mTORC1 and mTORC2 regulate insulin secretion through Akt in INS-1 cells

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Abstract
Regulated associated protein of mTOR (Raptor) and rapamycin-insensitive companion of mTOR (rictor) are two proteins that delineate two different mTOR complexes, mTORC1 and mTORC2 respectively. Recent studies demonstrated the role of rictor in the development and function of β-cells. mTORC1 has long been known to impact β-cell function and development. However, most of the studies evaluating its role used either drug treatment (i.e. rapamycin) or modification of expression of proteins known to modulate its activity, and the direct role of raptor in insulin secretion is unclear. In this study, using siRNA, we investigated the role of raptor and rictor in insulin secretion and production in INS-1 cells and the possible cross talk between their respective complexes, mTORC1 and mTORC2. Reduced expression of raptor is associated with increased glucose-stimulated insulin secretion and intracellular insulin content. Downregulation of rictor expression leads to impaired insulin secretion without affecting insulin content and is able to correct the increased insulin secretion mediated by raptor siRNA. Using dominant-negative or constitutively active forms of Akt, we demonstrate that the effect of both raptor and rictor is mediated through alteration of Akt signaling. Our finding shed new light on the mechanism of control of insulin secretion and production by the mTOR, and they provide evidence for antagonistic effect of raptor and rictor on insulin secretion in response to glucose by modulating the activity of Akt, whereas only raptor is able to control insulin biosynthesis.

Key Words
- Raptor
- rictor
- mTORC1
- mTORC2
- insulin secretion

Introduction
The mammalian target of rapamycin (mTOR) is a multiprotein complex that integrates a wide range of stimuli including nutrients and growth factors to control cellular processes such as translation initiation and proliferation (Hay & Sonenberg 2004, Wullschleger et al. 2006). mTORC1 and mTORC2 are two distinct complexes sharing mTOR as a catalytic subunit. mTORC1 is composed of mTOR, regulated associated protein of mTOR (raptor), proline-rich Akt substrate of 40 kDa (PRAS40), and G-protein β subunit like protein (GβL)
and is sensitive to rapamycin (Hay & Sonenberg 2004, Wullschleger et al. 2006). Its activation leads to the phosphorylation of p70 S6 protein kinase-1 (S6K1) and eIF4E-binding proteins (4E-BPs), which allows cap-dependent translation initiation and protein synthesis (Wullschleger et al. 2006, Guertin & Sabatini 2007). mTORC1 also initiates a negative feedback on Akt1 activity through S6K1-mediated degradation of IRS1 and IRS2 (Wullschleger et al. 2006, Guertin & Sabatini 2007). mTORC2 contains mTOR, rapamycin-insensitive companion of mTOR (rictor), PRR5/proline-rich protein S (protor), mammalian stress-activated kinase interacting protein 1 (mSIN1), and GβL. This complex is insensitive to short-term rapamycin treatment and controls in particular cytoskeleton organization (Sarbassov et al. 2004) and Akt1 activity (Sarbassov et al. 2005, Jacinto et al. 2006).

Recently, studies demonstrated the role of rictor and mTORC2 in β-cell function and development. The long-term toxic effects of rapamycin in β-cells are closely related to the dissociation and inactivation of mTORC2 and the inhibition of Akt1 activity (Barlow et al. 2012). Furthermore, in the mouse, specific deletion of rictor in β-cells leads to hyperglycemia and glucose intolerance due to reduced β-cell mass and proliferation (Gu et al. 2011).

Several studies addressed the role of mTORC1 in pancreas development and β-cell function either by modulating the expression of proteins controlling its activity, such as TSC2 (Rachdi et al. 2008, Shigeyama et al. 2008), RHEB (Hamada et al. 2009), and AKT1 (Balcazar et al. 2009), or through rapamycin treatment (Bell et al. 2003, Zhang et al. 2006, Niclauss et al. 2011). Altogether, these studies depict a picture where mTORC1 inhibition leads to β-cell and pancreatic islet dysfunction and impaired survival (Bell et al. 2003, Zhang et al. 2006, Niclauss et al. 2011), whereas overactivation increases β-cell mass and function in mouse models (Rachdi et al. 2008, Shigeyama et al. 2008, Balcazar et al. 2009, Hamada et al. 2009). However, some studies described deleterious effects of mTORC1 overactivation. In the mouse, it has been demonstrated that specific deletion of TSC2 in β-cells induced β-cell apoptosis and reduced insulin secretion, after a first phase of β-cell mass expansion (Shigeyama et al. 2008). In the rat, β-leucine, an amino acid known to induce mTORC1 activation, negatively alters pancreatic β-cell differentiation and function (Rachdi et al. 2012).

In this study, we investigated the molecular effect of mTORC1 in the control of insulin secretion and synthesis as well as the possible interplay between mTORC1 and mTORC2 in INS-1 β-cell line.

Materials and methods

Materials and chemicals

All cell culture solutions and supplements were purchased from Invitrogen. All chemicals were purchased from Sigma–Aldrich. FBS was purchased from PAA Laboratories (Les Mureaux, France). Reagents for SDS–PAGE were purchased from Bio-Rad. The enhanced chemiluminescence kit was obtained from PerkinElmer (Courtaboeuf, France). Anti-mouse and anti-rabbit IgGs conjugated to HRP were purchased from Amersham Biosciences.

Cell culture and transfection of siRNA and plasmids

INS-1 cells (passage 35–40) were maintained in RPMI 1640 containing 11 mmol/l glucose, 1 mmol/l sodium pyruvate, 2 mmol/l glutamine, 50 mg β-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FCS, incubated at 37°C in 5% CO₂, and subcultured at 80% confluence. siRNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were transfected for 72 h with On-Target plus SMARTpool siRNA (Dharmacon, Epsom, UK) against raptor (L-086862-00-005) and rictor (L-087724-00-005). siGENOME nontargeting siRNA (D-001206-14-05) was used as a control. Dominant-negative Akt (K179A-Akt) and constitutively active (myr-Akt) constructs (Cong et al. 1997) were kindly provided by M Quon (University of Maryland, Baltimore, MD, USA). Constitutively active S473D-Akt construct (Rodrik-Outmezguine et al. 2011) was provided by N Rosen (Memorial Sloan-Kettering Cancer Center, New York, NY, USA).

Assay of insulin secretion and content

For acute insulin release, 72 h after transfection, INS-1 cells were washed and pre-incubated 2 h in Krebs–Ringer Bicarbonate buffer (KRB) containing 2 mmol/l glucose. The KRB was then replaced by KRB containing 2 mmol/l glucose for 45 min (basal), followed by an additional 45-min (stimulated) incubation in KRB containing 20 mmol/l glucose, and supernatants were collected to measure glucose-stimulated insulin secretion. Cells were then lysed in PBS 1×-0.5% Triton X-100 and sonicated to quantify intracellular insulin content. Insulin concentrations in the supernatant and intracellular fractions were assayed using an anti-rat insulin ELISA kit (Mercodia, Paris, France) according to the manufacturer’s instructions.
Immunoblot analysis
INS-1 cells were harvested in lysis buffer containing 40 mmol/l HEPES (pH 7.5), 120 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l pyrophosphate, 10 mmol/l glycerophosphate, 50 mmol/l NaF, 1.5 mmol/l sodium orthovanadate, 10 μmol/l okadaic acid, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After lysis, insoluble material was removed by centrifugation at 12 000 g for 10 min at 4 °C. Protein content was determined by the Bradford protein assay (Bio-Rad). Proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% nonfat dry milk in PBS containing 0.1% (v/v) Tween 20 and incubated overnight with primary antibody. Primary antibodies were detected with peroxidase-coupled secondary antibody and enhanced chemiluminescence (Amersham Biosciences). Antibodies against raptor, rictor, Akt1, thr308, and ser473-phosphorylated Akt1, S6K1, thr389-phosphorylated S6K1, ser21/9-phosphorylated GSK3α/β, and tubulin were purchased from Cell Signaling Technology (Danvers, MA, USA).

Real-time RT-PCR analysis
Total RNA was extracted from INS-1 cells with an RNeasy kit (Qiagen). cDNA was synthesized from total RNA using a high-capacity cDNA RT kit (Applied Biosystems). Real-time quantitative PCR analysis was evaluated in a sequence detector ABI7900 with specific primers and master mix (Applied Biosystems). The relative abundance of mRNAs was calculated with Rplp0 as housekeeping gene.

Cell death detection assay
Following cell culture and treatment, cell death was evaluated using the Cell Death Detection Elisa PLUS kit (Roche) according to the manufacturer’s instructions.

Statistical analysis
Data are presented as mean ± S.E.M. For gene expression results, we assessed the significances of differences between independent means by a two-tailed paired Student’s t-test. Glucose-stimulated insulin secretion (GSIS), insulin content, stimulatory index, and apoptosis results were analyzed with ANOVA and the Fisher’s least significant difference test using Statview 4.1 Software (Abacus, Berkeley, CA, USA). A P value < 0.05 was considered statistically significant.

Results
Raptor controls insulin secretion in INS-1 cells
We first investigated the effects of raptor on pancreatic β-cell viability and function in INS-1 cells using siRNA. Seventy-two hours after transfection, the raptor mRNA level was knocked down by 50% without affecting rictor or mTOR expression (Fig. 1A) and INS-1 viability (Fig. 1B). Reduced
raptor expression led to a reduced thr389 phosphorylation of S6K1 (a known target of mTORC1) in response to serum stimulation (Fig. 1C). To investigate the effects of raptor on insulin secretion, INS-1 cells were incubated in the presence of 2 mmol/l (basal) or 20 mmol/l glucose (stimulated). As shown in Fig. 1D, insulin secretion at 20 mmol/l glucose was increased in the raptor siRNA-treated cells (Fig. 1D, $P < 0.05$).

As a consequence, the stimulation index, calculated as the ratio of stimulated to basal insulin secretion, was increased from 5.8 ± 1.1 to 7.8 ± 1.3 ($P < 0.05$). Insulin secretion is influenced by many genes involved in insulin exocytosis, glucose sensing, and membrane transport. Quantitative PCR analysis was used to detect expression of these transcripts in scrambled or raptor siRNA-treated cells. As shown in Fig. 1E, knocking down raptor had no effect on the level of exocytosis genes. The expression of Glut2 (Slc2a2) and glucokinase (Gck) (two important genes in glucose metabolism, Im et al. (2006)) and the granule zinc transporter (ZNT8, Chimienti et al. (2006)) were unchanged in the raptor siRNA-treated cells (Fig. 1F). However, we observed a slight increase in ABCC8 expression ($P = 0.08$) and KCNJ11 expression ($P < 0.05$), two proteins that are part of the pancreatic β-cell $K_{ATP}$ channel (Gloyn et al. 2006).

**Raptor controls insulin production**

To assess a potential role of raptor in insulin production, we next measured the intracellular content in scrambled and raptor siRNA-treated INS-1 cells. Raptor siRNA induced a ≈60% increase in insulin content (Fig. 2A), which was associated with increased Ins1 and Ins2 mRNA expression (Fig. 2B). The homeodomain transcription factor PDX1 regulates the expression of several β-cell specific genes, including insulin and MafA (Petersen et al. 1994, Raum et al. 2006). Furthermore, MafA plays an important role in the regulation of insulin expression (Kataoka et al. 2002), and it was recently shown that PDX1 and FOXO1 mediate MafA transcription (Kitamura et al. 2005). Foxo1, Pdx1, and MafA mRNA expressions were increased by 40% ($P < 0.05$), 30% ($P < 0.05$), and 45% ($P < 0.01$) respectively in raptor siRNA-treated INS-1 cells.

**mTORC2 is involved in the control of insulin secretion by raptor**

It has been demonstrated that mTORC1 complex controls S6K1 activation (Kim et al. 2002, Hay & Sonenberg 2004), which then drives a negative feedback on insulin signaling leading to Akt inactivation (Hay & Sonenberg 2004, Wullschleger et al. 2006). Rictor, delineating the mTORC2 complex, is also involved in the control of Akt activity (Sarbassov et al. 2005, Jacinto et al. 2006), and interplay between mTORC1 and mTORC2 complexes has been documented (Sarbassov et al. 2006, Julien et al. 2010). Therefore, we next investigated the effect of rictor on insulin secretion in INS-1 cells. Knocking down rictor expression by siRNA decreased Akt activity as demonstrated by a reduced phosphorylation of ser473-Akt (Fig. 3A). We also observed a 30% decrease in stimulated insulin release (Fig. 3B), leading to a 30% reduction of the stimulatory index (5.2 ± 0.7–3.6 ± 0.5; Fig. 3C; $P < 0.05$). To investigate the interplay between mTORC1 and mTORC2, we reduced the expression of rictor in raptor siRNA-treated cells. In these conditions, rictor siRNA overrides the effect of rictor knockdown on insulin secretion (Fig. 3B and C). However, intracellular insulin content was not affected by rictor siRNA treatments neither in control nor in raptor siRNA-treated conditions (Fig. 3D). Western blot analysis reveals that rictor siRNA induces an increased Akt activity, as exemplified by its increased phosphorylation on ser473 (Fig. 3A). Rictor siRNA treatment overrides the effect of rictor siRNA on ser473-Akt phosphorylation (Fig. 3A).

**Figure 2**

Inhibition of raptor induces insulin production in INS-1 cells. (A) Insulin intracellular content in scrambled and raptor siRNA-treated INS-1 cells. Cells were transfected with scrambled or raptor siRNA and intracellular insulin content measured 72 h after transfection using ELISA kit. (B) Real-time PCR analysis of Ins1 and Ins2 mRNA expression. (C) Real-time PCR analysis of transcription factors involved in the regulation of insulin expression. Values are mean ± S.E.M. *$P < 0.05$, **$P < 0.01$ vs control. All results are representative of four independent experiments.
Raptor and rictor control insulin secretion by modulating Akt activity

As inhibition of rictor expression was able to correct the effect of raptor siRNA on insulin secretion, we then asked whether the effects of raptor and rictor on insulin secretion were mediated through Akt. To test this hypothesis, we first overexpressed a dominant-negative form of Akt (K179A-Akt) and several constitutively active forms of Akt (myr-Akt, S473D-Akt) in INS-1 cells. Overexpression of K179A-Akt reduced glucose-induced insulin secretion (Fig. 4). We then overexpressed myr-Akt or S473D-Akt in rictor siRNA-treated cells. As expected, overexpression of myr-Akt restored Akt activity as demonstrated by an increased phosphorylation of S21/9-GSK3α/β, a known target of Akt (Cross et al. 1997; Fig. 5A). Basal insulin secretion in
response to 2 mmol/l glucose was not modified by the inhibition of rictor expression or myr-Akt overexpression. However, overexpression of myr-Akt abolished the effect of rictor knockdown on the stimulated insulin release (Fig. 5B). A similar result was obtained when S473D-Akt was overexpressed in rictor siRNA-treated cells (Fig. 6). As a consequence, we observed an increase in the stimulatory index (Fig. 5C). Intracellular insulin content remained unaffected in these conditions (Fig. 5D).

Subsequent experiments focused on whether the effect of raptor knockdown on insulin secretion was also mediated by Akt. To test this, raptor siRNA-treated cells were co-transfected with a dominant-negative form of Akt (K179A-Akt; Cong et al. 1997), or pre-treated 4 h with wortmannin, a potent PI3K inhibitor, before glucose-induced insulin secretion assay. In agreement with the literature, inhibition of raptor expression led to a reduced

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**Figure 5**
Effect of rictor on insulin secretion is mediated by Akt. (A) Cells were transfected with scrambled or rictor siRNA with or without constitutively active Akt construct (myr-Akt). An immunoblot of scrambled, rictor siRNA-treated cells is shown. S21/9-pGSK3, phosphorylated ser21/9 of GSK3α/β. (B) Effect of rictor siRNA and myr-Akt on glucose-induced insulin secretion. (C) Insulin secretion stimulation index (SI). SI is calculated as the ratio of secreted insulin between stimulated (20 mmol/l glucose) and basal (2 mmol/l glucose) conditions. (D) Intracellular insulin content in rictor siRNA-treated cells with or without myr-Akt overexpression. Values are mean ± S.E.M. *P<0.05 vs control. $P<0.05 vs siRictor. All results are representative of four independent experiments.

**Figure 6**
S473D-Akt restores insulin secretion in rictor siRNA-treated INS-1 cells. (A) Effect of siRictor and S473D-Akt on glucose-induced insulin secretion. (B) Intracellular insulin content in rictor siRNA-treated cells with or without S473D-Akt overexpression. (C) Effect of S473Akt mutant overexpression and rictor siRNA on INS-1 cell apoptosis. Values are mean ± S.E.M. *P<0.05 vs control. $P<0.05 vs siRictor. All results are representative of four independent experiments.
thr389-S6K1 phosphorylation and increased phosphorylation of ser473-Akt (Figs 1C and 3A; Hay & Sonenberg 2004, Wullschleger et al. 2006). Raptor siRNA also induced increased S21/9-GSK3a/b phosphorylation (Fig. 7A). Both K179A-Akt overexpression and wortmannin treatment reduced Akt activity as exemplified by the reduced phosphorylation of GSK3a/b (Fig. 7A). These treatments inhibited the raptor siRNA-mediated increase in insulin secretion in response to glucose and stimulatory index (Fig. 7B and C) but had no effect on the intracellular insulin content (Fig. 7D).

Discussion
In this study, we investigated the role of the mTORC1 component raptor in insulin secretion and biosynthesis in INS-1 cells. We demonstrated that inhibition of raptor expression induces an increase in glucose-stimulated insulin secretion and insulin content that correlates with altered Pdx1 and MafA expression. We also provide evidence that mTORC2 and mTORC1 share a common mechanism to regulate insulin secretion through modulation of Akt activity.

Herein, we observed that raptor/mTORC1 is important for β-cell function as shown by the ~50% increase in glucose-stimulated insulin secretion after raptor knockdown. The increased insulin secretion does not seem to be associated with deregulation of genes involved in insulin exocytosis or glucose sensing. We demonstrated that Akt mediates the effect of raptor as the overexpression of a dominant-negative form of Akt or inhibition of PI3K activity by wortmannin completely abrogates its effect on insulin secretion. The diminished expression of raptor also results in an increased insulin content possibly mediated by the increased mRNA expression of Pdx1 and MafA, two transcription factors that control insulin production (Petersen et al. 1994, Kataoka et al. 2002, Raum et al. 2006). Interestingly, PDX1, an important determinant of β-cell function, is known to be regulated by Akt signaling (Wu et al. 1999, Elrick & Docherty 2001, Johnson et al. 2006). However, the reduction of rictor expression by siRNA (which leads to downregulation of Akt activity) or overexpression of DN-Akt do not correct the increased insulin content observed in raptor siRNA-treated cells. Furthermore, overexpression of constitutively active forms of Akt (myr-Akt or S473D-Akt) in rictor siRNA-treated cells did not alter insulin content. This suggests that insulin production is controlled by the mTORC1 complex rather than by Akt.

Several studies address the role of mTORC1 in islet function and survival. mTORC1 hyperactivation in vivo is associated with increased β-cell mass and function in vivo (Rachdi et al. 2008, Balcazar et al. 2009, Mori et al. 2009). However, in these studies, mTORC1 hyperactivation was driven by the expression of constitutively active form of AKT (Balcazar et al. 2009), RHEB (Hamada et al. 2009), or deletion of TSC2 (Rachdi et al. 2008, Mori et al. 2009). None of these studies investigated the direct role of raptor, except one where raptor knockdown did not alter insulin secretion in dispersed islets (Barlow et al. 2012). This latter result is in discrepancy with our data and might result from the biological material used for the experiment: INS-1 cell line vs islet. Pancreatic islets are a mix of different cell types, insulin-secreting β-cells, glucagon-secreting α-cells, and somatostatin-secreting δ-cells, and the secretion of these hormones is tightly controlled through paracrine processes (Bruni et al. 2001).
Furthermore, these cells are not proliferating. Whether the effect of raptor siRNA is dependent on the proliferative state or whether raptor controls insulin secretion through additive effects in α- and δ-cells will require further studies. mTORC1 overactivation might also be deleterious for β-cell development and function (Shigeyama et al. 2008, Rachdi et al. 2012). Rapamycin treatment has been shown to have deleterious effects on the function and survival of the murine β-cell line (Bell et al. 2003) and isolated islets from mouse, rat, or humans (Bell et al. 2003, Hui et al. 2005, Zhang et al. 2006, Johnson et al. 2009, Barlow et al. 2012). The results we obtained with a moderate reduction of mTORC1 activity by raptor siRNA are in discrepancy with these data. Loss of insulin secretion and biosynthesis is often associated with increased cell death; this is particularly true for islet transplantation where a significant fraction of the functional mass of islets is lost (Ryan et al. 2005). A short treatment with caspase inhibitor reduces apoptosis and promotes long-term islet graft function (Emamaullee et al. 2007, 2008). This suggests that the rate of apoptosis and outcome of islet functions are mechanistically linked. In agreement, transduction of islets with the antiapoptotic gene XIAP prevents the negative effects of sirolimus (a rapamycin analog) on synthesis and secretion of insulin (Hui et al. 2005). Recently, it was demonstrated that loss of MIN6 cell function and viability induced by rapamycin was associated with the dissociation of the mTORC2 complex, leading to the complete inhibition of Akt (Barlow et al. 2012), a key modulator of cell viability. Altogether, these results suggest the deleterious effects of rapamycin on islet and β-cell function are mainly mediated through impaired cell viability. Our results, where cell viability is unaffected after a partial mTORC1 inhibition by raptor siRNA, are in line with this hypothesis. Furthermore, our results suggest that Akt activity is able to control glucose-induced insulin secretion independently of its role in cell viability.

In conclusion, by inhibiting the expression of raptor and rictor in INS-1 cells, we demonstrate that both mTORC1 and mTORC2 are involved in β-cell function. Raptor and rictor have antagonistic effects on insulin secretion in response to glucose by modulating the activity of Akt, whereas only raptor is able to control insulin biosynthesis.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
O Le Bacquier, G Queniart, and V Gmyr planned and carried out experiments, analyzed data, and wrote the paper; J Kerr-Conte and B Lefebvre helped with experiments and analyzed data; B Lefebvre and F Pattou planned experiments and supervised all the work on this paper. All authors had final approval of the submitted and published versions.

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