Insulin Receptor Substrate-2 Proteasomal Degradation Mediated by a Mammalian Target of Rapamycin (mTOR)-induced Negative Feedback Down-regulates Protein Kinase B-mediated Signaling Pathway in β-Cells*

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Regulation of insulin receptor substrate (IRS)-2 expression is critical to β-cell survival, but the mechanisms that control this are complex and undefined. Here in pancreatic β-cells (INS-1), chronic exposure (>8 h) to 15 mM glucose and/or 5 μM IGF-1, increased Ser/Thr phosphorylation of IRS-2, which correlated with decreased IRS-2 levels. This glucose/IGF-1-induced decrease in IRS-2 levels was prevented by the proteasomal inhibitor, lactacystin. In addition, the glucose/IGF-1-induced increase in Ser/Thr phosphorylation of IRS-2 and the subsequent decrease in INS-1 cell IRS-2 protein levels was thwarted by the mammalian target of rapamycin (mTOR) inhibitor, rapamycin. Moreover, adenoviral-mediated expression of constitutively active mTOR (mTORΔ) further increased glucose/IGF-1-induced Ser/Thr phosphorylation of IRS-2 and decreased IRS-2 protein levels, whereas adenoviral-mediated expression of “kinase-dead” mTOR (mTOR-KD) conversely reduced Ser/Thr phosphorylation of IRS-2 and maintained IRS-2 protein levels. In adenoviral-infected β-cells expressing mTORΔ, the decrease in IRS-2 protein levels was also prevented by rapamycin or lactacystin, further indicating a proteasomal mediated degradation of IRS-2 mediated via mTOR-induced Ser/Thr phosphorylation of IRS-2. Finally, we found that chronic activation of mTOR leading to decreased levels of IRS-2 in INS-1 cells led to a significant decrease in PKB activation and consequently increased β-cell apoptosis. Thus, chronic activation of mTOR by glucose (and/or IGF-1) in β-cells leads to increased Ser/Thr phosphorylation of IRS-2 that targets it for proteasomal degradation, resulting in decreased IRS-2 expression and increased β-cell apoptosis. This may be a contributing mechanism as to how β-cell mass is decreased by chronic hyperglycemia in the pathogenesis of type-2 diabetes.

Insulin receptor substrate (IRS) proteins are adaptor molecules that act as signaling interfaces between activated tyrosine kinase receptors and downstream signaling molecules, such as the 85-kDa regulatory subunit of the phosphatidylinositol 3′-kinase (p85(PI3K)), and the growth factor receptor-bound protein-2 (Grb2)-mamalian son of sevenless (mSOS) complex (1, 2). The IRS protein family now contains at least six members, IRS-1 to 6, however, IRS-2 is the most prominently expressed and functionally important in pancreatic β-cells (1–3). In β-cells, IRS-2 controls cell growth and survival (4–9). Adenoviral-mediated increased expression of IRS-2 in INS-1 β-cells increases glucose/IGF-1-induced mitogenesis (7), and protects β-cells against free fatty acid (FFA)-induced apoptosis (9). Recently, it has been demonstrated that β-cell-specific transgenic expression of IRS-2 in vivo promotes β-cell survival (6). In contrast, specifically reducing IRS-2 expression in INS-1 cells, using adenoviral-mediated expression of IRS-2 antisense, causes spontaneous apoptosis and decreased β-cell survival (9). Likewise, IRS-2−/− mice have a marked decreased β-cell mass and consequently become diabetic (4, 5). Thus, both in vitro and in vivo studies indicate that regulation of IRS-2 expression levels in β-cells is critical for control of β-cell mass (10).

Regulation of β-cell mass has important implications for the pathogenesis of obesity-linked type 2 diabetes (1). Obesity is linked to an increase in peripheral insulin resistance, but this can be compensated by an increase in β-cell function and mass so that the onset of type 2 diabetes is avoided (10). However, with time and/or increased insulin resistance, β-cell mass eventually becomes insufficient to cope with the increased insulin demand and type 2 diabetes ensues (1, 10). Indeed, there is a decreased β-cell mass associated with the onset of type 2 diabetes in human and rodent models of type 2 diabetes, caused by increased β-cell apoptosis (10–13). The balance between β-cell mass and insulin resistance relative to the pathogenesis of type 2 diabetes is nicely illustrated by comparing the IRS-1 and IRS-2 knock-out mice phenotypes (4, 5, 14). The IRS-1−/− mice are insulin resistant but this is adequately compensated for by an increased β-cell mass and as such diabetes is avoided in these animals. In contrast, IRS-2−/− mice have a marked decrease in β-cell mass so that the inherent insulin resistance is not compensated for in these animals and they become severely diabetic (4, 5, 14).

Although the pathophysiological relevant factors that instigate a decrease in β-cell mass associated with the onset of type 2 diabetes have not been elucidated, chronic hyperglycemia...
and/or hyperlipemia are thought to be the prime suspects (10, 15, 16). Both chronic exposure to elevated glucose levels and/or FFA levels cause β-cell apoptosis (16–22). FFA are thought to decrease β-cell survival, at least in part, via inhibition of IGF-1-induced PKB activation (10, 22, 23). In skeletal muscle, FFA-induced inhibition of insulin-mediated PI3K/PKB activation has been suggested to be via FFA-induced activation of novel PKC isoforms that increase Ser/Thr phosphorylation of IRS-1 that, in turn, has a negative effect of dampening downstream IRS-1 signal transduction (24, 25). This is thought to make a significant contribution to the mechanism of insulin resistance. In β-cells, FFA also activate novel PKC isoforms that could increase the IRS-2 Ser/Thr phosphorylation status (22) and, in turn, contribute to FFA-induced inhibition of downstream PKB activation (22, 23). As such, it is possible to envisage commonalities between mechanisms of FFA-induced insulin resistance in muscle and FFA-induced β-cell apoptosis (10). However, it has been shown that other protein kinases, not just certain PKC isoforms, are capable of Ser/Thr phosphorylating IRS proteins, including mTOR (26–28), but this has yet to be examined in pancreatic β-cells.

Under normal circumstances, relatively short term fluctuations in glucose concentrations in the 3–15 mM range appear protective for β-cell (9, 22). Glucose can induce ERK-1/2 phosphorylation activation in β-cells, independently of IRS-2 (7, 29–31). However, activation of the ERK-1/2 signaling pathway in β-cells has yet to be associated with control of β-cell survival (10). Glucose can also activate mTOR independently of IRS-2 in β-cells (29, 30), which contributes to up-regulation of general protein synthesis in β-cell via phosphorylation of the eIF-4E-binding protein (4E-BP1) and p70S6K (32–35). However, it is possible that prolonged activation of mTOR by chronically elevated glucose concentrations could be detrimental to IRS-mediated signaling in β-cells. It has been described in insulin target tissues that chronically activated mTOR increased the Ser/Thr phosphorylation state of IRS-1 and IRS-2 (27, 28). This increased Ser/Thr phosphorylation of IRS-1 or IRS-2 via mTOR subsequently leads to increased degradation of IRS-1/2 proteins and a consequential down-regulation of insulin signaling transduction pathways, which also contributes to insulin resistance in peripheral tissues (27, 28, 36, 37). If chronic activation of mTOR leads to Ser/Thr phosphorylation of IRS-2 in β-cells, then this would decrease IRS-2 protein levels and be detrimental for β-cell survival. Indeed, in this present study we present experimental evidence to indicate that this might well be the case, and in turn may be a contributing mechanism as to how chronic hyperglycemia eventually leads to a decrease in β-cell mass in the pathogenesis of type 2 diabetes.

**Experimental Procedures**

Materials—Alkaline phosphatase was from Sigma. Lactacystin and rapamycin were from Calbiochem-Novabiochem (La Jolla, CA). The IRS-2 and p70S6K antisera were kindly provided by Drs. M. White and M. Myers Jr. (Joslin Diabetes Center, Boston, MA). The p85 subunit of the PI3K antibody was from Upstate (Lake Placid, NY). The total PHAS-I, phospho-(Ser57)PHAS-I, IGF-1 receptorThr1589/PKB, IGF-1 receptorThr2449/p70S6K, total PKB, phospho-(Ser2430)PKB, phospho-ERK-1/2, and the caspase-9-cleaved antibodies were from Cell Signaling (Beverly, MA). The total ERK-1/2 antibody was from Promega Corp. (Madison, WI). The antibody to AU1 tag was from Covance Inc. (Princeton, NJ). The anti-rabbit IgG horse-radish peroxidase conjugate was from Jackson ImmunoResearch (West Grove, PA). The anti-mouse IgG horse-radish peroxidase conjugate was from Upstate Biotechnology, Inc. IGF-1 was obtained from Calpep Pty Ltd. (Adelaide, Australia). The BCA protein assay kit was from Pierce.

**Cell Culture and Treatment**—The pancreatic β-cell line, INS-1, was maintained in the complete medium as previously described (38). In general, INS-1 cells were subcultured on 6-well plates to ~80% confluence and then incubated 16 h at 37 °C in serum-free modified RPMI 1640 medium containing 3 mM glucose and 1% (w/v) bovine serum albumin as described (7, 29). After the quiescent period the cells were incubated at 37 °C with fresh serum-free RPMI 1640 medium with 3 or 15 mM glucose ± 5 nM IGF-1 + inhibitors for 8 or 24 h as indicated.

**Adenovirus Infection**—All mTOR constructs have an NH2-terminal AU1 epitope tag. The “kinase-dead” mTOR KD variant contains an Asp3283 to Ala mutation that renders it catalytically inactive (39, 40). The mTORΔ variant is a mutant form of mTOR that was generated by deleting amino acids 2430–2450 (41). This region contains sites phosphorylated by Akt (41), as well as the epitope for the activating antibody. The intrinsic kinase activity of mTORΔ is ~5-fold higher than that of wild type mTOR when assayed in vitro (43) and overexpression of mTORΔ in cells results in constitutive phosphorylation of the mTOR targets, 4E-BP1 and p70S6K (41). Adenoviruses expressing wild type (AdV-mTOR-WT), constitutively activated (AdV-mTORA), and kinase-dead mTOR (AdV-mTOR-KD) were generated, amplified, and purified as previously described (7, 29), using the bacterial recombination method in BSH183 Escherichia coli cells (44). Adenoviruses expressing GFP (AdV-GFP) and luciferase (AdV-Luc) were used as control viruses. The determination of the appropriate titer of adenovirus and the adenovirus infection were as previously described (7, 29).

**Immunoblot Analysis**—Cell lysates were prepared as previously described and assayed for total protein levels as determined using the BCA protein assay kit (Pierce). Equivalent protein sample aliquots were then subjected to immunoblot analysis as previously described (7, 29). For adenovirus phosphate treatment, INS-1 cells were lysed in phosphate inhibitor-free lysis buffer. These lysates were then treated with 1.725 DEA units/100 μl of alkaline phosphate attached to agarose beads with shaking at 37 °C for 1 h. The alkaline phosphate was removed by microcentrifugation prior to the immunoblot analysis.

**Immunofluorescence**—AdV-infected INS-1 cells were subcultured on glass coverslips within 6-well plates to 60–70% confluence, and after incubation were washed with ice-cold PBS, fixed with 4% paraformaldehyde/PBS, washed with 0.1% Triton/PBS, and then re-washed with PBS. Fixed cells were first incubated for 1 h with 5% donkey serum in 5% bovine serum albumin/PBS prior to incubation with primary antibody against ERK-1/2 overnight at a 1:100 dilution in 5% bovine serum albumin/PBS at 4 °C. The cells were then washed 3 times with PBS and incubated with a secondary antibody conjugated to fluorescein isothiocyanate (1:500 in 5% bovine serum albumin/PBS) (Amersham Biosciences) for 1 h at 25 °C. The cells were then washed 3 times in PBS and mounted onto glass slides with mounting media (Molecular Probes). Activated caspase-9 was visualized using an Olympus FV500 confocal microscope.

**Expression of Data and Statistics**—Data are presented as mean ± S.E. Statistically significant differences between groups were analyzed using Student’s t test, where p < 0.05 was considered statistically significant.

**RESULTS**

**Chronic Exposure to Glucose and/or IGF-1 Induces Ser/Thr Phosphorylation of IRS-2 in Pancreatic β-Cells**—Quiescent INS-1 cells were incubated for 8 h in the presence of basal 3 mM glucose or stimulatory 15 mM glucose ± 5 nM IGF-1. Immunoblot analysis of IRS-2 indicated different stages of a decreased electrophoretic mobility of IRS-2 protein on a 7% polyacrylamide gel analysis (Fig. 1A). A stimulatory 15 mM glucose induced an upward mobility shift in IRS-2 protein migration compared with that at basal 3 mM glucose (Fig. 1A). IGF-1 added at 3 mM basal glucose also induced a slight upward shift in IRS-2 migration, which was further increased when IGF-1 was added together with a stimulatory 15 mM glucose concentration (Fig. 1A). IRS-2 immunoblot analysis by the 10% polyacrylamide gel was used to more tightly resolve IRS-2 electrophoretic migration and better evaluate total IRS-2 protein levels. Intriguingly, IRS-2 protein levels were increased 2–3-fold at 15 mM glucose compared with basal 3 mM glucose (Fig. 1A). The addition of IGF-1 at basal 3 mM glucose had no effect on IRS-2 protein levels, and addition of IGF-1 at a stimulatory 15 mM glucose tended to reduce IRS-2 protein levels compared with that at 15 mM glucose alone (Fig. 1A). Immunoblot analysis of the p85 subunit of PI3K was used as a loading control and its protein expression levels were unchanged under all
conditions emphasizing the specific effect on IRS-2 (Fig. 1A).

The time course of this glucose-induced IRS-2 gel mobility shift was examined. Quiescent INS-1 cells were incubated in the presence of basal 3 mM or stimulatory 15 mM glucose for 1, 4, 8, and 24 h. There was little change in IRS-2 electrophoretic mobility at basal 3 mM glucose during early time points, although IRS-2 protein levels were decreased by ~50% at 24 h compared with that at the preceding 8-h time point (Fig. 1B). At 15 mM glucose, there was an increased IRS-2 gel mobility shift apparent by 4 h and remained up to 24 h (Fig. 1B). Stimulatory 15 mM glucose increased IRS-2 protein levels, which reached a maximum 2-3-fold increase at 8 h (Fig. 1B). IRS-2 expression levels at 15 mM glucose remained elevated by 24 h compared with that at basal 3 mM glucose; although, this was actually decreased by about 50% compared with that at the preceding 8-h time point at a stimulatory 15 mM glucose concentration (Fig. 1B).

Glucose/IGF-1-induced IRS-2 Electrophoretic Mobility Shift Is Partly Because of the Increased IRS-2 Ser/Thr Phosphorylation State—To determine whether IRS-2 gel shift was because of an increase in the Ser/Thr phosphorylation state, INS-1 cell lysates were first treated with alkaline phosphatase to specifically dephosphorylate phosphoserine and phosphothreonine but not the phosphotyrosine residues on IRS-2. As a positive control it was found that both IGF-1 and the glucose-induced Ser/Thr phosphorylation state of ERK-1/2 and PKB was markedly decreased by alkaline phosphatase treatment (Fig. 2A). The IGF-1 or 15 mM glucose-induced IRS-2 electrophoretic upward mobility shift was decreased after alkaline phosphatase treatment, indicating that, at least in part, this shift was because of the increased Ser/Thr phosphorylation state of IRS-2. However, the shift was not completely resolved to that at basal 3 mM glucose by alkaline phosphatase treatment, suggesting that something else might have contributed to the glucose/IGF-1-induced IRS-2 electrophoretic mobility shift.

Glucose/IGF-1-induced IRS-2 Electrophoretic Mobility Shift Is Also Contributed by Targeting IRS-2 for Proteasomal Degradation—Previous studies in adipocytes have shown a controlled proteasome-mediated degradation of IRS-1 and IRS-2 proteins that correlated with the IRS-1 or IRS-2 Ser/Thr phosphorylation states (27, 36, 45, 46). Thus, it was investigated whether the mobility shift of IRS-2 might also be contributed by a ubiquitin-dependent targeting of IRS-2 for proteasomal degradation. Lactacystin, a specific proteasome inhibitor, was used to prevent ubiquitin-dependent proteasomal protein degradation in pancreatic β-cells. Quiescent INS-1 cells were incubated for 8 h in the presence of 3 mM glucose ± 5 mM IGF-1, or 15 mM glucose, in the presence or absence of 10 μM lactacystin. The addition of lactacystin rendered an increased mobility shift of IRS-2 in itself at basal 3 mM glucose, and further increased the IGF-1 and 15 mM glucose-induced shift in IRS-2 electrophoretic mobility (Fig. 3A). The IRS-2 gel mobility shift induced by lactacystin was then examined to see how much of it was contributed to by IRS-2 Ser/Thr phosphorylation. Alkaline phosphatase-mediated removal of the phosphates from IRS-2 Ser/Thr sites did not completely resolve the upward gel mobility shift of IRS-2 induced by IGF-1 or glucose in the presence of lactacystin (Fig. 3B). These results suggest that an alternative post-translational modification of IRS-2, other than Ser/Thr phosphorylation, induced by glucose/IGF-1 stimulation contributes to the IRS-2 upward electrophoretic mobility shift on SDS-PAGE. Although lactacystin inhibits ubiquitin-dependent proteasomal degradation it does not affect the ubiquitinylation of proteins. As lactacystin further increased the glucose/
Quiescent INS-1 cells were treated with 3 mM glucose ± 5 nM IGF-1 or 15 mM glucose for 8 h. Cell lysates were treated with alkaline phosphatase (see "Experimental Procedures") as indicated, resolved by 7 and 10% SDS-PAGE, and subjected to immunoblot (IB) analysis (as described under "Experimental Procedures") with IRS-2 and p85, and antibodies against phospho-Ser473-PKB and phospho-ERK-1/2. The same blots were reprobed with PKB and αERK-1/2 as indicated.

**mTOR Mediates IGF-1/Glucose-induced Ser/Thr Phosphorylation of IRS-2**—It was examined whether mTOR mediated the glucose/IGF-1-induced increase of IRS-2 Ser/Thr phosphorylation in β-cells, initially by using the relatively specific mTOR inhibitor, rapamycin. Quiescent INS-1 cells were cultured at basal 3 mM or stimulatory 15 mM glucose ± IGF-1, in the presence or absence of rapamycin (50 nM), for 8 h. The IRS-2 upward gel mobility shift induced by IGF-1 or glucose was reduced in the presence of rapamycin (Fig. 4). Interestingly, IRS-2 protein expression levels appeared to be enhanced by rapamycin, especially at stimulatory 15 mM glucose concentrations (Fig. 4). The P13K p85 subunit protein levels did not change indicating specific effects on IRS-2 protein phosphorylation levels (Fig. 4). The inhibitory effect of rapamycin on mTOR activation was confirmed by observing a prevention of glucose/IGF-1-induced phosphorylation activation of p70S6K (data not shown). To further examine mTOR-mediated phosphorylation of IRS-2 we investigated the effect of adenoviral-mediated expression of a wild type (AdV-mTOR-WT), constitutively active (AdV-mTORΔ), or a kinase-dead form (AdV-mTOR-KD) of mTOR in pancreatic β-cells on IRS-2 Ser/Thr phosphorylation. A GFP-expressing adenovirus (AdV-GFP) was used as a control. The mTOR adenoviral constructs all had a NH2-terminal AU1 tag that was used for specific immunoblot analysis of adenoviral-mediated expression of mTOR. Twenty-four hours after infection of INS-1 cells with increasing titer of AdV-mTOR-WT, AdV-mTORΔ, or AdV-mTOR-KD adenovirus, a corresponding increase in AU1-tagged mTOR expression was observed (Fig. 5A). The activity of adenoviral mediated expression of the mTOR variants was assessed by evaluating the phosphorylation state of the substrates of mTOR, p70S6K and 4E-BP1, in INS-1 cells infected with equivalent titer (750 plaque forming units/cell) of AdV-mTOR-WT, AdV-mTORΔ, and AdV-mTOR-KD or the control AdV-GFP. Infected cells were incubated for 16 h at basal 3 mM glucose and then for either 1 h (for p70S6K analysis (Fig. 5B)) or 30 min (for 4E-BP1 analysis (Fig. 5C)) at 3 or 15 mM glucose ± IGF-1, and ± rapamycin, as indicated (Fig. 5). Glucose-induced p70S6K phosphorylation was further increased by the addition of IGF-1 in AdV-GFP- and AdV-mTOR-WT-infected cells, as previously shown (29) (Fig. 5B). In AdV-mTORΔ-infected cells, p70S6K phosphorylation activation was significantly increased at basal 3 mM glucose as indicated by an increased upward mobility shift of total p70S6K, because of its increased phosphorylation state (Fig. 5B), consistent with the degree of constitutive activation of the mTORΔ variant (41, 43). Likewise, p70S6K activation by IGF-1 and/or 15 mM glucose was enhanced in AdV-mTORΔ-infected cells (Fig. 5B). In contrast, p70S6K phosphorylation induced by glucose and/or IGF-1 was decreased in AdV-mTOR-KD-infected cells, consistent with the lack of mTOR activity in the mTOR-KD variant (Fig. 5B). As a control to indicate these effects of p70S6K phosphorylation were mediated by mTOR. It was found that rapamycin inhibited glucose- and glucose/IGF-1-induced p70S6K phosphorylation in AdV-GFP and AdV-mTORΔ-infected cells (Fig. 5B). As previously described (33), a stimulatory 15 mM glucose concentration induced 4E-BP1 phosphorylation in β-cells, which was further increased by IGF-1, as indicated by an increase in the upper migrating band corresponding to the highly phosphorylated γ-form of 4E-BP1, in AdV-GFP and AdV-mTOR-WT-infected cells (Fig. 5C). In AdV-mTORΔ-infected cells, 4E-BP1 phosphorylation was slightly increased at basal 3 mM and enhanced further at 15 mM glucose, compared with that in AdV-GFP- and AdV-mTOR-WT-infected cells (Fig. 5C). This was also consistent with the constitutive activation of the mTORΔ variant (41, 43). In contrast, 4E-BP1 phosphorylation induced by glucose and/or IGF-1 was decreased in AdV-mTOR-KD-infected cells.
(Fig. 5C), again consistent with the lack of mTOR activity in the mTOR-KD variant. Rapamycin also inhibited glucose and IGF-1-induced 4E-BP1 phosphorylation in AdV-GFP and AdV-mTORΔ-infected cells (Fig. 5C), reaffirming that the 4E-BP1 phosphorylation was mediated by mTOR.

IRS-2 gel mobility shift was assessed in INS-1 cells infected with AdV-mTORWT, AdV-mTORΔ, and AdV-mTOR-KD or the control AdV-GFP recombinant adenoviruses. Infected cells were incubated for 16 h at basal 3 mM glucose and then for 8 h in the presence of basal 3 mM or stimulatory 15 mM glucose ± 5 nM IGF-1, as indicated (Fig. 6). Glucose and IGF-1 caused an upward electrophoretic mobility shift of IRS-2 on the 7% PAGE immunoblot analysis, in control AdV-GFP-infected cells (Fig. 6) and as shown previously in uninfected INS-1 cells (Fig. 1). This IGF-1/glucose-induced upward shift of IRS-2 electrophoretic mobility was further increased in both AdV-mTORWT- and AdV-mTORΔ-infected cells (Fig. 6). In contrast, in AdV-mTOR-KD-infected cells the glucose/IGF-1-induced upward mobility shift of IRS-2 was prevented (Fig. 6), similarly to that observed in rapamycin-treated cells (Fig. 4). These results further indi-
cated that mTOR mediated the electrophoretic mobility shift of IRS-2, presumably by Ser/Thr phosphorylation of IRS-2 induced by IGF-1 or glucose.

Chronic mTOR-mediated Ser/Thr Phosphorylation of IRS-2 Leads to Decreased IRS-2 Protein Levels—The expression level of IRS-2 was assessed in AdV-mTOR/H9004, and AdV-mTOR-KD or the control AdV-GFP-infected INS-1 cells treated with rapamycin or lactacystin. The AdV-mTOR-WT, AdV-mTORΔ, AdV-mTOR-KD, or AdV-GFP as a control were made quiescent and then incubated for either 1 h (for p70<sup>S6K</sup> analysis, panel B) or 30 min (for 4E-BP1 analysis, panel C) at basal 3 mM glucose or stimulatory 15 mM glucose ± 5 mM IGF-1, ± 50 nM rapamycin as indicated. The cell lysates were resolved by 7% SDS-PAGE and subjected to immunoblot analysis using antibodies against phospho-Thr<sup>389</sup>-p70<sup>S6K</sup> (Panel B) and phospho-Ser<sup>65</sup>-4E-BP1 (Panel C). Immunoblot analysis of the PI3K(p85) regulatory subunit (panel B) and syntaxin 1A (panel C) were used as loading control proteins.

FIG. 5. The effect of adenoviral-mediated expression of mTOR-WT, mTORΔ, and mTOR-KD on p70<sup>S6K</sup> and 4E-BP1 phosphorylation. Panel A, INS-1 cells were infected with increasing titer of AdV-mTOR-WT, AdV-mTORΔ, or AdV-mTOR-KD (see “Experimental Procedures”) as indicated. At 24 h after infection, cells were lysed and analyzed for adenovirally expressed mTOR by immunoblotting (IB), as described under “Experimental Procedures,” with antibody against AU1 tag present on each mTOR construct. Panels B and C, INS-1 cells infected with AdV-mTOR-WT, AdV-mTORΔ, AdV-mTOR-KD, or AdV-GFP as a control were made quiescent and then incubated for either 1 h (for p70<sup>S6K</sup> analysis, panel B) or 30 min (for 4E-BP1 analysis, panel C) at basal 3 mM glucose or stimulatory 15 mM glucose ± 5 mM IGF-1, ± 50 nM rapamycin as indicated. The cell lysates were resolved by 7% SDS-PAGE and immunoblotted (IB) with IRS-2 and p85. The results shown are representative of three independent experiments.

Fig. 6. mTOR mediates Ser/Thr phosphorylation of IRS-2. INS-1 cells infected with AdV-mTOR-WT, AdV-mTORΔ, AdV-mTOR-KD, or AdV-GFP as a control, were made quiescent and then incubated for 8 h with 3 mM glucose and 15 mM glucose ± 5 mM IGF-1. The cell lysates were resolved by 7% SDS-PAGE and immunoblotted (IB) with IRS-2 and p85. The results shown are representative of three independent experiments.
infected cells, IRS-2 protein levels were generally better preserved and significantly increased at 15 mM glucose and 15 mM glucose/IGF-1 compared with that in AdV-GFP control cells ($p < 0.05$; Fig. 7A). Inhibition of endogenous mTOR by rapamycin had no additional effect on IRS-2 expression levels in AdV-mTOR-KD-infected INS-1 cells (Fig. 7A). These observations on IRS-2 protein expression levels in AdV-GFP, AdV-mTORΔ, or AdV-mTOR-KD-infected β-cells were specific because protein levels of p85(PI3K) were similar between samples (Fig. 7A). Thus, chronic activation of mTOR in mTORΔ-expressing β-cells decreased IRS-2 protein levels, while inhibiting mTOR Ser/Thr kinase activity, by either rapamycin or expression of mTOR-KD, preserved IRS-2 protein expression levels.

It was also investigated whether lactacystin prevented the mTOR-mediated decrease of IRS-2 protein levels. The AdV-mTORΔ and AdV-mTOR-KD or the control AdV-GFP-infected INS-1 cells were made quiescent and then incubated for 24 h with 3 mM glucose and 15 mM glucose/IGF-1, alone or in the presence of 50 nM rapamycin (A) or 10 μmol/liter of lactacystin for the last 6 h (B) as indicated. The cell lysates were resolved by 10% SDS-PAGE and immunoblotted (as described under “Experimental Procedures”) with IRS-2 and p85. The results shown are representative of four (A) or six (B) independent experiments. Quantification of IRS-2 protein levels was performed by densitometric scanning with Optiquant software analysis and expressed as mean ± S.E. of at least four individual experiments. IB, immunoblot.

**Fig. 7.** Chronic activation of mTOR decreases IRS-2 protein levels. INS-1 cells infected with AdV-mTORΔ, AdV-mTOR-KD, or AdV-GFP as a control were made quiescent and then incubated for 24 h with 3 mM glucose and 15 mM glucose/IGF-1, alone or in the presence of 50 nM rapamycin (A) or 10 μmol/liter of lactacystin for the last 6 h (B) as indicated. The cell lysates were resolved by 10% SDS-PAGE and immunoblotted (as described under “Experimental Procedures”) with IRS-2 and p85. The results shown are representative of four (A) or six (B) independent experiments. Quantification of IRS-2 protein levels was performed by densitometric scanning with Optiquant software analysis and expressed as mean ± S.E. of at least four individual experiments. IB, immunoblot.

**mTOR-mediated Decrease in IRS-2 Protein Levels Correlates with Down-regulation of IGF-1/Glucose-induced PKB Phosphorylation Activation**—To determine whether the mTOR-mediated decrease in IRS-2 protein levels results in downregulation of downstream signaling, we examined the
phosphorylation activation of PKB and ERK-1/2 in AdV-mTORΔ, AdV-mTOR-KD, or AdV-GFP as a control were made quiescent and then incubated for 24 h with 3 mM glucose and 15 mM glucose ± 5 nM IGF-1, alone or in the presence of 50 nM rapamycin (A) or 10 μM/liter of lactacystin for the last 6 h (B) as indicated. The cell lysates were resolved by 10% SDS-PAGE and immunoblotted (as described under “Experimental Procedures”) with antibodies against phospho-Ser473-PKB and phospho-ERK-1/2. The same blots were reprobed with PKB and ERK-1/2 as indicated. The results shown are representative of at least four independent experiments. IB, immunoblot.

Fig. 8. Proteasomal degradation of IRS-2 via mTOR down-regulates IGF-1/PI3K/PKB-mediated signaling pathway. INS-1 cells infected with AdV-mTORΔ, AdV-mTOR-KD, or AdV-GFP as a control were made quiescent and then incubated for 24 h with 3 mM glucose and 15 mM glucose/IGF-1, alone or in the presence of 50 nM rapamycin (A) or 10 μM/liter of lactacystin for the last 6 h (B) as indicated. The cell lysates were resolved by 10% SDS-PAGE and immunoblotted (as described under “Experimental Procedures”) with antibodies against phospho-Ser473-PKB and phospho-ERK-1/2. The same blots were reprobed with PKB and ERK-1/2 as indicated. The results shown are representative of at least four independent experiments. IB, immunoblot.

Chronic Activation of mTOR Decreases Cell Survival—Decreasing IRS-2 expression levels promotes β-cell apoptosis (4, 5, 9). Thus, it was investigated whether the mTOR-mediated decrease in IRS-2 protein levels was associated with an increase in β-cell apoptosis. The degree of apoptosis was indicated by immunoblot analysis of cleaved/activated caspase-9. The final stages of apoptosis are executed by the family of

FIG. 8. Proteasomal degradation of IRS-2 via mTOR down-regulates IGF-1/PI3K/PKB-mediated signaling pathway. INS-1 cells infected with AdV-mTORΔ, AdV-mTOR-KD, or AdV-GFP as a control were made quiescent and then incubated for 24 h with 3 mM glucose and 15 mM glucose/IGF-1, alone or in the presence of 50 nM rapamycin (A) or 10 μM/liter of lactacystin for the last 6 h (B) as indicated. The cell lysates were resolved by 10% SDS-PAGE and immunoblotted (as described under “Experimental Procedures”) with antibodies against phospho-Ser473-PKB and phospho-ERK-1/2. The same blots were reprobed with PKB and ERK-1/2 as indicated. The results shown are representative of at least four independent experiments. IB, immunoblot.

Chronic Activation of mTOR Decreases Cell Survival—Decreasing IRS-2 expression levels promotes β-cell apoptosis (4, 5, 9). Thus, it was investigated whether the mTOR-mediated decrease in IRS-2 protein levels was associated with an increase in β-cell apoptosis. The degree of apoptosis was indicated by immunoblot analysis of cleaved/activated caspase-9. The final stages of apoptosis are executed by the family of
caspases, including the initiator caspase-9 that is proteolytically cleaved from inert procaspase-9 (47). The amount of cleaved caspase-9 has been correlated to the degree of \( \text{H}9252 \text{-cell apoptosis} \) (9, 22). The AdV-mTOR and AdV-mTOR-KD or the control AdV-GFP-infected INS-1 cells were incubated at basal 3 mM glucose for 16 h and then for a further 24 h at basal 3 mM or stimulatory 15 mM glucose \( \pm 5 \text{nM IGF-1} \). A low degree of basal caspase-9 activation was seen in AdV-GFP-infected control INS-1 cells that was not significantly changed between 3 or 15 mM glucose, or 15 mM glucose \( \pm 5 \text{nM IGF-1} \) (Fig. 9, A and B). However, in AdV-mTORΔ-infected cells, activated caspase-9 was significantly increased compared with that in control AdV-GFP-infected cells \( (p < 0.05; \text{Fig. 9B}) \). In contrast, in AdV-mTOR-KD-infected cells minimal activated caspase-9 was detected, even below the degree of apoptosis found in AdV-GFP-infected control INS-1 cells, whether at basal 3 or 15 mM glucose, or 15 mM...
glucose + IGF-1 (Fig. 9, A and B). Phosphorylation activation of PKB was assessed in the same INS-1 cell lysates. Consistent with the observations in Fig. 8, PKB Ser473 phosphorylation activation induced by glucose/IGF-1 was reduced in AdV-mTORΔ-infected cells and remained unchanged in AdV-mTOR-KD -infected INS-1 cells compared with that in AdV-GFP-infected control cells (Fig. 9A). Total PKB protein levels were similar between samples (Fig. 9A), emphasizing the specific effect on PKB and caspase-9 activation. Immunocytochemical analysis of activated caspase-9 in AdV-mTORΔ, AdV-mTOR-KD, and AdV-Luc control-infected INS-1 cells incubated for 36 h at basal 3 mM glucose are shown in Fig. 9C. Immunofluorescence analysis of activated caspase-9 was markedly increased in AdV-mTORΔ-infected cells compared with that in AdV-Luc control cells. In contrast, the immunofluorescence analysis of activated caspase-9 revealed was almost undetectable in AdV-mTOR-KD-infected cells (Fig. 9C), in accordance with the low incidence of activated caspase-9 by immunoblot analysis even below that of AdV-Luc-infected control INS-1 cells (Fig. 9, A and B).

**DISCUSSION**

Here, we have demonstrated that chronic activation of mTOR leads to an increased Ser/Thr phosphorylation state of IRS-2 in pancreatic β-cells. Activation of IRS-mediated signaling pathways requires tyrosine phosphorylation of IRS molecules by certain tyrosine kinase activities (e.g. ligand-activated insulin or IGF-1 receptors (2, 48)). The phosphorylated tyrosine motifs on IRS proteins act as docking sites for Src homology domain 2 containing proteins (such as the p85 regulatory subunit of PI3K or Grb2) to associate with IRS and consequently become activated (2). There are actually more potential Ser/Thr phosphorylation sites on IRS proteins than there are tyrosine phosphorylation sites, but the functional consequence of these has not yet been well characterized. It appears that a few Ser/Thr phosphorylation sites on IRS might have a positive role in potentiating IRS-mediated signal transduction, but most appear to have a negative role opposing the effect of tyrosine phosphorylation of IRS proteins (26). Indeed, a common mechanism for down-regulating IRS-mediated signaling pathways is via Ser/Thr phosphorylation of IRS-1 or IRS-2 (26). In insulin responsive cell types, the Ser/Thr phosphorylation of IRS-1 can disrupt the association between the insulin receptor and IRS and inhibit tyrosine phosphorylation (49–51), and/or can facilitate a subcellular redistribution of IRS linked to its ubiquitin-mediated proteasomal degradation (27, 28, 52).

Several Ser/Thr kinases (e.g. certain PCK isoforms, mTOR, JNK, IKKβ, and mitogen-activated protein kinase (26)) have been described to promote Ser/Thr phosphorylation of the IRS proteins in insulin target tissues such as myocytes, hepatocytes, and adipocytes (24, 25, 27, 28, 53, 54). Here, we find in pancreatic β-cells that chronic activation of the mTOR Ser/Thr protein kinase by glucose/IGF-1, or via adenoviral-mediated expression of a constitutively active variant of mTOR, specifically increased the Ser/Thr phosphorylation state of IRS-2 that was correlated with a decrease in IRS-2 protein expression levels. Conversely, inhibition of mTOR activity, by either rapamycin or the expression of the kinase-dead mTOR variant, inhibited Ser/Thr phosphorylation of IRS-2 and correlated with increased IRS-2 protein levels in β-cells. Although we were unable to directly test whether IRS-2 is ubiquitinatated in β-cells, the use of the proteasomal degradation inhibitor, lactacystin, indicated that the increased Ser/Thr phosphorylation state of IRS-2 mediated by mTOR was associated with IRS-2 ubiquitination. Lactacystin upwardly shifted the electrophoretic mobility of IRS-2 caused by glucose or IGF-1, and this could never be completely resolved by alkaline phosphatase treatment. Thus, another post-translational modification was contributing to the apparent increased molecular mass of IRS-2 in addition to the Ser/Thr phosphorylation, and was most likely an ubiquitination. Moreover, lactacystin prevented the decrease in IRS-2 protein levels in AdV-mTORΔ-infected cells. This, in turn, indicated that the decrease in β-cell IRS-2 protein levels associated with mTOR-mediated Ser/Thr phosphorylation of IRS-2 was most likely via ubiquitin-dependent proteasomal degradation, rather than any effect at the level of IRS-2 protein synthesis. Intriguingly, a similar pathway has been described in adipocytes (45), where Ser/Thr-phosphorylated IRS-2 become ubiquitinated and are subsequently degraded by the 26 S proteasome in response to a chronic insulin stimulation or cellular stress (46).

Although it was observed that mTOR is involved in Ser/Thr phosphorylation of IRS-2, it is not yet clear whether mTOR directly phosphorylates IRS-2 in pancreatic β-cells. It has recently been revealed that other protein factors, such as raptor and mLST8, are required to modify mTOR activity and/or present substrates such as 4E-BP1 and p70S6K to mTOR for Ser/Thr phosphorylation (55–57). The increased expression of a constitutively active mTOR variant markedly increased IRS-2 Ser/Thr phosphorylation in β-cells, apparently independently of a need for protein cofactors of mTOR. Thus, these mTOR cofactors are expressed in excess in β-cells that cater for the chronic activation of mTOR, or that another protein kinase(s) lies between mTOR and IRS-2/Ser/Thr phosphorylation in an as of yet undefined signaling pathway. Nonetheless, the observation that rapamycin inhibits Ser/Thr phosphorylation of IRS-2 in β-cells, as well as preserves IRS-2 protein expression levels, indicates that mTOR plays a critical part in instigating Ser/Thr phosphorylation of IRS-2 and its subsequent proteasomal degradation. It should be noted that the mTORΔ variant was able to increase phosphorylation of p70S6K and 4E-BP1, particularly under basal conditions, consistent with its degree of constitutive activation. This suggested that there were sufficient protein cofactors of mTOR expressed in β-cells for suitable p70S6K and 4E-BP phosphorylation activation. Another note should be made that the constitutively activated mTORΔ variant was inhibited by rapamycin that is consistent with the so-called rapamycin-binding FRB domain being retained in the mTORΔ variant (41, 43, 57).

Maintenance of optimal IRS-2 expression levels in β-cells and downstream activation of the PI3K/PKB signaling is essential for promoting β-cell survival (10). The mTOR-mediated decrease in IRS-2 protein levels in β-cells was also associated with decreased glucose/IGF-1-induced PKB phosphorylation activation. Both rapamycin and lactacystin prevented the IRS-2 protein degradation and rescued PKB phosphorylation activation in response to glucose/IGF-1, indicating that the extent of PKB activation is linked to the level of IRS-2 expression. This is corroborated by previous findings that increased expression of IRS-2 enhanced PKB phosphorylation activation and prevented β-cells from FFA-increased apoptosis (9), while decreasing expression of IRS-2 using adeno viral-mediated expression of IRS-2 antisense-inhibited PKB phosphorylation activation and induced β-cell apoptosis (9). Adenoviral-mediated expression of constitutively active PKB in β-cells is also protective against FFA-induced apoptosis (22).

In this study, it was also found that decreased IRS-2 protein expression in β-cells mediated via chronic activation of mTOR correlated with decreased PKB phosphorylation activation and that this, in turn was associated with increased caspase-9 activation, a marker of β-cell apoptosis. Thus, there is a general interrelationship between IRS-2 expression and downstream activation of PKB in β-cells, which is key to maintaining β-cell
survival. However, a small proviso should be made in regard to the combination of stimulatory glucose and IGF-1 that occasion-ally causes a modest decrease in IRS-2 protein levels (Fig. 8), yet normal PKB phosphorylation activation was maintained (Fig. 8). In this instance it should be noted that glucose can activate mTOR independently of IRS-2 in β-cells (30), and is a relatively poor activating stimulus for PKB requiring >4-h exposure to glucose (29). In contrast, IGF-1 induced activation of PKB and mTOR in β-cells is IRS-2 dependent, and IGF-1 renders a rapid and sustained activation of PKB in β-cells within minutes (7, 29, 30). As such, although there may be reduced IRS-2/glucose protein expression levels, mediated by chronic glucose/IGF-1 induced activation of mTOR, IGF-1 (but not necessarily glucose) is still able to give an effective activation of PKB via the residual IRS-2 present in β-cells. Notwithstanding, in contrast to the mTOR-mediated decrease in IRS-2 protein levels generally correlating with decreased PKB phosphorylation, there was no effect on ERK-1/2 phosphorylation activation in the same β-cells. This underlines the importance of the specific glucose-induced activation of ERK-1/2 in β-cells that is independent of IRS-2 (30, 31). Moreover, it reaffirms that the ERK-1/2 signaling pathway is not necessarily involved in maintaining β-cell survival (9, 10).

The reduction of β-cell mass via increased β-cell apoptosis is key to the pathogenesis of type 2 diabetes (10, 11, 13). Chronic hyperglycemia has been proposed to be at least one of the major factors that instigates β-cell apoptosis. Indeed, in vitro studies have shown that chronically elevated concentrations of glucose induce β-cell apoptosis (17–20). In contrast, over a shorter duration of exposure. It has been previously shown that glucose can independently activate mTOR in β-cells and this is further augmented by IGF-1-induced signaling via IRS-2 (29, 30). It is therefore possible to envisage that chronic exposure to elevated glucose concentrations might induce a decrease in IRS-2 protein levels, at least in part, via chronic activation of mTOR to mediate increased IRS-2 Ser/Thr phosphorylation, leading to proapoptotic degradation of IRS-2, decreased β-cell IRS-2 levels, and increased β-cell apoptosis. This potential mechanism has yet to be confirmed in primary β-cells and/or in models of type 2 diabetes. However, it raises the prospect that inhibition of mTOR using rapamycin might have a role in preserving β-cell mass as a potential therapy for type 2 diabetes. In this regard, it should be noted that as well as having immunosuppressive activity, rapamycin should also promote β-cell survival of transplanted islets used as a therapy for type 1 diabetes (59). Finally, considering that hyperglycemia and/or hyperinsulinemia also promotes mTOR-mediated Ser/Thr phosphorylation of IRS-1/2 and subsequent IRS-1/2 degradation in insulin target tissues that in turn contributes to the mechanism of insulin resistance (26–28, 36, 37), a therapeutic strategy to protect β-cells by inhibiting mTOR should also alleviate a degree of insulin resistance and perhaps even delay the onset of type 2 diabetes. Notwithstanding, our findings in this study further emphasize the importance that control of IRS-2 levels in pancreatic β-cells is critical for maintaining their survival and plays a pivotal point in the pathogenesis of type 2 diabetes.

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Insulin Receptor Substrate-2 Proteasomal Degradation Mediated by a Mammalian Target of Rapamycin (mTOR)-induced Negative Feedback Down-regulates Protein Kinase B-mediated Signaling Pathway in β-Cells

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