Regulation of Peroxisome Proliferator–Activated Receptor-γ Activity by Mammalian Target of Rapamycin and Amino Acids in Adipogenesis

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Adipocyte differentiation is a developmental process that is critical for metabolic homeostasis and nutrient signaling. The mammalian target of rapamycin (mTOR) mediates nutrient signaling to regulate cell growth, proliferation, and diverse cellular differentiation. It has been reported that rapamycin, the inhibitor of mTOR and an immunosuppressant, blocks adipocyte differentiation, but the mechanism underlying this phenomenon remains unknown. Here we show that mTOR plays a critical role in 3T3-L1 preadipocyte differentiation and that mTOR kinase activity is required for this process. Rapamycin specifically disrupted the positive transcriptional feedback loop between CCAAT/enhancer-binding protein-α and peroxisome proliferator–activated receptor-γ (PPAR-γ), two key transcription factors in adipogenesis, by directly targeting the transactivation activity of PPAR-γ. In addition, we demonstrate for the first time that PPAR-γ activity is dependent on amino acid sufficiency, revealing a molecular link between nutrient status and adipogenesis. The results of our further investigation have led us to propose a model in which the mTOR pathway and the phosphatidylinositol 3-kinase/Akt pathway act in parallel to regulate PPAR-γ activation during adipogenesis by mediating nutrient availability and insulin signals, respectively. It is interesting that troglitazone (a thiazolidinedione drug) reversed the inhibitory effects of rapamycin and amino acid deprivation, implicating therapeutic values of thiazolidinedione drugs to counter certain side effects of rapamycin as an immunosuppressant. *Diabetes* 53: 2748–2756, 2004

Adipose tissue plays major roles in energy homeostasis, lipid metabolism, and insulin actions. It also acts as an endocrine organ to regulate the secretion of a wide range of factors such as leptin, adiponectin, tumor necrosis factor-α, plasminogen activator inhibitor-1, and various cytokines, some of which are key regulators of energy homeostasis (1). The 3T3-L1 cell line is commonly used as an adipocyte differentiation model system for the investigation of the molecular mechanisms that regulate adipogenesis. In 3T3-L1 preadipocytes, the addition of insulin, glucocorticoids, and cAMP enhancer initiates a transcriptional regulatory cascade that results in a gene expression profile specific for adipocyte functions (2). Several critical transcription factors and the cross-talks among those factors have been well studied, including the CCAAT/enhancer-binding proteins C/EBP-β, C/EBP-δ, and C/EBP-α and peroxisome proliferator–activated receptor-γ (PPAR-γ). Exogenous hormonal stimuli induce the expression of C/EBP-β and -δ in preadipocytes rapidly and transiently (3,4), which in turn mediate the induction of PPAR-γ and C/EBP-α expression (5).

Once activated upon initiation of differentiation, PPAR-γ and C/EBP-α form a positive feedback loop to reinforce and maintain each other's expression (6); this cooperative interplay activates the essential adipogenic genes required for adipocyte functions and maintains the terminally differentiated state (1,7,8). Whereas C/EBP-α cannot promote adipogenesis in the absence of PPAR-γ, PPAR-γ can in the absence of C/EBP-α (9). Therefore, PPAR-γ is both necessary and sufficient for adipogenesis, and C/EBP-α induces adipocyte differentiation at least partly by acting through PPAR-γ (9). PPAR-γ is also the target of a group of antidiabetic thiazolidinedione (TZD) drugs, which potently activates PPAR-γ activity (10,11).

The bacterial macrolide rapamycin is a potent immunosuppressant in clinical use and an inhibitor of cell growth and proliferation in organisms ranging from yeast to human (12). The mammalian target of rapamycin (mTOR) (also named FRAP/RAFT1) is a member of the phosphatidylinositol kinase–related kinase family of Ser/Thr kinases (13). Numerous studies have established a role of the mTOR pathway in mediating nutrient signaling to regulate cell growth and proliferation (14). In particular, amino acids have been shown to activate an mTOR-dependent pathway that leads to the phosphorylation of two downstream effectors, eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal S6 kinase 1 (S6K1) (15–18). In adipocytes, mTOR is thought to regulate protein synthesis (19), adipose tissue morphogenesis (20), and leptin synthesis/secretion (21).

Rapamycin has been reported to inhibit the differentiation of 3T3-L1 cells (22,23) and human preadipocytes in primary culture (24), implicating mTOR in adipogenesis,

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4E-BP1, eukaryotic initiation factor 4E-binding protein 1; CMV, cytomegalovirus; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; MIX, isobutylmethylxanthine; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator–activated receptor; PPRE, PPAR-γ responsive element; RXR, retinoid X receptor; S6K1, ribosomal S6 kinase 1; TZD, thiazolidinedione.

but the mechanisms of mTOR signaling in this process remain poorly understood. Our studies reported here suggest that mTOR regulates adipocyte differentiation and adipocyte functions by controlling the transactivation activity of PPAR-γ toward its target genes. In addition, we have found that PPAR-γ activity is dependent on amino acid sufficiency, and this nutrient-sensing signal is most likely mediated by mTOR.

RESEARCH DESIGN AND METHODS

Materials and reagents. 3T3-L1 cells were obtained from American *Type Culture* Collection. Dexamethasone (DEX), isobutylmethylxanthine (MIX), insulin, Oil Red O, polybrene, and puromycin were purchased from Sigma. mTOR antibody was previously described (25). All other antibodies were obtained from commercial sources: anti–C/EBP-α, C/EBP-β, C/EBP-δ, PPAR-γ, adipsin, and $p21^{CIP}$ from Santa Cruz Biotechnology; and anti-Foxo1, phospho-Ser256 Foxo1, and phospho-Thr24 Foxo1/Thr32 Foxo3 from Cell Signaling.

Plasmids. The retroviral constructs for mTOR and S6K1 were generated by inserting mutant mTOR cDNAs into pQCXIP (Clontech) via the *Not*I site or truncated S6K1 cDNA (corresponding to amino acids 24–398) via the *Eco*RI site. pBabe-puro-C/EBP-α (retroviral vector), luciferase reporters for PPAR-γ and C/EBP-α containing −603 to 62 bp of the PPAR-γ proximal promoter fragment in pXP2 (26), and −343 to 125 bp of the C/EBP-α proximal promoter in pGL3-BA (27) all were gifts from Dr. G.S. Hotamisligil's laboratory (Harvard University School of Public Health). pMSV-C/EBP-α, -β, and -δ plasmids (28) were provided by Dr. A.D. Friedman (Johns Hopkins University). The PPAR-γ responsive element (PPRE) reporter was generated by inserting three copies of DR1 sequence (TTCTGACCTATGACCTGG) into pGL2-based basal reporter plasmid pTK-luc.

Cell culture. Both HEK293 and 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that contained 10% FBS at 37°C with 5% CO_2. For 3T3-L1 preadipocyte differentiation, 2 days after the cultures were confluent, they were induced to differentiate (defined as day 0) by incubation in DMEM with 10% FBS, 1 μmol/l DEX, 0.5 mmol/l MIX, and 1 μg/ml insulin. At day 2, the induction medium was replaced by DMEM with 10% FBS plus 1 μg/ml insulin only, and cells were then fed every 2 days with the same medium. At the time points indicated, the cells were either harvested for Northern or Western analysis or stained with Oil Red O.

Retroviral infection. BD EcoPack2-293 packaging cells (Clontech) were grown in 10-cm plates in DMEM with 10% FBS until they reached 70% confluence and then were transfected with 5 μg of pBabe-puro-C/EBP-α, pQCXIP-mTOR, or pQCXIP-RR-S6K1 plasmid using SuperFect transfection reagent (Qiagen). Six hours after transfection, the medium was replaced with fresh medium, and after another 48 h of incubation, the virus-containing medium was collected, filtrated, and either stored at −80°C or used immediately for viral infection as follows: 5 ml of the viral suspension was mixed with 5 ml of fresh DMEM that contained 10% FBS and 16 μg/ml polybrene and added to 3T3-L1 cells in a 10-cm plate at 50% confluence. After 24 h, the cells were further infected by replacing 5 ml of cell medium with 5 ml of viral suspension. After 48 h, the cells were split at 1:6 and seeded under selection of 200 μg/ml puromycin. Five to 7 days later, the selected cells were pooled and cultured for Western analysis or for differentiation experiments.

Transient transfection and luciferase assay. HEK293 cells grown in 24-well plates to 60–70% confluence were transfected with 0.25 μg of luciferase reporter plasmids using PolyFect (Qiagen). In each transfection, 0.25 μg of pMSV-C/EBP plasmid (28) or 0.05 μg of PPAR-γ/retinoid X receptor (RXR) plasmid was transfected. After 3 h, cells were incubated in fresh medium for an additional 24 h. Cells were treated with rapamycin for 3 h before lysis when indicated. Cells were lysed in 100 μl/well Passive Lysis Buffer and subjected to luciferase assay using Luciferase Assay System (Promega).

For insulin stimulation, cells were incubated with DMEM with 0.3% FBS for 24 h and then stimulated with 1 μg/ml insulin for 3 h. Rapamycin and/or wortmannin were added 30 min before insulin stimulation, when indicated. 3T3-L1 adipocytes at day 6 were transfected with 0.5 μg of reporter plasmid using Lipofectamine Plus (Invitrogen). Rapamycin treatment and luciferase assays were performed as described above. For amino acid withdrawal, cells were washed once with Dulbecco's phosphate-buffered saline and incubated in the same buffer that contained 4.5 g/l glucose, 1 mmol/l sodium pyruvate, 0.375% sodium bicarbonate, and 1× MEM vitamin solution (Cambrex) for 3 h. Readdition of amino acids was achieved by changing to the same medium for amino acid starvation plus a mixture of amino acids, which contained 2 mmol/l glutamine, 2× MEM amino acids solution, and 2× MEM nonessential amino acids solution (Cambrex). Troglitazone (10 μmol/l) was added along with rapamycin or amino acid withdrawal when indicated.

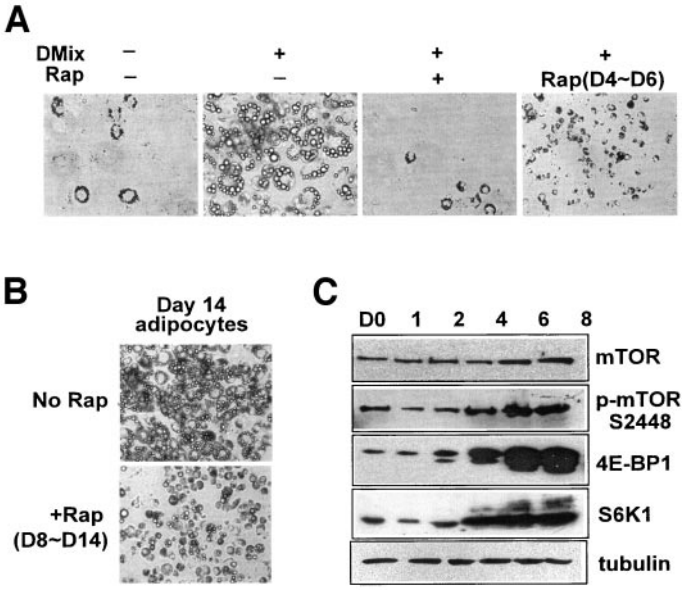

FIG. 1. Effect of rapamycin on differentiation of 3T3-L1 preadipocytes. *A*: 3T3-L1 cells were induced to differentiate with the differentiation medium (DMix) as described in RESEARCH DESIGN AND METHODS. Some cells were treated with 100 nmol/l rapamycin during the entire differentiation process or only from days 4 to 6. After 8 days, the cells were fixed, stained with Oil Red O, and visualized by phase-contrast microscopy. *B*: 3T3-L1 adipocytes at day 8 were treated with 100 nmol/l rapamycin and continued to be cultured until day 14 before fixation and staining with Oil Red O. *C*: Total cell lysates prepared at the indicated time points during differentiation were subjected to Western blot analyses. The antibodies used are indicated.

RESULTS

Preadipocyte differentiation requires mTOR and its kinase activity. 3T3-L1 preadipocytes were induced to differentiate by addition of the differentiation cocktail (DEX, MIX, and insulin). Typically, changes in cell morphology and the accumulation of lipid droplets were evident 3–4 days after induction, and adipocytes were fully differentiated in 7–8 days. Rapamycin completely blocked the differentiation and lipid accumulation, as shown by the Oil Red O staining in Fig. 1*A*. Rapamycin also effectively attenuated terminal differentiation when added at day 4 and removed at day 6 (Fig. 1*A*), suggesting that a rapamycin-sensitive pathway is involved in later stages of differentiation after the clonal expansion phase, consistent with previous reports (22,23). Furthermore, rapamycin treatment of day 8 adipocytes for 4 days led to a reduction of lipid droplets and smaller adipocytes (Fig. 1*B*), indicating that rapamycin affects the maintenance of adipogenic characteristics of mature adipocytes. This effect is apparently not due to increased cell death (data not shown). It is not known whether rapamycin induces de-differentiation or promotes lipolysis in adipocytes.

The protein level of mTOR in fully differentiated adipocytes was higher than that in preadipocytes, accompanied by an increase in phosphorylation at the putative Akt site Ser2448, the functional significance of which is yet to be determined (29,30) (Fig. 1*C*). It is interesting that the expression of S6K1 and 4E-BP1, two well-known downstream targets of mTOR, underwent even more drastic increase upon adipogenesis (Fig. 1*C*), as seen by other

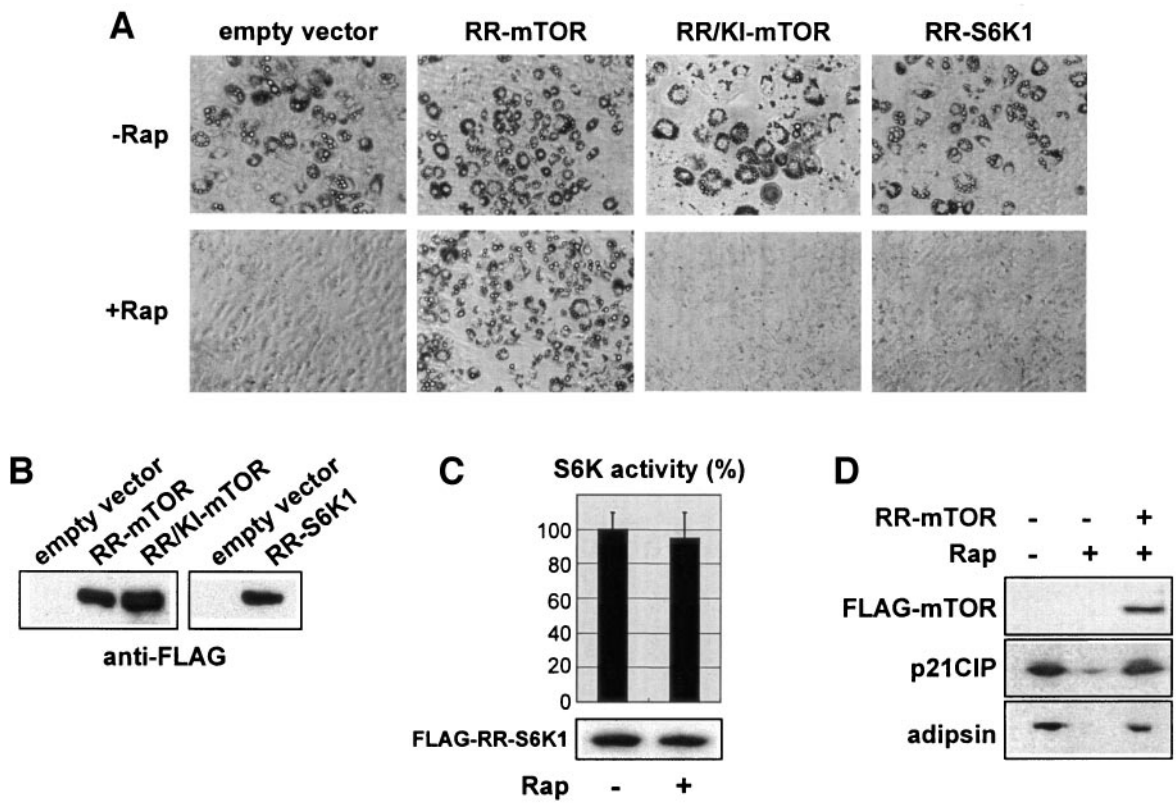

FIG. 2. Restoration of preadipocyte differentiation by rapamycin-resistant mTOR in the presence of rapamycin. *A*: 3T3-L1 preadipocytes were infected with retrovirus vectors expressing RR-mTOR, rapamycin-resistance kinase-inactive mTOR (RR/KI-mTOR), or RR-S6K1 as described in RESEARCH DESIGN AND METHODS. The cells were induced to differentiate with or without 100 nmol/l rapamycin for 8 days, followed by Oil Red O staining. *B*: Whole-cell lysates from infected cells were analyzed by Western blotting to verify the expression of FLAG-tagged recombinant proteins. *C*: FLAG-RR-S6K1 was immunoprecipitated from the virally infected cells with or without rapamycin treatment and subjected to S6 kinase assays. *D*: 3T3-L1 cells with or without RR-mTOR expression were differentiated as in *A*, followed by Western analyses of the cell lysates.

groups (23,31). Because S6K1 is a positive regulator of protein synthesis downstream of mTOR, whereas 4E-BP1 is a negative regulator, the upregulation of both proteins did not provide clear insights into the involvement of mTOR signaling in adipogenesis. Obviously, a direct examination of mTOR would be necessary.

We next probed the involvement of mTOR in adipogenesis directly. A point mutation, S2035T, confers rapamycin resistance to mTOR functions by abolishing rapamycin binding to mTOR (32,33). This rapamycin-resistant mTOR (RR-mTOR) was introduced into 3T3-L1 cells via retroviral infection followed by puromycin selection. The expression of the FLAG-tagged recombinant proteins from the viral source was confirmed by Western blots shown in Fig. 2*B*. The infected cells were induced to differentiate with or without rapamycin. Cells expressing RR-mTOR differentiated fully in the presence of rapamycin, as indicated by the appearance of rounded adipocytes and lipid droplets (Fig. 2*A*), as well as expression of the adipogenic markers adipsin and $p21^{CIP}$ (Fig. 2*D*). However, cells expressing a kinase-inactive mutant (D2357E) of mTOR containing the rapamycin-resistant mutation (RR/KI-mTOR) or infected with empty vector control did not differentiate in the presence of rapamycin (Fig. 2*A*), suggesting that the kinase activity of mTOR is required for adipocyte differentiation. We then asked whether S6K1 mediates the adipogenic signaling of mTOR. A rapamycin-resistant mutant of S6K1 (RR-S6K1; NH_2-terminal 23 amino acids and COOH-terminal 104 amino acids deleted [34]) was introduced into 3T3-L1 cells by viral infection, but expression of this mutant S6K1 did not rescue adipocyte differentiation from rapamycin inhibition (Fig. 2*A* and *B*), although the recombinant protein displayed full rapamycin-resistant S6 kinase activity (Fig. 2*C*). Thus, if S6K1 is involved, it is not the only effector downstream of mTOR in the adipogenic process.

Rapamycin inhibits the expression of C/EBP-α and PPAR-γ. To understand the mechanism by which mTOR regulates adipocyte differentiation, we focused on the transcription factors C/EBPs and PPAR-γ, which are known to play major regulatory roles in adipogenesis (4,7,8). As demonstrated by Western analyses shown in Fig. 3*A*, the protein levels of C/EBP-β and C/EBP-δ were highly induced after 1 day of differentiation and gradually decreased from day 4 to day 8; rapamycin treatment did not affect the early expression of those two proteins but seemed to prevent their subsequent decrease. Contrary to the rapid induction and transient expression profile of C/EBP-β and -δ, the protein levels of PPAR-γ and C/EBP-α, which were significantly blocked by rapamycin treatment (Fig. 3*A*), rose later during differentiation. At the same time, rapamycin also inhibited the expression of the adipogenic markers adipsin and $p21^{CIP}$ (Fig. 3*A*), which are thought to be the direct or indirect targets of PPAR-γ (35,36). Thus, rapamycin seems to block a later stage of adipogenesis by potentially targeting transcription factors C/EBP-α and PPAR-γ. Importantly, these rapamycin effects on the protein expression were closely mirrored by

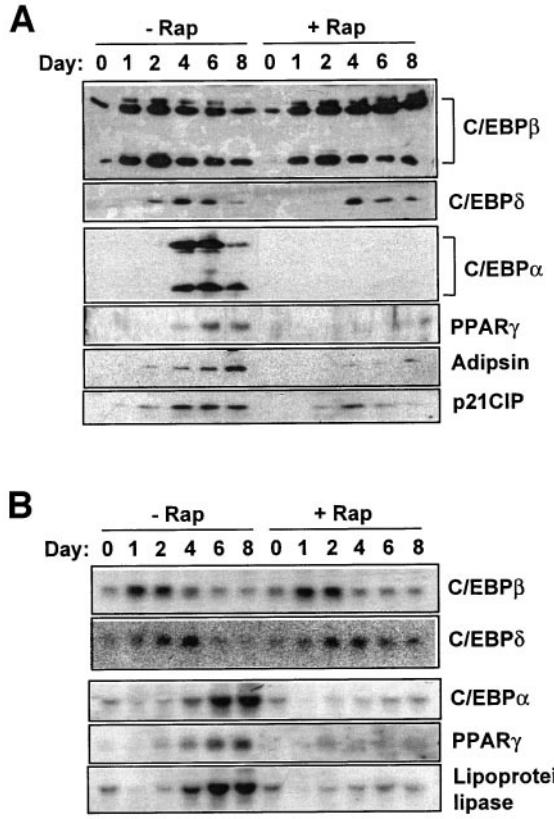

FIG. 3. Inhibition of C/EBP-α and PPAR-γ expression by rapamycin. 3T3-L1 cells were induced to differentiate for 8 days. Whole-cell extracts were prepared at the indicated time points and analyzed by Western blotting (*A*) or Northern blotting (*B*) with the indicated antibodies or probes.

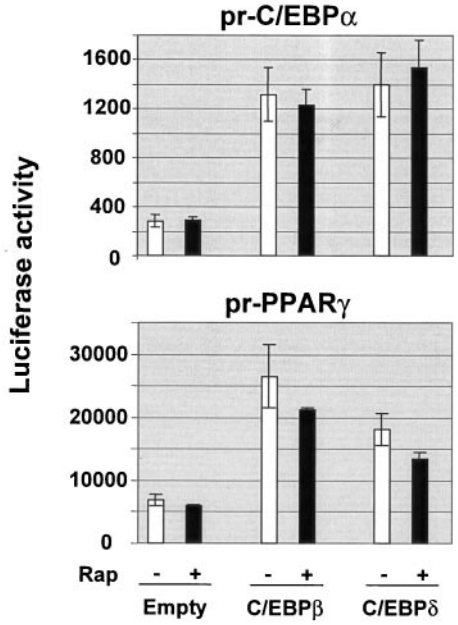

FIG. 4. Effect of rapamycin on C/EBP-β and C/EBP-δ activity. HEK293 cells were transfected with luciferase reporter for the C/EBP-α or PPAR-γ promoter (pr-C/EBP-α or pr-PPAR-γ) along with C/EBP-β or -δ expression plasmid as indicated. Cells were treated with 100 nmol/l rapamycin for 3 h when indicated, followed by lysis and luciferase assays.

those on the corresponding mRNA levels, as demonstrated by the Northern analyses shown in Fig. 3*B*, suggesting that the inhibition likely occurred at the transcriptional level.

The activity of C/EBP-β and C/EBP-δ is not affected by rapamycin. It has been reported that C/EBP-β and -δ are essential for adipocyte differentiation (37) and at least partly responsible for the expression of C/EBP-α and PPAR-γ (5). It was thus possible that the activity of C/EBP-β and C/EBP-δ toward the transcription of C/EBP-α and PPAR-γ could be affected by rapamycin. To test this possibility, we transfected 3T3-L1 preadipocytes with a luciferase reporter driven by the C/EBP-α promoter (27), along with C/EBP-β or C/EBP-δ expression plasmid. As shown in Fig. 4, overexpression of C/EBP-β and -δ enhanced the reporter activity as expected, but rapamycin had no effect, suggesting that the activation of C/EBP-α promoter by C/EBP-β or C/EBP-δ is independent of mTOR function. Next, 3T3-L1 preadipocytes were transfected with a luciferase reporter for the PPAR-γ promoter (26) along with the C/EBP-β or -δ expression plasmid, and the reporter activity was measured with or without rapamycin treatment of the cells. Although rapamycin caused a moderate decline in PPAR-γ promoter activity (Fig. 4), it is unlikely that this effect accounts for the complete absence of adipogenesis in the presence of rapamycin.

Ectopic expression of C/EBP-α and PPAR-γ does not reverse the rapamycin effect. Because rapamycin significantly blocked the expression of C/EBP-α and PPAR-γ transcription, we asked whether ectopic expression of C/EBP-α and PPAR-γ would rescue adipogenesis in the presence of rapamycin. To address this question, we generated 3T3-L1 cells stably expressing C/EBP-α by retroviral infection. The endogenous C/EBP-α mRNA yields two isoforms of the protein, p42 and p30, by utilizing alternative open reading frames (38). Here we used a C/EBP-α cDNA engineered to remove the start codon for the p30 isoform (38); as a result, only the p42 isoform was expressed from the retroviral vector, allowing us to distinguish the recombinant C/EBP-α from its endogenous counterpart. It was reported that constitutive expression of C/EBP-α p42 confers spontaneous adipocyte differentiation without hormonal induction (38). Indeed, we observed phenotypic differentiation in C/EBP-α p42-expressing cells even before hormonal addition (data not shown), and insulin further stimulated adipogenesis (Fig. 5*A*). Rapamycin completely blocked the insulin-induced accumulation of lipid droplets, as shown by Oil Red O staining in Fig. 5*A*. We analyzed the protein expression profiles and found that the expression of PPAR-γ was intact in C/EBP-α p42-expressing cells in the presence of rapamycin, but the adipogenic marker $p21^{CIP}$ was not induced under the same conditions (Fig. 5*B*). Thus, rapamycin inhibits adipogenesis even when both C/EBP-α and PPAR-γ proteins are expressed.

Rapamycin specifically inhibits PPAR-γ activity. It is generally believed that C/EBP-α and PPAR-γ induce each other's expression in a positive feedback loop to promote and/or maintain adipocyte differentiation (1,7,8). Because rapamycin inhibited C/EBP-α and PPAR-γ expression without affecting C/EBP-β and C/EBP-δ activity, it seemed likely that rapamycin's target would be the positive feedback loop between C/EBP-α and PPAR-γ. The data in Fig. 5*B* suggest that in the activation feedback loop between C/EBP-α and PPAR-γ, the induction of PPAR-γ by C/EBP-α

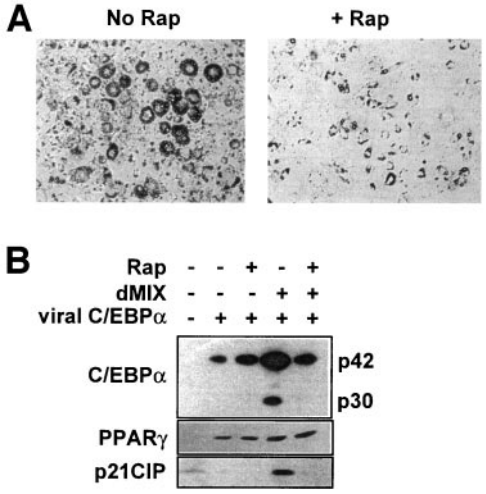

FIG. 5. Effect of ectopic expression of C/EBP-α on rapamycin inhibition of adipogenesis. *A*: 3T3-L1 preadipocytes were infected with retrovirus that contained C/EBP-α expression vector. The cells were induced to differentiate ("dMIX") with or without 100 nmol/l rapamycin for 8 days, followed by Oil Red O staining. *B*: Adipocytes at day 8 were lysed and subjected to Western blot analysis with the indicated antibodies.

remained intact in the presence of rapamycin, but apparently PPAR-γ activity was impaired as suggested by the lack of expression of PPAR-γ target $p21^{CIP}$ and the endogenous C/EBP-α p30 isoform despite the presence of C/EBP-α and PPAR-γ. A simple model that would explain all of these observations is that mTOR specifically regulates PPAR-γ transactivation activity.

To examine this possibility, we transfected HEK293 cells with a luciferase reporter that contained triple repeats of the DR1 sequence, a potent PPRE (39), along with PPAR-γ and RXR expression plasmids. This reporter activity was stimulated by insulin in serum-starved cells, which was mostly eliminated by rapamycin (Fig. 6*A*), suggesting that mTOR may be required for the stimulation of PPAR-γ activity by insulin on PPRE-driven transcription. The activity of PPAR-γ monitored by this reporter was also inhibited by wortmannin, a specific inhibitor for phosphatidylinositol 3-kinase (PI3K) (Fig. 6*A*). Indeed, PI3K and its downstream effector Akt have been reported to play crucial roles in adipogenesis (40,41). One of the downstream targets of Akt is the Foxo subfamily of Forkhead transcription factors. The phosphorylation and subsequent inhibition of Foxo proteins by Akt is critical for the insulin action on adipocyte differentiation (42,43). Recently, it has been reported that Foxo1 antagonizes PPAR-γ activity through its direct interaction and disruption of DNA binding activity of PPAR-γ (44). Because mTOR is intimately linked to the PI3K pathway, we asked whether mTOR would also have an impact on PPAR-γ activity through Foxo. To this end, we analyzed the phosphorylation of various Foxo transcription factors during adipogenesis. As shown in Fig. 6*B*, phosphorylations of Foxo1 at Ser256, Foxo3 at Thr32, and Foxo1 at Thr24 all were inhibited by wortmannin treatment, but none of them was affected by rapamycin. Thus, mTOR and PI3K seem to regulate PPAR-γ transactivation via different mediators.

Amino acid sufficiency regulates PPAR-γ activity, possibly through mTOR. To further verify the regulation

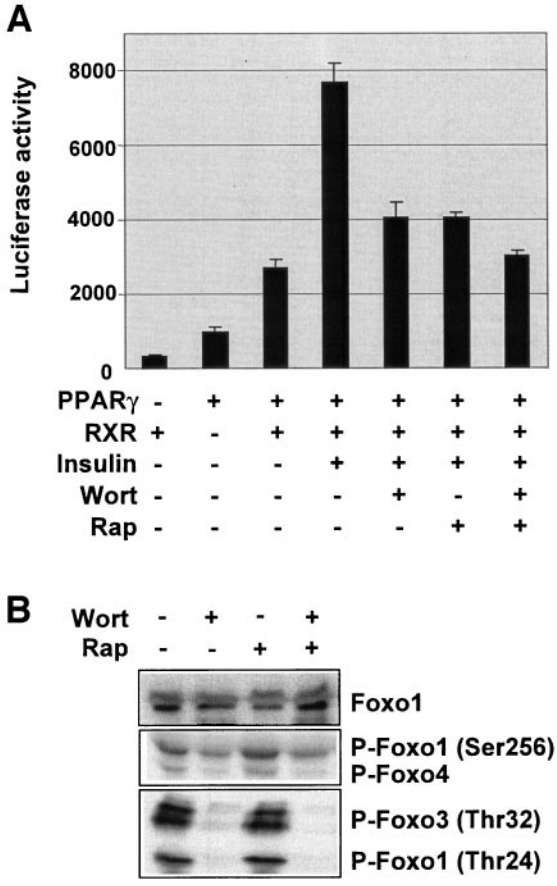

FIG. 6. Effect of rapamycin on PPAR-γ activity toward PPRE-dependent transcription. *A*: HEK293 cells were transfected with the PPRE reporter (3xDR1) along with PPAR-γ and/or RXR plasmids and incubated in DMEM with 0.3% FBS for 24 h. The cells were then treated with 100 nmol/l rapamycin or wortmannin for 30 min, and 1 μg/ml insulin was added for 3 h. Cell lysates were prepared and subjected to luciferase assays. *B*: 3T3-L1 cells were induced to differentiate. At day 2, some of the cells were treated with 100 nmol/l rapamycin or wortmannin for 1 h. Total cell lysates were prepared and subjected to Western blotting with the indicated antibodies. The anti–phospho-Foxo1-Ser 256 and phospho-Foxo1-Ser24 antibodies tend to cross-react with phospho-Foxo4 and phospho-Foxo3, respectively.

of PPAR-γ activity by mTOR in adipocytes, we transfected 3T3-L1 adipocytes (at day 6 differentiation) with the DR1 luciferase reporter. This PPRE-driven activity was significantly reduced by rapamycin treatment for 3 h (Fig. 7*A*). The short duration of rapamycin treatment ensured that the decrease in luciferase activity was not due to a general inhibition of protein synthesis by rapamycin, and, indeed, a cytomegalovirus (CMV) promoter-driven luciferase reporter was minimally affected under similar conditions (data not shown). The level of PPAR-γ protein was unchanged upon rapamycin treatment (Fig. 7*A*). Furthermore, the reporter activity was resistant to rapamycin treatment in RR-mTOR–expressing cells (Fig. 7*B*), confirming the role of mTOR in the regulation of PPAR-γ activity.

Previous studies have established that the mTOR pathway transduces nutrient availability signals, especially amino acid sufficiency. Therefore, we examined whether amino acid sensing is involved in the regulation of PPAR-γ activity by mTOR. As shown in Fig. 7*A*, day 6 3T3-L1 adipocytes transfected with DR1 luciferase reporter were

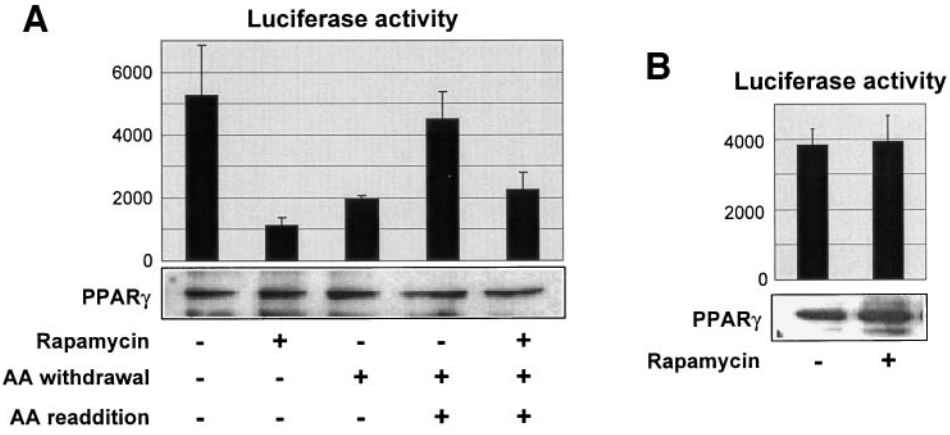

FIG. 7. Effects of rapamycin and amino acid withdrawal on PPAR-γ activity toward PPRE-dependent transcription in adipocytes. *A*: Differentiated 3T3-L1 cells at day 6 were transfected with the PPRE reporter (3xDR1-luc) for 24 h. Cells were treated with 100 nmol/l rapamycin for 3 h before harvest. Amino acid withdrawal and resupply were performed as described in RESEARCH DESIGN AND METHODS. The cells were lysed and subjected to luciferase assays as well as Western blot analysis for PPAR-γ. *B*: 3T3-L1 cells expressing RR-mTOR were differentiated, transfected, and treated as in *A*, followed by luciferase assay and Western blot analysis.

incubated in amino acid–deficient medium for 3 h. The reporter activity was significantly reduced by amino acid starvation, which was mostly restored when amino acids were replenished for 3 h in these cells. The restoration of PPAR-γ activity by amino acid readdition was abolished by rapamycin treatment. As a control, the CMV-driven luciferase activity was not significantly affected by such short-term amino acid starvation (data not shown). Taken together, these data suggest that PPAR-γ transactivation activity may be directly controlled by a nutrient-sensing signaling pathway, likely involving mTOR.

Troglitazone reverses the effect of rapamycin treatment. TZD drugs, as specific ligands for PPAR-γ (10), have been used to improve insulin sensitivity in various models of diabetes and obesity (11). It is interesting that when day 6 3T3-L1 adipocytes were treated with 10 μmol/l troglitazone, a TZD drug, the inhibitory effect of rapamycin on PPAR-γ activity (as measured by the DR1 reporter) was completely reversed (Fig. 8*A*). Likewise, the reduction of PPAR-γ activity upon amino acid starvation was reversed by troglitazone (Fig. 8*A*). Once again, the CMV-driven luciferase reporter was mostly unaffected under the same conditions. Accordingly, the expression of various adipogenic markers, including C/EBP-α, adipsin, and $p21^{CIP}$, was completely rescued by troglitazone treatment in the presence of rapamycin (Fig. 8*B*), and so was phenotypic differentiation including lipid formation and morphological changes (data not shown). These findings suggest that troglitazone neutralizes the inhibitory effect of rapamycin and amino acid deficiency by enhancing PPAR-γ activity. Troglitazone seems to act independent of the S6K1 pathway, because S6K1 phosphorylation at Thr398 (a putative mTOR site and an indication of S6K1 activity) was not rescued by troglitazone in rapamycin-treated cells (Fig. 8*B*). An obvious implication of the troglitazone effect on reversing rapamycin-inhibited PPAR-γ activity was that mTOR controls the production of a PPAR-γ ligand. However, conditioned media from normally differentiating adipocytes did not rescue differentiation in rapamycin-treated 3T3-L1 cells (data not shown). Thus, the major function of mTOR in the regulation of PPAR-γ activity is unlikely to be through the production of a ligand but rather through its direct impact on PPAR-γ transactivation activity, as suggested by the rapid inhibitory effect of rapamycin (Figs. 6 and 7). It is possible that troglitazone, as an unnatural

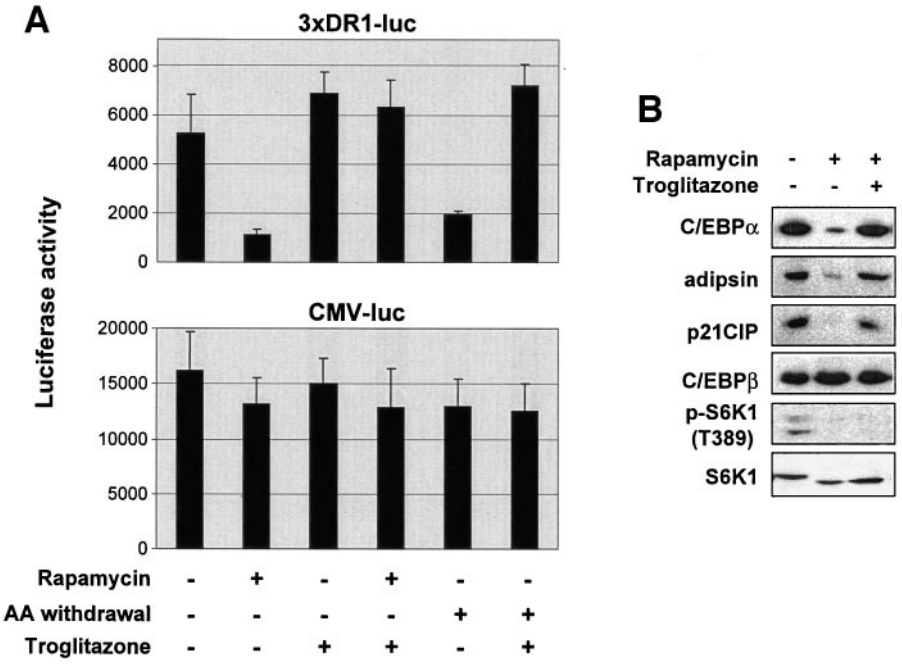

FIG. 8. Effect of troglitazone on rapamycin inhibition of PPAR-γ activity. *A*: 3T3-L1 adipocytes at day 6 transfected with 3xDR1 reporter were treated with 100 nmol/l rapamycin or subjected to amino acid withdrawal as described in the legend of Fig. 7, with or without 10 μmol/l troglitazone. The cells were lysed and subjected to luciferase assays. A CMV promoter-driven luciferase plasmid (CMV-luc) was used as a control. *B*: 3T3-L1 were differentiated in the presence of 100 nmol/l rapamycin with or without 10 μmol/l troglitazone for 8 days, followed by cell lysis and Western blot analysis with the indicated antibodies.

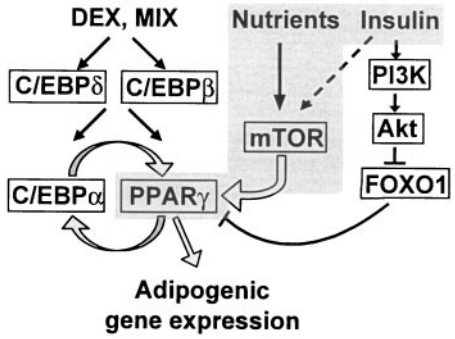

FIG. 9. A proposed model of adipogenic regulation by mTOR. See text for details.

ligand, overrides the normal requirements for PPAR-γ activation and thus counters rapamycin inhibition.

DISCUSSION

A number of studies were published reporting the inhibitory effect of rapamycin on adipogenesis (22–24). However, the role of mTOR was not directly examined, and the molecular mechanism of rapamycin inhibition was not explored in this important biological process. In our current study, we provide the first direct demonstration that mTOR is required for adipogenesis in the system of 3T3-L1 cells and that the catalytic activity of mTOR is indispensable for its adipogenic functions. To unravel the mechanisms of mTOR signaling in adipogenesis, we focused on the transcriptional regulatory network in adipocyte differentiation. Our findings have led us to propose a novel model (Fig. 9) in which the mTOR pathway specifically regulates the transactivation activity of PPAR-γ, which is essential for a positive feedback control of C/EBP-α expression as well as the adipogenic gene expression program and thus critical for both initiating and maintaining adipogenesis. Furthermore, mTOR signaling may serve to transduce nutrient availability signals to control the activity of PPAR-γ.

mTOR signaling is best known for its role in translational regulation via two major downstream effectors, S6K1 and 4E-BP1. The vast majority of cellular mTOR is found in the cytoplasm at steady state, but we previously identified a cytoplasmic-nuclear shuttling behavior for mTOR (45), which may suggest a nuclear function for mTOR. Several lines of evidence have implicated the direct involvement of mTOR in the regulation of transcription (for examples, see references 46–48), but the mechanisms are not well understood. So far, the only transcriptional regulator identified as a target of mTOR is STAT3, which is reportedly phosphorylated by mTOR at Ser727 and activated (48). In this study, we have revealed another transcriptional regulator as a potential target of mTOR (PPAR-γ) further extending the complexity of mTOR signaling. How mTOR functionally interacts with PPAR-γ is currently unknown and warrants future investigations. mTOR did not seem to affect the DNA binding affinity of PPAR-γ for the DR1 sequence (data not shown). The direct target of mTOR could be a nuclear cofactor of PPAR-γ, or as a potential scaffold protein mTOR could participate in the recruitment of a coactivator complex for PPAR-γ.

The PI3K/Akt pathway, downstream of insulin signaling, has previously been shown to be critically involved in preadipocyte differentiation. The activities of PI3K and Akt kinase rise during differentiation (40), and constitutively active Akt causes spontaneous differentiation of 3T3-L1 without hormone stimulation (41). Most recently, Akt has been demonstrated to regulate adipogenesis via phosphorylating and thus inactivating Foxo1 (42,43), and Foxo1 is known to regulate directly PPAR-γ activity (44). The mTOR and PI3K pathways are intricately linked, and at least two distinct models have been proposed for their relationship (discussed in ref 49). In one model, the two pathways adopt a parallel relationship, and they converge on common downstream targets such as S6K1 and 4E-BP1. The alternative model places mTOR downstream of PI3K/Akt in a linear manner. Our observations suggest that in adipogenesis, these two pathways most likely work in parallel and converge on the activation of PPAR-γ: whereas the PI3K/Akt pathway seems to mediate insulin signals to downregulate Foxo1, the mTOR pathway most likely transduces nutrient-availability signals to regulate PPAR-γ via a target distinct from Foxo1 (Fig. 9).

The functions of the adipose tissue, including metabolism, energy homeostasis, and endocrine actions, are dependent on cellular energy status and nutrients (1). The mTOR pathway is known to sense amino acid availability (14) and more recently was shown to respond to cellular energy levels (50,51). Our data suggest that adipocytes may utilize the mTOR pathway to sense nutrient availability and modulate the activity of PPAR-γ, revealing the first molecular link between nutrients and adipogenesis/adipogenic functions. It is interesting to note that skeletal myogenesis also requires mTOR signaling. Nutrient sensing through mTOR controls the autocrine production of insulin-like growth factor-II that instructs the initiation of the differentiation program in skeletal myoblasts (47). Hence, the mTOR pathway seems to be a master mediator of nutrient signals, governing a wide range of biology from cell growth to various types of cellular differentiation.

Rapamycin (also known as Rapamune or sirolimus) has been widely used as an immunosuppressant to prevent graft rejection in transplant recipients (52,53). Recent clinical studies have demonstrated that one of the major side effects associated with rapamycin treatment is dyslipidemia, characterized by increased serum levels of triglyceride and cholesterol (54,55). This toxicity of rapamycin is potentially the result of deregulated energy and lipid homeostasis in peripheral tissues including adipose and hepatic tissues. Our findings that rapamycin specifically inhibits PPAR-γ activity and subsequently disrupts the adipogenic functions provide a possible molecular explanation for this toxic effect of rapamycin. Furthermore, it would be reasonable to explore the possibility of mTOR regulation of other PPAR isoforms such as PPAR-α and PPAR-β/δ, because these factors are also critical for lipid metabolism and control of serum lipid levels (56). A significant implication of our observation that troglitazone reversed the inhibitory effect of rapamycin on PPAR-γ transactivation activity is the rationale for testing the possibility of using TZD drugs to counter the dyslipidemia side effect of rapamycin in transplant recipients.

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