A Combination of HNF-4 and Foxo1 Is Required for Reciprocal Transcriptional Regulation of Glucokinase and Glucose-6-phosphatase Genes in Response to Fasting and Feeding

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Glucokinase (GK) and glucose-6-phosphatase (G6Pase) regulate rate-limiting reactions in the physiologically opposed metabolic cascades, glycolysis and gluconeogenesis, respectively. Expression of these genes is conversely regulated in the liver in response to fasting and feeding. We explored the mechanism of transcriptional regulation of these genes by nutritional condition and found that reciprocal function of HNF-4 and Foxo1 plays an important role in this process. In the GK gene regulation, Foxo1 represses HNF-4-potentiated transcription of the gene, whereas it synergizes with HNF-4 in activating the G6Pase gene transcription. These opposite actions of Foxo1 concurrently take place in the cells under no insulin stimulus, and such gene-specific action was promoter context-dependent. Interestingly, HNF-4-binding elements (HBEs) in the GK and G6Pase promoters were required both for the insulin-stimulated GK gene activation and insulin-mediated G6Pase gene repression. Indeed, mouse in vivo imaging showed that mutating the HBEs in the GK and G6Pase promoters significantly impaired their reactivity to the nutritional states, even in the presence of intact Foxo1-binding sites (insulin response sequences). Thus, in the physiological response of the GK and G6Pase genes to fasting/feeding conditions, Foxo1 distinctly decodes the promoter context of these genes and differentially modulates the function of HBE, which then leads to opposite outcomes of gene transcription.

The liver plays an important role in maintaining plasma glucose homeostasis by adjusting a delicate balance between hepatic glucose utilization and production via the glycolytic and gluconeogenic pathways. These physiologically opposed cascades are regulated, at least in part, at the transcriptional level of the glucokinase (GK) and glucose-6-phosphatase (G6Pase) genes, which catalyze the first and last rate-limiting steps in glycolysis and gluconeogenesis, respectively. A number of studies have shown that fasting/feeding (or hormone) controls transcription of these two enzymes in the opposite direction. Fasting is associated with inhibition of GK transcription, whereas feeding or insulin treatment activates its transcription (1–3). Conversely, G6Pase transcription is negatively regulated by insulin or feeding and markedly increased in a fasting state (4).

Previous studies have established that Foxo1, a member of the FOXO family of forkhead transcription factors, is important in mediating the effects of insulin on G6Pase gene expression in cultured cells (5, 6). Foxo1 is phosphorylated by protein kinase B (also called Akt) in the presence of insulin signal and then excluded from the nucleus, resulting in inhibition of the expression of target genes (7–10). On the other hand, unphosphorylated form of Foxo1 localizes to the nucleus and interacts with insulin response sequences (IRSs) present within the promoter regions of multiple target genes, including the G6Pase gene, and activates their transcription. Recent reports have demonstrated that Foxo1, in some cases, can also be recruited to a promoter through a DNA-binding transcription factor and act as a transcriptional repressor (11–15). However, little is known about how these two modes switch and what determines its state as activator or repressor in vivo.

The HNF-4, expressed mainly in the liver, kidney, and intestine, binds to a specific DNA element (HNF-4-binding element (HBE)) as a homodimer and activates transcription of many genes that are involved in glucose, fatty acid, and cholesterol metabolism (16–20). Of particular interest, HNF-4 activates
the transcription of both GK and G6Pase genes, which, as mentioned above, are oppositely regulated by nutritional manipulation (21–24). However, it has not yet been determined how HNF-4 is involved in the nutritional regulation of these genes. If involved, because HNF-4 itself acts exclusively as an activator, it is reasonable to hypothesize that another nutritional status-regulated factor may modulate the function of the HNF-4 in an opposite way in regulating GK and G6Pase gene transcription. Previously, we have reported that HNF-4 directly interacts with Foxo1 (11), which is regulated in its localization in response to nutritional transition. Therefore, we hypothesized that Foxo1 is involved in the HNF-4-dependent regulation of these genes in response to metabolic status.

Here, we show in cell transfection assays that Foxo1 represses the HNF-4-potentiated expression of the GK gene and simultaneously exerts synergistic activation on the HNF-4-dependent transcription of the G6Pase gene. Both of these HNF-4-dependent effects are abrogated by treating cells with insulin, which translocates Foxo1 to the cytosol. Importantly, the gene-specific effects of Foxo1 on HNF-4 functions were promoter context-dependent (i.e. different combination of HBEs and IRSs on the promoters). To gain insight into this regulation in a more physiological situation, we employed in vivo imaging technology and demonstrated that HBEs are in fact required for the regulation of these two genes in response to fasting and feeding. These data strongly imply that opposite effects of Foxo1 on GK and G6Pase gene expression require HNF-4 activity and that reciprocal action of Foxo1 and HNF-4 is an important determinant of the metabolic shift toward the glycolysis or gluconeogenesis.

EXPERIMENTAL PROCEDURES

Western Blotting—HepG2, HeLa, and CV1 cells were lysed in a lysis buffer (20 mM Hepes, pH 7.9, 0.1% Nonidet P-40, 100 mM NaCl, 1 mM dithiothreitol, and protease inhibitors), cell extracts were resolved by SDS-PAGE, electrotransferred onto polyvinylidene difluoride membrane. Chemiluminescent detection was performed with horseradish peroxidase-conjugated secondary antibodies.

Northern Blot Analysis—Total RNA was isolated from the livers of 8-week-old male C57BL6 mice or HepG2 cells using ISOGEN (Nippon Gene). After electrophoresis, RNA was blotted onto GeneScreen Plus (PerkinElmer Life Sciences) and subsequently hybridized with specific 32P-labeled cDNA probes. Mouse G6Pase (14–682 bp), mouse glucokinase (517–958 bp), subsequently hybridized with specific 32P-labeled cDNA probes. Mouse G6Pase (14–682 bp), mouse glucokinase (517–958 bp), mouse glyceraldehyde-3-phosphate dehydrogenase (25), and β-actin (26) were used as hybridization probes (Figs. 1A and 3B). The probe for human G6Pase was generated by PCR using forward primer (5′-gagattggatcttcggtg-3′) and reverse primer (5′-ctccctttctctgtcagaggt-3′).

qRT-PCR—Total RNA was isolated from HepG2 cells with ISOGEN (Nippon Gene) according to the manufacturer’s instructions. Total RNA (0.5 μg) was reverse transcribed using random hexamers, oligo(dT) primer, and PrimeScript™ RT regent kit (TaKaRa Bio Inc.) according to the manufacturer’s protocol. Quantitative real time PCR analysis was performed in 25-μl reactions using the Thermal Cycler Dice™ TP860 (TaKaRa Bio) and SYBR Premix EX TaqII (TaKaRa Bio). Relative gene expression was determined by the ΔΔCt method. G6Pase mRNA levels were normalized to β-actin expression. For PCR amplification, the specific primers (5′-taagtccttctccctacagctc-3′ and 5′-tccctgtggtccagttcaca-3′) were used for hG6Pase. Primers for β-actin were purchased from Takara.

siRNA—For transfection of siRNAs, HepG2 cells at 30–50% confluence were transfected with siRNA using Lipofectamine RNAi max (Invitrogen) according to the manufacturer’s protocol. Thirty-six h after siRNA transfection, cells were transfected with DNA using GeneJuice® Transfection Regent (Merck). Green fluorescent protein siRNAs were purchased from B-Bridge International, Inc. The siRNA duplexes were used as follows; CBP (sense, 5′-aauccacaguacagaaattt-3′; antisense, 5′-uuuucggguuguaguggattt-3′), HNF-4, and PGC1α (Dharmacon). Protein and RNA were extracted at 72 h after transfection of siRNA. Expression levels of HNF-4 were analyzed by immunoblot, and G6Pase expression levels were analyzed by qRT-PCR.

Cell Culture, Transfections, and Reporter Gene Assays—HepG2 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. CV1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 0.25% glucose. Transfections were performed using Fugene-6 (Roche Applied Science) or Genejuice® Transfection Regent (Merck). Thirty-three ng of pCMV-β-gal or pRSV-β-gal plasmid was included in each experiment to control for transfection efficiency. To ensure equal DNA amounts, empty plasmids were added to each transfection. Luciferase activity was measured with an ARVO™SX 1420 Multilabel Counter (Wallac Berthold) and normalized for β-galactosidase activity in the same sample. Luciferase and β-galactosidase assays were performed in triplicate.

ChIP Assay—The liver from a C57BL/6 mouse was excised, rinsed in cold phosphate-buffered saline, and suspended in 5 ml of buffer A (10 mM Hepes, pH 7.6, 25 mM KCl, 1 mM sodium EDTA, 2 mM sucrose, 10% (v/v) glycerol). The livers were homogenized in a Dounce homogenizer. The homogenate was filtered through a cell strainer, and the sample was centrifuged at 24,000 rpm for 1 h at 4 °C. The pellet was used as hepatic nuclei. ChIP assays were performed as described by the manufacturer (Upstate Biotechnology, Inc., Lake Placid, NY) with some modifications. Cross-linking between transcription factors and chromatin was achieved by adding formaldehyde (final concentration 1%) to hepatic nuclei for 15 min for 37 °C. Chromatin solutions were sonicated and incubated with anti-HNF-4 antibody (H171; Santa Cruz), with anti-Foxo1 antibody (11), or with control IgG and rotated for 5 h. At 4 °C. Immune complexes were collected with a salmon sperm DNA/protein G-Sepharose slurry for 4 h with rotation, washed, and then incubated at 65 °C for 6 h for reverse cross-linking. Chromatin DNA was extracted using QIAquick PCR purification kit (Qia- gen, Inc.) and subjected to the quantitative real time PCR analysis. To amplify the G6Pase promoter regions, the following primer sets were used: 5′-CCTTGCCCCTGTATTATGCC-3′ and 5′-CGTAAATCACCTGACATGTGGT-3′. For the GK promoter regions, the following primer sets were...
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were used: 5′-AGCACGCTCCAACAGGTGTTCAG-3′ and 5′-CAGCCAAGGACTTCTGCACTAAT-3′. The quantitative real time PCR was performed in the ABI 7700 with cycling as follows: an initial cycle for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Hydrodynamic Infusion of DNA and in Vivo Optical Imaging—Hydrodynamic infusion was performed under optimal conditions for expression as previously reported (26, 27). Eight- to 10-week-old male ICR mice were given a rapid (5–6 s) tail vein injection of FLuc plasmids 2 μg (rGK-WT, rGK-ΔHBE, hG6Pase-WT, hG6Pase-mutHBE, or hG6Pase ΔIRS-LUC plasmid) combined with RLuc plasmid 1 μg (pRLSV40) and pCMV-βgal 22 μg in Ringer’s solution in a volume of 2.5 ml. The pRLSV40 plasmid (Renilla luciferase reporter; Toyo-ink) was used as an internal control to normalize for infusion efficiency. To measure RLuc bioluminescence signals, mice were anesthetized by isofluran and given a tail vein injection of native coelenterazine (0.2 mg/ml in 100 μl). One min after coelenterazine administration, mice were imaged for 1 min with a Xenogen imaging system. Timed images were quantitatively analyzed by Living image version 2.5 software. To measure FLuc bioluminescence signals, mice were anesthetized by isofluran. Anesthetized mice were intraperitoneal injected with firefly d-luciferin (30 mg/ml luciferin in 150 μl of sterile saline (Promega)). Five and 10 min after d-luciferin administration, mice were imaged for 1 min with a Xenogen imaging system. Timed images were quantitatively analyzed by Living image version 2.5 software. For GK, the first group of mice was allowed free access to food ad libitum, and the second group of mice was subjected to a fast for 8 h. Mice were then given an intraperitoneal injection of firefly d-luciferin, and glucose and insulin levels were measured from tail vein blood. After imaging with a Xenogen imaging system, mice were allowed free access to food ad libitum, given a tail vein injection of native coelenterazine, and imaged. For G6Pase, the first group of mice was allowed free access to food ad libitum, and the second group of mice was subjected to a fast for 5 h. Mice were given a tail vein injection of native coelenterazine. After imaging with a Xenogen imaging system, mice were either allowed free access to food ad libitum or subjected to a fast for 19 h again. Then mice were given an intraperitoneal injection of firefly d-luciferin and imaged, and glucose levels in tail vein blood and insulin levels in blood from the inferior vena cava were measured. Animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba and in accordance with the Regulations for Animal Experiments at our university and Fundamental Guideline for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology.

Statistical Analysis—The data were analyzed by Student’s t test. p < 0.05 was considered significant. Results are expressed as the means ± S.E.

Plasmids—pcDNA3-HA-HNF-4a2 WT, pcDNA3-FLAG-Foxo1 WT, and pcDNA3-FLAG-Foxo1 3A plasmids were described previously (11, 28). The critical residues in the DNA binding domains of HNF-4a2 and Foxo1 were mutated by PCR mutagenesis. The mPGC1α cDNA was subcloned into pcDNA3 tagged with the HA epitope (pcDNA3-HA). Genomic fragments encompassing the human and rat glucokinase promoters extending from −1492 to +31 bp and from −1431 to +23 bp relative to the transcription initiation sites, respectively, were cloned into pGV-Basic vector II (firefly luciferase reporter; Toyo-ink) to generate hGK- and rGK-Luc constructs. To construct hG6Pase-WT LUC plasmid, an 800-bp (from −826 to +7 bp) DNA fragment containing the hG6Pase gene promoter was cloned into pGV-Basic vector II (firefly luciferase reporter; Toyo-ink). The hG6Pase-ΔIRS123 and hG6Pase-mutHBE plasmids were generated within the context of hG6Pase-WT Luc. The mutant sequences (sense strand) utilized were the following: hG6Pase-mutHBE 5′-gatgcttgacctcctgtctcggttcggagcgggaccaggagccatg-3′. The underlined sequences represent the substituted nucleotides. A PCR strategy was used to delete an IRS region (−235 to −157) from hG6Pase-WT Luc plasmid. To construct GK plus IRS-Luc, a 50-bp (from −196 to −149 bp of the human G6Pase promoter) IRS-DNA fragment was introduced into nucleotide -52 of hGK-WT-LUC. To generate the rGK-ΔHBE-Luc plasmid, a PCR strategy was used to delete an HBE region (−62 to −36) from rGK-Luc plasmid.

Antibodies—Antibodies used for immunoblot analysis were as follows: FLAG tag (M2; Sigma); β-actin (AC-74; Sigma); HNF-4 (C-19, Santa Cruz Biotechnology).

RESULTS

HNF-4 Potentiates Two Gene Promoters Involved in Physiologically Opposite Metabolic Pathways—We first examined the GK and G6Pase mRNA accumulation in the liver of C57BL/6 mice under conditions of fasting and feeding. As expected from previous results (1–4), the G6Pase mRNA level was higher in the fasting state and lower in the feeding state. On the other hand, GK mRNA level was lower in the fasting state and higher in the feeding state (Fig. 1A). These data demonstrate that activity of these two enzymes is conversely controlled by nutritional status, at least in part at the gene expression level. Expression levels of HNF-4 and Foxo1 were largely not affected (Fig. S1, A and B). Since it is established that HNF-4 binds and activates the rat GK promoter (29), we identified an HBE in the human GK promoter at the corresponding region of the rat gene (Fig. S2, A–C). We next examined an effect of HNF-4 on the transcriptional activity of GK and G6Pase genes. Although HNF-4 strongly potentiated the promoter activity of these two genes (Fig. 1B) (21, 23, 24), the possible function of HNF-4 with respect to nutritional conditions of these two contrarily regulated genes remained unclear. We therefore explored whether HNF-4 plays any role in these processes.

HNF-4-potentiated GK Promoter Activity Is Modulated by Foxo1 in Response to Insulin—A previous study showed that hepatic expression of constitutively active Foxo1 in transgenic mice was related to reduced GK gene expression (30). A genetic study with liver-specific disruption of Foxo1 in mice also suggests that Foxo1 suppresses GK expression in the liver when insulin signaling is disrupted (31). However, it remains unclear how Foxo1 is involved in a repression of the GK gene expression. On the other hand, we have previously shown, by using an
artificial model promoter composed of eight tandem HBEs plus the TK promoter, that Foxo1 repressed HNF-4-potentiated transcriptional activity, and this repression was abrogated by treating the cells with insulin (11). Here, we tested whether Foxo1 repression of the HNF-4-mediated transcriptional activity also took place in the GK promoter context. In the cell transfection assay into HepG2 cells, HNF-4 strongly activated the GK promoter, regardless of insulin addition. When Foxo1 WT was cotransfected with HNF-4 in the absence of insulin, GK promoter activity was remarkably repressed, although no repression was observed in the absence of transfected HNF-4 (Fig. 1C). Furthermore, insulin stimulation significantly canceled the repression of HNF-4-dependent GK promoter activity in the presence of Foxo1 WT but not of the 3A mutant, in which three putative Akt phosphorylation sites (Thr-24, Ser-253, and Ser-316) were replaced by nonphosphorylatable alanine residues (Fig. 1C and Fig. S3, B and C). We revealed that the Foxo1 WT was much more abundantly expressed than Foxo1 3A mutant (Fig. 1D); however, the 3A mutant repressed the HNF-4-dependent GK-LUC activity to the same extent as the WT did. Considering that Foxo1 3A mutant dominantly localized in the nucleus, we speculate that the Foxo1 WT and 3A variants are equally expressed in the nucleus.

Next, to investigate the recruitment of Foxo1 to the GK promoter in vivo, we performed ChIP analysis using primers that amplify the HBE of the GK promoter on hepatic nuclei from fasted and refed mice (Fig. 1E). The results obtained by qRT-PCR indicated that HNF-4 binds to the HBE either in the fasted or refed state. On the other hand, Foxo1 is preferentially recruited to the HBE on GK promoter in the liver of fasted mice but not in one of refed mice. Together, these results suggest that Foxo1 is recruited to the HBE of the GK promoter through the binding to HNF-4 and represses its transcriptional activity, whereas feeding or insulin signal results in the dissociation of Foxo1 from the HBE through the phosphorylation of Foxo1, leading to enhancement of the HNF-4-dependent transcriptional activity. These results support our idea
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FIGURE 2. HNF-4 and Foxo1 synergistically activate the G6Pase promoter. A, HepG2, HeLa, and CV1 cells were cotransfected with hG6Pase (100 ng) and Foxo1 expression plasmids (3, 10, or 20 ng). The luciferase activity was presented as -fold induction above the activity obtained with the transfection of hG6Pase reporter plasmids alone, respectively. B, cell extracts from HepG2, HeLa, and CV1 cells were probed with anti-HNF-4 and β-actin antibodies. C, HeLa or CV1 cells were transfected with Foxo1 expression plasmid (50 ng) in the absence or presence of HNF-4 expression plasmid (3 ng). The luciferase activity was presented as -fold induction above the activity obtained with the transfection of hG6Pase reporter plasmids alone, respectively. D, HepG2 cells were transfected with Foxo1 expression plasmid (10 ng) in the absence or presence of HNF-4 DN expression plasmid (3 ng). The luciferase activity was presented as -fold induction above the activity obtained with the transfection of hG6Pase reporter plasmids alone.

that interplay between HNF-4 and Foxo1 plays a role in the insulin response of GK gene expression.

Foxo1 Synergizes with HNF-4 on the G6Pase Promoter—Next, we explored the effect of functional interplay between Foxo1 and HNF-4 on the G6Pase gene transcription. Previous transfection studies in HepG2 cells have shown that Foxo1 activates the G6Pase promoter through the IRSs (Fig. 2A) (5, 6). We found in HeLa and CV1 cells that the G6Pase reporter construct was only minimally activated by transfected Foxo1, in comparison with HepG2 cells (Fig. 2A). These data prompted us to consider that an important factor(s) for Foxo1-mediated transcriptional activation is missing from HeLa and CV1 cells. Western blot analysis with an antibody against HNF-4 revealed an intensive immunoreactive band in HepG2 cells but not in HeLa or CV1 cells at all (Fig. 2B). To test if HNF-4 is essential for Foxo1-mediated, high level G6Pase promoter activation, we co-transfected Foxo1 and HNF-4 into HeLa or CV1 cells and found that the G6Pase reporter was far more abundantly activated in comparison with transfection of Foxo1 alone (Fig. 2C). The high level of activation by Foxo1 was HNF-4-dependent; coexpression of either HNF-1α or C/EBPβ, which binds and activates the G6Pase promoter (32–34), produced little or no increase in the G6Pase transcriptional activity (Fig. 5A and B). In HepG2 cells, we examined the effect of the dominant-negative form of HNF-4 (HNF-4dn, C106R) (35), which forms defective heterodimers with wild-type HNF-4, thereby preventing DNA binding, on the transcriptional potential of Foxo1 and found that the addition of the HNF-4dn caused a marked reduction of Foxo1-stimulated G6Pase promoter activity (Fig. 2D and Fig. S6C). Consistent with these data, depletion of endogenous HNF-4 by RNA interference significantly reduced the expression of the G6Pase gene in serum deprivation conditions, which increases nuclear localization of Foxo1 (Fig. S3E and Fig. S5). Together, these results demonstrate that HNF-4 is an integral component of the Foxo1-responsive, synergistic G6Pase promoter activation.

Insulin Abolishes the Synergy between Foxo1 and HNF-4 in G6Pase Promoter Activation—We examined the effect of insulin signaling on the transcriptional synergy between HNF-4 and Foxo1 in the G6Pase promoter activation. In HepG2 cells under no insulin stimulation, the G6Pase reporter was synergistically activated by Foxo1 and HNF-4 (Fig. 3A). Insulin treatment of the cells significantly abolished the synergistic G6Pase promoter activation exerted by Foxo1 WT but not by the Foxo1 3A mutant (Fig. 3A). We next tested if transfected HNF-4 and Foxo1 also activate the endogenous G6Pase gene, in a synergistic manner, in the chromatin environment. HepG2 cells were transfected with HNF-4 along with Foxo1 WT or 3A mutant expression plasmids, and endogenous G6Pase gene expression was analyzed by Northern blot hybridization (Fig. 3B). Transfection of HNF-4, Foxo1 WT, or Foxo1 3A mutant alone produced little or no increase in G6Pase mRNA accumulation. However, abundantly accumulated G6Pase mRNA was observed when HNF-4 and either Foxo1 WT or Foxo1 3A mutant expression plasmids were cotransfected. Moreover, insulin treatment of the cells significantly canceled the synergistic activation of the endogenous G6Pase gene exerted by forced HNF-4 and Foxo1 WT expression. Synergistic activation exerted by HNF-4 and Foxo1 3A mutant was not modulated by insulin treatment. These results demonstrate that Foxo1 and HNF-4 synergistically activate both transfected and endogenous G6Pase gene promoters.

We next examined whether this synergy requires the DNA binding of Foxo1 and HNF-4 to IRSs and HBEs, respectively. We performed the reporter assay in HepG2 cells using HNF-4dn or Foxo1 GP mutant, in which two amino acid residues that are essential for DNA binding (Trp-206 and His-212) were replaced by Gly and Pro, resulting in a significant reduction of DNA binding activity (8, 36). The results showed that no synergistic activation was observed when HNF-4dn or Foxo1 GP mutant was cotransfected (Fig. S6, A–C). These results showed that the recruitment of both Foxo1 and HNF-4 to the G6Pase gene is essential for the synergistic activation. Moreover, to
confirm the binding of HNF-4 and Foxo1 to the HBE(s) and the IRS(s) of the G6Pase promoter in vivo, respectively, ChIP assays were performed using mouse liver. Whereas HNF-4 was recruited to the HBE(s) on the G6Pase promoter in both the liver of fasting and refeeding mice, Foxo1 binds to the IRS(s) under the fasting but not refeeding condition (Fig. 3C). These results suggest that under the fasting conditions, both HNF-4 and Foxo1 bind and exert the synergistic effect on the G6Pase gene transcription. On the other hand, insulin signal or feeding remarkably decreases the synergistic effect, probably mediated by Foxo1 phosphorylation, supporting our idea that interplay between HNF-4 and Foxo1 may play a crucial role in the regulation of G6Pase gene expression in response to fasting/feeding states.

Next, we attempted to determine the participation of cofactors in the synergy. CBP is a well-known coactivator that functions as a key integrator in various enhanceosome (37). We have reported that CBP binds to both HNF-4 and Foxo1 (28, 38). Hence, to investigate the possible involvement of CBP in the HNF-4- and Foxo1-induced synergistic activation, we examined the RNA interference-mediated knockdown of CBP. As shown in Fig. 3D, knockdown of CBP significantly abrogated the synergy by Foxo1 and HNF-4 in a dose-dependent manner, whereas it showed marginal effects on the transactivation when HNF-4 or Foxo1 alone was cotransfected (Fig. 3D and Fig. S5). Further, we examined the ability of PGC1 to synergize with Foxo1 and HNF-4 in the regulation of G6Pase transcription. Expression of PGC1 has been found to be induced under the fasting state and to stimulate gluconeogenic enzymes (e.g. G6Pase) mediated by HNF-4 (24). RNA interference-mediated knockdown of PGC1 dramatically decreased the transcrip tional activity of HNF-4 and Foxo1 (Fig. 3E and Fig. S5). These results illustrate that CBP and PGC-1α are essential for the synergistic activation of the G6Pase gene induced by forced HNF-4 and Foxo1 expression.

The Presence or Absence of IRSs on the Promoter Determines the Properties of Foxo1—Next, we investigated the mechanism by which Foxo1 oppositely modulates HNF-4-dependent transcriptional activity. The G6Pase promoter carries two HBEs and three IRSs (6, 23). A composite HBE-IRS element on the G6Pase promoter is required for the synergistic activation of the gene (Fig. S7). Since the GK gene only carries the HBEs in the promoter, these observations prompted us to consider a model in which Foxo1 synergizes with the HNF-4 on the promoters carrying both Foxo1- and HNF-4-binding sites (e.g. G6Pase promoter), whereas it inhibits HNF-4-dependent promoters carrying no adjacent IRS in cis (e.g. GK promoter). To directly verify this hypothesis, we generated test reporter con-
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The HNF-4-binding Sites Are Required for Nutritional Response of GK and G6Pase Gene Promoters in Vivo—We found, in a cell transfection assay, that Foxo1 is an important mediator of insulin action on both GK and G6Pase gene regulation, and its effects require HNF-4-dependent transcriptional activities. To assess the physiological significance of these observations, we conducted in vivo imaging LUC assays by using a whole animal imaging technique (Fig. 5). Assuming that Foxo1-mediated insulin action of GK and G6Pase gene expression requires HNF-4 activities in vivo, the destruction of HBEs on each gene leads to a decreased response to nutritional status in the expression of these genes. Initially, mice were injected with the wild-type GK promoter construct (GK-WT-LUC) and subjected to fasting and then refeeding, and then bioluminescence intensities were measured. Plasma insulin level was 8-fold higher in the fed than in the fasted states (data not shown). Consistent with the reported transcriptional activity of the endogeneous GK gene (Fig. 1A) (1–3), the bioluminescence level of the GK-WT-LUC was lower in the fasted state and increased in the refed state, validating this experimental system in measuring transcriptional activity of the test construct in living mice (WT in Fig. 5A). We then tested the mutant construct (GK-ΔHBE-LUC (ΔHBE in Fig. 5A)), which lacks HBEs from the GK-WT-LUC. The luminescence level in the fasted state was slightly lower in the ΔHBE mice, in comparison with that in the WT mice. In the ΔHBE mice, although the luminescence level was increased in the refed state, the extent of the induction rate was statistically significant compared with that in the WT mice (Fig. 5B), indicating that the HBEs are required for the proper regulation of GK gene expression in response to nutritional signaling in vivo. This result also implies that HNF-4–Foxo1 interaction is a functional mediator of insulin action on GK gene expression in vivo.

We next determined the in vivo role of the HBEs in the G6Pase promoter activity. Relative luciferase bioluminescence level in the G6Pase-WT-LUC (WT in Fig. 5C) between fasted/fed conditions was consistent with relative transcriptional activities of the endogeneous G6Pase gene (Figs. 1A and 5C). Deletion of the IRSs from the G6Pase-WT-LUC resulted in lower basal activity in the fasted state and no transcriptional repression in response to refeeding (ΔIRS in Fig. 5, C and D), demonstrating that IRSs are essential for G6Pase gene activa-
The expression of GK and G6Pase genes is inversely regulated in response to fasting/feeding. Although it is well known that HNF-4 potentiates promoter activities of these genes (21–24), it has not yet been established how HNF-4 is involved in the regulation of these genes by nutritional status. To elucidate the regulatory mechanism of these genes by fasting/feeding, we focused here on the functional interplay between HNF-4 and Foxo1. Our results showed that Foxo1 represses HNF-4–potentiatted transcription of the GK gene (Fig. 1C) and that it synergizes with HNF-4 in G6Pase gene transcription (Figs. 2C and D) and 3 (A and B) and Fig. S3E). These reciprocal reactions, exerted by Foxo1, took place simultaneously, and importantly, insulin treatment of the cells abrogated both reactions. These results prompted us to propose that HNF-4 plays essential roles in the reciprocal regulation of GK and G6Pase genes in that Foxo1 translates the insulin signal, in response to fasting/feeding, into two opposite transcriptional outcomes when these genes are potentiatted by HNF-4. Shown in Fig. 6 is a possible model that is based on our current results. In the absence of insulin (fasted state; left), Foxo1 localizes to the nucleus, where it represses HNF-4–dependent activity of the GK promoter as a corepressor. Simultaneously, DNA-bound Foxo1 and HNF-4 synergistically activate the G6Pase gene promoter, presumably by the interaction with CBP and PGC1α. These together shift the balance between glycolysis and gluconeogenesis toward the latter in the fasted state. When cells are stimulated by insulin (fed; right), Foxo1 is phosphorylated and excluded from the nucleus, resulting in its dissociation from the HNF-4 (11). Accordingly, HNF-4 can activate the GK gene promoter through HBE, whereas the G6Pase gene promoter is moderatley activated by HNF-4 alone. Thus, insulin signaling shifts the metabolic balance in favor of glycolysis, by suppressing G6Pase gene expression and activating GK gene expression. In this model, Foxo1 plays a key role in switching between glycolysis and gluconeogenesis, and HNF-4 is an indispensable component of this switching mechanism.

According to the recent reports (11–15), Foxo1 functions as transcriptional activator in some situations and repressor in others. It has not been understood, however, what determined the nature of Foxo1 in each case. In this study, we showed that Foxo1 conferred either positive or negative transcriptional influence on HNF-4–potentiatted gene activity in a promoter context-dependent manner; Foxo1 inhibited transactivation by HNF-4 in promoters that do not contain adjacent IRSs (e.g. GK promoter) and synergized with HNF-4 in activating promoters containing binding sites for both HNF-4 and Foxo1 (e.g. G6Pase promoter). Consistent with this notion, removing the IRSs from the G6Pase promoter (Fig. 4A and Fig. S7) converted the nature of Foxo1 from the activator to the repressor. We thus propose that combination or arrangement of factors binding sites in a promoter (combinatorial code) determines how transcription factors modulate gene transcription, and the data presented here are a good example of this mechanism. Importantly, inversion of the response to the nutritional status in the

**FIGURE 5.** The HBE(s) are required for nutritional response of GK and G6Pase gene promoters in vivo. A, mice were subjected to a 16-h fast and hydrodynamically infused with plasmids bearing the indicated GK reporter constructs and both SV40-RLUC plasmid and CMV-β-galactosidase plasmid. After injection, mice were divided into two experimental groups, fasted and refeed groups. Top, quantification of data obtained from mice is shown. Total flux (photons/s) of firefly luciferase signal was normalized to that of Renilla luciferase. Bottom, a pseudocolored image 5 min after injection of α-luciferin is represented with the intensity of red > orange > yellow > green > blue (n = 12–15 per group). B, total flux ratio of FLuc/RLuc in the refeed mice was normalized to that in the fasted mice. C, mice were subjected to a 10-h fast and then hydrodynamically infused with plasmids bearing the indicated hG6Pase reporter constructs and both SV40-RLUC and CMV-β-galactosidase plasmids. After injection, mice were divided into two experimental groups, fasted and refeed groups. Top, quantification of data obtained from mice is shown. Total flux (photons/s) of firefly luciferase signal was normalized to that of Renilla luciferase. Bottom, a pseudocolored image 5 min after injection of α-luciferin is represented with the intensity of red > orange > yellow > green > blue (n = 9–15 per group). D, total flux ratio of FLuc/RLuc in the fasted mice was normalized to that in the refeed mice.
G6Pase promoter was also observed in vivo. When the IRSs were deleted from the G6Pase promoter (ΔIRS in Fig. 5C), the luciferase signal in fed mice tended to be more intense than that in fasted mice (Fig. 5, C and D), although the difference was not statistically significant. These observations support the notion that IRSs are not only essential for G6Pase gene activation in the fasting state but also in determining the nature of Foxo1 as an activator or repressor.

Diaz Guerra et al. (18) have shown that promoter region of the liver-type pyruvate kinase (L-PK) gene, an important regulatory enzyme in the glycolytic pathway, carries single HBE and no IRS. When our model was applied to this gene, Foxo1 may modulate the HNF-4-potentiated transcriptional activity of the L-PK gene and confer fasting/refeeding response on it. Indeed, Bergot et al. (39) have identified the fasting/refeeding response element in the L-PK gene, and this element contained the HBE. This observation agrees well with our hypothesis that Foxo1 represses the HNF-4-potentiated activity of the L-PK gene in the fasting state, and the repression is canceled in the feeding state. On the other hand, a composite HBE-IRS element was found in the promoter region of the phosphoenolpyruvate carboxykinase gene, which is a key enzyme in the regulation of flux through the gluconeogenic pathway (40). Interestingly, we found that Foxo1 also exerts synergistic activation with HNF-4 on the phosphoenolpyruvate carboxykinase promoter in the cell transfection assay (data not shown). Thus, a distinct combination of HBE and IRS may be found in the promoter region of important regulatory enzymes in the glycolytic/gluconeogenic pathways, play a key role in regulating transcription of the genes in response to nutritional status, and consequently orchestrate a whole process of glycolysis/gluconeogenesis.

G6Pase gene expression is found in selected tissues (liver, kidney, and intestine), where it is dynamically regulated upon nutritional conditions (41). Previous transfection studies have shown that Foxo1 mediates transcriptional repression of the G6Pase gene in response to the insulin signaling, conferring temporal specificity on gene expression (42). However, since Foxo1 is ubiquitously expressed (43, 44), it has been a challenge to determine how Foxo1 confers cell type specificity on the regulation. This study revealed that HNF-4 functions as a synergistic activator of Foxo1-dependent G6Pase gene transcription. Intriguingly, HNF-4 expression is mainly restricted to the liver, kidney, and intestine, which agrees well with the tissue distribution of G6Pase. In view of our results, HNF-4 is an indispensable partner of the Foxo1 in abundantly activating G6Pase transcription in the expressing tissues. In tissues where HNF-4 is not expressed, transcriptional potential of the G6Pase gene may not be high enough, although Foxo1 binds to IRSs in the G6Pase promoter. This idea is consistent with the finding that mutation of the HBEs in the G6Pase promoter abrogated transcription of the gene in the fasting state, despite the presence of intact IRSs (Fig. 5, C and D). Thus, it is likely that a combination of insulin-regulated Foxo1 and tissue-restricted HNF-4 transcription factors is necessary for establishing time-dependent and tissue-specific G6Pase promoter activity.

Although the complex spatio-temporal pattern of gene expression seemed to require a vast number of transcriptional regulators, it is in fact achieved by a specified set of regulators, the molecular basis of which is not fully understood. Our results provide a concept that the combination of two factors could generate even the opposite outcome in transcription when further combined with the promoter DNA context.

Acknowledgments—We thank Dr. Shunsuke Ishii (RIKEN) for the pact-C/EBPβ plasmid and Dr. Kazuya Yamagata (Kumamoto University) for HNF-1α-pcDNA3.1 plasmid. We thank Dr. Ritsuko Shimizu and Mikiko Suzuki for excellent technical advice and members of the Fukamizu laboratory for helpful discussion and encouragement.

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doi: 10.1074/jbc.M806179200 originally published online September 19, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M806179200

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