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IGF-1-induced phosphorylation and altered distribution of TSC1/TSC2 in C2C12 myotubes

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Abstract

Insulin like growth factor-1 (IGF-1) is established as an anabolic factor that can induce skeletal muscle growth through activating the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway. While this signaling pathway has been heavily studied, the molecular mechanisms linking IGF-1 binding to mTOR activation are still poorly defined in muscle. The purpose of this study was to test the hypothesis that IGF-1 activation of mTOR in C2C12 myotubes requires a phosphorylation dependent, altered distribution of the tuberous sclerosis complex (TSC)1/TSC2 complex from the membrane to the cytosol. We found that IGF-1 treatment does not affect complex formation between TSC1 and TSC2, but rather IGF-1 induces an altered distribution of the TSC1/TSC2 complex in C2C12 myotubes. In response to IGF-1 treatment, there was a relative re-distribution of the TSC1/TSC2 complex, composed of TSC1 and phosphorylated TSC2, from the membrane to the cytosol. IGF-1 stimulated TSC1/TSC2 phosphorylation and re-distribution were completely prevented by the PI3K inhibitor wortmannin, but were not with the downstream mTOR inhibitor, rapamycin. When a non-phosphorylatable form of TSC2 (S939A) was overexpressed, phosphorylation-dependent binding of the scaffold protein 14-3-3 to TSC2 was diminished and no re-distribution of the TSC1/TSC2 complex was observed following IGF-1 stimulation. These results indicate that TSC2 phosphorylation in response to IGF-1 treatment is necessary for the altered distribution of the TSC1/TSC2 complex to the cytosol and we suggest that this translocation is likely critical for mTOR activation by dissociating the interaction between the GAP activity of the TSC1/TSC2 complex and its downstream target Rheb.

Keywords

mTOR; TSC1; TSC2; wortmannin; rapamycin

Introduction

Skeletal muscle mass is generally thought to be determined by the net balance between the rates of protein synthesis and degradation [1,2]. To date, numerous studies have shown that the mammalian target of rapamycin (mTOR) plays a critical role in regulating the rate of protein synthesis and cell hypertrophy in skeletal muscle [3–6]. mTOR is a serine/threonine kinase of the phosphatidylinositol kinase-related kinase family and is highly conserved from yeast to mammals [7]. In skeletal muscle cells, mTOR serves as a central integrator of a wide range of signals that function to either activate or inhibit protein synthesis and cell growth [2]. The most well defined signaling mechanism regulating mTOR activity in

skeletal muscle is the insulin like growth factor-1 (IGF-1)/insulin-dependent pathway [5,6]. Stimulation of muscle cells with growth factors such as IGF-1 lead to activation of phosphoinositide 3-kinase (PI3K) and its downstream effector Akt, triggering multiple downstream signaling events including mTOR activation [8].

Studies in both *Drosophila* and mammalian cells have indicated that Akt influences mTOR signaling through regulation of the protein complex of tuberous sclerosis complex (TSC) 1/TSC2 [9-12]. The TSC1/TSC2 protein complex is composed of the heterodimer TSC1 and TSC2, and is a direct target of Akt phosphorylation. Within the TSC1/TSC2 protein complex, TSC2 functions as a GTPase activating protein (GAP) for Ras homolog enriched in brain (Rheb), a small G protein. The GTP-bound active form of Rheb strongly stimulates mTOR and its downstream targets, such as the ribosomal S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E-binding protein (4EBP-1) [13-16].

It is now generally recognized that the primary function of the TSC1/TSC2 protein complex is to inhibit mTOR signaling. Cells or tissues with depleted levels of TSC1 or TSC2 have higher mTOR activity, resulting in a robust cell growth and tumorigenesis [9,10,17,18]. Although it is clear that growth factor-induced activation of Akt blocks TSC1/TSC2 inhibition of mTOR signaling [9-11], the molecular mechanism by which Akt inhibits the function of TSC1/TSC2 protein complex as a cell growth suppressor is still undefined. A study using *Drosophila* cells provided evidence that the TSC1/TSC2 protein complex became dissociated upon insulin stimulation and was dependent on TSC2 phosphorylation by Akt [9]. Cai and co-workers [19] have reported that phosphorylation of TSC2 by Akt caused the translocation of TSC2 from the membrane to the cytosolic fraction. The movement of phosphorylated TSC2 to the cytosol, away from membrane-bound TSC1, relieved TSC1/TSC2 protein complex inhibition of Rheb [19]. In contrast, Dong and colleagues found that neither insulin stimulation nor a non-phosphorylatable TSC2 mutant (S924A/T1518A) altered the complex formation of TSC1/TSC2 in *Drosophila* cells [20]. In addition, other groups have also reported that in mammalian cells Akt-mediated phosphorylation of TSC2 had no effect on the TSC1/TSC2 complex [11,21].

To date, few studies have examined the role of TSC1 or TSC2 during IGF-1-induced skeletal muscle hypertrophy. The objective of the current study was to test the hypothesis that IGF-1 activation of mTOR in C2C12 myotubes requires the altered distribution of TSC1/TSC2 complex within the cell. We found that IGF-1 treatment does not affect complex formation between TSC1 and TSC2, but rather IGF-1 induces an altered distribution of TSC1/TSC2 protein complex from the membrane to the cytosolic fraction in C2C12 myotubes. We suggest that this step is likely critical for mTOR activation by dissociating the interaction between the GAP activity of the TSC1/TSC2 complex and Rheb.

RESULTS AND DISCUSSION

IGF-1-induced activation of mTOR signaling is prevented by PI3K inhibitor wortmannin and mTOR inhibitor rapamycin

In this study, the C2C12 cell line from mouse skeletal muscle was used as a model system because this cell line has been widely used to study the signaling pathways involved in IGF-1 hypertrophic stimulation [6]. Previous studies have demonstrated that IGF-1-induced muscle cell growth is mediated through PI3K/Akt/mTOR-dependent signaling pathway [5,6]. To confirm these earlier reports, we examined the phosphorylation states of five different targets of PI3K/Akt/mTOR signaling following IGF-1 stimulation in C2C12 myotubes. As shown in Fig 1A, each of the PI3K/Akt/mTOR targets was phosphorylated upon IGF-1 stimulation. The PI3K inhibitor wortmannin blocked phosphorylation of each PI3K/Akt/mTOR target which included Akt (T308 and S473), S6K1 (T389 and T421/S424),

IRS-1 (S636/639), PRAS40 (T246) and GSK3- α/β (S21/9). A relatively high concentration of wortmannin (10 μ M) was required to inhibit insulin-induced activation of mTOR signaling in C2C12 myotubes (Fig S1A) so we can not rule out the possibility of inhibition of other lipid or protein kinases besides class-I PI3K. Treatment of the IGF-1 stimulated cells with the mTOR inhibitor, rapamycin (50 nM), blocked phosphorylation of S6K1 and IRS-1 but not phosphorylation of upstream or independent signaling molecules such as Akt, PRAS40 and GSK3.

In addition to the PI3K/Akt-dependent signaling, it is known that IGF-1 also activates the Ras-Raf-mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway [22,23]. Recent studies have reported that a MEK/ERK-dependent pathway may contribute to cell growth and tumor progression through modulating mTOR signaling [24,25]. To investigate whether or not this pathway was operative in muscle cells, the effects of IGF-1 stimulation on the MEK/ERK-dependent signaling cascade was examined in C2C12 myotubes. IGF-1-induced ERK1/2 phosphorylation was detected rapidly and was transient; phosphorylation of ERK1/2 was induced within 10 min and phosphorylation levels were back to basal by 60 min, unlike phosphorylation of Akt and S6K1, following IGF-1 stimulation (Fig S1B and Fig 3A). The rapid phosphorylation of ERK1/2 was completely prevented by the MEK inhibitor U0126 (10 μ M). In contrast, there were no inhibitory effects of U0126 treatment on IGF-1-induced phosphorylation of either Akt or S6K1 (Fig S1C). These results support the hypothesis that IGF-1-induced activation of mTOR signaling is mediated through PI3K/Akt-dependent signaling pathway in skeletal muscle cells.

IGF-1 induced TSC2 phosphorylation and subcellular localization of TSC1/TSC2 are modulated by a PI3K/Akt-dependent mechanism

Previous studies have demonstrated that mTOR activity is modulated via Akt-dependent phosphorylation of the TSC1/TSC2 protein complex [10,11,19], though the precise molecular mechanism remains undefined. We examined the protein complex formation of TSC1 and TSC2 as well as the TSC2 phosphorylation status following IGF-1 stimulation. It has been shown that the two conserved phosphorylation sites on TSC2, S939 and T1462, are directly phosphorylated by Akt [21]. As shown in Fig 1B, both S939 and T1462 sites on TSC2 were phosphorylated in C2C12 myotubes following IGF-1 stimulation. IGF-1-induced phosphorylation of TSC2 was blocked by treatment with wortmannin, the PI3K inhibitor, but no effect was observed using rapamycin, the mTOR inhibitor. Furthermore, co-immunoprecipitation with either TSC1 or TSC2 revealed that IGF-1 stimulation or inhibitor treatment did not alter the complex formation between TSC1 and TSC2, or the amount of either TSC1 or TSC2 protein levels (Fig 1B). This finding is contrary to previous studies which reported phosphorylation of TSC2 caused a dissociation of the TSC1/TSC2 protein complex and ubiquitin-mediated degradation of TSC2 protein [9,10,25,26]. One possible explanation for the difference between our results and others is that because the levels of phosphorylated to total TSC2 are so low that we might not have been able to detect selective loss of the phosphorylated TSC2 from the TSC1/TSC2 protein complex. In C2C12 myotubes, however, we found no effect of IGF-1 stimulation on the functional interaction between TSC1 and TSC2, even though the phosphorylation level of TSC2 was altered. Stability of the protein complex between TSC1 and phosphorylated TSC2 was confirmed by co-immunoprecipitation experiments with each phospho-specific antibody toward TSC2. IGF-1 treatment resulted in the TSC2 phosphorylation (S939 and T1462 residues) and a concomitant increase in the relative amount of total-TSC1 which was bound to phosphorylated TSC2 protein (Fig 1C). The failure to detect a change in the stability of the TSC1/TSC2 complex was not specific to muscle cells as complex formation between TSC1 and TSC2 was not changed in response to IGF-1 treatment in human embryonic kidney

(HEK)293 cells (Fig S2). These observations suggest that the stability of the complex between TSC1 and TSC2 is not a critical factor in determining the function of the TSC1/TSC2 protein complex in response to IGF-1 treatment.

To investigate the subcellular localization profile of TSC1 and TSC2 in C2C12 myotubes, the C2C12 lysates were fractionated to yield the membrane and the cytosolic fractions by ultracentrifugation techniques. A previous study by Cai et al. (2006) demonstrated that phosphorylation of TSC2 by Akt caused the translocation of TSC2 protein from the membrane to the cytosolic fraction [19]. Based on these findings, we examined whether or not IGF-1 stimulation caused a re-distribution of TSC1 and/or TSC2 from the membrane to the cytosol in skeletal muscle cells. Under control conditions, TSC1 and TSC2 proteins were detected in both membrane and cytosolic fractions (Fig 2A). Following 1 hr stimulation with IGF-1, there was an increase in the relative amount of both TSC1 and TSC2 proteins in the cytosolic fraction which was associated with a concomitant decrease in the relative abundance of TSC1 and TSC2 protein in the membrane fraction; compared to control, IGF-1 stimulation decreased membrane TSC1 and TSC2 by 36% and 51%, respectively, while cytosolic TSC1 and TSC2 increased 57% and 63%, respectively (Fig 2B - 2E). This IGF-1-induced alteration in the subcellular distribution of TSC1 and TSC2 was completely blocked by wortmannin but not by rapamycin treatment (Fig 2B - 2E). The re-distribution of the TSC1/TSC2 protein complex to the cytosol was confirmed by co-immunoprecipitation experiments with either TSC1 or TSC2 which revealed more TSC1/TSC2 protein complex within the cytosolic fraction following IGF-1 stimulation (Fig 2F). The alteration in the distribution of TSC1 and TSC2 proteins was detected within the 10 min of IGF-1 stimulation which is consistent with the timeframe of Akt and S6K1 phosphorylation (Fig 3). These findings suggest that IGF-1 treatment leads to a change in the distribution of the TSC1/TSC2 protein complex from the membrane to the cytosol that is mediated by a PI3K/Akt-dependent phosphorylation of TSC2. The localization of the downstream target of TSC2, the small G-protein Rheb, was not affected by IGF-1 stimulation, remaining localized to the membrane fraction (Fig 2A). One implication of these results is that mTOR activity is increased as the TSC1/TSC2 protein complex moves away from the membrane fraction. To support this idea, it has been shown that the phosphorylation state of TSC2 does not affect its GAP activity towards recombinant Rheb [21,27]. Huang and Manning have indicated that phosphorylation status of TSC2 on S939 and T1462 were significantly changed with serum-starvation or growth-factor-stimulation, but they could not detect any differences in its GAP activity toward G-protein Rheb [21]. Cai et al. also reported that the TSC2 mutant lacking Akt-dependent phosphorylation sites (S939A and S981A) maintains the same level of GAP activity toward Rheb compared to the wild-type TSC2, even after insulin treatment [19]. Collectively, our findings support a mechanism whereby TSC2 phosphorylation leads to its translocation from the membrane to the cytosol and it is this physical separation, and not changes in GAP activity, that results in increased GTP-bound active form of Rheb and higher mTOR activity. It is interesting to note, however, that the amount of TSC1 and TSC2 in the membrane fraction is still quite high after IGF-1 stimulation (Fig 2). Further studies will be necessary to determine whether or not the remaining TSC1/TSC2 protein complex in the membrane fraction does act as a GAP for Rheb.

Phosphorylation-dependent 14-3-3 interaction is associated with increased cytosolic pool of TSC1/TSC2

Prior studies have reported that the 14-3-3 protein can directly bind to phosphorylated TSC2 and modulate its subcellular localization [19,28-31]. In C2C12 myotubes, 14-3-3 protein is almost exclusively localized in the cytosol suggesting a similar mechanism may underlie the change in distribution of TSC2 with IGF-1 stimulation (Fig 2). To test this idea, we performed co-immunoprecipitation assays to determine whether or not 14-3-3 and TSC2

interact in C2C12 myotubes and, if so, does this interaction change upon IGF-1 stimulation. As shown in Fig 4, IGF-1 treatment resulted in a 2.4-fold increase in the amount of total-TSC2 which was bound to 14-3-3 protein. We found that the phosphorylation level of TSC2 protein at S939 and T1462 sites, which was associated with 14-3-3 protein, was increased after IGF-1 stimulation. Furthermore, the IGF-1-induced increase in 14-3-3/TSC2 interaction and phosphorylation of TSC2 were blocked by wortmannin but not rapamycin (Fig 4A). These findings suggest that the interaction between 14-3-3 and TSC2 is through a PI3K/Akt-dependent phosphorylation.

According to the motif scanning analysis (<http://scansite.mit.edu/>), TSC2 contains three putative sites which are both 14-3-3 recognition motifs and Akt phosphorylation sites (S939, S981 and S1130; Fig S3). To test the idea that S939 site in TSC2 is a phosphorylation-dependent 14-3-3 binding site, we examined the ability of a non-phosphorylatable form of TSC2 (S939A) to interact with 14-3-3. As a control, we used a second TSC2 mutant that eliminated a putative Akt phosphorylation site (T1462A) but did not harbor a 14-3-3 binding site. It was confirmed that both mutant forms of TSC2 were not recognized by the phosphospecific antibodies (data not shown). As shown in Fig 5A, the interaction between TSC2 mutant S939A and 14-3-3 was clearly decreased compared to wild-type TSC2. In contrast, there was no change in 14-3-3 interaction with the T1462A TSC2 mutant from wild-type TSC2. These results provide evidence that phosphorylation of TSC2 at the S939 site is the primary recognition motif of 14-3-3 protein binding.

Having established the importance of S939 site in TSC2 for phosphorylation-dependent 14-3-3 interaction, we next wanted to know if this site had a role in the increased cytosolic pool of TSC1/TSC2 protein complex upon IGF-1 stimulation. As shown in Fig 5B, protein abundance of myc-tagged TSC1 and Flag-tagged TSC2 (wild-type) in the cytosolic fraction were increased by IGF-1 stimulation. The increased cytosolic pool of TSC2 required an intact S939 phosphorylation site, as alanine substitution completely eliminated subcellular re-distribution of TSC2 to the cytosolic fraction with IGF-1 stimulation; cytosolic myc-TSC1 and wild-type Flag-TSC2 increased by 51% and 44%, respectively, following IGF-1 stimulation whereas no change was detected when the mutant Flag-TSC2 (S939A) was used (Fig 5B). These observations clearly show that Akt-dependent phosphorylation and 14-3-3 protein binding to the TSC2 on S939 site is required for the sequestration away from the membrane fraction in C2C12 myotubes.

Summary

In this study, we have shown in skeletal muscle cells that 1) IGF-1 stimulation leads to Akt-dependent phosphorylation of TSC2 on residue S939, 2) IGF-1 stimulation does not affect complex formation between TSC1 and TSC2, but results in re-distribution of the TSC1/TSC2 protein complex from the membrane to the cytosol and 3) alteration in the subcellular distribution of TSC1/TSC2 protein complex was mediated by the phosphorylation-dependent binding of 14-3-3 proteins to the TSC2 S939 site. Collectively, these findings provide a plausible Akt-dependent mechanism by which IGF-1 stimulates mTOR activity; Akt phosphorylation of TSC2 on S939 promotes interaction with 14-3-3 scaffold protein which results in the translocation of the TSC1/TSC2 protein complex to the cytosol, thus limiting TSC2:GAP activity toward Rheb. The results of the current study represent the first evidence in skeletal muscle cells showing how growth factor-induced PI3K/Akt activation regulates mTOR activity by modulating TSC1/TSC2 protein complex and Rheb interaction.

MATERIALS AND METHODS

Materials

C2C12 mouse myoblasts and HEK293 cells were purchased from ATCC (Manassas, VA). High-glucose DMEM, fetal bovine serum and horse serum were from GIBCO (Grand Island, NY). FuGENE 6 Transfection Reagent was from Roche (Indianapolis, IN). Immobilized protein A plus was from PIERCE (Rockford, IL). Long R³ IGF-1 (recombinant analog) and protease inhibitor cocktail for mammalian tissues (P8340) were from Sigma-Aldrich (St. Louis, MO). Wortmannin and rapamycin were from Calbiochem (San Diego, CA). U0126 was from LC Laboratories (Woburn, MA). Protein assay dye reagent concentrated was from Bio-Rad Laboratories (Hercules, CA). Glutathione sepharose 4B, ECL and ECL plus solutions were obtained from GE Healthcare (Piscataway, NJ). *Antibodies*: phospho-S6K1 (T389), phospho-S6K1 (T421/S424), Akt, phospho-Akt (T308), phospho-Akt (Ser473), IRS, phospho-IRS (S636/S639), PRAS40, phospho-PRAS40 (T246), phospho-GSK3 α/β (S21/9), TSC1, phospho-TSC2 (S939), phospho-TSC2 (T1462), phospho-ERK1/2 (T202/T204), ERK1/2 and Rheb were from Cell Signaling Technology (Danvers, MA). GSK3 β was from BD Transduction Laboratories (San Jose, CA). Tuberin (C-20) and S6K1 (C-18) were from Santa Cruz Biotechnology (Santa Cruz, CA). 14-3-3, Pan Ab-4 (CG15) was from Thermo Scientific (Fremont, CA). The ANTI-FLAG polyclonal antibody was from Sigma-Aldrich (Saint Louis, MO). Rabbit polyclonal to myc tag and mouse monoclonal (CH-19) to pan-cadherin were from abcam (Cambridge, MA). GST antibody was from Bethyl Laboratories (Montgomery, TX). Peroxidase labeled anti-rabbit IgG and anti-mouse IgG secondary antibodies were from Vector Laboratories (Burlingame, CA). *Plasmids*: pcDNA3.1-myc-TSC1 (12133), pcDNA3-Flag-TSC2 (14129), pcDNA3-Flag-TSC2-S939A (14132), pcDNA3-Flag-TSC2-T1462A (14130) and pGEX-2TK-14-3-3 beta GST (13276) were purchased from Addgene (Cambridge, MA).

Cell culture and transfection

All cell culture experiments were performed in a humidified environment at 37 °C in a 5% CO₂. The skeletal muscle cell line C2C12 myoblasts were grown in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at low confluence. Myoblasts were transfected while the cells were in suspension [32]. To induce differentiation, culture medium was switched to 2% horse serum when cells were fully confluent. *IGF-1 treatment*: C2C12 myotubes at 4 days of differentiation were treated with serum/antibiotics free media for 120 min and then stimulated for 60 min with recombinant IGF-1 (100 ng/ml in serum/antibiotics free DMEM), co-incubated with/without wortmannin (10 μ M), rapamycin (50 nM) or U0126 (10 μ M). Series of the experiments were repeated at least 3 times with using different passage of C2C12 myotubes.

Immunoprecipitation assay and western blotting

To study the functional interactions between TSC1, TSC2 and/or 14-3-3, co-immunoprecipitation assays were performed as described previously [33]. CHAPS based buffer (0.3% CHAPS, 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM B-glycerophosphate, 50 mM NaF and 10 μ l/ml protease inhibitor cocktail) was used to produce total cell lysates. One milligram of total protein was used from cell lysates and samples were immunoprecipitated with each antibody and immobilized protein A. Immunocomplexes were washed for three times with CHAPS based buffer and then washed once with wash buffer (50 mM HEPES (pH 7.5), 40 mM NaCl and 2mM EDTA). Precipitated protein samples were then subjected to SDS-PAGE. Western blotting assay was carried out as previously described [33].

Subcellular fractionation

The C2C12 myoblasts were plated in 60 mm diameter cell culture dish and then differentiated to myotubes for 4 days. C2C12 myotubes were washed twice with PBS and then scraped off in 3 ml of ice-cold buffer (20 mM Tricine (pH 7.8), 250 mM sucrose, 1 mM EDTA (pH 8.0) and 10 µl/ml protease inhibitor cocktail). The samples were homogenized with using a dounce homogenizer for 20 times, and then centrifuged at 1,000 xg for 10 min at 4 °C. The pellet was collected as nuclei-enriched fraction, and the supernatant was then separated by ultracentrifugation at 300,000 xg for 30 min at 4 °C. After ultracentrifugation, the supernatant (cytosolic fraction) was removed and the pellet (membrane fraction) was directly lysed in 200 µl of 1× SDS-PAGE sample buffer. The supernatant (cytosolic fraction) protein sample was concentrated by acetone precipitation and then re-suspended in 1× SDS-PAGE sample buffer at 1.0 µg/µl concentration. 10 µl of each fractionated sample was loaded onto the gel.

GST-14-3-3 pull-down assay

For the GST-14-3-3 binding experiments, expression and purification of GST fusion protein was performed according to the manufacturer's instructions (GST Gene Fusion System Handbook, Amersham Biosciences, Piscataway, NJ). C2C12 myotubes were washed twice with PBS and then lysed in the buffer containing 20 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, 1 mM β-glycerolphosphate, 1 mM Na₃VO₄, 1 mM PMSF and protease inhibitor cocktail. Cleared myotube protein lysates were mixed with the purified GST fusion protein bound to sepharose beads and then incubated for 3 hours at 4 °C. Sepharose beads-protein complex were washed for four times with lysis buffer and then re-suspended in SDS-PAGE sample buffer.

Statistical analysis

All results are reported as means ± SE. Two groups comparison was determined by student's T-test, and multi-group comparisons were performed by one-way analysis of variance followed by Tukey's post-hoc test. For all comparisons, the level of statistical significance was set at p<0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

mTOR	mammalian target of rapamycin
IGF-1	insulin like growth factor-1
PI3K	phosphoinositide 3-kinase
TSC	tuberous sclerosis complex
GAP	GTPase activating protein
Rheb	Ras homolog enriched in brain

S6K1	S6 kinase 1
4EBP-1	4E-binding protein-1
MEK	mitogen-activated protein kinase kinase
ERK	extracellular signal-regulated kinase
HEK	human embryonic kidney

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Fig 1. IGF-1 induced activation of mTOR signaling is prevented by PI3K inhibitor wortmannin and mTOR inhibitor rapamycin

C2C12 myotubes at 4 days of differentiation were treated with serum/antibiotics free DMEM for 120 min and then stimulated for 60 min with recombinant IGF-1 (100 ng/ml in serum/antibiotics free DMEM), co-incubated with/without wortmannin (10 μ M) or rapamycin (50 nM). Series of the experiments were repeated at least 3 times with using different passage of C2C12 myotubes. A) Phosphorylation states of PI3K-dependent and mTOR-dependent signaling pathway were determined with using phospho-specific antibodies (Akt at T308 and S473 sites, S6K1 at T389 and T421/S424 sites, IRS-1 at S636/639 sites, PRAS40 at T246 site and GSK3- α/β at S21/9 sites). Each of the PI3K/Akt/mTOR targets was phosphorylated with IGF-1 stimulation. Co-incubation with the PI3K inhibitor wortmannin blocked all measured phosphorylation in the PI3K/Akt-dependent pathway. Treatment with mTOR inhibitor rapamycin did not affect upstream Akt phosphorylation nor branches of this pathway (PRAS40 and GSK3), but did block the phosphorylation of downstream mTOR effectors including S6K1 and IRS1. B) Total lysates were immunoprecipitated with either anti-TSC1 or anti-TSC2 antibody. Phosphorylation levels of both S939 and T1462 sites on TSC2 were increased in C2C12 myotubes with IGF-1 stimulation, and were completely prevented by the PI3K inhibitor wortmannin but not by rapamycin. Co-immunoprecipitation assay showed that IGF-1 stimulation or the inhibitor treatments (wortmannin and rapamycin) did not affect the physical association between TSC1 and TSC2. GAPDH was used as a loading control among the experimental conditions. C) Total lysates were immunoprecipitated with each phospho-specific antibody (TSC2-S939 and TSC2-T1462). IGF-1 treatment resulted in the TSC2 phosphorylation (S939 and T1462 residues) and concomitant increase in the relative amount of TSC1 which was co-immunoprecipitated with phosphorylated-TSC2.

Fig 2. IGF-1 induced subcellular distributions of TSC1 and TSC2 in the membrane vs. the cytosolic fraction

C2C12 myotubes at 4 days of differentiation (60 mm culture dishes) were treated with serum/antibiotics free DMEM for 120 min and then stimulated for 60 min with recombinant IGF-1 (100 ng/ml in serum/antibiotics free DMEM), co-incubated with/without wortmannin (10 μ M) or rapamycin (50 nM). A) Cleared protein lysates were fractionated by ultracentrifugation at 300,000 $\times g$ for 30 min at 4 $^{\circ}$ C. Pellet sample (membrane fraction) was directly re-suspended in 200 μ l of 1 $\times$ SDS-PAGE sample buffer. Protein samples of the supernatant (cytosolic fraction) were concentrated by acetone precipitation and then re-suspended in SDS-PAGE sample buffer at 1.0 μ g/ μ l concentration. 10 μ l of each fractionated sample was loaded onto the gel. Pan-cadherin antibody was used for the membrane fraction control, and S6K1 was used as cytosolic fraction control. B) - D) Distributions of TSC1 and TSC2 proteins in the membrane fraction and the cytosolic fraction. In this study, alterations in relative amount of both TSC1 and TSC2 proteins were quantified as a percentage of control for each fraction. Image-J software (National Institute of Health) was used for the quantification. $n = 4$ plates per condition. Each value is means $\pm$ SE. *, significant difference versus control group ($p < 0.05$), #, significant difference versus wortmannin group ($p < 0.05$). The relative amount of both TSC1 and TSC2 proteins in the membrane fraction were significantly decreased (36% and 51% decreases, B and D) whereas significant increased in the cytosolic TSC1 and TSC2 (57% and 63% increases, C and E) with IGF-1 stimulation. IGF-1-induced re-distribution of TSC1/TSC2 complex was completely prevented by PI3K inhibitor wortmannin, but was not by mTOR inhibitor rapamycin. F) Protein samples of the supernatant after ultracentrifugation (cytosolic fraction) were immunoprecipitated with either anti-TSC1 or anti-TSC2 antibody. Co-immunoprecipitation assay revealed that there is indeed more TSC1/TSC2 protein complex within the cytosolic fraction following IGF-1 stimulation.

Fig 3. Time course alterations in PI3K/Akt-dependent signaling with IGF-1 stimulation
C2C12 myotubes at 4 days of differentiation were treated with serum/antibiotics free DMEM for 120 min and then stimulated with recombinant IGF-1 (100 ng/ml in serum/antibiotics free DMEM). Protein samples were collected at 10 min, 30 min and 60 min after the initiation of IGF-1 stimulation. A) Changes in phosphorylation states of Akt (T308 and S473 sites) and S6K1 (T389 and T421/S424 sites) were determined. B) Subcellular distributions of TSC1/TSC2 protein were determined. Membrane and cytosolic protein samples were fractionated by ultra-centrifugation. Pan-cadherin antibody was used for the membrane fraction control, and S6K1 was used as cytosolic fraction control. C) - F) Distributions of TSC1 and TSC2 proteins in the membrane fraction and the cytosolic fraction. In this study, alterations in relative amount of both TSC1 and TSC2 proteins were quantified as a percentage value compared to the control group in each fraction. Image-J software (National Institute of Health) was used for the quantification. $n = 3$ plates per condition. Each value is means $\pm$ SE. *: significant difference versus control group ($p < 0.05$). Phosphorylation of Akt and S6K1 were induced within 10 min, and were maintained for at least 60 min. Altered distributions of TSC1 and TSC2 proteins were also detected on the same time scale as Akt and S6K1 phosphorylations. The relative amount of both TSC1 and TSC2 proteins in the membrane fraction were significantly decreased whereas significant increases were observed in cytosolic TSC1 and TSC2 over the time course after IGF-1 stimulation.

Fig 4. TSC1/TSC2 protein complex interact with 14-3-3 proteins under phosphorylation dependent manner of TSC2

C2C12 myotubes at 4 days of differentiation were treated with serum/antibiotics free DMEM for 120 min and then stimulated for 60 min with recombinant IGF-1 (100 ng/ml in serum/antibiotics free DMEM), co-incubated with/without wortmannin (10 μ M) or rapamycin (50 nM). A) Total lysates were immunoprecipitated with anti-pan-14-3-3 antibody. Immuno-protein complex with pan-14-3-3 antibody precipitates both TSC1 and TSC2. B) Levels of TSC2 which is bound to 14-3-3 proteins were quantified. Image-J software (National Institute of Health) was used for the quantification. $n = 4$ plates per condition. Each value is means $\pm$ SE. *, significant difference versus control group ($p < 0.05$), #; significant difference versus wortmannin group ($p < 0.05$). IGF-1 treatment resulted in a phosphorylation-dependent increase (2.4-fold) in the amount of total-TSC2 which was bound to 14-3-3 protein. TSC2 phosphorylation and increased interaction between TSC2 and 14-3-3 were prevented by wortmannin treatment but not with rapamycin.

Fig 5. Protein interaction between TSC2 and 14-3-3 protein is mediated through phosphorylation of TSC2 at S939 site

C2C12 myoblasts were transfected and then differentiated for totally 4 days. A) *in vitro* GST-14-3-3 pull-down assay. Total cell lysates containing myc-TSC1 (wild type) and Flag-TSC2 (wild-type, S939A or T1462A) proteins were pulled-down with batch-purified GST-14-3-3-beta. Affinity-purified protein complex with Glutathione sepharose beads were then subjected to SDS-PAGE. The interaction between TSC2 mutant S939A and 14-3-3 was clearly decreased compared to wild-type TSC2 or mutant TSC2-T1462A. B)

Unphosphorylatable mutant of TSC2 (S939A) showed no increase in the cytosolic relative abundance following the IGF-1 stimulation. Cleared protein lysates were fractionated into the membrane and the cytosolic fraction by ultra-centrifugation. Pan-cadherin antibody was used for the membrane fraction control, and S6K1 was used as cytosolic fraction control. Image-J software (National Institute of Health) was used for the quantification. Protein contents in IGF-1 stimulated sample were quantified as relative amount compared to the simultaneously transfected control. $n = 4$ per condition. Each value is means $\pm$ SE. Amounts of myc-tagged TSC1 and Flag-tagged TSC2 (wild-type) in the cytosolic fraction were increased by IGF-1 stimulation compared to control. In contrast, no changes in the cytosolic pool of myc-TSC1 and Flag-TSC2 (S939A) were observed after IGF-1 stimulation. We could not see significant alterations in the distributions of myc-TSC1 or Flag-TSC2 in the membrane fraction.