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A role for Raptor phosphorylation in the mechanical activation of mTOR signaling

John W. Frey, Brittany L. Jacobs, Craig A. Goodman, Troy A. Hornberger *

Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, Madison, 2015 Linden Dr., Madison, WI 53706, USA

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ABSTRACT

The activation of mTOR signaling is necessary for mechanically-induced changes in skeletal muscle mass, but the mechanisms that regulate the mechanical activation of mTOR signaling remain poorly defined. In this study, we set out to determine if changes in the phosphorylation of Raptor contribute to the mechanical activation of mTOR. To accomplish this goal, mouse skeletal muscles were subjected to mechanical stimulation via a bout of eccentric contractions (EC). Using mass spectrometry and Western blot analysis, we found that ECs induced an increase in Raptor S696, T706, and S863 phosphorylation, and this effect was not inhibited by rapamycin. This observation suggested that changes in Raptor phosphorylation might be an upstream event in the pathway through which mechanical stimuli activate mTOR. To test this, we employed a phospho-defective mutant of Raptor (S696A/T706A/S863A) and found that the EC-induced activation of mTOR signaling was significantly blunted in muscles expressing this mutant. Furthermore, mutation of the three phosphorylation sites altered the interactions of Raptor with PRAS40 and $p70^{S6k}$, and it also prevented the EC-induced dissociation of Raptor from $p70^{S6k}$. Combined, these results suggest that changes in the phosphorylation of Raptor play an important role in the pathway through which mechanical stimuli activate mTOR signaling.

1. Introduction

Skeletal muscles make up the largest proportion of the body's mass, and they are not only the motors that drive locomotion, but they also play a crucial role in whole body metabolism [1,2]. Accordingly, it has been well recognized that the maintenance of skeletal muscle mass contributes significantly to disease prevention and issues associated with the quality of life [3].

Skeletal muscle mass is known to be regulated by a variety of stimuli, one of which is mechanical loading. For instance, chronic mechanical loading induces an increase in muscle mass, while chronic mechanical unloading results in a loss of muscle mass [4]. Although it is well recognized that mechanical loads play a role in the regulation of muscle mass, the molecular mechanisms that regulate these changes remain poorly defined [5].

One molecule that has been widely implicated in the regulation of skeletal muscle mass is the Serine/Threonine kinase called the mammalian [or mechanistic] Target of Rapamycin (mTOR). For example, previous studies have shown that mechanically-induced muscle hypertrophy is associated with increases in mTOR signaling, while atrophy resulting from mechanical unloading is associated with decreased mTOR signaling [6,7]. Furthermore, recent studies have provided genetic evidence which indicates that the activation of mTOR is sufficient to induce skeletal muscle hypertrophy, and that signaling through mTOR is necessary for a mechanically-induced hypertrophic response [8,9].

mTOR exists in at least two distinct multi-protein complexes called mTORC1 and mTORC2 and signaling by mTORC1, but not mTORC2, can be inhibited by the drug rapamycin [10]. This is important because rapamycin has been shown to inhibit mechanical load-induced increases in mTOR-dependent signaling events, protein synthesis, and growth [9,11,12]. Thus, it has been widely concluded that signaling by mTORC1 is critical for mechanically-induced changes in muscle mass.

The mTORC1 complex contains mTOR and other accessory proteins such as Raptor and PRAS40 [13–16]. Previous studies have shown that Raptor, which binds directly to mTOR, acts as a scaffold protein and is important in the regulation of mTOR-dependent signaling events. For example, knockdown of Raptor using RNAi results in a reduction of cell size *in-vitro*. Furthermore, it has been demonstrated that skeletal muscle specific ablation of Raptor *in-vivo* results in the loss of muscle mass, and it also prevents mechanical load-induced hypertrophy

Abbreviations: EC, eccentric contraction; GβL, G-protein β-subunit-like protein; GFP, green fluorescent protein; JNK, c-jun n-terminal kinase; MAPK, mitogen-activated protein kinase; mLST8, lethal with sec13 protein 8; mTOR, mammalian [or mechanistic] target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; p38, p38 mitogen-activated protein kinase; $p70^{S6k}$, ribosomal S6 kinase 1; PI3K, phosphotidylinositol-3-kinase; PRAS40, proline-rich Akt substrate of 40 kDa; Raptor, regulatory associated protein of mTOR; TA, tibialis anterior muscle.

* Corresponding author at: Department of Comparative Biosciences, 2015 Linden Drive, Madison, WI 53706, USA. Tel./fax: +1 608 890 2174.
E-mail address: thornb1@svm.vetmed.wisc.edu (T.A. Hornberger).

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[17–19]. Hence, it seems apparent that Raptor plays an important role in the regulation of mTOR signaling and muscle mass, but a complete understanding of the mechanisms through which Raptor regulates these processes is only beginning to be established.

Changes in the phosphorylation state of Raptor appear to be one mechanism through which Raptor can regulate mTOR signaling. For example, alterations in Raptor phosphorylation have been implicated in the regulation of mTOR signaling that occurs in response to insulin, osmotic stress and energetic stress [20–23]. It has also been shown that signaling through the Ras/MAPK pathway can induce changes in mTOR signaling by promoting alterations in the phosphorylation of Raptor [24,25]. Combined, these studies have provided strong evidence to support the conclusion that mTOR signaling can be regulated through alterations in the phosphorylation state of Raptor. However, to date, the potential role of Raptor phosphorylation in the regulation of mTOR by mechanical stimuli has not been explored. Thus, the goal of this study was to determine if changes in the phosphorylation of Raptor contribute to the mechanical activation of mTOR signaling.

2. Experimental procedures

2.1. Materials

Primary antibodies, anti-total $p70^{S6k}$ (#2708), phospho Serine/Threonine – Proline (S^*/T^*–P) (#9391), phospho Akt substrate ($RxRxxS^*/T^*$) (#10001), total Raptor (#2280), phospho Raptor (S792) (#2083), total mTOR (#2972) and PRAS40 (#2691) were purchased from Cell Signaling Technology (Danvers, MA, USA). The primary antibody anti-phospho $p70^{S6k}$(T389) (#sc-11759-R) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-specific Raptor antibodies (S696, T706, S859, S863, and S877) have been previously described [20] and were kindly provided by Dr. Diane Fingar (University of Michigan Medical School, Ann Arbor, Michigan, USA). The peroxidase labeled anti-rabbit IgG (H + L) secondary antibody (#PI-1000) was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). Peroxidase labeled anti-rabbit IgG (Light Chain Specific) (#211-032-171) and anti-mouse IgG (Light Chain Specific) (#115-035-174) antibodies were purchased from Jackson ImmnunoResearch Laboratories Inc. (West Grove, PA, USA).

2.2. Animals

C57BL6 mice (Jackson Laboratories, Bar Harbor, MA, USA) 8–10 weeks of age were randomly assigned to different experimental groups. Before all surgical procedures, the mice were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After tissue extraction, the mice were sacrificed by cervical dislocation. All animals were housed in a room maintained with a 12–12 h light–dark cycle and received food and water ad libitum. The Institutional Animal Care and Use Committee at the University of Wisconsin-Madison approved all of the methods employed in this study.

2.3. Plasmid constructs and purification

The GFP and GST-$p70^{S6k}$ plasmid constructs have been previously described [8]. Wild type FLAG-Raptor and FLAG-Raptor (S696A, T706A, S863A) have been previously described [21] and were kindly provided by Dr. Sung Ho Ryu (Pohang University of Science and Technology, Pohang, Kyungbuk, Korea). Myc-$p70^{S6k}$ was generated by using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) to insert a stop codon before the region that encodes for the GST tag on the Myc-$p70^{S6k}$-GST plasmid that has been previously described [8]. All plasmid DNA was grown in DH5α E. Coli, purified with an Endofree plasmid kit (Qiagen, Valencia, CA) and resuspended in sterile phosphate buffered saline (PBS).

2.4. Skeletal muscle transfection (electroporation)

As previously described [8], a small incision was made through the skin covering the TA muscle and then a 27-gauge needle was used to inject 12 μl of plasmid DNA solution containing 7.5–25 μg of GFP, wild type FLAG-Raptor (WT) or mutant FLAG-Raptor (S696A, T706A, S863A) (3A) and, when indicated, 2 μg of GST- or Myc-tagged $p70^{S6k}$. After the injections, electric pulses were applied through two stainless steel pin electrodes (1-cm gap; Harvard Apparatus, Holliston, MA, USA) laid on top of the proximal and distal myotendinous junctions. Eight 20 ms square-wave electric pulses at a frequency of 1 Hz were delivered with an ECM 830 electroporation unit (BTX; Harvard Apparatus) at a field strength of 160 V/cm. After the electroporation procedure, the incision was closed with Vetbond surgical glue (3 M Animal Care Products, St. Paul, MN, USA). Following the surgery, mice were given an intraperitoneal injection of buprenorphine (0.05 μg/g) dissolved in 100 μl of PBS.

2.5. Eccentric contractions and insulin injections

The model previously described by O'Neil et al. (2009) was used to induce eccentric contractions (EC) in the TA muscle [26]. Specifically, electrodes were placed on the sciatic nerve of the right leg and contractions were elicited by stimulating the sciatic nerve with an SD9E Grass stimulator (Grass Instruments, Quincy, MA, USA) at 100 Hz, 4–8 V pulse, for 10 sets of 6 contractions. Each contraction lasted 3 s and was followed by a 10 s rest period, and a 1 min rest period was provided between each set. The left (control) TA muscle and the right (EC) TA muscle were collected 40 min after the last set of contractions. When indicated, mice were also given an intraperitoneal injection of 20 U/kg Novolog® insulin (Novo Nordisk, Princeton, NJ, USA) dissolved in 100 μl of PBS at 30 min prior to the collection.

2.6. Rapamycin injections

Rapamycin was purchased from LC laboratories (Woburn, MA, USA) and was dissolved in DMSO to generate a 5 μg/μl stock solution. Rapamycin was administered at a dose of 2.0 mg/kg and dissolved in a final volume of 200 μl PBS. For the vehicle condition, an equal amount of DMSO was dissolved in a final volume of 200 μl PBS. The vehicle or rapamycin solutions were administered via an intraperitoneal injection 100 min prior to the initiation of the EC protocol.

2.7. Sample preparation for immunoprecipitations and Western blot analysis

Upon collection, TA muscles were either immediately homogenized or frozen in liquid nitrogen. In some cases, the samples were homogenized with a Polytron for 20 s in ice-cold buffer A (40 mM Tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 25 mM β-glycerophosphate, 25 mM NaF, 1 mM Na3VO4, 10 mg/ml leupeptin, and 1 mM PMSF) and the whole homogenate was used for further analysis. In other cases (e.g. preservation of the mTORC1 complex) the samples were homogenized with a Polytron for 20 s in ice-cold CHAPS lysis buffer (40 mM HEPES (pH. 7.4), 2 mM EDTA, 0.3% CHAPS, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and 1 tablet of EDTA-free protease inhibitors (Roche) per 25 ml) and the whole homogenate was used for further analysis. Sample protein concentration was determined with a DC protein assay kit (Bio-Rad, Hercules, CA, USA), and unless otherwise noted (e.g. immunoprecipitation), equivalent amounts of protein from each sample were dissolved in 2× Laemmli buffer, heated to 100 °C for 5 min and then subjected to Western blot analysis as described below.

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