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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′-CACCATGGATTACAAGGATGACGACGATAAGATCAGGGAAGGGGATTTCCTGGCTCCCAAGC-3′
	R	5′-TTATAATGGTCTGCCCAGGGGACCCTCTGTG-3′
BHLHB2 (flag)	F	5′-CACCATGGATTACAAGGATGACGACGATAAGATCGAACGGATCCCCAGCGCGCAACC -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′-CGCGGTCCAACACGTGAGGCTCATGTGATGAAGGTCCAACACGTGAGGCTCATGTGATGAAGGTCCAACACGTGAGGCTCATGTGATGAAG-3
	R	5′-GATCCTTCATCACATGAGCCTCACGTGTTGGACCTTCATCACATGAGCCTCACGTGTTGGACCTTCATCACATGAGCCTCACGTGTTGGAC-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 luciferase 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

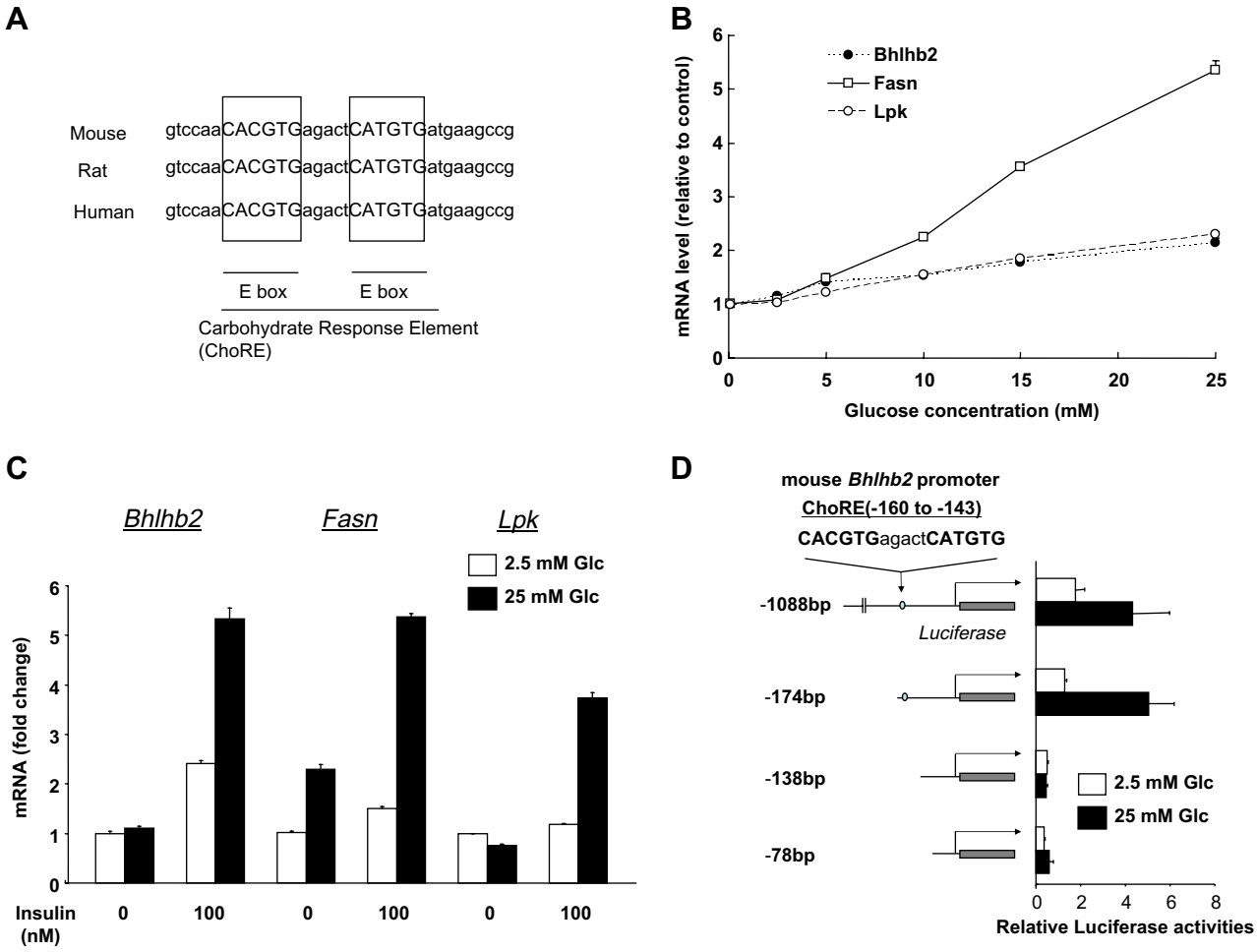

Fig. 1. Regulation of the mouse *Bhlhb2* promoter by glucose in rat primary hepatocytes. (A) A schematic representation of the mouse Bhlhb2 promoter with the sequences and locations of the carbohydrate response element (ChoRE). (B) Dose-dependent effects of glucose on *Bhlhb2, Fasn*, and *Lpk* mRNA expression 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 *Renillla* 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

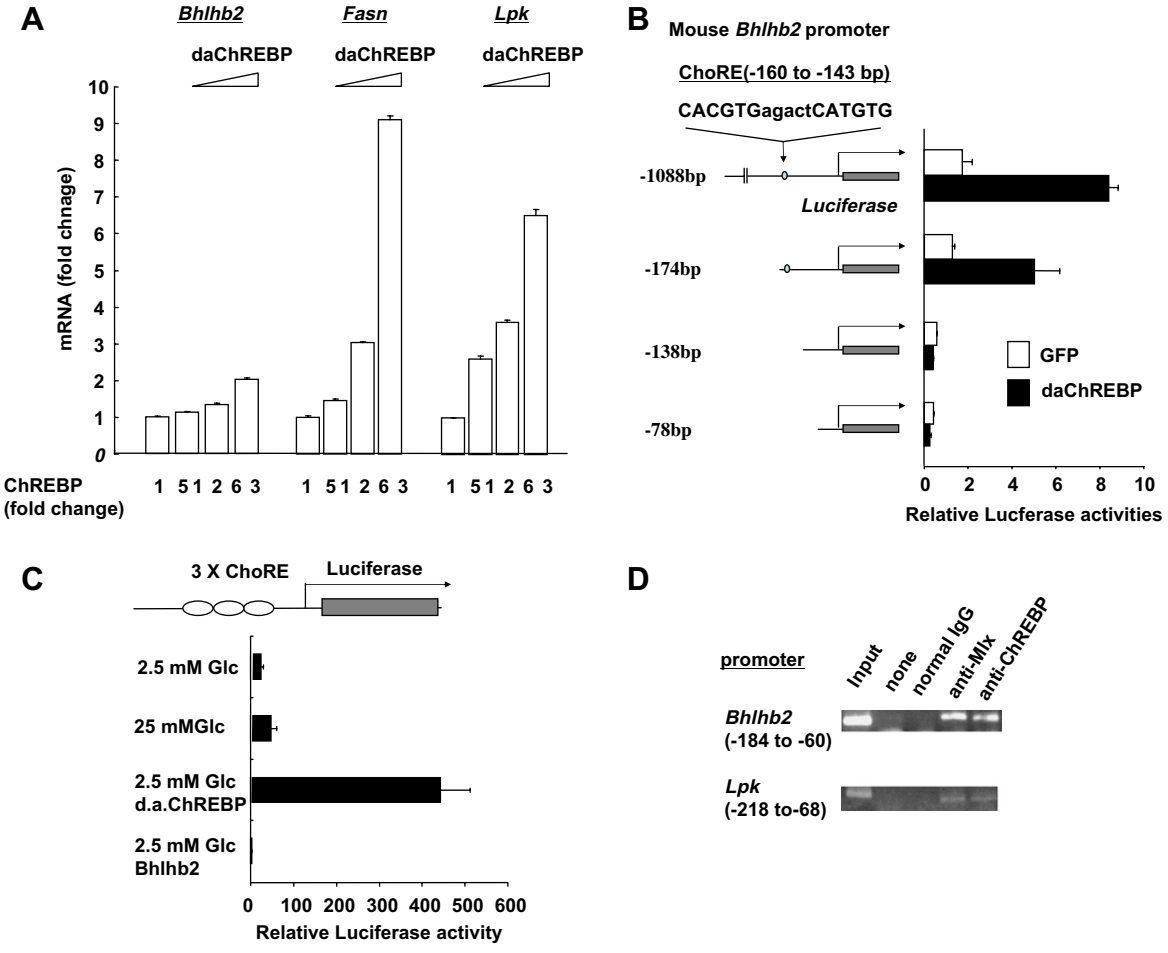

Fig. 2. Regulation of the mouse *Bhlhb2* promoter by ChREBP in rat primary hepatocytes. (A) Adenoviral overexpression of daChREBP activates mouse *Bhlhb2* mRNA expression. In hepatocytes infected with 5, 20, and 50 m.o.i. of Ad daChREBP or 50 m.o.i. of Ad-GFP, ChREBP mRNA was increased 5-, 12-, and 63-fold, 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 *Renillla* 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

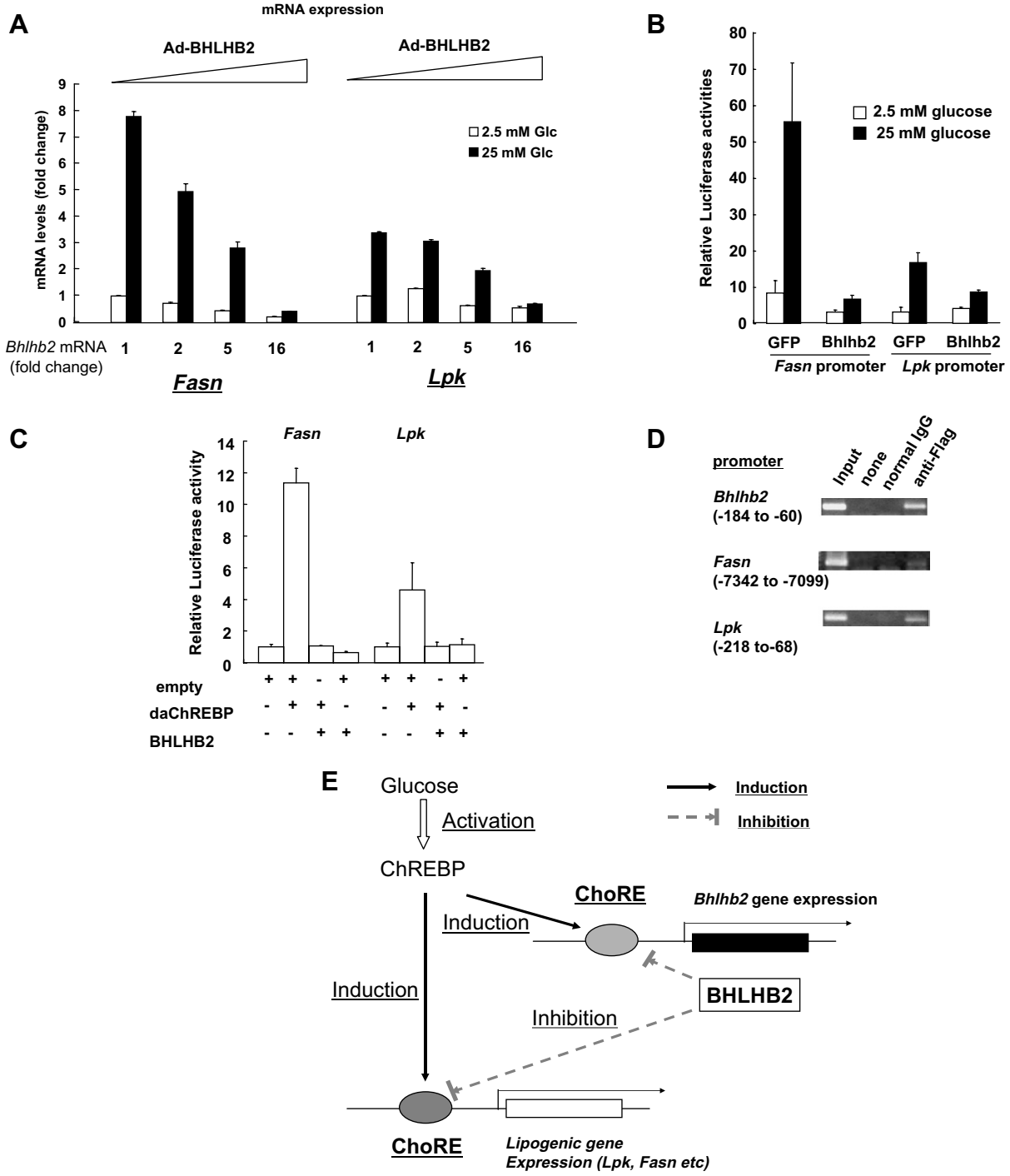

Fig. 3. Bhlhb2 antagonizes the effect of ChREBP on the transactivities of the *Lpk* and *Fasn* promoter. (A) Bhlhb2 dose-dependently inhibited glucose-induced gene expression in rat primary hepatocytes. In hepatocytes infected with 5, 20, or 50 m.o.i. of Ad-BHLHB2 and Ad-GFP adenovirus, *Bhlhb2* mRNA 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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