Isolation of the Gene for Murine Glucose-6-phosphatase, the Enzyme Deficient in Glycogen Storage Disease Type 1A*

(Received for publication, July 15, 1993)

Leslie L. Shelly, Kei-Jian Lei, Chi-Jiunn Pan, Shigeko F. Sakata, Siegfried Ruppert, Gunther Schutz, and Janice Yang Chou

From the Human Genetics Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892 and the Institute of Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany

Glycogen storage disease (GSD) type 1a (von Gierke disease) is caused by a deficiency in glucose-6-phosphatase, the key enzyme in glycogen homeostasis catalyzing the terminal step in gluconeogenesis and glycogenolysis. Despite its clinical importance, this membrane-bound enzyme has eluded molecular characterization. Here we report the cloning and characterization of a murine glucose-6-phosphatase cDNA by screening a mouse liver cDNA library differentially with mRNA populations representing the normal and the albino deletion mouse known to express markedly reduced glucose-6-phosphatase activity. Additionally, we identified the gene that consists of 5 exons. Biochemical analyses indicate that the in vitro expressed enzyme is indistinguishable from mouse liver microsomal glucose-6-phosphatase exhibiting essentially identical kinetic constants, latency, thermal lability, and vanadate sensitivity. The characterization of the murine glucose-6-phosphatase gene opens the way for studying the molecular basis of GSD type 1a in humans and its etiology in an animal model.

Glucose-6-phosphatase (G6Pase, EC 3.1.3.9), the key enzyme in the homeostatic regulation of blood glucose levels, catalyzes the terminal step in both gluconeogenesis and glycogenolysis (1-3). Deficiency of G6Pase causes glycogen storage disease (GSD) type 1a (von Gierke disease), the most severe form of GSD. This autosomal recessive disorder is characterized by hypoglycemia, hepatomegaly, growth retardation, delayed puberty, lactacidemia, hyperuricemia, and in adults, a high incidence of hepatic adenomas (1, 2). Despite extensive clinical and biochemical investigations, G6Pase has eluded molecular characterization due primarily to its tight association with the endoplasmic reticulum (ER) and nuclear membranes (3). Characterization of the G6Pase protein and gene is critical for understanding the molecular basis of GSD type 1a and for the development of novel therapeutic approaches to this genetic disorder.

To isolate cDNAs encoding G6Pase, we took advantage of an albino deletion mutant mouse that is known to express markedly reduced levels of G6Pase activity (4). The primary defect of this mutant mouse is the loss of the fumarylacetoacetate hydrolase gene located around the albino locus on chromosome 7 (5). Fumarylacetoacetate hydrolase is the final enzyme in the tyrosine degradation pathway, and a deficiency of this enzyme leads to the accumulation of toxic tyrosine metabolites resulting in reduced expression of a group of liver-specific proteins, including G6Pase (5, 6). Newborn homozygous deletion mice develop hypoglycemia shortly after birth, correlating with undetectable levels of G6Pase activity (4). In the present study, we have isolated and characterized a full-length cDNA (pmG6Pase) encoding murine liver microsomal G6Pase by screening a normal mouse liver cDNA library differentially (6) with probes representing mRNA populations from the normal and the albino deletion mutant mouse. Moreover, we have characterized the murine G6Pase transcription unit that spans approximately 10 kilobases and consists of 5 exons.

MATERIALS AND METHODS

Library Screening and Characterization of cDNA and Genomic Clones—A cDNA library in agt10 representing wild-type homozygote (cCh/cCh) mouse liver mRNA was screened differentially (6) with probes representing the mRNA populations from the wild-type and the albino deletion mutant mouse. pmG6Pase-1 that contains nucleotides 12-2259 of the murine G6Pase cDNA was one of the differentially expressed genes extensively characterized. The murine G6Pase gene was obtained by screening a mouse liver genomic library in Lambda Dash (Stratagene) with the pmG6Pase-1 probe. The cDNA and genomic inserts of murine G6Pase were subcloned into pGEM (Promega Biotech, Madison, WI) vectors for further characterization. Both strands of the cDNA and genomic clones were sequenced by the Sanger dideoxy chain termination method (7), and the genomic sequences were compared with cDNA sequences to identify intron-exon junctions.

Expression in COS-1 Cells and Isolation of Microsomal Membranes—Nucleotides 12-1814 of the pmG6Pase cDNA (pSVLmG6Pase), which contains the entire coding region at nucleotides 83-1153, was subcloned in a pSVL vector (Pharmacia LKB Biotechnology Inc.) and transfected into COS-1 cells by the DEAE-dextran/chloroquine method (8). Mock transfections of COS-1 cultures with the pSVL vector were used as controls.

Microsomal membranes were isolated by the method of Burchell et al. (9) either from Swiss Webster mice that had been fasted overnight or from freshly prepared homogenates of pSVLmG6Pase-transfected COS-1 cells. Disrupted microsomal membranes were prepared by incubating intact membranes in 0.2% deoxycholate for 20 min at 37 °C. The latency or intactness of microsomal preparations was assessed by assaying mannose-6-phosphorylase in intact versus detergent-disrupted membranes (10).

Phosphorylase and Phosphotransferase Assays—Phosphorylase activity was determined essentially as described by Burchell et al. (9). Phosphotransferase activity was determined by a modification of the method described by Jorgenson and Nordlie (11). The reaction mix-
ture (10 μl) contained 100 mM HEPES buffer, pH 6.5, 50 mM glucose, α-[U-14C]glucose (106 cpm/reaction, 256 mCi/mmol, ICN Biochemicals, Irvine, CA), 4 mM carbamyl-P, and deoxoycholate-disrupted microsomal proteins. After incubation at 30 °C for 10 min, reactions were stopped by heating at 80 °C for 5 min. The samples were then centrifuged at 10,000 x g for 5 min, and 2-4 μl of supernatant was applied to a polyethyleneimine cellulose plate (T. Baker, Inc.). Glucose-6-P was separated from glucose by thin-layer chromatography developed in water. Spots were quantitated on an AMBIS radioanalytic imaging system (San Diego, CA).

Northern Hybridization Analysis—RNA was isolated by the guanidinium thiocyanate-CsCl method (12), separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde (13), and transferred to Nytran membranes (Schleicher & Schuell). The hybridization and washing conditions were performed as previously described (6).

RESULTS AND DISCUSSION

Using differential cDNA screening of a mouse liver cDNA library, a group of liver genes affected in lethal albino mice has been isolated and characterized. Nucleotide sequence analysis of a 2248-bp cDNA (nucleotides 12–2259, designated pmG6Pase-1) revealed an open reading frame of 1071 nucleotides that encodes a 357-amino acid polypeptide (Fig. 1A). The pmG6Pase-1 cDNA probe was used to screen a Lambda Dash mouse genomic library, and a genomic clone containing the entire murine G6Pase transcription unit was isolated and extensively characterized. The structural organization of the murine G6Pase transcription unit (Fig. 1B) was defined by restriction endonuclease mapping, Southern blot hybridization, and DNA sequencing. This murine gene spans approximately 10 kilobases and consists of 5 exons: I(311 bp), II (110 bp), III (106 bp), IV (116 bp), and V (1615 bp). The transcription initiation site of murine G6Pase was demonstrated by primer extension experiments (data not shown); the first 11 base pairs of the pmG6Pase cDNA were identified by sequencing the genomic clone.

Comparison of the nucleotide and deduced amino acid sequences of pmG6Pase cDNA with that in the data bases indicated no significant identity to any sequence reported to date. The predicted pmG6Pase-encoded protein has a calculated molecular mass of 40 kDa and contains an ER protein retention signal, motifs for protein glycosylation, and several membrane-spanning segments. This suggested that this cDNA may encode microsomal G6Pase glycoprotein of 35–36.5 kDa (14, 15). The identity of the cDNA was confirmed by performing detailed biochemical studies after transient expression of the pSVLmG6Pase plasmid (nucleotides 12–1814 of the pmG6Pase cDNA) in COS-1 cells.

The G6Pase, produced by translation of murine G6Pase mRNA synthesized by in vitro transcription of the pmG6Pase-1 cDNA, migrated on a SDS-polyacrylamide gel as a 34-kDa polypeptide (Fig. 2A), which is considerably smaller than the predicted molecular size of 40 kDa. The anomalous electrophoretic mobility of the murine G6Pase protein may reflect the extremely hydrophobic nature of the encoded polypeptide. The hydropathy index analysis (16, 17) predicts that the G6Pase protein contains six putative membrane-spanning segments (Fig. 2B).

The ER localization of murine G6Pase is predicted by the presence of two lysines, positioned 3 and 4 amino acids from the carboxyl terminus of the deduced protein (Fig. 1A), a consensus motif for the retention of proteins in the ER (18). In addition, three potential asparagine-linked glycosylation sites are present in the predicted murine G6Pase polypeptide at amino acids 96–98, 203–205, and 276–278 (Fig. 1A), suggesting that G6Pase is a glycoprotein.

To demonstrate the functional identity of the pmG6Pase cDNA, detailed biochemical studies were performed in microsomal preparations of pSVLmG6Pase-transfected COS-1 cells and compared with activities in microsomes isolated from adult mouse livers. COS-1 cells, which are monkey cells transformed with a replication origin-defective SV40 DNA molecule, produce high levels of the SV40 large T antigen in culture (19). The pSVL vector, which contains a SV40 replication origin, is engineered to express genes efficiently after transient transfection in COS-1 cells (20, 21).

Hepatic G6Pase is known to exhibit latency, referring to the portion of enzymatic activity that is not expressed unless the microsomes are disrupted (3). Microsomal G6Pase has varying degrees of latency depending on the substrate utilized. Both glucose-6-P and mannose-6-P are rapidly hydrolyzed in disrupted microsomes; only glucose-6-P is hydrolyzed in intact microsomes (22). Therefore, mannose-6-P phosphohydrolysis is in intact versus detergent-disrupted microsomes is used to measure the latency or intactness of microsomal preparations. Latencies for mannose-6-P phosphohydrolysis are 95% or greater in rat liver microsomes (3) and are about 40–54% in microsomes derived from isolated rat hepatocytes or hepatoma cells (11). In agreement with these observations, G6Pase of the rat, mannose-6-P phosphohydrolyase activity in microsomes isolated from mouse livers and pSVLmG6Pase-transfected COS-1 cells exhibited latency values of 97 and 50%, respectively (Table I). Therefore, microsomes of cultured cells have similarly reduced latencies.
Isolation of the Gene for Murine Glucose-6-phosphatase

Characteristics of microsomal G6Pase activity in pSVLmG6Pase-transfected COS-1 cells and adult mouse livers

| Parameters (pSVLmG6Pase-transfected COS-1 cells) | Mouse liver |
|-------------------------------------------------|-------------|
| Latency                                          | 50%         | 97%          |
| Thermal stability                                | 1.6%        | <1%          |
| Glucose-6-P, $K_m$                               | 0.65 ± 0.09 | 0.68 ± 0.03  |
| Mannose-6-P, $K_m$                               | 0.67 ± 0.07 | 0.67 ± 0.04  |
| Glucose-6-P, $V_{max}$                           | 0.21 ± 0.05 | 0.35 ± 0.06  |
| Mannose-6-P, $V_{max}$                           | 0.20 ± 0.06 | 0.30 ± 0.08  |
| Vanadate, $K_v$                                  | 0.34        | 0.35         |
| Phosphotransferase                               | 0.22        | 0.3          |
| Carbamyl-P: Glucose                             |             |              |

$V_{max}$ values for both microsomal preparations are virtually identical (Table I) and are in agreement with those reported previously for rat liver microsomes (10).

Vanadate is a potent inhibitor of glucose-6-P phosphohydrolase activity (25). Microsomal preparations from both pSVLmG6Pase-transfected COS-1 cells and adult mouse livers were equally sensitive to vanadate, giving nearly identical inhibition curves of glucose-6-P hydrolysis (Fig. 3B). Moreover, vanadate was a competitive inhibitor of glucose-6-P phosphohydrolase in both microsomal preparations (Fig. 3, C and D), yielding essentially identical $K_i$ values (Table I). The $K_i$ values (0.33–0.34 mM) for vanadate observed in the present study differed considerably from the $K_i$ for vanadate (1.5 μM) reported for permeable hepatocytes or rat microsomes (25). The reason for this discrepancy is unknown. However, a similar vanadate inhibition curve to that for the mouse G6Pase reported here was observed for commercially obtained crude microsomal preparations of rabbit G6Pase obtained from Sigma (data not shown).

In addition to displaying phosphohydrolatic activity, G6Pase is capable of catalyzing the formation of glucose-6-P from glucose and a variety of phosphate substrate donors (3). Phosphotransferase activities in microsomes of pSVLmG6Pase-transfected COS-1 cells and adult mouse livers were evaluated using carbamyl-P and glucose as substrates (Table I). Similar specific transferase activities were observed in both microsomal preparations, in good agreement with the value (0.294 μmol/min/mg of microsomal protein) reported for carbamyl-P-glucose phosphotransferase activity in rat hepatocyte microsomes (11).

The expression of G6Pase mRNA was examined in livers of normal and albino deletion mice by Northern blot hybridization analysis (Fig. 4). As expected, G6Pase mRNA was detected only in normal mouse liver; little or no G6Pase transcripts were detected in the liver of the albino deletion mouse. Both liver...
rupted microsomes isolated from pSVLmGGPase-transfected COS-1 cells (C) and adult mouse livers (D). A, phosphohydrolase activity was assayed at various pH values using glucose-6-P (10 mM) as the substrate in sodium acetate containing 50 mM cacodylate buffer, pH 6.5-8.5) buffers. B, inhibition by vanadate was assayed in reactions containing 50 mM cacodylate buffer, pH 6.5, and glucose-6-P (10 mM), at various concentrations of vanadate. C and D, competitive inhibition of glucose-6-P hydrolysis by vanadate using microsomes of adult mouse livers (C) or pSVLmGGPase-transfected COS-1 cells (D). K values for vanadate were determined in reactions containing 50 mM cacodylate buffer, pH 6.5, glucose-6-P (0.6-5 mM), and vanadate (1.5 mM) in microsomes isolated from adult mouse livers (C) or in microsomes isolated from pSVLmGGPase-transfected COS-1 cells (A).

FIG. 3. The pH dependence (A) and vanadate inhibition (B-D) of glucose-6-P phosphohydrolytic activity of deoxycholate-disrupted microsomes isolated from pSVLmGGPase-transfected COS-1 cells (C) and adult mouse livers (D). A, phosphohydrolase activity was assayed at various pH values using glucose-6-P (10 mM) as the substrate in sodium acetate (50 mM, pH 5.0) or cacodylate (50 mM, pH 5.5-8.5) buffers. B, inhibition by vanadate was assayed in reactions containing 50 mM cacodylate buffer, pH 6.5, and glucose-6-P (10 mM) at various concentrations of vanadate. C and D, competitive inhibition of glucose-6-P hydrolysis by vanadate using microsomes of adult mouse livers (C) or pSVLmGGPase-transfected COS-1 cells (D). K values for vanadate were determined in reactions containing 50 mM cacodylate buffer, pH 6.5, glucose-6-P (0.6-5 mM), and vanadate (1.5 mM) in microsomes isolated from adult mouse livers (C) or in microsomes isolated from pSVLmGGPase-transfected COS-1 cells (A).

FIG. 4. Expression of G6Pase in livers of newborn wild-type and albino deletion mice (A) and in adult mouse tissues (B). Total RNAs (5 μg/plane) were analyzed by Northern blot hybridization with either an antisense pmG6Pase-1 probe (A) or an antisense probe containing nucleotides 750-1820 of the pmG6Pase cDNA (B). The filters were rehybridized either with a transferin (TP) or a heat shock protein 84 (HSP84) probe, which was used as an internal standard.

and kidney are known to express high levels of G6Pase enzyme activity (1-3). G6Pase mRNA was evident in the liver and kidney but was not detectable in testes, brain, muscle, or lung, demonstrating that G6Pase mRNA expression is also restricted to the liver and kidney (Fig. 4).

Two models for G6Pase catalysis have been proposed to account for the relationship between G6Pase and the membrane with which it is intimately associated. The conformation-substrate-transport model (26) proposes that G6Pase represents a single membrane channel protein capable of both transport and catalytic functions. The translocase-catalytic unit model (3, 10) proposes that G6Pase is a multicomponent complex consisting of a G6Pase catalytic unit and associated translocases. The translocase-catalytic unit model has been used to explain the existence of GSD type 1a, 1b, 1c, and 1d patients, which correspond to defects in G6Pase, the putative glucose-6-P translocase, phosphate/pyrophosphate translocase, and glucose translocase (1-3, 27, 28). The absence of mutations in the G6Pase gene in GSD type 1b, 1c, or 1d patients will support the translocase-catalytic unit model, suggesting the existence of translocases.

The present study demonstrates that the pmG6Pase cDNA encodes the multifunctional G6Pase, the enzyme deficient in GSD type 1a in humans. Cloning and characterization of the murine gene will greatly facilitate the isolation of the human G6Pase gene and the identification of mutations in GSD type 1a patients. Accordingly, understanding the molecular basis for GSD type 1a and the development of new therapies is now possible. Finally, an animal model for studying the etiology of GSD type 1a in humans can be easily established by homologous gene targeting to disrupt the murine G6Pase gene.

Acknowledgments—We thank Drs. M. Brantly, M. Chamberlin, L. Charnas, W. A. Gahl, B. Mansfield, A. Mukherjee, I. Owens, J. Ritter, M. J. Shields, and J. B. Sidbury for critical reading of the manuscript and Drs. T. Hoffmann and B. Hovemann for the gift of the HSP84 cDNA clone.

REFERENCES

1. Hers, H.-G., Van Hoof, F., and de Barys T. (1969) in The Metabolic Basis of Inherited Disease (Server, C. R., Beaudet, A. L., Charles, R., Sly, W. S., and Valle, D., eds) pp. 425-452, McGraw-Hill, New York.

2. Beaudet, A. L. (1991) in Harrison's Principles of Internal Medicine (Wilson, J. D., Braunwald, E., Isselbacher, K. J., Petersdorf, R. G., Martin, J. B., Fauci, A. S., and Root, R. K., eds) 12th Ed., pp. 1854-1860, McGraw-Hill, New York.

3. Fialkow, R. C., and Sukakelis, R. A. (1985) in The Enzymes of Biological Membranes (Martoens, A. N., ed.) 2nd Ed., pp. 349-398, Plenum Press, New York.

4. Gluecksohn-Walsch, S. (1979) Cell 16, 225-237.

5. Ruppert, S., Kelsey, G., Scheld, A., Schmid, E., Thies, E., and Schutz, G. (1992) Genes Dev. 6, 1430-1433.

6. Ruppert, S., Boshart, M., Bosch, F. X., Schmid, W., Fournier, K., and Schutz, G. (1990) Cell 61, 895-904.

7. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467.

8. Ausasdel, P. M., Breen, R., Kingston, R. E., Moore, D. D., Seizman, J. G. Smith, J. A., and Strubh, K. (1992) Current Protocols in Molecular Biology, pp. 9.2.1-9.2.6, Greene Publishing and Wiley-Interscience, New York.

9. Burchell, A., Hume, R., and Burchell, B. (1990) Clin. Chem. Acta 173, 183-192.

10. Arion, W. J., Lange, A. J., Walls, E. H., and Ballas, L. M. (1980) J. Biol. Chem. 255, 10396-10406.

11. Jorgensen, R. A., and Nordlie, R. C. (1980) J. Biol. Chem. 255, 5907-5915.

12. Chirpew, J. M., Przybyla, E. A., MacDonald, P. R., and Kutter, W. J. (1972) Biochemistry 11, 5294-5299.

13. Luehr, H., Diamond, D., Wozney, J. M., and Keedler, H. (1977) Biochemistry 16, 4743-4751.

14. Speth, M., and Schulze, H. U. (1992) Eur. J. Biochem. 206, 643-650.

15. Countaway, J. L., Waddell, I. D., Burchell, A., and Arion, W. J. (1980) J. Biol. Chem. 255, 2673-2678.

16. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132.

17. Klein, P., Kanehisa, M., and DeLasa, C. (1985) Biochim. Biophys. Acta 815, 468-475.

18. Jackson, M. R., Nilsson, T., and Peterson, P. A. (1990) EMBO J. 9, 3153-3162.

19. Gluzman, Y. (1981) Cell 23, 175-182.

20. Mellon, P. K., Parker, V., Gluzman, Y., and Maniatis, T. (1981) Cell 27, 279-288.

21. Kaufman, R. J. (1990) Methods Enzymol. 185, 487-511.

22. Arion, W. J., Wallin, B. K., Carlson, P. W., and Lange, A. J. (1972) J. Biol. Chem. 247, 2558-2565.

23. Hers, H. G. (1964) Adv. Metab. Disord. 1, 1-44.

24. Lange, A. J., Arion, W. J., Burchell, A., and Buchell, B. (1986) J. Biol. Chem. 261, 101-107.

25. Singh, J., Nordlie, R. C., and Jorgensen, R. A. (1981) Biochim. Biophys. Acta 678, 477-482.

26. Schulze, H. U., Nele, B., and Kannler, R. (1986) J. Biol. Chem. 261, 16571-16578.

27. Lange, A. J., and Arion, W. J. (1980) J. Biol. Chem. 255, 8381-8384.

28. Nordlie, R. C., Sukakelis, R. A., Munoz, J. M., and Baldwin, J. J. (1983) J. Biol. Chem. 258, 9739-9744.

29. Von Heijne, G. (1986) Nucleic Acids Res. 14, 4683-4690.