mTOR function in skeletal muscle hypertrophy: increased ribosomal RNA via cell cycle regulators

Gustavo A. Nader, Thomas J. McLoughlin, and Karyn A. Esser

School of Kinesiology, The University of Illinois at Chicago, Chicago, Illinois

Submitted 7 April 2005; accepted in final form 27 July 2005

Nader, Gustavo A., Thomas J. McLoughlin, and Karyn A. Esser. mTOR function in skeletal muscle hypertrophy: increased ribosomal RNA via cell cycle regulators. Am J Physiol Cell Physiol 289: C1457–C1465, 2005.—The purpose of this study was to identify the potential downstream functions associated with mammalian target of rapamycin (mTOR) signaling during myotube hypertrophy. Terminally differentiated myotubes were serum stimulated for 3, 6, 12, 24, and 48 h. This treatment resulted in significant myotube hypertrophy (protein/DNA) and increased RNA content (RNA/DNA) with no changes in DNA content or indices of cell proliferation. During myotube hypertrophy, the increase in RNA content was accompanied by an increase in tumor suppressor protein retinoblastoma (Rb) phosphorylation and a corresponding increase in the availability of the ribosomal DNA transcription factor upstream binding factor (UBF). Serum stimulation also induced an increase in cyclin D1 protein expression in the differentiated myotubes with a concomitant increase in cyclin D1-dependent cyclin-dependent kinase (CDK)-4 activity toward Rb. The increases in myotube hypertrophy and RNA content were blocked by rapamycin treatment, which also prevented the increase in cyclin D1 protein expression, CDK-4 activity, Rb phosphorylation, and the increase in UBF availability. Our findings demonstrate that activation of mTOR is necessary for myotube hypertrophy and suggest that the role of mTOR is in part to modulate cyclin D1-dependent CDK-4 activity in the regulation of Rb and ribosomal RNA synthesis. On the basis of these results, we propose that common molecular mechanisms contribute to the regulation of myotube hypertrophy and growth during the G1 phase of the cell cycle.

mTOR function in skeletal muscle hypertrophy: increased ribosomal RNA via cell cycle regulators

THE MAMMALIAN TARGET OF RAPAMYCIN (mTOR) has recently emerged as a central controller of cell growth in both proliferating and differentiated cell types (9, 10, 37). In skeletal muscle, the activity of mTOR has been shown to be required for mechanical overload-induced hypertrophy (3, 17). These studies demonstrated that inhibition of mTOR by administration of rapamycin was effective in preventing mechanical overload-induced skeletal muscle hypertrophy of the rat plantaris muscle (3, 17). Similarly, rapamycin administration prevented the hypertrophic action of IGF-1 in skeletal muscle myotubes (36), demonstrating that, both in vivo and in vitro, the activity of mTOR is required for skeletal muscle hypertrophy.

Although it is clear that mTOR is important for the regulation of skeletal muscle hypertrophy, the mechanisms by which this regulation occurs are not fully understood. Several lines of evidence have demonstrated that mTOR exerts its role in protein synthesis at the initiation step by phosphorylating and inhibiting the eukaryotic initiation factor 4 (eIF4E)-binding protein 1 (4E-BP1) and thereby relieving repression of eIF4E and cap-dependent translation (10, 35, 37). Another mTOR-regulated mechanism important for protein synthesis is the regulation of ribosome biogenesis (12, 26). mTOR controls this process by at least two different mechanisms: 1) modulation of the activation of the 70-kDa ribosomal protein S6 kinase (S6K-1/p70S6K), which in turn plays a role in the translation of ribosomal proteins and other 5’-TOP mRNA (5, 21, 22); and 2) modulation of the synthesis of ribosomal RNA (rRNA) (12, 26).

Ribosome accumulation during cellular hypertrophy has been studied well in several systems and is mainly regulated by de novo ribosome synthesis (27, 28). Transcription of the 45S ribosomal DNA (rDNA) genes is thought to be the rate-limiting step of ribosome synthesis (30, 32). Efficient transcription of rDNA genes is supported by the RNA polymerase I (RNA Pol I) holoenzyme complex, and a series of accessory proteins such the trans-activating factors selectivity factor 1 (SL-1) and upstream binding factor (UBF) (23, 32). The best characterized of these factors is UBF, and its availability for transcriptional activity at the 45S rDNA promoter is regulated in part through sequestration by the tumor suppressor protein retinoblastoma (Rb) (13). Rb is in turn modulated by the concerted action of cyclin-dependent kinases (CDK); their catalytic partners, cyclins (e.g., cyclin D); and cyclin-dependent kinase inhibitors (CKI; e.g., p21) (29, 46).

Given the importance of mTOR in skeletal muscle hypertrophy, the goal of this project was to identify downstream molecular steps by which mTOR may regulate components of protein synthesis and subsequent muscle cell growth. The general hypothesis was that signaling through mTOR modulates increases in rRNA associated with myotube growth. Experiments were performed to determine whether mTOR signaling is necessary for the accumulation of rRNA during growth and whether steps such as availability of UBF, inhibition of Rb, and activation of cyclin D1-dependent CDK-4 activity are mTOR sensitive. The results presented herein demonstrate that mTOR-dependent growth of myotubes is associated with the accumulation of rRNA and an increase in cyclin D1-dependent CDK-4 activity, Rb phosphorylation, and UBF availability. On the basis of these findings, we suggest that the mechanisms modulating mTOR-dependent hypertrophy of differentiated myotubes resemble those necessary for growth during the G1 phase of the cell cycle.

METHODS

Materials. L6 rat myoblasts were purchased from the American Type Culture Collection (Manassas, VA). High-glucose DMEM,

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
FBS, and horse serum (HS) were purchased from GIBCO (Grand Island, NY), and rapamycin was obtained from Calbiochem (La Jolla, CA). Antibodies used were anti-Rb antibody, which was purchased from BD Biosciences Pharmingen (San Diego, CA); anti-CDK-4, obtained from Neomarkers (Fremont, CA); anti-ERK, phospho-ERK, rpS6, phospho-rpS6, Rb-Sepharose 780, and Rb fusion protein, which were purchased from Cell Signaling Technology (Beverly, MA); and anti-cyclin D1, anti-p21, anti-mycosin heavy chain (anti-MHC), and secondary antibodies, which were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-UBF polyclonal antibody was a generous gift from Dr. L. Rothblum (Weiss Center for Research, Geisinger Clinic, Danville, PA). Protein Sepharose beads and ECL solutions were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The bromodeoxyuridine (BrdU) labeling kit was purchased from Roche Biochemicals (Mannheim, Germany), TRIZol reagent was obtained from Invitrogen (Carlsbad, CA), the DNA Wizard isolation kit was purchased from Promega (Madison, WI), the reagent was obtained from Invitrogen (Carlsbad, CA). Antibodies used were anti-Rb antibody, which was purchased from Cell Signaling Technology (Beverly, MA), and anti-cyclin D1, anti-p21, anti-mycosin heavy chain (anti-MHC), and secondary antibodies, which were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-UBF polyclonal antibody was a generous gift from Dr. L. Rothblum (Weiss Center for Research, Geisinger Clinic, Danville, PA). Protein Sepharose beads and ECL solutions were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The bromodeoxyuridine (BrdU) labeling kit was purchased from Roche Biochemicals (Mannheim, Germany), TRIZol reagent was obtained from Invitrogen (Carlsbad, CA), the DNA Wizard isolation kit was purchased from Promega (Madison, WI), the DC protein assay was obtained from Bio-Rad Laboratories (Hercules, CA), and mounting media were purchased from Vector Laboratories (Burlingame, CA).

**Cell culture.** Myoblasts were grown to confluence in differentiation medium (GM: DMEM supplemented with 10% FBS) and were induced to fuse into myotubes by being incubated in differentiation medium (DM; DMEM supplemented with 2% HS) for 4 days. At day 4 postdifferentiation, myotubes were either maintained in DM (control group) or stimulated with GM high-serum medium (20% FBS) for 3, 6, 12, 24, and 48 h. All experiments were performed in a humidified environment at 37°C in a 5% CO2-95% O2 atmosphere. For RNA, DNA, and protein analysis, cells were cultured in six-well plastic plates, of which three wells were used for protein or RNA and the other three were used for DNA quantitation. Protein and RNA experiments were performed in triplicate (n = 3/plate). For commouniprecipitation and kinase assays, cells were grown in 10-mm plates.

**RNA, DNA, and protein quantitation.** Cells were washed with cold PBS and homogenized in TRIZol reagent by being passed several times through a 21-gauge needle. RNA concentration was determined spectrophotometrically at 260 nm. The integrity of the RNA was assessed visually using agarose gel fractionation of the 28S and 18S rDNA subunits. RNA was isolated using the Trizol Wizard kit (Promega) according to the manufacturer’s guidelines and quantified spectrophotometrically at 260 nm. Total protein was isolated after the myotubes were washed as described above, collected in lysis buffer [50 mM HEPES-NaOH, pH 7.5, 1% Nonidet P-40 (NP-40), 150 mM NaCl2, 1 mM EDTA, 2.5 mM EGTA, 50 mM β-glycerophosphate, 200 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM okadaic acid, 100 μM Na3VO4, and 1 μM microcystin-LR] and homogenized by being passed several times through a 27-gauge needle. Total protein content was determined using the Bio-Rad DC protein assay.

**Western blot analysis.** Protein homogenates were mixed 1:1 with 2× Laemmli sample buffer for SDS-PAGE and Western blot analysis. Primary antibody dilutions were for Rb (1:500 dilution), UBF (1: 5,000 dilution), cyclin D1 (1:2,000 dilution), p21 (1:1,000 dilution), and CDK-4 (1:500 dilution), and rpS6, P03-rpS6, ERK, and P03-ERK (1:1000 dilution). After being incubated with primary antibodies, membranes were washed three times with Tris-buffered saline with Tween 20 and incubated with the corresponding anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:10,000 dilution). Protein immunoblots were visualized using ECL, and bands were quantified by performing scanning densitometry.

**Analysis of BrdU-UBF interaction.** Myotubes were lysed in 50 μl of immunoprecipitation buffer (50 mM HEPES-NaOH, pH 7.5, 0.1% NP-40, 150 mM NaCl2, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 50 mM β-glycerophosphate, 200 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM okadaic acid, and 100 μM sodium orthovanadate) and homogenized by being passed through a 21-gauge needle. Homogenates were rotated for 30 min at 4°C and spun at 10,000 g for 10 min, and protein content was determined. After centrifugation, 500 μg of protein were precleared for 1 h with 50 μl of 50% protein Sepharose A slurry. After preclearing, 5 μg of anti-Rb antibody were added and incubated overnight with gentle rotation at 4°C. The antigen-antibody complex was precipitated by adding 50 μl of protein A beads with rotation for 30 min at 4°C and spun at 12,000 g for 20 s. Pellets were washed three times with immunoprecipitation buffer, once with 50 mM Tris-C12, pH 8.0, boiled in 2× Laemmli sample buffer, and resolved using SDS-PAGE as described above. A negative control with a nonspecific antibody was prepared in each individual experiment.

**Analysis of CDK-4 activity.** CDK-4 activity was determined as described by Pestov et al. (33). Myotubes were lysed, and 500 μg of lysate were precleared for 1 h with 50 μl of 50% protein Sepharose G slurry. After preclearing, 2 μg of anti-CDK-4 antibody were added and incubated for 2.5 h with gentle rotation at 4°C. The antigen-antibody complex was precipitated by adding 50 μl of protein G beads, rotated for 30 min at 4°C, and spun at 12,000 g for 20 s, and then the pellets were washed three times with 1 ml of kinase buffer (Cell Signaling Technology). The kinase reaction was performed by incubating the immune complex in 30 μl of kinase buffer with the addition of 1 mM ATP and 2 μg of maltose-binding protein Rb fusion protein (amino acids 701–928) as a substrate. After being incubated for 10 min at 30°C, the reaction was stopped by boiling the sample in 30 μl of 2× Laemmli sample buffer (25). Reaction products were resolved by performing SDS-PAGE (12%), and CDK-4 activity was determined using Western blot analysis with a phosphospecific anti-Rb antibody (1:2,000 dilution) that detects Rb phosphorylation only at Ser780 and does not cross react with any other phosphorylation site in Rb. A negative control with a nonspecific antibody was prepared in each individual experiment.

**Rapamycin treatment.** Rapamycin was resuspended in absolute ethanol and used at a final concentration of 5 ng/ml. Before being incubated with rapamycin, myotubes were washed twice with PBS and incubated with serum-free media, rapamycin or medium, and vehicle for 30 min. Myotubes were then treated for 24 h under one of the following four conditions: DM plus vehicle, DM plus rapamycin, GM plus vehicle, or GM plus rapamycin.

**BrdU labeling and confocal microscopy.** For BrdU visualization, both myoblasts and myotubes were grown on collagen-coated glass plates. BrdU labeling (10 μM) of myotubes was performed for 1 h after 24 h of serum stimulation. As a positive control, asynchronously proliferating myoblasts at ~50–60% confluence were incubated for 1 h with BrdU after 24 h of serum stimulation. After being incubated with BrdU, cells were washed with ice-cold PBS followed by fixation in 15 mM glycine-methanol solution for 20 min at ~20°C. Fixed cells were incubated with working solution for 30 min at 37°C, incubated with an anti-BrdU mouse antibody for 30 min at 37°C, allowed to dry at room temperature, and then covered with VectaShield medium containing 1.5 μg/ml 4',6-diamidino-2-phenylindole HCl (DAPI). Visualization was performed using a multichannel Zeiss LSM-510 confocal microscope.

**Immunohistochemical localization of cyclin D1.** Localization of cyclin D1 expression was performed as previously described (43). Briefly, myotubes were grown as described above and coimmunostained for cyclin D1, MHC, and nuclei (DAPI staining). Cultured cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked, and incubated overnight at 4°C with antibodies against cyclin D1 (1:50 dilution) and MHC (1:50 dilution). Cells were then washed and incubated with mouse anti-rabbit rhodamine-conjugated (tetramethylrhodamine isothiocyanate; 1:200 dilution) and donkey anti-goat FITC (1:200 dilution) secondary antibodies for cyclin D1 and MHC, respectively. Nuclei were stained using DAPI, and immunoreactivity was visualized using a Nikon LabShot-2 fluorescence microscope with Spot RT imaging software (Diagnostic Imaging, Sterling Heights, MI).
RESULTS

Serum stimulation resulted in myotube hypertrophy and increased RNA content. Serum stimulation induced myotube hypertrophy and a significant increase in RNA content. There was no change in myotube protein accumulation or protein:DNA between control and serum-stimulated myotubes at 3, 6, and 12 h (84 ± 3.32, 97 ± 5.33, 94 ± 3.47, and 105 ± 6.02 μg/μg; P > 0.05), respectively, but protein content was significantly increased at 24 and 48 h after serum stimulation (131 ± 6.04 and 178 ± 8.51 μg/μg, respectively; P < 0.05) (Fig. 1A). Similarly to the protein changes, there was no difference in total RNA or RNA:DNA between control and serum-stimulated myotubes at 3, 6, or 12 h (5.1 ± 0.18, 4.73 ± 0.18, 5.10 ± 0.25, and 5.73 ± 0.33 μg/μg; P > 0.05), respectively. However, by 24 and 48 h after serum stimulation, there was a significant increase in RNA:DNA (7.68 ± 0.23, and 9.43 ± 1.20 μg/μg; P < 0.05) respectively (Fig. 1B).

Importantly, no change in the quantity of DNA in the serum-stimulated myotubes was detected at any time point. The average DNA content per well was not different between groups, and the values were 4.84 ± 0.25, 5.09 ± 0.77, 5.17 ± 0.64, 5.15 ± 0.64, 5.07 ± 0.42, and 5.26 ± 0.76 μg for control at 3, 6, 12, 24, and 48 h, respectively (P > 0.05). Consistent with the lack of changes in DNA content, BrdU uptake was not detected in differentiated myotubes in response to serum stimulation (Fig. 2). These findings were important because they demonstrated that the L6 myotubes were terminally differentiated, and any unfused myoblasts did not reenter the cell cycle in response to serum stimulation.

Serum stimulation increased Rb phosphorylation and disrupted Rb-UBF interaction. Serum stimulation induced the phosphorylation of Rb. The ratio of hyperphosphorylated to hypophosphorylated Rb (ppRb:pRb) was not different from control at either 3 or 6 h [0.61 ± 0.03, 0.55 ± 0.03, and 0.73 ± 0.05 optical density (OD); P > 0.05], but there was a significant increase in Rb phosphorylation at 12, 24, and 48 h (1.09 ± 0.08, 1.84 ± 0.06, and 2.16 ± 0.14 OD; P < 0.05) after serum stimulation. Coimmunoprecipitation of the Rb-UBF complex with an anti-Rb antibody revealed a decrease in Rb-UBF interaction at 24 and 48 h after serum treatment (Fig. 3A). The changes in Rb phosphorylation were detectable before the changes in Rb-UBF interaction. The lag between the initial increase in Rb phosphorylation and UBF availability likely reflects the complex nature of Rb regulation. Specifically, Rb contains 16 phosphorylation consensus sites for cyclin/CDK activity; therefore, the initial increase in Rb phosphorylation detected using Western blot analysis may not represent the phosphorylation necessary to release UBF from Rb. It is important to note that serum stimulation did not have any detectable effect on UBF protein content at any of the time points studied (1,081 ± 149, 1,116 ± 165, 1,017 ± 207, 1,129 ± 167, 1,109 ± 175, and 1,064 ± 177 OD/μg of protein; P > 0.05) (Fig. 3B).

Serum stimulation increased cyclin D1 protein expression and associated CDK-4 activity. Serum stimulation resulted in increased cyclin D1 protein expression by 12, 24, and 48 h after serum stimulation (1,358 ± 82, 2,349 ± 51, and 3,133 ± 136 OD/μg of protein; P < 0.05). In contrast to the increase in cyclin D1, no changes in CDK-4 or p21 protein levels were detected at any time point after serum treatment (Fig. 4A). Consistent with the increase in cyclin D1 protein expression, increases in cyclin D1-dependent CDK-4 activity was observed at 24 and 48 h after serum stimulation. Coimmunoprecipitation analysis with an anti-CDK-4 antibody revealed that both cyclin D1 and p21 existed in a functional complex with CDK-4 (Fig. 4B). Because not all myoblasts may fuse into myotubes in vitro, it was important to determine the localization of cyclin D1 after serum stimulation. As shown in Fig. 4C, and consistent with the lack of cellular proliferation in the system used in the present study, the increases in cyclin D1 protein detected using Western blot analysis were localized to nuclei within differentiated myotubes. This finding is demonstrated by the colocalization of cyclin D1 protein in cells that stained positive for sarcomeric MHC.

Fig. 1. Serum stimulation resulted in myotube hypertrophy and increased RNA content. Serum stimulation of terminally differentiated myotubes resulted in (A) myotube hypertrophy (protein:DNA) and (B) an increase in RNA content (RNA:DNA). Statistically significant differences were detected at 24 and 48 h after serum stimulation. Data are expressed as means ± SE; n = 9. *P < 0.05. Inset: agarose gel fractionation of total RNA. Note the increased density for 18S and 28S ribosomal RNA (rRNA) subunits at 24 and 48 h after serum stimulation.
Rapamycin blocked myotube hypertrophy and RNA accumulation, increase in cyclin D1-dependent CDK-4 activity, inhibition of RB, and increase in UBF availability. Treatment of myotubes with rapamycin (5 ng/ml) prevented both serum-induced protein accumulation (68.43 ± 7.82, 54.01 ± 5.33, 131.02 ± 8.81, and 62.14 ± 5.92 μg/μg for 2%, 2%+R, 20%, and 20%+R, respectively; *P < 0.05) and RNA accumulation (5.22 ± 0.48, 3.55 ± 0.33, 8.83 ± 0.37, and 4.57 ± 0.47...
μg/μg for 2%, 2%+R, 20%, and 20%+R, respectively; P < 0.05). There was no statistical difference in the protein or RNA values between the 2%, 2%+R, and 20%+R groups (Fig. 5A). The effects of rapamycin were specific to the mTOR/p70S6K/rpS6 pathway because rapamycin blocked rpS6 phosphorylation but did not prevent serum-stimulated ERK phosphorylation (Fig. 5B). Rapamycin treatment also inhibited Rb phosphorylation and the increase in UBF availability without affecting UBF protein content (Fig. 5C). In addition, rapamycin inhibited the serum-stimulated increase in cyclin D1 pro-
Fig. 5. Rapamycin blocked myotube hypertrophy and the increase in RNA, in part, through the regulation of the cyclin D1/cdk-4/Rb/UBF pathway. A: rapamycin treatment blocked the serum-induced increases in protein:DNA. *P < 0.05, 20% vs. 2%; †P < 0.05, 20% vs. 20%+R. Data are expressed as means ± SE, n = 6 per group. B: rapamycin treatment blocked the serum-induced increases in RNA:DNA increase. *P < 0.05, 20% vs. 2%; †P < 0.05, 20% vs. 20%+R. Data are expressed as means ± SE, n = 6 per group. C: rapamycin treatment blocked the serum-induced increase in rpS6 phosphorylation but did not prevent the phosphorylation of ERK. D: rapamycin inhibited serum-stimulated increase in Rb phosphorylation and the associated increase in UBF availability. Serum stimulation in the presence of rapamycin could not induce an increase in Rb phosphorylation and consequently did not result in an increase in UBF availability. E: rapamycin inhibited the serum-induced increase in cyclin D1 protein expression and its associated increase in cdk-4 kinase activity. Data for C–E are representative of 3 individual experiments as described in METHODS.
tein expression and consequently the corresponding increase in CDK-4 kinase activity, with no detectable changes in CDK-4 or p21 protein levels or CDK-4/p21 functional interaction (Fig. 5D).

**DISCUSSION**

Signaling through mTOR has been shown to be necessary for both in vitro and in vivo skeletal muscle growth (3, 17, 36). Therefore, the main goal of the present study was to determine potential mechanisms downstream of mTOR that are involved in myotube hypertrophy. Our initial hypotheses were that mTOR activity modulates myotube hypertrophy through 1) the accumulation of rRNA and 2) regulating the availability of the rDNA transcription factor UBF via inhibition of Rb in a cyclin D1-dependent CDK-4 activity manner. The results of the experiments described herein support these hypotheses and are consistent with the previously established model of mTOR function during growth in the G1 phase of the cell cycle.

Serum stimulation of terminally differentiated L6 myotubes resulted in myotube hypertrophy as evidenced by an increase in protein mass without detectable changes in DNA content or BrdU uptake. This finding is consistent with previous reports in which stimulation of myotubes with IGF-1 induced an increase in myotube diameter without an increase in DNA synthesis as measured by [3H]thymidine uptake (36, 38), and it indicates that hypertrophy of differentiated myotubes does not require an increase in DNA accumulation.

As demonstrated during in vivo skeletal muscle hypertrophy (1), myotube hypertrophy was associated with a significant increase total RNA levels. Because >85% of total cellular RNA is rRNA, the increase in RNA levels represents an increase in ribosomal RNA. This finding can be interpreted as an increase in ribosome content, because previous studies have shown that ribosome biogenesis is regulated in part by an increase in rRNA synthesis (6, 8, 11). The relative contribution of rRNA synthesis and degradation to rRNA accumulation has been studied during cardiomyocyte hypertrophy (27, 28). The results from these studies showed that RNA accumulation resulted from accelerated rates of rRNA synthesis because assessment of 45S rRNA transcription using nuclear run-on analysis revealed that the increase in rRNA could be accounted for by increased transcriptional activity (27, 28). Therefore, assuming similarities between models of striated muscle cell growth, the increase in total RNA observed during serum-induced myotube hypertrophy is likely a consequence of increased rates of rRNA transcription.

Consistent with increased rDNA transcription, the results from this study show an increased availability of the rDNA transcription factor UBF at times associated with increased total RNA levels. This finding is consistent with other findings reported for cardiomyocyte hypertrophy in which an increase in rDNA transcription by RNA Pol I was modulated by an increase in UBF availability (14–16) or by changes in UBF phosphorylation (44, 45). Increased amounts of free UBF can be achieved by increasing UBF protein content or by increasing its availability by hyperphosphorylation of Rb. In the present study, UBF protein content did not increase in parallel with the increase in rRNA. However, increases in Rb phosphorylation were detected and were associated with an increase in UBF availability. This suggests that in myotubes, the increase in RNA is in part a result of an increase in rDNA transcription via UBF availability.

In the absence of cellular proliferation, serum-stimulated myotube hypertrophy was associated with a significant increase in cyclin D1 protein expression and an increase in cyclin D1-dependent CDK-4 activity. In agreement with previous studies, the increase in cyclin D1 protein expression was detected in the nuclei of myotubes, indicating that reexpression of this cell cycle gene can occur in the multinucleated myotube and is not limited to unfused myoblasts (20). Coimmunoprecipitation analysis of CDK-4 demonstrated that both cyclin D1 and p21 were functionally associated with CDK-4. Even though p21 has been viewed as a CDK inhibitor (39, 40), the identification of p21 in the CDK-4 complex was not surprising, because recent evidence has suggested that p21 is required for cyclin D1/CDK-4 activity (7). Fibroblasts lacking p21 are unable to form active cyclin D1/CDK-4 complexes, and p21 has been found to remain bound to active cyclin/CDK complexes in proliferating cells (47), indicating that the functional interaction between p21 and cyclin D1/CDK-4 complexes is required for their kinase activity.

An association between cell cycle genes and cellular hypertrophy has previously been demonstrated in cardiomyocytes (31, 42). For example, stimulation of cardiomyocytes with serum or ANG II resulted in cellular hypertrophy, upregulation of various G1 phase cyclin/CDK complexes, and increased kinase activity toward Rb even in the absence of DNA synthesis (31, 42). In animal and human models of skeletal muscle hypertrophy, increases in cyclin D mRNA and other cell cycle regulators have been shown (1, 2, 18, 19). These reports, together with the data in the present study, provide strong evidence for a role of cell cycle genes in the growth process of terminally differentiated myotubes/muscle fibers. Although in vitro the cell cycle genes likely function in the modulation of protein synthetic regulation, the in vivo expression of cell cycle regulators likely contribute to both hypertrophy and proliferation on the basis of localization studies (18, 19).

![Model of mammalian target of rapamycin (mTOR) regulation of myotube hypertrophy via cell cycle regulators.](http://ajpcell.physiology.org/)

Fig. 6. Model of mammalian target of rapamycin (mTOR) regulation of myotube hypertrophy via cell cycle regulators. Activation of mTOR leads to an increase in translation of cyclin D1 mRNA, resulting in increased cyclin D1 protein expression and a cyclin D1-dependent increase in CDK-4 activity toward Rb. These changes lead to the inhibition of Rb with a concomitant increase in UBF availability, thereby resulting in enhanced rDNA transcription and the accumulation of rRNA. The activity of mTOR also results in increased S6k-1 activity, which modulates the translation of ribosomal protein mRNA. Consequently, the coordinated increase in rRNA and ribosomal protein content results in an increased number of ribosomes, which support the protein synthetic demands of the hypertrophying cell.
Inhibition of serum-stimulated increases in RNA by rapamycin suggests that ribosomal biogenesis is one critical function regulated by mTOR in skeletal muscle. This conclusion in myotubes is consistent with the findings reported in other studies because rapamycin has been shown to 1) prevent the increase in muscle mass in response to muscle overload in both skeletal and cardiac muscles (3, 17, 41), 2) inhibit ribosome biogenesis in yeast (34), and 3) modulate RNA levels during cardiomyocyte hypertrophy (4).

On the basis of the results of the present study, a model is proposed for the increased protein synthetic changes that occur during skeletal muscle hypertrophy (Fig. 6). In this model, activation of mTOR leads to an increase in the translational efficiency of cyclin D1 mRNA, resulting in increased cyclin D1 content and an increase in cyclin D1-dependent CDK-4 activity. These changes lead to the phosphorylation of Rb with a concomitant increase in UBF availability, resulting in enhanced rDNA transcription and the accumulation of rRNA. The activity of mTOR also results in increased S6K-1 activity that in turn modulates an increased translation of ribosomal protein mRNA and mRNA associated with the regulation of translation, such as elongation factor-1 protein mRNA and mRNA associated with the regulation of rDNA transcription and the accumulation of rRNA. Consequently, the increase in rRNA and ribosomal protein content results in increased ribosomal biogenesis, which supports the protein synthetic demands of the growing cell.

In summary, the results of the present study provide evidence for the critical role of mTOR in skeletal muscle hypertrophy. Specifically, activation of mTOR signaling was associated with upregulation of cell cycle genes, increased availability of UBF, and subsequent increases in rRNA accumulation. These findings are consistent with the role of mTOR during cellular proliferation and suggest that there are common mechanisms involved in cellular growth control in both proliferative and differentiated states.

ACKNOWLEDGMENTS

Present address of G. A. Nader: Research Center for Genetic Medicine, Children’s National Medical Center, 111 Michigan Ave. NW, Washington, DC 20010 (e-mail: gnader@cmresearch.org).

Present address of T. J. McLoughlin: Department of Kinesiology, MS 201, The University of Toledo, 2801 W. Bancroft St., Toledo, OH 43606 (e-mail: tmclough@Umet.utoledo.edu).

GRANTS

This work was supported by an American College of Sports Medicine Graduate Student Research Award (to G. A. Nader) and by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-45617 (to K. A. Esser).

REFERENCES


