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Inhibition of p70S6K does not mimic the enhancement of Akt phosphorylation by rapamycin

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Abstract

It has been suggested that the mTOR complex 1 (mTORC1)/p70S6K axis represses upstream PI3K/Akt signaling through phosphorylation of IRS-1 and its subsequent degradation. One potential and current model that explains Akt activation induced by the mTOR inhibitor rapamycin is the relief of mTORC1/p70S6K-mediated feedback inhibition of IRS-1/PI3K/Akt signaling, although this has not been experimentally proven. In this study, we found that chemical inhibition of p70S6K did not increase Akt phosphorylation. Surprisingly, knockdown of p70S6K even substantially inhibited Akt phosphorylation. Hence, p70S6K inhibition clearly does not mimic the activation of Akt by rapamycin. Inhibition or enforced activation of p70S6K did not affect the ability of rapamycin to increase Akt phosphorylation. Moreover, inhibition of mTORC1 with either rapamycin or raptor knockdown did not elevate IRS-1 levels, despite potentially increasing Akt phosphorylation. Critically, knockdown or knockout of IRS-1 or IRS-2 failed to abolish the ability of rapamycin to increase Akt phosphorylation. Therefore, IRS-1 and IRS-2 are not essential for mediating rapamycin-induced Akt activation. Collectively, our findings suggest that Akt activation by rapamycin or mTORC1 inhibition is unlikely due to relief of p70S6K-mediated feedback inhibition of IRS-1/PI3K/Akt signaling.

Keywords: Biochemistry, Cancer research, Biological sciences

1. Introduction

The ribosomal protein, 70 kDa ribosomal S6 kinase (p70S6K), is one of the best characterized downstream effectors of mTOR complex 1 (mTORC1). In response to mitogenic stimuli or nutrient availability, mTORC1 is activated, leading to phosphorylation of p70S6K. The first identified substrate of p70S6K is the ribosomal protein S6, a component of the 40S ribosome. It is generally recognized that the mTORC1-p70S6K axis controls fundamental cellular processes, including transcription, translation, protein and lipid synthesis, cell growth/size, and cell metabolism. Hence, p70S6K plays important roles in a number of pathologies, including obesity, diabetes, ageing, and cancer [1, 2].

It is generally thought that mTORC1 functions downstream of the PI3K/Akt pathway and is phosphorylated (or activated) in response to stimuli that activate the PI3K/Akt pathway [3, 4, 5]. We and others have shown that the conventional mTOR inhibitor rapamycin and its analogues (rapalogs) activate Akt while suppressing mTORC1 signaling in different types of cancer cell lines and clinical human tumor samples [6, 7, 8]. Our previous study suggested that sustained Akt phosphorylation during rapamycin treatment seems associated with the development of rapamycin resistance [8]. Another study suggested that early increase in Akt phosphorylation predicts better response of cancer cells to rapalogs [9]. Moreover, prevention of Akt activation induced by mTOR inhibition exhibits enhanced cancer therapeutic efficacy in different preclinical studies [8, 10, 11, 12].

Insulin receptor substrate-1 (IRS-1) is an important mediator of insulin receptor-dependent activation of PI3K. Chronic insulin stimulation causes the phosphorylation and degradation of IRS-1 protein in a rapamycin-sensitive manner [13]. Thus, studies on insulin signaling in mammalian skeletal muscle cells, adipocytes, and fibroblasts have suggested that mTORC1 activation by insulin initiates the feedback inhibition of PI3K and Akt, likely through p70S6K activation and its subsequent phosphorylation of IRS-1. The phosphorylation of IRS-1 promotes IRS-1 degradation and reduces its abundance, leading to decreased activity of PI3K and Akt. Thus, it has been proposed that rapalogs suppress p70S6K, thereby relieving this negative feedback inhibition of Akt and leading to activation of the PI3K/Akt survival pathway [7, 13, 14]. However, our recent work has suggested that protein phosphatase 2A and DNA-dependent protein kinase are involved in mediating rapamycin-induced Akt phosphorylation [12]. Moreover, Src-family kinase (SFK)-dependent Akt activation also occurs during rapamycin treatment [11].

The importance of p70S6K function in human diseases has led to the development of p70S6K-specific inhibitors by a number of companies as potential therapeutic

agents [2, 15]. Considering the central role of p70S6K in feedback inhibition of PI3K/Akt signaling activation as discussed above, we were interested in addressing the question of whether inhibition of p70S6K (e.g., by a chemical inhibitor) exerts similar effect on Akt activation as rapalogs do. Interestingly, we found that both chemical and genetic inhibition of p70S6K fails to increase Akt phosphorylation, suggesting that inhibition of p70S6K does not necessarily result in the activation of PI3K/Akt signaling.

2. Results

2.1. Chemical inhibition of p70S6K does not increase Akt phosphorylation

FRI00705 is a specific inhibitor of p70S6K1 ($IC_{50} = 3$ nM) and p70S6K2 ($IC_{50} = 35$ nM) developed by Forest Research Institute (Jersey City, NJ). We first determined whether FRI00705 is able to increase Akt phosphorylation, as rapamycin does, by comparing the ability of FRI00705 and rapamycin to suppress p70S6K signaling and induce Akt phosphorylation. In A549 cells, rapamycin at both 1 nM and 10 nM clearly increased Akt phosphorylation while decreasing the levels of not only p-S6, but also p-ricor (T¹¹³⁵), another novel substrate of p70S6K [16, 17]. In contrast, FRI00705 at concentration ranges between 100 nM and 10 μ M effectively reduced the levels of p-S6 and p-ricor, but did not increase Akt phosphorylation (Fig. 1A and B). Similar results were also generated in other cancer cell lines including H157, Calu-1, H1299, Du145, and DLD1 (Fig. 1C). These results clearly show that inhibition of p70S6K with FRI00705 does not necessarily lead to increased Akt phosphorylation.

2.2. Genetic inhibition of p70S6K1 does not increase Akt phosphorylation or impair the ability of rapamycin to increase Akt phosphorylation

We then determined whether genetic inhibition of p70S6K1 using a small interfering RNA (siRNA) approach increases Akt phosphorylation and impacts rapamycin-induced Akt phosphorylation. p70S6K1 siRNA substantially reduced the level of p70S6 K, indicating a successful gene silencing effect (Fig. 2A). Moreover, the levels of p-S6 and p-Rictor were also reduced, indicating that p70S6K1 knockdown also inhibits downstream signaling. However, the level of basal p-Akt was decreased to a substantial degree in cells transfected with p70S6K1 siRNA (Fig. 2A and B). In addition, IRS-1 levels were not altered (Fig. 2B). Treatment with rapamycin increased the levels of p-Akt not only in control siRNA-transfected cells but also in p70S6K1 siRNA-transfected cells to comparable degrees. Rapamycin did not alter the level of IRS-1 (Fig. 2B). These results suggest that inhibition of p70S6K does not result in IRS-1 elevation or Akt

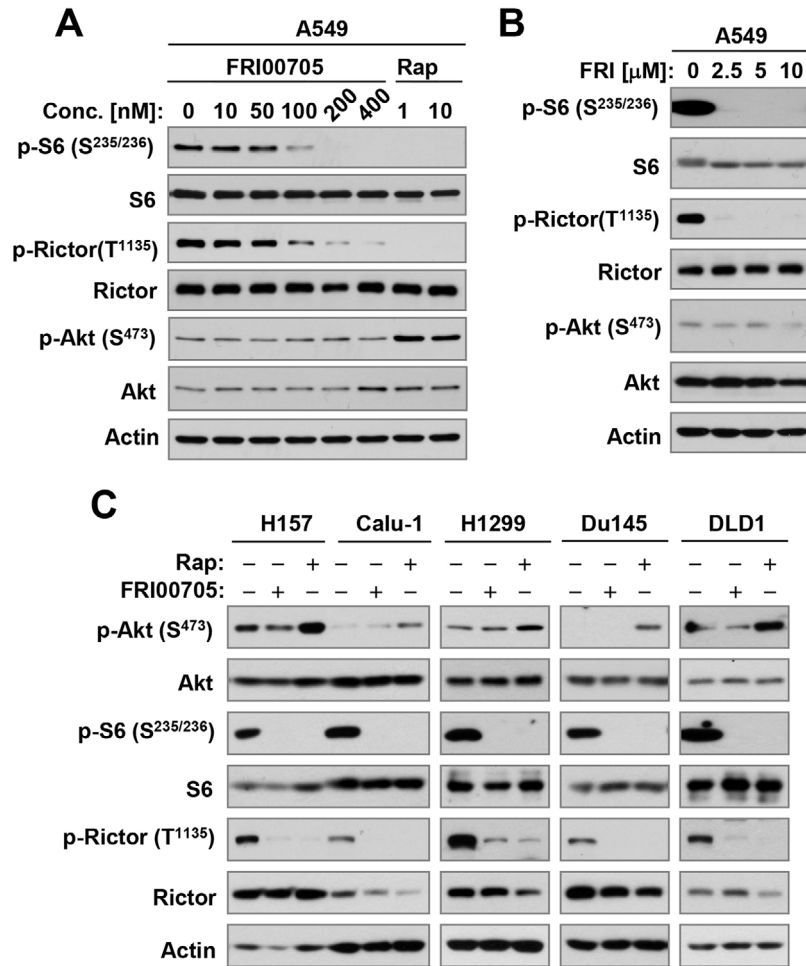


Fig. 1. Inhibition of p70S6 K with FRI00705 does not increase Akt phosphorylation in cancer cell lines. The indicated cells were exposed to the indicated concentrations of FRI00705 or rapamycin (Rap) for 8 h (A and B) or 1 μ M FRI00705 or 10 nM Rap for 8 h (C) and then harvested for preparation of whole-cell protein lysates and subsequent Western blotting. Full-length blots/gels are presented in Supplementary Figs. S1A, S1B, S1C-1, and S1C-2.

activation and that rapamycin increases Akt phosphorylation under the condition that p70S6K is depleted.

2.3. Enforced activation of p70S6K1 does not alter Akt phosphorylation or rapamycin-induced Akt phosphorylation

To further study the regulation of Akt by p70S6K, we transfected cells with constitutively active and rapamycin-resistant mutants of p70S6K1 (i.e., F5A-E389-R3A and E389- Δ CT) and then determined their impact on rapamycin-induced Akt phosphorylation. Overexpression of wild-type (WT) p70S6K1, and particularly F5A-E389-R3A and E389- Δ CT, abrogated the reduction of p-S6 by rapamycin;

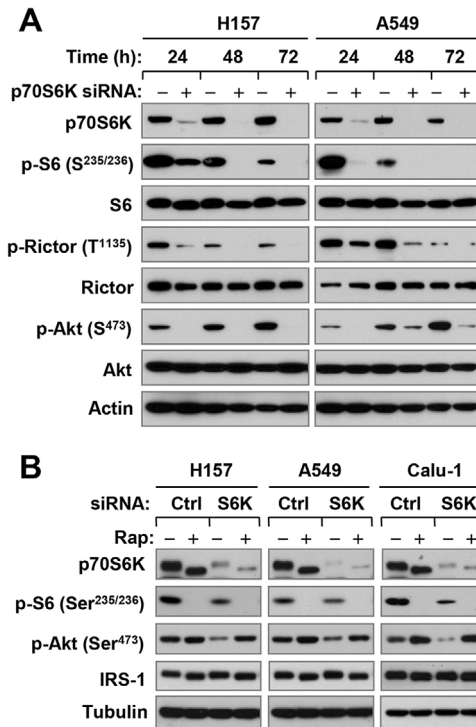


Fig. 2. Knockdown of p70S6 K suppresses Akt phosphorylation (A) and does not attenuate the ability of rapamycin to increase Akt phosphorylation (B). A, The indicated cell lines were transfected with control or p70S6 K siRNA for different times as indicated. B, The indicated cell lines were transfected with control (Ctrl) and p70S6 K (S6 K) siRNA, respectively. After 48 h, cells were treated with 10 nM rapamycin (Rap) for 1 h before harvesting for preparation of whole-cell protein lysates and subsequent Western blotting. Full-length blots/gels are presented in Supplementary Figs. S2A and S2B.

however, rapamycin was equally potent in increasing the level of p-Akt in these cells transfected with empty vector, WT p70S6K1, or mutant p70S6K1, regardless

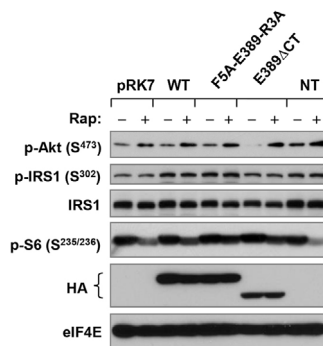


Fig. 3. Enforced activation of p70SK does not affect the ability of rapamycin to increase Akt phosphorylation. HEK293 cells were transfected with the given constructs. After 48 h, rapamycin (10 nM) was added to the cells for 1 h before the cells were harvested for preparation of whole-cell protein lysates. eIF4E was used as a loading control. NT, no transfection. Full-length blots/gels are presented in Supplementary Fig. S3.

of whether p70S6K signaling was inhibited or not (Fig. 3). In this experiment, we also did not see any effect of rapamycin on the level of IRS-1. Thus, these data also do not support a role for p70S6K in rapamycin-induced Akt phosphorylation.

2.4. Rapalogs or raptor knockdown increases Akt phosphorylation without altering IRS-1 levels

Following the above experiments, we determined whether IRS-1 protein levels are elevated while Akt phosphorylation is increased in cells exposed to rapalogs. We compared the effects of rapamycin and RAD001 on the modulation of p-Akt and IRS-1 in a group of lung cancer cell lines after either a short (i.e., 1 h) or long (24 h) treatment time. Among the 8 tested cell lines, IRS-1 was not detected in two cell lines (i.e., H460 and H226 cells). In the remaining cell lines in which IRS-1 was present, rapamycin or RAD001 treatment did not alter IRS-1 levels regardless of whether the cells were treated for 1 h or 24 h. However, the level of p-Akt was increased in all cell lines treated with rapamycin or RAD001 regardless of IRS-1 presence or modulation and exposure time (Fig. 4A and B). We also extended the study to other types of cancer cells and obtained similar results in HCT116 (colon cancer) and MCF-7 (breast cancer) cells. In Du145 (prostate cancer) cells, we

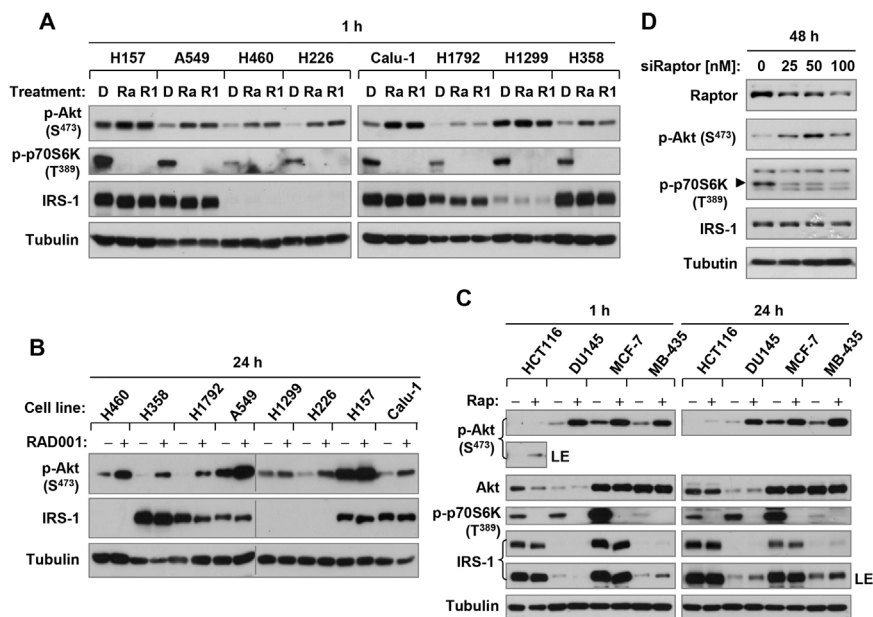


Fig. 4. Rapalogs (A-C) or raptor knockdown (D) increase Akt phosphorylation without elevating IRS-1 levels. A-C, The indicated human cancer cell lines were treated with DMSO (D), 10 nM rapamycin (Ra) or 10 nM RAD001 (R1) for 1 h (A), with 10 nM RAD001 for 24 h (B), or with 10 nM rapamycin for 1 h or 24 h (C). D, A549 cells were transfected with the indicated concentrations of raptor siRNA for 48 h. After these treatments, the cell lines were subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for the detection of proteins as presented. LE, long exposure. Full-length blots/gels are presented in Supplementary Figs. S4A-D.

detected increased p-Akt at both 1 h and 24 h, and observed a slight increase in IRS-1 only with 24 h of rapamycin treatment. In MDA-MB-345 (breast cancer) cells, we detected increased amounts of IRS-1 and p-Akt at both 1 h and 24 h (Fig. 4C). Moreover, we knocked down raptor in A549 cells and found that this did not increase the level of IRS-1, but effectively elevated p-Akt and reduced p-p70S6K (Fig. 4D). Taken together, these data clearly indicate that mTORC1 inhibition induces Akt phosphorylation without the apparent elevation of IRS-1 levels.

2.5. Knockdown or knockout of IRS-1, IRS-2, or both does not prevent rapamycin from increasing Akt phosphorylation

We examined the effects of rapamycin on Akt phosphorylation in cell lines in which IRS-1 was knocked down by IRS-1 siRNA or knocked out. Transfection of IRS-1 siRNA efficiently reduced the level of IRS-1 in three lung cancer cell lines (i.e., A549, H157 and Calu-1) as detected by Western blotting (Fig. 5A). Under such conditions, rapamycin treatment for 1 h did not alter the level of IRS-1, but

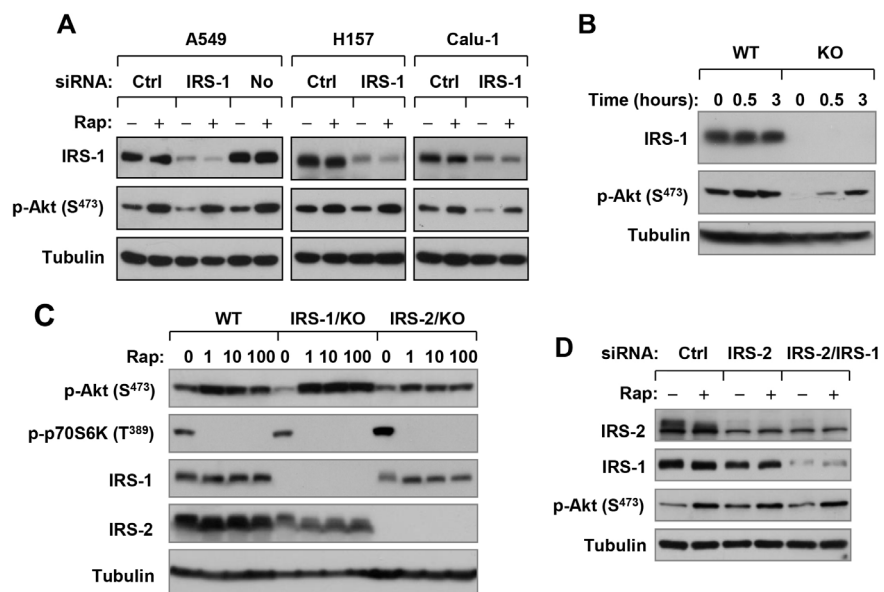


Fig. 5. Silencing (A and D) or deficiency (B and C) of IRS-1 and/or IRS-2 fails to abolish rapamycin-induced Akt phosphorylation. A, The indicated cell lines were transfected with control (Ctrl) or IRS-1 siRNA. After 48 h, the cell lines were then treated with 10 nM rapamycin (Rap) for 1 h. B, The IRS-1 WT and knockout (KO) murine 3T3 fibroblasts were treated with 10 nM rapamycin for the given times. C, The WT, IRS-1 knockout (IRS-1-KO) and IRS-2 knockout (IRS-2-KO) murine fibroblasts were treated with the given concentrations of rapamycin for 3 h. D, A549 cells were transfected with control (Ctrl), IRS-2 or IRS-1 plus IRS-2 siRNAs, and 48 h later were treated with 10 nM rapamycin for an additional 1 h. After these treatments, the cell lines were subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for the detection of proteins as presented. Full-length blots/gels are presented in Supplementary Figs. S5A-D.

increased p-Akt in both control siRNA- and IRS-1 siRNA-transfected cells to comparable degrees (Fig. 5A). We found that basal levels of p-Akt were lower in IRS-1/KO murine 3T3 cells than in IRS-1 WT cells. Treatment with rapamycin for 30 min or 3 h did not increase the levels of IRS-1 in IRS-1 WT cells, but increased p-Akt levels in both IRS-1 WT and knockout cell lines (Fig. 5B). We also examined the effects of rapamycin on Akt phosphorylation in WT, IRS-1/KO, and IRS-2/KO murine embryonic fibroblasts (MEFs) and found that rapamycin treatment increased the level of p-Akt in both IRS-1 and IRS-2 knockout cells (Fig. 5C). Furthermore, we knocked down IRS-2 or both IRS-1 and IRS-2 in human cancer cells and found that knockdown of IRS-2 or both IRS-1 and IRS-2 did not inhibit rapamycin-induced Akt phosphorylation (Fig. 5D). These results indicate that rapamycin is unlikely to increase Akt phosphorylation through IRS-1 and/or IRS-2.

3. Discussion

It has been suggested that mTORC1/p70S6K represses upstream PI3K signaling through phosphorylation of IRS-1 and its subsequent degradation [14, 18, 19]. Therefore, one potential and widely accepted model [15] that may explain rapamycin-induced Akt activation is the relief of an mTORC1/p70S6K-mediated feedback inhibition of IRS-1/PI3K [7, 19], although this has not been experimentally confirmed. If this model is correct, inhibition of p70S6K would mimic mTORC1 inhibition (e.g., by a rapalog) in activating Akt. However, we demonstrated that while both rapamycin treatment and chemical inhibition of p70S6K with FRI00705 effectively suppressed S6 and rictor phosphorylation, p-Akt levels were potently elevated by rapamycin but not by FRI00705 (Fig. 1). Surprisingly, knockdown of p70S6K1 substantially suppressed Akt phosphorylation (Fig. 2). Hence it is clear that inhibition of p70S6 K does not mimic the activation of Akt by rapamycin or mTORC1 inhibition.

Our previous study clearly showed that rapamycin increases p-Akt rapidly, within 1 h of treatment [6]. Moreover, Akt seemed to be more sensitive than p70S6K to mTORC1 inhibition, since the increase in Akt phosphorylation in response to mTORC1 inhibition occurred under conditions in which p-p70S6K levels were not reduced: e.g., transient knockdown of raptor as we demonstrated previously [8]. This notion is further supported by our finding that knockdown of p70S6K itself actually decreased the basal level of p-Akt, which could be increased upon rapamycin treatment (Fig. 2). In agreement, overexpression of constitutively active and rapamycin-resistant p70S6K1 mutants attenuated the ability of rapamycin to inhibit S6 phosphorylation, but did not affect its ability to increase Akt phosphorylation (Fig. 3). Collectively, it is clear that inhibition of p70S6K is unlikely to be the mechanism leading to increased Akt phosphorylation. In agreement with our finding, a recent study reported that inhibition of mTORC1 by

knocking down raptor in p70S6K1/p70S6K2 double knockout cells still increased Akt phosphorylation without affecting IRS-1 abundance [20].

Although O'Reilly et al. [7] reported that rapamycin increased IRS-1 abundance from 1 h to 24 h in the cell lines tested, we did not find that either rapamycin or RAD001 increased the level of IRS-1 in most of the tested cell lines, whereas p-Akt levels were increased in every tested cell line under our tested conditions (1 or 24 h treatment). Moreover, rapamycin still effectively increased p-Akt levels even in cell lines in which IRS-1 was not detected (e.g., H460 and H226 cells) or knocked down (e.g., A549, H157 and Calu-1). Importantly, rapamycin still increased Akt phosphorylation even in the IRS-1 knockout cell line; this finding is in agreement with a previous report by Wan et al. [21], showing that knockdown of IRS-1 failed to block the effects of mTOR inhibitors on increasing Akt phosphorylation. Collectively, we conclude that IRS-1 is not required for rapid Akt phosphorylation by rapalogs.

Since IRS-2 can also play a role in regulating PI3K and Akt signaling [22], we also examined whether IRS-2 is required for mTOR inhibitor-induced Akt phosphorylation. Both knockdown and knockout of IRS-2 failed to inhibit rapamycin-induced Akt phosphorylation. Even co-knockdown of both IRS-1 and IRS-2 did not suppress rapamycin-induced Akt phosphorylation. Thus, rapalogs also increase Akt phosphorylation independently of IRS-2.

We noted that rapamycin did increase the levels of p-Akt and IRS-1 in MDA-MB-435 cells after both 1 h and 24 h and in Du145 cells after 24 h of rapamycin treatment. Thus, we cannot rule out the possibility that sustained exposure to rapamycin may result in Akt phosphorylation through an IRS-1-dependent mechanism in certain cancer cell lines (e.g., MDA-MB-435).

Genetic inhibition of p70S6K through gene knockdown substantially decreased p-Akt levels in this study. The underlying mechanism is unknown. Rictor phosphorylation at T¹¹³⁵ by p70S6K does not affect mTORC2 assembly and kinase activity [16, 17, 23]. However, it has been suggested that this phosphorylation inhibits mTORC2 and Akt S⁴⁷³ phosphorylation albeit with no effects on phosphorylation of SGK1 or PKC α [16, 23]. Our results clearly show that both chemical inhibition and genetic knockdown of p70S6K effectively inhibited rictor T¹¹³⁵ phosphorylation, but generated different outcomes on Akt S⁴³⁷ phosphorylation: chemical inhibition did not alter Akt phosphorylation while genetic knockdown potently suppressed Akt phosphorylation. Hence, the suppression of rictor T¹¹³⁵ phosphorylation is not involved in the inhibition of

Akt phosphorylation. Further study of the mechanism accounting for the positive regulation of Akt activity by p70S6K is warranted.

4. Materials and methods

4.1. Reagents

Rapamycin was purchased from LC Laboratories (Woburn, MA). RAD001 was provided by Novartis Pharmaceuticals Corporation (East Hanover, NJ). FRI00705 was provided by Forest Research Institute. These agents were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, and aliquots were stored at -80° C. Stock solutions were diluted to the desired final concentrations with growth medium just before use. Antibodies against Akt, raptor, p70S6 K, S6, p-Akt (S⁴⁷³), p-p70S6 K (T³⁸⁹) and p-S6 (S^{235/236}), were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit polyclonal anti-IRS-1 and anti-IRS-2 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-actin and anti-tubulin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal anti-GAPDH antibody was purchased from Trevigen, Inc. (Gaithersburg, MD). The empty vector pRK7 and expression constructs for WT HA-p70S6K1 and constitutively active/rapamycin-resistant mutants, HA-p70S6K1-E389 Δ CT (E389 Δ CT) and HA-p70S6K1-F5A-E389-R3A (F5A-E389-R3A) [24, 25], were generously provided by Dr. John Blenis (Harvard Medical School, Boston, MA).

4.2. Cell lines and cell culture

Human cancer cell lines used in this study were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and were grown as described previously [6]. Murine WT and IRS-1 knockout (IRS-1-KO) 3T3 fibroblasts [26] were obtained from Dr. C. Ronald Kahn (Harvard Medical School, Boston, MA). WT, IRS-1-KO and IRS-2 knockout (IRS-2-KO) MEFs [27] were provided by Morris F. White (Harvard Medical School, Boston, MA). Except for H157 and A549 cells, which were authenticated by Genetica DNA Laboratories, Inc. (Cincinnati, OH) through analyzing short tandem repeat DNA profile, other cell lines have not been authenticated.

4.3. Western blot analysis

The procedures for preparation of whole-cell protein lysates from cells and for Western blotting were described previously [8]. Since rapalog treatment does not alter the total levels of Akt, p70S6 and S6 based on our previous studies [6, 8, 12, 28], we did not detect these proteins in some experiments while their phosphorylation was detected.

4.4. Gene knockdown by siRNA

IRS-1 (M-003015), IRS-2 (M-003554) and non-specific control (D-001206) SMARTpool[®] siRNAs were purchased from Upstate Biotechnology. p70S6K1 (Cat. #1027217) and control (non-silencing) (Cat. #1022076) siRNAs were ordered from Qiagen (Valencia, CA). Raptor siRNA that targets 5'-AAGGC-TAGTCTGTTTCGAAAT-3' [29] was synthesized by Qiagen. The transfection of siRNA was conducted in a 12-well plate or 24-well plate using Lipofectamine 2000 (Invitrogen) or HiPerFect (Qiagen) following the manufacturer's instructions. Forty-eight hours after transfection, cells were treated with DMSO or rapamycin for the given times and then subjected to detection of the given proteins by Western blot analysis.

Declarations

Author contribution statement

Xuerong Wang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Ping Yue, Hui Tao: Performed the experiments.

Shi-Yong Sun: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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References

- [1] B. Magnuson, B. Ekim, D.C. Fingar, Regulation and function of ribosomal protein S6 kinase (S6 K) within mTOR signalling networks, *Biochem. J.* 441 (2012) 1–21.
- [2] T.R. Fenton, I.T. Gout, Functions and regulation of the 70 kDa ribosomal S6 kinases, *Int. J. Biochem. Cell Biol.* 43 (2011) 47–59.
- [3] I. Ben-Sahra, B.D. Manning, mTORC1 signaling and the metabolic control of cell growth, *Curr. Opin. Cell Biol.* 45 (2017) 72–82.
- [4] R.A. Saxton, D.M. Sabatini, mTOR Signaling in Growth, Metabolism, and Disease, *Cell* 168 (2017) 960–976.
- [5] E. Ilagan, B.D. Manning, Emerging role of mTOR in the response to cancer therapeutics, *Trends Cancer* 2 (2016) 241–251.
- [6] S.Y. Sun, L.M. Rosenberg, X. Wang, Z. Zhou, P. Yue, H. Fu, F.R. Khuri, Activation of Akt and eIF4E Survival Pathways by Rapamycin-Mediated Mammalian Target of Rapamycin Inhibition, *Cancer Res.* 65 (2005) 7052–7058.
- [7] K.E. O'Reilly, F. Rojo, Q.B. She, D. Solit, G.B. Mills, D. Smith, H. Lane, F. Hofmann, D.J. Hicklin, D.L. Ludwig, J. Baselga, N. Rosen, mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt, *Cancer Res.* 66 (2006) 1500–1508.
- [8] X. Wang, P. Yue, Y.A. Kim, H. Fu, F.R. Khuri, S.Y. Sun, Enhancing mammalian target of rapamycin (mTOR)-targeted cancer therapy by preventing mTOR/raptor inhibition-initiated, mTOR/ricor-independent Akt activation, *Cancer Res.* 68 (2008) 7409–7418.
- [9] F. Meric-Bernstam, A. Akcakanat, H. Chen, K.A. Do, T. Sangai, F. Adkins, A.M. Gonzalez-Angulo, A. Rashid, K. Crosby, M. Dong, A.T. Phan, R.A. Wolff, S. Gupta, G.B. Mills, J. Yao, PIK3CA/PTEN mutations and Akt activation as markers of sensitivity to allosteric mTOR inhibitors, *Clin. Cancer Res.* 18 (2012) 1777–1789.
- [10] H. Ren, M. Chen, P. Yue, H. Tao, T.K. Owonikoko, S.S. Ramalingam, F.R. Khuri, S.Y. Sun, The combination of RAD001 and NVP-BKM120 synergistically inhibits the growth of lung cancer in vitro and in vivo, *Cancer Lett.* 325 (2012) 139–146.
- [11] J.L. Yori, K.L. Lozada, D.D. Seachrist, J.D. Mosley, F.W. Abdul-Karim, C. N. Booth, C.A. Flask, R.A. Keri, Combined SFK/mTOR inhibition prevents

- rapamycin-induced feedback activation of AKT and elicits efficient tumor regression, *Cancer Res.* 74 (2014) 4762–4771.
- [12] Y. Li, X. Wang, P. Yue, H. Tao, S.S. Ramalingam, T.K. Owonikoko, X. Deng, Y. Wang, H. Fu, F.R. Khuri, S.Y. Sun, Protein phosphatase 2A and DNA-dependent protein kinase are involved in mediating rapamycin-induced Akt phosphorylation, *J. Biol. Chem.* 288 (2013) 13215–13224.
- [13] B.D. Manning, Balancing Akt with S6 K: implications for both metabolic diseases and tumorigenesis, *J. Cell Biol.* 167 (2004) 399–403.
- [14] L.S. Harrington, G.M. Findlay, R.F. Lamb, Restraining PI3 K: mTOR signalling goes back to the membrane, *Trends Biochem. Sci.* 30 (2005) 35–42.
- [15] M.R. Tavares, I.C. Pavan, C.L. Amaral, L. Meneguello, A.D. Luchessi, F.M. Simabuco, The S6 K protein family in health and disease, *Life Sci.* 131 (2015) 1–10.
- [16] L.A. Julien, A. Carriere, J. Moreau, P.P. Roux, mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling, *Mol. Cell Biol.* 30 (2010) 908–921.
- [17] C. Treins, P.H. Warne, M.A. Magnuson, M. Pende, J. Downward, Rictor is a novel target of p70 S6 kinase-1, *Oncogene* 29 (2010) 1003–1016.
- [18] B.D. Manning, M.N. Logsdon, A.I. Lipovsky, D. Abbott, D.J. Kwiatkowski, L.C. Cantley, Feedback inhibition of Akt signaling limits the growth of tumors lacking Tsc2, *Genes Dev.* 19 (2005) 1773–1778.
- [19] J.B. Easton, R.T. Kurmasheva, P.J. Houghton, IRS-1: auditing the effectiveness of mTOR inhibitors, *Cancer Cell* 9 (2006) 153–155.
- [20] P.P. Hsu, S.A. Kang, J. Rameseder, Y. Zhang, K.A. Ottina, D. Lim, T.R. Peterson, Y. Choi, N.S. Gray, M.B. Yaffe, J.A. Marto, D.M. Sabatini, The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling, *Science* 332 (2011) 1317–1322.
- [21] X. Wan, B. Harkavy, N. Shen, P. Grohar, L.J. Helman, Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism, *Oncogene* 26 (2007) 1932–1940.
- [22] L.M. Shaw, Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the $\alpha 6 \beta 4$ integrin-dependent activation of phosphoinositide 3-OH kinase and promotion of invasion, *Mol. Cell Biol.* 21 (2001) 5082–5093.

- [23] C.C. Dibble, J.M. Asara, B.D. Manning, Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1, *Mol. Cell Biol.* 29 (2009) 5657–5670.
- [24] S.S. Schalm, J. Blenis, Identification of a conserved motif required for mTOR signaling, *Curr. Biol.* 12 (2002) 632–639.
- [25] S.S. Schalm, A.R. Tee, J. Blenis, Characterization of a conserved C-terminal motif (RSPRR) in ribosomal protein S6 kinase 1 required for its mammalian target of rapamycin-dependent regulation, *J. Biol. Chem.* 280 (2005) 11101–11106.
- [26] J.N. Winnay, J.C. Bruning, D.J. Burks, C.R. Kahn, Gab-1-mediated IGF-1 signaling in IRS-1-deficient 3T3 fibroblasts, *J. Biol. Chem.* 275 (2000) 10545–10550.
- [27] L. Rui, T.L. Fisher, J. Thomas, M.F. White, Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2, *J. Biol. Chem.* 276 (2001) 40362–40367.
- [28] X. Wang, P. Yue, C.B. Chan, K. Ye, T. Ueda, R. Watanabe-Fukunaga, R. Fukunaga, H. Fu, F.R. Khuri, S.Y. Sun, Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3 kinase-dependent and Mnk-mediated eIF4E phosphorylation, *Mol. Cell Biol.* 27 (2007) 7405–7413.
- [29] D.D. Sarbassov, D.A. Guertin, S.M. Ali, D.M. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex, *Science* 307 (2005) 1098–1101.