Induction of the ChREBPβ isoform is essential for glucose-stimulated beta cell proliferation.

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Short title: ChREBPβ and glucose-stimulated beta cell proliferation

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Abstract

Carbohydrate responsive element binding protein (ChREBP) is a glucose sensing transcription factor required for glucose-stimulated proliferation of pancreatic beta cells in rodents and humans. The full-length isoform (ChREBPα) has a low glucose inhibitory domain (LID) that restrains the transactivation domain when glucose catabolism is minimal. A novel isoform of ChREBP (ChREBPβ) was recently described that lacks the LID domain, and is therefore constitutively and more potently active. ChREBPβ has not been described in beta cells, nor has its role in glucose-stimulated proliferation been determined. We found that ChREBPβ is highly expressed in response to glucose, particularly with prolonged culture in hyperglycemic conditions. In addition, siRNAs that knocked down ChREBPβ transcripts without affecting ChREBPα expression or activity, decreased glucose-stimulated expression of ChoRE-containing genes and glucose-stimulated proliferation in INS-1 cells, and in isolated rat islets. Quantitative chromatin immunoprecipitation, EMSAs and luciferase reporter assays were used to demonstrate that ChREBP binds to a newly identified powerful carbohydrate response element in beta cells and hepatocytes, distinct from that in differentiated 3T3-L1 adipocytes. We conclude that ChREBPβ contributes to glucose-stimulated gene expression and proliferation in beta cells, with recruitment of ChREBPα to tissue-specific elements of the ChREBPβ isoform promoter.
Introduction

Carbohydrate responsive element binding protein (ChREBP; gene name, MLXIPL) is a nutrient sensing transcription factor that is activated by products of glucose catabolism (1). ChREBP is expressed in numerous tissues, and while it is clearly involved in lipogenesis in the liver and in adipocytes (2; 3), its role in other tissues is less well understood. Changes in blood glucose levels are sensed in pancreatic beta cells by corresponding changes in the rate of cellular glucose metabolism, which drives the secretion of insulin to maintain glucose homeostasis (4). In addition, circulating glucose is a systemic regulator of beta cell mass, which expands in response to persistent hyperglycemia and increased “workload.” This proliferative process also requires beta cell-specific glucose catabolism (5). We recently found that ChREBP is expressed in pancreatic beta cells at levels comparable to liver in rodents and humans, and that ChREBP is required for glucose-stimulated beta cell proliferation (6).

ChREBP is a large transcription factor with an N-terminal glucose sensing domain comprising conserved Mondo regions that can be broadly described as a low glucose inhibitory or LID domain that folds over and represses a glucose response activation conserved element or GRACE domain (7; 8). Glucose metabolism leads to molecular events including nuclear localization, binding to carbohydrate response elements (ChoREs), and conformational changes in the LID and GRACE domains that allow interaction with coactivators and transactivation of glucose responsive genes. The full-length form of ChREBP (ChREBPα) is transcriptionally inactive in low glucose and
exquisitely sensitive to increased glucose metabolism, making this factor a transcriptional sensor of glucose uptake and utilization (1).

Recently, Herman et al. described a novel isoform of ChREBP (ChREBP\(\beta\)) produced from a newly identified exon (ChREBP exon 1b), transcription start site and promoter, resulting in an alternatively spliced mRNA with the first translational start site located in exon 4 rather than exon 1a (2). The resultant, truncated ChREBP\(\beta\) lacks the LID domain (also containing the nuclear export signals), generating a constitutively active, constitutively nuclear, and transcriptionally potent transcription factor. The expression of ChREBP\(\beta\) is driven by a ChoRE, creating a feed forward amplification of the glucose signal wherein activation of ChREBP\(\alpha\) leads to the production of the more potent and constitutively active isoform. Expression of ChREBP\(\beta\) correlates positively with adipocyte lipogenesis and insulin sensitivity. By contrast, its expression in liver correlates with hepatic insulin resistance and steatosis (2; 9; 10). Given that ChREBP is necessary for glucose-stimulated beta cell proliferation (6), here we sought to determine the role of ChREBP\(\beta\) in beta cell gene expression and proliferation. We found that ChREBP\(\beta\) is present in primary islet cells under basal conditions at much lower levels than ChREBP\(\alpha\), but nonetheless contributes significantly to glucose-stimulated gene expression and beta cell proliferation, and does so through recruitment to novel and powerful tissue-specific genomic elements.
Research Design and Methods

Cell culture. INS-1–derived 832/13 rat insulinoma cells were provided by Dr. Christopher Newgard (Sarah W. Stedman Nutrition and Metabolism Center, Duke University, Durham, NC). Cells were maintained as described previously (11). Murine 3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were induced to differentiate using a standard induction protocol (12).

Isolation of rodent islets and liver. Islets were isolated, dispersed and cultured from 80-87 day-old Wistar rats (Charles River Laboratories, Wilmington, MA) as described previously (13; 14). Mouse liver was isolated, flash-frozen, and powderized as previously described (15). These animal studies were performed in compliance with, and with approval of, the University of Pittsburgh and the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committees.

Human islets. Human cadaveric islets were obtained through NIDDK-supported Integrated Islet Distribution Program (IIDP) (http://www.iidp.coh.org). The mean age of the donors was 38, average purity was 86%. Islets were cultured and dispersed by trypsinization as described previously (16).

RNA isolation and Quantitative RT-PCR analysis. RNA (0.5–1 µg) was used for reverse-transcription, and RT-PCR analysis was conducted using The iTaq™ Universal SYBR ® Green supermix (Bio-Rad,Cat#172-5124) on the 7500 Applied Biosystems Real-Time System. Primers are listed in Supplementary Table 1.
The efficiency of primer pairs in the PCR was determined as described (17). RNA from human and rat islet cells was extracted using RNeasy microRNA isolation kit (Qiagen cat#74004).

**Small interfering RNA duplex-mediated gene suppression.** Two siRNAs (Thermo scientific) were designed to target the rat ChREBP β coding region. siRNAs were transfected into 832/13 for 72 h in the presence of lipofectamine RNAi max (Life technologies, cat# 13778150) by reverse transfection. The sequences were: 01-5' CGAGGUCCCAGGAUCCAGUUU-3', and 02-5'-GUCCCAGGAUCCAGUCCG AUU-3'. The cells were treated with 2 mM or 20 mM glucose for 16 h, then RNA was extracted and RT-PCR was performed. A duplex with no known sequence homology or biological effect (siControl) was used as a control (cat # D-001810-01).

The Accell® siRNA treatment was as previously described (6). After incubation for 4 d, cells were fixed with 2% periformaldehyde and were stained with antibodies specific for Ki67 and insulin. Accell® siRNA sequences were: 01, 5' CGAGGUCCCAGGAUCCAGUUU-3', 5'ACUGGAUCCUGGGACCUCGUU3'; 02 5'GUCCCAGGAUCCAGUCCG AUU3', 5'UCGGACUGGAUCCUGGACUU3'.

**Cell Proliferation assay.** INS-1-derived 832/13 cells grown in Nunc® Lab-Tek® II Chamber Slide System (Thermo Fisher,cat#154534) were transfected with ChREBPβ siRNA for 48 h. Cells were then cultured in 2 mM or 20 mM glucose for 16 h. 100 µM Bromodeoxyuridine (BrdU) was added for 30 min. Cells were fixed with 2% paraformaldehyde, and treated with 1 N HCl for 30 min prior to treatment with 4% normal goat serum, 1% BSA, 0.5% Triton for 2 h. Cells were incubated overnight with a
BrdU antibody (Abcam, cat# ab6326) at (1:200). After washing with PBS twice, Alexa 594 anti-rat (1:10,000) was used as second antibody. The slides were mounted with mounting medium containing DAPI (VECTOR Laboratories, Inc.H-1500). To determine proliferation in dispersed rat islets, cells were stained for ki67 using rabbit anti-ki67 (Thermo fisher Scientific, cat# 9106). All images were acquired using a Zeiss Axioplan2 at the Microscopy Shared Resource Facility of Icahn School of Medicine at Mount Sinai.

**Electrophoretic Mobility Shift Assay (EMSA).** HeLa cells were transfected using Lipofectamine 2000 (Life technologies) to overexpress Flag-tagged ChREBP and HA-tagged Mlx, the obligate heterodimer binding partner for ChREBP [kindly provided by Dr. Towle, University of Minnesota (18)], or HeLa cells transfected with empty vector control. Nuclear extracts were obtained using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific). Nuclear extracts (15 µg) were added to the EMSA reaction buffer [20 mM HEPES (pH 7.9), 5 mM MgCl₂, 0.5 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 6.25% glycerol, 1 µg bovine serum albumin, 2 µg salmon sperm DNA, 2 µg poly(dI-dC)]. The Ebox/ChoRE oligonucleotides were fluorescently labeled with 5'IRD700 (Integrated DNA Technologies), and these oligos or unlabeled oligos (“unlabeled probes”) were annealed for 5 min at 95 °C. For antibody displacement experiments, nuclear extracts (15 µg) were preincubated with anti-Flag, anti-HA, or IgG antibodies (1:500) for 10 min at room temperature before the addition of labeled DNA probe. Oligonucleotide sequences can be found in Supplementary Table 2. Samples were loaded onto 4.5% TBE gels (Life Technologies) and scanned using the LiCor laser-based image detection method.
Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as previously described (14; 19). One mg of cross-linked 832/13 or NIH 3T3-L1 chromatin was incubated overnight (18 h) at 4°C with 3 µg of rabbit anti-ChREBP (abcam, ab157153) antibody, anti Myc antibody (Santa Cruz Biotechnology N-262, sc-764), or normal rabbit IgG (Santa Cruz Biotechnology). Immune complexes were captured with 60 µL of 50% protein A-Sepharose agarose slurry (Millipore #16157). DNA-protein crosslinking was reversed by adding 50% cellex beads and boiling 10 min. Five µl of supernatant containing immunoprecipitated DNA fragments were subjected to quantitative PCR using SYBR green as previously described (14; 19). For ChIP from frozen liver tissue, fine-powdered liver tissue was made and fixed with 1% formaldehyde. The powder was homogenized with a Dounce homogenizer with tight (A) pestle in a hypotonic solution (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2% Nonidet P-40, 0.2 mM sodium orthovanadate, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 5% sucrose, 1 mM dithiothreitol, and protease inhibitors), and layered onto a sucrose cushion buffer (10 mM Tris/HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% sucrose, and protease inhibitors) and sucrose gradient centrifugation was carried out for 1 h at 30,000 rpm. The pellet was washed once with ice-cold phosphate-buffered saline and resuspended in SDS lysis buffer. The sample was then sonicated with a BioRuptor and processed as described above. For rat islets, we used a Micro Chromatin Immunoprecipitation (ChIP) assay. Briefly, Rat islet cultured with 20mM glucose culture medium for 2 days were cross linked with 1% formaldehyde followed by
neutralization with 125 mM glycine. Following fixation, a ChIP assay was performed using Thermo Scientific Pierce Magnetic ChIP Kit™ (Thermo Scientific, Cat#26157) according to the manufacturers’ instructions. Antibodies to ChREBP (Thermo Scientific, Cat# PA5-22924) and IgG control antibody (Santa Cruz Biotechnology, Cat# 2027) were used to immunoprecipitate chromatin bound supernatants. The PCR primers for amplification of ChIP products are listed in Supplemental Table 1.

**Luciferase assay**

832/13 cells were transfected with ChREBPβ promoter luciferase wild type, ChoRE or E box mutation vectors (2) by Lipofectamine 2000 (Life Technologies cat. #11668-019). Cells were harvested after 16 h and luciferase activity was measured using the Luciferase Reporter Assay System (Promega cat. #E1500) on a MicroBeta² LumiJET™ Microplate Counter (Perkin Elmer). Firefly luciferase activity was normalized to protein concentration.

**Statistical Analysis**  
Results are represented as means with error bars being SEM. Comparisons between two means were conducted using unpaired Student’s t test. Comparisons between multiple groups were conducted using two-way ANOVA with Tukey’s post hoc test. Statistical significance was set at $P < 0.05$. 
Results
ChREBPβ is expressed in rat and human beta cells.

The ChREBPβ isoform was originally identified in adipose tissue and is also expressed in the liver (2). However, whether ChREBPβ is expressed or serves important functions in pancreatic beta cells has not been addressed. RT-PCR experiments were performed using oligonucleotide primers designed for ChREBP-specific sequences derived from exon 1b, exon 1a or primers that recognize both (Figure 1A, Supplemental Table 1). In INS1-derived 832/13 cells, we found robust induction of ChREBPβ mRNA in response to glucose in a time and dose-dependent manner (Figure 1B and C). Using the ∆∆CT method and adjusting to primer efficiencies and normalizing to beta actin, we found that the expression of ChREBPβ was approximately 8-fold lower than ChREBPα in low glucose (see Figure 5 below). But after glucose treatment, ChREBPβ mRNA increased 4-fold by 6 h, reaching 6-fold by 24 h, a level that was greater than ChREBPα after the glucose treatment. As expected, the induction of ChREBPβ mRNA by glucose was consistent with its being regulated by glucokinase, as the half maximal dose was near the $S_{0.5}$ of glucokinase (20). Thus, the rate of glucose metabolism through glucokinase correlated with the stimulation of ChREBPβ mRNA. Remarkably, we found that glucose inhibited ChREBPα specific transcripts, suggesting that transcription of exon 1b excludes the use of exon 1a in these cells (Figure 1B, C and D). We knocked down ChREBPβ to determine its role in beta cells. In order to minimize off target effects, two siRNAs were found to decrease ChREBPβ mRNA by approximately 50%, both in low and high glucose concentrations, without affecting ChREBPα mRNA levels (Figure 1D). The abundance of amplicons
derived from primers that recognize both isoforms (ChREBP-common) remained relatively stable, with small but significant changes observed after treatment with the siRNAs and glucose, reflecting diminished ChREBPβ in these cells (Figure 1D). Note that the 4 to 6-fold increase of ChREBPβ in response to glucose is accompanied by proportionately smaller changes in ChREBP-common, reflecting the small fraction of ChREBPβ relative to total ChREBP mRNA (see Figure 5 below). Treatment with these siRNAs led to decreased expression of direct ChREBP target genes Pklr and Acaca, and the indirect target gene Myc, but had no significant effect on Txnip mRNA levels (Figure 1E). Since Txnip gene expression is induced by glucose through the binding of ChREBP (21), these results suggest that ChREBPβ and ChREBPα may have different gene targets, or that glucose-mediated induction of Txnip mRNA is driven by other factors, such as MondoA (22).

In isolated rat islet cells, glucose stimulated the expression of several ChoRE-containing glucose responsive genes, ranging from 2-fold to 20-fold for Hbegf, Pklr, GDPH and Txnip [Figure 2 A-G, ref. (14)]. By contrast, ChREBPβ mRNA was increased about 2-fold after one day, and then increased exponentially to over 1,000-fold by day 4. Note that the abundance of ChREBPβ was approximately 1,000-fold lower than ChREBPα in low glucose (see Figure 5 below). ChREBPα did not increase significantly over the same time frame, and the ChREBP-common amplicon increased significantly, but less than 2-fold, only on day 4 (Figure 2 A-C). Thus, ChREBPβ is capable of enormous amplification if given enough time in hyperglycemic conditions. In addition, we found that a 2 to 4-day incubation with 15 mM glucose resulted in a 5-fold
increase in ChREBP\(\beta\) in isolated human islet cells, correlating with a robust increase in Tn nip, and small, but significant increases in the other ChoRE containing genes tested (Figure 2H). Importantly, we found that inhibition of ChREBP\(\beta\) using two individual siRNAs that did not significantly affect ChREBP\(\alpha\), led to a significant decrease in ChoRE-containing glucose responsive genes in dispersed rat islet cells (Figure 2 I). Note that Tn nip expression was significantly reduced by the siRNAs, highlighting a difference between rat islet and 832/13 cells, and that ChREBP\(\beta\) is important for at least part of the glucose-mediated induction of Tn nip in primary cells. Thus, the induction of ChREBP\(\beta\) is essential for the glucose-mediated stimulation of glucose-responsive genes in primary rat islet cells.

ChREBP binds to elements in the ChREBP\(\beta\) regulatory region in a tissue-specific manner. Herman et al. described a carbohydrate response element responsible for the glucose-mediated induction of ChREBP\(\beta\) at the 5’ end of exon 1b (2). The study also described an E-box responsible for approximately 50% of the glucose response located about 100 bp upstream of the start site (see Figure 3). We tested if the same arrangement of elements is used for the glucose-mediated expression of ChREBP\(\beta\) in pancreatic beta cells. First, we determined the relative importance of the E-box and ChoRE motifs for glucose-responsive gene expression in INS1-derived 832/13 cells using promoter-reporter constructs (Figure 3 A). Mutation of the upstream E-box completely blocked the very robust 80-fold transcriptional glucose response, as did mutating both the E-box and downstream ChoRE elements, while mutation of the downstream ChoRE decreased luciferase activity by approximately 50%. This result
suggested that the E-box motif is essential for ChREBPβ mRNA expression in beta cells, and that this element may be a carbohydrate response element. To test this idea, we examined the sequence surrounding the E-box and found an E-box-like element 5 bp upstream that is highly conserved in mammals (Figure 3B), an arrangement consistent with known ChoREs, but one that is unique due to the guanine in the third position (23-25). To confirm that ChREBP binds to this element, an EMSA was performed using extracts from HeLa cells transfected with vectors expressing Flag-tagged ChREBP and HA-tagged Mlx (the obligate heterodimer binding partner of ChREBP) together with a fluorescently labeled double stranded probe containing the upstream motif. A band representing ChREBP/Mlx was competed by a 500-fold molar excess of unlabeled probe, but not a mutant probe with the same sequence of the mutant used in Figure 3A. Further, antibodies against Flag or HA, but not an IgG control, abrogated the shifted band. Finally, the band was competed by an unlabeled probe with a consensus ChoRE from the Acaca gene promoter, but not with a mutant version of the Acaca ChoRE (probe sequences in Supplemental Table 2). We conclude that the upstream element is an authentic ChoRE, and will hereafter refer to this element as the upstream ChoRE motif. To demonstrate binding of ChREBP to the upstream ChoRE motif in cells, Figure 4 shows a chromatin immunoprecipitation (ChIP) assay from INS1-derived 832/13 cells cultured in low or high glucose (2 or 20 mM, respectively) using antibodies directed against ChREBP, or IgG as a negative control. The signal for bound protein was determined by quantitative PCR using primers for sites near the transcription start site of exon1b, and at intervals 5’ of exon1b and 3’ of exon1a. In response to glucose, we found robust recruitment of ChREBP to exon1b,
with the location of the greatest signal corresponding to a region near the upstream ChoRE motif. By contrast, the region with the previously defined carbohydrate response element at the 3’ end of exon 1b (2), did not bind ChREBP significantly in these cells in response to glucose. It is possible that different tissues use different regulatory elements to promote the glucose response. To test this, we performed ChIP experiments with rat islets, mouse liver tissue and 3T3-L1 cells after differentiation into adipocytes (Figures 4B-D). We found that ChREBP binds to regions containing both the upstream ChoRE motif and the exon1b ChoRE in mouse liver and rat islets, with a clear preference for the upstream ChoRE in these tissues. By contrast, ChREBP recruitment is restricted to a region near the exon1b ChoRE in 3T3-L1 cells, confirming the previous report (2). Thus, in supporting glucose-stimulated transcription, both elements remain important for a complete response to glucose, and ChREBP binds differentially to these two elements in a tissue-specific manner.

**ChREBPβ is required for glucose-stimulated proliferation in INS-1-derived 832/13 cells.**

A striking observation was that the relative abundance of ChREBPβ in response to increased glucose changes over time when compared to ChREBPα, particularly in primary tissues. After adjusting for primer efficiencies, we found that ChREBPβ is expressed at much lower levels than ChREBPα, and when cultured in high glucose concentrations, the abundance of ChREBPβ increases dramatically in the rodent models, while the abundance of ChREBPα does not change very much. Thus, the ratios of the absolute abundance of ChREBPβ to ChREBPα increases with increasing
time in culture supplemented with high concentrations of glucose (15 to 20 mM). For instance, in INS-1-derived 832/13 cells, the ratio of beta to alpha was 1:8 in low glucose and rapidly increased to 1:0.2 after 18 h. In isolated rat islet cells, the ratio of the beta to alpha isoform was 1:1,100 under basal conditions (5.5 mM), which increased to 1:454 by 18 h and reached 1:2 by 4 days. Furthermore, in human islet cells, ChREBPβ was expressed at a ratio of 1:28,700 compared to ChREBPα, which increased to 1:7,700 after 4 days in culture (Figure 5A). Interestingly, we found a strong correlation between the ratio of the ChREBP isoforms and the proliferative capacity of the cell systems examined, particularly in the primary cells (Figure 5B). When we plotted the relationship between each isoform of ChREBP independently, we found that ChREBPβ expression correlated with proliferation much better than ChREBPα (Figures 5 C and D).

We recently found that ChREBP is required for glucose stimulated beta cell proliferation in mice, rats and humans (6). These observations were made prior to the discovery of the beta isoform of ChREBP, and used tools that did not distinguish between the two isoforms. Indeed, since ChREBPα is required for the induction of ChREBPβ, depletion of ChREBPα automatically decreases the abundance of ChREBPβ (we confirmed this in Supplemental Figure 1). In Figure 6, we used siRNAs to deplete ChREBPβ mRNA to test if ChREBPβ is necessary for glucose-stimulated beta cell proliferation. As shown in Figure 1, two siRNAs were found that decreased ChREBPβ mRNA approximately 50% in INS-1-derived 832/13 cells, but had no effect on ChREBPα mRNA levels. In addition, these siRNAs had no effect on the abundance or glucose-stimulated translocation of the full-length ChREBPα protein (Fig 6 A and B).
Note that we were unable to measure endogenous ChREBPβ protein, as measurement of the alpha isoform is just detectable by western blot and the ChREBPβ isoform is expressed at approximately 2-10-fold lower levels (Figure 5). Depletion of ChREBPβ mRNA resulted in a significant 40% decrease in glucose-stimulated proliferation in 832/13 cells as determined by BrdU incorporation (Figure 6 C and D), demonstrating that a complete glucose response requires the expression of ChREBPβ in these cells.

**ChREBPβ is required for glucose-stimulated proliferation in primary rat beta cells.**

We used freshly isolated rat islets to test the importance of ChREBPβ in glucose-stimulated beta cell proliferation in primary cells. We used Accell® siRNA, a lipid-conjugated siRNA that is less toxic and provides a convenient way to deplete primary cells of specific mRNAs (6; 26). Accell® siRNA decreased ChREBPβ mRNA by approximately 40-50% in isolated rat islet cells, without affecting ChREBPα mRNA levels (Figure 7A and B). Importantly, depletion of ChREBPβ mRNA had no effect on the abundance or translocation of the full length ChREBPα as determined by immunoblotting and confocal microscopy (Figure 7C and D). Finally, depletion of ChREBPβ mRNA by two different Accell® siRNAs, to diminish the possibility of off-target effects, led to a 50% decrease in glucose-stimulated beta cell proliferation as measured by Ki-67 staining of insulin positive cells (Figure 7E-H). Taken together, these observations suggest that expression of ChREBPβ is required for a complete proliferative response to glucose in beta cells.

**Discussion**
ChREBPβ is a newly identified isoform of the glucose-sensing transcription factor, ChREBP, that is generated by a feed-forward loop and whose expression in adipocytes and hepatocytes correlates with alterations in insulin sensitivity (2; 9; 10). Exploration of the role of ChREBPβ in glucose-stimulated gene expression and proliferation in beta cells led to a number of novel observations: 1) ChREBPβ is expressed in beta cells, and is highly responsive to glucose; 2) depletion of ChREBPβ results in diminished expression of glucose responsive genes, with Txnip being an exception; 3) ChREBPβ is required for a complete proliferative response to glucose, and has a surprisingly strong control strength relative to the full-length, glucose-responsive isoform; 4) ChREBP is recruited to a newly described ChREBPβ promoter ChoRE in beta cells and liver, and therefore binds to the regulatory regions of ChREBP exon 1b in a tissue-specific manner.

Glucose is a natural beta cell mitogen and much attention has been focused on understanding the basic mechanisms by which glucose drives beta cell proliferation (5; 27-35). We recently demonstrated that ChREBP is required for glucose stimulated beta cell proliferation in mouse, rat and human beta cells (6). Islet cells isolated from ChREBP global knock out mice failed to proliferate in response to glucose. Further, siRNAs targeting the coding region of ChREBP attenuated glucose-stimulated beta cell proliferation in islet cells isolated from both rat and human islets. These observations were made prior to the description of a new isoform of ChREBP, ChREBPβ, which is formed by alternative promoter usage and splicing (2). Since ChREBPβ expression is stimulated by glucose, through a ChoRE that recruits both isoforms, the induction of
ChREBPβ is initially mediated by glucose-activated ChREBPα, and once ChREBPβ protein levels increase, the truncated ChREBPβ, missing its low glucose inhibitory domain, is constitutively active and may contribute to its own production as long as the glucose signal is present through a feed forward loop. We found that targeting ChREBP exon 1a with siRNA led to a decrease in glucose stimulated ChREBPβ mRNA, confirming this arrangement (Supplemental Figure 1). The feed-forward amplification was most clear in dispersed rat islet cells cultured for up to 4 days in high glucose where we saw a 27-fold increase in ChREBPβ after one day and a 1,000-fold increase after 4 days (Figure 2). This was made possible by the newly discovered beta cell-specific carbohydrate response element, upstream of exon 1b, which is very strong [80-fold response versus 3 to 4-fold response of the Pklr ChoRE (14; 19; 36)]. We propose a model (Figure 8), where small proportion of ChREBPα, which is primarily cytoplasmic in beta cells in low glucose, and only partially nuclear in high glucose (37-40), translocates to the nucleus in response to glucose and binds to a carbohydrate response element located upstream of the ChREBP exon 1b transcription start site, driving the expression of ChREBPβ. ChREBPβ is constitutively nuclear, and is more transcriptionally potent than ChREBPα (2). Thus, continued glucose metabolism drives a transcriptional amplification of the glucose signal. Our study suggests this feed forward amplification is required for glucose-stimulated beta cell proliferation. We propose that in a physiological setting a measured, intermittent and somewhat prolonged glucose signal, as might happen as an animal becomes increasingly insulin resistant on a high fat diet, is necessary for beta cell expansion. However, if hyperglycemia persists beyond a critical threshold, the signal becomes glucotoxic and
can lead to apoptosis, as demonstrated by overexpression of a truncated and constitutively active form, functionally identical to ChREBPβ in INS-1 cells and in mice (41).

A limitation of the current study was our inability to test the role of ChREBPβ in human beta cells. The reason for this is two-fold: one, for technical reasons we were unable to find an siRNA that would effectively knock down human ChREBPβ; exon1b for ChREBP is very short (100 bp) and there is only a short stretch that might accommodate repression by siRNA. Secondly, the expression level of ChREBPβ in human islet cells is extremely low – only 1:10,000 of the ChREBPα isoform. This may be in part due to the relatively lower percentage of beta cells in human islet compared to rodent islets (30-50% compared to 90%), but clearly there is less ChREBPβ expressed in human beta cells than in rodent beta cells. Possible implications of this observation include: 1) ChREBPβ plays a less important role in human compared to rodent beta cell biology; 2) ChREBPβ, by promoting lipogenesis and lipotoxicity (42) may be highly toxic to human beta cells and so its expression is highly repressed; or 3) alternatively, ChREBPβ expression, in a moderate and temporally controlled manner may potentiate glucose-stimulated beta cell proliferation in human beta cells, and it is difficult to measure ChREBPβ in human beta cells simply because so few human beta cell actually proliferate. Experiments are in progress to test these possibilities.

In summary, we found that ChREBPβ expression is strongly induced by glucose in beta cells. ChREBPβ binds to elements in the regulatory region of exon 1b in a tissue-
specific manner, binding to different ChoREs in different proportions in liver, beta cells and adipose cells. Finally, we found that CREBPβ is required for a complete glucose-stimulated beta cell proliferative response in 832/13 cells and in cultured rat beta cells. Clearly much more needs to be learned about ChREBPβ and its relationship to beta cell lipotoxicity and proliferation.
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Duality of Interest. No potential conflicts of interest relevant to this article was reported.

Author Contributions. P.Z. designed and performed most of the experiments, and contributed to the writing of the manuscript. A.K., L.S.K, researched data and contributed to the writing and editing of the manuscript. L.L. and M.P. researched data. M.A.H. contributed to the discussion and editing of the manuscript. D.K.S. conceived of the study, wrote the manuscript, and 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.

Prior Presentation. A portion of this study was presented at the 74th annual Scientific Meeting for The American Diabetes Association in June, 2014.
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Figure Legends

Figure 1. ChREBP-β is expressed in beta cells and is responsive to glucose.  A) Diagram of the primers used in RT-PCR assays.  Primers were designed to be specific for exon 1b, exon 1a, or for a region that is common to both isoforms.  B) Time course of ChREBPβ expression in response to 20 mM glucose.  832/13 INS-1-derived rat insulinoma cells were cultured in media containing either 2 or 20 mM glucose for the indicated times and RT-PCR was performed using primers specific for the 3 forms of ChREBP or for β-actin as a control.  C) Dose response of ChREBPβ expression.  832/13 cells were cultured in the indicated concentrations of glucose for 18 h and RT-PCR performed as in B.  D and E) 832/13 cells were cultured in the indicated concentrations of glucose for 18h and treated with either a scrambled control siRNA (siCon) or with siRNAs directed against ChREBPβ (siChREBP01 -02).  RT-PCRs were performed using primers for the indicated genes. Data are presented as the fold change relative to starting time point (B), or the lowest concentration (C), or to the low glucose scramble control (D and E) for each primer pair after normalizing to beta actin using the ΔΔCT method. All experiments were performed at least three times. Error bars represent SEM; *, P < 0.05.

Figure 2. Glucose induces ChREBP-β in primary rat and human islet cells.
A-G. Dispersed rat islet cells were incubated in media containing either 5.5 or 15 mM glucose for 1 to 4 days, and the expression of the indicated genes were determined and shown as fold change relative to each day after normalizing to β-actin using the ΔΔCT method. H. Isolated human islet cells were cultured in media containing either 5.5 or 15
mM glucose for 1, 2 or 4 days. Total RNA was isolated and subjected to RT-PCR using primers specific for the indicated genes. The data are expressed as a fold change from 5.5 mM glucose. I. Rat islets were isolated, dispersed and incubated with lipid-conjugated Accell siRNA for 4 days. Total RNA was isolated and subjected to RT-PCR. Data are presented as relative to the scramble control (SiCon) 15 mM treatment, after normalization to β-actin using the ΔΔCT method. Error bars are SEM, n=3 to 4 for rat islets, n=5-9 for human islets, *, P < 0.05.

**Figure 3. ChREBP binds to tissue specific ChoREs of Exon 1b.**

A. Luciferase assays identify the exon 1b E-box as a functional ChoRE. 832 cells were transfected with luciferase reporter plasmids driven by exon1b and upstream sequences containing wild type or mutant versions of an upstream E-box element and a previously defined downstream ChoRE, as indicated, or an empty vector control (pGL3). Cells were treated for 18h with 2 or 20 mM glucose and luciferase activity was measured from cell lysates. Results shown are relative fold luciferase activity, normalized to protein. n=3, +/- SEM; *, P < 0.05 when comparing 20 mM to 2 mM glucose; #, P < 0.05 when compared to the WT ChREBPβpromoter construct. B. Conservation of flanking sequence of the upstream E-box of ChREBP exon1b were aligned from data obtained from the UCSC genome browser (http://genome.ucsc.edu/). Sequence deviation from human is denoted with bolding and underlining. Together, the conserved sequences are consistent with a consensus ChoRE, with 2 E-box-like elements separated by 5 bp. C. EMSA demonstrates ChREBP binding to the newly identified upstream ChoRE. HeLa cells were transfected with plasmids expressing flag-tagged ChREBP and HA-tagged
Mlx (the obligate heterodimer binding partner of ChREBP) and cell lysates were used for gel shift assays using the upstream ChoRE and flanking sequences as fluorescently labeled double-stranded probes with the indicated incubation parameters. The gel was visualized with a LI-COR Odyssey System and is representative of 3 independent experiments with essentially identical results.

**Figure 4. Chromatin immunoprecipitation assays reveal tissue specific recruitment of ChREBP to exon1b ChoREs.** A. INS1-derived 832/13 cells were treated with 2 or 20 mM glucose for 18 h and cells were fixed with formaldehyde and subjected to a ChIP assay using antibodies directed against either ChREBP or an IgG control. The results are presented as fold enrichment over the control IgG signal. Numerous primer pairs were chosen to scan regions of the ChREBP gene upstream of exons 1a and 1b as indicated. The results are from 4 to 7 independent experiments. Chromatin isolated from rat islets cultured for 4 days in 15 mM glucose (B) or from *ad lib* fed frozen mouse liver (C) was sheared and subjected to a ChIP assay using antibodies against either ChREBP or IgG as control. Results relative to the IgG control and are from 3 mice and 4 rats, respectively. D. NIH 3T3 cells were differentiated into mature adipocytes, cultured for 18 h in 20 mM glucose and processed for a quantitative ChIP assay. Results of 3 to 4 independent experiments are expressed as the signal from an antibody directed against ChREBP relative to the IgG control. Error bars are SEM; *, P < 0.05.
Figure 5. Correlation of proliferation and ChREBPβ expression pancreatic beta cells. A. The ratios of ChREBPβ to ChREBPα are displayed in relation to percent BrdU incorporation for each of the indicated model systems after expression for the indicated times in 15 or 20 mM glucose was measured in absolute terms, using the ∆∆CT method relative to beta actin and adjusting for primer efficiency. Data are listed in the order of highest to lowest BrdU incorporation, corresponding to highest to lowest ChREBPβ to ChREBPα ratio. Errors represent SEM; n=3 to 4, *, P < 0.05. B. Data from primary rodent and human cells from Figure 5A were plotted on a double log scale and fitted with a power least squares fit and R² value, calculated in Excel. We note that inclusion of data from INS1-driven insulinoma cells decreased the R² value to 0.84. C and D. data from ChREBPα and ChREBPβ and their relation to proliferation are presented separately. N=3-5, error bars represent SEM.

Figure 6. ChREBPβ depletion attenuates glucose-stimulated beta cell proliferation in INS1-derived 832/13 cells. A. INS1-derived 832/13 cells were treated with control siRNAs (siCon), or siRNAs targeted against ChREBPβ mRNA (Siβ-01, -02) and 24 h later, cultured for 16 h in either 2 or 20 mM glucose. Immunoblots were performed using antibodies against ChREBP (ChREBPα is visualized) and beta actin (note that these siRNAs decreased ChREBPβ mRNA, but had no effect on ChREBPα, Fig 1). pos, positive control. B. After 48 h of the siRNA treatment, cells were treated for 2 h with 2 or 20 mM glucose and fixed and stained with an antibody recognizing ChREBPα. C. After 48 h, cells were cultured in either 2 or 20 mM glucose for 16 h and BrdU was added 30 min prior to fixation and staining for BrdU (red) and DAPI (blue). D.
Quantification of the results in C, wherein at least 1,000 cells were counted. Results are from 4 independent experiments, error bars are SEM, *, P < 0.05.

**Figure 7. Depletion of ChREBPβ attenuates glucose-stimulated beta cell proliferation in isolated rat islet cells.** A,B. Isolated rat islet cells were treated with lipid-conjugated (Accell®) siRNAs and cultured in either 5.5 or 15 mM glucose for 96 h. Total RNA was collected and subjected to RT-PCR using primers specific for either ChREBPβ or ChREBPα mRNAs. C. Protein extracts were subjected to immunoblots using an antibody against ChREBPα and β-actin. D. To determine the effects of the siRNAs on ChREBP translocation, cells were treated with control or Accell® siRNA against ChREBPβ cultured in 5.5 mM or 15 mM glucose for 32 h and were fixed and stained for insulin, ChREBP and DNA (DAPI). E. Isolated rat islet cells were treated with Accell® siRNAs and cultured in either 5.5 or 15 mM glucose for 96 h and fixed and stained with Ki67 and insulin. F. Quantification of the results in E, wherein at least 1,000 insulin positive cells were counted. Results are from 4 different rat islet isolations, siCon, scramble control siRNA, error bars are SEM, *, P < 0.05.

**Figure 8. A model of ChREBPβ-mediated glucose-stimulated beta cell proliferation.** ChREBPα is mostly cytoplasmic in beta cells, and only a small percentage actually enters the nucleus in response to increased glucose metabolism (37; 39). ChREBPβ is a target gene of ChREBPα and is constitutively nuclear and more transcriptionally potent than ChREBPα (2). Thus glucose drives a feed forward amplification signal that continues as long as glucose metabolism remains elevated.
We propose that elevation of glucose metabolism for a period of time is required for glucose-stimulated proliferation, but that hyperglycemia for too long results in ChREBPβ-contributed glucose toxicity.
Figure 1

A. Exon 1b
Exon 1a
Exons 2-15

B. Beta specific

C. Alpha specific
Common

D. ChREBPβ
ChREBPα
ChREBP common

E. 2 mM
20 mM

Glucose (mM)

Relative mRNA Expression

ns

ChREBPb
ChREBPa
ChREBP common

Pklr
Acaca
Myc
Txnip
Figure 2

**Relative mRNA Expression**

- **H**: ChREBP
  - 5 mM Glucose
  - 15 mM Glucose

- **I**: Relative mRNA Expression
  - 5 mM Glucose
  - 15 mM Glucose

*Note: Graphs show changes in mRNA expression levels for different conditions, with significant differences indicated by asterisks.*
Figure 3

Diabetes

A

Relative Luciferase Activity (fold)

WT ChREBPβ

Downstream ChoREMut

EboxMut

Double

PGL3

E-box

ChoRE

Exon 1b

ChoRE

Exon 1b

ChoRE

Exon 1b

ChoRE

Exon 1b

ChoRE

Exon 1b

ChoRE

Exon 1b

ChoRE

2 mM

20 mM

0 20 40 60 80 100

Relative Luciferase Activity (fold)
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1. Probe
2. " + HeLa, untransfected
3. " + Flag ChREBP, HA-Mlx
4. " + " + cold probe x500
5. " + " + cold mut probe x500
6. " + " + HA Ab
7. " + " + Flag Ab
8. " + " + HA, Flag Abs
9. " + " + IgG
10. " + " + cold ACC probe x500
11. " + " + cold mut ACC x500
**Figure 4**

A. ChREBP ChIP INS1 cells (rat insulinoma)

- Binding Signal Relative to IgG Control
- * indicates significance
- * indicates nonsignificance
- Exon 1α
- Exon 1β
- Upstream ChoRE
- Downstream ChoRE

B. ChREBP ChIP rat islets

- Binding Signal Relative to IgG Control
- * indicates significance
- Exon 1α
- Exon 1β
- Upstream ChoRE
- Downstream ChoRE
- Coding region

C. ChREBP ChIP mouse liver

- Binding Signal Relative to IgG Control
- * indicates significance
- Exon 1β
- α-actin

D. ChREBP ChIP 3T3L1 adipocytes

- Binding Signal Relative to IgG Control
- * indicates significance
- Exon 1α
- Exon 1β
- Upstream ChoRE
- Downstream ChoRE
- Coding region

**Diabetes**
### Figure 5

**A**

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<td>Rat islet cells 18 h</td>
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<td>1:28,700 ± 8,200</td>
<td>0.3 ± 0.02 (ref 6)</td>
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**B**

![](image1.png) \( R^2 = 0.8648 \)

**C**

![](image2.png) \( R^2 = 0.1264 \)

**D**

![](image3.png) \( R^2 = 0.8935 \)
Figure 6

A. Glucose (mM)  
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ChREBPα

β-actin

B.  

2 mM

Scr

20 mM

Si-01

Si-02

ChREBP/DAPI
Figure 6

C.

siCon 2 mM  siCon 20 mM

siChREBPβ 2 mM  siChREBPβ 20 mM

D.

siChREBPβ-01

Percent BrdU Positive Cells (relative to 20 mM)

2 mM  20 mM  2 mM  20 mM

siCon  siCon  siChREBPβ  siChREBPβ

siChREBPβ-02

Percent BrdU Positive Cells (relative to 20 mM)

2 mM  20 mM  2 mM  20 mM

siCon  siCon  siChREBPβ  siChREBPβ
Figure 7

A.

Accell siChREBPβ -01

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B.

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C.

Glucose (mM) | Scr | Si-01 | Si-02
--- | --- | --- | ---
5.5 | 15 | 5.5 | 15 | 5.5 | 15

ChREBPα

β-actin
Figure 7 D

5.5mM Glucose

15 mM Glucose

Si-Scr

Si-ChREBP-01

Si-ChREBP-02

Insulin  ChREBP  DAPI
E. siCon 5.5 mM

siCon 15 mM

F. siCon 5.5 mM

siCon 15 mM

siChREBPβ 5.5 mM

siChREBPβ 15 mM

G. Fold response BrdU+/Ins+ Cells

H. Fold response BrdU+/Ins+ Cells

siCon 5.5 mM

siCon 15 mM

siChREBPβ 5.5 mM

siChREBPβ 15 mM

*
Glucose-regulated gene expression and proliferation
## Supplemental Table 1. RT-PCR and ChIP oligonucleotides

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### Supplemental Table 2. EMSA oligonucleotides

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All sequences listed are from 5’ to 3’
Supplemental Figure 1.

**Depletion of ChREBPα abrogates expression of ChREBPβ.** An assumption of the relationship between ChREBPα and ChREBPβ is that ChREBPα must be expressed and activated by glucose to activate the ChoRE of ChREBPβ in order to drive expression of ChREBPβ. A prediction of this assumption is that depletion of ChREBPα would block expression of ChREBPβ. INS-1-derived 832/13 cells were treated with siRNA directed against ChREBPα exon 1a for 48 h and then treated with either 2 or 20 mM glucose. Total RNA was collected and subjected to RT-PCR using primers specific for the indicated exons. Depletion of ChREBPα was confirmed in 2 mM glucose since 20 mM glucose decreases its expression in these cells (Figure 1). The siRNA against ChREBPα significantly decreased its target and ChREBP as well as a Pklr, a ChoRE-containing target gene of ChREBP.
Supplemental Figure 1

A.

Relative mRNA Expression

B.

Relative mRNA Expression

- 2 mM siCon
- 20 mM siCon
- 2 mM siExon1a
- 20 mM siExon1a

ChREBPβ

PkIr

* indicates significance.