

Opposite Translational Control of GLUT1 and GLUT4 Glucose Transporter mRNAs in Response to Insulin

ROLE OF MAMMALIAN TARGET OF RAPAMYCIN, PROTEIN KINASE B, AND PHOSPHATIDYLINOSITOL 3-KINASE IN GLUT1 mRNA TRANSLATION*

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Prolonged exposure of 3T3-L1 adipocytes to insulin increases GLUT1 protein content while diminishing GLUT4. These changes arise in part from changes in mRNA transcription. Here we examined whether there are also specific effects of insulin on GLUT1 and GLUT4 mRNA translation. Insulin enhanced association of GLUT1 mRNA with polyribosomes and decreased association with monosomes, suggesting increased translation. Conversely, insulin arrested the majority of GLUT4 transcripts in monosomes. Insulin inactivates the translational suppressor eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) through the mammalian target of rapamycin (mTOR). Hence, we examined the effect of rapamycin on GLUT1 mRNA translation and protein expression. Rapamycin abrogated the insulin-mediated increase in GLUT1 protein synthesis through partial inhibition of GLUT1 mRNA translation and partial inhibition of the rise in GLUT1 mRNA. 4E-BP1 inhibited GLUT1 mRNA translation *in vitro*. Because phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB), in concert with mTOR, inactivate 4E-BP1, we explored their role in GLUT1 protein expression. Cotransfection of cytomegalovirus promoter-driven, hemagglutinin epitope-tagged *GLUT1* with dominant inhibitory mutants of PI3K or PKB inhibited the insulin-elicited increase in hemagglutinin-tagged GLUT1 protein. These results unravel the opposite effects of insulin on GLUT1 and GLUT4 mRNA translation. Increased GLUT1 mRNA translation appears to occur via the PI3K/PKB/mTOR/4E-BP1 cascade.

Glucose transport into most tissues occurs through the action of members of a family of facilitative diffusion glucose transport proteins designated GLUT1–5 (1). GLUT1 is ubiquitously distributed and has been proposed to act as a constitutive transport protein (1). In contrast, the GLUT4 isoform is expressed almost exclusively in adipose cells and skeletal muscles, tissues responsible for the major portion of insulin stim-

ulation of glucose transport after a meal (2, 3). An acute insulin challenge results in an increase in glucose uptake via the translocation of GLUT proteins, mainly GLUT4, from internal stores to the plasma membrane (2, 4–6).

In addition to its acute effect on the redistribution of glucose transporters, insulin also exerts long-term regulation of glucose transporter concentration. Prolonged exposure to insulin, a condition that occurs in type II diabetes, which is characterized by insulin resistance and compensatory hyperinsulinemia, results in an increase in GLUT1 protein levels (7). In cells in culture, prolonged exposure to insulin also increases GLUT1 protein content and reduces GLUT4 protein (8–11). In 3T3-L1 fibroblasts and adipocytes, the elevation in GLUT1 protein expression is due in part to an elevation in GLUT1 mRNA transcription (11) and to a rise in the GLUT1 mRNA half-life (12). Conversely, chronic insulin treatment of 3T3-L1 adipocytes decreases GLUT4 protein levels as a result of a reduction in mRNA levels (13) and a decrease in the half-life of GLUT4 protein (8). Therefore, the pathways involved in *GLUT1* and *GLUT4* gene expression are complex, and insulin appears to exert both transcriptional and post-transcriptional regulation. It remains unknown, however, whether the hormone can also regulate the expression of these two transporters at the level of their mRNA translation.

Translational control usually occurs at the rate-limiting step of initiation. Eukaryotic cellular mRNAs (except organellar) contain a cap structure (m⁷GpppX, where X is any nucleotide) at their 5' termini, and initiation involves recognition of this structure by the mRNA cap-binding protein eIF-4E¹ (14). eIF-4E, together with eIF-4A (an RNA helicase) and eIF-4G (a bridge between eIF-4E and eIF-4A), forms the eIF-4F initiation complex (15, 16). eIF-4E activity is regulated through the formation of complexes with inhibitory eIF-4E-binding proteins (14). In mammals, the eIF-4E-binding proteins (4E-BPs) compose a family of three members termed 4E-BP1 (17), 4E-BP2 (18), and 4E-BP3 (19). 4E-BPs compete with eIF-4G for interaction with eIF-4E, thereby inhibiting cap-dependent translation (14). Whether this mechanism affects equally all mRNAs has not been determined.

In response to insulin, 4E-BP1 (also termed PHAS-I (phos-

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¹ The abbreviations used are: eIF-4, eukaryotic initiation factor 4; 4E-BP, eIF-4E-binding protein; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; HA, hemagglutinin; GST, glutathione S-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase.

phorylated heat- and acid-stable protein I) becomes hyperphosphorylated, leading to its dissociation from eIF-4E to relieve translational inhibition. This phenomenon has been demonstrated in rat adipose tissue (17, 20), 3T3-L1 adipocytes (18), rat skeletal muscle (21), and L6 myoblasts (22). The phosphorylation of 4E-BP1 by insulin is inhibited by rapamycin (23), a drug that forms a complex with the immunophilin FKBP12 to inhibit the kinase mammalian target of rapamycin (mTOR). mTOR phosphorylates 4E-BP1 both *in vitro* and *in vivo* (24). Subsequent studies have demonstrated that phosphatidylinositol 3-kinase (PI3K) and its downstream effector, protein kinase B (PKB; also known as Akt), are critical intermediates in the signal transduction pathway leading from the insulin receptor to the activation of mTOR and phosphorylation of 4E-BP1 (25–27).

In addition to stimulating overall protein synthesis, insulin preferentially regulates the biosynthesis of certain proteins over and above its general anabolic effects on protein synthesis and proteolysis. A large number of these specific effects on protein synthesis are dependent upon continued mRNA synthesis (28), whereas few others occur without changes in mRNA levels (28–30). In this study, we show that insulin specifically up-regulates GLUT1 mRNA translation, in contrast to GLUT4 mRNA and the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. Furthermore, the increase in GLUT1 mRNA translation appears to occur via the PI3K/PKB/mTOR/4E-BP1 pathway.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium and serum were obtained from Life Technologies, Inc. Porcine insulin was purchased from Sigma. Rapamycin was obtained from Calbiochem. Anti-GLUT1 antibody used for immunoprecipitation was generated as described previously (8). Polyclonal anti-GLUT1 (RaGLUTRANS) antibody used for immunoblotting was purchased from East Acres Biologicals (Southbridge, MA). Monoclonal anti-hemagglutinin (HA) antibody (HA.11) was purchased from Babco (Berkeley, CA). Monoclonal antibody 6H to the α_1 -subunit of the Na^+/K^+ -ATPase was a kind gift from Dr. M. Caplan (Department of Cellular and Molecular Physiology, Yale University). GST-4E-BP1 was generated as described previously (17). C_{12}E_8 (octaethylene glycol dodecyl ether) was purchased from Fluka (Ronkonkoma, NY).

Cell Culture, Incubations, Plasmids, and Transfections—3T3-L1 fibroblasts were grown and differentiated as described previously (8). Cells were grown in 10-cm dishes for transfections, total membrane preparation, RNA isolation, and polysome profile analysis and in 60-mm dishes for labeling with [^{35}S]methionine and immunoprecipitation. Mature adipocytes were used between days 12 and 14 after the initiation of differentiation. Adipocytes were treated with or without 100 nM insulin in the presence or absence of 30 ng/ml rapamycin for 18 h as described in the figure legends. Plasmid containing full-length cDNA for GLUT1 (prGT4-12) used in Northern blot hybridization was kindly provided by Dr. M. Birnbaum (University of Pennsylvania School of Medicine). Plasmid containing full-length cDNA for GLUT4 (IRGT2⁺) was kindly provided by Dr. D. E. James (University of Queensland, Queensland, Australia). Plasmid containing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was kindly given by Dr. H. Elsholtz (University of Toronto). The HA epitope-tagged GLUT1 construct was subcloned into the pcIS2 mammalian expression vector. Dominant-negative AAA-PKB/Akt was created by substituting alanine residues at the two major regulatory phosphorylation sites of PKB α /Akt (Thr-308 and Ser-473) and the phosphate transfer residue in the catalytic site (Lys-179) as described previously (31, 32). The AAA-PKB construct was subcloned into the eukaryotic expression vector pcDNA3. The construct pSG5p85 α SH2-N, commonly referred to as Δ p85 α , the dominant-negative mutant of type I PI3K (33), was a kind gift from Dr. J. Downward (Imperial Cancer Research Fund, London, United Kingdom). The cDNA insert of Δ p85 α was subcloned into pcDNA3 for experimentation. Parental L6 myoblasts were used for transfections. Cells were cotransfected with 2 μg of HA-GLUT1 cDNA and 2 μg of empty vector (pcDNA3), AAA-PKB, or Δ p85 α construct/dish according to the Effectene product manual (QIAGEN Inc.). L6 myoblasts were seeded in 10-cm dishes at 2×10^6 cells/dish and incubated

overnight. DNA complexes were made at an 8:1 enhancer/DNA ratio in all cases. The Effectene reagent was used at 25 μl /dish. DNA was introduced into the cells at the start of the day for 6 h and then removed. Cells were maintained for another 42 h until experimentation. In the final 18 h of the 48-h post-transfection period, cells were treated with or without 100 nM insulin. Total membranes were then prepared, and HA-GLUT1 was detected by immunoblotting.

Polysome Profiles: Analysis of Polysomes by Sucrose Density Gradients—Polysome profiles were generated as described by Jain *et al.* (34). Briefly, three 10-cm dishes of 3T3-L1 adipocytes were used for each polysome distribution analysis. Following the 18-h treatment with or without insulin and/or rapamycin, cells were washed twice with ice-cold phosphate-buffered saline containing 100 $\mu\text{g}/\text{ml}$ cycloheximide and lysed by the addition of 200 μl of polysome lysis buffer (100 mM KCl, 5 mM MgCl_2 , 10 mM HEPES, pH 7.4, 100 $\mu\text{g}/\text{ml}$ cycloheximide, 0.5% Nonidet P-40, and 1000 units/ml RNasin)/dish. The lysate was transferred to a 1.5-ml microcentrifuge tube and passed three to four times through a 27-gauge needle to ensure cell lysis. Nuclei were pelleted by centrifugation at 4 $^\circ\text{C}$ and $12,000 \times g$ for 5 min. The supernatant was then subjected to centrifugation one more time to ensure the removal of any nuclei. The resulting supernatant was layered on a linear 15–45% (w/v) sucrose gradient in polysome gradient buffer (100 mM KCl, 5 mM MgCl_2 , and 10 mM HEPES, pH 7.4), and gradients were centrifuged at 35,000 rpm for 2 h at 4 $^\circ\text{C}$ in a Beckman SW 41 rotor. Gradient fractions were collected. RNA was purified from the sucrose density fractions by proteinase K digestion. Each sample was diluted with an equal volume of a proteinase K solution (0.2 M Tris-HCl, pH 7.5, 25 mM EDTA, 0.3 M NaCl, 2% SDS, and 250 $\mu\text{g}/\text{ml}$ proteinase K). Samples were incubated at 45 $^\circ\text{C}$ for 30 min and then extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v). The aqueous phase was recovered, and the RNA was precipitated with 0.3 M sodium acetate, pH 5.2, and 2.5 volumes of ethanol. GLUT1, GLUT4, and GAPDH mRNAs were detected by Northern blot analysis as described below. The distinction between monosomes and polysomes was made based on the ethidium bromide staining of the ribosomal RNA. By definition, the 80 S subunit contains the highest amount of 18 S and 28 S rRNAs (*i.e.* the brightest staining); the 60 S subunit has a higher level of 28 S rRNA, whereas the 40 S subunit has a higher amount of 18 S rRNA. Using ethidium bromide staining, we identified the fractions that contain the brightest staining of both 18 S and 28 S rRNAs (*i.e.* the 80 S subunit-containing fractions). These 80 S subunit-containing fractions and the preceding ones were collectively designated “monosomes,” and the heavier subsequent fractions were designated “polysomes.” This criterion was previously used by Siomi *et al.* (35).

In Vitro Translation—GLUT1 protein was produced *in vitro* from full-length GLUT1 cDNA as follows. The pUC19 plasmid containing full-length GLUT1 cDNA (prGT4-12, 5292 base pairs) was digested with *Eco*RI and *Bgl*II to release the entire GLUT1 cDNA insert (2.6 kilobase pairs). The GLUT1 cDNA insert was then subcloned into a pGEM vector downstream of the T7 RNA polymerase promoter. Coupled transcription/translation was performed using a TNT reticulocyte lysate coupled transcription/translation system (Promega) as instructed by the manufacturer. Briefly, rabbit reticulocyte lysates were preincubated for 10 min without or with purified GST (400 ng) or GST-4E-BP1 (600 ng). The following reagents were then added to the rabbit reticulocyte lysates: transcription/translation reaction buffer, amino acid mixture minus methionine, [^{35}S]methionine, RNasin ribonuclease inhibitor, 1 μg of GLUT1 cDNA template, nuclease-free water, and T7 RNA polymerase. The reaction was incubated at 30 $^\circ\text{C}$ for 90 min. Translation products were resolved by SDS-polyacrylamide gel electrophoresis, and the gels were processed for fluorography.

Total Membrane Preparation and Immunoblotting—Total membranes were isolated as described previously (36). GLUT1 and the α_1 -subunit of the Na^+/K^+ -ATPase were detected by immunoblot analysis as described previously (36).

Labeling with [^{35}S]Methionine, Solubilization, and Immunoprecipitation—Following 14 h of treatment with or without insulin in the presence or absence of rapamycin, adipocytes were incubated in methionine-free Dulbecco's modified Eagle's medium in the continued presence or absence of insulin and/or rapamycin. After 2 h, this medium was removed, and the adipocytes were pulsed for 2 h in methionine-free Dulbecco's modified Eagle's medium supplemented with 200 μCi of [^{35}S]methionine/dish. The labeling medium also contained insulin and/or rapamycin, and the total incubation period was 18 h. The labeling medium was then removed; cells were washed twice with ice-cold phosphate-buffered saline and solubilized; and GLUT1 was immunoprecipitated according to Sargeant and Paquet (8).

RNA Isolation and Northern Blot Hybridization—Total RNA was

isolated, and Northern blots were performed as described previously (10).

Statistical Analysis—Quantitative analysis of the relative amount of mRNA in every fraction in Figs. 1 and 2 was performed using a Molecular Dynamics PhosphorImager system. Autoradiograms of Figs. 3 and 4 were quantified by laser scanning densitometry using a PDI Model DNA 35 scanner with Version 1.3 of the Discovery Series one-dimensional gel analysis software. Statistical analysis was performed using the analysis of variance test (Fisher, multiple comparisons).

RESULTS

Effect of Insulin on Distribution of GLUT1 and GLUT4 mRNAs in Polysome Profiles—To analyze the effect of insulin on GLUT1 and GLUT4 mRNA translation, we examined their mRNA distribution between monosomes/preinitiation complexes and polysomes. Sucrose density gradients were used to separate monosomes/preinitiation complexes from polysomes as described under “Experimental Procedures.” Total RNA from each fraction of these profiles was extracted and analyzed by agarose gel electrophoresis. The locations of monosomes and polysomes were determined by ethidium bromide staining, and a typical profile is illustrated in Fig. 1A. The separation of monosomes and polysomes was not altered with insulin treatment (data not shown). The results in Fig. 1B reveal that under basal conditions, GLUT1 mRNA was almost uniformly distributed throughout the gradient. Following insulin treatment, a shift in the profile was observed showing that the proportion of GLUT1 mRNA dropped in the monosomes (fractions 1–5) and augmented in the denser portion of the gradient that contains heavier polysomes (fractions 6–15) (Fig. 1B). The results of four independent experiments were quantitated to calculate the proportion of GLUT1 mRNA associated with monosomes and with polysomes in the presence or absence of insulin. This analysis revealed that both the reduction in GLUT1 mRNA in the monosomes and the increase in polysomes in response to insulin were statistically significant at $p < 0.01$. The progression of GLUT1 mRNA from preinitiation complexes to polysomes elicited by insulin suggests an increased efficiency of ribosome loading and acceleration in the rate of translation.

Like the GLUT1 mRNA from unstimulated cells, GLUT4 mRNA was distributed uniformly throughout the sucrose gradient (Fig. 1C). In contrast to its effect on GLUT1, however, prolonged insulin treatment resulted in an arrest of the majority of GLUT4 transcripts in the monosome fractions with a concomitant reduction of the message in the polysome fractions (Fig. 1C). Similar results were obtained in two independent experiments. To address the specificity of insulin action on the translation of GLUT1 and GLUT4 mRNAs, we examined the mRNA distribution of the GAPDH housekeeping gene. Fig. 1D reflects the active translation of this housekeeping gene since the majority of the transcript was polysome-bound under basal conditions. This distribution was minimally affected by insulin treatment (Fig. 1D). Similar results were obtained in two independent experiments.

Effect of Rapamycin on the Insulin-mediated Increase in GLUT1 mRNA Translation—Because the rapamycin-sensitive mTOR pathway mediates an increase in the rate of translation of certain mRNAs, we tested whether this pathway was also involved in the insulin-dependent increase in GLUT1 mRNA translation. Indeed, rapamycin reduced the insulin-elicited increase in GLUT1 transcripts in polysomes (Fig. 2B), without affecting the separation of monosomes and polysomes (Fig. 2A). The results of four independent experiments showed that the rapamycin-induced increase in GLUT1 mRNA associated with monosomes and the decrease in polysomes from insulin-stimulated cells were statistically significant at the $p < 0.05$ level.

Effect of 4E-BP1 on GLUT1 mRNA Translation in Vitro—To address the possibility that the mTOR pathway might regulate

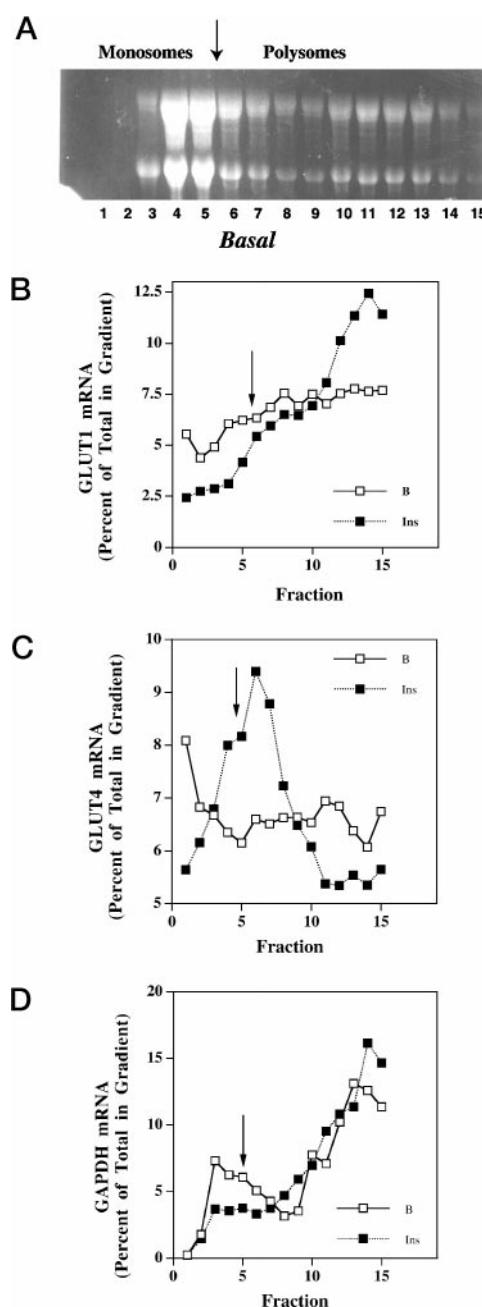


FIG. 1. Effect of insulin on the translation of GLUT1, GLUT4, and GAPDH mRNAs. Cells were treated without (basal) or with insulin for 18 h. Cytoplasmic extracts were then prepared and applied to 15–45% (w/v) sucrose density gradients. The gradients were fractionated, generating a polysome profile. Total RNA from each fraction was extracted and analyzed by agarose gel electrophoresis and ethidium bromide staining. A, a typical segregation of ribosomal RNA along the sucrose gradient is illustrated. The arrow shows the separation between monosomes and polysomes. B–D, GLUT1, GLUT4, and GAPDH mRNAs, respectively, in each fraction were detected by Northern blot analysis. Densitometric analysis of the relative amount of mRNA in every fraction in basal (B) or insulin (Ins)-treated cells was performed. Values are expressed as percentage of the total mRNA recovered in the gradient. The separation between monosomes and polysomes is indicated by the arrows.

GLUT1 mRNA translation by removing the inhibitor 4E-BP1, we examined the efficiency of GLUT1 mRNA translation *in vitro* in rabbit reticulocyte lysates in the presence or absence of GST-4E-BP1. As shown in Fig. 3, the presence of GST-4E-BP1 reduced the efficiency of GLUT1 mRNA translation by 45%, suggesting a role of mTOR/4E-BP1 in GLUT1 protein expres-

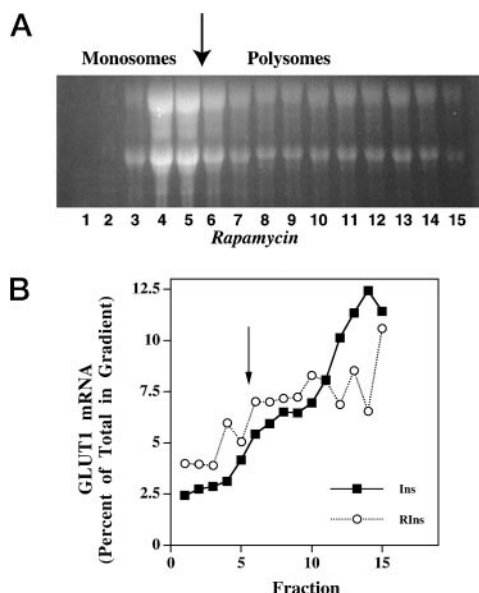


FIG. 2. Effect of rapamycin on the translation of GLUT1 mRNA. Cells were treated with insulin alone or with rapamycin for 18 h. Polysome profiles were analyzed for GLUT1 mRNA as described in the legend of Fig. 1. A, separation between monosomes and polysomes in the presence of rapamycin. B, densitometric analysis of the relative amount of mRNA in every fraction in insulin (*Ins*)- or insulin plus rapamycin (*RIIns*)-treated cells.

sion. This 45% inhibition by 4E-BP1 is similar to the previously reported 40% inhibition of cap-dependent translation caused by rapamycin (23).

Effect of Rapamycin on the Insulin-mediated Increase in GLUT1 Protein and mRNA Expression.—To further understand the involvement of the rapamycin-sensitive mTOR pathway in GLUT1 protein expression, we assessed the impact of rapamycin on the insulin-stimulated increase in the steady-state content of GLUT1 protein. Prolonged exposure to insulin resulted in a 97% increase in the steady-state GLUT1 content above the basal value, and rapamycin completely eliminated this increase (Fig. 4A). Steady-state levels reflect a balance of the rate of synthesis and the rate of degradation. To test whether the abrogation of the insulin-mediated elevation in the total amount of GLUT1 protein by rapamycin was due to inhibition of the synthesis of this transporter, adipocytes were labeled with [35 S]methionine, and GLUT1 protein was then immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. Continuous insulin stimulation for 18 h raised the level of newly synthesized GLUT1 protein by 114% above basal levels (Fig. 4B). This increase was almost completely abolished in the presence of rapamycin (Fig. 4B). These results suggest that the mTOR pathway is essential for the stimulation of GLUT1 protein synthesis by insulin.

The insulin-dependent elevation in GLUT1 protein synthesis is the result of increases in both GLUT1 mRNA abundance and mRNA translation. We therefore assessed whether the rapamycin-sensitive pathway also participates in the elevation in GLUT1 mRNA in response to insulin. Chronic insulin treatment raised the amount of GLUT1 mRNA by 176% above basal levels (Fig. 4C). In contrast to its effect on the steady-state levels and synthesis of GLUT1 protein, rapamycin only partially inhibited the insulin-mediated elevation in GLUT1 mRNA (Fig. 4C). These observations support the notion that the rapamycin-sensitive mTOR signaling pathway is necessary for the insulin-dependent stimulation of GLUT1 protein synthesis beyond its participation in elevating GLUT1 mRNA levels.

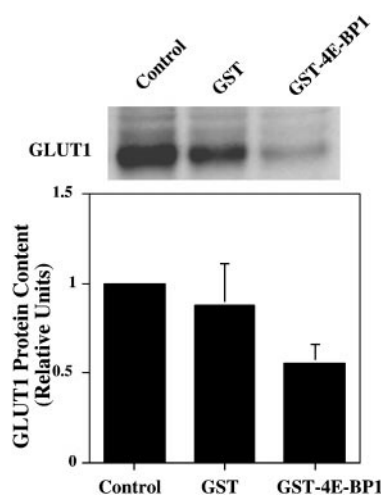


FIG. 3. Effect of 4E-BP1 on GLUT1 mRNA translation *in vitro*. Coupled transcription/translation of full-length GLUT1 cDNA in rabbit reticulocyte lysates was carried out as described under "Experimental Procedures." The lysates were untreated (*Control*; first lane) or were preincubated for 10 min with purified GST (400 ng) (*second lane*) or GST-4E-BP1 (600 ng) (*third lane*) before the addition of GLUT1 cDNA. Translation products were resolved by SDS-polyacrylamide gel electrophoresis, and the gels were processed for fluorography. Shown is a representative blot of five independent experiments. The results of five independent experiments were densitometrically scanned. The amount of GLUT1 protein produced in the control state (*first bar*) is assigned a value of 1.0, and other values are expressed in relative units. Values represent means \pm S.E.

Role of PI3K and PKB in GLUT1 mRNA Translation.—Recent reports have provided direct evidence for a linear signaling pathway leading from the insulin receptor to PI3K, PKB, mTOR, and 4E-BP1 (25–27). The activity of PI3K is an obligatory step in PKB activation by insulin as products of PI3K bind to PKB (37). Full activation of PKB by insulin also requires hierarchical phosphorylation on two residues (Thr-308 and Ser-473) by 3-phosphoinositide-dependent protein kinases 1 and 2, respectively (38). Activation of PKB results, in turn, in mTOR phosphorylation and activation (27) and 4E-BP1 phosphorylation (25, 26). We have recently demonstrated that a kinase-inactive, phosphorylation-deficient PKB α construct with the mutations K179A, T308A, and S473A (AAA-PKB) behaves as a dominant-negative inhibitor of endogenous PKB in L6 myoblasts (32). To determine the role of PKB in GLUT1 mRNA translation, the cDNA of HA-GLUT1 under the control of the cytomegalovirus promoter was cotransfected with empty vector alone (pcDNA3) or AAA-PKB at a 1:1 DNA ratio in L6 myoblasts. As shown in Fig. 5A, insulin caused an increase in the amount of HA-GLUT1 protein as detected by anti-HA antibody. Cotransfection of AAA-PKB inhibited this increase, suggesting a participation of PKB in this phenomenon.

To assess the role of the upstream player PI3K in GLUT1 protein expression, the cDNA of HA-GLUT1 was cotransfected with the Δ p85 α construct at a 1:1 DNA ratio in L6 myoblasts. The Δ p85 α construct is a dominant-negative construct of the p85 α regulatory subunit of PI3K lacking the region that binds to the p110 catalytic subunit on PI3K (33). We have recently shown that expression of Δ p85 α in L6 myoblasts inhibits insulin responses that are dependent on PI3K (32). As illustrated in Fig. 5B, expression of Δ p85 α inhibited the insulin-mediated increase in HA-GLUT1 protein. This rise in HA-GLUT1 by insulin is suggested to be post-transcriptional since actinomycin D, an inhibitor of transcription, did not eliminate the elevation in HA-GLUT1 protein (data not shown), and the pcIS2 mammalian expression vector containing the HA-GLUT1 construct lacks the GLUT1 promoter. Taken together, these obser-

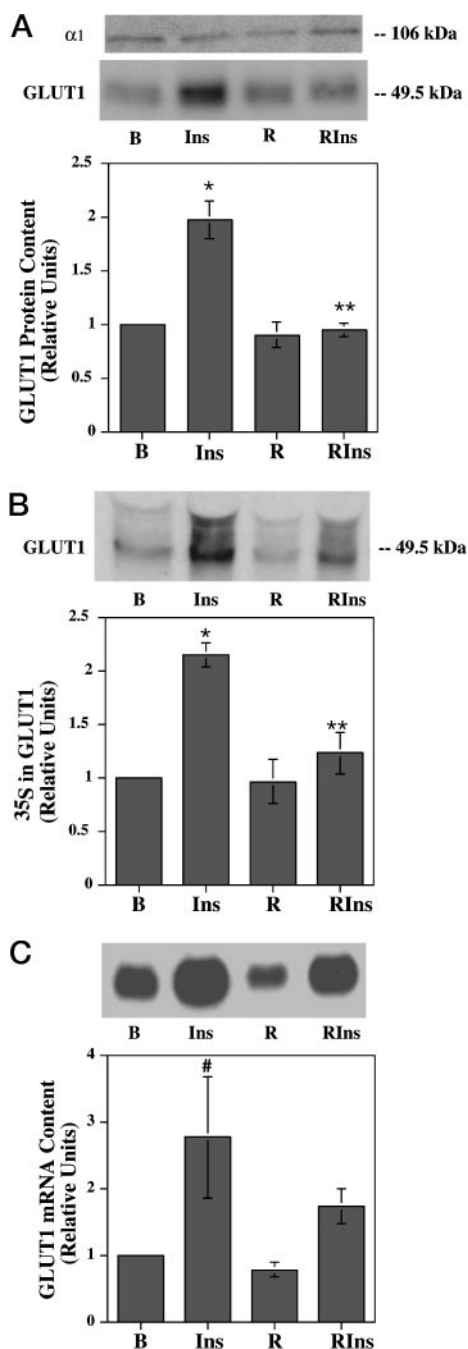


FIG. 4. Effect of rapamycin on the insulin-mediated increase in the total cellular content of GLUT1 protein, rate of synthesis of GLUT1 protein, and GLUT1 mRNA abundance. Cells were treated without (basal (B)) or with 100 nM insulin (Ins), with 30 ng/ml rapamycin (R), or with 100 nM insulin plus 30 ng/ml rapamycin (RIns) for 18 h. A, total membranes were prepared and immunoblotted. The content of the Na⁺/K⁺-ATPase α_1 -subunit was monitored to ensure equality of protein loading. B, GLUT1 protein synthesis was measured based on the rate of [³⁵S]methionine incorporation. C, total RNA isolation and Northern blot hybridization were performed. The results of five independent experiments in A, seven in B, and five in C were densitometrically scanned. The content of GLUT1 in the basal state is assigned a value of 1.0, and other values are expressed in relative units. Values represent means \pm S.E. * and #, statistically significant ($p < 0.01$ and $p < 0.05$, respectively) compared with the basal state; **, statistically significant ($p < 0.01$) compared with insulin-stimulated cells.

variations are consistent with the hypothesis that PI3K/PKB/mTOR participate in the insulin-dependent elevation in GLUT1 protein expression at a post-transcriptional level.

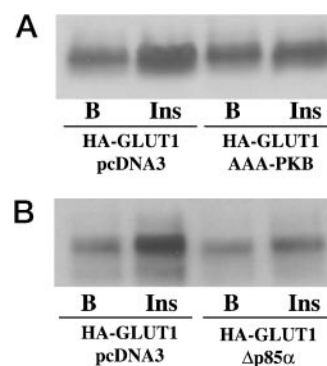


FIG. 5. Effect of dominant inhibitory mutants of PKB/Akt and PI3K on HA-GLUT1 protein expression in response to insulin. L6 myoblasts were cotransfected with HA-GLUT1 (2 μ g) and empty vector (pcDNA3; 2 μ g) (A and B), with HA-GLUT1 (2 μ g) and AAA-PKB (2 μ g) (A), or with HA-GLUT1 (2 μ g) and $\Delta p85\alpha$ (2 μ g) (B) and incubated in culture for 48 h. In the final 18 h, cells were treated without (basal (B)) or with 100 nM insulin (Ins). Total membranes were prepared and immunoblotted using anti-HA antibody.

DISCUSSION

Translational Control of GLUT1 and GLUT4 mRNAs by Insulin—Insulin is an anabolic hormone that increases the overall rate of protein synthesis. Insulin also preferentially regulates the biosynthesis of certain proteins above and beyond its general effect on global protein synthesis. However, the list of these preferentially regulated proteins remains short and thus far includes only ornithine decarboxylase (29), elongation factor 2 (28), and 20 other unidentified proteins (28). A small number of these preferential effects can take place in the absence of ongoing mRNA transcription (29), whereas a large number of selective effects on protein synthesis are dependent upon continued mRNA synthesis (28).

Much has been learned about the preferential induction of GLUT1 expression at the transcriptional and post-transcriptional levels. In this report, we observed that GLUT1 mRNA translation is yet another level that insulin regulates. Indeed, insulin promoted an increase in the percentage of GLUT1 transcripts associated with heavy polysomes and resulted in a concomitant decrease in GLUT1 mRNA associated with monosomes. The association of GLUT1 mRNA in 3T3-L1 cells with polysomes under unstimulated conditions was previously shown by Jain *et al.* (34) and suggests that ongoing translation of this mRNA may be required to fulfill the basal needs of glucose uptake. A similar behavior has been reported for the mRNAs of tumor necrosis factor α and the α_1 - and β_1 -subunits of the Na⁺/K⁺-ATPase (39, 40). Considering that insulin also increases the rate of synthesis of GLUT1 protein (Fig. 4B), the effect of insulin on the distribution of GLUT1 transcripts in the polysome profile may indicate an acceleration of the initiation of translation rather than inhibition of elongation. The increase in GLUT1 protein synthesis caused by insulin (114% above basal levels) (Fig. 4B) exceeded the [³⁵S]methionine incorporation into total trichloroacetic acid-precipitable protein (~5% increase above basal levels following 18 h of insulin treatment) (8). This suggests that GLUT1 protein is preferentially regulated in response to the hormone above the level of elevation in global protein synthesis.

Despite a clear recruitment of GLUT1 mRNA from monosomes to polysomes by insulin, the hormone did not alter the separation of rRNA between monosomes and polysomes. Interestingly, a similar scenario was encountered by Nielsen *et al.* (41), who observed that exponential growth enhances insulin-like growth factor II mRNA translation, yet the monosome/polysome sedimentation profiles of growth-arrested and exponentially growing cells were identical.

Features of mRNAs that are translationally regulated include the following: (i) a 5'-terminal polypyrimidine tract, which is found mainly in mRNAs of ribosomal proteins (42); (ii) a high GC content in the 5'-untranslated region (UTR), indicating the potential for extensive secondary structure formation (43); (iii) the presence of *cis*-acting elements in the mRNA to interact with *trans*-acting cytosolic factors; and (iv) the presence of AUUUA motifs in the 3'-UTR, which are recognition sequences for factors regulating mRNA stability/degradation (44). The 5'-UTR of GLUT1 mRNA is GC-rich (73%), and potential hairpin-loop structures have been proposed to form in this region (45). The *cis*-acting elements in the GLUT1 5'-UTR involved in the translational control of this transcript have been mapped, and deletion of these elements produced a marked decrease in the translational efficiency of the GLUT1 mRNA (45). Conversely, transfection of brain endothelial cells with a luciferase construct containing these elements of the GLUT1 5'-UTR resulted in a >3-fold increase in luciferase expression (45). Finally, the destabilizing motif AUUUA in the 3'-UTR of GLUT1 mRNA in 3T3-L1 preadipocytes binds to RNA-binding proteins, thus increasing GLUT1 mRNA stability and potential for translation (44). Hence, features typical of mRNAs susceptible to regulation at the level of translation are displayed by GLUT1 mRNA. This prediction is borne out by our observation that GLUT1 expression is regulated by insulin at the translational level.

Although insulin caused *both* an increase in the amount of GLUT1 mRNA (Fig. 4C) and an increase in GLUT1 mRNA translation (Fig. 1B), the elevation in GLUT1 protein did not exceed that of the message. It is possible that the accompanying decrease in the half-life of GLUT1 protein in response to insulin in these cells (8) prevents any cumulative elevation in GLUT1 protein. Interestingly, a similar scenario has been observed by Goalstone and Draznin (46), who demonstrated that insulin increases farnesyltransferase mRNA levels without a matching rise in farnesyltransferase protein.

Unlike its effect on GLUT1 mRNA, chronic exposure to insulin inhibited the translation of GLUT4 mRNA. This distinct effect on GLUT1 and GLUT4 translation may be a reflection of their specific structural mRNA features. For example, the 5'-UTR of GLUT4 mRNA is shorter than that of GLUT1 (105 nucleotides compared with 179) and is only 47% GC-rich compared with 73% for GLUT1 (2).

Role of mTOR/4E-BP1 and the Upstream Regulators PI3K/PKB in GLUT1 mRNA Translation—The insulin-dependent stimulation of GLUT1 mRNA translation occurs, at least in part, via the rapamycin-sensitive mTOR pathway. It is conceivable that insulin increases GLUT1 mRNA translation in 3T3-L1 adipocytes via the phosphorylation of 4E-BP1 by mTOR. This is based on the following findings. (i) In 3T3-L1 cells, insulin phosphorylates 4E-BP1 (17, 18). (ii) Rapamycin blocks cap-dependent translation by preventing phosphorylation of 4E-BP1 (18, 23). (iii) Rapamycin inhibited the insulin-induced up-regulation of GLUT1 mRNA translation (this study). (iv) 4E-BP1 reduced the translation of GLUT1 transcripts *in vitro* (this study). Our observation that rapamycin did not affect the polysome profile (Fig. 2A) supports the notion that rapamycin exhibits a minor effect on the translation of the majority of RNAs and further highlights the specificity of its effect on GLUT1 translation. Our observation is similar to that made by Pedersen *et al.* (47), who reported that the overall sedimentation profile, the relative distribution of ribosomes between monosomes and polysomes, and, in turn, the average number of ribosomes on mRNAs are essentially unchanged following rapamycin treatment.

The role of this pathway in GLUT1 mRNA translation and

protein expression was further confirmed by the ability of rapamycin to abrogate the insulin-elicited increase in *de novo* GLUT1 protein synthesis. The partial inhibitory effects of rapamycin on GLUT1 mRNA translation and the partial inhibition of increases in GLUT1 mRNA abundance, combined, may explain the complete abrogation of GLUT1 protein expression.

The stimulation by insulin of p70 S6 kinase, a serine/threonine kinase that phosphorylates the S6 ribosomal protein, is also prevented by rapamycin (48). As p70 S6 kinase lies downstream of mTOR, it is conceivable that insulin could increase GLUT1 mRNA translation via the mTOR → p70 S6 kinase axis. However, this prediction may not hold since expression of an active, but rapamycin-resistant, p70 S6 kinase cannot protect 4E-BP1 from dephosphorylation upon treatment with rapamycin (49, 50). Furthermore, p70 S6 kinase fails to directly phosphorylate 4E-BP1 *in vitro* (51), whereas mTOR functions as a 4E-BP1 kinase both *in vivo* and *in vitro* (24). Hence, the two mTOR targets, namely p70 S6 kinase and 4E-BP1, are regulated in a parallel rather than sequential manner. Thus, it is conceivable that the up-regulation of GLUT1 mRNA translation by insulin lies downstream of mTOR and not of p70 S6 kinase. Additionally, GLUT1 mRNA lacks an oligopyrimidine tract at its transcriptional start site (5'-TOP), a hallmark of mRNAs whose translation is regulated by p70 S6 kinase (42). In the future, further verification of the lack of involvement of p70 S6 kinase in GLUT1 mRNA translation will stem from using dominant-interfering constructs of p70 S6 kinase.

In this study, we also provide evidence for a role of PI3K and PKB in GLUT1 protein expression. Given the difficulty in introducing foreign DNA into 3T3-L1 cells without viral infection, which may affect protein synthesis, and considering our recent characterization of the behavior of AAA-PKB and $\Delta p85\alpha$ constructs in L6 myoblasts (32), the latter cell line was chosen for the transfection experiments. We found that dominant inhibitory mutants of PI3K and PKB inhibit the insulin-mediated increase in HA-GLUT1 protein. These observations support our hypothesis that the PI3K/PKB cascade, in concert with mTOR, participates in GLUT1 protein expression at a post-transcriptional level.

Translational control has been well described for mRNAs encoding components of the translational apparatus itself (*e.g.* ribosomal proteins and elongation factors). Regulation of translation by the mTOR/4E-BP1/eIF-4E pathway has so far been described only for mRNAs encoding growth-related proteins such as ornithine decarboxylase (29), cyclin D1 (52), and p23 (53). Our observation that GLUT1 mRNA is governed by this type of regulation indicates that GLUT1 is a new member of the family of growth-related proteins and adds a new perspective to the functions of mTOR.

Although our results suggest a need for the PI3K/PKB/mTOR signaling pathway in linking the insulin receptor to GLUT1 protein expression, the involvement of other signals is not excluded. For example, an inhibitor of MEK1/MEK2, PD098059 (54), also inhibited the insulin-stimulated elevation in the total cellular content of GLUT1 protein in 3T3-L1 adipocytes.² Additionally, microinjection of dominant inhibitory forms of Ras or neutralizing antibodies directed against Ras in 3T3-L1 adipocytes blocks the gain in GLUT1 protein expression at the cell surface caused by exposure of these cells to insulin (55).

Relevance to Diabetes—Studies have shown that the suppressed responsiveness to insulin in type II diabetes is due to decreased abundance of as well as functional defects in GLUT4 (56–58). Other studies have also shown an elevation in GLUT1

² C. Taha and A. Klip, unpublished observations.

protein levels associated with prolonged hyperinsulinemia (7). The increase in GLUT1 mRNA translation and protein expression and the decrease in that of GLUT4 in response to prolonged insulinemia could contribute to several deleterious consequences of diabetes such as (i) a chronic rise in the flux of glucose under basal conditions (7, 59), (ii) an elevation in the risk of glucose toxicity and glucose-induced tissue damage known as "diabetic complications," and (iii) the failure of insulin to further stimulate glucose uptake (insulin resistance) (7, 59). Understanding and thereby manipulating the signal transduction pathway that leads to GLUT1 and GLUT4 expression may serve as a means to manage type II diabetes.

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