Mammalian Target of Rapamycin Promotes Vincristine Resistance through Multiple Mechanisms Independent of Maintained Glycolytic Rate

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Abstract

Deregulation of the phosphoinositide 3-kinase-Akt pathway is a major contributor to oncogenesis and resistance to cancer therapy. Recent work has shown Akt to be a major target of rapamycin (mTOR) to be a major target of Akt that contributes to both transformation and therapeutic resistance. Although inhibitors of Akt are not yet clinically available, rapamycin, a mTOR-specific inhibitor, has long been used as an immunosuppressant, and several rapamycin analogues are now in clinical trials in oncology. Recent data indicate that a mTOR complex phosphorylates Akt, and this complex is insensitive to rapamycin. We show that dominant-negative mTOR diminishes phosphorylation of endogenous Akt and exogenous myristoylated Akt (mAkt), that prolonged exposure to rapamycin also inhibits Akt activation, and that this inhibition is dependent on new protein synthesis. These data suggest that mTOR facilitates Akt activation through mechanisms other than direct phosphorylation. A constitutively active mTOR mutant that fails to enhance Akt phosphorylation nevertheless promotes resistance to multiple antimitrotubule agents, indicating that mTOR also mediates survival independent of Akt. Although Akt- and mTOR-mediated survival has been linked to regulation of cellular metabolism, we also show that survival and metabolic control are separable. The hexokinase inhibitor 5-thioglucose markedly inhibits glycolytic rate but does not diminish vincristine resistance mediated by mAkt or mTOR, and it has only a minor effect on mTOR- or mAkt-mediated resistance to growth factor withdrawal, suggesting that Akt-mTOR-mediated resistance is largely independent of maintenance of glycolytic rate. We conclude that mTOR activity can promote resistance through multiple mechanisms independent of maintained glycolytic rate. (Mol Cancer Res 2005; 3(11):635–44)

Introduction

Tumor cells frequently acquire independence from exogenous growth and survival signals in the process of carcinogenesis (1). The phosphoinositide 3-kinase (PI3K) pathway is normally regulated by multiple growth factor receptors, and this pathway is deregulated in numerous cancer types (2). The downstream serine/threonine kinase Akt, first identified as a viral oncogene homologue, is overexpressed in many solid tumors (3, 4). Loss of the tumor suppressor PTEN, a negative regulator of this pathway, allows persistent activation of Akt. Recently, Samuels et al. have found that the catalytic subunit of PI3K is mutated in up to 32% of colon cancers (5). Further understanding of the PI3K pathway and how it contributes to cancer cell survival will enhance the development of therapies targeting various components of this pathway.

Activation of the survival kinase Akt is a major component of PI3K function. Several targets downstream of Akt have been proposed as contributors to Akt-mediated survival and therapeutic resistance. Akt phosphorylates the proapoptotic Bcl-2 family member Bad, which can then be bound by 14-3-3 proteins, sequestered in the cytosol, and prevented from promoting apoptosis at the mitochondria (6). Akt also activates mammalian target of rapamycin (mTOR), a PI3K-related protein kinase family member that has been implicated in both transformation and therapeutic resistance. Akt can directly phosphorylate a repressor domain of mTOR, promoting its activity (7). In addition, Akt indirectly activates mTOR through phosphorylation and inhibition of the negative regulator complex TSC1/TSC2 leading to activation of the positive mTOR regulator Rheb (8, 9). mTOR phosphorylates 4E-BP and p70S6K, key regulators of protein translation, and also has effects on transcription, cell cycle progression, and nutrient sensing (10). The macrolide rapamycin is a specific inhibitor of mTOR. Rapamycin and its analogues are being actively studied in clinical trials involving a variety of hematologic malignancies and solid tumors (11).

mTOR associates with regulatory-associated protein of mTOR and G3P to promote translation of a subset of proteins via phosphorylated 4E-BP and p70S6K. Elucidation of mTOR function has largely been done using rapamycin, which disrupts mTOR-regulatory-associated protein of mTOR function. The
ability of rapamycin to inhibit all mTOR function equally has been controversial, however (12, 13). Recent work has identified rapamycin-insensitive companion of mTOR (rictor), a new mTOR-binding partner, and a mTOR-rictor complex is insensitive to rapamycin (14). When associated with rictor and G3p, mTOR phosphorylates and activates Akt (15). These data show a positive feedback relationship between mTOR and Akt.

Events downstream of mTOR activation have been shown to promote survival and therapeutic resistance. An activated mTOR mutant has been shown to maintain nutrient transport and promote survival in the absence of growth factor in a murine hematopoietic cell line (16). IF-4E is bound and inhibited by 4E-BP. mTOR activation leads to phosphorylation and neutralization of 4E-BP, allowing IF-4E to promote cap-dependent translation of a subset of proteins. Overexpression of IF-4E in a murine leukemia model has been shown to promote tumor growth and drug resistance (17). Given recent reports on the complex relationship between mTOR-rictor and Akt, the ability of mTOR to promote survival could be through positive feedback on Akt and/or through Akt-independent mechanisms.

Activation of Akt has been shown to promote survival in the absence of serum and growth factors (18) and to increase resistance to several chemotherapeutic agents (19). In contrast to survival mediated by overexpression of Bcl-2 or Bcl-xL, Akt-mediated resistance to growth factor withdrawal is characterized by maintenance of high-level glucose consumption (20). Akt regulates several key steps in glycolytic metabolism: expression of glucose transporters, translocation of transporters to the plasma membrane, localization of hexokinase, and activation of phosphofructokinase 1 (21, 22). Data from our laboratory and others suggested that the ability of Akt to promote survival might be linked to its ability to up-regulate glycolytic activity and that both these activities require mTOR for maximal function (23-26).

It has long been known that transformed cells consume more glucose than their normal counterparts and that this glucose is used preferentially for glycolysis and lactate production rather than oxidative phosphorylation and consumption of oxygen (27). This observation, termed the Warburg effect, is the basis for [18F]fluorodeoxyglucose positron emission tomography scanning as a clinical staging modality. Tumors take up this nonmetabolizable glucose analogue, which is then phosphorylated by hexokinase and retained in the cell, where it can be visualized on positron emission tomography scan. Otto Warburg proposed that the altered metabolism associated with malignant growth is due to loss of functional mitochondria (27), but subsequent studies show that oxidative pathways are functional in cancer cells. The consistent confirmation of the Warburg effect across tumor types suggests that preferential use of glycolytic metabolism by cancer cells is of functional significance.

We sought to investigate the relationship between mTOR activity and the activity of endogenous or membrane-targeted Akt and the contribution of this relationship to therapeutic resistance. We show that mTOR increases vincristine resistance through at least two distinct mechanisms. First, mTOR activation can feed back to augment Akt phosphorylation and activity. This mTOR function is sensitive to prolonged exposure to rapamycin and this sensitivity is dependent on new protein synthesis. Second, a constitutively active mTOR mutant can promote vincristine resistance independent of effects on Akt.

We previously used rapamycin to show that mTOR activity is a major determinant of Akt-mediated vincristine resistance and is required for Akt-mediated up-regulation of glycolysis following exposure to vincristine (24). This correlation suggested that mTOR-dependent glycolytic activity might be required for Akt-mTOR-mediated vincristine resistance. Using the hexokinase inhibitor 5-thioglucose (5TG), we sought to investigate further the mechanistic basis of Akt-mTOR-mediated chemotherapeutic resistance. Data presented here show that Akt-mTOR-mediated vincristine resistance is in fact not dependent on up-regulated hexokinase activity or on maintenance of an elevated rate of glycolysis. We conclude that mTOR contributes to Akt-mediated vincristine resistance through multiple mechanisms independent of maintained glycolytic activity. Further definition of the feedback mechanisms within the PI3K-Akt-dependent signaling pathway regulating chemotherapeutic resistance will be essential in the rational incorporation of agents targeting downstream components of this pathway into cancer therapy.

**Results**

**Inhibition of mTOR Activity Inhibits Akt Phosphorylation**

Previous results from our laboratory and others showed that Akt activity promotes resistance to microtubule-directed chemotherapeutic agents and that inhibition of mTOR activity diminishes Akt-mediated survival (17, 24, 28). Recent results suggest that mTOR regulates Akt through a feedback mechanism, whereby inhibition of mTOR leads to a subsequent decrease in Akt phosphorylation (15, 29). These reports show that inhibition of mTOR activity can lead to down-regulation of phosphorylation of endogenous Akt. We have shown that rapamycin diminishes the ability of membrane-targeted, constitutively active myristoylated Akt (mAkt) to confer therapeutic resistance. To determine if mTOR activity is required for maximal phosphorylation of the active mutant mAkt, we stably expressed wild-type (WT) or dominant-negative mTOR with point mutations in the rapamycin-binding and kinase domains in FL5.12 cells expressing mAkt or a control vector. Lysates from these cells were probed for total and phosphorylated 4E-BP proteins. Dominant-negative mTOR decreases 4E-BP phosphorylation in control and mAkt cells (lane 2 versus lane 1 and lane 4 versus lane 3, respectively).

We used these cells to evaluate whether inhibition of mTOR decreases Akt activation. mAkt cells expressing dominant-negative mTOR showed a decrease in Akt phosphorylation at S473 (Fig. 1B, lanes 1 and 2), indicating a positive feedback relationship between mTOR and Akt. Sarbassov et al. have shown that a mammalian complex homologous to the yeast TORC2 complex phosphorylates Akt at S473 (15). Unlike the association between mTOR and regulatory-associated protein of mTOR (contained in a TORC1-like complex), the association between mTOR and rictor (in the TORC2-like complex) is not disrupted by exposure to rapamycin (14). To determine if mTOR-rictor is responsible for the positive feedback of mTOR on constitutively active mAkt, we exposed mAkt cells to...
translocation and subsequent gene transcription. To determine if rapamycin also affects phosphorylation of FKHR family members by Akt, the Akt-specific phosphorylation site (S253) of FKHR was examined by immunoblot. In contrast to phosphorylation of GSK-3β and Bad, we detected no change in FKHR phosphorylation following exposure to rapamycin in either control or mAkt cells (Fig. 2C). These data suggest that the sensitivity of Akt signaling to rapamycin may be target specific.

Rapamycin-Mediated Inhibition of Akt Is Time Dependent and Requires New Protein Synthesis

In complex with GβL and rictor, mTOR has been shown to directly phosphorylate Akt on S473 (15). Because mTOR is the kinase mediating this direct phosphorylation, one would expect that inhibition of mTOR would lead to a rapid decrease in Akt phosphorylation and that it would not depend on new protein synthesis. To determine if duration of mTOR inhibition affects the degree of Akt inhibition, we examined Akt phosphorylation following exposure to rapamycin over time. We wanted to examine the effect of mTOR inhibition on both endogenous Akt and mAkt. Phosphorylation of Akt was not decreased following exposure to rapamycin for 1 hour in either control or mAkt cells (Fig. 3A). Exposure to rapamycin for ≥4 hours resulted in progressive inhibition of mAkt phosphorylation. In contrast, endogenous Akt was not detectably inhibited by rapamycin unless length of exposure was >8 hours.

![FIGURE 1. mTOR modulates phosphorylation of Akt. A. Lysates of control (ctrl) or mAkt FL5.12 cells containing either WT or dominant-negative (DN) mTOR were probed for phosphorylated (T37/T46) or total GSK-3β and Akt. B. Lysates of mAkt FL5.12 cells exposed to 20 nmol/L rapamycin overnight or left untreated or expressing WT or dominant-negative mTOR were probed for phosphorylated (S473) or total Akt. Representative of one of three independent experiments.](image)

![FIGURE 2. Rapamycin inhibits phosphorylation of GSK-3β and Bad but not FKHR. mAkt or control FL5.12 cells were exposed to 20 nmol/L rapamycin overnight. Lysates were made and probed with antibodies for phosphorylated (S9) GSK-3β, total GSK-3β, and β-actin (A); phosphorylated (S136) Bad or total Bad (B); or phosphorylated (S256) FKHR, total FKHR, and β-actin (C). Bottom, the ratio of phosphorylated to total protein for each sample relative to that of untreated control cells; – , not distinguishable from background. B and C. Experiments were repeated with similar results.](image)
mTOR is known to regulate translation of a subset of proteins, and the effect of rapamycin on cell cycle progression is thought to be mediated by its inhibition of mTOR-dependent translation. To determine if translation of new proteins is required for inhibition of Akt by rapamycin, cells were treated with cycloheximide, a global inhibitor of protein synthesis. Cells treated with rapamycin, cycloheximide, both, or neither were examined by immunoblot for Akt phosphorylation (Fig. 3B). Cycloheximide alone somewhat enhanced Akt phosphorylation in control cells, and the inhibition of endogenous Akt phosphorylation by rapamycin in these cells seemed to be entirely blocked by cycloheximide (Fig. 3B, lane 4). In contrast, the effect of rapamycin on mAkt activation seemed to be at least partially maintained in the presence of cycloheximide (Fig. 3B, lane 8). These data suggest that although endogenous Akt and exogenous mAkt are both inhibited by rapamycin these effects may be mechanistically distinct. The requirement for prolonged rapamycin exposure (>8 hours) to inhibit endogenous Akt, together with the reversal of this effect by cycloheximide, suggests that translation of new proteins is required for rapamycin-mediated inhibition of endogenous Akt. Furthermore, the requirement for new protein synthesis suggests that the positive feedback of mTOR on Akt is not due to direct phosphorylation of S473.

mTOR Promotes Resistance to Microtubule-Directed Chemotherapeutics through Akt-Dependent and Akt-Independent Mechanisms

Rapamycin inhibits mTOR and diminishes mAkt-mediated vincristine resistance. Rapamycin also inhibits phosphorylation and activation of Akt. One hypothesis to account for these observations is that mTOR promotes Akt-mediated survival solely through its activation of Akt. Alternatively, mTOR may have additional effects on survival independent of a feedback regulation of Akt. To differentiate between these two possibilities, we used two subclones expressing a constitutively active mTOR mutant containing a deletion in a COOH-terminal repressor domain (ΔTOR). To evaluate whether this construct is functional in maintaining mTOR activity, we measured the ability of these subclones to maintain phosphorylation of 4E-BP following withdrawal of the growth factor interleukin-3 (IL-3; Fig. 4A). Although subclones expressing ΔTOR show some decrease in phosphorylation of 4E-BP following IL-3 withdrawal, 4E-BP phosphorylation is preserved relative to control transfectants, indicating constitutive, although submaximal, mTOR activity in these subclones. Because Akt also activates mTOR, leading to phosphorylation of 4E-BP, as a control, we also examined the ability of mAkt to maintain phosphorylation of 4E-BP. mAkt maintains 4E-BP phosphorylation in the absence of growth factor. The shift in mobility of total 4E-BP is indicative of its maximal phosphorylation in the presence of mAkt.

Inhibition of mTOR leads to diminished Akt phosphorylation, indicating positive feedback between mTOR and the upstream kinase Akt. To determine if up-regulated activation of mTOR leads to superactivation of Akt, ΔTOR lysates were probed with antibodies to phosphorylated or total Akt. There was no apparent change in Akt phosphorylation in ΔTOR cells (Fig. 4B), suggesting that, although mTOR activity may be necessary for full Akt activation, expression of a constitutively active mTOR construct does not further enhance this effect.

The inability of the ΔTOR mutant to superactivate Akt allowed us to examine the protective effects of activated mTOR independent of Akt. To determine if ΔTOR cells are resistant to multiple antimicrotubule agents, two independent ΔTOR subclones were exposed to paclitaxel or nocodazole (Fig. 4C and D). The ΔTOR subclones exhibited increased resistance to both antimicrotubule agents. The ΔTOR mutation also confers
resistance to vincristine (see Fig. 6C). Resistance of these clones to vincristine, paclitaxel, or nocodazole was not as robust as that of mAkt-expressing cells (24). There are many potential explanations for this difference, including the probable presence of downstream targets of Akt unaffected by mTOR and/or relative weakness of Akt-mediated vincristine resistance to microtubule-directed chemotherapeutics through both Akt-dependent and Akt-independent mechanisms.

5TG Inhibits Glycolytic Rate and Production of Lactate

mTOR feeds back to promote maximal Akt phosphorylation. Inhibition of mTOR activity leads to down-regulation of Akt activity. In addition, mTOR promotes survival following exposure to chemotherapeutics independent of Akt activation. The ability of both Akt and mTOR to promote survival has been linked to effects on cellular metabolism. Previous work by our laboratory and others showed that Akt promotes resistance to antitubulin agents, and this Akt-mediated survival is dependent on activity of the downstream kinase mTOR. The mTOR inhibitor rapamycin inhibited both Akt-mediated survival and Akt-mediated glycolytic maintenance following exposure to vincristine. This correlation suggested that these two Akt-mediated, mTOR-dependent activities might be linked.

To determine if Akt is dependent on increased glycolytic rate to promote vincristine resistance, we examined the ability of Akt to promote survival following withdrawal of glucose or following exposure to the glucose analogue and glycolytic inhibitor 2-deoxyglucose. We found these treatments to be inherently cytotoxic and hence confounded analysis of the additional effects of the cytotoxic agents (data not shown). We hypothesized that inhibition of hexokinase, the key catabolic enzyme of the first step of glycolysis and an Akt target, would also result in a reduction in glycolytic rate. 5TG is a competitive inhibitor of hexokinase. To determine the effectiveness of 5TG in inhibiting glycolysis, the rate of conversion of [5-3H]glucose to H2O was measured in the presence of 1 mmol/L glucose and increasing concentrations of 5TG (Fig. 5A). In mAkt or control FL5.12 cells, 5TG decreased glycolytic rate in a dose-dependent manner. At 1- or 10-fold molar ratio of 5TG to glucose, the glycolytic rate of control and mAkt cells was decreased by ~70% or 90%, respectively, without having a significant effect on cell survival.

The inhibition of glycolysis by 5TG that we observed would be expected to greatly diminish cell growth and proliferation. In addition, functional inhibition of glycolysis would be expected to increase the shunting of available pyruvate into the citric acid cycle and oxidative phosphorylation to maintain cellular ATP production rather than being used to generate lactate, an end product of aerobic glycolysis (30).

We evaluated the effects of 5TG on cellular proliferation and lactate production of mAkt and control cells. As shown in Fig. 5B, 5TG substantially inhibited cell proliferation of both control and mAkt-expressing cells. Lactate production in both control and mAkt-expressing cells was almost completely shutdown in the presence of 5TG (Fig. 5C). These data suggest that residual cellular proliferation in the presence of 5TG is dependent on nearly complete oxidative consumption of available pyruvate derived from the minimal remaining glycolytic activity and/or from other cellular sources.

Increased Hexokinase Activity and Glycolytic Rate Are Not Required for Akt-Mediated Resistance to Antimicrotubule Agents

To assess the contribution of hexokinase activity to Akt-mediated vincristine resistance, mAkt or control cells were exposed to vincristine in the presence or absence of 5TG. Because maintenance of glycolytic rate is thought to contribute

**FIGURE 4.** mTOR contributes to vincristine resistance through Akt-independent mechanisms. A. Two independent subclones of FL5.12 cells expressing constitutively active mTOR (ΔTOR 1 or ΔTOR 2) or an empty vector control (ctrl 1 and ctrl 2) were probed with antibodies to phosphorylated (T37/T46) or total 4E-BP. B. Lysates of two ΔTOR subclones (Δ1 and Δ2) or two control subclones (C1 and C2) were probed with antibodies to phosphorylated (S473) or total Akt. Representative of one of four independent experiments with similar results. C and D. FL5.12 cells expressing a constitutively active mTOR mutant or an empty vector control were exposed to 1 μg/mL paclitaxel (C) or 100 nmol/L nocodazole (D). Cell viability was measured every 24 hours via propidium iodide exclusion. Experiments were repeated with similar results. Bars, SD.
to mAkt-mediated resistance to growth factor withdrawal, for comparison, cells were also withdrawn from the growth factor IL-3 in the presence or absence of 5TG. Inhibition of hexokinase had no effect on mAkt-mediated vincristine resistance (Fig. 6A). Surprisingly, inhibition of hexokinase also had very little effect on the viability of mAkt cells following growth factor withdrawal. Although 5TG slows proliferation somewhat, the cells remain viable and maintain therapeutic resistance. 5TG inhibits not only hexokinase activity but also the overall glycolytic rate of mAkt and control cells (Fig. 6A). These data imply that mAkt can promote resistance to growth factor withdrawal independent of glycolytic rate.

Voltage-dependent anion channel–associated mitochondrial hexokinase has been shown to promote resistance to Bax-mediated release of cytochrome c (31). Replacing glucose in medium with glucose-6-phosphate, the product of the reaction catalyzed by hexokinase, bypasses the need for hexokinase, and the inactive hexokinase dissociates from voltage-dependent anion channel and the mitochondria. Pastorino et al. found that exogenous glucose-6-phosphate sensitized mitochondria to Bax-mediated cytochrome c release (31). To further assess whether hexokinase activity is required for Akt-mediated vincristine resistance, mAkt or control cells were grown in medium in which glucose was entirely replaced by glucose-6-phosphate or galactose, both of which are substrates that enter the glycolytic pathway downstream of the hexokinase-mediated reaction. mAkt cells showed similar resistance to vincristine whether maintained in glucose or in either of the alternative carbohydrate substrates. Control cells showed similar vincristine sensitivity, again irrespective of substrate (Fig. 6B).

We have shown previously that an activated mTOR construct promotes resistance to vincristine (24). Activated mTOR also has been shown to support growth factor withdrawal but not withdrawal from glucose or amino acids and to maintain membrane localization of nutrient transporters (16). To assess the contribution of hexokinase activity to mTOR-mediated resistance, we exposed FL5.12 cells expressing ΔTOR or an empty vector control to vincristine, or withdrew them from growth factor, for 48 hours in the presence or absence of 5TG (Fig. 6C). The presence of 5TG had a minimal effect on the ability of ΔTOR to promote resistance to vincristine or growth factor withdrawal.

Taken together, these data show that neither increased hexokinase activity specifically nor elevated glycolytic rate in general is required for mAkt- or ΔTOR-mediated vincristine resistance. The up-regulation of aerobic glycolysis and elevated production of lactate characteristic of cancer cells are not required for, and seem unlikely to contribute to, the vincristine resistance we have observed. In this model, mAkt, ΔTOR, or control cells can adapt to a significant inhibition of glycolytic rate without a loss of viability and without markedly altering sensitivity to apoptotic stimuli.

**Discussion**

Deregulated activation of Akt-dependent signaling, due to up-regulation of PI3K, or more commonly loss of the negative regulator PTEN, has been found to be a common feature of many solid tumors, including cancers of the lung, breast, liver,

![FIGURE 5.](image_url) The hexokinase inhibitor 5TG inhibits glucose consumption. A. The glycolytic rates of FL5.12 cells expressing mAkt or a control vector were measured as the conversion of [5-3H]glucose to H2O. Cells were incubated in the presence of tritiated glucose, 1 mmol/L nonradioactive glucose, and the indicated concentrations of 5TG. B and C. The same cells were grown in FL5.12 medium with (+5TG) or without (-5TG) 10 mmol/L 5TG. Every 24 hours, measurements were made to determine cell concentration (B) and the amount of lactate in the medium (C). Experiments were repeated with similar results. Bars, SD.
gastrointestinal tract, and ovary, and the transforming factor of a murine thymoma virus is an Akt homologue with an activating mutation (2). Expression of a dominant-negative Akt allele has been shown to suppress tumor growth in vivo (32), confirming the significance of this oncprotein in the development of cancer.

In several experimental systems, Akt-mediated function is mTOR dependent, establishing mTOR as a target downstream of Akt (17, 33). New data indicate, however, that this signaling network is complex and that a feedback relationship exists between mTOR and Akt. The mechanism whereby rapamycin diminishes Akt phosphorylation and activation is just beginning to unfold. Recent developments implicate a new mTOR-binding partner and a new mTOR complex, which modulates Akt activity. Rictor binds mTOR in the presence of GαL, and together, this complex has been reported to facilitate phosphorylation of Akt on T308 and to mediate S473 phosphorylation (15). Contrary to the modulation of Akt that we have observed, however, this mTOR complex is said to be insensitive to rapamycin (14). It is possible that, with prolonged exposure to rapamycin, rapamycin-insensitive complexes exhibit indirect inhibition.

Rapamycin has been shown to inhibit Akt phosphorylation in a mouse model of ErbB2-dependent breast tumors. Blockade of mTOR signaling lead to down-regulation of ErbB3 at the mRNA and protein levels. ErbB3 alone is not ligand activated, but in complex with ErbB2 this tyrosine kinase initiates PI3K activation and subsequent Akt phosphorylation (29). Inhibition of ErbB3 by rapamycin is a recent observation, and it seems likely that mTOR modulates expression of other receptor signaling molecules.

Inhibition of mTOR by rapamycin has been shown to activate the protein phosphatase 2A, and protein phosphatase 2A dephosphorylates and inactivates multiple substrates, including Akt (34, 35). The phosphatase PHLPP has been shown recently to specifically dephosphorylate S473 of Akt (36), although any regulation of PHLPP by mTOR is unknown. Although rapamycin has not been shown to inhibit Akt activity by facilitating its dephosphorylation, this constitutes a third possible mechanism for the feedback relationship between mTOR and Akt.

Because exposure to rapamycin diminishes activation of membrane-targeted Akt, which is less dependent on upstream activation, it seems unlikely that the feedback observed in our system is due to modulation of upstream receptor tyrosine kinases, such as ErbB3. We observed that the ability of rapamycin to diminish phosphorylation of endogenous Akt required 16 hours. Combined with the rapamycin sensitivity of this feedback relationship, it seems unlikely that it occurs through a direct mTOR/rictor-Akt interaction. Protein phosphatase 2A is a general phosphatase, acting as a negative regulator of multiple signaling pathways, and it has been shown...
that rapamycin-mediated inhibition is specific for Akt (13), suggesting that the mTOR-Akt relationship we have observed is not mediated by protein phosphatase 2A. Investigation into these complex Akt-mTOR feedback relationships is a relatively new topic of research, however, and more work needs to be done to determine if they occur through the same mechanism as we describe here.

Negative regulation of Akt by mTOR has also been described recently. The PI3K-Akt-mTOR pathway is a major mediator of insulin signaling, and it has been shown that prolonged S6K activity leads to phosphorylation and down-regulation of the insulin receptor substrate 1 or 2, resulting in diminished Akt activity (37). It seems that mTOR activity can have opposite effects on similar, but distinct, receptor signaling pathways, and this finding again highlights the complex relationship between mTOR and Akt.

Constitutive up-regulation of mTOR activity by COOH-terminal deletion does not further augment phosphorylation of Akt. Nevertheless, independent of Akt, constitutive activation of mTOR by this mechanism promotes resistance to multiple antimicrotubule agents. These data suggest that the dependence of Akt-mediated resistance on mTOR is due to both direct effects of mTOR on downstream targets and feedback up-regulation of Akt activity.

Because of the dual effects of Akt and mTOR on cell survival and metabolism, much work has focused on how these functions are related, especially in maintaining survival following growth factor withdrawal. Cells expressing constitutively active Akt maintain an elevated glycolytic rate following growth factor withdrawal relative to control cells in part because Akt increases expression of hexokinase and glucose transporters and promotes the translocation of these transporters to the plasma membrane (20). Expression of hexokinase and glucose-transporter 1 has been shown to increase resistance to Bax-mediated cell death (38). Both elevated glycolytic rate and maintenance of transporter translocation have been shown to be mTOR dependent (13, 26).

Following the initial observations of Warburg seven decades ago that tumor cells produce more lactate and consume less oxygen than nontransformed cells (27), others have confirmed that tumor cells preferentially use aerobic glycolysis compared with their normal counterparts. Recent work has shown that Akt alone increases aerobic glycolysis. It remains unclear what advantage aerobic glycolysis bestows on a cell, and in fact, Akt-mediated elevated glycolytic rate seems to sensitize cells to glucose withdrawal (39). Increased glycolytic rate has been hypothesized to confer a survival advantage on the cell in vivo, where glucose availability is generally not limiting. Hexokinase in particular has been reported to support survival and resistance to apoptotic stimuli. Activated Akt promotes an increase in the fraction of hexokinase located at the mitochondria (23). Active mitochondrial hexokinase has been shown to compete with the proapoptotic factor Bax for voltage-dependent anion channel binding and to prevent cleavage of Bid (31, 40). Glucokinase, a form of hexokinase found in the liver, has been localized to the mitochondria in a complex with Bad (41), further suggesting a link between regulation of glycolysis and control of cell death.

The elevated glucose metabolism of cancer cells is the basis for the use of [18F]fluorodeoxyglucose positron emission tomography scans to visualize tumor location. [18F]Fluorodeoxyglucose is concentrated in tumor cells because of their increased rate of glucose uptake relative to background or in nontransformed cells. This large difference in glucose uptake suggests a possible therapeutic window. One hypothesis is that inhibiting glucose uptake in cancer cells would enhance their sensitivity to other cytotoxic therapies. Our data, however, do not support that model, because marked inhibition of glycolytic rate with 5TG did not sensitize cells to antimicrotubule chemotherapeutic agents. Exposure of mAkt or control cells to 5TG slowed cell proliferation and nearly eliminated production of lactate. Surprisingly, however, 5TG had essentially no effect on viability in WT cells and in cells expressing activated Akt or activated mTOR. Indeed, although 5TG inhibits glycolytic rate to a much greater extent than rapamycin (13, 24), 5TG did not diminish mAkt-mediated growth factor independence or vincristine resistance to the same degree as did rapamycin (ref. 24; data not shown). The association between Akt-mediated survival and metabolic control has been based largely on the ability of Akt to increase glucose consumption and its inability to permit survival following the complete withdrawal of glucose, and we, too, have seen that Akt cannot substantially increase the survival of cells in which inhibition of glycolysis is virtually complete, by either withdrawing glucose or adding 2-deoxyglucose. We have shown, however, that mAkt cells can adapt to even a sharply decreased rate of glucose consumption by addition of 5TG without suffering a loss of viability or therapeutic resistance. This is an important distinction if one is to consider overcoming Akt-mediated resistance through inhibition of glycolysis, and it shows that Akt-mediated effects on glycolysis can be separated from its effects on survival.

Based on multiple lines of evidence implicating the PI3K-Akt pathway as a critical factor in oncogenic transformation and therapeutic resistance, several candidate Akt inhibitors are being evaluated in preclinical models. In addition, rapamycin and related mTOR inhibitors are being actively tested in multiple ongoing phase I and II clinical trials. In the optimal implementation of these agents in clinical development, it will be essential to determine if the feedback relationship between mTOR and Akt is limited to certain cell types or represents a general mechanism of Akt regulation. Consideration of the mechanisms of action of mTOR and Akt and of the feedback regulation within this critical signaling pathway may influence the development and testing of potentially synergistic combinatorial therapies involving mTOR- and Akt-directed agents.

Materials and Methods

Materials

Reagents were purchased from the following companies: rapamycin (EMD Biosciences, Inc., San Diego, CA); 5TG (MP Biomedicals, Irvine, CA); galactose (Acros Organics, Morris Plains, NJ); protease and phosphatase inhibitor cocktails, glucose-6-phosphate, β-actin antibody, and lactate detection reagents (Sigma, St. Louis, MO); phosphorylated Akt (S473), Akt, phosphorylated GSK-3α/β, GSK-3β, phosphorylated FKHR (S256), FKHR, phosphorylated Bad (S136), Bad, phosphorylated 4E-BP (T37/T46), and 4E-BP antibodies (Cell Signaling Technologies, Beverly, MA); [5-3H]glucose...
(Perkin-Elmer, Boston, MA); H₂O and Enhanced Chemiluminescence and Enhanced Chemiluminescence Plus kits (Amer sham Biosciences, Piscataway, NJ); and polyvinylidene difluoride membrane (Millipore, Bedford, MA).

**Cell Lines and Reagents**

The nontransformed murine fetal liver cell line FL5.12 is dependent on IL-3 for proliferation and survival (42). FL5.12 cells were cultured in FL5.12 medium (RPMI 1640 supplemented with 10% fetal bovine serum, 20 mmol/L HEPES, penicillin/streptomycin, 2 mmol/L L-glutamine, 55 mmol/L β-mercaptoethanol, and 10% supernatant from WEHI-3B cells as a source of IL-3). To make medium lacking glucose, RPMI 1640 with no glucose, dialyzed fetal bovine serum, and recombinant murine IL-3 were substituted in. FL5.12 cells were transfected with pRevTRE containing constitutively active Akt, mAkt, or empty vector; pEF6 containing WT mTOR; dominant-negative mTOR containing point mutations in the rapamycin-binding and kinase domains; a constitutively active mTOR mutant containing a deletion encompassing the COOH-terminal Akt phosphorylation site; or empty vector. All plasmids have been described previously (13, 16, 20).

**Determination of Glycolytic Rate**

Glycolytic rate was measured as conversion of [5-3H]glucose to H₂O as described previously (43, 44). Briefly, 10⁶ cells were washed in Dulbecco’s PBS and resuspended in 500 μL Krebs buffer [25 mmol/L NaHCO₃, 115 mmol/L NaCl, 2 mmol/L L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, and 0.25% bovine serum albumin (pH 7.4)] containing 1 mmol/L nonradioactive glucose, the indicated amount of STG, and 1 μCi [5-3H]glucose and incubated for 60 minutes in 5% CO₂ at 37°C. Aliquots (100 μL) of each treatment group were added to 0.2 N HCl (50 μL) in open tubes that were placed upright in scintillation vials containing 1 mL H₂O. The vials were sealed and H₂O produced by glucose consumption was allowed to equilibrate with H₂O outside the tube for 24 hours at room temperature. The amount of ⁴¹H retained within the tube and the amount that had diffused into the surrounding H₂O by evaporation and condensation were determined separately. [5-3H]glucose and H₂O standards were included in each experiment, allowing calculation of the rate of conversion of [5-3H]glucose to H₂O (43).

**Immunoblotting**

Whole-cell lysates were prepared in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktails, and protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Each lysate (50 μg) was run on a SDS-PAGE gel and electrophoretically transferred to polyvinylidene difluoride membrane. Following antibody incubation, blots were developed on film using an Enhanced Chemiluminescence kit (Perkin-Elmer, Boston, MA); H₂O and Enhanced Chemiluminescence Plus kit according to the manufacturer’s instructions. Densitometry of immunoblot bands was carried out using FluorChem software (Alpha Innotech Corp., San Leandro, CA). The ratio of phosphorylated protein to total protein for each treatment group, normalized to that of the control treatment group, was calculated.

**Determination of Cell Death**

Cells were resuspended in buffer containing Dulbecco’s PBS, 1% fetal bovine serum, 0.1% sodium azide, and 2 μg/mL propidium iodide. Cells were run on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed using CellQuest Pro software (Becton Dickinson). Viable cells were distinguished from dead cells based on exclusion of propidium iodide.

**Measurement of Lactate Levels**

Protein was precipitated from samples of cell cultures using trichloroacetic acid, and supernatant was added to a mixture of NAD⁺, lactate dehydrogenase, and glycine buffer according to the manufacturer’s instructions. Following 15-minute incubation at 37°C, lactate concentration in each sample was determined based on A₃₄₀ on a BioMate 3 spectrophotometer (Thermo Spectronic, Sumerset, NJ).

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**References**


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