Published in final edited form as:

Cell Signal. 2014 March ; 26(3): 461–467. doi:10.1016/j.cellsig.2013.11.035.

RhoA modulates signaling through the mechanistic target of rapamycin complex 1 (mTORC1) in mammalian cells

Bradley S. Gordon, Abid A. Kazi, Catherine S. Coleman, Michael D. Dennis, Vincent Chau, Leonard S. Jefferson, and Scot R. Kimball*

Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, PO Box 850, Hershey, PA 17033, USA

Abstract

The mechanistic target of rapamycin (mTOR) in complex 1 (mTORC1) pathway integrates signals generated by hormones and nutrients to control cell growth and metabolism. The activation state of mTORC1 is regulated by a variety of GTPases including Rheb and Rags. Recently, Rho1, the yeast ortholog of RhoA, was shown to interact directly with TORC1 and repress its activation state in yeast. Thus, the purpose of the present study was to test the hypothesis that the RhoA GTPase modulates signaling through mTORC1 in mammalian cells. In support of this hypothesis, exogenous overexpression of either wild type or constitutively active (ca)RhoA repressed mTORC1 signaling as assessed by phosphorylation of p70S6K1 (Thr389), 4E-BP1 (Ser65) and ULK1 (Ser757). Additionally, RhoA-GTP repressed phosphorylation of mTORC1-associated mTOR (Ser2481). The RhoA-GTP mediated repression of mTORC1 signaling occurred independent of insulin or leucine induced stimulation. In contrast to the action of Rho1 in yeast, no evidence was found to support a direct interaction of RhoA-GTP with mTORC1. Instead, expression of caRheb, but not caRags, was able to rescue the RhoA-GTP mediated repression of mTORC1 suggesting RhoA functions upstream of Rheb to repress mTORC1 activity. Consistent with this suggestion, RhoA-GTP repressed phosphorylation of TSC2 (Ser939), PRAS40 (Thr246), Akt (Ser473), and mTORC2-associated mTOR (Ser2481). Overall, the results support a model in which RhoA-GTP represses mTORC1 signaling upstream of Akt and mTORC2.

Keywords

Akt; TSC2; mTORC2; RAPTOR; RICTOR; 4E-BP1

1. Introduction

The mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase that plays a key role in the regulation of cell and organismal growth [1]. In metazoans, it exists in two complexes referred to as mTOR complex 1 and 2 (mTORC1 and mTORC2, respectively) [1,2]. The complexes are distinguished by the presence of proteins such as RAPTOR (regulatory-associated protein of mTOR, complex 1) and PRAS40 (proline-rich Akt/PKB substrate 40 kDa) in mTORC1 and RICTOR (RAPTOR-independent companion of mTOR) and the stress-activated map kinase-interacting protein 1 (Sin1) in mTORC2. The presence

© 2013 Elsevier Inc. All rights reserved.

*Corresponding author at: Department of Cellular and Molecular Physiology, H166, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033, USA. Tel.: +1 717 531 8970; fax: +1 717 531 7667. skimball@psu.edu (S.R. Kimball).

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cellsig.2013.11.035>.

of RAPTOR and RICTOR is thought to determine the distinct substrate specificity of the two-kinase complexes [3]. For example, mTORC1 phosphorylates proteins such as eukaryotic initiation factor 4E binding protein 1 (4E-BP1), the 70 kDa ribosomal protein S6 kinase 1 (p70S6K1), and uncoordinated-51-like kinase 1 (ULK1), whereas mTORC2 phosphorylate proteins such as Akt (aka protein kinase B) [1,4]. The kinase activity of mTORC1 is enhanced in the presence of growth factors (e.g. insulin) and nutrients (e.g. amino acids) [5] whereas mTORC2 signaling is activated in the presence of growth factors through an undefined mechanism [2]. As both complexes play key roles in a variety of physiological and pathological conditions [6–9], it is expected that a greater understanding of their regulation will identify novel targets for therapies that address these pathological conditions.

Signaling through mTORC1 is regulated in part by its interaction with several small GTPases including the Ras homolog enriched in brain (Rheb) and a family of Ras-related GTP-binding (Rag) proteins, RagA, RagB, RagC, and RagD [10–12]. Activation of Rheb is controlled by the Tuberous Sclerosis Complex (TSC), consisting of TSC1 (hamartin) and TSC2 (tuberin) [12], which enhances Rheb GTPase activity resulting in reduced activation of mTORC1 [13]. The GTPase activator protein (GAP) activity of TSC2 is repressed by hormones such as insulin and IGF1 as a result of Akt-mediated phosphorylation of TSC2 on multiple residues, including Ser939 [12]. In addition to Rheb, the Rag proteins also modulate signaling through mTORC1. Though the mechanism is not completely understood, amino acids both activate a guanine nucleotide exchange factor, referred to as Ragulator [14], and inhibit a GAP complex, referred to as GATOR [15], thereby increasing the GTP loading of Rag A and B. RagA/B-GTP recruits mTORC1 to the lysosomal membrane where it can interact with Rheb [11]. In addition to the Rheb and Rag GTPases, work in yeast has recently shown that the ortholog of the mammalian RhoA GTPase, Rho1, interacts with and represses TORC1 activity when Rho1 is bound to GTP [16]. However, regulation of mTORC1 by RhoA in mammalian cells is not known. Therefore, we sought to determine whether or not RhoA modulates signaling through mTORC1 in mammalian cells, and if so, whether the regulation occurs through a similar or different action than that described for Rho1 in yeast.

2. Experimental procedures

2.1. Cell culture and transfections

HEK 293E cells were maintained in DMEM supplemented with 10 mM HEPES, 10% FBS (Atlas Biologicals, Fort Collins, CO), and 1% penicillin/streptomycin. Rat2 fibroblasts were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Twenty-four hours prior to transfection, HEK 293E cells or Rat2 fibroblasts were seeded in either 6 or 12 well plates, or 100 mm culture dishes. Transfection of HEK 293E cells was carried out for 48 h using Lipofectamine 2000 (Life Technologies, Grand Island, NY) in Opti-MEM reduced serum medium (Life Technologies, Carlsbad, CA) at a reagent to DNA ratio ($\mu\text{l}/\mu\text{g}$) between 2:1 and 2:5. After 4–5 h in Opti-MEM, an equal volume of DMEM supplemented with 10 mM HEPES and 20% FBS was added to each well. Cells were maintained in this medium until the day of treatment and harvest. Transfection of Rat2 fibroblasts was carried out overnight using Xtremegene 9 Transfection Reagent (Roche Applied Science, Indianapolis, IN) with a 3:1 ($\mu\text{l}:\mu\text{g}$) reagent to plasmid ratio in DMEM supplemented with 10% FBS without antibiotics. The following plasmids were purchased from Addgene (Cambridge, MA) and used in the transfections: pRK7-Myc-RhoA (wild-type RhoA (wtRhoA)) and pRK7-Myc-RhoA-Q63L (constitutively active (caRhoA)) (both plasmids were deposited by the late Dr. Gary Bokoch, The Scripps Research Institute and the caRhoA plasmid has been previously used by this group to investigate RhoA signaling [17]), constitutively active RagB (caRagB; pRK5-HA-GST-RagB-Q99L) and RagC

(caRagC; pRK5-HA-GST-RagC-S75L) (both deposited by Dr. David Sabatini, Massachusetts Institute of Technology). The plasmid encoding constitutively active Rheb (caRheb; pRK5-Myc-Rheb-S16H) was kindly provided by Dr. Kun-Liang Guan (University of California San Diego). A pRK5 Empty Vector was used as a control plasmid. Eighteen hours or 48 h after transfection, cells were incubated in medium lacking serum and leucine (Custom formula from Atlanta Biologicals, Lawrenceville, GA) [18] for 2 h followed by treatment with 10 nM insulin, 0.76 mM leucine, or the combination for 30 min to activate mTORC1 signaling. Cells were collected in 1× SDS sample buffer for analysis of whole cell lysate or in buffer appropriate for immunoprecipitation as described below.

2.2. Immunoprecipitation

Following transfection, HEK 293E cells were washed twice with PBS prior to immunoprecipitation of RAPTOR or RICTOR. For immunoprecipitation of RAPTOR, cells were lysed with 800 µl of buffer consisting of 10 mM KPO₄ (pH 7.2), 1 mM EDTA, 0.3% CHAPS, 5 mM EGTA, 10 mM MgCl₂, 50 mM β-glycerophosphate, 1.5 mM sodium vanadate, 1 µM microcystin, and protease inhibitor cocktail at a concentration of 10 µl/ml (Sigma-Aldrich, St. Louis, MO). For immunoprecipitation of RICTOR, cells were lysed with 800 µl of buffer consisting of 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, 0.3% CHAPS, 1 µM microcystin, 1.5 mM sodium vanadate, and protease inhibitor cocktail at a concentration of 10 µl/ml. Cells were collected by scraping and then incubated for 20 min at 4 °C with rocking followed by centrifugation for 3 min at 1000 × g at 4 °C. Immunoprecipitations were performed by incubating supernatants with 0.5 µg of anti-RAPTOR antibody (Millipore, Billerica, MA), 1 µg of anti-RICTOR antibody (Bethyl Laboratories, Inc, Montgomery, TX), or an equal volume of lysis buffer as a negative control for 1.5 h at 4 °C with rocking. The antibody-antigen complexes were then incubated for 1.5 h at 4 °C with rocking with either 80 µl of protein A/G agarose bead slurry (Pierce Thermo Fisher Scientific, Rockford, IL) or 100 µl goat anti-rabbit magnetic beads (Pierce Thermo Fisher) previously blocked with 1% non-fat dry milk in Raptor extraction buffer. The beads were collected by centrifugation and then boiled in 1× SDS buffer.

2.3. Western blotting

Equal volumes of whole cell lysate were fractionated by electrophoresis using Bio-Rad Criterion precast gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes as described [19]. Primary antibodies against phospho-p70S6K1 (Thr389), mTOR (Ser2481), 4E-BP1 (Ser65), ULK1 (Ser757), Akt (Ser473), and TSC2 (Ser939); total RhoA, ULK1, mTOR, PRAS40, Akt, RAPTOR, RICTOR, and TSC2; and anti-Myc were obtained from Cell Signaling (Danvers, MA). Antibodies to total p70S6K1 and total 4E-BP1 were obtained from Bethyl Laboratories (Montgomery, TX). Anti-phospho-PRAS40 (Thr246) was obtained from Invitrogen (Grand Island, NY). Anti-HA antibodies were obtained from Santa Cruz (Dallas, TX). Secondary antibodies were obtained from Bethyl Laboratories (Montgomery, TX). The antibody-antigen interaction was visualized via ECL using a ProteinSimple Fluorchem M imaging system (Santa Clara, CA). All blots were quantified by using ImageJ software (NIH, Bethesda, MD).

2.4. Statistics

Data are expressed as mean ± standard error of the mean (SEM). Student's *t*-test and a Two-way ANOVA (Treatment × Vector) were used for analysis. Significance was set at *p* < 0.05 for all experiments.

3. Results

3.1. Increasing RhoA expression and cellular Rho-GTP content modulates mTORC1 signaling

To assess a possible role for RhoA in modulating mTORC1 signaling, expression of the GTPase was increased by a plasmid expressing wild type RhoA (wtRhoA). Overexpression of wtRhoA resulted in a modest repression of the insulin and leucine induced phosphorylation of three direct targets of mTORC1, p70S6K1 (Thr389), 4E-BP1 (Ser65), and ULK1 (Ser757), compared to cells transfected with empty vector (Fig. 1A). Thus, mTORC1 signaling was altered in response to changes in expression of RhoA.

As RhoA function is determined by changes in its association with GTP [20], we sought to determine whether or not altering the cellular content of RhoA associated with GTP modulated mTORC1 signaling. HEK 293E cells were transfected with either a control plasmid or a plasmid encoding RhoA that preferentially binds GTP (caRhoA), and mTORC1 signaling was stimulated by insulin and leucine administration. Expression of caRhoA repressed the insulin and leucine-induced phosphorylation of p70S6K1 (Thr389), 4E-BP1 (Ser65), and ULK1 (Ser757) to less than 60% of the control values (Fig. 1B). The reduction in p70S6K1, 4E-BP1, and ULK1 phosphorylation occurred in parallel with repressed phosphorylation of mTOR on Ser2481 from RAPTOR immunoprecipitates (Fig. 1C), a biomarker for the catalytic activity of mTOR [21]. Therefore, enhancing the cellular content of RhoA-GTP significantly represses mTORC1 signaling likely through inhibition of the catalytic activity of mTORC1, which would be similar to the action of Rho1 in yeast.

3.2. RhoA-GTP mediated repression of mTORC1 occurs independent of insulin or leucine-induced signaling but is rescued by constitutively active Rheb

The data presented thus far demonstrates that RhoA-GTP was able to overcome the stimulation of mTORC1 signaling that occurs in response to treatment with a combination of insulin and leucine. However, insulin and leucine modulate mTORC1 activity through distinct signaling pathways, and therefore, the ability of RhoA to repress the effect of individual inputs from the two pathways was investigated. For these studies, Rat2 fibroblasts were utilized because unlike HEK293 cells, mTORC1 is activated in Rat2 fibroblasts in response to treatment with near physiological concentrations of insulin. Notably, previous studies have shown that signaling through mTORC1 in Rat2 fibroblasts responds equally upon exposure to near physiological levels of insulin or leucine [5]. In the present study, Rat2 cells were transfected with a control plasmid or a plasmid encoding caRhoA. Eighteen h following transfection, cells were deprived of serum and leucine for 2 h prior to stimulation of mTORC1 by insulin, leucine, or the combination. As shown in Fig. 2A, mTORC1 signaling was significantly diminished in cells expressing RhoA-GTP regardless of the treatment condition, suggesting that RhoA acts to overcome stimulating inputs through both the Rag (i.e. amino acid) and Rheb (i.e. insulin) signaling pathways. To assess whether activation of endogenous RhoA also leads to repression of insulin- and leucine-induced activation of mTORC1, Rat2 cells were treated with a chemical activator of the GTPase. As shown in Supplementary Fig. 1, activation of RhoA attenuated insulin- and leucine-induced phosphorylation of both p70S6K1 and 4E-BP1. Direct binding of RhoA to the mTORC1 complex as described in yeast [15] would explain these results. However, in the present study, RhoA was not detected in RAPTOR immunoprecipitates, although both mTOR and PRAS40 were present (Fig. 2C). Moreover, we were unable to detect RhoA in RAPTOR immunoprecipitates under other immunoprecipitation conditions, including intracellular crosslinking prior to harvesting cells (data not shown). To examine other possible points of regulation within the insulin or leucine stimulatory inputs, HEK 293E cells were transfected with a control plasmid or with plasmids encoding either caRheb or a

combination of constitutively active Rag B and C (caRags) alone or in combination with caRhoA. Consistent with previous studies [5], expression of either caRheb or caRags effectively maintained p70S6K1 (Thr389) phosphorylation in cells deprived of serum and leucine (Fig. 3). However, only caRheb was able to overcome the repressive effect of caRhoA indicating that RhoA represses mTORC1 upstream of Rheb, but not Rags. Such an action would not be consistent with that reported for Rho1 in yeast.

3.3. RhoA-GTP represses signaling upstream of mTORC1

The data in Fig. 3 suggest that the RhoA-GTP repression of mTORC1 signaling occurs upstream of Rheb. Thus, we measured the phosphorylation of various intermediates within the Akt signaling pathway. Following stimulation of mTORC1 signaling by insulin and leucine, Akt (Ser473) phosphorylation was significantly repressed in cells expressing caRhoA compared to control (Fig. 4A). Concurrent with this reduction in Akt phosphorylation, phosphorylation of PRAS40 (Thr246) and TSC2 (Ser939), both direct downstream targets of Akt [12,22], was also significantly repressed by expression of caRhoA (Fig. 4B and C). Lastly, phosphorylation of mTOR (Ser2481) from RICTOR immunoprecipitates was measured to determine whether the RhoA-GTP-mediated repression occurred upstream of Akt. RhoA-GTP significantly repressed mTOR (Ser2481) phosphorylation (Fig. 4D). Thus, increasing the cellular content of RhoA-GTP repressed signaling through Akt, at least in part through inhibition of mTORC2.

4. Discussion

The results of the experiments presented herein demonstrate that RhoA acts to modulate mTORC1 signaling in mammalian cells. Thus, in the present study, inducing RhoA expression repressed mTORC1 signaling. Moreover, enhancing the cellular content of RhoA-GTP resulted in further repression of mTORC1 signaling. The RhoA-GTP-mediated repression occurred upstream of Rheb as the expression of caRheb, but not caRags, maintained mTORC1 signaling in cells expressing caRhoA. Consistent with this conclusion, phosphorylation of Akt (Ser473), PRAS40 (Thr246), TSC2 (Ser939), and mTORC2-associated mTOR (Ser2481) were all repressed in the presence of elevated levels of RhoA-GTP. The data corroborate findings in yeast showing that Rho1 represses TOR [16], but suggests that the mechanism through which the repression occurs is distinct in mammalian cells.

In yeast, the TOR protein kinase activity in Kog1 (the yeast ortholog of raptor) immunoprecipitates was repressed by incubation with Rho1-GTP γ S, but not by incubation with Rho1-GDP. The effect was specific for Rho1, because no inhibition was observed when immunoprecipitates were incubated with Rho2, Cdc42, or Ras1. Moreover, Rho1 variants with mutations in the Kog1 binding domain failed to repress TOR kinase activity [16], suggesting that Rho1 acts by binding directly to the TORC1 complex.

The results of the present study are consistent with those from the findings in yeast [16] in that increasing RhoA-GTP represses mTORC1 signaling, as assessed by insulin and leucine-induced changes in phosphorylation of three direct mTORC1 targets: p70S6K1, 4E-BP1, and ULK1. This conclusion is also supported by the observation that exogenous expression of caRhoA, that preferentially binds GTP, led to impaired stimulation of mTOR phosphorylation on Ser2481 when the kinase was present in complex 1. Upon activation, mTOR undergoes autophosphorylation at Ser2481, and increased phosphorylation of that residue is used as a biomarker of mTOR catalytic activity [21]. The finding that caRhoA expression reduced the insulin and leucine-induced phosphorylation of mTOR on Ser2481 in RAPTOR immunoprecipitates therefore indicates that RhoA-GTP represses mTORC1 kinase activity.

Unlike the mechanism described in yeast where Rho1 binds directly to Kog1, a direct interaction between RhoA and RAPTOR was not detected in the present study using a variety of immunoprecipitation conditions including intracellular crosslinking prior to harvesting cells. However, the results of the studies depicted in Fig. 2A nevertheless suggest that RhoA might act to directly repress mTORC1 signaling, because caRhoA attenuated both insulin and leucine-induced phosphorylation of p70S6K1. It was therefore surprising to find that expression of caRheb, but not caRags, blocked caRhoA-induced inhibition of mTORC1 signaling. The inability of caRags to overcome the repressive effect of caRhoA on mTORC1 signaling is unlikely to be due to inadequate expression of the proteins, because the level of expression was sufficient to overcome the effect of leucine deprivation on mTORC1 signaling in control cells, i.e. cells transfected with the control plasmid. Moreover, the amount of caRags expressed in cells transfected with caRhoA was significantly greater than controls. Instead, the caRhoA-mediated repression of mTORC1 signaling in cells expressing caRags is more likely a consequence of a RhoA-mediated reduction in the availability of Rheb-GTP. A similar conclusion was presented to explain why amino acid-induced activation of mTORC1 is blunted in serum-deprived cells [5,23]. Thus, it has been proposed that recruitment of mTORC1 to the lysosomal membrane by Rags is not sufficient on its own to fully activate mTORC1 kinase as an interaction between mTORC1 and Rheb-GTP is essential for activation. Therefore, the limiting step in the amino acid-induced activation of mTORC1 is its binding to Rheb-GTP. Consistent with this proposal, in the present study, caRheb effectively overcame the caRhoA-mediated inhibition of p70S6K1 phosphorylation, suggesting that RhoA-GTP acts upstream of Rheb to suppress mTORC1 signaling. Further support for the proposal that RhoA-GTP represses mTORC1 signaling by decreasing the amount of Rheb-GTP is provided by the observation that expression of caRhoA caused a reduction in the insulin-induced phosphorylation of TSC2 on Ser939 and PRAS40 on Thr246, suggesting that the GTPase might repress signaling through mTORC2, an upstream regulator of Akt [3]. In support of this conclusion, the insulin-stimulated phosphorylation of Akt on Ser473, a site directly phosphorylated by mTORC2 [3], was significantly repressed in cells expressing caRhoA compared to control cells. Decreased Akt phosphorylation has been reported previously in HUVEC cells, pre-osteoblasts, and HEK cells expressing caRhoA [24–26]. The conclusion is also supported by the novel observation in the present study that the insulin and leucine-induced phosphorylation of mTOR on Ser2481 in RICTOR immunoprecipitates was significantly reduced in cells expressing caRhoA compared to control cells. Notably, RhoA-mediated repression of mTORC2 signaling likely contributed to a reduction in mTORC1 activation as a consequence of reduced Akt-mediated TSC2 phosphorylation on Ser939 and PRAS40 phosphorylation on Thr246. A potential mechanism for the RhoA-mediated repression of mTORC2 could be through disruption of early events in the insulin-signaling pathway. For example, expression of caRhoA in mammary epithelial cells repressed insulin-induced tyrosine phosphorylation of insulin receptor substrate 1 [27]. Alternatively, expression of caRhoA stimulated phosphatase and tensin homolog (PTEN) activity, resulting in reduced Akt phosphorylation likely due to reduced formation of phosphatidylinositol (3,4,5)-triphosphate [26]. Further studies are needed to determine whether this, or another mechanism is involved in RhoA-induced repression of mTORC2.

The results of Yan et al. [16] place Rho1 upstream of TOR, and the data from the present study are in support of that conclusion and extend it to place RhoA upstream of mTORC2 in mammalian cells. This conclusion contrasts with the results of other studies in which the GTPase is suggested to be a downstream target of mTORC1/2 signaling. For example, Jacinto et al. [2] found that disrupting mTORC2 resulted in depletion of F actin stress fibers and the disruption could be overcome by expression of caRhoA. Other studies have shown the GTP loading of RhoA depends upon mTORC1 and mTORC2 activation in cancer cells [8,20]. Moreover, reloading previously atrophied skeletal muscle following hind limb

suspension results in increased phosphorylation of p70S6K1 in parallel with increased expression of RhoA [28]. Such results, together with the data presented here, are consistent with the conclusion that RhoA both regulates, and is regulated by, mTORC1/2. In such a model, RhoA GTP loading is enhanced by mTORC1, mTORC2, or other signaling molecules such as reactive oxygen species [29], and RhoA·GTP subsequently feeds back to repress mTORC1/2 signaling. An understanding of this feedback system could be important for the development of cancer therapies as colorectal cancer cell migration depends on mTORC1/2-mediated activation of RhoA [8]. Inhibiting activation or reducing the expression of both mTORC1 and mTORC2 in colorectal cancer cells repressed GTP loading of RhoA and the subsequent metastasis [8]. Thus, impaired negative feedback between RhoA and mTORC1/2 may be partially responsible for the hyperactive mTORC1 and subsequent metastasis that occurs with cancer [30].

The physiological significance of RhoA·GTP-mediated repression of mTORC1 and mTORC2 is thus far unexplored. However, it is known that the GTP loading of RhoA occurs with activation of Gα12 and Gα13 proteins. Furthermore, the AT1 angiotensin II G protein-coupled receptor acts through Gα12 and Gα13 [31]. Moreover, angiotensin II and the AT1 receptor cause muscle-wasting concomitant with decreased rates of protein synthesis and decreased signaling through mTORC1 and mTORC2 [31,32]. Thus, a possible mechanism that would explain angiotensin II-induced muscle wasting involves hormone-mediated activation of RhoA and subsequent repression of mTORC1 and mTORC2.

5. Conclusion

In conclusion, the results of the present study show that in mammalian cells RhoA·GTP acts in a dominant fashion to repress insulin and leucine-induced stimulation of mTORC1 signaling. The repressive effect of RhoA·GTP occurs through a decrease in the amount of Rheb·GTP available to stimulate mTORC1 through RhoA·GTP-mediated repression of mTORC2 leading to reduced Akt signaling to TSC2 (Fig. 5). These results differ from those reported for yeast in which Rho1 interacts directly with TORC1 to repress its activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants DK15658 and DK13499 (LSJ). The authors would like to thank Lydia Kutzler for the technical assistance, and Dr. Kun-Liang Guan for kindly providing the plasmid encoding caRheb.

Abbreviations

4E-BP1	eukaryotic initiation factor 4E binding protein 1
caRags	constitutively active Rags B and C
caRheb	constitutively active Rheb
caRhoA	constitutively active RhoA
mTOR	mechanistic target of rapamycin
mTORC	mechanistic target of rapamycin complex
p70S6K1	70 kDa ribosomal protein S6 kinase 1
PRAS40	proline-rich Akt/PKB substrate 40 kDa

RAPTOR	regulatory-associated protein of mTOR complex 1
RICTOR	RAPTOR-independent companion of mTOR
Sin1	stress-activated map kinase-interacting protein 1
TSC	tuberous sclerosis complex
ULK1	uncoordinated-51-like kinase 1
UTR	untranslated region
wtRhoA	wild type RhoA

References

1. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. *Mol. Cell.* 2006; 22:159–168. [PubMed: 16603397]
2. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN. *Nat. Cell Biol.* 2004; 6:1122–1128. [PubMed: 15467718]
3. Toschi A, Lee E, Xu L, Garcia A, Gadir N, Foster DA. *Mol. Cell. Biol.* 2009; 29:1411–1420. [PubMed: 19114562]
4. Kim J, Kundu M, Viollet B, Guan KL. *Nat. Cell Biol.* 2011; 13:132–141. [PubMed: 21258367]
5. Dennis MD, Baum JI, Kimball SR, Jefferson LS. *J. Biol. Chem.* 2011; 286:8287–8296. [PubMed: 21239491]
6. Baar K, Esser K. *Am. J. Physiol.* 1999; 276:C120–C127. [PubMed: 9886927]
7. Dennis MD, Shenberger JS, Stanley BA, Kimball SR, Jefferson LS. *Diabetes.* 2013; 62:2204–2214. [PubMed: 23434932]
8. Gulhati P, Bowen KA, Liu J, Stevens PD, Rychahou PG, Chen M, Lee EY, Weiss HL, O'Connor KL, Gao T, Evers BM. *Cancer Res.* 2011; 71:3246–3256. [PubMed: 21430067]
9. White JP, Baynes JW, Welle SL, Kostek MC, Matesic LE, Sato S, Carson JA. *PLoS One.* 2011; 6:e24650. [PubMed: 21949739]
10. Duran RV, Hall MN. *EMBO Rep.* 2012; 13:121–128. [PubMed: 22240970]
11. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM. *Science.* 2008; 320:1496–1501. [PubMed: 18497260]
12. Inoki K, Li Y, Zhu T, Wu J, Guan KL. *Nat. Cell Biol.* 2002; 4:648–657. [PubMed: 12172553]
13. Li Y, Inoki K, Guan KL. *Mol. Cell. Biol.* 2004; 24:7965–7975. [PubMed: 15340059]
14. Bar-Peled L, Schweitzer LD, Zoncu R, Sabatini DM. *Cell.* 2012; 150:1196–1208. [PubMed: 22980980]
15. Bar-Peled L, Chantranupong L, Cherniack AD, Chen WW, Ottina KA, Grabiner BC, Spear ED, Carter SL, Meyerson M, Sabatini DM. *Science.* 2013; 340:1100–1106. [PubMed: 23723238]
16. Yan G, Lai Y, Jiang Y. *Mol. Cell.* 2012; 45:743–753. [PubMed: 22445487]
17. Subauste MC, Von Herrath M, Benard V, Chamberlain CE, Chuang TH, Chu K, Bokoch GM, Hahn KM. *J. Biol. Chem.* 2000; 275:9725–9733. [PubMed: 10734125]
18. Dennis MD, Jefferson LS, Kimball SR. *J. Biol. Chem.* 2012; 287:42890–42899. [PubMed: 23105104]
19. Kelleher AR, Kimball SR, Dennis MD, Schilder RJ, Jefferson LS. *Am. J. Physiol. Endocrinol. Metab.* 2013; 304:E229–E236. [PubMed: 23193052]
20. Liu L, Luo Y, Chen L, Shen T, Xu B, Chen W, Zhou H, Han X, Huang S. *J. Biol. Chem.* 2010; 285:38362–38373. [PubMed: 20937815]
21. Soliman GA, Acosta-Jaquez HA, Dunlop EA, Ekim B, Maj NE, Tee AR, Fingar DC. *J. Biol. Chem.* 2010; 285:7866–7879. [PubMed: 20022946]
22. Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. *Nat. Cell Biol.* 2007; 9:316–323. [PubMed: 17277771]

23. Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, Byfield MP, Backer JM, Natt F, Bos JL, Zwartkruis FJ, Thomas G. *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102:14238–14243. [PubMed: 16176982]
24. Li Z, Dong X, Wang Z, Liu W, Deng N, Ding Y, Tang L, Hla T, Zeng R, Li L, Wu D. *Nat. Cell Biol.* 2005; 7:399–404. [PubMed: 15793569]
25. Ming XF, Viswambharan H, Barandier C, Ruffieux J, Kaibuchi K, Rusconi S, Yang Z. *Mol. Cell. Biol.* 2002; 22:8467–8477. [PubMed: 12446767]
26. Yang S, Kim HM. *Biomaterials.* 2012; 33:2902–2915. [PubMed: 22244698]
27. Lee YJ, Hsu TC, Du JY, Valentijn AJ, Wu TY, Cheng CF, Yang Z, Streuli CH. *J. Cell. Physiol.* 2009; 220:476–484. [PubMed: 19391109]
28. McClung JM, Thompson RW, Lowe LL, Carson JA. *J. Appl. Physiol.* 2004; 96:1341–1348. [PubMed: 15016791]
29. Aghajanian A, Wittchen ES, Campbell SL, Burrige K. *PLoS One.* 2009; 4:e8045. [PubMed: 19956681]
30. Guigon CJ, Fozzatti L, Lu C, Willingham MC, Cheng SY. *Carcinogenesis.* 2010; 31:1284–1291. [PubMed: 20299527]
31. Siehler S Br. *J. Pharmacol.* 2009; 158:41–49.
32. Burks TN, Andres-Mateos E, Marx R, Mejias R, Van Erp C, Simmers JL, Walston JD, Ward CW, Cohn RD. *Sci. Transl. Med.* 2011; 3:82ra37.

Fig. 1.

Increasing RhoA expression and cellular content of RhoA associated with GTP represses mTORC1 signaling in mammalian cells. (A) HEK 293E cells were transfected with control (Empty), wild type RhoA plasmids —pRK7-myc-RhoA-WT (wtRhoA) or (B) plasmid expressing constitutively active RhoA (caRhoA; pRK7-myc-RhoA-Q63L). Forty-eight hours later, cells were exposed to serum/leucine free medium for 2 h prior to stimulation of mTORC1 with insulin (10 nM) and leucine (0.76 mM) for 30 min. The ratio of phosphorylated p70S6K1 (Thr389), 4E-BP1 (Ser65), and ULK1 (Ser757) to total p70S6K1, 4E-BP1, and ULK1, respectively, were assessed by Western blot analysis. (C) mTORC1 was isolated by immunoprecipitation of RAPTOR and the ratio of phosphorylated mTOR (Ser2481) to total mTOR was assessed by Western blot analysis. Representative blots are shown. Data are means $\pm$ SEM; $n = 6$ /group (A) and $n = 6$ /group (B) from two independent experiments. $*p < 0.05$ by Student's t -test.

Fig. 2. RhoA·GTP mediated repression of mTORC1 occurs independent of insulin or leucine stimulation without directly binding to themTORC1 complex. (A) Rat2 cells were transiently transfected with a control plasmid (empty) or a plasmid encoding constitutively active RhoA (caRhoA) and exposed to serum/leucine free medium for 2 h. Cells were treated for 30 min with insulin (10 nM), leucine (0.76 mM), or the combination for 30 min. The phosphorylation of p70S6K1 (Thr389) was assessed by Western blot analysis, and the ratio of phosphorylated p70S6K1 (Thr389) to total p70S6K1 is depicted in the graph. Bars with different letters indicate significant difference at $p < 0.05$ by Two-way ANOVA. * $p < 0.05$ by Student's t -test. (B) HEK 293E cells were transfected with a control plasmid

(Empty) or a plasmid encoding constitutively active RhoA (caRhoA). Forty-eight hours following transfection, cells were incubated in serum/leucine free medium for 2 h prior to stimulation of mTORC1 with insulin (10 nM) and leucine (0.76 mM) for 30 min. mTORC1 was isolated by immunoprecipitation of RAPTOR and the presence of mTOR, PRAS40, and myc-RhoA were assessed by Western blot analysis. Samples were run on the same gel but in non-contiguous lanes. Black lines denote non-contiguous lanes.

Fig. 3.

RhoA·GTP mediated repression of mTORC1 occurs upstream of the Rheb GTPase. HEK 293E cells were transfected with a control plasmid (Empty), or plasmids expressing either constitutively active Rheb (Myc-caRheb; pRK5-Myc-Rheb-S16H) or a combination of constitutively active RagB (pRK5-HA-GST-RagB-Q99L) and constitutively active RagC (pRK5-HA-GST-RagC-S75L) (collectively denoted as caRags B/C) alone or in combination with constitutively active RhoA (caRhoA; pRK7-myc-RhoA-Q63L). Forty-eight hours following transfection, cells were deprived of serum and leucine for 2 h prior to stimulation of mTORC1 with insulin (10 nM) or leucine (0.76 mM) for 30 min where indicated.

Expression of caRhoA and caRheb were assessed by anti-Myc antibodies (Cell Signaling, Danvers, MA) while expression of caRags B/C were assessed by anti-HA antibodies (Santa Cruz Biotechnology, Dallas, TX). Representative blots are shown.

Fig. 4.

RhoA-GTP represses signaling upstream of mTORC1. HEK 293E cells were transfected with control plasmid (Empty) or constitutively active RhoA (caRhoA; pRK7-myc-RhoA-Q63L) and deprived of serum and leucine for 2 h prior to stimulation of mTORC1 with insulin (10 nM) and leucine (0.76 mM) for 30 min. The ratio of (A) phosphorylated Akt (Ser473) to total Akt, (B) phosphorylated PRAS40 (Thr246) to total PRAS40, and (C) phosphorylated TSC2 (Ser939) to total TSC2 was determined by Western blot analysis. (D) mTORC2 was isolated by immunoprecipitation of RICTOR and the ratio of phosphorylated mTOR (Ser2481) to total mTOR was assessed by Western blot analysis. Representative blots are shown. Data are means $\pm$ SEM; $n = 4-6$ /group from two independent experiments.

* $p < 0.05$ by Student's t -test.

Fig. 5. Proposed model of RhoA-GTP-mediated repression of mTORC1/2 signaling in mammalian cells. The data presented within suggests that RhoA-GTP represses mTORC1 signaling by reducing the amount of Rheb-GTP. This effect likely occurs through RhoA-GTP-mediated inhibition of mTORC2 signaling to Akt, leading to repressed phosphorylation of Akt (Ser473) and TSC2 (Ser939) and ultimately to decreased Rheb GTP loading.