

Glucose-stimulated Protein Synthesis in Pancreatic β -Cells Parallels an Increase in the Availability of the Translational Ternary Complex (eIF2-GTP-Met-tRNA_i) and the Dephosphorylation of eIF2 α *

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In pancreatic β -cells, glucose causes a rapid increase in the rate of protein synthesis. However, the mechanism by which this occurs is poorly understood. In this report, we demonstrate, in the pancreatic β -cell line MIN6, that glucose stimulates the recruitment of ribosomes onto the mRNA, indicative of an increase in the rate of the initiation step of protein synthesis. This increase in the rate of initiation is not mediated through an increase in the availability of the initiation complex eIF4F, because glucose is unable to stimulate eIF4F assembly or, in the absence of amino acids, modulate the phosphorylation status of 4E-BP1. Moreover, in MIN6 cells and isolated islets of Langerhans, rapamycin, an inhibitor of the mammalian target of rapamycin, only partially inhibited glucose-stimulated protein synthesis. However, we show that glucose stimulates the dephosphorylation of eIF2 α in MIN6 cells and the assembly of the translational ternary complex, eIF2-GTP-Met-tRNA_i, in both MIN6 cells and islets of Langerhans. The changes in the phosphorylation of eIF2 α are not mediated by the PKR-like endoplasmic reticulum eIF2 α kinase (PERK), because PERK is not phosphorylated at low glucose concentrations and overexpression of a dominant negative form of PERK has no significant effect on either glucose-stimulated protein synthesis or the phosphorylation of eIF2 α . Taken together, these results indicate that glucose-stimulated protein synthesis in pancreatic β -cells is regulated by a mechanism largely independent of the activity of mammalian target of rapamycin, but which is likely to be dependent on the availability of the translational ternary complex, regulated by the phosphorylation status of eIF2 α .

The pancreatic β -cell releases insulin in response to glucose. Additionally, there is a rapid 2-fold increase in total protein synthesis, a 20-fold increase in proinsulin synthesis, and a co-ordinate increase in the synthesis of a small subset of pro-

teins, including the prohormone convertases PC2 and PC3 (1–9). These rapid increases in protein synthesis are almost entirely mediated at the translational level (1–4, 6, 7, 10) and ensure the replenishment of secreted proteins and the maintenance of β -cell mass. However, the mechanism by which glucose regulates protein synthesis in pancreatic β -cells is poorly understood.

One key step in the regulation of protein synthesis is the recruitment of the 40 S ribosome to the mRNA. This is promoted by the initiation complex, eIF4F,¹ composed of the initiation factors eIF4G, a large scaffolding protein, eIF4E, the protein that binds to the 5'-cap structure (7mGTP) of the mRNA, and eIF4A, an RNA helicase (11). An important mechanism regulating eIF4F assembly involves a family of eIF4E-binding proteins (4E-BPs, archetype 4E-BP1) (12, 13). In their hypophosphorylated forms, 4E-BP1 competes with eIF4G for binding to eIF4E and can therefore inhibit eIF4F complex formation. Phosphorylation of 4E-BP1 leads to its dissociation from eIF4E, therefore allowing eIF4E to bind eIF4G, potentially leading to an increase in mRNA translation (14–16). In islets and in the pancreatic β -cell lines RINm5F and β TC6-F7, glucose can, in synergy with amino acids and insulin released into the medium, increase the phosphorylation of 4E-BP1 (9), indicating that the regulation of eIF4F assembly may be important in glucose-stimulated protein synthesis. However, in islets, glucose can stimulate protein synthesis in the absence of amino acids (17–19), under conditions that do not lead to the dissociation of 4E-BP1 from eIF4E (20), a step considered a prerequisite for eIF4F assembly.

Another important rate-limiting step in the regulation of protein synthesis is the binding of the methionyl-initiator tRNA (Met-tRNA_i) to the 40 S ribosomal subunit, regulated by the availability of the translational ternary complex (eIF2-GTP-Met-tRNA_i), consisting of the heterotrimeric eukaryotic initiation factor 2 (eIF2), GTP, and Met-tRNA_i (21). The binding of the Met-tRNA_i to the 40 S ribosomal subunit is dependent on the formation of the translational ternary complex that assembles only when eIF2 is in its GTP-bound state (21, 22). The 40 S ribosomal subunit, in association with a number of

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¹ The abbreviations used are: eIF, eukaryotic initiation factor; 4E-BP1, eIF4E binding protein 1; MIN6, mouse insulinoma cell line 6; Erk, extracellular signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium minus glucose; KRB, Krebs-Ringer bicarbonate buffer; mTOR, mammalian target of rapamycin; PKR, protein kinase activated by RNA; PERK, PKR-like endoplasmic reticulum kinase; eIF2, eukaryotic elongation factor-2; ER, endoplasmic reticulum; mGCN2, mammalian general control non-derepressible 2; UTR, untranslated region; EGFP, enhanced green fluorescent protein; PABP, poly(A)-binding protein.

initiation factors (including the eIF4F complex), delivers the eIF2-GTP-bound Met-tRNA_i to the AUG start codon. This is followed by the joining of the 60 S ribosomal subunit, leading to the formation of the 80 S ribosome. The GTP bound to eIF2 is then hydrolyzed to GDP and P_i, yielding the inactive eIF2-GDP complex, which is unable to bind Met-tRNA_i. The recycling of the inactive eIF2-GDP complex to the active eIF2-GTP complex is catalyzed by the guanine nucleotide exchange factor, eIF2B (21, 23). eIF2B plays a crucial role in ternary complex formation and is regulated in response to a wide range of conditions by several mechanisms, including phosphorylation, allosteric regulation, and competitive inhibition by the phosphorylated form of the α -subunit of eIF2 (eIF2 α) (21). Interestingly, eIF2B activity is transiently up-regulated in isolated islets of Langerhans in response to glucose (20). Therefore, glucose may be an important modulator of ternary complex formation and hence protein synthesis in pancreatic β -cells.

Competitive inhibition of eIF2B by the phosphorylated form of eIF2 α is the most studied and best characterized example by which eIF2B activity is modulated. Several eIF2 kinases have been identified in mammals: the double-stranded RNA-dependent eIF2 kinase (PKR), the mammalian orthologue of the yeast GCN2 protein kinase (mGCN2), the heme-regulated eIF2 kinase (HRI), and the eIF2 kinase-like endoplasmic reticulum kinase (PERK). These kinases respond to different physiological stimuli. For example, PKR is induced by interferon and regulated by double-stranded RNA (24, 25). (m)GCN2 is activated under conditions of amino acid and glucose starvation (21, 26, 27). HRI is regulated by heme in erythroid cells (28). PERK is highly expressed in the pancreas and is activated by endoplasmic reticulum stress (29–31). Interestingly, PERK plays an important role in pancreatic β -cell survival, because PERK knock-out mice develop diabetes mellitus due to the loss of pancreatic β -cells (32). Additionally, islets isolated from prediabetic PERK knock-out mice have increased glucose-stimulated proinsulin synthesis (32), indicating that eIF2 α phosphorylation may play a role in the specific regulation of proinsulin synthesis.

In this report, we demonstrate that glucose-stimulated protein synthesis in pancreatic β -cells is accompanied by an increase in the rate of initiation. This increase is not mediated through an increase in the assembly of the eIF4F complex and is only partially dependent on the activity of mTOR. However, we show that glucose stimulates an increase in the availability of the translational ternary complex and the dephosphorylation of eIF2 α . Therefore, taken together, glucose-stimulated protein synthesis in pancreatic β -cells is likely the result of an increase in the rate of initiation mediated through an increase in the availability of the translational ternary complex, brought about by the dephosphorylation of eIF2 α .

EXPERIMENTAL PROCEDURES

Chemicals and Materials—Rapamycin was purchased from Calbiochem. m⁷GTP-Sepharose and RNAGuard™ were from Amersham Biosciences. Dulbecco's modified Eagle's medium without glucose (DMEM) and fetal calf serum were obtained from Invitrogen. All other chemicals (unless stated) were obtained from Sigma.

Cell Culture and Treatment—In this study, MIN6 cells (kindly provided by Prof. Jun-Ichi Miyazaki) were used between passages 16 and 35 at ~80% confluence. MIN6 cells were grown in DMEM containing 25 mM glucose (DMEM full media) supplemented with 15% heat-inactivated fetal calf serum, 100 μ g/ml streptomycin, 100 units/ml penicillin sulfate, and 75 μ M β -mercaptoethanol, equilibrated with 5% CO₂, 95% air at 37 °C. Prior to treatment, the medium was removed and the cells washed twice with either HEPES-balanced Krebs-Ringer bicarbonate buffer (115 mM NaCl, 5 mM KCl, 10 mM NaHCO₃, 2.5 mM MgCl₂, 2.5 mM CaCl₂, 20 mM HEPES, pH 7.4) containing 0.5% bovine serum albumin (KRB buffer) or DMEM minus glucose (DMEM). The cells were then incubated for 1 h at 37 °C in KRB buffer or DMEM containing 2 mM

glucose (unless otherwise stated) prior to incubation in KRB buffer or DMEM containing 2 or 20 mM glucose for a further hour (unless otherwise stated). Full details of treatments are provided in the figure legends. After treatment, cells were washed twice in ice-cold phosphate-buffered saline, and the cells were lysed by the addition of ice-cold lysis buffer containing 1% Triton X-100, 10 mM β -glycerophosphate, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM benzamidine-HCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 μ g/ml each of leupeptin and pepstatin, 0.1% β -mercaptoethanol, and 50 mM sodium fluoride. The lysates were then centrifuged for 10 min at 16,000 \times g. The supernatants were kept, and total protein concentrations were determined by the Bradford assay (Bio-Rad) using bovine serum albumin as standard. The protein lysates were stored at -80 °C until further analysis.

Sedimentation Gradients and Polysome Analysis—MIN6 cells were incubated for 1 h in either KRB or DMEM supplemented with 2 mM glucose prior to the cells being incubated for a further 1 h in either KRB or DMEM supplemented with either 2 or 20 mM glucose. Cycloheximide was added for the last 5 min to a final concentration of 0.1 mg/ml. After treatment, the cells were scraped into polysome extraction buffer (1% Triton X-100, 20 mM HEPES, pH 7.6, 2 mM MgCl₂, 130 mM KCl, 1 mg/ml heparin, 1 mg/ml cycloheximide, 1 mM dithiothreitol, 1 μ l/ml RNAGuard) and lysed using 15 strokes of a potter homogenizer. Cell lysates were centrifuged for 10 min at 13,000 \times g at 4 °C to remove nuclei and cellular debris. The supernatants were then layered onto 7–47% sucrose gradients (sucrose, 20 mM HEPES, pH 7.6, 2 mM MgCl₂, 130 mM KCl, 1 mg/ml heparin, 1 mg/ml cycloheximide, 1 mM dithiothreitol) and centrifuged for 2 h at 39,000 rpm at 4 °C in a TH64.1 rotor (Sorvall). The gradients were pumped, by upward displacement, through a UV monitor, and the absorbance was read at 254 nm using an ISCO gradient collector.

Islet Isolation and Culturing—Pancreatic islets were isolated from 200- to 250-g female Wistar rats by collagenase digestion and Hispotaque density gradient centrifugation as previously described (3). Islets were either used immediately or were cultured in RPMI 1640 containing 5.6 mM glucose with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin (see figure legends for details). Prior to treatment, islets were washed twice with either KRB buffer or DMEM minus glucose. The cells were then incubated for 2 h at 37 °C in KRB buffer or DMEM containing 2 mM glucose prior to incubation in KRB buffer or DMEM containing 2 or 20 mM glucose for the times indicated in the figure legends (full details of treatments are provided in the figure legends). After treatment, islets were collected by centrifugation for 1 min at 16,000 \times g and lysed by the addition of ice-cold lysis buffer.

Adenovirus Generation and Adenoviral Infection—cDNA encoding the firefly luciferase reporter downstream of the 5'-UTR of ATF4 and cDNA encoding amino acids 1–583 of PERK (PERK Δ C) were subcloned from the plasmids TK::ATF4UTR::LUC (30) and PERK.WT.9E10.pCDNA.amp (29), respectively, kindly provided by Prof. David Ron (Skirball Institute, New York University School of Medicine), into the shuttle vector pAd-Track-CMV prior to the generation of recombinant adenoviruses. Recombinant adenoviruses expressing ATF4 luciferase (Ad-ATF4luc) and PERK Δ C (Ad-PERK Δ C) were then prepared using the Ad-easy system (33) as previously described (34). The adenoviral and shuttle vectors were provided by Prof. Tong-Chuan He, Howard Hughes Medical Institute & Johns Hopkins Oncology Center. Recombinant adenoviruses containing EGFP (Ad-emptyEGFP) were a gift from Dr. Jo Carter (University of Leicester).

To infect MIN6 cells or islets of Langerhans, the recombinant adenoviruses were mixed with culture medium, and cells were exposed to the viruses with a 100–200 multiplicity of infection for 24–48 h prior to the experiment. Under these conditions, >90% of cells were infected, as determined by EGFP expression.

Protein Synthesis Measurements—Protein synthesis measurements were performed as described previously (35). In these experiments, the cell treatments were performed in the presence of [³⁵S]methionine for 1 h. All treatments were stopped by washing the cells with ice-cold phosphate-buffered saline and lysing them in ice-cold extraction buffer (as described under "Cell Culture and Treatment"). Cell lysates were prepared, and equal amounts of protein (about 20 μ g) were spotted onto 3MM Whatman filters. The filters were then washed by boiling them for 1 min in 5% trichloroacetic acid containing 0.1g/liter methionine. This was repeated three times. The filters were then dried and immersed in scintillant before determining radioactivity by scintillation counting.

SDS-PAGE, Western Blotting, and Immunoprecipitation—SDS-PAGE and Western blotting were performed as described previously (35). The anti-eIF2 α , anti-4E-BP1, anti-eIF4G1, anti-eIF4A, and anti-

poly(A)-binding protein (PABP) antibodies were generous gifts from Chris Proud (Dundee University), Adri Thomas (Utrecht University), Simon Morley (Sussex University), H. Trachsel (Bern University), and Amelia Nieto (Centro Nacional de Biotecnología Cantoblanco, Madrid), respectively. Anti-phospho (Ser-65 and Thr-36/45) 4E-BP1, anti-eIF4E, anti-phospho (Thr-980) PERK, anti-phospho (Ser-235/236) ribosomal protein S6 and anti-Erk2 antisera were purchased from New England Biolabs. Anti-luciferase antiserum was purchased from Chemicon. Anti-phospho (Ser-51) eIF2 α was purchased from Biosource. Detection was by horseradish peroxidase-linked anti-rabbit/mouse secondary antibodies and enhanced chemiluminescence (Amersham Biosciences). Briefly, immunoprecipitations of luciferase were performed as follows. Anti-luciferase antibody was coupled to Protein G-Sepharose for 1 h at room temperature. The bound antibody/protein G complexes were then added to [³⁵S]methionine-labeled MIN6 protein lysates and rotated for 2 h at 4 °C. The beads were then washed three times in radioimmune precipitation assay buffer, and the bound proteins were finally eluted into Laemmli sample buffer (36) prior to being separated by SDS-PAGE. The [³⁵S]methionine-labeled luciferase was then visualized by autoradiography.

m⁷GTP-Sepharose Chromatography—*m⁷GTP-Sepharose* chromatography was performed as described previously (37). Briefly, equal amounts of protein (0.3–0.4 mg) were incubated with 30 μ l of *m⁷GTP-Sepharose* CL-6B for 1 h at 4 °C. The beads were then washed three times in lysis buffer, and the bound proteins were separated on a 12.5% SDS-polyacrylamide gel and visualized by immunoblotting using antisera against eIF4E, eIF4G1, 4E-BP1, eIF4A, and PABP.

RESULTS

Glucose-stimulated Protein Synthesis Is Mediated through an Increase in the Rate of Initiation—To investigate the mechanism by which glucose regulates protein synthesis in pancreatic β -cells, we initially determined the rates of protein synthesis at either low or high glucose concentrations in the pancreatic β -cell line MIN6, a cell line that synthesizes and secretes insulin in response to physiologically relevant glucose concentrations (6, 38).

MIN6 cells were preincubated for 1 h in either KRB or DMEM (*i.e.* in the absence or presence of amino acids) containing 2 mM glucose and then further incubated for 1 h at either 2 or 20 mM glucose in the presence or absence of the transcriptional inhibitor actinomycin-D. Increasing glucose concentration from 2 to 20 mM led to a significant increase in [³⁵S]methionine incorporation into total protein in cells incubated in either KRB or DMEM. These increases were unaffected by the addition of actinomycin-D, demonstrating that glucose-stimulated protein synthesis is mediated by a post-transcriptional mechanism (Fig. 1). These results are in agreement with and extend previous findings in MIN6 cells (6).

To establish which step in protein synthesis may be important in glucose-stimulated protein synthesis, we determined the polysome profile of MIN6 cells incubated at both 2 and 20 mM glucose in either KRB or DMEM (Fig. 2). In cells incubated in either KRB or DMEM, increasing glucose concentration from 2 to 20 mM led to a decrease in the 80 S monosomal peak along with an increase in the number of polysomes, indicative of an increase in the rate of the initiation phase of protein synthesis. Additionally, the polysome to monosome ratio at either low or high glucose concentrations is greater when cells are incubated in DMEM compared with those incubated in KRB. This indicates that the basal rates of protein synthesis are higher in cells incubated in DMEM than those incubated in KRB.

The Role of mTOR in Glucose-stimulated Protein Synthesis in MIN6 Cells—Amino acids and glucose have been shown to influence the rate of protein synthesis in a number of cell types through the modulation of the phosphorylation status of 4E-BP1 and p70 S6 kinase via a mechanism that is dependent on mTOR. We therefore investigated the role of mTOR in glucose-stimulated protein synthesis in MIN6 cells. MIN6 cells were preincubated for 1 h in either KRB or DMEM supplemented with 2 mM glucose and then further incubated for 1 h in the

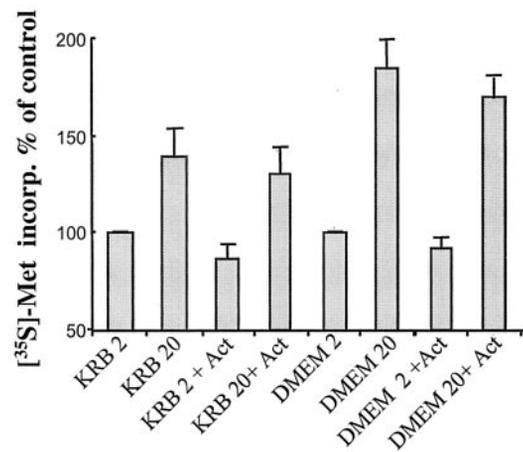


FIG. 1. **Glucose regulation of protein synthesis in the pancreatic β -cell line, MIN6.** MIN6 cells were preincubated for 1 h in KRB or DMEM supplemented with 2 mM glucose. The cells were then treated for 1 h in the same medium, KRB or DMEM, containing either 2 or 20 mM (2 or 20) glucose and [³⁵S]methionine. Where indicated, MIN6 cells were also incubated in the presence of the transcriptional inhibitor actinomycin-D (1 μ g/ml, *Act*) for the last 30 min of the preincubation and during the hour treatment. Total protein was precipitated from cell lysates with trichloroacetic acid (see “Experimental Procedures”), and the incorporation of [³⁵S]methionine into protein was expressed as the percentage of control (KRB 2 mM) ($n = 3 \pm$ S.D.).

same medium (KRB or DMEM) at either 2 mM or 20 mM glucose in the presence or absence of rapamycin, a specific inhibitor of mTOR (Fig. 3). Increasing glucose concentration in cells incubated in KRB (*i.e.* in the absence of amino acids) had no effect on the phosphorylation of either p70 S6 kinase or 4E-BP1 as assessed by determining: 1) the mobility of both 4E-BP1 and p70 S6 kinase on SDS-PAGE; 2) the phosphorylation of specific residues within 4E-BP1, Thr-36/45 and Ser-65; and 3) the phosphorylation of ribosomal protein S6, a substrate for p70 S6 kinase (Fig. 3a). In contrast, in MIN6 cells incubated in DMEM, an increase in glucose concentration led to: 1) an increase in the phosphorylation of both 4E-BP1 and p70 S6 kinase, demonstrated by their decrease in mobility on SDS-PAGE; 2) the phosphorylation of residues Thr-36/45 and Ser-65 within 4E-BP1; and 3) an increase in the phosphorylation of ribosomal protein S6 at Ser-235/236 (Fig. 3a). In this latter condition (*i.e.* in the presence of amino acids), rapamycin completely blocked both the basal and glucose-stimulated phosphorylation of p70 S6 kinase, 4E-BP1, and S6, indicating that mTOR plays an important role in these events (Fig. 3a). Indeed, it has been previously demonstrated that amino acids are required for glucose-stimulated phosphorylation of downstream targets of mTOR in the pancreatic β -cell line RINm5F cells and in islets (39), and this explains why glucose cannot stimulate the phosphorylation of the downstream targets of mTOR in KRB.

Because the phosphorylation of 4E-BP1 can lead to its release from eIF4E, an increase in eIF4F complex assembly (*i.e.* an increase in the association of eIF4G and eIF4A with eIF4E) and hence the rate of initiation, we therefore examined the effect of glucose on eIF4F assembly by pulling down eIF4E and associated proteins using 7mGTP-Sepharose beads (Fig. 3b). In KRB (*i.e.* in the absence of amino acids), an increase in glucose concentration from 2 to 20 mM had no detectable effect on the dissociation of 4E-BP1 from eIF4E, on eIF4F assembly, or on the association of PABP with the eIF4F complex. Additionally, treatment of cells with rapamycin had no significant effect on glucose-stimulated protein synthesis in KRB (Fig. 3c). Therefore, the activation of downstream targets of mTOR and the stimulation of eIF4F assembly play no detectable role in glu-

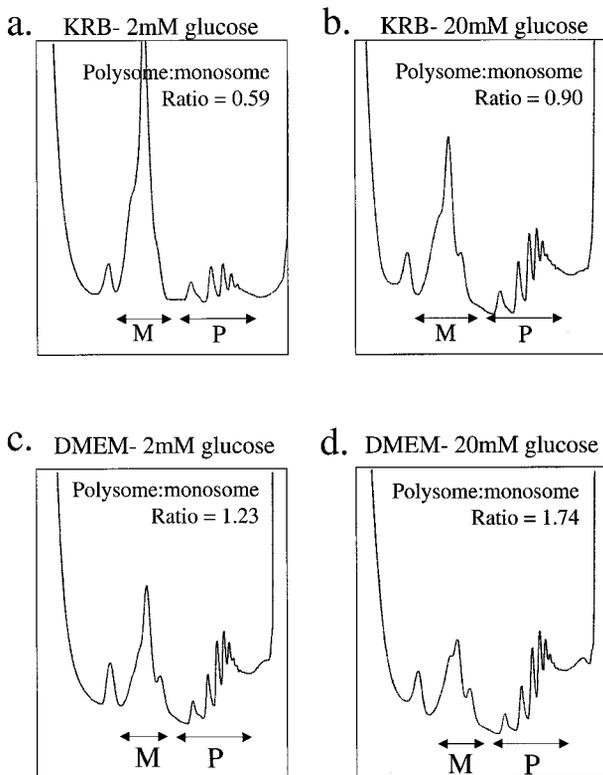


FIG. 2. Glucose stimulates protein synthesis in MIN6 cells through an increase in the rate of initiation. Analysis of polysome profiles from MIN6 cells using sucrose density gradient centrifugation. MIN6 cells were incubated for 1 h in either KRB (*a* and *b*) or DMEM (*c* and *d*) supplemented with 2 mM glucose prior to being incubated in the same medium, KRB or DMEM, at either 2 or 20 mM glucose for a further 1 h. Cell extracts were layered onto a 7–47% sucrose gradient and centrifuged at 39,000 rpm for 2 h in a TH64.1 rotor. Gradients were then fractionated while scanning at 254 nm, and the resulting profiles are shown. *M* indicates the position of 80 S monosomes. *P* indicates the position of the polysomes. The ratio of polysomes to 80 S monosomes was determined by measuring the area under the peaks. These results are representative of at least three separate experiments.

glucose-stimulated protein synthesis in KRB (*i.e.* in the absence of amino acids). In DMEM, basal levels of the eIF4F complex and the levels of associated PABP were greater than in KRB. An increase in glucose concentration from 2 to 20 mM in DMEM caused a moderate release of 4E-BP1 from eIF4E. However, glucose had no detectable effect on eIF4F assembly or the association of PABP with the eIF4F complex (Fig. 3*b*). Treatment of cells with rapamycin led to an increase in the association of 4E-BP1 with eIF4E but no dissociation of the eIF4F complex. Additionally, rapamycin inhibited glucose-stimulated protein synthesis by only 26% in DMEM (*i.e.* in the presence of amino acids) (Fig. 3*c*). Therefore, the activation of downstream targets of mTOR and the stimulation of eIF4F assembly is not the major determinant in glucose-stimulated protein synthesis in DMEM (*i.e.* in the presence of amino acids).

Glucose Stimulates the Rapid, Dose-dependent Dephosphorylation of eIF2 α —One important and well characterized mechanism by which the initiation of translation and hence protein synthesis is regulated is via the phosphorylation of the α subunit of eIF2 (eIF2 α) at Ser-51.

We therefore investigated the phosphorylation of eIF2 α in response to glucose in MIN6 cells. MIN6 cells were preincubated for 1 h in either KRB or DMEM supplemented with 2 mM glucose and then further incubated for 1 h in the same medium (*i.e.* KRB or DMEM) at either 2 or 20 mM glucose (Fig. 4*a*). Using a phospho-specific antibody to eIF2 α Ser-51, we show that eIF2 α is phosphorylated at low glucose concentration (Fig.

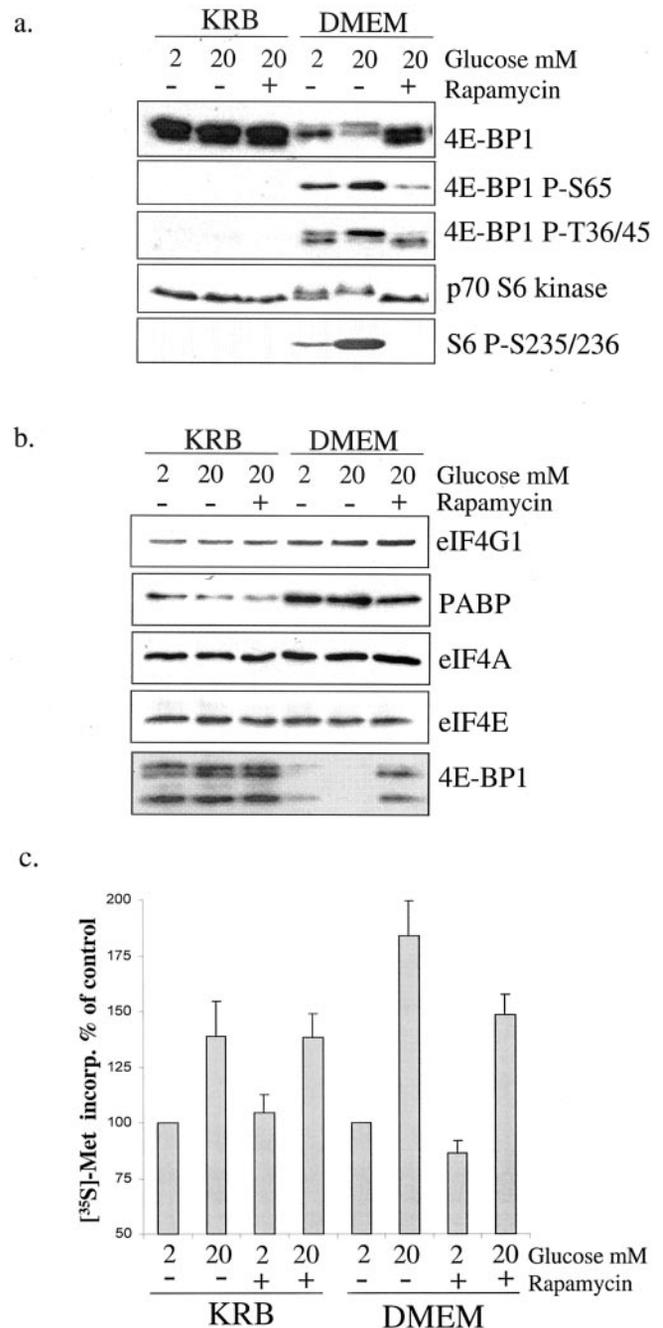
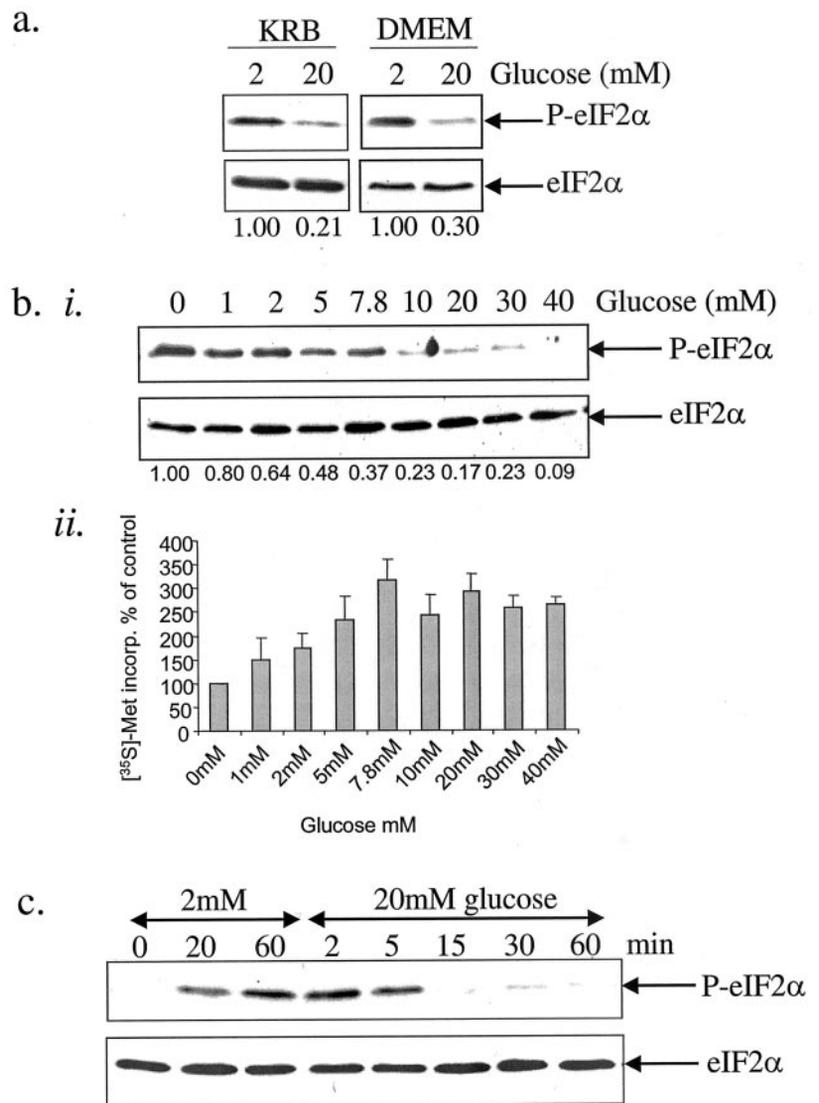


FIG. 3. The role of mTOR in glucose-stimulated protein synthesis. MIN6 cells were preincubated for 1 h in KRB or DMEM supplemented with 2 mM glucose. Where indicated, the cells were also pre-treated with 200 nM of the mTOR inhibitor rapamycin for the last 30 min. The cells were then incubated for 1 h in the same medium, KRB or DMEM, containing either 2 or 20 mM glucose and [³⁵S]methionine, in the presence or absence of 200 nM rapamycin. *a*, samples of cell lysates were run on a SDS-polyacrylamide gel followed by Western-blotting using different antisera; anti-4E-BP1 antibody (4E-BP1); anti-phospho 4E-BP1 (Ser-65) antibody (4E-BP1 P-S65); anti-phospho 4E-BP1 (Thr-36/45) (4E-BP1 P-T36/45) antibody; anti-p70 S6 kinase antibody (p70 S6 kinase); anti-phospho ribosomal protein S6 (Ser-235/236) antibody (S6 P-S235/236). *b*, analysis of proteins isolated by m⁷GTP-Sepharose pull-down and separated on a SDS-polyacrylamide gel followed by Western blotting using antisera against eIF4E, eIF4G1, eIF4A, 4E-BP1, and PABP. *c*, incorporation of [³⁵S]methionine into total protein expressed as percentage of control (KRB 2 mM) ($n = 3 \pm$ S.D.). These results are representative of three separate experiments.

4*a*). However, at high glucose concentration, eIF2 α is dephosphorylated (Fig. 4*a*). The dephosphorylation of eIF2 α was shown to occur between 2 and 10 mM, within the physiological

FIG. 4. Glucose regulates eIF2 α phosphorylation in MIN6 cells. *a*, MIN6 cells were preincubated for 1 h in KRB or DMEM supplemented with 2 mM glucose. Cells were then incubated in the same medium (KRB or DMEM) supplemented with either 2 or 20 mM glucose for a further hour. Lysates were separated on SDS-PAGE and Western-blotted using anti-phospho eIF2 α (Ser-51) (P-eIF2 α) and anti-eIF2 α (eIF2 α). The intensities of the bands were quantified by ImageJ, and the phosphorylation levels of eIF2 α were normalized to the expression levels of total eIF2 α . The results are expressed as a ratio of eIF2 α phosphorylation relative to eIF2 α phosphorylation at low glucose. *b*, MIN6 cells were preincubated in KRB buffer containing no glucose for 1 h, before incubation in KRB containing the indicated concentrations of glucose for 1 h in the presence of [³⁵S]methionine. *Panel i*, cell lysates were separated on SDS-polyacrylamide gel and Western blotted using anti-phospho eIF2 α (Ser-51) (P-eIF2 α) and anti-eIF2 α as a loading control. The intensities of the bands were quantified by ImageJ, and the phosphorylation levels of eIF2 α were normalized to the expression levels of total eIF2 α . The results are expressed as a ratio of eIF2 α phosphorylation relative to eIF2 α phosphorylation at low glucose. *Panel ii*, incorporation of [³⁵S]methionine into total protein. Results are indicated as a percentage of control (0 mM glucose). ($n = 3 \pm$ S.D.). *c*, MIN6 cells incubated in DMEM complete media (containing 25 mM glucose) were transferred to KRB buffer supplemented with 2 mM glucose for the time shown. After 60 min, the cells were then incubated in KRB supplemented with 20 mM glucose for the times indicated. Cell lysates were separated on SDS-polyacrylamide gel and Western blotted using anti-phospho eIF2 α (Ser-51) (P-eIF2 α) antibody, and as a loading control anti-eIF2 α antiserum. These results are representative of three separate experiments.



range of glucose concentrations (Fig. 4*b*, *panel i*). Additionally, decreases in the phosphorylation of eIF2 α in response to glucose paralleled increases in the rates of protein synthesis (Fig. 4*b*, *panel ii*).

The temporal kinetics of the regulation of eIF2 α phosphorylation in response to glucose were also investigated (Fig. 4*c*). Transfer of cells from DMEM complete media containing 25 mM glucose into KRB supplemented with 2 mM glucose led to the phosphorylation of eIF2 α within 20 min. After a 1-h incubation in KRB supplemented with 2 mM glucose, the cells were then further incubated with KRB supplemented with 20 mM glucose. This led to a rapid (within 15 min) dephosphorylation of eIF2 α (Fig. 4*c*).

Glucose Stimulates the Assembly of the Translational Ternary Complex—A likely consequence of eIF2 α dephosphorylation is an increase in the assembly of the translational ternary complex via an increase in the activity of eIF2B. To determine whether ternary complex formation is stimulated by glucose *in vivo*, we monitored the expression of luciferase in cells infected with recombinant adenovirus encoding luciferase downstream of the 5'-UTR of ATF4 and under the control of a cytomegalovirus promoter (Ad-ATF4Luc). ATF4 is a transcription factor whose translation is regulated by short open reading frames in its 5'-UTR by a mechanism analogous to that which regulates GCN4 expression in yeast (30). Briefly, upon an increase in ternary complex availability, ATF4 expression is repressed.

However, when the availability of ternary complex is low, ATF4 expression is up-regulated (30).

MIN6 cells were mock infected or infected with the recombinant adenovirus Ad-ATF4Luc. 24 h post-infection, the cells were preincubated for 1 h in KRB or DMEM supplemented with 2 mM glucose. The cells were then further incubated for 2 h in the same medium (*i.e.* KRB or DMEM) at either 2 or 20 mM glucose in the presence of [³⁵S]methionine during the final hour of treatment. As a positive control, MIN6 cells were treated with thapsigargin, a compound that induces endoplasmic reticulum stress and the phosphorylation of eIF2 α via the activation of PERK (29). eIF2 α phosphorylation in turn leads to a decrease in ternary complex availability. Luciferase was immunoprecipitated from the cell lysates and separated by SDS-PAGE, and the incorporation of [³⁵S]methionine into luciferase was determined by autoradiography (Fig. 5). As expected, the incorporation of [³⁵S]methionine into luciferase increased in cells incubated in the presence of thapsigargin in contrast to total protein synthesis, which decreased significantly (Fig. 5). In either KRB or DMEM, the incorporation of [³⁵S]methionine into luciferase decreased in cells incubated at 20 mM glucose compared with those incubated at 2 mM glucose, indicative of an increase in ternary complex availability at high glucose (Fig. 5). This was accompanied by an increase in the incorporation of [³⁵S]methionine into total protein in cells incubated at 20 mM glucose compared with those incubated at 2 mM (Fig. 5). We

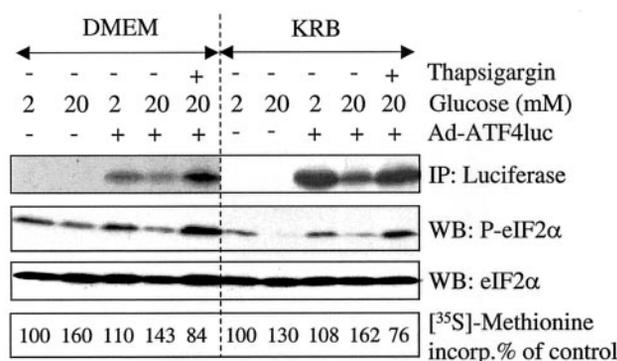


FIG. 5. Glucose regulates ternary complex formation in MIN6 cells. MIN6 cells were either mock infected or infected with adenovirus expressing luciferase downstream of the 5'-UTR of ATF4 (Ad-ATF4luc). After 24 h, MIN6 cells were preincubated in either DMEM or KRB containing 2 mM glucose for 1 h. Cells were then transferred to the same medium (DMEM or KRB) containing 2 or 20 mM glucose for 2 h. As a control, cells were also treated (at 20 mM glucose) with 1 μ M thapsigargin. During the final hour of treatment, cells were labeled with [³⁵S]methionine. Cells were then lysed, and luciferase was immunoprecipitated. Immunoprecipitated luciferase was run out on SDS-PAGE gels, and the incorporation of [³⁵S]methionine into luciferase was detected by autoradiography (IP:Luciferase). Total cell lysates were also separated on SDS-PAGE and Western blotted (WB) using anti-eIF2 α and anti-phospho eIF2 α (Ser-51) (P-eIF2 α) antisera. Incorporation of [³⁵S]methionine into total protein was expressed as a percentage of the mock infected 2 mM glucose control. Results presented are representative of at least three separate experiments.

also investigated eIF2 α phosphorylation in identical samples used to investigate changes in ternary complex formation using a phospho-specific antibody to eIF2 α Ser-51 (Fig. 5). In the presence of thapsigargin and at low glucose concentrations, either in the presence or absence of amino acids, eIF2 α is phosphorylated. However, upon an increase in glucose concentration, eIF2 α is dephosphorylated. Taken together, these results indicate that glucose stimulates ternary complex formation in MIN6 cells incubated in either KRB or DMEM and that these changes parallel the dephosphorylation of eIF2 α .

Role of PERK in Glucose-stimulated Protein Synthesis—In the pancreatic β -cell line MIN6, eIF2 α phosphorylation is regulated in response to changes in glucose concentration. At low glucose concentration (2 mM), eIF2 α is phosphorylated, whereas at high glucose concentration (20 mM), eIF2 α is dephosphorylated. These changes in the phosphorylation of eIF2 α parallel changes in both the rate of protein synthesis and changes in ternary complex formation. However, the kinase responsible for the phosphorylation of eIF2 α upon glucose deprivation is unknown. One possible candidate is the eIF2 α kinase PERK (29, 40).

Upon activation, PERK is auto-phosphorylated at Thr-980. Therefore, we investigated the phosphorylation status of PERK in response to glucose in MIN6 cells using a phospho-specific antibody to PERK (Thr-980). MIN6 cells were preincubated for 1 h in KRB supplemented with 0.5 mM glucose and then further incubated for 1 h in the same medium at either 0.5 mM glucose, 20 mM glucose, or 20 mM glucose plus thapsigargin as control (Fig. 6a). As expected, treatment of MIN6 cells with thapsigargin led to a large increase in the phosphorylation of PERK (Fig. 6a). However, no significant change in the phosphorylation of PERK was detected between low (0.5 mM) and high (20 mM) glucose concentrations (Fig. 6a).

As a corollary of the above, MIN6 cells were mock infected or infected with recombinant adenoviruses overexpressing either a dominant negative mutant of PERK (Ad-PERKAC) (29) or EGFP (Ad-emptyEGFP) only as control. 24 h post-infection, the cells were incubated in KRB containing 0.5 mM glucose for 1 h prior to incubation in the same buffer at either 0.5 or 20 mM

glucose for a further 1 h. MIN6 cells were also treated with thapsigargin as control. Following treatment, the phosphorylation status of eIF2 α (Fig. 6b, panel ii) and the rates of protein synthesis (Fig. 6b, panel i) were determined. Overexpression of the dominant negative PERK inhibited thapsigargin induced eIF2 α phosphorylation and blocked a significant proportion of the inhibitory effect of thapsigargin on the rate of protein synthesis (Fig. 6b). However, expression of the dominant negative PERK had only a partial effect on the phosphorylation of eIF2 α at low glucose and had no effect on glucose-stimulated protein synthesis (Fig. 6b).

Glucose-stimulated Protein Synthesis Is Independent of mTOR and Parallels an Increase in Ternary Complex Formation in Islets of Langerhans—To confirm whether the results obtained in MIN6 cells also occur in primary β -cells, islets of Langerhans were isolated from rats and preincubated for 2 h in KRB or DMEM supplemented with 2 mM glucose and then further incubated in the same medium (*i.e.* KRB or DMEM) for 1 h at either 2 or 20 mM glucose in the presence or absence of rapamycin. Increasing glucose concentration from 2 to 20 mM led to a significant increase in [³⁵S]methionine incorporation into total protein in islets incubated in either KRB or DMEM (Fig. 7a, panel i). Additionally, glucose-stimulated increases in the rate of protein synthesis were only partially affected in KRB and unaffected in DMEM by the addition of rapamycin (Fig. 7a, panel i). As a control, the phosphorylation of ribosomal protein S6 was also monitored using a phospho-specific antibody to S6 (Ser-235/236) (Fig. 7a, panel ii). As in MIN6 cells, S6 phosphorylation was stimulated at 20 mM glucose in DMEM but not in KRB, and glucose-stimulated phosphorylation of S6 was completely inhibited by rapamycin (Fig. 7a, panel ii).

To investigate changes in ternary complex availability, rat islets of Langerhans were infected 48 h with the Ad-ATF4luc adenovirus. 48 h post-infection, the islets were preincubated for 2 h in KRB containing 2 mM glucose. The cells were then further incubated for 2 h in KRB supplemented with either 2 or 20 mM glucose, or as control thapsigargin, in the presence of [³⁵S]methionine during the final hour of treatment. Luciferase was immunoprecipitated from the cell lysates and separated by SDS-PAGE, and the incorporation of [³⁵S]methionine into luciferase was visualized by autoradiography. As expected, thapsigargin treatment led to a large increase in the incorporation of [³⁵S]methionine into luciferase, indicating a large decrease in the availability of translational ternary complexes. Importantly, increasing the glucose concentration to 20 mM also led to a decrease in the incorporation of [³⁵S]methionine into luciferase, indicating an increase in the availability of translational ternary complexes (Fig. 7b). The phosphorylation status of eIF2 α was also investigated using a phospho-specific antibody to eIF2 α (Ser-51). However, no consistent change in eIF2 α phosphorylation was observed in response to either glucose or thapsigargin (Fig. 7b).

DISCUSSION

Pancreatic β -cells are almost unique in their ability to sense and respond to changes in extracellular glucose, with increases in glucose concentration leading not only to rapid increases in proinsulin synthesis, but also to a rapid increase in the rate of total protein synthesis. This short term glucose-stimulated protein synthesis (within 1 h) is almost entirely regulated at the post-transcriptional level through an increase in the rate of translation (Refs. 1, 2, 6, and 7 and this report, Fig. 1). However, the one or more mechanisms by which glucose stimulates an increase in protein synthesis in pancreatic β -cells are poorly understood.

In this report, we demonstrate, in the pancreatic β -cell line MIN6, that glucose stimulates protein synthesis in the presence or absence of amino acids and that these increases are

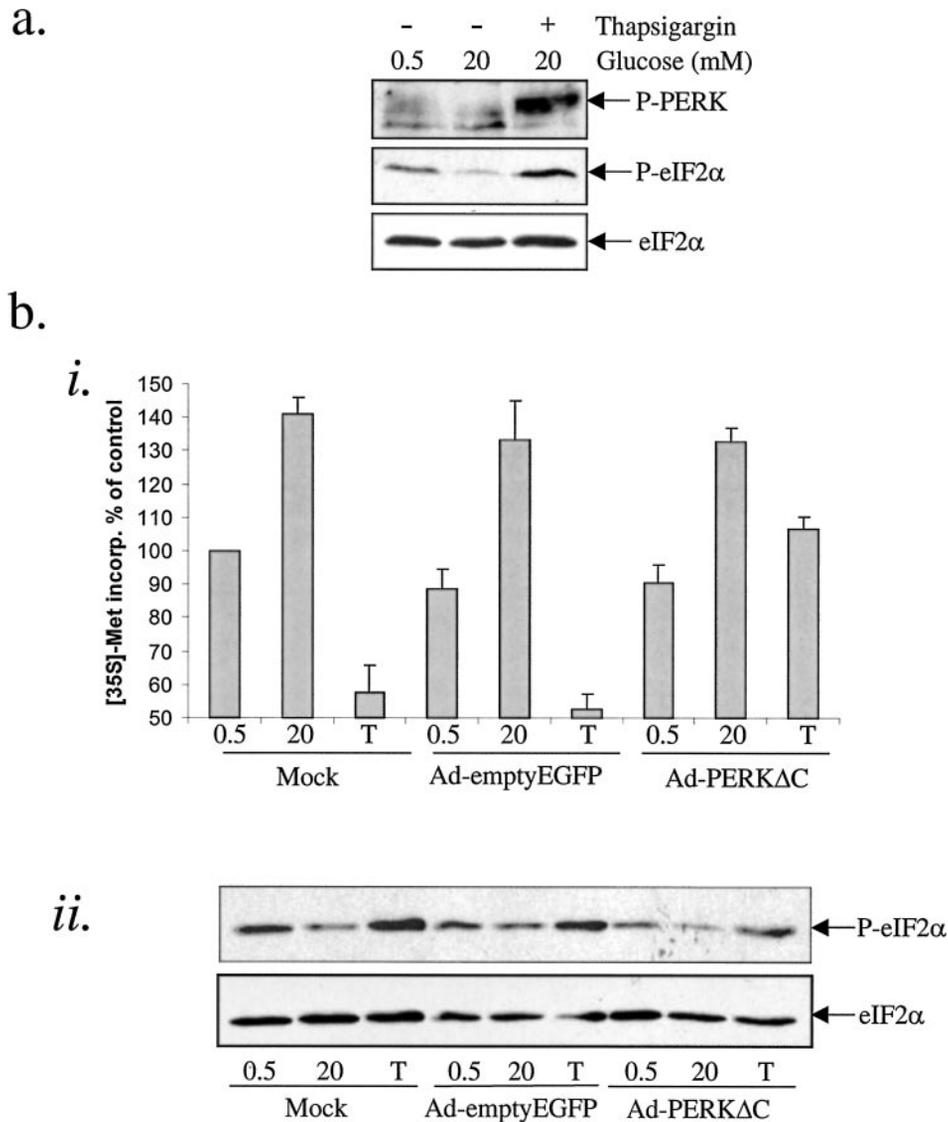


FIG. 6. Role of PERK in glucose-stimulated protein synthesis. *a.* MIN6 cells were preincubated in KRB supplemented with 0.5 mM glucose for 1 h. The cells were then incubated for a further 1 h at 0.5, 20 mM glucose or 20 mM plus 1 μ M thapsigargin. After treatment, cell lysates were separated by SDS-PAGE and Western blotted using anti-phospho-PERK (Thr-980) (*P-PERK*) and anti-phospho-eIF2 α (Ser-51) (*P-eIF2 α*) antisera. *b.* MIN6 cells were mock-infected, infected with the control adenovirus Ad-Empty.EGFP, or infected with the recombinant adenovirus expressing a dominant negative form of PERK (*Ad-PERK Δ C*). 24 h post-infection, the cells were preincubated for 1 h in KRB supplemented with 0.5 mM glucose prior to further incubation for 1 h in the presence of [³⁵S]methionine in KRB containing 0.5 or 20 mM glucose and thapsigargin as a control. *Panel i.*, incorporation of [³⁵S]methionine into total protein expressed as percentage of control ($n = 3 \pm$ AvD). *Panel ii.*, samples of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using antisera specific to phospho-eIF2 α Ser-51 (*P-eIF2 α*) and to total eIF2 α as control.

likely primarily mediated through an increase in the rate of initiation of translation (Fig. 2). These increases in protein synthesis occur via a mechanism that is independent of the activation of mTOR in the absence of amino acids and only partially dependent on mTOR in the presence of amino acids. In contrast, we show that glucose stimulates both the assembly of the translational ternary complex, eIF2-GTP:Met-tRNA_i, and the dephosphorylation of eIF2 α , both in the absence or presence of amino acids. Importantly, these changes parallel an increase in the rate of protein synthesis in response to glucose. The role of mTOR in glucose-stimulated protein synthesis and glucose-stimulated changes in ternary complex formation were confirmed in islets. Therefore, taken together, our data show that eIF2 α phosphorylation is likely to be key in regulating glucose-stimulated protein synthesis in pancreatic β -cells. However, we cannot definitively conclude that the results obtained in MIN6 cells or islets occur in primary β -cells.

It has been reported that glucose stimulates the de-phospho-

rylation of eEF2 in both islets and MIN6 cells between 1 and 10 mM, indicating that glucose may also stimulate an increase in the rate of elongation (41). Indeed, it has also been reported that the rate of protein elongation is specifically up-regulated between 0 and 3.3 mM glucose in islets (42). However, these increases in the elongation rate are fairly modest and occur only at these low non-physiological glucose concentrations (42). Hence, these changes are not thought to play a major role in glucose-stimulated protein synthesis. Therefore, it is likely that the primary mechanism by which glucose stimulates protein synthesis in pancreatic β -cells is through an increase in the rate of initiation.

In a number of other cell types, nutrients such as glucose or amino acids have been shown to stimulate protein synthesis. In many of these cases, this has been linked to an increase in the activity of the protein kinase mTOR (for reviews see Refs. 43–45), which is required for the phosphorylation of both 4E-BP1 and p70 S6 kinase, two important modulators of protein

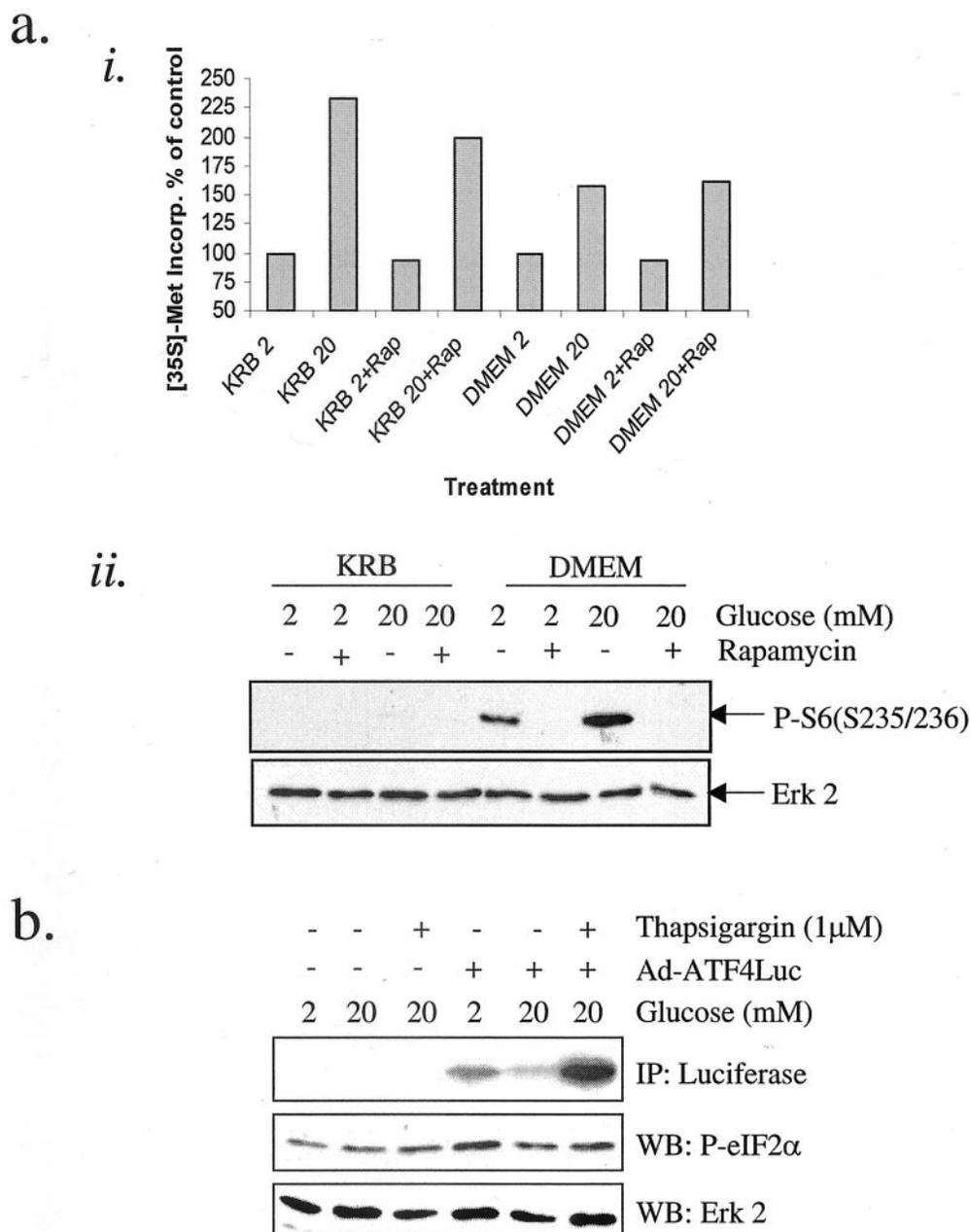


FIG. 7. Glucose-stimulated protein synthesis in islets. *a*, islets of Langerhans (~150 per treatment) were preincubated for 2 h in KRB or DMEM supplemented with 2 mM glucose. Where indicated, the cells were also pre-treated with 200 nM rapamycin for the last 30 min. The cells were then incubated for 1 h in the same medium, KRB or DMEM, containing either 2 mM (*KRB 2* and *DMEM 2*) or 20 mM glucose (*KRB 20* and *DMEM 20*) and [³⁵S]methionine, in the presence or absence of 200 nM rapamycin (*Rap*). *Panel i*, incorporation of [³⁵S]methionine into total protein expressed as percentage of control (*KRB 2* mM). *Panel ii*, samples of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using anti-phospho ribosomal protein S6 (Ser-235/236) antibody (*P-S6*) and as a loading control, anti-Erk2 antibody. These results are representative of three separate experiments. *b*, 48 h before treatment islets of Langerhans (~150 per treatment) were mock infected or infected with Ad-ATF4Luc. Following infection, islets were preincubated for 2 h in KRB buffer containing 2 mM glucose. Islets were then incubated for a further 2 h in the same medium at either 2 or 20 mM glucose in the presence of [³⁵S]methionine for the final hour of treatment. Islets were also treated with 1 μ M thapsigargin (at 20 mM glucose) as a control. Luciferase was immunoprecipitated from the cell lysates and separated on SDS-PAGE, and the incorporation of [³⁵S]methionine into luciferase was visualized by autoradiography (*IP:Luciferase*). Identical total cell lysates were also separated on a SDS-PAGE and Western blotted (*WB*) using anti-phospho-eIF2 α (Ser-51) and anti-Erk2 antisera as a loading control. Results presented are representative of three separate experiments.

synthesis. Hyperphosphorylation of 4E-BP1 stimulates its dissociation from eIF4E and the assembly of the eIF4F complex and hence the rate of initiation from cap-dependent mRNAs (14–16). The phosphorylation and activation of p70 S6 kinase leads to the phosphorylation of ribosomal protein S6, which has been reported to lead to an increase in the rate of initiation of a specific subset of mRNAs, which have a tract of polypyrimidines in their 5'-UTR (46–48). Indeed, in this report, we demonstrate in MIN6 cells that glucose, in the presence of amino

acids, stimulates the phosphorylation of both 4E-BP1 and p70 S6 kinase through a mechanism dependent on mTOR (Fig. 3). This is in agreement with previous reports demonstrating that amino acids are required for glucose-stimulated phosphorylation of 4E-BP1 and p70 S6 kinase in islets and in the pancreatic β -cell line RINm5F (9, 39). We additionally demonstrate that glucose stimulates the phosphorylation of 4E-BP1 on amino acid residues Ser-65 and Thr-36/45 (Fig. 3*a*). Importantly, the phosphorylation of these residues is known to be critical in

stimulating the release of 4E-BP1 from eIF4E (49, 50). Accordingly, we also show that glucose stimulates the release of 4E-BP1 from eIF4E (Fig. 3). Surprisingly, these changes do not lead to any detectable increase in eIF4F assembly (Fig. 3b). Therefore, the functional significance of the phosphorylation of the downstream targets of mTOR is unclear. However, treatment of MIN6 cells or islets with rapamycin, a specific inhibitor of mTOR, did lead to a partial inhibition of glucose-stimulated protein synthesis (Figs. 3c and 7a). In the absence of amino acids, under conditions where there is no detectable phosphorylation of the downstream targets of mTOR or any changes in eIF4F assembly, glucose can still stimulate protein synthesis in both MIN6 cells and islets (Figs. 3 and 7a). Furthermore, this glucose-stimulated protein synthesis is unaffected by the addition of rapamycin in MIN6 cells and only partially affected in islets (Figs. 3 and 7a). Clearly, these data show that glucose can stimulate protein synthesis through a mechanism independent of mTOR. Therefore, we believe that glucose-stimulated protein synthesis, either in the presence or absence of amino acids, is likely to be primarily regulated by a mechanism independent of the activation of mTOR.

An alternative mechanism by which the rate of initiation and hence protein synthesis could potentially be up-regulated in response to glucose is through an increase in the availability of the translational ternary complex. Interestingly, we demonstrate, both in islets and MIN6 cells, that the availability of the translational ternary complex is up-regulated at high glucose concentrations *in vivo*, either in the presence or absence of amino acids (Figs. 5 and 7b). It is likely that glucose stimulates the assembly of the translational complex, at least in part, through the activation of the guanine nucleotide exchange factor eIF2B, because eIF2B activity is transiently increased in response to glucose in rat islets (20). The mechanism by which eIF2B is regulated by glucose is unknown, but one well characterized mechanism by which eIF2B can be regulated is through the phosphorylation of eIF2 α on Ser-51. In this report, we demonstrate that eIF2 α becomes rapidly dephosphorylated in response to increasing glucose concentrations in MIN6 cells and that these changes in eIF2 α phosphorylation parallel changes in glucose-stimulated protein synthesis (Fig. 4, a and b). Moreover, glucose-stimulated dephosphorylation of eIF2 α occurs in both DMEM and KRB (Fig. 4a). In contrast, in islets, we couldn't detect any consistent change in the phosphorylation status of eIF2 α in response to glucose (in agreement with Gilligan *et al.* (20)) (Fig. 7b). Additionally, we couldn't detect any increase in the phosphorylation of eIF2 α after treatment with thapsigargin (an agent known to induce eIF2 α phosphorylation) (Fig. 7b). However, increased glucose concentration clearly led to an increase in the assembly of the translational ternary complex, whereas thapsigargin treatment resulted in a large inhibition of ternary complex formation (Fig. 7b). One potential reason that could explain why we cannot detect changes in eIF2 α phosphorylation in islets (even after treatment with thapsigargin) is that phosphorylated eIF2 α in non- β -cells, or perhaps much more likely, highly phosphorylated eIF2 α in a population of stressed cells, may mask the effects of glucose or thapsigargin on the phosphorylation of eIF2 α in β -cells. Indeed, cells at the center of cultured islets can suffer from hypoxia, a stress known to induce eIF2 α phosphorylation in other cell types (51). Therefore, given that glucose stimulates the dephosphorylation of eIF2 α in MIN6 cells, we believe that it is likely that glucose-stimulated assembly of the translational ternary complex in islets is also mediated by the dephosphorylation of eIF2 α .

Two regulatory subunits of protein phosphatase 1 have been implicated in eIF2 α dephosphorylation. These include

GADD34, whose expression is up-regulated in response to cell stress (*i.e.* after a few hours) to stimulate the recovery from the shut-off of protein synthesis (52, 53) and CReP, which is constitutively active and acts to maintain the basal phosphorylation state of eIF2 α (54). Given the temporal kinetics of eIF2 α dephosphorylation in response to glucose in MIN6 cells (Fig. 4c), it is unlikely that this event is regulated through the up-regulation of GADD34 expression. We believe it is more likely that these changes in phosphorylation of eIF2 α are regulated through the activity of an eIF2 kinase in combination with a constitutively active phosphatase.

Glucose starvation is known to stimulate eIF2 α phosphorylation in C6 glial cells (55) and whole mouse pancreata (56). It is also known that glucose starvation can cause endoplasmic reticulum stress, hence it was previously speculated that PERK may phosphorylate eIF2 α in response to glucose deprivation (57). However, we show that overexpression of a dominant negative mutant of PERK (Ad-PERK Δ C) (29) in MIN6 cells had only a partial effect on the phosphorylation of eIF2 α at either low or high glucose concentrations and had no effect on glucose-stimulated protein synthesis (Fig. 6b). Additionally, no increase in the phosphorylation of PERK was detected at low glucose compared with high glucose (Fig. 6a). These data demonstrate that PERK is unlikely to be the primary eIF2 kinase responsible for eIF2 α phosphorylation in response to low glucose concentration in pancreatic β -cells. However, the eIF2 kinase mGCN2 is regulated by nutrients. For example, mGCN2 is activated by amino acid starvation (26). Additionally, its yeast orthologue, GCN2, is also activated in response to glucose starvation (27). However, it has not been demonstrated that mGCN2 activity is regulated by glucose in mammalian cells. Nevertheless, it is possible that mGCN2 is the kinase responsible for the phosphorylation of eIF2 α in pancreatic β -cells under conditions of low glucose concentration and this will be the subject of further investigation.

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Glucose-stimulated Protein Synthesis in Pancreatic β -Cells Parallels an Increase in the Availability of the Translational Ternary Complex (eIF2-GTP-Met-tRNAi) and the Dephosphorylation of eIF2 α

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