Myogenic Differentiation Is Dependent on Both the Kinase Function and the N-terminal Sequence of Mammalian Target of Rapamycin*

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The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase known to control initiation of translation through two downstream pathways: eukaryotic initiation factor 4E-binding protein 1 (4E-BP1)/eukaryotic initiation factor 4E and ribosomal p70 S6 kinase (S6K1). We previously showed in C2C12 murine myoblasts that rapamycin arrests cells in G1 phase and completely inhibits terminal myogenesis. To elucidate the pathways that regulate myogenesis, we established stable C2C12 cell lines that express rapamycin-resistant mTOR mutants (mTORrr; S2035I) that have N-terminal deletions (∆10 or ∆91) or are full-length kinase-dead mTORrr proteins. Additional clones expressing a constitutively active S6K1 were also studied. Our results show that ∆10mTORrr signals 4E-BP1 and permits rapamycin-treated myoblasts to differentiate, confirming the mTOR dependence of the inhibition of myogenesis by rapamycin. C2C12 cells expressing either ∆91mTORrr or kinase-dead mTORrr(D2338A) could not phosphorylate 4E-BP1 in the presence of rapamycin and could not abrogate the inhibition of myogenesis. Taken together, our results indicate that both the kinase function of mTOR and the N terminus (residues 11–91, containing part of the first HEAT domain) are essential for myogenic differentiation. In contrast, constitutive activation of S6K1 does not abrogate rapamycin inhibition of either proliferation or myogenic differentiation.

Myogenic differentiation entails a cascade of intracellular events that coordinate muscle-specific gene expression, induce withdrawal from the cell cycle, and generate terminally differentiated myotubes (1). The MyoD family (MyoD (2), myogenin (3), myf-5 (4), and MRF-4 (5)) belongs to the basic helix-loop-helix superfamily of transcription factors that act as transcriptional activators of genes that encode skeletal muscle-specific proteins (6, 7). These proteins bind to a consensus E box sequence, CANNTG (8), upon heterodimerization with other basic helix-loop-helix factors such as the ubiquitously expressed E2A proteins E12 and E47 (9).

A rapamycin-sensitive pathway is required for differentiation of C2C12 and L6 myoblasts (10–12). This finding is consistent with reports that wortmannin, an inhibitor of phoshatidylinositol 3-kinase upstream of the mammalian target of rapamycin (mTOR), FRAP, inhibits IGF-I-stimulated differentiation. Conversely, however, Jayaraman and Marks (13) reported that rapamycin induces terminal differentiation. This discrepancy may arise partially from the use of different clones and different end points for assessing differentiation. Whereas several reported studies have used myoblast fusion as the marker of differentiation, the single study that showed rapamycin induction of differentiation used α-actin expression in a nonfusing C3H clone to identify differentiation (13). To date, the mechanism by which rapamycin inhibits myogenesis has not been established.

The rapamycin target, mTOR (14), links mitogen stimulation to translation through control of ribosomal S6K1 and 4E-BP1, the suppressor of eukaryotic initiation factor 4E (eIF4E) (15). The S6K1 pathway controls synthesis of proteins, such as IGF-II and ribosomal proteins, whereas the 4E-BP1 pathway controls many proteins involved in cell cycle regulation. The two pathways regulate the initiation of translation of distinct classes of mRNA. Mitogen-induced phosphorylation and activation of S6K1 appear to play an important role during the G1 phase of the cell cycle (16, 17). Phosphorylation of the S6 protein, the small ribosomal subunit, by S6K1 permits efficient translation of mRNAs containing terminal oligopyrimidine tracts in their 5′-untranslated regions (18). Phosphorylation of 4E-BP1 controls cap-dependent translation of mRNAs with extensive secondary structure. Growth factors stimulate phosphorylation of 4E-BP1, thereby reducing its affinity for the cap-binding protein eIF4E and releasing the blockade of cap-dependent translation (19). mTOR phosphorylates at least two residues of 4E-BP1: Thr37 and Thr46 (20). mTOR-dependent phosphorylation of these residues blocks 4E-BP1 association with eIF4E in vitro, and phosphorylation of Thr46 appears to be the main regulator of the 4E-BP1-eIF4E interaction in vivo (21). By preventing the phosphorylation of specific residues on S6K1 and 4E-BP1, rapamycin inhibits mitogen-stimulated activation of S6K1 and the resultant phosphorylation of S6 (22, 23) and prevents dissociation of 4E-BP1 from eIF4E (24, 25). Rapamycin negates mitogen-induced activation of S6K1 by preventing the acute phosphorylation of a specific subset of sites, including Thr329, Thr389, Ser404, and Ser411, Thr389, which resides in the linker region coupling the catalytic and autoinhibitory domains, has been identified as the principal site of rapamycin-induced dephosphorylation that leads to S6K1 inactivation (26).

Although the molecular mechanisms involved in rapamycin

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1 The abbreviations used are: mTOR, mammalian target of rapamycin; IGF, insulin-like growth factor; S6K1, ribosomal p70 S6 kinase; eIF4E, eukaryotic initiation factor 4E; 4E-BP1, eIF4E-binding protein 1; mTORrr, rapamycin-resistant mTOR; DM, differentiation medium; GM, growth medium; PBS, phosphate-buffered saline.
inhibition of cell proliferation are coming to light, the precise mechanisms by which rapamycin inhibits myogenesis have remained elusive. A recently reported study by Erbay and Chen (27) concluded that the kinase function of mTOR is not required for myogenic differentiation. In that study, rapamycin treatment of C2C12 myoblasts prevented differentiation and inhibited S6K1, but it did not induce significant hypophosphorylation of 4E-BP1. Here we confirm the finding that rapamycin inhibits myogenesis through inhibition of mTOR. However, unlike Erbay and Chen (27), we show that rapamycin induces hypophosphorylation of 4E-BP1 and that the kinase function of mTOR is required for differentiation. Our results indicate that the extreme N terminus of mTOR (residues 1–10) is not required for differentiation, whereas residues 11–91, which include part of the first HEAT sequence, are essential.

**EXPERIMENTAL PROCEDURES**

**Cell Line and Cultures**—Mouse C2C12 myoblasts were purchased from American Type Culture Collection (Manassas, VA) and routinely grown in antibiotic-free Dulbecco’s modified Eagle’s medium with 15% fetal calf serum (growth medium (GM)). Cells were induced to differentiate by growth in differentiation medium (DM; Dulbecco’s modified Eagle’s medium with 2% horse serum supplemented with 4 mM l-glutamine) at 37 °C and 5% CO2. After 2 days, mature myotubes were used in those studies, and myotube formation was only partial inhibited. We used C2C12 cells to examine the cellular role of rapamycin in terminal differentiation. Cells were

**Establishment of Stable C2C12 Cell Lines Expressing ΔαmTORrr, Δ91mTORrr, or Kinase-dead mTORrr**—C2C12 cells were transfected with plasmids expressing Δα10, Δ91, or kinase-dead mTORrr (Sida) or with pcDNA3 control vector by using the TransIT™ LTI kit. Cells were selected for G418 resistance and cloned. Individual clones were screened for expression of mTOR constructs by immunoprecipitation with M2 anti-FLAG or anti-Au1 antibodies and by Western blot with anti-mTOR (26E3) mouse monoclonal antibody.

**Cell Lysis, Immunoprecipitation, and Western Blot**—Cells were lysed in lysis buffer containing 1% Triton, 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 μM phenylmethylsulfonyl fluoride. The lysates were cleared by centrifugation for 10 min at 12,000 × g at 4 °C and used for immunoprecipitation or Western blots.

For immunoprecipitation, cell lysates were preclarified with 2.5 μg/ml normal rabbit IgG and 30 μl of protein A/G plus-agarose at 4 °C for 30 min and then centrifuged at 10000 × g for 5 min. Primary antibody (2 μg/ml) was added to the supernatant, and samples were rotated at 4 °C overnight. After centrifugation, the collected beads were washed three times with lysis buffer, 30 μl of 1× protein loading buffer were added, and samples were boiled for 5 min and centrifuged for 5 min at 10000 × g.

Electrophoresis was performed under conditions that do not resolve 4E-BP isoforms, as described previously (28). Isoforms of 4E-BP1 were separated in parallel experiments that used 15% Tris-HCl denaturing gel. Electrophoresis was performed at a constant 100 V at 4 °C for 2 h. The separated proteins were transferred to an Immobilon-P membrane by electrophoresis at 4 °C for 1 h. Non-specific binding was blocked by incubation with 5% nonfat milk at room temperature for 1 h, and the membrane was incubated overnight with primary antibody at 4 °C. The membrane was washed three times with phosphate-buffered saline with 0.1% Tween 20, incubated with secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h, washed three times in PBS-T, incubated with Supersignal substrate, and exposed to Kodak BioMax.

**Detection of Myosin Heavy Chain by Immunofluorescence**—C2C12 cells were seeded on 35-mm plates and grown to 80–90% confluence in GM. The next day, cells were washed once, and the membrane was replaced with DM with or without rapamycin (0–1000 ng/ml) for 24 h. Cells were fixed in B treadmillaldehyde-fresh solution (Fisher Scientific) for 30 min, permeabilized with 0.25% Triton X-100 in PBS for 30 min, and blocked with non-specific antibody binding. Cells were rinsed thoroughly, incubated with mouse monoclonal anti-myosin heavy chain antibody (Sigma; 15 μg/ml in 1% swine serum-PBS) for 2 h, rinsed with PBS, and incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Inc.; 4 μg/ml in 1% swine serum-PBS) for 2 h. All procedures were carried out at room temperature. The cells were examined under an inverted fluorescence microscope. Both phase-contrast and fluorescence images (at least 50 fields) were recorded by a digital camera. Nonfluorescent immunohistochemical detection of myosin heavy chain was performed as described previously (31).
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Expression of Myc-tagged Constitutively Active S6K1(D3E-E389) — The target of rapamycin, mTOR, is a serine/threonine kinase that signals to S6K1 and 4E-BP1. To further understand which downstream pathway is necessary for differentiation, we derived stable C2C12 cell lines expressing the Myc-tagged, constitutively active S6K1 mutant D3E-E389 by co-transfecting C2C12 with S6K1(D3E-E389) and pcDNA3 plasmids. The proteins expressed in the presence or absence of rapamycin were immunoprecipitated with anti-Myc monoclonal antibody and detected by Western blot with polyclonal anti-S6K1 (Fig. 2A). High-level expression of S6K1-D3E-E389 protein was found in five clones and was not altered by treatment of cells with rapamycin (100 ng/ml).

Rapamycin Does Not Inhibit the S6 Kinase Activity of Myc-tagged S6K1(D3E-E389) — To determine whether rapamycin inhibits IGF-I-induced stimulation of S6K1, we assayed S6 kinase activity in vector control cells (C2C12pcDNA3) and in C2C12 cells expressing S6K1 mutant D3E-E389. The D3E-E389 clone had a higher basal level of S6K1 activity than the vector control clone (Fig. 2B) and had 50% greater total S6K1 activity than the control clone after IGF-I stimulation. Importantly, the basal kinase activity (derived from endogenous and mutant S6K1) was only slightly inhibited (∼30%) by rapamycin (100 ng/ml). These results suggest that the constitutively active D3E-E389 mutant functioned in C2C12 cells. If rapamycin inhibits differentiation through inhibition of the S6K1 pathway, the C2C12(D3E-E389) clones would be expected to continue to differentiate in the presence of rapamycin.

Rapamycin Inhibits Differentiation of C2C12 Clones Expressing Constitutively Active S6K1-D3E-E389 — To determine whether C2C12(D3E-E389) clones could terminally differentiate, we cultured the cells in DM in the presence or absence of rapamycin (100 ng/ml). After 3 days, cells were fixed and incubated with a monoclonal antibody to myosin heavy chain (Fig. 2C). The C2C12(D3E-E389) clones differentiated into myotubes, which stained positively for myosin heavy chain. However, differentiation was completely inhibited by rapamycin. Similar results were obtained with four additional clones that we examined (data not shown). These results indicate that the S6K1 pathway downstream of mTOR may not be crucial in the regulation of C2C12 myoblast differentiation. Therefore, we suspect that rapamycin inhibits myogenesis through the mTOR/4E-BP1 pathway. In support of this conjecture, S6K1 activity decreased in C2C12 cells undergoing normal differentiation. In vector control and D3E-E389 clones, S6K1 activity decreased by 55% and 61%, respectively, when cells were cultured in differentiation medium without rapamycin (data not shown).
Rapamycin Induces Dephosphorylation of 4E-BP1 in C2C12 Cells—Erbay and Chen (27) reported that whereas rapamycin suppressed activation of S6K1, it had a minor effect on dephosphorylation of 4E-BP1 in C2C12 cells. We examined the effect of rapamycin on 4E-BP1 phosphorylation and function by using three approaches. C2C12 cells were cultured in DM with or without rapamycin (100 ng/ml) for up to 48 h. First, we determined the phosphorylation state of 4E-BP1 by Western blot analysis under electrophoretic conditions that separate isoforms. Rapamycin-induced dephosphorylation of 4E-BP1 was observed in the parental C2C12 cells at all times tested (3–48 h of culture; Fig. 3A); this result is inconsistent with those of Erbay and Chen (27). Second, because mTOR has been reported to phosphorylate 4E-BP1 at residues Thr37 and Thr46 (20), we examined the phosphorylation status of Thr 37 and Thr 46 of 4E-BP1 by using phospho-specific antibodies. The phosphorylation of Thr 37 and Thr 46 was slightly decreased (∼20–30%) in rapamycin-treated C2C12 cells (Fig. 3A). Third, we independently determined whether rapamycin affected the binding activity of 4E-BP1 to eIF4E protein. We used the 7-methyl-GTP-Sepharose binding assay of Gingras et al. (32) to detect 4E-BP1 associated with eIF4E. Under control conditions, virtually no 4E-BP1 was associated with eIF4E (Fig. 3B). In contrast, 4E-BP1 was associated with eIF4E in rapamycin-treated cells. These results indicate that rapamycin inhibits mTOR signaling to 4E-BP1 under conditions in which it inhibits differentiation.

Rapamycin-resistant mTOR Signaling to 4E-BP1 Requires mTOR Kinase Function and N-terminal Sequences—To further explore how mTOR signaling regulates C2C12 myogenesis, we derived stable C2C12 cell lines expressing mTORrr constructs with N-terminal truncations of 10 (∆10mTORrr) or 91
with eIF4E in C2C12 larly, rapamycin induced only a weak association of 4E-BP1/H9004 ing C2C12 was hypophosphorylated in rapamycin-treated clones express-

antibodies (β-tubulin is shown as a loading control). B, changes in phosphorylation were assessed by binding of 4E-BP1 to 7-methyl-GTP-Sepharose as described in Fig. 3A. All blots were processed using similar conditions for exposure.

**FIG. 4.** Effect of expression of kinase-dead (mTORrrSIDA), Δ10mTORrr, or Δ91mTORrr rapamycin-resistant mutants on rapamycin-induced dephosphorylation of 4E-BP1 in C2C12 myoblasts. Clones of C2C12 that stably expressed rapamycin-resistant mTOR mutants were grown and processed as described in Fig. 3A. Changes in phosphorylation were assessed by electrophoretic mobility and by phospho-specific antibodies. C2C12mTORrr or the kinase-dead mTORrrSIDA. Similarly, rapamycin induced only a weak association of 4E-BP1 with eIF4E in C2C12mTORrr cells, whereas 4E-BP1 associated with eIF4E in the presence of rapamycin in the cells expressing C2C12mTORrr or kinase-dead mTORrrSIDA (Fig. 4B). These results further indicate that both the N-terminal 11–91 amino acids of mTOR and the kinase domain of mTOR are crucial for phosphorylation of 4E-BP1.

**Rapamycin Inhibits Proliferation and Differentiation of C2C12 Cells Expressing Δ91mTORrr or Kinase-dead mTORrrSIDA**—To determine whether C2C12Δ91mTORrr or C2C12mTORrrSIDA were resistant to the proliferation-inhibitory effects of rapamycin, vector control and clones expressing mTORrr mutants were grown in GM for 5 days with or without rapamycin, and the IC50 was calculated for each cell line (Fig. 5A). Vector control cells and C2C12 cells expressing Δ91mTORrr or mTORrrSIDA were equally sensitive to rapamycin. Thus, neither mutant signals to 4E-BP1 in the presence of rapamycin or confers resistance to the growth-inhibitory action of this agent. To determine whether the expression of Δ91mTORrr or the kinase-dead mTORrrSIDA allowed myogenic differentiation in the presence of rapamycin, we cultured clones in DM with or without rapamycin (1, 10, or 100 ng/ml) for 3 days and used immunofluorescence to detect myosin heavy chain. C2C12 cells that expressed Δ91mTORrr or mTORrrSIDA differentiated normally in the absence of rapamycin, although cell death was slightly increased in clones expressing the kinase-dead construct (Fig. 5B). Differentiation of both clones was markedly suppressed by 1 ng/ml rapamycin and completely inhibited by 10 ng/ml rapamycin. These results indicate that both the N-terminal 11–91 amino acids and the kinase function of mTOR are essential for the regulation of myogenesis.

**DISCUSSION**

Myogenic differentiation is a highly complex process regulated by the balance between positive and negative effectors. The insulin-like growth factors (IGF-I and IGF-II) are unique among growth factors in that they stimulate both proliferation and differentiation of muscle cells in culture (10, 33). We have shown previously (29, 34) that the macrolide antibiotic rapamycin inhibits one signaling pathway downstream of the IGF-I receptor. A rapamycin-sensitive pathway is increasingly thought to be required for myogenic differentiation. Whereas rapamycin potently inhibits cell growth and induces G1 arrest, it also prevents myogenesis in both L6 and C2C12 myoblasts. However, the precise mechanism by which rapamycin inhibits myogenesis has not been elucidated. Rapamycin, when bound to its cytosolic receptor FK506-binding protein 12, potently inhibits signaling by the serine/threonine kinase mTOR. Although it has been established that inhibition of mTOR is responsible for the growth-inhibitory effect of rapamycin (29), there are only two reports that rapamycin inhibits myogenesis via inhibition of mTOR (12, 27). The investigators in the more recent study (27) concluded that the kinase function of mTOR was not essential for myogenic differentiation of C2C12 myoblasts and that rapamycin did not cause hypophosphorylation of 4E-BP1. If these findings are substantiated, they will have identified the first kinase-independent function of mTOR.

To further understand which domains and functions of mTOR are required for myogenesis, we investigated the ability of a rapamycin-resistant mutant mTOR (S2035I) with N-terminal deletions to support downstream signaling and myogenesis in the presence of rapamycin. In parental C2C12 myoblasts, rapamycin potently inhibited differentiation and suppressed or delayed the expression of muscle-specific proteins, confirming previously reported results (10, 12, 27). C2C12 myoblasts that expressed Δ10mTORrr were highly resistant to inhibition of proliferation by rapamycin under growth conditions (GM), and they differentiated normally when shifted to DM in the absence of rapamycin. However,
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FIG. 5. Rapamycin inhibits the proliferation and differentiation of C2C12 cells that stably express Δ10mTORrr and kinase-dead mTORrrSIDA mutants. A, C2C12Δ91mTORrr (●), C2C12Δ12mTORrrSIDA (■), or vector control (○) cells were cultured in GM in the presence or absence of increasing concentrations of rapamycin. After 5 days, cells were counted as described under “Experimental Procedures” (n = 3; error bars < symbol size). B, C2C12Δ91mTORrr (top panels) and C2C12Δ12mTORrrSIDA (bottom panels) cells were cultured in DM for 3 days in the presence or absence of rapamycin (1, 10, or 100 ng/ml) and examined by immunofluorescence for expression of myosin heavy chain.

Unlike parental and vector control clones, C2C12 myoblasts expressing Δ10mTORrr differentiated normally in the presence of high concentrations of rapamycin. This result strongly suggests that inhibition of mTOR by rapamycin is the mechanism responsible for the suppression of differentiation, as reported previously (12, 27). Furthermore, deletion of the N-terminal 10 amino acids does not compromise mTOR-dependent myogenesis.

mTOR controls initiation of translation through two downstream pathways: 4E-BP1/eIF4E and ribosomal S6K1. mTOR directly phosphorylates S6K1 at Thr389, a residue whose phosphorylation is rapamycin-sensitive in vitro and is necessary for S6 kinase activity (21). When we exposed myoblasts that stably expressed the constitutively active S6K1 mutant to rapamycin, none of five clones examined differentiated, although differentiation occurred in DM in the absence of rapamycin. The basal level of S6K1 activity was increased in these cells and was relatively resistant to inhibition by rapamycin. These findings suggest that inhibition of S6K1 activity is not required for inhibition of myogenesis.

We next assessed inhibition of the mTOR/4E-BP1 pathway by rapamycin. mTOR phosphorylates 4E-BP1 at Thr37 and Thr46 and blocks its association with the cap-binding protein eIF4E in vitro. Phosphorylation of Thr46 appears to be the major regulator of the 4E-BP1-eIF4E interaction in vitro (21). Most studies of the mTOR-4E-BP1 pathway have focused on its role in cell growth and proliferation, although a single report concluded that 4E-BP1 is unlikely to be involved in the rapamycin-sensitive regulation of differentiation in C2C12 cells (27). In our present study, Thr37 and Thr46 of 4E-BP1 were hyperphosphorylated in C2C12 cells cultured in either GM or DM, and their phosphorylation was slightly decreased in rapamycin-treated cells. We observed previously (28) that relatively small changes in 4E-BP1 phosphorylation were associated with inhibition of tumor growth by the rapamycin analogue CCI-779. Consistent with this finding, 4E-BP1 associated with eIF4E in rapamycin-treated C2C12 myoblasts. These results differ from those of Erbay and Chen (27). Our study demonstrates potent inhibition of mTOR signaling to 4E-BP1 in rapamycin-treated C2C12 cells. Residues Thr37 and Thr46 remained hyperphosphorylated in C2C12 cells expressing Δ10mTORrr, and 4E-BP1 was not associated with eIF4E in the presence of rapamycin. Importantly, C2C12Δ10mTORrr cells continued to differentiate in the presence of rapamycin, whereas neither C2C12Δ91mTORrr nor kinase-dead mTORrr-SIDA prevented rapamycin-induced hypophosphorylation of 4E-BP1 and association of 4E-BP1 with eIF4E.

It has been reported that a kinase-inactive rapamycin-resistant mTOR mutant (D2357E) can support myogenic differentiation of C2C12 myoblasts in the presence of rapamycin (27). That report is the first to describe an mTOR function that is independent of mTOR kinase activity. It has been shown that mTOR signaling to 4E-BP1 is dependent on its kinase function (15). Signaling to S6K1 was also shown to require kinase activity and was abrogated by deletion of 70 residues from the N terminus of mTOR (35). We therefore examined the ability of two other rapamycin-resistant deletion mutants to support proliferation and myogenesis in rapamycin-treated C2C12 cells. Proliferation of C2C12Δ91mTORrr and kinase-dead mTORrrSIDA cells was as sensitive to inhibition by rapamycin as that of control or parental C2C12 cells. The Δ91mTORrr and kinase-dead mTORrrSIDA cells differentiated normally in DM medium, but neither differentiated in the presence of rapamycin. These results, which indicate that the kinase function of mTOR is required for myogenic differentiation, are contradictory to those of Erbay and Chen (27).

In our study, C2C12Δ10mTORrr myoblasts were rapamycin resistant and differentiated in the presence of rapamycin, but C2C12Δ91mTORrr myoblasts were sensitive to rapamycin, and their differentiation was inhibited. This result indicates that the region of amino acids 11–91 in the N terminus of mTOR has a functional domain that is crucial for mTOR downstream signaling to both 4E-BP1 and S6K1 (35). The N-terminal 1200 amino acids of mTOR proteins comprise a HEAT domain (named for the first proteins found to possess such a motif: Huntington, elongation factor 3, the regulatory A subunit of PP2A, and Tor1p; Refs. 36–38). HEAT domains form curved rods that consist of α-loop-α repeats and provide a large hydrophobic surface area for potential protein-protein interactions (39–42). Because Δ91mTORrr is truncated in this HEAT sequence, protein interactions may be disturbed.

Taken together, our study substantiates the finding that mTOR plays a crucial role in controlling myogenesis in C2C12 cells. mTOR-dependent activation of the S6K1 pathway does not appear to be essential for muscle cell differentiation, whereas signaling to 4E-BP1 appears to be important. The N-terminal amino acids 11–91 and the kinase domain of mTOR appear to be essential for regulating myogenesis.

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REFERENCES
1. Arnold, H. H., and Braun, T. (1996) Int. J. Dev. Biol. 40, 345–353
2. Davis, R. L., Weintraub, H., and Lassar, A. B. (1987) Cell 51, 987–1000
3. Wright, W. E., Sassoon, D. A., and Lin, V. K. (1989) Cell 56, 607–617
4. Braun, T., Buschhausen-Denker, G., Beber, E., Tannich, E., and Arnold, H. H. (1989) EMBO J. 8, 701–709
5. Rhodes, S. J., and Konieczny, S. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 93, 4076–4080
6. Jefferies, H. B., Reinhard, C., Kozma, S. C., and Thomas, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 93, 6649–6653
7. Price, D. J., Grove, J. R., Calvo, V. C., Avruch, J., and Bierer, B. E. (1992) Science 257, 170–172
8. Hoekstra, M. F., Aebersold, R., and Sonenberg, N. (1999) Genes Dev. 13, 1422–1437
9. Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H., and Sabatini, D. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1432–1437
10. Kuo, C. J., Chung, J., Firestone, D. F., Planagan, W. M., Blenis, J., and Crabtree, G. R. (1992) Cell 69, 1227–1236
11. Cuenda, A., and Cohen, P. (1999) Mol. Cell 27, 6653–6662
12. Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J., and Florini, J. R. (1996) J. Biol. Chem. 271, 6653–6662
13. Cuenda, A., and Cohen, P. (1999) Cancer Res. 59, 886–894
14. Liu, N., Dias, P., and Houghton, P. J. (1998) Cell Growth Differ. 9, 699–711
15. Florini, J. R., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., and Schreiber, S. L. (1995) Cancer Res. 55, 115–116
16. Groves, M. R., and Barford, D. (1999) Curr. Opin. Struct. Biol. 9, 383–389
17. Groves, M. R., Hanlon, N., Turweski, P., Hemnings, B. A., and Barford, D. (1999) Cell 96, 99–110
18. Chook, Y. M., and Blobel, G. (1999) Nature 399, 230–237
19. Maretigianio, J., Lonakina, I. B., Sonenberg, N., Pestova, T. V., Hellen, C. U. T., and Burley, S. K. (2001) Mol. Cell 7, 183–203