Hyperglycemia Mediates a Shift From Cap-Dependent to Cap-Independent Translation Via a 4E-BP1–Dependent Mechanism

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Diabetes and its associated hyperglycemia induce multiple changes in liver function, yet we know little about the role played by translational control of gene expression in mediating the responses to these conditions. Here, we evaluate the hypothesis that hyperglycemia-induced O-GlcNAcylation of the translational regulatory protein 4E-BP1 alters hepatic gene expression through a process involving the selection of mRNA for translation. In both streptozotocin (STZ)-treated mice and cells in culture exposed to hyperglycemic conditions, expression of 4E-BP1 and its interaction with the mRNA cap-binding protein eIF4E were enhanced in conjunction with downregulation of cap-dependent and concomitant upregulation of cap-independent mRNA translation, as assessed by a bicistronic luciferase reporter assay. Phlorizin treatment of STZ-treated mice lowered blood glucose concentrations and reduced activity of the cap-independent reporter. Notably, the glucose-induced shift from cap-dependent to cap-independent mRNA translation did not occur in cells lacking 4E-BP1. The extensive nature of this shift in translational control of gene expression was revealed using pulsed stable isotope labeling by amino acids in cell culture to identify proteins that undergo altered rates of synthesis in response to hyperglycemia. Taken together, these data provide evidence for a novel mechanism whereby O-GlcNAcylation of 4E-BP1 mediates translational control of hepatic gene expression.

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R egulation of gene expression at the level of translation initiation plays a critical role in biological processes such as cellular proliferation, development, and response to biological cues and environmental stresses. Recruitment of ribosomes to mRNA is the rate-limiting step in translation initiation, which in mammalian cells occurs through both cap-dependent and cap-independent mechanisms. According to the traditional model proposed for cap-dependent translation, recruitment of the ribosome onto eukaryotic mRNA occurs upon recognition of the m7GTP cap at the 5′-end of mRNA by eIF4F, followed by binding of the eIF4F-mRNA complex to the 40S ribosomal subunit (1). Alternatively, some cellular mRNAs contain unique RNA elements known as internal ribosomal entry sites (IRES) that enable them to use a cap-independent mechanism of translation initiation (2). An IRES is a highly structured nucleotide sequence located in the 5′-untranslated region (UTR) that promotes binding of 40S ribosomal subunits to a portion of the mRNA at or near the AUG start codon (3). The inclusion of IRES elements in the 5′-UTR of messages allows them to be translated under physiological stress conditions wherein cap-dependent protein synthesis is compromised (4–7).

With the exception of eIF4E, the same canonical initiation factors that facilitate cap-mediated loading of ribosomes onto mRNA are required for cap-independent initiation (8). Further, eIF4E has been shown to function as a negative modulator of IRES-mediated translation by increasing competition from capped mRNAs for initiation factor complexes (9). The interaction of eIF4E and eIF4G is of critical importance for cap-dependent initiation and is principally regulated by eIF4E sequestering proteins, such as 4E-BP1. Binding of 4E-BP1 to eIF4E is mutually exclusive of eIF4E interaction with eIF4G and is controlled by the sequential phosphorylation of serine/threonine residues on 4E-BP1 (10). Hypophosphorylated 4E-BP1 binds eIF4E strongly; however, upon phosphorylation 4E-BP1 releases eIF4E, allowing eIF4E to interact with eIF4G, which promotes ribosome loading onto the mRNA 5′-cap. Thus, conditions that promote 4E-BP1 binding to eIF4E potentially act as a switch between cap-dependent and cap-independent translation. Intriguingly, we have recently showed that under hyperglycemic conditions 4E-BP1 is also modified by addition of N-Acetylglucosamine to Ser or Thr residues (O-GlcNAcylation), which enhanced its interaction with eIF4E independent of phosphorylation status (11).

Hyperglycemia increases the flux of glucose through the hexosamine biosynthetic pathway (HBP) to increase the production of uridine diphosphate (UDP) N-Acetylgalactosamine, which promotes protein O-GlcNAcylation (12) and contributes to the pathophysiology of diabetes (13). O-linked GlcNAcylation has been shown to influence protein function by altering subcellular localization, protein-protein interactions, DNA binding, enzyme activity, and turnover rates (14–18). O-GlcNAcylation modification is dynamic, cycling rapidly on and off proteins in a manner that is more reminiscent of protein phosphorylation than other forms of common glycosylation (19). The O-GlcNAcylation cycling reactions are catalyzed by the enzymes O-GlcNAcylation transferase and O-GlcNAcase (20–22), which strongly associate with subpopulations of cytosolic ribosomes, suggesting that they play an important role in regulating mRNA translation (23). In the current study, we evaluated the hypothesis that hyperglycemia causes elevated
O-GlcNAcylation of 4E-BP1 and enhanced binding of 4E-BP1 to eIF4E, which in turn causes a shift from cap-dependent to cap-independent mRNA translation. Overall, the results support the conclusion that O-GlcNAcylation of 4E-BP1 expression underlies a hyperglycemia-mediated shift in gene expression.

RESEARCH DESIGN AND METHODS

Male RFL12xBLK6 F1 mice expressing a bicistronic Renilla luciferase (LucR) and firefly luciferase (LucF) IRES-driven luciferase (LucF) mRNA were treated with 50 mg/mL streptozotocin (STZ) for 5 days to induce diabetes. RFL12 mice have previously been described (24). Diabetes phenotype was confirmed by blood glucose concentrations >400 mg/dL. Four weeks after STZ treatment, phosphor-1R was administered subcutaneously twice daily for 7 days (11). Procedures were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. Analysis of protein phosphorylation in liver homogenates and immunoprecipitations was performed as previously described (11). LucR and LucF activity was measured by Dual-Glo Luciferase Assay (Promega).

Cell culture and transfections. Primary cultures of hepatocytes were prepared from the liver of RFL12xBLK6F1 and previously described Eif4ebp1 or Eif4ebp2 knockout mice (25) using the Worthington Hepatocyte Isolation System (Worthington). Prior to transfection, Eif4ebp1 or Eif4ebp2 to hepatocytes were transfected with a bicistronic reporter plasmid containing the vascular endothelial growth factor (VEGF) IRES using an Amazix Hepatocyte Nucleofector kit (Lonza). Hepatocytes were seeded onto plates coated with rat tail collagen in Williams E Medium (Gibco) supplemented with 5% FBS (Atlas), 1% penicillin/streptomycin, 100 U/mL sodium pyruvate and containing either 25 or 5 mmol/L glucose plus 20 mmol/L mannitol as an osmotic control supplemented with 10% FBS and 1% penicillin/streptomycycin. Transfections were performed using X-tremeGène HP (Roche). Where indicated, cells were treated with 3 mmol/L glucosamine (Sigma) or 50 mmol/L thiamet G (Cayman) to enhance protein O-GlcNAcylation. Pulsed stable isotope labeling by amino acids in cell culture. Cultures of wild-type or Eif4ebp1;Eif4ebp2 double knockout mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco’s modified Eagle’s medium lacking sodium pyruvate and containing either 25 or 5 mmol/L glucose plus 20 mmol/L mannitol as an osmotic control supplemented with 10% FBS and 1% penicillin/streptomycin. Transfections were performed using X-tremeGène HP (Roche). Where indicated, cells were treated with 3 mmol/L glucosamine (Sigma) or 50 mmol/L thiamet G (Cayman) to enhance protein O-GlcNAcylation.

RESULTS

Diabetes promotes cap-independent translation and enhances 4E-BP1 expression. In the current study, we used a transgenic mouse model that expresses a bicistronic mRNA containing two open reading frames (ORFs) encoding two distinct luciferase enzymes: LucR and LucF. Translation of the first ORF (LucR) occurs in a cap-dependent manner, whereas translation of the second ORF (LucF) is driven by the FGF-2 IRES that is located between the two cistrons (Fig. 1A). Similar transgenic mice have been used to demonstrate that diabetes leads to upregulated expression of FGF-2 by increased utilization of the FGF-2 IRES in the aorta (30). Four weeks after the induction of diabetes, blood glucose levels were raised from 180 ± 12 to 540 ± 26 mg/dL and the activity of the LucR reporter was reduced by 62% in the liver of mice with STZ-induced diabetes compared with nondiabetic littermates (Fig. 1B), whereas LucF activity was elevated by 192% (Fig. 1C). As a result, the relative LucF-to-LucR activity ratio was enhanced by 633% (Fig. 1D). To investigate a potential mechanism for mediating the shift from cap-dependent to cap-independent translation in the liver of STZ-treated mice, the interaction of 4E-BP1 and eIF4F with eIF4E was evaluated. The amount of 4E-BP1 bound to eIF4E was elevated in the liver of diabetic compared with control mice (Fig. 1E), whereas the amount of eIF4E bound to eIF4E was reduced (Fig. 1F). These changes were accompanied by decreased 4E-BP1 phosphorylation (Fig. 1G) and increased O-GlcNAcylation (Fig. 1H). Whereas the expression of eIF4E in the liver of STZ-treated and control mice was not different (Fig. 1D), total hepatic 4E-BP1 expression was increased (Fig. 1J) and likely contributed to increased interaction with eIF4E.

Role of hyperglycemia in promoting cap-independent translation. The role of hyperglycemia in promoting the diabetes-induced shift toward cap-independent translation was assessed using phlorizin treatment, which rapidly lowers blood glucose concentrations by blocking intestinal glucose absorption and producing renal glucosuria (31). Phlorizin treatment reduced the nonfasting blood glucose of mice with STZ-induced diabetes from a concentration of 539 ± 22 to 300 ± 16 mg/dL (Fig. 2A). LucR activity was repressed in the liver of diabetic compared with control mice, and upon phlorizin treatment its activity was elevated by 47% (Fig. 2A). Whereas LucF activity was elevated by 99% in diabetic compared with control mice, after treatment with phlorizin LucF activity was elevated by only 42% (Fig. 2C). Lowering blood glucose levels reduced the relative LucF-to-LucR ratio observed in the liver of diabetic mice by 52% (Fig. 2D). Phlorizin treatment of diabetic mice also reduced the amount of 4E-BP1 in eIF4E immunoprecipitates (Fig. 2E) and concomitantly elevated the interaction of eIF4E with eIF4G (Fig. 2F). The reduced
interaction of 4E-BP1 with eIF4E upon phlorizin treatment was likely in part due to lower 4E-BP1 expression (Fig. 2G) as well as increased phosphorylation (Fig. 2H) and decreased O-GlcNAcylation (Fig. 2I) of 4E-BP1 compared with untreated diabetic mice. Similar to previous findings with Ins2Akita/+ diabetic mice (11), phlorizin treatment alleviated the elevated hepatic expression of glutamine-fructose-6-phosphate amidotransferase (GFAT) (Fig. 2J) but did not alter total eIF4E expression (Fig. 2K).

**Hyperglycemia promotes cap-independent translation by increasing flux through the HBP.** GFAT is the rate-limiting enzyme of the HBP and as such regulates the production of UDP N-Acetylglucosamine, which serves as the substrate for protein O-GlcNAcylation. Changes in GFAT expression suggest that flux through the HBP plays an important role in mediating the effects of hyperglycemia on 4E-BP1 O-GlcNAcylation and expression. For further evaluation of the role of hyperglycemia and the HBP in cap-dependent and cap-independent translation, hepatocytes isolated from the liver of the transgenic bicistronic reporter mice were incubated in medium containing either 11 or 33 mmol/L glucose to match blood glucose concentrations observed in control and STZ-treated mice. Hyperglycemic conditions dramatically enhanced the relative cap-independent translation (Fig. 3A) and 4E-BP1 expression (Fig. 3B) without altering eIF4E expression (Fig. 3C). Immunoprecipitation of eIF4E revealed elevated interaction of 4E-BP1 with eIF4E in hepatocytes maintained in the presence of high compared with low glucose (Fig. 3D). When hepatocytes maintained in low glucose medium were treated with glucosamine to directly stimulate the HBP or thiamet G, a potent and selective inhibitor of O-GlcNAcase that dramatically elevates protein O-GlcNAcylation (32) by bypassing GFAT, total protein O-GlcNAcylation was enhanced (Fig. 3E). Similarly, both glucosamine and thiamet G enhanced the LucF-to-LucR activity ratio in hepatocytes when maintained in low glucose medium (Fig. 3F). Both glucosamine and thiamet G enhanced expression of 4E-BP1 (Fig. 3G) but not eIF4E (Fig. 3H), and immunoprecipitation of eIF4E revealed elevated interaction of 4E-BP1 with eIF4E (Fig. 3I). Overall, these findings support the conclusion that the hyperglycemia-induced shift from cap-dependent to cap-independent luciferase reporter activity was mediated by changes in protein O-GlcNAcylation that coincide with alterations in the expression of 4E-BP1 and its interaction with eIF4E.

**Ablation of 4E-BP1/2 prevents hyperglycemia-mediated shift from cap-dependent to cap-independent translation.** The role of hyperglycemia in modulating the shift from cap-dependent to cap-independent translation was further assessed using MEFs transiently transfected with bicistronic luciferase constructs in which the activity of LucR occurs in a cap-dependent manner and the activity of LucF is regulated by either the FGF-2 or the VEGF. Under high-compared with low-glucose conditions, the ratio of LucF to LucR activity was examined by immunoprecipitating eIF4E and measuring the amount of 4E-BP1 and eIF4G in the immunoprecipitate (IP) by Western blot analysis. Phosphorylation (G) and O-GlcNAcylation (H) of 4E-BP1 as well as total eIF4E (I) and 4E-BP1 (J) content was measured by treating supernatant fractions with λ-phosphatase (PPase) followed by Western blot analysis as previously described (11). Values are means ± SE for a single experiment (n = 8). Statistical significance of the differences between means was assessed by Student t test and is denoted by *P < 0.05.
to LucR activity increased by 20% for the FGF-2 IRES and 77% for the VEGF IRES (Fig. 4A). Further, the ratio of LucF to LucR activity with the VEGF IRES also increased by 79% when cells maintained in low-glucose medium were treated with glucosamine (Fig. 4B). When wild-type and 4E-BP1/2 knockout (Eif4ebp1;Eif4ebp2) MEFs were analyzed after transient transfection with the bicistronic luciferase construct containing the VEGF IRES, the expression of 4E-BP1 was increased by 72% in cells maintained in medium containing high glucose and treated with glucosamine compared with the low-glucose condition (Fig. 4C). There was no detectable 4E-BP1 under either condition in knockout cells. Exposure to high glucose and glucosamine increased the ratio of LucF to LucR activity in wild-type cells to 333% that of the low-glucose condition. In contrast, there was no effect on the ratio of LucF to LucR activity with treatment of 4E-BP1/2 knockout cells (Fig. 4D). For confirmation of the role of 4E-BP1 specifically, hepatocytes from 4E-BP1/2 knockout mice were transfected with a bicistronic plasmid containing the IRES for VEGF and maintained in medium containing either low glucose or high glucose with glucosamine. There was no significant difference in the ratio of LucF to LucR activity under the high- and low-glucose conditions for 4E-BP1 knockout hepatocytes, whereas high glucose increased the ratio of LucF to LucR activity in 4E-BP2 knockout hepatocytes to 234% that of the low-glucose condition (Fig. 4E). These results demonstrate that expression of 4E-BP1 but not 4E-BP2 is necessary for the hyperglycemia-induced shift from cap-dependent to cap-independent translation.
PsILAC identifies proteins with altered synthetic rates under hyperglycemic conditions. To identify hyperglycemia-induced changes in the translational control of protein expression on a global scale, we used PsILAC to evaluate newly synthesized proteins and quantitate changes in their accumulation rates in MEFs in the presence of either low glucose or high glucose supplemented with glucosamine, as these conditions produced the largest increase in the LucF-to-LucR ratio (Fig. 4B). In total, 1,355 and 2,905 proteins were identified with high confidence in two independent runs. While the majority of proteins had medium-to-heavy ratios of ~1:1, indicating that their translation was not significantly altered by hyperglycemic conditions, the synthesis of a number of proteins was significantly altered between the two conditions. The top-scoring proteins whose synthesis was either upregulated or repressed under high- compared with low-glucose condition are listed in Tables 1 and 2, respectively.

In the classic model of cap-dependent translation initiation, ribosome scanning of the 5'-UTR is impaired by strong secondary structures. Characteristics that decrease the efficiency of cap-dependent translation include the length of the 5'-UTR and the complexity of secondary structure (33). Thus, we predicted that the high-glucose condition would repress the translation of mRNAs with
less complex 5' UTRs in favor of mRNAs with more structured 5' UTRs. Most cellular IRES elements are 150–300 nucleotides in length, although some are as short as 22 nucleotides (34). The mRNA-encoding proteins whose synthesis was upregulated by high glucose averaged 185.3 nucleotides in length and were 74% longer than the average number of nucleotides in 5' UTRs of mRNAs encoding proteins whose synthesis was downregulated by high glucose (Fig. 5A). To investigate the complexity of these 5' UTRs, we used mfold (http://mfold.rna.albany.edu/?) to compute secondary structures and calculate a corresponding free energy change for folding ($\Delta G$). The top-scoring proteins whose synthesis was downregulated by high-glucose conditions have an average $\Delta G$ of $-41.1$ kcal/mol, whereas the top-scoring proteins whose synthesis was upregulated by high-glucose conditions have a significantly lower average $\Delta G$ value of $-77.5$ kcal/mol, indicative of more stable secondary structures (Fig. 5B). To verify that the hyperglycemia-induced enhancement in the synthetic rates of proteins with longer 5' UTRs was not
| Accession no. | RefSeq ID | Name | %Cov (95) | Peptides (95%) | M:H | P |
|--------------|-----------|------|-----------|----------------|-----|---|
| 8393135      | NM_016904 | Cyclin-dependent kinases regulatory subunit 1 | 32.91 | 2 | 0.01 | 0.017 |
| 27734154     | NM_173413 | ras-related protein Rab-8B | 27.05 | 5 | 0.01 | 0.015 |
| 6756037      | NM_011738 | 14–3-3 protein η | 24.80 | 6 | 0.01 | 0.00 |
| 31542499     | NM_007544 | BH3-interacting domain death agonist | 18.46 | 2 | 0.01 | 0.014 |
| 6755100      | NM_011119 | Proliferation-associated protein 2G4 | 17.77 | 5 | 0.01 | 0.016 |
| 110625776    | NM_027412 | Tetrapropeptide repeat protein 9C | 16.96 | 2 | 0.01 | 0.014 |
| 21313588     | NM_024499 | Small glutamine-rich tetrapropeptide repeat-containing protein α | 15.56 | 4 | 0.01 | 0.018 |
| 6755478      | NM_011358 | Serine/arginine-rich splicing factor 2 | 14.93 | 3 | 0.01 | 0.014 |
| 47059484     | NM_009030 | Histone-binding protein RBBP4 | 14.59 | 7 | 0.01 | 0.014 |
| 169790999    | NM_023514 | 28S ribosomal protein S9, mitochondrial precursor | 13.33 | 4 | 0.01 | 0.015 |
| 295789090    | NM_001177965 | N-acetyltransferase 10 isoform 2 | 13.33 | 2 | 0.01 | 0.014 |
| 32441290     | NM_027133 | Protein lunapark isoform a | 12.94 | 3 | 0.01 | 0.014 |
| 9789907      | NM_019719 | STIP1 homology and U box-containing protein 1 | 10.53 | 3 | 0.01 | 0.013 |
| 21313618     | NM_025336 | Coiled-coil-helix-coiled-coil-helix domain-containing protein 3 | 8.81 | 2 | 0.01 | 0.014 |
| 258679524    | NM_001164838 | Leucine-rich repeat flightless-interacting protein 2 isoform 1 | 7.95 | 2 | 0.01 | 0.014 |
| 6671549      | NM_007453 | Peroxiredoxin-6 | 7.59 | 1 | 0.01 | 0.017 |
| 160707921    | NM_013469 | Annexin A11 | 7.16 | 3 | 0.01 | 0.014 |
| 30409972     | NM_178398 | WD repeat domain phosphinositide-interacting protein 2 | 6.52 | 2 | 0.01 | 0.014 |
| 18278501     | NM_027879 | Pre-mRNA-processing factor 17 | 5.70 | 2 | 0.01 | 0.014 |
| 31980866     | NM_019586 | Ubiquitin-conjugating enzyme E2 J1 | 5.35 | 1 | 0.01 | 0.014 |
| 21225951     | NM_146093 | UBX domain-containing protein 1 | 4.40 | 1 | 0.01 | 0.016 |
| 255683374    | NM_145610 | Suppressor of SW41 homolog | 3.83 | 1 | 0.01 | 0.014 |

Cultures of wild-type MEFs were maintained in Dulbecco’s modified Eagle’s medium containing either 5 or 25 mmol/L glucose. For the low-glucose condition, cells were then labeled for 6 h by pSILAC using 5 mmol/L glucose supplemented with 20 mmol/L mannitol and labeled with 4,4,5,5-D4-L-lysine monohydrochloride and L-arginine-13C6. For the high-glucose condition, MEF cultures were maintained in 25 mmol/L glucose supplemented with 3 mmol/L glucosamine and labeled with media containing L-lysine-13C6-15N2 monohydrochloride and L-arginine-13C6-15N4 monohydrochloride. Cells were harvested, combined, and analyzed by mass spectrometry to compare labeled peptide peaks. Statistical significance was assessed by Student t test on the average of all ratio values for the peptides associated with each particular protein. %Cov (95), the percentage of matching amino acids from identified peptides having confidence greater than or equal to 95 divided by the total number of amino acids in the sequence.
The result of elevated mRNA transcription, we evaluated the abundance of mRNAs corresponding to six proteins from Table 1 with the longest 5′-UTRs (Fig. 5C). Of the six analyzed, only one exhibited increased abundance, and that was 50-fold less than the accumulation of newly synthesized protein. Changes in protein degradation may also impact the M-to-H ratio observed during pSILAC; however, its contribution is relatively small (35). These findings suggest that hyperglycemia favors the translation of messages with longer/more highly structured 5′-UTRs. Although no proteins encoded by mRNAs containing known IRES structures were identified among the top-scoring upregulated proteins, several proteins were identified whose mRNAs are listed on IRESite, the database of experimentally verified IRES structures (36). Among these, cellular inhibitor of apoptosis 1 (c-IAP1) (gi|133922596), heat shock 70-kDa protein 1A (HSPA1A) (gi|40254361), cold shock domain containing E1 (UNR) (gi|240255574), and cytochrome c oxidase assembly protein 15N4 (COX15 homolog) (gi|356995938) were all elevated by nearly twofold in cells exposed to high-glucose conditions.

Cultures of wild-type MEFs were maintained in Dulbecco’s modified Eagle’s medium containing either 5 or 25 mmol/L glucose. For the low-glucose condition, cells were then labeled for 6 h by pSILAC using 5 mmol/L glucose supplemented with 20 mmol/L mannitol and labeled with [15N4]L-lysine monohydrochloride and L-arginine-13C6. For the high-glucose condition, MEF cultures were maintained in 25 mmol/L glucose supplemented with 3 mmol/L glucosamine and labeled with media containing L-lysine-13C6.

### TABLE 2

Top proteins identified by pSILAC with repressed synthetic rates in cells exposed to hyperglycemic conditions

| Accession no. | RefSeq ID | Name                                      | %Cov (95) | Peptides (95%) | M:H  | P   |
|---------------|-----------|-------------------------------------------|-----------|----------------|------|-----|
| 71480098      | NM_001024622 | PEST proteolytic signal–containing nuclear protein | 27.53     | 3              | 100  | 0.016 |
| 18702317      | NM_016900  | Caveolin-2                                 | 22.22     | 2              | 100  | 0.015 |
| 21426823      | NM_030609  | Histone H1.1                               | 20.19     | 6              | 100  | 0.015 |
| 13384730      | NM_025364  | SAP domain-containing ribonucleoprotein    | 20.00     | 3              | 100  | 0.000 |
| 6677691       | NM_009307  | Reticulocalbin-1 precursor                 | 13.55     | 5              | 100  | 0.000 |
| 19705424      | NM_009439  | 26S proteasome non-ATPase regulatory subunit 3 | 13.02    | 3              | 100  | 0.014 |
| 6753412       | NM_011801  | Craniofacial development protein 1         | 12.88     | 3              | 100  | 0.014 |
| 170632536     | NM_007916  | ATP-dependent RNA helicase DDX19A          | 12.55     | 6              | 100  | 0.015 |
| 160707909     | NM_009496  | Vasodilator-stimulated phosphoprotein       | 11.20     | 3              | 100  | 0.015 |
| 152963551     | NM_177374  | tRNA [adenine(58)-N(1)]-methyltransferase   | 10.69     | 2              | 100  | 0.015 |

For the high-glucose condition, cells were then labeled for 6 h by pSILAC using 5 mmol/L glucose supplemented with 20 mmol/L mannitol and labeled with [15N4]L-lysine monohydrochloride. Cells were harvested, combined, and analyzed by mass spectrometry to compare labeled peptide peaks. Statistical significance was assessed by Student’s t test on the average of all ratio values for the peptides associated with each particular protein. %Cov (95), the percentage of matching amino acids from identified peptides having confidence greater than or equal to 95 divided by the total number of amino acids in the sequence.
in two independent runs, while BCL2 (gi|6753200) increased by nearly 80-fold in the second run but was not detected in the initial run.

Functional relationships between genes that were differentially expressed in low and high glucose were investigated with IPA. IPA showed that the proteins whose accumulation was upregulated are associated with pathways that regulate eIF2, eIF4F, and S6K1 signaling; protein ubiquitination; and aminoacyl-tRNA biosynthesis (Table 3). Further, the most significant toxicologic list pathways were hypoxia-inducible factor (HIF) signaling, oxidative stress mediated by nuclear factor erythroid 2-related factor 2 (Nrf2), mitochondrial dysfunction, and DNA damage checkpoint regulation of cell cycle phase G2/M (Table 3). Both HIF-1 and Nrf2 contain IRES sequences in their 5'-UTR that allow their translation to be maintained under stress conditions that are inhibitory to cap-dependent translation (6,37). IPA was also used to predict which mRNAs encoding transcription factors were most likely responsible for the altered pattern of expression under low- and high-glucose conditions. The top transcription factor altered by high glucose was the proto-oncogene myc (Table 3), whose 5'-UTR contains an IRES. Activation of myc was predicted under high-glucose conditions based on a broad network of interacting proteins (Supplementary Fig. 1).

**DISCUSSION**

The findings of the current study provide insight into a novel mechanism through which diabetes-induced hyperglycemia alters the selection of mRNA for translation. In the liver of diabetic mice, 4E-BP1 exhibited reduced phosphorylation, elevated O-GlcNAcylation, and enhanced interaction with eIF4E compared with non-diabetic mice. These alterations in 4E-BP1 were associated with a shift from cap-dependent to cap-independent translation using bicistronic luciferase reporter assays. For demonstration of the component of the diabetic state responsible for this shift, diabetic mice were treated with phlorizin to reduce blood glucose concentrations. When the blood glucose concentration was lowered, the interaction of 4E-BP1 with eIF4E returned to nondiabetic levels and the shift from cap-dependent to cap-independent reporter activity was reversed. A similar shift toward cap-independent mRNA translation was observed with cells in culture upon exposure to hyperglycemic conditions or under conditions that promoted protein O-GlcNAcylation. O-GlcNAcylation of 4E-BP1 correlated with the hyperglycemia-induced shift from cap-dependent to cap-independent translation, and expression of 4E-BP1 was necessary for this effect. We extended these findings using pSILAC to identify novel proteins that undergo altered rates of synthesis in high- versus low-glucose conditions. Examination of the expression pattern of these proteins revealed a hyperglycemia-induced shift toward proteins with more complex 5'-UTRs. Overall, the results are consistent with a model wherein the O-GlcNAcylation of 4E-BP1 results in elevated expression and promotes its interaction with eIF4E (11), thereby altering gene expression in response to hyperglycemia and diabetes.
Regulation of the initiation complex eIF4F by post-translational modification is of critical importance in the selection of mRNAs for cap-dependent translation initiation. Binding of hypophosphorylated 4E-BP1 to eIF4E, which is mutually exclusive of interaction with eIF4G, prevents assembly of functional eIF4F complexes to repress loading of ribosomes onto the mRNA 5′-cap. The interaction of 4E-BP1 with eIF4E is not only regulated by phosphorylation; O-GlcNAcylation of 4E-BP1 also enhances its binding to eIF4E independent of 4E-BP1 phosphorylation status (10). Thus, hyperglycemia-induced sequestration of eIF4E potentially downregulates the synthesis of a broad array of proteins. However, under conditions where eIF4E is limiting, the same canonical initiation factors can facilitate the cap-independent loading of ribosomes onto mRNA that contain IRES elements (8). Therefore, by reducing the competition from capped mRNAs, hyperglycemia-induced sequestration of eIF4E potentially acts as a positive regulator of cap-independent translation in response to conditions of stress.

A critical role for 4E-BP1 in upregulating cap-independent translation has previously been demonstrated (38). Viral IRES-mediated translation is promoted when cellular cap-dependent translation is diminished under conditions of cellular stress (38). However, 4E-BP1 knockdown prevents upregulation of viral cap-independent translation under conditions of amino acid starvation (39). Furthermore, 4E-BP1 plays a crucial role in mediating differential protein expression during hypoxia (40). Overexpressed 4E-BP1 and eIF4G mediate a hypoxia-activated switch from cap-dependent to cap-independent mRNA translation that promotes increased tumor angiogenesis and growth (41). It has been previously demonstrated that the presence of 4E-BP1 is also necessary for increased expression of VEGF in both the retina during diabetes and cells maintained under hyperglycemic conditions (42). The results of the current study suggest that increased VEGF expression occurs through increased use of internal ribosome entry sites in response to increased flux through the HBP. This finding likely extends to other mRNAs that are translated through an IRES-dependent mechanism, as overexpression of O-GlcNAcylation transferase produces an accumulation of 80S monosomes, suggesting that excessive O-GlcNAcylation suppresses translation initiation (23).

To explore the effect of hyperglycemia on mRNA translation, we used pSILAC to identify proteins that undergo altered rates of synthesis. Hyperglycemia promoted translation of mRNAs that contained long and structured 5′-UTRs, a characteristic associated with mRNAs containing IRES. This observation does not directly indicate that hyperglycemic conditions favor translation of all mRNAs with IRES domains. Instead, it is more likely that hyperglycemia-induced modification of the translational machinery enhances translation of a specific subset of messages, some of which contain IRES domains. In this study, hyperglycemia specifically enhanced IRES-mediated translation from luciferase reporters driven by either the FGF-2 or VEGF IRES sequence. Using pSILAC, we also observed increased translation of the following cellular mRNAs that have been previously shown to contain IRES structures: B-cell lymphoma 2 (BCL2), 78-kDa glucose-regulated protein (BiP), c-IAP1, HSPA1A, Runt-related transcription factor 1 (RunX1), UNR, and UTRA. IRES containing mRNAs often encode proteins with crucial biological functions, such as the major vascular growth factors VEGF, FGF-2, platelet-derived growth factor, and RunX1. Thus, enhanced hyperglycemia-mediated translation of angiogenic proteins potentially disrupts the relative balance of inducers and inhibitors of angiogenesis to promote the neurovascular complications associated with diabetes. Furthermore, our data support a previous microarray analysis of the retinal transcriptome where normalization of systemic glycemia in diabetic rats primarily restored pathways associated with growth factor signaling (43).

A functional link between the metabolic abnormalities associated with disease and regulation of IRES sequences has previously been proposed (44), yet direct evidence remains limited. In the current study, we find that hyperglycemia and conditions that promote protein O-GlcNAcylation enhance IRES-mediated mRNA translation through a mechanism that is dependent on 4E-BP1. Thus, regulation of 4E-BP1 expression by O-GlcNAcylation represents a novel mechanism for altering the selection of mRNAs for translation under pathophysiological conditions. Elucidation of molecular mechanisms underlying cap-independent translation initiation will not only enhance our understanding of gene expression but also impact the development of treatment strategies for addressing the pathophysiology of diabetes.

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M.D.D. researched data and wrote and edited the manuscript. J.S.S. contributed to discussion and reviewed the manuscript. B.A.S. researched data, wrote the manuscript, contributed to discussion, and reviewed and edited the manuscript. S.R.K. and L.S.J. designed experiments, contributed to discussion, and reviewed and edited the manuscript. M.D.D. is the guarantor of this work and as such had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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2214 DIABETES, VOL. 62, JULY 2013 diabetes.diabetesjournals.org