ARTÍCULO

TSC1-TSC2 complex on the crossroad of pancreatic β cell signaling. Role on cell proliferation, death and survival

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ABSTRACT

TSC1-TSC2 complex has emerged as a signal interaction core within the cell. This complex integrates both nutrient and growth factor signaling and is a critical negative regulator of mTORC1. mTORC1 signaling leads to increased protein biosynthesis, which is essential for cell proliferation. Other cellular events such as endoplasmic reticulum stress and authophagy are intimately linked with TSC/mTORC1 pathway and play an important role in pancreatic β cell death or survival. We found that either insulin, glucose independent signaling or the energetic status of the cell are able to modulate TSC2 phosphorylation in pancreatic β cell lines. To show the central role of TSC2 for these cells, we conducted siRNA-mediated TSC2 silencing. Downregulation of TSC2 leads to an increase in mTORC1/p70S6K signaling, this produces resistance to insulin action. However, specific expression of insulin receptor isoform A restored insulin signalling under these conditions. Moreover, we have

explored other processes related to the TSC/mTORC1 pathway and their effect on cell death or survival.

Keywords: TSC2; mTORC1; beta cell; diabetes; insulin.

RESUMEN

Papel del complejo TSC1-TSC2 en la señalización de la célula β pancreática: prolifreración, muerte y supervivencia celular

El complejo TSC1-TSC2 ha emergido como un núcleo de integración de la señalización de factores de crecimiento y del estado energético celular. Este complejo funciona como un regulador negativo de la actividad de mTORC1. La señalización a través de mTORC1 dirige la síntesis proteica, esencial para la proliferación celular. Otros procesos como el estrés de retículo y la autofagia están íntimamente ligados a la ruta TSC/mTORC1 y juegan un importante papel en la muerte o supervivencia de las células β pancreáticas. Encontramos que tanto la insulina, como la acción independiente de la glucosa o el estatus energético celular modulan la fosforilación de TSC2 en líneas celulares β pancreáticas. Para demostrar el papel central de TSC2 en la regulación de estas células, hemos silenciado su expresión mediante transfección con ARNi. Una menor expresión de TSC2 lleva a un aumento de la señalización de mTORC1/p70S6K, lo cual produjo un estado de resistencia a la acción de la insulina. Sin embargo, la expresión selectiva de la isoforma A del receptor de insulina consigue salvar esta resistencia. Por otro lado, hemos explorado otros procesos modulados por la ruta como la autofagia o el estrés de retículo, y su efecto en la supervivencia o muerte celular.

Palabras Clave: TSC2; mTORC1; célula beta; diabetes; insulina.

1.   INTRODUCTION

β cells are a highly specialized cell type for the production and secretion of insulin, which is required for maintaining metabolic homeostasis. Through a tight regulation of insulin levels, β cells are the en-

ergetic sensor for the metabolic control of the whole organism. Insulin demand is increased under different physiological and pathological conditions, such as pregnancy, obesity or aging (1, 2). These various conditions enforce the β cell mass to adapt to higher insulin synthesis and secretion requirements. Thus, β cell plasticity can be accomplished by an increase in β cell mass, which is the net result of both cell hyperplasia and cell hypertrophy (3). The failure of these compensatory mechanisms results in an insulin deficiency that leads to hyperglycemia and clinical manifestations of type 2 diabetes (3).

Autocrine insulin signaling on β cells has shown to be essential in these compensatory mechanisms (4). On this regard, downstream effectors of insulin signaling such as Akt and the 70-kDa ribosomal protein S6 kinase (p70S6K) have proven to be positive key regulators of β cell mass (5, 6).

Glucose is the main effector of insulin secretion in β cells, but it also plays a role in β cell proliferation. Mice haploinsufficient for glucokinase are unable to increase β cell mass in response to high-fat diet (7). Furthermore, glucose has been found to stimulate ERK 1/2 activation (8, 9). This activation is independent of insulin signaling as seen in IR -/- β cells (10). Glucose directly increases ATP/AMP ratio inhibiting AMP-activated protein kinase (AMPK) activity.

The TSC1-TSC2 complex is located in the crossroad of this signaling network, integrating different signals to determine the cellular fate through regulation of protein synthesis, cell size and proliferation (11). The GTPase-activating protein (GAP) activity of TSC2 towards Ras homolog enriched in brain (Rheb) negatively regulates mTORC1 signaling. Akt and ERK directly phosphorylates TSC2 leading to an increase in mTORC1 signaling (12, 13). In contrast, AMPK activation has been found to phosphorylate TSC2 and stabilize the TSC1-TSC2 complex, leading to a diminished mTORC1 activity under low energetic states (14). TSC/mTORC1/p70S6K signaling pathway is tightly regulated. Although a diminished mTORC1/p70S6K activity can lead to a decrease in β cell mass and diabetes, its hyperactivity induces an insulin resistance state by a negative feedback loop on IRS-1/2 (15). As the key regulator of protein synthesis, mTORC1 hyperactivity can also lead to endoplasmic reticulum associated stress (ER stress) (16). β cell ER has a high capacity of pro-

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tein synthesis and folding, which in turn makes them extremely prone to misfolded protein accumulation (17). Moreover, mTORC1 regulates the autophagic activity of the cell, which has proved to be essential for β cell homeostasis (18).

Just recently, animal models have shown the key role of TSC1-TSC2 complex in pancreatic β cells. Specific disruption of the complex in β cells by either TSC1 (19), TSC2 deletion (20, 21) or Rheb overexpression (22), results in hyperinsulinemia and improved glucose tolerance mainly due to an increase in β cell size.

Alternative splicing of the IR (insulin receptor) results in two isoforms, one encoding the 36 nucleotide exon 11 (IRB) and the other one without it (IRA) (23). The relative expression of the two isoforms varies among different tissues (24), and may play a role in different pathological conditions such as cancer or insulin resistance (25). We have recently observed an increase of IRA in pancreatic islets from inducible-liver IR knock-out (iLIRKO) mice showing compensatory β cell hyperplasia in response to hepatic insulin resistance (26).

Here, we report a differential regulation of TSC2 phosphorylation and mTORC1 signaling by insulin, glucose, or energetic status in pancreatic β cell lines. Also are described its consequences on proliferation, and the involvement of autophagy or ER stress in β cell death or survival.

2. MATERIALS AND METHODS

2.1. Antibodies and reagents

All antibodies were from Cell Signaling Biotechnology unless stated otherwise. Exceptions were IR-β (Santa Cruz), TSC1 (Bethyl Laboratories), P-mTOR Ser[2448] (Biosource), β-actin (Sigma), anti-mono and poly-ubiquitinated protein conjugates FK2 mAb (Enzo Life Sciences). Antibody against P-TSC2 Ser[664] used for Western-blot was a generous gift from P.P. Pandolfi (Beth Israel Deaconess Medical Center, Boston, MA). Chemicals were from Calbiochem (U0126, wortmannin and rapamycin) or Sigma-Aldrich.

2.2.   Origin of pancreatic β cell lines and cell culture

IR +/+ and IR -/- pancreatic β cells from *IR loxP* mice were generated as described before (10). From IR -/- cells, expression of a single IR isoform was reconstituted by retroviral infection, and the differential mRNA splicing was assessed by RT-PCR as previously described (27). TSC2 -/- and TSC2 +/+ MEFs were provided by D. J. Kwiatkowsky (Dana-Farber Cancer Institute, Boston, MA).

2.3.   Cell signalling, Western-blotting and immunofluorescence assays

For cell signaling experiments, cells were serum and glucose starved for 3 h in DMEM containing 0.2 mM glucose and 0.5% bovine serum albumin (BSA), and subsequently stimulated with insulin 10 nM or glucose 5 mM for 5 or 15 min respectively. Inhibitors were added 30 min prior to stimulus. Cells were washed and lysed for protein extraction, protein concentration was determined and samples were subjected to SDS-PAGE for Western-blot according to standard procedures (10). Immunofluorescence, cell cycle and violet crystal assays assays were carried as decribed previously (10).

2.4.   siRNA trasnfection

siRNA against the expression of mouse TSC2 was designed and chemically synthesized by Bionova Científica SL. For siRNA transfection, cells were electroporated using Nucleofector II and Cell Line Kit T (Amaxa) following the instructions from the manufacturer. A pool of three different siRNA (2 µg) was added to the transfection reagent per $2x10^6$ cells. After transfection, cells were seeded and protein expression was checked after 24-48 h

2.5.   λ-Phosphatase treatment

100 µg of total lysates were mixed in λ-Phosphatase Buffer (50 mM Tris-HCl pH7.5, 0.1 mM Na_2EDTA, 5 mM dithiothreitol, 0.01% BRIJ

35, 2 mM MnCl$_2$) with 400 U of λ-Phosphatase (Sigma), and then the mixture was incubated for 1 h at 30 °C.

2.6.   Statistics

Statistically significant differences between mean values were determined using paired Student *t*-test for paired comparisons. One-way ANOVA and Tukey tests were used for multiple comparisons. Differences were considered statistically significant at *P* < 0.05.

3.   RESULTS

3.1.   Characterization of pancreatic β cell lines

Different β cell lines were generated and used in our study (IR +/+, IR -/-, Rec A and Rec B). From an IR -/- β cell line generated in a previous work (10), Rec A and Rec B cell lines were reconstituted by retrovirus-mediated transfection with a human IRA or IRB encoding plasmids. IR levels were measured by Western-blotting (Figure 1A), and RT-PCR of IR exon 11 mRNA determined the specific expression of both isoforms (Figure 1B).
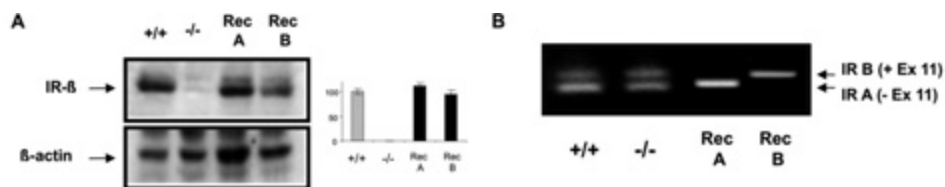

Figure 1.   Specific recostitution of IR isoforms. **A)** IR expression by Western-blot in the different pancreatic β cell lines: IR +/+, IR -/-, and reconstituted cell lines stably expressing IRA (Rec A), or IRB (Rec B). A densitometric analysis of the IR levels is shown, data are presented as means ± S.E.M. (n = 5). **B)** Total RNA was isolated, and IR exon 11 expression was checked by RT-PCR.

3.2. Insulin stimulates TSC/mTORC1 signaling pathway through PI3K/Akt in pancreatic β cells

To assess the signaling pathway specificity of insulin or glucose-mediated TSC2 phosphorylation, we used selective inhibitors of the class I PI3K (wortmannin 40 nM), and MEK 1/2 (U0126 5 µM). Use of wortmannin blocked insulin-stimulated Akt Ser^{473} phosphorylation and subsequently TSC2 Ser^{939} and Thr^{1462} phosphorylation and mTORC1 activity. Inhibition of MEK/ERK pathway did not affect either Akt or TSC2 phosphorylation levels. Surprisingly, although both reconstituted cell lines express similar levels of IR (Figure 1A), wortmannin 40 nM only partially impaired Akt/TSC2 phosphorylation in IRA expressing β cell line. However, this phosphorylation was totally blunted in IR +/+ or IRB expressing β cells (Figure 2). Time-course stimulation also revealed a more sustained phosphorylation of Akt/TSC2 in Rec A cells versus Rec B cells (data not shown). In IR -/- cells, glucose did not stimulate either Akt or TSC2 phosphorylation in these residues. However, glucose stimulated p70S6K Thr^{389} phosphorylation (Figure 2).

3.3. Glucose activates TSC/mTORC1 signaling via ERK 1/2 independently from insulin signaling in pancreatic β cells

To test whether glucose by itself was capable to stimulate TSC2 Ser^{664} phosphorylation in pancreatic β cells, we submitted IR -/- β cells to 5 mM glucose stimulation. We found a glucose-mediated activation of TSC2 Ser^{664} phosphorylation by Western-blot analysis (Figure 3A). This phosphorylation was completely blunted by the addition of U0126. TSC2 Ser^{664} phosphorylation has been described as a marker of hyperactivation of MEK/ERK signaling in tuberous sclerosis, breast and colon human cancer (28).
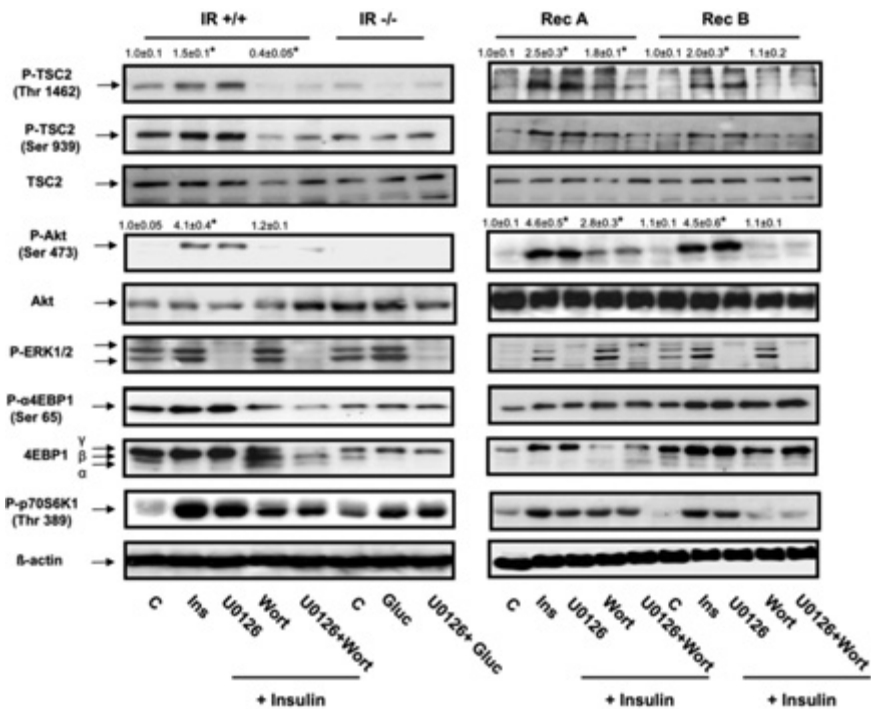
Figure 2. Insulin and glucose action on the PI3K/TSC/mTORC1 pathway. Cells were stimulated with insulin 10 nM for 5 min (IR +/+, Rec A and Rec B), or glucose 5 mM (IR -/-) for 15 min. Specific inhibitors were added 30 min prior to stimulation (wortmannin 40 nM, U0126 5 μM). Protein extracts were collected and submitted to Western-blot with the use of specific antibodies. Blots are representative of three independent experiments. Quantification of the most significative points is shown. Data are presented as means ± S.E.M. $^{*}P < 0.05$ compared to control points.

3.4. Energetic status-dependent modulation of mTORC1

AMPK is the energetic sensor of the cell and it is activated by a rise in AMP/ATP ratio (29). Thus, AMPK-Thr^{172} phosphorylation is decreased by glucose and increased by 2 deoxylucose (2-DG) in a dose-dependent manner as well as by 4 mM AICAR in IR +/+ β cells. Activation of AMPK by 2-DG or AICAR addition to the culture medium inhibited mTOR and p70S6K phosphorylation. Conversely, inac-

tivation of AMPK by glucose resulted in an enhancement of mTOR and p70S6K phosphorylation (Figure 3B). Thus, activation of AMPK can directly regulate mTORC1 inhibition. In a similar manner, AMPK activation caused Raptor-Ser[792] phosphorylation, which has been described as an additional mechanism to downregulate mTORC1 signaling, independently from TSC1-TSC2 complex (30). The role of the TSC complex in energy sensing response was observed, as the phosphorylation of TSC2 by AMPK activation causes a mobility shift of the TSC2 protein in poliacrylamide gel electrophoresis, which is reverted by λ-phosphatase treatment of the samples (Figure 3C).
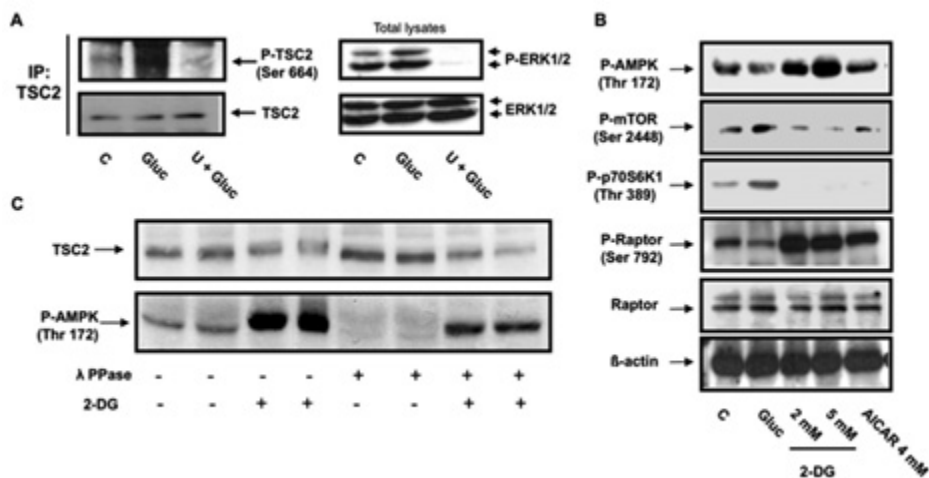

Figure 3.   Glucose and energy dependent modulation of TSC2 and mTORC1. A) IR -/- were stimulated with glucose 5 mM for 15 min. U0126 5 µM was added 30 min prior to the stimulus. Protein extracts were submitted to immunoprecipitation with anti-TSC2 antibody, and blotted with a phosphospecific anti TSC2-Ser[664] antibody. Total lysates were used for Western-blot against P-ERK 1/2. **B)** IR +/+ cells were stimulated with glucose 5 mM, 2-DG (2-5 mM) or AICAR 4 mM. **C)** IR +/+ cells were stimulated or not with 2-DG 5 mM for 15 minutes, protein extracts were collected and part of the samples were treated with λ-phosphatase. Samples were subjected to SDS-PAGE and incubated with specific antibodies. Blots are representative of at least three independent experiments.

3.5.   Effect of TSC2 interference on pancreatic β cell signaling

To assess the importance of TSC complex in the integration of β cell signaling, we targeted the expression of TSC2 with siRNA. A similar rate of TSC2 knockdown was achieved in all the cell lines, close to 70% of protein expression interference (Figure 4B). TSC2 knockdown induced p70S6K basal phosphorylation in all cell lines studied (Figure 4A). TSC2 interference generated insulin resistance, decreasing Akt and p70S6K phosphorylation in response to insulin in IR +/+ (Figure 4, A and C). However, this insulin resistance effect was dependent on the IR isoform expression. Thus, Rec A, but not Rec B cells overcome insulin resistance on Akt Ser[473] and p70S6K phosphorylation (Figure 4, A, C and D). In addition, TSC2 interference generated insulin resistance, decreasing ERK 1/2 phosphorylation in IR +/+, Rec A, or Rec B cell lines (Figure 4A). The insulin resistance induced by TSC inactivation in IR +/+ or Rec B cells was blunted by rapamycin treatment, indicating a requirement for mTORC1 signaling. In contrast, the effect observed on ERK 1/2 was rapamycin-independent in all cell lines studied (Figure 4A). In IR -/- cells, TSC2 interference impaired glucose mediated ERK 1/2 phosphorylation. However, glucose induced p70S6K signaling regardless of the presence of the MEK 1/2 inhibitor U0126 through ERK 1/2 independent pathway (Figure 4A, upper right panel). To further investigate the differences between IRA or IRB expression on insulin resistance, we stimulated cells with insulin 10 nM for 15 minutes, and observed a different modulation of IRS-1 Ser[307] phosphorylation. This phosphorylation is mediated by p70S6K, as described before (15), reverted by rapamycin, and upregulated in Rec B cells compared with Rec A or IR +/+ cells (Figure 5).

3.6.   Role of the TSC2/mTORC1 pathway in the proliferation of pancreatic β cells bearing IRA or IRB isoforms

To assess the role played by mTORC1 in the proliferation rate of β cell lines studied, we submitted those cells to 10% FBS in the presence or absence of rapamycin 40 nM (Figure 6). The rate of proliferation, as estimated by cell counting, increased by 8-fold in IR +/+ or in Rec B and by 4-fold in IR -/- cells (Figure 6, A and B). In addition, Rec A cells showed a much higher rate of proliferation under the same experimen-
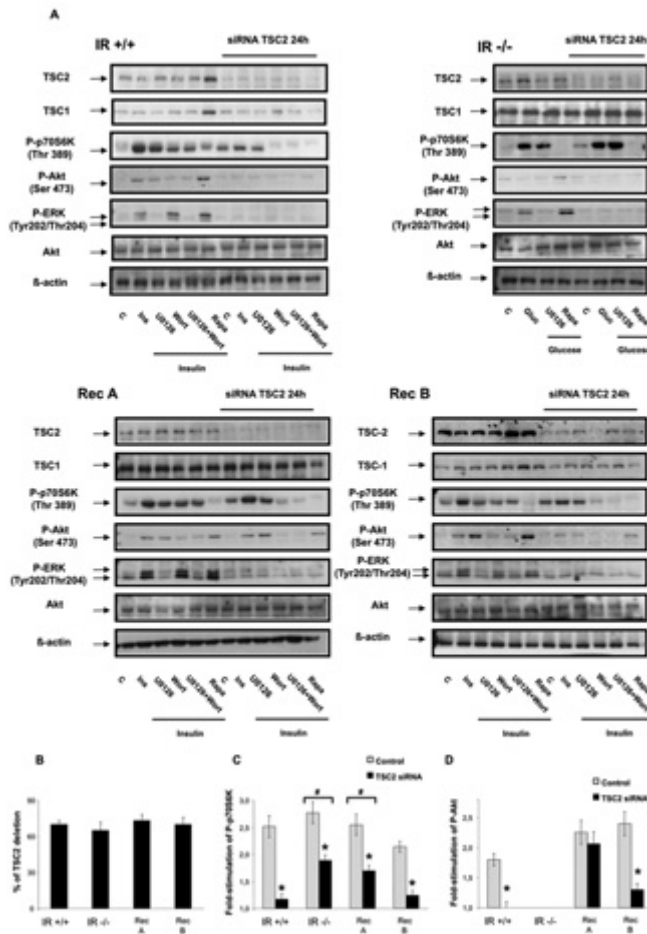
Figure 4.   Effect of TSC2 interference on insulin and glucose signaling. A) 24 h after siRNA transfection cells were stimulated with insulin 10 nM for 5 min (IR +/+, Rec A and Rec B), or glucose 5 mM for 15 min (in IR -/- cells, right upper pannel). Specific inhibitors were added 30 min before stimulation. Blots are representative of four independent experiments. **B)** Densitometric analysis showing the percentage of TSC2 deletion in TSC2-interfered cells compared to control cells. Results are means ± S.E.M. of the level of interference reached. **C)** Fold-increase of p70S6K Thr^{389} phosphorylation after insulin or glucose stimulation in control and TSC2-interfered cells. Results are means ± S.E.M. $^*P < 0.05$ compared to control cells. The differences between the ratios of fold-stimulation between control and TSC2-interfered cells were calculated and significant differences are indicated $^#P < 0.05$. **D)** Fold-increase of Akt Ser473 phosphorylation after insulin or glucose stimulation in control and TSC2-interfered cells. Results are means ± S.E.M. $^*P < 0.05$ compared to control cells.
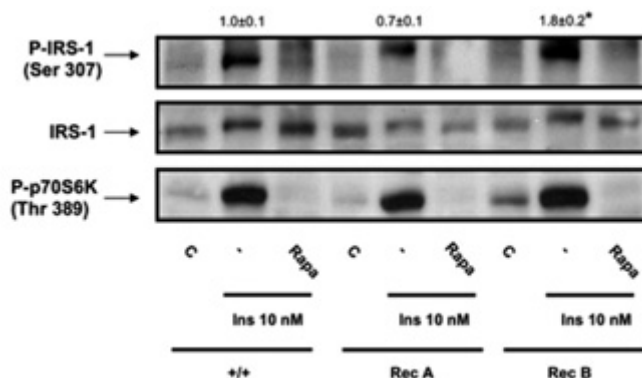
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Figure 5. Feedback loop on IRS-1 Ser[307] is differentially activated depending on IR isoform expression. Cells were glucose and serum deprived for 3 h, and then were stimulated with insulin 10 nM for 15 min. Rapamycin 40 nM was added 30 minutes before stimulation. Blots are representative of three independent experiments. Results are means ± S.E.M. [*]$P < 0.05$ compared to IR +/+ cells.

tal conditions (10-fold increase). Addition of rapamycin lowered the proliferation rate in all the cell lines studied, but this effect was of different magnitude. In Rec A cells the effect of rapamycin on proliferation was a 4-fold decrease while in Rec B cells only a 2-fold decrease was observed (Figure 6, A and B). Flow cytometry assays showed a tendency in cell size increase after TSC2 interference, although it was not statistically significant (data not shown). In addition, TSC2 interference increased the number of cells in S/G2-M phase in all cell lines studied (Figure 6C). These data were confirmed by crystal violet assays (Figure 6D). Rapamycin treatment reverted the increased proliferation mediated by TSC2 knockdown in all cell lines studied (Figure 6, A, B and C).

3.7. Critical roles of autophagy and ER-stress in β cell death or survival

ER-stress and autophagy are closely related to TSC2/mTORC1 signaling. TSC deficiency is known to lead to ER-stress (16), and mTORC1 blocking with rapamycin is a common way for inducing autophagy in vitro (31). Both processes are also interplaying, as autophagy has a role

in misfolded protein clearance (31). β cells turned out to be more sensible to apoptotic death mediated by chemical ER-stressors when compared to fibroblasts (Figure 7A). Immunofluorescence assays showed that both basal and rapamycin-induced autophagy is sensibly higher in β cells than the observed in fibroblasts, observed as the number of LC3B puncta on cells (Figure 7C). Even more shocking are the differences between both cell types when stained with FK2 mAb against ubiquitin-protein conjugates, which showed higher levels of protein ag-
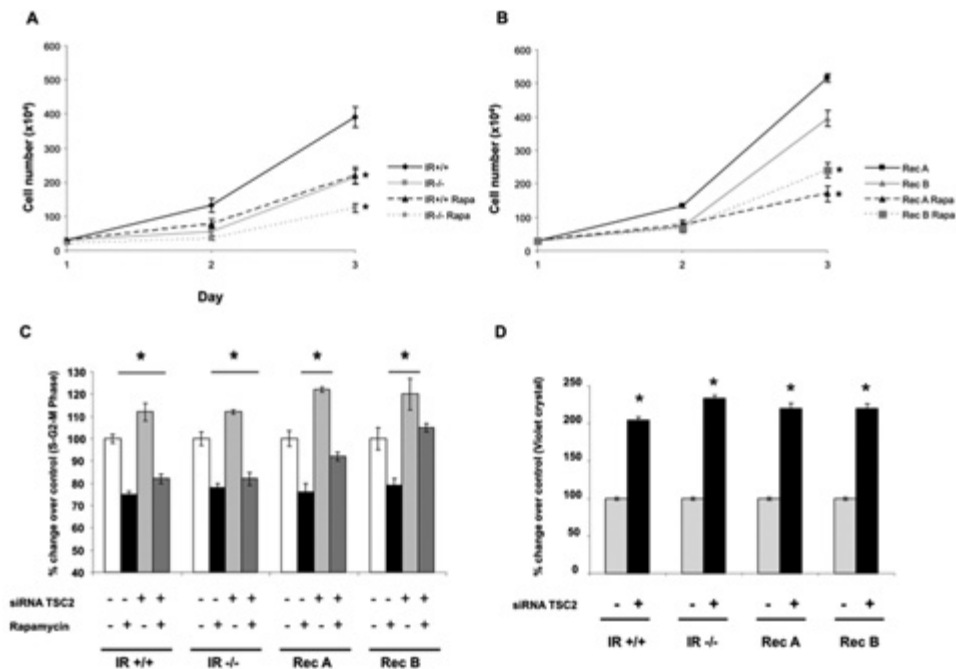

Figure 6. Effect of mTORC1 inhibition or overactivation on β cell proliferation. A) IR +/+ and IR -/- cells were counted and seeded at the same density, and grown in glucose 1 g/L 10% FBS-DMEM. At 24 and 48 h, cells were counted. **B)** Same experiment for Rec A and Rec B cells. Results are means ± S.E.M. $^*P < 0.05$ compared to control points. **C)** Transfected and control cells were grown in glucose 1 g/L 10% FBS-DMEM. DNA content was analyzed by flow cytometry. The bars represent the percentual change of cells in S/G_2-M phase compared to controls for each cell line. **D)** Cells were seeded at the same density. After 24 and 48 h violet crystal stain was measured as described. The differences between absorbances in 24 and 48 h, in control or TSC2 downregulated cells, are represented. Results are means ± S.E.M. $^*P < 0.05$ compared to controls for each cell line.
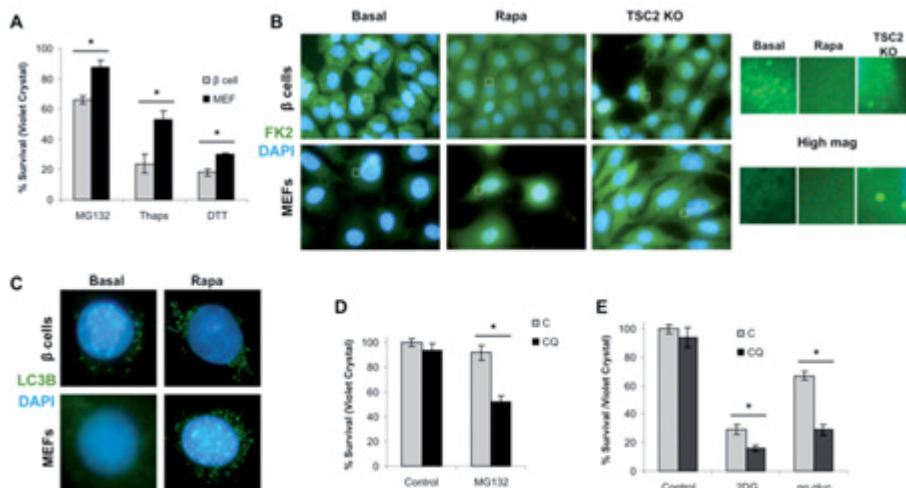
Figure 7.   ER-stress and autophagy interplay, effect on β cell death or survival.
A) Cell survival after 24h treatment with MG132 0,5 µM; Thaps: Thapsigargin 0,1 µM;
or DTT: dithiothreitol 5 mM, measured by violet crystal assay. **B)** Immunofluoresecence
against ubiquitin-protein conjugates with FK2 mAb antibody (green), nuclei are conun-
tersestained with DAPI (blue). Rapamycin 40 nM treatment for 24 h. TSC2 was inter-
fered with siRNA for 24 h in β cells, TSC2 -/- MEFs were used. **C)** Immunofluorescence
against LC3B (green), puncta is indicative of lipidated LC3B accumulation in au-
tophagosomes, while cytoplasmic staining is indicative of no autophagic activity.
Rapamycin 40 nM was used for 24 h. **D)** Pancreatic β-cell survival after 24 h treatment
with a lower concentration of MG132 (0,1 µM), and or CQ; chloroquine 25 µM by vi-
olet crystal assay. **E)** β-cell survival after 48 h of glucose deprivation, or 2-DG 5 mM;
and or CQ 25 µM by violet crystal assay. Results are means ± S.E.M. significative dif-
ferences are indicated $^*P < 0.05$.

gregates in β cells. Interestingly, mTORC1 modulation rendered differ-
ent outcomes in the accumulation of these conjugates. Rapamycin-me-
diated blocking of mTORC1 diminished the accumulation observed in
β cells. On the other hand, TSC2 interference and the consequent
mTORC1 hyperactivation did not further increased staining in β cells.
In contrast, TSC2 -/- MEFs showed higher staining when compared
with TSC2 +/+ MEFs (Figure 7B). Chemical inhibition of autophagy
enhances ER-stress, or nutrient starvation mediated cell death in β cells
(Figures 7, D and E).

4. DISCUSSION

Recent animal models have pointed out the relevance of TSC1-TSC2 complex acting as a repressor of mTORC1 for the control of β cell mass. Thus, β cell specific knockout models of TSC1 or TSC2, or Rheb overexpression results in an augmented β cell mass, mainly because of a huge cell hypertrophy (19-22). The importance of mTORC1 functioning in human β cells is also patent; progressive impairment in β cell function was observed in patients who received β cell transplantation and were treated with rapamycin as an immunosuppressant (32). We previously demonstrated that insulin or glucose independently induced proliferation signaling in pancreatic β cells of fetal origin (10). Now, we address the important issue of the integration of both independent signaling in the regulation of β cells proliferation.

Insulin stimulation mediates TSC2 phosphorylation in a PI3K/Akt-dependent manner leading to diminished TSC2 activity towards Rheb, and subsequently producing downstream mTORC1 activation (12). More importantly, insulin stimulates Akt/TSC2/p70S6K and MEK/ERKs pathways in either IRA or IRB expressing cell lines. These results contrast with previous data showing specific signaling for each of the IR isoforms (33, 34). However, insulin induced a more sustained signaling in Rec A as compared with Rec B cell lines. In fact, wortmannin did not completely block either Akt or TSC2 phosphorylation in Rec A as compared with Rec B β cells. These results suggest that IRA confers a stronger proliferation capability as compared with IRB in response to insulin, as we recently published in iLIRKO primary islets (26).

Glucose is an essential proliferation and survival factor for pancreatic β cells (7, 10). Glucose stimulation in vitro is able to induce ERK 1/2 phosphorylation, independently from insulin secretion in β cells (10). ERK 2 directly phosphorylates TSC Ser[664] in HEK293T cells (13), and was increased in several human tumours leading to an upregulation of mTORC1 signaling (28). Neither TSC2 Ser[939] nor Thr[1462] phosphorylation was observed upon glucose stimulation in β cells. However, TSC2 Ser[664] was stimulated by glucose in a MEK/ERK-dependent manner. Glucose can also exert its effects on mTORC1 signaling by modulation of ATP levels and therefore by AMPK inhibition. AMPK, on one hand, directly stabilizes TSC1-TSC2 complex (14)

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and on the other, modulates an essential component of mTORC1, Raptor (30). AMPK activation implicates a strong inhibition of mTORC1, which can be reverted by glucose addition. We have proven that AMPK activation can induce Raptor Ser[792] phosphorylation in pancreatic β cells, contributing to energy deprivation-mediated impairment in mTORC1/p70S6K signaling.

TSC2 interference causes an increase in p70S6K activity and insulin resistance in IR +/+ or Rec B β cells on Akt stimulation. Lately, some animal and cellular models have shown the importance of the regulation of mTORC1 in the physiology of β cells. Although mTORC1/p70S6K hyperactivity can lead to IRS-mediated insulin resistance, trials failed to globally diminish it by blocking mTORC1 with the use of rapamycin, because a functional mTORC1 is required for β cell adaptation (35). The inhibition of mTORC1 with rapamycin reverted the insulin resistance on Akt stimulation in our cell lines. More importantly, IRA reconstituted cells overcome the insulin resistance seen above showing insulin-mediated Akt stimulation even though TSC2 knockdown. Our data suggest that the inhibition of the upstream insulin signaling mediated by p70S6K overactivation feedback mechanism, may be differentially modulated in IRA or IRB expressing cells. Phosphorylation of IRS-1 Ser[307] is considered a marker of insulin resistance (36). The negative loop concerning IRS-1 Ser[307] phosphorylation was upregulated in Rec B cells as compared to Rec A cells in response to insulin. This might explain the differences observed on Akt Ser[473] phosphorylation between Rec A and Rec B cells when we interfered TSC2 expression.

In our β cell lines, we found an increase of proliferation in all cell lines when TSC2 was interfered. This increase was dependent on mTORC1 activity as rapamycin addition blocked the stimulatory effect of TSC2 interference on cell proliferation. Our results point out the higher mitogenic effect of IRA versus IRB isoform in the regulation of β cell proliferation.

Other processes related with TSC2/mTORC1 are also critical for β cell death or survival. We found β cells to be more susceptive to cell death in response to ER-stressors compared with fibroblasts. Both autophagy and ER-stress is basally increased in β cells, observed as the increased number of puncta of LC3B and ubiquitin-protein conjugates, respectively. This may be explained as β cells have a highly deve-
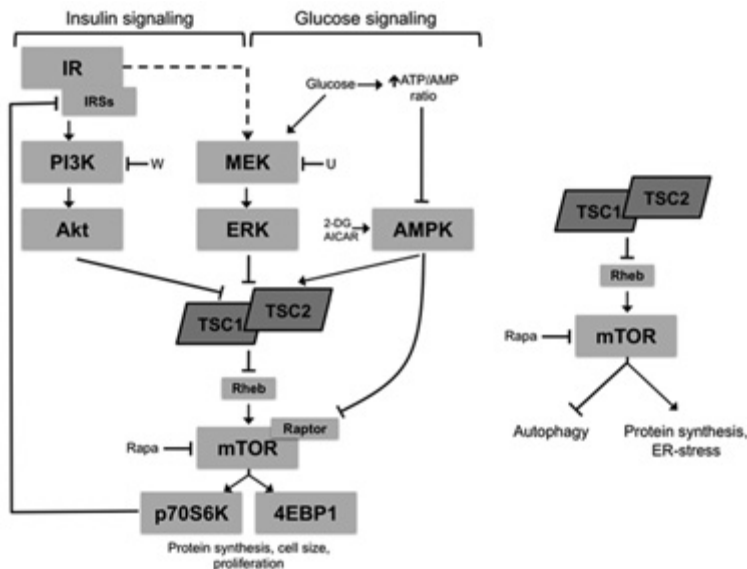
Figure 8. Insulin and glucose signaling toward TSC complex and mTORC1. Integration of insulin or glucose-independent signaling by TSC1-TSC2 complex converging on mTORC1/p70S6K proliferation pathway in pancreatic β-cells. Also is depicted the effect on autophagy and ER-stress.

loped ER specialized for the production of insulin, and any stress such as in vitro culture makes them prone to misfolded protein accumulation, and extremely sensible to ER-stress mediated cell death. We believe that the basal autophagic level observed in β cells may be a direct consequence of the higher misfolded protein accumulation. Finally, we found autophagy to be protective under certain conditions such as glucose starvation or ER-stress.

In conclusion, here we described the relevance of TSC1-TSC2 complex in the integration of insulin and glucose independent signaling in pancreatic β cell proliferation (Figure 8). This complex participates coordinating multiple signals from either energetic or hormonal status, determining the functioning of protein synthesis through mTORC1. This route has been described as critical for β cell mass maintenance, and may play a very important role in the β cell mass hyperplasia associated with insulin resistance states. Also, the role of autophagy and

ER-stress may be involved in β cell death or survival. At this stage, the relative expression of IRA versus IRB within the β cells may play an essential role in the regulation of pancreatic β cell proliferation. A better knowledge of the molecular mechanisms involved in β cell plasticity is needed to understand its role in type 2 diabetes progression.

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