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IGF-II transcription in skeletal myogenesis is controlled by mTOR and nutrients

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Insulin-like growth factors (IGFs) are essential for skeletal muscle development, regeneration, and hypertrophy. Although autocrine actions of IGF-II are known to initiate myoblast differentiation, the regulatory elements and upstream signaling pathways for myogenic expression of IGF-II remain elusive. Here, we report the regulation of IGF-II transcription by mTOR, as well as by amino acid sufficiency, through the IGF-II promoter 3 and a downstream enhancer during C2C12 myoblast differentiation. Furthermore, we present evidence that IGF production, and not IGF signaling, is the primary target for mTOR's function in the initiation of differentiation. Moreover, myogenic signaling by mTOR is independent of its kinase activity and mediated by the PI3K–Akt pathway. Our findings represent the first identification of a signaling pathway that regulates IGF-II expression in myogenesis and implicate the mTOR–IGF axis as a molecular link between nutritional levels and skeletal muscle development.

Introduction

The insulin-like growth factors (IGF-I and IGF-II) are critically involved in skeletal muscle development (Florini et al., 1991a) as well as adult muscle regeneration and hypertrophy (Barton-Davis et al., 1999), which require muscle satellite cell activation and differentiation (Rosenblatt et al., 1994). The autocrine actions of IGF-II are essential for the differentiation of satellite cells in culture (Florini et al., 1991c). However, the regulation of IGF-II expression in these cells is poorly understood. The IGF-II gene is complex in structure and its expression can be regulated at many levels, including transcription from multiple promoters, alternative translation initiation, mRNA stability, and genomic imprinting. IGF-II expression during skeletal muscle differentiation is regulated at the transcriptional level, and the transcripts are derived from promoter 3 (P3; Kou and Rotwein, 1993). However, the activity of this promoter was not increased in myotubes compared with myoblasts, and further analyses of a 25-kb segment around this promoter region did not reveal any element responsible for myogenic regulation of IGF-II transcription (Kou and Rotwein, 1993). Partly due to the lack of information on the relevant cis-acting regulatory elements, the upstream pathways that directly instruct the expression of IGF-II in muscle differentiation are unexplored.

The cellular target of the bacterial macrolide rapamycin, mTOR, belongs to the phosphatidylinositol kinase (PI3K)-related family of Ser/Thr kinases and functions as a master regulator of cell growth and proliferation by regulating multiple downstream effectors (Jacinto and Hall, 2003). In cell proliferation, the best-characterized function of mTOR is the regulation of translation initiation through eIF-4E binding protein 1 (4EBP1) and S6 kinase 1 (S6K1), and the PI3K pathway acts in parallel with mTOR to regulate 4EBP1 and S6K1 (Fumagalli and Thomas, 2000; Gingras et al., 2001). The mTOR pathway is believed to mediate nutrient signals such as amino acid sufficiency (Fumagalli and Thomas, 2000; Gingras et al., 2001), as well as directly receive mitogenic signals through a lipid second messenger (Fang et al., 2001).

Implicated by the inhibitory effect of rapamycin on the differentiation of a variety of myoblasts in culture (Coolican et al., 1997; Cuenda and Cohen, 1999; Conejo et al., 2001; Erbay and Chen, 2001), mTOR's essential role in skeletal myogenesis has been demonstrated by the ability of a rapamycin-resistant mTOR mutant to rescue rapamycin-inhibited differentiation in C2C12 myoblasts (Erbay and Chen, 2001; Shu et al., 2002). Remarkably, the kinase activity of mTOR is not required for initiation of differentiation, and both

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Abbreviations used in this paper: 4EBP1, eIF-4E binding protein 1; c.a., constitutively active; IGF, insulin-like growth factor; ME, muscle enhancer; MHC, myosin heavy chain; P3, promoter 3; PI3K, phosphatidylinositol 3-kinase; RPA, RNase protection assay; RR, rapamycin resistant; RR/KI, RR and kinase inactive; S6K1, S6 kinase 1.

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S6K1 and 4EBP1 have therefore been excluded as downstream mediators of mTOR's myogenic signaling (Erbay and Chen, 2001). The molecular events regulated by mTOR in myogenesis are currently unknown. In this report, we provide strong evidence that mTOR governs myoblast differentiation by controlling the transcription of IGF-II, potentially through a nutrient-sensing pathway.

Results and discussion

Myogenic transcription of IGF-II is regulated through P3 and a skeletal muscle enhancer (ME) in a rapamycin-sensitive manner

To facilitate the investigation of IGF-II regulation during myogenesis, we established C2C12 cell pools stably expressing a luciferase reporter for P3 of the IGF-II gene. P3 activity was found slightly decreased in fully formed myotubes when compared with myoblasts (unpublished data), this finding is in agreement with a previous work (Kou and Rotwein, 1993). However, a closer examination revealed an acute activation of P3 3 h after the induction of differentiation by growth factor deprivation, which returned to the basal level by 6 h (Fig. 1 A). As a control, a CMV promoter reporter displayed no change in luciferase activity under the same conditions (unpublished data). Remarkably, this increased activity was completely blocked by rapamycin treatment (Fig. 1 A), whereas the CMV-driven luciferase activity was not affected (not depicted).

This brief activation of P3 alone is insufficient to account for the continued increase of IGF-II mRNA levels throughout differentiation (see Fig. 2 A), which prompted us to consider other potential regulatory elements. A recently identified skeletal ME shared by *IGF-II* and *H19*, a gene closely linked to the *IGF-II* gene and reciprocally imprinted, appeared as a likely candidate (Kaffer et al., 2001). Indeed, the luciferase activity in C2C12 cells stably expressing the ME reporter under the control of H19 promoter increased steadily during differentiation, reaching ~5-fold by 24 h and ~40-fold by 48 h when compared with 0 h (Fig. 1 B). The activity of an enhancerless reporter for H19 promoter did not change under similar conditions (unpublished data). Importantly, the ME reporter activity was highly sensitive to rapamycin (Fig. 1 B). Thus, a rapamycin-sensitive pathway may play a distinct role in the regulation of IGF-II transcription through the sequential activation of P3 and ME.

In addition to the mTOR pathway, the PI3K pathway and p38 MAPK are also required for skeletal myocyte differentiation (Coolican et al., 1997; Kaliman et al., 1998; Cuenda and Cohen, 1999). However, both P3 and ME activity during differentiation were insensitive to the PI3K inhibitor wortmannin and the p38 inhibitor SB202190 (Fig. 1, C and D).

mTOR is required for IGF-II mRNA production in C2C12 cells

Consistent with rapamycin's effect on P3 and ME of IGF-II, the drastic increase of IGF-II mRNA levels during differentiation was significantly blocked by rapamycin treatment of differentiating cells, as shown by the results of RNase protection assays (RPAs) in Fig. 2 A. This inhibition was not due to changes in IGF-II mRNA stability because during a 12-h window of actinomycin D treatment to suppress transcription, IGF-II mRNA remained highly stable, regardless of the presence of rapamycin (Fig. 2 B).

To assess whether the rapamycin effect on IGF-II expression was directly attributable to mTOR, we examined IGF-II mRNA in C2C12 cell lines stably expressing various recombinant FLAG-tagged mTOR proteins including wild type, rapamycin resistant (RR; S2035T), and RR and kinase inactive (RR/KI; S2035T/D2357E; Brown et al., 1995). Several independent clones for each cell line were examined. The expression of IGF-II mRNA in both RR and RR/KI cells, but not in wild-type mTOR cells, was insensitive to rapamycin treatment. Fig. 2 (C–E) shows the RPA results from two representative clones for each cell line. Thus, rapamycin-resistant mTOR, both kinase active and inactive, fully rescued IGF-II expression from rapamycin inhibition. Autophosphorylation assays indicated no detectable mTOR catalytic activity from the recombinant RR/KI proteins in

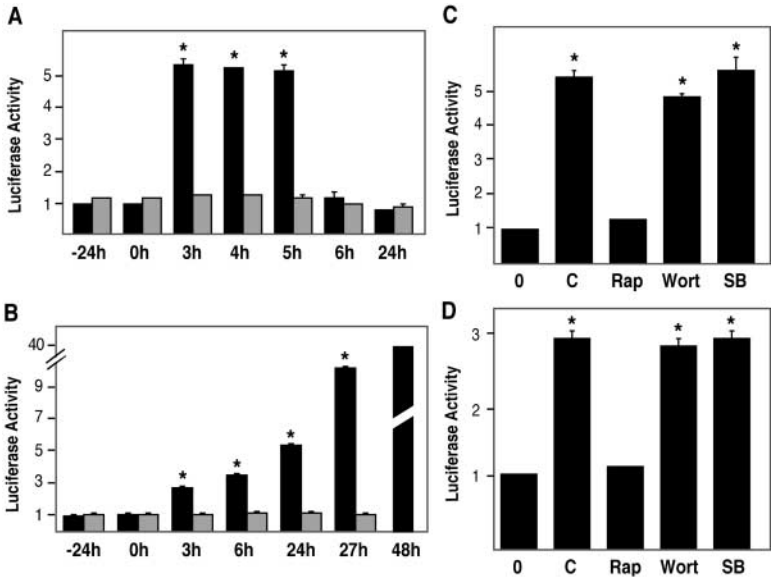

Figure 1. **IGF-II transcription during C2C12 differentiation is regulated through P3 and a skeletal muscle enhancer in a rapamycin-sensitive manner.** (A and B) C2C12 cells stably expressing the P3 (A) or ME (B) reporter were induced to differentiate in the absence (black bars) or presence (gray bars) of 100 nM rapamycin. At the indicated times, cells were lysed and luciferase assays were performed. Proliferating myoblasts, 1 d before differentiation (−24 h), were also examined. Relative luciferase activities are shown, with those at 0 h set at 1. (C and D) Cells stably expressing P3 (C) or ME reporter (D) were induced to differentiate for 3 h in the absence of any inhibitor (C) or in the presence of 100 nM rapamycin (Rap), 100 nM wortmannin (Wort), or 10 μM SB202190 (SB). Relative luciferase activities are shown, with those at 0 h (0) set at 1. All the data are the mean results of three independent experiments; SD are shown as error bars. *t* tests were performed to compare each data with that at 0 h. *, $P < 0.001$.

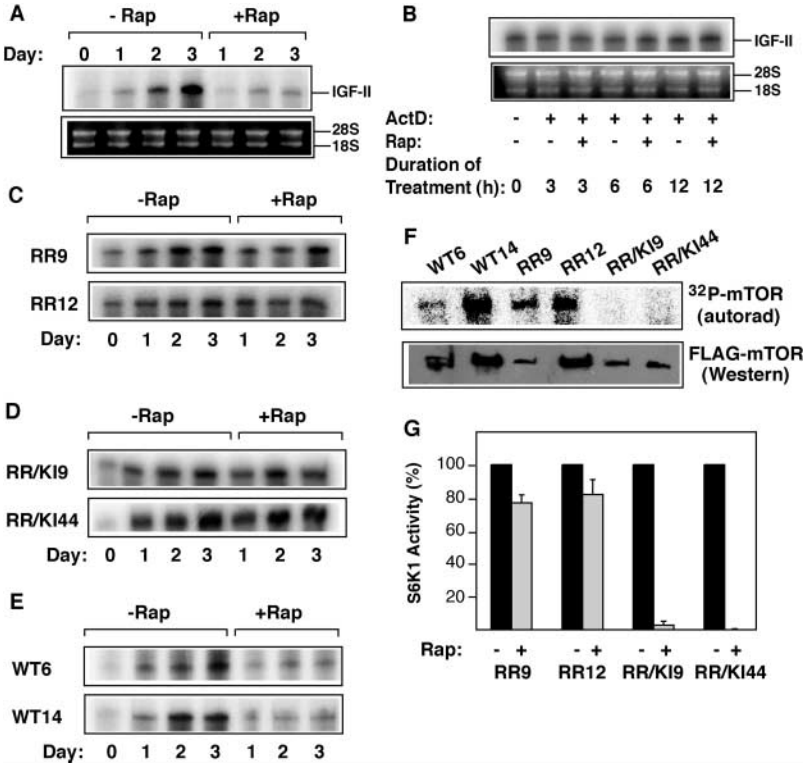

Figure 2. **mTOR controls IGF-II mRNA production in C2C12 cells.** Parental C2C12 (A and B) and cells stably expressing RR (C), RR/KI (D), or wild-type mTOR (E) were induced to differentiate in the presence or absence of 100 nM rapamycin (Rap). (B) On day 1 of differentiation, the cells were treated with 2 μg/ml actinomycin D (ActD) in the presence or absence of 100 nM rapamycin for the indicated durations. (C–E) The experiments were performed with two independent clones for each mTOR cell line as indicated. Total RNA was extracted from the cells and IGF-II mRNA levels were detected by RPA analyses. The consistency in RNA sample loading was confirmed by visualizing rRNAs with ethidium bromide staining (A and B only). Recombinant mTOR (F) and endogenous S6K1 (G) were immunoprecipitated from cells stably expressing various mTOR proteins, followed by mTOR autokinase assays and S6 kinase assays, respectively. (G) Relative S6K1 activities are shown for each cell line comparing without or with rapamycin treatment of the cells (100 nM for 45 min). All experiments were repeated at least three times with similar results. The data are the mean results of three independent experiments; SD are shown as error bars.

the stable cells (Fig. 2 F). As expected, the endogenous S6K1 activity was abolished by rapamycin treatment in wild-type and RR/KI cells, whereas the RR cells yielded a significant portion of RR S6K1 activity (Fig. 2 G).

Together, our data suggest that during skeletal myogenesis mTOR regulates the production of IGF-II mRNA. Moreover, this myogenic function of mTOR is independent of its kinase activity, which is consistent with our previous observations (Erbay and Chen, 2001). This conclusion contradicts that of Shu et al. (2002) who reported that a C2C12 cell line stably expressing a kinase-inactive mTOR did not differentiate in the presence of rapamycin. It is noted that a different mutation (D2338A) was used to inactivate the kinase by Shu et al. Although D2357E and D2338A are equally effective in inactivating mTOR's catalytic activity (Brown et al., 1995), it cannot be ruled out that D2338A may affect an additional biochemical property of mTOR. The possibility of clonal variation may also be considered. To avoid such a potential problem, multiple stable clones were examined in our studies as described in the previous paragraph, and transient transfection was previously used to express mTOR (Erbay and Chen, 2001).

Amino acid sufficiency is required for acute activation of IGF-II transcription

Because the mTOR pathway is known to sense the availability of amino acids in proliferating cells (Fumagalli and Thomas, 2000; Gingras et al., 2001), we hypothesized that mTOR may mediate amino acid signals in the regulation of IGF-II transcription during myogenesis. Indeed, the acute activation of P3 normally seen at 3 h was abrogated when amino acids were depleted from the differentiation medium (Fig. 3). When normal concentrations of amino acids were replenished for 3 h, activation of P3 was recovered, and this

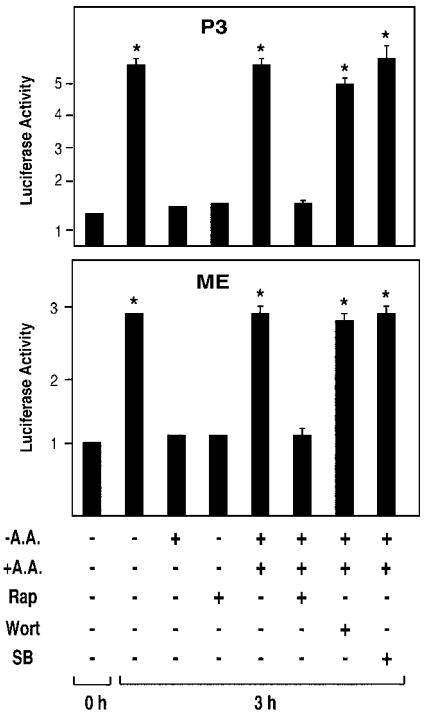

Figure 3. **IGF-II transcription during myogenesis is acutely sensitive to amino acid deprivation.** C2C12 cells stably expressing the P3 (top) or ME reporter (bottom) were induced to differentiate for 3 h. In some of the cells, amino acids were depleted from the differentiation medium (−A.A.) and followed by their resupply (+A.A.) for 3 h in the presence of inhibitors when indicated; 100 nM rapamycin (Rap), 100 nM wortmannin (Wort), and 10 μM SB202190 (SB). Relative luciferase activities are shown, with those at 0 h of differentiation set at 1. The data are the mean results of three independent experiments; SD are shown as error bars. *t* tests were performed to compare each data with that at 0 h. *, $P < 0.001$.

reactivation was completely blocked by rapamycin, but not by wortmannin or SB202190 (Fig. 3). Similarly, the induction of ME activity at 3 h was ablated by amino acid deprivation and stimulated upon readdition of amino acids (Fig. 3). Again, the reactivation of ME by amino acids was inhibited by rapamycin and was insensitive to wortmannin and SB202190. The short duration of amino acid withdrawal ensured that the effect would be the result of affecting a signaling cascade rather than a general stoppage of protein synthesis. Indeed, the CMV-driven luciferase activity was unaffected by amino acid deprivation under similar conditions (unpublished data).

Circulating IGF levels in animals have previously been proposed to provide a critical link between the nutritional status and animal/organ growth (Pilistine et al., 1984), but molecular mechanisms underlying nutritional regulation of IGF levels are unknown. Our results provide the first direct demonstration of IGF-II transcriptional regulation by cellular amino acid levels, and implicate a potential role for mTOR in controlling nutritional regulation of IGF-II expression in skeletal muscle development.

mTOR regulates C2C12 differentiation by controlling IGF production

The regulation of IGF-II transcription by mTOR led us to hypothesize that mTOR might exert its myogenic function by controlling IGF-II secretion that is critical for initiation of the differentiation program. To probe this possibility, conditioned medium from normally differentiating "donor" C2C12 cells were transferred daily to "recipient" C2C12 cells subjected to rapamycin treatment. The recipient cells proceeded to terminally differentiate despite the presence of rapamycin, evidenced by the formation of sarcomeric myosin heavy chain (MHC)–positive myotubes (Fig. 4 A, a). Thus, it appears that rapamycin's inhibitory effect on differentiation is through the elimination of a secreted factor. This factor was confirmed to be IGF-II by the observation that preincubation with an anti–IGF-II antibody, but not a control rabbit IgG, completely eliminated the myogenic potency of the donor medium (Fig. 4 A, b and c). Moreover, conditioned media from rapamycin-treated RR-mTOR cells, as well as RR/KI-mTOR cells, conferred rapamycin resistance to the differentiation of the recipient cells (Fig. 4 A, d and e), further validating the requirement of mTOR and a kinase-independent mechanism. The neutralizing IGF-II antibody also abolished the rapamycin-resistant myogenic effects of the media from the recombinant mTOR-expressing cells (unpublished data).

Furthermore, exogenous IGF-II (at 300 ng/ml) restored C2C12 differentiation in the presence of rapamycin as shown by myotube formation (Fig. 4 B) with a differentiation index indistinguishable from that of control cells (Fig. 4 C). Similar rescuing effects were also observed with 150

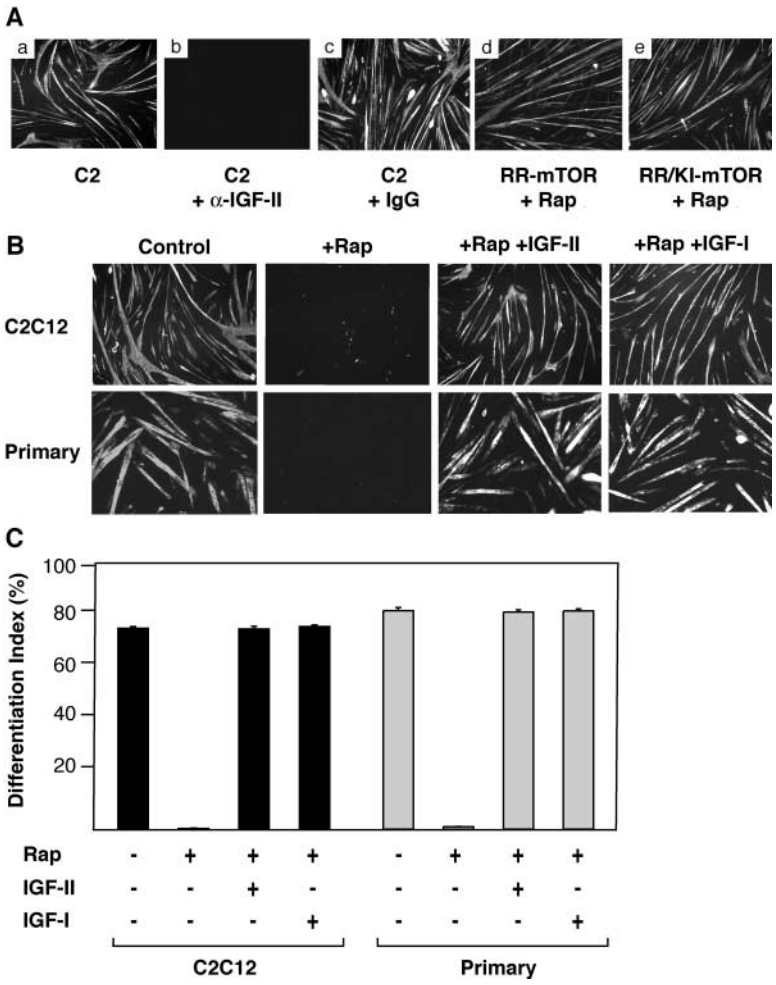

Figure 4. **mTOR regulates C2C12 and primary myoblast differentiation by controlling IGF-II secretion.** (A) Donor cells (not depicted) were induced to differentiate 24 h earlier than the recipient cells. After every 24 h, the media from the donor cells were transferred to the recipient cells in the continuous presence of 100 nM rapamycin. The donor cells were either parental or stable C2C12 cells, whereas all recipient cells were parental cells. The recipient cells, fixed and stained for MHC after 72 h of differentiation, are shown. The identity of donor cells and the treatments of the donor media are indicated under the micrographs. (B) C2C12 cells (top) or mouse primary myoblasts (bottom) were induced to differentiate normally or in the presence of 100 nM rapamycin, 300 ng/ml IGF-II, or 100 ng/ml IGF-I. The cells were fixed and stained for MHC after 72 h of differentiation. (C) Quantification of the results in B by differentiation index, calculated as the percentage of nuclei in MHC-positive myocytes (~500 total nuclei counted for each sample). The mean results of three independent experiments are shown, with error bars representing SD.

ng/ml IGF-II (unpublished data), a concentration comparable to that reportedly secreted by C2C12 cells (Tollefsen et al., 1989). Interestingly, 100 ng/ml IGF-I was equally effective (Fig. 4, B and C). The apparently thinner myotubes in the IGF-rescued culture suggests that mTOR may play a role in myotube morphology independent of IGF and the initiation of differentiation. Importantly, the differentiation of mouse primary myoblasts was inhibited by rapamycin, which was also reversed by exogenous IGF-I or IGF-II (Fig. 4, B and C), suggesting that the molecular mechanisms delineated in C2C12 cells are most likely applicable to primary cells. In differentiating C2C12 cells, IGF-II expresses to a much higher level than IGF-I (Tollefsen et al., 1989), and IGF-II antisense oligonucleotides are sufficient to abolish differentiation (Florini et al., 1991c). The collective evidence is consistent with the notion that IGF-II is the primary myogenic factor in these cells, whereas IGF-I can compensate for the lack of IGF-II; both IGFs presumably signal through the IGF-I receptor. Together, it is apparent that the primary function of mTOR in the initiation of skeletal muscle differentiation is controlling IGF-II production and not mediating IGF downstream signaling.

PI3K–Akt pathway is a major mediator of mTOR's myogenic function

The PI3K–Akt pathway has been shown to be critical for IGF's myogenic signaling (Coolican et al., 1997; Kaliman et al., 1998). We found that expression of a constitutively active (c.a.) Akt reversed the inhibition of C2C12 differentiation by the PI3K inhibitor LY294002 (Fig. 5 A), suggesting that Akt is the main effector for PI3K signaling in myogenesis. Remarkably, c.a.Akt also rescued differentiation from rapamycin inhibition (Fig. 5 A). The results of RPA analysis indicated that IGF-II mRNA levels were significantly inhibited by rapamycin in c.a.Akt-expressing cells (Fig. 5 C), which excluded the possibility of enhanced IGF-II production independent of mTOR in c.a.Akt cells. These results imply that the PI3K–Akt pathway is sufficient to mediate IGF's myogenic signaling, and they further confirm that regulation of IGF production is a primary function for mTOR in myogenesis. It is interesting to note the contrast between the relationship of these two pathways in myogenesis and mitogenesis. In cell proliferation, the mTOR pathway and the PI3K pathway cooperate in a parallel manner to transduce growth factor signals and regulate the same downstream targets such as S6K1 (Fumagalli and Thomas, 2000); whereas in myogenesis, they regulate two sequential processes: production of IGF and subsequent IGF signaling, respectively (Fig. 5 D).

In conclusion, we have shown that the autocrine IGF-II transcription required for skeletal myocyte differentiation is regulated by mTOR and the availability of amino acids. Although the expression of various myogenic markers, including myogenin, $p21^{CIP}$, and MHC, is also blocked by rapamycin (Cuenda and Cohen, 1999), they are regulated by IGF signaling (Florini et al., 1991b), and are therefore unlikely to be directly controlled by mTOR. To the best of our knowledge, this is the first identification of an upstream signaling pathway in the regulation of IGF production in myogenesis. Importantly, our findings also suggest a potential role for the mTOR–IGF axis as a molecular bridge between nutritional status and skeletal muscle development.

Materials and methods

Antibodies and other reagents

M2 anti-FLAG and HA.11 anti-HA were obtained from Sigma-Aldrich, anti–IGF-II was obtained from R&D Systems, anti-S6K1 was obtained from Upstate Biotechnology, and MF20 antisarcomeric MHC was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. IGF-I,

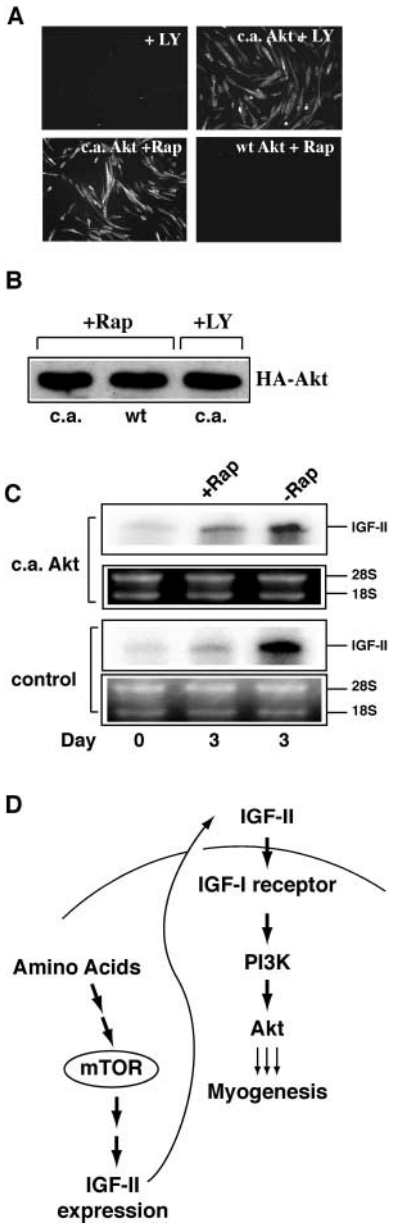

Figure 5. **Akt is a major mediator of mTOR's myogenic function.** C2C12 cells were transfected with wild-type (wt) or constitutively active (c.a.) Akt and differentiated in the presence of 100 nM rapamycin (Rap) or 10 mM LY294002 (LY). (A) The cells were fixed and stained for MHC after 72 h. (B) Recombinant protein expression was confirmed by Western blot analysis using an anti-HA antibody. (C) IGF-II mRNA levels were analyzed by RPA in c.a.Akt-expressing cells and untransfected C2C12 cells (control). (D) A proposed model for mTOR's myogenic signaling. See Results and discussion for details.

IGF-II, gelatin, actinomycin D, and LY294002 were obtained from Sigma-Aldrich. Rapamycin, wortmannin, and SB202190 were acquired from Calbiochem. All other cell culture reagents were obtained from Invitrogen.

Plasmids

The following plasmids were gifts from various laboratories: pCEFL-HA-Akt (wild type) and pCMV6-myristoylated-HA-Akt (c.a.) were gifts from N. Ahmed and J. Blenis (Harvard Medical School, Boston, MA), mouse IGF-II cDNA was a gift from P. Rotwein (Oregon Health and Science University, Portland, OR), P3-luc reporter and basic-luc (promoterless) were gifts from A. Murrell (The Babraham Institute, Cambridge, UK; Murrell et al., 2001), and H19-luc was a gift from K. Pfeifer (National Institute of Child Health and Human Development, Bethesda, MD; Pfeifer et al., 1996). CMV-luc was as previously reported (Kim and Chen, 2000). To assemble the skeletal ME reporter H19-luc-ME, the SpeI–EagI enhancer fragment (+23 kb to +27 kb) was inserted into a SpeI–EagI linker present in the XhoI site 3′ of the luciferase in H19-luc.

Cell culture and immunofluorescence microscopy

C2C12 myoblasts were maintained and differentiated as described previously (Erbay and Chen, 2001). Primary skeletal myoblasts were isolated from 2-wk-old mice, grown, and differentiated as previously reported (Rando and Blau, 1994). Transfections were performed using FUGENE-6 (Roche), and stable clones or pools were selected in 1 mg/ml G418. For amino acid deprivation and readdition, the cells were cultured in Dulbecco's PBS with 4.5 g/liter glucose and 2% dialyzed serum for 3 h and switched to normal differentiation medium for 3 h. For immunocytochemistry, cells were fixed in 3.7% formaldehyde and stained with MHC antibody and FITC–anti–mouse IgG. Microscopy was performed on a microscope (model DMIL; Leica) with CPLAN 10×/0.22 NA lenses. The images were captured using a monochrome charged-couple device camera (model SPOT RT; Diagnostic Instruments), and processed as 8-bit RGB images using Adobe Photoshop 7.0.

RPA

Total RNA from C2C12 cells (in 60-mm plates) was isolated using the RNeasy mini kit (QIAGEN), and 4 μg of each RNA was applied to RPA using the RPA III kit (Ambion). Radioactive probes (used at 10^6 cpm/ml) were generated from linearized IGF-II cDNA construct by in vitro transcription using the MAXIscript kit (Ambion). The samples were run on 5% denaturing polyacrylamide gels and analyzed on a phosphorimager (model Cyclone; Packard Instrument Co.). To confirm loading consistency, 1-μg RNA samples were run on 0.8% agarose-formaldehyde gels, followed by ethidium bromide staining.

Reporter assays

C2C12 cells stably expressing CMV-luc, P3-luc, basic-luc, H19-luc, or H19-luc-ME were grown to 100% confluence and induced to differentiate in 2% horse serum. Various drug treatments were performed as described in the figure legends. The cells were lysed at the indicated times, and luciferase assays were performed using the Luciferase Assay Systems kit (Promega).

Kinase assays

mTOR autokinase assays and S6K1 kinase assays were performed as described previously (Erbay and Chen, 2001).

Statistical analysis

t tests were performed for all data comparisons. Unless specifically indicated in figure legends, significant difference was defined by $P < 0.05$.

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