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Prolonged Rapamycin Treatment Inhibits mTORC2 Assembly and Akt/PKB

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Summary

The drug rapamycin has important uses in oncology, cardiology, and transplantation medicine, but its clinically relevant molecular effects are not understood. When bound to FKBP12, rapamycin interacts with and inhibits the kinase activity of a multiprotein complex composed of mTOR, mLST8, and raptor (mTORC1). The distinct complex of mTOR, mLST8, and rictor (mTORC2) does not interact with FKBP12-rapamycin and is not thought to be rapamycin sensitive. mTORC2 phosphorylates and activates Akt/PKB, a key regulator of cell survival. Here we show that rapamycin inhibits the assembly of mTORC2 and that, in many cell types, prolonged rapamycin treatment reduces the levels of mTORC2 below those needed to maintain Akt/PKB signaling. The proapoptotic and antitumor effects of rapamycin are suppressed in cells expressing an Akt/PKB mutant that is rapamycin resistant. Our work describes an unforeseen mechanism of action for rapamycin that suggests it can be used to inhibit Akt/PKB in certain cell types.

Introduction

The mammalian TOR (mTOR) signaling network regulates cell growth, proliferation, and survival (reviewed in Sarbassov et al. [2005a]). The central component of the pathway, the mTOR protein kinase, nucleates two distinct multiprotein complexes that regulate different branches of the mTOR network. The mTOR complex 1 (mTORC1) consists of mTOR, raptor, and mLST8 (also known as GβL) and regulates cell growth through effectors such as S6K1 and 4E-BP1. The mTOR complex 2 (mTORC2) contains mTOR, rictor, and mLST8, and recent work shows that it regulates the prosurvival kinase Akt/PKB by phosphorylating it on S473 (Hresko and Mueckler, 2005; Sarbassov et al., 2005b). Together with the phosphorylation of T308 by PDK1, S473 phosphorylation is necessary for full Akt/PKB activation (Alessi et al., 1996).

Because deregulation of the mTOR pathway occurs in diverse human diseases (reviewed in Guertin and Sabatini [2005]), small molecules that target mTOR are attracting increasing clinical interest. One mTOR-targeting molecule, called rapamycin, is already an approved drug and is in use to prevent the rejection of transplanted organs and to block restenosis after angioplasty (reviewed in Chueh and Kahan [2005], Di Mario et al. [2004]). Rapamycin works through a gain-of-function mechanism in which it binds to the intracellular protein FKBP12 to generate a drug-receptor complex that then binds to and inhibits the kinase activity of mTORC1. Because FKBP12-rapamycin does not bind to mTORC2 (Jacinto et al., 2004; Sarbassov et al., 2004), rapamycin is thought to inhibit only mTORC1. Rapamycin has been reported to cause an array of effects that suggest it could be a useful antitumor agent (reviewed in Guertin and Sabatini [2005]). It slows the proliferation of many cancer cell lines grown in culture; it promotes apoptosis in some cancer lines, usually in combination with another agent; and, lastly, it has antiangiogenic properties. These findings have led to the current interest in the potential of rapamycin and its analogs (CCI-779, AP23573, SDZ-RAD) for the treatment of sporadic human cancers. However, it increasingly clear that rapamycin does not have universal antitumor effects in people and that only a fraction of patients respond to the drug (reviewed in Panwalkar et al. [2004], Sawyers [2003]). Predictors of tumor sensitivity to rapamycin are not available, although recent work indicates that tumors missing the PTEN or VHL tumor suppressors may be particularly sensitive to the drug (Neshat et al., 2001; Podsypanina et al., 2001; Thomas et al., 2006). To inform the clinical use of rapamycin and to understand why it exhibits tissue- and tumor-specific effects, it is necessary to fully elucidate its mechanism of action and the signaling pathways it perturbs. Here, we describe the unexpected finding that rapamycin suppresses the assembly and function of mTORC2 to inhibit Akt/PKB and that this property of rapamycin contributes to the in vitro and in vivo effects of the drug.

Results

Although FKBP12-rapamycin cannot bind to preformed mTORC2 (Jacinto et al., 2004; Sarbassov et al., 2004), it does bind to free mTOR (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). Because mTOR molecules should be free when newly synthesized and when mTOR complexes turn over, long-term exposure of cells to rapamycin should lead to the binding of FKBP12-rapamycin to a large fraction of the mTOR molecules within cells. As the binding of FKBP12-rapamycin to free mTOR may prevent the subsequent binding of rictor, we hypothesized (Sarbassov et al., 2005b) that prolonged rapamycin treatment may inhibit Akt/PKB signaling by interfering with the assembly of mTORC2.

To determine if rapamycin can alter the levels of intact mTORC2, we treated HeLa or PC3 cells with 100 nM rapamycin for 0.5, 1, 2, or 24 hr and compared the amounts of rictor and raptor bound to mTOR. Rapamycin had little effect on the expression levels of mTOR, raptor, or

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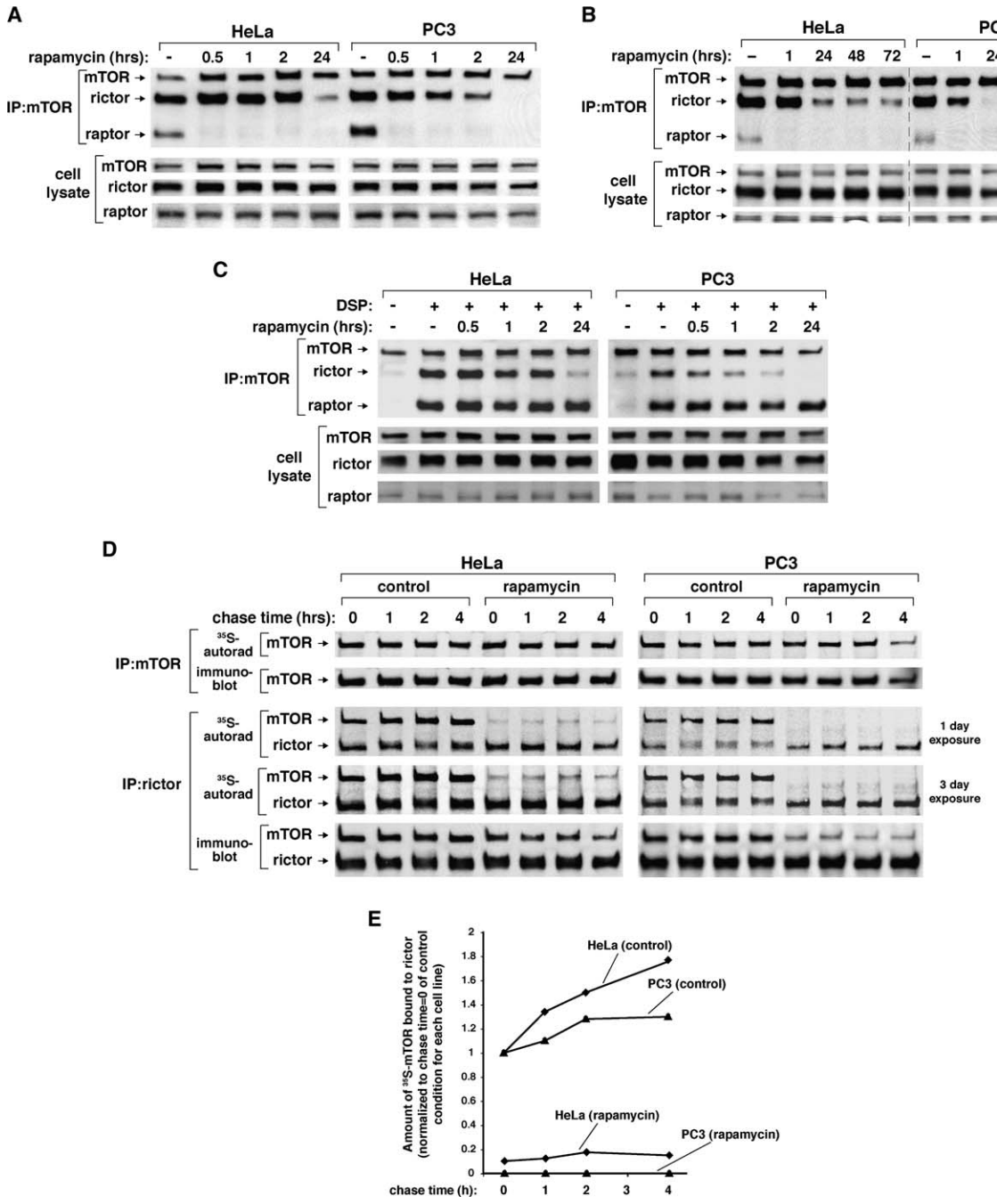

Figure 1. Prolonged Treatment of Cells with Rapamycin Inhibits Assembly of mTORC2

(A) HeLa and PC3 cells were treated with 100 nM rapamycin for the indicated times. Cell lysates and mTOR immunoprecipitates prepared from the lysates were analyzed by immunoblotting for the levels of mTOR, rictor, and raptor.

(B) HeLa and PC3 cell lines were treated with 100 nM rapamycin for 1, 24, 48, or 72 hr and analyzed as above.

(C) In rapamycin-treated cells, use of a reversible crosslinker preserves the raptor-mTOR but not the rictor-mTOR interaction. Experiment was performed as in (A) except that, where indicated, cells were treated with the DSP crosslinker before lysis with a buffer containing Triton X-100.

(D) Pulse-chase experiment indicates that rapamycin inhibits assembly of the mTORC2 without suppressing mTOR or rictor synthesis. Cells were pretreated with 100 nM rapamycin or vehicle control for 20 min and then pulsed with ^{35}S-methionine/cysteine and chased with cold amino acids for the indicated periods of time. Rictor and mTOR immunoprecipitates were prepared from cell lysates and analyzed by autoradiography and immunoblotting for the levels of newly synthesized and total mTOR and rictor.

(E) Quantification of the amount of newly synthesized mTOR bound to rictor in control or rapamycin-treated HeLa and PC3 cells.

rictor, but, as expected (Kim et al., 2002), it strongly reduced the amounts of raptor recovered with mTOR within 30 min of addition to HeLa or PC3 cells (Figure 1A). In contrast, at early time points after addition to HeLa cells, rapamycin did not reduce the amounts of rictor bound to mTOR, but after 24 hr the drug did cause a partial loss of rictor from mTOR. Rapamycin treatment had a similar but more pronounced effect in PC3 cells,

with an almost complete loss of mTOR bound rictor after 24 hr. Concentrations of rapamycin ranging from 5 nM to 1 μM produced identical effects to 100 nM on the raptor-mTOR and rictor-mTOR interactions in HeLa and PC3 cells (data not shown). In addition, rapamycin treatment times of 48 and 72 hr gave identical results to 24 hr treatments in HeLa and PC3 cells (Figure 1B).

Using a cross-linking assay, we previously demonstrated that the binding of FKBP12-rapamycin to mTORC1 does not break the raptor-mTOR interaction within cells but only weakens it so that it cannot survive biochemical isolation (Kim et al., 2002). A similar mechanism cannot explain the loss of the rictor-mTOR interaction in rapamycin-treated cells, because FKBP12-rapamycin cannot bind to a formed mTORC2 (Jacinto et al., 2004; Sarbassov et al., 2004). Instead, we suspected that, after prolonged rapamycin treatment, a large fraction of the rictor and mTOR molecules within cells are no longer associated with each other. We tested this possibility in a modified version of the experiment in Figure 1A. We first treated cells with a reversible crosslinker that covalently links mTOR to associated proteins and then lysed the cells with a buffer that breaks noncovalent interactions. As expected (Kim et al., 2002; Sarbassov et al., 2004), in untreated cells, raptor and rictor coimmunoprecipitated with mTOR only when the crosslinker had been added (Figure 1C). In cells treated with rapamycin, the crosslinker preserved the interaction of raptor with mTOR but did not prevent the loss of the rictor-mTOR association caused by prolonged rapamycin treatment (Figure 1C). These results confirm that rapamycin affects mTORC1 and mTORC2 in different ways. mTORC1 is destabilized at all times after drug addition, consistent with the capacity of FKBP12-rapamycin to bind to it and weaken the raptor-mTOR interaction (Kim et al., 2002). On the other hand, prolonged treatment of cells with rapamycin leads to a progressive loss of the rictor-mTOR interaction to an extent that varies with cell type. Prolonged incubation of cell lysates with rapamycin did not disrupt the rictor-mTOR interaction (see Figure S1 in the Supplemental Data available with this article online), suggesting that rapamycin exerts its effects on a process that occurs within cells, such as mTORC2 assembly.

To test this, we pulse labeled HeLa and PC3 cells with ^{35}S-methionine/cysteine in the presence or absence of rapamycin and followed the amount of newly synthesized (i.e., ^{35}S-labeled) mTOR bound to rictor during a chase period with unlabeled amino acids. In the absence of rapamycin and at all times during the chase period, we readily detected newly synthesized mTOR bound to immunoprecipitated rictor in both HeLa and PC3 cells (Figure 1D). Strikingly, rapamycin prevented the binding of newly synthesized mTOR to rictor in PC3 cells and greatly reduced it in HeLa cells (Figure 1D). Quantification of these results revealed that rapamycin prevented 100% and 80% of the detectable interaction between newly synthesized mTOR and rictor in PC3 and HeLa cells, respectively (Figure 1E). Rapamycin does not inhibit mTOR or rictor protein synthesis because the drug did not reduce the amount of radiolabeled mTOR or rictor immunoprecipitated by the mTOR or rictor antibody, respectively (Figure 1D). These results indicate that, in HeLa cells, a fraction of mTORC2 assembles even in the presence of rapamycin, a result consistent with the finding that some rictor remains bound to mTOR in HeLa cells grown for 72 hr in the presence of rapamycin (Figure 1B). On the other hand, rapamycin completely blocks detectable mTORC2 assembly in PC3 cells.

Because the interaction of mTOR with rictor is necessary for mTOR to phosphorylate S473 of Akt/PKB (Hresko and Mueckler, 2005; Sarbassov et al., 2005b), we asked if a 24 hr treatment with rapamycin inhibits Akt/PKB phosphorylation. In several cell lines, we compared the effects of rapamycin on the phosphorylation of S473 of Akt/PKB and of T389 of S6K1, a well-known mTORC1 phosphorylation site (Burnett et al., 1998) (Figure 2A). In PC3, HEK-293T, HeLa, and H460 cells, 1 or 24 hr treatments with rapamycin eliminated S6K1 phosphorylation, consistent with inhibition of mTORC1. Because S6K1 normally suppresses the PI3K/Akt pathway (Harrington et al., 2004; O'Reilly et al., 2006; Tremblay and Marette, 2001; Um et al., 2004), inhibition of S6K1 by rapamycin should lead to an increase in Akt/PKB phosphorylation, and, indeed, this happened in HeLa and H460 cells. However, in PC3 cells, the drug strongly decreased Akt/PKB phosphorylation, while, as previously reported (Edinger et al., 2003), it caused a weak inhibition in HEK-293T cells. Changes in the phosphorylation of T308 of Akt/PKB paralleled those occurring on S473, as expected from the proposed role of S473 phosphorylation in regulating the phosphorylation of T308 by PDK1 (Scheid et al., 2002; Yang et al., 2002). FKBP12 is necessary to mediate the effects of rapamycin on Akt/PKB and mTORC2, as treatment of PC3 cells with excess FK506, a small molecule that competes with rapamycin for binding to FKBP12, blocked the effects of rapamycin (Figure 2B). The initial cell survey suggested that a 24 hr treatment with rapamycin can cause either (1) a strong inhibition, (2) a partial inhibition, or (3) an increase in Akt/PKB phosphorylation. To determine the frequency of these responses, we tested the effects of 1 and 24 hr of rapamycin treatment on Akt/PKB phosphorylation in 33 cancer or transformed cell lines (Table S1). In about one third of the cell lines, rapamycin caused a strong or partial inhibition of Akt/PKB phosphorylation, while the drug either did not affect or increased Akt/PKB phosphorylation in the others. Figure 2C shows three representative cell lines for each type of response. We also examined a variety of primary and nontransformed cell lines and found several, including endothelial and muscle cells, with rapamycin-sensitive Akt/PKB phosphorylation (Figure 2D and Table S1). Lastly, we showed that rapamycin can inhibit Akt/PKB phosphorylation in vivo, as mice treated daily for 1 week with the drug had decreased Akt/PKB phosphorylation in the thymus, adipose tissue, heart, and lungs (Figure S2). These findings indicate that rapamycin-sensitive Akt/PKB phosphorylation is common and occurs in cultured normal and cancer cell lines as well as in vivo.

The experiments in Figure 1 clearly showed that long-term rapamycin treatment does not always lead to the total loss of intact mTORC2 (Figures 1A and 1B) and that mTORC2 assembly is not completely blocked in all cell types (Figures 1D and 1E). Considering this, we hypothesized that cell lines with rapamycin-sensitive

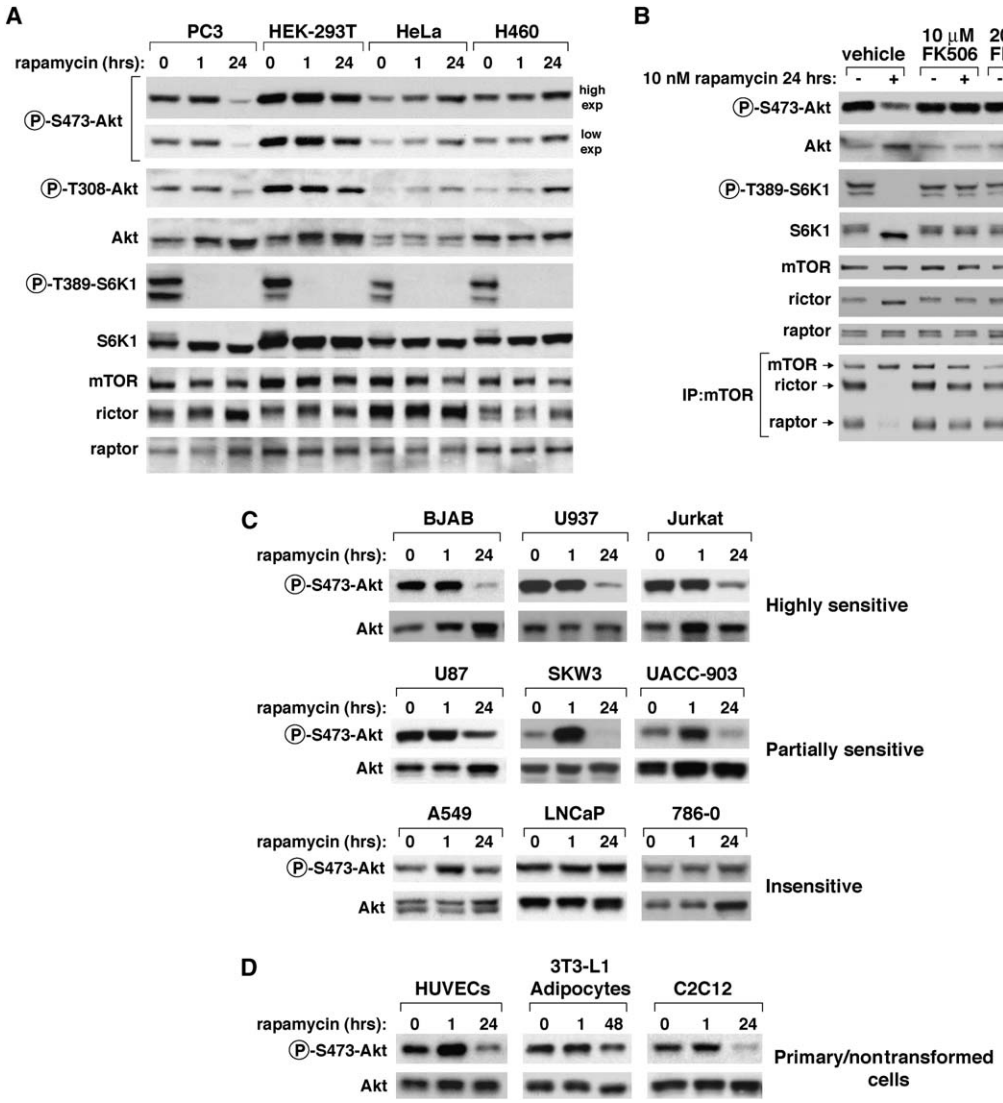

Figure 2. Rapamycin Is a Cell-Type-Dependent Inhibitor of Akt/PKB S473 Phosphorylation

(A) Indicated cell lines were treated with 100 nM rapamycin for the indicated times and analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states.

(B) PC3 cells were treated with 10 nM rapamycin or vehicle for 24 hr and with or without the indicated concentrations of FK506. Cell lysates were analyzed for the indicated phosphorylation states and protein levels, and mTOR immunoprecipitates were analyzed by immunoblotting for the levels of mTOR, rictor, and raptor.

(C–D) Experiments were performed as in (A).

Akt/PKB phosphorylation should have very low amounts of intact mTORC2 complexes after prolonged rapamycin treatment. Indeed, cell lines with rapamycin-sensitive Akt/PKB phosphorylation (PC3, BJAB, Jurkat) had less intact mTORC2 following 1 hr of drug treatment and an almost complete loss of complexes by 24 hr (Figure 3A). In contrast, cell lines with rapamycin-insensitive Akt/PKB phosphorylation (H460, HeLa, LNCaP, 768-0) showed stable levels of intact mTORC2 after 1 hr of drug treatment and only a partial loss by 24 hr (Figure 3A). The degree of loss of the complexes after rapamycin treatment correlated with their residual in vitro kinase activity toward Akt/PKB (Figure 3B). Rictor immunoprecipitates prepared from PC3, BJAB, and Jurkat cells treated with rapamycin for 24 hr had almost background levels of kinase activity, consistent with the large loss of mTOR from these immunoprecipitates. On the other hand, in HeLa and H460 cells treated with rapamycin for 24 hr, a greater amount of mTOR remained bound to rictor, and this correlated with a higher level of kinase activity toward Akt/PKB. Thus, our results suggest that, in certain cell types, the small amount of mTORC2 assembled in the presence of rapamycin is sufficient to mediate Akt/PKB phosphorylation.

To test this hypothesis, we asked if it is possible to confer rapamycin-sensitive Akt/PKB phosphorylation to a cell line by partially decreasing the expression of mTOR. A reduction in total mTOR should decrease the levels of mTORC2 in the cells so that rapamycin-mediated suppression of mTORC2 assembly will leave insufficient amounts of mTORC2 to mediate Akt/PKB phosphorylation. This is exactly what we observe. A partial

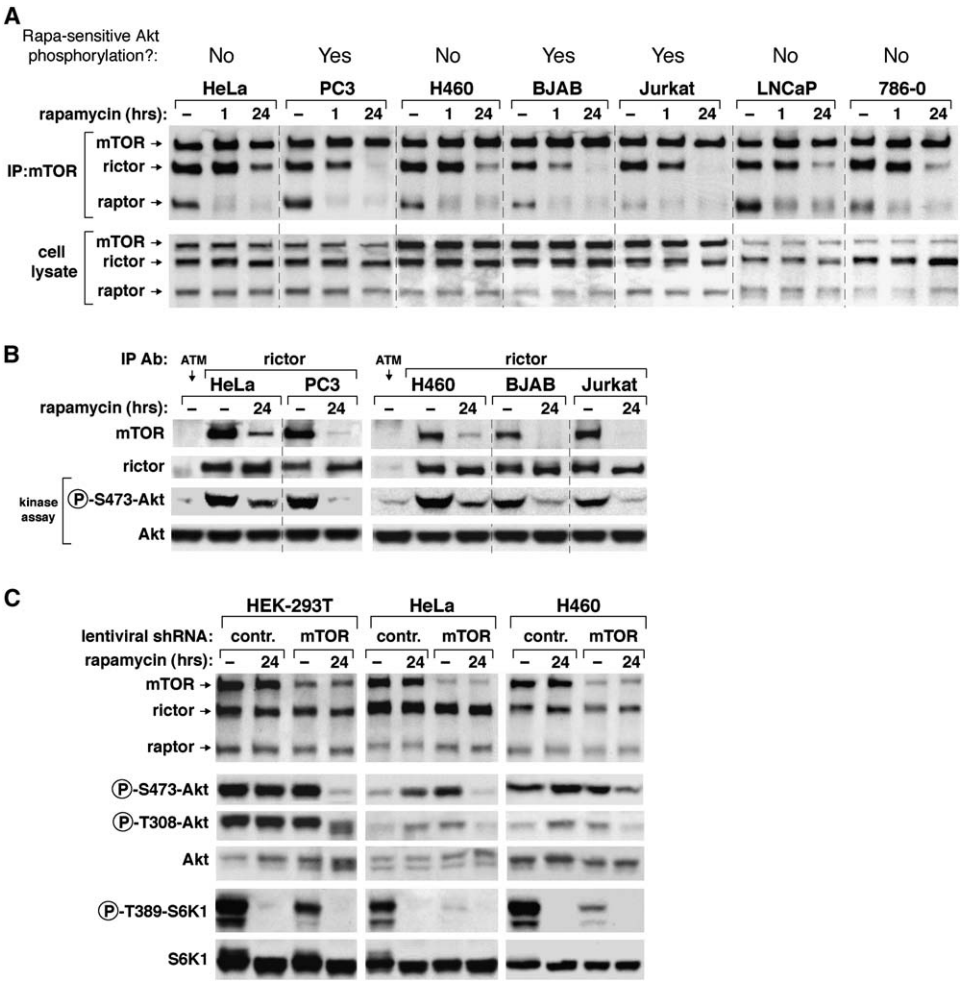

Figure 3. Rapamycin Causes an Almost Complete Loss of Intact mTORC2 in Cell Lines with Rapamycin-Sensitive Akt/PKB Phosphorylation

(A) The indicated cells lines were treated with 100 nM rapamycin for 1 or 24 hr. Cell lysates and mTOR immunoprecipitates prepared from the lysates were analyzed by immunoblotting for levels of mTOR, rictor, and raptor.

(B) Rictor immunoprecipitates prepared from cell lines with rapamycin-sensitive Akt/PKB phosphorylation have baseline levels of in vitro kinase activity toward Akt/PKB when isolated from cells treated for 24 hr with rapamycin. Indicated cell lines were treated with or without 100 nM rapamycin for the indicated times, and rictor immunoprecipitates prepared from cell lysates were used in kinase assays using Akt1/PKB1 as a substrate as described in the Experimental Procedures.

(C) Partial suppression of mTOR expression converts a cell line with rapamycin-insensitive Akt/PKB phosphorylation to one with rapamycin-sensitive phosphorylation. HEK-293T, HeLa, and H460 cells were infected with lentiviruses expressing a control or mTOR-targeting shRNA and after 1 day in culture were selected for an additional 2 days with puromycin. Equal cell numbers were then treated with 100 nM rapamycin for the indicated times and cell lysates analyzed as in Figures 1 and 2 for the levels of the indicated proteins and phosphorylation states. The phosphorylation state of S6K1 was used as a marker of the activity of the mTORC1 pathway. Akt/PKB phosphorylation is less sensitive than S6K1 phosphorylation to a decrease in mTOR expression, because a partial loss of mTOR removes the inhibitory signal on PI3K/Akt signaling that is normally mediated by S6K1.

knockdown of mTOR in HEK-293T, HeLa, and H460 cells is sufficient to render Akt/PKB phosphorylation rapamycin sensitive in these cell lines (Figure 3C). Strikingly, in HeLa and H460 cells, a partial knockdown of mTOR induced a strong increase in Akt/PKB phosphorylation—a finding consistent with removal of the inhibitory signal coming from S6K1—and this increase was suppressed by rapamycin. That only low amounts of mTORC2 are sufficient to mediate phosphorylation of S473 of Akt/PKB is consistent with the finding that only 10% of normal PDK1 levels are needed for the full phosphorylation of T308 of Akt/PKB (Lawlor et al., 2002) and that levels of mTOR must be strongly reduced to affect S473 phosphorylation (Sarbassov et al., 2005b).

We next turned to assessing the physiological significance of rapamycin-mediated inhibition of Akt/PKB phosphorylation. Because S473 phosphorylation is required for full Akt/PKB activation (reviewed in Scheid and Woodgett [2003]), we expected inhibition of S473 phosphorylation by rapamycin to suppress Akt/PKB activity and signaling. In fact, rapamycin inhibited the kinase activity of Akt/PKB in PC3 but not HeLa cells (Figure 4A). In PC3 cells, rapamycin inhibited the phosphorylation of FKHR (Foxo1) and AFX (Foxo4a) (Figure 4B), forkhead family transcription factors that are direct substrates of Akt/PKB (Brunet et al., 1999; del Peso et al., 1999; Kops et al., 1999; Rena et al., 2002; Takaishi et al., 1999; Tang et al., 1999). Expression

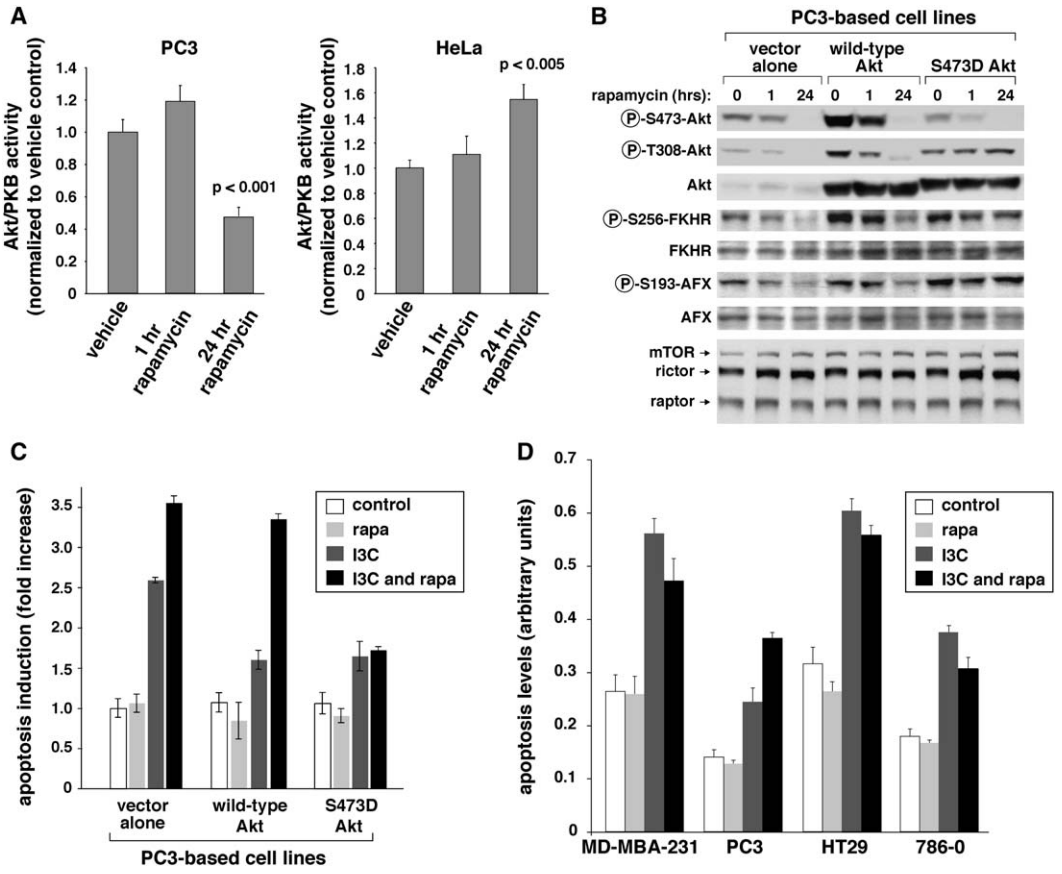

Figure 4. In Tissue Culture Cells, Rapamycin Inhibits Akt/PKB Activity, Signaling, and Its Prosurvival Function, and this Inhibition Requires the Dephosphorylation of S473

(A) PC3 or HeLa cells were treated with vehicle or 100 nM rapamycin for the indicated times. Akt/PKB was immunoprecipitated from cell lysates and activity determined as described in the Experimental Procedures. Values were normalized to vehicle treated condition for each cell line, and p value is for comparison with the vehicle treated condition. Means ± standard deviations for n = 3 are shown.

(B) Vector-alone PC3 cells or PC3 cells stably expressing wild-type or S473D Akt1/PKB1 were treated with 100 nM rapamycin for 1 or 24 hr, and cell lysates were analyzed by immunoblotting for the indicated proteins and phosphorylation states. Note that exposure times of the Akt/PKB, phospho-S473 Akt/PKB, and phospho-T308 Akt/PKB blots were chosen to show expression levels and phosphorylation states of the recombinant Akt1/PKB1 protein. Also, the S473D Akt1/PKB1 mutant is not recognized by the anti-S473 Akt/PKB antibody.

(C) Indicated cell lines were cultured in serum-free medium in the presence of vehicle (DMSO), 100 nM rapamycin (rapa), 125 μM indole-3-carbinol (I3C), or both rapamycin and indole-3-carbinol. After 72 hr, the cells were harvested and apoptosis measured by quantifying DNA fragmentation. Results are represented as fold induction of apoptosis compared to the vector-alone cells grown in the absence rapamycin or indole-3-carbinol. Means ± standard deviations for n = 3 are shown.

(D) Experiment was performed as in (C) using the indicated cell lines. Means ± standard deviations for n = 3 are shown.

of either the phosphomimetic S473D mutant of Akt1/PKB1 or wild-type Akt1/PKB1 increased the phosphorylation of FKHR and AFX (Figure 4B). However, only expression of S473D Akt1/PKB1 prevented the inhibition of FKHR, AFX, and T308 Akt1/PKB1 phosphorylation caused by rapamycin (Figure 4B). The capacity of the S473D mutant to prevent the effects of rapamycin indicates that rapamycin-mediated inhibition of S473 phosphorylation leads to the decrease in the phosphorylation of FKHR, AFX, and T308 Akt/PKB. Because Akt/PKB has a well-known prosurvival role, we asked if rapamycin could potentiate a cell death signal as well as prevent the capacity of Akt/PKB to suppress apoptosis. Indeed, treatment of PC3 cells with rapamycin and submaximal concentrations of indole-3-carbinol, a small molecule known to induce apoptosis in PC3 cells (Chinni and Sarkar, 2002), showed greater levels of apoptosis than treatment with indole-3-carbinol alone (Figure 4C). In PC3 cells, the expression of either wild-type or S473D Akt1/PKB1 decreased the capacity of indole-3-carbinol to induce apoptosis. However, the addition of rapamycin strongly reduced the prosurvival effect conferred by wild-type Akt1/PKB1 but not by the S473D mutant (Figure 4C). Rapamycin did not potentiate the proapoptotic effects of indole-3-carbinol in three cell lines, MD-MBA-231, HT29, and 786-0, that do not exhibit rapamycin-sensitive Akt/PKB phosphorylation (Figure 4D). Thus, our data indicate that inhibition of mTORC2 by rapamycin contributes to the proapoptotic effects of rapamycin.

Inhibition of Akt/PKB also contributes to the antigrowth effects of rapamycin against PC3 cell tumor xenografts in immunocompromised mice. In established tumors derived from control PC3 cells, rapamycin strongly decreased the phosphorylations of S473 and T308 of Akt/PKB without affecting Akt/PKB expression

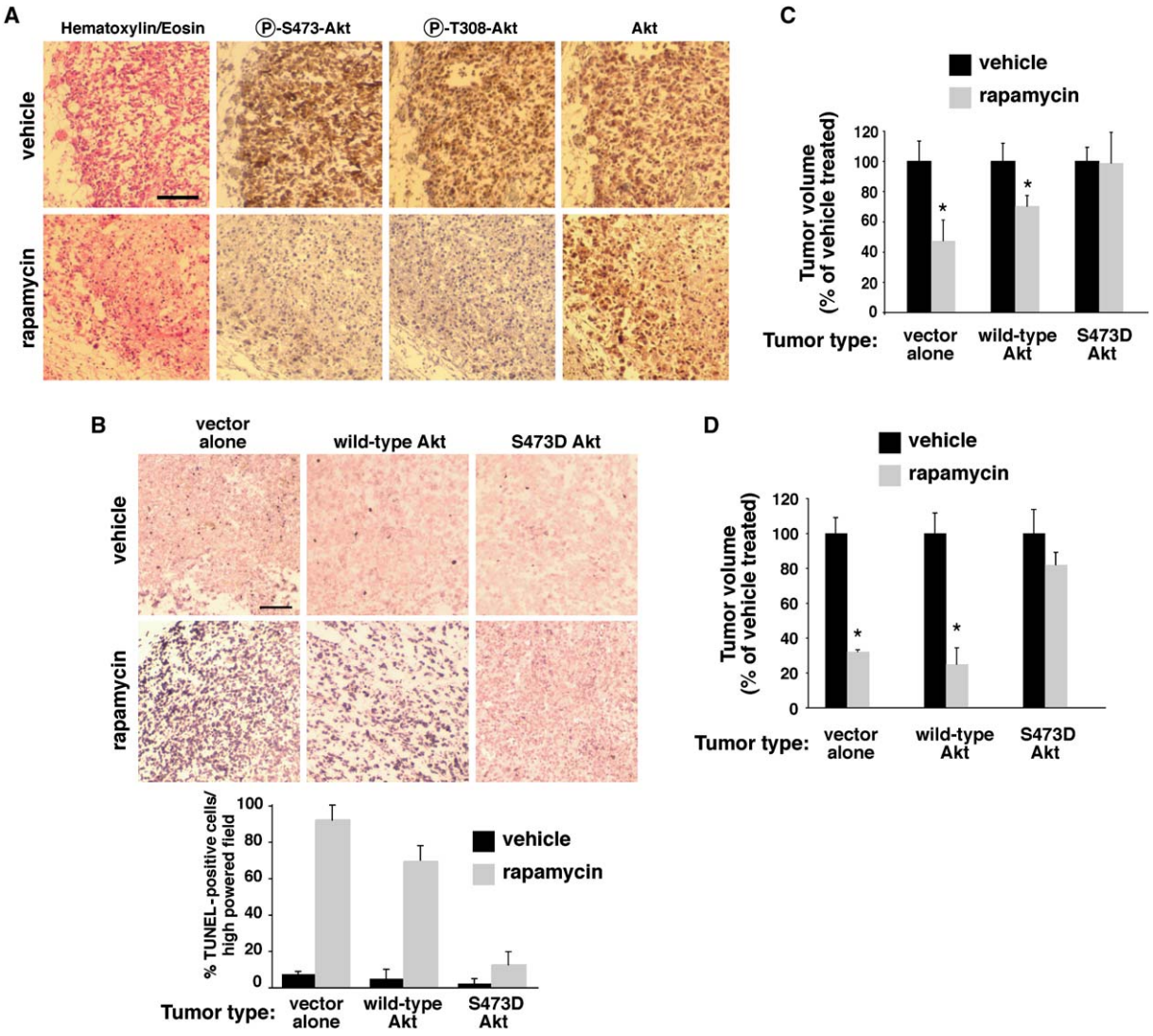

Figure 5. Rapamycin-Mediated Inhibition of Akt/PKB Contributes to the Antitumor Effects of Rapamycin

(A) Mice with tumor xenografts of vector-alone PC3 cells were treated with rapamycin or vehicle for 2 days, and tumor sections were analyzed with immunohistochemistry for levels of Akt/PKB, phospho-S473 Akt/PKB, and phospho-T308 Akt/PKB.

(B) Mice with tumor xenografts made from vector-control PC3 cells or PC3 cells stably expressing wild-type or S473D Akt1/PKB1 were treated with rapamycin or vehicle for 2 days, and tumor sections were analyzed for the presence of apoptotic cells using TUNEL staining (images). Means ± standard deviations for n = 4 are shown for percentage of apoptotic cells in each tumor type (graph).

(C) Mice with tumor xenografts made from the indicated PC3 cell lines were treated with rapamycin or vehicle for 2 days. Mice were then sacrificed, and tumor volumes were measured at the time of tumor harvest. Graph indicates means ± standard deviations for tumor volume as percentage of vehicle-treated mice at the end of the 2 day treatment (n = 6 per condition). *$p < 0.05$ for difference between rapamycin- and vehicle-treated conditions.

(D) Mice were injected with 1×10^6 cells of the indicated PC3 cell lines and treated daily with rapamycin or vehicle for 4 days beginning the day of cell line injection. After 11 days of growth, the volumes of tumor xenografts were determined, and the graph shows means ± standard deviations for tumor volume as the percentage of vehicle-treated mice (n = 6 per condition). *$p < 0.05$ for difference between rapamycin- and vehicle-treated conditions.

(Figure 5A). The drug also caused a sharp increase in the number of cells undergoing apoptosis within the tumor, and this effect was strongly suppressed by the expression of the S473D Akt1/PKB1 mutant but only partially by wild-type Akt1/PKB1 (Figure 5B). In addition, expression of the S473D mutant but not wild-type Akt1/PKB1 suppressed the capacity of rapamycin to decrease the size of established tumors (Figure 5C). Lastly, treatment of mice with rapamycin at the same time that PC3 cells were xenografted into the mice greatly reduced the size of the resulting tumors, an effect that was largely blocked by expression of the S473D mutant but not by wild-type Akt1/PKB1 (Figure 5D). Thus, our results indicate that Akt/PKB inhibition by rapamycin plays an important role in the antitumor effects of the drug.

Discussion

Our work indicates that rapamycin inhibits Akt/PKB signaling in cells where the drug decreases the levels of

intact mTORC2 below those needed to maintain the phosphorylation of S473 of Akt/PKB. It will be important to identify biomarkers that can predict if Akt/PKB is sensitive to rapamycin in a particular cell type and to design dosing regimens that ensure Akt/PKB inhibition. To obtain a biomarker, it will be necessary to understand why in certain cell lines (e.g., HeLa) a small fraction of mTORC2 is able to assemble even in the presence of rapamycin, while, in other cell lines, this does not happen. A possible mechanism is that, in certain cell types, a fraction of the mTORC2s assembles in such a way that the FKBP12-rapamycin binding site is never accessible to the drug, perhaps because an unidentified protein or posttranslational modification blocks the binding site. We have ruled out PTEN status and FKBP12 expression levels as possible modulators of rapamycin-mediated inhibition of Akt/PKB phosphorylation (Figures S3 and S4).

We suggest that rapamycin, and likely its analogs (CCI 779, RAD001, AP23573), are cell-type-dependent inhibitors of mTORC2 function as well as universal inhibitors of the mTORC1 pathway. Rapamycin is in clinical trials as a treatment for cancer and has established uses in preventing vascular restenosis and the immune rejection of transplanted organs. It is interesting to note that Akt/PKB has important roles in the pathological processes implicated in all these conditions. A high fraction of tumors have activated Akt/PKB signaling as a result of PTEN loss and these cancers may be particularly sensitive to rapamycin (reviewed in Guertin and Sabatini [2005]). Rapamycin is known to have antiangiogenic effects (Guba et al., 2002), and we find that the drug strongly inhibits Akt/PKB in endothelial cells (Figure 2D). Akt/PKB plays key roles in T lymphocytes (reviewed in Kane and Weiss [2003]) and smooth muscle cells (reviewed in Zhou et al. [2003]), the cellular targets of rapamycin in its immunosuppressive and antirestenotic uses, respectively. In addition, rapamycin-mediated inhibition of Akt/PKB may help explain the side effects of the drug. For example, rapamycin strongly inhibits Akt/PKB phosphorylation in adipose tissue (Figure S2), a tissue type in which insulin-stimulated Akt/PKB activity plays an important role in suppressing lipolysis (Elks and Manganiello, 1985; Wijkander et al., 1998). Inhibition of Akt/PKB by rapamycin in adipocytes may allow lipolysis to remain high even in the presence of insulin, resulting in the accumulation of free fatty acids in the plasma that can be used by the liver to generate triglycerides, perhaps providing a molecular mechanism for the hyperlipidemia commonly seen in patients treated with rapamycin (Morrisett et al., 2003). Thus, we propose that rapamycin-mediated inhibition of mTORC2 contributes to the clinical effects of the drug and must be considered when rapamycin is administered to patients.

Experimental Procedures

Materials

Reagents were obtained from the following sources: protein G-Sepharose and Dithiobis[succinimidyl propionate] (DSP) from Pierce; rapamycin from Calbiochem; FK506 from LC Laboratories; DMEM, RPMI, F12, and MCDB 131 from Life Technologies; fetal bovine serum (FBS), heat-inactivated fetal bovine serum (IFS), and indole-3-carbinol (I3C) from Sigma; EGM-2 media from Cambrex; antibodies to mTOR, S6K1, and ATM as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; and antibodies to phospho-T389 S6K1, phospho-S6, phospho-S473 and phospho-T308 Akt/PKB, Akt/PKB (all three Akt/PKB-directed antibodies recognize the three known Akt/PKB isoforms), phospho-S256 FKHR (also recognizes phospho-S193 of AFX), and AFX from Cell Signaling Technologies. Antibodies to rictor and raptor were previously described (Sarbassov et al., 2004).

Cell Lines and Culture

Cell lines were cultured in the following media: Jurkat, BJAB, SKW3, U937, Ishikawa, HepG2, A375, A549, and H460 cells in RPMI with 10% IFS; OPM2, Δ47, LNCaP, UACC-903, Kym-1, Rd88SC.10, rh30, and rSMC cells in RMPI with 10% FBS; PC3, HeLa, HeLa S3, U2OS, Mel-STR, u87, 786-0, HEK-293T, MD-MBA-231, MD-MBA-468, HT29, c2c12, and MEFs ($p53^{-/-}$) cells in DMEM with 10% IFS; CACO2, 827, and SW480 cells in DMEM with 10% FBS; BJ fibroblasts in DMEM/F12 with 10% IFS; HUVECs in MCDB 131 media supplemented with EGM-2 and 5% FBS; and HMLE cells in 1:1 DMEM/F12 supplemented with insulin, epidermal growth factor (EGF), and hydrocortisone. All of the above cell lines were cultured at a density that allowed cell division throughout the course of the experiment. 3T3-L1 cells were cultured and differentiated as described (Frost and Lane, 1985). Parental, vector control, and PTEN-null DLD1 cells were cultured as described (Lee et al., 2004), as were Jurkat cells having a doxycycline-inducible PTEN (Xu et al., 2002).

Cell Lysis, Immunoblotting, and Crosslinking Assay

Cells growing in 10 cm diameter dishes were rinsed once with cold PBS and lysed on ice for 20 min in 1 ml of ice-cold buffer A (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and EDTA-free protease inhibitors [Roche]) containing 1% Triton X-100. After clearing of the lysates by centrifugation at 13,000 × g for 10 min, samples containing 50–100 μg of protein were resolved by SDS-PAGE and proteins transferred to PVDF and visualized by immunoblotting as described (Kim et al., 2002). For experiments with FKHR and AFX, the Triton X-100 insoluble materials were solubilized in 1% SDS in 10 mM Tris-HCl [pH 7.4] by heating at 100°C for 3 min followed by a brief sonication. Equal protein amounts were then analyzed by immunoblotting. For standard immunoprecipitation experiments, the cell lysis buffer consisted of buffer A containing 0.3% CHAPS instead of 1% Triton X-100 in order to preserve the integrity of the mTOR complexes (Kim et al., 2002; Sarbassov et al., 2004). When used, DSP was prepared as a stock solution of 50 mg in 200 μl of DMSO and added to a final concentration in the cell culture medium of 1 mg/ml (2.5 mM) (Kim et al., 2002; Sarbassov et al., 2004). Cells were then incubated at 37°C, 5% CO_2, and after 10 min the DSP was quenched by adding Tris-HCL (pH 8.0) to a final concentration of 100 mM. After a further 10 min incubation at 37°C, 5% CO_2 cells were lysed in buffer A containing Triton X-100. On occasion, DSP used at these high concentrations can form a precipitate, but this has no effect on the performance of the crosslinking assay. Reducing conditions were used during the SDS-PAGE analysis of immunoprecipitates prepared from DSP-treated cells to ensure breaking of the crosslinking disulfide bonds.

Immunoprecipitations and Kinase Assays

To the cleared lysates prepared as above, 4 μg of mTOR, rictor, or ATM antibodies were added per 1.2 mg of soluble protein, and immune complexes were allowed to form by incubating with rotation for 90 min at 4°C. A 50% slurry (25 μl) of protein G-Sepharose was then added and the incubation continued for 1 hr. Immunoprecipitates captured with protein G-Sepharose were washed four times with CHAPS-containing buffer A and analyzed by immunoblotting as described (Sarbassov et al., 2004). Immunoprecipitates used in kinase assays were also washed once with the rictor-mTOR kinase buffer (25 mM HEPES [pH 7.5], 100 mM potassium acetate, 1 mM $MgCl_2$). In kinase reactions, immunoprecipitates were incubated in a final volume of 15 μl for 20 min at 37°C in the rictor-mTOR kinase buffer containing 500 ng inactive Akt1/PKB1 (Akt1/PKB1, Upstate Biotechnology, #14–279) and 500 μM ATP. The reaction was stopped by the addition of 200 μl ice-cold enzyme dilution buffer (20 mM MOPS [pH 7.0], 1 mM EDTA, 0.01% Brij 35, 5% glycerol,

0.1% 2-mercaptoethanol, 1 mg/ml BSA). After a quick spin, the supernatant was removed from the protein G-Sepharose, and a 20 μl portion was analyzed by immunoblotting (Kim et al., 2002). Akt/PKB kinase assays were performed with the Akt1/PKBα Immunoprecipitation Kinase Assay Kit (Upstate) as described by the manufacturer.

^{35}S-Labeling and Pulse-Chase Experiments

HeLa or PC3 cells (4×10^6) growing in 100 mm dishes were treated with 100 nM rapamycin or vehicle control for 20 min, rinsed once in methionine- and cysteine-free DMEM, and then incubated in 3.5 ml of the same medium containing 10% dialyzed serum and 0.1 mCi/ml of ^{35}S-methionine/^{35}S-cysteine (Express Protein Labeling Mix, Perkin Elmer). After allowing the cells to label for 30 min, the cells were washed once in the normal culture medium and incubated in fresh medium for the periods of time indicated in the figures. Cells were then lysed in CHAPS lysis buffer and rictor and mTOR immunoprecipitates prepared as described above. Quantification was performed using images acquired with a phosphoimager.

Lentiviral shRNA Cloning, Production, and Infection

Lentiviral shRNAs were generated and used as described (Sarbassov et al., 2005b).

Apoptosis Induction and Detection

Cell lines stably expressing wild-type or S473D human Akt1/PKB1 were generated by infecting PC3 cells with retroviruses made from the MSCV vector system (Clontech). cDNAs were cloned into pMSCV-hygro at the Xho1/EcoR1 site, and retroviruses were generated as described (Ali and Sabatini, 2005). Cells were selected for 1 week in 200 μg/ml hygromycin before use. PC3 (10,000) (MSCV controls, wt Akt1/PKB1, or 473D Akt1/PKB1) cells were seeded in the wells of a 96-well plate and cultured overnight. The next day, the cells were rinsed once in serum-free medium and then cultured for 72 hr in serum-free medium containing 0.001% BSA and either DMSO (the small molecule vehicle), 100 nM rapamycin, 125 μM indole-3-carbinol, or both rapamycin and indole-3-carbinol. Indole-3-carbinole was prepared at 200 mM concentration, aliquoted, and stored at −20ºC. After 72 hr in culture, all cells (adherent and floating) were processed with the Cell Detection Elisa Plus (Roche, catalog number 1774425) as described by the manufacturer. For similar studies in MD-MBA-231 and 786-0 cells, the indole-3-carbinole was used at 100 μM, and in HT29 cells the indole-3-carbinole was used at 125 μM.

Tumor Xenografts, Immunohistochemistry, and In Situ Apoptosis Assays

PC3 cell lines stably expressing wild-type or S473D human Akt1/PKB1 or the empty vector were xenografted into 6-week-old immunodeficient mice (Ncr nu/nu mice; Taconic). All animal studies were performed according to the official guidelines from the MIT Committee on Animal Care and the American Association of Laboratory Animal Care. For Figures 5A–5C, CDE 3×10^6 PC3 cells were injected subcutaneously in the upper flank region of mice that had been anaesthetized with isoflurane. Tumors were allowed to grow to at least 50 mm^3 in size and then treated with rapamycin (10 mg/kg) for 2 days before sacrificing the mice. For Figure 5D, 1×10^6 PC3 cells were injected subcutaneously in the upper flank region of mice that had been anaesthetized with isoflurane. At the same time and for an additional 3 days postinjection of cells, mice were treated with rapamycin (10 mg/kg). Mice were sacrificed 11 days after xenografting of PC3 cells. At completion of all xenograft studies, the tumors were excised and tumor volumes estimated with the following formula: volume = $(a^2 \times b)/2$, where a = short and b = long tumor lengths, respectively, in millimeters. Sections of paraffin-embedded tumors on slides were processed for immunohistochemistry using the following primary antibodies and dilutions: 1:50 Akt1 (2H10, Cell Signaling Technology), 1:50 phospho-S473 Akt (736E11, Cell Signaling Technology), and 1:100 phospho-T308 Akt (244F9, Cell Signaling Technology). Briefly, sections were dewaxed and incubated in 3% H_2O_2 for 10 min at room temperature to quench endogeneous peroxidases and then processed for antigen retrieval by incubating in 10 mM sodium citrate buffer (pH 6) for 10 min in a subboiling water bath in a microwave oven. The sections were then incubated in blocking solution (5% horse serum in 1× TBST buffer) for 30 min at room temperature, washed three times, and then incubated overnight at 4ºC with primary antibody diluted in blocking solution. The next day, sections were incubated with the biotinylated secondary antibody for 1 hr at room temperature, washed three times, incubated 30 min with streptavidin-HRP (DakoCytomation), rewashed, and developed with DAB reagents (DakoCytomation) for 5–20 min until staining appeared. The slides were counterstained with hematoxylin, dehydrated, and mounted with coverslips. All washes were for 5 min in 1× TBST wash buffer (1 × TBS with 0.1% Tween-20). An in situ cell death detection kit (Roche) was used as described by the manufacturer to detect apoptotic cells in tumors. Percentages of apoptotic cells per high-power field were quantified in a blinded fashion.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://www.molecule.org/cgi/content/22/2/159/DC1/.

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