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GLUCOSE REGULATES MTORC1 THROUGH A MECHANISM INVOVLING THE SESTRINS.

A Thesis in

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by

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ABSTRACT

The mammalian target of rapamycin (mTOR) is a protein kinase that forms two distinct complexes referred to as mTOR complexes 1 and 2 (mTORC1 and mTORC2, respectively). mTORC1 controls cellular growth and metabolism. There is a growing interest in understanding the underlying mechanism of the Sestrin family of proteins in regulating mTORC1. The Sestrins are highly conserved, stress-induced proteins, that act as negative regulators of mTORC1. Specifically, recent studies have shown that the Sestrins suppress mTORC1 activity by binding to a heteropentameric complex referred to as GATOR2 (GTPase-activating protein (GAP) activity toward Rags (Ras related GTP binding) 2) that itself acts to repress mTORC1. The interaction of all three Sestrin proteins with GATOR2 occurs in in an amino-acid-dependent manner. Interestingly, a recent study showed that the Rag proteins are required not only for amino acid-induced activation of mTORC1, but also for activation of mTORC1 by glucose, suggesting that glucose may activate mTORC1 through the same pathway utilized by amino acids. Based on these observations, we hypothesized that glucose acts through the Sestrin proteins to activate mTORC1. Initially, we confirmed the results of previous studies and showed that mTORC1 activity is suppressed in cells deprived of leucine and that restoration of leucine to deprived cells restores mTORC1 activity to control values. We also showed that leucine-induced changes in mTORC1 activity require the Sestrins, because in cells lacking all three Sestrin proteins (referred to hereafter as Sestrin triple knockout cells (Sestrin TKO cells)) mTORC1 activity unaffected by changes in leucine availability. We extended the initial studies to show that in wild type cells glucose deprivation suppressed mTORC1 activity and resupplementation with glucose activated mTORC1. However, mTORC1 activity was unaffected by changes in glucose availability in Sestrin TKO cells. In part, leucine activates mTORC1 by promoting Sestrin2 phosphorylation, an event that can be detected as decreased electrophoretic mobility during gel electrophoresis. In agreement with the previous studies, leucine deprivation caused a decrease in Sestrin2 electrophoretic mobility

and leucine supplementation increased its mobility. In contrast, Sestrin2 electrophoretic mobility was unaffected by changes in glucose availability. The results suggest that although glucose regulates mTORC1 through a Sestrin-dependent pathway, it does so through a mechanism that is independent of Sestrin2 phosphorylation at the sites regulated by leucine.

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List of Abbreviations

1. Add Back (AB)
2. AMPK (adenosine monophosphate-activated protein kinase)
3. Dulbecco's high glucose growth medium (DMEM)
4. GAP (GTPase activator protein)
5. GATOR1/2 (GTPase-activating activity towards Rags (Ras related GTP binding))
6. Glucose (Gluc)
7. Leucine (Leu)
8. Mammalian target of rapamycin (mTOR)
9. Mammalian target of rapamycin complex 1 (mTORC1)
10. Rag (Ras related GTP binding)
11. Rheb (ras homolog enriched in brain)

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Chapter 1: Introduction to the topic

mTOR is a serine/threonine protein kinase that, when present in complex 1, phosphorylates a plethora of downstream targets to control cell growth and many aspects of cellular metabolism. The mTORC1 complex is regulated through a variety of upstream signaling pathways that transmit signals from growth hormones and nutrients to the complex. Previous studies have shown that glucose activates mTORC1 and that the effect is associated with suppression of AMPK (adenosine monophosphate-activated protein kinase), a negative regulator of mTORC1. However, a recent study (1) showed that glucose-mediated regulation of mTORC1 was maintained in cells lacking the two catalytic subunits of AMPK. Thus, AMPK is not necessary for the effect. Surprisingly, the study by Efeyan et al. also showed that glucose-induce activation of mTORC1 required the Rag proteins. The Rags are integral components of the pathway through which amino acids act and are located immediately upstream of mTORC1 in the pathway. Therefore, the purpose of the present study was to assess a potential role for components of the amino acid signaling pathway upstream of the Rags in glucose-mediated activation of the kinase.

Chapter 2: ABSTRACT

Based on a recent study (1) showing that glucose-mediated regulation of mTORC1 was dependent upon the Rag proteins, we hypothesized that the signaling pathway through which glucose acts to regulate mTORC1 activity overlaps with the pathway utilized by amino acids. Our initial focus was on the Sestrin family of proteins that have been shown to mediate mTORC1 activation by the amino acid leucine. The results show that in wild type cells containing Sestrins, deprivation of either leucine or glucose causes suppression of mTORC1 activity and resupplementation with the deprived nutrient restores mTORC1 activity to control levels. In contrast, in cells lacking all three Sestrins (Sestrin triple knockout (TKO) cells), mTORC1 is completely resistant to deprivation of either leucine or glucose. In part, leucine activates mTORC1 by promoting dephosphorylation of Sestrin2 (2). In agreement with the previous study, leucine deprivation promoted Sestrin2 phosphorylation and leucine resupplementation caused Sestrin2 dephosphorylation, as assessed by increased and decreased electrophoretic mobility of the protein, respectively. However, glucose deprivation and resupplementation had no apparent effect on Sestrin2 electrophoretic mobility, suggesting that, if it occurs, glucose-induced changes in Sestrin2 phosphorylation occurs on sites distinct from those modulated in response to changes in leucine availability.

INTRODUCTION

The mammalian target of rapamycin complex 1 (mTORC1) is a serine/threonine protein kinase that acts as a master regulator of protein synthesis. It also regulates a diverse set of metabolic pathways that respond to changes in nutrients, e.g. glucose and amino acids, hormones, e.g. insulin, and energy status. For example, mTORC1 activity is potently suppressed by the protein kinase referred to as AMPK (adenosine monophosphate-activated protein kinase) (3). AMPK is referred to as an "energy sensor" that is activated under conditions that promote a decrease in the intracellular/AMP ratio (3). In cells deprived of glucose, AMPK is activated (4) and thus it is assumed that the inhibition of mTORC1 associated with glucose deprivation is a consequence of AMPK activation. However, a recent study (1) showed that in cells lacking the catalytic subunit of AMPK glucose-mediated regulation of mTORC1 was unimpeded, suggesting that the effect occurs through a mechanism distinct from AMPK. Surprisingly, that study also showed that glucose-mediated regulation of mTORC1 occurred through the Rag proteins. The Rags (RagA, B, C, and D) are a family of small GTPases that localize to the late endosomal/lysosomal membrane and recruit mTORC1 to the membrane in an amino acid-dependent manner (5). At the membrane, mTORC1 binds to another small GTPase, ras homolog enriched in brain (Rheb) and is activated. The active form of the Rags consists of either RagA or RagB in the GTP-bound form in a complex with either RagC or RagD in the GDP-bound form. The GTPase activity of RagA and RagB is controlled by a GTPase activating protein (GAP) complex referred to as GATOR1 (GAP activity toward Rags). Consequently, GATOR1 inhibits mTORC1. A second complex referred to as GATOR2 opposes GATOR1 function through an incompletely defined mechanism, and thus, when it is active GATOR2 activates mTORC1.

In a recent study (6), proteins that interact with, and thus potentially regulate, GATOR2 were investigated. Among the proteins identified were the Sestrins. The Sestrins are a highly conserved family of three stress-inducible proteins that act to repress mTORC1 (7). In part, the Sestrins act to inhibit mTORC1 by binding to and inhibiting GATOR2. Notably, Chantranupong and coworkers (6) showed that the binding of the Sestrins to GATOR2 was inhibited by amino acids. Thus, amino acids activate mTORC1 by promoting the release of Sestrin from GATOR2. A more recent study (8) showed that leucine binds directly to GATOR2 and is the most potent amino acid in promoting dissociation of the Sestrin•GATOR2 complex.

Another mechanism through which leucine acts to activate mTORC1 is through changes in Sestrin2 phosphorylation. A recent study (2) showed that the electrophoretic mobility of Sestrin2 was decreased in cells deprived of leucine compared to cells in leucine replete medium and that leucine resupplementation rapidly (i.e. within 10 min) returned Sestrin2

electrophoretic mobility to control levels. That study also showed that the changes in Sestrin2 electrophoretic mobility were due to changes in phosphorylation of the protein. Thus, leucine deprivation promotes increased Sestrin2 phosphorylation whereas leucine resupplementation induces Sestrin2 dephosphorylation. Changes in Sestrin2 phosphorylation were specific for leucine as other amino acids that activate mTOR, e.g. arginine, had no effect on Sestrin2 electrophoretic mobility. Notably, leucine had no effect on the electrophoretic mobility of Sestrin1 or 3.

The current studies were designed to assess whether the Sestrin proteins were required for glucose-mediated regulation of mTORC1, and if so, whether glucose promoted changes in the electrophoretic mobility of Sestrin2.

Chapter 3

MATERIALS AND METHODS

Cell Culture

HEK293T were obtained from the ATCC (Manassas, VA) and the HEK293T cells in which Sestrins 1, 2, and 3 were knocked out were provided by Dr. David Sabatini (Whitehead Institute for Biomedical Research, Cambridge, MA) (8). The HEK293T and Sestrin TKO cells were maintained in high glucose medium (Dulbecco's Modified Eagle's; ATCC) and supplemented with 10% FBS (Atlas Biologicals) and 1% penicillin/streptomycin (growth medium). Cells were seeded at 4 x 10^5 cells/well in 6 well plates and incubated for 48 hours in a CO2 incubator at 37°C.

To assess the effect of leucine on mTORC1 activity, growth medium was replaced with either fresh growth medium or medium lacking leucine and the cells were incubated for an additional 2-hours. Leucine was added to the medium of one-half of the leucine deprived cells at a final concentration of 0.8 mM and 30 minutes later all of the cells were harvested. The medium was removed from the wells and each well was washed with PBS. Lysis buffer (50 mM HEPES (pH 7.4), 2.5% Triton X-100, 0.25% deoxycholate, 1mM DTT, 1mM benzamidine, 0.5 mM sodium vanadate and Protease Inhibitor Cocktail 10 µl/ml (Sigma Aldrich #P8340)) was added to each well, the cells were scraped, and the suspension was incubated on ice for 20 minutes and harvested in 1X SDS sample buffer and boiled at 100°C for 5 minutes.

To assess the effect of glucose on mTORC1 activity, the approach described in the previous paragraph was utilized except DMEM lacking glucose and containing dialyzed serum was used instead of medium lacking leucine. 50 minutes after replacing the medium, glucose was restored to one-half of the glucose deprived cells to a final concentration of 5 mM and 10 minutes later cells were harvested and lysed as described above.

Samples were rocked for 20 minutes at 4°C to ensure cell lysis and then centrifuged at 1000 x g for 10 minutes at 4°C. The protein content of the supernatant fractions was assessed using the Bradford protein assays to provide a means to normalize samples for SDS-PAGE gel. Samples were diluted in SDS sample buffer and boiled at 100°C for 5 minutes and subjected to electrophoresis as described in the following section.

Western Blot Analysis

Protein content was assessed using a Bradford assay and equal amounts of protein from each sample were subjected to SDS-PAGE. For analysis of p70S6K1 phosphorylation, samples were resolved on Criterion Tris-HCl Pre-cast Gels. For analysis of Sestrin2 phosphorylation, samples were resolved on 7.5% home-made gels as described in previous studies from the Kimball laboratory (Kimball 2016). Proteins were transferred

to a PVDF membrane and the membrane was stained using a kit (Pierce Reversible Protein Stain Kit for PVDF Membranes (Cat. #24585) purchased from Thermo Scientific. The membrane was then blocked and incubated overnight at 4°C with primary antibodies with gentle rocking. (p70S6K1 (Thr389) (Cat. #9205L) was purchased from Cell Signaling, Total p70S6K1 was purchased from Bethyl, GAPDH and Sestrin2 (Cat. #SC2233) was purchased from Santa Cruz Biotechnology). Following incubation with appropriate secondary antibodies. Antibody- reactive bands were visualized with enhanced chemiluminescence using a ProteinSimple Fluorchem M imaging system.

Statistical Analysis

Data are expressed as means $\pm$ SEM. One-way ANOVA was used to analyze the data. Significance was set at $p < 0.05$ for all analyses for both leucine and glucose experiments. The leucine and glucose treatment conditions were independent experiments.

Chapter 4

RESULTS

Experiment 1. The effects of leucine deprivation on mTORC1 activity in wildtype and Sestrin TKO HEK293T cells

In an initial study, the effect of leucine deprivation and resupplementation on mTORC1 activity was assessed in wildtype and Sestrin TKO HEK293T cells as a positive control. For all studies, changes in phosphorylation of p70S6K1 on Thr389 was used as a readout of mTORC1 activity because p70S6K1 is directly phosphorylated on that residue by mTORC1 (9). As shown in Figure 1, in wildtype cells mTORC1 activity was significantly suppressed by leucine deprivation and resupplementation of the medium with leucine rapidly restored activity. Interestingly, mTORC1 activity in the Sestrin TKO cells did not change upon leucine deprivation or resupplementation, demonstrating that the mTORC1 pathway is active regardless of leucine availability. This result confirms that the Sestrins are required for leucine-mediated regulation of mTORC1 activity.

Experiment 2. Glucose-mediated regulation of mTOR activity

After confirming that the Sestrins were required for leucine-induced modulation of mTORC1 activity, the study was repeated except that wildtype and Sestrin TKO cells were deprived of or resupplemented with glucose instead of leucine. As shown in Figure 2, mTORC1 activity was suppressed in wildtype cells deprived of glucose and resupplementation restored mTORC1 activity to control values. Similar to the effect observed with leucine, in Sestrin TKO cells, glucose deprivation and resupplementation had no effect on mTORC1 activity. Thus, mTORC1 activity in HEK293T cells requires both leucine and glucose.

Experiment 3. Effect of leucine and glucose deprivation and resupplementation on Sestrin2 electrophoretic mobility

Previous studies have shown that leucine deprivation promotes Sestrin2 phosphorylation and that phosphorylation of the protein causes its electrophoretic mobility to decrease. Initially, we sought to reproduce this finding in HEK293T cells. As shown in Figure 3, leucine deprivation caused a shift in the electrophoretic mobility of Sestrin2 such that the amount of the protein present in the hyperphosphorylated δ form was increased and the amount in the hypo phosphorylated α form was decreased. Resupplementation of the medium with leucine restored Sestrin2 phosphorylation to the pattern observed in cells maintained in leucine-replete medium. In contrast, glucose deprivation and resupplementation had no apparent effect on Sestrin2 electrophoretic mobility, suggesting that, in contrast to leucine, glucose either has no effect on Sestrin2 phosphorylation. Alternatively, glucose alters the phosphorylation of sites distinct from those phosphorylated in response to changes in leucine availability and phosphorylation of the glucose-regulated sites has no effect on the electrophoretic mobility of the protein.

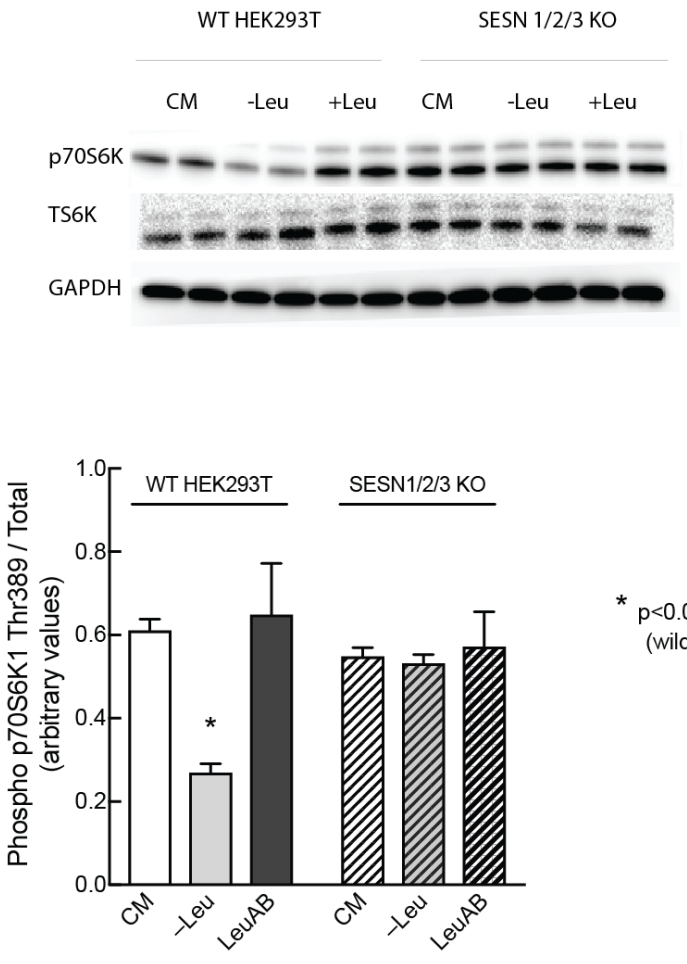

Figure 1. Leucine-dependent regulation of mTORC1 in HEK293T cells. Wildtype (WT HEK293T) or Sestrin triple knockout (SESN1/2/3 KO) HEK293T cells were incubated in complete media (CM) or medium lacking leucine (-Leu) for 2 hours. Leucine was then returned to the medium of one-half of the cells deprived of the amino acid (+Leu or LeuAB) for 30 minutes prior to harvesting. Samples were subjected to western blot analysis for p70S6K1 phosphorylated on Thr389 (p70S6K1), total p70S6K1 (TS6K), and GAPDH. (A) Representative blots. (B) Relative phosphorylation of p70S6K1 was expressed as a ratio of the signal obtained for the phosphorylated protein to the total p70S6K1. The results represent the mean $\pm$ SEM from 3 independent experiments; within each experiment two

independent samples/condition were analyzed. *p<0.02 vs either complete medium or leucine addback in wildtype HEK293T cells.

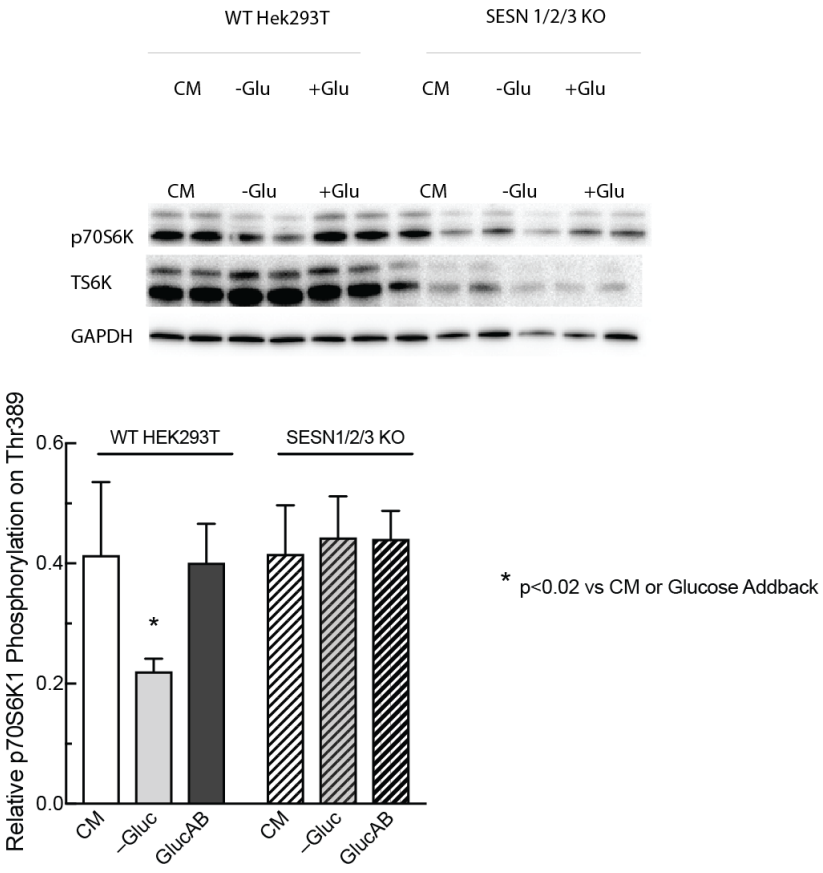

Figure 2. Glucose-dependent regulation of mTORC1 in HEK293T cells. Wildtype (WT HEK293T) or Sestrin triple knockout (SESN1/2/3 KO) HEK293T cells were incubated in complete media (CM) or medium lacking glucose (-Glu or -Gluc) for 50 min. Glucose was then returned to the medium of one-half of the deprived cells (+Glu or GlucAB) for 10 minutes prior to harvesting. Samples were subjected to western blot analysis as described in the legend to Figure 1. (A) Representative blots. (B) Relative phosphorylation of p70S6K1 on Thr389. The results represent the mean $\pm$ SEM from 3 independent experiments; within each experiment two independent samples/condition were analyzed. *p<0.02 vs either complete medium or glucose addback in wildtype HEK293T cells.

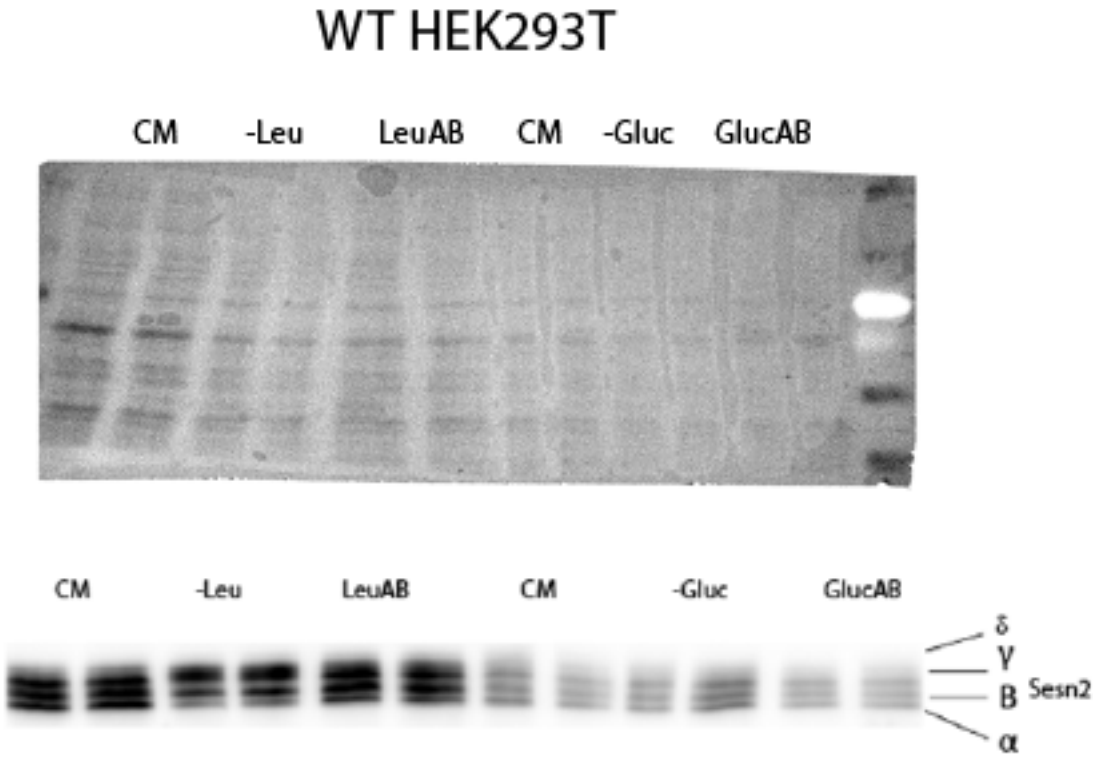

Figure 3. Leucine, but not glucose, alters Sestrin2 phosphorylation in HEK293T cells. HEK 293T cells were incubated in complete medium (CM), medium lacking leucine (-Leu), or medium lacking glucose (-Gluc) and either leucine (LeuAB) or glucose (GlucAB) was returned to the medium of one-half of the deprived cells before harvest as described in the legends to Figures 1 and 2. Sestrin2 (Sesn2) electrophoretic mobility was assessed by western blot analysis. The blot shown is representative of two independent experiments.

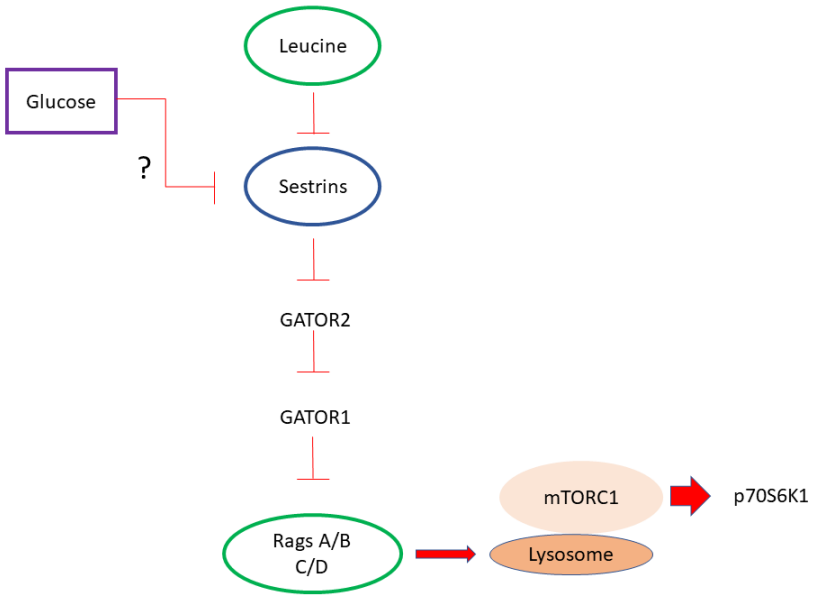

Figure 4. Illustration of the upstream inputs, such as leucine of glucose, that can promote or repress the activation of mTORC1.

DISCUSSION

Leucine-mediated regulation of mTORC1

Overall our data confirmed the findings of previous studies and showed that mTORC1 is responsive to changes in leucine availability, that leucine-induced activation of mTORC1 is dependent upon the Sestrins, and that Sestrin2 phosphorylation is regulated by leucine. In these studies, we used phosphorylation of p70S6K1 as a surrogate for changes in mTORC1 activity. In wildtype cells deprived of leucine, p70S6K1 phosphorylation was decreased but when leucine was returned to the medium of the deprived cells p70S61 phosphorylation was increased. However, leucine deprivation and resupplementation had no effect in cells lacking the three Sestrin proteins. Thus, our results confirm previous findings showing that leucine activates mTORC1 in a Sestrin-dependent manner.

Glucose-mediated regulation of mTORC1

Our results confirm those of previous studies and show that in wildtype cells, glucose deprivation suppresses mTORC1 activity and the resupplementation activates the kinase. We extend the previous studies to show that in Sestrin TKO HEK293T cells, mTORC1 activity is insensitive to glucose availability. We also present the novel finding that glucose deprivation and resupplementation had no effect on the electrophoretic mobility of Sestrin2. These results suggest that, although the Sestrins are required for glucose-mediated regulation of mTORC1, the mechanism involved in the regulation is likely to be distinct from that of leucine.

Limitations of the research

Previous work (2) showed that leucine deprivation and resupplementation had no effect on the electrophoretic mobility of Sestrin1 or Sestrin3. However, in unpublished studies, the Kimball laboratory has discovered that both Sestrin1 and Sestrin3 are phosphorylated on multiple sites, as assessed by mass spec analysis. Thus, unlike Sestrin2, phosphorylation of Sestrin1 and Sestrin3 does not alter their electrophoretic mobility and we were therefore

unable to assess potential changes in Sestrin1 or Sestrin3 in the present study. The initial study investigating Sestrin2 phosphorylation identified three sites by mass spec analysis (2). Subsequent, unpublished work from the Kimball laboratory has identified nine more phosphorylation sites. Only four-five electrophoretic forms are evident during SDS-polyacrylamide gel electrophoresis (Figure 3). Thus, not all phosphorylation events lead to altered electrophoretic mobility. Consequently, it is possible that glucose alters the phosphorylation of sites distinct from those modulated by changes in leucine availability and that those sites do not engender alterations in electrophoretic mobility under the conditions used in this study.

FUTURE DIRECTIONS

Future research should address potential roles for Sestrin 1 and 3 in glucose-mediated regulation of mTORC1. A potential approach is to perform immunoprecipitation experiments to determine whether glucose alters the interaction of GATOR2 with Sestrin1 and/or 3. In addition, possible glucose-induced changes in phosphorylation of any of the Sestrins could be assessed by mass spec analysis. If such changes occur, the potential role of the individual sites could be assessed by expression of Sestrin mutants bearing Ser/Thr to alanine mutations to prevent phosphorylation.

REFERENCES

1. Efeyan A, Zoncu R, Chang S, Gumper I, Snitkin H, Wolfson RL, Kirak O, Sabatini DD, Sabatini DM. Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. *Nature*. 2013;493(7434):679-83. PMC4000705
2. Kimball SR, Gordon BS, Moyer JE, Dennis MD, Jefferson LS. Leucine induced dephosphorylation of Sestrin2 promotes mTORC1 activation. *Cell Signal*. 2016;28(8):896-906. PMC4899281
3. Herzig S, Shaw RJ. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nat Rev Mol Cell Biol*. 2018;19(2):121-35. PMC5780224
4. Chopra I, Li HF, Wang H, Webster KA. Phosphorylation of the insulin receptor by AMP-activated protein kinase (AMPK) promotes ligand-independent activation of the insulin signalling pathway in rodent muscle. *Diabetologia*. 2012;55(3):783-94. PMC4648248
5. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science*. 2008;320(5882):1496-501. PMC2475333
6. Chantranupong L, Wolfson RL, Orozco JM, Saxton RA, Scaria SM, Bar-Peled L, Spooner E, Isasa M, Gygi SP, Sabatini DM. The Sestrins interact with GATOR2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1. *Cell Rep*. 2014;9(1):1-8. PMC4223866
7. Budanov AV, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. *Cell*. 2008;134(3):451-60. PMC2758522
8. Wolfson RL, Chantranupong L, Saxton RA, Shen K, Scaria SM, Cantor JR, Sabatini DM. Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science*. 2016;351(6268):43-8. PMC4698017
9. Pearson RB, Dennis PB, Han JW, Williamson NA, Kozma SC, Wettenhall RE, Thomas G. The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. *EMBO J*. 1995;14(21):5279-87. PMC394637