The Mammalian Target of Rapamycin Regulates C2C12 Myogenesis via a Kinase-independent Mechanism*

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Rapamycin inhibits differentiation of mouse C2C12 myoblasts, a tissue culture model for skeletal muscle differentiation. The mechanism by which a rapamycin-sensitive signaling pathway regulates myogenesis is largely unknown. The mammalian target of rapamycin (mTOR) is a central regulator of cell growth and proliferation, but its role in myogenesis has not been examined directly. Here we report the investigation of the function of mTOR and its downstream effectors in muscle differentiation. Rapamycin exerts an inhibitory effect on C2C12 myogenesis at different stages, implying that a rapamycin-sensitive pathway may be required for multiple processes during muscle differentiation. The mTOR protein level increases 10-fold during differentiation, via a post-transcriptional mechanism. As the first direct demonstration of the essential role of mTOR in muscle differentiation, we show that a rapamycin-resistant mTOR, but not S6 kinase 1, can rescue rapamycin-inhibited myogenesis. Remarkably, the myogenic function of mTOR does not require its kinase activity. Two downstream effectors of the rapamycin-sensitive pathway, S6 kinase 1 and eIF4E-binding protein 1, undergo differential regulation during myogenesis, but neither protein is the relevant effector for the myogenic signaling of mTOR. Taken together, our observations suggest a novel mTOR signaling mechanism essential for skeletal muscle differentiation.

Skeletal muscle differentiation entails mononucleated myoblasts exiting the cell cycle and fusing to form multinucleated myotubes. This well orchestrated process of cell cycle withdrawal and initiation of differentiation is largely regulated by the MyoD and myocyte enhancer factor-2 families of transcription factors (1, 2). Most mitogens promote myoblast proliferation and inhibit differentiation, whereas insulin and insulin-like growth factors (IGF-I and IGF-II) stimulate both processes in muscle cell cultures (3, 4). In cultured myoblasts serum withdrawal often initiates the differentiation program, and it is believed that autocrine/paracrine actions of IGF-II in response to growth factor deprivation stimulate muscle differentiation (5, 6). The signaling pathways regulating myogenesis are beginning to be elucidated, one of which is the PI3K-Akt pathway (reviewed in Ref. 4). Other PI3K downstream effectors, including S6K1, may also participate in myogenic regulation, but the signaling mechanisms remain poorly understood.

Rapamycin, a potent immunosuppressant of bacterial origin, inhibits cell growth and proliferation in a variety of organisms ranging from yeast to mammals. When complexed with the ubiquitous cellular protein FKBP12, rapamycin directly binds and inhibits mTOR (also named FRAP [FKBP12-rapamycin-associated protein] and RAFT1 [rapamycin and FKBP12 target]) (7–9). mTOR belongs to the PIKK family of novel Ser/Thr kinases and functions as a master regulator of cell growth and cell cycle progression (10, 11). A protein of likely pleiotropic functions, mTOR is best known for its role in regulating translation initiation (12). Among several downstream effectors of the mTOR pathway, two best characterized ones are 4E-BP1 and S6K1, which regulate 5′-cap-dependent and 5′-terminal oligopyrimidine tract-dependent translation initiation, respectively (13, 14). Both 4E-BP1 and S6K1 are regulated by mTOR and the PI3K pathway in parallel (12, 14); each pathway is indispensable for the stimulation of both proteins. It has been well established that the kinase activity of mTOR is essential for S6K1 activation and 4E-BP1 phosphorylation (15–17).

The effect of rapamycin on myogenesis has been examined in several muscle cell culture systems, and the results have been controversial. Although rapamycin inhibits differentiation of some myoblasts (18–20), promotion of myogenesis by rapamycin has also been reported (21), and in some systems no rapamycin effect is observed (22). As the downstream effector of both the PI3K pathway and the mTOR pathway, S6K1 activity has been shown to increase during skeletal muscle differentiation in several cases (18–20). However, whether S6K1 is required for myogenesis remains an unanswered question, and whether mTOR has an essential function in skeletal muscle differentiation has not been examined directly. In C2C12 muscle cell culture, we investigated the role of mTOR and its downstream effectors in muscle differentiation. Here we provide definitive evidence to support the essential role of mTOR in myogenesis and to exclude the requirement of S6K1. Furthermore, our observations reveal a novel signaling mechanism that governs the myogenic function of mTOR, which does not require the kinase activity of mTOR and most likely involves downstream effectors other than S6K1 and 4E-BP1.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Rapamycin was purchased from Calbiochem. The following antibodies were obtained from commercial sources: M2 anti-FLAG, Sigma; anti-4E-BP1, Zymed Laboratories Inc.; anti-S6K1 (for Western blotting), New England Biolabs; MF20 anti-MHC, the Hybridoma Bank (University of Iowa); all secondary antibodies, Jack-to-3-kinase; PIKK, phosphatidylinositol kinase-related kinase; S6K1/2, S6 kinase 1/2; 4E-BP1, eIF4E-binding protein 1; MHC, myosin heavy chain; FBS, fetal bovine serum; RR, rapamycin-resistant; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

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1 The abbreviations used are: IGF, insulin-like growth factor; mTOR, mammalian target of rapamycin; FKBP12, FK506-binding protein; FRB, FKBP12-rapamycin-binding (domain); PI3K, phosphatidylinositol-
son Immunoresearch Laboratories. A rabbit polyclonal anti-mTOR antibody raised against the FRB domain (23), a mouse polyclonal anti-mTOR antibody raised against amino acids 1235–1292, and 9E10.2 anti-Myc ascites were generated by the Immunological Research Facilities at the University of Illinois at Urbana-Champaign.

All expression plasmids were constructed in pCDNA3 (Invitrogen). FLAG-mTOR, FLAG-mTOR-2035T, and FLAG-mTOR-2035T2357E were described previously (24). Myc-S6K1 (amino acids 1–698) and Myc-S6K1-BR (amino acids 55–398) (25) were generated by amplifying the corresponding cDNAs by polymerase chain reaction from a cDNA library, which were then inserted into pCDNA3-Myc (23) via NolI and Xhol sites.

**Cell Culture and Transfection**—C2C12 myoblasts were maintained in DMEM containing 20% FBS and 0.5% chicken embryo extract were induced to differentiate in differentiation medium (DMEM with 2% horse serum), and fed with fresh differentiation medium every day until myotubes were fully formed (3 days).

C2C12 cells grown on 6-well plates to 40–50% confluence were transfected with 3 μg of DNA per well using PolyFect (Qiagen). After 24 h, the transfected cells were trypsinized and re-plated into growth medium containing 1 mg/ml G418 on 12-well plates. When the cells reached 100% confluence, differentiation was induced by incubation in differentiation medium with or without rapamycin for 3–4 days.

**Phase-contrast and Immunofluorescence Microscopy**—C2C12 myoblasts or myotubes grown on tissue culture plates were fixed in 3.7% formaldehyde (made in PBS), permeabilized in 0.1% Triton X-100, and stained with MF20 anti-MHC antibody (26). Both phase contrast and fluorescent images were recorded using a CCD camera.

**Kinase Assays**—Transfected or nontransfected C2C12 cells were lysed in lysis buffer (20 mM Tris-Cl, pH 7.5, 0.1 mM Na3VO4, 25 mM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.3% Triton X-100). mTOR autokinase assays were performed as described previously (24) with immunoprecipitated recombinant proteins (using anti-FLAG) or the endogenous protein (using the mouse polyclonal anti-mTOR). Endogenous S6K1 and recombinant S6K1 were immunoprecipitated using a polyclonal anti-S6K1 antibody and the anti-Myc antibody, respectively. S6 kinase assays were carried out with the immune complexes using a peptide substrate as described previously (23).

**Northern Analysis**—Total RNA from a 60-mm plate of C2C12 cells was isolated using the RNeasy mini kit (Qiagen). 20 μg of RNA per lane were run on 0.8% formaldehyde agarose gels, followed by visualizing rRNAs by ethidium bromide staining and transferring to Hybond-N membranes (Amersham Pharmacia Biotech). Hybridizations were carried out in Church buffer (0.5 M sodium phosphate, pH 7.4, 1 mM EDTA, 7% SDS) at 65 °C overnight, with a radioactive probe generated by random priming using a mouse mTOR cDNA 5’ fragment (nucleotide 1–698) as template. Membranes were washed in 40 mM sodium phosphate (pH 7.4), 1 mM EDTA, and 5% SDS at 65°C and analyzed by phosphorimaging.

**RESULTS AND DISCUSSION**

**Rapamycin Inhibits C2C12 Cell Differentiation**—C2C12 myoblasts grown to 100% confluence in medium containing 20% FBS and 0.5% chicken embryo extract were induced to differentiate by serum withdrawal (2% horse serum). Typically, cell fusion and formation of small myofibers were evident 2 days after induction, and myofibers were fully formed in 3 days. When added at the beginning of induction, 20 nM rapamycin completely blocked C2C12 differentiation (Fig. 1), consistent with observations reported by others (18–20). To assess the time window of the inhibitory effect of rapamycin, rapamycin was added at various time points or removed after certain periods. Myotube formation and the presence of MHC, a skeletal muscle marker, were examined 3–4 days after induction. As shown in Fig. 1, rapamycin effectively blocked terminal differentiation even when added as late as 36 h (1.5 days) after induction. After 2 days of differentiation induction, rapamycin addition no longer had any effect on the complete formation of myofibers. These observations imply that the rapamycin-sensitive pathway is required during the mid-phase of muscle differentiation.

Because myogenin is an early differentiation marker for skeletal muscle (26), and its production is inhibited by rapamycin in C2C12 cells (20), we further examined the effect of rapamycin during the early stage of differentiation. When cells were exposed to rapamycin during the first 24 h only, subsequent myofiber formation was delayed for at least 24 h (Fig. 1), suggesting that the rapamycin-sensitive pathway is also required for the early stage of myogenesis. Therefore, mTOR may
be involved in regulating multiple events during muscle differentiation.

**Differential Regulation of S6K1 and 4E-BP1 during C2C12 Myogenesis**—To investigate the molecular mechanisms of the rapamycin-sensitive pathway in C2C12 differentiation, we set out to determine the activity and phosphorylation of S6K1 and 4E-BP1. As shown in Fig. 2A, S6K1 activity increased steadily during differentiation, and it was inhibited by rapamycin, consistent with previous reports in both C2C12 and L6A1 myoblasts (18–20). In contrast, 4E-BP1 phosphorylation monitored by gel mobility shift remained elevated (Fig. 2B). Both S6K1 and 4E-BP1 protein levels were unchanged during differentiation. Surprisingly, 4E-BP1 phosphorylation during the differentiation period was mostly resistant to rapamycin (up to 100 nM), whereas S6K1 activity was completely inhibited by 5 nM rapamycin (data not shown). Considering that 20 nM rapamycin is sufficient to abolish differentiation (Fig. 1), 4E-BP1 is unlikely to be involved in the rapamycin-sensitive regulation of differentiation in C2C12 cells. S6K1, on the other hand, remains a candidate regulator of differentiation in the rapamycin-sensitive pathway.

**mTOR Is Up-regulated Post-transcriptionally during C2C12 Differentiation**—To gain further insight into the role of the rapamycin-sensitive pathway in myogenesis, we examined mTOR, the cellular target of rapamycin. The mTOR protein level increased drastically during differentiation (Fig. 3A); when compared in the same number of cells, the amount of mTOR was 10-fold higher on day 3 of differentiation than on day 0 (Fig. 3B), whereas the increase of total cellular protein content was ~1.5-fold. The in vitro kinase activity of mTOR from myoblasts and differentiated cells was also compared. Although the total activity of mTOR increased upon differentiation, its specific activity did not increase (Fig. 3C). Northern analysis revealed that the mRNA level of mTOR remained constant during differentiation (Fig. 3D). Thus, it is possible that mTOR, the master regulator of translation, may itself be regulated at the translational level during the transition from myoblasts to myotubes. However, a protein stability change has not been ruled out.

**mTOR Regulates Myogenesis via a Kinase-independent Mechanism; S6K1 Is Not Required**—To probe a direct link between mTOR function and myogenesis, we next asked whether a rapamycin-resistant mTOR could reverse the inhibitory effect of rapamycin on C2C12 differentiation. A point mutation, S2035T, confers rapamycin resistance to mTOR function by abolishing rapamycin binding to the FRB domain (15, 27). C2C12 cells transfected with various mTOR cDNAs constructed in pCDNA3 (under the control of a cytomegalovirus promoter) were selected in G418-containing medium and induced to differentiate in the presence of rapamycin (Fig. 4A). As shown in Fig. 4A, cells expressing the rapamycin-resistant mutant of mTOR differentiated in the presence of rapamycin, as indicated by the appearance of myofibers and the presence of MHC. Expression of the wild-type mTOR or transfection of the empty pCDNA3 vector did not protect C2C12 from the inhibitory effect of rapamycin. As further controls, transfection and G418 selection did not affect normal differentiation.

**Fig. 3.** mTOR is up-regulated at a post-transcriptional level during myogenesis. C2C12 cells were induced to differentiate in differentiation medium for 3 days, during which time cell lysates were generated at various times for subsequent analyses. A, lysates from differentiating cells on various days were simultaneously analyzed for mTOR and MHC by Western blotting. B, lysates from the same number of cells before (day 0) and after (day 3) differentiation were analyzed for mTOR by Western blotting. Chemiluminescent signals from the Western blots were recorded by x-ray films and quantified using the software NIH Image (version 1.62). C, mTOR protein was immunoprecipitated from day 0 myoblasts and day 3 myotubes, followed by in vitro autokinase assays. Samples were analyzed for protein levels by Western blotting and for autophosphorylation by phosphorimaging. The specific activity was calculated as the ratio of radioactive signal versus Western signal. Results shown are relative specific kinase activities with that on day 0 as 1. D, 20 μg of total RNA from day 0 myoblasts and day 3 myotubes were analyzed by Northern blotting using a mouse mTOR cDNA fragment as probe. Ethidium bromide staining of rRNAs is shown as a control for equal RNA amounts.

**Fig. 4.** Rapamycin-resistant mTOR rescues rapamycin-inhibited myogenesis independent of kinase activity. A, C2C12 myoblasts were transfected with various cDNA constructs, selected by G418, and induced to differentiate in the absence or presence of 20 nM rapamycin (Rap). After 3 days of induction, cells were fixed and immunostained for MHC. Phase contrast (light) and immunofluorescent (FITC) images are shown. The designations of constructs are as follows: WT, wild-type; KI, kinase-inactive. B, Myc-tagged wild-type and rapamycin-resistant S6K1 (S6K1-RR) were expressed in C2C12 cells with or without treatment by 50 nM rapamycin for 45 min prior to lysis, followed by 86 kinase assays with immunoprecipitated recombinant Myc-S6K1.
differentiation in the absence of rapamycin (Fig. 4A). These observations provide the first direct evidence that mTOR is required for C2C12 myogenesis. Most unexpectedly, a kinase-inactive mutant (D2357E) of mTOR (15, 23) containing the rapamycin-resistant mutation also fully rescued C2C12 myogenesis in the presence of rapamycin (Fig. 4A), clearly indicating that the myogenic function of mTOR does not require its kinase activity. This is the first example of a kinase-independent function conferred by a PIKK protein; until now, all the reported functions associated with each member of the PIKK family required catalytic activity of the kinase. Whether rapamycin inhibits mTOR kinase activity in vivo has been a controversial issue (discussed in Ref. 12); our observation of the rapamycin-resistant and kinase-inactive mTOR behavior definitively dissociates the effect of rapamycin from mTOR kinase inhibition in myogenesis. The fact that differentiation proceeded normally in the presence of enough rapamycin to abolish S6K1 activity when a kinase-inactive and rapamycin-resistant mTOR is overexpressed, definitively indicates that S6K1 activity is not required for myogenesis. Consistently, a rapamycin-resistant S6K1 mutant (N-terminal 54 amino acids and C-terminal 104 amino acids deleted; see Ref. 25) was unable to reverse the inhibitory effect of rapamycin on C2C12 differentiation (Fig. 4A). The rapamycin-resistant activity of this mutant was confirmed in C2C12 cells (Fig. 4B). A functional homologue of S6K1, S6K2, is another downstream effector of mTOR (reviewed in Ref. 14) and has not been examined in this system. However, S6K2 activation also requires kinase activity of mTOR,2 and thus is unlikely to be the relevant target of mTOR in myogenesis.

What is the biological end point of mTOR pathway in myogenesis? Although the translational regulation of factors required for myogenesis is a possibility for mTOR function, the obvious candidate mediators of this function, S6Ks and 4E-BP1, have already been ruled out. In addition to translation, the yeast TOR proteins are involved in regulating other processes, including transcription and cytoskeleton reorganization (11). Hence, it is conceivable that mTOR may regulate myogenesis via modulating cellular processes other than translation. Skeletal muscle differentiation involves several concerted and temporally separable events (26). The ability of rapamycin to inhibit more than one stage of the differentiation (Fig. 1) implies that mTOR may be involved in regulating multiple events of differentiation. Identification of the downstream effectors responsible for the myogenic signaling of mTOR will not only provide a better understanding of molecular mechanisms controlling myogenesis but also further unravel the multifunctionality of mTOR.

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REFERENCES

1. Olsen, E. N., Perry, M., and Schulz, R. A. (1995) Dev. Biol. 172, 2–14
2. Weintraub, H. (1993) Cell 75, 1241–1244
3. Florini, J. R., Ewton, D. Z., and Magri, K. A. (1991) Annu. Rev. Physiol. 53, 201–216
4. Perry, R. L. S., and Rudnicki, M. A. (2000) Front. Biosci. 5, 750–767
5. Florini, J. R., Magri, K. A., Ewton, D. Z., James, P. L., Grindstaff, K., and Rotwein, P. S. (1991) J. Biol. Chem. 266, 15917–15923
6. Tollefson, S. E., Sadow, J. L., and Rotwein, P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1543–1547
7. Brown, E. J., Abers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) Nature 369, 756–758
8. Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S. H. (1994) Cell 78, 35–43
9. Sabers, C. J., Martin, M. M., Brunn, G. J., Williams, J. M., Dumont, F. J., Wiederrrecht, G., and Abraham, R. T. (1995) J. Biol. Chem. 270, 815–822
10. Keith, C. T., and Schreiber, S. L. (1995) Science 270, 50–51
11. Schmelzle, T., and Hall, M. N. (2000) Cell 103, 253–262
12. Gingras, A. C., Raught, B., and Sonenberg, N. (2001) Genes Dev. 15, 807–826
13. Gingras, A. C., Raught, B., and Sonenberg, N. (1999) Ann. Rev. Biochem. 68, 913–963
14. Pumagalli, S., and Thomas, G. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 695–718, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
15. Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., and Schreiber, S. L. (1995) Nature 377, 441–446
16. Brunn, G. J., Hudson, C. C., Sekulic, A., Williams, J. M., Houghton, P. J., Lawrence, J. C. Jr., and Abraham, R. T. (1997) Science 277, 98–101
17. Harati, Y., Yonezawa, K., Kozlowski, M. T., Sugimoto, T., Andrabi, K., Weng, Q. P., Kanuga, M., Nishimoto, I., and Avruch, J. (1997) J. Biol. Chem. 272, 26457–26463
18. Cione, R., Valerde, A. M., Benito, M., and Lorenzo, M. (2001) J. Cell. Physiol. 186, 82–94
19. Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J., and Florini, J. R. (1997) J. Biol. Chem. 272, 6653–6660
20. Cuenda, A., and Cohen, P. (1999) J. Biol. Chem. 274, 4341–4346
21. Jayaraman, T., and Marles, A. R. (1993) J. Biol. Chem. 268, 25385–25388
22. Canicio, J., Gallardo, E., Illa, I., Testar, X., Palacin, M., Zorzano, A., and Kaliman, P. (1998) Endocrinology 138, 5042–5049
23. Kim, J. E., and Chen, J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 14340–14345
24. Villalba-Bach, M., Nuzzi, F., Fang, Y., and Chen, J. (1999) J. Biol. Chem. 274, 4266–4272
25. Dennis, P. B., Pullen, N., Kozma, S. C., and Thomas, G. (1996) Mol. Cell. Biol. 16, 6242–6251
26. Anders, V., and Walsh, K. (1996) J. Cell Biol. 132, 657–666
27. Chen, J., Zheng, X. F., Brown, E. J., and Schreiber, S. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4947–4951
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