A Molecular Link between the Common Phenotypes of Type 1 Glycogen Storage Disease and HNF1α-null Mice

The clinical manifestations of type 1 glycogen storage disease (GSD-1) in patients deficient in the glucose-6-phosphatase (G6Pase) system (e.g. growth retardation, hepatomegaly, hyperlipidemia, and renal dysfunction) are shared by Hnf1α−/− mice deficient of a transcriptional activator, hepatocyte nuclear factor 1α (HNF1α). However, the molecular mechanism is unknown. The G6Pase system, essential for the maintenance of glucose homeostasis, is comprised of glucose 6-phosphate transporter (G6PT) and G6Pase. G6PT translocates G6P from the cytoplasm to the lumen of the endoplasmic reticulum where it is metabolized by G6Pase to glucose and phosphate. Deficiencies in G6Pase and G6PT cause GSD-1a and GSD-1b, respectively. Hnf1α−/− mice also develop noninsulin-dependent diabetes mellitus caused by defective insulin secretion. In this study, we sought to determine whether there is a molecular link between HNF1α deficiency and function of the G6Pase system. Transactivation studies revealed that HNF1α is required for transcription of the G6Pase gene. Hepatic G6PT mRNA levels and microsomal G6P transport activity are also markedly reduced in Hnf1α−/− mice as compared with Hnf1a+a/+ and Hnf1a+a−/− littermates. On the other hand, hepatic G6Pase mRNA expression and activity are up-regulated in Hnf1α−/− mice, consistent with observations that G6Pase expression is increased in diabetic animals. Taken together, the results strongly suggest that metabolic abnormalities in HNF1α-null mice are caused in part by G6PT deficiency and by perturbations of the G6Pase system.

Glycogen storage disease type 1 (GSD-1), also known as von Gierke disease, is a group of autosomal recessive disorders that occurs approximately once in every 100,000 live births (1, 2). These disorders are caused by deficiencies in the activity of the glucose-6-phosphatase (G6Pase)1 system that consists of two integral membrane proteins, glucose 6-phosphate transporter (G6PT) and the G6Pase catalytic unit (1–3). G6PT translocates glucose 6-phosphate (G6P), the terminal product of glucoseogenesis and glycogenolysis, from the cytoplasm to the lumen of the endoplasmic reticulum (ER). Inside the ER, G6Pase with its active site facing the lumen (4) catalyzes the hydrolysis of G6P to glucose and phosphate. Therefore, G6PT and G6Pase work in concert to maintain glucose homeostasis. Deficiencies in G6Pase and G6PT cause GSD-1a and GSD-1b, respectively (1, 2). Both groups of patients manifest growth retardation, hepatomegaly, hyperlipidemia, and renal dysfunction, clinical features associated with HNF1α (HNF1α), a dimeric homeodomain-containing transcriptional activator (8–10). HNF1α is expressed in the liver, kidney, pancreas, and digestive tract (5, 8, 10) and is required for the expression of many liver genes (11–14). In this study, we establish a molecular link between HNF1α deficiency and function of the G6Pase system. We show that HNF1α binds to the G6PT promoter and is required for activation of G6Pase gene transcription. Further, we show that hepatic G6PT mRNA expression in Hnf1α−/− mice is markedly reduced, resulting in a near abolishment of microsomal G6P transport activity. These data indicate that Hnf1a−/− mice, similar to GSD-1b patients, are deficient in the G6PT.

Hnf1α−/− mice also develop noninsulin-dependent diabetes mellitus (NIDDM) (6) caused by defective insulin secretion and β-cell glycolytic signaling (15, 16). This finding is consistent with observations showing that mutations in the Hnf1α−/− gene in humans cause type 3 maturity-onset diabetes of the young, an autosomal dominant form of NIDDM characterized by impaired insulin secretion (17–20). It has been speculated that overexpression of G6Pase might contribute to the pathophysiology of NIDDM. In animal models of diabetes, G6Pase mRNA expression and enzymatic activity are increased, resulting in an elevation in hepatic glucose production (21, 22). Moreover, rats overexpressing the G6Pase gene exhibit several metabolic abnormalities associated with NIDDM, including glucose intolerance and hyperinsulinemia (23). Insulin has been shown to inhibit G6Pase gene transcription, and this effect is mediated through a cluster of insulin-response elements in the G6Pase promoter (24). Further, HNF1α is required for maximal repression of G6Pase gene transcription by insulin (25). In this study, we show that G6Pase activity and mRNA levels are elevated in Hnf1α−/− mice. Taken together, our data indicate that metabolic abnormalities in Hnf1a−/− mice are caused in part by perturbation of the G6Pase system.

MATERIALS AND METHODS

Construction of Promoter-CAT Fusion Genes, Transfection, and CAT Assays—The G6PT promoter-chloramphenicol acetyltransferase (CAT) fusion gene constructs were synthesized by polymerase chain reaction using the G6PT gene (26) as the template. The 3′ primer for the G6PT 5′ deletion mutants consisted of nucleotides −21 to −1, and the 5′ primers consisted of nucleotides −609 to −589, −200 to −180, and

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1 The abbreviations used are: G6Pase, glucose-6-phosphatase; HNF1, hepatocyte nuclear factor; ER, endoplasmic reticulum; CAT, chloramphenicol acetyltransferase; G6P, glucose 6-phosphate; NIDDM, noninsulin-dependent diabetes mellitus; GSD-1, type 1 glycogen storage disease.
HepG2 human hepatoma cells were grown at 37 °C in α-modified minimal essential medium supplemented with 4% fetal bovine serum. Cells in 25-cm² flasks were transfected with the G6PT promoter-CAT constructs by the calcium phosphate-DNA coprecipitation method as previously described (27). The CAT activity was assayed by incubating total cellular protein in a buffer containing 250 mM Tris-HCl, pH 7.8, 4 mM acetyl coenzyme A, and 0.1 mM ATP. Total cellular protein was determined by electrophoresis through 1.2% agarose gels containing 2.2 M formaldehyde and transferred to a Nytran membrane by electrophoresis. The amount of CAT activity was determined by autoradiography and quantitated by densitometry.

Electromobility Shift Assays—HepG2 nuclear extracts were prepared essentially as described (28). End-labeled oligonucleotide probes (2 ng; 0.2–0.5 × 10⁶ cpm) were incubated for 20 min at room temperature in binding reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.005% Nonident P-40, 1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol) containing 0.1 μg of poly(dI-dC) and 5 μg of nuclear extracts. Following binding, the mixture was electrophoresed through a 5% nondenaturing polyacrylamide gel, dried, and autoradiographed. For competition experiments, probe DNA was incubated in the mixture prior to the addition of probe. For gel supershift assays, specific antisera were preincubated with HepG2 extracts at 4 °C for 20 min before the addition of probe.

Northern Blot, Phosphorylase, and G6P Uptake Analyses—Total RNA was isolated by the guanidinium thiocyanate/CsCl method, fractionated by electrophoresis through 1.2% agarose gels containing 2.2 M formaldehyde, and transferred to a Nytran membrane by electrophoresis. The membranes were hybridized to cDNA probes for G6Pase, G6PT, or β-actin. Phosphorylase assays were performed as previously described (4). Disrupted microsomal membranes were prepared by incubating intact microsomes in 0.2% deoxycholate for 20 min at 0 °C. Non-specific phosphorylase activity was estimated by preincubating microsomal preparations at pH 5 for 10 min at 37 °C, a condition that inactivates the thermally labile G6Pase. G6P uptake measurements were performed as previously described (26). Microsomes permeabilized with 0.2% deoxycholate, which abolished G6P uptake, were used as negative controls. Statistical analysis using the unpaired t test was performed with the GraphPad Prism Program (GraphPad Software, San Diego, CA).

**RESULTS**

**HNF1α Binds to the G6PT Promoter and Transactivates G6PT Gene Transcription.—**To determine whether HNF1α regulates G6PT gene expression, we analyzed the 5′-flanking region of the gene and identified a HNF1 motif at nucleotides 165 to 153 followed by a TATA-box at nucleotides −141 to −136 upstream of the translation start site at +1 (Fig. 1A). To determine whether HNF1α activates transcription of the G6PT gene, we cotransfected the CAT gene directed by the G6PT promoter. Whereas both G6PT−609/−1CAT and G6PT−200/−1CAT constructs directed significant levels of CAT expression, CAT activity was found to be barely detectable with the G6PT−152/−1CAT construct (Fig. 1B). These data indicate that nucleotides −200 to −1 constitute a minimal G6PT promoter, which contains an activating element at nucleotides −200 to −153 encompassing the HNF1 motif (−165/−153).

**Fig. 1. The G6PT promoter.** A, nucleotides −200 to +3 of the 5′-flanking region of the human G6PT gene. The numbers indicate the distance in nucleotides from the translation start site (+1). The TATA-box and motifs for HNF1, HNF3, and C/EBP are boxed. The transcription start site is denoted by an arrow. B, promoter activity of the G6PT 5′-flanking region. The G6PT promoter-CAT fusion genes (10 μg/25-cm² flask) were transfected into HepG2 cells, and CAT activity was expressed as percent activity expressed by the G6PT−609/−1CAT construct. Specific CAT activities directed by G6PT−609/−1CAT, p53VCA, and pCAT-Basic-N plasmids were 7.6, 4.8, and 0.02 nmol/min/mg protein, respectively. Five independent experiments were conducted with two preparations of each construct.

To demonstrate whether HNF1α is the protein factor that binds to this activating element, electromobility shift assays were performed using HepG2 nuclear extracts. A protein-DNA complex, C1, was formed between the G6PT(−173/−145) oligo and HepG2 extracts (Fig. 2A, lane 2). The formation of complex C1 was efficiently blocked by the addition of unlabeled target DNA (lanes 3–5) and by an oligonucleotide containing the HNF1 motif (lanes 6–8), but not by an unrelated HNF4 (lane 21) and C/EBP oligonucleotide (lane 22). An HNF1-M1 oligonucleotide containing a mutated HNF1 site (TAA→GGG) had markedly reduced ability to block complex C1 formation (lanes 9–13) and an HNF1-M2 oligonucleotide that disrupts the entire DNA binding motif (TAA→GGG and TAA→GGG conversions) was completely incapable of blocking complex C1 formation (lanes 14–18). Further, an antiserum to HNF1α (lane 19), but not HNF1β (lane 20), caused a shift in the mobility of complex C1, demonstrating that a protein factor in this complex is HNF1α.
hepatic G6PT mRNA were markedly reduced in Northern blot analysis. The results show that the levels of Hnf1\(^{-}\)G6PT mRNA expression in the liver of Hnf1\(^{-}\)mice are likely to be perturbed in these mice. We therefore examined Hnf1\(^{-}\)G6P uptake activities in intact hepatic microsomes from (TAA\(^{+}\)-null Mice—Hnf1\(^{-}\)mice: Total RNAs (10 \(\mu\)g/lane), isolated from livers of 2-3 month old Hnf1\(^{-}\) mice and their Hnf1\(^{-}\) and Hnf1\(^{-}\)/Hnf1\(^{-}\) littermates, were separated on formaldehyde-agarose gels and hybridized with a uniformly labeled antisense probe of G6PT, G6Pase, or \(\beta\)-actin.

activation of the G6Pase gene (27), and it acts as an accessory factor for maximal suppression of G6Pase transcription by insulin (25). In this study, we show that levels of hepatic G6Pase mRNA were increased by 2- to 6-fold in Hnf1\(^{-}\)/G6Pase expression is increased in diabetic animals (21, 22) and required (Table I). The results suggest that HNF1\(^{-}\)activation is required for transcription of the G6Pase gene in vivo. On the other hand, the results are consistent with observations that G6Pase expression is increased in diabetic animals (21, 22) and that HNF1\(^{-}\) is required for suppression of G6Pase transcription by insulin (25).

The active site of G6Pase faces the lumen of the ER (4) and for G6Pase catalysis in vivo, G6P must be translocated from the cytoplasm into the lumen by the G6PT (3, 26). Biochemically, G6Pase activity in intact hepatic microsomes of GSD-1b patients, deficient in G6PT, is low or nondetectable, consistent with a functional G6Pase deficiency manifested by these patients. On the other hand, high levels of G6Pase activity were detected in disrupted hepatic microsomes of GSD-1b patients where G6PT function was abolished. The difference in G6Pase enzymatic activity in deoxycholate-disrupted microsomes where the G6PT function is not required (Table I). The results suggest that HNF1\(^{-}\) is not required for transcription of the G6Pase gene in vivo. On the other hand, the results are consistent with observations that G6Pase expression is increased in diabetic animals (21, 22) and that HNF1\(^{-}\) is required for suppression of G6Pase transcription by insulin (25).

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terize the G6Pase system in these mice. As expected, G6Pase activity in intact hepatic microsomes of Hnf1α−/− mice was only 53% of that found in Hnf1α+/+ or Hnf1α+/− mice (Table I). Moreover, hepatic G6Pase latency value was 87.9% in Hnf1α−/− mice, which was markedly higher than the value of 41.7% found in Hnf1α+/+Hnf1α+/− mice (Table I). Taken together, these results indicate that Hnf1α−/− mice, like GSD-1b patients, are deficient in the G6PT.

**DISCUSSION**

In this study, we have investigated the molecular mechanisms of phenotypic similarities between GSD-1 patients deficient in the G6Pase system (1, 2) and Hnf1α−/− mice lacking the transactivator, HNF1α (5–7). We demonstrate that Hnf1α−/− mice, like GSD-1b patients, are deficient in G6PT, an ER-associated membrane protein that transports G6P from cytoplasm to the lumen of ER and a member of the G6Pase system required for the maintenance of glucose homeostasis (1–3). The results establish for the first time a molecular link between the common phenotypes of GSD-1 and Hnf1α−/− mice. Further, we show that the expression of the G6Pase gene is also perturbed in Hnf1α−/− mice.

G6PT, encoded by a single copy gene located on human chromosome 11q23 (35), is expressed in nearly all tissues examined, including liver, kidney, pancreas, and digestive tract (36). In this study, we demonstrate that nucleotides −200 to −1 upstream of the translation start site constitute a minimal G6PT promoter and HNF1α is required for transcription of the G6PT gene. The minimal G6PT promoter contains an activating element at nucleotides −200 to −153 encompassing the HNF1 motif at nucleotides −165 to −153. We show that HNF1α activates transcription of the G6PT gene following binding to its cognate site. Consistent with this, hepatic G6PT mRNA expression was inhibited and microsomal G6P transport function in the liver was impaired in Hnf1α−/− mice. In GSD-1b patients, deficiency in G6PT results in an increase in hepatic G6Pase latency values (34). Likewise, G6Pase latency values in Hnf1α−/− mice are also markedly increased. Taken together, these data demonstrate that the G6PT function in Hnf1α−/− mice is impaired, resulting in a phenotype that closely resembles that of GSD-1b.

In addition to functional G6Pase deficiency, GSD-1b patients suffer additional infectious complications because of heritable neutropenia and functional deficiencies of neutrophils and monocytes (37), clinical features not associated with Hnf1α−/− mice. The results of a recent study showed that GSD-1b patients carrying either a homozygous splicing (794G→A) mutation or heterogeneous G339D and R415X mutations suffer no impairment in their polymorphonuclear leukocyte functions (38). The 794G→A mutation was shown to be leaky because the mutated G6Pase gene of the patient directed the expression of both mature and truncated G6PT transcripts (38). Likewise, the R415X mutation was shown to only partially inactivate the transporter (39). These studies strongly suggest that neutropenia as well as neutrophil and monocyte dysfunctions occur only in patients that harbor null G6PT mutations. Therefore, Hnf1α−/− mice, which express a low level of the G6PT gene, do not manifest neutropenia or polymorphonuclear leukocyte dysfunction.

It is noteworthy that overexpression of G6Pase in primary hepatocytes creates the metabolic profile of liver cells derived from NIDDM patients (40). Moreover, rats overexpressing the G6Pase gene manifest glucose intolerance and hyperinsulinemia (23). Transient expression studies have shown that HNF1α is required for transcription of the G6Pase gene (27). However, HNF1α is also required for the maximal repression of G6Pase gene transcription by insulin (24, 25). The increase in G6Pase expression in Hnf1α−/− mice strongly suggests that the in vivo role of HNF1α is to act as an accessory factor to enhance the inhibitory action of insulin on G6Pase gene transcription (25). It has been shown that in diabetic rats, prolonged hyperglycemia increases G6Pase gene expression independent of insulin (22) and that the glucose-stimulated increase in G6Pase mRNA depending upon the presence of glucokinase (41). It appears that HNF1α deficiency compounded with impaired insulin secretion and hyperglycemia contributes to G6Pase overexpression in Hnf1α−/− mice. Whether perturbations in G6Pase expression contribute to the pathogenesis of NIDDM in these mice remains to be elucidated.

Glucose-6-phosphate, the substrate of the G6Pase system, plays a pivotal role in metabolism. It is at the branch point of lipid biosynthesis and glycogen biosynthesis as well as facilitating energy homeostasis through glucose. Kinetic studies have suggested that G6P uptake is the rate-limiting step in G6Pase catalysis (3). This notion is further supported by functional G6Pase deficiency manifested by GSD-1b patients carrying inactivating mutations in the G6PT gene (26). Similarly, in Hnf1α−/− mice, G6P generated by glycolysis and glucogenesis could not be efficiently translocated to the lumen of the ER, resulting in an increase in hepatic glycogen deposition and stimulation of cholesterol and fatty acid synthesis. Taken together, our study demonstrates, for the first time that metabolic abnormalities in Hnf1α−/− mice are caused in part by G6PT deficiency and disruption in the balance of the G6Pase system.

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**Table I**

| Genotype       | Control | Deoxycholate | Latency |
|----------------|---------|--------------|---------|
|                | nmol/min/mg | nmol/min/mg | %       |
| (+/+) (−/−)    | 153.9 ± 12.9 (n = 5) | 277.3 ± 11.8 (n = 5) | 41.7 ± 2.7 |
| (−/−)          | 81.6 ± 7.9 (n = 5) | 709.5 ± 30.4 (n = 5) | 87.9 ± 5.8 |

*Latency was assessed by G6P phosphorylase in intact (I) versus detergent-disrupted (D) microsomes, defined as [(1 − I/D) × 100] (33).

*Hepatic G6Pase activity is similar in Hnf1α−/− and Hnf1α−/− mice.*
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