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HUMAN MUTATION Mutation in Brief #1029, 29:E271–E277, (2008) Online

MUTATION IN BRIEF

Mutations in the Small Heterodimer Partner Gene Increase Morbidity Risk in Japanese Type 2 Diabetes Patients

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Mutations in the small heterodimer partner gene (NR0B2; alias SHP) are associated with high birth weight and mild obesity in Japanese children. *SHP* mutations may also be associated with later obesity and insulin resistance syndrome that induces diabetes. To investigate this possibility, the prevalence of *SHP* mutations in Japanese with and without type 2 diabetes mellitus and the functional properties of the mutant proteins were evaluated. Direct sequencing of two exons and flanking sequences of *SHP* in 805 diabetic patients and 752 non-diabetic controls identified 15 different mutations in 44 subjects, including 6 novel mutations. Functional analyses of the mutant proteins revealed significantly reduced activity of nine of the mutations. Mutations with reduced activity were found in 19 patients (2.4%) in the diabetic group and in 6 subjects (0.8%) in the control group. The frequency difference between DM and control subjects adjusted for sex and age was statistically significant (P=0.029, odds ratio 2.67, 95% CI 1.05 – 6.81, 1-β=0.91). We conclude that *SHP* mutations associated with mild obesity in childhood increase susceptibility to type 2 diabetes in later life in Japanese. © 2008 Wiley-Liss, Inc.

KEY WORDS: SHP, type 2 diabetes, obesity, fatty liver, NASH

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INTRODUCTION

Type 2 diabetes mellitus is characterized by defects of insulin secretion in pancreatic b-cells and insulin action in peripheral tissues. Failure of pancreatic β-cells to compensate for insulin resistance by increasing insulin secretion is thought to underlie the development of type 2 diabetes (Reaven, 1988, Polonsky, 2000).

We have previously shown that mutations in the gene encoding small heterodimer partner (*NR0B2*, alias *SHP*; MIM# 604630), an orphan nuclear receptor that interacts with a number of other receptors (Seol et al., 1996, Masuda et al., 1997, Seol et al., 1998, Johansson et al., 1999), are associated with high birth weight and mild obesity in Japanese children, although the molecular mechanisms by which the *SHP* mutations cause these disorders are unknown (Nishigori et al., 2001).

Nuclear receptors such as SHP and peroxisome proliferator-activated receptor (PPAR) α that regulate lipid metabolism in liver are potential contributors to fatty liver. It should be noted that the storage of lipids in liver can trigger inter-organ crosstalk systems that affect insulin sensitivity in muscle. Farnesoid X receptor (FXR)-null mice, with reduced levels of SHP, develop severe fatty liver and elevated circulating FFAs, which is associated with elevated serum glucose and impaired glucose and insulin tolerance resulting from attenuated inhibition of hepatic glucose production by insulin and reduced peripheral glucose disposal (Ma et al., 2006). Some patients with *SHP* mutations exhibit liver dysfunction due to fatty liver (Nishigori et al., 2001). Accordingly, mutations in *SHP* may be associated with insulin resistance due to both later obesity and also to fatty liver in Japanese subjects.

Nonalcoholic fatty liver disease (NAFLD) is a polygenic disease caused by a combination of environmental and genetic factors. Potential candidate genes contributing to NAFLD, a condition comprising a spectrum of pathological liver conditions ranging from steatosis alone to non-alcoholic steatohepatitis (NASH), include those involved in fat deposition, insulin sensitivity, and hepatic lipid oxidation, synthesis, storage, and export. NASH is believed to be a hepatic expression of metabolic syndrome (Ono and Saibara, 2006). In this regard, genetic abnormalities manifested in obesity and fatty liver might well act in concert to induce diabetes.

To evaluate the influence of *SHP* mutations on risk of later development of type 2 diabetes, we examined the frequencies of these mutations in Japanese subjects with and without type 2 diabetes mellitus as well as in patients with NASH.

MATERIALS AND METHODS

Patient populations

The ADA definitions of type 2 diabetes were used. Obesity is defined in these studies as BMI of >25 kg/m^2, in accord with the criteria of the Japan Society for the Study of Obesity (Japanese Society for the Study of Obesity, 2000) and the report by WHO (Western Pacific Region) and IASO/IOTF (International Association for the Study of Obesity/International Obesity Task Force) (WHO and IASO/IOTF, 2000). We evaluated the prevalence of SHP mutations in 805 Japanese patients with type 2 diabetes (male/female, 432/373: age, 60.3±11.8 yr.; BMI, 24.1 ± 4.0 kg/m^2) and 752 non-diabetic controls (male/female, 418/334; age, 59.7±13.3 yr.; BMI, 22.9±2.9 kg/m^2). Informed consent was obtained from all of the diabetic subjects and volunteer controls. NASH patients with nonalcoholic fatty liver disease underwent liver biopsy after signed informed consent and thorough clinical evaluation. Liver biopsy was analyzed by a pathologist (H.E.) and the diagnosis of NASH was based on Brunt's criteria (Brunt et al., 1999). Laboratory blood tests and BMI were analyzed in 93 biopsy-proved NASH patients (48 males and 45 females, age: 29.2 ± 5.4 years old, BMI: 29.2 ± 5.4 kg/m2, ALT: 102.6 ± 66.6 IU/L, T Chol: 5.25 ± 1.05 mmol/L, TG: 1.74 ± 0.88 mmol/L, HDL-C: 1.24 ± 0.35 mmol/L, HbA1c: 5.6 ± 1.0 %, FPG: 6.03 ± 1.79 mmol/L).

Mutation analysis

The two exons and flanking regions of the SHP gene were screened for mutations by direct DNA sequencing of the amplified polymerase chain reaction (PCR) products, using specific primer pairs and an ABI PRISM BigDye Terminator Cycle Sequencing FS ready Reaction Kit (Applied Biosystems, Foster City, CA). Primer pairs and PCR conditions used for screening of the SHP gene are as follows. Exon1: 5'-CATGACTTCTGGAGTCAAGG-3' and 5'-GTCCCTTTCAGGCAGGCATA-3',

5'-CATCCTTCTGGCAGCTGCCT-3' and 5'-TTAGAAGCTACCTTCCCTGGCT

GG-3' Exon2: 5'-CAGATCTTGGGCCAGTCTTG-3' and 5'-CTCCAGGAGCATTG GGTCAC-3'. Genomic DNA extracted from diabetic and control subjects was initially denatured at 95° C for 1 min, followed by 35 cycles of denaturation at 94° C for 30 sec, annealing at 60° C or 62° C for 30 sec, extension at 72° C for 30 sec, and a final extension step of 7 min. The sequencing reactions were analyzed by automatic DNA sequencers (Applied Biosystems models 3100 and 3700).

Mutation Nomenclature

The cDNA NM_021969.1 and protein NP_068804.1 sequences were used for mutation nomenclature, with DNA +1 corresponding to the A of the ATG translation initiation codon. Descriptions of all sequence variants were checked using the Mutalyzer program (http://www.LOVD.nl/mutalyzer/).

Functional analysis of SHP mutant proteins

Analysis of the functional properties of mutant and wild-type proteins was performed as described previously (Nishigori et al., 2001). Briefly, the *SHP* mutations newly identified in this study were generated by PCR-based site-directed mutagenesis and cloned in the expression pCMV-6b vector. The sequences for wild-type and mutant SHP proteins, and HNF-4α were cloned in pCMV-6b and pcDNA3.1 (Invitrogen, Groningen, The Netherlands), respectively. For luciferase reporter assays, the promoter region of the human HNF-1α gene was inserted into the pGL3-Basic Reporter vector (Promega, Madison, WI).

HepG2 cells ($1x10^5$) were grown in 6-well plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum. The cells were transfected with ExGen 500 solution (6.6 ml) (Fermentas, Ontario, Canada), 333 ng of HNF-1α-promoter/reporter construct, 100 ng of HNF-4α-expression plasmid, 0-125 ng of test DNA, and 17 ng of pRL (Renilla luciferase)-TK. Luciferase reporter activity was measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Renilla luciferase activity was used to normalize transfection efficiencies among experiments.

Statistical analyses

Statistical difference in frequencies of *SHP* mutations between the diabetic and control groups was analyzed by logistic regression analysis, using a package of STATVIEW 5.0 (SAS Institute Inc., Cary, NC). Data obtained by luciferase reporter assay were analyzed by the Student's *t*-test.

RESULTS

Eight hundred five Japanese patients with adult-onset type 2 diabetes (T2DM), 752 non-diabetic controls, and 93 patients with NASH were examined. Screening of the SHP gene (*NR0B2*) by direct sequencing resulted in the identification of fifteen different mutations (c.100C>T [p.Arg34X], c.112C>T [p.Arg38Cys], c.134G>C [p.Arg45Pro], c.157_166del [p.His53AlafsX50], c.160C>T [p.Arg54Cys], c.169C>T [p.Arg57Trp], c.292_300delinsAC [p.Leu98ThyfsX6], c.314T>G [p.Val105Gly], c.512G>C [p.Gly171Ala], c.532G>A [p.Asp178Asn], c.566G>A [p.Gly189Glu], c.583G>T [p.Ala195Ser], c.618G>A [p.Trp206X], c.637C>T [p.Arg213Cys], and c.647G>A [p.Arg216His]) including six novel mutations in type 2 diabetic patients (Table 1),

eight of which were previously identified in obese children (Nishigori et al., 2001) and one of which, p.Gly171Ala, was reported as a polymorphism in a study of Caucasians (Hung et al., 2003, Echwald et al., 2004, Mitchell et al., 2003). In NASH patients, only one mutation, p.Arg45Pro, was identified. We could not find any variants in flanking sequences.

Table 1: Mutations identified in the human SHP gene (*NR0B2*).

Exon	Codon	Nucleotide change	Designation	Patients (n=805)	Controls (n=752)
Mutations with reduced activity					
1	34	c.100C>T	p.Arg34X [a)b)c)d)e)]	2	0
1	53	c.157_166del	p.His53AlafsX50 [a)b)c)e)]	2	0
1	54	c.160C>T	p.Arg54Cys*	0	1
1	57	c.169C>T	p.Arg57Trp [a)]	1	0
1	98	c.292_300 delinsAC	p.Leu98ThyfsX6 [a)c)e)]	6	1
1	105	c.314T>G	p.Val105Gly*	1	0
2	189	c.566G>A	p.Gly189Glu [a)]	3	0
2	195	c.583G>T	p.Ala195Ser [a)c)d)e)]	1	3
2	206	c.618G>A	p.Trp206X*	2	1
2	213	c.637C>T	p.Arg213Cys [a)b)c)e)]	1	0
			sum	19 (2.4%)	6 (0.8%)
Mutations with normal activity					
1	38	c.112C>T	p.Arg38Cys*	1	0
1	45	c.134G>C	p.Arg45Pro*	1	0
1	171	c.512G>C	p.Gly171Ala	0	1
1-2	178	c.532G>A	p.Asp178Asn*	1	0
2	216	c.647G>A	p.Arg216His	6	9
			sum	9 (1.1%)	10 (1.3%)

* indicates six novel variants identified in the present study.

To determine if the mutations alter the function of the SHP protein, the effect of the wild-type and mutant proteins on HNF-4α-mediated transactivation of HNF-1α gene transcription in HepG2 cells was examined by luciferase reporter assay (Fig. 1 and Nishigori et al., 2001). a) early-onset obesity, b) high birth weight, c) diabetes, d) fatty liver, e) decreased insulin sensitivity (Nishigori et al., 2001) Mutations were numbered according to GenBank NM_021969.1 and NP_068804.1. Nucleotide +1 is A of the ATG initiation codon.

Functional analyses of the novel mutant proteins showed significantly reduced activity of transcriptional regulation of HNF-4α, except in the case of p.Arg38Cys, p.Arg45Pro, and p.Asp178Asn. The results of functional analyses of the mutations newly identified in this study are shown in Fig. 1. The mutations with reduced activity were found in nineteen subjects (2.4%) in the diabetic group, six (0.8%) in the control group, and none in the NASH group (Table 1). The frequency difference between DM and control groups was statistically significant by logistic regression analysis considering gender and age (P=0.029; 1-β =0.91) with odds ratio of 2.67 [95% CI, 1.05-6.81]. This frequency difference between DM and control groups came to be not statistically significant by logistic regression analysis when considering gender, age, and BMI (P=0.078), with odds ratio of 2.26 [95% CI, 0.87-5.86]. Furthermore, subjects with mutations of reduced activity showed significantly higher BMI than subjects without the mutations (25.6 ± 4.6 vs 23.5 ± 3.6, P=0.0039 in combined subjects, and 24.0 ± 4.0 vs. 26.5 ± 5.0, P=0.012 in diabetic patients). In control subjects, those with mutations of reduced activity showed similar BMI to those without mutations (22.9 ± 2.9 vs. 23.1 ± 2.7, P=0.87).

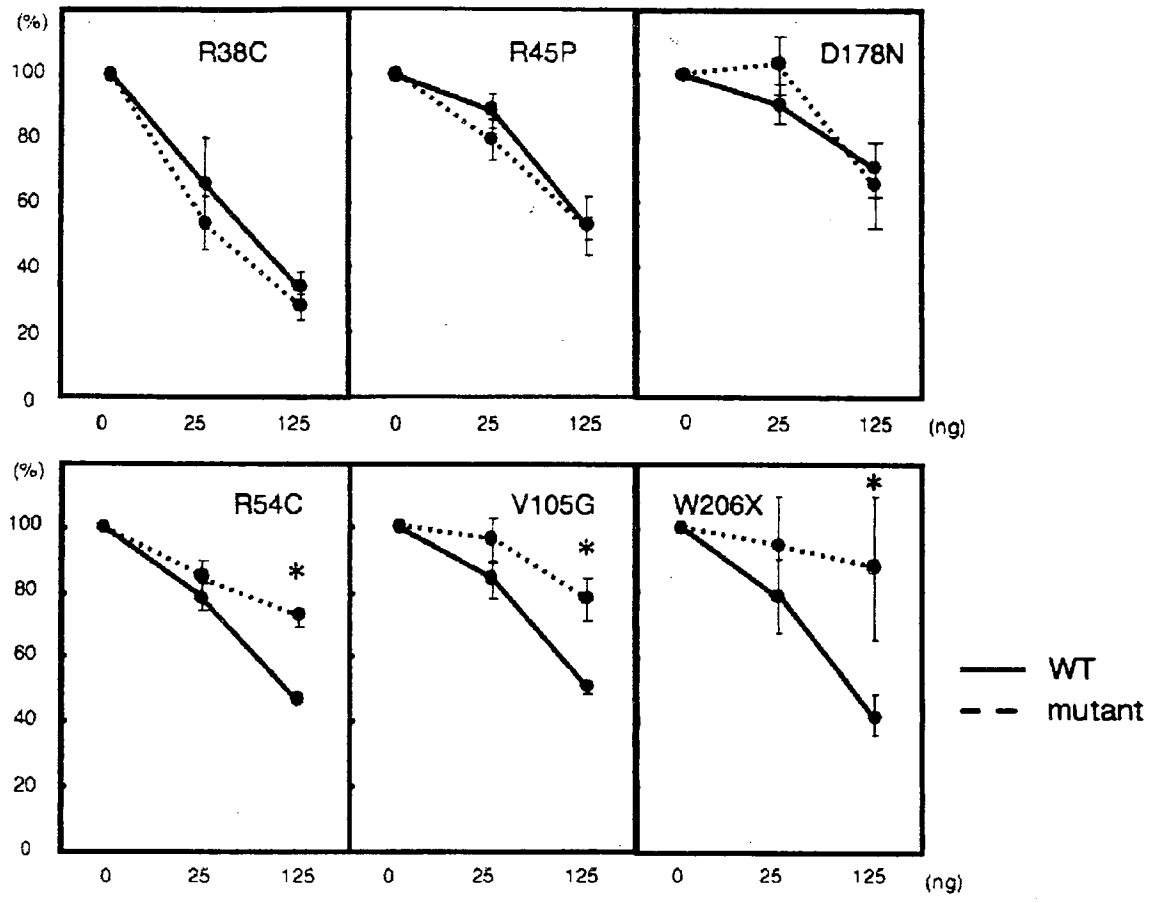

Figure 1: Inhibition of transactivation activity of HNF-4α by wild-type and mutant SHP proteins. It has been shown previously that expression of wild-type SHP significantly decreases HNF-4α transactivation of the HNF-1α gene prompter in HepG2 cells, indicating that SHP is a negative regulator of HNF-4α (Nishigori et al., 2001, Lee et al., 2000). Transcriptional regulation of the novel six mutations of p.Arg38Cys, p.Arg45Pro, p.Arg54Cys, p.Val105Gly, p.Asp178Asn and p.Trp206X was examined by luciferase reporter assay (n=3 in each experiment). Functional properties of the other mutations identified have been examined previously (Nishigori et al., 2001, Echwald et al., 2004). The relative luciferase activity (firefly/Renilla) of each construct at 0 ng, 25 ng, and 125 ng of wild-type and mutant SHP proteins was measured in HepG2 cells. Percent activity in relation to basic HNF-4 α activity is shown as mean ± SD. * $P<0.05$.

DISCUSSION

Mutations in the SHP gene have been shown to be associated with high birth weight and early-onset mild obesity in Japanese. Although the molecular mechanism by which these mutations increase body weight is unknown at present, one possibility is suggested by the fact that pancreatic β cells express SHP mRNAs at high levels. Since SHP inhibits HNF-4α (MODY1 protein) (Nishigori et al., 2001,Lee et al., 2000), functional defects of SHP might well increase the activity of HNF-4α and other downstream components of glycolytic signal transduction (Dukes et al., 1998), resulting in increased insulin secretory response to glucose (Wang et al., 2006). In addition, since insulin is a key hormone in fetal growth, high levels of fetal insulin may well be associated with high birth weight and postnatal obesity.

As adult-onset type 2 diabetes is a polygenic disorder requiring interaction of multiple genetic and environmental factors, and Japanese patients exhibit a lesser insulin secretory capacity due to pancreatic β–cell

dysfunction (Kosaka et al., 1977, Kosaka and Akanuma, 1980, Yoshinaga and Kosaka, 1999), the increased insulin secretory demand associated with *SHP* mutations might increase susceptibility to type 2 diabetes in this population. Since other nuclear receptors that interact with SHP in peripheral tissues (Seol et al., 1996, Masuda et al., 1997, Seol et al., 1998, Johansson et al., 1999) may be involved in the pathogenesis of insulin resistance due to obesity or fatty liver, the secondary demand for compensatory insulin secretion might also promote the development of overt diabetes.

FXR-null mice, which show reduced levels of SHP, exhibit elevated plasma cholesterol and triglyceride levels and excessive accumulation of fat in the liver (Ma et al., 2006). Fatty liver also was observed in some early-onset obesity patients with *SHP* mutations (Nishigori et al., 2001). In addition, increased insulin secretion derived from *SHP* mutations accelerates fat accumulation in the liver. Accordingly, we examined 93 NASH patients, and found one mutation. However, none of the mutations associated with reduced activity was found in the NASH group, suggesting that the effect of SHP on the accumulation of fat in the liver may be of little clinical importance.

While we cannot link the etiology of NASH to mutation of *SHP*, the finding that *SHP* mutations increase morbidity risk for type 2 diabetes due to mild obesity in later life in Japanese suggests a genetic link between obesity and type 2 diabetes in human. To clarify the complex relationship of type 2 diabetes with SHP deficiency, further genetic analysis and characterization of the diabetogenic factors involved is required.

According to previous epidemiological studies, low birth weight and fetal thinness are associated with insulin resistance syndrome and were therefore thought to be related to later risk of type 2 diabetes (Hales et al., 1991, Eriksson et al., 2003). In contrast, we demonstrate here an increased morbidity risk of type 2 diabetes due to *SHP* mutations associated with high birth weight and mild obesity in Japanese children. Further analysis of the functional properties of mutant SHP proteins in energy expenditure should provide new insight into the relationship between birth weight and the development of type 2 diabetes.

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Regulation of lipogenesis via BHLHB2/DEC1 and ChREBP feedback looping

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ABSTRACT

BHLHB2/DEC1 is a transcription factor implicated in cell proliferation, apoptosis, and metabolism, and is also known to play an important role in the regulation of the mammalian circadian rhythm. However, its precise role in metabolism remains unclear. We investigated the link between BHLHB2 and ChREBP, a glucose-activated transcription factor involved in the regulation of lipogenesis. Glucose stimulation and overexpression of dominant active ChREBP induced *Bhlhb2* mRNA expression in rat hepatocytes. Deletion studies showed that ChoRE (−160 to −143 bp) in the mouse *Bhlhb2* promoter region is functional in vivo. Overexpression of BHLHB2 inhibited glucose and ChREBP-mediated induction of rat *Fasn* and liver pyruvate kinase (*Lpk*) mRNA. ChIP assay demonstrated that BHLHB2 bound to ChoRE in the *Fasn*, *Lpk*, and *Bhlhb2* promoter regions in vivo. In conclusion, BHLHB2 and ChREBP constitute a novel feedback loop involved in the regulation of lipogenesis.

Excess carbohydrate intake leads to fat accumulation, insulin resistance, and development of metabolic syndrome. Glucose and insulin coordinately regulate de novo lipogenesis in the liver [1]. Insulin regulates lipogenic enzymes and gene expression by the activation of several transcription factors such as sterol regulating element binding protein 1c (SREBP1c) [2]. Glucose also activates lipogenic gene expression by activating the carbohydrate response element binding protein (ChREBP) [1]. ChREBP and Mlx form a heterodimer and bind to the carbohydrate response element (ChoRE) in the promoter of glycolytic and lipogenic enzymes such as liver-type pyruvate kinase (*Lpk*) and fatty acid synthase (*Fasn*) [3,4].

We previously reported that deletion of the ChREBP gene in *ob/ob* mice decreased obesity, hyperglycemia, and fatty liver [4]; however, there was a high amount of glycogen accumulation and hepatomegaly, indicating the need for an alternative therapy. ChoRE is composed of two E-boxes separated by 5 bp [1]. As basic-helix-loop-helix (Bhlh) transcription factors can bind to the E-box, we hypothesized that some of these transcription factors might compete with ChREBP for binding to ChoRE, thereby antagonizing ChREBP transcriptional activity. Therefore, among the transcription factors bound to the E-box, we focused on the transcription repressor basic helix-loop-helix binding Protein 2 (BHLHB2).

BHLHB2 (DEC1 or STRA13) encodes a deduced 412-amino acid protein containing a basic helix-loop-helix (BHLH) domain [5]. BHLHB2 represents a large and diverse class of transcription factors implicated in cell proliferation, apoptosis, and metabolism [5], and is known to play an important role in the regulation of the mammalian circadian rhythm [6]. BHLHB2 and BHLHB3 are helix-loop-helix transcription factors that repress Clock/BMAL1-induced trans-activation of the mouse *Per1* promoter through direct protein–protein interactions with Bmal1 and/or competition for E-box elements [6]. Recently, deletion of the *Clock* gene was reported to cause metabolic syndrome [7], and *Bhlhb2* mRNA was reported to be increased in muscles of diabetic and insulin-resistant humans [8]. These findings suggest that *Bhlhb2* plays an important role in the development of metabolic syndrome.

In this study, we show that glucose induces *Bhlhb2* gene expression through ChREBP activation. Moreover, in rat hepatocytes, overexpression of BHLHB2 inhibits the expression of glucose-induced lipogenic genes by inhibiting the binding of CHREBP to the carbohydrate response element (ChoRE). Thus, this study helps to clarify the relationship between ChREBP and BHLHB2 in glucose regulation, lipid metabolism, and the pathology of metabolic syndrome.

Materials and methods

Animals, isolation of rat primary hepatocytes, and cell culture. The protocols for all animal experiments were approved by the Institutional Animal Care and Use Committee of Gunma University Medical School (code no. 08–025). Rat primary hepatocytes were isolated from 6-week-old male Wister rats by the collagenase perfusion methods [4]. Isolated hepatocytes were suspended with DMEM supplemented with 10% fetal calf serum (FCS), 100 nM insulin, 100 nM dexamethasone (dex), 10 nM triiodothronine (T_3), and 100 μg/ml penicillin/streptomycin (pen/strep). Cells were seeded

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in 6-well plates or 10-cm dishes and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After the cells were incubated for 4 h, the media was removed and exchanged into DMEM containing 10 nM T_3.

Construction of plasmid and adenovirus vectors. PCR was performed using Prime star DNA polymerase (Takara) and Primers (Sigma–Aldrich). Rat FLAG-tagged dominant active ChREBP deleting 1–196 amino acids (daChREBP) and mouse FLAG-tagged BHLHB2 full length cDNA were amplified from rat and mouse liver cDNA [9]. The nucleotide sequences of PCR primers are shown in Table 1. These PCR fragments were cloned into the pENTR vector (Invitrogen) to produce pENTR-daChREBP and pENTR-BHLHB2. The adenovirus vectors bearing BHLHB2 and daChREBP cDNA were constructed to recombine pENTR-daChREBP or pENTR-BHLHB2 vectors into pAd/CMV/V5-DEST using the LR Clonase II master mix (Invitrogen) according to manufacturer's protocol. The pcDNA6.2 vectors bearing BHLHB2 cDNA (pcDNA-BHLHB2) and daChREBP cDNA (pcDNA-daChREBP) were constructed in the same manner as the adenovirus. The series of 5′-deletions in *Bhlhb2* promoter, termed −1088, −174, −138, and −78 bp vectors were inserted into the pGL3 basic vector (Promega). These promoter fragments were amplified using PCR primer (−1088 F, −174 F, −138 F, −78 F and -R) and Ex Taq DNA polymerase (Takara) (Table 1). The PCR products were digested with HindIII and ligated with pGL3 basic vectors precut by Hind III. The reporter vectors, pGL3-*Fasn* and pGL3-*Lpk*, were the same vectors as used previously [3]. pGL4.74[hRLuc/TK] was purchased from Promega. pGL3 promoter vector was digested with MluI and BglII and dephosphorylated with calf intestinal alkaline phosphatase (Takara). Two oligonucleotides of BHLHB2 (3XChoRE) were denatured at 90 °C for 10 min and annealed at room temperature for 1 h (Table 1). These double stranded oligonucleotides were ligated into the pGL3 promoter vector predigested by MluI and BglII.

Table 1
Oligonucleotide sequences for PCR primers and double stranded DNA inserted into pGL3 promoter vector

Gene name		Nucleotide sequences
DaChREBP (flag)	F	5′-CACCATGGATTACAAGGATGACGACGATAAGATCAGGGA AGGGGATTTCCTGGCTCCCAAGC-3′
	R	5′-TTATAATGGTCTGCCCAGGGGACCCTCTGTG-3′
BHLHB2 (flag)	F	5′-CACCATGGATTACAAGGATGACGACGATAAGATCGAACG GATCCCCAGCGCGCAACC -3′
	R	5′-TTAGTCTTTGGTTTCTAAGT-3′
BHLHB2 promoter	−1088 bp F	5′-gcaagcttTGCAAGTGAGCCGAGCTCCGGCCCG-3′
	−174 bp F	5′-gcaagcttCACTTCGCAGCCGCCAGAGCG-3′
	−138 bp F	5′-gcaagcttGGGAGGGCGGGCAGGTCG-3′
	−78 bp F	5′-gcaagcttACCCACTCGCTCCCATTTAACCC-3′
	R	5′-CGCCGTGCGAGCCAAGTGAAT-3′
BHLHB2 (3XChoRE)	F	5′-CGCGGTCCAACACGTGAGGCTCATGTGATGAAGGTCCAA CACGTGAGGCTCATGTGATGAAGGTCCAACACGTGAGGCTC ATGTGATGAAG-3
	R	5′-GATCCTTCATCACATGAGCCTCACGTGTTGGACCTTCATC ACATGAGCCTCACGTGTTGGACCTTCATCACATGAGCCTCAC GTGTTGGAC-3′
LPK for CHIP	F	5′-CTTTGATCCGAGGCTCTGCAGAC-3′
	R	5′-TGAGTCCTGGTTAAAGTATAACC-3′
BHLHB2 for CHIP	F	5′-GCAGCCGCAGACACCTGGGGCCCGAGG-3′
	R	5′-CGTGTTCTACCCTGTGACTCCAAGCAC-3′
FASN for CHIP	F	5′-AAAGGCCTGCTCTGGAATCATTCTC-3′
	R	5′-CAGAGAGGCTTGCTGAAGCTGAGACC-3′

Mammalian transfection and Reporter assay. Primary hepatocytes were cultured in 6 plates in 2 ml DMEM medium without anibiotics. After 20 multiplicity of infection (m.o.i.) of adenovirus bearing GFP, BHLHB2 or daChREBP cDNA was infected into the hepatocytes for 2 h, and the cells were transfected with lipofectamine2000 (10 μl), pGL3-*Fasn*, or -*Lpk* (3.6 μg), or a deletion series of BHLHB2 vectors and the pGL4.74[hRLuc/TK] vector (0.4 μg). After 24 h of incubation, the cells were collected and used to measure luciferase activity (Dual Luciferase assay system; Promega) according to Manufacture's protocol. To measure the effect of BHLHB2 and daCHREBP, cells were cotransfected with the pGL3-*Fasn* or -*Lpk* vector (3.0 μg), pGL4.74[hRLuc/TK] vector (0.4 μg), pcDNA-BHLHB2 (0.2 μg), and/or pcDNA-daChREBP (0.2 μg). The total amount of DNA was adjusted by the addition of an empty vector.

RNA isolation and semi-quantitative RT-PCR. Total RNA was isolated using the RNeasy Mini Plus kit (Qiagen) according to the manufacturer's protocol. Single-stranded cDNA was synthesized from the RNA of each sample (0.1 μg), 0.01 mM DTT, 0.5 mM of each dNTP, random primer (0.5 μg), 40 U RNase Out ribonuclease inihibitor, and 200 U SUPERSCRIPT III reverse transcriptase (Invitrogen). The resulting cDNA products (1 ng), water (5 μl), and PCR master mix (10 μl) were used in the Taqman PCR assay with an ABI prism 7900 sequencing detector (Applied Biosystems) on the cDNA samples. Taqman PCR probes for semi-quantitative RT-PCR were purchased from Applied Biosystems.

Chromatin immunoprecipitation (ChIP) assay. Rat hepatocytes were isolated and cultured in 10-cm culture dishes in DMEM supplemented with 10% FCS, 100 nM insulin, 100 nM dex, 10 nM T_3 and 100 μg/ml pen/strep for 4 h and then for 2 h in DMEM supplemented with 10 nM T_3 and 100 μg/ml pen/strep. After the incubations, 10% formaldehyde (270 μl) was added to the culture dishes and the cells were used in the chromatin immunoprecipitation (ChIP) assay according to the manufacturer's protocol. The purified DNA was dissolved in Tris/EDTA (50 μl, pH 8.0) and used with gene specific primers for the PCR (Table 1). The ChIP assays were performed using anti-Mlx (Santa Cruiz Biotechnology), anti-ChREBP (Cayman Chemical) and anti-FLAG antibody (Sigma–Aldrich). Buffer only and rabbit normal IgG (Wako Chemical) were used as the negative control.

Statistical analyses. Results are reported as means ± SD. The comparison of different groups was carried out using two-tailed unpaired Student's *t* test. Differences were considered statistically significant at $p < 0.05$.

Results

Glucose activation of Bhlhb2 gene expression

Increases in *Bhlhb2* mRNA have been reported for diabetic and insulin-resistant patients, and insulin is known to increase *Bhlhb2* expression [5,8]; however, whether glucose activates *Bhlhb2* expression remains unclear. Mouse, rat, and human proximal *Bhlhb2* promoters contain the conserved ChoRE, which is composed of one perfect E-box and one imperfect E-box, separated by 5-bp spaces (Fig. 1A). *Lpk* and *Fasn* are well known to be glucose-response genes targeted by ChREBP [1].

We therefore used *Lpk* and *Fasn* as positive controls for our experiments. Glucose dose-dependently activated *Bhlhb2* mRNA expression in rat primary hepatocytes, and there was a 2.15-fold increase in *Bhlhb2* mRNA at a glucose concentration of 25 mM compared with that at 0.1 mM glucose (Fig. 1B). Moreover, increases of 2.4-, 1.1-, and 5.3-fold were found in cells treated with insulin alone, high-concentration glucose alone, or in combination, respectively (Fig. 1C). We then constructed a series of deletion mutants of the pGL3-BHLHB2 vectors to test the role of putative ChoRE on glucose-activated *Bhlhb2* expression. Glucose activated only the −1088 and −174 bp vectors, including putative ChoRE (Fig. 1D).

ChREBP regulatation of Bhlhb2 gene expression

ChREBP is activated by glucose and binds to ChoRE of the lipogenic gene promoter region. The ChoRE of mouse *Bbhlhb2* is similar to ChoRE of *Lpk* and *Fasn* [1]. We then tested whether ChREBP regulates mouse *Bhlhb2* mRNA expression by binding directly to ChoRE of the *Bhlhb2* gene promoter. The *Bhlhb2* mRNA level was dose-dependently up-regulated by the overexpression of daChREBP (Fig. 2A). By increasing the dose of adenovirus bearing daChREBP cDNA, ChREBP mRNA was increased 5-, 12-, and 63-fold compared with endogenous ChREBP mRNA. Like *Fasn* and *Lpk*, *Bhlhb2* mRNA was also increased 2.1-fold. To test whether daChREBP could directly activate mouse *Bhlhb2* gene promoter, we ran the reporter assay against the series of deleted pGL3-Bhlhb2 vectors (−1088, −174, −138, −78 bp) (Fig. 1D). Overexpression of daChREBP increased the transactivity of −1088 and −174, which contain putative ChoRE (Fig. 2B). We also used pGL3 promoter vectors containing tandem 3XChoRE (from −166 to −138 bp) and tested whether a putative ChoRE possessed glucose responsiveness. The luciferease activity at a glucose concentration of 25 mM was 2-fold higher than that of the control cells (Fig. 2C) and the daChREBP overexpression was 18-fold higher than that in the control cells; however, BHLHB2 alone potently inhibited reporter activity to one-tenth the level of the control cells. Thus, ChoRE in *Bhlhb2* gene promoter possessed glucose responsiveness. Consistent with these data, the ChIP assay showed that ChREBP and Mlx bound to ChoRE of mouse *Bhlhb2* and *Lpk* (Fig. 2D).

BHLHB2 antagonizes the effect of ChREBP on the transactivities of LPK and FAS promoter

BHLHB2 is known to be a transcriptional repressor of gene expression by binding to the E-box (consensus sequence: CACGTG) in the promoter region. ChoRE is composed of two E-boxes separated by a 5-bp insert. We then examined the role of BHLHB2 in glucose activation of lipogenic gene expression. Adenoviral overexpression of BHLHB2 cDNA dose-dependently suppressed glucose-induced mRNA expression of *Fasn* and *Lpk* (Fig. 3A). Interestingly, the inhibitory effect of BHLHB2 on the *Fasn* gene was more potent than that on *Lpk*, probably due to

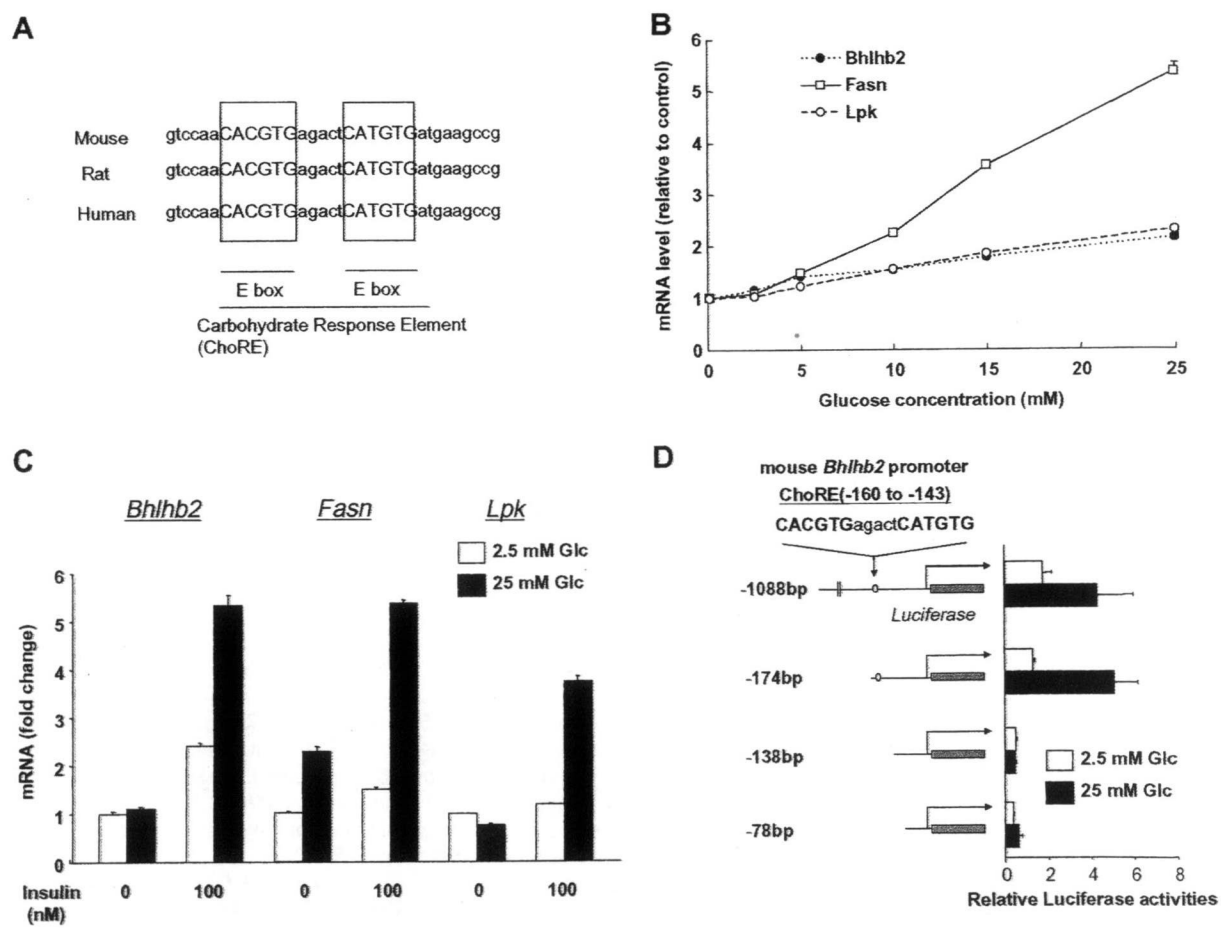

Fig. 1. Regulation of the mouse *Bhlhb2* promoter by glucose in rat primary hepatocytes. (A) A schematic representation of the mouse Bhlhb2 promoter with the sequences and locations of the carbohydrate response element (ChoRE). (B) Dose-dependent effects of glucose on *Bhlhb2*, *Fasn*, and *Lpk* mRNA expression in rat primary hepatocytes. Primary hepatocytes isolated from 6-week-old male Wister rats were cultured for 18 h in culture medium containing 0.1, 2.5, 5.0, 10, 15, or 25 mM glucose. Relative mRNA levels were determined by real-time RT-PCR and normalized to rat RNA polymerase II (*Pol2*) mRNA as the invariant control. The -fold change in expression level of each normalized enzyme mRNA level was determined with reference to the value for hepatocytes at 0.1 mM glucose, which was arbitrarily defined as 1. (C) Synergistic effects of glucose and insulin on *Bhlhb2*, *Fasn*, *and Lpk* mRNA expression. Rat hepatocytes were incubated for an additional 18 h in serum-free culture medium containing either a low (2.5 mM, open bars) or high (25 mM, filled bars) glucose (Glc) concentration in the absence or presence (100 nM) of insulin. (D) A schematic representation of the mouse Bhlhb2 promoter luciferase deletion constructs and the effect of these deletions on relative luciferase activity with low (2.5 mM) or high (25 mM) Glc concentration. Various reporter gene plasmids (−1088, −174, −138, and −78 bp) were cotransfected into rat hepatocytes with pGL4.74[hRLuc/TK] as a reference, and luciferase activity was normalized to *Renilla* luciferase activity. The values are represented as means and SD ($n = 6$). The figure represents data from two independent experiments.

the difference of nucleotide sequence in ChoRE. At 25 mM glucose, cotransfection of BHLHB2 suppressed luciferase activity of the *Fasn* and *Lpk* promoters (Fig. 3B). Moreover, BHLHB2 antagonized ChREBP-mediated *Fasn* and *Lpk* promoter activities in rat hepatocytes (Fig. 3C). The ChIP assay showed that BHLHB2 bound ChoRE in the promoter region of *Fasn*, *Lpk*, and the *Bhlhb2* gene (Fig. 3D). Thus, BHLHB2 antagonized ChREBP-mediated lipogenic gene expression by competing for binding to the E-box (Fig. 3E).

Discussion

In this study, we show that the glucose-activated transcription factor, ChREBP, regulates mouse *Bhlhb2* gene expression by directly binding to ChoRE in the mouse *Bhlhb2* promoter. BHLHB2 competes with ChREBP for binding to ChoRE and suppresses the transactivities of *Fasn*, *Lpk*, *and Bhlhb2* promoter mediated by ChREBP. These data indicate that BHLHB2 and ChREBP coordinately regulate de novo lipogenesis in the rat liver.

We also show that CHREBP induces *Bhlhb2* mRNA expression by binding to ChoRE in the *Bhlhb2* promoter region. In the presence of insulin, glucose is converted to Xu-5-P, a metabolite of the pentose phosphate pathway, and activates protein phosphatase 2A and ChREBP by dephospohorylation of the ChREBP protein [1]. In the absence of insulin, glucose was not metabolized into Xu-5-P, and did not activate ChREBP. Consistent with these results, glucose alone did not induce *Bhlhb2* mRNA (Fig. 1C). Moreover, 25 mM xylitol, which is immediately converted to Xu-5-P, induced a 6.4-fold increase *Bhlhb2* mRNA in rat hepatocytes (data not shown). These results indicate that glucose and Xu-5-P induce *Bhlhb2* mRNA expression. Previous studies have reported that *Bhlhb2* mRNA levels in the liver are suppressed during fasting and induced 36 h later during refeeding [10]. These results indicate that *Bhlhb2* expression is regulated by glucose both in vitro and in vivo.

Mouse, rat, and humans share the same ChoRE in the Dec1 promoter region (Fig. 1A). According to the deletion studies of the reporter assay, ChoRE in the *Bhlhb2* promoter region is required for ChREBP-mediated induction of this gene. Moreover, ChREBP

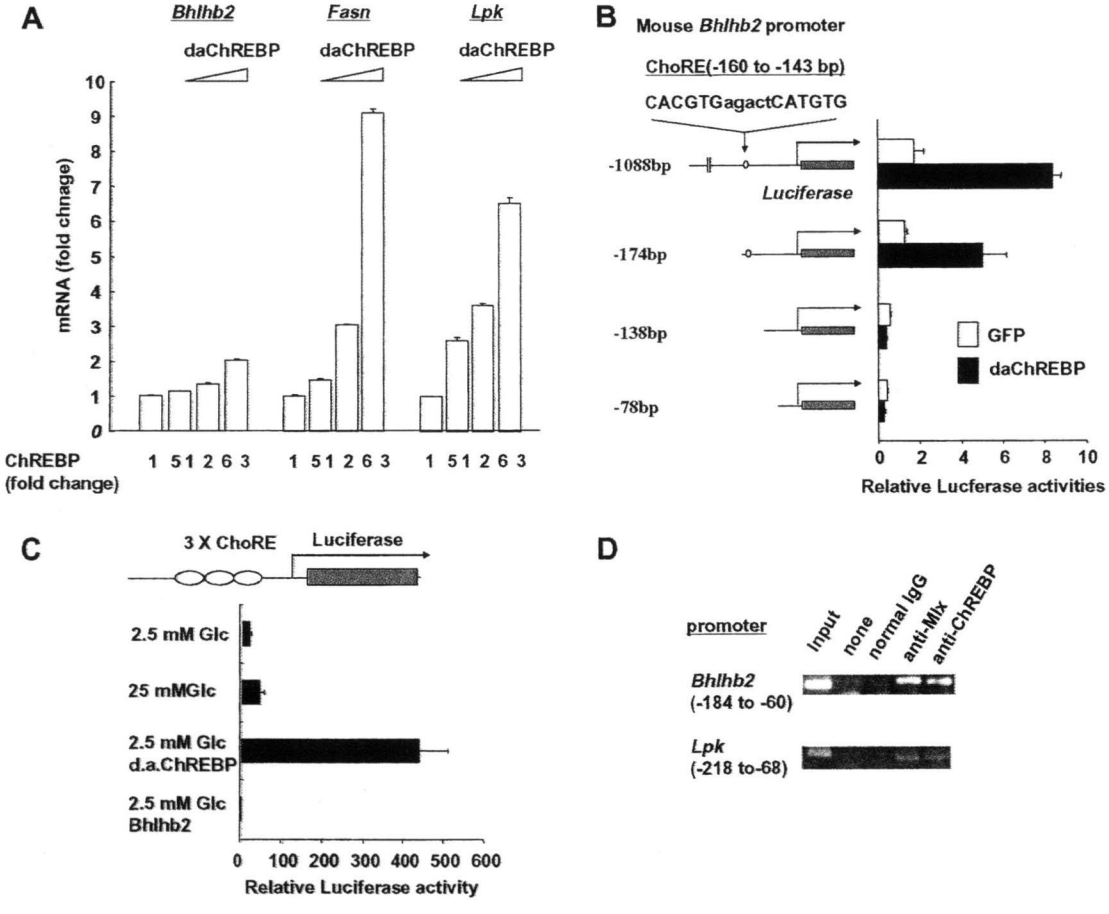

Fig. 2. Regulation of the mouse *Bhlhb2* promoter by ChREBP in rat primary hepatocytes. (A) Adenoviral overexpression of daChREBP activates mouse *Bhlhb2* mRNA expression. In hepatocytes infected with 5, 20, and 50 m.o.i. of Ad daChREBP or 50 m.o.i. of Ad-GFP, ChREBP mRNA was increased 5-, 12-, and 63-fold, respectively. The ChREBP, *Bhlhb2*, *Fasn*, *and LPK* mRNA levels were analyzed by Taqman RT-PCR and corrected with pol2. Data are means and SD. ($n = 3$) of two independent experiments. (B) A schematic representation of mouse *Bhlhb2* promoter luciferase deletion constructs and the effect of these deletions on relative lucieferase activity with overexpression of daChREBP. Various pGL3 plasmids (−1088, −174, −138, and −78 bp.) were cotransfected into rat hepatocytes with pGL4.74[hRLuc/TK] as a reference. The luciferase activity was normalized to *Renilla* luciferase activity. Data are the means ± SD ($n = 6$) of two independent experiments. (C) Putative ChoRE in the mouse *Bhlhb2* gene is a response element activated by glucose and ChREBP. The pGL3 promoter vector containing tandem 3X BHLHB2 ChoRE was cotransfected into rat primary hepatocytes with the pGL4.74[hRLuc/TK], pcDNA6.2-daCHREBP vector, and/or pcDNA6.2-BHLHB2 vector. Total DNA was adjusted with pcDNA 6.2 empty vectors. The relative luciferase activity was expressed as -fold change with reference to the pcDNA6.2 empty vector. Data are the means ± SD ($n = 6$) of two independent experiments. (D) Cells were incubated for 4 h in media and subjected to chromatin immunoprecipitation assay (CHIP assay) using the anti-ChREBP or Mlx antibody. Buffer alone and non-specific rabbit IgG were used as the negative control. Immunoprecipitated samples were subjected to PCR using primers to amplify ChoRE containing regions of the rat *Bhlhb2* or *Lpk* promoters. All experiments were performed in duplicate.

directly bound to ChoRE in the mouse *Bhlhb2* promoter, and overexpression of ChREBP increased *Bhlhb2* mRNA. Some groups have reported that USF2 binding to ChoRE prevents activation of the rat *Bhlhb2* promoter [11]. As with the *Bhlhb2* gene promoter, when USF bound to ChoRE in the *Fasn* and *Lpk* promoters, it did not induce expression of these genes [12]. SREBP1c belongs to the bHLH transcription factor family and binds to their E-box in vitro [2]. While insulin induces *Bhlhb2* expression, the precise mechanism is unclear. In preliminary data, a 20-fold increase in the overexpression of SREBP1c increased *Bhlhb2* mRNA 6-fold in mouse

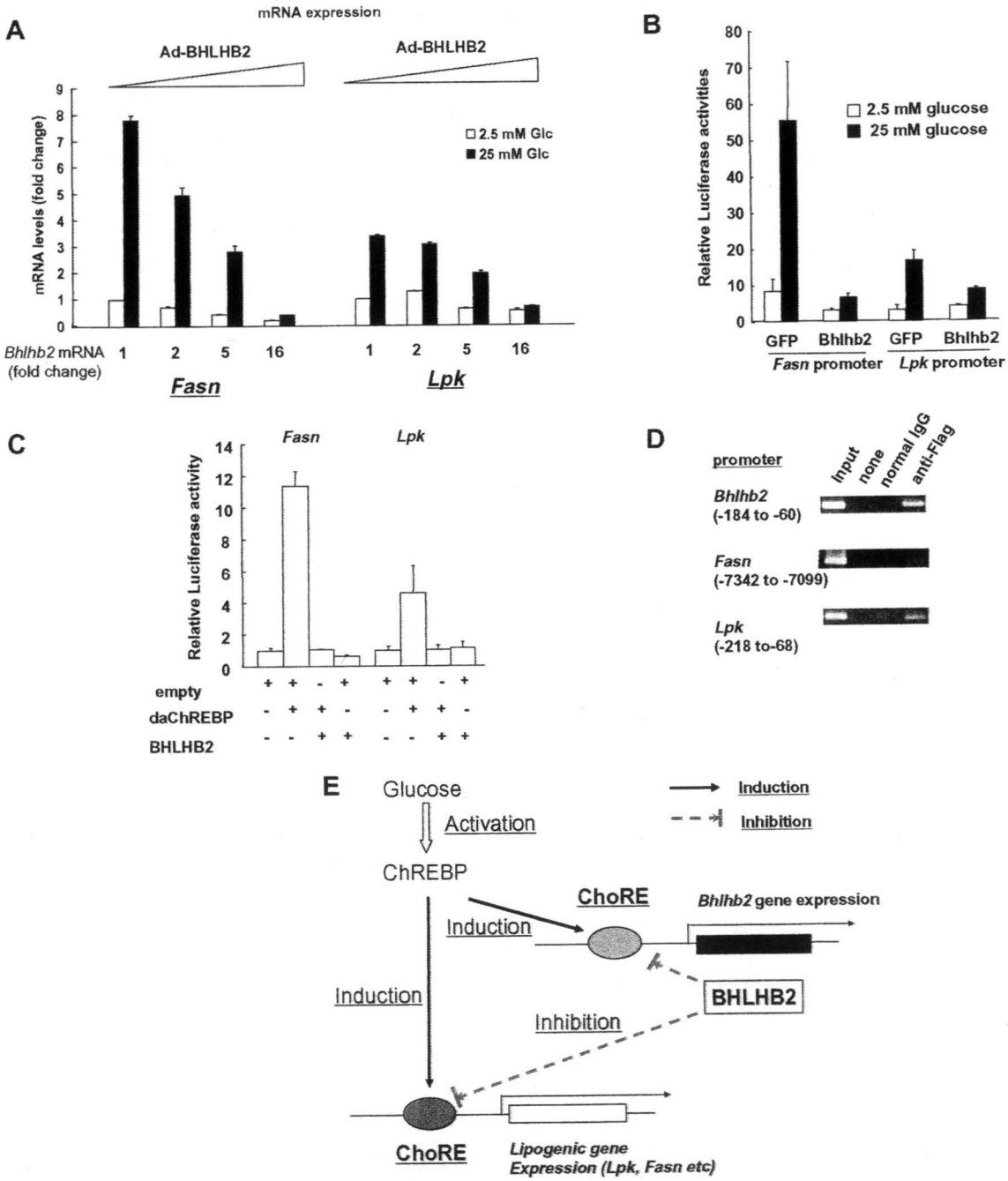

Fig. 3. Bhlhb2 antagonizes the effect of ChREBP on the transactivities of the *Lpk* and *Fasn* promoter. (A) Bhlhb2 dose-dependently inhibited glucose-induced gene expression in rat primary hepatocytes. In hepatocytes infected with 5, 20, or 50 m.o.i. of Ad-BHLHB2 and Ad-GFP adenovirus, *Bhlhb2* mRNA increased 5-, 12-, and 63-fold, respectively. *Fasn* and *Lpk* mRNA levels were detected by Taqman RT-PCR and corrected with pol2 mRNA. Data are represented as means and SD. (B) Overexpression of *Bhlhb2* antagonized glucose-mediated *Fasn* and *Lpk* promoter activities in rat hepatocytes. pGL3-*Fas* or -*Lpk* and pGL4.74[hRLuc/TK] was cotransfecetd into rat primary hepatocytes infected with 50 m.o.i. of Ad GFP or BHLHB2. (C) BHLHB2 inhibited ChREBP-mediated transcription activities in *Fasn* and *Lpk* promoters. pGL3 *Fasn* or *Lpk* and pGL4.74[hRLuc/TK] was cotransfected into hepatocytes with pcDNA-BHLHB2 and/or pcDNA-daChREBP. (D) Rat hepatocytes overexpressing BHLHB2 cDNA were subjected to ChIP assay with an anti-FLAG monoclonal antibody and rabbit IgG as the negative control. Immunoprecipitated samples were subjected to PCR analysis using primers to amplify ChoREs in the *Lpk*, *Fas*, and *Bhlhb2* promoter. (E) A schematic representation of the feedback loop model for the de novo coordinate regulation of lipogenesise in rat hepatocytes by ChREBP and BHLHB2.

hepatocytes. Further investigation is required to determine whether SREBP1c induces *Bhlhb2* mRNA by binding to ChoRE and the E-box. These results indicate that ChREBP regulates *Bhlhb2* expression by binding to ChoRE in the promoter region of the *Bhlhb2* gene.

We have shown that BHLHB2 inhibits glucose- and ChREBP-mediated lipogenic gene expression in rat hepatocytes. ChREBP transactivity is known to be partly regulated by a phosphorylation/dephosphorylation mechanism, although the details remain unclear [1]. We show here that the link between ChREBP and BHLHB is a novel feedback loop involved in the regulation of lipogenesis (Fig. 3E). A similar feedback system is also seen between BHLHB2 and Clock/BMAL. The Clock/BMAL1 complex recognizes the perfect E-box in ChoRE of the *Bhlhb2* promoter region, and induces *Bhlhb2* mRNA expression [6]. In contrast, BHLHB2 is a transcriptional repressor that binds to the E-box in the promoter region of *Per1* and *Bhlhb2*, and represses the gene induction mediated by Clock/BMAL1. Thus, a negative feedback loop between BHLHB2 and Clock/BMAL1 is involved in the regulation of the circadian rhythm [6]. Based on these results, we tested the hypothesis that ChREBP and BHLHB2 compete for binding to the *Fasn* and *Lpk* promoters of ChoRE to form a negative feedback loop that regulates lipogenesis. The results of our ChIP and reporter assays supportes this hypothesis. BHLHB2 has also been shown to bind to ChoRE at the *Bhlhb2* promoter region, potentially inhibiting its promoter activity [13]. Our data also show that overexpression of BHLHb2 inhibits the promoter activity of the pGL3 promoter vector with 3X Bhlhb2 ChoRE in hepatocytes, and that BHLHB2 binds to ChoRE in its promoter. For this reason, while the luciferase activity of PGL3-3XBHLHB2 ChoRE is dramatically induced by ChREBP, the effect of ChREBP on the induction of *Bhlhb2* mRNA may be only modest.

The physiological significance of the negative feedback loop between ChREBP and BHLHB2 remains unclear. In muscles from diabetic patients, *Bhlhb2* mRNA was found to be remarkably increased [8]. Consistent with this data, we found that ob/ob mice and 6-month high fat diet-loaded mice showed *Bhlhb2* mRNA in the liver 4.2 and 3.6 times higher than that in control mice, respectively ($n=4$, $p<0.05$). Moreover, a 6-fold overexpression of BHLHB2 inhibited glucose-mediated induction of *Fasn* and *Lpk* by 40% and 60%, respectively. Thus, the inhibitory effect of BHLHB2 on the FAS gene is probably more important than the inhibition on the *Lpk* gene. Furthermore, the 6-fold overexpression of BHLHB2 potently inhibited ChREBP-mediated induction of *Fasn* and *Lpk* expression. These results indicate that BHLHB2 regulates glucose activation of lipogenic enzyme gene expression and plays an important role in preventing overshoot of ChREBP-mediated lipid synthesis. Analysis of the effect of fasting and refeeding on *Fasn* and *Lpk* mRNA expression in ChREBP and Bhlhb2 knockout mice should permit identification of the physiological role of *Bhlhb2* in glucose-mediated lipogenic gene expression.

In conclusion, we have demonstrated that BHLHB2 is regulated by glucose and ChREBP and that it is inhibited by glucose and ChREBP-mediated *Fasn* and *Lpk* expression. Thus, ChREBP and BHLHB2 constitute a negative feedback loop involved in the regulation of lipogenesis. Further studies to clarify the in vivo relationship between BHLHB2 and ChREBP will be helpful for understanding the mechanism of regulation of lipogenesis and the development of treatments for metabolic syndrome.

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Hepatic overexpression of dominant negative Mlx improves metabolic profile in diabetes-prone C57BL/6J mice

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ABSTRACT

Mlx and ChREBP form a heterodimer to regulate glucose-mediated gene expression in the liver. This study was performed to determine if the metabolic syndrome might be improved using dominant negative Mlx (dnMlx). An adenovirus bearing dnMlx was constructed and used to test the inhibitory effect of dnMlx on lipogenesis both *in vitro* and *in vivo*. Adenoviral overexpression of dnMlx in rat hepatocytes inhibited expression of glucose-regulated genes, including *Chrebp* and *Transketolase*, which constitute a positive feedback loop in the regulation of *Chrebp* gene expression. Adenoviral overexpression of dnMlx in 25-week-old male C57BL/6J mice reduced hepatic triglyceride contents and improved glucose intolerance by inhibiting expression of *Glucose-6-phosphatase* and *Elovl6* mRNA in addition to lipogenic enzymes. In conclusion, overexpression of dnMlx improves glucose intolerance by inhibiting expression not only of lipogenic enzymes but also other important genes such as *Glucose-6-phosphatase* and *Elovl6*.

Nonalcoholic fatty liver disease is associated with metabolic syndrome and poses increased risk of cardiovascular disease. Development of non-alcoholic fatty liver disease is caused by decreased fatty acid oxidation and/or increased triglyceride synthesis. In the fed state, excess glucose uptake is converted to liver triglyceride storage. Hepatic *de novo* lipogenesis is regulated by insulin and glucose. Insulin and glucose induces glycolytic and lipogenic gene expression by sterol regulatory element binding protein (SREBP1c) and carbohydrate response element binding protein (ChREBP), respectively, [1,2]. Experiments in knockout mice indicate that SREBP1c and ChREBP exhibit *de novo* lipogenesis of 50% and 60%, respectively, [1]. Inhibition of these transcription factors reverses hepatic steatosis in obesity [1]. Thus, SREBP1c and ChREBP coordinately regulate hepatic lipogenesis. ChREBP heterodimerizes with Mlx to bind to the carbohydrate response elements (ChoRE) in the promoter of glycolytic and lipogenic genes [1].

We previously reported that gene deletion of ChREBP in ob/ob mice improved metabolic syndrome compared with ob/ob mice [3]. Consistent with our previous study, adenoviral delivery of ChREBP short hairpin RNA (shRNA) into the liver improved hepatic steatosis and insulin resistance by inhibiting hepatic lipogenesis [4]. In contrast, liver-specific inhibition of SREBP1c was found to improve hepatic steatosis but not glucose intolerance [1]. Liver-specific inhibition of PPARγ also improved hepatic steatosis, but increased glucose intolerance [1]. Clarification of the differences between ChREBP and SREBP1c or PPARγ in the development of the metabolic syndrome thus might facilitate development of clinical treatment options for the condition [1].

In this study, we investigate the mechanism of the improvement by liver-specific inhibition of ChREBP transactivity of glucose intolerance. An adenovirus bearing dominant negative Mlx (dnMlx) was constructed to test the transactivity of ChREBP *in vitro* and *in vivo*. Our findings in Ad-dnMlX-injected mice suggest that drugs disrupting the association between ChREBP and Mlx might be useful in preventing metabolic syndrome.

Materials and methods

Animals, isolation of rat primary hepatocytes, and cell culture. The protocols for all animal experiments were approved by the Institutional Animal Care and Use Committee of Gunma University Medical School (code no. 08-025). Six-week-old male Wistar rats were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan) and male C57BL/6J mice were purchased from Charles River Japan (Yokohama, Kanagawa, Japan). Rat hepatocytes were isolated from 6-week-old male Wistar rats by the collagenase perfusion method. Isolated hepatocytes were suspended in DMEM supplemented with 10% fetal calf serum (FCS), 100 nM insulin, 100 nM dex, 10 nM T_3, and 100 μg/ml pen/strep [5]. Cells were seeded in 6-well plates or 10-cm dishes and grown in a humidified atmosphere of

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5% CO_2/95% air at 37 °C. After incubation for 4 h, the medium was replaced with DMEM containing 10 nM T_3.

Construction of plasmid and adenovirus vectors. PCR to construct pENTR vectors was performed using PrimeSTAR DNA polymerase (Takara) and primers (Sigma–Aldrich). A series of Mlx deletion mutants with an N-terminal Flag tag were amplified from mouse liver cDNA (Fig. 1A). PCR fragments (2–245aa, 67–245aa, and 88–245aa) were cloned into the pENTR vector (Invitrogen), yielding pENTR-Mlx1-1, -Mlx2-1, or -Mlx4-1, respectively. Adenovirus vectors bearing Mlx4-1 were constructed by recombining pENTR-Mlx4-1 vectors into pAd/CMV/V5-DEST using LR Clonase II Master Mix (Invitrogen) according to the manufacturer's protocol. The pcDNA6.2 vectors bearing Mlx1-1, Mlx2-1, or Mlx4-1 (pcDNA-Mlx1-1, pcDNA-Mlx2-1, or pcDNA-Mlx4-1, respectively) were constructed in the same manner as the adenovirus. pGL3-Lpk and AddaChREBP were the same vector and adenovirus used previously [5]. pGL4-TK-RLuc vector was purchased from Promega.

Treatment with recombinant adenovirus in rat hepatocytes. Rat isolated hepatocytes were cultured in 6-well plates in 2 ml of DMEM. Hepatocytes were infected with adenovirus bearing GFP, Mlx4-1, or dominant active ChREBP (daChREBP) at m.o.i. of 2, 10, or 50 for 2 h, media were removed, and infected cells were incubated in media with 2.5 or 25 mM glucose for 18 h. The cells were then collected and used for RT-PCR analysis.

RNA extraction and quantitative real-time PCR. Total RNA was extracted from hepatocytes or liver samples using an RNeasy Mini Plus kit (Qiagen) according to the manufacturer's protocol [5]. Taqman PCR probes for semi-quantitative RT-PCR were purchased from Applied Biosystems.

Mammalian transfection and reporter assay. Primary hepatocytes were cultured in 6-well plates in 2 ml of DMEM without antibiotics. The cells were transfected with Lipofectamine 2000 (10 μl), pGL3-Lpk (3.6 μg), or the series of pcDNA-Mlx vectors (empty, Mlx1-1, Mlx2-1, or Mlx4-1) (0.4 μg) and the pGL4-TK-Renilla luciferase vector (0.4 μg) [5]. After 24 h of incubation with 2.5 or 25 mM glucose, the cells were collected and used to measure luciferase activity (Dual Luciferase Assay System; Promega) according to the manufacturer's protocol.

Establishment of Ad dominant negative Mlx4-1-infected mice. Twenty-five-week-old male C57BL/6J mice were anesthetized with pentobarbital and injected *via* the tail vein with 3×10^{13} particle/kg body weight (BW) of Ad-GFP or Ad-Mlx4-1 in a final volume of 300 μl of phosphate buffered saline. After 5 days, the mice were sacrificed and the liver was used for extraction of total RNA and liver metabolites.

Measurement of plasma profile and liver metabolites and oral glucose tolerance test. Plasma concentrations of glucose, insulin, and triglyceride were measured as described previously [3]. Total cholesterol, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured using Cholesterol and Transaminase Wako test kits (Wako). Liver glycogen, triglyceride, and cholesterol contents were measured as described previously [3]. Oral glucose tolerance tests at 3 days were performed by oral injection of glucose at a dose of 1 g glucose/kg BW after 18-h fast. Blood glucose levels were measured at the designated times using a FreeStyle Kissei Meter (Kissei, Tokyo, Japan).

Data presentation and statistical methods. All data are expressed as means ± SD. The listed *n* values represent the number of single experiments performed (each experiment was duplicated). Comparisons between two groups were performed by Student's *t*-test, and $P < 0.05$ was considered significant.

Results

Mlx4-1 acts as dominant negative Mlx to inhibit glucose-induced Lpk and Fas mRNA expression

As our and other groups reported previously, inhibition of ChREBP transactivity was found to be beneficial for treating metabolic disorders in ob/ob mice [3,4]. A series of pcDNA6.2-Mlx mutants was constructed as shown in Fig. 1A and screened for dnMlx by analyzing Lpk promoter activity. Consistent with Fig. 1A, Mlx4-1 inhibited glucose-induced Lpk promoter activity by 60% (Fig. 1B). In contrast, Mlx2-1 increased Lpk promoter activity by 60% (Fig. 1B). As with the localization of Mlx mutants, Mlx2-1 was mainly localized in the nucleus, while Mlx4-1 and Mlx1-1 were localized in both the cytosol and nucleus (see Supplementary Fig. S1).

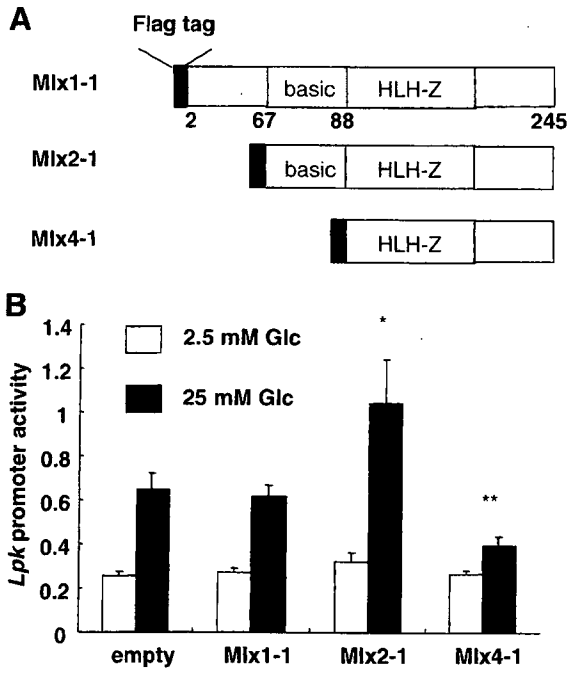

Fig. 1. (A) Schematic representation of wild-type Mlx (Mlx1-1), dominant active Mlx (Mlx2-1), and dominant negative Mlx (Mlx4-1). (B) Effects of Mlx1-1, Mlx2-1, or Mlx4-1 on Lpk promoter activity. Isolated hepatocytes were transfected with 3.6 μg of pGL3-Lpk, 0.4 μg of pGL4-TK-RLuc, and 0.4 μg of pcDNA6.2 empty, Mlx1-1, Mlx2-1, or Mlx4-1 vector using Lipofectamine 2000. Transfected cells were cultured for 24 h and used for analysis of luciferase activity. Data are means ± S.D. ($n = 6$ per group). *, **, $p < 0.05$ *vs.* pcDNA6.2 empty.

Mechanism by which Mlx4-1 inhibits ChREBP-mediated Lpk and Fasn gene expression

In the present study, a deletion mutant, Mlx4-1, was established that inhibits glucose-induced *Lpk* and *Fasn* gene expression. The effects of this mutant on *Lpk* and *Fasn* mRNA gene expression were evaluated. Consistent with Fig. 1B, Mlx4-1 dose-dendently inhibited glucose-induced *Lpk* and *Fasn* mRNA expression (Fig. 2A). When the Mlx4-1 mRNA level was increased 1680-fold, induction of these genes by glucose was completely blocked. The pGL3-3X*Lpk* and 3X*Fasn*-ChoRE vector were then used to clarify whether these inhibitory effects are due to binding of ChREBP/Mlx4-1 to ChoRE. Mlx4-1 completely inhibited ChREBP-regulated *Lpk* and *Fasn* reporter activity in rat hepatocytes (see Supplementary Fig. S2A). The inhibitory effects of Mlx4-1 on glucose-induced expression of *Chrebp* and *Transketolase* (*Tkt*) in rat primary hepatocytes were then examined. *Tkt* is an enzyme that forms xylulose-5-phosphate (Xu-5-P) from two glycolytic intermediates, glyceraldehyde 3-P (GAP) and fructose-6-P (Fru 6-P) (GAP + Fru 6-P ↔ Xu 5-P + erythrose 4-P) in the pentose phosphate pathway [1]. Xu-5-P activates

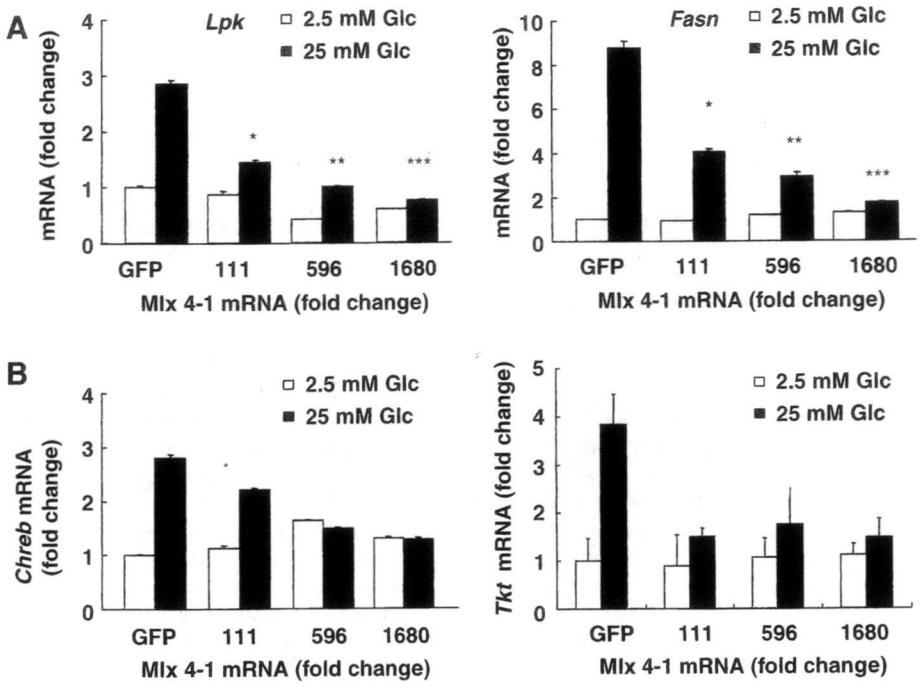

Fig. 2. (A) Overexpression of Mlx4-1 in rat hepatocytes inhibited glucose-induced *Lpk* and *Fasn* mRNA expression in a dose-dependent manner. *, **, *** $p < 0.05$ *vs.* GFP. (B) Mlx4-1 inhibited glucose-mediated *Chrebp* and *Tkt* mRNA induction in rat hepatocytes.

protein phosphatase 2A (PP2A), which in turn activates ChREBP by dephosphorylation [1]. Glucose stimulation was found to increase *Chrebp* and *Tkt* mRNA expression (see Supplementary Fig. S2B). Consistent with these data, a 1680-fold increase in Mlx4-1 completely inhibited glucose-induced *Chrebp* and *Tkt* mRNA expression in rat hepatocytes (Fig. 2B). These findings suggest that ChREBP and Tkt constitute a positive feedback loop involved in the regulation of glucose-mediated gene expression such as *Lpk* and *Fasn*.

Overexpression of Mlx4-1 improves glucose and lipid metabolism in diabetes-prone C57BL/6J mice

C57BL/6J mice develop metabolic syndrome with obesity and diabetes in response to a diet high in carbohydrate and/or fat [6]. Moreover, C57BL/6J mice develop glucose intolerance with age [6]. In this study, 25-week-old male C57BL/6J mice were used as a model diabetes-prone mouse to determine whether overexpression of Mlx4-1 improves glucose intolerance and other metabolic parameters in middle-aged C57BL/6J mice. Mice infected with Ad-GFP were used as controls. To minimize the side effects of adenovirus infection in the liver, all experiments were terminated after 5 days. Ad-Mlx4-1-injected mice appeared normal without hepatomegaly (Table 1). As shown in Table 1, body and tissue weights in Ad-Mlx4-1-infected mice were similar to those in controls. Ad-Mlx 4-1 overexpression did not affect food intake, as shown by stomach weight and food intake (Table 1). As shown in Table 2, AST and ALT concentrations were only slightly changed as compared with previously reported observations [4]. The delivery of 3×10^{13} particles/kg BW of Ad-Mlx4-1 or Ad-GFP adenovirus into C57BL/6J mice was liver-specific (data not shown). Increases of about 170-fold in endogenous Mlx mRNA levels were observed in livers infected with Ad-Mlx4-1 as compared with Ad-GFP (Fig. 3A). In liver overexpressing Ad-Mlx4-1, expression levels of the ChREBP target genes *Lpk* and *Fasn* were decreased by 84% and 65%, respectively, (Fig. 3A), suggesting that Mlx4-1 also functions as a dominant negative form of Mlx *in vivo*.

Liver triglyceride contents in Ad-Mlx4-1-injected mice were decreased by 40% due to decreased hepatic lipogenesis, but liver cholesterol contents were unchanged (Fig. 3B). Plasma lipid profile of Ad-Mlx4-1 mice was similar to that of controls (Table 2). Liver glycogen contents of Ad-Mlx4-1 mice were similar to controls (Fig. 3B). Plasma glucose concentration in Ad-Mlx4-1-injected mice was significantly decreased in both fasted and fed states (Table 2). In contrast, plasma insulin, triglyceride, and total cholesterol

Table 1
Phenotypic characteristics of 5-day-treated Ad-Mlx4-1 C57BL/6J mice.

	Ad-GFP	Ad-Mlx4-1
BW (g)	30.6 ± 2.1	30.4 ± 1.9
Food intake (g)	3.68 ± 0.26	3.60 ± 0.28
Stomach (%)	2.24 ± 0.26	2.04 ± 0.54
Liver (%)	5.8 ± 0.24	6.6 ± 0.73
White adipose tissue (%)	2.4 ± 1.4	1.6 ± 0.6
Kidney (%)	1.36 ± 0.14	1.45 ± 0.03

Data are means ± S.D. (n = 5 per group).

Table 2
Metabolic characteristics of 5-day-treated Ad-Mlx4-1 C57BL/6J mice.

	Ad-GFP	Ad-Mlx4-1	*P*-value
5-h fast			
Plasma glucose (mg/dl)	115 ± 6.2	81.4 ± 10.8*	$P = 0.014$
Plasma TG (mg/dl)	97.8 ± 22.1	89.6 ± 16.3	
Plasma T.Chol (mg/dl)	78.7 ± 10.1	81.5 ± 14.0	
18-h fast			
Plasma glucose (mg/dl)	86.2 ± 11	67.8 ± 6.6*	$P = 0.010$
Plasma insulin (ng/ml)	0.63 ± 0.21	0.43 ± 0.07	
Ad. lib. Fed			
Plasma glucose (mg/dl)	178 ± 34	111 ± 7.9*	$P = 0.019$
Plasma insulin (ng/ml)	0.83 ± 0.26	0.80 ± 0.26	
ALT (Units/l)	47.5 ± 16.6	93.9 ± 20.0*	$P = 0.010$
AST (Units/l)	32.3 ± 7.9	87.2 ± 14.2*	$P = 0.005$

Data are means ± S.D. (n = 5 per group). *$p < 0.05$ vs GFP. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase.

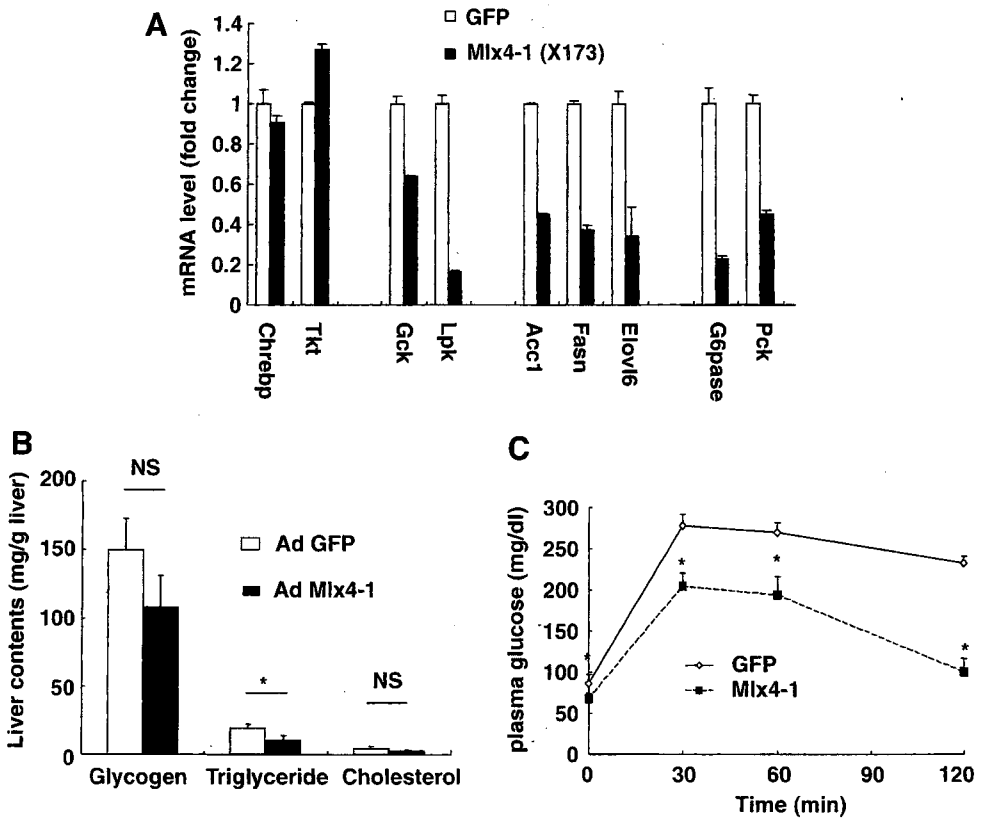

Fig. 3. (A) RT-PCR analysis of glycolytic, lipogenic, gluconeogenic, and lipolytic enzyme mRNA levels in liver of C57BL/6J mice infected with Ad-GFP or Ad-Mlx4-1. Results are means±SD ($n = 5$ per group). $^{*}p < 0.05$ vs. Ad-GFP injected C57BL/6J mice. (B) Determination of liver glycogen, triglyceride, and cholesterol contents in Ad-GFP or Ad-Mlx4-1 injected mice. Results are means±SD ($n = 5$ per group) $^{*}p < 0.05$ *vs.* Ad-GFP-injected mice. (C) Glucose tolerance tests (1 g/kg BW) were performed in C57BL/6J mice treated for 3 days with Ad-GFP or Ad-Mlx4-1 adenovirus. Animals were fasted for 18 h before OGTT ($n = 5$ per group). Data are means ± SD ($n = 5$ per group). $^{*}p < 0.05$ *vs.* Ad-GFP-injected C57BL/6J mice.

concentrations were similar to controls (Table 2). Consistent with these observations, oral glucose tolerance test showed that overexpression of Mlx4-1 improved glucose clearance from the body (Fig. 3C). In addition, glycolytic, lipogenic, and gluconeogenic gene mRNA expression levels were measured. As described above, glycolytic genes such as *Lpk* and lipogenic genes such as *Fasn*, *Acc1*, and ELOVL family member 6 (*Elovl6*) were suppressed by overexpression of Ad-Mlx4-1. In the gluconeogenesis pathway, glucose-6-phosphatase (*G-6-Pase*) and phosphoenolpyruvate carboxykinase (*Pepck*) mRNA levels were decreased by 80% and 50%, respectively, (Fig. 3C).

ChREBP induces G-6-Pase gene expression in rat hepatocytes

G-6-Pase gene expression is regulated by glucose signaling [7]. Moreover, Elovl6 is involved in lipid metabolism, and deletion of *Elovl6* improves glucose intolerance in ob/ob mice [8]. As shown in Fig. 3A, *G-6-pase* and *Elovl6* mRNA were down-regulated in the liver of Ad-Mlx4-1-injected mice. We therefore examined whether glucose and ChREBP can induce *G-6-Pase* and *Elovl6* mRNA in rat hepatocytes. Glucose increased hepatic *G-6-Pase* and *Elovl6* mRNA expression in a dose dependent manner (see Supplementary Fig. S4). Moreover, overexpression of dominant active ChREBP increased *G-6-Pase* and *Elovl6* mRNA expression in rat primary hepatocytes (Fig. 4A). Consistent with the findings shown in Fig. 4A, overexpression of dominant negative Mlx4-1 suppressed glucose-induced *G-6-Pase* and *Elovl6* mRNA expression (Fig. 4B). Thus, ChREBP regulates *G-6-Pase* and *Elovl6* gene expression in rat hepatocytes.

Discussion

This study was performed to determine whether hepatic inhibition of ChREBP transactivity can improve glucose intolerance in C57BL/6J mice. Overexpression of dnMlx/Mlx4-1 inhibited glucose-induced *Lpk*, *Fasn*, *Chrebp*, and *Tkt* gene expression in rat hepatocytes. Moreover, adenoviral delivery of dnMlx/Mlx4-1 into the liver improved glucose intolerance. Thus, blocking the association between ChREBP and Mlx is a promising therapeutic strategy to cure metabolic syndrome.

Mlx and ChREBP form the ChREBP-Mlx complex that binds ChoRE in glycolytic and lipogenic gene promoters such as *Lpk* and *Fasn*. Mlx4-1 is a dominant negative form of Mlx without the N-terminal and DNA-binding domains (Fig. 1A). Mlx4-1 is localized in both the cytosol and nucleus (see Supplementary Fig. S1). In contrast, Mlx2-1 is a dominant active form lacking only the N-terminal domain and is localized mainly in the nucleus. Consistent with our data, some groups have reported that the N-terminal domain of ChREBP has an important role in functions of ChREBP such as nuclear translocation and glucose sensitivity [9]. These observations suggest that the N-terminal domain of Mlx plays a role in determining glucose sensitivity and localization.

dnMlx/Mlx4-1 inhibits glucose-mediated *Lpk* and *Fasn* mRNA expression in rat hepatocytes. Towle et al. reported that Mlx plays an important role in glucose regulation of lipogenic enzymes, using their original Mlx mutant lacking only the DNA-binding domain, and obtained results compatible with ours [10]. Unlike their mutant, we constructed a mutant lacking both the N-terminal and the DNA-binding domains. Our observations suggest that the

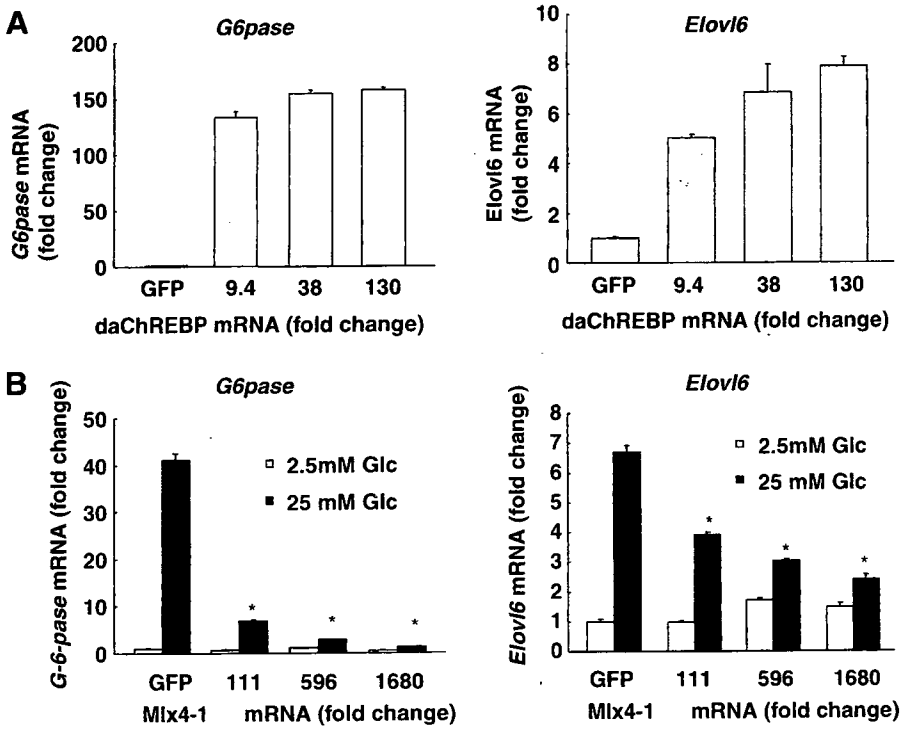

Fig. 4. (A) Adenoviral overexpression of dominant active ChREBP (daChREBP) induced *G-6-Pase* and *Elovl6* mRNA expression in rat hepatocytes. Adenovirus expressing either GFP or daChREBP was transduced into rat hepatocytes at m.o.i. of 2, 10, or 50. As control, a recombinant adenovirus expressing GFP was used at m.o.i. of 50. Two h after infection, cells were kept in DMEM including 2.5 mM glucose for an additional 18 h. Total RNA was then extracted from hepatocytes and RT-PCR analysis was performed. Values represent means±S.D. $^{*}p < 0.05$ *vs.* Ad-GFP. (B) Adenoviral overexpression of Mlx4-1 inhibited glucose-stimulated *G-6-Pase* and *Elovl6* mRNA expression in rat hepatocytes. Adenovirus overexpressing either GFP or Mlx4-1 was transduced into rat hepatocytes at m.o.i. of 2, 10, or 50. As control, a recombinant adenovirus expressing GFP was used at m.o.i. of 50. Two h after infection, cells were kept in DMEM including glucose concentrations of 2.5 or 25 mM for an additional 18 h. Total RNA was then extracted from hepatocytes and RT-PCR analysis was performed. Values represent means±S.D. $^{*}p < 0.05$ *vs.* Ad-GFP.

DNA-binding domain is critical in transactivity of Mlx. Moreover, dnMlx/Mlx4-1 inhibited activation of *Chrebp* and *Tkt* mRNA expression by glucose. In the pentose pathway, glucose is converted to Xu-5-P, an activator of ChREBP [1]. As Xu-5-P is produced by Tkt, ChREBP and Tkt constitutes a positive feedback loop by increasing *Tkt* and *ChREBP* mRNA in rat hepatocytes. It is surprising that glucose increases *Chrebp* mRNA expression in rat hepatocytes and the mechanism remains unknown. Using dnMlx-expressing adenovirus vector, ChREBP was found to directly regulate *Chrebp* gene expression. However, *Tkt* and *Chrebp* mRNA levels were not decreased in the livers of Ad-Mlx4-1-injected mice. In contrast, overexpression of daChREBP caused 40- and 14-fold increases in endogenous *Chrebp* and *Tkt* mRNA levels, respectively, (data not shown). Thus, while glucose regulates *Tkt* and *ChREBP* mRNA expression in rat hepatocytes, other factors may regulate their gene expression more potently *in vivo*.

We and other groups have independently reported that gene deletion or shRNA of *Chrebp* improves metabolic disorders in ob/ob mice [3,4]. However, complete deletion of *Chrebp* causes massive hepatomegaly due to liver glycogen accumulation [3]. Moderate inhibition of ChREBP transactivity thus might improve the symptoms of metabolic syndrome without hepatomegaly. In rat hepatocytes, Mlx4-1 functions only at higher glucose concentrations. In contrast, *Chrebp* gene deletion or *Chrebp* shRNA works at both lower and higher glucose concentrations. Since this is a unique characteristic of dnMlx/Mlx4-1, we tested 25-week-old diabetes-prone C57BL/6J mice. The C57BL/6J mouse is a model of diet-induced obesity and diabetes [6]. As C57BL/6J mice develop glucose intolerance with age similarly to humans, middle-aged C57BL/6J mice were used to test the effects of dnMlx on improvement of glucose intolerance. Overexpression of dnMlx/Mlx4-1 improved glucose intolerance and liver lipid contents without hepatomegaly. These observations suggest that drugs disrupting the association between ChREBP and Mlx may be promising for preventing the development of metabolic syndrome.

dnMlx/Mlx4-1 inhibited expression of genes encoding the glycolytic enzyme *Lpk*, lipogenic enzymes *Acc1*, *Fasn*, and *Elovl6*, and gluconeogenic enzyme *G-6-Pase*, although the precise mechanism by which inhibition of ChREBP transactivity improves metabolic syndrome remains unknown. Elovl6 catalyzes conversion of palmitate to stearate [8]. Consistent with Fig. 4B, dnMlx inhibited glucose-induced *Elovl6* gene expression in primary hepatocytes [11]. Gene deletion of *Elovl6* in high-fat-loaded mice or ob/ob mice improved glucose intolerance by modifying hepatic fatty acid composition, but did not improve hepatic steatosis [8]. We did not analyze hepatic fat composition in Ad-Mlx4-1-injected mice, but fat composition in Chrebp$^{-/-}$ mice was similar to that in normal controls (unpublished data). Moreover, glucose clearance in *Elovl6* knockout mice was similar to that in controls [8]. Thus, *Elovl6* mRNA may contribute to improvement of glucose intolerance, even though its effect in Ad-dnMlx/Mlx4-1 mice is limited.

DnMlx inhibited glucose induction of *G-6-pase* mRNA expression. Acute inhibition of the G-6-Pase system lowers the plasma glucose concentration in rats [12]. These observations suggest that G-6-Pase plays an important role in glucose homeostasis in rodents. Some groups have reported the possibility that ChREBP binds to ChoRE in the rat *G-6-Pase* promoter region [7]. Fig. 4A and B indicates that glucose and ChREBP strongly regulate *G-6-pase* gene expression in rat hepatocytes. As SREBP1c did not regulate *G-6-Pase* mRNA expression, and deletion of SREBP1c did not improve glucose intolerance, the difference in *G-6-pase* gene regulation be-

tween ChREBP and SREBP may underlie the improvement of glucose intolerance by ChREBP but not by SREBP [1].

In conclusion, experiments using dnMlx/Mlx4-1 indicate that ChREBP is regulated by *Chrebp* and *Tkt* gene expression, and forms a positive feedback loop in rat hepatocytes. Moreover, overexpression of dominant negative Mlx improves glucose intolerance in diabetes-prone C57BL/6J mice with metabolic syndrome. These observations suggest that drugs capable of dissociating the ChREBP-Mlx complex may be a promising approach to treatment of metabolic syndrome including glucose intolerance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.100.

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