mTORC1 Activation Regulates β-Cell Mass and Proliferation by Modulation of Cyclin D2 Synthesis and Stability*§

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Growth factors, insulin signaling, and nutrients are important regulators of β-cell mass and function. The events linking these signals to the regulation of β-cell mass are not completely understood. The mTOR pathway integrates signals from growth factors and nutrients. Here, we evaluated the role of the mTOR/raptor (mTORC1) signaling in proliferative conditions induced by controlled activation of Akt signaling. These experiments show that the mTORC1 is a major regulator of β-cell cycle progression by modulation of cyclin D2, D3, and Cdk4 activity. The regulation of cell cycle progression by mTORC1 signaling resulted from modulation of the synthesis and stability of cyclin D2, a critical regulator of β-cell cycle, proliferation, and mass. These studies provide novel insights into the regulation of cell cycle by the mTORC1, provide a mechanism for the antiproliferative effects of rapamycin, and imply that the use of rapamycin could negatively impact the success of islet transplantation and the adaptation of β-cells to insulin resistance.

The defects that result in diabetes are diverse, but the loss of pancreatic β-cell mass is a critical determinant for the development of this disease (1, 2). The capacity for β-cells to expand in response to insulin resistance is required to maintain glucose homeostasis and prevent type 2 diabetes. Pancreatic β-cell mass is regulated by a dynamic balance of neogenesis, proliferation, hypertrophy, and apoptosis (3). In particular, β-cell proliferation (determined by the number of mature β-cells entering the cell cycle) has a major role in the maintenance of β-cell mass in adult life and after proliferative stimuli (4). Although there has been much research showing the role of β-cell mass in diabetes, there is a lack of knowledge pertaining to how β-cells enter the cell cycle, proliferate, and increase mass.

In pancreatic β-cells, glucose, amino acids, and growth factors have been shown to induce G1-S progression (5–7). Recent studies have demonstrated that mTOR integrates growth factors and nutrient signals and is essential for cell growth and proliferation (8). One of the major mechanisms by which nutrient and growth factors regulate mTOR activity involves the tuberous sclerosis complex 2 (TSC2) gene product (tuberin) as well as TSC1 (hamartin) and the small G protein Ras homolog enriched in brain. Phosphorylation of TSC2 by the serine-threonine kinase AKT induces mTOR signaling by derepressing the TSC2 GTPase-activating protein activity toward Ras homolog enriched in brain, (9–13). Recent findings indicate that mTOR is a part of two distinct complexes: mTORC1 and mTORC2 (14, 15). The mammalian mTORC1 contains Raptor and the G protein β-subunit-like protein (GβL). mTORC1 activates key regulators of protein translation; ribosomal S6 kinase (S6K), eukaryote initiation factor 4E-binding protein 1, and eukaryote initiation factor 4E (16). The mTORC2 complex includes mTOR and rictor and is insensitive to rapamycin (14, 15). This complex is potentially important for the regulation of β-cell mass and function, because it is responsible for the phosphorylation/activation of Akt on Ser473 (17). Evidence for the importance of mTOR signaling on the modulation of β-cell mass and proliferation in vivo comes from genetically modified mice. Decreased β-cell mass and hyperglycemia in mice deficient for S6K and mutant for ribosomal protein S6 provide evidence for the importance of this pathway in these processes (18–20). Moreover, activation of mTOR signaling by conditional deletion of TSC2 in β-cells induces β-cell proliferation and hypertrophy (21, 22) The contribution and potential role for mTOR signaling and the mTORC complexes in β-cell mass and function have yet to be adequately explored.

The current experiments delineate some of the molecular mechanisms involved in β-cell G1-S transition by the mTOR arm of Akt signaling. In these studies, the hypothesis that the mTORC1 (mTOR/Raptor) is a major regulator of β-cell cycle progression and mass in vivo was tested. To test this hypothesis, we studied the effects of inhibition of the mTORC1 complex under proliferative conditions induced by controlled activation of Akt signaling in β-cells. To activate Akt signaling in a controlled fashion, we developed a doxycycline (dox)-inducible mouse model. This animal model allowed us to induce β-cell proliferation.

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1–54.

2 The abbreviations used are: TSC, tuberous sclerosis complex; S6K, ribosomal S6 kinase; dox, doxycycline; Cdk, cyclin-dependent kinase; DT, double transgenic; ST, single transgenic; GST, glutathione S-transferase; GFP, green fluorescent protein; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; Rap, rapamycin.
proliferation and mass without disturbing peripheral tissues. These studies showed that the mTORC1 complex mediates the regulation of cell cycle in β-cells in vivo and does so by activation of cyclin-dependent kinase-4 (Cdk4). The regulation of cell cycle progression by mTORC1 signaling resulted from modulation of the synthesis and stability of cyclin D2, a critical regulator of β-cell cycle, proliferation, and mass. These studies indicate that the mTORC1 is major component relating proliferative signals induced by nutrients and growth factors and uncover the molecular mechanisms implicated in the regulation of β-cell cycle by this signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Mice**—RIP-rtTA mice express the reverse tetracycline transactivator under the control of the rat insulin II gene and are in C57Bl/6 (B6)/CBA background (23). The tetOAkt1 mice have been previously described (24) and contain the myristoylated AKT1 gene under the regulation of tetracycline-responsive element. Generation and phenotypic characterization of myr-Akt transgenic (ST) was used as control and included RIP-rtTA mice. Experiments were performed in 2-month-old males. Control and experimental animals were on comparably mixed background. Doxycycline treatment was performed by adding 2 mg/ml doxycycline to the drinking water. Mice overexpressing AKT1 gene under the regulation of tetracycline-responsive element. Generation and phenotypic characterization of myr-Akt transgenic (ST) was used as control and included RIP-rtTA mice. Experiments were performed in 2-month-old males. Control and experimental animals were on comparably mixed background. Doxycycline treatment was performed by adding 2 mg/ml doxycycline to the drinking water. Mice overexpressing a constitutively active form of Akt under the control of the rat insulin promoter (caAktRIP) have been previously described (25). All of the procedures were performed in accordance with the Washington University Animal Studies Committee.

**Islet and MIN6 Cell Culture**—MIN6 cells were stably infected with a lentivirus containing a constitutively active Akt mutant (MIN6-caAkt) or GFP (MIN6-GFP) as control. These lines were maintained as described previously (26). For experiments with rapamycin, MIN6 cells were cultured with rapamycin for 16 h. The *in vitro* experiments in islets were performed in islets from ST or DT mice treated with vehicle or dox in the drinking water for 3 weeks. After isolation, the islets were cultured in medium with vehicle (ST) or dox (DT) for 40 h. Rapamycin (50 nM) or vehicle was added to the medium for the last 16 h of culture before harvesting. This experimental protocol reproducibly inhibit mTORC1 complex *in vitro*. These experimental conditions were used for all of the islet experiments except for the studies in Fig. 2C.

**Islet Isolation and Western Blot Analysis**—Islet isolation was accomplished by collagenase digestion as described previously (25). The following morning after isolation, the islets were hand picked and treated with 2 μg/μl doxycycline and/or 50 nM rapamycin for 16 h as indicated in the figures and results. Isolated islets were lysed in a buffer containing 0.3% CHAPS, 150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 8.0, and protease and phosphatase inhibitors (Roche Applied Science). Islet lysates were subjected to immunoblotting using the following antibodies: Akt, pGSK3 α/β, p70S6K (phospho-p70 S6K Thr 389) phospho-S6 ribosomal protein (Ser235/236), cyclin D1, cyclin D3, and p27 were from Cell Signaling (Beverly, MA), and cyclin D2 was obtained from Lab Vision Corporation (Fremont, CA). Cdk4 was from Santa Cruz Biotechnology (Santa Cruz, CA), and α-tubulin was from Sigma. Secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were from Cell Signaling.

**Immunohistochemistry, Islet Morphometry, and Analysis of Proliferation and Apoptosis**—Pancreatic tissue was fixed overnight in 3.7% formalin solution and embedded in paraffin using standard techniques. Immunostaining for insulin was done as describe previously (25). Immunofluorescence for phospho-pS6 ribosomal protein (Ser235/236) (Cell Signaling) and insulin was performed as described previously (25). Assessment of β-cell mass was performed by point counting morphometry from five insulin-stained sections (4 μm) separated by 200-μm National Institutes of Health Image software (v1.3.8x) as described previously (27, 28). Proliferation was performed in insulin-and Ki67-stained sections (Novocastra, Burlingame, CA) from ST and DT mice. Proliferating cells were identified by co-staining for Ki67 and insulin. Apoptosis was determined in pancreatic sections stained for insulin and cleaved-caspase 3 (Cell Signaling). Co-staining for insulin and cleaved-caspase 3 identified apoptotic cells. At least 1000 insulin-stained cells were counted for each animal.

**Quantitative Reverse Transcription-PCR**—Total RNA was isolated using RNeasy (Qiagen). cDNA was synthesized using random hexamers and reverse transcribed with Superscript II (Invitrogen) according to the manufacturer’s protocol. Real time PCR was performed on ABI 7000 sequence detection system using TaqMan gene expression assays (Applied Biosystem, Foster City, CA). Primers from cell cycle components were purchased from Applied Biosystem with reference numbers p21 (Mm 00432448), p27 (Mm 00432359), Cdk4 (Mm 01273583), Ccnd1 (Mm 00432359), Ccnd2 (Mm 00438071), and Ccnd3 (Mm 01273583).

**In Vitro Cdk4 Kinase Assays**—*In vitro* Cdk4 activity were performed as described previously (29). Four-week-old ST and DT mice were placed on vehicle or dox treatment in their drinking water for 3 weeks. After isolation, DT islets were cultured for 40 h in medium containing dox (2 μg/ml). Rapamycin (50 nM) or vehicle was added for 16 h before harvesting. Lysates from islets or MIN6 cells were immunoprecipitated using anti-Cdk4 antibody (Santa Cruz Biotechnology) and 50 μl of protein G-Sepharose beads (Sigma-Aldrich). The final kinase reaction was carried out in 50 mmol/liter HEPES, pH 7.5, 10 mmol/liter MgCl₂, 1 mmol/liter dithiothreitol, 2.5 mmol/liter EDTA 10 mmol/liter glycero phosphate, 0.1 mmol/liter Na₂VO₃, 1 mmol/liter NaF, 5 μmol/liter ATP, 6 Ci/reaction of [γ-32P]ATP (Amersham Biosciences), and GST-Rb 769–921 (Santa Cruz Biotechnology). The samples were incubated at 30 °C for 30 min and separated by polyacrylamide gel electrophoresis. The amount of 32P-labeled GST-Rb was visualized and quantified by autoradiography using a PhosphorImager. The levels of immunoprecipitated Cdk4 were used as loading control.

**In Vitro Kinase Assay for Akt**—Akt kinase activity was measured using the Akt kinase assay kit from Cell Signaling. Four-week-old ST and DT mice were placed on vehicle or dox treatment in their drinking water for 3 weeks. After isolation, ST and DT islets were cultured for 40 h with vehicle or dox (2 μg/ml), respectively. Rapamycin (50 nm) or vehicle was added for the last 16 h before harvesting. Resuspended immobilized Akt anti-
body slurry (20 μl) was added to 100 μg of lysates to selectively immunoprecipitate Akt by gentle rocking 2 h at 4 °C. The pellet was washed twice with 500 μl of 1× lysis buffer and twice with 500 μl of 1× kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, and 10 mM MgCl₂). The immunoprecipitated pellet was then incubated with 40 μl of 1× kinase buffer supplemented with 200 μM ATP and 1 μg of GSK-3 fusion protein for 30 min at 30 °C allowing immunoprecipitated Akt to phosphorylate GSK-3. The reaction was terminated with 20 μl of 3× SDS sample buffer. The samples were boiled for 5 min and loaded on 12% SDS-PAGE gel. Band intensity was quantified using National Institutes of Health ImageJ software (v1.3.8x). Input protein was used as control for quantification of the Akt activity levels. Akt-induced phosphorylation of GSK-3 was detected by Western blotting using phospho-GSK-3/ (Ser21/9) antibody.

**Pulse-Chase Analysis**—Islets from four ST and four DT mice/time point were washed in Dulbecco’s modified Eagle’s medium without methionine and cysteine for 30 min at 37 °C. The islets were pulse-labeled for 30 min with [35S]Protein labeling mix without methionine and cysteine for 30 min at 37 °C allowing pulse-labeled protein to phosphorylate GSK-3. The reaction was terminated with 20 μl of 3× SDS sample buffer. The samples were boiled for 5 min and loaded on 12% SDS-PAGE gel. Band intensity was quantified using National Institutes of Health ImageJ software (v1.3.8x). Input protein was used as control for quantification of the Akt activity levels. Akt-induced phosphorylation of GSK-3 was detected by Western blotting using phospho-GSK-3/ (Ser21/9) antibody.

**RESULTS**

**Development of an Inducible System to Activate Akt/mTOR Signaling in β-Cell**—To determine the molecular mechanisms involved in β-cell G1-S transition by the mTOR arm of Akt signaling, we developed a dox-inducible system. DT mice were treated with doxycycline for 6 and 24 h, treated with doxycycline for 6 and 24 h, and treated with doxycycline for 6 and 24 h, and then treated with doxycycline for 6 and 24 h. Intra-peritoneal glucose tolerance tests were performed in ST and DT mice after initiation of dox treatment. C, intraperitoneal glucose tolerance tests performed in ST and DT mice after 20 weeks of doxycycline treatment. D, 6-h fasting glucose measurements obtained from ST or DT mice. E, insulin levels in 6 h fasted ST and DT mice. F, β-cell mass measurements in ST and DT mice after 20 weeks of doxycycline or vehicle treatment. G, proliferation rate assessed by Ki67 in insulin-stained sections from ST and DT mice that received doxycycline treatment for 20 weeks. The data are presented as the means ± S.E. (n = 5), *p < 0.05.

**FIGURE 1. Overexpression of Akt in β-cells using a doxycycline-inducible system.** A, immunoblotting for Akt and pGSK3α/β (Ser21/9) in islets from ST (RIP-rTA) and DT (RIP-rTA/tetOcaAkt) mice treated with doxycycline for 6 and 24 h. B, intraperitoneal glucose tolerance test after overnight fasting in ST and DT mice before initiation of dox treatment. C, intraperitoneal glucose tolerance tests performed in ST and DT mice after 20 weeks of doxycycline treatment. D, 6-h fasting glucose measurements obtained from ST or DT mice. E, insulin levels in 6 h fasted ST and DT mice. F, β-cell mass measurements in ST and DT mice after 20 weeks of doxycycline or vehicle treatment. G, proliferation rate assessed by Ki67 in insulin-stained sections from ST and DT mice that received doxycycline treatment for 20 weeks. The data are presented as the means ± S.E. (n = 5), *p < 0.05.
The improved glucose tolerance in DT mice was maintained after 20 weeks of dox treatment (Fig. 1C). Assessment of 6-h fasting glucose levels in ST and DT mice at different time points during dox treatment demonstrated that glucose levels in DT mice during doxycycline treatment were not different from those obtained from ST mice (Fig. 1D). Serum concentrations of insulin in DT mice increased after 2 weeks of dox administration (Fig. 1E). Compared with ST mice, insulin levels in DT mice remained elevated after 16 weeks of dox administration (Fig. 1E).

The histology of the pancreas and quantitation of β-cell mass were assessed by islet morphometry. After 20 weeks of doxycycline treatment, β-cell mass was augmented more than 5-fold in DT mice compared with ST mice (Fig. 1F). β-Cell proliferation by determined Ki67 immunostaining in insulin-stained pancreatic sections showed a 3-fold increase in proliferative rate in DT mice (Fig. 1G). These results showed that the inducible system could be used as a powerful tool to study the molecular mechanisms involved in cell cycle progression and β-cell mass under proliferative conditions induced by Akt.

**Rapamycin Treatment Inhibits the Activation mTORC1 Signaling by Akt**—The importance of the mTORC1 in the metabolic and morphologic phenotype observed in DT mice was assessed using the following experimental design (Fig. 2A): 1) 4-week-old ST and DT mice were placed on vehicle or dox treatment in their drinking water for 3 weeks. 2) After 3 weeks of dox treatment, rapamycin was injected intraperitoneally for 2 weeks in half of the ST and DT mice. The other half of the ST and DT mice continued vehicle and dox treatment (Fig. 2A). To assess the inhibition of mTOR signaling by rapamycin treatment in ST and DT mice, we performed immunofluorescence staining using anti-phospho-S6 ribosomal protein (Ser235/236) antibody (pS6rp) (Fig. 2B). DT mice exhibited a significant increase in pS6rp staining when compared with ST mice (Fig. 2B). Staining for pS6rp in the pancreas from rapamycin-treated ST and DT mice was completely absent, suggesting that the dose of rapamycin was effective in inhibiting mTOR activation by Akt (Fig. 2B). To complement these studies, we performed immunoblotting in islet lysates from these mice. After dox administration, Akt levels were higher in DT compared with ST mice (Fig. 2C). Rapamycin treatment had no effect on Akt levels from ST or DT mice (Fig. 2C). Similar to the results shown on Fig. 2B, levels for pS6rp were higher in DT than ST mice. Rapamycin treatment of ST and DT mice completely abolished the phosphorylation of S6rp (Fig. 2C).

**FIGURE 2.** Assessment of mTOR signaling in islets from ST and DT mice. **A**, experimental design used to assess rapamycin effect on ST and DT mice. **B**, immunostaining for insulin (green) and pS6 ribosomal protein (red) in islets from ST and DT mice at the end of the experimental protocol. **C**, immunoblotting for total Akt, p70S6K (phospho-p70 S6K Thr 389), phospho-S6 ribosomal protein (Ser235/236) (pS6) and tubulin in islet lysates from ST and DT mice at the end of the experimental protocol described in A. Islets from all groups were harvested immediately after isolation and subjected to immunoblotting. Scale bar, 50 μm.

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Rapamycin Treatment Partially Reverses the Akt-mediated Improvement in Carbohydrate Metabolism—Assessment of carbohydrate metabolism in ST and DT mice treated with vehicle or rapamycin was then performed. Before administration of dox, glucose tolerance in 4-week-old ST and DT mice was comparable (Fig. 3A). In contrast to ST mice, DT mice treated with dox for 3 weeks exhibited lower glucose levels at 30, 60, and 120 min after glucose injection (Fig. 3B). Glucose tolerance after rapamycin treatment showed impaired glucose tolerance in ST+Rap mice when compared with ST mice (Fig. 3C). Glucose levels at 30, 60, and 120 min in DT+Rap mice were higher than those of DT mice treated with vehicle (Fig. 3C). Glucose tolerance in DT+Rap mice showed that glucose levels at 30 and 60 min were comparable with those of ST+Rap (Fig. 3C). The metabolic alterations induced by rapamycin treatment reversed after discontinuation of treatment (supplemental Fig. S2). These results indicate that the improvement in carbohydrate metabolism observed by activation of Akt in β-cells was partially reversed by the inhibition of the mTORC1.

Assessment of islet morphometry at the end of the experimental protocol showed that the β-cell mass in ST and ST+Rap mice was comparable (Fig. 3D). In contrast, the β-cell mass in DT mice was higher than that of ST mice (Fig. 3D; p < 0.05). Rapamycin treatment of DT mice reduced β-cell mass to the levels found in ST and ST+Rap (Fig. 3D). Proliferation assessed by Ki67 staining demonstrated that ST and ST+Rap exhibited similar rates of proliferation (Fig. 3E). The proliferative rate observed in DT mice was 2-fold greater than that of ST mice (p < 0.05; Fig. 3E). The increased proliferative rate observed in DT mice was completely inhibited by rapamycin treatment (Fig. 3E). Assessment of apoptosis by cleaved caspase 3 staining demonstrated that the apoptotic rate was increased in DT mice when compared with ST mice (p < 0.05; Fig. 3F). The apoptotic rate in DT mice was reduced by rapamycin treatment (Fig. 3F). Rapamycin treatment had no effect on apoptosis in ST mice (Fig. 3F). Taken together, these experiments suggest that over-expression of Akt in β-cells induces β-cell mass by increased proliferation in an mTORC1-dependent mechanism.

Rapamycin Treatment Had No Effect on mTORC2 and Akt Activity—Recent experiments suggest that the mTORC2 complex phosphorylates Akt on Ser^{473} (17). Although the mTORC2 complex was initially described as rapamycin-insensitive, recent evidence suggests that rapamycin treatment can modulate mTORC2 activity in some systems (30, 31). To test whether the effects of rapamycin treatment in β-cell mass and proliferation resulted from inhibition of mTORC2/Akt signaling, we assessed the phosphorylation status of Akt and Akt kinase activity in islets from ST and DT mice treated with vehicle or rapamycin. Phosphorylation of Akt on Thr^{308} was increased in islets from DT mice (Fig. 4A). Rapamycin treatment had no effect on phosphorylation of Akt on Thr^{308} in islets lysates from ST and DT mice (Fig. 4A). Similarly, Akt phosphorylation on

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**FIGURE 3.** Assessment of carbohydrate metabolism β-cell mass, proliferation, and apoptosis. A, intraperitoneal glucose tolerance test after overnight fasting in ST and DT mice before initiation of dox treatment. B, after 3 weeks of doxycycline treatment. C, after 2 weeks of Rap treatment (5 mg/kg) daily. D, β-cell mass in ST and DT mice treated with vehicle or Rap (5 mg/kg) daily for 2 weeks as indicated in Fig. 2A. Co-staining for insulin and Ki67 defined proliferating cells. E, apoptosis measured by cleaved-caspase 3 staining in insulin-stained sections from ST and DT mice at the end of the experimental protocol described for Fig. 2A. Apoptotic cells were determined as cells that were co-stained for insulin and cleaved-caspase 3. The data are presented as the means ± S.E. (n = 5). *, p < 0.05; #, p < 0.05 DT + Rap versus DT.
Ser473 was increased in DT mice compared with ST mice, and these changes were not affected by rapamycin (Fig. 4A). \textit{In vitro} Akt kinase activity in islets from ST and DT mice showed increased Akt kinase activity in DT mice (Fig. 4B). Akt activity in ST or DT mice was not altered by rapamycin (Fig. 4B). Similar to islets from ST and DT mice, \textit{in vitro} Akt kinase activity was increased in MIN6 cells stably transfected with a constitutively active mutant of Akt (MIN6-caAkt), and this activity was not altered by rapamycin treatment (Fig. 4C). Phosphorylation of endogenous GSK3α/β on Ser21/9 was increased in islet lysates from DT mice when compared with ST mice (4.2 ± 1.0, p < 0.05). In contrast to the \textit{in vitro} Akt kinase activity, rapamycin treatment of DT islets inhibited the phosphorylation of GSK3α/β on Ser21/9, suggesting that a downstream target of mTOR could phosphorylate GSK3α/β on the same residue as Akt (Fig. 4D; p < 0.05). These experiments suggest that rapamycin treatment did not alter the phosphorylation status or activity of Akt in islets or cell lines overexpressing a constitutively active form of Akt.

\textit{Rapamycin Treatment Inhibited Cdk4 Activity by Reducing Cyclin D2 and D3 Levels}—The effect of rapamycin in β-cell proliferation in DT islets was further investigated by analysis of the cyclin D-Cdk4 complex. \textit{In vitro} kinase activity assays were conducted using recombinant GST-Rb (amino acids 769–921) as substrate. This exogenous substrate contains the phosphorylation site for Cdk4. The incorporation of radioactive phosphate to this substrate is proportional to Cdk4 activity in the immunoprecipitate. The activity of Cdk4 in islets from DT mice was increased when compared with that of ST mice (Fig. 5C). The activation of Cdk4 observed in islets from DT mice was inhibited by rapamycin treatment (Fig. 5C; 1.4 ± 0.1 versus 0.8 ± 0.07, p < 0.005). Rapamycin also inhibited Cdk4 activity in islets from ST mice (Fig. 5C). No differences in the amount of immunoprecipitated Cdk4 among the different experimental conditions were observed (Fig. 5C, lower panel). The increased Cdk4 activity observed in islets from mice expressing a constitutively active form of Akt under the control of the insulin promoter (caAkt) was also inhibited by rapamycin (Fig. 5A). Similar inhibition of Cdk4 activity was obtained when MIN6 cells stably transfected with a caAkt mutant or GFP control were exposed to rapamycin treatment (Fig. 5B). Assessment of the cyclin D-Cdk4 complex components involved in regulation of Cdk4 activity showed that cyclin D2 and D3 but not D1 protein levels were increased in DT mice (3.1 ± 0.7 and 1.5 ± 0.1, respectively, p < 0.05) (Fig. 5D). Rapamycin treatment inhibited the induction of cyclin D2 and D3 in DT islets (Fig. 5D; p < 0.05). In contrast, cyclin D1 levels were not affected by rapamycin treatment of ST or DT islets (Fig. 5D). The levels of Cdk4 were no different among all the conditions (Fig. 5D). P27 levels in islets from DT mice were decreased when compared with those of ST mice (0.6 ± 0.1, p < 0.05). Rapamycin treatment had not effect on p27 levels in islets from both ST and DT mice (Fig. 5D). To assess cyclin D2 levels \textit{in vivo}, we performed immunofluorescence staining in ST and DT mice treated and not treated with rapamycin (Fig. 5E). DT mice exhibited a significant increase in nuclear cyclin D2 staining when compared with ST mice (Fig. 5E). Staining for cyclin D2 in the pancreas from rapamycin-treated DT mice was absent, suggesting that mTORC1 regulates cyclin D2 protein levels.

\textit{Assessment of mRNA Levels of Cyclin D-Cdk4 Complex Components}—Rapamycin treatment induced down-regulation of cyclin D2 and D3 in islets from DT mice (Fig. 5D). To determine whether the decrease in protein levels resulted from alterations in transcription, we performed real-time PCR in islets from ST and DT mice treated with vehicle or rapamycin. Cyclin
D1 and D2 mRNA levels were reduced in islets from DT mice (Fig. 6). Rapamycin treatment of ST and DT islets resulted in induction of cyclin D1 and D2 levels when compared with those obtained from ST or DT islets. In contrast to cyclin D1 and D2, no changes among the different groups were observed for cyclin D3 (Fig. 6). Cdk4 mRNA levels were increased in DT mice compared with ST mice. Rapamycin treatment inhibited Cdk4 mRNA levels in DT mice (Fig. 6). p21 mRNA levels were no different between ST, ST+Rap, and DT islets. Islets from DT+Rap exhibited lower p21 mRNA levels when compared with those of DT islets (Fig. 6). Compared with islets from ST mice, p27 mRNA levels were decreased in islets from DT mice, and rapamycin had no effect (Fig. 6). These observations suggest that the increases in cyclin D2 and cyclin D3 levels observed in islets from DT mice occurred primarily by translational regulation and/or by an effect on protein stability.

Rapamycin Inhibits Cyclin D2 Synthesis—The reduction in cyclin D2 protein could be caused by a decrease in protein synthesis, a decrease in protein stability, or both. We therefore designed pulse-chase experiments to determine the rate of incorporation of radiolabeled methionine into cyclin D2 in the presence or absence of rapamycin treatment for 30 min (Fig. 7A). The pulse-labeling was followed by wash out (chase) for 1 h and subsequent immunoprecipitation with cyclin D2 antibodies. The rate of synthesis of cyclin D2 was elevated in islets from DT mice when compared with ST mice (Fig. 7A). Rapamycin treatment of DT islets inhibited the synthesis of cyclin D2 (Fig. 7A). The levels of cyclin D2 after 1 h of the chase were higher in
DT mice when compared with ST mice (Fig. 7A). Pulse-label experiments in MIN6 cells were performed to corroborate the islet data (Fig. 7, B and C). Rapamycin treatment inhibited cyclin D2 synthesis in control (MIN6-GFP) and in MIN6 cells expressing a constitutively active Akt (MIN6-caAkt) ($p < 0.05$). To confirm that activation of Akt signaling in DT islets regulates the stability of cyclin D2, we pulse-labeled islets from ST and DT mice for 1 h and performed a 30-min chase in the presence of cyclohexamide (Fig. 7D). Cyclin D2 levels in DT mice were higher after 30 min of culture with cyclohexamide, indicating that Akt regulates cyclin D2 stability (Fig. 7D).

**Rapamycin Affects the Stability of Cyclin D2 and D3 Protein**—The previous experiments indicated that activation of Akt signaling increases cyclin D2 stability. To assess whether the mTORC1 mediated the augmented cyclin D2 stability induced by Akt, the stability of this cyclin in islets from DT mice treated

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with control vehicle or rapamycin was determined (Fig. 7E). Compared with vehicle control, the levels of cyclin D2 were lower after 6 h of treatment with rapamycin (Fig. 7E). The decreased in cyclin D2 levels induced by rapamycin could be explained by reduction in protein synthesis or by decreased stability. To determine the effect of rapamycin treatment on cyclin D2 stability, we determined the half-life of cyclin D2 by treating islets from DT mice with cycloheximide for different times in the presence or absence of rapamycin (Fig. 7F). Similar to the results shown on Fig. 7C, cyclin D2 levels were not altered during the first 4 h of rapamycin treatment (Fig. 7F). In contrast, cyclin D2 was not detected after 4 h of treatment with cycloheximide (Fig. 7F). Treatment with cycloheximide and rapamycin further decreased the cyclin D2 levels. Decrease in steady state levels of cyclin D3 were observed only after 6 h of rapamycin treatment (supplemental Fig. S3). In contrast to cyclin D2, cyclin D3 expression was not affected by cycloheximide treatment (supplemental Fig. S3). Treatment with cycloheximide and rapamycin further decreased the half-life of cyclin D3 protein. These results indicate that rapamycin affected cyclin D2 and D3 stability.

DISCUSSION

The current studies provide new insights into the molecular mechanisms that govern cell cycle and β-cell proliferation, a critical component for maintenance of β-cell mass. Here, we present evidence showing that mTORC1 signaling is a major modulator of β-cell expansion and cell cycle progression by regulating cyclin D2 and D3 levels and Cdk4 activity. These studies show for the first time that the mTORC1 modulates cyclin D2 synthesis and stability and suggest that the mTORC1 is an important component in post-transcriptional regulation of cell cycle components in β-cells. These observations are clinically relevant because they suggest that rapamycin has deleterious effects on β-cells. Rapamycin treatment may attenuate the adaptive responses of β-cells in patients with insulin resistance and in other states that require the expansion of β-cells. This work can positively affect treatment of human diabetes, because it uncovers potential targets to develop new pharmacologic agents designed to augment proliferation of β-cells in vivo and in vitro. In addition, the fundamental knowledge obtained on the role of the mTORC1 pathway in β-cell cycle progression provides a better understanding of the effects of immunosuppressant medications used in islet transplantation protocols and suggests that the use of rapamycin could negatively impact the success of islet transplantation.

The serine-threonine kinase Akt has been demonstrated to be an important mediator of growth signals in β-cells. Induction of Akt by dox administration resulted in progressive improvement in glucose tolerance and hyperinsulinemia as a result of increased β-cell mass and proliferation. The inhibition of mTORC1 signaling by rapamycin partially reverted the improved glucose tolerance induced by activation of Akt in β-cells. Interestingly, the effect of rapamycin in glucose tolerance was also observed in ST mice, suggesting that rapamycin alters insulin secretion and/or insulin sensitivity. We cannot discard the possibility of decreased glucose-stimulated insulin secretion by rapamycin in our model, but insulin levels in the fed state were comparable among the groups (data not shown). Insulin tolerance test demonstrated that rapamycin induced insulin resistance in ST mice (supplemental Fig. S4). The alterations of insulin sensitivity in DT mice were less apparent, because DT mice showed some degree of insulin resistance, perhaps as a response of chronic hyperinsulinemia (supplemental Fig. S4). Therefore, we can conclude that the reversal of the metabolic phenotype in rapamycin-treated DT mice could be explained in part by a combination of development of insulin resistance, inhibition of β-cell expansion, and possibly a component of altered insulin secretion. However, the expected adaptive response to insulin resistance would be an expansion of β-cell mass. In contrast, we observed a reduction in β-cell mass in rapamycin-treated DT mice for 15 days as a result of decreased proliferation, suggesting that the effect of rapamycin on β-cells is a major component of the phenotype. Rapamycin treatment was not associated to increased in apoptotic rate. Interestingly, DT mice exhibited augmented apoptosis most likely associated to increased proliferation and enhanced turnover. The changes in β-cell mass after of rapamycin could be explained by reduced cell size, because 15 days of treatment is a short time to observe an effect from inhibition of proliferation. It is also possible that the impaired glucose phenotype observed in rapamycin-treated ST and DT mice could be caused by alterations in β-cell function because rapamycin treatment has been shown to reduce mitochondrial potential and affect insulin secretion (32, 33).

Akt signaling regulates several downstream targets that regulate multiple biological processes. In these experiments, we used rapamycin to assess the role of mTORC1 in Akt signaling. Rapamycin has been used extensively in the literature as specific inhibitor of the mTORC1 complex. However, recent evidence suggests that rapamycin can also disturb the mTORC2 complex (31). Therefore, it was plausible that the rapamycin effect resulted solely from inhibition of Akt signaling. However, rapamycin treatment of islets from DT mice had no effect on phosphorylation of Akt in Ser473 and Thr308 and Akt activity. These observations suggest that in our in vitro experimental conditions, mTORC2 activity in β-cells was not altered by rapamycin treatment and that alterations in mTORC2 activity were not responsible for the rapamycin effects. These results are consistent with the concept that the mTORC1 is the major regulator of β-cell mass and proliferation in this system. The importance of the mTORC1 in β-cell proliferation and mass has also been demonstrated recently in mice conditionally deficient for Tsc2 in β-cells (βTsc2). In these mice, activation of mTOR signaling by deletion of Tsc2 in β-cells resulted in increased β-cell mass and proliferation, and these changes were completely suppressed by rapamycin treatment (22). Experiments using rapamycin in pancreatectomy models and pregnancy provide further evidence for a role of the mTORC1 in adaptive responses of β-cells to stress conditions. The results of the current experiments provide new insights into the importance of mTORC1 in proliferative conditions and unravel the mechanisms whereby inhibition of the mTORC1 regulates the cell cycle and mass in β-cells. The novel findings on the regulation of cyclin D2 synthesis and stability by the mTORC1 also provides critical information for the use of rapamycin in malig-
nancies and tissues in which cell cycle progression is driven mainly by increases in cyclin D2 and D3.

Previous studies suggest that modulation of the cyclin D-Cdk4 complex activity is a major step in controlling the cell cycle progression, proliferation, and maintenance of β-cell mass (27, 34–37). Genetic experiments indicated that cyclin D2 and to a lesser extent cyclin D1 are critical for maintenance of β-cell mass and proliferation post-natally (36, 38). In the current experiments, we showed mTORC1 activity regulates Cdk4 activity by modulation of cyclin D2 and D3 levels. The pulse-chase experiments showed that the activity of the mTORC1 plays an important role in regulation of cyclin D2 synthesis. The mechanisms involved in regulation of cyclin D2 synthesis by the mTORC1 are unclear, but it is possible that this complex favors the translation of cyclin D2 by regulating elements in the 5′- or 3′-untranslated region of the mRNA. In addition to synthesis, our observations showed for the first time that the mTORC1 also regulates the stability of cyclin D2 and D3 (Fig. 7F and supplemental Fig. S3). The mechanisms involved in this process are not entirely clear, but rapamycin treatment of islets from DT mice inhibited GSK3β phosphorylation. The inhibition in GSK3 phosphorylation could explain in part the reduction in cyclin D2 stability because activation of this kinase phosphorylates D-type cyclins and induces ubiquitin-proteosome-dependent degradation (39–43). The inhibition of GSK3β phosphorylation was surprising because it suggests that a downstream target of mTOR could phosphorylate GSK3β at the same residue as Akt. Interestingly, evidence in TSC2-deficient fibroblast demonstrated that S6K1 phosphorylates GSK3β on the site normally phosphorylated by Akt, and this was inhibited by rapamycin (44). Because the inhibition of GSK3β by rapamycin was observed in the context of constitutively activation of Akt, it is reasonable to conclude that S6K phosphorylation of GSK3 could be a more important mechanism. Future experiments with proteosome inhibitors will provide useful information to determine the mechanisms involved in cyclin D2 stability by Akt/mTORC1 signaling. In summary the current experiments suggest that the mTORC1 activity is a major regulator of the stability of cyclin D2 and D3 by regulation of GSK3β.

These studies uncovered key novel pathways controlling cell cycle progression in β-cells in vivo. This information can be used to develop alternative approaches to expand β-cell mass in vivo and in vitro without the risk of oncogenic transformation. The acquisition of such knowledge is critical for the design of improved therapeutic strategies for the treatment and cure of diabetes as well as to understand the effects of mTOR inhibitors in β-cell function. A better understanding of the effects of rapamycin in β-cells mass and function will be important to the advancement and improvement of the success of islet transplantation.

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