Glucose Mediates the Translocation of NeuroD1 by O-Linked Glycosylation*5,6

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O-Linked GlcNAc modification of nuclear and cytosolic proteins has been shown to regulate the function of many cellular proteins. Increased O-linked glycosylation, observed under chronic hyperglycemia conditions, has been implicated in the pathogenesis of diabetes. However, the exact role of O-GlcNAc modification in regulating glucose homeostasis remains to be established. We report here that the subcellular localization of the pancreatic beta cell-specific transcription factor NeuroD1 is regulated by O-linked glycosylation in the mouse insulinoma cell line MIN6. Under low glucose conditions, NeuroD1 is mainly in the cytosol. However, treatment of MIN6 cells with high glucose results in O-linked GlcNAc modification of NeuroD1 and its subsequent translocation into the nucleus. Consistent with these data, treatment of MIN6 cells with O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino N-phenylcarbamate, an inhibitor of O-GlcNAcase, causes NeuroD1 localization to the nucleus and induction of insulin gene expression even on low glucose. Furthermore, we demonstrate that NeuroD1 interacts with the O-GlcNAc transferase, OGT only at high concentrations of glucose and depletion of OGT by using small interfering RNA oligos interferes with the nuclear localization of NeuroD1 on high glucose. On low glucose NeuroD1 interacts with the O-GlcNAcase and becomes deglycosylated, which is likely to be important for export of NeuroD1 into cytosol in the presence of low glucose. In summary, the presented data suggest that glucose regulates the subcellular localization of NeuroD1 in pancreatic beta cells via O-linked GlcNAc modification of NeuroD1 by OGT.

Regulation of insulin production and secretion in accordance with blood glucose levels is critical for maintaining glucose homeostasis (1, 2). Defects in insulin production are associated with Type 1 as well as Type 2 diabetes. Induction of insulin gene transcription in response to elevated blood glucose levels plays an important role in maintaining normoglycemia. Glucose regulation of insulin gene transcription in the pancreas has been shown to be regulated by three beta cell-specific transcription factors, Pdx-1, MafA, and NeuroD1 (3–7). However, the exact mechanisms by which glucose modulates the function of these transcription factors to induce insulin gene expression remains to be established.

In this report we demonstrate that changes in glucose concentration regulate the subcellular localization of NeuroD1 by causing its modification by O-linked glycosylation. NeuroD1/BETA2 belongs to the basic helix loop helix family of transcription factors and functions in a complex with the ubiquitously expressed E2A proteins (5). NeuroD1 is expressed in pancreatic islet cells (5), the intestine (8), and a subset of neurons in the central and peripheral nervous system (9). NeuroD1 binds to the E-box of the insulin promoter and interacts with the p300 co-activator in pancreatic beta cells and regulates insulin gene expression (10). Mutations in NeuroD1 have been linked to the MODY6 locus (11, 12). Homozygous NeuroD1 knock-out mice fail to develop mature islets and die shortly after birth due to severe diabetes (13). NeuroD1 plays an important role also in the development of the nervous system and can convert epidermal cells to neurons in Xenopus (9). Although, NeuroD1 is important for stimulation of insulin gene expression, the exact mechanism by which NeuroD1 activates transcription in pancreatic beta cells is unknown.

O-Linked GlcNAc transferase (OGT)2 modifies nuclear and cytoplasmic proteins by attachment of a single N-acetylglucosamine (GlcNAc) sugar to serine and threonine residues of proteins. This modification is dynamic and reversible and in many cases reciprocally regulated with phosphorylation (14–19). Deletion of OGT in mice results in embryonic lethality, suggesting an essential role for OGT in cell survival (20). O-Linked GlcNAc modification of proteins has been shown to regulate their subcellular localization, protein–protein interactions, DNA binding activity, and proteolytic processing (14, 17, 18, 21, 22). A role for O-GlcNAc modification in regulation of gene transcription is evident from the fact that several transcription factors including c-Myc and p53 are modified by O-linked GlcNAc (14, 18). In addition, the CTD domain of RNA polymerase

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5 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S7.

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2 The abbreviations used are: OGT, O-linked GlcNAc transferase; MIN6, mouse insulinoma 6; PUGNAc, O-(2-acetamido-2-deoxy-o-glucopyranosylidene)amino N-phenylcarbamate; DON, 6-diazoo-5-oxonolaneucleic; ChiP, chromatin immunoprecipitation; siRNA, small interfering RNA; GFP, green fluorescent protein; HBP, hexosamine biosynthetic pathway; O-GlcNAcase, N-acetyl-β-O-glucosaminidase.
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II is also modified by O-GlcNAc in a reciprocal manner with phosphorylation (14, 23). In the case of transcription factors Sp1 and the estrogen receptor, O-GlcNAc modification has been shown to modulate their turnover rate and ability to undergo protein-protein interactions (24–27). It has been previously suggested that O-linked GlcNAc modification plays a role in the assembly of protein complexes. Consistent with this idea OGT has been found in a complex with the co-repressors Hdac1 and mSin3A in the liver cell line HepG2 and is required for repression of gene expression by this complex (28, 29).

Several lines of evidence indicate that elevation of O-linked GlcNAc protein modification results in symptoms associated with diabetes. First, overexpression of OGT in muscle and fat results in insulin resistance and hyperleptinemia (30, 31). Second, overexpression of glutamine fructose-6-phosphate amidotransferase, which catalyzes the rate-limiting step in the synthesis of UDP- N-acetylglucosamine (UDP-GlcNAc) is a substrate for OGT) in various tissues, demonstrates an important role for this pathway in glucose- and satiety-sensing (32). Third, chronic hyperglycemia is associated with increased levels of O-linked GlcNAc modification in pancreatic beta cells (21, 34). The exact mechanism(s) by which increased levels of O-linked GlcNAc modification leads to diabetes is unknown.

In this report we demonstrate that high concentrations of glucose mediate the O-linked GlcNAc modification of the transcription factor NeuroD1 in pancreatic beta cells, leading to its translocation from the cytosol into the nucleus and thereby, to the activation of insulin gene expression. These findings contribute to the understanding of how O-GlcNAc modification regulates pancreatic beta cell function and insulin gene transcription.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse insulinoma 6 (MIN6) cells of passage 22 through 35 were cultured in Dulbecco’s modified Eagle’s media containing 25 mM glucose, 15% (v/v) fetal bovine serum, 1% penicillin/streptomycin, 2 mM glutamine, and 100 μg/ml β-mercaptoethanol (35). For the glucose regulation experiments, MIN6 cells were grown overnight in Dulbecco’s modified Eagle’s media with 25 mM glucose and then washed with 1× phosphate-buffered saline and transferred to low (3 mM) or high (30 mM) glucose containing media for the indicated times.

Cells were treated with the following chemicals: 100 μM O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino N-phenylcarbamate (PUGNAc) (Toronto Research Chemicals Inc.), 20 μM 6-diazo-5-oxonorleucine (DON), and 2 mM glucosamine. The last two chemicals were obtained from Sigma.

Construction and Electroporation of Plasmids—For generation of the enhanced green florescent protein (EGFP)-NeuroD1 fusion construct, the full-length hamster NeuroD1 cDNA was amplified by PCR using the primers: OS269 (forward primer, starts at nucleotide +2 with HindIII site attached (underlined)), CCAAGGCTTTGACAAAATCATACAGCGAG, and OS270 (reverse primer, starts at Stop with BamHI site attached (underlined)), CGGGATCCCTAATCGTGAAA-GATGGCCATTG. After restriction with HindIII and BamHI, the PCR product was subcloned into the EGFP-C1 vector (Clontech). Following purification, 10–50 μg of plasmid was introduced into MIN6 cells by electroporation using GenePulser II (Bio-Rad). The electroporation was performed with potential difference of 0.25 kV and 650 microfarad capacitance. The efficiency of electroporation was 40–70%.

Chromatin Immunoprecipitation (ChIP) Assay—Chromatin isolation was performed as previously described (36, 37). The NeuroD1 antibodies used for immunoprecipitation in ChIP assays were from Santa Cruz (sc-1086). Real time PCR amplification was performed using the Brilliant SYBR Green QPCR Master Mixture according to the manufacturer’s protocol (Stratagene) using the machine Mx4000 (Stratagene). The primers used were GAAGGTCTCACCTTTCTGG and GGGGGTTACTGGATGCC for the mouse insulin I gene (from −10 to −281). Each PCR was performed in duplicates at least two independent times for each sample. The PCR products obtained with the immunoprecipitated DNA were normalized to the products obtained with the total input DNA.

Preparation of Cell Extracts, Co-immunoprecipitation Assays, and Western Blot Analysis—Cell extracts from MIN6 cells were prepared in lysis buffer (10 mM Tris-Cl, pH 8.0, 140 mM NaCl, 5 mM MgCl, 0.2 mM EDTA, 0.5% Nonidet P-40, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors). Co-immunoprecipitation assays were performed with about 0.5 to 1 mg of MIN6 whole cell extracts as described previously (38). The antibodies used in this study are: NeuroD1 (sc-1086, Santa Cruz), CTD 110.6 (MMS-248R, Covance Research Products), RL2 (MA1–076, Affinity Bioreagents), histone H3 (382157, Calbiochem), OGT (38), O-GlcNAcase and thio- lase (kindly provided by Dr. Whiteheart, University of Kentucky).

Immunohistochemical Analysis—MIN-6 cells were grown on acid washed coverslips. Cells were fixed in para-formaldehyde and permeabilized in 0.1% Triton as described previously (39) and incubated with the primary antibody overnight at 4 °C. Then, the cells were incubated in secondary antibody conjugated to Alexa Fluor 594 (Molecular Probes). Coverslips were mounted using Vectashield containing 4,6-diamidino-2-phenylindole to visualize the cell nuclei. GFP-NeuroD1 and OGT were visualized by immunofluorescence microscopy using a laser scanning confocal microscope (Leica).

Nuclear Fractionation—Cells were grown to 70% confluence and resuspended in 1 ml of isolation buffer (10 mM Tris-HCl, pH 7.5, 320 mM sucrose, 1.5 mM MgCl, 1 mM EDTA, protease inhibitors). The cells were lysed using a Dounce homogenizer (about 40–50 strokes) and centrifuged at 1000 × g for 7 min at 4 °C. The pellets containing the nuclei were washed 3 times in 1 ml of isolation buffer and resuspended in 200 μl of isolation buffer. After addition of 0.1% Triton X-100, the samples were sonicated for about 12 s at 10% amplitude.

RNA Isolation and Reverse Transcriptase-PCR—Total RNA and poly(A) RNA were isolated as described previously (40). The poly(A) RNA was reverse transcribed using Enhanced avian myeloblastosis virus reverse transcriptase (Sigma). The resulting cDNAs were used as template for PCR with oligonu- cleotides to amplify the insulin I and β-actin genes. The primers used were CCGTGGTTTGTCATCTCCTAC and TGGCAGTAGCTTCTCAGCTGC for the mouse insulin I gene (41) and CGTGGGCGGGGGTACGGGCAAACC and TTGGCCCTTAAAGGCGCATTG.
TTCAGGGGGG for the β-actin gene (42). The PCR amplification conditions are the same as described previously (40).

Electroporation of siRNA Oligos—OGT and negative control (Silencer® negative control 1) siRNA oligos were purchased from Ambion Inc. (Austin, TX). The transfection of the siRNA oligos into MIN6 cells was carried out as described previously (40). Briefly, a total of 2 µg of siRNAs per 2 × 10⁶ MIN6 cells and 50 µg of NeuroD1-GFP construct were co-transfected using the transfection reagent Nucleofector kit (Amaxa Inc.) and the Nucleofector II Device (Amaxa Inc.) according to the manufacturer’s instructions. Approximately 60 h after transfection, cells were treated with 30 mM glucose without serum, and afterward fixed with para-formaldehyde to detect OGT levels and NeuroD1-GFP localization by immunofluorescence microscopy.

Statistical Analysis—Statistical analysis was carried out using the two-tailed, unpaired Student’s t test. A p value less than 0.05 was considered statistically significant. Data are expressed as mean ± S.D.

RESULTS

NeuroD1 Binds to the Insulin Promoter Mainly on High Glucose—Because NeuroD1 is one of the transcription factors important for glucose regulation of insulin gene expression, we tested whether changes in glucose levels affect NeuroD1 binding to the insulin promoter. We analyzed the binding of NeuroD1 to the insulin I promoter by using the ChIP assay with NeuroD1 antibodies in MIN6 cells incubated with 3 or 30 mM glucose (Fig. 1A). NeuroD1 was associated with the insulin I promoter mainly on high glucose in the insulinoma cell line MIN6 (Fig. 1A). NeuroD1 binding to the insulin promoter was very weak in low glucose-incubated MIN6 cells. Quantification of NeuroD1 binding by real time PCR indicated a 6-fold increase in NeuroD1 binding to the insulin promoter in MIN6 cells incubated with 30 mM glucose (Fig. 1B).

Glucose Causes Changes in Subcellular Localization of NeuroD1—In Fig. 1A we have demonstrated that NeuroD1 binds to the insulin I promoter mainly at high concentrations of glucose. This may be due to changes in DNA binding affinity, expression levels, or subcellular localization of NeuroD1 in response to glucose. To address this issue, we first analyzed the levels of NeuroD1 protein in low and high glucose-incubated MIN6 cells. However, we could not detect any significant differences in NeuroD1 protein levels in MIN6 cells incubated with low versus high glucose (supplemental Fig. S1A).

Next, we investigated whether glucose regulates the subcellular localization of NeuroD1 and thereby determines its access to the insulin promoter. To test this idea, we generated a GFP-NeuroD1 fusion construct, in which the enhanced GFP protein was attached to the amino terminus of NeuroD1. After transfection of the GFP-NeuroD1 construct into the MIN6 cell line, the cells were incubated on low or high glucose for varying times and the subcellular localization of NeuroD1 was determined by indirect immunofluorescence microscopy (Fig. 1C).

We found that NeuroD1 was mainly cytosolic in MIN6 cells incubated with low glucose and nuclear in cells exposed to high glucose (Fig. 1C, first and second rows). High concentrations of L-glucose did not affect NeuroD1 localization (Fig. 1C, third row), indicating that the translocation of NeuroD1 into the nucleus on high glucose was not due to osmotic stress.

After counting the number of cells with nuclear localization of NeuroD1, we found that about 80% of the high glucose-incubated MIN6 cells displayed an almost complete nuclear localization of NeuroD1, whereas in the remaining 20% of cells NeuroD1 was localized both in the cytosol and nucleus (supplemental Fig. S1B). In low glucose-incubated MIN6 cells, about 35% of the cells displayed both nuclear and cytosolic localization of NeuroD1 and in about 65% of the cells NeuroD1 was solely in the cytosol (supplemental Fig. S1B). Complete nuclear localization of NeuroD1 was observed in less than 1% of low glucose-incubated cells. Nuclear localization of NeuroD1 was increased in MIN6 cells starting at 5 mM glucose and more than 60% of cells treated with 15 mM glucose displayed a mainly nuclear localization of NeuroD1. In MIN6 cells incubated with 20–40 mM pyruvate and 1 µM insulin or 50 mM KCl in the presence of low glucose, NeuroD1 was mainly localized in the cytosol (data not shown). These data suggest that exposure to high glucose causes NeuroD1 to translocate from the cytosol...
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**FIGURE 2. Increased flux via the hexosamine biosynthetic pathway regulates the translocation of NeuroD1 into nucleus in MIN6 cells.**

GFP-NeuroD1-transfected MIN6 cells were treated overnight with 3 mM glucose in the presence or absence of 2 mM glucosamine (GlcN) (A) or 30 mM glucose with or without 20 μM DON (B) and GFP-NeuroD1 localization was visualized by fluorescence microscopy. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

into the nucleus and that this translocation event occurs independent of the changes in ATP and insulin secretion levels.

**Nuclear Localization of NeuroD1 on High Glucose Is Mediated by the Hexosamine Biosynthetic Pathway**—The hexosamine biosynthetic pathway (HBP) has been suggested to be a sensor of glucose levels inside the cell. To determine whether this pathway was important for nuclear localization of NeuroD1, we increased the UDP-GlcNAc levels on low glucose by incubating the MIN6 cells with glucosamine. In addition, we blocked the synthesis of UDP-GlcNAc by inhibiting the first and rate-limiting enzyme L-glutamine:D-fructose-6-phosphate amidotransferase of this pathway using DON. Treatment of MIN6 cells with glucosamine resulted in translocation of NeuroD1 into the nucleus even on low glucose (Fig. 2A). Whereas 40% of the low glucose-treated cells displayed both nuclear and cytosolic localization of NeuroD1, in 70% of the low glucose and glucosamine-treated cells, NeuroD1 was almost completely localized to the nucleus (supplemental Fig. S2).

Inhibition of glutamine fructose-6-phosphate amidotransferase with DON blocked the nuclear translocation of NeuroD1 on high glucose (Fig. 2B). After treatment with DON the number of cells with nuclear-localized NeuroD1 drastically decreased and in about 55% of the cells NeuroD1 was localized partly in the nucleus and cytosol compared with high glucose grown cells without DON treatment, where 80% of the cells showed almost a complete nuclear localization of NeuroD1 (supplemental Fig. S3A). The addition of 2 mM glucosamine to MIN6 cells incubated with high glucose in the presence of the glutamine fructose-6-phosphate amidotransferase inhibitor DON restored the nuclear localization of NeuroD1 (supplemental Fig. S3B). Almost 50% of the cells showed both nuclear and cytosolic localization of NeuroD1 when treated with high glucose and DON. However, after addition of glucosamine to high glucose and DON-treated cells, NeuroD1 was almost exclusively localized to the nucleus in 75% of the cells, whereas only 25% of the cells displayed both nuclear and cytosolic localization (supplemental Fig. S3A). These data clearly indicate that the flux through the hexosamine biosynthetic pathway determines the subcellular localization of NeuroD1.

**Increased O-Linked Glycosylation Levels Mediate the Nuclear Localization of NeuroD1 Independent of Glucose**—We found that increased flux through the HBP causes the nuclear localization of NeuroD1. The end product of the HBP (UDP-GlcNAc) is used as substrate for O-linked GlcNAcylation, which can regulate the subcellular localization of transcription factors. Therefore, we tested the role of O-GlcNAc modification in nuclear localization of NeuroD1 in response to high glucose. MIN6 cells transfected with the GFP-NeuroD1 construct were treated with 100 μM PUGNAc, which inhibits the activity of the O-linked glucosaminidase (O-GlcNAcase) and thereby increases O-linked glycosylation of proteins. As shown in Fig. 3A, NeuroD1 was mainly localized to the nucleus in low glucose-incubated MIN6 cells treated with PUGNAc. 80% of the MIN6 cells treated with low glucose and PUGNAc displayed a complete...
nuclear localization of NeuroD1 (supplemental Fig. S4). Although about 40% of the low-glucose-incubated MIN6 cells showed a partial localization of NeuroD1 to the nucleus, the number of cells where NeuroD1 could only be found in the nucleus was less than 1% (supplemental Fig. S4).

Using the GFP-NeuroD1 construct we could demonstrate that high glucose or increased O-GlcNAc modification (low glucose with PUGNAc) causes NeuroD1 to translocate from the cytosol into the nucleus. To test whether the endogenous NeuroD1 protein also changed its localization in a glucose and O-linked glycosylation dependent manner, MIN6 cells were treated with high or low glucose in the presence or absence of PUGNAc. Following these treatments, nuclear fractions were isolated and immunoblotted with NeuroD1 antibodies (Fig. 3B). Whereas only a small amount of endogenous NeuroD1 was present in the nucleus when cells were incubated with low glucose, the level of nuclear NeuroD1 significantly increased after treatment with high glucose or low glucose with PUGNAc (Fig. 3B). These data demonstrate that the subcellular localization of endogenous NeuroD1 changes in response to glucose similarly as observed with the GFP-NeuroD1 fusion protein.

Because treatment of MIN6 cells with PUGNAc caused NeuroD1 to localize to the nucleus even in the presence of low glucose, we analyzed the binding of NeuroD1 to the insulin I promoter after treatment with PUGNAc using the ChIP assay (Fig. 3C). We found that endogenous NeuroD1 was able to bind to the insulin I promoter in MIN6 cells incubated with low glucose in the presence of PUGNAc, indicating that nuclear localization of NeuroD1 is sufficient for its binding to the insulin I promoter.

NeuroD1 Is O-GlcNAc Modified in MIN6 Cells Treated with High Glucose—The data obtained with PUGNAc-treated MIN6 cells suggest that nuclear localization of NeuroD1 in the presence of high glucose requires an O-linked glycosylation event. Therefore, we first tested whether NeuroD1 itself was directly modified by O-linked glycosylation. For this purpose, MIN6 cells were incubated with low or high glucose containing media. After lysis of the cells, the obtained protein extracts were used for immunoprecipitation experiments with the NeuroD1 antibody. The immunoprecipitated proteins were then immunoblotted with CTD 110.6 (Fig. 4A) or RL2 (Fig. 4B) antibodies, which recognize O-GlcNAc modification on proteins. Normal IgG was used as negative control. The amount of NeuroD1 immunoprecipitated under various conditions as determined using the NeuroD1 antibodies is similar. Input represents 10% of the total cell lysate used for immunoprecipitation.

We also carried out the reverse co-immunoprecipitation assay in which we first immunoprecipitated all of the O-GlcNAc-modified proteins using the RL2 antibody and immunoblotted for NeuroD1 protein. Consistent with our previous co-immunoprecipitation experiment, NeuroD1 was O-GlcNAc modified mainly on high glucose (supplemental Fig. S5).

NeuroD1 Interacts with OGT on High Glucose and with O-GlcNAcase on Low Glucose—Our data suggest that NeuroD1 becomes O-GlcNAc modified and translocates into the nucleus in response to high glucose, whereas on low glucose, NeuroD1 is deglycosylated and exits the nucleus. Thus, we determined the interaction of NeuroD1 with OGT and O-GlcNAcase in the presence of low (3 mM) or high (30 mM) glucose. We first immunoprecipitated OGT (Fig. 5A) or O-GlcNAcase (Fig. 5B) from protein extracts and blotted the immunoprecipitated proteins with NeuroD1 antibodies. As shown in Fig. 5, NeuroD1 interacts with OGT mainly on high glucose (Fig. 5B) and with O-GlcNAcase only on low glucose (Fig. 5B). A reverse co-immunoprecipitation assay in which we immunoprecipitated NeuroD1 with specific antibodies first and blotted with antibodies against OGT or O-GlcNAcase yielded similar results (supplemental Fig. S6).

O-GlcNAc Modification of NeuroD1 by OGT Is Essential for Its Nuclear Localization in the Presence of High Glucose—To confirm the role of O-GlcNAc modification in glucose-regulated nuclear translocation of NeuroD1, we utilized siRNA oligos to knockdown the expression of OGT. As demonstrated in Fig. 6, NeuroD1 is localized to the nucleus in MIN6 cells transfected with a random siRNA oligo after treatment with high glucose. However, depletion of OGT by siRNA treatment blocks the nuclear localization of NeuroD1 by high glucose (Fig
6). NeuroD1 was exclusively localized to the nucleus in 70% of the MIN6 cells transfected with the random siRNA oligo and incubated on high glucose. The remaining 30% of cells had both nuclear and cytosolic localization of NeuroD1 (supplemental Fig. S7). After transfection of the OGT siRNA oligos, NeuroD1 was found in both the nucleus and cytosol in 47% of the cells and in the remaining 53% of the cells NeuroD1 was mainly in the cytosol, when cells were treated with high glucose (supplemental Fig. S7).

Treatment with PUGNAc in the Presence of Low Glucose Increases Insulin Gene Expression—We have demonstrated that NeuroD1 is localized to the nucleus also in low glucose-treated MIN6 cells in the presence of PUGNAc, which is an inhibitor of O-GlcNAcase and increases the level of O-GlcNAc-modified proteins. To test whether the nuclear localization of NeuroD1 on low glucose by PUGNAc treatment is sufficient to increase insulin gene expression, we treated low glucose-incubated MIN6 cells with or without PUGNAc and quantified insulin I mRNA levels using real time reverse transcriptase-PCR (Fig. 7). We observed a 3-fold increase in insulin I mRNA levels in low glucose-incubated MIN6 cells after treatment with PUGNAc, which was similar to the insulin I mRNA levels observed with high glucose-incubated MIN6 cells (Fig. 7).

**DISCUSSION**

In this report we demonstrate that the insulin gene transcription factor NeuroD1 is O-linked GlcNAc modified in response to high concentrations of glucose, which causes its translocation from the cytosol into the nucleus to activate insulin gene transcription. On low glucose, NeuroD1 interacts with O-GlcNAcase and becomes deglycosylated, which is likely to be required for its nuclear export. Consistent with these data, treatment of MIN6 cells with PUGNAc (an inhibitor of O-GlcNAcase) leads to O-GlcNAc modification of NeuroD1 even on low glucose and to its translocation to the nucleus and increased insulin gene expression on low glucose. Moreover, depletion of OGT, which is responsible for O-linked GlcNAc modification of proteins by using siRNA oligos in MIN6 cells, blocks the nuclear localization of NeuroD1 in response to high glucose. This demonstrates the importance of O-GlcNAc modification of NeuroD1 for its translocation into the nucleus. Consistent with this idea, the effects of high glucose on NeuroD1 localization can be mimicked by treatment of low glucose-incubated MIN6 cells with glucosamine. This again suggests an important role for the hexosamine biosynthetic pathway and O-linked GlcNAc modification in regulation of NeuroD1 localization.

Changes in subcellular localization following O-GlcNAc modification has been demonstrated for several transcription factors (43, 44). The transcription factor Elf-1, which is expressed in hematopoietic cells has been shown to contain O-GlcNAc modification when it is in the nucleus (45). Stat5, another transcription factor that mediates the induction of interferon gene expression is also O-GlcNAc modified and only the nuclear form of Stat5 has this modification after cytokine activation (46). Furthermore, nuclear localization of transcription factors c-Myc, Tau, Sp1, and Pax-6 has been shown to correlate with O-GlcNAc modification of these proteins (47–50). The unmodified form of these transcription factors appears to be mainly localized in the cytosol. These observations are consistent with our data obtained with the GFP-NeuroD1 fusion construct as well as with endogenous NeuroD1 using nuclear fractionation, which indicate that NeuroD1 becomes translocated into the nucleus after O-linked GlcNAc modification in response to high glucose.

NeuroD1 has been reported to be phosphorylated as well as acetylated by the histone acetyltransferase p300. Acetylation of NeuroD1 appears to be important for its binding to the insulin promoter, but it is not clear if the acetylation of NeuroD1 occurs in the cytosol or nucleus (51). It is likely that NeuroD1 becomes acetylated only if it is in the nucleus. We demonstrate in this report that NeuroD1 translocates into the nucleus and binds to the insulin promoter on low glucose, after treatment of the cells with PUGNAc, which keeps NeuroD1 glycosylated even on low glucose. These findings suggest that NeuroD1
binding to the insulin promoter is not regulated by high glucose because we observe binding on low glucose with PUGNAc treatment. Therefore, we propose that once NeuroD1 is in the nucleus it becomes acetylated by p300, which increases its binding affinity for the insulin promoter, leading to activation of insulin gene transcription.

Our data indicate that the nuclear localization of NeuroD1 by O-GlcNAc modification in the presence of high glucose requires the HBP. This finding is not surprising given the fact that flux through HBP is required to synthesize UDP-GlcNAc, which is the substrate used by OGT to modify proteins by O-GlcNAcylation. Consistent with these data, depletion of OGT levels by siRNA treatment caused cytosolic localization of NeuroD1 in MIN6 cells incubated with 30 mM glucose. Flux through the HBP has been suggested to serve as a signal for nutritional status of a cell and therefore the HBP has been implicated to regulate many cellular processes (52–55). In agreement with the idea that increased flux through the HBP is required for the nuclear localization of NeuroD1 and thereby for activation of insulin gene expression, addition of glucosamine to MIN6 cells in the presence of low glucose causes the translocation of NeuroD1 into the nucleus. Furthermore, treatment with PUGNAc in the absence of high glucose induces insulin gene expression similar to levels observed under high glucose conditions.

Although our data are consistent with the idea that high glucose mediates the O-GlcNAc modification of NeuroD1 and its translocation into the nucleus, we cannot completely exclude the possibility that O-linked GlcNAc modification of another protein involved in glucose signaling or nuclear import may be involved in nuclear localization of NeuroD1. To demonstrate the importance of O-linked GlcNAc modification of NeuroD1 for its nuclear translocation in response to high glucose, it is necessary to identify and mutate the modification site(s) on NeuroD1.

The translocation of NeuroD1 from the cytosol into the nucleus in response to high glucose has been previously reported (56). Furthermore, it was shown that inhibition of the mitogen-activated protein kinase pathway by using the MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) inhibitor PD98059 disrupts the translocation of NeuroD1 into the nucleus. Moreover, mutating serine 274 in NeuroD1 into alanine resulted in accumulation of NeuroD1 in the cytosol (56). From these data it was concluded that phosphorylation of Ser274 in NeuroD1 is required for its translocation into the nucleus. Interestingly, the same Ser274 residue in NeuroD1 is predicted to be one of the high potential O-GlcNAc modification sites (57). If NeuroD1 becomes O-GlcNAc modified by high glucose at Ser274, then mutating this site to alanine would abolish the translocation of NeuroD1 into the nucleus as reported (56). Therefore, it is likely that O-GlcNAc modification of Ser274 is involved in regulation of NeuroD1 localization.

We have recently shown that increased flux through the HBP is required for glucose induction of MafA expression in the pancreatic beta cell line MIN6 (40). Like NeuroD1, MafA is also a beta cell-specific transcription factor required for insulin gene expression. Moreover, previous data indicate that Pdx-1, another beta cell-specific insulin gene transcription factor is also modified by O-linked GlcNAcylation, which appears to regulate the ability of Pdx-1 to bind to the insulin gene promoter (33). These findings suggest that HBP and O-linked glycosylation play an important role in modulation of the function of insulin gene transcription factors mediated by changes in glucose levels. Thus, the flux through HBP and O-linked glycosylation appear to be major sensors of glucose in pancreatic beta cells and regulate insulin gene transcription and beta cell function by glucose.

In summary, the data presented in this report demonstrate that high concentrations of glucose mediate the O-GlcNAc modification of NeuroD1 via its interaction with OGT resulting in NeuroD1 localization to the nucleus and activation of insulin gene expression. On low glucose, NeuroD1 interacts with O-GlcNAcase and becomes deglycosylated and exits the nucleus. These findings contribute to our understanding of how O-linked GlcNAc modification regulates pancreatic beta cell function.

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