Akt and Bcl-xL Promote Growth Factor-independent Survival through Distinct Effects on Mitochondrial Physiology*

A comparison of Akt- and Bcl-xL-dependent cell survival was undertaken using interleukin-3-dependent FL5.12 cells. Expression of constitutively active Akt allows cells to survive for prolonged periods following growth factor withdrawal. This survival correlates with the expression level of activated Akt and is comparable in magnitude to the protection provided by the antiapoptotic gene Bcl-xL. Although both genes prevent cell death, Akt-protected cells can be distinguished from Bcl-xL-protected cells on the basis of increased glucose transporter expression, glycolytic activity, mitochondrial potential, and cell size. In addition, Akt-expressing cells require high levels of extracellular nutrients to support cell survival. In contrast, Bcl-xL-expressing cells deprived of interleukin-3 survive in a more vegetative state, in which the cells are smaller, have lower mitochondrial potential, reduced glycolytic activity, and are less dependent on extracellular nutrients. Thus, Akt and Bcl-xL suppress mitochondrion-initiated apoptosis by distinct mechanisms. Akt-mediated survival is dependent on promoting glycolysis and maintaining a physiologic mitochondrial potential. In contrast, Bcl-xL maintains mitochondrial integrity in the face of a reduced mitochondrial membrane potential, which develops as a result of the low glycolytic rate in growth factor-deprived cells.

There is increasing evidence that tissue homeostasis in multicellular organisms is controlled by the availability of growth factors (1). Within a given tissue, high levels of relevant growth factors promote increased cellular mass, metabolism, and proliferation. In contrast, when the availability of growth factor becomes limiting, cellular atrophy and an increased rate of apoptosis are observed. Many growth factors affect cellular responses through receptor-mediated recruitment and activation of the phosphoinositide 3-kinase (PI3K) and the serine/threonine kinase Akt (2, 3). Once activated, Akt can phosphorylate and inactivate pro-apoptotic proteins, including Bad and Forkhead family transcription factors (4). Akt phosphorylates and inactivates pro-apoptotic proteins, including Bad and Forkhead family transcription factors (9, 10). In addition, Akt has been shown to stimulate the expression of anti-apoptotic Bcl-2 proteins, such as Bcl-xL and Mcl-1, through the activation of NF-kB (11, 12). However, it is not clear if these targets entirely account for the effects of Akt on cell survival.

Members of the Bcl-2 family are attractive Akt targets, as they have been shown to be potent regulators of apoptosis following growth factor withdrawal. Transgenic overexpression of anti-apoptotic family members prevents the induction of programmed cell death and leads to an accumulation of cells, whereas transgenic overexpression of pro-apoptotic family members can result in decreased cell numbers within an organ (13, 14). Anti-apoptotic Bcl-2 family proteins, such as Bcl-xL, are localized to the outer mitochondrial membrane and function to maintain mitochondrial homeostasis upon growth factor withdrawal. Bcl-xL and Bcl-2 have been reported to promote mitochondrial homeostasis by promoting continued transport of metabolites across the outer membrane, despite decreases in cellular metabolism (15, 16). In the absence of growth factor, Bcl-xL facilitates cell survival by preserving cellular ATP production following the decrease in glycolysis that accompanies growth factor withdrawal.

Since both Akt and Bcl-xL can regulate cell survival and cellular metabolism, we have compared the bioenergetic properties of growth factor-deprived FL5.12 cells expressing either Akt or Bcl-xL. FL5.12 cells are nontransformed pro-B cells that depend on IL-3 for survival and proliferation. The results demonstrate that cells expressing Akt or Bcl-xL maintain distinct metabolic states in the absence of growth factor. Akt expression sustains sufficient glucose uptake and glycolysis to maintain a physiologic mitochondrial membrane potential in growth factor withdrawn cells. In contrast, Bcl-xL promotes the survival of growth factor-deprived cells by maintaining mitochondrial integrity and function, despite a decrease in mitochondrial potential that results from a decline in glucose-derived substrates. These differences in cellular metabolism suggest that...
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EXPERIMENTAL PROCEDURES

Cell Culture—FL5.12 cells were cultured as described previously (17). Myristylated Akt (mAkt) gene expression was induced for 18 h with 1 μg/ml of doxycycline (Sigma) treatment. Cell volume measurements were made using a Coulter Z2 instrument (Beckman Coulter). Where indicated, wortmannin (Calbiochem) and LY294002 (Sigma) were added to cultures at final concentrations of 100 nm and 10 μM, respectively. Glucose- and glutamine-free medium was made using the RPMI 1640 Select Amine Kit (Life Technologies, Inc.) and 10% dialyzed fetal bovine serum (Life Technologies, Inc.). Glucose and glutamine were supplemented to final concentrations as indicated.

Plasmid Constructs and Retroviral Transductions—pUHD172–1Neo expressing rtTA was generously provided by J. Leiden, Abbott. Akt constructs were generously provided by N. Hay, University of Illinois, Chicago. mAkt was hemagglutinin (HA)-tagged at the C terminus, and both mAkt-HA and K179M Akt were cloned into pRevTRE (pRT, CLONTECH). Bcl-xL was ligated into pBabeMN-IRESGFP (Bcl-x L1, generously provided by G. Nolan, Stanford University) or pRT (Bcl-xL2). Retroviral expression vectors were virally transduced using the Phoenix packaging cell line (generously provided by G. Nolan, Stanford University) or pRevTRE (Bcl-xL, 2). Retroviral expression vectors were virally transduced using the Phoenix packaging cell line (generously provided by G. Nolan) as described previously (18). Briefly, target cells were combined with the retrovirus and 4 μg/ml hexadimethrine bromide (Sigma), spun at 2500 rpm for 1.5 h, and selected with 3 mg/ml hygromycin (CLONTECH). Cell populations were cloned by limiting dilution. Three independently isolated clones expressing the epitope-tagged mAkt in an inducible manner (mAkt1, mAkt2, and mAkt3) were obtained and subjected to further study.

Protein Expression—Lysates from cell lines were standardized for protein content and separated by SDS-polyacrylamide gel electrophoresis (Invitrogen). Blots were probed with either rabbit anti-Bcl-xL (13.6) (19), rabbit anti-Akt (New England Biolabs), or mouse anti-tubulin (Santa Cruz Biotechnology) and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Northern Blot—Total RNA was prepared using TRIZOL (Life Technologies, Inc.) from cells cultured with or without IL-3 for 14 h. 10 μg of RNA were separated on a 1% formaldehyde agarose gel and probed with rat Glut1 cDNA (generously provided by M. Birnbaum, University of Pennsylvania). Loading was assessed by visualizing gels stained with ethidium bromide.

Flow Cytometry—Cell viability assays were performed using propidium iodide as described (17). For mitochondrial potential determination, live cells were enriched by centrifugation over Ficoll, rested for 1 h, and incubated for 30 min at 37 °C with 200 nM tetramethylrhodamine ethyl ester (Molecular Probes). Analysis was performed in a FacsCalibur flow cytometer (Becton Dickinson).

Glycolysis and ATP Assays—After 6 days without IL-3, 1 × 10^6
Ficoll-enriched live cells were resuspended in pre-warmed CO₂-buffered Krebs solution (115 mM NaCl, 2 mM KCl, 25 mM NaHCO₃, 1 mM MgCl₂, 2 mM CaCl₂, 0.25% bovine serum albumin, pH 7.4) lacking glucose for 30 min at 37 °C. Cells were then washed and resuspended in 500 μl of Krebs buffer containing 10 mM glucose supplemented with 20 μCi/ml [5-3H]glucose (PerkinElmer Life Sciences) for 1 h at 37 °C, and the reaction was stopped by adding equal volume 0.2 N HCl. 3H₂O was separated from [3H]glucose by evaporative diffusion of 3H₂O in a closed chamber, as described (20). Total ATP levels were determined from 1 × 10⁶ live cells, enriched by Ficoll, using the ATP bioluminescence assay kit HS II (Roche Molecular Biochemicals) (15).

**RESULTS**

Akt is involved in transmitting intracellular signals from growth factor receptors (21). We sought to determine whether Akt is involved in IL-3-dependent signal transduction in FL5.12 cells. FL5.12 cells were deprived of growth factor for 12 h, and recombinant IL-3 was reconstituted for the indicated time intervals. Addition of IL-3 resulted in rapid and transient phosphorylation of Akt, which was maximal 15 min after IL-3 addition (Fig. 1A). Activation of Akt was dependent on PI3K, since the PI3K inhibitors wortmannin or LY294002 prevented the induction of Akt phosphorylation (Fig. 1A and data not shown). Thus, Akt is a component of the proximal signal transduction machinery of the IL-3 receptor.

When FL5.12 cells are withdrawn from growth factor, they undergo progressive atrophy until they initiate programmed cell death. Bcl-xL protects from cell death in response to growth factor withdrawal, but it does not prevent cellular atrophy (22). Since the PI3K/Akt pathway is an important regulator of cell size in *Drosophila* (23), we investigated whether the PI3K/Akt pathway is required for cell growth following IL-3 readdition. Reconstitution of IL-3 in cultures that had been withdrawn from growth factor resulted in rapid recovery of cell size (Fig. 1B). Addition of the PI3K inhibitor LY294002 simultaneously with IL-3 resulted in a delay in the recovery of cell size, indicating that the PI3K pathway regulated cell growth in response to stimulation with IL-3. These data indicate that the PI3K/Akt pathway is required for cell growth following IL-3 readdition.
pathway is important in transmitting cell growth signals in mammalian cells, as has been observed in *Drosophila* cells (23, 24).

In addition to promoting cell growth, the PI3K/Akt pathway also transmits cell survival signals from growth factor receptors (2, 25). To assess the role of Akt in promoting cell survival in FL5.12 cells, we established cell lines expressing constitutively active, mAkt (26). Addition of the Src myristoylation sequence to the N terminus of Akt targets it to the plasma membrane, conferring constitutive activity to the kinase (27). FL5.12 cells were infected with a retrovirus encoding an epitope-tagged mAkt under the control of a tetracycline response element. Three independent clones were isolated which expressed doxycycline-induced mAkt at a level comparable to the level of total Akt expressed in parental FL5.12 cells (Fig. 2). In the absence of induction, all three clones expressed low levels of mAkt. Withdrawal of IL-3 from wild-type FL5.12 cells resulted in loss of viability within 48 h, as determined by the ability of cells to exclude propidium iodide (Fig. 2, A and B). All three mAkt clones maintained viability over 6 days, whereas cells expressing kinase-deficient Akt died with kinetics similar to wild-type cells (Fig. 2A). As has been shown previously, cells expressing high and low levels of Bcl-xL (Bcl-xL1 and 2, respectively) maintained viability in the absence of growth factor in a dose-dependent manner (28). After 6 days of growth factor withdrawal, the remaining viable cells could be recovered quantitatively from mAkt- and Bcl-xL-expressing populations as assayed by IL-3 readdition and cloning by limiting dilution (data not shown). Thus, increased viability over 6 days represented true cell survival over the entire time course. Constitutively active Akt promotes cell survival in a dose-dependent manner, as doxycycline addition increased mAkt expression and cell survival concomitantly (Fig. 2, B and C).

Akt signal transduction has been implicated in the control of cell cycle progression by stimulating increased translation of cyclin D (29). To determine whether Akt-dependent survival following growth factor withdrawal involved continued cell proliferation, we assessed the rates of DNA synthesis in cells growing in IL-3 and in cells that had been withdrawn from IL-3. Incorporation of the nucleotide analog bromodeoxyuridine (BrdUrd) was similar for all clones growing in IL-3 (Fig. 3). Following growth factor withdrawal, cells expressing either mAkt or Bcl-xL lacked significant levels of DNA synthesis, while maintaining significant viability (Fig. 3). In contrast, vector control cells also lack the ability to incorporate BrdUrd, but this reflects the fact that all these cells have died as indicated by their sub-diploid DNA content and inability to exclude propidium iodide (Fig. 2, A and data not shown). These data suggest that Akt-dependent survival in FL5.12 cells does not require cell cycle progression.

FL5.12 cells undergo progressive atrophy when deprived of growth factor (22). Since Akt transmits cell growth signals from the IL-3 receptor, expression of constitutively active Akt could prevent cellular atrophy upon growth factor withdrawal. Cell size was assessed in cells that had been withdrawn from IL-3 by measuring forward scatter of equal numbers of live cells in a flow cytometer. Cells expressing mAkt maintained a larger cell size in the absence of growth factor, compared with cells expressing Bcl-xL (Fig. 4). Cell size correlated with mAkt expression, as cells induced with doxycycline were larger than uninduced cells. Although cells expressing mAkt remained larger than Bcl-xL cells, they still atrophied after growth factor withdrawal (data not shown). This indicates that mAkt can transmit a cell growth signal in the absence of IL-3, but it does not completely substitute for the cell growth and proliferative signals transmitted from the IL-3 receptor. Thus, activated Akt both promotes cell survival and prevents cellular atrophy in the absence of growth factor.

Cellular atrophy is correlated with decreases in cellular metabolism and macromolecular synthesis (30–32). Since Akt signaling attenuates cellular atrophy, the metabolic status of
mAkt and Bcl-xL cells was compared in the absence of growth factor. Total cellular ATP levels were assessed in cells that had been withdrawn from IL-3. Cells expressing mAkt contained more ATP than cells expressing Bcl-xL (Fig. 5), suggesting that cells expressing mAkt remained metabolically more active than cells expressing Bcl-xL. Furthermore, the prevention of cellular atrophy by Akt is correlated with increased metabolism in FL5.12 cells.

In insulin receptor signal transduction, Akt regulates several aspects of glucose uptake, metabolism, and storage (33–35). The elevated amounts of ATP in mAkt cells may be due in part to the ability of mAkt to maintain glucose metabolism in the absence of growth factor. Akt can increase glucose metabolism in response to insulin by inducing the expression of the glucose transporters in insulin-responsive cell types (35). In contrast to insulin-responsive tissues, which primarily express Glut4, the major glucose transporter in lymphocytes is Glut1.2 Analysis of Glut1 mRNA in cells growing in the presence of IL-3 revealed no difference in mRNA levels between vector control cells and cells expressing mAkt or Bcl-xL (Fig. 6A). Following IL-3 withdrawal, Glut1 mRNA was undetectable in vector control and Bcl-xL cells but was still detectable in cells expressing mAkt (Fig. 6A). In addition to promoting increased Glut1 expression, mAkt sustained an overall increase in glycolysis in the absence of IL-3 (Fig. 6B). Following growth factor withdrawal, cells expressing mAkt mediated significantly increased glycolytic rates compared with cells expressing Bcl-xL (Fig. 6B). However, constitutively active Akt did not completely substitute for growth factor receptor signaling, since the rates of glycolysis in Akt-protected cells were lower than in cells growing in IL-3.

Mitochondrial potential is indicative of mitochondrial activity, and perturbations in inner membrane potential following growth factor withdrawal have been associated with the commitment to cell death (15). Therefore, mitochondrial membrane potential was assessed using the potentiometric dye tetramethylrhodamine ethyl ester (TMRE). Following IL-3 withdrawal, mitochondrial membrane potential was significantly reduced in cells expressing Bcl-xL (Fig. 7A). In contrast, there was no reduction in the mitochondrial potential of cells expressing mAkt following IL-3 withdrawal. TMRE fluorescence was reflective of mitochondrial potentials in these cells, since addition of agents that collapse the mitochondrial potential resulted in a decrease in TMRE staining (Fig. 7B). Thus, the difference in staining with TMRE alone was due to the difference in mitochondrial potential in cells expressing mAkt or Bcl-xL. These data indicate that cells expressing mAkt contain sufficient electron transport substrates to maintain mitochondrial potential, despite the absence of growth factors.

To survive growth factor withdrawal, cells must maintain cellular metabolism at a level sufficient to sustain viability. The data indicate that Akt-expressing cells are metabolically more active than Bcl-xL-expressing cells in the absence of growth factor, as determined by total cellular ATP, glycolytic rates, and mitochondrial potentials. One difference between Akt and Bcl-xL is the ability of Akt to regulate glucose metabolism. Glucose and glutamine are the major carbon sources for lymphocytes in culture medium (36, 37), and limiting concentrations of these nutrients resulted in decreased cellular glycolysis (data not shown). To determine whether the increased

\[ \text{J. C. Rathmell, unpublished observations.} \]
rate of glycolysis was necessary for Akt-dependent survival, we tested cell survival under conditions of nutrient limitation. As the concentrations of glucose and glutamine were decreased in the culture medium, a progressive impairment in the ability of mAkt to promote growth factor-independent survival was evident (Fig. 8). In contrast, Bcl-xₐ cells were unaffected by decreasing concentrations of carbon sources. Cells expressing mAkt also decreased in cell size prior to undergoing apoptosis as nutrients became more limiting (data not shown). These data suggest that Bcl-xₐ promotes cell survival by regulating a cell intrinsic effect on bioenergetics, rather than by regulating glucose uptake or utilization. In contrast, Akt is reliant on the availability of exogenous nutrients to promote cell survival.

**DISCUSSION**

The data indicate that Akt can function to promote both cell growth and cell survival downstream of growth factor receptors. Akt is rapidly phosphorylated following stimulation with IL-3, identifying the kinase as a component of the proximal IL-3 receptor signaling pathway. Inhibition of the PI3K/Akt pathway prevents the rapid induction of cell growth upon re-stimulation of FL5.12 cells with IL-3. Constitutively active Akt both promotes cell survival and attenuates cellular atrophy in the absence of growth factor. Thus, Akt regulates both cell survival and cell size in mammalian cells.

Increases in Akt activity have been shown to result in increases in cyclin D expression, which results in cell cycle progression (29). However, constitutively active Akt does not support IL-3-independent proliferation in FL5.12 cells. Cells expressing Akt are slower to exit the cell cycle than cells expressing Bcl-xₐ following growth factor withdrawal (data not shown). Nonetheless, cells expressing constitutively active Akt do exit from the cell cycle while maintaining cellular viability. This indicates that mAkt is not sufficient to transform FL5.12 cells and that cellular proliferation is not necessary for Akt-dependent cell survival.

The dual role of Akt in controlling both cell growth and cell survival has been suggested by genetic studies of Akt in Drosophila. Ablation of Akt in embryos results in increases in...
apoptosis throughout the embryo (38). Overexpression of Akt in eye and wing imaginal discs results in increases in cell size, without increases in cell number (23). Importantly, overexpression of Akt does not increase cellular proliferation and does not over-}


do not cover cell cycle arrest in the zone of nonproliferating cells in the wing imaginal disc (23). This indicates that Akt can promote increases in cell size without altering cell proliferation in Drosophila, in agreement with the findings in FL5.12 cells.

How can Akt simultaneously control both cell growth and cell survival? The answer may be linked to the control of cellular metabolism. Cellular growth requires the uptake and utilization of energy-rich metabolites. Similarly, to survive growth factor withdrawal, cells must establish a mechanism to sustain their metabolism. It is possible that Akt influences both cell growth and cell survival by sustaining increased cellular bioenergetics. In the absence of growth factor, constitutively active Akt promotes increases in cellular ATP levels, glycolytic rates, and mitochondrial potential, indicating that Akt mediates a global increase in cellular metabolism. This global increase may attenuate the alterations in cellular metabolism that are associated with growth factor withdrawal-induced programmed cell death.

Akt can control cellular metabolism on a number of levels. Here we report that IL-3 signal transduction is required to maintain Glut1 expression in FL5.12 cells. However, Akt activation is sufficient to induce Glut1 expression in these cells, even in the absence of IL-3. In addition to stimulating glucose uptake, Akt also controls glucose utilization within cells. Constitutively active Akt is sufficient to increase the overall rate of glycolysis in cells surviving growth factor withdrawal. Akt may increase glucose utilization by phosphorylating GSK-3\(\beta\) or PFK-2 (33, 34). The finding that GSK-3\(\beta\) overexpression results in apoptosis lends support to the possibility that Akt control of glucose metabolism contributes to its ability to promote cell survival (39).

The ability of Akt to maintain the glycolytic rate of a cell is sufficient to explain how Akt overexpression maintains the mitochondrial membrane potential. A higher glycolytic rate will result in greater substrate availability for mitochondrial electron transport. Consistent with this hypothesis, the ability of Akt to maintain the mitochondrial potential was found to be dependent on glucose. In contrast, Bcl-2 family proteins have been reported to maintain mitochondrial integrity following growth factor withdrawal by facilitating mitochondrial exchange of metabolites and ATP/ADP (15). This allows mitochondria to sustain coupled respiration in the face of a fall in mitochondrial potential that occurs as a result of the decrease in glycolytic substrates.

Biochemical analysis of cells surviving growth factor withdrawal in an Akt-dependent manner indicates that these cells are metabolically more active than their Bcl-x\(_L\) counterparts. To sustain high levels of metabolism and prevent atrophy, cells expressing Akt are reliant on external sources of energy. Akt promotes cell survival in the context of insufficient external energy sources. In contrast, cells that express Bcl-x\(_L\) can maintain viability even in the context of limited external nutrient sources. Since Akt is a component of growth factor receptor signal transduction, constitutively active Akt may promote survival by transmitting partial cell growth signals that sustain cellular metabolism, whereas Bcl-x\(_L\) promotes survival by allowing cells to adapt to a reduced metabolic state.

The data suggest that Akt does not promote cell survival solely by inactivating pro-apoptotic factors. If Akt functioned exclusively as an anti-apoptotic protein, cells surviving growth factor withdrawal would adopt a low energy pheno-


type, as do cells expressing Bcl-x\(_L\). The difference in the metabolic requirements of cells surviving due to Akt or Bcl-x\(_L\) indicates cellular contexts in which each gene might be functional. Bcl-x\(_L\) can mediate survival under limiting concentrations of nutrients. Thus, newly transformed cells that have not yet established an effective blood supply would be better served by expressing Bcl-x\(_L\) than by expressing Akt. Vascularized tumors, in contrast, can survive the absence of specific growth factors by expressing Akt. These cells would maintain greater levels of metabolism, which might contribute to their oncogenic potential. The requirement for a rich source of nutrients may partially explain why mutations in PTEN, which result in elevations in Akt activity, are correlated with late stage, aggressive tumors (6, 7). In contrast, overexpression of Bcl-2 proteins correlates with low grade tumors with low mitotic indices (40).

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