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Akt Stimulates Aerobic Glycolysis in Cancer Cells

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ABSTRACT

Cancer cells frequently display high rates of aerobic glycolysis in comparison to their nontransformed counterparts, although the molecular basis of this phenomenon remains poorly understood. Constitutive activity of the serine/threonine kinase Akt is a common perturbation observed in malignant cells. Surprisingly, although Akt activity is sufficient to promote leukemogenesis in nontransformed hematopoietic precursors and maintenance of Akt activity was required for rapid disease progression, the expression of activated Akt did not increase the proliferation of the premalignant or malignant cells in culture. However, Akt stimulated glucose consumption in transformed cells without affecting the rate of oxidative phosphorylation. High rates of aerobic glycolysis were also identified in human glioblastoma cells possessing but not those lacking constitutive Akt activity. Akt-expressing cells were more susceptible than control cells to death after glucose withdrawal. These data suggest that activation of the Akt oncogene is sufficient to stimulate the switch to aerobic glycolysis characteristic of cancer cells and that Akt activity renders cancer cells dependent on aerobic glycolysis for continued growth and survival.

INTRODUCTION

The pathogenesis of cancer involves numerous genetic and epigenetic lesions, resulting in the critical alteration of multiple cellular restraints. Some of these changes include the acquisition of cell-autonomous growth and proliferation, resistance to apoptosis, and immortalization (1). Additionally, genomic instability has been recognized both as a force for new mutations and as a manifestation of checkpoint loss (2). Invasive and metastatic cells typically reside at ectopic sites, demanding neovascularization and stromal remodeling (3). Furthermore, cancer cells have been noted to shift their metabolism away from respiration toward aerobic glycolysis (4, 5). Many of these cancer phenotypes are thought to result from the derepression of pathways normally tightly regulated during embryogenesis and homeostasis.

Individual oncogenes and tumor suppressors have been classified based on their contributions to these cancer pathways (6). For example, telomerase appears to promote transformation because of its ability to confer immortalization. Bcl-2 is thought to contribute to malignancy because it renders cells resistant to apoptosis (7).

The serine/threonine kinase Akt falls into the category of oncogenes promoting cell survival (8). Despite the fact that Akt can promote cell survival, *in vivo* studies of leukemogenesis have demonstrated that Akt synergizes with the more potent antiapoptotic oncogene Bcl-x_L, suggesting that Akt has additional functions that affect the biology of transformed cells (9). Consistent with this possibility, Akt has pleiotropic effects that might contribute to tumorigenesis. For instance, Akt has been reported to regulate cell proliferation (10). Akt has also been implicated in the regulation of glucose uptake in nontransformed cells (11, 12). Additionally, Akt has been shown to maintain mitochondrial membrane potential and hexokinase activity of nontransformed cells under apoptogenic conditions (13, 14).

Activation of Akt is commonly observed in cancer cells. Akt was first described as the cellular homologue of a viral oncogene (15), and it has been shown to be amplified in a number of human tumors (15–17). Cancer cells frequently attain constitutive Akt activity through indirect means such as amplification of phosphatidylinositide-3-kinase (PI3K), an upstream activator of Akt, or, more commonly, deletion of PTEN, a PI3K antagonist (18). Akt is also an important downstream effector of numerous oncogenes, including Bcr-Abl, Her2/*neu*, and Ras.

Previous studies have demonstrated that Akt-transfected immortalized hematopoietic cells are tumorigenic. We now report that, in these transformed cells, Akt exerts a direct influence on glucose metabolism that recapitulates the effect first observed by Warburg in 1924 (19). Akt induces a dose-dependent stimulation of glycolysis that correlates with a more aggressive malignancy *in vivo*. The effects of Akt on glucose metabolism appear to dominate over effects of Akt on proliferation and survival of these transformed cells. Activation of Akt in established human glioblastoma cells showed similar effects of promoting aerobic glycolysis and glucose dependence.

MATERIALS AND METHODS

Cell Culture. Parental FL5.12 cell lines expressing Bcl-x_L, myristoylated Akt1, and vector controls have been described previously (13, 20). Cells were maintained in RPMI (Invitrogen) supplemented with 1 mg/ml G418 (Invitrogen), 10% dialyzed fetal bovine serum (Invitrogen), 3.5 mM glucose, 0.05 ng/ml recombinant interleukin 3 (R&D Systems), 10 mM HEPES (Invitrogen), 50 μM 2-mercaptoethanol (Sigma), and penicillin/streptomycin (Invitrogen). Leukemic subclones were isolated as described previously (9). Leukemic cell lines were termed myrAkt-3-D1, myrAkt-3-D2, and myrAkt-3-D3 for doxycycline-treated animals 1, 2, and 3. The glioblastoma cell lines LN18 and LN229 were kindly provided by M. Celeste Simon. These cell lines have been described previously (21). These cells were grown in RPMI at a final concentration of 10 mM glucose, supplemented with 10% fetal bovine serum (Gemini Bioproducts, Woodland, CA), penicillin/streptomycin, and 2 mM L-glutamine. Experiments were performed culturing cells in RPMI with or without 10% fetal bovine serum as stated in the text. For experiments involving glucose deprivation, cells were cultured in RPMI lacking glucose but containing L-glutamine. LY294002 was obtained from Cell Signaling Technologies (Beverly, MA). Cell concentration was determined with a Coulter Z2 particle analyzer. Cell viability was determined by the exclusion of 2 μg/ml propidium iodide (Molecular Probes) by a LSR flow cytometer (BD Biosciences).

Plasmids and Transfection. The myristoylated Akt construct is composed of murine Akt1 with the Src myristoylation signal fused to the NH_2 terminus. The construct was cloned into pSFFV plasmid by standard PCR-based strategies and confirmed by sequencing. Transfection was performed with Lipofectamine 2000 (Life Technologies, Inc., Gaithersburg, MD), and transfected cells were selected in medium containing G418.

Immunoblotting. Lysates were standardized for protein content and resolved by SDS-PAGE. Nitrocellulose blots were probed with rabbit anti-Akt, anti-phospho-Akt Ser^{473} (Cell Signaling), or anti-actin I19 (Santa Cruz Biotechnology) antibodies.

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Note: R. Elstrom and D. Bauer contributed equally to this work.

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Table 1 *FL5.12 cell tumorigenicity*

Cell line	Induction	Mice with leukemia/ mice injected
vec	n/a	0/4
Bcl-x_L	n/a	0/4
myrAkt-2	−	0/5
myrAkt-2	+	5/6
myrAkt-3	−	0/6
myrAkt-3	+	4/8

Glucose and Lactate Measurements. Media samples were collected and stored at −20°C until the time of the assay. Glucose and lactate were measured using colorimetric kits according to manufacturer's instructions (CMA/Microdialysis).

NADH Measurement. Cells were cultured in the absence of interleukin 3 for 12 h, then washed and resuspended in Krebs buffer [115 mM NaCl, 2 mM KCl, 25 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.25% BSA (pH 7.4)] with 10 mM glucose. NAD(P)H fluorescence was measured at an excitation of 340 ± 2.5 nm and an emission of 461 ± 2.5 nm using a Fluoromax 2 spectrofluorimeter (Jobin Yvon-Spex) with constant stirring. To improve the readability of the tracing noise, editing was used. The final concentrations of carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (Sigma) and rotenone (Sigma) were 5 μM. The final concentration of iodoacetic acid (Sigma) was 1 μM. The final concentration of potassium cyanide (Sigma) was 500 μM.

Total NAD was measured after lysis in perchloric acid and neutralization with potassium hydroxide. An enzymatic cycling assay was performed as described previously (22). Briefly, samples were mixed with reagent containing 0.1 M bicine, 0.5 M ethanol, 4.17 mM EDTA, 0.8 mg/ml BSA, 0.42 mM 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, and 1.66 mM phenazine ethosulfate. The reaction was started by addition of alcohol dehydrogenase and carried out in the dark for 30 min. Absorbance at 540 nm was measured on a microplate reader and compared with a NAD standard curve.

Glycolysis. Glycolytic rate was determined by measuring the conversion of 5-^{3}H-glucose to $[^3H]_2O$. Cells were incubated with 10 μCi of 5-^{3}H-glucose (Perkin-Elmer Life Sciences) at 37°C for 1 h. After incubation, the reaction was stopped with 0.2 N HCl, and $[^3H]_2O$ was separated from 5-^{3}H-glucose by diffusion in an airtight container. Diffused and undiffused counts were measured using a 1450 Microbeta scintillation counter (Wallac) and compared with controls of 5-^{3}H-glucose and $[^3H]_2O$ alone.

Oxygen Consumption. Measurement of oxygen consumption of whole cells was performed as described previously (23). Briefly, oxygen consumption was measured using a water-jacketed, airtight 3-ml chamber with a polarographic oxygen electrode. Measurements were made at 37°C with constant stirring.

Tumorigenesis Assay. Parental FL5.12 cell lines expressing Bcl-x_L, myristoylated Akt1, and vector controls have been described previously (13, 20). For leukemogenesis studies, 1×10^8 cells were injected i.v., and mice were monitored by physical examination and histological analysis as described previously (9). For leukemic reintroduction studies, cells were expanded *in vitro* in the absence of doxycycline. Forty-eight h before reintroduction, appropriate cells were treated with 1 μg/ml doxycycline (Clontech) and then 1–5 × 10^8 cells were injected i.p. to athymic Balb/C nu^+/nu^+ mice (Taconic). Drinking water was supplemented with 5% sucrose ± 1 mg/ml doxycycline. Mice were sacrificed when visibly ill. Mice expiring immediately after positron emission tomography (PET) scanning or sacrificed when not visibly ill were excluded from the overall survival analysis.

^{18}F-fluorodeoxyglucose (FDG)-PET. A dedicated PET scanner (G-PET) using gadolinium orthosilicate crystals incorporated into an Anger-logic detector developed for human brain imaging was used to acquire the images. The G-PET scanner uses small 4 × 4 × 10-mm gadolinium orthosilicate crystals to ensure good energy resolution (10% at 511 keV), high density, and short decay time. The scanner has a diameter of 42 cm and an axial field-of-view of 25 cm. The camera has a spatial resolution of 3.7 mm full-width half-maximum. Food and water were withdrawn from the mice four h prior to imaging. The images were acquired 60 min following the administration of 30 μCi of ^{18}F-FDG by tail vein or retro-orbital injection. Mice were anesthetized for image acquisition. PET studies consisted of 12 min of static acquisition, yielding approximately 2 × 10^7 true events. The images were reconstructed using a fully 3D iterative image reconstruction algorithm.

RESULTS

Akt Activity Promotes Leukemogenesis. Resistance to apoptosis has been described as one of the hallmarks of cancer. Immortalized hematopoietic cells stably transfected with either Bcl-x_L or Akt are profoundly resistant to a variety of apoptogenic stimuli (13, 20). However, animals receiving Bcl-x_L-expressing cells fail to develop leukemia (Table 1). In contrast, when mice were administered either of two independently derived FL5.12 clones expressing myristoylated Akt under the control of a tetracycline promoter (termed myrAkt-2 and myrAkt-3), the animals treated with doxycycline developed leukemia at a high frequency (Table 1). Because Akt promotes transformation while Bcl-x_L did not, these results suggest that Akt contributes to transformation by an additional mechanism beyond inhibition of apoptosis.

The latency associated with the development of leukemia implied that additional genetic lesions promoting oncogenesis had occurred. To determine whether these lesions had supplanted the initial need for Akt or whether maintenance of Akt activity was still required for the phenotype, cells derived from one of the myrAkt-3-leukemic animals (termed myrAkt-3-D1) were reintroduced i.p. into animals in the presence or absence of systemic treatment with doxycycline. As determined from the tumor-free survival of the recipient animals, maintenance of Akt activity was required for the rapid dissemination of malignancy (Fig. 1*A*). Recipients of the leukemic cells treated with doxycycline had an average survival time that was 58% of the average survival time of untreated recipients ($P < 0.05$).

The abdominal tumor burden of the animals administered the leukemic subclones was imaged by FDG-PET. FDG-PET, an imaging modality increasingly used in the clinical diagnosis and management of cancer, measures the *in vivo* uptake of a radiolabeled tracer glucose analogue. The tracer typically accumulates at sites of high glucose metabolism such as the heart and brain. In addition, the tracer is

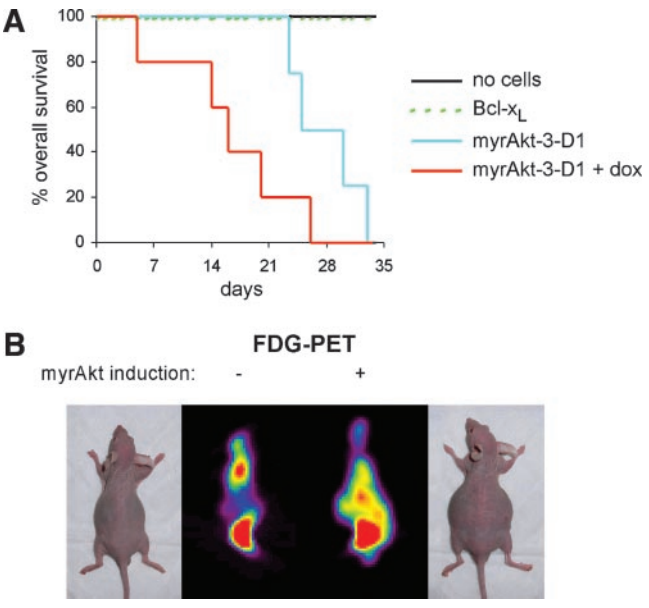

Fig. 1. Maintenance of Akt activity is required for the aggressive, glucose-avid cancer cell phenotype. The myrAkt-3-D1 cell line was derived from a leukemic myrAkt-induced mouse by culturing cells in the presence of G418 drug selection. The myrAkt-3-D1 cells were expanded *in vitro* and then transferred to naïve nude mice. *A*, mice receiving no cells, Bcl-x_L-expressing cells, or myrAkt-3-D1 cells with or without doxycycline exposure were monitored for overall survival. Mice were sacrificed when visibly ill. *P* (for survival of recipients of myrAkt-3-D1 cells with *versus* without doxycycline treatment, $P < 0.05$) was determined by Student's *t* test. *B*, mice receiving myrAkt-3-D1 cells with or without doxycycline treatment were imaged by ^{18}F-fluorodeoxyglucose-positron emission tomography (FDG-PET) 14–21 days after transfer of cells. Mice were i.v. administered 30 μCi of FDG, and 60 min later, PET images were acquired. Representative images are shown.

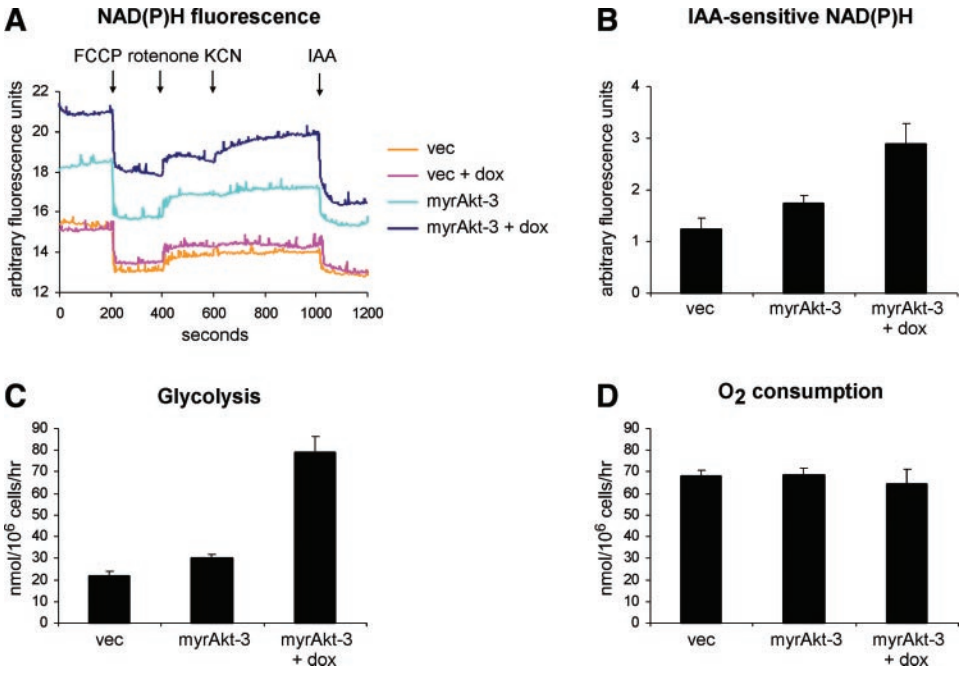

Fig. 2. Akt stimulates aerobic glycolysis. *A*, vector control or myrAkt-3 cells in the absence or presence of doxycycline were assayed for whole cell NAD(P)H fluorescence. Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), rotenone, potassium cyanide (KCN), and iodoacetic acid (IAA) were added at 200, 400, 600, and 1000 s, respectively. The traces from a representative experiment are shown. *B*, IAA-sensitive NAD(P)H was calculated as the change in fluorescence in response to IAA treatment. *C* and *D*, vector control or myrAkt–3 cells in the absence or presence of doxycycline were assayed for rates of glycolysis and oxygen consumption after 48 h in culture. *C*, glycolytic rate was measured in cells incubated with a 5-^{3}H-glucose tracer by the production of $[^3H]_2O$ by enolase. *D*, cellular oxygen consumption was measured in an airtight chamber with an oxygen electrode. Values shown are the means of three independent experiments. *Error bars* represent SDs.

excreted renally and thus accumulates in the bladder. Animals harboring the Akt-induced leukemic cells displayed avid abdominal uptake of the FDG tracer, consistent with high glucose utilization by cancer cells located in the peritoneal cavity, liver, and spleen (Fig. 1*B*). In contrast, animals that were not treated with doxycycline did not display evidence of abdominal FDG uptake in excess of that in surrounding tissue despite the fact that all of these animals ultimately succumbed to their tumor burden (Fig. 1*B*).

Akt Activity Stimulates Aerobic Glycolysis. The failure to image tumors in the absence of doxycycline was surprising. We had expected to be able to use PET imaging to quantify the differences in size of the tumors in the presence or absence of doxycycline. This result suggested the possibility that Akt-induced tumors might be more readily imaged because of cancer cell-autonomous stimulation of glucose uptake by Akt. Alternatively, Akt could have induced FDG uptake indirectly by promoting an increased rate of proliferation. To attempt to additionally understand the unique contributions of Akt to the initiation and maintenance of cellular transformation, the effects of Akt on cellular proliferation, survival, and glucose metabolism were studied. Parental nontransformed myrAkt-3 cultures have an enhanced level of Akt expression and activation (as assessed by phosphorylation of serine 473) that is significantly increased in the presence of doxycycline (data not shown). By comparing vector control cells to myrAkt-3 cells in the absence and presence of doxycycline, we were able to assess the dose-dependent effects of Akt. Although at neither level did Akt activity promote increased cell accumulation *in vitro*, Akt transfection and induction resulted in a dose-dependent stimulation of glucose consumption and lactate production (data not shown and Ref. 11).

These data suggest that, in growing cells, activated Akt can stimulate glucose metabolism to a level that is in excess of that needed to simply support cellular demand. To more rigorously test this hypothesis, we measured the redox state of the bioenergetic cofactor NAD. If metabolic throughput exceeded cellular bioenergetic demand, then NAD, the end product of glycolysis and the tricarboxylic acid cycle, would be expected to accumulate in its reduced state. Therefore, we assessed the redox state of NAD by measuring cellular fluorescence emission at 461 nm, which detects only the reduced form, NADH. Akt promoted a dose-dependent increase in this fluorescence (Fig. 2*A*). However, other molecular species, notably NADPH, also autofluoresce at this same wavelength, so a variety of inhibitors were used to verify the specificity of the measurement for NADH. Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone is an uncoupler that drives mitochondrial consumption of NADH; rotenone and potassium cyanide inhibit mitochondrial electron transport at complexes I and IV, respectively, and thus prevent consumption of NADH by the electron transport chain; iodoacetic acid is an inhibitor of glyceraldehyde 3-phosphate dehydrogenase, the enzyme that generates glycolytic NADH. Akt expression caused a dose-dependent increase in the production of NADH from glycolysis, as assessed by iodoacetic acid-sensitive NAD(P)H fluorescence (Fig. 2*B*). Therefore, despite robust lactate production, cells expressing Akt still performed glycolysis in excess of their ability to recycle glycolytic end products.

Because Akt stimulated the metabolism of glucose by the cells, it seemed plausible that Akt would also stimulate the complete oxidation of glucose via the mitochondria. Direct measurement of glycolysis demonstrated a ~4-fold increase in the glycolytic rate of myrAkt-3 cells in the presence of doxycycline as compared with vector control cells (Fig. 2*C*). Surprisingly, under these same conditions, Akt did not stimulate the rate of cellular oxygen consumption (Fig. 2*D*). Therefore, Akt specifically activates proximal glucose metabolism in nontransformed cells without activating mitochondrial oxidative phosphorylation.

Akt Renders Cells Dependent on Aerobic Glycolysis for Survival. Strikingly, this pattern of aerobic glycolysis is precisely what has been described as characteristic of the metabolism of spontaneously transformed cells (4). Therefore, we wished to determine whether the maintenance of Akt activity in the leukemic cells was sufficient to cause such a cell-autonomous alteration in metabolism. Leukemic cell lines (termed myrAkt-3-D1, myrAkt-3-D2, and myrAkt-3-D3) were established from three individual animals that had acquired Akt-dependent leukemia (Table 1). These cell lines were tested in culture for their cell accumulation, cell survival, glucose consumption, and lactate production properties. These studies revealed identical findings in all three lines. Maintenance of Akt activity did not enhance cell accumulation (Fig. 3*A*). In fact, cell viability in

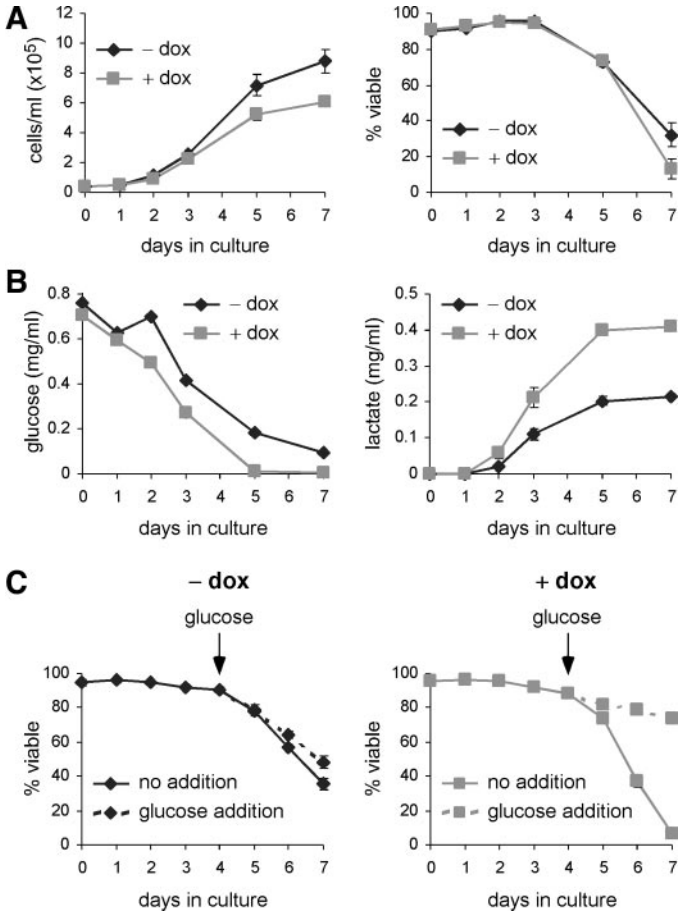

Fig. 3. Leukemic Akt-expressing cells demonstrate increased rates of glucose metabolism and require continuous glucose supply for a survival advantage. *A* and *B*, cell lines obtained from three leukemic mice (myrAkt-3-D1, myrAkt-3-D2, and myrAkt-3-D3) were cultured without passage in the absence or presence of doxycycline. Cells were seeded at 5×10^4 cells/ml. *A*, cell concentration was determined with a Coulter Z2 particle analyzer. Cell viability was determined by propidium iodide exclusion. *B*, glucose and lactate were quantified using colorimetric reactions. Glucose and lactate concentrations were normalized to cell number in the presence of doxycycline. Results were averaged for the three lines. Values shown are the means of three independent experiments. *Error bars* represent standard errors of the mean. *C*, the myrAkt-3-D1-leukemic cells were cultured without passage in the absence (*left*) or presence (*right*) of doxycycline. Cells were seeded at 5×10^4 cells/ml. Cultures were either supplemented or not after 4 days with 3.5 mM glucose. Cell viability was determined by propidium iodide exclusion. Values shown are the means of three independent experiments. *Error bars* represent SDs.

culture without passage became compromised after 5–7 days in both the doxycycline-treated and untreated cells. Induction of Akt not only failed to prevent this cell death but, in each of the cell lines, actually accelerated the death observed between 5 and 7 days in culture (Fig. 3*A*). However, all three lines more rapidly exhausted their media of glucose in the Akt-induced as compared with the Akt-uninduced state. The excess glucose consumed was neither used for synthesis nor oxidized but rather secreted as lactate (Fig. 3*B*).

The increased consumption of glucose suggested that the cause of the leukemic cell death *in vitro* might be the depletion of glucose from the culture. The myrAkt-3-D1 cultures were supplemented with glucose after 4 days, the time point immediately preceding complete depletion. Glucose resupply had only a marginal effect on the survival of the cells in which enforced Akt activation was not maintained. In contrast, the readdition of glucose almost completely abrogated the decline in viability of the Akt-maintained leukemic lines (Fig. 3*C*). Therefore, glucose availability is sufficient to render the leukemic cells with sustained Akt activity resistant to cell death and thus to promote net cell accumulation in the absence of a stimulatory effect of Akt on cell proliferation.

Human Glioblastoma Cell Lines with Different Glycolytic Rates. To determine whether this phenomenon of Akt-regulated aerobic glycolysis could occur in naturally occurring tumors, we investigated a series of human glioblastoma cell lines, which have been described previously (21, 24, 25). The cell lines varied in their rate of proliferation in culture and most displayed a high rate of glucose consumption that correlated poorly with their proliferative capacity. In the absence of serum-derived growth factors, two cell lines, LN18 and LN229, were found to have a comparable proliferative capacity while displaying distinct differences in glucose uptake. As shown in Fig. 4*A*,

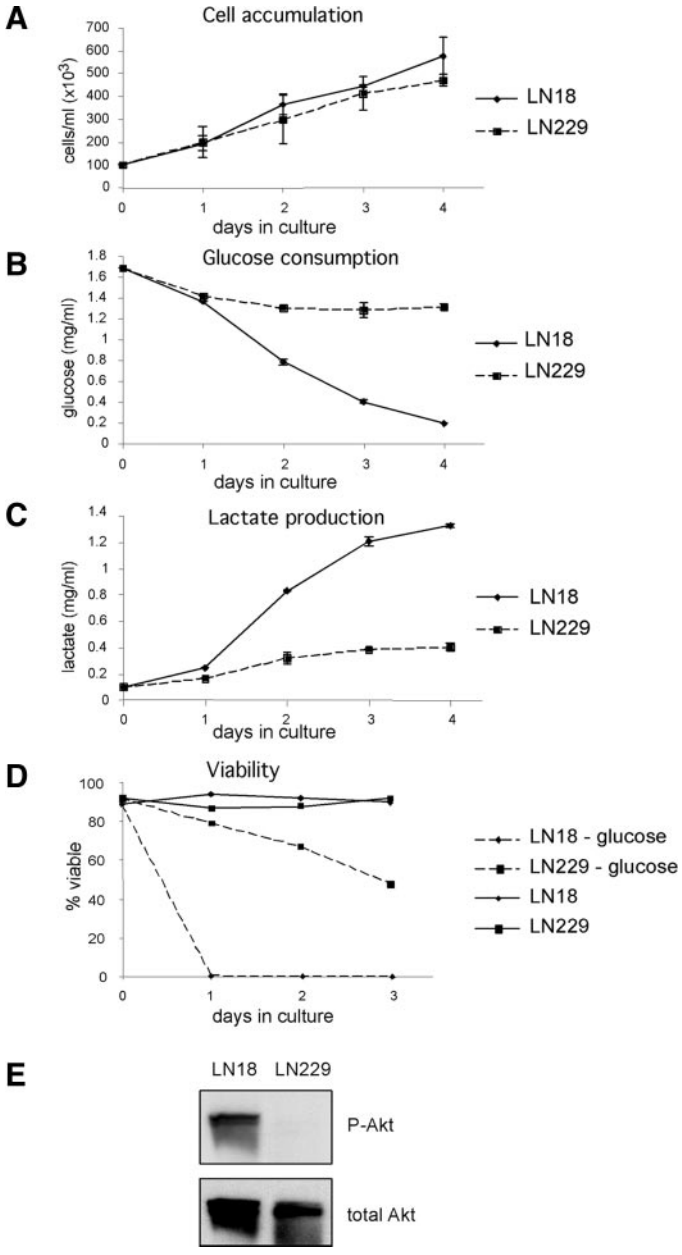

Fig. 4. LN18 cells take up glucose and secrete lactate to a greater extent than LN229 cells, and LN18 cells are dependent on glucose for viability. LN18 or LN229 glioblastoma cells were plated at 10^5/ml and cultured for 4 days in the absence of serum. Cells were counted daily (*A*). Aliquots of medium were removed at each time point and tested for glucose (*B*) and lactate (*C*). *D*, viability was tested by propidium iodide exclusion. Data are representative of three independent experiments. *E*, Akt activity was evaluated by Western blot with antibody against phospho-Akt Ser^{473} (P-Akt) in cells deprived of serum.

these two cell lines proliferated at a similar rate. Although LN18 consumed glucose from the medium at a high rate, LN229 demonstrated a much lower level of glucose consumption (Fig. 4*B*). This excess glucose consumption was closely paralleled by increased lactate production in the culture medium (Fig. 4*C*). Thus, at a first approximation, both cells used a comparable amount of glucose to support cell growth and bioenergetics, but they differed dramatically in how much additional glucose was processed during a 4-day culture period. NADH levels were higher in LN18 than LN229, whereas total NAD levels were comparable. The two lines consumed oxygen at similar rates (data not shown). In addition, upon inhibition of glycolysis by iodoacetic acid, NADH levels dropped 75% more in LN18 than in LN229, indicating that more NADH was generated through glycolysis in LN18 than in LN229.

LN18 Cells, in Contrast to LN229, Require Glucose for Viability. The observation of high glucose metabolism in LN18 raised the question of whether these cells are dependent on glucose for support of bioenergetics and cellular survival. The viability of LN18 cells was compared with that of LN229 cells upon glucose withdrawal. As shown in Fig. 4*D*, the viability of LN229 cells remained high upon glucose withdrawal, declining only gradually over the course of several days. LN18 cells, in contrast, died rapidly in the absence of glucose, with essentially all cells dead after 1 day in culture. Therefore, not only do LN18 cells demonstrate a high rate of glucose metabolism, but also they are dependent on glucose for viability.

Akt Activity Correlates with High Rate of Glucose Metabolism. Given the ability of Akt to promote glucose metabolism in the murine leukemia model, we wished to determine whether Akt activity might account for the metabolic differences observed between these cells. By Western blot analysis (Fig. 4*E*), in the absence of exogenous serum, LN18 had a high level of active Akt, as measured by activation-associated phosphorylation at serine 473. LN229 cells, however, showed minimal to no detectable Akt phosphorylation. Upon addition of serum, however, both LN18 and LN229 induced Akt phosphorylation markedly and to a similar level (data not shown). Therefore, consistent with the findings in the leukemic cells, LN18, which showed a high level of constitutively active Akt, metabolized glucose at a high rate and was dependent on glucose for survival. In contrast, LN229, which had a low level of Akt under unstimulated conditions, performed glycolysis at a lower rate and was much less dependent on glucose for its survival. Although correlative, these findings supported the hypothesis that Akt activity accounts for the strikingly different metabolic phenotypes observed in these cancer cells.

Because serum stimulation promoted Akt activation in LN229 to a level comparable with that in LN18, glucose uptake and lactate production were measured in these cells in the presence of serum. No difference in cellular proliferation between the cell lines was observed in the presence of serum. However, glucose uptake and lactate production of LN229 cells were dramatically stimulated, resulting in identical levels of glucose uptake and lactate production upon serum stimulation (data not shown).

Disruption of the PI3K/Akt Pathway in LN18 Inhibits Glucose Metabolism. To additionally elucidate the role of Akt in promotion of aerobic glycolysis in these cells, the effect on glucose metabolism of inhibition of the upstream activator, PI3K, by treatment with LY294002 was studied. Cells were incubated under identical conditions to those in Fig. 4, except that 10 μM LY294002 were added. The presence of the inhibitor abolished Akt phosphorylation (Fig. 5*A*). Furthermore, LY294002 inhibited the glucose uptake and lactate production observed in LN18 cells to the level of LN229 cells (Fig. 5, *B* and *C*). PI3K inhibition also compromised the viability of LN18 to a greater extent than that of LN229 at later time points (data not shown), suggesting that LN18 cells, in addition to their dependence on glucose, are dependent on the activity of PI3K for viability.

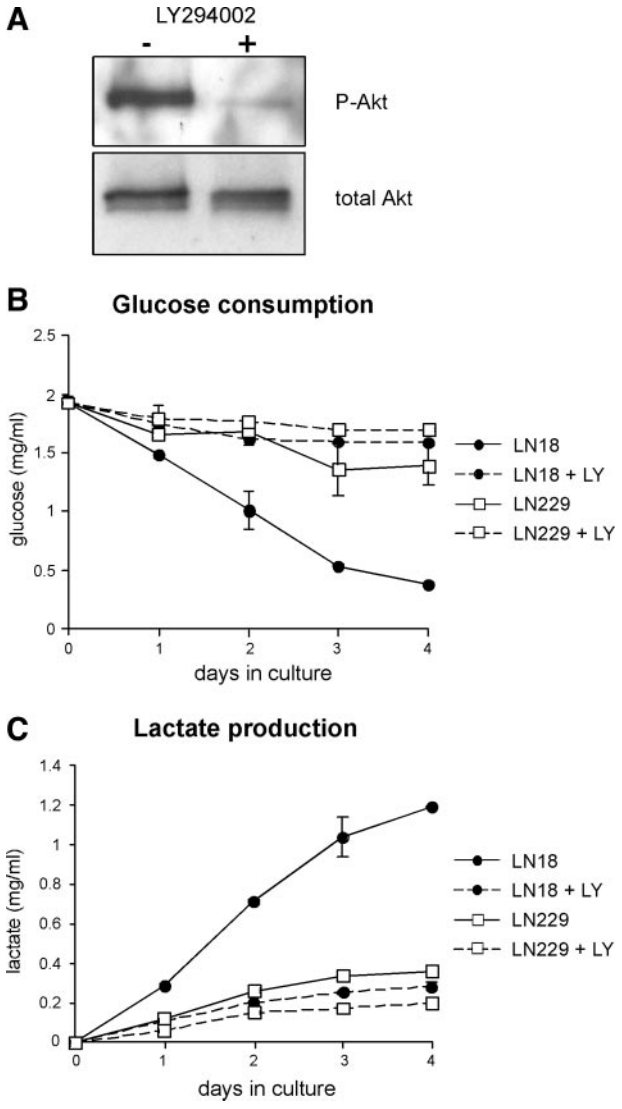

Fig. 5. The phosphatidylinositide-3-kinase inhibitor LY294002 (LY) causes dephosphorylation of Akt in LN18 cells and inhibits glucose uptake and lactate production. *A*, Akt phosphorylation in LN18 cells was assayed by Western blot for phospho-Ser^{473} in the absence or presence of LY. *B* and *C*, cells were cultured as in Fig. 4 in the presence or absence of LY and glucose (*B*) and lactate (*C*) levels were assayed every day.

Expression of Active Akt in LN229 Promotes Glycolytic Metabolism. To clarify the role of Akt in the metabolic differences observed in these cells, we chose to study the effect of Akt activation in LN229. LN229 cells were transfected with pSFFV vector alone or pSFFV containing a myristoylated Akt transgene. As shown in Fig. 6*A*, LN229 cells transfected with myrAkt (myrAkt-1 and myrAkt-2) expressed a higher level of total and phosphorylated Akt than either vector control (vec-1 and vec-2) or parental LN229, with some clones approaching the phospho-Akt levels seen in LN18 cells. This Akt phosphorylation was resistant to LY294002, showing PI3K independence of Akt activity.

The effect of myrAkt expression on glucose metabolism in LN229 was investigated by measuring glucose consumption and lactate production. Vector control and myrAkt-expressing cells proliferated at a rate similar to parental LN229 (Fig. 6*B*). As shown in Fig. 6*C*, myrAkt-expressing cells, myrAkt-1 and myrAkt-2, consumed glucose at a higher rate than either vector control or parental LN229 cells. In

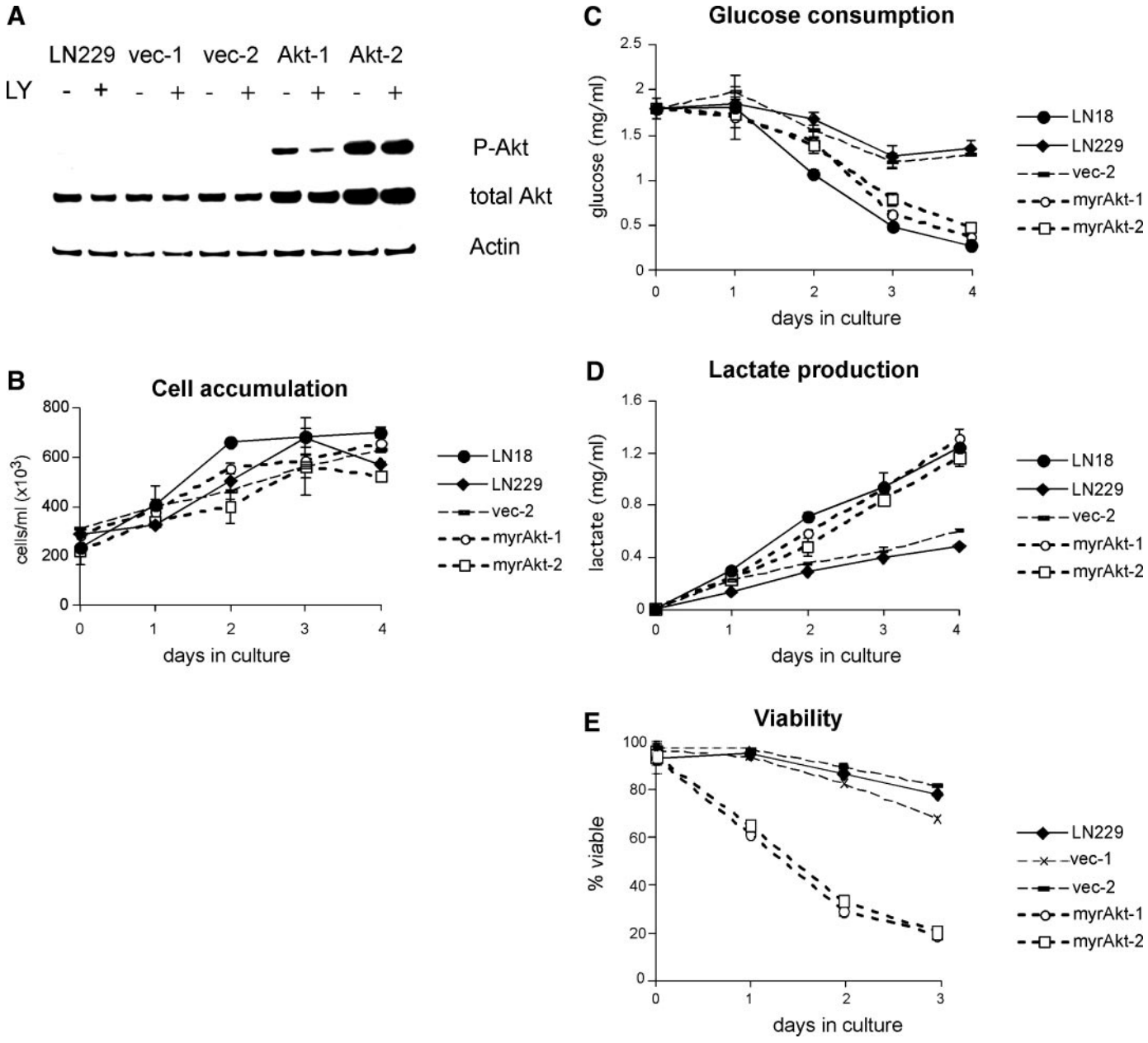

Fig. 6. Expression of myrAkt in LN229 cells drives glucose uptake and lactate production. *A*, LN229 cells were transfected with pSFFV plasmid alone or pSFFV containing a myrAkt construct. Levels of phosphorylated (P-Akt) and total Akt were determined by Western blot analysis. LN229 represents the parental cell line; myrAkt-1 and myrAkt-2 represent two independent clones stably transfected with myrAkt; vec-1 and vec-2 are two independent clones stably transfected with the empty vector. Cells were cultured in the presence or absence of LY294002 (LY) for 8 h before protein isolation. *B*, control and transfected cells were cultured as in Fig. 4, and cell numbers were determined every day. Glucose (*C*) and lactate (*D*) levels were assayed at each time point of the experiment presented in *B*. *E*, cells were cultured in the absence of glucose, and viability was determined by propidium iodide exclusion. Data are representative of three independent experiments.

addition, lactate production was higher in the myrAkt transfectants, similar to that seen in LN18 cells (Fig. 6*D*). MyrAkt transfectants had a higher ratio of NAD(P)H/NAD(P) than control transfectants or parental LN229 cells despite similar total NAD levels, and inhibition of glycolysis by iodoacetic acid resulted in a 2-fold greater drop in NADH in the myrAkt-expressing cells than in controls (data not shown). Nevertheless, the myrAkt-expressing cells and vector controls displayed similar rates of oxygen consumption. Therefore, activated Akt expression is sufficient to convert LN229 cells to a glycolytic form of metabolism.

Akt Activation Renders LN229 Cells Susceptible to Glucose Withdrawal-Induced Death. The susceptibility of LN18 cells to death upon glucose starvation could either be due to a specific adaptation of LN18 cells or it could be due to a detrimental effect of Akt itself under conditions of glucose deprivation. To test these two alternatives, the effect of Akt expression in LN229 cells on survival in the absence of glucose was assessed. If the poor survival of LN18 cells was due to a cell-specific adaptation, Akt-transfected LN229 cells would be expected to survive similarly to parental cells in the absence of glucose. Surprisingly, Akt-expressing LN229 cells showed a marked acceleration of death upon removal of glucose (Fig. 6*E*), indicating that Akt activity itself is detrimental to survival under conditions of glucose starvation.

DISCUSSION

Inhibition of apoptosis has been implicated as a major contributor to cellular transformation. Two major antiapoptotic proteins are

Bcl-x_L and Akt, and both potently generate apoptotic resistance *in vitro*. Despite this, Akt promoted the malignant transformation of nontransformed hematopoietic cells whereas Bcl-x_L did not (9). Therefore, there are functions of Akt in addition to its effects on cell survival that are instrumental in the development of malignancy. Consistent with this hypothesis, Bcl-x_L and Akt have been shown to have synergistic effects on cellular transformation (9). The purpose of this study was to define the properties of Akt that might contribute to transformation beyond apoptosis inhibition. The data presented above demonstrate the ability of Akt to promote increased glucose utilization by tumors that is independent of any effects Akt has on stimulation of cell proliferation.

Although Akt expression did not promote increased accumulation of malignant hematopoietic cells *in vitro*, Akt caused a dose-dependent stimulation of glycolysis. Equivalent results were observed in human glioblastoma cells. The difference in rates of glucose metabolism between LN18 and LN229 cells correlated with the activity of Akt. Furthermore, inhibition of Akt activation prevented the increased utilization of glucose by LN18 cells, whereas activation of Akt converted LN229 cells to a high level of aerobic glycolysis.

Several possibilities may explain why the glucose utilization of a cell might increase. The first is to meet an elevated demand for byproducts of glucose metabolism such as energy equivalents or biosynthetic substrates. Second, cells may acquire defects in their ability to carry out oxidative phosphorylation. Although both of these possibilities have been extensively studied in cancer cell lines, they appear to provide an inadequate explanation for most tumor-associated increases in aerobic glycolysis. The present data suggest a third potential explanation for a tumor-associated increase in glucose utilization: direct stimulation of the cellular rate of glycolysis. Unlike liver, muscle, and fat cells, most cancer cells are unable to store excess glucose as either glycogen or fat. Therefore, stimulation of cellular glucose uptake in excess of demand will result in increases in lactate production to allow the cell to rid itself of excess carbon and to recycle NADH to NAD^+. The increased production of lactate and NADH by glycolysis that we observed provides evidence in favor of the latter model. That is, Akt activity appeared to directly promote the use of glucose in proliferating cells.

Furthermore, Akt prompted cells to increase glucose utilization without increasing oxygen consumption. This shift toward aerobic glycolysis has been noted as characteristic of many types of malignant cells. Many of the previous explanations of this so-called Warburg effect have invoked mutations in the mitochondrial respiratory apparatus or in glycolytic enzymes themselves (4, 26). We suggest that activation of a single oncogene, the serine/threonine kinase Akt, is sufficient to account for this switch to glycolysis. Activation of the PI3K/Akt pathway is prevalent in many types of malignancies (15, 17, 18, 27, 28) and thus may constitute a more universal explanation of tumor cell metabolism.

The observation that an oncogene may directly alter the metabolism of cancer cells is surprising. Oncogenes are generally considered to direct cellular processes such as proliferation or survival, with metabolic changes considered as secondary effects of oncogene activation. This present finding raises the possibility that metabolic transformation may be a key step in the development of cancer. Although Akt is one mediator of this phenomenon, it is possible that other signaling pathways may also drive similar phenotypes. The observation that nearly all lymphomas, including very low-grade tumors, have a high rate of glucose uptake and utilization, as demonstrated by FDG-PET scanning (29), suggests that this metabolic transformation may play a fundamental role in tumorigenesis. Other investigators have demonstrated the ability of c-Myc to promote glucose metabolism (30, 31), and c-Myc-transformed cells have also been demonstrated to be susceptible to glucose deprivation-induced apoptosis (32). The specificity of this finding is unclear because c-Myc expression renders cells susceptible to death by various stimuli, whereas Akt promotes survival under many conditions not associated with glucose depletion. Hypoxia-inducible factor (HIF) has also been implicated in promotion of glycolytic metabolism in cancer cells. Although HIF can be activated under conditions of hypoxia present in large tumor masses, some investigators have suggested that HIF may also be activated in signaling pathways, including the PI3K/Akt pathway (33–35), although this finding is controversial (36, 37). HIF activity does not appear to account for the increased aerobic glycolysis observed in the current study, however, because there was no difference in HIF expression in Akt-transfected cells in our system (data not shown).

The potential benefit to a cancer cell of a high glycolytic rate is speculative but could derive from several processes. First, a continuous supply of substrates for mitochondrial electron transport may prevent Bax activation, mitochondrial catastrophe and programmed cell death. A high rate of glucose uptake ensures a constant supply of substrates for biosynthetic processes such as nucleic acid and amino acid synthesis to fuel the growth and proliferation induced by other oncogenes. Finally, the availability of glucose to enter the oxidative arm of the pentose phosphate cycle provides ample substrate for production of NADPH, which may be important in maintaining the redox state of a cell under oxidative stress. A high availability of glucose has been shown to provide protection against oxidative stress in some cell types (38).

Previous work has suggested that a major factor regulating cell growth is the ability of cells to take up sufficient amino acids and lipids to supply the production of proteins and membranes necessary for cell doubling. Akt-transfected cells have been shown to up-regulate transporters for both amino acids and lipids (39). Despite this, malignant cells expressing Akt are unable to survive in the absence of extracellular glucose. Thus, the finding that Akt activity may actually be detrimental to transformed cells under conditions of glucose starvation is unexpected because Akt has been shown to protect cells from numerous causes of death. The mechanism of this susceptibility to death is unclear. It is possible that Akt drives downstream biosynthetic pathways such as protein synthesis that require a constant supply of substrates. The observation that Akt promotes mTOR activity and, subsequently, translation supports this possibility (39). Under Akt stimulation, amino acids and lipids may be channeled into cell growth, and their catabolism may be reciprocally suppressed. In favor of this model, Akt-expressing cells dramatically up-regulate the rate of glycolysis but do not increase total oxygen consumption, as would be expected if catabolism of nonglucose substrates made a significant contribution to cellular energetics. Thus, Akt would render cells dependent on glucose catabolism to maintain bioenergetics. Alternatively, Akt may suppress cellular programs that allow survival in the face of nutrient deprivation. Although Akt activation renders cells susceptible to cell death upon glucose limitation, cancer cells at the growing edge of a tumor are unlikely to experience such a limitation because vascularized regions of the body are constantly resupplied with glucose.

In conclusion, these results indicate that the expression of the active Akt oncogene is sufficient to promote tumor cell-autonomous increases in aerobic glycolysis. These Akt-expressing cancer cells require a continuous glucose supply for survival. Therefore, Akt-activated aerobic glycolysis appears to provide a therapeutic target that may have selectivity for transformed cells.

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