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Leucine Induced Dephosphorylation of Sestrin2 Promotes mTORC1 Activation

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Abstract

The studies described herein were designed to explore the role of Sestrin2 in mediating the selective action of leucine to activate mTORC1. The results demonstrate that Sestrin2 is a phosphoprotein and that its phosphorylation state is responsive to the availability of leucine, but not other essential amino acids. Moreover, leucine availability-induced alterations in Sestrin2 phosphorylation correlated temporally and dose dependently with the activation state of mTORC1, there being a reciprocal relationship between the degree of phosphorylation of Sestrin2 and the extent of repression of mTORC1. With leucine deprivation, Sestrin2 became more highly phosphorylated and interacted more strongly with proteins of the GATOR2 complex. Notably, in cells lacking the protein kinase ULK1, the activation state of mTORC1 was elevated in leucine-deficient medium, such that the effect of re-addition of the amino acid was blunted. In contrast, overexpression of ULK1 led to hyperphosphorylation of Sestrin2 and enhanced its interaction with GATOR2. Neither rapamycin nor Torin2 had any effect on Sestrin2 phosphorylation, suggesting that leucine deprivation-induced repression of mTORC1 was not responsible for the action of ULK1 on Sestrin2. Mass spectrometry analysis of Sestrin2 revealed three phosphorylation sites that are conserved across mammalian species. Mutation of the three sites to phospho-mimetic amino acids in exogenously expressed Sestrin2 promoted its interaction with GATOR2 and dramatically repressed mTORC1 even in the presence of leucine. Overall, the results support a model in which leucine selectively promotes dephosphorylation of Sestrin2, causing it to dissociate from and thereby activate GATOR2, leading to activation of mTORC1.

Graphical abstract

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Keywords

mechanistic target of rapamycin; amino acids; signaling; ULK1; Rag GTPases

1. Introduction

Earlier studies employing isolated tissues and intact animals demonstrated that of the twenty naturally occurring amino acids, the branched-chain ones, i.e. leucine, isoleucine, and valine, not only serve as substrates for protein synthesis, but also act to stimulate the process [1,2,3,4]. Subsequent studies showed that the stimulatory effect was due to an enhancement of the translation initiation phase of protein synthesis [3] that resulted from the activation of the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway [5]. Moreover, stimulation of translation initiation and activation of mTORC1 were found to be due specifically to leucine [5].

More recent investigations into mechanisms and pathways through which amino acids signal to activate mTORC1 have for the most part relied on cell culture model systems. In general, the approach used in these studies has been to expose cells to either complete amino acid depletion or repletion [e.g. 6,7], although some have investigated the role of individual amino acids [e.g. 8]. Based on these studies, the present understanding is that amino acids act to modulate the nucleotide binding state of the heterodimeric Ras-related GTP binding (Rag) guanosine triphosphatases (GTPases) and thereby facilitate the localization of mTORC1 to its site of activation, i.e. the lysosome [9]. Moreover, the studies suggest two separate pathways through which amino acids act to regulate the Rag GTPases. One is thought to involve a lysosomal membrane associated complex consisting of the Regulator complex [10], the vacuolar ATPase (v-ATPase) [11], and the putative arginine transporter referred to as solute carrier (SLC) 38A9 [12,13]. The other involves a hetero-octomeric complex referred to as GTPase activator protein (GAP) activity toward Rags (GATOR) [14]. The eight proteins that comprise GATOR form two subcomplexes known as GATOR1 and GATOR2. GATOR1 binds directly to the Rag complex and stimulates the GTPase activity of

RagA/B, leading to accumulation of RagA/B in the inactive GDP-bound form. Inhibition of GATOR1, for example through reduced expression of one of its five subunits, leads to complete insensitivity of mTORC1 to amino acid deprivation [14], suggesting that amino acids act by repressing GATOR1 GAP activity. In contrast to the inhibitory action of GATOR1, GATOR2 is a positive regulator of mTORC1, and shRNA-mediated knockdown of the individual GATOR2 subunits dramatically attenuates amino acid-induced activation of mTORC1 [14]. Epistasis studies place GATOR2 upstream of GATOR1 in the amino acid signaling pathway to mTORC1, leading to the proposal that amino acids signal to mTORC1 by promoting GATOR2-mediated repression of GATOR1 GAP activity toward RagA/B [14].

Although the mechanism through which amino acids might act to promote GATOR2-mediated repression of GATOR1 GAP activity is incompletely defined, recent studies have implicated the Sestrin proteins in the response [6,7]. Thus, the association of Sestrins with GATOR2 is enhanced in cells deprived of amino acids and readdition of amino acids to deprived cells rapidly, i.e. within 10 min, promotes dissociation of the Sestrin•GATOR2 complex [6]. Moreover, ectopic expression of Sestrin2 dramatically attenuates amino acid-induced activation of mTORC1 in a Rag- and GATOR1-dependent manner, whereas concomitant knockdown of Sestrins 1, 2, and 3 leads to resistance to amino acid deprivation-mediated repression of mTORC1 [6,7]. However, in both studies [6,7], cells were deprived of all amino acids, and the effect of individual amino acids was not examined. Therefore, whether or not the Sestrin proteins might act in a selective manner to mediate leucine-induced activation of mTORC1 was not determined.

Two papers published subsequent to completion of the studies reported herein presented the conclusion from cell culture experiments that Sestrin2 is a leucine “sensor” [15,16]. However, the results presented in the Wolfson et al. publication [16] showed that methionine and isoleucine also bind directly to Sestrin2. Moreover, they demonstrated that the dissociation constant for leucine to disrupt the Sestrin2•GATOR2 interaction is 20 μ M, well below the concentration of leucine in human plasma or tissue, 157 and 167 μ M, respectively [17]. Thus, if the dissociation constant determined in *in vitro* studies is a valid representation of its affinity for leucine *in vivo*, binding of the amino acid to Sestrin2 would be fully saturated under physiological conditions. Therefore, more information is required to fully understand how leucine selectively induces activation of mTORC1.

The objective of the present study was to assess whether one or more of the Sestrin proteins might be involved in the selective effect of leucine to activate mTORC1. In the studies described herein, cells were deprived of single or multiple amino acids followed by restoration of the deprived amino acids either singly or in pairs. The results show that phosphorylation of Sestrin2 was inversely correlated with leucine concentration in culture medium. The effect was specific for leucine, as arginine or glutamine, two other amino acids implicated in the regulation of mTORC1 in cells in culture [8,13,18], had no discernable effect. Interestingly, phosphorylation of Sestrin2 correlated with enhanced interaction with the GATOR2 complex, a key upstream regulator of mTORC1 activity. Notably, the activation state of mTORC1 in cells deprived of leucine was elevated in cells lacking the protein kinase uncoordinated movement-51-like kinase 1 (ULK1), whereas exogenous expression of ULK1 promoted Sestrin2 phosphorylation and interaction with GATOR2.

Three Sestrin2 phosphorylation sites were identified by mass spectrometry, and expression of a Sestrin2 mutant in which the sites were converted to phospho-mimetic amino acids demonstrated enhanced interaction with GATOR2, even in the presence of leucine, and dramatically repressed leucine-induced mTORC1 activation. Overall, the results of the present study demonstrate that changes in Sestrin2 phosphorylation are key in mediating the selective effect of leucine to activate mTORC1.

2. Materials and Methods

2.1. Cell culture

HEK293 (ATCC; Manassas, VA), HeLa cells (ATCC; Manassas, VA), and ULK1^{+/+} and ULK1^{-/-} mouse embryo fibroblasts (MEF; a kind gift of Dr. Mondira Kundu, St. Jude Children's Research Hospital, Memphis, TN) were maintained in high glucose DMEM supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO), and 1% penicillin/streptomycin (Growth Medium) unless otherwise indicated. When ~90% confluent, cells were incubated with either fresh growth medium or other formulated media (see section 2.2 below) for the indicated time. Rapamycin (NIH; Bethesda, MD) or Torin2 (Selleckchem; Houston, TX) were added at concentrations of 100 nM and 10 nM, respectively, for the indicated time prior to treatment or harvest. Cells were harvested either in 1X SDS sample buffer, or where indicated, in buffer consisting of 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM Na₄P₂O₇, 100 mM β-glycerophosphate, 25 mM NaF, 5mM Na₃VO₄, and 10 μl/ml Protease Inhibitor Cocktail (Sigma Aldrich #P8340, St. Louis, MO), rocked for 20 min at 4 °C, and centrifuged at 1,000 × *g* for 3 min at 4 °C. The protein content of the supernatant fraction was quantified by the Bradford method and equal quantities of protein were used for analysis. No differences were observed in the leucine-induced alteration in electrophoretic migration of Sestrin2 between the two methods of extraction. Alternatively, cells were harvested in appropriate buffer for lambda protein phosphatase treatment or immunoprecipitation as described below.

2.2. Custom formulated cell culture medium

Medium lacking L-leucine is a custom formula (Atlanta Biologicals, Lawrenceville, GA). Medium lacking L-leucine and phosphate and medium lacking L-glycine, L-glutamine, L-leucine, L-arginine, and L-histidine were made in house using the ingredient formulation listed for high glucose DMEM (Life Technologies, Grand Island, NY; Cat. #11965092) with the following exceptions: 1) the indicated amino acids were omitted from the medium, 2) MEM vitamin mix (Life Technologies, Grand Island, NY; Cat. #11120-052) was used in place of the listed formulated vitamins, and 3) medium lacking leucine and sodium phosphate contained 103.7 mM sodium chloride and 25 mM HEPES. Unless otherwise stated, the final concentration of amino acids restored to the medium during experimental procedures was identical to the concentration in the high glucose DMEM formulation.

2.3. Lambda phosphatase treatment

HEK293 cells were plated in 100 mm dishes and the next day were incubated in medium containing or lacking leucine for 2 h prior to collection in 800 μl of buffer consisting of 50 mM Tris-base (pH 7.5), 100 mM NaCl, 100 μM EGTA, 0.01% Brij 35, 2 mM DDT, 1 mM

benzamidine, and 10 μ l/ml Protease Inhibitor Cocktail. Extracts were centrifuged at $1,000 \times g$ for 3 min at 4 °C and 200 μ l of the supernatant fraction were incubated with or without 3200 U of Lambda Protein Phosphatase (New England Biolabs, Ipswich, MA; Cat. #P0753S) for 1 h at 37 °C. The sample was then boiled for 3 min, suspended in 2X SDS sample buffer, and boiled for an additional 5 min prior to storage at -80 °C until analysis.

2.4. Transient transfection and [$^{32}\text{P}_i$] incorporation

HEK293 cells were transfected with plasmids encoding FLAG-tagged Sestrin2 with a Myc and FLAG-tag at the C-terminus (pCMV6-Sestrin2-myc-DDK, catalog #RC501386, Origene Technologies, Inc.) in Opti-MEM reduced serum medium (Life Technologies, Carlsbad, CA) at a reagent to DNA ratio (μ l/ μ g) of 4:1 as previously described [19]. After 5 h in Opti-MEM, an equal volume of DMEM supplemented with 20% FBS was added. Eighteen hours post transfection, cells were incubated in complete medium or medium lacking leucine prior to harvest. For $^{32}\text{P}_i$ incorporation, cells were washed with medium lacking leucine and phosphate followed by incubation in complete medium lacking phosphate or medium lacking leucine and phosphate for 1 h. Cells were then incubated with 0.5 mCi/ml [$^{32}\text{P}_i$] orthophosphate for 30 min at 37 °C followed by a single wash with PBS. Cells were harvested in buffer consisting of 20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM NaF, 100 mM KCl, 200 μ M EDTA, 50 mM β -glycerophosphate, 2.5% Triton X-100, 0.25% deoxycholate, 1 mM DTT, 1 mM benzamidine, 500 μ M NaVO₄, and 10 μ l/ml Protease Inhibitor Cocktail. Samples were rocked for 20 min at 4 °C followed by centrifugation at $1,000 \times g$ for 3 min at 4 °C. A portion of the supernatant fraction was incubated for 2 h with EZview Red Anti-Flag M2 Affinity Gel (Sigma-Aldrich; St. Louis, MO; Cat. #F2426) that was previously blocked in Buffer A consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, and 0.1% β -mercaptoethanol containing 1% bovine serum albumin. The bound fraction was then washed twice with buffer A followed by a final wash with a buffer consisting of 50 mM Tris-HCl (pH 7.4), 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 0.04% β -mercaptoethanol. The bound fraction was then eluted with 1X SDS sample buffer. The eluate was fractionated on a 7.5% Criterion Pre-cast gel (Bio-Rad Laboratories, Inc; Hercules, CA) (see below). Gels containing $^{32}\text{P}_i$ -labeled samples were stained with Coomassie brilliant blue dye, destained, and incorporation of radioactivity into the immunoprecipitated protein was visualized using a Typhoon imager (GE Healthcare Life Sciences).

2.5. Western blot analysis

Samples were fractionated using either Criterion Pre-cast gels (Bio-Rad Laboratories, Inc; Hercules, CA) as previously described [19] or 7.5% polyacrylamide gels with 0.19% bis-acrylamide to permit resolution of Sestrins into multiple electrophoretic forms as previously demonstrated for the 70 kDa ribosomal protein S6 kinase 1 (p70S6K1) [e.g. 20]. Proteins were transferred to PVDF membranes, blocked, and incubated overnight at 4°C with appropriate antibodies. Following incubation with appropriate secondary antibodies (Bethyl Laboratories; Montgomery, TX; Cat. #A120-101P or #A90-116P), the antigen-antibody interaction was visualized with enhanced chemiluminescence (Clarity Reagent; Bio-Rad Laboratories, Inc; Hercules, CA) using a ProteinSimple Fluorchem M imaging system (Santa Clara, CA). Blots were quantified using Image J software (NIH, Bethesda, MD). The

antibody against Sestrin2 (Cat. #10795-1-AP) was purchased from ProteinTech (Chicago, IL). Antibodies against Mios (Cat. #D12C6) and phosphorylated p70S6K1 (Thr389) (Cat. #9234) and Akt (Thr308) (Cat. #9275) were purchased from Cell Signaling (Danvers, MA). Antibodies against GAPDH (Cat. #sc-32233) and Tubulin (Cat. #sc-32293) were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against Sestrin1 were obtained from either Novus (Cat. #NBP1-96045; Littleton, CO) or Abcam (Cat. #ab134091; Cambridge, MA) and antibodies against Sestrin3 (Cat. #AP12471C) were from Abgent (San Diego, CA). The antibody against the FLAG-Tag (Anti-DDK; Cat. #TA50011-100) was obtained from Origene (Rockville, MD).

2.6. Identification of phosphorylation sites

Cells were plated in 15 cm dishes and maintained until approximately 90-95% confluent. Cells (16 dishes/condition) were incubated in medium containing or lacking leucine for 2 h, and then harvested and lysed in the buffer described in section 2.4 above. Endogenous Sestrin2 was immunoprecipitated using a polyclonal antibody (0.24 µg antibody/mg protein; ProteinTech), and the immunoprecipitate was resolved by SDS-PAGE. The gel was stained with SimplyBlue Safe Stain (Invitrogen) and the band corresponding to Sestrin2 was excised and sent to the Taplin Mass Spectrometry Facility (Harvard University) for analysis.

2.7. Exogenous expression of ULK1 and Sestrin2

Site directed mutagenesis of the phosphorylation sites identified by mass spectrometry analysis was performed using a kit (Quick-Change Lightning; Agilent Technologies) and the plasmid expressing human Sestrin2 described in section 2.4 above. The primers used are listed in Table 1. Mutations were confirmed by sequence analysis. Cells were transfected in 12-well plates with 0.5 µg of either a control plasmid (pCMV5) or a plasmid expressing either wild type Sestrin2 or the phosphorylation site mutants using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Twenty-five h later, cells were incubated in leucine-free medium for 2 h, followed by addition of leucine to one-half of the cells at a final concentration of 760 µM. The cells were harvested 30 min later by scraping into SDS sample buffer; the cell extracts were then boiled for 5 minutes at 100 °C and subjected to Western blot analysis. Alternatively, cells were transfected with a plasmid expressing human ULK1 (a gift from Dr. Do-Hyung Kim (Addgene plasmid # 31963)) with or without co-transfection of the Sestrin2 plasmids or a plasmid expressing p70S6K1 (a kind gift from John Blenis (Addgene plasmid # 8984)).

2.8. Analysis of Mios interaction with exogenously expressed Sestrin2

Cells were transfected with plasmids as described in the previous paragraph, and 24 h later were incubated in either leucine-replete or leucine-free medium for 2.5 h. Cells were then harvested by scraping in ice-cold Buffer B consisting of 40 mM HEPES, pH 7.5, 0.3% CHAPS, 120 mM NaCl, 1 mM EDTA (disodium salt), 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and 50 mM NaF supplemented with 1 µM microcystin, 1.5 mM sodium vanadate, and 10 µg/mL Protease Inhibitor Cocktail. Homogenates were centrifuged at 1,000 × g for 3 min, the protein concentration of the supernatant fraction was assessed using a kit (BioRad Protein Assay), and exogenously expressed Sestrin2 was immunoprecipitated using EZview Red Anti-FLAG M2 Affinity Gel. Before use, the

Affinity Gel was washed thrice in buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% β -mercaptoethanol, and then incubated for 1 h at 4°C in the same buffer containing 1% bovine serum albumin. The Affinity Gel was washed one more time and resuspended in ice-cold Buffer B to a final concentration of 0.1 ml of Affinity Gel/ml of Buffer B. Cell supernatants containing 500 μ g of protein were incubated with 10 μ l of Affinity Gel for 2 h at 4°C, and the Affinity Gel was collected by centrifugation and washed twice with ice-cold buffer B. The Affinity Gel was suspended in SDS sample buffer, boiled for 5 min, and the Affinity Gel was removed by centrifugation. The final supernatants were subjected to Western blot analysis as described above.

2.9. Statistical analysis

Data are expressed as mean $\pm$ SEM. Student's *t*-test or one- or two-way ANOVA was used to analyze the data. All analyses passed the Brown Forsythe test for equal variance. Fischer's LSD test was used to identify specific differences if a significant overall F-value was observed following one-way ANOVA. Relationships were determined by Pearson Product Moment Correlation analysis. Significance was set at $p < 0.05$ for all analyses. All data were collected from at least two independent experiments unless noted otherwise.

3. Results

3.1. Leucine alters Sestrin2 electrophoretic mobility

A recent study [21] showed that prolonged leucine deprivation (i.e. 8 h or more) led to increased expression of the mTORC1 repressor Sestrin2. To assess whether changes in Sestrin2 expression might be involved in the acute response of mTORC1 to changes in leucine availability, we examined whether or not its expression was altered in response to 2 h of leucine deprivation. Although the activation state of mTORC1 was repressed in response to leucine deprivation, no change in Sestrin2 expression was observed in HEK293 cells incubated in leucine-free medium for 2 h compared to cells maintained in leucine-replete medium (Fig. 1A). Thus, alterations in Sestrin2 expression are not responsible for the acute modulation of mTORC1 by leucine. Another mechanism through which leucine might act to modulate mTORC1 activity is through covalent modification of Sestrin2. We have previously shown that some phosphorylated proteins, e.g. p70S6K1 and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), separate into multiple electrophoretic forms when resolved on a polyacrylamide gel with reduced bisacrylamide concentration [e.g. 20]. When homogenates from cells incubated in complete medium were resolved under such conditions, Sestrin2 separated into three distinct electrophoretic bands, which we refer to as α , β , and γ in order of decreasing electrophoretic mobility (Fig. 1B). Strikingly, incubation of cells in leucine deficient medium caused a shift in electrophoretic mobility of the protein, resulting in the appearance of a slower migrating band termed δ (Fig. 1B). Thus, leucine deprivation led to a reduction in the relative amount of Sestrin2 present in the α band to $63 \pm 4\%$ ($p < 0.001$) of the value observed in cells incubated in complete medium, with a concomitant increase in the relative amount in the δ band by $131 \pm 9\%$ ($p < 0.001$). In contrast to Sestrin2, no change in migration of either Sestrin1 or Sestrin3 was observed in response to leucine deprivation or repletion (Fig. 1C). The altered electrophoretic mobility

that occurred in response to alterations in leucine availability was also observed in HeLa cells (Fig. 1D), showing that the effect was not specific for HEK293 cells.

3.2. Leucine-induced alteration in Sestrin2 electrophoretic mobility is independent of the activation state of mTORC1

To assess a possible role for mTORC1 in the leucine-induced changes in Sestrin2 electrophoretic mobility, two different approaches were pursued. In the first approach, serum- and leucine-deprived cells were treated with insulin and/or leucine because these agents have been previously shown to act in an additive manner to activate mTORC1 [e.g. ^{22,23}]. In the absence of leucine, insulin had no effect on mTORC1 activity or Sestrin2 electrophoretic mobility, although it effectively enhanced Akt phosphorylation (Fig. S1A). In contrast, addition of leucine to serum- and leucine-deprived cells resulted in both activation of mTORC1 and a shift in Sestrin2 electrophoretic mobility into more rapidly migrating forms. As previously reported a combination of leucine and insulin activated mTORC1 to a greater extent than leucine alone [^{22,23}], but as shown here the combination had no additional effect on Sestrin2 electrophoretic mobility beyond that seen with leucine alone. In the second approach, two different mTORC1 inhibitors, Torin2 and rapamycin, were used. As shown in Fig. S1B, neither inhibitor had any effect on Sestrin2 electrophoretic mobility in cells incubated in leucine-replete medium, although both were highly effective in repressing mTORC1. Moreover, Torin2 did not prevent the leucine deprivation- or repletion-induced alterations in Sestrin2 electrophoretic mobility (Fig. S1C). Together, these results suggest that leucine-mediated alterations in Sestrin2 electrophoretic mobility occur in a mTORC1-independent manner.

3.3. Changes in Sestrin2 electrophoretic mobility are leucine specific

To determine whether alterations in Sestrin2 electrophoretic mobility were specific to leucine, or if other amino acids might produce a similar effect, cells were exposed to medium lacking five amino acids: leucine, arginine, glutamine, histidine, and glycine (-5AA). Cells incubated in medium lacking the 5 amino acids exhibited repressed mTORC1 activity, as assessed by a reduction in phosphorylation of p70S6K1 on Thr389 (Fig. 2A). Restoration of either leucine or arginine, but not glutamine or histidine, to cells incubated in the -5AA medium was sufficient to activate mTORC1, with a combination of both leucine and arginine having an additive effect (Fig. 2A). The arginine-induced activation of mTORC1 was blunted by co-addition of glutamine. In part, the blunting effect may be due to exchange of intracellular leucine for extracellular glutamine by the SLC7A5 antiporter when cells are incubated in glutamine-containing, but leucine-deficient medium [²⁴], leading to a decrease in intracellular leucine concentrations. Interestingly, although leucine and arginine were similarly effective in activating mTORC1, only restoration of leucine promoted a shift in the electrophoretic mobility of Sestrin2 (Fig. 2B). Thus, leucine, either alone or in combination with arginine or glutamine, caused a reduction in the proportion of Sestrin2 present in the δ band with a corresponding increase in the proportion in the α band.

3.4. Sestrin2 electrophoretic mobility correlates with mTORC1 activity

If the modification of Sestrin2 that produces changes in electrophoretic mobility functionally alters its ability to regulate mTORC1, then changes in Sestrin2 mobility should correlate

both temporally and in a dose-dependent manner with changes in the activation state of mTORC1. In support of this idea, depriving cells of leucine for 1 h was sufficient to induce both maximal repression of mTORC1 and altered electrophoretic mobility of Sestrin2 (Fig. 3A). Moreover, within 10 min of returning leucine to leucine-deprived cells, both mTORC1 activity and Sestrin2 electrophoretic mobility were restored to levels observed in cells incubated in complete medium (Fig. 3B). Incubation of leucine-deprived cells with increasing concentrations of leucine led to a shift in the pattern of Sestrin2 electrophoretic mobility to faster migrating bands, with a corresponding increase in mTORC1 activity (Figs. 3C and 3D, respectively). Indeed, the activation state of mTORC1 was directly proportional to changes in the amount of Sestrin2 present in the α band and inversely proportional to the amount in the δ band (Figs. 3E and 3F, respectively).

3.5. Leucine deprivation promotes Sestrin2 phosphorylation

An altered electrophoretic mobility during SDS-PAGE is often due to changes in protein phosphorylation. To assess the possibility that the leucine deprivation-induced alteration in electrophoretic mobility of Sestrin2 might be due to enhanced phosphorylation of the protein, the incorporation of $^{32}\text{P}_i$ into FLAG-tagged Sestrin2 was assessed in cells incubated in medium lacking or containing leucine. As shown in Fig. 4A, $^{32}\text{P}_i$ incorporation into FLAG-Sestrin2 was greater in cells deprived of leucine compared to cells maintained in complete medium. Note that only a single band was observed for Sestrin2 in Fig. 4A because it was not resolved on a gel containing lower bisacrylamide concentration. To provide additional evidence supporting the conclusion that the multiple electrophoretic bands represent different phosphorylated forms of Sestrin2, homogenates from cells incubated in medium containing or lacking leucine were incubated in the presence or absence of lambda protein phosphatase prior to electrophoresis. The effectiveness of phosphatase treatment was confirmed by a shift in the electrophoretic migration of 4E-BP1 into a single band (Fig. 4B). Notably, treatment with lambda protein phosphatase led to a shift in the electrophoretic migration of Sestrin2 into a single, rapidly migrating band, suggesting that the β , γ , and δ bands correspond to multiply phosphorylated forms of the protein.

3.6. Identification of Sestrin2 phosphorylation sites

Mass spectrometry analysis of immunoprecipitates of endogenous Sestrin2 identified three phosphorylation sites on the protein: Thr232, Ser249, and Ser279 (Fig. 5). All three sites are well conserved among mammals, as is the amino acid sequence surrounding the phosphorylation sites (although Thr232 is replaced by a Ser in rodents). Interestingly, neither Ser249 nor Ser279 are conserved in the zebra fish protein, and although the protein has a Ser in the position corresponding to the human Thr232 residue, the surrounding amino acid sequence is poorly conserved (Fig. 5A). Moreover, although all three Sestrin proteins have either a Ser or Thr at the positions corresponding to Sestrin2 Thr232 and Ser279, the neighboring amino acids are poorly conserved for Ser232 and only partially conserved for Ser279 (Fig. 5B). The lack of conservation among Sestrins 1, 2, and 3 in the vicinity of the identified phosphorylation sites may in part explain the lack of a change in their electrophoretic mobility in response to changes in leucine availability.

3.7. A phospho-mimetic Sestrin2 variant represses mTORC1 signaling and binds to GATOR2 in the presence of leucine

A plasmid expressing wild type Sestrin2 was mutated to change individually the three phosphorylation sites to either alanine or glutamate/aspartate. Transient expression of the single amino acid variants in HEK293 cells had no discernable effect on activation of mTORC1 in response to leucine resupplementation of leucine-deprived cells (Fig. S2A). Similarly, expression of a mutant form of the protein with all three sites mutated to alanine (Sestrin2AAA) was without apparent effect. In contrast, expression of either wild type Sestrin2 or a phospho-mimetic mutant (Sestrin2EDE) repressed mTORC1 in cells incubated in leucine-free medium (Fig. 6A). However, expression of wild type Sestrin2 had no effect on the magnitude of the leucine-induced activation of mTORC1, whereas expression of Sestrin2EDE prevented the leucine-induced phosphorylation of p70S6K1 (Fig. 6A). Identical results were obtained in HeLa cells (Fig. S2B). In contrast to wild type FLAG-Sestrin2 which migrated as four bands during SDS-PAGE, both Sestrin2AAA and Sestrin2EDE migrated as a doublet. Moreover, the two Sestrin2AAA bands exhibited electrophoretic mobility similar to that of the hypophosphorylated wild type protein, whereas Sestrin2EDE electrophoretic mobility was similar to that of the hyperphosphorylated protein (Fig. S2C).

Both wild type Sestrin2 and the Sestrin2EDE variant interacted with Mios, a subunit of the GATOR2 complex (Fig. 6B). However, whereas the interaction of wild type Sestrin2 with Mios was enhanced in cells deprived of leucine compared to cells incubated in leucine-replete medium, Sestrin2EDE interaction with Mios was unaltered by leucine availability (Fig. 6B). Indeed, the amount of Mios present in Sestrin2EDE immunoprecipitates from cells incubated in leucine-containing medium was similar to that observed in cells expressing wild type Sestrin2 under leucine depletion conditions.

3.8. ULK1 promotes Sestrin2 phosphorylation and association with GATOR2

It was recently reported that the electrophoretic mobility of Sestrin2 was reduced in cells co-expressing the protein and ULK1 [25]. Therefore, to assess a possible role for ULK1 in leucine-induced alterations in mTORC1, wild type (ULK1^{+/+}) and ULK1 knockout (ULK1^{-/-}) MEF were deprived of leucine and serum for 2 h and leucine was returned to one-half of the cells for 30 min. As shown in Fig. 7A, relative phosphorylation of p70S6K1 was significantly lower in ULK1^{+/+} cells deprived of serum and leucine compared to ULK1^{-/-} cells exposed to the same conditions. Moreover, although phosphorylation of p70S6K1 was increased upon repletion of the amino acid in both ULK1^{+/+} and ULK1^{-/-} MEF, the effect was significantly greater in wild type (3.2-fold increase) compared to knockout (1.5-fold) cells. Interestingly, although the human and mouse Sestrin2 proteins exhibit significant sequence homology, the mouse protein resolves into fewer electrophoretic forms during SDS-PAGE (Fig. 7A). Despite this difference, mouse Sestrin2 still manifested an increase in electrophoretic mobility in response to leucine repletion in ULK1^{+/+} MEF. In contrast, there was no apparent effect of leucine repletion in ULK1^{-/-} MEF.

To assess whether ULK1 might be acting to repress the leucine-induced activation of mTORC1 by altering the phosphorylation state of Sestrin2, ULK1 was expressed in

HEK293 cells with or without co-expression of Sestrin2. As shown in Fig. 7B, when co-expressed with ULK1, Sestrin2 phosphorylation increased dramatically, even though the cells were maintained in leucine replete medium. Moreover, expression of ULK1 led to repression of mTORC1 as assessed both by phosphorylation of p70S6K1 on Thr389 (Fig. 7C) and by a reduction in the electrophoretic mobility of p70S6K1 (Fig. S3). Notably, when co-expressed with ULK1, Sestrin2 interaction with Mios was significantly enhanced (Fig. 7D). Together, the results support a model in which leucine deprivation leads to a ULK1-dependent increase in the phosphorylation state of Sestrin2 and interaction with GATOR2, leading to a repression of mTORC1 (Fig. 8).

4. Discussion

The results of the present study identify a novel mechanism through which leucine acts to modulate selectively the activation state of mTORC1, i.e. through regulation of Sestrin2 phosphorylation. Leucine-induced changes in Sestrin2 phosphorylation were initially detected as a reduction in electrophoretic mobility in cells deprived of the amino acid. Retrospective examination of Sestrin2 Western blots from previous studies reveals that a similar phenomenon occurred in cells deprived of either all amino acids or leucine alone [see e.g. Fig. 1A in reference ¹⁶]. A reduction in Sestrin2 electrophoretic mobility was also evident in cells co-expressing ULK1 and Sestrin2 [²⁵]. The reduction in electrophoretic migration in the latter study was reversed by treatment of cell extracts with calf intestinal phosphatase, suggesting that Sestrin2 phosphorylation occurred in an ULK1-dependent manner. The results of the present study extend those of the earlier ones to show that ULK1-induced phosphorylation of Sestrin2 promotes its interaction with GATOR2, leading to a repression of mTORC1 even in leucine-replete cells. The results also show that in cells deficient in ULK1, the inhibition of mTORC1 caused by leucine deprivation is severely attenuated, suggesting that ULK1 is involved in mediating the effect of the amino acid.

Previous studies showed that mTORC1 phosphorylates and inhibits ULK1, leading to inhibition of autophagy [^{26,27}]. Conversely, repression of mTORC1 is associated with upregulated autophagy [28]. These and similar studies place ULK1 downstream, rather than upstream of mTORC1. However, the results of the present study instead place ULK1 upstream of mTORC1 in the leucine signaling pathway. Interestingly, a recent study [29] suggests that in addition to the canonical mTORC1-dependent mechanism, amino acids may also regulate ULK1 in an mTORC1-independent manner. In that study, amino acid deprivation led to dissociation of protein phosphatase 2A (PP2A) from its inhibitor $\alpha 4$, dephosphorylation of ULK1, and activation of autophagy. Knockdown of either the catalytic subunit of PP2A or its regulatory subunit B55 α using shRNA attenuated the amino acid starvation-induced dephosphorylation of the kinase. Notably, the amino acid deprivation-induced dephosphorylation of ULK1 occurred only on certain residues. Based on these findings, it is tempting to propose that leucine deprivation might lead to dephosphorylation of certain residues on ULK1, allowing it to phosphorylate Sestrin2. Subsequent inhibition of mTORC1 by Sestrin2-mediated activation of GATOR1 would then reduce the phosphorylation of other residues, allowing ULK1 to phosphorylate other proteins. Such a model is also supported by the observation that the association of Sestrin2 with ULK1 can occur independent of two other ULK1-interacting proteins that play important roles in the

induction of autophagy, autophagy-related 13 (Atg13) and the 200 kilodalton focal adhesion kinase family kinase-interacting protein (FIP200) [25]. Thus, we speculate that in response to amino acid deprivation, ULK1 phosphorylates Sestrin2 in an ATG13/FIP200-independent manner leading to its association with GATOR2 and inhibition of mTORC1. Reduced mTORC1 activity would subsequently lead to reduced phosphorylation of ULK1 in the heterotrimeric complex with ATG13 and FIP200, leading to activation of autophagy.

Based on the observation that Sestrin2 binds to amino acids including leucine [16], it is tempting to speculate that the phosphorylation of Sestrin2 might be controlled in part through the binding of amino acids to the protein. A recent report showed that the interaction of Sestrin2 with GATOR2 was reduced in response to readdition of leucine, isoleucine, or methionine, and to a lesser extent by readdition of valine to cells deprived of all amino acids [16]. However, in contrast to leucine which was maximally effective at a concentration of approximately 10 μ M, ten times that concentration of either isoleucine or methionine was required to completely disrupt the interaction between the two proteins. Interestingly, the K_d of another protein that has been proposed to be a leucine sensor, i.e. the leucyl-tRNA synthetase [LARS, 30,31], is also approximately 10 μ M [31,32]. Thus, the K_d of both Sestrin2 and LARS for leucine is approximately 10-fold lower than the concentration observed in skeletal muscle of humans subjected to three days of protein deprivation [i.e. 103 μ M, 33]. In contrast, in freely-fed individuals the intramuscular isoleucine and methionine concentrations are 5-6-fold lower than the K_d value reported for Sestrin2 [16]. Thus, under physiological conditions, both Sestrin2 and LARS would be expected to be saturated with leucine, while the binding of isoleucine and methionine to Sestrin2 would be minimal.

Another recent publication reported the crystal structure of Sestrin2 in complex with leucine [15]. In that study, the N- and C-terminal domains were found to be structurally similar and to be linked by a domain that consists of a short helical domain flanked on either side by two partially disordered regions, findings that were confirmed in a second study [34]. One of the disordered regions is predicted to be proximal to a loop that serves as a “lid” on the leucine-binding pocket in the C-terminal domain [15]. Individual mutation of Thr374 or Thr377 in the loop region prevents leucine binding, and allows constitutive interaction with GATOR2. The other disordered region in the linker domain abuts two residues, Asp406 and Asp407, that are crucial for the interaction of Sestrin2 with GATOR2. Interestingly, the three phosphorylation sites identified in the present study are located in the two disordered regions of the linker domain; two in the N-terminal region and one in the C-terminal region.

It is noteworthy that although neither Sestrin1 nor Sestrin3 exhibited a change in electrophoretic mobility in response to changes in leucine availability, either a serine or a threonine is present in those proteins at positions corresponding to the Thr232 and Ser279 Sestrin2 phosphorylation sites identified in the present study. Thus, it might have been expected that both Sestrins 1 and 3 would undergo phosphorylation on at least two residues in response to leucine deprivation. However, in Sestrins 1 and 3 the amino acid sequence surrounding the residue corresponding to Sestrin2(Thr232) is poorly conserved, suggesting that, at most, leucine deprivation might promote phosphorylation of the residue corresponding to Sestrin2(Ser279). Moreover, it is possible that additional, as yet to be

identified, phosphorylation sites might be present in Sestrin2, and that are not conserved in Sestrin1 or 3 but are necessary for its repressive action on mTORC1 signaling. This possibility is supported by the observation that both Sestrin2AAA and SestrinEDE migrate as at least two, and in some studies, three electrophoretic bands during SDS-PAGE.

Overall, the results of the present study support a model in which leucine acts to repress Sestrin2 phosphorylation, perhaps in part by inhibiting ULK1, and that phosphorylation of Sestrin2 alters its interaction with GATOR2 to selectively modulate mTORC1 activity (Fig. 8). In this model, when leucine is bound to Sestrin2, the lid domain interaction with the C-terminal disordered region is potentially altered, attenuating phosphorylation of Thr232 and Ser249, as well as that of Ser279 in the N-terminal disordered region. Although speculative, when Ser279 is unphosphorylated, it might interact, e.g. through hydrogen bonds, with Asp406 and/or Asp407 to occlude interaction of Sestrin2 with GATOR2. Phosphorylation of Ser279 would prevent such an interaction, potentially displacing the disordered region from Asp406/407, thereby allowing GATOR2 to bind to the protein. Further elucidation of the precise mechanism through which leucine acts to repress Sestrin2 phosphorylation and promote activation of mTORC1 is critical for the development of therapeutics that address dysregulation of the kinase in conditions such as ageing, diabetes, and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4E-BP1	eukaryotic initiation factor 4E binding protein 1
AMPK	AMP-activated protein kinase
Atg13	autophagy-related 13
FIP200	200 kilodalton focal adhesion kinase family kinase-interacting protein
GAP	GTPase activator protein
GATOR	GAP activity toward Rags
LARS	leucyl-tRNA synthetase
MEF	mouse embryo fibroblasts
mTOR	mechanistic target of rapamycin
mTORC1	mTOR complex 1
p70S6K1	70 kDa ribosomal protein S6 kinase 1
PP2A	protein phosphatase 2A
Rag	Ras-related GTP binding

Rheb	ras homolog enriched in brain
SLC	solute carrier
TSC	tuberous sclerosis complex
ULK1	uncoordinated movement-51-like kinase 1
v-ATPase	vacuolar ATPase

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Highlights

- Deprivation of leucine led to a reduction in the electrophoretic mobility of Sestrin2
- Reduced electrophoretic mobility was due to increased phosphorylation
- Sestrin2 phosphorylation was independent of mTORC1, but dependent upon ULK1
- Sites of Sestrin2 phosphorylation were identified as Thr232, Ser249, and Ser279
- A phospho-mimetic Sestrin2 variant repressed mTORC1 in a leucine-independent manner

Figure 1.

Leucine alters the electrophoretic migration of Sestrin2. (A) HEK293 cells were incubated in complete medium (CM) or medium lacking leucine (-L) for 2 h prior to harvest and samples were resolved on a BioRad Criterion gel prior to Western blot analysis for p70S6K1 (phospho-Thr389 (p-p70) and total (p70)), Sestrin2 (Sesn2), and GAPDH. (B and C) HEK293 cells were incubated in complete medium, medium lacking leucine for 2 h prior to stimulation with 760 μ M leucine (LAB) for 30 min prior to harvest and cell extracts were fractionated on 7.5% polyacrylamide gels with 0.19% bisacrylamide. (D) HeLa cells were incubated as in panels B and C. All samples were analyzed on the same blot, but not in contiguous lanes; a white line separates non-contiguous lanes. * indicates significantly different than CM, $p < 0.05$. Results represent the mean $\pm$ SEM of 3 independent experiments with 2 replicates performed in each experiment.

Figure 2.

The altered migration of Sestrin2 is specific to changes in leucine content. (A and B) HEK293 cells were incubated in complete medium (CM) or medium lacking leucine, arginine, glutamine, glycine, and histidine (–5AA) for 2 h prior to stimulation with either 760 μ M leucine (+L), 400 μ M arginine (+R), 4 mM glutamine (+Q), or 200 μ M histidine (+H) alone or in combination for 30 min prior to harvest. Samples were subjected to Western blot analysis for (A) total p70S6K1 (p70), p70S6K1 phosphorylated on Thr389 (p-p70), and GAPDH, or (B) Sestrin2. Letters above the bars indicate significant differences. Significance was set at $p < 0.05$ for all analysis. Results represent the mean $\pm$ SEM of 3 independent experiments with 1-2 replicates performed in each experiment.

Figure 3.

Time course and dose response analysis of leucine-induced changes in Sestrin2 electrophoretic migration. (A) HEK293 cells were incubated in medium lacking leucine (-L) for the indicated time prior to harvest. (B) HEK293 cells were incubated in complete medium (CM) or medium lacking leucine for 2 h prior to stimulation with 760 μ M leucine (LAB) for the indicated time. (C and D) HEK293 cells were incubated in CM or medium with the indicated concentration of leucine for 2 h prior to harvest. Sestrin2 (Sesn2) and GAPDH protein content and phosphorylation of p70S6K1 on Thr389 (p-p70) were determined by Western blot analysis. * indicates significantly different than 0 μ M leucine. Significance was set at $p < 0.05$ for all analyses. Results represent the mean $\pm$ SEM of 3 independent experiments with 2 replicates performed in each experiment.

Figure 4.

Leucine-induced changes in Sestrin2 electrophoretic mobility are due to phosphorylation. (A) HEK293 cells were transiently transfected with a control plasmid or one encoding FLAG-tagged Sestrin2 18 h prior to incubation in phosphate-free medium containing (CM) or lacking leucine (-L) for 1 h. [^{32}P]orthophosphate was added to the medium and cells were harvested 30 min later. FLAG-tagged Sestrin2 was immunoprecipitated, subjected to SDS-PAGE, and the gel was dried and $^{32}\text{P}_i$ incorporation was imaged on a Typhoon Imager. Incorporation of $^{32}\text{P}_i$ was from a single experiment. An aliquot of the immunoprecipitate was also subjected to Western blot analysis for FLAG content. (B) HEK293 cells were incubated in medium containing or lacking leucine for 2 h prior to harvest. A portion of the cell extracts were incubated with lambda protein phosphatase prior to Western blot analysis for Sestrin2 (Sesn2) and 4E-BP1 as indicated in the figure.

Figure 5.

Identification of Sestrin2 phosphorylation sites by mass spectrometry. Sestrin2 was isolated from cells incubated in the presence or absence of leucine as described under “Methods” and sent to the Taplin Mass Spectrometry Facility for analysis. Three sets of samples were independently analyzed. Ser249 was identified as being phosphorylated in cells incubated in medium containing leucine and Thr232 and Ser279 were identified as being phosphorylated in cells deprived of leucine. (A) Alignment of Sestrin2 phosphorylation sites across species. Hs, Homo sapiens; Pt, Pan troglodytes; Bt, Bos Taurus; Mm, Mus musculus; Rn, Rattus norvegicus; Dr, Danio rerio. (B) Multisequence alignment of human Sestrin2 phosphorylation sites with Sestrin1 and Sestrin3.

Figure 6.

Exogenous expression of a Sestrin2 phospho-mimetic variant results in repressed p70S6K1 phosphorylation and interaction with Mios in a leucine-independent manner. HEK293 cells were transiently transfected with either an empty vector (EV), a plasmid expressing wild-type Sestrin2 (WT Sesn2), or a plasmid expressing a triple phospho-mimetic Sestrin2 variant (Sesn2EDE). (A) Cells were incubated in medium lacking serum and leucine for 2 h (-SL) prior to stimulation with 760 μ M leucine for 30 min (LAB). Phosphorylation of p70S6K1 on Thr389 (p-p70), FLAG-tagged Sestrin2 (FLAG-Sesn2), and GAPDH were evaluated by Western blot analysis. * indicates significantly different than LAB, # indicates significantly different than EV. Results represent the mean $\pm$ SEM of 5 independent experiments with 2-3 replicates performed in each experiment. (B) Cells were incubated in complete medium (CM) or deprived of serum and leucine for 2 h (-SL). FLAG-tagged Sestrin2 was immunoprecipitated from the supernatant fraction of cell lysates. Co-immunoprecipitation of Mios with FLAG-tagged Sestrin2 was assessed by Western blot analysis. Letters above the bars indicate significant differences. Results represent the mean $\pm$ SEM of 3 independent experiments with 2 replicates performed in each experiment.

Figure 7.

ULK1 promotes Sestrin2 phosphorylation and interaction with GATOR2. (A) Wild type (ULK1^{+/+}) and ULK1 knockout (ULK1^{-/-}) MEF were incubated in medium lacking serum and leucine for 2 h (-SL) prior to stimulation with 760 μ M leucine for 30 min (LAB). Phosphorylation of p70S6K1 on Thr389 (p-p70), total p70S6K1 (p70), ULK1, Sestrin2 (Sesn2), and GAPDH were evaluated by Western blot analysis. Results are representative of 2 independent experiments with 2-3 replicates performed in each experiment. Letters above the bars indicate significant differences. (B) HEK293 cells were transfected with the indicated plasmids, and the next day cell lysates were subjected to Western blot analysis for FLAG-Sestrin2 (FLAG-Sesn2) and HA-ULK1. Results are representative of 3 independent experiments with 2 replicates performed in each experiment. (C) HEK293 cells were transfected with an empty vector (EV) and/or plasmids expressing HA-p70S6K1 (HA-p70) and HA-ULK1. The next day cell lysates were immunoprecipitated using anti-HA beads and immunoprecipitates were subjected to Western blot analysis for HA and p70S6K1 phosphorylated on Thr389 (p-p70). Results represent 1 experiment with 2 replicates. (D) HEK293 cells were transfected with the plasmids indicated in the figure, and the next day FLAG-Sestrin2 was immunoprecipitated from the supernatant fraction of cell lysates. Co-immunoprecipitation of Mios with FLAG-tagged Sestrin2 was assessed by Western blot

analysis. Results are representative of 2 independent experiments with 2 replicates performed in each experiment.

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Figure 8.

Working model for the mechanism whereby leucine availability acts to repress mTORC1 activity via phosphorylation of the Sestrin2 linker domain at Thr232, Ser249, and Ser279. CTD, C-terminal domain; NTD, N-terminal domain; linker, unstructured region between the C-terminal and N-terminal domains.

Table 1
Primer sequences for site directed mutagenesis of Sestrin2

Primer	Sequence
T232E Forward	5'-ttcactaggggctcaggtgcctggggggcagg-3'
T232E Reverse	5'-ctgccccccaggcacctgagccccctagtga-3'
S249D Forward	5'-gactcaaagccccatcgttgttaacgggtccct-3'
S249D Reverse	5'-agggacccgttgaacaacgatgggggctttgagtc-3'
S279E Forward	5'-ctccatctctctctgctccgtccctcatccg-3'
S279E Reverse	5'-cgggatgaggggacggagcaggaggagatggag-3'
T232A Forward	5'-ccccaggcacctgcacccctagtgc-3'
T232A Reverse	5'-cactagggggtgcaggtgcctgggg-3'
S249A Forward	5'-ggaccggttgaacaacgtgggggctttg-3'
S249A Reverse	5'-caaagccccagcgttgttcaacgggtcc-3'
S279A Forward	5'-atctctctctggcctgcccctcatcc-3'
S279A Reverse	5'-ggatgaggggacggcccaggaggagat-3'