CHREBP: INSIGHTS INTO THE

MECHANISM OF ACTION BY GLUCOSE

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Dedication

This thesis is dedicated to my family,

from whom all of my successes and accomplishments stem.

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Chapter 1:

INTRODUCTION

The development of obesity and onset of obesity-related illnesses

Obesity has increased at an alarming rate in the United States over the past 20 to 30 years. According to the National Health and Nutritional Examination Survey (NHANES), over 30% of adults are currently considered obese, having a body mass index (BMI) of 30 or higher. Additionally, the Center for Disease Control (CDC) reports that obesity among children has increased dramatically, ranging from 10% in infants to 18% in teenagers. Having such a large population of obese individuals not only has social and behavioral implications, it is also a major contributor to public health trends and concerns in our society. Research over the last decade has consistently shown a strong correlation between obesity and metabolic diseases such as hypertension, dyslipidemia, coronary heart disease and type 2 diabetes. A population based study by the CDC revealed that 70% of obese children aged 5-17 have at least one risk factor for cardiovascular disease and that 39% have at least 2 risk factors. With such staggering trends in our society, it is and will be essential to further elucidate the underlying mechanisms and dysregulation of metabolic processes that drive obesity and obesity-related diseases.

The onset of obesity stems from a multitude of factors that involve environmental elements and genetic components of an individual. However, a major component of obesity in our society is due to an energy imbalance, in which calories consumed are much greater than calories burned. A study by NHANES reports that between the years 1970-2000, overall caloric intake increased by an average of 300 calories per day per person, yet approximately 50% of the population report that they participate in little or no physical activity. In conjunction with dietary consumption trends, weight gain and

obesity has been frequently attributed to high levels of fat and cholesterol in the diet. However, carbohydrates make up approximately 50% of our diet and its consumption has increased dramatically over the last half-century. In addition, with the introduction of sweeteners such as sucrose and high fructose corn syrup (HFCS), carbohydrate intake has shifted from largely complex polysaccharides to a high proportion of simple sugars. Current literature strongly supports the theory that simple sugars such as fructose play a significant role in weight gain and obesity-related illnesses (1-3).

Carbohydrates are essential nutrients that are primarily used as fuel for immediate energy needs. When energy requirements are fulfilled, excess carbohydrates are either stored within the liver and muscle tissue as glycogen or are converted to fat for long term energy storage. While most fat storage occurs in adipose tissue, in individuals with dyslipidemia associated with obesity, fat accumulation in muscle, liver and other tissues occurs and likely contributes to the development of insulin resistance. Although pathways of glucose metabolism such as glycolysis have been well defined for decades, it is becoming apparent that nutrients such as carbohydrates have additional roles in signaling pathways and gene regulation. Additionally, dysregulation of carbohydrate metabolism is at the center of many metabolic diseases such as diabetes and many cancers. Thus, understanding the relationship between carbohydrate metabolism, metabolic diseases and obesity will potentially lead to the discovery of new and innovative therapeutic targets to help fight the epidemic of obesity-related diseases.

Hormonal regulation of energy homeostasis

Over the span of evolution, mammals have developed very complex and intricate regulatory pathways to adjust to the flux between energy utilization and consumption. It

is crucial for an organism to maintain relatively constant levels of fuel during times when resources are either scarce or plentiful. Dysregulation of these pathways is at the crux of many metabolic diseases. Because glucose is the preferred substrate for energy production in many tissues, including brain, regulation of its blood concentration is a critical component of maintaining energy homeostasis. The main organ responsible for monitoring glucose homeostasis is the endocrine pancreas, which controls blood glucose levels acutely by releasing two hormones, insulin and glucagon, that perform opposite functions.

Insulin is synthesized by β-cells, which are located in the Islets of Langerhans of the pancreas. β-cells express a specialized form of hexokinase, glucokinase, that allows these cells to sense physiologic changes in blood glucose concentrations. Upon glucose elevation, β-cells secrete insulin to peripheral tissues such as the liver and muscle to stimulate glucose uptake and suppress glucose production, decreasing blood glucose concentrations. Once in the cell, glucose is funneled through the glycolytic pathway to generate ATP to be utilized for a variety of cellular processes such as amino acid synthesis and ribose formation for nucleotide synthesis. An additional fate of insulin-stimulated glucose uptake is glycogen synthesis. Glycogen is a branched polysaccharide consisting of glucose monomers and functions as a cellular storage form for excess glucose molecules. Thus, insulin acts to decrease blood glucose levels by promoting its utilization and storage, while consistently suppressing hepatic production of glucose.

Alternatively, when blood glucose concentrations are low during times of sleep, exercise and fasting, glucagon is secreted into circulation by the pancreatic α-cell. Glucagon binds to the glucagon receptor found on the plasma membrane of the liver and

subsequently activates gluconeogensis, a glucose producing pathway that synthesizes glucose from metabolic intermediates such as pyruvate and alanine. Glucagon also stimulates the breakdown of liver glycogen into glucose, which in turn is secreted into the bloodstream to increase blood glucose levels and provide fuel for peripheral tissues. Additionally, glucagon functions to turn off glucose utilization pathways such as glycolysis to aid in increasing net glucose production. Thus, the interplay between insulin and glucagon actions is critical to maintain healthy glucose levels within the blood. One major risk factor of type 2 diabetes that correlates strongly with obesity is the gradual desensitization of metabolic tissues to the actions of insulin. Insulin resistance results in a decrease in glucose uptake and utilization by tissues, an increase in hepatic glucose production and a prolonged state of high blood glucose concentrations called hyperglycemia. In early stages, hyperglycemia is countered by increased production of insulin from β-cells. However, over time the capacity of the β-cell to compensate is lost and type 2 diabetes ensues. Type 2 Diabetes can lead to heart, kidney and liver complications and eventual failure.

De novo lipogenesis: the conversion of excess carbohydrates to fat

A primary fate of excess glucose is glycogen storage. However, glycogen stores are limited and when they reach their full capacity, a pathway called *de novo* lipogenesis provides a secondary fate for excess carbohydrates. The lipogenic pathway converts excess carbohydrates to fatty acids and triglyceride predominantly in the liver and adipose tissue. Triglycerides synthesized in the liver by lipogenesis are transported to the white adipose tissue (WAT) by very low density lipoproteins (VLDL) for long term energy storage. Free fatty acids are released from VLDL triglyceride by lipases and are

taken up by WAT. Upon entering WAT, free fatty acids are re-esterified into triglycerides. Alternatively, tissues such as the muscle can utilize free fatty acids released from VLDL as a substrate for beta-oxidation and energy production. Under conditions of prolonged and elevated lipogenesis, VLDL transport becomes compromised and results in accumulation of fat in the liver, leading to non-alcoholic fatty liver disease (NAFLD) and hepatic steatosis (4).

During ancient times, lipogenesis likely provided an advantageous adaptive response by allowing storage of energy for extended periods when nutrients were scarce. Evolutionarily, this trait was selected for and is now an inherent characteristic of all mammals. For many mammals, especially species that hibernate during winter, the ability to efficiently store excess energy is critical for survival (5). However, in a society that consumes enormous quantities of carbohydrates and is showing increasing trends of reduced exercise, humans no longer require the same degree of adaptive lipogenesis. In fact, recent evidence strongly suggests that lipogenesis is a key component contributing to obesity and many obesity-related diseases such as non-alcoholic fatty liver disease and dyslipidemia (6-9).

Nutrient regulation of lipogenic gene expression in the liver

Consuming a diet with high carbohydrate levels stimulates lipogenesis (10). This stimulation occurs at two levels: activation of pre-existing enzymes in the lipogenic pathway and transcriptional induction (11). Many enzymes involved in critical steps of fatty acid synthesis and triglyceride formation are induced by high carbohydrate diets. For example, expression of key regulatory enzymes such as liver pyruvate kinase (L-PK), fatty acid synthase (FAS), acetyl CoA carboxylase (ACC) and steroyl CoA desaturase

(SCD-1) are upregulated in response to dietary carbohydrate (12). Transcriptional induction of lipogenic genes has been largely attributed to the hormonal actions of insulin and subsequent activation of the transcription factor Sterol Response Element Binding Protein-1c (SREBP-1c) (13,14). SREBP-1c is a member of the basic-helix-loop-helix/leucine zipper (bHLH/LZ) family of transcription factors. A role for SREBP-1c in lipid metabolism was first identified in a study that overexpressed this protein in adipose tissue of mice, which resulted in hyperglycemia, fatty liver and increased triglyceride serum levels (15). Additionally, overexpression of SREBP-1c resulted in increased lipogenic gene expression even during a fasted state. Further analysis in rats revealed that high carbohydrate feeding after a fast, increased SREBP-1c mRNA levels and activity (8,9). SREBP-1c mRNA levels were also decreased in rats with streptozotocin-induced diabetes, a toxic chemical that eliminates insulin production. Thus, it was concluded from these studies that SREBP-1c is an insulin-dependent regulator of lipogenic gene expression.

Although insulin is a key regulator of lipogenesis, research over the last decade has established that SREBP action is not sufficient to stimulate lipogenesis. For example, studies performed with diabetic rat livers revealed that increased fructose levels stimulate L-PK mRNA expression (16). Unlike glucose, fructose metabolism is not tightly regulated by insulin. Thus, fructose is capable of stimulating expression of lipogenic genes in an insulin resistant state. Additional studies were performed in which glucokinase was overexpressed in the rat liver (17). Glucokinase expression in the liver is upregulated by insulin and functions to phosphorylate glucose to glucose-6-phosphate, a critical substrate for glucose metabolism. Overexpression of glucokinase stimulated

expression of genes such as L-PK and ACC in a fasted state to levels comparable to that observed in a fed state (17). Additionally, it was observed in primary rat hepatocytes that both insulin and glucose are required for the full induction of lipogenic enzyme genes (18-20). These results suggest that metabolism of glucose is capable of stimulating expression of lipogenic enzyme genes, independent of insulin.

The Carbohydrate Response Element and its role in glucose-dependent regulation of transcription

With the demonstration of a distinct role for glucose in the induction of lipogenic genes, subsequent studies revealed a promoter element essential to mediate a glucose-response. This glucose-dependent promoter element was termed the Carbohydrate Response Element (ChoRE). A ChoRE consists of two E-boxes that have a CACGTG consensus sequence that are separated by 5 bases. ChoRE sites have been identified in multiple lipogenic genes such as L-PK, ACC, Spot 14 and FAS (Figure 1) (21-24). The first ChoRE was identified in the promoter region of the L-PK gene and mutational analysis of this ChoRE revealed that both E-boxes are required for glucose-dependent induction (25,26). Additionally, the 5 base pair spacing between the E-boxes is critical for ChoRE function (27).

ChoRE elements are distinct from the Sterol Response Element (SRE), recognized by SREBP, which consists of divergent E-box motifs TCACNCCAC (28). Both elements are found on most lipogenic gene promoters and are necessary for maximal induction. CACGTG-type E-boxes are generally recognized by bHLH/LZ transcription factors such as Myc and Max. Heterodimer formation of Myc and Max is required for CACGTG-type E-box binding within promoters of genes involved in cell proliferation, while

homodimerization of SREBP is required for recognition of the SRE upon insulin stimulation (28-30).

Carbohydrate Response Element Binding Protein interacts with the ChoRE

Once the ChoRE was identified, a large effort was put into determining the transcription factor responsible for glucose-dependent gene activation. Based on the presence of CACGTG-type E-boxes in the ChoRE, it was postulated that the carbohydrate-regulated factor would be a member of the bHLH/LZ family of transcription factors. Eventually, through the use of DNA affinity purification techniques and 300 rat livers, a novel factor that bound to the ChoRE was identified and termed the Carbohydrate Response Element Binding Protein (ChREBP) (31). Consistent with other E-box binding proteins, computational sequence analysis of ChREBP revealed a bHLH/LZ homology domain in the C-terminal region of ChREBP. Additionally, yeast two-hybrid experiments identified a protein that interacts with the C-terminal region of ChREBP called Max-like factor X (Mlx) (32). Mlx is a member of the Myc/Max family of bHLH/LZ transcription factors and shows a high level of sequence conservation not only with the bHLH/LZ domain of ChREBP, but also with a second region just downstream of the bHLH/LZ region termed the Mlx homology region (Figure 2) (33). Electrophoretic Mobility Shift Assays (EMSAs) using DNA probes containing either the L-PK or ACC ChoRE established that ChREBP interaction with Mlx is required for DNA binding (32).

Single E-boxes require dimer formation of DNA-interacting proteins for binding. ChREBP/Mlx is capable of binding to a perfect CACGTG motif as a dimer (34). However, few natural ChoREs contain perfect CACGTG motifs. The two E-box nature of

the ChoRE suggested heterotetramer binding. In fact, EMSA revealed that the ChREBP/Mlx complex binds cooperatively to the ChoRE of lipogenic genes as a heterodimer and a heterotetramer (34). In isolated rat hepatocytes, disruption of one E-box within the ChoRE by substitution mutagenesis inhibited glucose-dependent activation, suggesting that heterotetramer formation between ChREBP/Mlx dimers is required (25,35). Additionally, mutations introduced into Mlx that disrupt heterotetramer, but not heterodimer formation, inhibit ChREBP/Mlx binding to the ChoRE in vitro and ChREBP/Mlx-dependent ChoRE activation in vivo, suggesting that heterotetramer formation is critical (34). The five base pair spacing between the two E-boxes is also essential for heterotetramer formation, allowing two heterodimers of ChREBP/Mlx to bind optimally to the ChoRE (32). From these results, it was concluded that heterotetramer formation between two ChREBP/Mlx heterodimers with the two E-box ChoRE is mediated by Mlx and is required for glucose-dependent activation of lipogenic genes (Figure 1). The use of imperfect CACGTG motifs and cooperative binding of heterodimers may provide greater specificity to the actions of ChREBP/Mlx.

Physiological role of the Carbohydrate Response Element Binding Protein

ChREBP was identified by its ability to directly interact with a ChoRE found within the promoter region of the L-PK gene (31). Moreover, ChREBP is expressed at highest levels in the liver and adipose tissue of rat and its binding to the L-PK ChoRE in rat livers is induced by a high carbohydrate diet (36). Thus, it was hypothesized that ChREBP was responsible for glucose-dependent transcriptional regulation in the liver. As mentioned previously, ChREBP requires a DNA binding partner called Mlx to bind to DNA. This interaction provided a critical tool for understanding the role of ChREBP in liver gene

expression. To determine the range of ChREBP action in the liver, microarray analysis was performed using isolated rat liver hepatocytes (22). Isolated hepatocytes were transduced with an adenoviral vector expressing a dominant negative form of Mlx (dn Mlx), which is incapable of binding to DNA yet still maintains its interaction with ChREBP (22,37). As such, overexpressed dn Mlx will bind to and squelch endogenous ChREBP within hepatocytes, preventing its ability to interact with DNA and inhibit ChREBP-dependent transcription. Microarray analysis revealed expression levels of 229 genes were induced by 1.8-fold or higher when hepatocytes were switched from low glucose (5.5 mM) to high glucose (27.5 mM). Of these 229 genes, 139 were repressed by the dn Mlx. The genes identified as ChREBP-dependent are involved all aspects of the lipogenic pathway, inducing glycolysis, NADPH production, and fatty acid synthesis and maturation. Thus, it was concluded that the ChREBP/Mlx complex is the major mediator of glucose-dependent transcription in the liver and a critical regulator of the lipogenic pathway.

The physiological importance of ChREBP was also explored by studies performed with ChREBP whole body knockout mouse models (ChREBP$^{-/-}$) (36). In mice fed a standard chow diet, it was observed that ChREBP$^{-/-}$ mice had decreased epididymal fat and brown fat compared to wild type mice. Significant increases in blood glucose and glycogen levels were also observed. These results were amplified in ChREBP$^{-/-}$ mice fed a high starch diet. Additionally, triglyceride levels in the liver and plasma cholesterol levels were markedly decreased in the ChREBP$^{-/-}$ compared to wild type mice when fed a high starch diet. The induction of lipogenic enzyme genes was greatly impaired in the

knockout mice. These results suggested that ChREBP is important for maintaining glucose homeostasis by driving triglyceride synthesis in the liver.

The role of ChREBP was also evaluated in the ob/ob mouse background, a model for obesity, insulin resistance and type 2 diabetes. Ob/Ob mice that lacked ChREBP showed a decrease in hepatic lipid synthesis, plasma free fatty acids and plasma triglyceride levels (38). Additionally, ob/ob mice with a ChREBP knockout showed reductions in weight and general adiposity compared to the ob/ob control mice. Liver specific knockdown of ChREBP using small interfering RNA in the ob/ob background showed reductions in hepatic steatosis and improved insulin sensitivity in only seven days (39). These studies provided strong evidence that ChREBP plays a significant role in the onset of obesity and insulin resistance resulting from dysregulation of glucose metabolism due to the effects of a high caloric diet.

A recent human genome-wide array study has revealed a strong correlation between triglyceride levels and a specific single nucleotide polymorphism (SNP) located within the ChREBP gene (40,41). Since this study, two more SNPs have been identified within the ChREBP gene and in close proximity, and these polymorphisms were associated with the risk of coronary heart disease (42). Thus, the animal studies and the genome-wide array studies strongly suggest that ChREBP contributes to the development obesity and has a key role in the onset of dyslipidemia and insulin resistance.

Max-like factor X and its role in ChREBP-dependent transcription

Mlx is expressed ubiquitously and functions as a heterodimer for several transcriptional repressors and activators (33). Mlx is a member of the Myc/Mad/Max family of transcription factors and was initially characterized as interacting with Mad1 and Mad4 (33). Myc, Mad and Max have been implicated in processes such as cell growth and proliferation. However, the role of Mlx in these cellular processes is completely undefined. A novel interaction for Mlx was identified with the discovery of a bHLH/LZ transcription factor called MondoA (43). Subsequent studies revealed that the MondoA/Mlx interaction is required for DNA binding to E-boxes, and that MondoA specifically interacts with Mlx and not Myc, Mad or Max, suggesting Mlx is involved in a transcriptional network separate from its close relatives.

The role of MondoA and Mlx remained unclear for years until the discovery of ChREBP and eventually its interaction with Mlx (32). MondoA and ChREBP share a high level of sequence identity, especially within the N-terminal region (Figure 2). Based on this conservation, the N-terminal domain of ChREBP was termed the MondoA Conserved Region (MCR). Demonstration of the lipogenic role of ChREBP/Mlx prompted examination of whether MondoA might have a similar function. Subsequent studies discovered that MondoA is expressed at highest levels in the skeletal muscle and localizes with the outer mitochondrial membrane (43). Nuclear translocation of MondoA and subsequent glycolytic gene activation was stimulated by the glucose analog, 2-deoxy-glucose (2-DOG) (44). From these results, it was inferred that MondoA/Mlx acts as a cellular energy sensor and regulator of glycolysis in the muscle. As such, ChREBP and MondoA were considered functionally homologous transcription factors in which their

physiological activity was determined by their tissue distribution. Mechanistic studies determined that ChREBP and MondoA are the targets of carbohydrate signaling and that the role of Mlx is to mediate DNA binding. The roles of MondoA and ChREBP in glucose regulation provide strong evidence that the Mlx transcriptional network is a key mediator of cellular energy homeostasis, a separate function from the Myc, Mad and Max transcriptional network.

Understanding the mechanism for glucose-dependent ChREBP activation

As ChREBP was being established as a mediator of glucose signaling in the liver, much effort was focused on determining its mechanism of activation. It has been known for many years that increased cAMP levels inhibited lipogenic enzyme gene expression, presumably in response to increased glucagon secretion (10). Subsequent activation of cAMP-dependent kinase (PKA) was proposed to lead to the phosphorylation of three phospho-acceptor sites in ChREBP: two (Ser626 and Thr666) near the bHLH/Lz domain at the C-terminal end and one (Ser196) near a putative nuclear localization signal (NLS) located in the MCR domain (31,45). In vitro phosphorylation studies and DNA binding assays revealed that PKA is capable of phosphorylating ChREBP at these three specific residues and inferred that increases in cAMP levels stimulate this process. Nuclear localization studies using a GFP-tagged ChREBP suggested that ChREBP is predominantly cytoplasmic in basal conditions and translocates to the nucleus in response to high carbohydrate levels. Based on these observations, a model was proposed in which ChREBP is inactive and cytoplasmic under basal conditions (Figure 3). In this model, ChREBP would require dephosphorylation of these specific residues for activation.

To determine the mechanism of activation, in vitro analysis was performed using cytosolic liver extracts and a ChREBP N-terminal peptide containing a phosphorylated residue at S196, the putative phospho-acceptor site located near the NLS (46). This analysis revealed that dephosphorylation of S196 is dependent on the activity of an isoform of Protein Phosphatase 2A (PP2A). This PP2A isoform activity was shown to be activated in response to increased xylulose 5-phosphate levels, a metabolite of glucose in the pentose phosphate pathway. Thus, it was proposed that xylulose 5-phosphate is the ChREBP activating metabolite that stimulates PP2A-dependent dephosphorylation of phopho-acceptor sites near the putative NLS and promotes DNA binding (Figure 3).

Although this model for glucose-dependent ChREBP activation follows a logical sequence of events, it does not account for all aspects of ChREBP regulation. The cAMP-dependent model for ChREBP inactivation cannot account for the basal level of ChREBP activity observed in isolated rat hepatocytes. When hepatocytes are isolated from the whole body, they are removed from circulating hormones such as glucogon (47). Thus, cAMP levels are not increased in basal glucose conditions, due to the lack of glucagon. However, in basal glucose conditions (5.5 mM) without cAMP, ChREBP remains inactive. Additionally, mutational analysis of the three putative PKA phospho-acceptor sites was performed, substituting serine/threonine residues with alanine. According to the proposed model, mutation of the phospho-acceptor sites should result in a constitutively active transcription factor. However, the triple mutant (Ser196A/Ser626A/T666A) showed a response to glucose comparable to wild-type ChREBP, suggesting that these residues are not critical targets of glucose regulation and are not sufficient for glucose-dependent activation (47). The ChREBP phospho-mutant, which eliminates the

requirement for PP2A action at these sites, also suggests that ChREBP activation is not dependent on xylulose 5-phosphate. However, subsequent studies have yet to identify an alternative signaling metabolite for ChREBP activation. Consequently, while PKA-mediated phosphorylation of ChREBP may account for its inactivation in fasting conditions, it does not appear to explain the glucose-stimulated pathway for activation.

Rationale

ChREBP is a glucose-regulated transcription factor that stimulates the expression of lipogenic enzyme genes in mammals. While the physiological role of ChREBP is well substantiated, the mechanism responsible for its glucose control is controversial. Hence, my thesis research will be focused on gaining an understanding of the molecular mechanism of glucose-stimulated ChREBP activation. When I initiated this study, the current model proposed that glucose signaling regulated ChREBP activity by controlling its nuclear localization and DNA binding through PKA-dependent phosphorylation. However, amino acid substitution at these proposed phospho-acceptor sites did not affect ChREBP activation. In addition, PKA-dependent phosphorylation cannot account for repression of ChREBP in low glucose conditions in isolated hepatocytes, as cAMP levels are not sufficient to activate PKA in these conditions. Finally, the net phosphorylation state of ChREBP was unchanged by glucose levels. All of this evidence suggested that the model for glucose-stimulated ChREBP activation needed to be revised. As such, my first experimental goal is to determine the intracellular localization of ChREBP in both basal and stimulatory conditions. I will use immunoflourescence techniques to evaluate the localization of ChREBP and various mutants in INS-1 cells, an immortalized rat pancreatic β-cell line that is highly responsive to glucose. The goal of my studies is to determine whether cellular localization plays a critical role in regulating ChREBP activity in response to glucose.

A second goal of my thesis will be to evaluate the importance and role of MCR domains in ChREBP regulation. The MondoA Conserved Region of ChREBP contains

five highly conserved domains (MCRs 1-5), suggesting that this region is important for ChREBP activation. As such, I will construct amino acid substitution mutants and deletion mutants throughout the MCR and test the functional consequences. I intend to establish the specific function of each MCR in glucose-stimulated ChREBP activation. Defining the regions of ChREBP essential for glucose control will provide critical insight for the mechanism of ChREBP action. These studies will also establish a foundation that will be essential for future endeavors that will focus on identifying the elusive, glucose-stimulatory signaling pathway.

Figure 1: Model for ChREBP binding to the ChoRE.

Two E box-like motifs identified from carbohydrate-responsive genes are depicted in capital letters. Five nucleotide bases between the two E boxes are in lower case letters. Functional DNA binding to a ChoRE requires heterotetramer formation of two ChREBP/Mlx heterodimers. ChREBP/Mlx heterodimers are capable of binding to a perfect CACGTG E box. However, heterotetramer formation of ChREBP and Mlx is required for transcriptional activation.

Figure 1

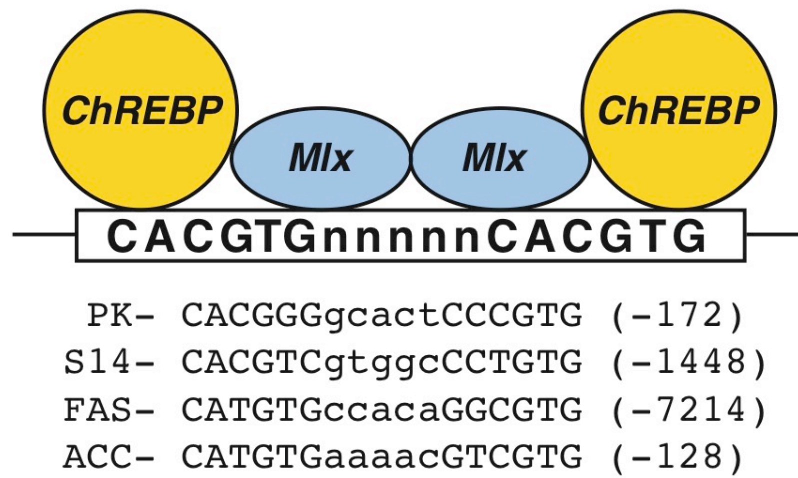


Figure 2: Conservation between ChREBP, MondoA and Mlx

ChREBP, MondoA and Mlx show their highest level of conservation in the basic Helix-Loop-Helix/Leucine Zipper domain and the Mlx homology region at the C-terminal end of each protein. ChREBP and MondoA also share a high level of conservation in the MondoA Conserved Region (MCR) of the N-terminal segment of both proteins. The MCR domain of ChREBP and MondoA can be further divided into 5 sub-domains (MCRs 1-5) that have approximately 90-100% conservation between the two proteins. ChREBP and MondoA also contain a proline-rich region within the internal region of each protein. Percentages are based on the level of sequence identity between the indicated regions.

Figure 2

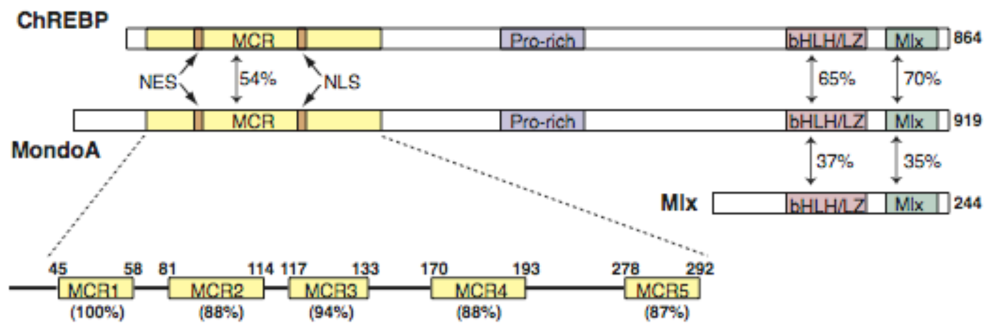
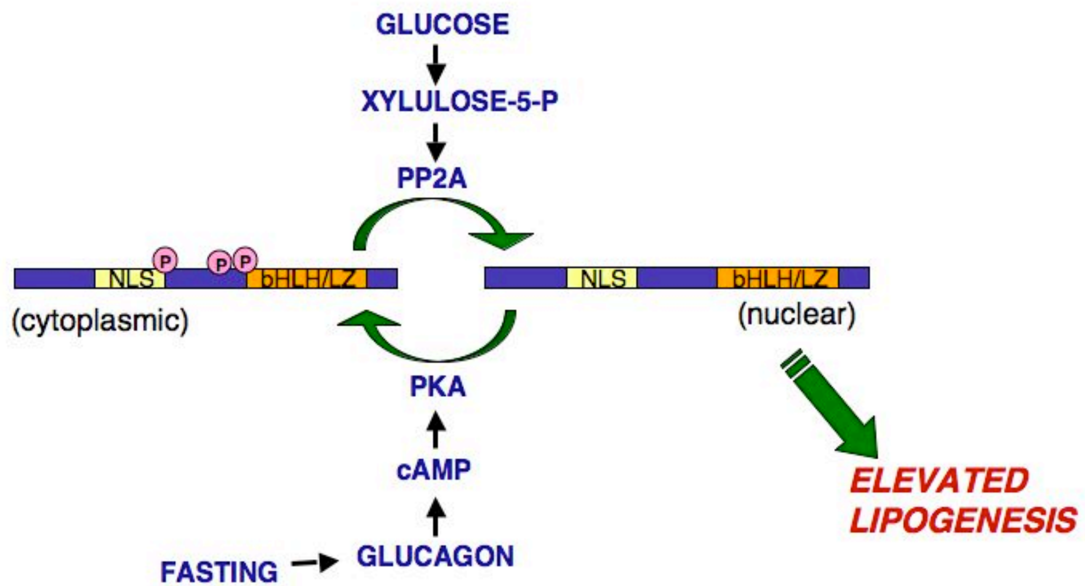


Figure 3: Proposed model for glucose-stimulated ChREBP activation.

In the proposed Uyeda model (45) for glucose-stimulated ChREBP activation, ChREBP is localized to the cytoplasmic compartment and is inactive in low (5 mM) glucose conditions. This inactivation is due to Protein Kinase A (PKA)-dependent phosphorylation of residues (Ser196) near the putative Nuclear Localization Signal (NLS) and (Ser626, Thr666) near the DNA binding (bHLH/LZ) domain of ChREBP. Phosphorylation was hypothesized to prevent nuclear translocation and DNA binding of ChREBP in basal conditions. Upon increased glucose metabolism, xyulose-5 phosphate levels are increased, resulting in Protein Phosphatase 2A (PP2A) activation. Once activated by xyulose-5 phosphate, PP2A then dephosphorylates the phospho-acceptor sites of ChREBP, resulting in nuclear translocation, DNA binding and subsequent transcriptional activation of lipogenic gene expression.

Figure 3



Chapter 2

Glucose Activates ChREBP by Increasing its Rate of Nuclear Entry and Relieving Repression of its Transcriptional Activity*

This chapter is unmodified from the published journal article:

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Carbohydrate Response Element Binding Protein (ChREBP) is a glucose-responsive transcription factor that activates genes involved in *de novo* lipogenesis in mammals. The current model for glucose activation of ChREBP proposes that increased glucose metabolism triggers a cytoplasmic to nuclear translocation of ChREBP that is critical for activation. However, we find that ChREBP actively shuttles between the cytoplasm and nucleus in both low and high glucose in the glucose-sensitive β cell-derived line, 832/13. Glucose stimulates a three-fold increase in the rate of ChREBP nuclear entry, but trapping ChREBP in the nucleus by mutagenesis or with a nuclear export inhibitor does not lead to constitutive activation. In fact, mutational studies targeting the nuclear export signal of ChREBP also identified a distinct function essential for glucose-dependent transcriptional activation. From this, we conclude that an additional event independent of nuclear translocation is required for activation. The N-terminal segment of ChREBP (amino acids 1-298) has previously been shown to repress activity under basal conditions. This segment has five highly conserved regions - Mondo Conserved Regions (MCRs) 1-5. Based on activating mutations in MCR2 and MCR5, we propose that these two regions act coordinately to repress ChREBP in low glucose. In addition, other mutations in MCR2 and mutations in MCR3 were found to prevent glucose activation. Hence, we conclude that both relief of repression and adoption of an activating form are required for ChREBP activation.

Introduction

The mammalian liver plays a critical role in maintaining energy homeostasis of an organism in response to its dietary state. When food is abundant, excess dietary carbohydrates are converted to triglycerides in the liver through the pathway of *de novo* lipogenesis for long-term energy storage. Lipogenic enzymes, such as L-type pyruvate kinase (48), acetyl-CoA carboxylase (49), fatty acid synthase (50) and stearoyl-CoA desaturase (51), involved in the conversion of glucose to triglycerides are induced upon feeding of a high carbohydrate diet. Transcriptional induction of these genes requires signals from insulin, acting through the Sterol Response Element Binding Protein-1c (8,28,52,53), and a second signaling pathway initiated in response to increased metabolism of simple carbohydrates, such as glucose (12,18,54,55). Lipogenic genes responsive to glucose contain a DNA element called the Carbohydrate Response Element (ChoRE)[1] (21,23-25,35). The ChoRE consists of two E box sequences (CACGTG) separated by five base pairs and serves as the recognition site for two heterodimeric transcription factors: Carbohydrate Response Element Binding Protein (ChREBP) and Max-like protein X (Mlx) (31,32,37,45,56). Both ChREBP and Mlx are required for binding to the ChoRE, but recent evidence establishes ChREBP as the direct target of glucose signaling. ChREBP is highly expressed in glucose-responsive tissues such as the liver, adipose and pancreas, while Mlx expression is ubiquitous (33,36,57). High carbohydrate-fed ChREBP$^{-/-}$ mice do not induce *de novo* lipogenesis or lipogenic enzyme gene expression (36). Similarly, cultured mouse hepatocytes in which ChREBP expression has been inhibited by small interfering RNA do not induce lipogenic gene expression in response to glucose (58). Finally, a fusion protein of the Gal4 DNA

Binding Domain and ChREBP can activate an appropriate reporter gene in response to glucose, even when the Mlx-interacting region of ChREBP is deleted (59-61). Hence, ChREBP is the major target of the glucose signaling pathway, while Mlx serves to help recruit ChREBP to appropriate target genes (34).

The originally proposed and widely accepted model for ChREBP activation suggests that its activity is regulated by reversible phosphorylation (56,62). In this model Protein Kinase A (PKA) phosphorylates residues Ser196, Ser626 and Thr666 of ChREBP in low glucose conditions, restricting it to the cytoplasm and inhibiting its DNA binding (45). An increase in glucose metabolism stimulates protein phosphatase 2A activity via direct binding to the glucose metabolite xyulose-5-phosphate (46). Dephosphorylation of ChREBP at these critical PKA sites allows ChREBP to translocate to the nucleus and bind to the ChoRE, which is sufficient for activation of lipogenic gene expression.

Subsequent studies performed by our laboratory and others have questioned the current model for glucose regulation of ChREBP. The level of phosphorylation of ChREBP did not decrease in response to high glucose, as predicted by the current model (47). Mutating residues Ser196, Ser626, and Thr666 to alanines did not lead to constitutive activation, suggesting that dephosphorylation of these residues is not sufficient for activation (47,59,60). Finally, inhibition of protein phosphatase 2A in 832/13 cells by addition of cantharidic acid did not interfere with the activation of ChREBP by glucose (60). Thus, we conclude that while PKA-mediated phosphorylation may repress ChREBP activity under fasting conditions, reversal of this phosphorylation is not sufficient for glucose activation.

To address the mechanism of ChREBP activation, we further evaluated the connection between ChREBP localization and glucose-stimulated activity. We present evidence that ChREBP shuttles between the nucleus and cytoplasm in both low and high glucose conditions, but accumulation in the nucleus occurs more rapidly in high glucose conditions than in low. However, nuclear localization is not sufficient for ChREBP activation. Thus, we suggest that there are additional events, independent of nuclear localization, required for glucose activation of ChREBP.

EXPERIMENTAL PROCEDURES

Construction of mutant ChREBP plasmids - Site-specific mutations of ChREBP were constructed with the QuickChange^{TM} site-directed mutagenesis kit (Stratagene) using mouse FLAG-tagged ChREBP in the expression plasmid CMVS4 as a template (32). All mutations and coding sequences were confirmed by DNA sequencing. Immunoblotting of ChREBP from transfected HEK293 cells was performed using FLAG monoclonal antibody (Sigma) to ensure that each construct was expressed. Only mutants with comparable expression to wild-type (WT) ChREBP were used for subsequent analysis.

Transcriptional reporter gene assays for measuring ChREBP activity - 832/13 cells (a gift from Dr. C. Newgard, Duke University) were cultured in RPMI media containing 11 mM glucose in 24-well plates as previously described (63). Cells were transduced with adenovirus expressing dominant negative ChREBP (dnChREBP) (61). The amount of virus used was determined empirically to give approximately 90% inhibition of the glucose response. After 2 h of transduction, 832/13 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) with a mixture of firefly luciferase reporter (700 ng) driven by the ACC ChoRE-containing promoter region (47) and a *Renilla* luciferase control plasmid (pRL-CMV, Promega; 15 ng). In addition where indicated, cells were also co-transfected with 60 ng of each ChREBP expression plasmid and 30 ng of Mlx expression plasmid. After 18 h, cells were cultured in RPMI media containing low (2.5 mM) or high (25 mM) glucose for 24 h and lysates were prepared in Passive Lysis Buffer (Promega). Dual luciferase assays were performed following the manufacturer's instructions. Values represent the ratio of firefly/*Renilla* luciferase from triplicate samples and are expressed as mean (±SD).

Immunolocalization of ChREBP - 832/13 cells were cultured in RPMI media containing 11 mM glucose on glass slides and grown to 70% confluence (61). Cells were co-transfected with 0.15 μg each of FLAG-tagged WT or mutant ChREBP and HA-tagged Mlx using Lipofectamine 2000 (Invitrogen) and 1.1 μl Virofect (Targeting Systems, San Diego, CA). After overnight transfection, cells were refed RPMI media containing 2.5 mM glucose for 4 h. Cells were then incubated in RPMI media containing 2.5 mM or 25 mM glucose for 2 h. Where indicated, leptomycin B was added at 3.6 μM. After treatment, cells were fixed in a 1.6% formaldehyde solution containing Phosphate Buffered Saline (PBS) and 0.2% Triton. Cells were subsequently washed in PBS, 0.2% Triton and incubated overnight in a humidity chamber at 4°C. The slides were washed two additional times and then blocked with 10 mg/ml BSA in PBS, 0.2% Triton and 5 μl/ml donkey serum (Jackson ImmunoResearch Laboratories). Following the block, fixed cells were incubated with 0.25 μg FLAG monoclonal antibody for 1 h at 37°C, washed five more times followed by incubation with a secondary FITC-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) for an additional hour. Nuclei were stained with TO-PRO3 (Molecuar Probes) during the final wash steps. Cells were imaged using a multi-photon confocal microscope (Fluoview 1000, Olympus).

Co-immunoprecipitation – 832/13 cells were co-transfected with FLAG-tagged ChREBP and Mlx in RMPI media containing 11 mM glucose, as described above. After 36 h, cells were collected in 50 mM Tris-HCl, pH 8.0, 10% glycerol, 150 mM NaCl, 0.5% Triton X-100 with protease inhibitors (Roche) and then lysed in a glass-teflon homogenizer. Anti-FLAG agarose beads (Sigma) were added and samples were processed according to the manufacturer’s instructions. Immunoadsorbed proteins were separated on a 10%

polyacrylamide gel and immunoblotted with horseradish peroxidase-conjugated 14-3-3β antibody (Santa Cruz Biotechnology).

Measurement of mRNA by quantitative RT-PCR - Total cellular RNA was isolated from 832/13 cells using Trizol reagent (Invitrogen), and selected ChREBP target gene products were measured by quantitative RT-PCR performed by a two-step procedure described previously (34). Primers were designed using MacVector (Accelrys Software, Inc.). RT-PCR results are expressed as fold induction by normalizing the mean of the Ct values from the high glucose treated cells relative to the mean of Ct values from low glucose cells. All samples were analyzed in triplicate and expressed as mean (± SEM).

RESULTS

ChREBP shuttles between the nucleus and cytoplasm under low and high glucose conditions - The human homolog of ChREBP, Wbscr14 (64), and the ChREBP paralog, MondoA (43), have been shown to shuttle between the cytoplasm and nucleus in several cell lines such as mouse 3T3, monkey COS7 and human 293 cells (65,66). However, these cell lines are not known to respond transcriptionally to changes in glucose metabolism. Thus, we decided to examine ChREBP nucleocytoplasmic shuttling and the effect of glucose on this process in a glucose-sensitive cell line. Immunofluorescence was performed in 832/13 cells, an INS-1 derived cell line with robust glucose-stimulated insulin secretion (63). These cells express ChREBP and support a transcriptional activation of ChREBP in response to elevated glucose (57,59,60). 832/13 cells were co-transfected with plasmids expressing FLAG-tagged ChREBP and Mlx. Mlx was incorporated in these studies because of its known interaction with ChREBP (32,34), its requirement for ChREBP-dependent gene transcription (34,37), and its requirement for ChREBP nuclear entry (see Supplemental Fig 1). Transfected cells were incubated in low glucose media for 4 h, and then treated with either low or high glucose media for 2 h. At this time optimal induction of several ChREBP target genes is observed (34,60). Immunoflourescence was performed with anti-FLAG primary and FITC-conjugated secondary antibodies and visualized by confocal microscopy. As expected, ChREBP was predominantly localized to the cytoplasm under low glucose conditions (Fig 1a). However, as previously reported (61), treatment of cells with high glucose to induce ChREBP activity did not result in a major accumulation of ChREBP in the nucleus. The majority of fluorescent signal in these cells was still found within the cytoplasm.

Quantification of signals from a large number of cells revealed that 94% displayed cytoplasmic localization and 6% had signals from both cytoplasm and nucleus in low glucose (Fig 2A). In high glucose, ChREBP was cytoplasmic in 78%, both cytoplasmic and nuclear in 18% and nuclear in 2% of cells. Hence, while there was a shift to a more nuclear pattern in high glucose, this trend was only observed in a small fraction of the total cells. Similar results were reported for a GFP-tagged fusion of ChREBP in 832/13 cells at 6 h of glucose treatment (60).

Since ChREBP was located in the cytoplasm under high glucose conditions in the majority of cells, shuttling between the cytoplasm and the nucleus must occur to support its transcriptional function. The domains of *Wbscr14* and MondoA found to contain the Nuclear Export Signal (NES) are highly conserved in ChREBP, suggesting that it may be subject to Crm-1 dependent export. To confirm this, localization of ChREBP was observed in 832/13 cells treated with low or high glucose conditions for 2 h with the addition of leptomycin B, a Crm-1 nuclear export inhibitor (Fig 1b). ChREBP accumulated in the nucleus in high glucose conditions with leptomycin B treatment, as expected. However, ChREBP was found predominantly in the nucleus in low glucose as well. The observation that ChREBP actively shuttles under low glucose conditions in which it is not transcriptionally active suggests an additional event is required for its transcriptional activation.

The rate of ChREBP nuclear entry is increased under high glucose conditions - The fact that ChREBP is predominantly cytoplasmic under stimulating conditions indicates that only a fraction of total cellular ChREBP needs to be in the nucleus for gene activation. Therefore, it seemed plausible that glucose might act by transiently increasing the rate of

ChREBP entry to the nucleus without a dramatic shift in the cellular pool of ChREBP to the nuclear compartment. To measure its rate of nuclear accumulation, 832/13 cells were co-transfected with ChREBP and Mlx. Subsequently, cells were treated with leptomycin B and the time course of ChREBP nuclear accumulation in low or high glucose conditions was compared. ChREBP localization was classified as cytoplasmic, both cytoplasmic and nuclear or nuclear. Cells that displayed either nuclear or both nuclear and cytoplasmic localization were combined to represent cells that had initiated nuclear accumulation. An increased nuclear accumulation was observed in high glucose conditions at the 15 and 30 minute time points relative to cells maintained in low glucose (Fig 2). At 45 minutes the difference in ChREBP accumulation between treatments was less evident, and at 1 h ChREBP was predominantly nuclear in both low and high glucose conditions. From the time required to achieve 50% nuclear accumulation, we estimate that the rate of nuclear entry was three-fold greater in high glucose compared to low glucose. Thus, we conclude that glucose regulates ChREBP at the level of nuclear entry.

The L86A/L93A ChREBP mutant is deficient in nuclear export and is not transcriptionally active - The observation that ChREBP enters the nucleus more rapidly in high glucose raises the question of whether this event is sufficient to account for the elevated ChREBP activity in these conditions. To further examine this question, a mutant ChREBP deficient in nuclear export was constructed and tested for functional activity. This ChREBP mutant was prepared by mutating two conserved leucine residues (Leu86 and Leu93) in the NES to alanines. The comparable mutations in Wbscr14 and MondoA result in proteins that accumulate in the nucleus (65,66). To test whether the L86A/L93A ChREBP mutant is deficient in nuclear export, we performed immunofluorescence in

832/13 cells (Fig 3a). The L86A/L93A ChREBP mutant localizes to the nucleus under both low and high glucose conditions without the addition of leptomycin B, indicating this mutant was indeed deficient in nuclear export. We then tested the activity of the L86A/L93A mutant using a functional rescue assay that our laboratory has previously established (47). To accomplish this, 832/13 cells were transduced with an adenoviral construct expressing a dominant negative form of ChREBP. This form of ChREBP is able to dimerize with Mlx, but the resultant dimer cannot bind to DNA. Hence, this mutant ChREBP competes with endogenous ChREBP for Mlx heterodimerization. Subsequently, 832/13 cells were transfected with a plasmid expressing WT or mutant ChREBP, together with a ChoRE-containing reporter construct. The activity of the reporter gene reflects the ability of the overexpressed ChREBP to support a glucose response.

As shown in Figure 3b, overexpressing WT ChREBP rescued the glucose response, although not to the level of endogenous ChREBP. The extent of rescue in different experiments varied from 30 to 80% depending on the effectiveness of the viral transduction of dnChREBP. However, the L86A/L93A ChREBP mutant did not rescue the glucose response, indicating that it is not functional for transcriptional activation. Western blotting showed that L86A/L93A ChREBP is expressed equivalently to WT ChREBP (see Supplemental Fig 2) and bound to a ChoRE-containing oligonucleotide in an electrophoretic mobility shift assay.[2] Thus, trapping ChREBP within the nucleus did not result in constitutive transcriptional activation, as might have been expected, but rather a loss of function.

Preventing nucleocytoplasmic shuttling of ChREBP does not inhibit glucose activation - We considered two possible explanations for why the L86A/L93A mutant was inactive despite its nuclear localization: a) ChREBP may need to be activated by an event in the cytoplasm and nucleocytoplasmic shuttling may be required to maintain this active state or b) a separate event, independent of nuclear localization and shuttling, may be required to activate ChREBP and stimulate gene transcription. In the latter circumstance, the L86A/L93A mutant would have altered both NES function and the distinct event required for activation. To test whether nucleocytoplasmic shuttling is required to maintain activity, mRNA levels of ChREBP target genes were measured in 832/13 cells pretreated with leptomycin B for 90 min in low glucose to trap ChREBP in the nucleus. Cells were subsequently maintained in low glucose or shifted to high glucose for 4 h. A short time point was selected to avoid potential secondary effects of inhibiting Crm-1-dependent export. If nucleocytoplasmic shuttling were essential, then leptomycin B treatment should inhibit ChREBP activation in high glucose conditions. mRNA from two ChREBP-responsive genes, thioredoxin-interacting protein and aldolase B, were measured. Expression of both gene products was higher in control cells treated with high glucose compared to low glucose at 4 h (Fig 4). In cells treated with leptomycin B, the ability of high glucose to induce the ChREBP-target mRNAs was not diminished. Therefore, preventing cytoplasmic shuttling did not inhibit ChREBP activity. It is also noteworthy that mRNA expression levels in low glucose plus leptomycin B were not different from those observed in the low glucose alone. These observations are consistent with the earlier conclusion that localizing ChREBP to the nucleus is not sufficient for activation.

Glucose-stimulated ChREBP activation requires an event independent of nuclear localization - The inability of the L86A/L93A mutant of ChREBP to support a glucose response suggested that this region is involved in two separate functions, nuclear export and glucose-activation, both of which were inactivated in the mutant. If this is the case, then it should be possible to find other mutations in this region that effect only one or the other of these two functions. Consequently, conserved residues within the NES region were individually mutated and these mutants were tested for nuclear export and transcriptional activation.

Of the ChREBP mutants in this region, several yielded informative results. For example, ChREBP mutants at Thr85 and Leu95 localized predominantly to the cytoplasm in both low and high glucose treated 832/13 cells, similar to wild-type ChREBP (Fig 5a).[2] To ensure that these mutations did not interfere with normal nuclear accumulation, their localization in the presence of leptomycin B was analyzed. Both T85A and L95A ChREBP mutants accumulated in the nucleus by 60 min in low and high glucose conditions (see Supplemental Fig 3). When the function of T85A and L95A ChREBP mutants was tested, however, they did not successfully rescue ChREBP activity (Fig 5b). Thus, the T85A and L95A ChREBP mutants shuttle between the nucleus and cytoplasm, but are transcriptionally inactive, separating nuclear export function from activation. In contrast, mutations at residues Leu89 and Phe90 resulted in nuclear accumulation of ChREBP regardless of glucose treatment (Fig 5a). Both L89A and F90A ChREBP mutants were able to rescue ChREBP activity in high glucose conditions (Fig 5b). It is noteworthy that the F90A ChREBP mutant consistently displayed a two- to three-fold higher increase in high glucose conditions than WT ChREBP. This increased activity of

the F90A mutant was also observed when tested in the context of rat primary hepatocytes (see Supplemental Fig 4). Thus, L89A and F90A ChREBP mutants are nuclear export deficient, but maintain transcriptional activity, again separating the two functions. Together, these data support the conclusion that the NES region of ChREBP plays distinct roles in both nuclear export and activation.

Roles of conserved MCR domains in repression and activation of ChREBP - Previous work from Li et al. (60) used deletion mutagenesis to distinguish two separable functions in the N-terminal segment (amino acids 1-298) of ChREBP. Amino acids 37-196 were found to be responsible for repression of ChREBP activity in low glucose, since deletion of these residues resulted in constitutive activation of ChREBP. This region was termed the LID for low-glucose inhibitory domain. Amino acids 197-298 were found to contain transcriptional activation function that is repressed by the LID in low glucose and was designated GRACE for glucose response activation conserved element. The N-terminal segment of ChREBP contains five highly conserved domains designated MondoA Conserved Regions (MCR) 1-5 based on their striking homology (>90% identity) with the ChREBP paralog, MondoA (Fig 6a). These conserved domains range in size from 14 to 31 amino acids. MCRs 1-4 are located in the LID of ChREBP, whereas MCR5 is found in the GRACE. We were interested in further exploring the role of each of these highly conserved domains.

We have previously shown that deletion of MCR1 alone results in a form of ChREBP that cannot be activated, suggesting that this domain is critical for receiving the glucose signaling event (61). The MCR4 domain contains the Nuclear Localization Signal of ChREBP and a mutation introduced into this region blocked nuclear import and

correspondingly gave an inactive form of ChREBP.[2] The NES function of ChREBP is found in MCR2. However, the mutations in MCR2 described above indicated that this domain is also involved in glucose-dependent transcriptional activation. To evaluate the roles of MCR3 and MCR5 domains, several additional mutants of ChREBP were constructed.

Two mutants in the MCR5 domain gave a particularly interesting phenotype. Residues Tyr275/Glu276/Gly277 (275-277) or Leu289/Gln290/Pro291 (289-291) were mutated to alanines resulting in two separate ChREBP triple mutants. When functionally tested in 832/13 cells, these mutants displayed increased activity in both low and high glucose (Fig 6b). These mutants are found in the nucleus in a greater percentage of cells than wild type ChREBP (Supplemental Fig 5). This result indicates that the MCR5 domain is not an essential part of the trans-activation function of the GRACE region, but instead may be involved in repression of transcriptional activity. The increased activity of the two MCR5 mutants was in fact similar to that observed with the F90A mutation of the MCR2 domain. These results suggested that these two domains may be involved in the same event to repress ChREBP activity. If these two domains act together in a repressive manner, then mutating both regions simultaneously should result in further increase in activity. To address this possibility, the F90A mutation was combined with either the ChREBP mutations at 275-277 or 289-291 and tested functionally. Both combined mutant forms showed a further increase in activity in low and high glucose conditions when compared to the F90, 275-277 and 289-291 ChREBP mutant counterparts (Fig 6a). The increased activity observed with both combined mutants in the low glucose treatment was particularly striking and was synergistic compared to the effects of the individual

mutants (Fig 6c). These data suggest that MCR2 and MCR5 act in a coordinate manner to repress ChREBP activity.

In addition to the mutations made in MCR5, we also targeted the MCR3 domain using site-directed mutagenesis. Mutations were made at residues Asn123, Phe126 and Trp130. When these mutants were tested using the rescue assay, all three failed to support a glucose response (Fig 7a). Hence, this domain appears to play an important role in activation of ChREBP. However, this domain in Wbscr14 and MondoA has previously been shown to interact with 14-3-3 proteins (65,66). It was suggested that the interaction with 14-3-3 might be responsible for the predominantly cytoplasmic localization of ChREBP. We therefore asked whether the MCR3 mutations disrupted the ability of ChREBP to interact with 14-3-3. In co-immunoprecipitation experiments, WT ChREBP did interact with 14-3-3β (Fig 7b). The W130A mutation disrupted the interaction between ChREBP and 14-3-3β, confirming the importance of this region for interaction. This disruption corresponded to a greater localizaton of N130A to the nucleus in both low and high glucose, suggesting that the 14-3-3 interaction may be important for cytoplasmic retention of WT ChREBP (Supplemental Fig 5). However, neither of the other two ChREBP mutants (N123F, F126Q) altered the binding of ChREBP to 14-3-3. Hence, the inactivity of these two MCR3 mutants cannot be attributed to their inability to bind to 14-3-3. These data suggest that the MCR3 domain plays an essential role in supporting glucose activation that is independent of its interaction with 14-3-3 proteins.

DISCUSSION

ChREBP, a glucose responsive transcription factor, plays a critical role in converting excess carbohydrates to triglycerides through *de novo* lipogenesis. While the importance of ChREBP in *de novo* lipogenesis and hepatic energy utilization is strongly supported (36,38,39,67,68), the mechanism driving its activation remains controversial and not fully understood. To address the mechanism of ChREBP activation, we focused on the importance of cellular localization. Under both low and high glucose conditions, ChREBP localized to the cytoplasm in the majority of 832/13 cells. Li *et al* (60) also found that a GFP-fused form of ChREBP was primarily cytoplasmic in these cells. However, leptomycin B treatment trapped ChREBP in the nucleus under either basal or stimulating conditions. These results demonstrate that ChREBP continuously shuttles between the cytoplasmic and nuclear compartments. The ability of ChREBP to transit through the nucleus in low glucose conditions, in which lipogenic gene expression is minimal, suggests nuclear localization is not solely responsible for its activation. However, one potential explanation for activation of ChREBP could be that glucose stimulates a transient increase in the rate of nuclear entry. Indeed, ChREBP nuclear entry was three-fold greater in high glucose conditions than in low glucose, consistent with a modest increase in nuclear localization observed at 2 h (Fig 2a). Previous studies using cell fractionation also reported increased levels of ChREBP in the nucleus acutely after glucose stimulation (39,59,69). Thus, we conclude that glucose regulates the rate of nuclear entry.

Glucose control of ChREBP trafficking could be due to an altered association with a cytoplasmic anchor protein such as 14-3-3. Increased glucose metabolism could reduce

the strength of this interaction, perhaps in response to post-translational modifications of ChREBP (61). Both the human homolog of ChREBP, Wbscr14, and the paralog, MondoA, have been shown to interact with multiple 14-3-3 isoforms (64,65). Disruption of this interaction by mutating the 14-3-3 binding site of these proteins led to increased nuclear localization, as we have also observed for the W130A ChREBP mutant. Alternatively, glucose could regulate nuclear entry by promoting the interaction of ChREBP with its nuclear import receptor. Characterization of the pathway of nuclear translocation will clearly be required to further elucidate this mechanism and its importance to the overall regulation of ChREBP.

Even though glucose regulates the nuclear entry of ChREBP, we cannot conclude that this step is sufficient for its activation. In fact, mutation of two residues within the NES not only trapped ChREBP in the nucleus, it also prevented glucose activation. This result indicates that nuclear localization is not sufficient for activation and that the NES region plays a critical role distinct from its nuclear export function. In addition, trapping ChREBP in the nucleus with leptomycin B did not interfere with induction of target genes. Hence, active shuttling is not required for glucose stimulation. Together, these data indicate that an additional event independent of ChREBP trafficking is required for activation.

If subcellular localization is not the key event in regulating ChREBP activity, then what is the purpose of nucleocytoplasmic shuttling? In fact, many transcription factors actively shuttle under both basal and stimulating conditions. For example, despite nuclear localization being the primary means of controlling their activity, STAT and SMAD transcription factors shuttle in the absence of ligand binding to their activating receptors

(70,71). In the case of SMADs, this shuttling has been suggested to increase the dynamic regulation affecting the duration and magnitude of signaling (72). For STATs, shuttling has been suggested to be important for cytokine sensitivity (73). In the case of ChREBP, it is yet unknown whether the critical cell signal that triggers activation in response to glucose metabolism occurs in the cytoplasm, nucleus or both. However, all of our studies have been carried out under conditions where glucose levels were saturating with respect to activation. Under conditions of intermediate and changing glucose levels that normally occur, nuclear-cytoplasmic shuttling may be important for sensitization of ChREBP to the metabolic status of the cell.

The N-terminal segment of ChREBP (amino acids 1-298) contains five highly conserved domains (MCRs 1-5) and is critical for its ability to respond to glucose (60,61). Deletion of MCRs 1-3 or MCRs 1-4 yields constitutively active forms of ChREBP, indicating that regulation involves repression in basal conditions mediated through this region. In the present study, the behavior of certain mutations in MCR2 and MCR5 suggested a possible mechanism for the repressive event. Mutation of residue F90 in the NES region (MCR2) increased ChREBP activity, while also blocking nuclear export. A similar mutation of residue L89 blocked nuclear export but had normal activity, suggesting that the behavior of the F90 mutant was not simply due to its nuclear accumulation. Interestingly, two independent mutations made in MCR5 (residues 275-277 and 289-291) gave a similar phenotype to the F90 mutation. Thus, we speculate that MCR2 and MCR5 function coordinately to repress ChREBP activation. Repression could result from an intramolecular interaction of MCR2 and MCR5 or from a simultaneous interaction of these domains with an independent repressor. In either case, we would

predict that a combined mutation would further disrupt the repressive interactions of MCR2 and MCR5 and amplify activity. In fact, both combined mutations yielded super active forms of ChREBP. This result was particularly dramatic in low glucose conditions in which a synergistic effect of combining mutations was observed. Thus, MCR2 and MCR5 play a role in the repressive mechanism of ChREBP under basal conditions. It is worth noting that deletion of MCRs 1-5 results in an inactive form of ChREBP, while deletion of MCRs 1-4 yields a constitutively active protein, as previously mentioned. Hence, we also conclude that MCR5 must have a dual role in promoting both inactivating and activating conformations.

While several MCR mutations resulted in increased ChREBP activity, others yielded forms that could not be activated by glucose. These mutations were found in both MCR2 and MCR3. Since deletion of the N-terminal segment that includes MCR2 and MCR3 yielded a constitutively active protein, these observations seem paradoxical. If the only function of the N-terminal region were in repression, then one would expect most point mutations would yield active forms of ChREBP. These data suggest that the relief of repression is not sufficient for activation and a second step involving recruitment of co-activating factors is required. Inactivating mutations in MCR2 and MCR3 could directly or indirectly prevent recruitment of these co-activating factors.

These studies were carried out in 832/13 cells, a β cell-derived line. An important question is whether the mechanism of regulation in 832/13 cells is the same as that in hepatocytes or adipocytes, two major target organs for glucose control. Previous studies have reported increased accumulation of GFP-tagged ChREBP in the nucleus of cultured hepatocytes treated with high glucose (45,46). This accumulation, however, occurs with

relatively slow kinetics (lag of 3 h, half maximal at 5 h) compared to the rapid activation of ChREBP-targeted genes following addition of high glucose. Thus, the importance of increased nuclear accumulation of ChREBP in hepatocytes to activation is uncertain. Because of the high auto-fluorescence of cultured hepatocytes, we have been unable to examine the effects of glucose on nuclear entry and shuttling in these cells. However, mutating Ser196 of ChREBP does not result in increased activity in low glucose conditions despite its proposed role in controlling localization (47,60,74). We have also tested the functional activity of each of the ChREBP mutants used in the present study in primary hepatocytes and found that all behave identically in the two cell types. Based on these observations, we surmise that control of ChREBP activity in response to glucose in the two cells is likely to be similar. While this work was in revision, a manuscript from the laboratory of L. Chan appeared that reached several similar conclusions regarding the regulation of ChREBP. This work, which was also performed in 832/13 cells, showed that MCR2 and 3 were involved in critical functions for activation independent of their role in controlling subcellular localization (75).

In conclusion, glucose regulation of ChREBP involves at least two distinct processes. One event is to accelerate the rate of nuclear entry. This may contribute to rapid effects of ChREBP in stimulating transcription of many metabolic enzyme genes, but is not sufficient for ChREBP activation. In addition, high glucose triggers a second process involving both relief of repression and adoption of an activating form. The MCR2 domain plays a particularly important role in both supporting NES function and in the transition from the repressive to the active state.

FOOTNOTES

*This work was supported by grants from the National Institutes of Health DK26919 and P30 DK50456 (the Minnesota Obesity Center).

[1]The abbreviations used are: ChoRE, carbohydrate response element; ChREBP, carbohydrate response element binding protein; Mlx, Max-like factor X; PKA, protein kinase A; NES, nuclear export signal; WT, wild-type.

[2]Davies, M. N. and Towle, H. C., unpublished results

ACKNOWLEDGEMENTS

The assistance of Eric Refsland in preparing adenoviruses and the University of Minnesota Biomedical Image & Processing Laboratory in confocal microscopy is greatly appreciated. We thank Drs. Nikolas Tsatsos, Do-Hyung Kim and Douglas Mashek for helpful comments in the preparation of this manuscript.

Fig 1. **ChREBP shuttles between the cytoplasm and nucleus under both low and high glucose conditions.** *a.* 832/13 cells were co-transfected with expression plasmids for FLAG-tagged ChREBP and Mlx overnight in 11 mM glucose and subsequently incubated in RPMI media containing 2.5 mM (low) glucose for 4 h. Cells were then continued in the same media or switched to RPMI media containing 25 mM (high) glucose for 2 h. Immunofluorescence was performed using an anti-FLAG primary antibody and a FITC-conjugated secondary antibody and images were obtained by confocal microscopy. FITC immunoflourescence represents ChREBP localization and is shown in green. Nuclei were stained with TO-PRO3 and are shown in blue. The panels labeled FITC represent ChREBP localization, while the panels labeled FITC+Nuclei show both ChREBP localization and nuclear staining. *b.* Cells were treated as described above except that leptomycin B was added simultaneously with the glucose treatment for 2 h.

Figure 1

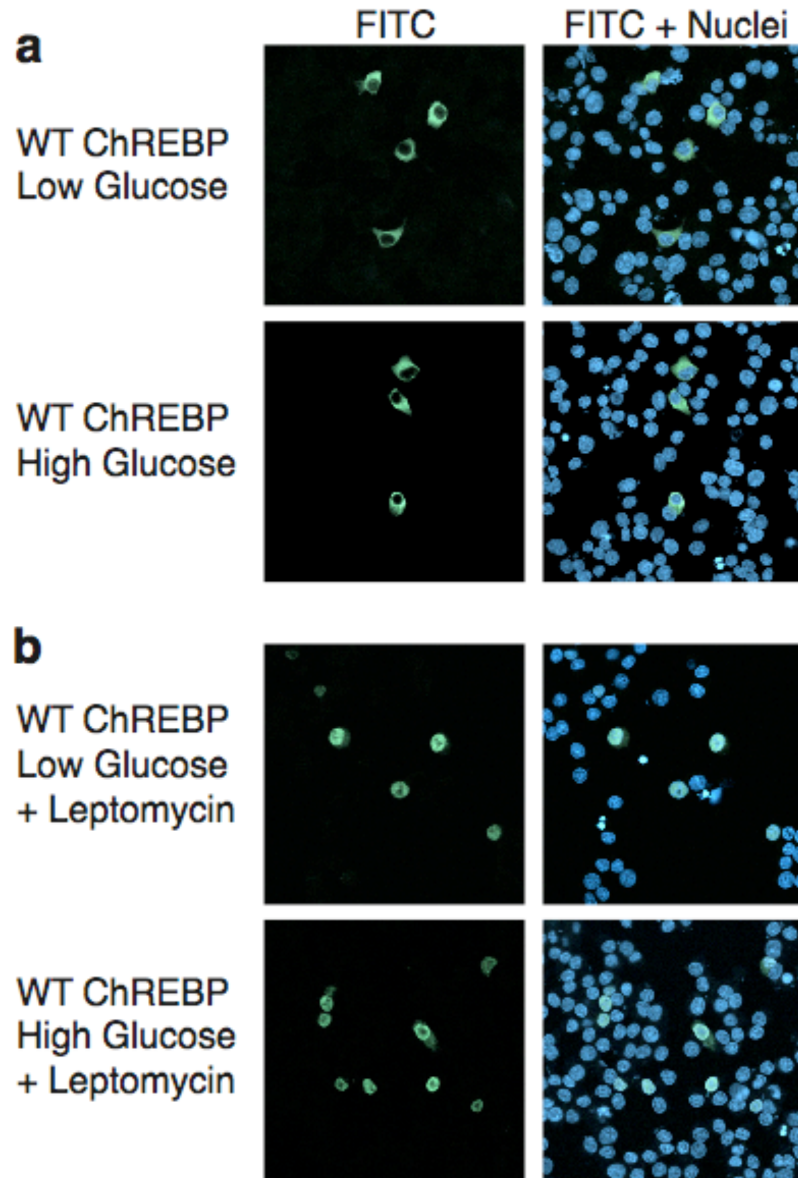
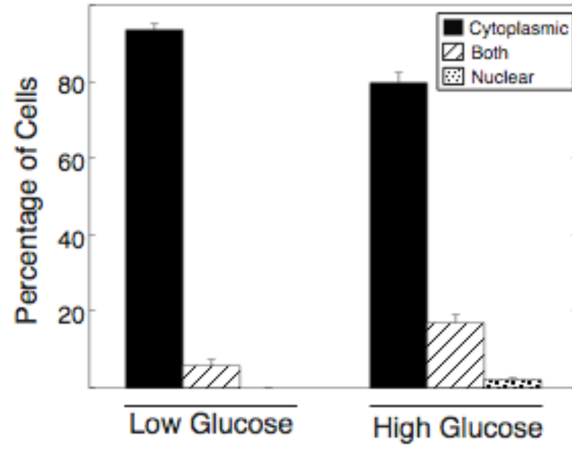


Fig. 2 **The rate of ChREBP nuclear entry is increased in high glucose conditions.** *a.* 832/13 cells were co-transfected with FLAG-tagged ChREBP and Mlx overnight in RPMI media containing 11mM glucose and were then incubated in low glucose media for 4 h. Cells were then treated with either low or high glucose for 2 h and immunofluorescence was performed as described in Experimental Procedures. Over 100 cells in each treatment were scored as predominantly cytoplasmic, both cytoplasmic and nuclear or predominantly nuclear by two observers. Results represent the means (±SEM) for three experiments. *b.* Cells were treated with low (open triangles) or high glucose (closed squares) media with the addition of leptomycin B at various time points as indicated. Immunofluorescence and quantification was performed as described above. Cells displaying either predominantly nuclear or both nuclear and cytoplasmic localization were combined and expressed as a percentage of the total cells.

Figure 2

a



b

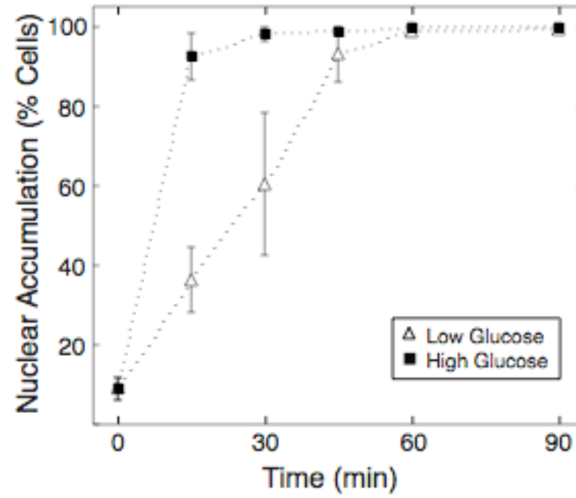
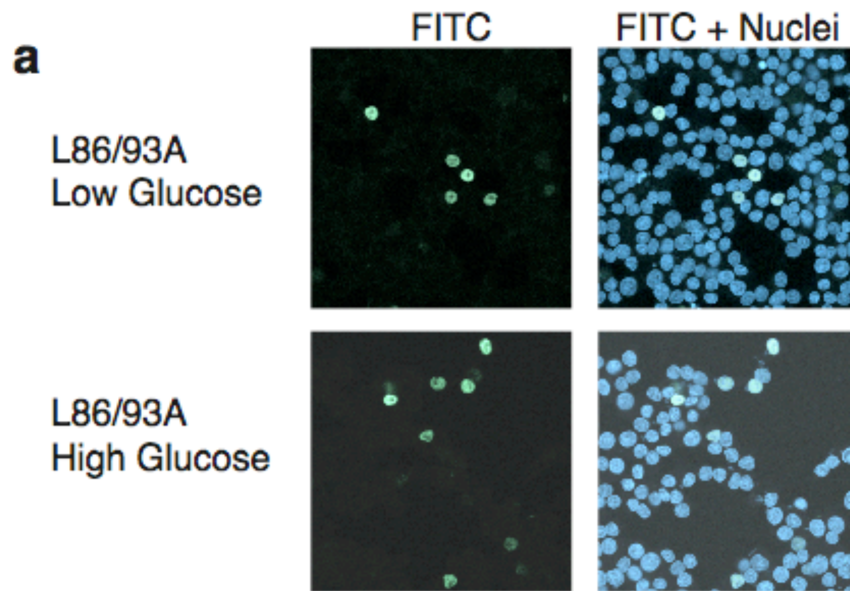


Fig 3. **The L86A/L93A mutant of ChREBP is trapped in the nucleus, but is transcriptionally inactive.** *a.* Immunofluorescence images of 832/13 cells co-transfected with FLAG-tagged L86A/L93A ChREBP mutant and Mlx in low and high glucose conditions are shown. See legend to Fig 1 for details. *b.* A functional assay for ChREBP activity was performed in 832/13 cells. 832/13 cells were transduced with an adenoviral vector expressing dnChREBP. Cells were then co-transfected with a luciferase reporter plasmid containing two copies of the ACC ChoRE, a *Renilla* luciferase reporter and expression plasmids for either WT ChREBP or the L86A/L93A ChREBP mutant and WT Mlx. After 18 h, cells were treated with low or high glucose for 24 h and extracts were prepared. Values are relative light units (firefly/*Renilla*) and represent the means (±SD) for triplicate samples.

Figure 3



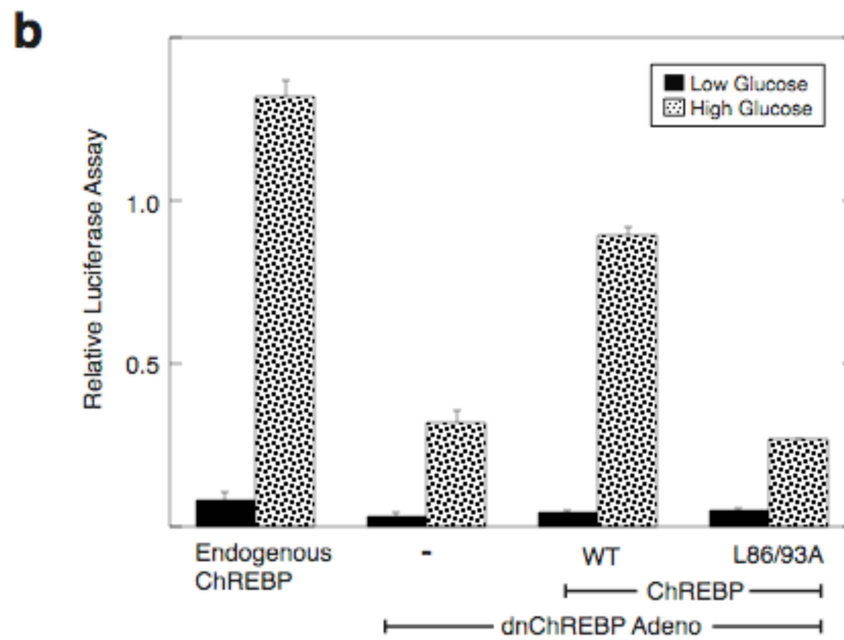


Fig 4. **Leptomycin B does not inhibit the induction of ChREBP target genes in 832/13 cells.** 832/13 cells were pretreated in low glucose RPMI media for 4 h with or without leptomycin B for the last 90 min. Cells from both experimental groups were then incubated with low or high glucose for 4 h. RNA was isolated and converted into cDNA using reverse transcriptase. mRNA levels of ChREBP target genes, thioredoxin-binding protein (Txnip) and aldolase B (AldoB), were measured by quantitative RT-PCR. mRNA levels in low glucose without the addition of leptomycin B were set to 1 and all values are normalized to this group. Values represent the mean (±SEM) of triplicate samples.

Figure 4

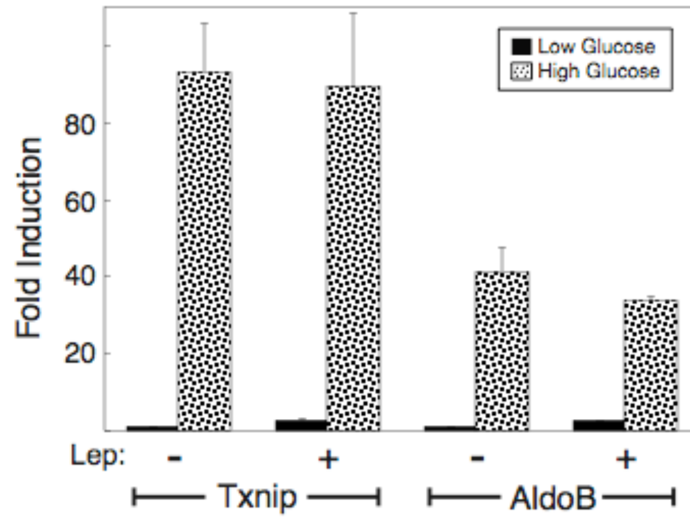


Fig 5. **NES region mutants of ChREBP separate NES function from transcriptional activation.** *a*. Immunofluorescence images of 832/13 cells transfected with FLAG-tagged L89A, F90A and L95A ChREBP mutants in low and high glucose conditions are shown. See legend to Fig 1 for details. *b*. Functional activities of ChREBP mutants were tested using the reporter assay as described in the legend to Fig 3b. Values are shown as relative light units (firefly/*Renilla*) and represent the means (±SD) for triplicate samples.

Figure 5

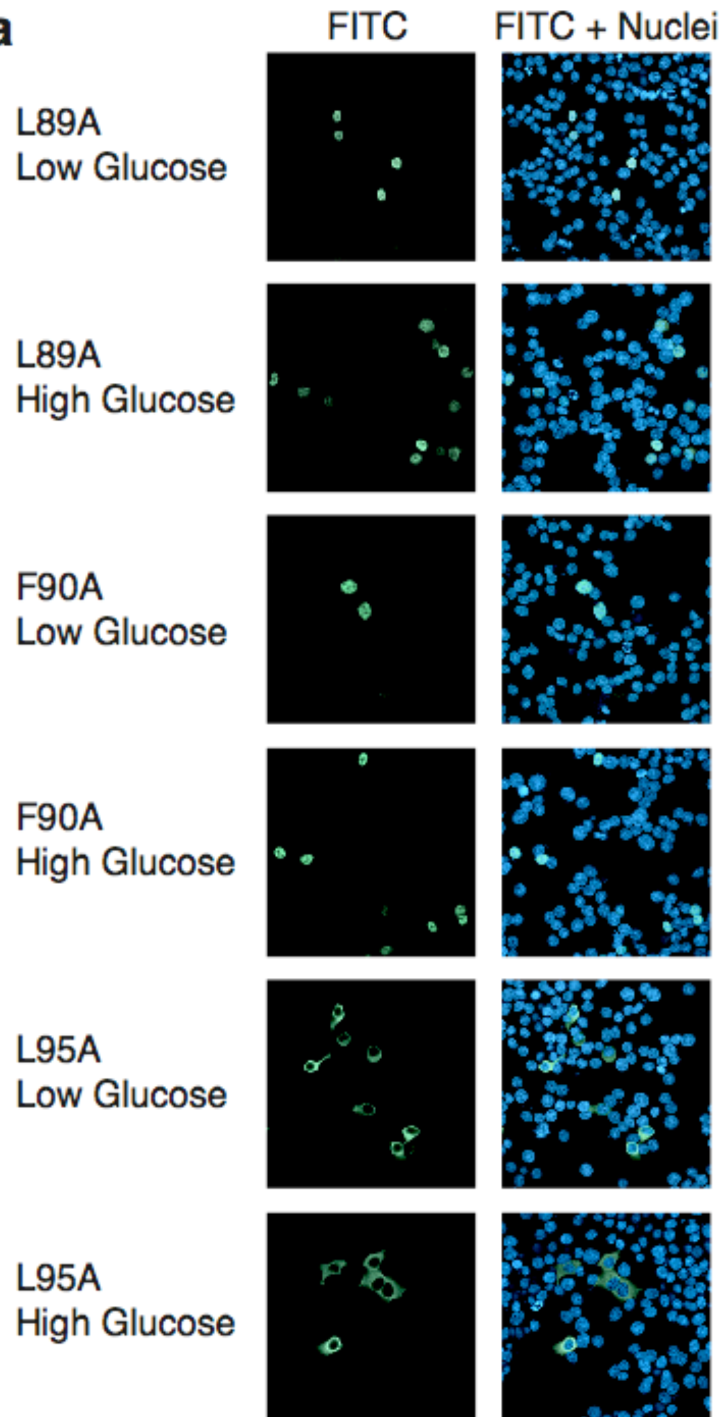


Figure 5

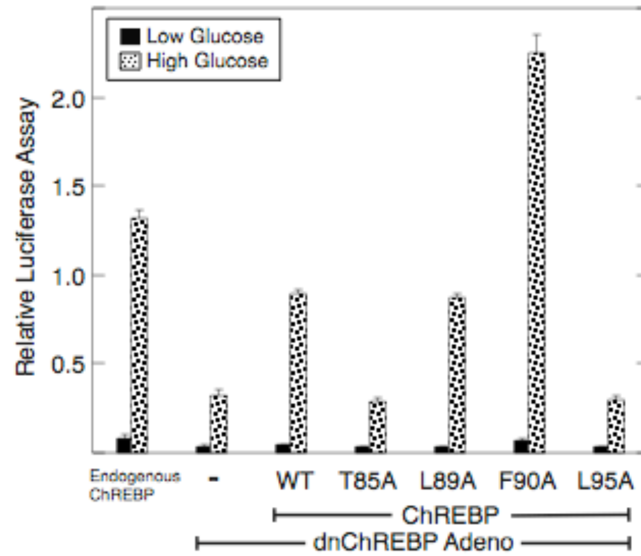


Fig 6 **MCR5 and MCR2/MCR5 mutants of ChREBP display increased transcriptional activity.** *a.* Conserved regions in the amino-terminal segment of ChREBP. The locations of five highly conserved regions in the amino-terminal region of ChREBP are indicated. Numbering is based on mouse ChREBP. These regions are highly conserved (>90% identity) with the ChREBP paralog, MondoA, as well as with ChREBP orthologs from pufferfish to human. Previously proposed functions for these conserved domains are indicated below. GSD = Glucose Sensing Domain; NLS = nuclear localization signal; LID = low-glucose inhibitory domain; GRACE = glucose response activation conserved element. *b.* Functional activity of ChREBP was tested using the rescue assay described in the legend to Fig 3b. WT, F90A, Y275A/E276A/G277A (275-277), L289A/Q290A/P291A (289-291), F90A/275-277 and F90A/289-291 were introduced and tested for activity. Values are shown as relative light units (firefly/*Renilla*) and represent means (±SD) for triplicate samples. *c.* Luciferase values measured in low glucose conditions of the mutants above are compared. The data presented in this panel is from the experiment shown in panel *b*. * = $p<0.05$ compared to WT ChREBP; ** = $p<0.01$ compared to WT ChREBP.

Figure 6

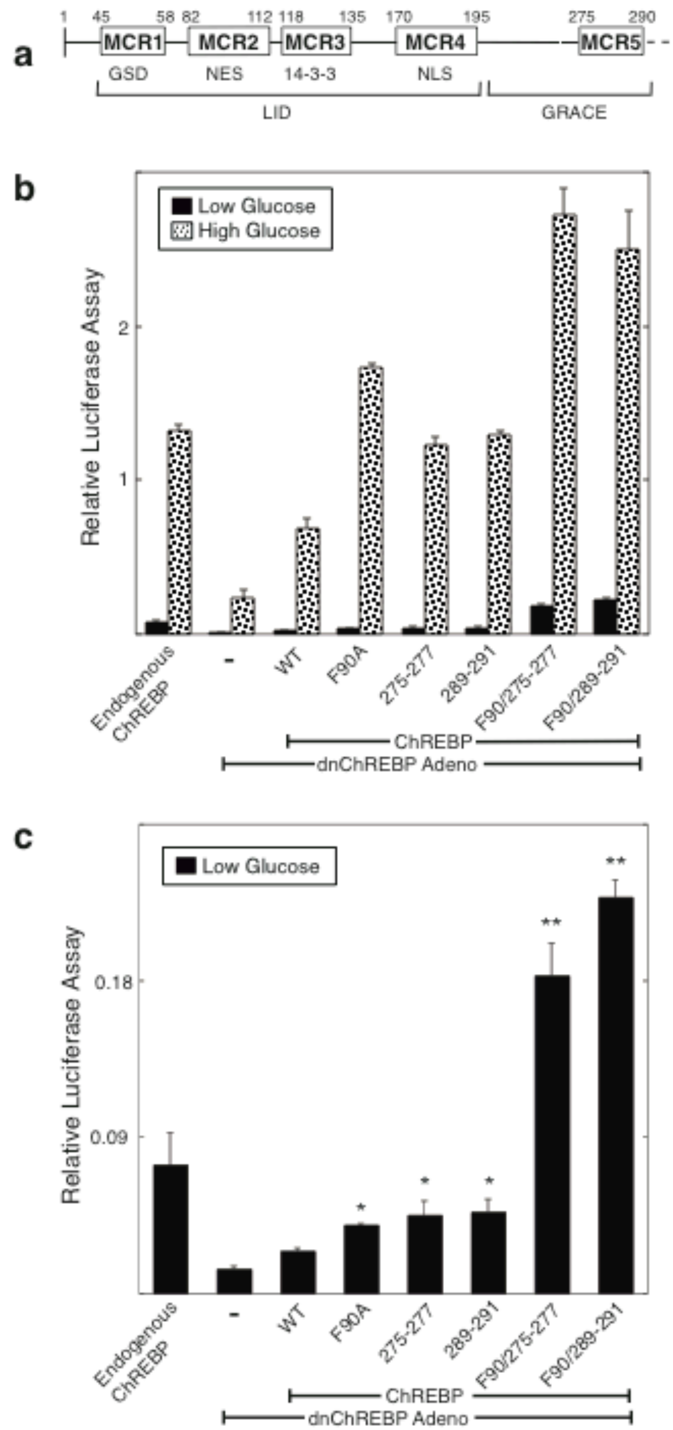


Fig 7. **MCR3 mutants of ChREBP inhibit glucose activation.** *a.* Functional activity of ChREBP was tested using the reporter assay described in the legend to Fig 3b. WT ChREBP or ChREBP mutants N123F, F126Q, and W130A were introduced and tested for activity. Values are shown as relative light units (firefly/*Renilla*) and represent the means (±SD) for triplicate samples. *b.* 832/13 cells were co-transfected with FLAG-tagged WT or mutant ChREBP and Mlx and incubated overnight in RPMI media with 11 mM glucose. Cell extracts were prepared and co-immunoprecipitations performed with anti-FLAG immunobeads as described in Experimental Procedures. Immunoblotting was carried out with a 14-3-3β antibody. Lane 1 represents extracts from untransfected 832/13 cells. Lanes 2-5 represent cells transfected with WT ChREBP and MCR3 mutations N123F, F126Q, and W130A, respectively. Lane 6 is an aliquot of the cell extract without co-immunoprecipitation. The arrow indicates the position of 14-3-3β protein and the asterisk indicates a background band that cross-reacts with the 14-3-3β antibody.

Figure 7

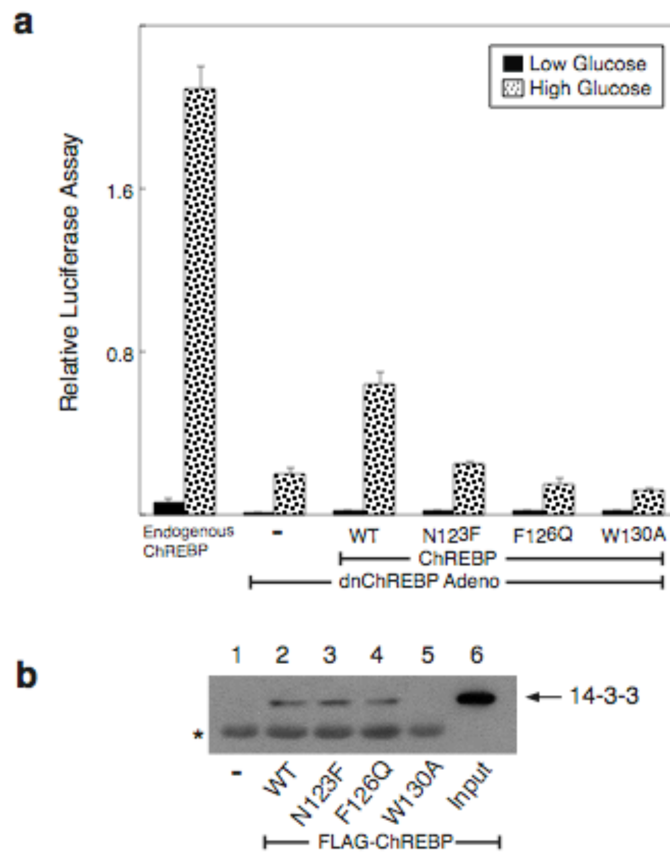


Fig S1. **Mlx is required for nuclear import of ChREBP in 832/13 cells.** 832/13 cells were transfected with an expression plasmid for FLAG-tagged ChREBP overnight and subsequently incubated in RPMI media containing 2.5 mM (low) glucose for 4 h. Leptomycin B (3.6 μM) was added to cells and incubation was continued for 2 h in 2.5 mM glucose or in 25 mM (high) glucose. Immunofluorescence was performed using an anti-FLAG primary antibody and a FITC-conjugated secondary antibody. Nuclei were stained with TO-PRO3 and images were obtained on a confocal microscope. Note that in both low and high glucose ChREBP remains predominantly cytoplasmic even in the presence of leptomycin B. This is in contrast with the results shown in Fig 1b, in which ChREBP is localized to the nucleus after treatment with leptomycin B in cells co-transfected with both ChREBP and Mlx. From this result, we conclude that Mlx is required for nuclear localization of ChREBP.

Figure S1

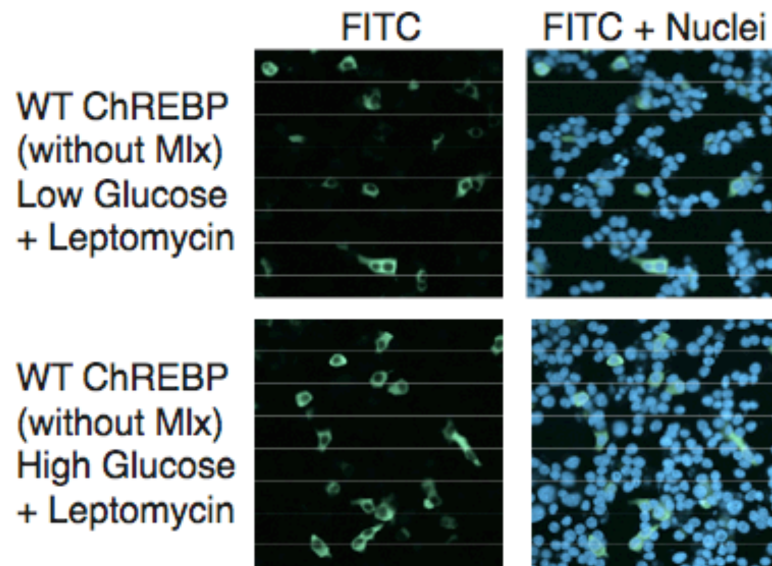


Fig S2. **Expression of various ChREBP mutants.** HEK293 cells were transfected with an expression plasmid for FLAG-tagged ChREBP (wild-type or mutant) (1.6 µg). Cells were incubated for 24 h and then cellular extracts were prepared as described previously (29). Aliquots from each cell extract were subjected to SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes. ChREBP was detected using a monoclonal antibody to FLAG and a secondary horseradish peroxidase-conjugated anti-mouse IgG antibody (Santa Cruz). ChREBP ran at approximately 95 kDa, as previously observed (29). *a.* ChREBP mutant tested in Fig 3. *b.* ChREBP mutants tested in Fig 5. *c.* ChREBP mutants tested in Fig 6. *d.* ChREBP mutants tested in Fig 7.

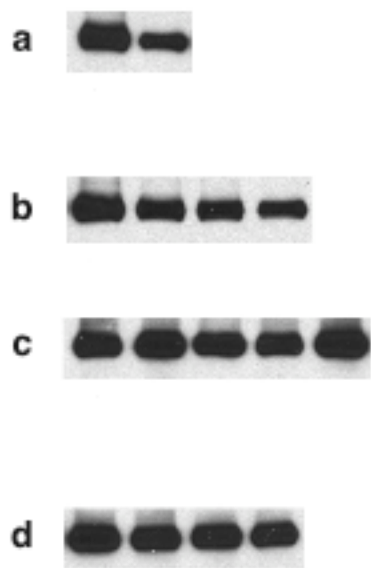


Figure S2
a
b
c
d

Fig S3. **Rate of nuclear entry of ChREBP mutants T85A and L95A.** 832/13 cells were co-transfected with expression plasmids for FLAG-tagged ChREBP mutants and Mlx overnight. Cells were treated and counted as described in legend to Fig 2. Note that both of these mutant ChREBP forms show similar kinetics of nuclear accumulation in low glucose conditions, reaching half-maximal in approximately 25 to 30 min. In high glucose, both forms show a modest reduction in nuclear accumulation at 15 min, but by 30 to 45 min these mutants are observed in the nucleus of transfected cells. Since functional assays are carried out for 24 h, it is unlikely that this delay at early time points would account for the lack of activity of these mutants.

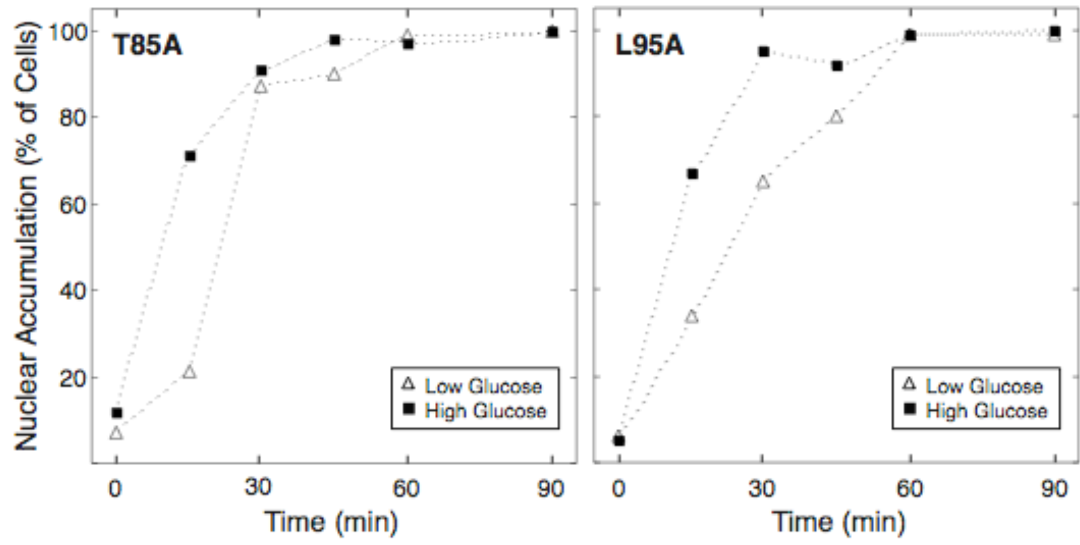

T85A
L95A
Nuclear Accumulation (% of Cells)
100
80
60
40
20
0
30
60
90
Time (min)
Low Glucose
High Glucose

Fig S4. **Functional activity of several ChREBP mutants tested in primary hepatocytes.** To test whether the phenotype of ChREBP mutants observed in 832/13 cells was cell-specific, we tested several mutants using the same rescue assay in primary rat hepatocytes, as described previously (29). Note that the activity of all mutants followed a similar pattern to that observed in 832/13 cells. The combined F90A/275-277 and F90A/289-291 mutants did not show an additive effect in high glucose conditions, perhaps because the transcriptional response has been maximized in these cells. However, the synergistic effect of these combined mutations in low glucose conditions is evident.

Figure S4

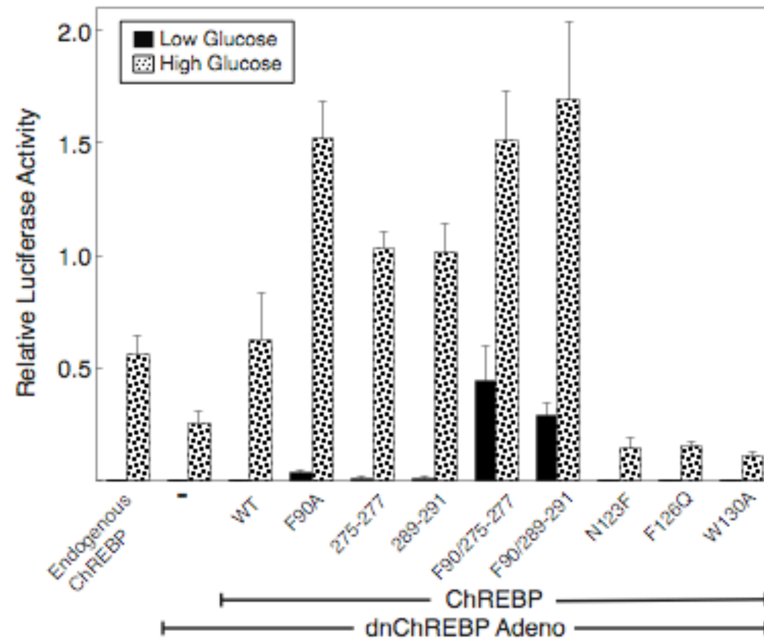
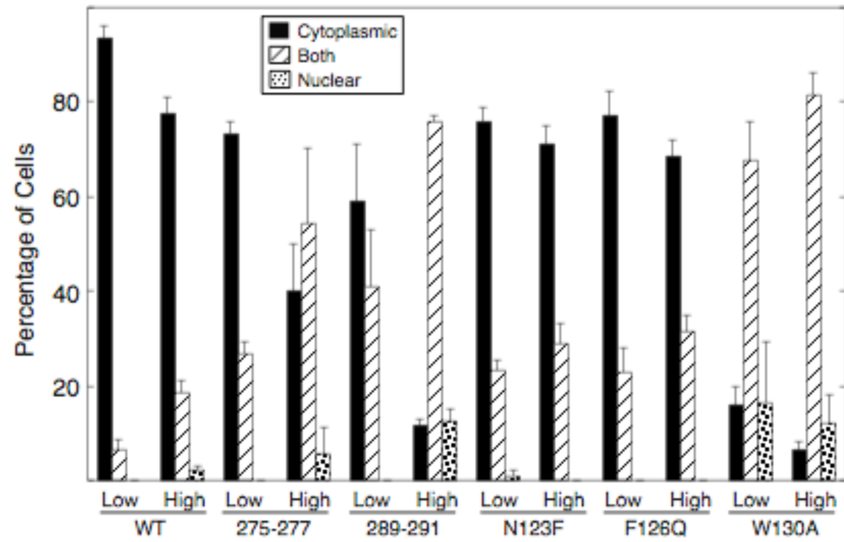


Fig S5. **Cellular distribution of MCR3 and MCR5 domain mutants.** 832/13 cells were co-transfected with FLAG-tagged ChREBP mutants and Mlx and cells were treated as described in the legend to Fig 1. The distribution of FLAG-tagged ChREBP was scored as either predominantly cytoplasmic, both cytoplasmic and nuclear or predominantly nuclear after incubation in low or high glucose for 2 h. Means (±SEM) from two independent experiments, each was scored by two observers, are displayed. Note that mutants 275-277 and 289-291 have higher proportion of cells with nuclear staining, especially in high glucose conditions. The basis of the increased nuclear accumulation is not known, but has been observed previously for other mutations that caused increased transcriptional activity (29). Also, mutant W130A shows a much higher localization to the nucleus than wild type ChREBP in either low or high glucose conditions. This mutation causes a disruption of the interaction between ChREBP and 14-3-3, which may account for this effect.

Figure S5:



CHAPTER 3

ACTIVATION AND REPRESSION OF GLUCOSE-STIMULATED CHREBP REQUIRES THE CONCERTED ACTION OF MULTIPLE DOMAINS WITHIN THE MONDOA CONSERVED REGION

This Chapter is currently in preparation for submission

Michael N. Davies, Brennon L. O'Callaghan and Howard C. Towle

Carbohydrate Response Element Binding Protein (ChREBP) is a glucose-dependent transcription factor that stimulates the expression of lipogenic genes in mammals. Glucose regulation of ChREBP has been mapped to its conserved N-terminal region of 300 amino acids, designated the MondoA Conserved Region (MCR). Within the MCR, five domains (MCRs 1-5) have a particularly high level of conservation and are likely to be important for glucose regulation. We carried out a large-scale deletion and substitution mutational analysis of the MCR domain of ChREBP. This analysis revealed that MCRs 1-4 function in a concerted fashion to repress ChREBP activity in basal (non-stimulatory) conditions. Deletion of the entire MCR 1-4 segment or combining four specific point mutations (Quad mutant) throughout this region lead to a highly active, glucose-independent form of ChREBP. However, deletion of any individual MCR domain and the majority of point mutations throughout MCRs 1-4 rendered ChREBP inactive. These observations suggest that MCRs 1-4 interact with a factor required for activation and that this interaction occurs after repression is relieved. This possibility is supported by the observation that the MCR 1-4 region can compete for activity with wild type ChREBP and the derepressed Quad mutant in both basal and stimulatory conditions. In contrast, mutations in the MCR 5 domain result in increased activity, suggesting that this domain may be the target of intramolecular repression in basal conditions. Thus, the MCR domains act in a complex and coordinated manner to regulate ChREBP activity in response to glucose.

Introduction

Mammals have evolved highly regulated mechanisms to adapt to their dynamic nutritional states. For example, when dietary carbohydrates are in excess, they are converted to triglycerides in the liver by the *de novo* lipogenic pathway for energy storage. Expression of lipogenic genes, such as L-type pyruvate kinase (L-PK), acetyl-CoA carboxylase (ACC), fatty acid synthase and stearoyl-CoA desaturase are up-regulated in response to increased carbohydrate levels to promote this process (10). Increased transcription of lipogenic enzymes occurs in response to increased glucose metabolism and is dictated in large part by the Carbohydrate Response Element (ChoRE), a DNA regulatory element found within the promoter regions of lipogenic genes (37). A ChoRE is composed of two consensus CACGTG E-boxes separated by 5 bases. Each E-box motif is recognized by a heterodimer consisting of Carbohydrate Response Element Binding Protein (ChREBP) and Max-like factor X (Mlx) (31,32). ChREBP is a member of the basic helix-loop-helix zipper (bHLH/LZ) family of transcription factors that is expressed at highest levels in the liver and adipose tissue (36). Mlx is a ubiquitously expressed bHLH/LZ factor that serves as a DNA binding partner of ChREBP (33,76).

Of the two heterodimer partners, ChREBP is the component that responds to increased glucose metabolism (47). It shows sequence conservation with its paralog, MondoA, a glucose-dependent transcription factor predominantly expressed in the skeletal muscle (43,44). In particular, the first 300 amino acids of ChREBP and MondoA share a remarkably high level of conservation. As such, the N-terminal segment of ChREBP has been termed the MondoA Conserved Region (MCR). Deletion of the MCR of ChREBP or MondoA completely disrupts the glucose response, resulting in

constitutive activity (44,60,61). Thus, this region is critical for glucose regulation and acts in a repressive manner in basal conditions. Within the N-terminal segment of ChREBP, five smaller regions, designated MCRs 1-5, ranging from 14 to 33 amino acid residues have nearly complete conservation with MondoA. MCR 2 contains a well-characterized nuclear export sequence (NES) and is responsible for the nucleocytoplasmic shuttling of ChREBP that occurs in both basal and stimulatory conditions (65,77,78). MCR 3 contains a binding site for the 14-3-3 protein family (65,78). Binding of 14-3-3 may be involved in the nuclear shuttling of ChREBP (75,79). However, certain mutations of MCR3 that do not disrupt 14-3-3 binding are inactive, suggesting an additional role of this domain in glucose activation (75,77). A putative nuclear localization sequence (NLS) has been identified in the MCR 4 region (45). This finding led to the hypothesis that glucose regulation of ChREBP function occurs by controlling its intracellular localization through phosphorylation of Ser196, adjacent to the putative NLS. Two additional PKA-dependent phospho-acceptor sites were identified near the DNA binding domain of ChREBP at Ser626 and Thr666. Thus, it was proposed that phosphorylation of these residues in low glucose conditions inhibited DNA binding and caused cytoplasmic retention of ChREBP. It was further postulated that its dephosphorylation was mediated by a xylulose-5-phosphate-stimulated protein phosphatase 2A isoform in response to elevated glucose metabolism, resulting in nuclear localization and DNA binding (45,46). However, ChREBP mutations that block phosphorylation at the proposed regulatory sites still require glucose for transcriptional activity (47,59,60). Furthermore, ChREBP mutations of the NES region that result in its nuclear accumulation under basal conditions are also dependent on increased glucose for

activation (77). Hence, we concluded that glucose regulation of ChREBP requires an additional event beyond nuclear localization. Mutations have also been identified in the MCR 2 region that do not disrupt NES function, but block glucose activation (77). Therefore, like MCR 3, the MCR 2 domain plays an additional role beyond nuclear export in the process of activation. No specific functions have been ascribed to either MCR 1 or MCR 5 regions.

Li et al. (75) have recently shown that the MCR 4 region is necessary and sufficient for repression of ChREBP activity in basal conditions. This conclusion was based on a deletional analysis of MCR domains of ChREBP and functional testing of these deletion mutants in the context of a Gal4 DNA binding domain (DBD) fusion protein. These authors found that only deletions including MCR4 were capable of relieving the repression of ChREBP activity observed in low glucose. A caveat of this study was that ChREBP function was evaluated in the context of the Gal4 DBD, thus bypassing any influence of ChREBP DNA binding or nucleocytoplasmic shuttling on the activation process.

In this study, we have performed a comprehensive analysis of the N-terminal segment of ChREBP, using both deletion and substitution mutagenesis, to evaluate functions of individual MCRs. ChREBP mutants were tested in the context of its natural DNA binding domain. These analyses revealed that multiple MCR regions (MCRs 1-4) in the N-terminal region function coordinately to mediate repression in basal conditions and relief of repression upon glucose stimulation. Activation of ChREBP requires an additional factor that interacts with the MCR 1-4 region.

Materials and Methods

Construction of mutant ChREBP plasmids- Site-specific mutations of ChREBP were constructed with the QuickChange^{Tm} site-directed mutagenesis kit (Stratagene) using mouse FLAG-tagged ChREBP (zeta isoform) in the expression plasmid CMV4 (32). All mutations and coding sequences were confirmed by DNA sequencing. To ensure that each construct was expressed, plasmids were transfected with HEK 293 cells and 24 h later, whole cell extracts were prepared. Western blotting was performed using anti-FLAG monoclonal antibody (Sigma). Amino acid substitutions at the following residues were constructed and tested for analysis: S49F, H51A, H51F, F52A, S55A, S56D, H58F, T85A, L86A, F90A, E91A, L95A, YSG97-99AAA, K105A, N108A, N123F, F126Q, W130A, LQG149-151AAA, L149A, E166A, E169A, W184A, YYK197-189AAA, KR190-191AA, YVG275-277AAA, N278A, IQP283-285AAA, Q285A, L286A, LQP289-291AAA, D294A.

Deletion mutants of ChREBP were constructed with PCR techniques using the Pfu Ultra II polymerase (Stratagene) and mouse FLAG-tagged ChREBP in the expression plasmid CMV4. Coding sequences and expression levels were confirmed in the same manner as the amino acid substitutions. The ChREBP deletion mutants constructed and tested were: ΔMCR 1 (Δ1-58), ΔMCR 2 (Δ86-114), ΔMCR 3 (Δ116-136), ΔMCR 4 (Δ141-197), ΔMCR 5 (Δ277-294), ΔMCR 1-2 (Δ1-114), ΔMCR 1-3 (Δ1-136), ΔMCR 1-4 (Δ1-197), ΔMCR 2-4 (Δ85-197) and MCR 3-4 (Δ116-197).

Construction of ChREBP competition plasmids- Various regions from the N-terminal segment of ChREBP were inserted into the pEYFP-C1 expression vector to form a Yellow Flourescent Protein-ChREBP fusion protein (YFP ChREBP). A fragment of

ChREBP containing MCRs 1-4 (aa 45-197) and MCR 5 (219-304) were PCR amplified using Pfu Ultra II polymerase with a 5' Bgl II site and a 3' Hind III site engineered into the amplifying primers. A double digest of the PCR product and the pEYP-C1 expression vector was performed using Bgl II and Hind III restriction enzymes. The digested MCR 1-4 and MCR 5 PCR products was then inserted into the pEYP-C1 expression vector by ligation (Roche). Oligonucleotides for each individual MCR domain 1-4 were synthesized. The upper oligonucleotide for each MCR was engineered with a 5' Bgl II site overhang and the lower oligonucleotide contained a 5' Hind III site overhang. Insertion into the pEYFP-C1 expression vector was performed as described above. The YFP ChREBP fusion proteins constructed are as follows: YFP MCR 1 (aa 45-58), YFP MCR 2 (81-114), YFP MCR 3 (115-135), YFP MCR 4 (166-192) and YFP MCR 5 (219-304), YFP MCR 1-4 (45-197).

Functional reporter gene assays- 832/13 cells (a gift from Dr. C. Newgard, Duke University) were cultured in RPMI media containing 11 mM glucose in 24-well plates as described (63). Once cells reached 70% confluence, they were transduced with a dominant negative ChREBP (dn ChREBP) expressing adenovirus for 2 h to inhibit endogenous ChREBP activity (61). The amount of dn ChREBP needed to inhibit endogenous ChREBP activity in high glucose by approximately 90% was determined empirically. After transduction for 2 h, cells were transfected using Lipofectamine LTX (Invitrogen) with 400 ng of a reporter plasmid containing the firefly luciferase gene fused to a promoter region consisting of 2 copies of the ACC ChoRE (47). The transfection mixture, where indicated, also contains 100 ng of an expression plasmid containing either wild type ChREBP or the various ChREBP mutants constructed. A control plasmid

containing the *Renilla* luciferase gene (15 ng pRL-CMV, Promega) was also included. After 18 h of transfection, cells were cultured in RPMI media containing either low (2.5 mM) glucose or high (25 mM) glucose for 24 h. Cellular lysates were prepared in Passive lysis buffer (Promega) and dual luciferase assays were performed following manufacturer's instructions (Promega). Values represent the ratio of firefly/*Renilla* luciferase from triplicate samples and are expressed as mean (+SD). All experiments were repeated at least three times with comparable results.

Competition Assay- 832/13 cells were transfected with elements of the Gal4 reporter gene system (60) using Lipofectamine LTX (Invitrogen). The transfection mixture consisted of 100 ng of an expression plasmid expressing a Gal4 DNA binding domain-ChREBP fusion protein (Gal4-ChREBP) or the Gal4-ChREBP Quad mutant, 200 ng of a reporter gene consisting of a five copy Gal4 Response Element driving firefly luciferase gene and 15 ng of the control plasmid containing the *Renilla* luciferase gene. The rest of the mixture contained various combinations of the YFP expression plasmid and the plasmids containing YFP MCR 1-4 ChREBP or the other individual YFP MCR fragments. Total transfected DNA remained constant at 615 ng. YFP ChREBP fragments were transfected in increments of 50 ng, 100 ng, 200 ng and 300 ng. The YFP expression plasmid without ChREBP was added to keep the total amount of DNA constant. After 18 h of transfection, cells were cultured with RPMI media containing low or high glucose conditions for 24 h and assayed for luciferase activity as described above. Values represent the ratio of firefly/*Renilla* luciferase from multiple experiments normalized to ChREBP control in high glucose and are expressed as the mean (+SEM) of 9 to 12 samples.

Immunolocalization of ChREBP- INS-1 cells were cultured in RPMI media containing 11 mM glucose on glass slides and grown to 70% confluence (61). Cells were cotransfected with 150 ng of each Flag-tag ChREBP or the various Flag-tag ChREBP mutants with HA-tagged Mlx using Lipofectamine LTX (Invitrogen) and 1.1 μl Virofect (Targeting Systems, San Diego, CA). After overnight transfection, cells were refed with RPMI media containing 2.5 mM glucose for 4 h. Cell were then cultured in either low or high glucose for 2 h. Where indicated Leptomycin B was added at 3.6 μM. Fixing and immunoflourescence techniques were performed as described (77).

Elctrophoretic Mobility Shift Assay (EMSA)- EMSAs were performed as described previously (21). HEK 293 cells were cultured in DMEM to 70% confluence and transfected with equal molar ratios of wild type ChREBP or the various ChREBP deletion mutants and wild type Mlx using Lipofectamine LTX (Invitrogen). Whole cell extracts were made with the Active Motif Cellular Extraction Kit (Carlsbad, CA). The DNA probe was labeled with ^{32}P-dCTP using the large fragment (Klenow) of DNA polymerase (New England Biolabs). The DNA-Protein binding reaction consisted of 10 μg of total protein from the cellular extracts and 100,000 cpm of ^{32}P-labled oligonucleotide.

Results

Repression of ChREBP in basal conditions involves multiple residues that span the N-terminal segment of ChREBP **-** Previous studies have demonstrated that the N-terminal segment of ChREBP containing the highly conserved MCR 1-5 regions is responsible for glucose regulation of its transcriptional activity (60,61). Notably, deletion of the region containing MCRs 1-4 from a Gal4-ChREBP fusion protein yielded a constitutively active transcription factor (60,61). This observation suggested that ChREBP activity is repressed under basal (low glucose) conditions and that this repression requires the MCR 1-4 region. Although this experiment provides a dramatic result, its interpretation requires some caution due to the use of a Gal4 fusion protein in which DNA binding was directed by a heterologous factor and in which large regions of ChREBP were removed. As an alternative approach, we have examined the behavior of amino acid substitution mutations of full-length ChREBP. We have previously identified several activating mutations within the N-terminal segment of ChREBP (S56D and F90A) (77) and more recently found others at His51 (H51A) and Asn278 (N278A). The activity of each of these ChREBP mutants was measured in INS-1 cells using a previously described functional assay in which endogenous ChREBP is inhibited by introduction of a dominant negative ChREBP mutant (77). The ability of exogenously expressed, amino acid substitution mutants to restore the glucose response of a ChoRE-containing reporter plasmid was monitored and compared to the rescue provided by wild type ChREBP. Each of the activating mutations caused an increase in transcriptional activity in both low (1.6- to 3.2-fold) and high (3.2- to 5.2-fold) glucose, but still retained a strong response to

glucose (Fig 1A). We reasoned that each individual mutation may not be sufficient to completely disrupt the repressive complex that inhibits ChREBP activity and hence would still retain a substantial response to glucose. If this were the case, then combining the individual activating mutations should be more effective and potentially result in constitutive activation. In fact, combining individual point mutations successively enhanced ChREBP activity, particularly in low glucose conditions (Figure 1B). A mutant containing all four activating mutations at His51, Ser56, Phe90 and Asn278 (Quad mutant) displayed potent transcriptional activity that was not affected by glucose. This mutant showed activity that was increased 10-fold in high glucose and over 250-fold in low glucose compared to wild type ChREBP. These results support the model that ChREBP is repressed under low glucose and furthermore, demonstrate that multiple residues spanning the N-terminal segment (MCRs 1, 2 and 5) are involved in this repression. The dramatic and unregulated activity of the Quad mutant resembles the phenotype observed with the MCR 1-4 deletion mutant (60,77), and suggests that the activity of wild type ChREBP does not reach its maximal potential in high glucose conditions.

Maximal repression in basal conditions requires the coordinate action of multiple MCRs - The results from the previous section suggest that repression of ChREBP in basal conditions requires a region of ChREBP spanning MCRs 1-5. However, a report by Li et al. suggested that the MCR 4 region is necessary and sufficient for ChREBP repression (75). To address these conflicting results, we performed a deletional analysis of the N-terminal segment of ChREBP. However, instead of using the Gal4-ChREBP fusion proteins as previously reported (75), we tested the function of these mutants with a rescue

assay in the context of binding to a natural ChoRE. Deletion of MCRs 1-4 of ChREBP resulted in constitutive activation, a similar phenotype to that observed with the ΔMCR 1-4 Gal4-ChREBP fusion protein (Fig 2). If the MCR 4 domain is sufficient for repression of ChREBP in basal conditions, then all deletion mutants that retain the MCR 4 domain should remain inactive in basal condition. However, the basal activity of the ΔMCR 1-3 ChREBP deletion mutant, which contains the MCR 4 region, was significantly higher than that of wild type ChREBP (Fig 2, Inset). In fact, the basal activity of ΔMCR 1-3 was comparable to its activity in high glucose conditions. Thus, the MCR 4 domain is not sufficient for repression of ChREBP activity in basal conditions.

We also examined the phenotype of several other mutants in which different combinations of MCRs 1-4 were deleted. Deletion of MCRs 3-4 resulted in a phenotype similar to ΔMCR 1-3: increased activity in basal conditions and no significant activation by glucose. Additionally, deletion of MCRs 2-4 was constitutively active. However, the MCR 2-4 deletion mutant had overall activity levels dramatically higher than that of ΔMCR 3-4 and ΔMCR 1-3. Alternatively, deletion of MCRs 1-2 did not result in increased basal activity. This result, in conjunction with ΔMCR 3-4, suggests that MCRs 3 and 4 are the minimal regions for repression. However, when comparing the activities of the ΔMCR 1-4 and ΔMCR 2-4 to the rest of the deletion mutants, deletion of the entire MCR segment resulted in the most highly active mutant. These results suggest that each MCR contributes to the overall repression of ChREBP and that the individual MCR 1-4 domains function in a coordinate manner to regulate ChREBP activity in response to glucose.

In addition to the multiple MCR deletions, individual MCR deletion mutants were constructed. If MCR 4 is the critical domain for repression, then deletion of this domain should result in increased activity in basal conditions. However, the ΔMCR 4 mutant failed to rescue ChREBP activity in either low or high glucose (Fig 3A). In fact, none of the deletion mutants of MCR 1, MCR2, MCR3 or MCR4 was capable of activating transcription despite ΔMCR 1-4 being constitutively active. Each mutant was tested to ensure that it was expressed at comparable levels (Fig 3C), bound to DNA in an EMSA (Fig 3B) and was capable of localizing in the nucleus in the presence of leptomycin B (data not shown). No defects were detected in any of these functions. We therefore conclude that each MCR region must contribute to ChREBP activity in the context of the full length protein and that the MCR 1-4 region is critical for glucose-stimulated activation in addition to its repressive role in basal conditions.

The majority of amino acid substitutions within the N-terminal segment of ChREBP result in inactivation - The remarkable contrast between the inactivity of individual MCR region deletions and the constitutive, high level activity of the combined deletion of MCRs 1-4 was surprising. Given the potential disruptive nature of deletion mutations to the overall protein conformation, we wanted to confirm these results using a more subtle approach. Consequently, we analyzed the effects of introducing amino acid substitution mutations throughout the MCR 1-5 region. Residues that showed high conservation between multiple species and MondoA were chosen for mutation. Individual mutations were tested for activity in the rescue assay and the results are summarized in Figure 4. Each arrow represents a single amino acid substitution mutant located at that particular residue. Upward arrows represent mutations that increased activity in both low and high

glucose conditions and have been discussed previously. Downward arrows represent inactive mutations. The predominant phenotype of mutations throughout MCR 1-4 was inactivating. The expression and localization patterns were tested for each mutant and were comparable to WT ChREBP (data not shown). Hence, these individual point mutations phenocopy the individual MCR region deletions in being unable to support ChREBP activity in low or high glucose. These mutants likewise contrast dramatically with the high level of the constitutively active MCR 1-4 deletion. As mentioned in the previous section, these data suggest that the MCR 1-4 region has a dual role in the regulation of ChREBP that involves both repression and activation. The range of inactivating mutations observed over a region spanning 200 amino acids additionally suggests that activation of ChREBP requires the coordination of multiple MCRs for activation.

In contrast, point mutations in the MCR5 region behaved quite differently. In this region, most mutations yielded increased activity of ChREBP as observed with the N278A mutation (see Fig 1). Hence, the role of MCR 5 appears to be distinct from that played by MCR 1-4. One possibility is that this region represents the target for repression in low glucose conditions through an intramolecular interaction with MCR 1-4 or an extragenic repressor. If this were the case, then the individual mutations in this region would weaken the repressive interaction to increase activity.

The N-terminal segment of ChREBP competes for the activation of ChREBP – Given that most of the point mutations and the individual MCR domain deletions were inactive, we hypothesized that the N-terminal region interacts with a factor necessary for ChREBP activity. To test this hypothesis, we performed a competition assay in which increasing

amounts of a truncated version of ChREBP containing MCRs 1-4 were transfected into INS-1 cells. If MCRs 1-4 interact with a factor required for activation, then increasing the amount of the MCR 1-4 fragment should result in a decrease in ChREBP activation. This experiment was performed using a Gal4 reporter gene system in which we co-transfected a Gal4-ChREBP fusion protein with a reporter gene consisting of 5 copies of the Gal4 Response Element fused to the firefly luciferase gene. In support of our hypothesis, increasing amounts of the MCR 1-4 fragment decreased Gal4-ChREBP activity in a dose-dependent manner in high glucose conditions (Fig 5). Additionally, expressing the MCR 1-4 fragment did not increase Gal4-ChREBP activity in low glucose conditions, as would be expected if a repressor bound to this region. These results suggest that the N-terminal region of ChREBP interacts with a co-regulatory factor(s) required for activation and does not interact with a repressor, which supports a model for intramolecular repression that was initially proposed by Li et al. (60).

In an attempt to determine which MCRs are responsible for this interaction(s), we constructed individual MCR competing fragments and tested their ability to compete for Gal4-ChREBP activation. MCR 3 slightly competed for Gal4-ChREBP activation (Fig 6), but not to same extent as MCR 1-4. However, MCR 1, MCR 2, MCR 4 and MCR 5 did not compete with Gal4-ChREBP for activation. Thus, we conclude that individual MCRs are not sufficient for interacting with a co-regulatory factor. These observations are consistent with a model in which activation of ChREBP requires coordination of multiple MCRs within the N-terminal segment of ChREBP.

Two potential mechanisms can be envisioned for the role of MCRs 1-4 and its interaction with a co-regulatory factor. In one mechanism, this factor could be

responsible for relief of the repressive intramolecular interaction involving MCRs 1-4. If mutations within the N-terminal region of ChREBP prevent this interaction, the repressive complex will remain intact and activation will not occur. Alternatively, relief of repression could occur through an interaction with a signaling molecule, resulting in a conformational change that allows for binding to co-activators required for transcriptional activation. Mutations that disrupt binding to transcriptional co-activators would also prevent activation. To distinguish between these two possibilities, a competition assay was performed in which the ability of the MCR 1-4 fragment to compete against the constitutively active Gal4-Quad mutant was tested. The mutations in the Quad mutant have presumably disrupted the intramolecular repressive interaction. If the role of the co-regulatory factor is to relieve the intramolecular repressive complex of ChREBP upon glucose stimulation, then increasing the amount of MCR 1-4 fragment in a dose-dependent manner should not affect the activity of the Gal4-Quad mutant. However, a decrease in Gal4-Quad mutant activity occurred in low and high glucose conditions when concentrations of the MCR 1-4 fragment were increased (Fig 7). This result suggests that the MCR 1-4 segment of ChREBP is interacting with a co-regulatory protein that is required for transcriptional activation and that this event is distinct from relief of repression.

Discussion

It is well established that ChREBP is a glucose-dependent transcription factor that is critical for the induction of lipogenic genes in the liver and beta cells (36,37,59). However, the molecular mechanism driving ChREBP activation is controversial. Recent work from our laboratory (77) and that of L. Chan (60,75) suggested that ChREBP is negatively regulated. It was postulated that in basal conditions, the transcriptional activity is repressed due to an intramolecular interaction involving the N-terminal MCR region and upon increased glucose metabolism this repression is reversed. Thus, the purpose of this study was to further evaluate this model and the mechanism of ChREBP activation.

The N-terminal segment of ChREBP was first implicated in repression by the observation that deletion of MCRs 1-4 in the context of Gal4-ChREBP resulted in a high level of activity in both low and high glucose conditions (60,61). This result suggested that the N-terminal segment of CHREBP is responsible for this repression, either through interaction with a repressor or through an intramolecular interaction that prevents DNA binding and co-activator recruitment. Thus, removing the domain of ChREBP critical for this repressive interaction led to an unregulated activator. We have found that introducing four specific point mutations at residues His51, Ser56, Phe90 and Asn278 that span the N-terminal region of ChREBP resulted in a similar phenotype. We postulate that these mutations disrupt the repressive interaction of ChREBP, resulting in a conformation similar to that found in activated wild type ChREBP. These mutated residues were located in MCRs 1, 2 and 5 of the N-terminal region of ChREBP, suggesting that

repression involves multiple domains within this region. It should be noted that the MCR 4 region containing the putative NLS and the PKA-dependent phospho-acceptor sites at Ser196, Ser626 and Thr666 are intact in the Quad mutant. Thus, the constitutive activation of the Quad mutant demonstrates that repression of ChREBP is not due to controlling its nuclear localization and DNA binding through phosphorylation of these residues, as proposed (45). It is also noteworthy that the activity of either the MCR 1-4 deletion or the Quad mutant are considerably higher than that observed with the wild type ChREBP in high glucose conditions. This observation implies that wild type ChREBP activity does not reach maximal levels in high glucose conditions in our experimental conditions. One possible explanation is that other signaling pathways could act in parallel with glucose to further stimulate ChREBP activity under appropriate physiological conditions. Alternatively, nuclear retention of the mutant forms could promote increased duration of action over the time of reporter gene accumulation. In this case, the nucleocytoplasmic shuttling of wild type ChREBP would serve to limit the extent of transcriptional enhancement and provide a more dynamic response to changing nutritional conditions.

In an effort to map the role of individual MCR domains in the repression of ChREBP, we constructed a number of deletion mutants containing varying combinations of MCRs 1-4. Each of these MCR domains appears to play a significant role in repression. For example, deletion of MCRs 3-4 resulted in glucose-independent activity, suggesting these domains are required for repression of basal activity and that MCRs 1-2 are not sufficient. However, increasing the deleted region to include MCR 2 (ΔMCR 2-4) or MCRs 1 and 2 (ΔMCR1-4) greatly increased the activity of the corresponding

ChREBP mutants. Furthermore, a deletion of MCRs 1-3, which only retains the MCR 4 domain, displays a similar constitutively active phenotype as ΔMCR 3-4. This result is strikingly different from a recent paper by Li et al. (75) that suggested that the MCR 4 domain is necessary and sufficient for repression. The basis for this difference is not understood, but may reflect the different contexts in which the deletions were constructed (i.e., Gal4 fusions in the Li et al. study versus the context of natural ChREBP in this study). We propose that the individual MCR domains are not acting independently, but rather act in a concerted manner to form the repressive conformation of ChREBP. In support of this concept, it is noteworthy that not only are the sequences of individual MCR domains conserved, but that the spacing between each MCR domain is highly conserved in both ChREBP and MondoA. This conclusion is also consistent with the Quad mutant in which mutations of residues throughout the N-terminal region contribute to the constitutively active phenotype. In the Quad mutant, the MCR 3 and 4 domains are unaltered, providing additional evidence indicating that no individual MCR domain is sufficient to repress ChREBP activity in basal conditions.

If the MCR 1-4 domains act in a concerted manner, then deletion of any individual domain should disrupt the repressive interaction and result in increased basal activity. However, in striking contrast, each individual MCR deletion was inactive. The loss of function observed with each individual MCR deletion mutants suggests that these domains have an additional role critical for glucose-stimulated activation. Consistent with this result, the great majority of single amino acid substitution mutations within the MCR 1-4 domain are inactivating and quite distinct from the phenotype of the MCR 1-4 deletion or Quad mutant. In contrast, the majority of mutations within the MCR 5 domain

result in increased activity levels in both low and high glucose conditions. These results suggest that MCR 5 is acting in a distinct manner from MCRs 1-4 and may be the target of repression in basal conditions. However, a simple model in which glucose activation involves release of a repressor from ChREBP cannot explain these results. If this were the case, the majority of mutations throughout the N-terminal segment should yield an activating phenotype. Instead, these results suggest that the MCR 1-4 region plays a second, distinct role in the process of glucose activation. For example, this region could interact with a modifying enzyme in response to glucose and mutations that disrupt this interaction should prevent activation. Alternatively, if the MCR 1-4 region functions as an allosteric binding site for a glucose metabolite, then mutations that disrupted this site would prevent activation. In either case, these results support a more complex role for the MCR 1-4 region of ChREBP.

A potential concern of these experiments was that deletions of large regions of ChREBP, in particular MCR 4, would disrupt nuclear translocation and thus indirectly lead to inactivity. It was proposed by Sakiyama et al. (79) using computational sequence analysis that the MCR 4 domain contains a putative nuclear localization signal (NLS) that is critical for nuclear translocation of ChREBP. In vitro analysis has shown direct binding of importin α and β to ChREBP, but no experiments were done to demonstrate this interaction occurs through the putative NLS in MCR 4 (79). Due to the constitutive activity of the MCR 1-4 deletion of ChREBP, it appears that this region is not essential for nuclear import. Furthermore, immunoflourescence images of Leptomycin B-treated INS-1 cells show nuclear accumulation of all deletion mutants constructed in both low and high glucose conditions, including the MCR 4 deletion (data not shown). Thus, we

conclude that nuclear import of ChREBP can be mediated by an NLS located outside of the MCR 1-4 region. Whether the putative NLS in the MCR 4 region also functions as an NLS remains to be established.

To test whether regulation of ChREBP through the MCR 1-4 domain requires interaction with an additional factor, a competition experiment was performed with the N-terminal segment of ChREBP. Expressing the MCR 1-4 fragment decreased ChREBP activity in a dose-dependent manner in high glucose. In contrast, no increase in ChREBP activity was observed in low glucose, indicating that the N-terminal region does not interact with a repressor in these conditions. This result supports a model for intramolecular repression which was originally proposed by Li et al. (60). Due to the competition observed in high glucose, we conclude activation requires a distinct factor that interacts with the MCR 1-4 region of ChREBP. Competing fragments that included individual MCRs 1, 2, 3 and 4 fragments did not compete with ChREBP activity to the same extent as the combined MCR 1-4 fragment, supporting the previous observation that regulation requires the coordinated action of multiple MCRs within the N-terminal segment. In a previous study, it was reported that the MCR 3 region alone could compete for ChREBP activation (75). Although we observe partial competition with this fragment, it is much less effective than the MCR 1-4 region.

Two potential functions of the MCR 1-4 interacting factor could be envisioned. In one, the role of this factor is to relieve repression upon glucose stimulation. In the second, upon relief of repression by a glucose metabolite, this factor could be a co-activator required for transcriptional activation. To distinguish between these two possibilities, a competition experiment was performed with the constitutively active Quad mutant. The

point mutations introduced into the Quad mutant presumably disrupted the intramolecular interaction that represses activity in basal conditions. Hence, if the role of this factor is simply to relieve repression, then the MCR 1-4 fragment should not compete for activity of this mutant. However, competition was observed with the Quad mutant in both low and high glucose. These data suggest that the MCR 1-4 interacting co-regulatory protein plays a role in transcriptional activation in a step subsequent to glucose-dependent relief of repression. Thus, we propose a model in which the MCR 1-4 domain plays a role in two distinct steps in the activation of ChREBP (Fig 8). In this model, repression of ChREBP activity in basal conditions occurs through an intramolecular interaction that involves MCRs 1-5. MCRs 1-4 are essential for this repressive complex and appear to target MCR 5. Upon increased glucose metabolism, repression is relieved through an interaction with a signaling molecule that promotes a conformation to allow for binding of a co-regulatory protein critical for activation. The interaction with the putative co-regulatory protein is an event distinct from glucose-dependent relief of repression and is essential for activation of ChREBP.

Figure 1: Combining activating mutations in the N-terminal segment of ChREBP. INS-1 cells were transduced with a dn ChREBP expressing adenovirus for 2 h. After transduction, cells were co-transfected with an expression plasmid containing either Flag-tag wild type ChREBP or various ChREBP mutants and a reporter gene containing two copies of the ACC ChoRE fused to the firefly luciferase gene. Transfection was performed overnight in RPMI media containing 11 mM glucose. Cells were then treated with media containing low (2.5 mM, open bars) or high (25 mM, filled bars) glucose for the following 24 h. After 24 h, extracts were prepared and firefly luciferase levels were measured. *Renilla* luciferase was included in the co-transfection and used as an internal control. Values are shown as relative light units (firefly*/Renilla)* and represent the means (+SD) for triplicate samples. A, Activity of single mutations with increased activity compared to wild type ChREBP. B, Activity of mutants with various combinations of activating mutants C, Western blotting with an anti-Flag antibody performed to detect expression of Flag-tag wild type ChREBP and various ChREBP mutants.

Figure 1:

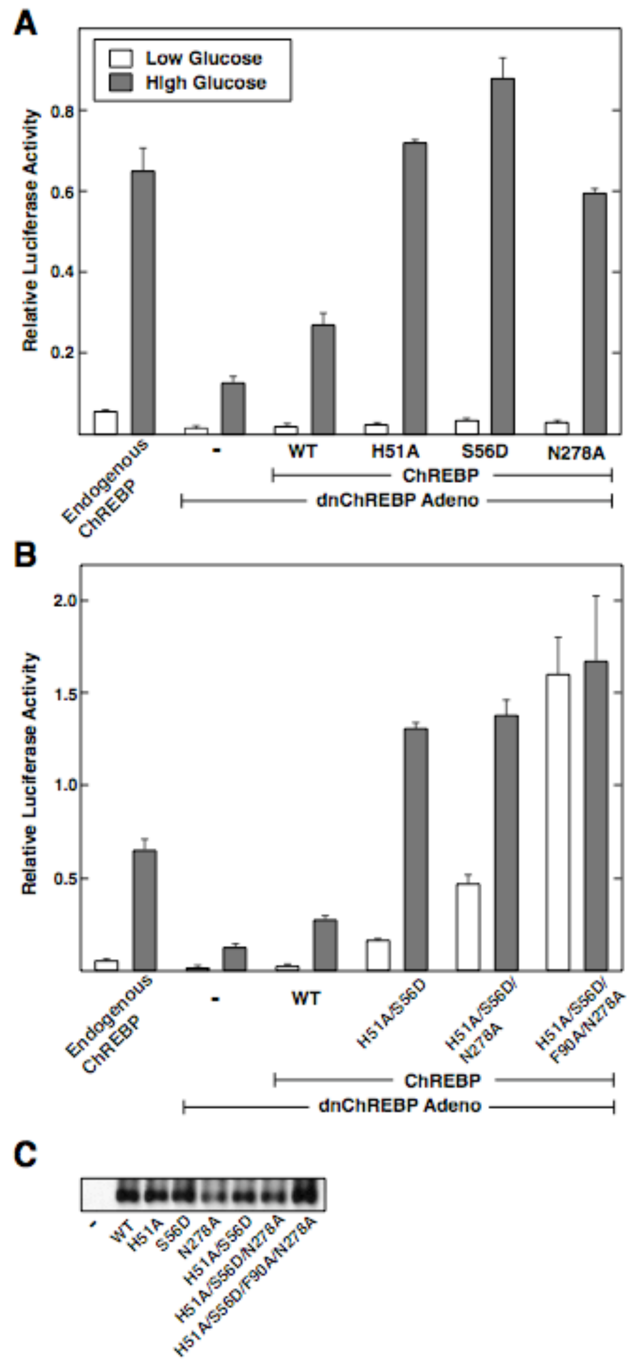


Figure 2: Deletion of multiple MCR domains result in constitutive activation.

Function of deletion mutants was tested using the rescue assay described in the legend to Figure 1. Cells were treated with media containing low (2.5 mM, open bars) or high (25 mM, filled bars) glucose for 24 h. Values are shown as relative light units (firefly/*Renilla)* and represent the means (+SD) for triplicate samples. Inset is a replot of data omitting ΔMCR 2-4 and ΔMCR 1-4. Western blotting demonstrated that all mutants were expressed comparably to WT ChREBP, except ΔMCR 1-3 and ΔMCR 1-4. These latter mutants were expressed at lower levels, but were active in the functional assay.

Figure 2:

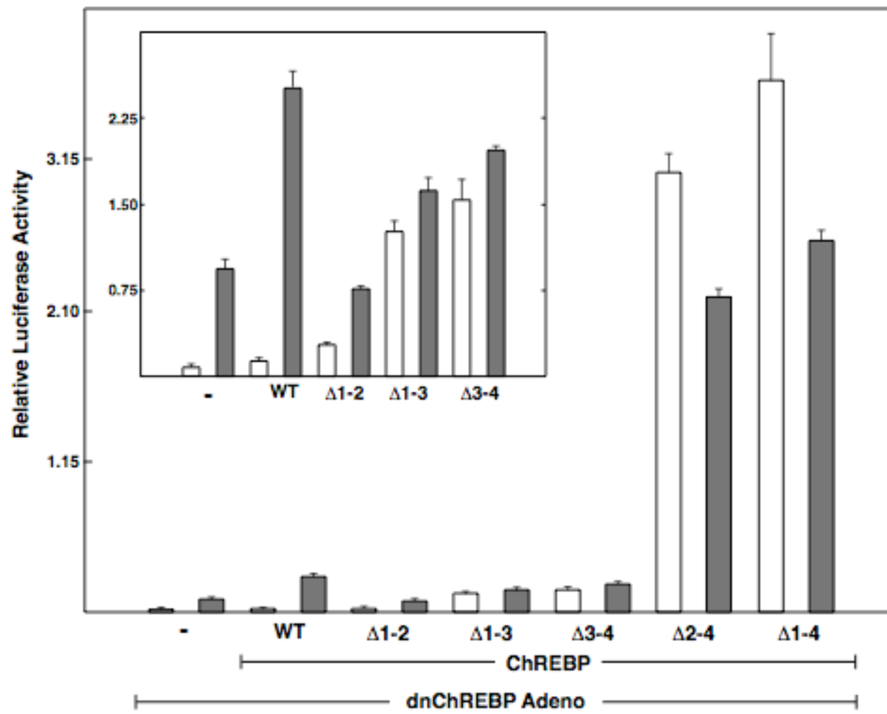


Figure 3: Deletions of individual MCRs result in inactivation of ChREBP. A, Function of deletion mutants of individual MCR domains was tested using the rescue assay described in the legend to Figure 1. Cells were treated with media containing low (2.5 mM, open bars) or high (25 mM, filled bars) glucose for 24 h. Values are shown as relative light units (firefly*/Renilla)* and represent the means (+SD) for triplicate samples. B, EMSA was performed using extracts of HEK 293 cells transfected with various ChREBP deletion mutants and an ACC ChoRE probe. The band adjacent to the star represents a background band. The bands adjacent to the right-pointing arrow represents the gel shift observed with wild type ChREBP and the ChREBP deletion mutants. C, Western blotting of the extracts used for the EMSA was performed using an anti-Flag antibody.

Figure 3:

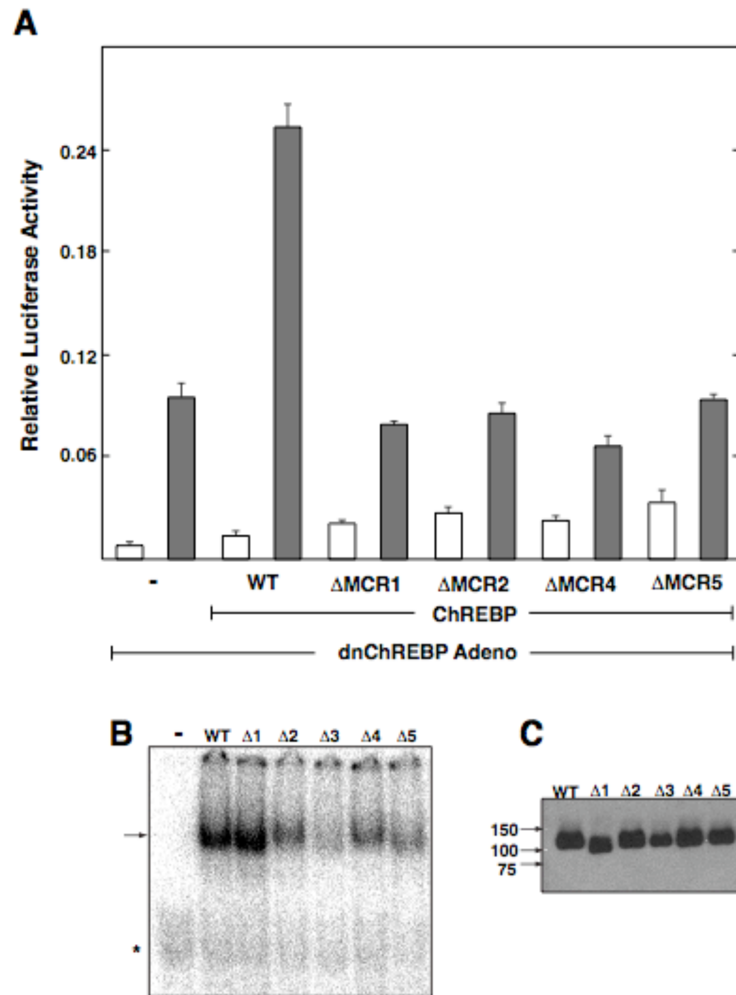


Figure 4: Effect of amino acid substitutions spanning MCRs 1-5 on the functional activity of ChREBP.

A, Schematic of the amino acid substitutions of the N-terminal segment of ChREBP. Upward arrows represent mutants that have increased activity in low and high glucose compared to wild type ChREBP. Downward arrows represent inactive mutants. The function of mutants was tested in INS-1 cells using the rescue assay described in the legend to Figure 1. Individual mutations are listed in Materials and Methods. B, A representative functional assay of single mutants from each MCR domain. Cells were treated with media containing low (2.5 mM, open bars) or high (25 mM, filled bars) glucose for 24 h. Values are shown as relative light units (firefly/*Renilla)* and represent the means (+SD) for triplicate samples. C, Expression of the Flag-tagged mutants in panel B from HEK 293 transfected cells was measured by Western blotting as described in the legend to Figure 1.

Figure 4:

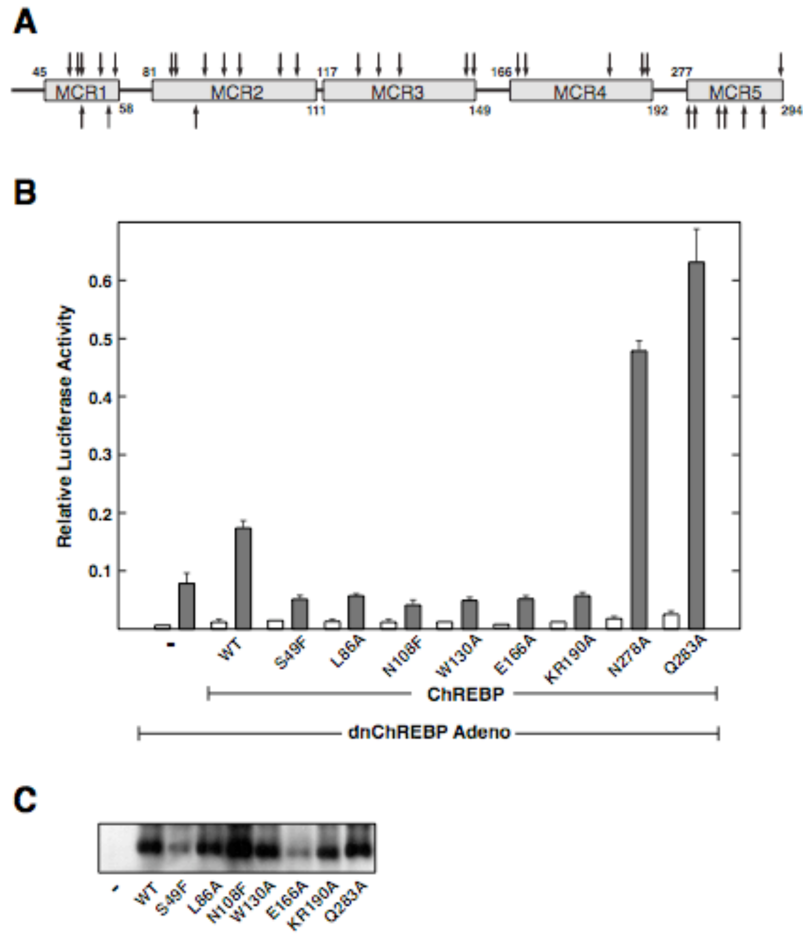


Figure 5: Increasing amounts of the MCR 1-4 fragment decrease ChREBP activity in a dose-dependent manner. INS-1 cells were co-transfected with an expression plasmid containing Gal4-ChREBP and a reporter gene plasmid consisting of the firefly luciferase gene fused to a promoter region containing five copies of the Gal4 response element. INS-1 cells were also transfected with increasing amounts of YFP MCR 1-4 competitor fragment. Total DNA concentrations were the kept constant for each test point by balancing total DNA with a plasmid expressing only YFP. Cells were transfected overnight in RPMI media containing 11 mM glucose. After approximately 18 h, cells were treated with low or high glucose for 24 h. The open circles represent low glucose and the closed circles represent high glucose. Values are shown as relative light units (firefly/*Renilla)* normalized to the activity of cells treated with no competitor and high glucose and represent the means (+SEM) for 8-12 samples from four separate experiments.

Figure 5:

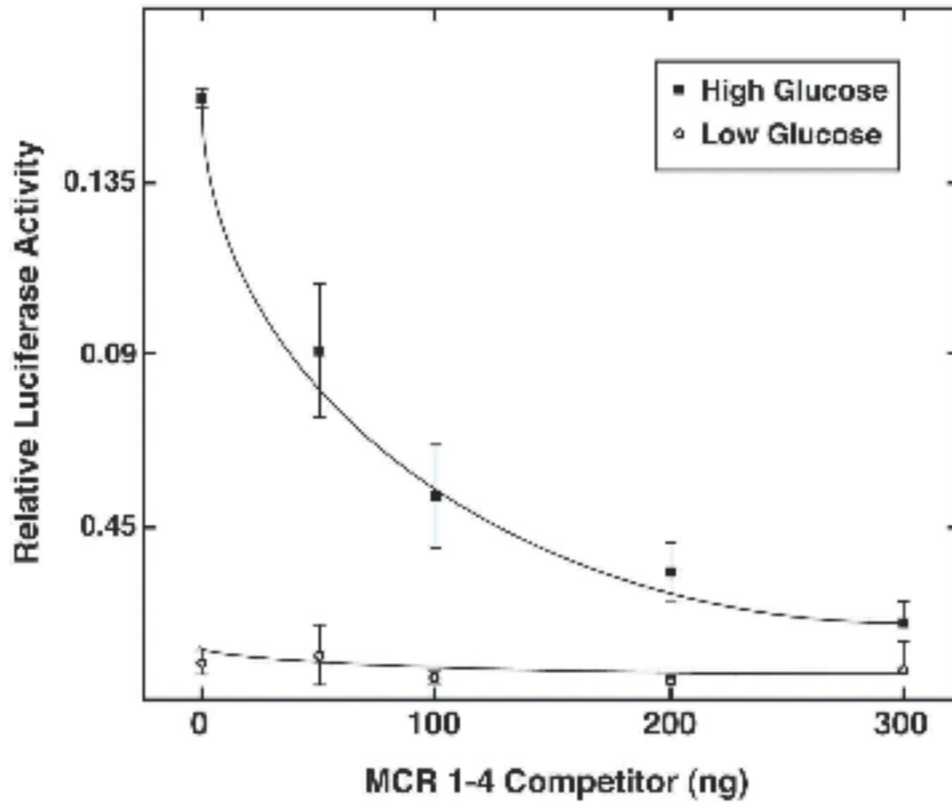


Figure 6: Individual MCR fragments do not effectively compete for ChREBP activity. A, Function of Gal4-ChREBP in the presence of increasing amounts of various competitor fragments was measured, as described in the legend to Figure 5. Individual MCR fragments consisting of MCRs 1, 2, 3, 4 and 5 were added in increasing amounts and ChREBP activity was measured. The white and dark gray bars represent Gal4-ChREBP activity in low and high glucose conditions without competitor. B, Western blot of YFP competing fragments expressed in HEK 293 cells, as described in the legend to Figure 1, except that an antibody to YFP was used. The specific regions used for each YFP fragment are stated in the materials and methods section. The migration pattern for each band represents the appropriate size of each fragment.

Figure 6:

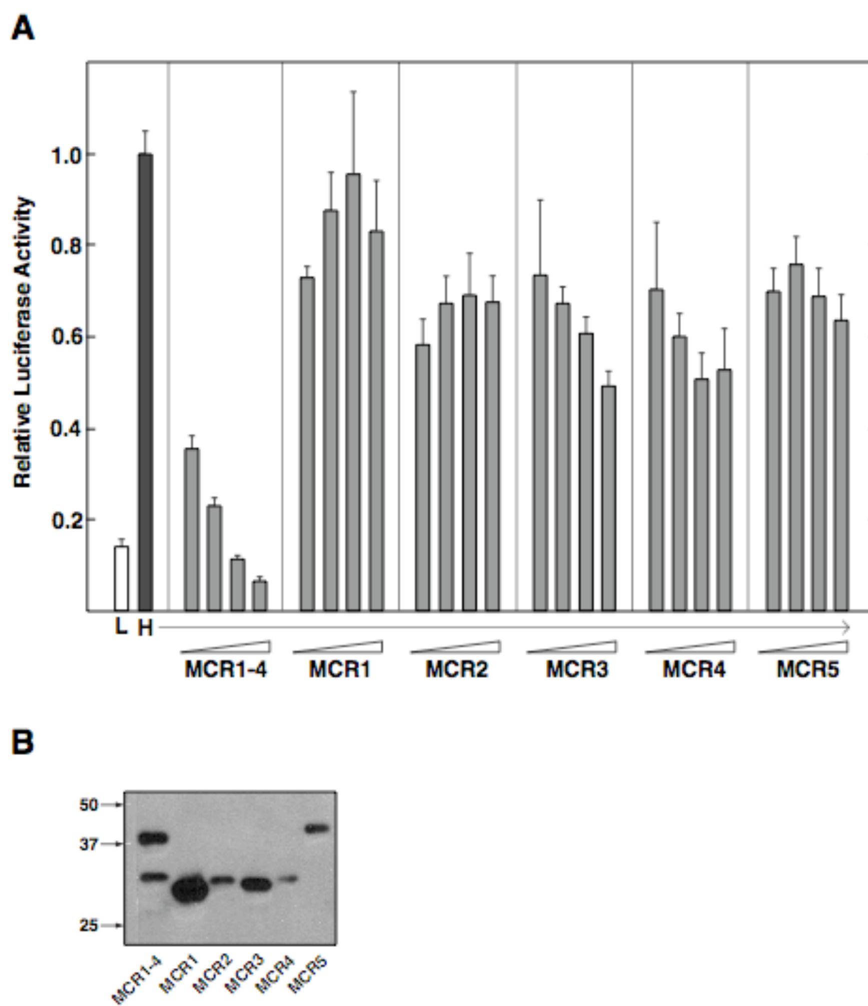


Figure 7: The MCR 1-4 fragment competes with the Quad mutant for activity in both low and high glucose conditions. Activity of the Gal4-Quad mutant in the presence of the MCR 1-4 competitor fragment was measured, as described in the legend to Figure 5. The bars with an "L" or an "H" below represent Gal4-Quad mutant activity in low and high glucose conditions without competitor, respectively. The bars that have an MCR 1-4-labeled gradient depicted below represent Gal4-Quad mutant activity in the presence of 100, 200 or 300 ng of plasmid. White bars represent low glucose and dark gray bars represent high glucose.

Figure 7:

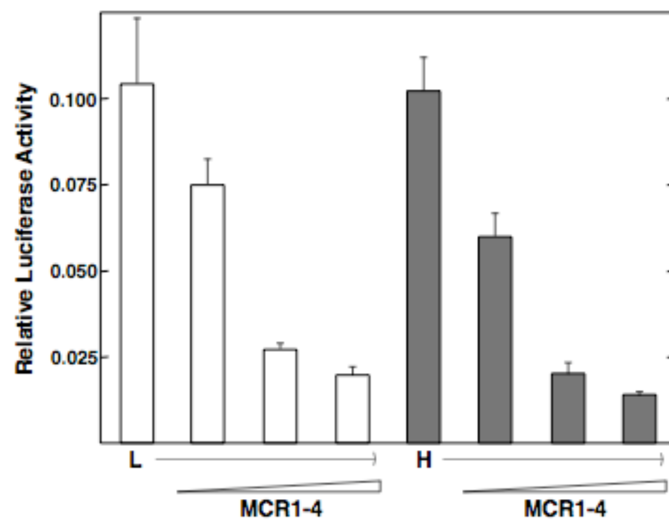
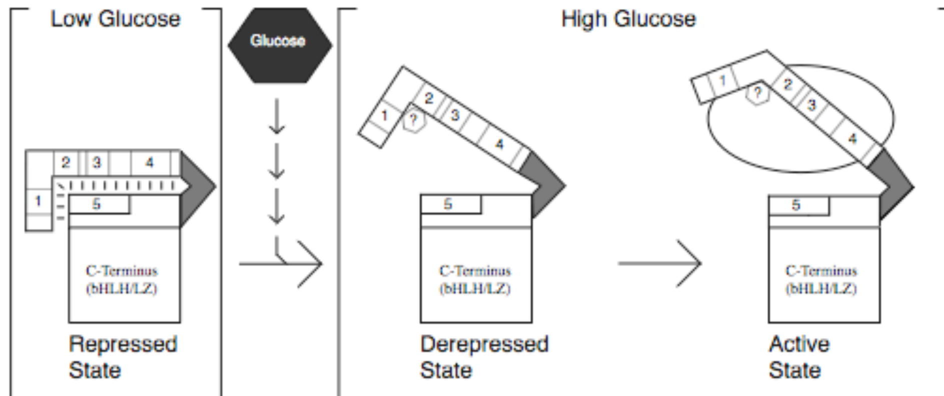


Figure 8: Model of glucose-stimulated ChREBP activation.

In low glucose conditions, ChREBP is repressed by an intramolecular interaction that involves MCRs 1-5 of the N-terminal segment of ChREBP. MCRs 1-4 are depicted as the repressor and MCR 5 the target of repression. The resulting conformation is incapable of binding to DNA and stimulating transcription. As glucose metabolism increases (depicted by the small arrows emanating from glucose), ChREBP is targeted and modified in a manner that relieves the repressive intramolecular interaction. One possibility is that a glucose metabolite binds directly to the MCR 1-4 region, as depicted by the hexagonal structure containing a question mark, representing this derepressed state. Although the model of the derepressed state emphasizes MCRs 1 and 2, the exact region that is targeted for derepression is unknown. As repression of ChREBP is relieved, the conformation that results allows for MCRs 1-4 to interact with a co-regulatory protein required for activation. This protein is represented by the open circle in the active state conformation.

Figure 8:



CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Discussion

The purpose of this thesis was to gain further insight into the glucose-dependent mechanism of ChREBP activation. At the start of my graduate studies, a model for ChREBP activation had been proposed by the Uyeda laboratory and was widely cited in the literature (45). In this model, it was postulated that ChREBP is inactivated by PKA-dependent phosphorylation of three specific phospho-acceptor sites. Phosphorylation of one phospho-acceptor site (Ser196), located adjacent to the putative NLS, was proposed to inhibit nuclear translocation of ChREBP. Phosphorylation of the other two phospho-acceptor sites (Ser626, Thr666), located near the DNA binding region of CHREBP, inhibited DNA binding. Upon glucose stimulation, it was proposed that the three critical phospho-residues are dephosphorylated by a xyulose-5 phosphate-dependent isoform of Protein Phosphatase 2A (PP2A) (46). In this model, increased metabolism of glucose leads to xyulose-5 phosphate accumulation and dephosphorylation of the three proposed phospho-sites resulting in nuclear accumulation and DNA binding of ChREBP to activate gene transcription.

Consistent with this model, Chromatin Immunoprecipitation (ChIP) assays revealed that ChREBP occupancy of the ChoRE within the L-PK promoter region increases significantly in response to high glucose conditions (59). However, research in our laboratory demonstrated that this model for glucose-stimulated ChREBP activation could not account for the actions of glucose on ChREBP activity. First, blocking phosphorylation of the three inhibitory phospho-sites did not yield a constitutively active form of ChREBP, as would be predicted by the model (47). Second, phospho-staining of

ChREBP revealed that ChREBP is phosphorylated to a similar extent in high glucose and low glucose conditions (47). This result suggests that phosphorylation of ChREBP might be critical for activation by glucose in addition to its proposed inhibitory role in basal glucose conditions. Finally, due to the absence of glucagon in isolated rat hepatocytes, repression of ChREBP activity in basal (low glucose) conditions cannot be attributed to increased cAMP levels and PKA-dependent phosphorylation. This result implies that ChREBP can be inhibited by a mechanism independent of PKA phosphorylation in basal glucose conditions. While it is possible that glucagon-stimulated PKA phosphorylation of ChREBP may serve to repress its activity during times of fasting or starvation, this mechanism appears distinct from the glucose stimulation of activity. Thus, the goal of my thesis was to further elucidate the mechanism of glucose-stimulated ChREBP activation.

The MondoA Conserved Region (MCR) within the N-terminal segment of ChREBP is a region that shows high conservation with MondoA, a paralog of ChREBP most highly expressed in the skeletal muscle (43). Five sub-domains within this region, MCRs 1-5, show 90-100% conservation between the two transcription factors. Furthermore, comparison of ChREBP sequences from a variety of vertebrate species revealed complete conservation within these five subdomains. The high degree of conservation within MCRs 1-5 suggested that the N-terminal segment of ChREBP has a significant role in the function of ChREBP. In support of this idea, deletion of the region containing MCRs 1-4 resulted in a mutant with constitutive activity, eliminating the requirement of glucose for ChREBP activation (60,80). Therefore, the MCR is clearly involved in the mechanism of glucose control. This experiment was performed with a Gal4 DNA binding domain fusion with ChREBP. Such fusion proteins would not require Mlx for DNA binding.

Thus, it was concluded that ChREBP, and not Mlx, is the glucose-dependent transcription factor. Additionally, this experiment suggested that glucose regulation involves repression of ChREBP activity in basal conditions. However, a caveat of the MCR 1-4 deletion experiment is that the putative NLS of ChREBP is located within MCR 4 (45). As such, nuclear localization of the Gal4 DNA binding domain ChREBP (Gal4-ChREBP) fusion protein was dependent on the NLS within the Gal4 DBD. Thus, any aspect of CHREBP regulation that acted through controlling nuclear localization would have been circumvented in this Gal4-CHREBP fusion protein.

To address the controversies concerning the model for ChREBP activation, the first goal of my thesis was to identify the cellular localization pattern of ChREBP and determine whether nuclear translocation of ChREBP is glucose-regulated. Due to the low transfection efficiency and high level of auto-flourescence of isolated rat hepatocytes, I used INS-1 cells for these studies. INS-1 cells are an immortalized rat pancreatic β-cell line that is widely used to study glucose regulation of insulin secretion. ChREBP has been shown to be active and glucose-regulated in these cells (59,60). Furthermore, all ChREBP mutants tested in INS-1 cells show a similar pattern of activity when compared to hepatocytes (M. N. Davies, unpublished data). Thus, the ChREBP localization patterns observed in INS-1 cells are likely to be a fair representation of the mechanism that occurs within the liver. Immunoflourescence of an overexpressed Flag-tagged version of ChREBP in INS-1 cells revealed a predominantly cytoplasmic localization pattern in both low and high glucose conditions. This result was surprising because ChREBP must be present in the nucleus to some extent to stimulate transcription in high glucose, as has been shown by ChiP experiments (59). This result suggests that only a small percentage

of the total cellular ChREBP is required in the nucleus for glucose-dependent induction of lipogenic genes. This observation also contradicted a key component of the existing model for ChREBP activation. Based on the model, we would have expected to see significant accumulation of ChREBP in the nucleus in high glucose conditions. Therefore, regulation of ChREBP does not appear to involve a major change in its cellular translocation, as has been found for several other transcription factors such as the FoxO or STAT family members (70,81). One reservation of this experiment is that the observed cytoplasmic localization of ChREBP could be a result of its overexpression, which might lead to saturation of a critical cellular component for nuclear import or retention. To address this concern, further analysis of nuclear localization was performed in the presence of the nuclear export inhibitor Leptomycin B. With the addition of Leptomycin B, ChREBP accumulated in the nucleus in both low and high glucose conditions, indicating that the cellular machinery is capable of fully transporting overexpressed ChREBP to the nucleus. More importantly, this result demonstrated that ChREBP shuttles between the nuclear and cytoplasmic compartments independent of glucose. The nuclear shuttling of ChREBP in low glucose conditions when it is inactive strongly implies that an event or events additional to nuclear localization is necessary for activation.

Although nucleocytoplasmic shuttling suggests that nuclear localization is not sufficient for ChREBP activation, further analysis revealed that the rate of ChREBP nuclear entry increases approximately 3-fold in high glucose conditions compared to low glucose. The molecular basis for the increased rate of nuclear import is not understood. The ChREBP paralog, MondoA, has been found to associate with the outer mitochondrial

membrane (44). Treatment of cultured epithelial or primary skeletal muscle cells with the glucose analog, 2-deoxy glucose (2-DOG), promoted movement of MondoA to the nucleus. Thus, in the context of MondoA, glucose might act to alter the equilibrium between a mitochondrial-associated pool of MondoA that cannot enter the nucleus and a cytoplasmic pool that can undergo shuttling. In a preliminary experiment, I found that only a small fraction of ChREBP associated with the mitochondria and that this association was not influenced by changes in glucose levels (M. N. Davies, unpublished data). Further analysis will be required to determine the relationship between ChREBP, mitochondrial association and nucleocytoplasmic shuttling. While the mechanism driving ChREBP nuclear localization is not understood, the increased rate of nuclear entry by glucose could account for the increased activity of ChREBP in high glucose conditions. In INS-1 cells and primary hepatocytes, we observed low levels of CHRBEP activity even in basal conditions (2.5-5 mM glucose). Additionally, expression of a dominant negative ChREBP decreases ChREBP activity in low glucose. These results suggest that ChREBP is entering the nucleus and is active, albeit at minimal levels in basal conditions. This hypothesis would still be consistent with the model in which nuclear localization is necessary and sufficient for ChREBP activation.

To further evaluate this hypothesis, nuclear export deficient ChREBP mutants were constructed and tested functionally. If increasing the entry rate of ChREBP in the nucleus is necessary and sufficient for activation, then nuclear export deficient mutants that are constitutively nuclear should be active in both low and high glucose conditions. A putative Crm-1 dependent nuclear export signal (NES) was identified within MCR 2 of the N-terminal segment of WBSCR14, the human homolog of ChREBP (78). Based on

this assessment, conserved residues within the putative NES of ChREBP were targeted for amino acid substitution. Three of the NES mutants were deficient in nuclear export, resulting in constitutive nuclear accumulation. This observation established the putative NES of the mouse homolog as a *bona fide* NES. However, constitutive activation was not observed with any of the NES mutants. In fact, one NES mutant was inactive, while the other two NES mutants showed a response to glucose comparable to wild type ChREBP. It was concluded from these results that the increased rate of ChREBP nuclear entry is not sufficient for activation. Additionally, the inactive NES mutant also indicates that the MCR 2 region has a role in glucose-stimulated activation of ChREBP in addition to its role in nuclear export. Thus, ChREBP requires an additional event, independent of nuclear localization for glucose-dependent activation.

While nuclear translocation is not sufficient to switch ChREBP from an inactive to an active state, a mechanism in which ChREBP is in constant flux through both nuclear and cytoplasmic compartments might be advantageous in a tissue that requires fine adjustments to an organism's nutritional state. For instance, other transcription factors such as SMAD and STAT have been shown to shuttle between both cytoplasmic and nuclear compartments in the absence of a stimulatory ligand (70,72). Nucleocytoplasmic shuttling of these transcription factors was proposed to control the magnitude and duration of activation upon ligand stimulation. One particular difference between SMAD, STAT and ChREBP is that the rate of ChREBP nuclear entry is regulated by its stimulatory signal, glucose. Regulation of nuclear entry by glucose could provide direct control of the magnitude and duration of ChREBP action in the nucleus in a mechanism separate from switching ChREBP from an inactive to an active state. Such a mechanism

would allow for the finer control of ChREBP activity necessary for tissues such as the liver, as glucose levels are in flux due to the variable intake of carbohydrates and metabolic demands during the day.

Although the mechanism that drives nucleocytoplasmic shuttling of ChREBP is critical for the overall mechanism of ChREBP activity, it is not sufficient for glucose-dependent activation. To continue with the goal of my thesis, I focused on determining the event driving ChREBP activation. As mentioned previously, it was concluded that ChREBP is repressed under basal conditions and that this repression involves the region encompassing MCRs 1-4 (60,80). In an attempt to determine the specific MCR domain(s) responsible for repression, serial deletion analysis of the N-terminal segment was performed and the various mutants were tested functionally. If relief of repression is necessary and sufficient for ChREBP activation, then deletion of MCR domains critical for this repression should result in increased activity in basal conditions, similar to that observed with the Gal4 MCR 1-4 deletion. Additionally, these mutants were constructed in the context of full length ChREBP, which include aspects of ChREBP activation such as nucleocytoplasmic shuttling and DNA binding that is bypassed in the Gal4 context. Deletion mutants ΔMCR 1-3, ΔMCR 2-4, ΔMCR 3-4 and ΔMCR 1-4 all showed a significant increase in basal activity levels, resulting in glucose unregulated transcriptional activity. These results imply that MCRs 3 and 4 are the minimal regions sufficient for repression of ChREBP activity. However, the activity levels of ΔMCR 1-4 and ΔMCR 2-4 were markedly greater than that of ΔMCR 3-4 ChREBP in high glucose conditions, suggesting that MCRs 1 and 2 contribute to the overall repressive capabilities of ChREBP as well. As such, these results support a mechanism in which repression

involves the entire N-terminal segment of ChREBP. Additionally, the dramatically high levels of activity observed with ΔMCR 1-4 and ΔMCR 2-4 suggest that wild type ChREBP does not reach maximal activity in high glucose conditions. This observation supports a potential role for the mechanism of nucleocytoplasmic shuttling controlling the magnitude and duration of ChREBP activation as previously mentioned.

One caveat to deletion mutants that span large regions is that they can have dramatic structural effects, which can potentially result in misinterpretation of experimental outcomes. Amino acid substitution mutations generally have a milder effect on the overall structure of a protein. As such, a strategy involving individual amino acid substitutions was performed in parallel with the deletion mutants. Residues that showed a high level of conservation between multiple species and MondoA throughout the N-terminal region of ChREBP were chosen for mutagenesis and tested for function. If regulation of ChREBP does in fact involve a simple repressive mechanism, then the individual mutants should have a similar phenotype as the deletion mutants. A few individual mutants identified in MCRs 1, 2 and 5 showed a slight increase in basal activity levels when compared to wild type ChREBP. However, these mutants still required glucose for activation. It was hypothesized from this experiment that these specific residues are important for repression, but their mutation did not completely disrupt the repressive complex. In fact, combining four of these mutations from MCRs 1, 2 and 5 (Quad mutant) resulted in constitutive activation, a similar phenotype to the MCR 1-4 deletion mutant. It is worth noting that MCRs 3 and 4 remain intact in the Quad mutant. This provides additional evidence that individual MCRs are not sufficient for

repression. Thus, we propose a model in which repression of ChREBP requires the coordination of multiple MCRs within the N-terminal segment of ChREBP.

However, the majority of individual point mutations from the large-scale mutagenesis were inactiving, suggesting that the N-terminal segment of ChREBP has a role in activation. Furthermore, deletions that removed individual MCR domains (MCRs 1, 2, 3 and 4) were inactive as well. Thus, in addition to having a repressive role in basal conditions, the multiple MCRs within the N-terminal segment of ChREBP are critical for activation in high glucose conditions. These results suggest two possible mechanisms for the role of the MCR 1-4 domain in activation. In the first possibility, MCRs 1-4 could form an allosteric binding site for a stimulatory metabolite. Binding of this metabolite would result in a conformational change that relieves repression without the action of an additional protein. In the second possibility, MCRs 1-4 could be the target of an additional regulatory protein. This protein could function either to disrupt the repressive conformation, resulting in activation, and/or interact with this region to recruit co-activators.

To test whether the MCR 1-4 region interacts with a co-regulatory protein, a competition experiment was performed with the N-terminal segment of ChREBP. A fragment of ChREBP containing MCRs 1-4 was introduced to the Gal4 reporter gene system in a dose-dependent manner. If this region of ChREBP interacts with a protein required for activation, then ChREBP activity should decrease as the amount of competing MCR 1-4 fragment is increased. We anticipated that co-regulatory proteins would be present at low, stoichiometric levels comparable to ChREBP and thus could be titrated out with increasing amounts of competitor fragment. Alternatively, signaling

metabolites would be present in μM to mM quantities. As such, we would predict that these levels would be too high to titrate with the concentration of protein we could achieve by transfection. In fact, the MCR 1-4 fragment does compete for ChREBP activity, indicating that a co-regulatory protein is involved in activation. However, fragments of individual MCR 1, 2, 3, 4 and 5 fragments do not compete with ChREBP activity, again suggesting that this interaction(s) requires multiple MCRs within the N-terminal segment.

Although the competition assay with the MCR 1-4 fragment of ChREBP establishes an interaction with a co-regulatory protein, it does not distinguish whether this protein acts directly to disrupt the repressive conformation or is required for a subsequent step in activation. To address this question, a competition assay was performed with the MCR 1-4 competing fragment and the Quad ChREBP mutant. The constitutively active phenotype of the Quad mutant suggested that the repressive complex has been disrupted by the introduced mutations. As such, if relief of repression is necessary and sufficient for activation, then the MCR 1-4 fragment should not compete for the Quad mutant activation. However, a decrease in Quad mutant was observed with increasing amounts of the MCR 1-4 fragment in low glucose, suggesting that ChREBP interacts with a co-regulatory protein that is required for activation in a step subsequent to relief of repression.

As these experiments were being performed, much effort in the field was targeted towards assigning specific roles for each MCR. For example, MCRs 2, 3 and 4 were shown to be critical for nucleocytoplasmic shuttling. MCR 2 was defined as the nuclear export region. Our studies on the nuclear accumulation of mutants in the NES motif of

MCR 2 strongly support this role. MCR 3 was defined by its ability to interact with 14-3-3. Subsequent studies strongly suggest that this interaction is critical for nucleocytoplasmic shuttling of ChREBP. However, the role of 14-3-3 in the mechanism of glucose-regulated activation remains unclear. Additionally, MCR 4 was defined as the region critical for nuclear import because it contained a putative NLS. It was originally postulated that glucose-dependent dephosphorylation of a serine phospho-acceptor residue near this region was critical for nuclear translocation and subsequent activation of ChREBP. Although the sequence of this motif resembles an NLS, the function of this motif has never been confirmed in a nuclear localization assay. However, all of the deletion mutants that do not contain the putative NLS are capable of entering the nucleus (M. N. Davies, unpublished observations). One potential explanation for this result is that the putative NLS in MCR 4 is not a *bona fide* NLS and that the functional NLS lies outside the MCR 1-4 region. Alternatively, ChREBP may contain two redundant NLS motifs. As the field focused on identifying specific roles for each MCR at the time I started my thesis, the majority of my experiments were garnered towards this goal as well. Although individual MCRs might have specific roles in nucleocytoplasmic shuttling such as nuclear import and export, the majority of my results suggest that that the entire N-terminal segment of ChREBP is acting in a coordinated fashion in both repression and activation of ChREBP.

Altogether, the results of my thesis support the following model for glucose-stimulated ChREBP activation. In basal conditions, ChREBP is repressed by an intramolecular interaction. This intramolecular interaction is mediated by MCRs 1-5, which function in a concerted fashion to create a repressive conformation. In this state,

ChREBP is incapable of binding to DNA or recruiting co-activators and is rapidly exported to the cytoplasm. Upon increased glucose levels, a glucose-generated metabolite accumulates and acts as an allosteric regulator to initiate ChREBP activation. This metabolite could act indirectly by binding to a modifying enzyme such as a protein kinase that acts on ChREBP. Alternatively, the MCR 1-5 region of ChREBP could form a binding pocket for this glucose metabolite. In either case, the consequence of this indirect or direct allosteric regulation would disrupt the intramolecular repressive complex. However, relief of repression is not sufficient for ChREBP activation and a subsequent interaction with a co-regulatory protein is required for ChREBP-dependent transcriptional activation of lipogenic genes.

Future Directions

Identification of the factor(s) required for activation

Deletion and amino acid substitution mutagenesis revealed that the N-terminal segment of ChREBP is critical for the mechanism of repression and activation. Further analysis with the competition assay determined that the N-terminal segment of ChREBP containing MCRs 1-4 inhibits ChREBP activity in a dose-dependent manner. This result suggested that MCRs 1-4 interact with a co-regulatory protein required for ChREBP activation in addition to its binding to components necessary for nucleocytoplasmic shuttling such as Crm-1 and 14-3-3. The individual MCR segments did not compete with ChREBP activity to the same extent as MCR 1-4 and it was concluded that glucose-dependent activation of ChREBP required the coordination of the entire region spanning MCRs 1-4. It was additionally postulated in Chapter 3 that the inactivating mutations identified within this region disrupt the interaction with the activating factor(s). Although these experiments strongly suggest that this region interacts with a heterologous protein important for ChREBP activation, further experiments are needed to provide direct evidence confirming this interaction.

To identify potential factor(s) that interact with the N-terminal region of ChREBP, I attempted an affinity purification technique that involves peptide identification through mass spectroscopy. I constructed a triply Flag-tagged MCR 1-4 fragment of ChREBP and introduced it into an adenoviral vector. Primary rat hepatocytes were transduced with the Flag-MCR 1-4 adenovirus expression plasmid and subsequently were treated with low or high glucose for 1 h. After treatment, I harvested the cells, prepared a cell lysate and incubated the lysate with sepharose beads conjugated with an anti-Flag antibody to

immunoprecipitate interacting proteins. The eluate of proteins from the sepharose beads were then digested with trypsin into peptide fragments for mass spectroscopy analysis. Control cells that were untransfected were processed in parallel. By using this technique, we anticipated that if a particular protein interacted with the N-terminal fragment of ChREBP, then we would see peptides of this protein consistently in multiple experiments in Flag-MCR 1-4-treated cells, but not in the control cells. We were particularly interested in proteins that showed a glucose-dependent interaction with ChREBP. Many peptides of the 14-3-3 family of proteins were found in Flag-MCR 1-4 transduced cells, but not in control cells. Since 14-3-3 proteins have been shown to interact with the MCR 3 domain of ChREBP, this result indicated that the affinity purification procedure was effective. However, the 14-3-3 proteins were detected in both low and high glucose conditions.

Although many interacting proteins were detected in each experiment, multiple experiments failed to conclusively reveal additional proteins that were present in each experiment or that showed differences in low versus high glucose. A limitation of this strategy is that low abundant and weakly interacting proteins are difficult to identify. The types of proteins that we anticipate interacting with ChREBP to stimulate activation in high glucose include modifying enzymes such as protein kinases. The transient nature of these interactions increases the difficulty in the immunoprecipitation and detection steps. In an attempt to detect such transiently interacting proteins, I tried using formaldehyde-crosslinking to stabilize weak interactions. This procedure did increase the number of proteins detected, but again did not reveal any interactors in repeated experiments. Due to the amount of trouble-shooting required to pursue this strategy and the time constraints of

my thesis, this project was not completed. However, identification of the factor(s) required for ChREBP activation will be critical to further elucidate the mechanism of glucose-stimulated ChREBP activation.

Discovering the ChREBP stimulatory molecule

The original model for glucose-regulated ChREBP activation postulated that dephosphorylation of three critical phospho-acceptor sites in high glucose promoted ChREBP activation. Subsequent experiments identified a Protein Phosphatase 2A (PP2A) isoform present in a fraction of rat hepatocyte lysate capable of dephosphorylating a PKA-phosphorylated peptide of ChREBP in vitro. Increased levels of xyulose-5 phosphate (82) stimulated this PP2A activity. Thus, it was proposed that xyulose-5 phosphate is the stimulatory molecule of ChREBP. Interstingly, the same PP2A isoform has been suggested to be responsible for glucose activation of the bifunctional enzyme, Phosphofructokinase 2/Fructose Bisphosphatase-2 (PFK2/FBPase2), to enhance glycolyisis (82,83). This might provide a mechanism to couple regulation acting at metabolic and transcriptional levels. Although this has been the accepted model for many years, there has not been a single experiment reported that has directly shown xyulose-5 phosphate to be the activating molecule of ChREBP. Additionally, the results of my thesis have shown that the original model for ChREBP activation was over simplified. As such, the conclusion that xyulose-5 phosphate is the stimulatory molecule needs to be reconsidered.

A second glucose metabolite that has been proposed as a potential regulator of ChREBP is glucose-6 phosphate. Upon entry into the liver, glucose is phosphorylated by glucokinase to form glucose-6 phosphate. Glucose-6 phosphate has multiple fates and is a

critical substrate for glycolysis, ribose synthesis and glycogen metabolism. The hypothesis that glucose-6 phosphate could be the critical signaling molecule came from experiments using the glucose analog, 2-deoxy glucose (2-DOG). 2-DOG is transported into the cell by the same transporters as glucose and phosphorylated by hexokinases to form 2-DOG-6-phosphate. However, 2-DOG-6-phosphate is poorly metabolized by glycolysis or the pentose phosphate shunt and accumulates as 2-DOG-6-phosphate in most cell types. Early studies showed that 2-DOG could support the induction of fatty acid synthase in adipose explants from suckling rats and did so at a much lower concentration than glucose (1 mM versus 20 mM) (84). Recent studies with MondoA have shown that increasing 2-DOG levels stimulate nuclear accumulation and activation of MondoA in cultured epithelial and primary skeletal muscle cells (85). This experiment implies that glucose-6 phosphate is the signaling molecule for MondoA activation. However, in parallel experiments with ChREBP, I was unable to observe nuclear accumulation or activation of ChREBP upon treatment with 2-DOG in primary hepatocytes or INS-1 cells. A potential limitation of using 2-DOG in hepatocytes is that they contain glucose-6 phosphatase, which is a critical enzyme in the final step of glycogenolysis and could prevent accumulation of 2-DOG-6-phosphate. INS-1 cells are a transformed cell line and such cell lines have altered properties of glucose metabolism. Hence, the potential of glucose-6 phosphate as a signaling molecule is unresolved.

In a study by Wu et al., it was observed that mRNA levels of lipogenic enzymes such as ACC and FAS were decreased in response to increased levels of fructose-2,6 bisphosphate levels ($F26P_2$) (86). $F26P_2$ is an allosteric regulator of phosphofructokinase-1, a key regulatory step of glycolysis. In this experiment, increased $F26P_2$ levels were

driven by overexpression of a kinase-active form of the bifunctional enzyme, PFK-2/FBPase-2. When $F26P_2$ levels remain high, glucose metabolites are fluxed through glycolysis at a high rate. As such, metabolites upstream of phosphofructokinase-1 action would not be expected to accumulate. In the same study, it was also shown that overexpression of glucokinase in mice livers, led to an increase in mRNA levels of ACC and FAS, the same lipogenic genes that were decreased by elevated $F26P_2$ levels. Thus, the observations of these two experiments suggest that the activating metabolite is present upstream of phosphofructokinase-1 action. These experiments are consistent with glucose-6 phosphate or xyulose-5 phosphate as signaling metabolites, but would appear to eliminate metabolites downstream of fructose-6-phosphate. Another pathway that has been overlooked in the regulation of ChREBP activation is glycogen synthesis. Theoretically, a link between glycogen synthesis and lipogenesis is plausible, as both are major pathways for glucose disposal and energy storage. As glycogen stores become saturated in the liver, a logical step would be to stimulate lipogenesis. Identifying the stimulatory glucose metabolite will provide incredible insight into the mechanism of ChREBP activation. Additionally, elucidating the stimulatory event of glucose-regulated ChREBP activation will provide further comprehension of the relationship between carbohydrate metabolism and *de novo* lipogenesis.

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