Direct Role of ChREBP/MLx in Regulating Hepatic Glucose-responsive Genes*

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Enzymes required for de novo lipogenesis are induced in mammalian liver after a meal high in carbohydrate. In addition to insulin, increased glucose metabolism initiates an intracellular signaling pathway that transcriptionally regulates genes encoding lipogenic enzymes. A cis-acting sequence, the carbohydrate response element (ChoRE), has been found in the promoter region of several of these genes. ChREBP (carbohydrate response element binding protein) was recently identified as a candidate transcription factor in the glucose-signaling pathway. We reported that ChREBP requires the heterodimeric partner Max-like factor X (MLx) to bind to ChoRE sequences. In this study we provide further evidence to support a direct role of MLx in glucose signaling in the liver. We constructed two different dominant negative forms of MLx that could dimerize with ChREBP, but block its binding to DNA. When introduced into hepatocytes, both dominant negative forms of MLx inhibited the glucose response of a transfected ChoRE-containing promoter. The glucose response was rescued by adding exogenous wild type MLx or ChREBP, but not MondoA, a paralog of ChREBP that can also form a heterodimer with MLx. Furthermore, dominant negative MLx blocked the induction of glucose-responsive genes from their natural chromosomal context under high glucose conditions. In contrast, genes induced by the insulin and thyroid hormone signaling pathways were unaffected by dominant negative MLx. MLx was present in the glucose-responsive complex of liver nuclear extract from which ChREBP was purified. In conclusion, MLx is an obligatory partner of ChREBP in regulating lipogenic enzyme genes in liver.

Mammals have evolved to maintain energy balance under varying nutritional conditions. Excess dietary carbohydrate can be converted into triglycerides, the principal energy storage form, when food is plentiful. The synthesis of triglycerides from carbohydrate is termed de novo lipogenesis and regulated lipogenesis occurs most abundantly in the liver in rodents. After a meal high in carbohydrate, many genes encoding enzymes involved in the lipogenic pathway are transcriptionally induced in liver, including L-type pyruvate kinase (PK), fatty acid synthase (FAS), acetyl-CoA carboxylase α (ACC) and S14 (1-3). Two signaling pathways are required for this response. Increased insulin secretion in response to elevated blood glucose levels activates the basic helix-loop-helix/leucine zipper (bHLH/LZ) transcription factor sterol regulatory element-binding protein-1c (SREBP-1c) (4-6). SREBP-1c binds to a regulatory sequence in the promoter region of most lipogenic enzyme genes to activate their transcription (7,8). Overexpressing SREBP-1c in mice leads to an increased rate of lipogenesis and increased mRNA levels of lipogenic enzyme genes (9,10). However, both in vitro
and in vivo data suggest that SREBP-1c alone is not sufficient to support the full induction of lipogenic enzyme genes in response to high carbohydrate diet (11,12). In addition to the insulin-signaling pathway, increased uptake and metabolism of glucose is now recognized to initiate a second signaling pathway responsible for controlling the synthesis of lipogenic enzymes. Increased glucose metabolism is postulated to generate a metabolite that activates the downstream signaling pathway. Both glucose-6-phosphate and xylulose-5-phosphate have been proposed as the critical metabolite in this pathway (2,13,14). However, the intracellular mechanism of the glucose-signaling pathway is not fully understood. A carbohydrate response element (ChoRE) that mediates the transcriptional response to glucose has been mapped within the promoter regions in several lipogenic enzyme genes (15-19). This element is composed of two E-box (CACGTG) or E-box-like sequences that are separated by 5 bp. The presence of E-box motifs in these response elements suggests that a bHLH protein family member recognizes the ChoRE and mediates the response to glucose.

A candidate transcription factor was purified based on its ability to bind to the PK ChoRE (20). This factor, designated as carbohydrate response element binding protein (ChREBP), is a member of the bHLH/LZ family (21). ChREBP is expressed at high levels in the liver and is also abundant in adipose, kidney, and small intestine (20-23). Overexpressing ChREBP in primary hepatocytes induces activity of the ChoRE-containing PK promoter in high glucose conditions, but not in low glucose conditions (20). However, ChREBP does not effectively homodimerize or bind to the ChoREs as a homodimer (22,24). Using a yeast-two hybrid screen, Max-like protein X (Mlx) was identified as a bHLH/LZ protein in liver that interacts with the bHLH/LZ domain of ChREBP (24). Mlx is a member of the Myc/Max/Mad family of transcription factors and, similar to Max, it can serve as a common interaction partner of a transcription factor network (25-27). In addition to ChREBP, Mlx can also interact with Mad1, Mnt, Mad4 and MondoA (a paralog of ChREBP) (25,26,28). However, no target genes have been identified for Mlx and these heterodimeric partners. Recently, we demonstrated that ChREBP can bind to various ChoREs only in the presence of Mlx (24). Moreover, activation of ChoRE-containing promoters depends upon both ChREBP and Mlx in human embryonic kidney 293 cells (24). The correlation between the binding and functional data suggested that Mlx is a functional interaction partner of ChREBP. However, 293 cells are not glucose-responsive. Whether ChREBP and Mlx function together to directly regulate the glucose-responsive lipogenic enzyme genes remains a question.

To further explore the role of ChREBP/Mlx in the glucose-signaling pathway, we developed dominant negative forms of Mlx to block the function of ChREBP in the context of a glucose-responsive cell environment. In this report, we provide evidence supporting a direct role of ChREBP/Mlx heterodimer in regulating glucose-responsive genes in liver and supporting an obligatory role for Mlx in this process.

**Experimental Procedures**

*Primary Hepatocyte Culture and Transfection* - Male Harlan Sprague-Dawley rats (200-300 g) were fed ad libitum, and primary hepatocytes were isolated by the collagenase perfusion method described previously (29). After a 3-h attachment to 35-mm Primaria plates, cells were transduced with recombinant adenovirus for 2 hours in Williams' E medium containing 5.5 mM glucose, 23 mM HEPES, 0.01 µM dexamethasone, 2 mM glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 26 mM sodium bicarbonate, and 0.1 unit/ml insulin (low glucose medium). Cells were then transfected using F1 reagent (Targeting Systems, San Diego, CA) with a mixture of a firefly luciferase reporter driven by
an ACC ChoRE-containing promoter region (18) and an internal control plasmid encoding Renilla luciferase (pRL-CMV, Promega). In some experiments, expression plasmids for ChREBP, Mlx or MondoA were cotransfected with the reporter plasmid mixture. Expression plasmids for mouse ChREBP and Mlx were described previously (24). The human MondoA cDNA in the pcDNA3.1 vector was obtained from Dr. Donald Ayers, University of Utah, and contained a C-terminal His-tag (28). Four hours after transfection, Matrigel (Collaborative Biomedical Products, Bedford, MA) was added at 667 \( \mu \text{g} / 35 \text{ mm plate} \) and cells were kept in low glucose medium for 24-36 hours to allow expression of recombinant protein. Cells were then maintained in low glucose medium or switched to high (27.5 mM) glucose medium for an additional 24 hours. Hepatocytes were harvested using Promega Passive Lysis Buffer. Luciferase assays were performed using Promega Dual-Luciferase Assay System kit. Results of luciferase assays are expressed as relative firefly light units/Renilla light units. For experiments in which RNA was extracted, the procedure was identical except no transfections were performed.

293 Cell Culture and Transfections - Experiments were performed in a similar manner as previously described (24). Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and penicillin/streptomycin. For whole cell extracts used in electrophoretic mobility shift assays (EMSAs), 293 cells were transfected in 100-mm plates using the calcium phosphate method. We transfected 7.5 \( \mu \text{g} \) each of plasmids expressing double mutant ChREBP (S196A/T666A) (24) and Mlx with 22.5 \( \mu \text{g} \) of either form of dominant negative Mlx or empty vector plasmids into 293 cells. Whole cell lysate was prepared from cells 40 h post-transfection using the "whole cell extract" protocol in the nuclear extract kit from ActiveMotif (Carlsbad, CA).

EMSAs - EMSAs were conducted as described previously (30). A typical reaction contained 100,000 cpm of \( ^{32} \text{P} \)-labeled oligonucleotide and 5 \( \mu \text{g} \) of whole cell extract from 293 cells. For reactions with antibodies, proteins were first incubated with antibody for 30 min at 4ºC. Liver nuclear extracts were prepared from male Harlan Sprague-Dawley rats as described previously (11). Antibodies to Mlx and USF1 were obtained from Santa Cruz Biotechnology; Flag and HA antibodies were purchased from Sigma. After incubation with oligonucleotide for 30 min at room temperature, samples were loaded and separated on a 4.5% non-denaturing polyacrylamide gel. Results were obtained by phosphorimager analysis.

Construction of recombinant adenovirus - Dominant negative Mlx plasmids were constructed by site directed mutagenesis using the QuickChange kit (Stratagene). The starting plasmid was a 5'-flag tagged Mlx\( \beta \) cDNA described previously (24). Adenovirus constructs were prepared according to the Qbiogene AdenoVator kit. Both dominant negative Mlx forms, wild type Mlx and ChREBP were first cloned into the transfer vector (pAdenoVator-CMV5-IRES-GFP). The cDNA of the clone was linearized with \( \text{PmeI} \) and cotransformed with a plasmid containing the partial adenoviral genome (pAdenoVator \( \Delta E1/\Delta E3 \)) into BJ5183 bacterial cells. Colonies were selected and screened by plasmid size and restriction enzyme analysis. The recombined plasmid was linearized with \( \text{PacI} \) and transfected into 293 cells. In 4-10 days, cell lysates containing virus were collected. PCR was performed using the viral DNA to confirm the presence of appropriate sequences. Viruses were titered by immunofluorescence microscopy using the GFP gene integrated into the adenoviral genome.

Measurement of mRNAs by RT-PCR - Total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Concentrations of RNA were measured spectrophotometrically at \( A_{260} \). Real time RT-PCR was performed in two steps. First,
RNA was reverse transcribed into cDNA using the iScript kit (BioRad). Quantitative PCR was then performed on the iCycler Real-Time PCR Detection System using iQ SYBR Green Supermix kit (BioRad). Primers were designed using the software Beacon Designer (BioRad). Primer information is available upon request. The results of RT-PCR were expressed as the fold induction by normalizing the mean of Ct values from treated hepatocytes samples to the mean of Ct values from low glucose untreated or GFP virus treated hepatocyte samples. All samples were analyzed in triplicate and expressed as mean ± SD.

**Immunoblot Analysis** - Protein from hepatocyte crude extract or 293 cells was boiled in reducing buffer and separated on a 10% SDS-PAGE. Proteins were electrotransferred onto an Immobilon-P PVDF 0.45-µm filter membrane (Millipore, Bedford, MA). Dominant negative Mlx was detected with the M2 mouse monoclonal anti-flag antibody (Sigma) and anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.). MondoA was detected using a mouse monoclonal to polyhistidine (Santa Cruz Biotechnology). Signals were detected using an ECL Western blot detection kit (Amersham Biosciences).

**Measurement of lactate levels** - Hepatocytes were treated the same as for the total RNA preparation. Hepatocytes were transduced with either control virus or dominant negative virus for two hours and then maintained in low glucose conditions for 36 hours. Cells were then cultured in either low or high glucose conditions and samples of medium were collected in 4, 8, 12, and 24 hours to measure lactate levels. Lactate levels were measured using the Lactate Reagent kit (Sigma).

**Results**

**Construction of dominant negative Mlx** - Several lines of evidence have implicated ChREBP/Mlx as the critical factor affording glucose-responsive transcription to ChoRE-containing genes in hepatocytes. We sought to obtain definitive evidence in this regard. Testing the function of ChREBP and Mlx by overexpression is complicated by the presence of the endogenous proteins in hepatocytes. Alternatively, the role of these factors in the glucose-signaling pathway can be evaluated by inhibiting their function. For this purpose, we constructed dominant negative forms of Mlx. Three different spliced Mlx isoforms α, β, γ are widely expressed and capable of heterodimerizing with ChREBP (25,26). As Mlxβ is the most abundant form in liver (26), two dominant negative Mlxβ constructs were prepared. In the first dominant negative, the entire basic region of thirteen amino acids was deleted (Mlx Δb). In the second one, two basic residues that are conserved in the basic region of the bHLH/LZ family were mutated to non-basic residues (Mlx b/a) (Fig. 1A). These two mutant forms should be able to heterodimerize with ChREBP as their HLH/LZ domains are intact. However, the resulting heterodimers should not bind to DNA because of the mutations in the basic region. To ensure that these dominant negative Mlx forms do block DNA binding of ChREBP/Mlx, we performed an EMSA experiment in which equal amounts of ChREBP and wild type Mlx expression plasmids were cotransfected into 293 cells alone or with a three-fold excess of each dominant Mlx form. A double mutant (S196A/T666A) ChREBP was used in this experiment because it enhances DNA binding to the ChoREs (24). As predicted, both dominant negative Mlx forms interfere with the binding of ChREBP/Mlx to the ChoRE sequence with the Mlx b/a form being somewhat more effective than the Mlx Ab form (Fig. 1B). Therefore, the mutant Mlx forms should block the function of ChREBP/Mlx in regulating their target genes in hepatocytes.

**Dominant negative Mlx forms inhibit the glucose-response of a ChoRE containing-promoter** - To evaluate the role of ChREBP/Mlx in mediating the glucose-response, dominant
negative forms of Mlx were introduced into adenoviral expression vectors. Both dominant negative Mlx proteins were expressed in hepatocytes following the transduction of recombinant adenovirus into primary hepatocytes (Fig 1C). To test their effect on a glucose-responsive promoter, increasing amounts of either dominant negative Mlx virus were transduced into hepatocytes. Subsequently, cells were transfected with a reporter containing multiple copies of the ChoRE from ACC promoter region (18). Since this reporter plasmid contains only the ChoRE element and the basal promoter, it provides a direct readout for the glucose-responsive transcription factor. Cells were kept in low (5.5 mM) glucose medium for 36 hours to allow the expression of the dominant negative Mlx protein and then incubated in either low or high (27.5 mM) glucose medium for 24 hours. Insulin was present throughout the experiment at fixed levels, so that any changes in promoter activity would be due to altered glucose levels. In the absence of virus, reporter gene activity was greatly elevated in cells cultured in high glucose conditions. However, both forms of dominant negative Mlx progressively inhibited the ACC ChoRE promoter activity in high glucose conditions (Fig. 2). As a control, we used a virus expressing the coding sequences for an unrelated protein, GFP. Expression of this protein did not inhibit the glucose response, indicating that transduction by the adenoviral vector does not interfere with the glucose-signaling pathway. Although there was a modest increase in activity in high glucose conditions with the GFP-expressing virus, this was not routinely observed. Therefore, both dominant negative Mlx forms efficiently block the glucose response of ChoRE-containing promoter activity by inhibiting the endogenous ChREBP/Mlx heterodimer from binding to the ChoRE.

Overexpression of wild type ChREBP or Mlx can compensate for the effect of dominant negative Mlx - Mlx can interact with several other bHLH/LZ proteins in addition to ChREBP (25,26,28). Consequently, we wanted to ensure that the inhibition of the glucose response by dominant negative Mlx is the result of binding to endogenous ChREBP instead of another bHLH/LZ protein. Hepatocytes were treated with dominant negative Mlx virus or control virus expressing GFP for two hours. Increasing amounts of adenovirus expressing wild type ChREBP were transduced with the dominant negative Mlx virus. The ACC ChoRE-containing reporter was then transfected into these cells. Following incubation in low glucose conditions for 36 hours, the media was either maintained in low glucose or changed to high glucose for 24 hours to induce the glucose response. Addition of increasing amounts of wild type ChREBP progressively rescued the glucose response of the hepatocytes treated with the dominant negative Mlx in high glucose conditions (Fig. 3A). Little or no effect was observed in low glucose conditions. Control adenovirus did not induce the glucose response in the presence of dominant negative Mlx. Similarly, adenovirus expressing the bHLH/LZ factor SREBP-1c (12) was unable to restore a glucose response. Consequently, inhibition of the glucose response by dominant negative Mlx appears to be the result of blocking ChREBP function.

The ability of dominant negative Mlx to inhibit the glucose response in hepatocytes presumably results from its overexpression relative to the endogenous Mlx gene. Hence, the majority of endogenous ChREBP would be bound to the dominant negative Mlx protein rather than endogenous Mlx. If ChREBP/Mlx are the active glucose-responsive complex, then increasing expression of wild type Mlx in the presence of a fixed amount of dominant negative Mlx should be able to overcome the inhibition as well. To test this possibility, we carried out a rescue experiment with wild type Mlx. As a control, we used the ChREBP paralog, MondoA. This bHLH/LZ protein also heterodimerizes with Mlx. However, there is no evidence indicating that MondoA functions in a
glucose-responsive manner and it lacks several amino acid residues found critical for the response of ChREBP to glucose (28,31). As seen in Fig. 3B, expressing increasing amounts of an Mlx expression plasmid in cells previously transduced with the dominant negative Mlx does indeed lead to a restoration of the glucose response. However, cells expressing the Mlx partner MondoA remain unresponsive to glucose. To ensure that the MondoA expression plasmid was capable of producing the full-length protein, this plasmid was introduced into 293 cells and extracts were immunoblotted to detect the His-tagged MondoA. A band of running just above the marker of 105 kilodaltons was detected in these extracts, indicating that MondoA was indeed being produced. These experiments support the conclusion that ChREBP functions in a heteromeric complex with Mlx to provide a glucose-responsive transcription factor.

**Dominant negative Mlx efficiently blocks induction of endogenous glucose-responsive genes** - The above experiments indicate that blocking the function of ChREBP/Mlx with dominant negative Mlx can inhibit the ChoRE-directed transcriptional response to glucose in hepatocytes. We subsequently used dominant negative Mlx to assess the role of these factors in the glucose induction of lipogenic genes in their natural chromosomal context. Hepatocytes were untreated or transduced with adenovirus expressing Mlx b/a for two hours. As before, cells were kept in low glucose medium for 36 hours and then incubated with either low or high glucose medium for 24 hours in the presence of insulin. Total RNA was then extracted and analyzed for specific mRNA levels by real-time PCR.

Acetyl-CoA mRNA is induced in mammalian liver following a high carbohydrate diet (32,33). As we had found that dominant negative Mlx inhibited the promoter activity driven by the ACC ChoRE, we first tested the effect of dominant negative Mlx on its induction (Fig. 4). In the untreated hepatocytes, ACC mRNA levels were induced in high glucose conditions compared with low glucose conditions. Transduction with the control virus did not alter the induction of ACC mRNA by high glucose. However, when the Mlx b/a dominant negative virus was present, little or no change in ACC mRNA levels was observed in the high glucose conditions. Besides ACC, ChoREs have also been identified in the PK, S14 and FAS genes (15-17,19). We examined the effect of dominant negative Mlx on mRNA levels of these glucose-responsive lipogenic enzyme genes as well (Fig. 4). Similar to ACC, the mRNA levels for each of these gene products was induced to varying extents in high glucose conditions. The dominant negative Mlx virus efficiently blocked the induction of the mRNA of these three genes in high glucose conditions. Again, mRNA level in low glucose conditions were unaffected or only slightly inhibited. Hence, ChREBP/Mlx plays an important role in supporting induction of all lipogenic enzyme genes with previously characterized ChoREs.

After a meal high in carbohydrate, many other lipogenic enzyme genes for which carbohydrate response elements have not yet been characterized are also induced. The GLUT2 glucose transporter displays a high $K_M$ for glucose to allow the increased flux of glucose into hepatocytes as blood glucose levels rise after a meal. Malic enzyme is one of the enzymes that provide NADPH for lipogenesis. Stearoyl-CoA desaturase 1 (SCD1) catalyzes the synthesis of monounsaturated C16 and C18 fatty acids from unsaturated fatty acid precursors. Although no ChoRE has been identified in any of these genes, glucose has been indicated as an important regulator for their induction (1,2). Introduction of dominant negative Mlx into hepatocytes can also block the glucose response of these three genes (Fig. 4). As a control, RPL32 (ribosomal protein large subunit 32) mRNA was tested and was not affected by the dominant negative Mlx virus. Thus, the dominant negative Mlx can influence the
glucose induction of a large number of lipogenic enzyme genes, suggesting that ChREBP/Mlx may be a common regulator for these genes.

**Dominant negative Mlx does not affect induction of genes by other signaling pathways** - In addition to glucose, insulin plays a critical role in supporting the induction of lipogenic enzyme genes. If dominant negative Mlx acted to repress expression of any component in the insulin signaling pathway, then its effects on lipogenic enzyme genes could be indirect. To evaluate this possibility, we asked whether the dominant negative Mlx interfered with the ability of insulin to induce gene expression. SREBP-1c is the transcription factor that mediates the insulin regulation of many lipogenic enzyme genes (4-6). The mRNA level of this transcription factor is induced in hepatocytes by insulin (34-36). No difference in the insulin induction of SREBP-1c mRNA levels was observed in hepatocytes treated with dominant negative Mlx virus compared with untreated hepatocytes (Fig. 5A). The high K_M hexokinase, glucokinase, supports the rapid uptake and metabolism of carbohydrate in hepatocytes. However, the regulation of glucokinase is independent of glucose and is directly exerted by insulin (37,38). Dominant negative Mlx did not interfere with the insulin induction of glucokinase mRNA levels (Fig. 5B). Therefore, dominant negative Mlx does not inhibit the expression of genes involved in the insulin-signaling pathway.

Thyroid hormones can also increase the rate of lipogenesis by activating the expression of many lipogenic enzyme genes (39). Carbohydrate and thyroid hormones often have a synergistic effect on the regulation of these genes (40). To exclude the possibility that the inhibition of lipogenic enzyme genes by dominant negative Mlx is mediated by blocking the thyroid hormone pathway, we examined the effect of dominant negative Mlx on a thyroid hormone-responsive mRNA. The active form of thyroid hormones, T_3, is generated from the 'prohormone' T_4 by removal of an outer-ring iodine through the action of iodothyronine deiodinase. Interestingly, the product T_3 in turn induces the expression of the deiodinase 1 (41,42). As expected, no difference was observed in the type 1 deiodinase mRNA levels in either low or high glucose conditions when hepatocytes were treated with control virus (Fig. 5C). In contrast, the mRNA level of deiodinase 1 was significantly induced in the presence of T_3. Addition of high glucose did not further increase deiodinase 1 mRNA levels. Dominant negative Mlx did not affect the mRNA levels of deiodinase 1 in any of the four conditions tested. The small decrease observed in high glucose conditions was not statistically significant, nor was it observed in additional experiments. Therefore, neither insulin nor thyroid hormone signaling is blocked by the dominant negative Mlx and its effects are specific for the glucose-signaling pathway.

**Dominant negative Mlx does not affect the rate of glycolysis** - Although the intracellular signaling pathway that is responsible for activation by glucose remains in question, studies in primary hepatocytes suggest that increased glucose uptake and metabolism are necessary for the generation of the signal. If dominant negative Mlx blocks the intracellular glucose-signaling pathway by decreasing the transcription of glycolytic enzymes such as PK and subsequently reducing the rate of glucose metabolism, the regulation of downstream lipogenic enzyme genes by ChREBP/Mlx may be indirect. To address this question, we monitored the rates of glycolysis by measuring lactate levels (Fig. 6). Hepatocytes were transduced with either control virus or dominant negative virus for two hours and then maintained in low glucose conditions for 36 hours. Cells were then cultured in either low or high glucose conditions and samples of medium were collected in 4, 8, 12, and 24 hours to measure lactate levels. In cells cultured in high glucose, lactate accumulation is accelerated compared with low glucose conditions, due to the elevated rates of glycolysis. Dominant
negative Mlx did not decrease lactate levels in either low or high glucose conditions. This experiment demonstrates that dominant negative Mlx does not impair the rate of glucose metabolism during the course of these experiments. Together with previous experiments, these data provide strong evidence that ChREBP/Mlx directly regulate glucose-responsive lipogenic enzyme genes.

*Mlx is present in the glucose-responsive complex from liver nuclear extract* - The preceding experiments provide a strong case supporting the direct involvement of Mlx in a complex with ChREBP to mediate glucose-responsive transcription. However, Mlx was not reported in the purification that first yielded ChREBP (20). It could be argued that the inhibition by dominant negative Mlx was due to its ability to sequester ChREBP from the ChoRE and that ChREBP functions by itself or with another partner. To test whether Mlx was indeed part of the glucose-responsive complex, EMSA was performed using an antibody to Mlx. As a control, we used cell extracts from 293 cells expressing ChREBP with a Flag tag and wild type Mlx with an HA tag. As observed previously (24), a slow-migrating complex bound to the ACC ChoRE in extracts from 293 cells expressing ChREBP and Mlx (Fig. 7). The migration of this complex was retarded with either anti-Flag or anti-HA antibodies, indicating that this complex contains both ChREBP and Mlx. Addition of the Mlx antibody also shifted the migration of this complex, indicating that this antibody recognizes an exposed epitope in Mlx. A control USF1 antibody did not cause any change in migration of the ChREBP-Mlx complex. The glucose-responsive complex from liver nuclear extract migrated to approximately the same position as the ChREBP/Mlx complex from the 293 cell extract. More importantly, the migration of this complex was shifted by the Mlx antibody, but not USF1 antibody. This result indicates that Mlx is complexed with ChREBP to form the glucose-responsive complex.

**Discussion**

The glucose-stimulated induction of lipogenic enzymes has served as a useful model for exploring nutrient control of gene expression in mammals. Several lines of evidence support a role for the transcription factor ChREBP in this glucose-signaling pathway. First, ChREBP was purified using the PK ChoRE as an affinity reagent and is a member of the bHLH/LZ family that recognizes E box sites, such as those found in the ChoRE (20,21). Second, ChREBP is most abundantly expressed in liver, brown and white adipose tissue where lipogenesis is most active (20,21). Third, ChREBP shuttles between the cytoplasm and nucleus in a glucose-responsive manner in hepatocytes (31). Fourth, mice in which the ChREBP gene has been disrupted or hepatocytes treated with siRNA to reduce ChREBP expression do not induce lipogenic gene expression in response to carbohydrate feeding or glucose, respectively (23,43). Fifth, hepatocytes prepared from ChREBP knockout mice do not support a glucose response when transfected with a ChoRE-containing promoter, but this response can be restored by addition of a ChREBP expression vector (44). Finally, ChREBP has been shown by chromatin immunoprecipitation to bind to the ChoRE region of lipogenic enzyme genes (44).

Recently, we suggested that ChREBP functions in a heteromeric complex with another bHLH/LZ factor, Mlx (24). Binding of ChREBP to the ChoREs from several lipogenic enzyme genes was only observed in the presence of Mlx. Furthermore, while neither ChREBP nor Mlx could activate promoter activity from cotransfected ChoRE-containing promoters in 293 fibroblasts, the combination of these factors was effective. The correlation between binding and activation led us to conclude that Mlx serves as a partner of ChREBP in supporting the glucose response of lipogenic enzyme genes. This conclusion was tempered, however,
because 293 cells do not respond to glucose in a manner analogous to the hepatocyte. Furthermore, when ChREBP was first isolated by affinity chromatography, Mlx was not identified in the bound complex (20). Finally, a recombinant C-terminal fragment of ChREBP containing the bHLH/LZ domain was found to be capable of binding to the PK ChoRE (31). Thus, the role of Mlx remained unconfirmed.

For this reason, we sought to further assess the role of Mlx in the process of glucose-stimulated gene expression in the hepatocyte. The use of a dominant negative that would interfere with the endogenous ChREBP/Mlx interaction allowed us to inhibit this complex and monitor effects of this inhibition. In hepatocytes, we demonstrated that two different dominant negative forms of Mlx could inhibit glucose-stimulated transcription from a promoter driven by the ACC ChoRE. Moreover, the ability of glucose to induce ACC mRNA was completely abrogated in cells transduced with adenovirus expressing the dominant negative Mlx forms. Similarly, induction of several other lipogenic enzymes genes containing previously characterized ChoREs was blunted. Hence, we conclude that the ChREBP/Mlx complex directly regulates the induction of these genes by binding to their promoter regions. Several other genes in the pathway for which ChoREs had not been identified were also inhibited. These include products critical for glucose uptake, NADPH generation and fatty acid maturation. Hence, ChREBP/Mlx appears to play a wide role in regulating the pathway of de novo lipogenesis in the liver. It is possible that ChoREs exist in the promoter regions of these additional genes as well, but these sites have not yet been identified.

Inhibition of the glucose response likely occurs due to a simple binding competition between endogenous wild type Mlx and overexpressed dominant negative Mlx for the limiting pool of ChREBP in the cell. However, Mlx is known from protein interaction studies to dimerize with several other bHLH/LZ proteins, including Mad1, Mad4, Mnt and MondoA (25-28). To ensure that the effects of dominant negative Mlx were not due to interference with one of these other partners, we tested whether addition of ChREBP could restore the glucose response. Overexpression of ChREBP did indeed partially restore glucose-stimulated promoter activity in hepatocytes treated with the dominant negative Mlx. This rescue occurred only at high concentrations of recombinant ChREBP adenovirus that would provide sufficient ChREBP to titrate out the dominant negative Mlx and allow formation of functional heteromers with endogenous Mlx to activate transcription. We surmise that if enough ChREBP could be added, the response would be fully rescued.

An alternative explanation for the effect of the dominant negative Mlx is that ChREBP acts as a homodimer (or a heterodimer with a distinct protein) in supporting the glucose response. In this scenario, the ChREBP/Mlx heteromer would be inactive in promoting the glucose response and the ratio of ChREBP homodimers to ChREBP/Mlx heteromers would determine the amount of active glucose-responsive transcription factor. By adding dominant negative Mlx to the cell, we would be shifting this equilibrium towards the inactive ChREBP/Mlx heteromer. However, the differential abilities of Mlx and MondoA to rescue the glucose response argue against this possibility. When wild type Mlx is overexpressed in the presence of dominant negative Mlx, endogenous ChREBP could bind to either the overexpressed wild type Mlx or dominant negative Mlx. In this case, little ChREBP should be available to form a homodimer and no rescue of the glucose response should be observed. Hence, the ability of wild type Mlx to restore the glucose response argues for a direct role of Mlx in the glucose-responsive complex. We would also note that if ChREBP homodimers were active, then the wild type Mlx by itself should function as a dominant negative. We have not seen any inhibition of the
glucose response in hepatocytes in response to adding wild type Mlx (24). In support of this contention, we have also found that overexpression of MondoA, a paralog of ChREBP (28), did not rescue the glucose response. Since MondoA can also heterodimerize with Mlx, this observation indicates that simply binding to the dominant negative Mlx is not sufficient to provide rescue. Instead, only the active components in the glucose-responsive complex are able to reconstitute this activity, arguing for an essential role of ChREBP and Mlx. In addition, we have found that the ChoRE-binding complex from liver nuclear extracts reacts with an antibody to Mlx and migrates identically with the ChREBP/Mlx complex formed in extracts from 293 cells overexpressing these proteins. Together, these data strongly support an obligatory role for Mlx in the glucose-responsive complex.

A possible concern in interpreting these results was whether the dominant negative Mlx might interfere with other signaling pathways known to be important for supporting the response of lipogenic enzyme genes. The induction of lipogenic enzyme genes by glucose is dependent on effective insulin action. This requirement is due, at least in part, to the insulin induction of glucokinase, a key step in stimulating glucose metabolism in the hepatocyte in the range of glucose concentrations encountered in the liver (43). Insulin has also been shown to be an important signal for the induction of lipogenic enzyme genes in response to high carbohydrate feeding. The actions of insulin in this regard appear to be largely or exclusively mediated by the SREBP transcription factors, especially SREBP-1c (4-6). SREBP-1c transcription is itself induced by insulin and this factor binds to the promoters of most lipogenic enzyme genes to activate their expression (34-36). Mice ablated for the SREBP-1 gene show severely blunted responses to high carbohydrate feeding (45). Hence, any effect of the dominant negative Mlx that interfered with insulin signaling and SREBP-1c action might lead indirectly to an inhibition of the glucose effect. Thyroid hormones have also been shown to function together with glucose in supporting the induction of many lipogenic enzyme genes (39,40,46). Therefore, we tested the effect of introducing dominant negative Mlx on insulin and thyroid hormone signaling pathways. We found that insulin induction of both SREBP-1c and glucokinase gene expression were normal in hepatocytes treated with the dominant negative Mlx. Similarly, the ability of T₃ to induce type I 5'-iodothyronine deiodionase, which is responsive to thyroid hormone, but not glucose, was unaffected. Consequently, the effects of dominant negative Mlx appear to be highly specific to the glucose-signaling pathway.

Based on these observations, we conclude that ChREBP and Mlx function together to provide the essential transcription factors for supporting the glucose response of lipogenic enzyme genes. These factors are targets of an intracellular signaling pathway that is activated by increased glucose metabolism. They function to coordinately regulate a set of genes encoding proteins and enzymes necessary for the hepatocyte to store excess carbohydrate nutrients as triglycerides. The nature of the glucose metabolite that triggers this pathway has been the subject of intense investigation. Recently, xylulose-5-phosphate was proposed to be the key intermediate and to act by stimulating the action of a protein phosphatase 2a isoform that removes inhibitory phosphates from ChREBP (14). Whether Mlx is also directly regulated by glucose or is a silent partner remains to be determined.

Recently, mice deleted for the ChREBP gene have been generated (23). These mice are viable, but display a complex pattern of metabolic abnormalities. The ChREBP-deleted mice are mildly hyperglycemic and insulin-resistant and have greatly elevated hepatic glycogen depots. These effects are consistent with a defect in glycolysis in these animals. The
mRNA levels of several glycolytic and lipogenic enzymes are reduced on both normal and high starch diets. Most importantly, rates of lipogenesis were reduced by greater than 50% in the null mice fed high starch diet compared to their normal counterparts. This change may account for a decrease in both white and brown adipose fat pad weight. The phenotype of the ChREBP null mice provides strong evidence for an important physiological role of ChREBP in glucose utilization and lipogenesis.

For most genes in the hepatocyte, both ChREBP/MLX and SREBP are required for induction and the two signaling pathways function in a highly synergistic manner to support the full transcriptional response to high carbohydrate diet. This overlapping regulatory control of lipogenesis may serve to ensure that lipogenesis, an energy-requiring process, does not occur under inappropriate physiological conditions. However, a few exceptions exist. Glucokinase is not affected by glucose and is controlled in the hepatocyte exclusively by insulin (47). This may reflect the fact that glucokinase lies upstream of all glucose metabolism in the liver, including glycogen and hexose monophosphate shunt pathways. Control of glucokinase is critical for reducing hyperglycemia regardless of the final metabolic fate of the glucose and this control is a fundamental role of insulin. On the other hand, no direct effect of insulin on PK gene expression has been found and it appears to be regulated largely by glucose metabolism. This could reflect a critical role for PK in providing acetyl-CoA for lipogenesis, which is promoted only when glucose levels are greatly elevated. While glucose and insulin work coordinately in normal animals, conditions of elevated insulin and hyperglycemia that occur during states of impaired glucose tolerance might result in abnormal regulation of the SREBP and ChREBP pathways and contribute to the metabolic abnormalities associated with type II diabetes.

Footnotes

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1 The abbreviations used are: PK, pyruvate kinase; ACC, acetyl-CoA carboxylase, FAS, fatty-acid synthase; SCD1, stearoyl-CoA desaturase 1; bHLH/LZ, basic helix-loop-helix/leucine zipper; SREBP-1c, sterol regulatory element binding protein-1c; ChoRE, carbohydrate response element; ChREBP, carbohydrate response element binding protein; MLX, Max-like protein X; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; GFP, green fluorescent protein; GLUT2, glucose transporter 2; ME, malic enzyme; USF, upstream stimulatory factor; RPL32, ribosomal protein large subunit 32; T3, 3,5,3’-triiodothyronine.

2 Ma, L., Tsatsos, N. G. and Towle, H. C., unpublished observations

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Figure Legends

Fig. 1. Dominant negative Mlx efficiently blocks the binding of ChREBP/Mlx to the ACC ChoRE. A, Diagram of dominant negative Mlx forms. Mlx Δb was constructed by deleting the entire basic region (residues 76 – 88). Mlx b/a was constructed by mutating two highly conserved basic residues - R87A and R88S. B, The formation of ChREBP/Mlx complex is inhibited in the presence of either dominant negative Mlx form. EMSA was performed as described under Experimental Procedures with 5 µg of protein from untransfected 293 cells (lane 1) or 293 cells that were transfected with 7.5 µg each of ChREBP and wild type Mlx together with 22.5 µg of CMV4 expression vector (lane 2), 7.5 µg each of ChREBP and wild type Mlx together with 22.5 µg of Mlx Δb (lane 3), or 7.5 µg each of ChREBP and Mlx together with 22.5 µg of Mlx b/a (lane 4). The oligonucleotide used was the ACC ChoRE (18). The black arrow indicates the position of the ChREBP/Mlx complex and the asterisk indicates the position of a background band. These results are representative of several experiments. C, Both forms of dominant negative Mlx are expressed in hepatocytes. Hepatocytes were infected with increasing amount of recombinant adenovirus expressing wild type Mlx (wt Mlx), Mlx Δb or Mlx b/a. Proteins were extracted from these cells, separated by SDS polyacrylamide gel electrophoresis and immunoblotted with HA antibody.

Fig. 2. Dominant negative Mlx inhibits ACC ChoRE-containing promoter activity. Viruses that express either Mlx Δb or Mlx b/a were transduced into hepatocytes at multiplicities of infection of 1, 2.5 or 5. As a control, a recombinant adenovirus that expresses GFP was used at a multiplicity of infection of 5. Hepatocytes were then transfected with 2 µg of firefly luciferase reporter containing the ACC ChoRE and 40 ng of Renilla luciferase reporter. Four hours after transfection, Matrigel was added to the hepatocytes and cells were kept in low glucose medium for an additional 36 hours. Cells were then either refed with low glucose medium or switched to high glucose medium for 24 hours as indicated. Luciferase activity is shown as firefly luciferase light units/Renilla luciferase light units. Values represent the means of triplicate samples ± SD.

Fig. 3. ChREBP or Mlx, but not MondoA, can rescue the glucose response inhibited by dominant negative Mlx. A, Overexpression of ChREBP restores the glucose response. Hepatocytes were untreated or were treated with control GFP virus alone, Mlx b/a virus alone or Mlx b/a virus with increasing amounts of ChREBP virus for 2 hours. Mlx b/a virus was used at a multiplicity of infection of 8 and ChREBP virus was present at levels 3 or 6 times higher. Hepatocytes were then transfected with 2 µg of firefly luciferase reporter containing the ACC ChoRE and 40 ng of Renilla luciferase reporter. Cells were then treated as described in Fig. 2. Values represent the means of triplicate samples ± SD. B, Wild type Mlx can rescue the glucose response, but MondoA cannot. Dominant negative Mlx Δb virus was transduced into hepatocytes at a multiplicity of infection of 4 in low glucose medium for 2 hours, as above. Hepatocytes were then transfected with the reporter plasmids and, where indicated, with plasmids expressing ChREBP, Mlx, or MondoA. Cells were kept in low glucose medium for additional 24 hours before switching to either low or high glucose medium for 24 hours. Values represent the means of triplicate samples ± SD. C, MondoA is expressed from the pcDNA3 expression vector. Cells extracts were prepared from untransfected 293 cells (lane 1); or 293 cells transfected with either a His-tagged DP1 expression plasmid (lane 2) or the MondoA expression plasmid (lane 3). The DP1 transcription factor is an E2F heterodimer partner of 45 kilodaltons (48) that was used as a positive control for the His-tag antibody in this
experiment. After SDS polyacrylamide electrophoresis and blotting, the membrane was reacted with a His-tag antibody for detection of proteins.

Fig. 4. The mRNA level of endogenous glucose-responsive genes are repressed by dominant negative Mlx. The mRNA products measured were ACC, PK, S14, FAS, glucose transporter GLUT2, malic enzyme (ME), SCD1 and, as a control, RPL32. Hepatocytes were untreated or were treated with Mlx b/a virus in low glucose medium for 2 hours. Matrigel was added to the hepatocytes and cells were kept in low glucose medium for additional 36 hours. Cells were then either refed with low glucose medium or switched to high glucose medium for 24 hours. Quantitative PCR was performed on total RNA as described in Experimental Procedures. Values represent the means of triplicate samples ± SD.

Fig. 5. The mRNA levels of genes induced by insulin and T3 are unaffected by dominant negative Mlx. A,B, Insulin-responsive genes are not inhibited by dominant negative Mlx. Hepatocytes were treated with Mlx Δb virus or control GFP virus in low glucose medium without insulin for 2 hours and maintained in this medium for an additional 24 hours. Cells were then refed with low glucose medium without or with insulin for 24 hours. SREBP-1c and glucokinase (GK) mRNA levels were examined by quantitative PCR. Values represent the means of triplicate samples ± SD. C, The thyroid hormone pathway is not inhibited by dominant negative Mlx. Hepatocytes were treated with Mlx Δb virus or control virus in low glucose medium and maintained in this media for 24. Cells were then refed with low or high glucose medium without or with 0.5 µM T3. Type I 5’-iodothyronine deiodinase (DI) mRNA levels were measured by quantitative PCR. Values represent the means of triplicate samples ± SD.

Fig. 6. The rate of glycolysis in hepatocytes is unaffected by dominant negative Mlx. Cells were first transduced with either Mlx b/a virus or control virus for 2 h and then maintained in low glucose for 36 hours. Cells were then either refed with low glucose medium or switch to high glucose medium for 24 hours. Aliquots of the medium were collected during the 24 hour induction period at the times indicated. Lactate levels were measured using the Lactate Reagent kit (Sigma). Open squares = low glucose with control virus; closed triangles = low glucose with Mlx b/a; open diamonds = high glucose with control virus; closed squares = high glucose with Mlx b/a. Values represent the means of triplicate samples ± SD.

Fig. 7. Mlx is present in the glucose-responsive complex of rat liver nuclear extract. EMSA was performed as described under Experimental Procedures with 5 µg of protein from untransfected 293 cells (lane 1) or 293 cell transfected with flag-tagged ChREBP and HA-tagged wild type Mlx (lanes 2-6) or 10 µg of rat liver nuclear extract (lanes 7-10). The following antibodies were added: anti-Flag (lane 3), anti-HA (lane 4), anti-Mlx (lane 5), anti-USF (lane 6), 1 µl of anti-Mlx (lane 8), 3 µl of anti-Mlx (lane 9), anti-USF (lane 10). The oligonucleotide used was the ACC ChoRE. The black arrow indicates the position of ChREBP/Mlx complex and the white arrow indicates the position of the antibody-supershifted complexes. The asterisks indicate the position of background bands. These results are representative of several experiments.
References

Fig 1

A

Mlx b/a

wt Mlx

Mlx Δb

B

1 2 3 4

C

wt Mlx Mlx Δb Mlx b/a

*
Relative Light Units

Low Glucose
High Glucose

Adenoviral Construct

Fig 2

GFP  Mix Δb  Mix b/a

Low Glucose  High Glucose
Fig 3

A

B

C
Fig 4

Gene expression levels for various genes under different conditions:

- **ACC**
- **PK**
- **S_{14}**
- **FAS**
- **SCD-1**
- **ME**
- **GLUT2**
- **RPL32**
Fig 5

A

B

C

mRNA Fold Induction

SREBP-1c

GFP

Mlx Δb

GFP

Mlx Δb

mRNA Fold Induction

Gluc:    L     H     L     H               L     H      L     H

T3:        -     -     +     +               -     -     +     +

Ins:

GFP

Mlx Δb

DI
Fig 6
Fig 7
Direct role of ChREBP/MLx in regulating hepatic glucose-responsive genes
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