Novel Role for SHP-2 in Nutrient-Responsive Control of S6 Kinase 1 Signaling

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Amino acids are required for the activation of the mammalian target of rapamycin complex 1 (mTORC1), which plays a critical role in cell growth, proliferation, and metabolism. The branched-chain amino acid leucine is an essential nutrient that stimulates mTORC1 to promote protein synthesis by activating p70 S6 kinase 1 (S6K1). Here we show that the protein tyrosine phosphatase SHP-2 is required for leucine-induced activation of S6K1 in skeletal myoblasts. In response to leucine, S6K1 activation is inhibited in myoblasts either lacking SHP-2 expression or overexpressing a catalytically inactive mutant of SHP-2. Activation of S6K1 by leucine requires the mobilization of intracellular calcium (Ca2+), which we show is mediated by SHP-2 in an inositol-1,4,5-trisphosphate-dependent manner. Ectopic Ca2+ mobilization rescued the S6K1 activation defect in SHP-2-deficient myoblasts. SHP-2 was identified to act upstream of phospholipase Cβ4, linking it to the generation of nutrient-induced Ca2+ release and S6K1 phosphorylation. Consistent with these results, SHP-2-deficient myoblasts exhibited impaired leucine sensing, leading to defective autophagy and reduced myoblast size. These data define a new role for SHP-2 as a nutrient-sensing regulator in skeletal myoblasts that is required for the activation of S6K1.

The mammalian target of rapamycin (mTOR) is a central metabolic regulator that has been implicated in metabolic disease and is an important effector of metabolic signaling (1, 2). mTOR exists in two distinct complexes: mTORC1 and mTORC2. mTORC1 controls cell growth (increase in cell size and mass) (2), whereas mTORC2 is involved in actin cytoskeleton organization and Akt activation (3, 4). mTORC1 achieves its effect on cell growth primarily through phosphorylating p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), which results in increased protein translation and cell growth (5, 6). mTORC1 is regulated by Rheb (Ras homolog enriched in brain), a small GTP-binding protein that binds to and activates mTORC1 (7, 8). Like other small GTP-binding proteins, Rheb is negatively regulated by a GTPase-activating protein (GAP), and in this case tuberous sclerosis complexes 1 and 2 (TSC1 and TSC2) serve as the cognate GAPs for Rheb.

mTORC1 integrates extracellular signals that arise from growth factors, energy status, and nutrient availability. Although there has been substantial progress toward delineating how growth factors and hormones couple to mTORC1, relatively little is known about how amino acids are linked to mTORC1 activation. Recent studies have implicated the Rag GTPases (9, 10) and the class III phosphatidylinositol 3-kinase (PI3K) human vacuolar protein sorting 34 (hVps34) as important players of nutrient-responsive mTORC1 signaling (11, 12). Calcium (Ca2+) has also been implicated as playing an important role in the regulation of mTORC1/S6K1 activity (13, 14). However, a thorough understanding of how amino acids control Ca2+-mediated activation of S6K1 has yet to be fully attained.

SH2 domain-containing protein tyrosine phosphatase (SHP-2) functions as a major positive signal enhancer downstream of receptor tyrosine kinases, cytokine receptors, integrins (15, 16), and in some cases G-protein-coupled receptors (17, 18). SHP-2 is required for the regulation of small GTPases such as p21Ras, leading to the activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) (19–21). Although SHP-2 has been shown to be involved in promoting cell proliferation through its actions on ERK1/2, the first line of evidence for a role of SHP-2 in organismal growth (cell size and cell mass) was provided by observations indicating that when SHP-2 was deleted from skeletal muscle in mice, skeletal muscle growth was impaired (22). Moreover, it has been demonstrated that under conditions of growth factor deprivation, SHP-2 limits cell growth, which is achieved by negatively regulating S6K1 (23). Together, these observations suggest that SHP-2 has the capacity to couple to the energy-sensing machinery represented by the mTORC1/S6K1 axis.

To further delineate the function of SHP-2 in mTORC1 signaling, we have examined whether in muscle cells SHP-2 functions in the regulation of signals that are generated by nutrients that specifically target the mTORC1/S6K1 pathway. Here we show that SHP-2 is required for nutrient-induced activation of S6K1 in muscle cells. SHP-2 couples to S6K1 by mobilizing intracellular Ca2+, which is required for the activation of S6K1. We show that SHP-2 engages a pathway that couples nutrient sensing to the regulation of autophagy and cell mass. These data define a novel role for SHP-2 as an integral component in the nutrient-sensing pathway and control of mTORC1/S6K1 signaling.

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MATERIALS AND METHODS

Antibodies and reagents. Phospho-S6 kinase 1 (S6K1) (Thr389), 4E-BP1, phospho-4E-BP1 (Ser65), phospho-AKT (Thr382), phospho-AKT (Ser473), mTOR, and hVps34 antibodies were purchased from Cell Signaling Technologies. S6K1, ERK1/2, and Akt antibodies were from Santa Cruz Biotechnologies, and SHP-2 and PLCγ4 antibodies were from BD Biosciences. LAMPI rat kidney was kindly provided by Baehoon Kim (Yale University, CT). Dactronolsar and thapsigargin were kind gifts from Barbara Ehrlich (Yale University, CT).

Cell cultures. C2C12 mouse myoblasts were purchased from ATCC and cultured as described previously (22). TSC2-deficient fibroblast (TSC2−/−) cells were a kind gift from David J. Kwiatkowski (Harvard Medical School, MA). Fibroblasts derived from mice containing a deletion in exon 3 of S6K were cultured as described previously (23). C2C12 myoblasts were cultured as described previously (24). C2C12 and TSC2−/− cells were cultured at 37°C and 5% CO2 in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS) (Sigma), 1 mM sodium pyruvate (Invitrogen), 5 units/ml penicillin, and 50 μg/ml streptomycin (Sigma). For leucine deprivation, cells were incubated in leucine-free RPMI (catalog no. 1629149; MP Biomedicals) supplemented with 10% dialyzed FBS (Sigma), 2 mM MgCl2 (Invitrogen), 5 units/ml penicillin, and 50 μg/ml streptomycin (Sigma). For leucine stimulation, cells were incubated in leucine-containing RPMI (leucine-free RPMI supplemented with 52 ng/ml leucine [Sigma]).

Plasmids and siRNAs. pRRES-SHP-2 WT, SHP-2 C459S, and SHP-2 E76A constructs were described previously (25). IP3, sponge (IP3, Sponge-NES-mRFP) was a kind gift from Michael Nathanson (Yale University, CT). Transient transfections were performed using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) according to the manufacturer’s recommendations. Small interfering RNAs (siRNAs) for SHP-2 (BENAD-000009), hVps34 (J-063416-08), PLCγ4 (TSC1), and PLCγ3 (TSC2) were purchased from Dharmacon (TNSC). Transient transfections were performed using Lipofectamine 2000 (Invitrogen) at a concentration of 5 units/ml penicillin, and 50 μg/ml streptomycin (Sigma). For leucine stimulation, cells were incubated in leucine-containing RPMI (leucine-free RPMI supplemented with 52 ng/ml leucine [Sigma]).

Ca2+ measurements. Ca2+ measurements were conducted as previously described (26). Briefly, cells were incubated (30 min at 37°C in 5% CO2) in HEPES medium containing 5 μM Fluo-4/AM (Molecular Probes) together with 0.1% Pluronic F-127 (Molecular Probes). HEPES medium contained 130 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl2,1 mM MgSO4, 12 mM KH2PO4, 20 mM HEPES (pH 7.4), and 5 mM dextrose. Coverslips were mounted into chambers (Warner Instruments, Hamden, CT) and transferred to a Zeiss LSM 510 scanning laser confocal microscope equipped with a C-Apochromat 20× objective (Zeiss). Images were acquired at 0.2 Hz. All drugs were bath applied. Single-cell measurements of fluorescence intensity were performed using Zeiss LSM Software (Carl Zeiss Microimaging GmbH).

Cell size measurements. C2C12 myoblasts were transfected with either nontargeting or SHP-2-specific siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Seventy-two hours later, transfected myoblasts were trypsinized and fixed with 4% paraformaldehyde for 10 min on ice. Fixed cells were then filtered through BD Falcon 5-ml polystyrene round-bottom tubes with cell strainer cap (BD Biosciences). Cell size was determined using a FACSCalibur flow cytometer (BD Biosciences). The median forward scattering height in 20,000 events was calculated using FlowJo flow cytometry analysis software (Tree Star Inc., OR).

Immunofluorescence. Cells were plated on glass coverslips in 12-well tissue culture plates and incubated at 37°C for 8 h in growth medium. Cells were starved in leucine-free medium for 50 min and leucine stimulated in leucine-containing medium for the indicated times. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 5 min, and incubated with the indicated primary antibody for 45 min and the appropriate secondary antibodies for 30 min. Images were collected with a Zeiss LSM 510 Meta scanning laser confocal microscope.

Statistical analysis. Data are presented as the means ± standard errors of the means (SEM). Statistical analyses were performed using Microsoft Excel (paired Student’s t test with two tails) or Prism software (GraphPad Software). Statistical analyses in three or more groups were performed using a one-way analysis of variance (ANOVA) followed by the Bonferroni’s t test for multiple comparisons. Statistical differences between two groups were analyzed using unpaired two-sided t tests. P values of >0.05 were defined as not significant (NS).

RESULTS

SHP-2 positively regulates S6K1 phosphorylation in response to leucine stimulation. Previously, we demonstrated that SHP-2 promotes skeletal muscle growth in mice and that under conditions of nutrient deprivation SHP-2 negatively regulates S6K1 and cell growth (22, 23). To further investigate the role of SHP-2 in cell growth and nutrient sensing, we studied the essential branched-chain amino acid leucine, which specifically activates the nutrient-sensing arm of the mTORC1 pathway (10, 27, 28). We first examined whether SHP-2 was required for leucine-induced activation of S6K1. C2C12 myoblasts cultured in the presence of complete medium were selectively deprived of leucine for 30 min, which was followed by readddition of leucine, and as expected, phosphorylation of both S6K1 and 4E-BP1 was induced (Fig. 1A). However, in SHP-2 siRNA-treated myoblasts, S6K1 phosphorylation on Thr389 was significantly impaired following leucine readddition (Fig. 1A). In contrast, 4E-BP1 phosphorylation on Ser65 remained unaffected in SHP-2 siRNA-treated myoblasts (Fig. 1A). These results suggest that in myoblasts SHP-2 is required for leucine-induced phosphorylation of S6K1. Interestingly, we observed differential effects of SHP-2 interference on S6K1 and 4E-BP1 activation. Similar findings have been previously reported for the inhibition of mTORC1 by rapamycin (29), which supports the argument for the involvement of SHP-2 in mTORC1/S6K1 regulation.

To address whether the requirement for SHP-2 in leucine-induced S6K1 phosphorylation was a function of its catalytic activity, we overexpressed in C2C12 myoblasts wild-type SHP-2 (SHP-2 WT) and a dominant negative mutant of SHP-2 that lacks catalytic activity (SHP-2 CS). We found that when overexpressed in C2C12 myoblasts, SHP-2 CS but not SHP-2 WT inhibited S6K1 phosphorylation in response to leucine (Fig. 1B). Consistent with the results of SHP-2 siRNA knockdown, overexpression of a catalytically inactive mutant of SHP-2 did not affect either 4E-BP1, ERK1/2, or Akt phosphorylation in response to leucine (Fig. 1B). We tested whether the requirement for SHP-2 in leucine-induced S6K1 phosphorylation was specific to myoblasts. We also found that leucine-induced S6K1 phosphorylation was impaired in fibroblasts in which SHP-2 was knocked down (Fig. 1C) or in fibroblasts derived from mice containing a loss-of-function mutant of S6K1 (SHP-2 (30) (Fig. 1D and E). Taken together, these results support the interpretation that SHP-2 is essential for nutrient-induced S6K1 phosphorylation.

SHP-2 functions upstream from and/or parallel to Rheb and TSC1/2 in the mTORC1 pathway. We next attempted to position SHP-2 in the canonical mTORC1-S6K1 pathway. Rheb is a small GTPase that binds to and directly stimulates mTORC1 (7). When SHP-2 was knocked down by siRNA in myoblasts, we observed impaired S6K1 phosphorylation in response to leucine (Fig. 2A). However, upon coexpression of a constitutively active mutant of
Rheb (Rheb Q64L), which stimulated S6K1 phosphorylation in the absence of leucine, the effect of SHP-2 siRNA knockdown on S6K1 phosphorylation was rescued (Fig. 2A). This result indicates that SHP-2 lies upstream of, and/or parallel to, Rheb in the mTORC1/S6K1 pathway.

The TSC1/2 complex exists as a heterodimer and negatively regulates mTORC1 by serving as a GTPase-activating protein for Rheb (7, 31). As such, mouse embryo fibroblasts (MEFs) derived from TSC2-deficient mice exhibit constitutively active S6K1 phosphorylation that is unaffected by the removal of leucine for 30 min (Fig. 2B)(32). Under these conditions, we found that knockdown of SHP-2 by siRNA in TSC2-deficient fibroblasts did not alter the level of S6K1 phosphorylation (Fig. 2B). Similar results were obtained when a catalytically inactive mutant of SHP-2 was overexpressed in TSC2-deficient cells (Fig. 2C). Together, these results demonstrate that SHP-2 lies upstream of, and/or parallel to, the TSC1/2 complex.

We considered the possibility that SHP-2 acts in a parallel pathway to control the action of a protein phosphatase that directly or indirectly affects S6K1 phosphorylation. To test this, we monitored the dephosphorylation of S6K1 following leucine deprivation in the presence and absence of SHP-2 (Fig. 2D). We anticipated that the kinetics of S6K1 dephosphorylation would be altered in cells lacking SHP-2 if a downstream S6K1 phosphatase was being regulated by SHP-2. However, these experiments showed that the rate of S6K1 dephosphorylation upon leucine deprivation was similar in SHP-2 siRNA-treated cells and in nontargeting-siRNA-treated cells (Fig. 2D). We infer that SHP-2 is unlikely to be regulating S6K1 phosphorylation by controlling an S6K1 phosphatase.

**FIG 1** SHP-2 is required for S6K1 phosphorylation in response to leucine. (A) C2C12 myoblasts were transfected with either nontargeting (NT) or SHP-2 siRNA, leucine deprived for 30 min, and either lysed (0 min) or stimulated with leucine (Leu) for the indicated times. Cell lysates were subjected to immunoblotting with the indicated antibodies. The graphs show densitometric analyses of the relative S6K1 phosphorylation (pS6K1/S6K1) and 4E-BP1 phosphorylation (p4E-BP1/4E-BP1) levels representing the means ± SEM from 3 independent experiments (*, P < 0.05). (B) C2C12 myoblasts were transfected with vector, wild-type SHP-2 (SHP-2 WT), or SHP-2 C459S (SHP-2 CS). Cells were leucine deprived and restimulated with leucine for the indicated times, and immunoblotting assays were performed with the indicated antibodies. The graphs show densitometric analyses of the relative S6K1 phosphorylation (pS6K1/S6K1) and 4E-BP1 phosphorylation (p4E-BP1/4E-BP1) representing the means ± SEM from 3 independent experiments (*, P < 0.05). (C) NIH 3T3 cells were transfected either with nontargeting (NT) siRNA or SHP-2 siRNA. Cells were subjected to leucine deprivation for 30 min, followed by the readdition of leucine for the indicated times. Cell lysates were prepared and immunoblotted with the indicated antibodies. The graph presents the densitometric ratio of pS6K1/S6K1 for the means ± SEM from 3 independent experiments. (D and E) Fibroblasts derived from SHP-2 exon 3-deleted mice (Ex3+/−) were cultured as previously described (23). Cells cultured in growth medium (GM) were leucine deprived and restimulated for the indicated times. Cell lysates were resolved by SDS-PAGE and immunoblotted for pS6K1, S6K1, and SHP-2. Results represent the relative pS6K1/S6K1 ratios ± SEM from >3 independent experiments. RG, randomly growing.
The Ragulator-Rag complex is thought to recruit mTORC1 to the lysosomal surface, where it facilitates the binding of mTOR to GTP-loaded Rheb (10). Because our data suggested that SHP-2 functions upstream of and/or parallel to Rheb (Fig. 2A, B, and C) we asked whether SHP-2 might be involved in the regulation of mTORC1 localization with the Ragulator-Rag complex. We tested this by examining the subcellular localization of mTOR following leucine stimulation in myoblasts. mTOR was diffusively localized throughout the cytoplasm in leucine-deprived myoblasts (Fig. 2E). However, upon leucine stimulation mTOR subcellular localization adopted a more concentrated punctate staining pattern that was particularly prominent in perinuclear regions (Fig. 2E). Costaining of LAMP-1, which is an established lysosomal marker, confirmed that the greater proportion of mTOR was localized to the lysosomal surface.
lyosomal membranes in response to leucine (Fig. 2E). Myoblasts treated with SHP-2 siRNA showed similar patterns of mTOR subcellular localization in the absence and presence of leucine (Fig. 2E). Hence, SHP-2 does not appear to be involved in the redistribution of mTORC1 to lysosomal membranes in myoblasts in response to leucine.

Ca\(^{2+}\) resuces leucine-mediated SHP-2 inhibition of S6K1 phosphorylation. SHP-2 signals to ERK1/2 and Akt in response to stimulation by growth factors, cytokines, and engagement of the extracellular matrix by integrins (16, 21, 33–36). However, we found no evidence that either ERK1/2 or Akt was altered in its level of phosphorylation in response to leucine stimulation in myoblasts (Fig. 1). These observations suggest alternative mechanisms to stimulation by growth factors, cytokines, and engagement of the extracellular matrix by integrins (16, 21, 33–36). Moreover, agents that increase intracellular Ca\(^{2+}\) increase in response to various stimuli, including amino acids (13, 14, 37, 38). Moreover, agents that increase intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) activate S6K1 but not Akt (39), and it has been shown that amino acids can increase the levels of [Ca\(^{2+}\)]\(_i\) (38). Therefore, we embarked upon investigating whether SHP-2 mediates nutrient activation of S6K1 by promoting the mobilization of [Ca\(^{2+}\)]\(_i\).

First, we tested whether in myoblasts the cell-permeable Ca\(^{2+}\) chelator BAPTA-acetoxyethyl ester (BAPTA-AM) inhibits leucine-induced S6K1 phosphorylation (Fig. 3A). We performed a dose-response assay of BAPTA-AM in myoblasts followed by leucine withdrawal and restimulation for 10 min. Phosphorylation of both S6K1 and 4E-BP1 was inhibited in a dose-dependent manner with BAPTA following leucine stimulation (Fig. 3A). Quantitative assessment of phosphorylation of both S6K1 and 4E-BP1 revealed that S6K1 phosphorylation was approximately twice as sensitive to inhibition by BAPTA as to 4E-BP1 (Fig. 3A, right panel). These results indicate that in myoblasts with S6K1 and 4E-BP1, phosphorylation is dependent upon intracellular Ca\(^{2+}\). Interestingly, the differential sensitivity of S6K1 and 4E-BP1 activation to Ca\(^{2+}\) is consistent with that observed for SHP-2 inhibition (Fig. 1), suggesting that SHP-2 and Ca\(^{2+}\) may exist in a common pathway leading to S6K1 and 4E-BP1 phosphorylation.

To test whether SHP-2 and Ca\(^{2+}\) exist in a common pathway leading to S6K1 and 4E-BP1 phosphorylation, we postulated that if SHP-2 lies upstream of Ca\(^{2+}\), then ectopic mobilization of Ca\(^{2+}\) should rescue the leucine-induced S6K1 phosphorylation defect observed in SHP-2 siRNA-transfected myoblasts (Fig. 1A). To test this, SHP-2 siRNA-transfected myoblasts were treated with thapsigargin followed by stimulation with leucine (Fig. 3B). Thapsigargin causes an increase in [Ca\(^{2+}\)]\(_i\), by inhibiting the SERCA (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase) pump (40). As shown previously, SHP-2 siRNA-transfected myoblasts were inhibited in their ability to stimulate S6K1 phosphorylation following leucine treatment (Fig. 3B). In contrast, SHP-2 siRNA-transfected myoblasts treated with thapsigargin induced S6K1 phosphorylation to levels comparable to that of nontargeting-siRNA-treated myoblasts (Fig. 3B). These results demonstrate that SHP-2 siRNA-treated myoblasts was independent of Akt phosphorylation since phosphorylation of both Akt Ser473 and Thr308 was unaltered by thapsigargin in SHP-2 siRNA-treated myoblasts (Fig. 3B). Importantly, the ability of thapsigargin to rescue the S6K1 phosphorylation defect in SHP-2 siRNA-treated myoblasts was independent of Akt phosphorylation since phosphorylation of both Akt Ser473 and Thr308 was unaffected by thapsigargin in SHP-2 siRNA-treated myoblasts (Fig. 3B). These results demonstrate that SHP-2 functions upstream of Ca\(^{2+}\) to promote leucine-induced S6K1 phosphorylation in myoblasts.

**SHP-2 is required for leucine-induced Ca\(^{2+}\) signaling.** In order to determine whether nutrient stimulation induces [Ca\(^{2+}\)]\(_i\) increases in myoblasts, we performed single-cell Ca\(^{2+}\) imaging experiments. Myoblasts were loaded with the Ca\(^{2+}\) indicator dye Fluo-4 and were leucine deprived for 30 min. Leucine stimulation was found to induce a transient increase in [Ca\(^{2+}\)]\(_i\), in ATP-re-
responsive myoblasts (Fig. 4A). The increase in \([\text{Ca}^{2+}]_i\), was leucine specific, since isoleucine failed to elicit a \([\text{Ca}^{2+}]_i\) response under similar conditions (Fig. 4B and C). Previously, our lab has shown that SHP-2 is required for the mobilization of intracellular \([\text{Ca}^{2+}]_i\) in response to various stimuli (26, 41). We therefore tested whether SHP-2 could mediate leucine-induced \([\text{Ca}^{2+}]_i\) release as a potential mechanism for its effects on S6K1 phosphorylation. We found that leucine-induced mobilization of \([\text{Ca}^{2+}]_i\), was abolished in myoblasts transfected with SHP-2 siRNA (Fig. 4D and F; see Movie S1 in the supplemental material), whereas nontargeting siRNA-transfected myoblasts exhibited a robust \([\text{Ca}^{2+}]_i\) increase (Fig. 4D and E; see Movie S2 in the supplemental material). These results indicate that SHP-2 is required for leucine-induced \([\text{Ca}^{2+}]_i\) signaling in myoblasts.

**SHP-2 mediates leucine-induced \([\text{Ca}^{2+}]_i\) increase in an IP₃-independent manner.** Increases in \([\text{Ca}^{2+}]_i\) levels originate from the

FIG 4 SHP-2 is required for leucine-induced \([\text{Ca}^{2+}]_i\) signaling in myoblasts. (A to C) Single-cell \([\text{Ca}^{2+}]_i\) recordings of Fluo-4-loaded C2C12 myoblasts stimulated with leucine (A) or isoleucine (B). After either leucine or isoleucine stimulation, cells were stimulated with ATP (10 μM). (C) Percentage of ATP-responsive cells (n > 200) responding to either leucine or isoleucine stimulation from panels A and B. (D) Representative images from \([\text{Ca}^{2+}]_i\) imaging experiments performed on C2C12 myoblasts transfected with nontargeting (NT) or SHP-2 siRNA. (E and F) Single-cell \([\text{Ca}^{2+}]_i\) recordings of C2C12 myoblasts transfected with nontargeting (NT) (E) or SHP-2 (F) siRNA. (G) Percentage of ATP-responsive cells (n > 200) responding to leucine stimulation in panels E and F. F/F₀, fluorescence intensity over baseline.
influx of Ca\(^{2+}\) from extracellular sources and/or from the release of Ca\(^{2+}\) from intracellular stores (42). To determine the origin through which SHP-2 mediates leucine-induced [Ca\(^{2+}\)]\(_i\), increases in myoblasts, we performed single-cell Ca\(^{2+}\) imaging experiments in which extracellular Ca\(^{2+}\) was buffered with EGTA. In the absence of extracellular Ca\(^{2+}\) (Fig. 5B and C), leucine was capable of inducing a Ca\(^{2+}\) response as efficiently as in the presence of extracellular Ca\(^{2+}\) (Fig. 5A and C). These results demonstrate that the major component of leucine-induced [Ca\(^{2+}\)]\(_i\) increases mobilized by SHP-2 is derived from intracellular rather than extracellular sources. In myoblasts, increases in [Ca\(^{2+}\)]\(_i\) may also stem from the actions of Ca\(^{2+}\) channels such as the ryanodine receptors (RyR) (43). Treatment of C2C12 cells with the RyR inhibitor dantrolene had no effect on leucine-induced Ca\(^{2+}\) release (Fig. 5E and F) compared with that of the control cells (Fig. 5D and F), demonstrating that ryanodine receptors play a negligible role in nutrient-induced Ca\(^{2+}\) signaling in myoblasts.

Since we found that leucine was capable of mobilizing intracellular Ca\(^{2+}\) stores, we next asked whether the IP\(_3\) receptor (IP\(_3\)R), which is responsible for liberating Ca\(^{2+}\) from the endoplasmic reticulum, was a downstream target of leucine in myoblasts. We tested this by using the IP\(_3\)R inhibitor 2-aminoethoxydiphenyl borate (2-APB), which effectively inhibited leucine-induced increases in [Ca\(^{2+}\)]\(_i\) (Fig. 6B and C) compared with vehicle control...
These results suggest that leucine mobilizes intracellular Ca\(^{2+}\) stores via an IP\(_3\)-dependent mechanism. In order to specifically test the IP\(_3\) dependency of leucine-induced [Ca\(^{2+}\)]\(_i\), mobilization, we overexpressed an IP\(_3\)-interfering decoy referred to as an IP\(_3\) sponge. The IP\(_3\) sponge encodes the ligand-binding domain (residues 224 to 605) of the IP\(_3\) receptor (InsP\(_3\)R) type I fused to a nuclear exclusion signal (NES) and monomeric red fluorescent protein (mRFP) (44, 45). This fragment of the IP\(_3\)R binds to IP\(_3\) with sufficient affinity to outcompete the endogenous receptor, hence acting as a “sponge” to capture free IP\(_3\) in the cytoplasm (44). When expressed in myoblasts, the IP\(_3\) sponge was appropriately expressed and localized to the cytoplasm (Fig. 6D). Upon leucine stimulation, we found that IP\(_3\) sponge-expressing myoblasts were completely impaired in their ability to increase [Ca\(^{2+}\)]\(_i\) (Fig. 6E to G). Taken together, these results demonstrate that in myoblasts SHP-2 is responsible for mobilizing leucine-induced increases in [Ca\(^{2+}\)]\(_i\), from IP\(_3\)-sensitive stores.

**Leucine-induced S6K1 phosphorylation is dependent upon tyrosine kinase signaling.** One of the proposed targets for the increases in Ca\(^{2+}\) is the Ca\(^{2+}\)-calmodulin-regulated class III PI 3'-kinase, hVps34, which plays an essential role in mTORC1/S6K1 activation (38). We investigated whether hVps34 could serve as the target for Ca\(^{2+}\) in response to leucine stimulation in myoblasts. However, we found that when hVps34 was knocked down in myoblasts, leucine-induced S6K1 activation was unaltered (Fig. 7A). These results demonstrate that in response to leucine, SHP-2 couples to S6K1 phosphorylation in myoblasts independently of hVps34.
In order to examine alternative mechanisms for the involvement of SHP-2 in leucine-induced S6K1 phosphorylation, we tested whether leucine mediated changes in total tyrosyl phosphorylation and whether this was influenced by SHP-2. We found that in response to leucine, total tyrosyl phosphorylation levels were not dramatically altered in either nontargeting or SHP-2 siRNA-treated cells (Fig. 7B). However, pretreatment of myoblasts with the broad-specificity tyrosine kinase inhibitor PP2 (8 μM) blocked leucine-induced S6K1 phosphorylation (Fig. 7C). These results demonstrate that although SHP-2 does not globally alter tyrosyl phosphorylation levels in response to leucine, a PP2-sensitive tyrosine kinase likely participates in the activation of S6K1 phosphorylation in response to leucine.

Given the observation that IP3 and tyrosine kinase signaling are required for leucine-induced S6K1 phosphorylation (Fig. 6 and 7C), we sought to identify the phospholipase responsible for the generation of IP3 in response to leucine. It has been reported that phospholipase Cγ (PLCγ) is activated by tyrosine phosphorylation downstream of SHP-2 (21, 46). Therefore, we investigated whether SHP-2 regulated PLCγ activation in response to leucine. Interestingly, we found that leucine stimulation of myoblasts induced PLCγ tyrosyl phosphorylation at its activation site, and this was inhibited upon siRNA SHP-2 transfection (Fig. 7D). These results demonstrate the existence of a leucine-inducible tyrosine kinase that phosphorylates PLCγ, suggesting that PLCγ might be responsible for mediating leucine-induced Ca2+ release and subsequently S6K1 phosphorylation. Surprisingly, siRNA knockdown of PLCγ did not have an effect on leucine-induced S6K1 phosphorylation (Fig. 7E). These results indicate that although PLCγ is a target for tyrosyl phosphorylation in response to leucine, it is dispensable for the activation of S6K1. Therefore, other phospholipase C isoforms are likely responsible for engagement of the leucine-induced IP3/Ca2+ response to S6K1.

**PLCβ4 is required for leucine-induced S6K1 activation.** Phospholipase Cβ (PLCβ) isoforms represent major downstream phospholipases for the generation of Ca2+ in response to G-protein-coupled receptor (GPCR) activation (47–49). Moreover, SHP-2 has been reported to function downstream of GPCRs (17, 50). To test the possibility that leucine signals through SHP-2 to promote PLCβ activation, we investigated whether leucine-induced Ca2+ increases were pertussis toxin (PTX) sensitive. Myoblasts pretreated with PTX were approximately 50% less responsive to leucine-induced Ca2+ mobilization (Fig. 8A). Consistent with this, we observed that leucine-induced S6K1 phosphorylation was also sensitive to GPCR inhibition by PTX (Fig. 8B). Of the four PLCβ family members, PLCβ4 is most abundantly expressed in C2C12 myoblasts (51). In order to determine whether PLCβ4 has a role in leucine-induced activation of S6K1, we knocked down PLCβ4 in C2C12 myoblasts (Fig. 8C). Myoblasts lacking PLCβ4 significantly inhibited leucine-induced S6K1 phosphorylation (Fig. 8C). These results demonstrate that PLCβ4 contributes to leucine-induced S6K1 phosphorylation.

To examine whether SHP-2 signals upstream of PLCβ4 in the activation of S6K1, we knocked down PLCβ4 in the presence of a constitutively active mutant of SHP-2 (SHP-2 E76A) (25). Whereas overexpression of SHP-2 E76A enhanced leucine-induced S6K1 phosphorylation (Fig. 8D), knockdown of PLCβ4 along with SHP-2 E76A overexpression inhibited S6K1 phosphorylation (Fig. 8D), demonstrating that SHP-2 functions upstream of PLCβ4. Previously we found that activated mutants of SHP-2 were capable of potentiating Ca2+ responsiveness to growth factors (26). Therefore, we tested whether SHP-2 E76A potentiated leucine-induced Ca2+ signaling. We found that at subsaturating concentrations of leucine, Ca2+ signaling was potentiated in myoblasts expressing SHP-2 E76A (Fig. 8E). Collectively, these observations imply that SHP-2-regulated S6K1 activation is dependent upon PLCβ4.

**SHP-2 is required to couple amino acid sensing to autophagy and cell growth in myoblasts.** Autophagy is a nutrient-sensitive process induced upon starvation, and it is suppressed when nutrients are in sufficient supply (52). Microtubule-associated protein 1 light chain 3 (LC3) is a 17-kDa soluble cytoplasmic protein that is posttranslationally modified during autophagy from the cytosolic form of LC3 (LC3-I) to the LC3-phosphatidylethanolamine conjugate form (LC3-II) (53, 54). The conversion of LC3-I to LC3-II is indicative of autophagic activity and was used to monitor the effects of SHP-2 on autophagy during conditions of leucine sufficiency. When nontargeting-siRNA-transfected myoblasts were leucine deprived, both LC3-1 and LC3-II were readily detectable (Fig. 9A). In nontargeting-siRNA-transfected cells, LC3-II levels decreased upon leucine stimulation, indicating the suppression of autophagy (Fig. 9A). However, in SHP-2 siRNA-transfected myoblasts, leucine stimulation failed to appreciably alter LC3-II levels (Fig. 9A). These results provide further evidence that SHP-2 participates in nutrient sensing in the mTORC1 pathway and demonstrate that SHP-2 is required in myoblasts to couple nutrient sufficiency to autophagy.

In addition to regulating autophagy, nutrient sensing is tightly coupled to cell growth through the actions of mTORC1/S6K1 (55). Under conditions of nutrient sufficiency (growth medium), the mTORC1 inhibitor rapamycin inhibited cell size by ~10% (Fig. 9B and D). In contrast, SHP-2 siRNA-transfected myoblasts exhibited an ~5% reduction in cell size (Fig. 9C and D). Elimination of SHP-2 expression along with rapamycin treatment did not further decrease cell size (Fig. 9D). These data demonstrate that SHP-2 plays a positive role in cell size regulation under nutrient-sufficient conditions, which is consistent with the interpretation that SHP-2 coordinates S6K1 activation in response to nutritional status.

**DISCUSSION**

In this study, we have shown that SHP-2 serves as an upstream regulator of mTORC1 in the nutrient-responsive pathway. We propose a model whereby SHP-2 becomes activated in response to nutrient stimulation, resulting in the generation of IP3, from PLCβ4 (Fig. 9E). The involvement of PLCβ4 was consistent with leucine-induced S6K1 phosphorylation being sensitive to both PLCβ4 knockdown and pertussis toxin. Given that PLCβ4 can be activated by GPCRs (47), our data raise the possibility that SHP-2 provides additional signals that are required for optimal PLCβ4 activation in response to nutrients. In this regard, it has been shown that PLCβs can become tyrosyl phosphorylated in response to GPCR activation (56). However, the consequences of PLCβ4 tyrosyl phosphorylation in response to nutrients are unknown. Nevertheless, our model clearly invokes SHP-2, and by inference the involvement of tyrosine phosphorylation, as an essential element in the generation of Ca2+ as a mechanism leading to S6K1 activation in response to nutrients (Fig. 9E).

Our previous studies suggested that SHP-2 is involved in the regulation of cell mass (22, 53). Under conditions of growth factor
deprivation/low energy, SHP-2 negatively regulates the mTORC1 pathway and cell size (23). In addition, we have observed that in mice with a skeletal muscle-specific SHP-2 deletion there is a reduction in myofiber size (22). Although we attribute the reduction in myofiber size to impairment of the nuclear factor of activated T cell pathways, it is important to note that this pathway is also Ca\textsuperscript{2+}/H\textsubscript{11001} dependent. Therefore, it is conceivable that impaired Ca\textsuperscript{2+}/H\textsubscript{11001} signaling disrupts both S6K1 and nuclear factor of activated T cells signaling, resulting in reduced skeletal muscle growth. Collectively, in the context of these observations and those presented here, we suggest a dual role for SHP-2 in the mTORC1/S6K1 pathway. The first appears to be a negative one, in which under conditions of low energy SHP-2 suppresses S6K1 activity (23); and as shown here, a second role is proposed whereby SHP-2 positively

FIG 8 SHP-2 acts upstream of PLC\textsubscript{4} in the activation of S6K1. (A) C2C12 myoblasts were pretreated with pertussis toxin (PTX). Cells were leucine deprived and Fluo-4 loaded for 30 min. Single-cell Ca\textsuperscript{2+} measurements were recorded in C2C12 myoblasts upon leucine (Leu) stimulation. The graph represents the percentages of TG-responsive myoblasts (n > 200) responding to leucine stimulation. (B) C2C12 myoblasts were pretreated for 5 h with either vehicle or 1 \mu g/ml PTX. Myoblasts were subjected to leucine deprivation and stimulated with leucine (Leu) in the presence or absence of PTX. The graph shows the densitometric analyses of the relative S6K1 phosphorylation (pS6K1/S6K1) representing the means ± SEM from 3 independent experiments (*, P < 0.05). (C) C2C12 myoblasts transfected with either nontargeting (NT) or PLC\textsubscript{4} siRNA were leucine deprived and restimulated with leucine. Cell lysates were subjected to immunoblotting with pS6K1, S6K1, and PLC\textsubscript{4} antibodies. The graph shows the densitometric analyses of the relative S6K1 phosphorylation (pS6K1/S6K1) representing the means ± SEM from 3 independent experiments (*, P < 0.05). (D) C2C12 myoblasts were transfected with either nontargeting (NT) or PLC\textsubscript{4} siRNA along with either vector or a constitutively activated SHP-2 mutant (SHP-2 E76A). Myoblasts were leucine deprived and restimulated with leucine (Leu) for 10 min. Cell lysates were subjected to immunoblotting with pS6K1, S6K1, SHP-2, and PLC\textsubscript{4} antibodies. The graph shows the densitometric analyses of the relative S6K1 phosphorylation (pS6K1/S6K1) representing the means ± SEM from 3 independent experiments (*, P < 0.05; **, P < 0.01; *** P < 0.001; determined by one-way ANOVA). (E) C2C12 myoblasts were transfected with either vector control or SHP-2 E76A. Fluo-4-loaded leucine-deprived cells were stimulated with leucine, and single-cell Ca\textsuperscript{2+} measurements were performed. The graph represents the percentages of TG-responsive myoblasts (n > 200) responding to leucine stimulation.
regulates S6K1 activity under nutrient-sufficient conditions (Fig. 9E). Hence, SHP-2 appears to play a key physiological function in setting the status of protein synthesis dependent upon the nutritional status of the cell. Although the data generated here are consistent with the in vivo phenotype of reduced muscle mass in mice lacking SHP-2 in skeletal muscle, we have also demonstrated that SHP-2 is required for leucine-induced S6K1 activation in fibroblasts (Fig. 1). Therefore, the pathway proposed here (Fig. 9E) may represent a more general mechanism of nutrient-mediated regulation of S6K1 activation by SHP-2. One interesting observation from our study is that the phosphorylation status of 4E-BP1 is less responsive to SHP-2 knockdown in response to amino acid stimulation than to S6K1 knockdown. There have been other instances in the literature where S6K1 phosphorylation and 4E-BP1 phosphorylation are differentially regulated (29, 57). Here, the differential regulation between S6K1 and 4E-BP1 phosphorylation is likely to be a result of 4E-BP1 activation being less sensitive to the effects of Ca\textsuperscript{2+} (Fig. 3A). Therefore, the downstream effect of SHP-2-mediated Ca\textsuperscript{2+} signaling is a likely cause for the differential S6K1 and 4E-BP1 sensitivity.

Previous studies have shown that mTORC1 activation of S6K1 requires Ca\textsuperscript{2+} (13, 14, 38, 39). In HeLa cells, leucine can induce Ca\textsuperscript{2+} mobilization (38). Here we show that in myoblasts, leucine-induced S6K1 phosphorylation is Ca\textsuperscript{2+} dependent and that leucine induces a transient Ca\textsuperscript{2+} signal from IP\textsubscript{3}-regulated stores. We expected that the downstream SHP-2-mediated Ca\textsuperscript{2+} target that
signaled to mTORC1 was likely to be hVps34 (38). However, knockdown of hVps34 in myoblasts failed to impair leucine-induced S6K1 phosphorylation, suggesting that at least in myoblasts, hVps34 is dispensable for S6K1 activation in response to nutrients. As a result of these data, we investigated other potential targets for SHP-2/Ca\(^{2+}\). Amino acids promote the relocalization of mTORC1 to lysosomal membranes to the Rag-Ragulator complex in order to facilitate engagement of Rheb (9). We found that SHP-2 was not involved in regulating the relocation of mTORC1 to lysosomal membranes in myoblasts in response to leucine (Fig. 2E). These data suggest that SHP-2 does not engage the Rag-Ragulator complex in the control of S6K1 activation. Instead, it seems likely that SHP-2 engages a parallel pathway that is mediated by Ca\(^{2+}\). The identification of either the SHP-2/Ca\(^{2+}\) target or the SHP-2 substrate that is dephosphorylated in response to nutrients has yet to be achieved.

We also discovered that SHP-2 mobilizes intracellular Ca\(^{2+}\) stores from the endoplasmic reticulum in an IP\(_3\)-dependent manner. These findings differ from those of Gulati et al., who showed that the amino acid-induced Ca\(^{2+}\) increase emanates from extracellular sources (38). Once again, differences in cell type and possibly experimental conditions account for this discrepancy. It is conceivable that nutrients trigger the Ca\(^{2+}\) response from IP\(_3\)-dependent intracellular stores initially and extracellular Ca\(^{2+}\) influx is required for sustained Ca\(^{2+}\) signals. Nevertheless, the demonstration of disruption of IP\(_3\) binding to endogenous IP\(_3\)Rs in myoblasts using the IP\(_3\) sponge provides strong evidence for the pool of Ca\(^{2+}\) residing in the endoplasmic reticulum. Our data have uncovered a novel role for PLC\(_\beta\)4 in leucine-mediated activation of S6K1, since knockdown of PLC\(_\beta\)4 inhibited leucine-mediated of S6K1 activation. The activation of PLC\(_\beta\)4 and the subsequent generation of IP\(_3\) are dependent upon GPCRs (47). Consistent with this, pertussis toxin inhibited leucine-induced S6K1 phosphorylation. There exist GPCRs that serve as receptors for a broad spectrum of ligands, including amino acids (38, 59).

Interestingly, recent work suggests that the G-protein-coupled taste receptor T1R1/T1R3 is involved in mediated amino acid-induced activation of mTORC1/S6K1 (60). Whether SHP-2 is a downstream effector of T1R1/T1R3 remains to be established; although disruption of T1R1/T1R3 interferes with mTORC1 localization and involves ERK1/2, SHP-2 does not seem to affect either of these in response to leucine. Thus, GPCRs in addition to T1R1/T1R3 might play a role in transducing SHP-2-mediated nutrient responsiveness. Nevertheless, these data along with those presented here raise new possibilities for the role of GPCRs in mTORC1 signaling. Other targets for leucine sensing have also been uncovered; it has been found that leucine binds directly to mTORC1 signaling. Other targets for leucine sensing have also been uncovered; it has been found that leucine binds directly to mTORC1 signaling.

In summary, we have uncovered a novel role for SLP-2 in nutrient-responsive signaling in muscle cells. SLP-2 promotes Ca\(^{2+}\) mobilization in response to nutrient load and couples the activation of mTORC1/S6K1 to the controls of autophagy and cell growth (Fig. 9E). A number of studies have shown that mice lacking expression of SLP-2 either peripherally or centrally exhibit a variety of metabolic defects (64–67). Altered mTORC1 signaling in these scenarios could contribute to these metabolic defects, given the important role of mTORC1/S6K1 in metabolism (1). SLP-2 can exhibit oncogenic properties, and as such it would be instructive to explore the possibility that cancers driven by activating SLP-2 mutations can be treated with mTORC1 inhibitors such as rapamycin. Further mechanistic studies are required to identify both the upstream activators and the downstream substrates of SLP-2 in this pathway, which will ultimately shed further light into the mechanisms of nutrient signaling.

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REFERENCES


20. Reference deleted.


