, K., Hosoi, K., Kuniyoshi, T., Miyaajima, A., Houghton, P. J., Look, A. T., Ozawa, K., and Inaba, T. (2001) *Mol. Cell. Biol.* 21, 854–864

Putcha, G. V., Moulder, K. L., Golden, J. P., Bouillet, P., Adams, J. A., Strasser, A., and Ellenson, L. H. (1997) *Cancer Res.* 57, 3935–3940

Cantley, L. C., and Neel, B. G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 4240–4245

Stiles, B., Gilman, V., Khanzenzon, N., Lesche, R., Li, A., Qiao, R., Liu, X., and Wu, H. (2002) *Mol. Cell. Biol.* 22, 3842–3851

Sulis, M. L., and Parsons, R. (2003) *Trends Cell Biol.* 13, 478–483

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. L., Zhu, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* 96, 857–868

Basu, S., Totey, T. N., Irwin, M. S., Sudol, M., and Johnson, E. M., Jr. (2001) *Carr. Biol.* 11, 11–23

Kim, A. H., Yano, H., Cho, K. H., Meyer, D., Monks, B., Margolis, B., Birnbaum, M. J., and Chao, M. V. (2002) *Neuron* 35, 697–709

Figureira, C., Tarras, S., Taylor, J., and Vojtek, A. B. (2003) *J. Biol. Chem.* 278, 47922–47927

Mayo, I. D., and Donner, D. B. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 11598–11603

Hanahan, D., and Weinberg, R. A. (2000) *Cell* 100, 57–70

Edwards, S. W., Derouet, M., and Moots, R. J. (2004) *Biochem. Soc. Trans.* 32, 489–492

Schubert, K. M., and Duronio, V. (2001) *Biochem. J.* 356, 473–480

Derouet, M., Thomas, L., Cross, A., Moots, R. J., and Edwards, S. W. (2004) *J. Biol. Chem.* 279, 26915–26921

Wang, J.-M., Chao, J.-R., Chen, W., Kun, M.-L., Yen, J. J.-Y., and Yang-Yen, H.-F. (1999) *Mol. Cell. Biol.* 19, 6195–6206

Moulding, D. A., Akulov, C., Derouet, M., White, M. R. H., and Edwards, S. W. (2001) *J. Leuk. Biol.* 70, 783–792

Kuo, M.-L., Chuang, S.-F., Lin, M.-T., and Yang, S.-Y. (2001) *Oncogene* 20, 667–685
Protection by Loss of PTEN Requires Mcl-1

43. Liu, H., Perlman, H., Pagliari, L. J., and Pope, R. M. (2001) J. Exp. Med. 194, 113–125
44. Okaro, A. C., Deery, A. R., Hutchins, R. R., and Davidson, B. R. (2001) J. Clin. Pathol. 54, 927–932
45. Reynolds, J. E., Yang, T., Qian, L., Jenkinson, J. D., Zhou, P., Eastman, A., and Craig, R. W. (1994) Canc. Res. 54, 6348–6352
46. Zhou, P., Qian, L., Kozopas, K. M., and Craig, R. W. (1997) Blood 89, 630–643
47. Moulding, D. A., Giles, R. V., Spiller, D. G., White, M. R. H., Tidd, D. M., and Edwards, S. W. (2000) Blood 96, 1756–1763
48. Taniai, M., Grambihler, A., Higuchi, H., Werneburg, N., Bronk, S. F., Farrugia, D. J., Kaufmann, S. H., and Gores, G. J. (2004) Can. Res. 64, 3517–3524
49. Marani, M., Hancock, D., Lopes, R., Tenev, T., Downward, J., and Lemoine, N. R. (2004) Oncogene 23, 2431–2441
50. Martin, D. P., Schmidt, R. E., DiStefano, P. S., Lowry, O. H., Carter, J. G., and Johnson, E. M., Jr. (1988) J. Cell Biol. 106, 829 – 844
51. Puthalakath, H., Huang, D. C. S., O'Reilly, L. A., King, S. M., and Strasser, A. (1999) Mol. Cell 3, 287–296
52. Lei, K., and Davis, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2432–2437
53. Davies, M. A., Lu, Y., Sano, T., Fang, X., Tang, P., LaPushin, R., Koul, D., Bookstein, R., Stokoe, D., Yung, W. K., Mills, G. B., and Steck, P. A. (1998) Canc. Res. 58, 5285–5290
54. Tian, X., Pang, J. C., To, S. S., and Ng, H. K. (1999) J. Neuropathol. Exp. Neurol. 58, 472–479
55. Wagenknecht, B., Glaser, T., Naumann, U., Kügler, S., Isenmann, S., Bähr, M., Korneluk, R., Liston, P., and Weller, M. (1999) Cell Death Diff. 6, 370–376
56. Fulda, S., Wick, W., Weller, M., and Debatin, K.-M. (2002) Nat. Med. 8, 808–815
57. Egle, A., Harris, A. W., Bouillet, P., and Cory, S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6164 – 6169
58. Tagawa, H., Karnan, S., Suzuki, R., Matsuoka, K., Zhang, X., Ota, A., Morishima, Y., Nakamura, S., and Seto, M. (2005) Oncogene 24, 1348–1358
59. Reginato, M. J., Mills, K. R., Paulus, J. K., Lynch, D. K., Sgroi, D. C., Debnath, J., Muthuswamy, S. K., and Brugge, J. S. (2003) Nat. Cell Biol. 5, 733–740
60. Oplerman, J. T., Letal, A., Beard, C., Sorcinell, M. D., Ong, C. C., and Korsmeyer, S. J. (2003) Nature 426, 671–676
Increased Expression of Mcl-1 Is Required for Protection against Serum Starvation in Phosphatase and Tensin Homologue on Chromosome 10 Null Mouse Embryonic Fibroblasts, but Repression of Bim Is Favored in Human Glioblastomas

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Human Sco1 and Sco2 function as copper-binding proteins.
Yih-Chern Horng, Scot C. Leary, Paul A. Cobine, Fiona B. J. Young, Graham N. George, Eric A. Shoubridge, and Dennis R. Winge

In Fig. 6, A and B, and Fig. 7, A and B, the mutant listed as D239A on the far right of each panel should be H239A.
Low energy CD of RNA hairpin unveils a loop conformation required for λN antitermination activity.

Neil P. Johnson, Walter A. Baase, and Peter H. von Hippel

In the labeling of the χ axes on Figs. 2, 3, 5, 6, 7, and 8, the Greek lowercase epsilon was transformed into an exclamation point. The corrected figures are shown below.
Increased expression of Mcl-1 is required for protection against serum starvation in phosphatase and tensin homologue on chromosome 10 null mouse embryonic fibroblasts, but repression of Bim is favored in human glioblastomas.

Mark Austin and Simon J. Cook

Under “Experimental Procedures,” subheading “Real-time RT-PCR,” the human Bim and β-actin primers described are wrong. The published primers actually amplify murine Bim and β-actin transcript rather than the stated human transcript. The real-time RT-PCR data presented in Fig. 8C still remains factually correct, as it was derived using the human Bim and β-actin primers. The correct primers used in this study are described as follows:

- Human Bim forward, 5′-TGC AGA CAT TTT GCT TGT TCA A-3′, and reverse, 5′-GAA CCG CTG GCT GCA TAA-3′;
- human β-actin forward, 5′-CTC CTC CTG AGC GCA AGT ACT C-3′, and reverse, 5′-CGG ACT CGT CAT AGT CCT GCT T-3′.