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Rapamycin Inhibits Akt-Mediated Oncogenic Transformation and Tumor Growth

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Abstract. *Akt is a serine/threonine kinase that plays a critical role in cell survival and proliferation. Three isoforms of Akt have been identified and have been shown to be up-regulated in human malignancies. We examined the requirement of these pathways for Akt transformation. We generated NIH-3T3 cells over-expressing constitutively active Myr-Akt1 (3T3-Akt1 cells) or Myr-Akt2 (3T3-Akt2 cells). These cells are able to form colonies in soft-agar and 3T3-Akt1 cells formed tumors in SCID mice. Rapamycin efficiently inhibited the activation of the mTOR-p70S6K pathway and the anchorage-independent growth of both 3T3-Akt cells, demonstrating the importance of the mTOR-p70S6K pathway for transformation by Akt1 as well as by Akt2. Moreover, rapamycin dramatically inhibited the tumor formation by 3T3-Akt1 cells in SCID mice. Thus, we demonstrated the importance of mTOR-p70S6 kinase pathway in the transformation by Akt, both in tissue-cultured cells and in animal tumor models. In contrast, neither the MAPK pathway nor the p38 MAPK pathway is required for Akt-dependent transformation of NIH3T3 cells.*

Akt (PKB: protein kinase B) plays an important role in regulating cell growth and survival (1). Akt transduces signals from phosphatidylinositol 3-kinase (PI3-kinase) upon growth factor stimulation (2). The pleckstrin homology domain of Akt is able to bind to the products of PI3-kinase, phosphatidylinositol 3,4-biphosphate (PIP2) or phosphatidylinositol 3,4,5-triphosphate (PIP3). This binding targets Akt to the plasma membrane where it is then phosphorylated and activated by PDK1 and PDK2 (3-9). Activated Akt promotes cell survival by phosphorylating and inhibiting many pro-apoptotic proteins including Bad and caspase-9, and forkhead transcription factors such as AFX, FKHRL1 and FHKR (10-13). Activated Akt also phosphorylates glycogen synthase kinase 3α/β (GSK3α/β), which is involved in insulin-mediated glucose metabolism (14).

Akt1 was initially identified as the human homologue of viral oncogene in Akt8 virus (15-17). Since then, two other isoforms of Akt, Akt2 and Akt3, have been identified (18, 19). All three isoforms of Akt have been shown to be up-regulated by either over-expression or gene amplification in various human cancers including breast, cervical, prostate, ovarian and pancreatic carcinomas (19-22). Moreover, the tumor suppressor gene, *PTEN*, is frequently mutated in many advanced malignancies (23-25). PTEN is a phosphatase that reverses the effect of PI3-kinase by dephosphorylating the 3'-phosphate in PIP3 and PIP2. Thus, mutations of PTEN result in the activation of Akt (23-25). Over-expression of constitutively active Akt1 or wild-type Akt2 results in oncogenic transformation of the NIH3T3 fibroblast (19, 20). On the other hand, the antisense oligonucleotide for Akt1 can inhibit the anchorage-independent growth of many human cancer cells (26), while antisense for Akt2 can inhibit, specifically, the malignant phenotype of pancreatic cancer cell lines (21).

Rapamycin is a natural macrolide that induces G1 growth arrest by binding to FKBP12 and inhibiting the protein kinase FRAP/mTOR (27, 28). Two well-characterized downstream targets of mTOR are p70 S6 kinase (p70S6K) and 4E binding protein 1 (29). mTOR controls the activation of p70S6K, that facilitates the translation of a subfamily of mRNAs, by phosphorylating ribosomal S6 protein (30). Over-expression of constitutively active Akt1 results in the phosphorylation and activation of p70S6K, whereas rapamycin inhibits the process (31), suggesting the signal transduction from Akt to p70S6K is *via* mTOR. The tumor suppressor protein tuberous sclerosis 2 (Tsc2 or

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Tuberin) has been identified as an Akt substrate that bridges Akt and mTOR (32-35). Mutations of Tsc1 and Tsc2 are responsible for familial tuberous sclerosis, which is characterized by the presence of disorganized but differentiated benign growth in a variety of organs. Tsc2 phosphorylation by Akt destabilizes the Tsc1-Tsc2 complex and releases the inhibitory effect of the complex on mTOR (32-35).

Rapamycin has been shown to specifically inhibit Akt1- or PI3-kinase- induced transformation of chicken embryonic fibroblast (31). Rapamycin or its analogue, CCI-779, blocks preferentially the growth of PTEN null human cancer cells in tisuse culture as well as in animal tumor models (36, 37, 38). In addition, rapamycin induces G1 cell cycle arrest in breast cancer cell lines expressing high levels of phospho-Akt as well as phospho-p70S6K1 even when PTEN is wild-type (39). However, it is not known whether rapamycin also inhibits the tumor growth of Akt-transformed cells *in vivo*.

In this study, we generated stable NIH-3T3 cell lines that over-express constitutively active Akt1 and Akt2. These cells are able to form colonies in soft-agar and 3T3-Akt1 cells form tumors in SCID mice, indicating oncogenic transformation. Rapamycin blocked Akt1- and Akt2-mediated transformation in 3T3 cells. In addition, rapamycin inhibited the tumor growth of 3T3-Akt1 cells in SCID mice. In contrast, we showed that neither the MAPK pathway nor the p38 MAPK pathway is required for transformation by Akt1 or Akt2.

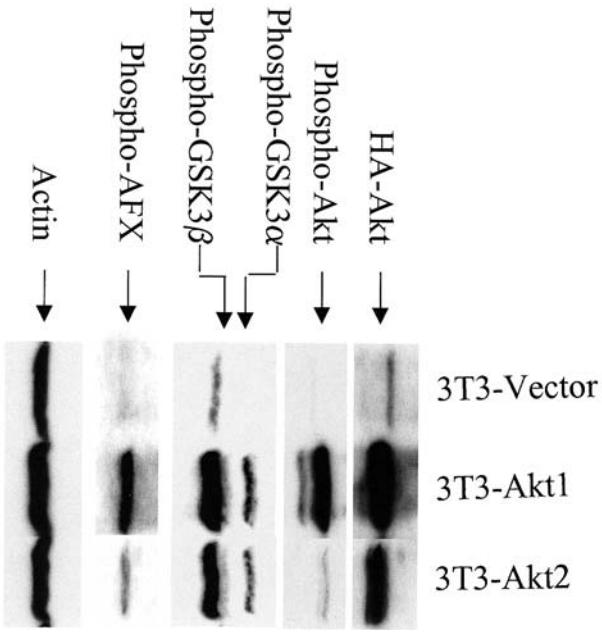

Figure 1. *Biochemical analysis of 3T3-Akt1 and 3T3-Akt2 cells. NIH3T3 cells over-expressing Akt1 and Akt2 were selected in the presence of G418 and cell extracts were prepared as described in Materials and Methods. Fifty μg of cell extracts were loaded onto 10% SDS gel (BioRad) and Western-blot analysis was carried out as described (26) using antibodies against HA-tag, Phospho-Akt (S473), phospho-GSK3α/β, phospho-AFX and Actin.*

Materials and Methods

Chemicals. LY294002, SB202190 and PD98059 were purchased from EMD Biosciences (La Jolla, CA, USA) while others were from Sigma (St. Louis, MO, USA). Protein concentration was determined using the BCA method (Pierce, Rockford, IL, USA).

Cell lines. NIH-3T3 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured under the conditions provided by the suppliers.

Western blot analysis. Cells from 10-cm dishes were harvested and lysed in 200 ml buffer B (20 mM Hepes, pH 7.5, 10 mM NaCl, 20 mM NaF, 1mM EDTA, 1mM EGTA, 5mM sodium pyrophosphate, 2mM sodium vanadate, 10mM β-glycerolphosphate and 1% NP-40) on ice for 30 min. The samples were centrifuged at 12,000g, at 4˚C for 10 min. Cell lysates were subjected to SDS-PAGE gel electrophoresis and Western analysis. Rabbit anti-Akt, anti-phospho-Akt (S473-P), anti-p70S6K1, anti-phospho-p70S6K1 (Thr421/Ser424), anti-phospho-GSK3α/β, anti-phospho-AFX and anti-phospho-S6 (Ser235/236) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Anti-HA antibody was from Bibco (Berkeley, CA, USA). Anti-Actin antibody was from Santa Cruz Biotechnology (Santa Crutz, CA, USA). Immunoblot analysis was performed using enhanced chemiluminescence (ECL) detection reagents (Amersham, Arlington Heights, IL, USA) according to the manufacturer's instructions.

Soft-agar assay. The assay was carried out in DMEM containing 20% calf serum. For the bottom layer of agar, 2 ml of 0.8 % low-melting agarose in complete medium was placed in each well of six-well plates. Two ml of 0.3% top low-melting agarose containing $2x10^4$ cells were layered on top of the solidified bottom layer of agar. After 25 days, the colonies were stained with *p*-iodonitrotetrazolium violet and the number of colonies was scored using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA).

cDNA cloning of Akt1 and Akt2. Full-length human cDNA for Akt1 and Akt2 was derived by PCR from human RNA. The expression plasmids pCIneo-Lck-Akt1 and pCIneo-lck-Akt2 were constructed so that an amino acid sequence (MGCGCSSHPEDDA AYPYDVPDYAPREFT) directly preceding the start methionine of the Akt sequences encodes the lck tag (MGCGCSSHPEDD) and HA epitope tag (YPYDVPDYAP).

Transfection of NIH3T3 cells and stable clone selection. On day 0, one million NIH3T3 cells were plated in a 10-cm dish. On day 1, the cells were transfected with pCIneo-lck-Akt1, pCIneo-lck-Akt2 or PCIneo vector using Lipofectamine Plus reagent according to the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA,USA). After 48 hours, the transfected cells were selected with 1mg/ml G418. Colonies were isolated and screened for the expression of Akt using anti-HA antibody (Bibco). The colonies expressing the highest amount of Akt1 (3T3-Akt1 cells) and Akt2 (3T3-Akt2 cells) were used for the study.

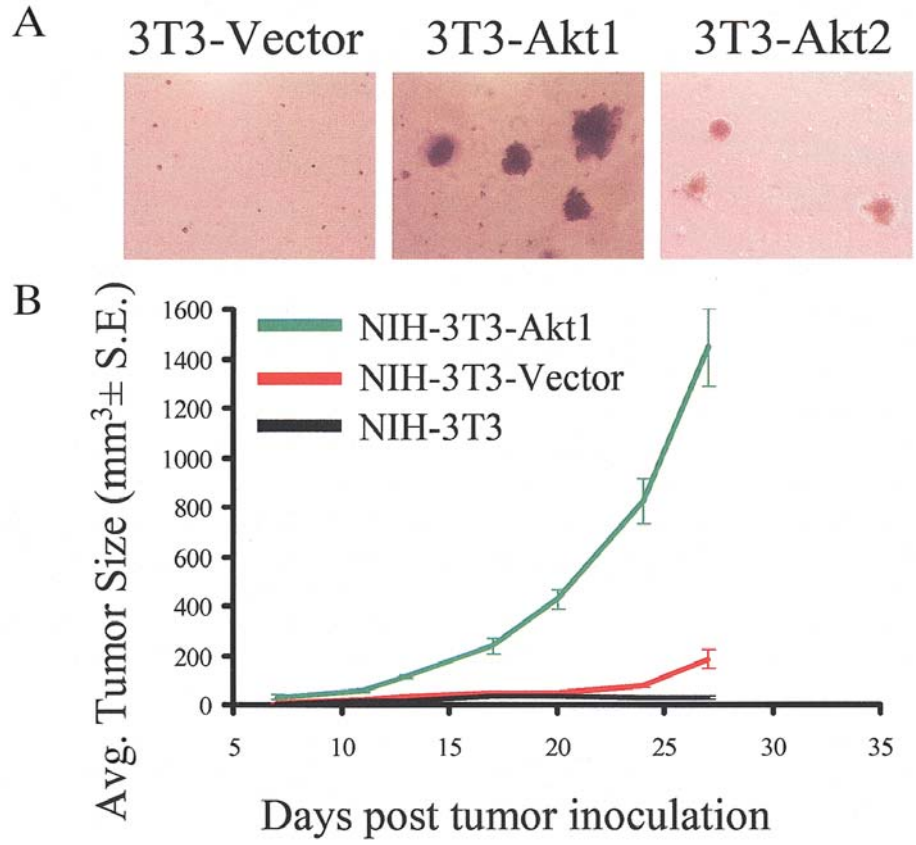

Figure 2. *3T3-Akt1 and 3T3-Akt2 cells form colonies in soft-agar and 3T3-Akt1 cells form tumors in SCID mice. (A) 3T3-Akt1 and 3T3-Akt2 cells were plated in a six-well plate for the soft-agar assay. Pictures of the colonies were taken after 25 days in culture. (B) Parental NIH-3T3 cells, NIH-3T3-vector cells, or 3T3-Akt1 cells were inoculated subcutaneously into SCID mice. The volumes of the tumors were measured twice weekly, as described in Materials and Methods.*

Xenograft tumor studies. Immunocompromised male SCID mice (C.B-17-*Prkdc*^{scid}) were obtained from Charles River Laboratories (Wilmington, MA, USA) at 6-8 weeks of age. Two million 3T3-Akt1 or 3T3-vector cells in 50% Matrigel (BD Biosciences, Bedford, MA, USA) were inoculated subcutaneously into the flank. The tumors were allowed to reach a size of approximately 250 mm^3 (~ 2 weeks post inoculation) before the mice were assigned to treatment groups of equal tumor size (N=10 mice per group). Tumor size was measured twice weekly with digital calipers. Tumor volume was estimated using the formula: $V=L \times W^2/2$. Rapamcyin was administered at a dose of 20 mg/kg/day given *i.p*, q.d.x21 starting the day after size matching. The vehicle was 4% EtOH, 10% solutol in saline.

Results

Over-expression of constitutively active Akt1 or Akt2 induces oncogenic transformation of NIH3T3 cells. In order to study the role of Akt in oncogenesis, we generated stable NIH3T3 cell lines that over-express constitutively active Akt1 (3T3-Akt1 cells) or Akt2 (3T3-Akt2 cells). As shown in Figure 1, these cell lines have elevated levels of Akt, phospho-Akt, phospho-GSK3α/β and phospho-AFX compared to the vector control, suggesting the over-expression of constitutively active Akt and the increased phosphorylation of Akt substrates in these cells. Soft-agar colony formation analysis was performed to test the transformation property in these cells. Consistent with previously published data (15), 3T3-Akt1 and 3T3-Akt2 cells formed colonies in soft-agar, while the vector control cells failed to do so (Figure 2A). In addition, 3T3-Akt1 cells also formed tumors in SCID mice. NIH3T3 cells transfected with pCIneo vector (3T3-vector control cells) also formed a tumor with a much longer latent time, which was much smaller in size (Figure 2B).

Rapamycin inhibits oncogenic transformation of 3T3-Akt1 and 3T3-Akt2 cells. The effect of rapamycin on the Akt signal transduction pathway in 3T3-Akt1 or 3T3-Akt2 cells was examined by Western blot analysis. Rapamycin inhibited the

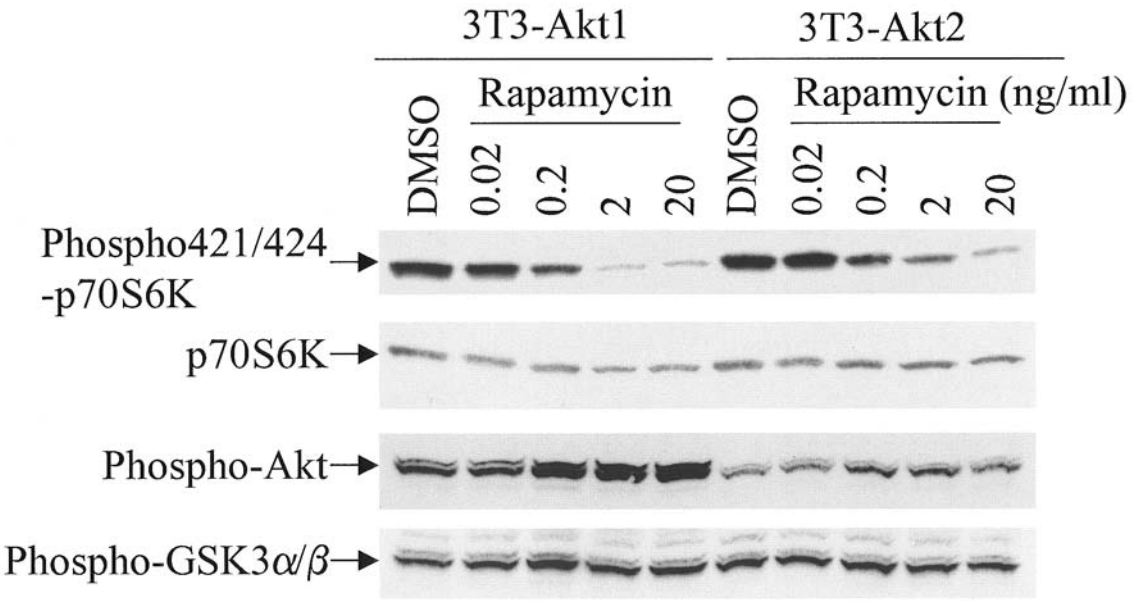

Figure 3. *Rapamycin inhibits the activity of p70S6K but not Akt. 3T3-Akt1 or 3T3-Akt2 cells were treated with the indicated concentrations of rapamycin for 4 hours. Cell extracts were prepared and Western blot analysis was carried out using antibodies against phospho-p70S6K1, p70S6K1, phospho-Akt, and phospho-GSK3α/β.*

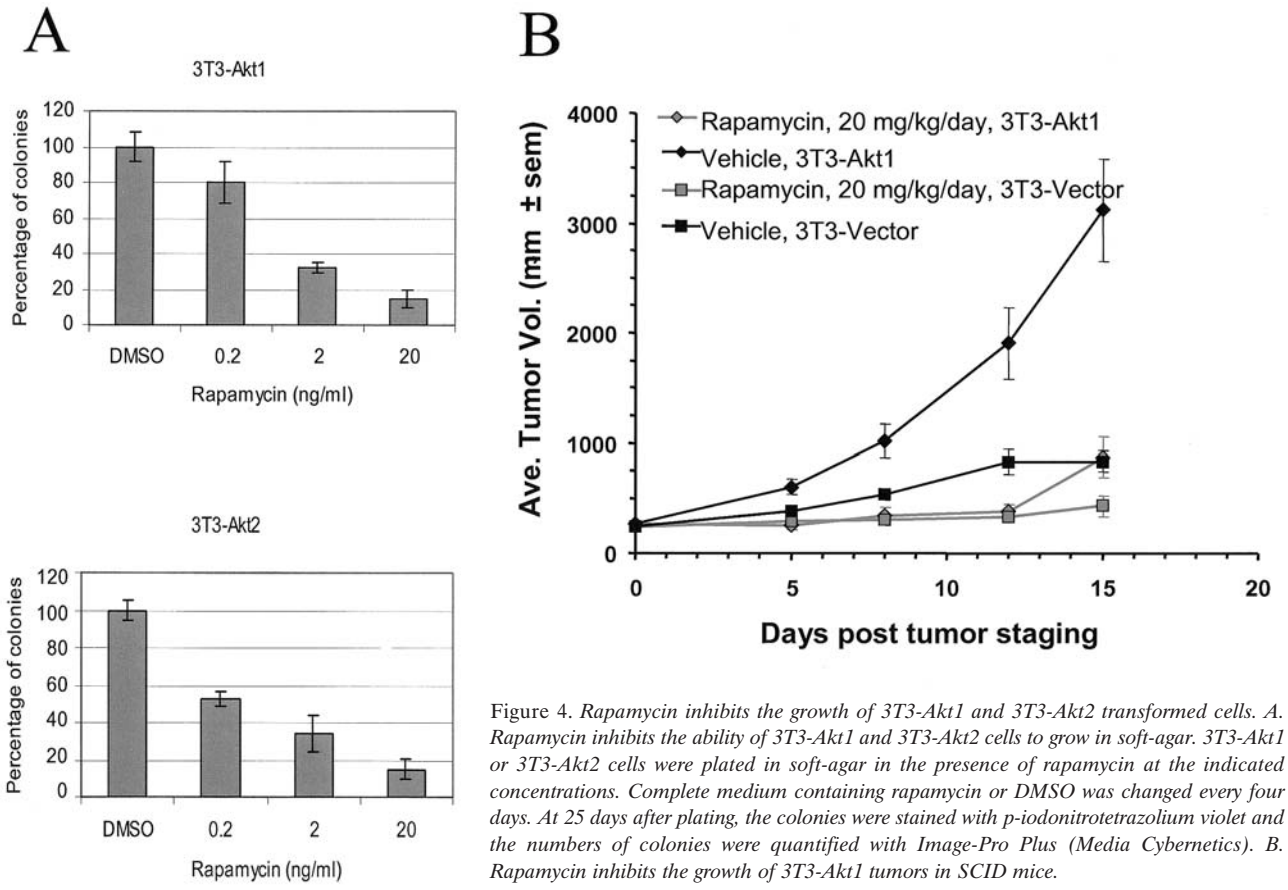

Figure 4. *Rapamycin inhibits the growth of 3T3-Akt1 and 3T3-Akt2 transformed cells. A. Rapamycin inhibits the ability of 3T3-Akt1 and 3T3-Akt2 cells to grow in soft-agar. 3T3-Akt1 or 3T3-Akt2 cells were plated in soft-agar in the presence of rapamycin at the indicated concentrations. Complete medium containing rapamycin or DMSO was changed every four days. At 25 days after plating, the colonies were stained with p-iodonitrotetrazolium violet and the numbers of colonies were quantified with Image-Pro Plus (Media Cybernetics). B. Rapamycin inhibits the growth of 3T3-Akt1 tumors in SCID mice.*

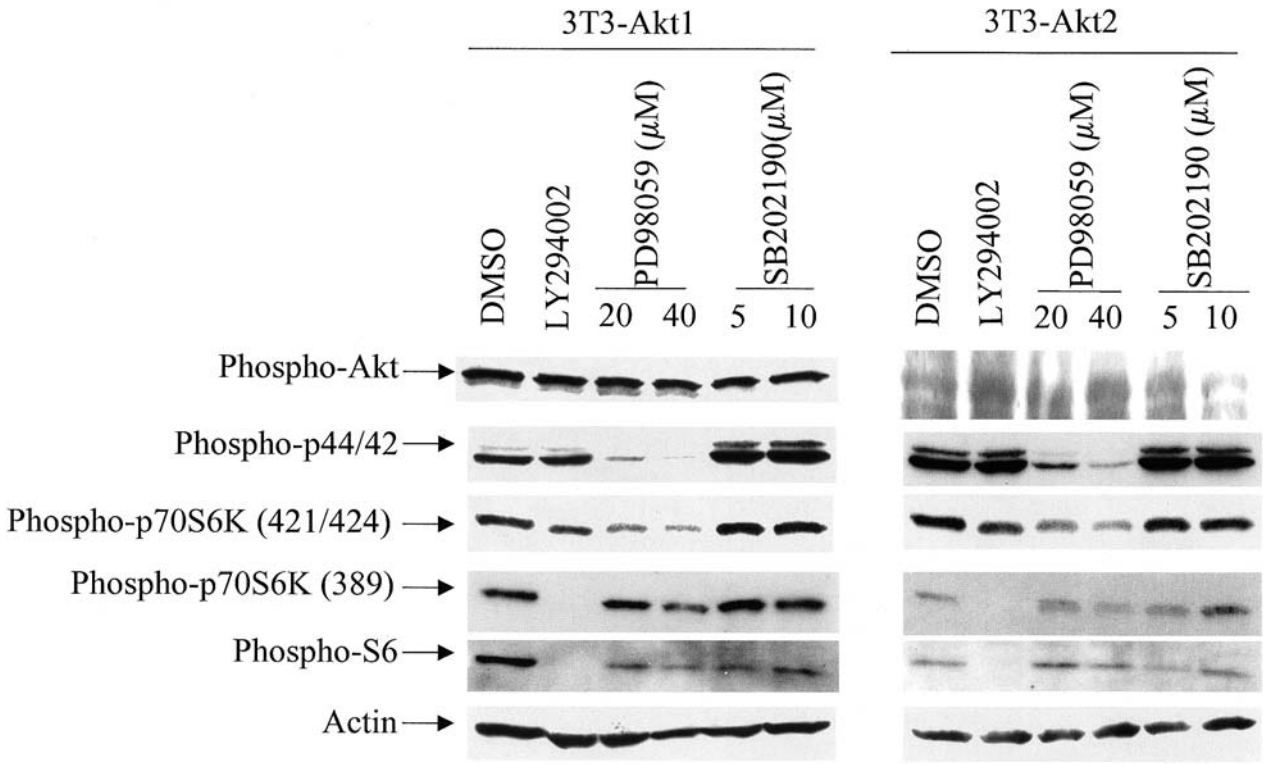

Figure 5. *Effect of LY294002, PD98059 and SB202190 on the phosphorylation status of Akt, p44/p42 MAP kinase, p70S6K and S6 phosphorylation. 3T3-Akt1 and 3T3-Akt2 cells were treated with indicated compounds for 12 hours. The cells were harvested and cell extracts were prepared. Fifty μg of cell extracts were loaded onto a 10% SDS ready gel and analyzed by Western blot.*

activation of p70S6K1 in a dose-dependent manner, without affecting its protein levels, in both 3T3-Akt1 and 3T3-Akt2 cells (Figure 3). However, the phosphorylation of Akt and GSK3α/β were not inhibited by rapamycin. In fact, Akt phosphorylation at Ser 473 increased slightly (Figure 3).

Since rapamycin has been shown to inhibit the transformation of chicken embryo fibroblasts over-expressing PI3-kinase or Akt1 (31), we tested whether rapamycin could also inhibit transformation by Akt1 or Akt2 in NIH3T3 cells. As shown in Figure 4A, rapamycin inhibited the formation of transformed foci by both 3T3-Akt1 and 3T3-Akt2 cells in soft-agar in a dose-dependent manner (Figure 4A). The concentration range of rapamycin required for p70S6K1 inhibition was similar to the concentration range needed for inhibiting the colony formation by either 3T3-Akt1 or 3T3-Akt2 cells, suggesting that mTOR-p70S6K activation is important for Akt-induced transformation.

Rapamycin was also able to inhibit the growth of 3T3-Akt tumors *in vivo*. Following inoculation, 3T3-Akt tumors were allowed to grow to approximately 250 mm^3 prior to their assignment to different treatment groups. Rapamycin was given at a dose of 20 mg/kg/day, *i.p.*, once a day for 21 days. Rapamcyin treatment resulted in up to 80% inhibition of tumor growth rate and greater than 100% increase in the time required for the tumor to reach 1.5 cm^3 endpoint relative to that for the vehicle controls (Figure 4B). Although some tumor growth was observed in the vector control animals, the effect of rapamycin on the growth rate of these tumors was minimal compared to that of 3T3-Akt tumors (Figure 4B).

MAPK and p38 MAPK pathways are not required for Akt-dependent transformation. We next tested whether the MAPK and p38 MAPK kinase pathways are required for Akt-induced transformation by treating 3T3-Akt1 and 3T3-Akt2 cells with the MEK1 inhibitor PD98059 and the p38 inhibitor SB202190. We also included the PI3-kinase inhibitor LY294002 as a control. These inhibitors were used at concentrations that specifically inhibit their target kinases (40). As shown in Figure 5, PD98059 effectively inhibited the phosphorylation of p44/p42 MAP kinase as expected. It also partially inhibited p70S6K1 phosphorylation at serine 421/424 and, to a lesser extent, at serine 389. As a result, S6

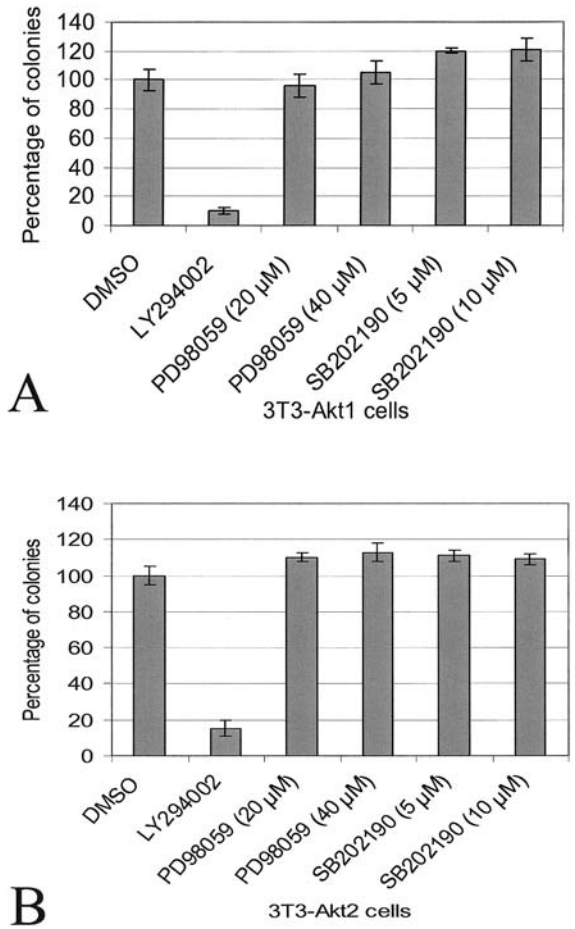

Figure 6. *Neither the MAPK nor the p38 MAPK pathway is required for Akt-dependent trnasformation. 3T3-Akt1 cells (A) or 3T3-Akt2 cells (B) were plated in soft-agar in the presence of LY294002 (10 μM), or PD98059 (20 or 40 μM), or SB20219 (5 or 10 μM). The number of colonies was scored at 25 days after plating, as described in Materials and Methods.*

phosphorylation was also partially inhibited by PD98059. However, PD98059 and SB202190 had no effect on Akt phosphorylation (Figure 5), GSK3 phosphorylation (data not shown) and colony formation in soft-agar by 3T3-Akt1 or Akt2 cells (Figure 6). This indicates that MEK1 and p38 MAP kinase are not required for Akt-induced transformation. Surprisingly, LY294002 significantly inhibited the ability of 3T3-Akt1 and 3T3-Akt2 cells to grow in soft-agar (Figure 6). We also examined the effect of LY294002 on signal transduction of the PI3' kinase-Akt pathway. LY294002 did not affect Akt phosphorylation (Figure 5) and GSK3 phosphorylation (data not shown), indicating that Myr-Akt1 and Myr-Akt2 are indeed constitutively active. However, LY294002 completely abolished the mTOR-dependent p70S6K1 phosphorylation at Thr 389, suggesting that LY294002 may inhibit mTOR activity as well (Figure 5). This is consistent with the study by Brunn *et al.* (41). Phosphorylation at Thr389 in p70S6K1 is required for its activity. Therefore, as expected, S6 phosphorylation was also abolished by LY294002 (Figure 6). These results suggest that the ability of LY294002 to affect 3T3-Akt1/2 growth may result from an inhibition of mTOR activity, rather than through direct inhibition on PI3'-kinase.

Discussion

We have shown that over-expression of constitutively active Akt1 or Akt2 induces oncogenic transformation of normal mouse fibroblast NIH3T3 cells. Aoki *et. al*. (31) showed that the mTOR-p70S6K pathway is required for Akt1-dependent transformation in chicken embryonic fibroblasts. Consistent with their study, we showed that the Akt1-induced transformation of NIH-3T3 cells also requires the mTOR-p70S6K pathway, since rapamycin completely blocked the colony formation by the 3T3-Akt1 cells at the concentrations that also inhibited p70S6 kinase activation (Figure 3 and Figure 4). Furthermore, rapamycin significantly inhibited the growth of 3T3-Akt1 tumors, indicating that the mTOR-p70S6K pathway is also required for Akt-induced tumorigenesis.

The phenotypes of Akt1 and Akt2 knockout mice suggest that Akt1 mainly modulates cell growth while Akt2 is important for insulin response (42-46). However, both Akt1 and Akt2 are up-regulated in various types of human malignancies, suggesting an important role for both Akt1 and Akt2 in tumorigenesis. We have shown that the molecular mechanisms by which Akt1 and Akt2 transform cells are similar. The mTOR-p70S6K pathway is critical for both Akt1 and Akt2-induced transformation of 3T3 cells. In contrast, neither the MAPK pathway nor the p38 MAPK pathway is required for Akt1- or Akt2-dependent transformation.

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