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Glucose Induces Protein Targeting to Glycogen in Hepatocytes by Fructose 2,6-Bisphosphate-Mediated Recruitment of MondoA to the Promoter

John L. Petrie,[a] Ziad H. Al-Oanzi,[a,b] Catherine Arden,[a] Susan J. Tudhope,[a] Jelena Mann,[a] Julius Kieswich,[c] Muhammad M. Yaqoob,[c] Howard C. Towle,[d] Loranne Agius[a]

Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom[a]; Department of Laboratory Medicine, Al-Jouf University, Sakaka, Saudi Arabia[b]; Centre for Translational Medicine and Therapeutics, William Harvey Research Institute, Queen Mary University of London, London, United Kingdom[c]; Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota, USA[d]

In the liver, a high glucose concentration activates transcription of genes encoding glucose 6-phosphatase and enzymes for glycolysis and lipogenesis by elevation in phosphorylated intermediates and recruitment of the transcription factor ChREBP (carbohydrate response element binding protein) and its partner, Mlx, to gene promoters. A proposed function for this mechanism is intracellular phosphate homeostasis. In extrahepatic tissues, MondoA, the paralog of ChREBP, partners with Mlx in transcriptional induction by glucose. We tested for glucose induction of regulatory proteins of the glycogenic pathway in hepatocytes and identified the glycogen-targeting proteins, G_L and PTG (protein targeting to glycogen), as being encoded by Mlx-dependent glucose-inducible genes. PTG induction by glucose was MondoA dependent but ChREBP independent and was enhanced by forced elevation of fructose 2,6-bisphosphate and by additional xylitol-derived metabolites. It was counteracted by selective depletion of fructose 2,6-bisphosphate with a bisphosphatase-active kinase-deficient variant of phosphofructokinase 2/fructosebisphosphatase 2, which prevented translocation of MondoA to the nucleus and recruitment to the PTG promoter. We identify a novel role for MondoA in the liver and demonstrate that elevated fructose 2,6-bisphosphate is essential for recruitment of MondoA to the PTG promoter. Phosphometabolite activation of MondoA and ChREBP and their recruitment to target genes is consistent with a mechanism for gene regulation to maintain intracellular phosphate homeostasis.

The liver maintains blood glucose homeostasis by conversion of dietary glucose to glycogen or triglyceride after a meal and by production of glucose from glycogen or gluconeogenic precursors in the postabsorptive state (1). The metabolic transition after a meal is mediated by rapid changes in the activities of key hepatic enzymes of glycogen metabolism, glycolysis, and gluconeogenesis by allosteric effectors and covalent modification. These mechanisms are regulated by the insulin-to-glucagon ratio and by the increase in the glucose concentration in the portal vein (1). Portal hyperglycemia after a carbohydrate-containing meal causes rapid activation of glucokinase by release from its inhibitory protein in the nucleus and translocation to the cytoplasm, with consequent elevation in glucose 6-phosphate (glucose 6-P) and downstream metabolites (2–4). Glucose 6-P is an allosteric effector of glycogen phosphorylase and glycogen synthase (5) and a precursor of fructose 2,6-bisphosphate (fructose 2,6-P_2), an allosteric regulator of glycolysis (4), and promotes increased flux through hepatic glycogen synthesis and glycolysis by feed-forward activation mechanisms. The action of glucose 6-P on glycogen metabolism involves binding to phosphorylase and stabilization of the tense conformation (T state), which is a better substrate for dephosphorylation by phosphorylase phosphatase (5). This comprises the catalytic unit of protein phosphatase 1 bound to the glycogen-targeting protein PTG (protein targeting to glycogen) (6–9). The phosphorylated form of phosphorylase is an allosteric inhibitor of the glycogen synthase phosphatase complex comprising protein phosphatase1 bound to the glycogen-targeting protein G_L. Accordingly, dephosphorylation of phosphorylase at high glucose levels leads to sequential activation of glycogen synthase and increased glycogen storage (5).

In addition to rapid control of metabolic flux by high glucose levels through allosteric mechanisms and covalent modification of enzymes, high glucose also induces transcription of various hepatic enzymes of glycolysis and lipogenesis through activation of the transcription factor ChREBP (carbohydrate response element binding protein) (10, 11). ChREBP is a member of the Mondo family of basic helix-loop-helix zipper domain transcription factors, which also includes MondoA (12). ChREBP and MondoA bind to DNA elements as heterodimers with Max-like protein X (Mlx), a 33-kDa protein that is expressed ubiquitously and also forms heterodimers with other transcriptional regulators (13–15). MondoA mediates glucose-regulated gene expression in muscle and other extrahepatic cell lines (12, 16, 17), whereas ChREBP is expressed at higher levels in liver than in extrahepatic tissues and is the major mediator of glucose-regulated gene expression in the liver (10). ChREBP knockdown studies confirmed its involvement in the induction of hepatic glycolytic and lipogenic enzymes (18–20), suggesting that a key function of ChREBP is the conversion of glucose to lipid (10, 18). However, gene microarrays in hepatocytes identified other potential target genes of ChREBP and Mlx, such as the genes encoding the gluconeogenic enzyme glucose

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Address correspondence to Loranne Agius, Loranne.Agius@ncl.ac.uk.

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6-phosphatase (G6pc) and the inhibitory protein of glucokinase (21). Induction of these genes by glucose is not consistent with a role for ChREBP in promoting hepatic glucose disposal (22, 23). This led to the hypothesis that a key function of ChREBP is to maintain intrahepatic homeostasis of phosphorylated intermediates and inorganic phosphate (22, 23). Both ChREBP and MondoA are activated by metabolism of glucose rather than by a direct effect of glucose itself (12). However, the exact mechanism has not been resolved. Two models have been proposed for ChREBP activation. Uyeda and colleagues proposed a role for xylulose 5-P through activation of a type 2A phosphatase that dephosphorylates ChREBP (10, 24), whereas other groups proposed a role for glucose 6-P rather than xylulose 5-P (25–27). Recent work from our laboratory has presented evidence for fructose 2,6-P_2 in mediating translocation of ChREBP and recruitment to its target promoters (23). The studies on MondoA activation in muscle and other nonhepatic cell lines suggest a role for glucose 6-P based on the similar or greater efficacy, compared with glucose, of 2-deoxyglucose, which is phosphorylated but not further metabolized (16, 17, 28).

If the function of glucose regulation of gene expression in the liver is to maintain intracellular phosphate homeostasis, then enzymes or regulatory proteins of other metabolic pathways involving phosphorylated intermediates, such as the glycogenic pathway, would also be expected to be regulated by high glucose levels. To advance our understanding of metabolite control of gene expression in the liver, we tested for glucose-responsive genes of the glycogenic pathway in hepatocytes. We identified three regulatory proteins of the glycogenic pathway that are induced by high glucose, including the glycogen-targeting protein PTG, which is a major regulator of glycogen metabolism in the liver (6, 7, 9, 29). We show that PTG is a target for Mlx and MondoA, but not ChREBP and, furthermore, that MondoA is activated in liver cells by a mechanism similar to that of ChREBP involving fructose 2,6-P_2.This suggests that regions conserved between MondoA and ChREBP mediate the fructose 2,6-P_2 mechanism, and it further supports a role for glucose regulation of hepatic gene expression in intracellular phosphate homeostasis.

MATERIALS AND METHODS

Hepatocyte isolation and culture. Hepatocytes were isolated from male Wistar rats fed *ad libitum* (23). Procedures conformed to Home Office regulations and were approved by the Newcastle University Ethical Committee. The hepatocytes were suspended in minimum essential medium (MEM) supplemented with 5% (vol/vol) newborn calf serum, seeded on gelatin-coated (1 mg/ml) multiwell plates or coverslips for immunostaining, and cultured for 2 to 4 h to allow cell attachment (23). Treatment with adenoviral vectors was in serum-free MEM between 2 and 4 h after cell attachment. Adenoviral vectors for expression of Mlx, MondoA, ChREBP, Mlx dominant negative (Mlx-DN), 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK2/FBP2) wild type (PFK-WT), and a bisphosphatase-active kinase-deficient S32D T55V variant (PFK-KD) were as described previously (21–23). After cell attachment, the medium was replaced with MEM containing 5 mM glucose, 10 nM dexamethasone, 10 nM insulin, and the hepatocytes were cultured for 18 h.

Hepatocyte incubations. Incubations for substrate-regulated gene expression were in minimum essential medium containing 5 mM glucose and with the additions indicated. Glucose analogues were from Sigma-Aldrich, and S4048, an inhibitor of the glucose 6-P transporter (30), was a gift from Aventis-Pharma Gmbh, Frankfurt, Germany. Parallel incubations were performed for RNA extraction (4-h incubation) and metabolite determination (1-h incubation).

C2C12 cells. C2C12 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% (vol/vol) fetal bovine serum, 75 mg/liter penicillin, 50 mg/liter streptomycin. They were treated with adenoviral vector for 4 h after seeding on multiwell plates and after removal of the vector were then cultured overnight in glucose-free medium containing 2% fetal bovine serum.

***In vivo* study.** Male Wistar rats (180 to 200 g) from Harlan Bicester United Kingdom were fasted for 18 h and then injected intraperitoneally with either glucose (0.2 g/100 g body weight as 22.5% glucose, 0.9% saline) or an equivalent volume of 0.9% saline. They were culled with a guillotine after 60 min, and the livers were snap-frozen in liquid nitrogen and stored on dry ice or at −80°C until analysis. Animal experiments were conducted in accordance with Home Office regulations and with Institutional Ethical Committee approval.

Metabolite determination. Incubations were stopped by draining the medium and either extraction in 0.15 M NaOH for determination of fructose 2,6-P_2 (23) or snap-freezing in liquid nitrogen for determination of other metabolites (22). Cells were extracted in 10% (wt/vol) $HClO_4$, and after deproteinization (9,000 × *g*; 15 min), the supernatant was neutralized with 3 M K_2CO_3, and glucose 6-P, fructose 6-P, and ATP were determined by coupling NADPH formation to reduction of resazurin with diaphorase and measured fluorimetrically (excitation, 530 nm; emission, 590 nm; Spectramax Me5) as described previously (22). Lowering of ATP (<20%) occurred in cells with high levels of overexpression of MondoA alone, but not with combined overexpression of MondoA and Mlx or Mlx-DN. None of the glucose analogues lowered ATP at the concentrations used in this study. Metabolites are expressed as pmol/mg protein (fructose 2,6-P_2) or nmol/mg protein (glucose 6-P or hexose 6-P).

mRNA determination. RNA was extracted in TRIzol (Invitrogen) (22), and cDNA was synthesized from 1 μg of RNA with random hexamers and Superscript (Invitrogen). Real-time reverse transcription (RT)-PCR was performed in a total volume of 10 μl containing 50 ng of reverse-transcribed RNA and 5 ng of forward and reverse primers in a Roche capillary light cycler, with initial denaturation at 95°C for 10 min, followed by 40 to 50 cycles of 95°C for 15 s, 58°C for 7 s, and 72°C for 15 s. The primer sequences are listed in Table 1. All mRNA values are expressed as the change relative to 5 mM glucose control.

ChIP assays. Hepatocytes were cultured in 150-cm^2 dishes and incubated under conditions similar to those for mRNA determination. Incubations were terminated by addition of formaldehyde (1% [vol/vol]; 10 min), followed by glycine (0.125 M; 5 min). The cells were washed, pelleted, extracted, and sonicated as described previously (22). For *in vivo* experiments, 80 mg frozen liver samples was homogenized in PBS containing 1% formaldehyde for 10 min before addition of 0.125 M glycine for 5 min. The cells were then pelleted, extracted, and sonicated as described previously (22). Binding of transcription factors to the gene promoters was determined using the Upstate Biotechnology chromatin immunoprecipitation (ChIP) assay kit (Millipore 17-295) essentially as described previously (22). After preclearing (22), the cell supernatants were incubated overnight (4°C) with 6 μg IgG against MondoA (Sigma; SAB2104303), ChREBP (Novus Biologicals; NB400-135), Mlx, or control IgG (sc-14705 or sc-2027; Santa Cruz Biotechnology). DNA was recovered by phenol-chloroform extraction and amplified by Touchdown real-time PCR with primer sequences amplifying promoter or coding regions as listed in Table 1.

Immunoblotting. Nuclear and cytoplasmic extracts were prepared as described previously (31). Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated overnight with antibodies to G_L, PTG, (32), FLAG (Sigma F7425), NF-Yα (Abd Serotec; AHP298), glyceraldehyde phosphate dehydrogenase (GAPDH) (Hytest; 5G4), and lamin (Cell Signaling; 2032). After washing, the membranes were incubated for 1 h with peroxidase-conjugated anti-rabbit (Dako) or anti-sheep (Stratech) IgG, and immunoreactive bands were visualized by enhanced chemiluminescence (GE Healthcare).

TABLE 1 Primers used for real-time RT-PCR

Region	Orientation	Primer
Coding		
Glucose 6-phosphatase (*G6pc*)	Forward	CTACCTTGCGGCTCACTTTC
	Reverse	ATCCAAGTGCGAAACCAAAC
Glycogen phosphorylase (*Pygl*)	Forward	GCAGAAGTGGTGAACAACGA
	Reverse	CATCCATAGTCCCGATGGTC
Glycogen synthase (*Gys2*)	Forward	GACACTGAGCAGGGCTTTTC
	Reverse	CCTCTCAGCCTCCTCTTCCT
G_L (*Ppp1r3b*)	Forward	CAGTCGTACTGGGACAGCAA
	Reverse	TAATAGGGCCCCAGCTTTTC
PTG (*Ppp1r3c*)	Forward	GAGGATTTGCTTGGCACATT
	Reverse	CTTGGAGTCAGCAAACACGA
R3D (*Ppp1r3d*)	Forward	TTGGCTCCCAGGTACACTTC
	Reverse	GTCTGCAGAGGTGGAAGCTC
R3E (*Ppp1r3e*)	Forward	GACCTGGCCTACGAGAAGC
	Reverse	CCGTTGTTGTCCCAGAACTC
Max-like protein X (*Mlx*)	Forward	TCTGTCCCCAACACAGATGA
	Reverse	ACGATGGCTTTGCTGAGTTT
ChREBP (*Mlxipl*)	Forward	GGGACATGTTTGATGACTATGTC
	Reverse	AATAAAGGTCGGATGAGGATGCT
MondoA (*Mlxip*)	Forward	ATCCACAGCGGCCACTTCATG
	Reverse	TCATGCACTCGAAGAGCTTGG
Pyruvate kinase (*Pklr*)	Forward	CTGGAACACCTCTGCCTTCTG
	Reverse	CACAATTTCCACCTCCGACTC
TXNIP (*Txnip*)	Forward	ACCAGTGTCTGCCAAAAAGG
	Reverse	GCCATTGGCAAGGTAAGTGT
PFKFB3 (*Pfkfb3*)	Forward	CACGGCGAGAATGAGTACAA
	Reverse	TTCAGCTGACTGGTCCACAC
Promoter		
G6pc (ChoRE region)	Forward	GCATCAGCCCTGTGTGAATA
	Reverse	GAGTTGAGGGCAAACAGAGC
TXNIP (ChoRE region)	Forward	CGCACCCGAACAACAACCAT
	Reverse	AAGCGGGAGCCGGAAACGG
PTG (−242 to −30)	Forward	CAGCCCCGCGGTGATCACGT
	Reverse	CCGCACCCAGCGCAGCTTCGCAC CAC
PTG (−395 to −203)	Forward	TAGAAGACCCCGCCAGGCTCG
	Reverse	ACGAGCCCCTGTCCCTGGAA
PTG (−635 to −366)	Forward	GGCCACTTCGTTTCACCGGC
	Reverse	AGCTTATGCGAGCCTGGCGG
Pklr (ChoRE region)	Forward	GGATGCCCAATATAGCCTCA
	Reverse	CCATGCTGCTACGTTGCTTA

Immunostaining. Hepatocytes or C2C12 cells on gelatin-coated coverslips overexpressing FLAG-tagged MondoA (at lower levels than for the gene expression studies) were fixed at the end of the incubation with 4% paraformaldehyde (22) and immunostained with anti-FLAG (Sigma; F7425) and Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes). Nuclei were counterstained using Hoechst 33342. The cells were imaged using a Nikon E400 microscope (60× oil immersion objective).

Statistical analysis. Results are means and standard errors (SE) for the number of hepatocyte preparations indicated. Statistical analysis on the raw data was by Student's paired *t* test or 2-way analysis of variance (ANOVA) with Bonferroni correction for ChIP assays.

RESULTS

Glycogen synthase, G_L, and PTG are induced by high glucose levels in hepatocytes. To identify hepatic genes that are induced by elevated glucose, we determined in primary hepatocytes the effects of 25 mM glucose versus 5 mM glucose in the absence or presence of insulin (10 nM) or S4048 (2 μM), an inhibitor of the glucose 6-P transporter that enhances the accumulation of phosphorylated intermediates at elevated glucose levels (22, 30). We used a 4-h incubation based on time course studies that showed significant induction of ChREBP target genes within this interval (21) and to minimize secondary changes in gene expression. We used G6pc as a positive control because induction of G6pc by high glucose levels is widely documented *in vivo* (18, 33) and in hepatocytes (21–23). G6pc mRNA was repressed (~90%) by insulin (Fig. 1A), as expected for a gluconeogenic enzyme, and it was elevated by 25 mM glucose (>5- to 20-fold) and further enhanced by S4048 in both the absence and the presence of insulin (Fig. 1A). Expression of glycogen phosphorylase was not significantly affected by high glucose levels (Fig. 1B). However, expression of glycogen synthase and the glycogen-targeting proteins, G_L and PTG (encoded by *Ppp1r3b* and *Ppp1r3c*), was significantly increased by insulin and by high glucose levels (Fig. 1C to E). Increased expression of G_L and PTG at the protein level was confirmed by immunoblotting (Fig. 1F and G). Expression of the glycogen-targeting proteins R3D and R3E (encoded by *Ppp1r3d* and *Ppp1r3e*) was not significantly affected by high glucose levels (Fig. 1H and I). These results identify three genes encoding regulatory proteins of the glycogenic pathway as glucose-inducible genes in the liver.

Induction of glycogenic genes requires Mlx. Since ChREBP-Mlx is a major mediator of glucose-regulated gene expression in the liver, we used a dominant-negative variant of Mlx (Mlx-DN) with a disrupted DNA-binding domain (21) to test for involvement of Mlx in the glucose induction of the genes identified in Fig. 1. Expression of Mlx-DN attenuated the glucose induction of genes for glycogen synthase, G_L, and PTG (Fig. 2A). Combined expression of ChREBP and Mlx-DN reversed the attenuation of glycogen synthase and G_L genes, suggesting involvement of Mlx and ChREBP in the regulation of these genes. However, surprisingly, the attenuation of PTG mRNA by Mlx-DN was not reversed by ChREBP expression (Fig. 2A).

Involvement of MondoA and ChREBP in glucose-regulated gene expression. To explore the mechanism of PTG regulation, we asked whether MondoA, which also forms a complex with Mlx (14), is involved. In hepatocytes, MondoA mRNA was expressed constitutively and was not induced by 25 mM glucose (4 h), unlike ChREBP mRNA, which was increased (Fig. 2B). The endogenous protein levels of ChREBP and MondoA were below the limits of detection by immunoblotting with commercially available antibodies. During forced overexpression of MondoA with an adenoviral vector, immunoactivity to FLAG-tagged MondoA was enhanced if Mlx was coexpressed (Fig. 2C). Forced overexpression of MondoA resulted in increased PTG mRNA expression at high glucose levels, and this was enhanced by coexpression of Mlx in parallel with the increase in MondoA immunoactivity, suggesting a role for MondoA in PTG expression at high glucose levels.

To test whether MondoA also affects the expression of ChREBP target genes, we compared the effects of forced overexpression of ChREBP, Mlx, and combined MondoA and Mlx on the glucose-responsive genes and on three established ChREBP target genes, the Pklr, G6pc, and TXNIP genes (Fig. 2D to I). Forced overexpression of Mlx by 3-fold at the protein level (corresponding to >10-fold at the mRNA level) did not affect mRNA expression of the ChREBP target genes, the Pklr, G6pc, and TXNIP genes, or the genes encoding the glycogen regulatory proteins (Fig. 2D to I). Forced overexpression of ChREBP (>30-fold at the mRNA level) significantly increased mRNA levels for Pklr and G6pc at 5 mM glucose and G6pc, glycogen synthase, and G_L at 25 mM glucose but did not affect the expression of either affect the expression of either the PTG gene or the TXNIP gene, which is a

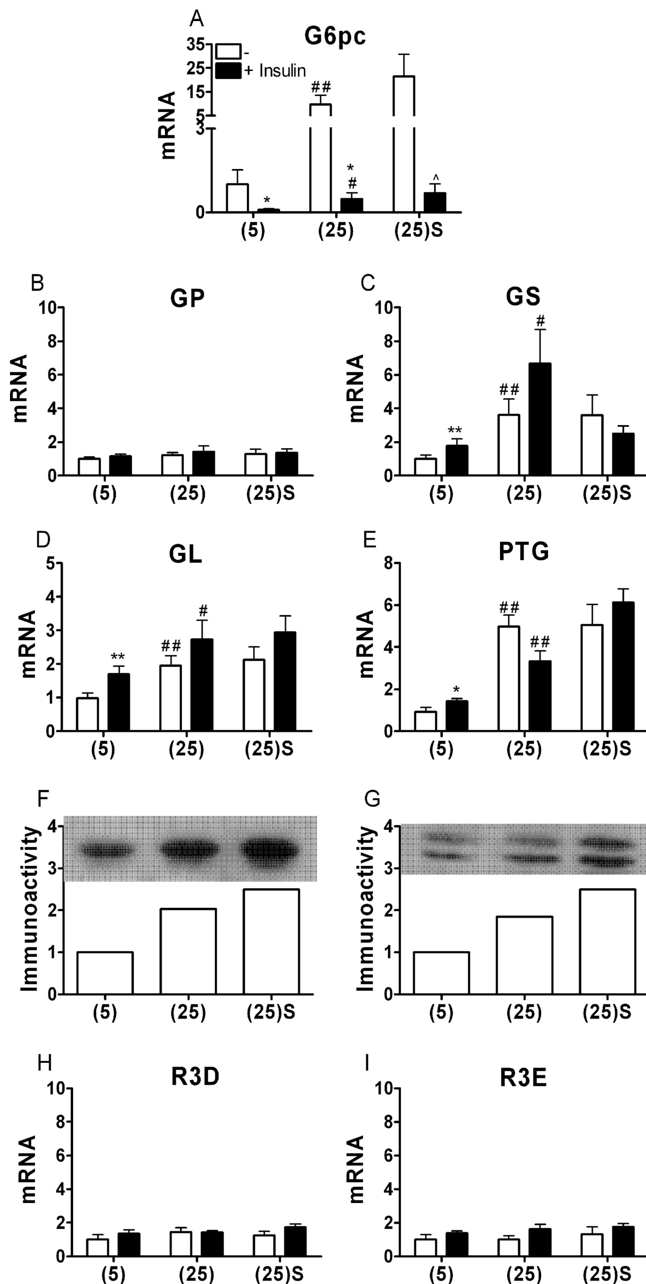

FIG 1 High glucose levels induce glycogen synthase, G_L, and PTG. Hepatocytes were precultured for 18 h and then incubated for 4 h in fresh medium without (− Insulin) or with (+ Insulin) 10 nM insulin and either 5 mM [(5)] or 25 mM glucose without [(25)] or with [(25)S] 2 μM S4048. (A to E, H, and I) mRNA levels of the indicated genes. GP, glycogen phosphorylase; GS, glycogen synthase. (F and G) Immunoactivity to G_L (F) and PTG (G) determined after 24 h of culture with 10 nM insulin and the indicated substrates. Shown are means and SE for 4 to 6 hepatocyte preparations (except for panels F and G, where $n = 2$). *, $P < 0.05$, and **, $P < 0.01$ for the effect of insulin; #, $P < 0.05$, and ##, $P < 0.01$ for the effect of 25 mM glucose; ^, $P < 0.05$ for the effect of S4048.

target gene for ChREBP in other cell types (34–36). Forced overexpression of MondoA plus Mlx increased the expression of PTG and TXNIP genes at 25 mM, but not at 5 mM, glucose, and it did not affect expression of the Pklr, G6pc, glycogen synthase, or G_L gene (Fig. 2D to I). These results suggest that these glucose-responsive genes are regulated either by MondoA or by ChREBP in hepatocytes. Further evidence for involvement of either MondoA or ChREBP was obtained from experiments with Mlx-DN and coexpression of MondoA or ChREBP (Fig. 2J and K). The attenuation by Mlx-DN of PTG mRNA was reversed by MondoA, but not ChREBP (Fig. 2J), and conversely, the G6pc attenuation was reversed by ChREBP, but not MondoA (Fig. 2K).

Recruitment of endogenous Mlx and MondoA to the PTG promoter in hepatocytes. We next used ChIP assays to test for recruitment of endogenous Mlx, MondoA, and ChREBP to the promoters of PTG and other glucose-responsive genes. Using primer sequences flanking established carbohydrate response elements (ChoRE) of the G6pc promoter (37), we confirmed glucose-dependent binding of Mlx and ChREBP, but not MondoA, to the G6pc promoter (Fig. 3A), and likewise, we confirmed recruitment of ChREBP and Mlx, but not MondoA, to the ChoRE region of the Pklr gene (see Fig. 8). Using primers flanking the two ChoRE regions of the TXNIP gene (38) that have been shown to bind MondoA (38), or ChREBP in the case of the proximal ChoRE (34, 36), we found glucose-dependent binding of Mlx and MondoA to the TXNIP gene promoter in hepatocytes (Fig. 3B). In contrast to the findings in HepG2 cells (35) and INS1 cells (34, 36), we did not find recruitment of ChREBP to the TXNIP gene promoter (Fig. 3B). This is consistent with the induction of TXNIP in hepatocytes by forced expression of MondoA, but not by ChREBP (Fig. 2F). We next used primers spanning from −635 to −30 of the PTG gene promoter and located a region proximal to the start site (−242 to −30) that showed significant recruitment at high glucose levels of both Mlx and MondoA (Fig. 3C). The proximal region that bound Mlx and MondoA contains a single CACGTG sequence corresponding to the Mlx-MondoA E box (39). ChREBP, unlike MondoA, did not bind to the PTG promoter (Fig. 3D). Interestingly, inspection of the proximal promoter sequences of the *Ppp1r3c* genes of 15 vertebrates showed that the CACGTG element is conserved throughout placental (eutherian) and marsupial (metatherian) mammals, but not in chicken, *Xenopus*, and zebrafish (Table 2).

Time course of the glucose induction of MondoA targets. The ChIP assays identified PTG and TXNIP as targets for endogenous MondoA in hepatocytes (Fig. 3). We next asked whether the temporal response to high glucose levels of MondoA targets differs from that of ChREBP target genes. During an 8-h time course after challenge with 25 mM glucose, PTG and TXNIP mRNAs showed rapid elevation within 1 h that was sustained but not further elevated over 7 h (Fig. 4A and B). This contrasts with the progressive increase over 8 h of the ChREBP target gene, *Pklr* (Fig. 4C). G6pc mRNA showed a marked increase after 1 h that was increased 3-fold over the next 5 h (Fig. 4D). These changes correlate with the progressive increase in ChREBP, but not MondoA, mRNA (Fig. 4E and F).

Glucose recruitment of MondoA to the PTG gene promoter *in vivo*. Having established the time course for glucose induction of PTG mRNA levels in hepatocytes (Fig. 4), we next tested whether this mechanism occurs *in vivo*. Rats were fasted for 18 h and then injected with either glucose or saline and culled after 60 min. The liver PTG, but not MondoA, mRNA levels were significantly higher in the glucose-treated rats (Fig. 5A and B), and this was associated with recruitment of MondoA, but not ChREBP, to the PTG gene promoter (Fig. 5C). This supports the validity of the

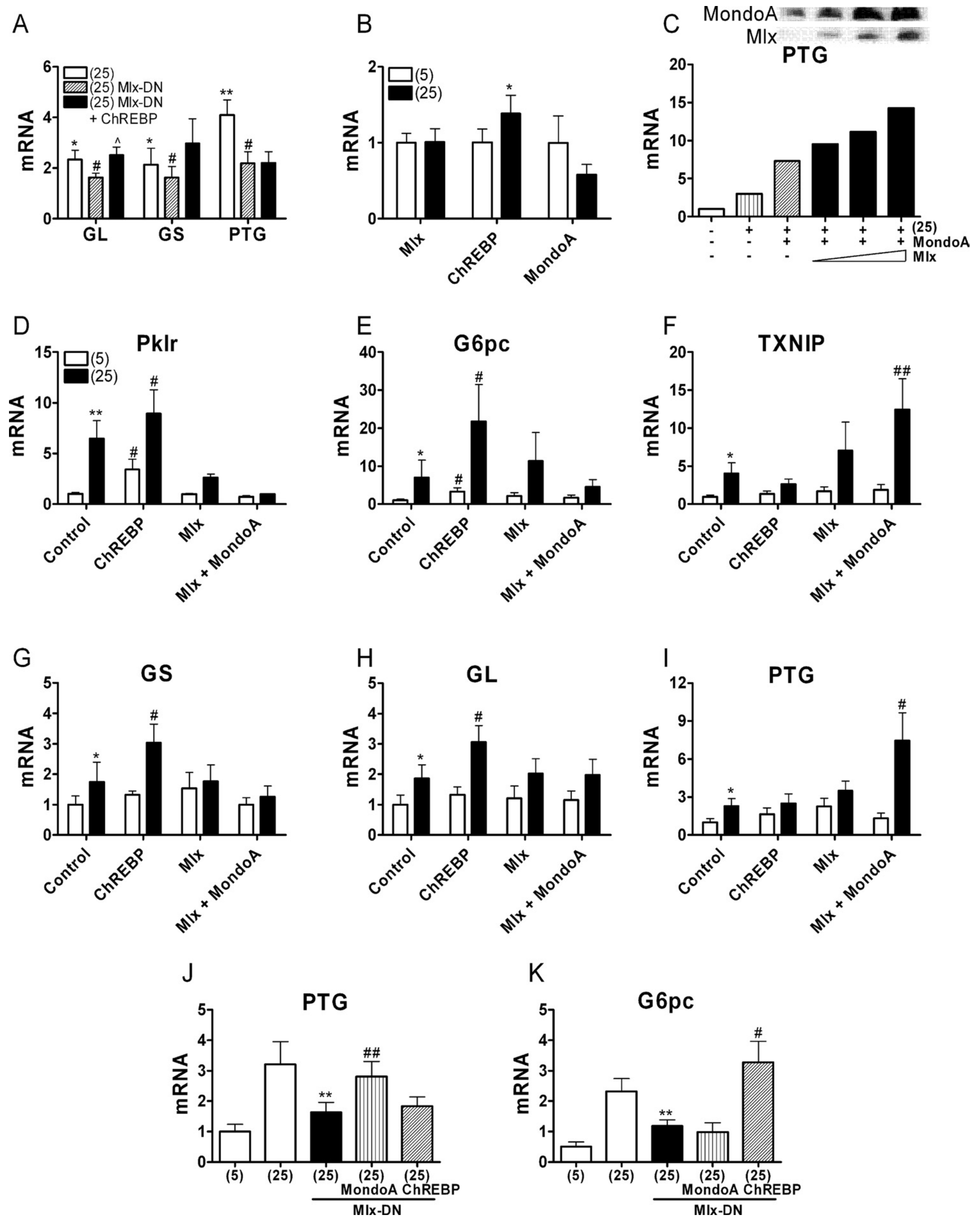

FIG 2 MondoA and ChREBP enhance glucose induction. (A) Hepatocytes were either untreated or treated with adenoviral vectors for expression of Mlx-DN either alone or combined with adenoviral vector for expression of ChREBP (+ ChREBP). They were then incubated either with 5 mM glucose (not shown) or with 25 mM glucose. mRNA levels of the indicated genes are expressed as the fold change relative to untreated hepatocytes with 5 mM glucose (1.0). Shown are means and SE for 4 hepatocyte preparations. *, $P < 0.05$, and **, $P < 0.01$ versus 5 mM glucose; #, $P < 0.05$, and ##, $P < 0.01$ versus 25 mM glucose; ^, $P < 0.05$ versus Mlx-DN. (B) mRNA levels for endogenous Mlx, MondoA, and ChREBP after 4 h of incubation with either 5 mM or 25 mM glucose. Shown are means and SE for 6 hepatocyte preparations. *, $P < 0.05$ for the effect of 25 mM glucose. (C) Hepatocytes were untreated or treated with adenoviral vectors for expression of MondoA alone or combined with Mlx and then incubated with 5 mM (−) or 25 mM (+) glucose as indicated for determination of PTG mRNA. Shown is immunoactivity to FLAG tag at 33 kDa (Mlx) or 98 kDa (MondoA).The data are representative of 2 experiments. (D to I) Hepatocytes were either untreated or treated with adenoviral vectors for overexpression of Mlx alone or ChREBP alone or combined Mlx and MondoA expression and cultured for 18 h in MEM containing 10 nM dexamethasone and then incubated for 4 h in fresh medium with either 5 mM or 25 mM glucose, as indicated, for determination of mRNA levels of the Pklr, G6pc, TXNIP, glycogen synthase, G_L, and PTG genes. Shown are means and SE for 3 to 6 hepatocyte preparations. *, $P < 0.05$, and **, $P < 0.01$ for the effect of 25 mM glucose; #, $P < 0.05$, and ##, $P < 0.01$ relative to untreated hepatocytes. (J and K) Hepatocytes were either untreated (open bars) or treated with adenoviral vectors for expression of Mlx-DN alone or combined with ChREBP or MondoA. They were incubated for 4 h with either 5 mM [(5)] or 25 mM [(25)] glucose for determination of mRNA for PTG (J) or G6pc (K). Shown are means and SE for 6 hepatocyte preparations. *, $P < 0.05$, and **, $P < 0.01$ versus 25 mM glucose; #, $P < 0.05$, and ##, $P < 0.01$ versus Mlx-DN.

hepatocyte model for replicating changes in the glucose-challenged liver *in vivo*. We next tested the effects of the glucose and insulin concentrations in the hepatocyte model (Fig. 5D and E). Glucose caused significant elevation of PTG mRNA at or above 10 mM glucose (Fig. 5D), suggesting responsiveness in the physiological range, as occurs in the portal vein in the postprandial state.

Glucose, but not 2-deoxyglucose, induces PTG mRNA and MondoA translocation in hepatocytes. Previous studies showed

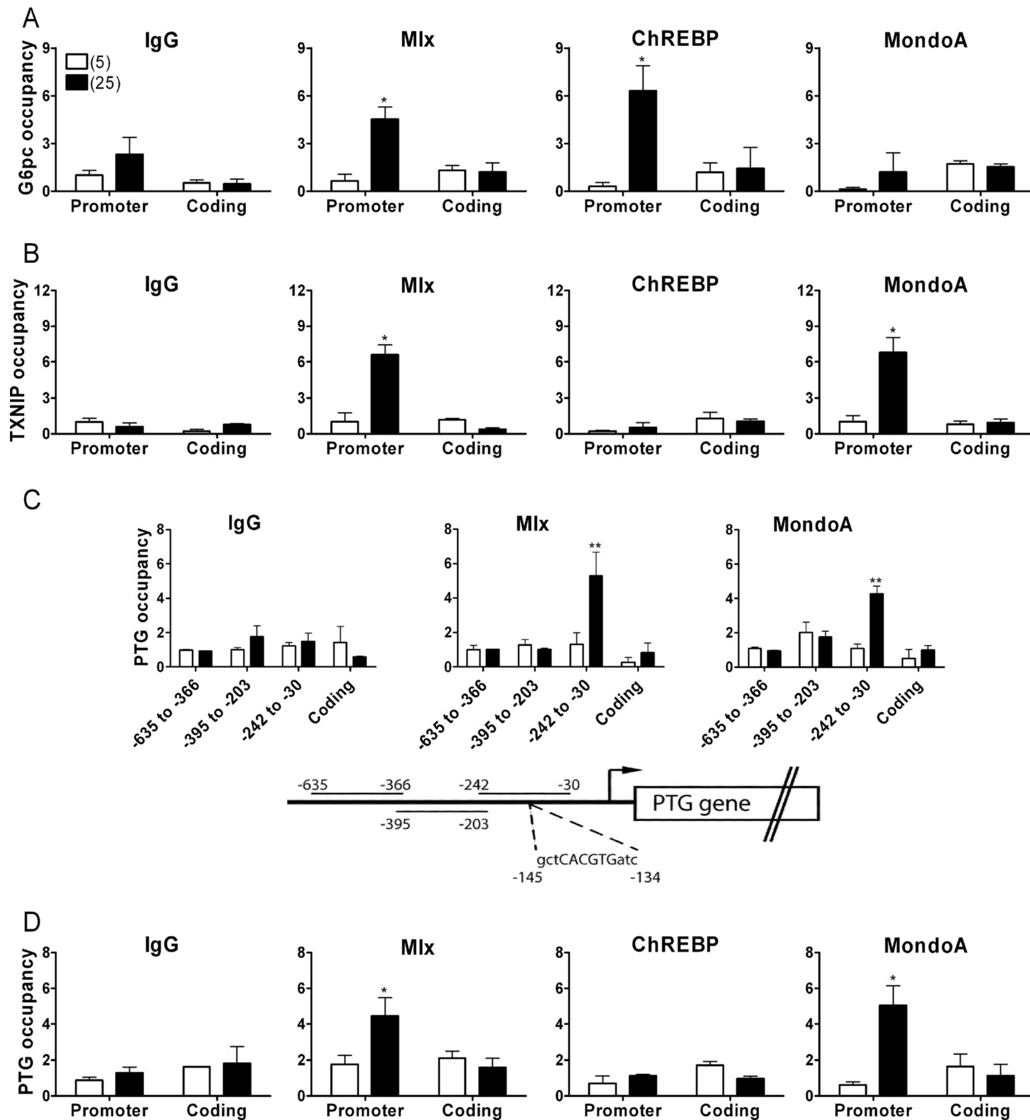

FIG 3 Glucose-dependent recruitment of endogenous Mlx and MondoA, but not ChREBP, to the proximal PTG promoter. Hepatocytes were precultured for 18 h and then incubated for 4 h in fresh medium with 10 nM insulin and either 5 mM [(5)] glucose or 25 mM glucose plus 2 μM S4048 [(25)], as described in the legend to Fig. 1. (A and B) ChIP assays were performed as described in Materials and Methods using control IgG or antibody to Mlx, MondoA, or ChREBP for determination of binding of these proteins to established elements in the G6pc (A) and TXNIP (B) gene promoters. (C) Binding to regions spanning the PTG gene promoter. (D) Binding to −242 to −30 of the PTG gene promoter. The results are expressed relative to control IgG with 5 mM glucose. Shown are means and SE for 3 hepatocyte preparations. *, $P < 0.05$, and **, $P < 0.01$ relative to 5 mM glucose.

that in hepatocytes, 2-deoxyglucose does not mimic the glucose induction of ChREBP target genes despite marked elevation in its 6-phosphate ester to concentrations exceeding those of glucose 6-P (23). In INS1 cells, 2-deoxyglucose mimicked the glucose induction of TXNIP, but not that of the ChREBP target gene, the ACC gene (40). In C2C12 cells and other cell lines, 2-deoxyglucose induced TXNIP more potently than glucose (16, 17, 28, 41, 42). To gain insight into the mechanism by which glucose induces PTG, we compared the effects of glucose with those of 2-deoxyglucose, 3-*O*-methylglucose, and allose, which have also been shown to induce TXNIP in various cell lines (17, 40). All glucose analogues tested induced TXNIP mRNA, but not PTG mRNA (Fig. 6A and B). Interestingly, forced overexpression of MondoA enhanced TXNIP expression with 25 mM glucose, but not with 2-deoxyglucose (Fig. 6C), and likewise, it enhanced PTG mRNA only with 25 mM glucose (Fig. 6D). The different responses of the TXNIP and PTG genes to 2-deoxyglucose (Fig. 6A to D) were unexpected, given that both genes are targets for endogenous MondoA at high glucose levels in ChIP assays (Fig. 3). We therefore tested the effects of 2-deoxyglucose on the PFKFB3 gene, which is a MondoA target in C2C12 cells (39) and, like the PTG gene, has a single CACGTG E box in the proximal promoter. PFKFB3 mRNA was significantly increased by overexpression of MondoA (Fig. 6E) but was not affected by 2-deoxyglucose (Fig. 6F). This suggests that induction by 2-deoxyglucose is not a property shared by the MondoA target PTG or PFKFB3 gene in hepatocytes. It is possible that 2-deoxyglucose activates TXNIP independently of MondoA in hepatocytes. To test for this possibility, we determined the

TABLE 2 Conservation of the *Ppp1r3c* gene CACGTG element in Eutherian and Metatherian mammals

Division	Superorder	Order; suborder	Species	Flanking regions of CACGTG[a]
Eutheria	Euarchontoglires	Primates; Catarrhini	Human, chimpanzee, gorilla, orangutan	TCGGGT**CACGTG**CTTGGG
	Euarchontoglires	Primates; Catarrhini	Gibbon, macaque	TCGGGT**CACGTG**CTTCGG
	Euarchontoglires	Primates; Strepsirrhini	Bush baby	TCTGCT**CACGTG**GTGCGG
	Euarchontoglires	Rodentia	Rat	CCAGCT**CACGTG**ATCGGG
	Euarchontoglires	Rodentia	Mouse	CCGGTT**CACGTG**ATCTGG
	Laurasiatheria	Artiodactyla	Cow	TCGGCT**CACGTG**GTCCCG
	Laurasiatheria	Carnivora	Dog	CGGGCT**CACGTG**GTCCGG
Metatheria		Dasyuromorphia	Sarcophilus	GGCTTT**CACGTG**AAAGCC

[a] The CACGTG element (boldface) is present in the proximal promoter region of the *Ppp1r3c* gene in the mammals listed but is absent in chicken, *Xenopus*, and zebrafish (http://www.ensembl.org/index.html).

MondoA location in hepatocytes by expressing FLAG-tagged MondoA and using an anti-FLAG antibody. Incubation with 25 mM glucose, but not 2-deoxyglucose, increased nuclear staining for MondoA in hepatocytes (Fig. 6G), consistent with the lack of effect of 2-deoxyglucose on PTG mRNA expression (Fig. 6B and D). Translocation of MondoA in response to 25 mM glucose, but not deoxyglucose, was also confirmed by immunoblotting of nuclear and cytoplasmic extracts for 'MondoA-FLAG' (Fig. 6H). Immunoactivity to lamin and glyceraldehyde phosphate dehydrogenase confirmed the integrity of the nuclear and cytoplasmic fractions, respectively. Because the carbohydrate-responsive elements of the TXNIP gene promoter are adjacent to NF-Y binding sites (38), we also tested the nuclear and cytoplasmic fractions for immunoactivity to NF-Yα. Immunoactivity to NF-Yα was predominantly nuclear and was not affected by 25 mM glucose or 2-deoxyglucose (Fig. 6H). In experiments on C2C12 cells transfected with FLAG-tagged MondoA (Fig. 6I), we confirmed the translocation of MondoA to the nucleus with 2-de-

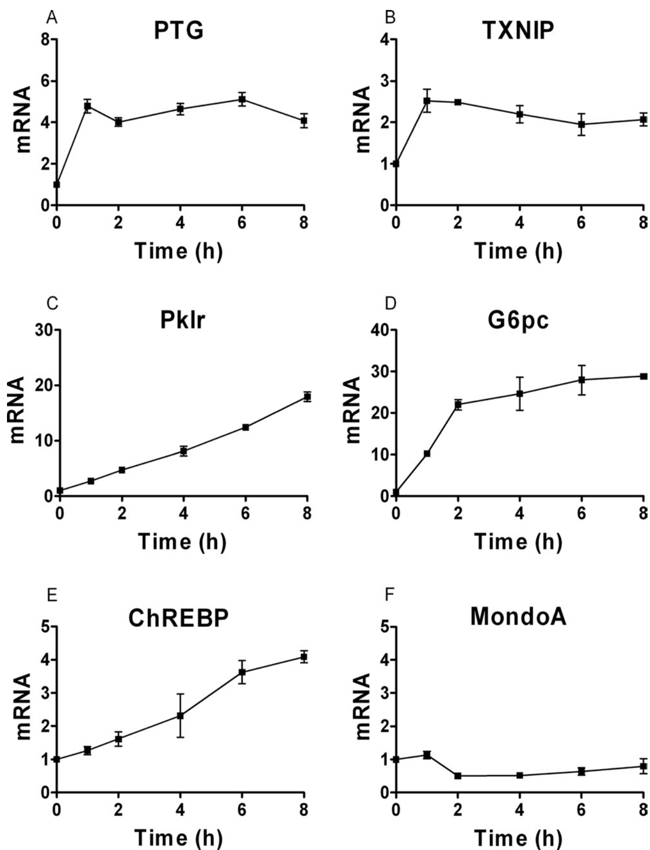

FIG 4 Time course of glucose induction. Hepatocytes were precultured for 18 h in MEM containing 10 nM dexamethasone and 10 nM insulin and then incubated with 25 mM glucose for the time intervals indicated. mRNA levels for PTG (A), TXNIP (B), Pklr (C), G6pc (D), ChREBP (E), and MondoA (F) are expressed as fold change relative to 5 mM glucose. The error bars indicate standard deviations (SD) for triplicate incubations.

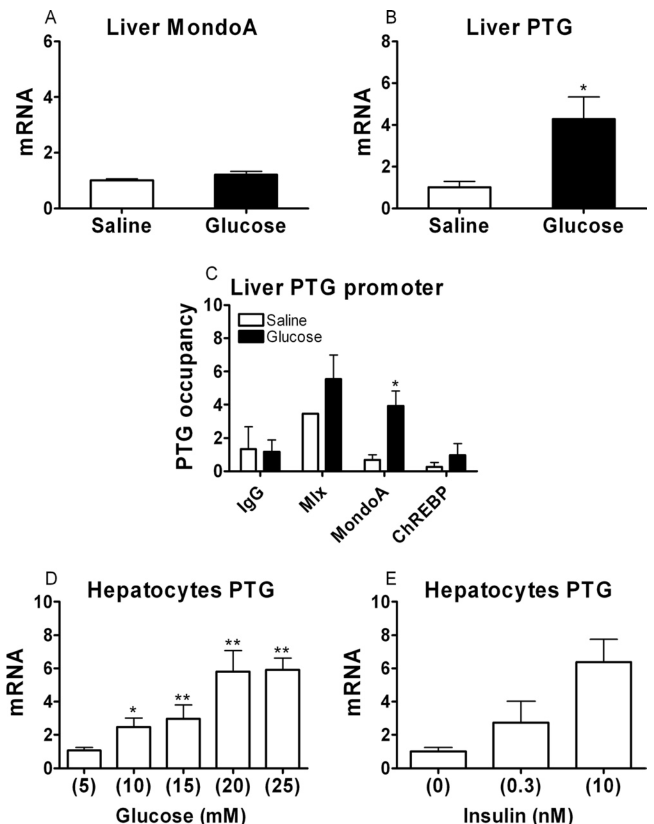

FIG 5 Regulation of liver PTG expression *in vivo* and *in vitro*. (A and B) Liver MondoA mRNA and PTG mRNA after treatment of fasted rats with saline or glucose for 1 h. Shown are means and standard errors of the mean (SEM); $n = 5$. (C) Recruitment of Mlx and MondoA to the PTG promoter by glucose (1 h) in fasted rats. Shown are means and SEM; $n = 4$. (D and E) Effects of glucose and insulin concentrations on PTG mRNA in hepatocytes. Shown are means and SEM; $n = 3$. *, $P < 0.05$ for the effect of glucose.

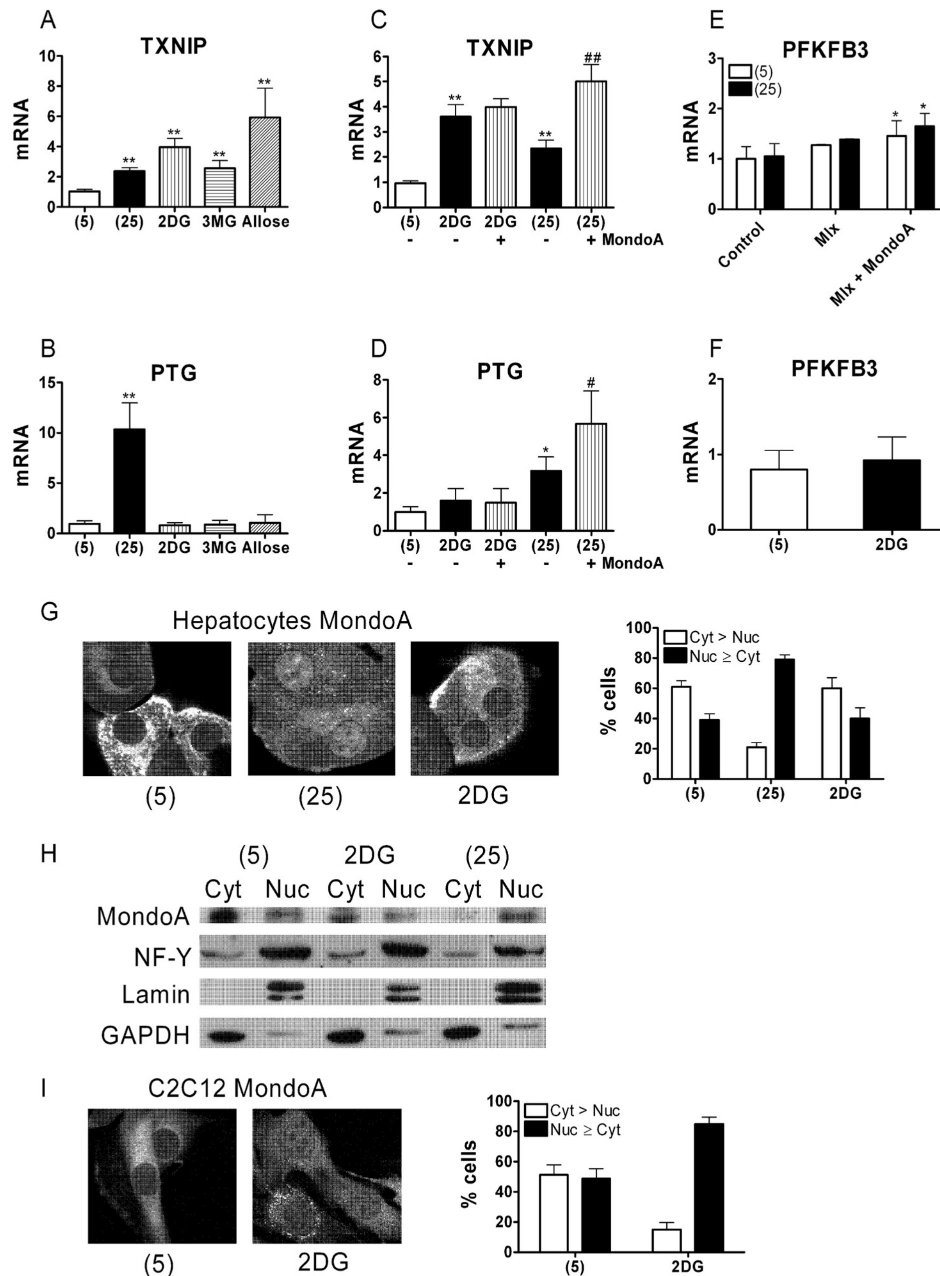

FIG 6 Glucose, but not 2-deoxyglucose, induces PTG mRNA and MondoA translocation in hepatocytes. (A and B) Hepatocytes were incubated for 4 h with 5 mM or 25 mM glucose and the glucose analogues indicated: 10 mM 2-deoxyglucose (2DG), 10 mM 3-*O*-methylglucose (3MG), and 5 mM allose for determination of TXNIP mRNA (A) and PTG mRNA (B). (C and D) Hepatocytes were either untreated or treated with adenoviral vector for expression of MondoA and then incubated for 4 h with 5 mM or 25 mM glucose or 10 mM 2-deoxyglucose as indicated for determination of TXNIP mRNA (C) or PTG mRNA (D). (E) Hepatocytes were untreated or treated with vectors for expression of Mlx and MondoA. They were then incubated for 4 h with 5 mM or 25 mM glucose for determination of PFKFB3 mRNA. (F) Effects of 10 mM 2-deoxyglucose on PFKFB3 mRNA. Shown are means and SE for 3 to 12 hepatocyte preparations. *, $P < 0.05$, and **, $P < 0.01$ relative to the control; #, $P < 0.05$, and ##, $P < 0.01$ relative to 25 mM glucose. (G) Hepatocytes were treated with FLAG-tagged MondoA vector, and after 18 h of culture, they were incubated for 1 h with 5 mM [(5)] or 25 mM [(25)] glucose or with 5 mM glucose plus 10 mM 2DG. Shown are representative images and combined data from 2 hepatocyte preparations. Cyt, cytoplasmic; Nuc, nuclear. (H) Immunoactivity to MondoA-FLAG, NF-Yα, lamin, and GAPDH in nuclear and cytoplasmic extracts of hepatocytes incubated for 1 h with 5 mM or 25 mM glucose or 10 mM 2-deoxyglucose. (I) C2C12 cells were treated with FLAG-tagged MondoA vector, and after 18 h of culture, they were incubated for 1 h with 5 mM glucose alone [(5)] or with 10 mM 2DG. Shown are means and SE for 3 experiments.

oxyglucose as reported previously (16, 17, 28). This suggests that the effect of 2-deoxyglucose on MondoA translocation is cell type dependent, as was also noted previously from the lack of translocation of MondoA in HEK cells (16).

Essential role for fructose 2,6-P_2 in the induction of PTG mRNA and MondoA translocation. The above-mentioned studies show induction of PTG mRNA by high glucose levels, but not by 2-deoxyglucose (Fig. 6), despite accumulation of 2-deoxyglucose 6-P in hepatocytes (23). Because 2-deoxyglucose is not metabolized by glycolysis, we next asked whether metabolism down-

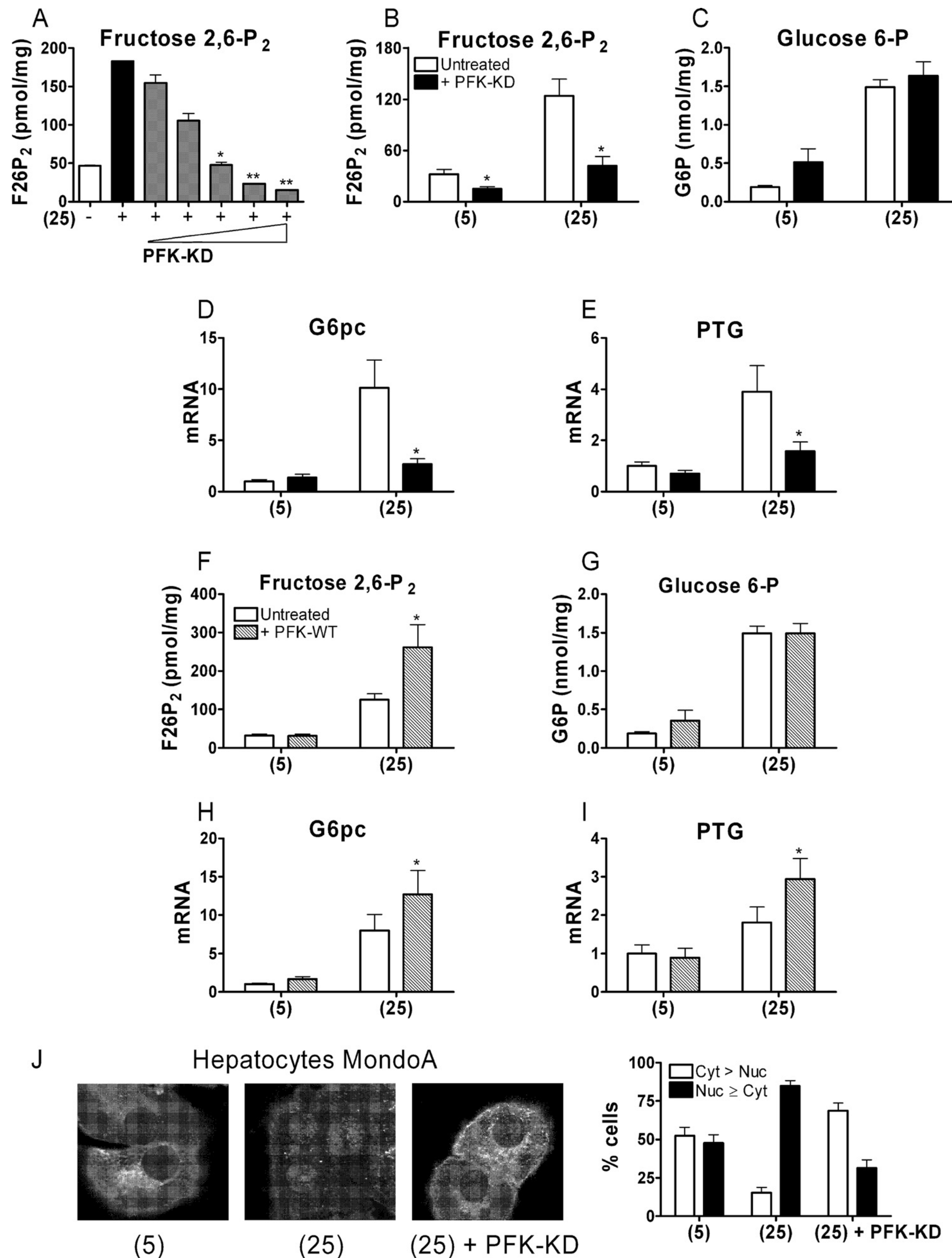

FIG 7 Essential role for fructose 2,6-P_2 in the glucose induction of PTG mRNA and MondoA translocation in hepatocytes. (A to E) Hepatocytes were untreated or treated with PFK-KD vector. After 18 h of culture, they were incubated with 5 mM or 25 mM glucose for 1 h for determination of fructose 2,6-P_2 (A and B) and glucose 6-P (C) or 4 h for G6pc mRNA (D) and PTG mRNA (E). Shown are means and SE for 3 to 8 hepatocyte preparations. *, $P < 0.05$ and **, $P < 0.01$, for the effect of PFK-KD. (F to I) Hepatocytes were untreated or treated with PFK-WT vector and after 18 h incubated with 5 mM or 25 mM glucose for 1 h for determination of fructose 2,6-P_2 (F) and glucose 6-P (G) or 4 h for determination of G6pc (H) and PTG (I) mRNAs. Shown are means and SE; $n = 4$. *, $P < 0.05$ for the effect of PFK-WT. (J) Hepatocytes were treated with FLAG-tagged MondoA vector and, where indicated, with PFK-KD vector. After 18 h of culture, they were incubated for 1 h with 5 mM or 25 mM glucose. Shown are representative images and means and SE for 4 hepatocyte preparations.

stream of fructose 6-P is involved in MondoA activation. Metabolism of fructose 6-P by glycolysis is regulated by fructose 2,6-P_2, a potent allosteric activator of phosphofructokinase 1 that is synthesized from fructose 6-P and degraded to fructose 6-P by the bifunctional enzyme PFK2/FBP2 (4). The cell content of fructose 2,6-P_2 depends on the fructose 6-P concentration, which is in equilibrium with glucose 6-P, and on the kinase/bisphosphatase activity ratio of the bifunctional enzyme (4). To test for involvement of fructose 2,6-P_2, we used a bisphosphatase-active kinase-deficient variant (PFK-KD) to selectively attenuate fructose 2,6-P_2 independently of its substrate, fructose 6-P. Incubation with 25 mM glucose raised fructose 2,6-P_2 levels by 4-fold, and this was attenuated by titrated expression of PFK-KD (Fig. 7A). PFK-KD also lowered fructose 2,6-P_2 levels at 5 mM glucose (Fig. 7B) but did not counteract the elevation at high glucose levels of glucose 6-P (Fig. 7C), which is in equilibrium with fructose 6-P. This

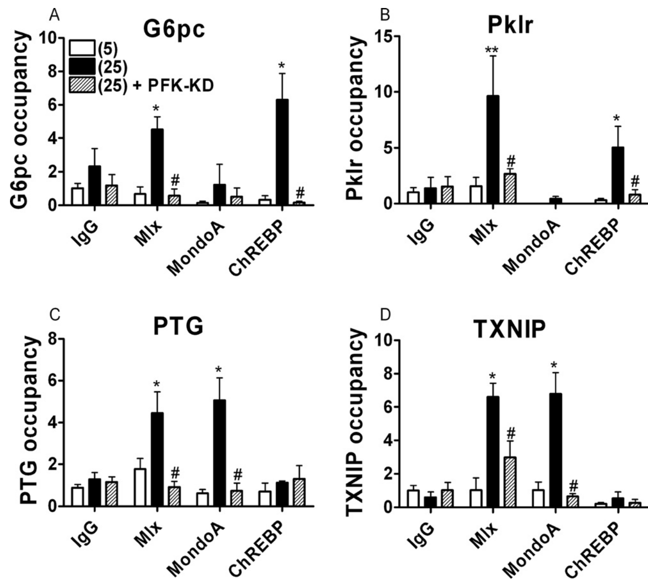

FIG 8 Fructose 2,6-P_2 is essential for glucose-induced recruitment of Mlx, MondoA, and ChREBP to their target promoters. Hepatocytes were either untreated or treated with PFK-KD vector as in Fig. 7. After 18 h of culture, they were incubated for 4 h with 5 mM glucose [(5)] or 25 mM glucose plus 2 μM S4048 [(25)]. Recruitment to the promoters of the G6pc (A), Pklr (B), PTG (C), and TXNIP (D) genes was determined as described in Materials and Methods. Shown are means and SE for 3 hepatocyte preparations. *, $P < 0.05$, and **, $P < 0.01$ relative to 5 mM glucose; #, $P < 0.05$ for the effect of PFK-KD.

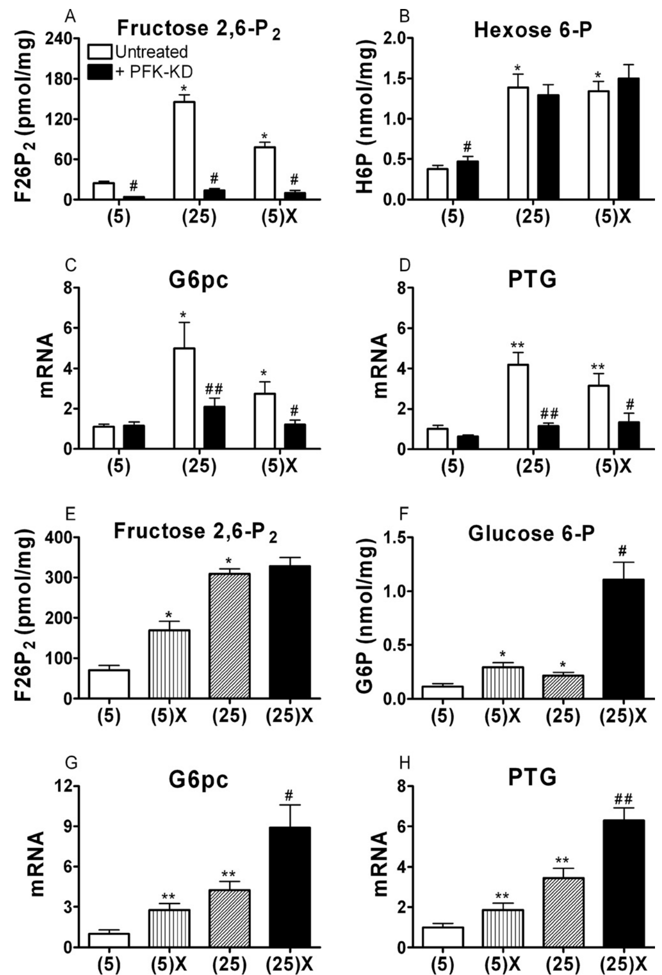

FIG 9 Glucose and xylitol induction of PTG and G6pc. (A to D) Hepatocytes were either untreated or treated with PFK-KD vector as in Fig. 7. After 18 h of culture, they were incubated with 5 mM glucose [(5)], 25 mM glucose [(25)], or 5 mM glucose plus 5 mM xylitol [(5)X] for determination of fructose 2,6-P_2; hexose 6-P, representing glucose 6-P plus fructose 6-P; G6pc mRNA; or PTG mRNA, as indicated. (E to H) Hepatocytes were incubated for 4 h with 5 mM or 25 mM glucose with or without 5 mM xylitol (X) for determination of fructose 2,6-P_2, glucose 6-P, G6pc mRNA, or PTG mRNA. Shown are means and SE for 3 to 12 hepatocyte preparations. *, $P < 0.01$, and **, $P < 0.01$ relative to 5 mM glucose; #, $P < 0.05$, and ##, $P < 0.01$ relative to untreated hepatocytes (A to D) or hepatocytes treated with 25 mM glucose (F to H).

supports the ability of PFK-KD to selectively attenuate fructose 2,6-P_2. Treatment with PFK-KD prevented both the glucose induction of the ChREBP target gene, the G6pc gene (Fig. 7D) and the glucose induction of PTG (Fig. 7E). To rule out a possible effect of PFK-KD protein independent of depletion of fructose 2,6-P_2, we expressed wild-type PFKFB1 (PFK-WT). This increased fructose 2,6-P_2 at 25 mM glucose, but not at 5 mM glucose (Fig. 7F), and did not increase glucose 6-P at 25 mM glucose (Fig. 7G). Expression of PFK-WT enhanced G6pc and PTG mRNAs at 25 mM glucose (Fig. 7H and I) in parallel with the increase in fructose 2,6-P_2 (Fig. 7F). The opposite effects of PFK-KD and PFK-WT on PTG mRNA confirm a role for fructose 2,6-P_2 in PTG induction. Blocking the elevation in fructose 2,6-P_2 with PFK-KD also inhibited translocation of MondoA to the nucleus at high glucose levels (Fig. 7J), indicating a role for fructose 2,6-P_2 in MondoA translocation.

Essential role for fructose 2,6-P_2 in the glucose-induced recruitment of MondoA and ChREBP to gene promoters. To further confirm that elevation in fructose 2,6-P_2 is essential for activation of MondoA, we determined the glucose-dependent recruitment of Mlx, MondoA, and ChREBP to their target genes in cells treated with PFK-KD to counteract elevation in fructose 2,6-P_2 levels, as shown in Fig. 7. PFK-KD inhibited the glucose-induced recruitment of Mlx and ChREBP to the G6pc and Pklr gene promoters (Fig. 8A and B), and also that of Mlx and MondoA to the PTG and TXNIP promoters (Fig. 8C and D). This confirms that elevation in fructose 2,6-P_2 levels is essential for glucose-induced recruitment of ChREBP and MondoA to their targets.

Dual effects of xylitol on PTG mRNA expression. Xylitol is metabolized to xylulose 5-P and then by the nonoxidative branch of the pentose phosphate pathway to fructose 6-P and glyceraldehyde 3-P via ribulose 5-P, ribose 5-P, sedoheptulose 7-P, and erythrose 4-P. Previous studies showed that while elevation of fructose 2,6-P_2 levels is essential for the induction of ChREBP target genes by high glucose levels, additional metabolites generated from xylitol enhance gene expression (23). To test whether the glucose induction of PTG by MondoA shares a similar mechanism with ChREBP targets, we determined the effects of xylitol either in combination with expression of PFK-KD to selectively knock down fructose 2,6-P_2 (Fig. 9A to D) or by coincubation with 25 mM glucose (Fig. 9E to H). Xylitol raised fructose 2,6-P_2 and hexose 6-P (glucose 6-P plus fructose 6-P) levels and also G6pc and PTG mRNA levels (Fig. 9A to D). PFK-KD counteracted the elevation in fructose 2,6-P_2, but not hexose 6-P, and attenuated PTG mRNA similarly to G6pc mRNA, indicating that elevated fructose 2,6-P_2 is essential for induction of PTG by xylitol.

When xylitol was combined with 25 mM glucose, it did not further increase fructose 2,6-P_2 levels relative to 25 mM glucose (Fig. 9E), but it further increased glucose 6-P (Fig. 9F) and also PTG mRNA in parallel with G6pc mRNA (Fig. 9G and H). Coincubation of 25 mM glucose and 2-deoxyglucose (10 mM), unlike combined 25 mM glucose and 5 mM xylitol, did not further increase expression of either PTG or G6pc mRNA relative to 25 mM glucose (results not shown). Cumulatively, these results show, first, that elevation in fructose 2,6-P_2 is essential for the induction of PTG by xylitol, as well as high glucose levels, and, second, that in addition to fructose 2,6-P_2, other metabolites enhance PTG expression.

DISCUSSION

The transcription factors ChREBP and MondoA are the main regulators of transcription in response to elevated glucose metabolism in liver and muscle, respectively (12). The function of ChREBP in the liver is thought to be the induction of glycolytic and lipogenic genes to convert surplus dietary glucose to lipid for energy storage (18–20, 25). However, another proposed function of ChREBP is the maintenance of intracellular homeostasis of phosphorylated metabolic intermediates under conditions of elevated glucose (22). This hypothesis is supported by the induction by ChREBP of G6pc, which lowers cellular levels of glucose 6-P and downstream intermediates when glucose is elevated (22, 23). In this work, we identified three regulatory proteins of the glycogenic pathway (glycogen synthase, G_L, and PTG) that are induced by high glucose levels. Glycogen synthase is a major determinant of the rate of glycogen synthesis (43). Overexpression of either the wild type or a constitutively active form of the enzyme enhances glycogen storage (43, 44). The glycogen-targeting proteins G_L and PTG function as molecular scaffolds for compartmentalizing protein phosphatase 1 and enzymes involved in glycogen metabolism with glycogen particles. G_L, in association with protein phosphatase 1, functions predominantly as a glycogen synthase phosphatase, whereas PTG associated with protein phosphatase 1 functions as a phosphorylase phosphatase (7, 8). Forced overexpression of G_L with adenoviral vectors in hepatocytes enhances glycogen synthase activity and glycogen storage severalfold (45), and forced titrated overexpression of PTG causes progressive inactivation of glycogen phosphorylase and consequent activation of glycogen synthase with progressive stimulation of glycogen storage that correlates inversely with the inactivation of phosphorylase (8, 29, 32, 46). Inactivation of phosphorylase attenuates the glucose-induced elevation in glucose 6-P levels (47). Accordingly, induction by high glucose levels of PTG and G_L is expected to favor increased glycogen storage (8, 29, 45, 46), but importantly, it would also restrain the elevation in glucose 6-P in response to elevated glucose, as occurs with pharmacological inhibitors of glycogen phosphorylase (47). Thus, the glucose induction of PTG has analogies to the glucose induction of glycolytic enzymes downstream of glucose 6-P in increasing the disposal of glucose 6-P to the end product glycogen compared with triglyceride while restraining the elevation in glucose 6-P.

The next issue addressed in this study was whether ChREBP-Mlx mediates the glucose induction of the glycogen regulatory proteins. This work demonstrated that the PTG gene is a target for MondoA and not for ChREBP. This was supported by four sets of evidence: first, high glucose levels induced the recruitment of MondoA and Mlx, but not ChREBP, to the PTG promoter in hepatocytes; second, overexpression of MondoA, but not ChREBP, induced PTG mRNA expression; third, expression of a dominant-negative variant of Mlx abrogated the glucose induction of PTG expression, and this effect was reversed by expression of MondoA, but not ChREBP; fourth, recruitment of MondoA to the PTG promoter and elevation of PTG mRNA were demonstrated in fasted rats *in vivo* after challenge with a glucose load for 1 h. The involvement of MondoA in the regulation of gene expression in the liver or hepatocytes has not been reported previously.

The selective targeting of MondoA or ChREBP to distinct target genes in hepatocytes implies discrete roles for these paralogs. One possible explanation for the expression of two glucose-responsive transcription factors in hepatocytes is the different temporal responses to glucose. Induction of the two identified MondoA targets (the PTG and TXNIP genes) by glucose occurred within 1 h and was sustained for 8 h. This contrasts with the expression of two ChREBP target genes (*Pklr* and *G6pc*), which was progressive over 8 h. This difference may be explained in part by the constitutive expression of MondoA compared with the induction of ChREBP by high glucose levels. The identification of a regulatory protein of glycogen metabolism that determines phosphorylase phosphatase activity (7–9, 32) as a MondoA target can be rationalized by the temporal response of glycogen storage in the postprandial state. Synthesis of glycogen after a meal is important to replenish the hepatic carbohydrate store that serves to maintain blood glucose homeostasis in the interval between meals. It is therefore a priority over conversion of glucose to fatty acid, which cannot be reconverted to glucose to maintain blood glucose homeostasis. However, the liver has a limited capacity for glycogen storage; thus, a sustained response for the duration of the postprandial state enables adequate but not excessive glycogen storage. Conversely, progressive induction of the glycolytic enzyme Pklr, which serves to divert glucose to the lipogenic pathway, is better suited for conversion of surplus dietary carbohydrate to lipid, which is exported by the liver for energy storage in adipose tissue. Induction of MondoA targets may therefore be characterized by a rapid rather than progressive response.

Previous studies identified candidate target genes for MondoA from gene microarray studies in cell lines transfected with a MondoA variant that is targeted constitutively to the nucleus (39) or from incubations with 2-deoxyglucose in cells with or without MondoA knockdown (16). The former study identified enzymes of glycolysis and showed binding of MondoA to promoter regions of PFKFB3, hexokinase II, and LDH4 containing single or multiple CACGTG elements (39). The latter study identified large numbers of genes induced by 2-deoxyglucose through MondoA-dependent or MondoA-independent mechanisms and showed a large induction of TXNIP by 2-deoxyglucose in cells without MondoA knockdown (16). In the present study, we demonstrate that in hepatocytes, both PTG and TXNIP genes are targets for MondoA, but not ChREBP. This observation raises the intriguing question of how targeting specificity is achieved for the two glucose-responsive transcription factors. One potential explanation is that differences in the nature of the E box-containing carbohydrate response elements are responsible. In all ChREBP target genes that have been evaluated, two E box elements that are separated by 5 bp are found (48). The spacing between these E box elements has been found to be essential for the ability of ChREBP-Mlx to form functional heterotetramers at the promoters of these genes. On the other hand, MondoA targets contain one or more E boxes, with no rigid spacing requirement observed in those con-

taining multiple E boxes (39). The PTG gene appears to fit this model, as only a single E box is found within the region interacting with MondoA/Mlx, and this region is highly conserved throughout placental and marsupial mammals, though, interestingly, not in other vertebrate classes. It is notable that the TXNIP gene, which is a target of both ChREBP and MondoA in different cells, may possess a "hybrid" response element capable of interacting with either ChREBP or MondoA. Additionally, given the wide array of E box binding factors (12, 26), and tissue differences in their abundance, competition of these factors with ChREBP or MondoA could also explain the tissue differences in recruitment of these regulators to the TXNIP gene promoter.

Given the role of MondoA as the mediator of the glucose induction of PTG and TXNIP, an unexpected finding was that TXNIP is induced by 2-deoxyglucose while PTG is not. A possible explanation for the effect of 2-deoxyglucose on TXNIP, but not PTG, mRNA is that this glucose analogue may regulate distinct transcriptional regulators that bind either remotely or near the MondoA binding site on the TXNIP gene promoter (38). This is supported by the observation that in hepatocytes, 2-deoxyglucose causes induction of TXNIP similar to that caused by glucose, but unlike glucose, it does not cause translocation of MondoA to the nucleus. The TXNIP promoter has two carbohydrate response elements, each consisting of two imperfect E boxes, and each is adjacent to an NF-Y binding site (38). NF-Y is a histone-like transcription factor that is tightly regulated by covalent modification and acts as a scaffold for other transcriptional regulators (49). Changes in covalent modification of NF-Y may therefore affect the binding of transcriptional regulators in the vicinity of the carbohydrate response element and could explain the tissue difference in binding of ChREBP to the TXNIP gene promoter in islet beta cells (34) but binding of MondoA to the TXNIP promoter in hepatocytes. Whether induction of TXNIP by 2-deoxyglucose is mediated by MondoA in conjunction with other regulators in muscle and by other regulators independently of MondoA in hepatocytes remains to be further explored. However, our study suggests that PTG is a more appropriate target for studying metabolite control of MondoA activation in hepatocytes.

The third issue addressed in this study was whether ChREBP and MondoA are activated by similar or distinct metabolite signals. The mechanism by which high glucose levels cause translocation of ChREBP and MondoA from the cytoplasm to the nucleus, recruitment to the respective gene promoters, and transcriptional activation is not completely understood. However, the region that is critical for glucose regulation has been mapped to the N-terminal 300 amino acids of both factors, termed the Mondo conserved region. This region is highly conserved between ChREBP and MondoA and likely serves to bind to the active glucose metabolite or glucose-sensing factor (50–53). Previous studies on the activation of ChREBP and MondoA have been performed in different cell models (42). A key finding from this study was that the induction of PTG by MondoA was similar to the induction of ChREBP targets in three key respects: first, it is induced by glucose and xylitol, but not by 2-deoxyglucose; second, elevation in fructose 2,6-P_2 is essential for substrate induction; third, combined exposure to high glucose and xylitol levels had a greater effect on gene induction than can be explained by the elevation in fructose 2,6-P_2. Previous studies suggested a role for glucose 6-P in the activation of ChREBP based on association studies demonstrating elevated expression of ChREBP target genes under conditions of elevated glucose 6-P (25). However, glucose 6-P is in equilibrium with glucose 1-P through phosphoglucomutase and with fructose 6-P via phosphoglucoisomerase. Fructose 6-P is a substrate for glycolysis, for PFK2/FBP2, and for the hexosamine pathway. Accordingly, changes in glucose 6-P are associated with changes in a wide range of downstream metabolites, and thus, association studies may not distinguish between glucose 6-P and downstream metabolites. Fructose 2,6-P_2, in contrast, is a dead-end metabolite that is synthesized from and degraded to fructose 6-P by PFK2/FBP2. Expression of a kinase-deficient variant of the protein enables selective attenuation of fructose 2,6-P_2 at high glucose levels without modulation of the hexose 6-phosphate pool. Using this approach, we demonstrate that induction of PTG mRNA at high glucose levels is critically dependent on the elevation in fructose 2,6-P_2 for the recruitment of MondoA to the PTG promoter and likewise for recruitment of ChREBP to its target gene promoters. It is noteworthy, however, that since elevation in fructose 2,6-P_2 occurs only under conditions of raised hexose 6-P as the substrate, a role for glucose 6-P, xylulose 5-P, or other linked metabolites that are raised by high glucose or xylitol levels cannot be excluded. Indeed the involvement of metabolites in addition to fructose 2,6-P_2 in the regulation of ChREBP and MondoA is supported by incubations with high glucose combined with xylitol. Involvement of multiple phosphorylated intermediates, as opposed to glucose 6-P *per se* (26), supports a role for these transcription factors in cellular homeostasis of phosphorylated intermediates (22).

A key question is whether fructose 2,6-P_2 has an analogous role in glucose-regulated gene expression in nonhepatic cells. Proliferating cells express hexokinase isoforms that are inhibited by the end product glucose 6-P and therefore respond to high glucose levels with smaller fluctuations in glucose 6-P. They also express the inducible isoform of PFK2/FBP2 (PFKFB3), which has a high kinase/bisphosphatase ratio compared with the liver isoform (54) and is present in the nucleus (55). These cell lines therefore differ from hepatocytes in the dynamic changes in these intermediates. Whereas hepatocytes respond to elevated glucose (25 mM versus 5 mM) with large and rapid changes in fructose 2,6-P_2, cells expressing PFKFB3 have a high basal level of fructose 2,6-P_2 constitutively (56). The nuclear location of PFKFB3 (55) also raises the possibility that fructose 2,6-P_2 may be generated in localized cell compartments. The present study shows that primary hepatocytes are a very good model for comparative studies of ChREBP and MondoA activation, because both transcriptional regulators have a role in the model and because phosphorylated intermediates of glucose metabolism show large adaptive changes in response to high glucose (3). Furthermore, glucose regulation of PTG expression in hepatocytes via MondoA recruitment replicates the changes in the liver *in vivo* after acute challenge of fasted rats with glucose. The hepatocyte model establishes that fructose 2,6-bisphosphate is essential for activation of both ChREBP and MondoA, but also that other metabolites are involved. Identification of these additional metabolites and also, importantly, how fructose 2,6-bisphosphate activates MondoA and ChREBP will further our understanding of the mechanisms by which the liver maintains intracellular phosphate homeostasis under conditions of absolute or relative glucose excess.

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