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RAPAMYCIN REGULATES THE PHOSPHORYLATION OF RICTOR

Argun Akcakanat^a, Gopal Singh^a, Mien-Chie Hung^{a,b}, and Funda Meric-Bernstam^a

^a Department of Surgical Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

^b Department of Molecular Cellular Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

Abstract

The mammalian target of rapamycin (mTOR) is a central regulator of cell growth. mTOR exists in two functional complexes, mTORC1 and mTORC2. mTORC1 is rapamycin-sensitive, and results in phosphorylation of 4E-BP1 and S6K1. mTORC2 is proposed to regulate Akt Ser473 phosphorylation and be rapamycin-insensitive. mTORC2 consists of mTOR, mLST8, sin1, Protor/PRR5, and the rapamycin insensitive companion of mTOR (rictor). Here, we show that rapamycin regulates the phosphorylation of rictor. Rapamycin-mediated rictor dephosphorylation is time and concentration dependent, and occurs at physiologically relevant rapamycin concentrations. siRNA knockdown of mTOR also leads to rictor dephosphorylation, suggesting that rictor phosphorylation is mediated by mTOR or one of its downstream targets. Rictor phosphorylation induced by serum, insulin and insulin-like growth factor is blocked by rapamycin. Rictor dephosphorylation is not associated with dephosphorylation of Akt Ser473. Further work is needed to better characterize the mechanism of rictor regulation and its role in rapamycin-mediated growth inhibition.

Keywords

Rictor; Mammalian target of rapamycin; mTOR complex 2; Rapamycin

Introduction

The mammalian target of rapamycin (mTOR) is a key regulator of cell proliferation and growth. Recently mTOR was found to exist in two distinct functional complexes, mTORC1 and mTORC2 [1,2]. mTORC1 consists of mTOR, mLST8 and raptor. Activation of mTORC1 results in phosphorylation of its effectors S6K1 and 4E-BP1, and this pathway is rapamycin-sensitive. In contrast, mTORC2 is thought to be rapamycin-insensitive [1,2]. mTORC2 regulates Akt Ser473 kinase phosphorylation and consists of mTOR, mLST8, sin1, Protor/PRR5, and the rapamycin-insensitive companion of mTOR (rictor) [1–6].

Rapamycin has variable effects on Akt activation in different models and cell lines. Rapamycin increases Akt activation in some cancer cell lines, and this has been attributed to the loss of mTOR/S6K-dependent feedback inhibition of signaling, with loss of phosphorylation and degradation of insulin response substrate-1 [7,8]. In animal models, rapamycin was found to

Corresponding author: Funda Meric-Bernstam, MD, Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Unit 444, Houston, TX 77030, USA; Phone: (713) 745 4453. Fax: (713) 745-4926; email: fmeric@mdanderson.org

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increase phosphorylated Akt (pAkt) in tumor lysates at doses that inhibit Akt signaling in nonmalignant tissues, including endothelial cells [9,10]. An increase in pAkt levels was also noted in tumors from patients treated with the rapamycin analogue everolimus (RAD001, Novartis, Basel, Switzerland) [7]. In contrast, in some cancer cell lines prolonged (24 hour) rapamycin treatment inhibits the assembly of mTORC2 and Akt Ser473 phosphorylation [11]. Human leukemia cells treated with temsirolimus have dissociation of mTORC2 as well as mTORC1 [12]. Furthermore, samples from patients with hematologic malignancies treated on Phase I/II trials with rapamycin analogues temsirolimus or everolimus demonstrated a decrease in pAkt Ser473 levels. Thus it appears that in some cells or under certain conditions rapamycin may affect mTORC2 assembly through a yet unknown mechanism.

Recently rapamycin analogue temsirolimus (Wyeth, PA) was approved by the Food and Drug Administration for renal cell carcinoma, and rapamycin and its analogues are currently in clinical trials for the treatment of many other tumor types. Better understanding the molecular consequences of mTOR inhibition is critical for rapamycin and its analogues to be most effectively used in the clinic.

Here we report that rictor, previously thought to be rapamycin-insensitive, is in fact regulated at the phosphorylation level by rapamycin. Knockdown of mTOR with siRNA also leads to a decrease in rictor phosphorylation, suggesting that phosphorylation of rictor may be mediated by mTOR or by one of its downstream targets. Rapamycin-mediated dephosphorylation of rictor is observed in multiple cell lines representing a spectrum of aberrations in Akt/mTOR signaling. Thus, the results of our study suggest that rapamycin regulates components of mTORC2 as well as mTORC1.

Materials and methods

Cell lines, cultures and reagents

MDA-MB-468, BT-549, BT-474 and MCF7, cell lines were obtained from the American Type Tissue Culture Collection (Manassas, VA). All cell lines were cultured in DMEM/F12 supplemented with 10% fetal bovine serum at 37°C and humidified in 5% CO₂.

Dimethyl sulphoxide (DMSO), insulin like growth factor-I and insulin were purchased from Sigma Chemical Company (St. Louis, MO).

Western blot analysis

Cultured cells were washed with cold PBS and lysed in lysis buffer as described elsewhere [13]. 25–50 µg of lysate was separated by SDS-PAGE on a 4% or 7.5% gel, and transferred to 0.2 µm polyvidine difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 0.1% casein or 5% bovine serum albumin in tris-buffered saline and probed with antibodies to rictor (1:2000) (#A300–459A, Bethyl Labs, Montgomery, TX) or (1:1000) (#2140), mTOR (1:250) (#2972), raptor (1:125) (#4978), Akt (1:1000) (#9272), phospho-Akt (Ser473) (1:1000) (#9271) (all from Cell Signaling Technology, Beverly, MA) and β-actin (1:10000) (Sigma Chemical Co.). The immunoblots were visualized by Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Immunoprecipitation

MDA-MB-468 cells (1×10^7) were lysed with 1 ml lysis buffer and immunoprecipitated following protein A agarose kit manufacturer's instructions (KPL, Inc., Gaithersburg, MD). One microgram of rictor antibody was added to protein extract.

Calf intestinal phosphatase (CIP) dephosphorylation

Cultured cells were lysed or immunoprecipitated. Samples were suspended in CIP buffer and incubated 60 minutes at 37°C with the enzyme (New England Biolabs, Ipswich, MA).

Small interfering RNA (siRNA)

To silence mTOR, a single transfection of either a single mTOR siRNA sequence or a siRNA pool duplex was performed using DharmaFECT 1 reagent according to manufacturer's protocol (Dharmacon, Inc., Lafayette, CO). The single mTOR siRNA target sequence used was CCCUGCCUUUGUCAUGCCU [2]. The pool siRNA target sequences were GGCCAUAGCUAGCCUCAUAUU, CAAAGGACUUCGCCCAUAAUU, GCAGAAUUGUCAAGGGAUAUU, CCAAAGCACUACACUACAAUU. The single and pool sequence mTOR and pool negative control siRNAs were purchased from Dharmacon, Inc. The single sequence negative control is purchased from Ambion, Inc (Austin, TX).

Results and discussion

In order to determine the effect of rapamycin on mTORC2 signaling, we initially studied the effect of rapamycin on MDA-MB-468 breast cancer cells, which are rapamycin-sensitive in vitro and in vivo [13,15,16]. On SDS-PAGE analysis and western blotting for rictor, we noted a significant and very reproducible increase in rictor electrophoretic mobility upon rapamycin treatment, suggestive of rictor dephosphorylation. Treatment of cell lysates with calf intestinal phosphatase (CIP) returned the mobility of rictor in the vehicle-treated cells to that of rapamycin-treated cells, suggesting that the mobility shift seen is indeed attributable to rictor phosphorylation (Fig. 1A). Similarly, when rictor was immunoprecipitated with anti-rictor antibodies, a mobility difference was observed between rictor protein immunoprecipitated from vehicle- and rapamycin-treated cells, and phosphatase treatment again equalized rictor mobility, confirming that the mobility shift seen is due to rictor dephosphorylation (Fig. 1B).

We hypothesized that rictor phosphorylation is stimulated through the mTOR signaling pathway. To test this hypothesis we exposed MCF7 cells to overnight serum starvation followed by stimulation with one of three mitogenic stimuli known to activate mTOR signaling: serum, insulin and insulin-like growth factor. Pretreatment of cells with rapamycin blocked rictor phosphorylation in the presence of all three mitogens (Fig. 1C). Knockdown of mTOR by either a pool siRNA duplex for mTOR, or a single mTOR siRNA sequence, also led to a decline in rictor phosphorylation, suggesting that rictor phosphorylation is mediated by mTOR or one of its downstream targets (Fig. 1D and Fig. 1E).

Rapamycin-induced rictor dephosphorylation in a time and dose-dependent fashion. Dephosphorylation of rictor in MDA-MB-468 cells was notable by 2 hours, and was significant by 4 hours and persisted for at least 24 hours (Fig. 2A). Rictor dephosphorylation occurred in physiologically relevant doses (1–100nM) (Fig. 2B).

As it has been proposed that mTORC2 regulates Akt phosphorylation, we next evaluated the effect of rapamycin on the phosphorylation of rictor as well as on pAkt Ser473 in additional cell lines known to have activation of mTOR signaling [15]. Rapamycin-mediated rictor dephosphorylation was observed in these cell lines as well (Fig. 2C). After 24 hours rapamycin treatment, no significant alteration in pAkt Ser473 levels were observed in MDA-MB-468 cells, while the pAkt Ser473 levels increased in the other three cell lines (MCF7, BT549 and BT474), although all four cell lines are sensitive to rapamycin's growth inhibitory effect [15]. Our finding that rictor undergoes dephosphorylation in the presence of persistent or even increased pAkt Ser473 suggests that either rictor phosphorylation is not critical for mTORC2-

mediated pAkt Ser473 phosphorylation, or that feedback loop activation of Akt upon rapamycin treatment may utilize another Akt kinase.

To date rictor has been thought to be the “rapamycin-insensitive companion of mTOR”. Due to the large size of rictor, the rapamycin-induced increase in rictor electrophoretic mobility is only apparent on low percentage gels (5% or less); this may have prevented recognition of its dephosphorylation in previous studies. Further, most studies on rictor have focused on the components of mTORC2 complex, and data from Yang et al suggests that the mTORC2 complex would primarily consist of phosphorylated rictor [4]. It should be mentioned that no increase in rictor mobility with rapamycin treatment was observed in an earlier report [1]. The discrepancy in findings may be attributable to cellular context, study time course, or other differences in experimental design.

Two other studies to date had suggested that rictor phosphorylation may be under regulation [1,4]. In their first report on rictor, Sarbassov et al. noted that in kinase assays with mTOR, a protein of the same apparent molecular weight as rictor gets phosphorylated. Further, in cells metabolically labeled with radioactive phosphate, a reduction in mTOR expression was associated with a decrease in the amount of radioactivity in rictor without decreasing its expression. In cells with reduced mTOR, rictor appeared as a doublet on SDS-PAGE analysis. These data suggested rictor may be a substrate of mTOR. However, they found that treatment of cells with PI3K and mTOR inhibitor LY294002, but not rapamycin, increased rictor mobility. Recently, Yang et al reported that the knockdown of sin1, an essential mTORC2 component required for complex formation and kinase activity, caused an increase in rictor electrophoretic mobility, suggesting that sin1 may modulate rictor phosphorylation [4]. Further they demonstrated that both hypo and hyperphosphorylated rictor was immunoprecipitated by rictor antibody while mTOR preferentially co-precipitated with hyperphosphorylated rictor. These data suggest that dephosphorylated rictor may have a weaker affinity to mTOR or alternately rictor needs to be in a complex with mTOR to be phosphorylated. Further study is needed to determine if modulation of rictor phosphorylation regulates the formation of mTORC2.

In summary, we report that rictor, previously thought to be rapamycin-insensitive, is in fact regulated at the phosphorylation level by rapamycin. Rictor phosphorylation is enhanced by known activators of mTOR signaling and decreased by rapamycin as well as mTOR siRNA. Taken together these data suggest that rictor phosphorylation is indeed regulated by mTOR or one of its downstream targets. Further, our results demonstrate that rapamycin may regulate rictor as well as mTORC1. Further study is needed determine the effect of rictor dephosphorylation on mTORC2 signaling and the role of rictor dephosphorylation in rapamycin-mediated growth inhibition.

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The abbreviations used are

mTOR	mammalian target of rapamycin
IGF-1	insulin-like growth factor-I
pAkt	phosphorylated Akt
mTORC 1 and 2	

mammalian target of rapamycin complex 1 and 2

Ser473

serine 473

rictor

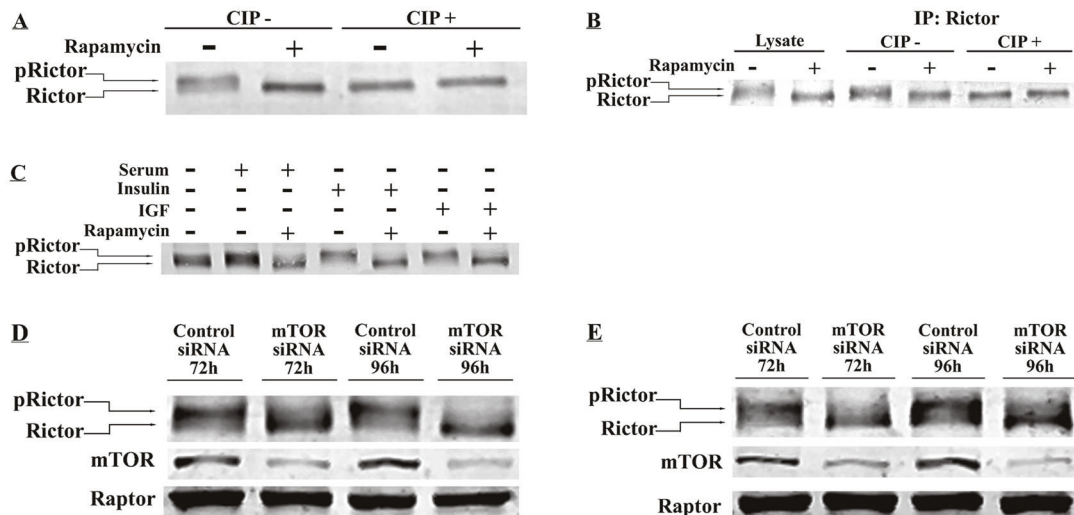
rapamycin-insensitive companion of mTOR

S6K1

ribosomal S6 kinase 1

PI3K

phosphoinositide-3 kinase

**Fig 1.**

Dephosphorylation of rictor with rapamycin treatment. (A) MDA-MB-468 cells were treated with vehicle (DMSO) or 100 nM rapamycin for 24 h. Cell lysate was incubated with or without CIP. 4% SDS-PAGE and western blotting with anti-rictor antibody demonstrates a migration shift with dephosphorylation. (B) Following immunoprecipitation (IP), IP product was incubated with or without CIP, and probed with anti-rictor antibody. Cell lysate was used as a control. (C) MCF7 cells were grown for 24 h in serum-free media. Cells were untreated or pre-treated with 100 nM rapamycin for 30 minutes and were cultured for another 24 h under the following conditions: serum-free medium, medium containing 10% serum, 10 μ g/ml insulin, or 100 ng/ml IGF-1. (D) MDA-MB-468 cells were transfected with a pool duplex siRNA sequence for mTOR or with a pool negative control siRNA. Cell lysates were prepared at 72 and 96 hours after transfection and western blotting was performed for rictor, mTOR and raptor. (E) MDA-MB-468 cells were transfected with single sequence mTOR siRNA or negative control siRNA. Western blotting was performed for rictor, mTOR and raptor.

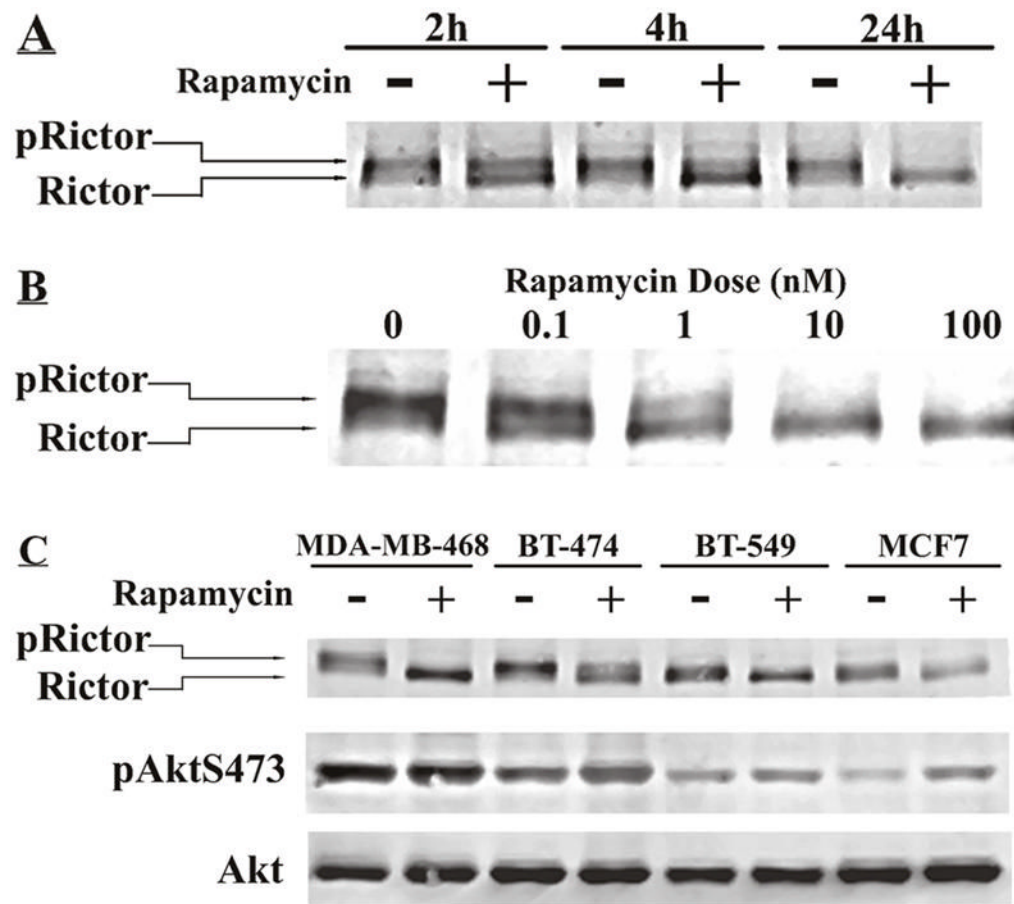


Fig 2. Effect of rapamycin concentration and duration of treatment on rictor dephosphorylation. (A) MDA-MB-468 cells were treated with DMSO or 100 nM rapamycin for 2, 4 or 24 h. Western blotting was performed for rictor. (B) Cells were treated with rapamycin at varying concentrations for 24 h. Western blotting was performed for rictor. (C) Four rapamycin sensitive breast cancer cell lines were treated with vehicle or 100 nM rapamycin for 24 h. Western blotting was performed for rictor, pAkt Ser473 and total Akt.