Activation of Akt and eIF4E Survival Pathways by Rapamycin-Mediated Mammalian Target of Rapamycin Inhibition

Shi-Yong Sun,[1] Laura M. Rosenberg,[1] Xuerong Wang,[1] Zhongmei Zhou,[1] Ping Yue,[1] Haian Fu,[2] and Fadlo R. Khuri[1]

[1]Department of Hematology and Oncology and Winship Cancer Institute and [2]Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia

Abstract

The mammalian target of rapamycin (mTOR) has emerged as an important cancer therapeutic target. Rapamycin and its derivatives that specifically inhibit mTOR are now being actively evaluated in clinical trials. Recently, the inhibition of mTOR has been shown to reverse Akt-dependent prostate intraepithelial neoplasia. However, many cancer cells are resistant to rapamycin and its derivatives. The mechanism of this resistance remains a subject of major therapeutic significance. Here we report that the inhibition of mTOR by rapamycin triggers the activation of two survival signaling pathways that may contribute to drug resistance. Treatment of human lung cancer cells with rapamycin suppressed the phosphorylation of p70S6 kinase and 4E-BP1, indicating an inhibition of mTOR signaling. Paradoxically, rapamycin also concurrently increased the phosphorylation of both Akt and eIF4E. The rapamycin-induced phosphorylation of Akt and eIF4E was suppressed by the phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002, suggesting the requirement of PI3K in this process. The activated Akt and eIF4E seem to attenuate rapamycin's growth-inhibitory effects, serving as a negative feedback mechanism. In support of this model, rapamycin combined with LY294002 exhibited enhanced inhibitory effects on the growth and colony formation of cancer cells. Thus, our study provides a mechanistic basis for enhancing mTOR-targeted cancer therapy by combining an mTOR inhibitor with a PI3K or Akt inhibitor. (Cancer Res 2005; 65(16): 7052-8)

Introduction

The mammalian target of rapamycin (mTOR), a 289-kDa serine/threonine kinase, belongs to the phosphatidylinositol kinase–related kinase family. It plays a central role in regulating cell growth, proliferation, and survival, in part by regulation of translation initiation (1–3). In response to mitogen stimulation, mTOR regulates translation initiation through two distinct pathways: ribosomal p70 S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs). In one pathway, mTOR phosphorylates and activates p70S6K, which in turn phosphorylates the 40S ribosomal protein S6, leading to the enhancement of translation of mRNAs with a 5′-terminal oligopyrimidine, including mRNAs that encode for ribosomal proteins and elongation factor-1. In another pathway, mTOR directly phosphorylates 4E-BP1, causing its dissociation from eIF4E, thereby increasing the availability of functional eIF4E, a rate-limiting component for cap-dependent translation. The free eIF4E then binds to eIF4G, promoting the assembly of the eIF4F initiation complex, which then leads to more efficient cap-dependent translation initiation, increasing the translation of mRNAs with long, highly structured 5′-untranslated regions, such as cyclin D1 and c-Myc (1–3).

The phosphatidylinositol-3 kinase (PI3K)/Akt signaling represents a major cell survival pathway. Its activation has long been associated with malignant transformation and apoptotic resistance (4). It has been well documented that mTOR functions downstream of the PI3K/Akt pathway and is phosphorylated (or activated) in response to stimuli that activate the PI3K/Akt pathway (1, 3). Normally, the phosphatase PTEN counters the PI3K activity and thus negatively regulates PI3K/Akt survival pathway. However, PTEN activity is frequently inactivated in many human tumor types through deletion, mutation, or silencing, leading to increased activation of Akt (4, 5). Additional mechanisms have also been found to induce the activation of the PI3K/Akt pathway, including oncogene (e.g., Ras) amplification and mutations, active mutations in the p110 and p85 subunits of PI3K, and Akt overexpression. Thus, mTOR signaling pathways are constitutively activated in many types of human cancer (1, 6). Recent studies have shown that the mutation of tuberous sclerosis complex and overexpression of Rheb that work downstream of Akt in regulating the mTOR signaling also occur in human cancers, contributing to mTOR activation (7). Moreover, eIF4E is overexpressed or amplified in multiple human cancers, which is often oncogenic (1, 6, 8). Therefore, mTOR signaling has emerged as an important and attractive therapeutic target for cancer therapy (1, 2). The potential applications of mTOR inhibitors for treating various types of cancer have been actively studied both preclinically and clinically. A recent animal study has shown that mTOR inhibition induces apoptosis of epithelial cells and reverses Akt-dependent prostate intraepithelial neoplasia (9). In the United States, several phase II or III trials are ongoing to test the effects of mTOR inhibitors on various cancers, including renal cell carcinoma, prostate, breast, pancreatic, and small cell lung cancers, recurrent brain tumors, recurrent mantle-cell lymphoma, and melanoma (1, 10).

The intrinsic sensitivity to mTOR inhibition by rapamycin among different cancer cell lines may vary by several orders of magnitude ranging from 1 to 5,000 nmol/L (IC_{50}; ref. 11), indicating that some cancer cell lines are actually resistant to mTOR inhibition. Therefore, understanding the mechanisms by which cells become resistant to mTOR inhibitors may guide the development of successful mTOR-targeted cancer therapy. Using

---

Requests for reprints: Shi-Yong Sun, Winship Cancer Institute, Emory University School of Medicine, 1365-C Clifton Road, C3088, Atlanta, GA 30322. Phone: 404-778-2170; Fax: 404-778-5520; E-mail: shi-yong_sun@emoryhealthcare.org.

doi:10.1158/0008-5472.CAN-05-0917

human non–small cell lung cancer (NSCLC) cells, here we report that mTOR inhibition by rapamycin induces activation of survival pathways involving increase of Akt and eIF4E phosphorylation. We show that prevention or disruption of the activation of Akt and eIF4E enhanced rapamycin-mediated growth inhibition, indicating that the induced activation of Akt and eIF4E survival pathways counteracts the mTOR inhibitor's effect on the growth of human cancer cells.

Materials and Methods

Reagents. Rapamycin was purchased from LKT Laboratories, Inc. (St. Paul, MN). LY294002 and U0126 were purchased from LC Laboratories (Woburn, MA). PD98059 and SB203580 were purchased from Biomol (Plymouth Meeting, PA) and Calbiochem (San Diego, CA), respectively. These agents were dissolved in DMSO at a concentration of 10 or 20 mmol/L, and aliquots were stored at −80°C. Stock solutions were diluted to the desired final concentrations with growth medium just before use. Rabbit polyclonal antibodies against Akt, mTOR, p70S6K, 4E-BP1, eIF4E, phospho-Akt (p-Akt, Ser^{473}), phospho-mTOR (p-mTOR, Ser^{2448}), phospho-GSK3β (p-GSK3β, Ser^{9}), phospho-p70S6K (p-p70S6K, Thr^{389}), phospho-4E-BP1 (p-4E-BP1, Ser^{65}), phospho-eIF4E (p-eIF4E, Ser^{209}), phospho-p44/p42 (p-p44/p42, Thr^{202}/Tyr^{204}), respectively, were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit polyclonal anti-actin antibody was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell lines and cell culture. Human NSCLC and other cancer cell lines used in this study were purchased from the American Type Culture Collection (Manassas, VA). They were grown in monolayer culture in RPMI 1640 supplemented with glutamine and 5% fetal bovine serum at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Growth inhibition assay. Cells were seeded in 96-well cell culture plates and treated on the second day with rapamycin, LY294002, or rapamycin combined with LY294002. At the end of a 3-day treatment, cell number was estimated by the sulforhodamine B (SRB) assay as previously described (12). The percentage of growth inhibition was calculated by using the equation: % growth inhibition = $(1 - A_t / A_c) \times 100$, where A_t and A_c represent the absorbance in treated and control cultures, respectively.

Colony formation assay. Cells (single-cell suspension) were plated in 12-well plates at a density of 200 to 300 cells per well. On the second day, cells were treated with rapamycin, LY294002, or rapamycin plus LY294002. Every 3 days, the medium was replaced with fresh medium containing the corresponding agents. After a 10-day treatment, the medium was removed and cell colonies were stained with SRB dye as described (12). Pictures were then taken using a digital camera to record the result.

Western blot analysis. The procedures for preparation of whole cell protein lysates and for Western blotting were described previously (13). Whole cell protein lysates (50 μg) were electrophoresed through 7.5%, 10%, or 12% denaturing polyacrylamide slab gels and transferred to a Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by electroblotting. The blots were probed or reprobed with the primary antibodies and then antibody binding was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's protocol.

Results

Inhibition of mammalian target of rapamycin signaling by rapamycin suppresses the growth of human non–small cell lung cancer cells. To determine whether NSCLC cells were sensitive to mTOR inhibitors, we examined the effects of rapamycin on the growth of NSCLC cell lines representing adenocarcinoma, squamous cell carcinoma, and large cell carcinoma cells. As shown in Fig. 1, rapamycin at concentrations of ≥1 nmol/L was effective in inhibiting the growth of NSCLC cells, albeit with varying degrees. However, at concentrations ranging from 1 to 1,000 nmol/L, rapamycin did not seem to exhibit a dose-dependent growth-inhibitory effect. Rapamycin at concentrations <1 nmol/L dramatically decreased its efficacy against the growth of NSCLC cells. Interestingly, rapamycin at concentrations up to 1,000 nmol/L inhibited the growth of NSCLC cells only by 50% to 75%. Even when rapamycin's concentration was increased to 10 μmol/L, its growth-inhibitory effects were not further increased (data not shown). These results suggest that certain portions of cells in the population are resistant to rapamycin or cancer cells have some resistant mechanisms to bypass growth inhibition caused by mTOR inhibitors. Under the microscope, cells exposed to rapamycin remained attached on dishes and had normal morphology in comparison with control cells, suggesting that rapamycin inhibits the growth of human NSCLC cells without apparently inducing cell death.

To determine whether the growth-inhibitory effects of rapamycin are due to impaired mTOR signaling, we examined the activation states of mTOR and the two downstream effectors of rapamycin-sensitive mTOR complex, p70S6K and 4E-BP1, in four representative NSCLC cell lines. Both mTOR and p70S6K were phosphorylated in these cell lines, whereas 4E-BP1 was phosphorylated in three (i.e., A549, H157, and H460) of the four cell lines (Fig. 2*A*). These results suggest that the mTOR signaling pathway is constitutively activated in human NSCLC cell lines. After treatment with rapamycin, the phosphorylation levels of p70S6K (p-p70S6K) and 4E-BP-1 (p-4E-BP1) were drastically decreased although the levels of p-mTOR was not apparently altered (Fig. 2*A*), revealing a potent inhibitory effect of rapamycin on the mTOR signaling. Thus, it is likely that rapamycin inhibits the growth of NSCLC cells through a blockade of the mTOR signaling pathway.

Inhibition of mammalian target of rapamycin by rapamycin increases the phosphorylation of Akt and eIF4E. To evaluate the effects of rapamycin on the status of molecules proximal to the mTOR axis, we probed the phosphorylation states of Akt and eIF4E. Because Akt has been placed upstream of mTOR in many cell types, we speculated that rapamycin would not alter the phosphorylation levels of Akt (p-Akt). Unexpectedly, all of the

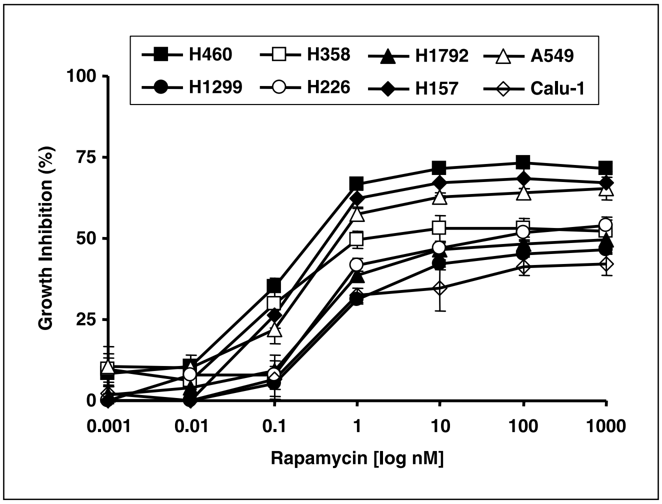

Figure 1. Effects of rapamycin on the growth of human NSCLC cells. The indicated cell lines were plated in 96-well plates and treated on the second day with the indicated concentrations of rapamycin. After 3 days, plates were subjected to determination of cell number using a SRB assay. *Points,* means of four replicate determinations; *bars,* ±SD.

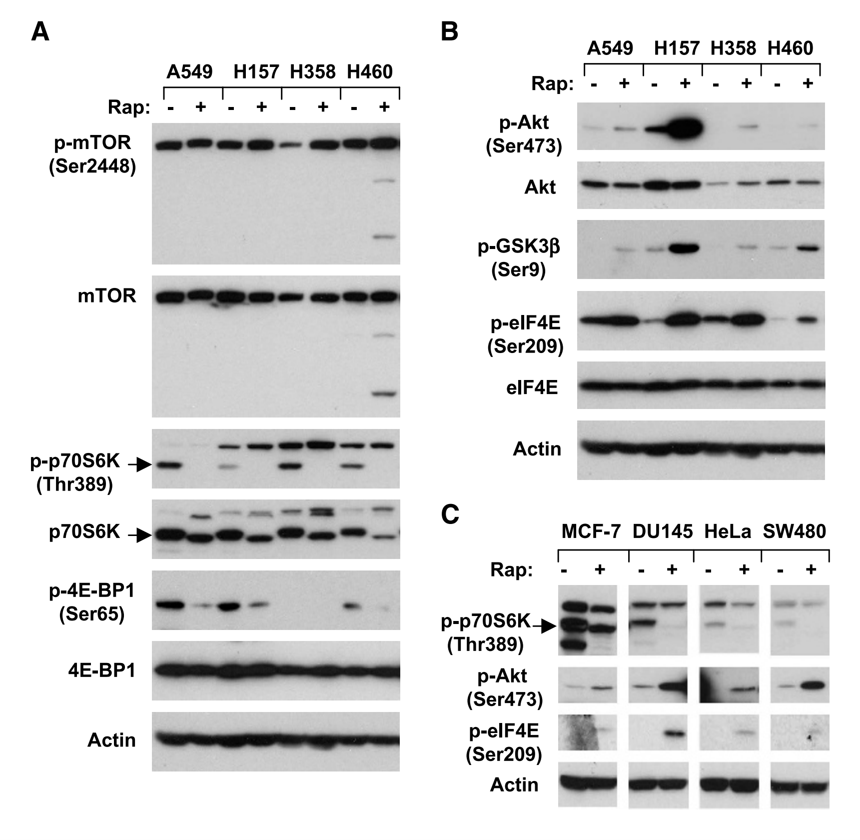

Figure 2. Rapamycin inhibits the mTOR signaling (*A-C*) and increases the phosphorylation of Akt and eIF4E (*B* and *C*) in human NSCLC (*A-B*) and other cancer (*C*) cell lines. The indicated cell lines were seeded in 10-cm-diameter cell culture dishes and treated on the second day with 100 nmol/L rapamycin for 24 hours (*A* and *B*) or 3 hours (*C*). The cells were then harvested for preparation of whole cell protein lysates and subsequent Western blot analysis.

tested cell lines exposed to rapamycin exhibited increased p-Akt at Ser^{473}, indicative of an activated Akt in these cells (Fig. 2*B*). In support of this notion, in response to rapamycin treatment, GSK3β, a well-established physiologic substrate of Akt, is also phosphorylated in these cells at Ser^{9}, a defined Akt phosphorylation site. These data suggest that rapamycin-induced mTOR inhibition results in the activation of the Akt survival pathway. In addition to Akt, the treatment of NSCLC cells with rapamycin also induced the phosphorylation of eIF4E (p-eIF4E), a molecule downstream of mTOR that is involved in promoting cell survival (8, 14). Phosphorylation of eIF4E may counteract the mTOR inhibition effect, leading to a decreased growth-inhibitory effect of rapamycin. Together, these results suggest that the inhibition of mTOR by rapamycin triggers a negative feedback mechanism by activating two survival pathways involving Akt and eIF4E. Such a mechanism may attenuate the rapamycin effects. This effect is not restricted to lung cancer cells. When a similar experiment was conducted in other cancer cell lines, we found that rapamycin also increased the levels of p-Akt and p-eIF4E whereas decreasing the levels of p-p70S6K (Fig. 2*C*), indicating that the increased phosphorylation of Akt and eIF4E by an mTOR inhibitor occurs commonly in cancer cells.

To correlate the dynamic changes in p-Akt and p-eIF4E with the inhibition of mTOR in response to rapamycin, we did a detailed time course analysis to examine rapamycin's effects on the alterations of p-p70S6K, p-4E-BP1, p-Akt, and p-eIF4E in two representative NSCLC cell lines. In both H157 and A549 cell lines, p-p70S6K and p-4E-BP1 levels decreased 3 hours after exposure to rapamycin; this decrease was sustained up to 24 hours. Concurrently, p-Akt and p-eIF4E levels increased soon after a 3-hour exposure to rapamycin; this increase was still evident up to 24 hours after treatment (Fig. 3*A*). We also examined the effects of rapamycin on the expression levels of p70S6K, Akt, and eIF4E and found that rapamycin did not markedly alter their expression (Fig. 3*A*). To get more information on the dynamic changes of p-p70S6K, p-Akt, and p-eIF4E in response to rapamycin, we further shortened the exposure time to rapamycin. As shown in Fig. 3*B*, suppression of p-p70S6K and increase of p-Akt were detected 15 minutes after the cells were exposed to rapamycin. The increase of p-eIF4E was also detected 30 minutes (A549) and 60 minutes (H157) after rapamycin treatment. Collectively, it seems that the decrease of p-p70S6K and the increase of p-Akt are rapid and concurrent events in cells treated with rapamycin.

To further explore the relationship between the suppression of mTOR signaling and the increased phosphorylation of Akt and eIF4E, we examined p-p70S6K, p-Akt, and p-eIF4E in cells exposed to different concentrations of rapamycin ranging from 0.01 to 10 nmol/L. Rapamycin at 0.01 and 0.1 nmol/L failed to alter the phosphorylation levels of either protein. However, in cells treated with 1 or 10 nmol/L rapamycin, p-p70S6K levels decreased, accompanied with increases of both p-Akt and p-eIF4E levels (Fig. 3*C*). These findings indicate a close relationship between the suppression of mTOR signaling and the activation of Akt and eIF4E.

Phosphatidylinositol 3-kinase is required for rapamycin-induced phosphorylation of Akt and eIF4E. To understand the mechanism by which rapamycin induces Akt activation, we investigated the involvement of PI3K, an upstream regulator of Akt, in this process. PI3K catalyzes the production of the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the cell membrane. PIP3 in turn recruits other pleckstrin homology domain–containing proteins, in particular Akt, to the membrane, where Akt is activated by PDK1 and a Ser^{473} kinase (4). If PI3K is involved in Akt activation induced by mTOR inhibition, the PI3K inhibitor LY294002 would block or suppress the Akt phosphorylation or activation by rapamycin. Therefore, we examined the effects of LY294002 on rapamycin-induced Akt activation and eIF4E phosphorylation. In the absence of LY294002, rapamycin at both 1 and 10 nmol/L increased p-Akt and p-GSK3β levels. In the presence of LY294002, rapamycin failed to increase Akt and GSK3β phosphorylation (Fig. 4*A*). These results suggest that rapamycin-induced Akt activation requires activated PI3K. Similarly, LY294002 also blocked the rapamycin-induced increase of eIF4E phosphorylation (Fig. 4*A*), suggesting that the rapamycin-induced increase of p-eIF4E is dependent on PI3K activation as well.

Both mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase (ERK) and p38 MAPK are known to regulate eIF4E phosphorylation through Mnk1 (15, 16). To determine whether MEK/ERK or the p38 MAPK pathway was involved in the rapamycin-mediated increase of p-eIF4E, we analyzed the p-eIF4E levels in cells treated with rapamycin in the presence of various inhibitors. These inhibitors include the MEK inhibitors U0126 and PD98059, the p38 MAPK inhibitor SB203580, and the PI3K inhibitor LY294002. As shown in Fig. 4*B*, the presence of U0129, PD98059, or SB203580 neither affected the rapamycin-induced increase of p-eIF4E and p-Akt, nor the decrease of p-p70S6K by rapamycin. Rapamycin did not increase p-p44/p42 levels at the time point tested, whereas both U0126 and PD98059 decreased basal levels of p-p44/p42, indicating that they indeed function to block MEK/ERK pathway. p38 MAPK levels were undetectable under the conditions tested (data not shown). These results suggest that either the MEK/ERK or the p38 MAPK pathway is unlikely involved in mediating the rapamycin-induced increase of p-eIF4E. As we have already shown, LY294002 did not block the rapamycin-mediated decrease of p-p70S6K but abrogated rapamycin-induced increases of both p-Akt and p-eIF4E. Thus, it seems that the rapamycin-induced increase of p-eIF4E is PI3K dependent but independent of the MEK/ERK and p38 MAPK pathway.

Combination of rapamycin with LY294002 exhibits enhanced inhibitory effects on the growth and colony formation of non–small cell lung cancer cells. It is well documented that Akt is a major survival kinase (4). Recently, eIF4E has also been shown to be a tumor survival factor (8, 14). Our data clearly show that suppression of mTOR by rapamycin activates Akt and elF4E survival pathways. Thus, we speculated that PI3K/Akt and eIF4E activation would counteract mTOR inhibitors' anticancer effects. Because rapamycin-induced Akt activation requires its upstream regulator, PI3K, (Fig. 4*A* and *B*), blocking the PI3K/Akt survival pathway by LY294002 would be expected to enhance the rapamycin effect. Thus, we examined the effects of rapamycin combined with LY294002 on the growth of human NSCLC cells. As shown in Fig. 4*C*, the combination of rapamycin and LY294002 in a 3-day growth inhibition assay apparently exhibit growth-inhibitory effects that are greater than those caused by each single agent alone. For example, in H157 cells, rapamycin at 1 nmol/L inhibited cell growth by 30%, whereas LY294002 at 0.5, 1.0, 2.5, and 5.0 μmol/L caused 4.6%, 3.2%, 11%, and 17.9% growth inhibition, respectively. However, their combinations led to growth inhibition by 46.5%,

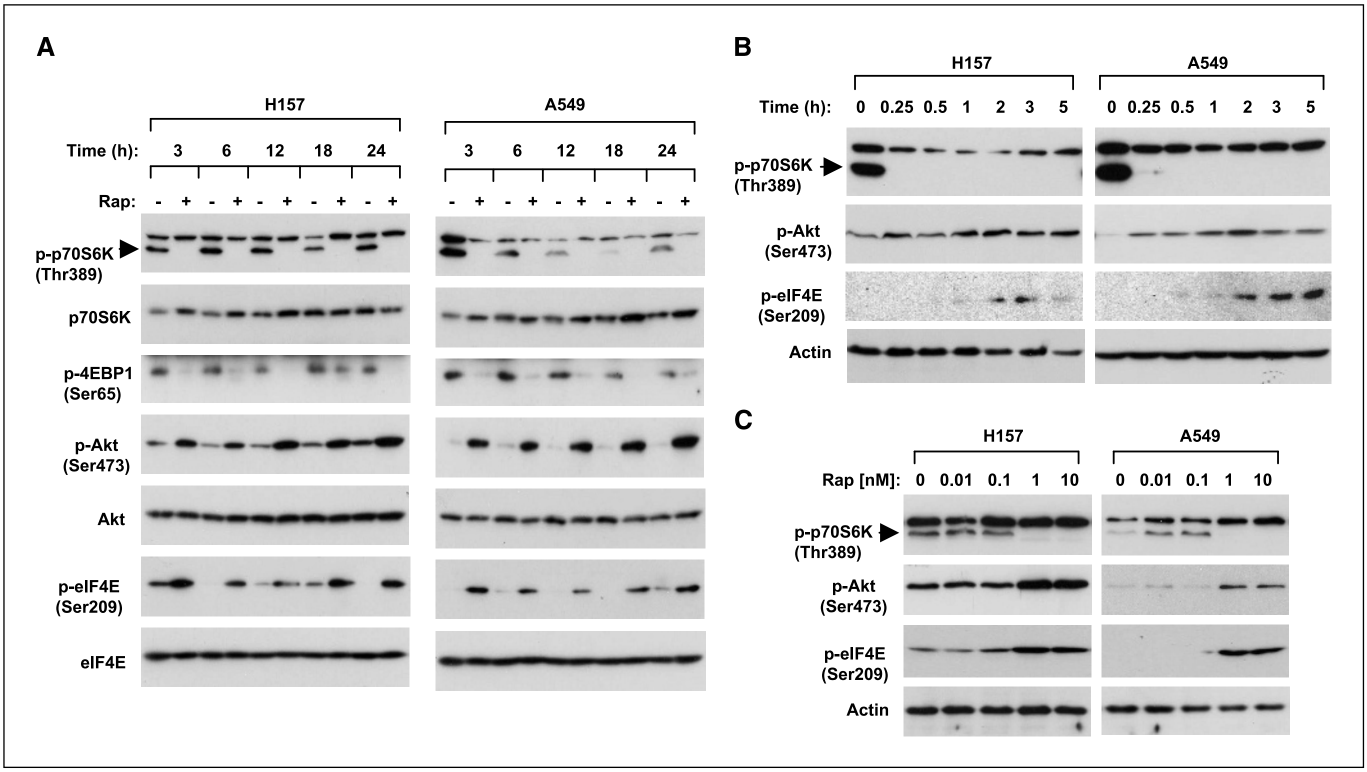

Figure 3. Increase of Akt and eIF4E phosphorylation by rapamycin is associated with suppression of the mTOR signaling in human NSCLC cells. The indicated cells lines were treated with 100 nmol/L rapamycin (*Rap*) for the given times (*A* and *B*) or with the indicated concentrations of rapamycin for 24 hours (*C*) and then subjected to preparation of whole cell protein lysates. The indicated proteins were detected by Western blot analysis.

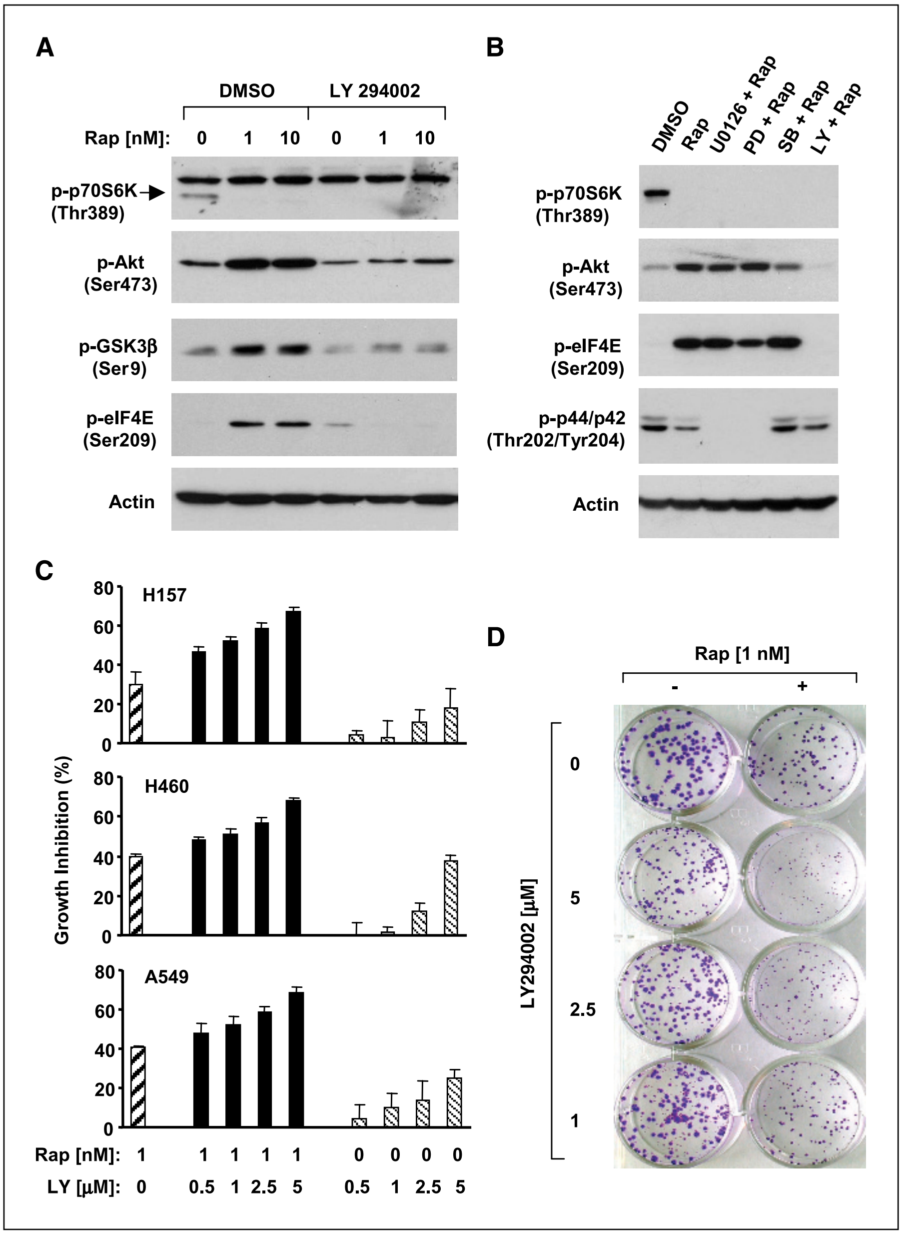

Figure 4. Involvement of PI3K in rapamycin-mediated increase of Akt and eIF4E phosphorylation (*A* and *B*) and enhancement of rapamycin-mediated growth inhibitor effects by LY294002 in NSCLC cells (*C* and *D*). *A,* H157 cells were pretreated with 5 μmol/L LY294002 for 30 minutes and then cotreated with the indicated concentrations of rapamycin (*Rap*) for 3 hours. The cells were subjected to preparation of whole cell protein lysates for detection of the indicated proteins using Western blotting. *B,* H157 cells were pretreated with 20 μmol/L U0126, PD98059 (*PD*), and SB203580 (*SB*), respectively, and 10 μmol/L LY294002 (*LY*) for 30 minutes, and then cotreated with 10 nmol/L rapamycin. After 3 hours, the cells were subjected to preparation of whole cell protein lysates for detection of the indicated proteins using Western blot analysis. *C,* the individual cell lines, as indicated, were seeded in 96-well plates. On the second day, they were treated with the indicated concentrations of LY294002 alone, 1 nmol/L rapamycin alone, and their respective combinations. After 3 days, plates were subjected to determination of cell number using a SRB assay. *Columns,* means of four replicate determinations; *bars,* ±SD. *D,* H460 cells at a density of ∼250 cells per well were seeded in 12-well plates. On the second day, cells were treated with the indicated concentrations of LY294002 alone, 1 nmol/L rapamycin alone, and their respective combinations. The same treatments were repeated every 3 days. After 10 days, the plates were stained for the formation of cell colonies with SRB dye. The picture of the colonies was then taken using a digital camera.

52.2%, 58.9%, and 67.3%, respectively. These effects are apparently greater than the sum of the inhibitory effects caused by each agent alone, indicating a more than additive or synergistic effect. In the long-term colony formation assay, we obtained similar results as determined from the 3-day assay. LY294002 and rapamycin alone did not decrease the number of colonies, although they reduced the sizes of the colonies. The combination of the two agents not only decreased the size of colonies but also reduced the number of colonies (Fig. 4*D*), indicating that the combination causes a greater growth-inhibitory effect than that of each single agent. Taken together, these results indicate that the combination of rapamycin and LY294002 results in an augmented growth-inhibitory effect.

Discussion

Using several human NSCLC cell lines, we have shown that rapamycin effectively inhibits cell growth to various degrees. However, rapamycin failed to completely inhibit cell growth even when its concentration was increased up to 10 μmol/L, suggesting that some cells remain resistant to mTOR inhibitors. Cell sensitivity to mTOR inhibitors has been linked to PTEN mutations or Akt activation (17–20). PTEN is a PIP3 phosphatase that negatively regulates the PI3K/Akt pathway. A recent animal study has shown that mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia (9). It has been well documented that human NSCLCs have rare or low frequencies of PTEN mutations (21, 22). Among tested NSCLC cell lines, H157 is the only cell line with PTEN mutation (21). Compared with most NSCLC cell lines, which have very low or undetectable basal levels of p-Akt, H157 cells constitutively express high levels of p-Akt, as we have shown previously (23). However, H157 cells were not more sensitive than other cell lines, such as A549 and H460, to rapamycin. Therefore, the effect of PTEN mutations or Akt activation on rapamycin's effects in NSCLC cells is not clearly apparent in NSCLC cells.

Rapamycin at concentrations (≥1 nmol/L) that exhibited growth inhibition rapidly and effectively suppressed the phosphorylation of p70S6K and 4E-BP1, indicating that rapamycin indeed blocks mTOR signaling. This blockage may well be the molecular basis for rapamycin to inhibit the growth of cancer cells. Unexpectedly, rapamycin rapidly induced the activation of Akt as shown by phosphorylation at Ser^{473} and the phosphorylation of its substrate, GSK3β, whereas suppressing the phosphorylation of p70SK and 4E-BP1. It seems that mTOR inhibition by rapamycin activates the Akt survival pathway. Even more surprisingly, cells treated with rapamycin within a concentration range that inhibits mTOR signaling increased eIF4E phosphorylation. To our knowledge, this is the first report to show that mTOR inhibition by rapamycin increases eIF4E phosphorylation.

Activation of Akt by mTOR inhibition was reported in *Drosophila* when studying the functional role of Rheb in regulation of p70S6K activity (24) and also in mammalian skeletal muscle cells, adipocytes, and fibroblasts when studying insulin signaling (25–27). Our study clearly shows that mTOR inhibition by rapamycin results in the activation of the Akt survival pathway in human NSCLC and other types of cancer cell lines. To the best of our knowledge, this is the first report to show mTOR inhibition–induced Akt activation in human cancer cells. Results from previous studies of insulin signaling suggest that the mTOR activation by insulin initiates a feedback inhibition of PI3K/Akt through p70S6K activation and its subsequent phosphorylation of insulin receptor substrate-1 (IRS-1). The phosphorylation of IRS-1 promotes IRS-1 degradation and reduces IRS-1 expression, leading to decreased activity of PI3K/Akt. Rapamycin suppresses p70S6K and thus relieves this negative feedback inhibition of Akt (28, 29). In our study, the presence of the PI3K inhibitor, LY294002, abrogated rapamycin-induced Akt activation (Akt and GSK3β phosphorylation), suggesting that PI3K activity is required for Akt activation by rapamycin. However, we currently do not know whether the Akt activation induced by rapamycin in human cancer cells is mediated by p70S6K suppression through the stabilization of IRS-1. It has been shown that there are two mTOR complexes in mammalian cells: the rapamycin-sensitive mTOR-raptor complex and the rapamycin-insensitive mTOR-rictor complex (30). A recent study has shown that the mTOR-rictor complex can directly phosphorylate Akt at Ser^{473} (31). Reduction of mTOR expression using small interfering RNA decreased Akt phosphorylation (31). Our data seem to favor the model that rapamycin induces Akt phosphorylation in a PI3K-dependent mechanism. However, it remains possible that rapamycin may indirectly stimulate the mTOR-rictor kinase activity to phosphorylate Akt. Elucidation of these mechanisms requires further investigation.

eIF4E plays a critical role in the regulation of cap-dependent-protein translation and thus its activity is integral in determining global translation rates (32). Consistent with this role, eIF4E is required for cell cycle progression, exhibits antiapoptotic or survival activity, and when overexpressed, transforms cells (8, 14), largely due to its critical role in initiating translation of mRNAs that encode cell cycle regulators or oncogenic proteins such as cyclin D1, ornithine decarboxylase, c-Myc, hypoxia-inducible factor 1α, fibroblast growth factor, and vascular endothelial growth factor (1, 8, 24). Therefore, it is not surprising that elevated levels of eIF4E are found in a broad spectrum of transformed cells and human cancers, including lung cancers, and is often associated with aggressive, poorly differentiated tumors (1, 8). eIF4E is phosphorylated (usually at Ser^{209}) in many systems in response to extracellular stimuli including growth factors, hormones, and mitogens (15, 16). Its phosphorylation increases its affinity for the cap and for mRNA and may also favor its entry into initiation complexes (15, 16, 33). Although mTOR inhibitors are expected to inhibit cap-dependent translation via activation of 4E-BP1 (i.e., promoting its dephosphorylation), we paradoxically found that cells treated with rapamycin exhibited increased eIF4E phosphorylation. Thus, it seems that rapamycin treatment generates conflicting signal to cap-dependent protein translation. Collectively, these findings suggest that rapamycin may promote cap-dependent protein translation, probably under certain conditions.

It has been documented that MEK/ERK and p38 MAPK signaling pathways activate eIF4E through Mnk1-mediated phosphorylation of eIF4E (15, 16). However, the MEK inhibitors U0126 and PD98059 or the p38 MAPK inhibitor SB203580 did not inhibit a rapamycin-induced elevation of p-eIF4E. Instead, LY294002 abolished the increase of p-eIF4E by rapamycin. Thus, it seems that the PI3K activity is required for mediating rapamycin-induced eIF4E phosphorylation. The investigation on the involvement of Mnk1 in rapamycin-induced eIF4E phosphorylation is ongoing.

Because Akt and eIF4E are often associated with cell survival and resistance to cancer therapy (4, 14), our findings imply that the activation of Akt and eIF4E through mTOR inhibition may counteract mTOR inhibitors' anticancer efficacy and confers resistance to mTOR-targeted cancer therapy. According to our results, Akt and eIF4E phosphorylation induced by rapamycin all occur downstream of PI3K. These findings may provide us the opportunity to interrupt or disrupt activation of the Akt and eIF4E survival pathways using a PI3K inhibitor or even an Akt inhibitor to enhance mTOR inhibitors' anticancer efficacy or mTOR-targeted cancer therapy. Our results indeed show that LY294002 in combination with rapamycin exhibits enhanced (synergistic) effects on the growth and colony formation of human NSCLC cells. Therefore, from a therapeutic point of view, our findings suggest a novel strategy to enhance mTOR-targeted cancer therapy through combining an mTOR inhibitor with an inhibitor of the PI3K/Akt pathway.

Acknowledgments

Received 3/18/2005; revised 5/16/2005; accepted 6/16/2005.

Grant support: Winship Cancer Institute faculty start-up research fund (S-Y. Sun), the Georgia Cancer Coalition Distinguished Cancer Scholar award (S-Y. Sun and F.R. Khuri), Department of Defense IMPACT grant W81XWH-05-0027 (Project 5 to F.R. Khuri and S-Y. Sun), and NIH R01 grant GM53165 (H. Fu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. Nat Rev Cancer 2004;4:335–48.
2. Sawyers CL. Will mTOR inhibitors make it as cancer drugs? Cancer Cell 2003;4:343–8.
3. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926–45.
4. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2:489–501.
5. Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 2004;22:2954–63.
6. Bjornsti MA, Houghton PJ. Lost in translation: dysregulation of cap-dependent translation and cancer. Cancer Cell 2004;5:519–23.
7. Inoki K, Corradetti MN, Guan KL. Dysregulation of the

TSC-mTOR pathway in human disease. Nat Genet 2005;37:19–24.

8. De Benedetti A, Graff JR. eIF-4E expression and its role in malignancies and metastases. Oncogene 2004;23: 3189–99.

9. Majumder PK, Febbo PG, Bikoff R, et al. mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nat Med 2004;10:594–601.

10. Rowinsky EK. Targeting the molecular target of rapamycin (mTOR). Curr Opin Oncol 2004;16:564–75.

11. Huang S, Bjornsti MA, Houghton PJ. Rapamycins: mechanism of action and cellular resistance. Cancer Biol Ther 2003;2:222–32.

12. Sun SY, Yue P, Dawson MI, et al. Differential effects of synthetic nuclear retinoid receptor-selective retinoids on the growth of human non-small cell lung carcinoma cells. Cancer Res 1997;57:4931–9.

13. Sun SY, Yue P, Wu GS, et al. Mechanisms of apoptosis induced by the synthetic retinoid CD437 in human non-small cell lung carcinoma cells. Oncogene 1999;18: 2357–65.

14. Wendel HG, De Stanchina E, Fridman JS, et al. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature 2004;428:332–7.

15. Raught B, Gingras AC. eIF4E activity is regulated at multiple levels. Int J Biochem Cell Biol 1999;31:43–57.

16. Pyronnet S. Phosphorylation of the cap-binding protein eIF4E by the MAPK-activated protein kinase Mnk1. Biochem Pharmacol 2000;60:1237–43.

17. Shi Y, Gera J, Hu L, et al. Enhanced sensitivity of multiple myeloma cells containing PTEN mutations to CCI-779. Cancer Res 2002;62:5027–34.

18. Neshat MS, Mellinghoff IK, Tran C, et al. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc Natl Acad Sci U S A 2001;98:10314–9.

19. Gera JF, Mellinghoff IK, Shi Y, et al. AKT activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-*myc* expression. J Biol Chem 2004;279:2737–46.

20. Noh WC, Mondesire WH, Peng J, et al. Determinants of rapamycin sensitivity in breast cancer cells. Clin Cancer Res 2004;10:1013–23.

21. Forgacs E, Biesterveld EJ, Sekido Y, et al. Mutation analysis of the PTEN/MMAC1 gene in lung cancer. Oncogene 1998;17:1557–65.

22. Yokomizo A, Tindall DJ, Drabkin H, et al. PTEN/MMAC1 mutations identified in small cell, but not in non-small cell lung cancers. Oncogene 1998;17:475–9.

23. Sun SY, Zhou Z, Wang R, Fu H, Khuri FR. The farnesyltransferase inhibitor Lonafarnib induces growth arrest or apoptosis of human lung cancer cells without downregulation of Akt. Cancer Biol Ther 2004; 3:1092–8.

24. Stocker H, Radimerski T, Schindelholz B, et al. Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*. Nat Cell Biol 2003;5:559–65.

25. Tremblay F, Marette A. Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem 2001;276: 38052–60.

26. Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology 2005;146:1328–37.

27. Harrington LS, Findlay GM, Gray A, et al. The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol 2004;166:213–23.

28. Manning BD. Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. J Cell Biol 2004;167:399–403.

29. Harrington LS, Findlay GM, Lamb RF. Restraining PI3K: mTOR signalling goes back to the membrane. Trends Biochem Sci 2005;30:35–42.

30. Sarbassov DD, Ali SM, Kim DH, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 2004;14:1296–302.

31. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307:1098–101.

32. Richter JD, Sonenberg N. Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature 2005;433: 477–80.

33. Lachance PE, Miron M, Raught B, Sonenberg N, Lasko P. Phosphorylation of eukaryotic translation initiation factor 4E is critical for growth. Mol Cell Biol 2002;22:1656–63.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Activation of Akt and eIF4E Survival Pathways by Rapamycin-Mediated Mammalian Target of Rapamycin Inhibition

Shi-Yong Sun, Laura M. Rosenberg, Xuerong Wang, et al.

Cancer Res 2005;65:7052-7058.