REDD2 is enriched in skeletal muscle and inhibits mTOR signaling in response to leucine and stretch

Mitsunori Miyazaki and Karyn A. Esser

Department of Physiology, College of Medicine, University of Kentucky, Lexington, Kentucky

Submitted 8 September 2008; accepted in final form 5 January 2009

Miyazaki M, Esser KA. REDD2 is enriched in skeletal muscle and inhibits mTOR signaling in response to leucine and stretch. Am J Physiol Cell Physiol 296: C583–C592, 2009. First published January 7, 2009; doi:10.1152/ajpcell.00464.2008.—The protein kinase mammalian target of rapamycin (mTOR) is well established as a key regulator of skeletal muscle size. In this study, we determined that the stress responsive gene REDD2 (regulated in development and DNA damage responses 2) is a negative regulator of mTOR signaling and is expressed predominantly in skeletal muscle. Overexpression of REDD2 in muscle cells significantly inhibited basal mTOR signaling and diminished the response of mTOR to leucine addition or mechanical stretch. The inhibitory function of REDD2 on mTOR signaling seems to be mediated downstream or independent of Akt signaling and upstream of Rheb (Ras homolog enriched in brain). Knock down of tuberous sclerosis complex 2 (TSC2) using small interfering (si)RNA potently activated mTOR signaling and was sufficient to rescue REDD2 inhibition of mTOR activity, suggesting that REDD2 functions by modulating TSC2 function. Immunoprecipitation assays demonstrated that REDD2 does not directly interact with either TSC1 or TSC2. However, we found that REDD2 forms a complex with 14-3-3 protein and that increasing expression of REDD2 acts to competitively dissociate TSC2 from 14-3-3 and inhibits mTOR signaling. These findings demonstrate that REDD2 is a skeletal muscle specific inhibitory modulator of mTOR signaling and identify TSC2 and 14-3-3 as key molecular links between REDD2 and mTOR function.

tuberosclerosis complex 1; tuberous sclerosis complex 2; 14-3-3 protein; S6 kinase 1; Ras homolog enriched in brain; mammalian target of rapamycin; regulated in development and DNA damage responses 2

MAINTENANCE OF SKELETAL MUSCLE MASS is important for human movement and health. Studies of skeletal muscle hypertrophy and atrophy in humans and rodents have indicated that regulation of the kinase mammalian target of rapamycin (mTOR) is one of the critical targets for muscle size. Hypertrophy of skeletal muscle requires activation of mTOR signaling, whereas atrophy of skeletal muscle is associated with significant downregulation of mTOR signaling in muscle (1, 12, 16, 26, 27).

mTOR is a serine/threonine kinase of the phosphatidylinositol kinase-related kinase family and is highly conserved from yeast to mammals. In muscle cells, mTOR functions as a central integrator of a wide range of signals that will function to either activate or inhibit protein synthesis and cell growth (25). Activation of mTOR occurs in response to growth factors such as insulin-like growth factor I (IGF-I) and treatment with branch chain amino acids such as leucine (5, 35, 43). Mechanical strain is another positive regulator of mTOR signaling in muscle cells (15, 17). In contrast, many laboratories have shown that mTOR signaling is inhibited in muscle under conditions of energy deprivation, disuse, sepsis, or inflammation (2, 22, 26, 33). Although these inputs into mTOR function have been well studied, the key mechanistic sites regulating activation/inhibition are less well understood.

Two hypoxia-induced genes, termed Scylla and Charybdis, were identified from a genetic screen for negative regulators of the Drosophila TOR pathway and cell size (32). In mammals, these genes are named REDD1 (regulated in development and DNA damage responses 1) and REDD2 (also called RTP801L/DDIT4L, respectively). These proteins share ~34% sequence identity with each other but have little homology to other known proteins. Coradetti et al. (6) found that both REDD1 and REDD2 inhibit mTOR signaling in human embryonic kidney (HEK)-293 cells and mouse embryonic fibroblasts. More recently, REDD1 has been shown to be important for inhibition of mTOR signaling in response to hypoxia, energy stress, and glucocorticoid treatment (3, 7, 37, 40, 42). The site of REDD1 function has been determined to be downstream of Akt through regulation of the tuberous sclerosis complex 1 (TSC1)/TSC2 complex (3, 6, 40). In skeletal muscle, work by Wang et al. (42) demonstrated that REDD1 is induced in skeletal muscle following dexamethasone treatment and contributes to inhibition of mTOR signaling. Much less is known about REDD2, but it is of interest because expression of REDD2 mRNA has been shown to be significantly induced in response to skeletal muscle unloading, a model of muscle atrophy that is associated with diminished mTOR activity (16, 30).

The TSC protein complex is a very well-studied signaling hub in cells and is linked to REDD1 inhibition of mTOR signaling (7). The TSC protein complex is a heterodimer of TSC1 and TSC2, also known as hamartin and tuberin, respectively. Studies in the cancer field have shown that mutations in either TSC1 or TSC2 are associated with the disease tuberous sclerosis, a dominantly inherited disorder characterized by the formation of a distinguishing type of benign tumor. Many studies have shown that the TSC1/TSC2 heterodimer regulates mTOR activity downstream of growth factor-phosphoinositide 3-kinase (PI3K)-Akt signaling. Cells null for TSC1 or TSC2, cells depleted of TSC1 or TSC2 by RNA interference, and human and mouse tissues deficient in TSC1 or TSC2 all have high mTOR activity (10, 13, 19, 31). TSC2 functions with its partner, TSC1, as a GTPase-activating protein (GAP) for a small G protein named Rheb (Ras homolog enriched in brain). GTP-bound Rheb strongly stimulates mTOR activity and
TSC2 functions to inactivate Rheb by increasing the intrinsic rate of GTP hydrolysis on Rheb (11, 18, 20, 41).

The goal of this study is to determine the molecular mechanisms through which REDD2 regulates mTOR activity in skeletal muscle cells. The primary hypothesis tested was that REDD2 is an mTOR inhibitor in skeletal muscle. Results from these experiments demonstrated that REDD2 is highly enriched in adult skeletal muscle and is a negative regulator of mTOR signaling in response to either leucine or mechanical stretch. REDD2 functions downstream or independently of Akt but upstream of Rheb, and loss-of-function experiments determined that the inhibitory function of REDD2 requires TSC2. Surprisingly, REDD2 and TSC2 do not directly interact, but our findings implicate the scaffold protein 14-3-3 in facilitating the effect of REDD2 on TSC complex function. These results identify that REDD2 expression is highly enriched in skeletal muscle and thus can be considered a muscle-specific negative regulator of mTOR signaling. It was also found that similar to the role of REDD1 in nonmuscle cells (3, 6, 40), REDD2 works as an inhibitor of mTOR by modulating the function of the TSC complex in skeletal muscle cells.

MATERIALS AND METHODS

Materials

C2C12 mouse myoblasts were purchased from ATCC (Manassas, VA). High-glucose DMEM and fetal bovine serum were obtained from GIBCO (Grand Island, NY). DMEM low glucose without l-glutamine, leucine, or phenol red was obtained from U.S. Biological (Swampscott, MA). FuGENE 6 transfection reagent was obtained from Roche (Indianapolis, IN). Lipofectamine 2000 reagent was obtained from Invitrogen (Carlsbad, CA). Immobilized Protein A Plus was obtained from Pierce (Rockford, IL). ECL and ECL Plus solutions were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Antibodies.

Phospho-S6K1 (Thr 389), Akt, and phospho-Akt (Ser473) were obtained from Cell Signaling Technology (Danvers, MA). Tuberin (C-20), S6K1 (C-18), and normal mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-hemagglutinin (HA) was obtained from Zymed Laboratories (South San Francisco, CA). MyD88 was obtained from Thermo Scientific (Fremont, CA). Anti-FLAG polyclonal was obtained from Sigma-Aldrich (St. Louis, MO). Anti-green fluorescent protein (GFP) and Texas red-labeled goat anti-rabbit IgG were obtained from Vector Laboratories (Burlingame, CA). Anti-14-3-3 was obtained from Abcam (Cambridge, MA). Peroxidase-labeled anti-rabbit IgG and anti-mouse IgG secondary antibodies were obtained from Vector Laboratories (Burlingame, CA).

Plasmids.

pcDNA3.1-myc-TSC1 and FLAG-TSC2-wt were purchased from Addgene (Cambridge, MA). pcIKS-myc-Rheb-wt and pRK5-myc-S6K1-GST were kindly provided from Dr. K. L. Guan’s laboratory (UCSD, La Jolla, CA). REDD2 construct was generated by PCR subcloning from IMAGE consortium clone 5254530 into the pcDNA3.1+ Zeo-3HA vector. Akt and 14-3-3q constructs were also subcloned into pcDNA3.1(+) vector (Invitrogen) from cDNA generated with total RNA from mouse liver tissue in C57BL/6 strain. Glutathione S-transferase (GST) sequence was subcloned from pGEX-2T (Amersham). All newly made constructs were verified by DNA sequencing (Davis Sequencing, Davis, CA).

Adenovirus production.

Recombinant adenovirus expressing REDD2 cDNA construct was created using the AdEasy adenoviral vector system (Stratagene, La Jolla, CA) according to the manufacturer’s directions. Ad5-CMV-EGFP adenovirus vector was provided from University of Iowa Gene Transfer Vector Core. Viral cultures from appropriate expression constructs were amplified in HEK-293 cells and then purified with using the AdEasy virus purification kits (Stratagene).

Animals and Experimental Procedures

All experimental procedures performed in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee. Animals were housed in temperature- and humidity-controlled holding facilities on a 14:10-h light-dark cycle and had access to food and water ad libitum. Male C57BL/6 mice (The Jackson Laboratory), 10 wk of age, were used in this study. For the tissue sample collection, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and the tissues were excised, weighed, quickly frozen in liquid nitrogen, and stored at −80°C.

Cell Culture and Transfection

C2C12 myoblasts were maintained in DMEM with 10% fetal bovine serum. All cell culture experiments were performed in a humidified environment at 37°C in a 5% CO2. Myoblasts were plated at 2×10⁴ cells/well on six-well polystyrene cell culture plates. Myoblasts were transfected while the cells were in suspension (9) and studied 2 days later.

Leucine treatment.

Myoblasts were incubated with serum/antibiotic-free DMEM or serum/antibiotic-free DMEM without leucine for 60 min, and leucine-depleted cells were restimulated with DMEM containing 50 μg/ml leucine for 20 min (36).

Mechanical stretch.

Myoblasts were plated on type I collagen-coated Bioflex membranes (Flexcell International, Hillsborough, NC) and grown to confluence. Cells were incubated with serum/antibiotic-free DMEM for 60 min and then stretched for 10 min of 15% multiaxial stretching using the FX-4000 system (Flexcell International) as previously published (15, 17).

RNA Isolation and RT-PCR

Total RNA was prepared from frozen tissue samples using TRIZol (Invitrogen) according to the manufacturer’s directions. RNA samples were treated with TURBO DNase (Ambion, Austin, TX) to remove genomic DNA contamination. Isolated RNA was quantified by spectrophotometry (A = 260 nm). First-strand cDNA synthesis from total RNA was performed with a mixture of oligo(dT) primer and random hexamers using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Tag DNA polymerase (Invitrogen) was used for PCR amplification of each gene. Primer sequences for each gene are as follows: REDD1, forward 5'-GAC GTG TGT GTG GAG CAA GCC-3', reverse 5'-CGG GTA CTT AGC GTG AG-3'; REDD2, forward 5'-GAA ACA GAG CCG TGC ACC ATC-3', reverse 5'-ATT AGC AAC TCA TTA GGG AC-3'; and RPL26 (ribosomal protein L26), forward 5'-CGA TGC CAG CGA GAG AGG G-3', reverse 5'-GCA GTT TTC AAT GAA AGC GT-3'. Quantification of RPL26 mRNA levels was used for normalization purposes. The PCR reaction was run onto a 5% polyacrylamide gel, stained with SYBR green I (Invitrogen), scanned with Storm 860 (GE Healthcare, Piscataway, NJ), and quantified using ImageQuant software.

Protein Extraction and Western Blotting

Samples were lysed in ice-cold RIPA buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaCl, 20 mM Tris-HCl (pH 7.6), 1 mM PMSF, 5 mM benzamidine, 1 mM EDTA, 5 mM N-ethylmaleimide, 50 mM NaF, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 10 μM protease inhibitor cocktail for mammalian tissues (P8340; Sigma-Aldrich)]. Homogenates were then centrifuged at 17,860 g for 10 min at 4°C, and the supernatant was collected for analysis. Protein concentration was determined using the Bradford method. Protein extracts were run on SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature and then incubated with the appropriate primary antibodies for 1 h at room temperature. Membranes were washed in TBST for 5 min and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000) in 5% nonfat dry milk in TBST for 1 h at room temperature. Membranes were again washed in TBST for 5 min and then developed using enhanced chemiluminescence (ECL or ECL Plus). Membranes were imaged using a Storm 860 (GE Healthcare, Piscataway, NJ) and analyzed using ImageQuant software.
gels and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were blocked in 5% nonfat dry milk and then incubated with dilutions of each primary antibody. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG were used as secondary antibodies. Bound antibody complexes were visualized using ECL or ECL Plus Western blotting detection reagents (Amersham) and exposed to X-ray film (Pierce). Subsequently, visualized protein bands were analyzed with Scion Image software (Frederick, MD).

**Immunoprecipitation Assay**

To study the functional interactions of REDD2, TSC1, TSC2, and 14-3-3, we performed coimmunoprecipitation assays (34). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-based buffer [0.3% CHAPS, 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, and 10 μl/ml protease inhibitor cocktail] was used to obtain total cell lysates. Total cell lysates were then immunoprecipitated with each antibody and immobilized protein A. Immunocomplexes were washed three times with lysis buffer and then once with wash buffer [50 mM HEPES (pH 7.5), 40 mM NaCl, and 2 mM EDTA]. Precipitated protein samples were then subjected to SDS-PAGE.

**Immunofluorescent Staining**

Intracellular localization of HA-tagged REDD2, myc-tagged 14-3-3, myc-tagged TSC1, and FLAG-tagged TSC2 were determined by immunofluorescent analysis of transfected myoblasts. Transfected cells were plated onto glass coverslips, and 2 days following transfection, cells were fixed in 4% paraformaldehyde and then permeabilized in 0.1% Triton X-100. Nonspecific antigens were blocked in 1% bovine serum albumin. Rabbit anti-HA and Texas red-conjugated goat anti-myc and anti-DDDDK (FLAG) antibodies were used for detecting HA-tagged REDD2, and rabbit anti-HA and Texas red-conjugated goat anti-rabbit antibodies were used for detecting HA-tagged REDD2, and FITC-conjugated goat anti-myc and anti-DDDDK (FLAG) antibodies were used for detecting myc-tagged 14-3-3, myc-tagged TSC1, and FLAG-tagged TSC2. Stained images were analyzed on a fluorescence microscopy system (ECLIPSE E600; Nikon).

**RNA Interference**

For the knockdown of TSC2, we used the predesigned small interfering (si)RNA reagents Stealth Select RNAi (Invitrogen). The specific sequence of the siRNA oligonucleotide for TSC2 was 5′-CCGCUGGACUACAAGUGCAACCUAU-3′. To confirm the silencing of TSC2 expression, we performed coimmunoprecipitation assays (34). For the detection of TSC2, we used Western blotting. Knockdown efficiency of TSC2 was determined by immunofluorescent analysis of transfected myoblasts. Transfected cells were plated onto glass coverslips, and 2 days following transfection, cells were fixed in 4% paraformaldehyde and then permeabilized in 0.1% Triton X-100. Nonspecific antigens were blocked in 1% bovine serum albumin. Rabbit anti-HA and Texas red-conjugated goat anti-myc and anti-DDDDK (FLAG) antibodies were used for detecting HA-tagged REDD2, and FITC-conjugated goat anti-myc and anti-DDDDK (FLAG) antibodies were used for detecting myc-tagged 14-3-3, myc-tagged TSC1, and FLAG-tagged TSC2. Stained images were analyzed on a fluorescence microscopy system (ECLIPSE E600; Nikon).

**Expression of REDD2 is Highly Enriched in Skeletal Muscle Tissue**

Little was known about REDD2 expression, but informative analysis from both human and mouse arrays queried at the Symatlas website (http://symatlas.gnf.org/SymAtlas/) suggested that REDD2 might be expressed in a skeletal muscle-specific manner (see Supplemental Data Fig. 1, A and B). In both human and mouse, REDD2 mRNA expression is at least 10 times above the median across all 80+ tissues and cells profiled. To verify the array data, we measured REDD2 mRNA levels in three different skeletal muscles and five different non-skeletal muscle tissues of the mouse using semiquantitative RT-PCR. As shown in Fig. 1, REDD2 mRNA was highly expressed in different types of skeletal muscle (plantaris, tibialis anterior, and diaphragm), with much lower expression in the brain and very low to undetectable levels in heart, lung, liver, and kidney. In our experiments, REDD2 gene expression was detectable in skeletal muscle with ×26 PCR amplification cycles using 50 ng of total RNA by semiquantitative RT-PCR. In contrast, REDD1 mRNA expression was ubiquitous, and compared with the results for REDD2, a greater number of PCR cycles (×34) was required to detect REDD1 expression in these tissues. Thus our RT-PCR results support the expression profiling data and confirm that REDD2, but not REDD1, is expressed in a skeletal muscle-specific manner.

**REDD2 Inhibits Basal mTOR Activity As Well As mTOR Signaling Following Leucine or Mechanical Strain Stimulation**

Leucine and mechanical strain are known to activate mTOR signaling through independent pathways in muscle cells (25). We used both these activators of mTOR to test 1) whether REDD2 would inhibit mTOR signaling and 2) if the effect on mTOR was common to independent activators. We cloned the mouse REDD2 cDNA into a mammalian expression vector and determined the effect of overexpression of REDD2 on mTOR signaling by measurement of S6K1 phosphorylation (T389) site in C2C12 myoblasts. We confirmed that basal and leucine/mechanical stimulation-induced S6K1 phosphorylation were completely inhibited by treatment with rapamycin (Supplemental Fig. 2). In Fig. 2A, a representative blot shows that overexpression of REDD2 significantly inhibited the basal phosphorylation state of cotransfected S6K1 (S6K1-GST) compared with control (empty control lane vs. REDD2 control lane). Quantification of these results is provided in Fig. 2B with
a 54% reduction in basal levels of S6K1 T389 phosphorylation \( (P < 0.05, n = 6) \). We also investigated the effects of REDD2 expression on leucine stimulation (Fig. 2, A and B) and mechanical stimulation (multiaxial stretching; Fig. 2, C and D) of mTOR activity. For both empty vector- and REDD2-transfected C2C12 cells, leucine deprivation (−leu) led to a 40–60% decrease in phosphorylation of S6K1 \( (P < 0.05) \). After restimulation with leucine (−leu+leu), the phosphorylation state of S6K1 in the empty vector cells increased back to control levels. In the REDD2-transfected cells, readdition of leucine was not sufficient to increase phosphorylation of S6K1 to empty vector control levels (almost 60% of empty, −leu+leu level: \( P < 0.05 \); Fig. 2B). The same pattern of REDD2 inhibition of mTOR signaling was also confirmed in C2C12 cells following stretch stimulation. In empty vector-transfected control myoblasts, mechanical stimulation in-
increased S6K1 T389 phosphorylation by 45% \( (P < 0.05) \). In contrast, overexpression of REDD2 resulted in almost a 50% reduction in S6K1 T389 phosphorylation both in the basal state and after stretching compared with empty vector-transfected controls (Fig. 2, C and D). These data demonstrate that REDD2 functions to suppress mTOR signaling in skeletal muscle cells. It is important to note that both activation of mTOR signaling with leucine or mechanical strain were equivalently diminished with REDD2 overexpression. As predicted from previous studies, there was no effect of REDD2 overexpression on the phosphorylation state of cotransfected Akt at the S473 site. There also was no effect of REDD2 overexpression on stretch-induced activation of Akt (Fig. 2C). These observations place REDD2 functions as a negative regulator of mTOR signaling at a molecular site downstream of Akt or independent of Akt.

Function of REDD2 is Mediated Upstream of Rheb

We determined that REDD2 functions to suppress mTOR signaling downstream or independent of Akt. It has been shown that the small G protein Rheb is a downstream regulator of Akt/TSC2 signaling and can directly modulate mTOR kinase activity. To determine whether REDD2 inhibits mTOR signaling through Rheb, we cotransfected plasmid vectors of REDD2 and Rheb into C2C12 cells. As shown in Fig. 3, A and B, overexpression of Rheb in C2C12 cells induced a significant increase (+3.8-fold) in phosphorylation of S6K1 at T389 site \( (P < 0.05, n = 6) \). As shown previously, REDD2 overexpression resulted in an \(~40\%\) reduction in basal S6K1 phosphorylation levels but did not alter the activation of mTOR signaling by Rheb overexpression. There were no effects of Rheb overexpression on the phosphorylation state of cotransfected Akt. These data indicate that in skeletal muscle cells, the inhibitory function of REDD2 on mTOR signaling is mediated upstream of Rheb.

Knockdown of TSC2 Rescues REDD2 Inhibition of mTOR Activity

The previous experiments demonstrated that the inhibitory function of REDD2 on mTOR signaling seems to be mediated downstream or independent of Akt and upstream of Rheb. The next experiment was designed to determine whether the inhibitory effect of REDD2 on mTOR signaling was mediated through TSC2 function. We used siRNA oligonucleotides to knockdown endogenous TSC2 levels in C2C12 myoblasts. We confirmed that the knockdown efficiency of TSC2 mRNA is \(~90\%\) compared with the negative control using GC content-matched non-sense RNA oligonucleotides (Supplemental Fig. 3). The results in Fig. 4A demonstrate that TSC2 protein expression is not detectable in cells transfected with TSC2 siRNA. In the absence of TSC2, mTOR signaling was potently activated, and this activation of mTOR signaling was not sensitive to loss of leucine. TSC2 knockdown did not affect the phosphorylation state of Akt at S473 site under either basal or leucine deprivation conditions.

In Fig. 4B, our results demonstrated that REDD2 inhibition of mTOR signaling requires the presence of TSC2 in C2C12 cells. As shown previously (Fig. 2A), REDD2 overexpression resulted in a \(30–40\%\) reduction in basal S6K1 phosphorylation (compare empty control and REDD2 control in the negative control experiment). In addition, similar to the endogenous S6K1 (Fig. 4A), cotransfected S6K1-GST was highly phosphorylated in myoblasts in which TSC2 was knocked down. In cells lacking TSC2, overexpression of REDD2 was not capable of inhibiting mTOR signaling as determined by the level of S6K1 phosphorylation (Fig. 4B). This was seen under control conditions and also in cells deprived of leucine. These results demonstrated that, in muscle cells, REDD2 function requires TSC2 for inhibition of mTOR signaling.

![Fig. 3. Overexpression of REDD2 is not sufficient to inhibit Rheb-induced phosphorylation of S6K1 (T389).](http://ajpcell.physiology.org/)

**A** C2C12 myoblasts were triple transfected with S6K1-GST or GST-Akt and empty or expression vector of Rheb and/or REDD2. **B**: relative phosphorylation levels of S6K1 (T389) in each group were quantified. Values are means ± SE; \( n = 6 \) in each group. \* \( P < 0.05 \), empty vector vs. REDD2-transfected group. \# \( P < 0.05 \), empty vector vs. Rheb-transfected group.
REDD2 Indirectly Effects TSC2 Function Via Competitive Interaction With 14-3-3

The overexpression studies indicated that REDD2 works through modulation of TSC2 function so we next performed coimmunoprecipitation assays to test whether REDD2 has any direct biochemical association with either TSC2 or TSC1. C2C12 myoblasts were cotransfected with plasmid vectors of HA-tagged REDD2 and myc-tagged TSC1 or FLAG-tagged TSC2, and total lysates were immunoprecipitated with specific antibodies. As shown in Fig. 5, A and B, we could not detect

- **Fig. 5.** REDD2 interacts with 14-3-3 protein but not with either TSC1 or TSC2. A and B: C2C12 myoblasts were cotransfected with plasmid vectors of HA-tagged REDD2 and myc-tagged TSC1 (A) or FLAG-tagged TSC2 (B). Total lysates were immunoprecipitated (IP) with each antibody. Reciprocal immunoprecipitation/immunoblotting with anti-HA and anti-myc/anti-FLAG antibodies showed that there was no direct interaction between REDD2 and TSC1 or TSC2. C and D: total cell lysates were immunoprecipitated with normal mouse IgG or pan-14-3-3 mouse monoclonal antibody. Endogenous TSC2 (C) and HA-tagged REDD2 (D) were coimmunoprecipitated with 14-3-3 antibody.
any direct interaction between exogenously expressed REDD2 and TSC1 or TSC2. Different buffers, detergents, and salt conditions were tried for the immunoprecipitation assays, but we were never able to detect any interaction between REDD2 and TSC1 or TSC2 (data not shown). Additional experiments found that overexpression of REDD2 did not affect the physical association or membrane/cytosolic localization profiles of the TSC1 and TSC2 complex (Supplemental Figs. 4 and 5). These results suggest that REDD2 likely modulates TSC2 function through an indirect mechanism.

Several studies have reported that 14-3-3 proteins can directly bind to TSC2, but not to TSC1, and modulate the signaling function of the TSC1/TSC2 complex (4, 23, 24, 28, 39). To test whether 14-3-3 protein interacts with REDD2 and/or TSC2 in skeletal muscle cells, we performed immunoprecipitation studies. As shown in Fig. 5, C and D, immunoprecipitation with an antibody to all 14-3-3 proteins pulled down both TSC2 (C) and REDD2 (D). These results demonstrate the potential for 14-3-3 proteins to provide a molecular link between REDD2 and TSC2. The next set of experiments tested whether REDD2 can competitively disrupt 14-3-3 and TSC2 binding. C2C12 cells were infected with increasing titer of adenovirus expressing HA-tagged REDD2. Using the adenoviral system, we were able to generate C2C12 cells that expressed different levels of REDD2 as demonstrated by the Western blots from the total lysates (Fig. 6). Immunoprecipitation of these lysates with an antibody to 14-3-3 showed that increasing REDD2 levels resulted in significant decreases in the TSC2 and 14-3-3 interaction. Direct protein interaction between TSC2 and 14-3-3 was completely dissociated with robust REDD2 expression. Total expression levels of TSC2 and 14-3-3 were not affected by REDD2 overexpression. In these same samples, mTOR signaling was also inhibited by the overexpression of REDD2 in a dose-dependent manner (Fig. 6). These findings demonstrate that REDD2 levels can act to competitively titrate the interaction between TSC2 and 14-3-3 proteins and that this is associated with an inhibition of mTOR signaling.

Immunofluorescent staining was performed in C2C12 cells to assess whether the biochemical association detected among REDD2, TSC2, and 14-3-3 was consistent with localization of the proteins in the C2C12 cells. As shown in representative images provided in Fig. 7A, exogenously expressed REDD2 (Texas red) is seen in a discrete, punctate pattern in the perinuclear region and cytoplasm of the cell. In contrast, exogenous 14-3-3β was detected diffusely throughout the nucleus and cytoplasm of the cell. Localization studies were also performed with antibodies to TSC1 (myc) and TSC2 (FLAG) in myoblasts. In these experiments we found that both TSC1 (Fig. 7B) and TSC2 (Fig. 7C) colocalized with REDD2. The localization of these proteins overlapped with REDD2 in discrete regions in the cytoplasm of myoblasts. Unlike 14-3-3, we did not detect any staining of these proteins in the nucleus. Thus, although we did not detect any direct biochemical association between REDD2 and TSC1 or TSC2, the immunocytochemical results suggest that REDD2 is localized to the same regions as TSC1 and TSC2 consistent with the function of REDD2 in mTOR signaling.

**DISCUSSION**

**REDD2 is a Unique Skeletal Muscle-Specific Negative Regulator of mTOR Signaling**

REDD2, also known as SMHS1/RTP801L/DDIT4L, was recently identified in a subtractive hybridization screen as one of the most highly upregulated genes in the soleus muscle following hindlimb unloading (30). The soleus muscle atrophies about 40% following 1–2 wk of hindlimb unloading, and this is concomitant with a significant inhibition of mTOR signaling (16, 30). In parallel, studies in nonmuscle cells indicate that REDD2, like REDD1, negatively regulates mTOR signaling (3, 6, 40) and that overactivation of REDD1 and REDD2 homologs in *Drosophila* leads to smaller cells (32). These observations suggest the possibility that REDD2 could be an important negative regulator of mTOR signaling in skeletal muscle with implications for muscle size. The results of this study determined that REDD2 is expressed in a skeletal muscle-specific pattern, but, unlike many muscle-specific genes, REDD2 is not detected in the heart. We also determined that REDD2 functions in muscle cells to negatively regulate mTOR signaling in response to leucine or mechanical strain stimulation in a TSC2-dependent mechanism. These findings highlight that the unique expression of REDD2 in skeletal muscle makes it a novel tissue-specific mTOR inhibitory molecule.

**REDD2 Functions as a General Modulator of mTOR**

One of the findings from this study is that REDD2 functions to decrease mTOR signaling in response to both leucine- and...
mechanical strain-induced activation of mTOR signaling. This observation is important given that mTOR receives signaling inputs from several independent pathways that communicate nutrient, growth factor, and mechanical status. Studies have demonstrated that mechanical strain activates mTOR independently of amino acids, and both of these factors activate mTOR signaling independently of the IGF-I-PI3K-Akt pathway (11, 17, 29). Our findings indicated that REDD2 functions as a...
potential inhibitory molecule for mTOR signaling across these two different activators. However, we did find that REDD2 overexpression did not completely block either amino acid or mechanical strain activation. This suggests that REDD2 likely acts more like a modulator to dampen mTOR signaling rather than as an on/off switch.

**REDD2 Function and TSC Protein Complex**

Although there have been a few studies documenting changes in expression levels of REDD2 (8, 30, 42), to date the function of REDD2 in skeletal muscle cells has not been reported. In this study we found that REDD2 inhibits mTOR signaling in a TSC2-dependent mechanism. When we knocked down TSC2 using siRNA, REDD2 had no effect on mTOR signaling. However, we did not detect any direct biochemical interaction between REDD2 and either TSC1 or TSC2. In addition, similar to results for REDD1 (21), we did not detect any changes in the formation of TSC1/TSC2 protein complex with REDD2 expression. Although Cai et al. (4) reported that TSC2 translocates from the membrane to the cytosol under growth factor stimulation, we did not detect any effects of leucine stimulation, mechanical strain, or REDD2 overexpression on TSC1/TSC2 localization profiles in membrane vs. cytosolic fractions (Supplemental Fig. 5). We did perform immunocytochemical studies in muscle cells and found that there was overlap in the localization of REDD2, TSC1, and TSC2. These localization profiles were also confirmed in cell fractionation assay with REDD2 and TSC2 detected in both membrane and cytosolic fractions in C2C12 myoblasts. Whether this colocalization is mediated indirectly by complex formation or through selective trafficking of these proteins is still to be determined.

**Potential Redundancy and Difference Between REDD1 and REDD2**

Our findings that REDD2 can inhibit mTOR signaling via modulation of TSC2 protein function are similar to what has recently been reported in mouse embryonic fibroblasts for REDD1 (7). The homology in amino acid sequence for REDD1 and REDD2 (in both mouse and human) is ~34% with both proteins sharing the protein domain referred to as RTP801_C in the COOH-terminal region. The precise function of RTP801_C domain is, to date, uncharacterized, but both REDD1 and REDD2 contain a consensus binding site for 14-3-3 proteins (Arg-X-X-Ser/Thr-X-Pro) in this protein domain. We have demonstrated that REDD2, like REDD1, can form a complex with 14-3-3, and with increasing amounts of REDD2 we can compete off binding of TSC2. Thus our findings suggest that REDD2 shares redundant functions with REDD1 and inhibits mTOR signaling via interactions with 14-3-3 and enhanced TSC function.

Whereas REDD2, like REDD1, functions as a negative regulator of mTOR signaling, the muscle-specific expression of REDD2 vs. the ubiquitous expression of REDD1 suggests that the molecular mechanisms regulating the expression of these two genes are quite different. Initially, REDD2 was identified as a REDD1-related gene; however, unlike REDD1, it is not inducible by hypoxia/HIF-1 in vitro or in vivo (30, 38). Recently, it was reported that the promoter sequences in *Drosophila Scylla* mammalian REDD1 contain putative FOXO recognition elements, and *Scylla*/REDD1 expression appeared to be regulated by FOXO transcription factors (14). In contrast, the promoter sequence of mouse REDD2 does not possess FOXO binding sites (determined using the CisView Mouse mm6 program; http://lgsun.grc.nia.nih.gov/cisview/). In addition, it has been reported that dexamethasone treatment increased REDD1 mRNA expression but decreased REDD2 mRNA expression in the rat gastrocnemius muscle and in L6 myoblast cells (42). The divergent regulation of REDD1 vs. REDD2 in skeletal muscle suggests that the factors regulating transcription of these genes are likely quite different.

**Summary**

In summary, results from these experiments demonstrate that REDD2 is highly enriched in adult skeletal muscle and is a negative regulator of mTOR signaling in response to either leucine or mechanical stretch. REDD2 functions downstream or independently of Akt but upstream of Rheb, and loss-of-function experiments determined that the inhibitory function of REDD2 requires TSC2. Surprisingly, REDD2 and TSC2 do not directly interact, but our findings implicate the scaffold protein 14-3-3 in facilitating the effect of REDD2 on TSC complex function. These results identify that REDD2 expression is highly enriched in skeletal muscle and thus can be considered a muscle-specific negative regulator of mTOR signaling. It was also found that, similar to the role of REDD1 in nonmuscle cells (3, 6, 40), REDD2 works as an inhibitor of mTOR by modulating the function of the TSC complex in skeletal muscle cells.

**ACKNOWLEDGMENTS**

We thank Dr. John J McCarthy and Dr. Xiping Zhang for valuable discussion and technical assistance.

**GRANTS**

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR45617 (to K. A. Esser) and American Heart Association Postdoctoral Fellowship 082566SD (to M. Miyazaki).

**REFERENCES**


