V490M, a Common Mutation in 3-Phosphoglycerate Dehydrogenase Deficiency, Causes Enzyme Deficiency by Decreasing the Yield of Mature Enzyme*

A deficiency of 3-phosphoglycerate dehydrogenase (PHGDH) is a disorder of serine biosynthesis identified in children with congenital microcephaly, seizures, and severe psychomotor retardation. We report here the identification of the 1468G→A (V490M) mutation of this gene in two siblings of an Ashkenazi Jewish family, providing further evidence that the V490M mutation is a common, panethnic cause of this deficiency. Using a novel, DNA-based diagnostic test, the mutation was not detected in 400 non-Jewish controls; one heterozygote was found among 400 persons of Ashkenazi Jewish ethnicity. Extensive biochemical studies were undertaken to characterize the effect of this mutation on enzyme activity, turnover, and stability. The V490M PHGDH yielded less than 35% of the activity observed for the wild-type enzyme when overexpressed by transient transfection or when comparing the endogenous activity in fibroblast cells from the patients with controls. Immunoblotting studies showed a comparable reduction in the level of immunoreactive PHGDH in cells expressing the mutant enzyme. Pulse-chase experiments with metabolically labeled PHGDH indicated that this resulted from an increased rate of degradation of the mutant enzyme following its synthesis. Thermolability analyses of mutant and wild-type enzyme activity revealed no significant differences. While others have proposed that the V490M mutation decreases the V_{max} of the enzyme, we conclude that this mutation impairs the folding and/or assembly of PHGDH but has minimal effects on the activity or stability of that portion of the V490M mutant that reaches a mature conformation.

A deficiency of the enzyme 3-phosphoglycerate dehydrogenase (PHGDH; EC 1.1.1.95) was identified as an inborn error of metabolism associated with congenital microcephaly (MIM 601815) by Jaeken et al. in 1996 (1). Siblings from a consanguineous family of Turkish origin were found to have abnormally low concentrations of serine and, to a lesser extent, glycine, in their cerebrospinal fluid. Fibroblasts from the probands displayed decreased activity of PHGDH (22 and 13% of control), the first enzyme in the de novo biosynthesis of serine from carbohydrates. Similar findings have since been reported on a second (unrelated) Turkish family (2), a Moroccan family (3), and another European family of undefined ethnicity (4). In addition to microcephaly, major clinical findings in patients with this disorder include severe psychomotor retardation, postnatal growth retardation, and intractable seizures. Magnetic resonance imaging of the brain shows cerebroatrophies and abnormal myelinization (1–3, 5).

Importantly, replacement therapy with serine can result in some neurological and developmental benefits, such as improved seizure control (reviewed in Ref. 6) and increased central nervous system myelination (5).

A reduced capacity to synthesize L-serine has potentially serious consequences for cellular metabolism. Serine is incorporated directly into proteins and is a precursor for the de novo biosynthesis of glycine, cysteine, and the nonstandard amino acid, selenocysteine. It is also essential for the biosynthesis of phosphatidylserine and sphingolipids. Serine can be converted to pyruvate in the liver and kidney, with subsequent utilization for gluconeogenesis, lipogenesis, or energy production. L-serine is also the precursor of D-serine (7, 8), an enantiomer prominent in the brain, where it functions as a neuromodulator of the strychnine-insensitive N-methyl-D-aspartate receptor (9–12). Finally, serine serves as the major intracellular source of one-carbon units in the cell, through the action of L-serine hydroxymethyltransferase. The products of this reaction, 5,10-methenyltetrahydrofolate and glycine, are critical metabolites involved in folate and bile acid metabolism and in the de novo biosynthesis of creatine, porphyrins, thymidylate, and purine nucleotides. Glycine also functions as a neurotransmitter in the central nervous system.

Serine is classified as a nutritionally nonessential amino acid because it can be synthesized de novo, from both glucose and glycine. PHGDH is the first enzyme in a widely distributed, cytoplasmic pathway that synthesizes serine from the glycolytic intermediate d-3-phosphoglycerate (reviewed in Refs. 13 and 14). PHGDH catalyzes the oxidation of this substrate to 3-phosphohydroxypyruvate, with NAD+ required as a cofactor. Transamination of 3-phosphohydroxypyruvate with glutamate genase; BHk, baby hamster kidney; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol.
by 3-phosphoserine aminotransferase yields 3-phosphoserine. In the final step, dephosphorylation of 3-phosphoserine by phosphoserine phosphatase produces L-serine. Only the final reaction in this pathway is irreversible. In some cells, serine can also be produced from glycine (13). This process requires the enzymes of the glycine cleavage complex, found in mitochondria, and serine hydroxymethyltransferase, an enzyme with one isoform localized in the cytoplasm and another in mitochondria. The mitochondrial isoform may operate reversibly, producing either serine or glycine, depending upon metabolite levels and the presence of the glycine cleavage system. In contrast, the cytoplasmic isoform appears to operate in one direction only, producing serine (15–17). While the relative contributions of these alternate synthesis pathways to serine homeostasis remain to be determined, the neurodevelopmental phenotype of patients with a deficiency of PHGDH highlights the importance of that pathway in the central nervous system.

The sequence of the cDNA encoding human PHGDH (4, 18), its localization to the 1p12 (19) or 1q12 (4) pericentromeric region of chromosome one, and the identification of two mutations causing PHGDH deficiency (4) have recently been reported. Klomp et al. (4) determined that a mutation in the Moroccan family resulted in a substitution of the amino acid valine at position 425 with methionine (V425M). The remainder of the families that have been described have a V490M substitution (4). The distribution and autosomal recessive inheritance pattern of these mutations support the conclusion that they are disease-causing. However, following synthesis in vitro, these mutations caused only modest reductions in PHGDH activity. These reductions were attributed to a reduced Vmax of the mutant enzymes, whereas the Km did not differ from the wild-type in this system. To reconcile the dramatic phenotype observed in the patients with the modest effects of these mutations on enzyme activity, the authors speculated that in addition to the lower catalytic turnover, the mutations may result in decreased expression of the enzyme in vivo. Measurements of PHGDH mRNA levels in fibroblasts showed no differences between patients and controls (4); other approaches to investigate reduced enzyme activity, such as measurements of protein levels, turnover, and stability were not reported.

In this paper, we report the independent identification of the V490M mutation in two siblings born to nonconsanguineous parents of Ashkenazi Jewish ethnicity from New England. Detailed characterization of the enzyme demonstrated an ~70% reduction of enzyme activity and a similar reduction of immunoreactive protein in cells expressing V490M PHGDH. In contrast to the work of Klomp et al. (4), we find that these reductions resulted from an increased degradation of the mutant protein, probably due to less efficient folding and/or assembly following synthesis. Our results provide a molecular explanation for the low levels of PHGDH and serine observed in our patients and confirm a role for this enzyme deficiency in the genesis of a neurodevelopmental phenotype of congenital microcephaly, severe psychomotor retardation, and seizures.

**EXPERIMENTAL PROCEDURES**

**Clinical Samples**—The probands, male and female siblings with congenital microcephaly, severe global developmental delays, seizures, and spastic quadriaparesis, were born to healthy, unrelated parents of Ashkenazi Jewish ethnicity. They were found to have low levels of serine in cerebrospinal fluid, as will be described in detail elsewhere.2 Heparinized blood and skin fibroblasts were obtained with informed consent.

Leukocyte pellets were prepared from heparinized blood as described (20). Blood specimens were obtained through informed consent from 400 non-Jewish and 400 Ashkenazi Jewish individuals. Samples were included in the latter group if all four grandparents were of Ashkenazi Jewish ethnicity.

**Culture**—Cells were cultured at 37 °C and 5% CO2 using reagents from Invitrogen. HeLa and BHK-21 cells (American Type Culture Collection, Manassas, VA) and skin fibroblasts were maintained in DMEM (high glucose) containing 8% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Control fibroblasts (GM 08399A) were obtained from the NIGMS (National Institutes of Health) Human Genetic Mutant Cell Repository (Camden, NJ).

**Isolation of cDNAs Encoding PHGDH**—Overlapping expressed sequence tags encoding the entire PHGDH cDNA were identified by sequence similarity to the rat liver cDNA (21) using the Basic Local Alignment Search Tool (available on the World Wide Web at www.ncbi.nlm.nih.gov/BLAST/) (22). Primers based upon this sequence were used to amplify the human cDNA by reverse transcriptase-PCR. 100 ng of total RNA prepared from HeLa cells and proband fibroblasts (Trizol; Invitrogen) were reverse transcribed using 200 units of SuperScript II RNase H− reverse transcriptase (Invitrogen) and 2.5 pmol of a primer complementary to the sequence 54–74 nucleotides downstream of the predicted stop codon (5′-TCT CTC ATT GAT CAC AGT GG-3′). The 5′ PCR primer (5′-TTA GGT ACT CCA GGT TAG-3′) begins 47 nucleotides upstream of the predicted start codon. The 3′ PCR primer (5′-CAT GAC TGG GAG TGT GAC GAC GGC CC-3′) is complementary to the carboxy-terminal region of the protein and contains a BamHI restriction site (in italics) added after the stop codon (underlined) for cloning purposes. Amplification conditions were as follows: 94 °C for 5 min, followed by 36 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 3 min 30 s, with a final 10 min at 72 °C, using FuJi DNA polymerase (Stratagene). A 1.6-kb product was purified (Qiagen II kit; Qiagen) from an agarose gel and digested with EcoRI and BamHI. The resulting clones were assessed by dyeoxy sequencing using T7 DNA polymerase (Amersham Biosciences, Inc.).

**Isolation of Genomic DNA**—Tissue culture cell pellets and white blood cell pellets were incubated overnight at 50 °C in 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS, and 200 μg/ml proteinase K (Roche Molecular Biochemicals). DNA was then purified by sequential extractions with phenol, phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (24:1), followed by precipitation with ethanol (modification of Ref. 23).

**Detection of the 1468G→A Mutation**—A PCR-based assay was developed to survey for the 1468G→A mutation in genomic DNA. Preliminary analysis indicated the presence of an ~800-bp intron close to this mutation site. This region was cloned, and the exon/intron boundaries (between 1447 and 1448) were determined.3 For detection of the 1468G→A mutation, the 5′ PCR primer (5′-CTC CGC TCA TTG CAC TTT GCA-3′) is complementary to nucleotides 1553–1572. A 238-bp product was amplified from ~100 ng of genomic DNA using Taq DNA polymerase (Invitrogen) and the following amplification conditions: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s, followed by a final 10-min extension at 72 °C. Aliquots were digested with Hsp92II (Promega), separated on 10% acrylamide gels, and stained with ethidium bromide. Fragments of 45 and 193 bp were observed for control DNA and 45, 57, and 136 bp if the 1468G→A mutation was present.

**Transient Expression and Enzyme Assays**—The activity of the V490M PHGDH was compared with the wild-type enzyme following transient expression in BHK-21 cells. In some experiments, the PHGDH-encoding vectors were cotransfected with pSV-β-galactosidase (Promega) in order to monitor the transfection efficiency. DNA–lipid complexes were prepared with 9 μg of pcDNA-PHGDH (wild-type or V490M), or 7 μg of pcDNA-PHGDH plus 2 μg of pSV-β-galactosidase and 20 μl of LipofectAMINE 2000 in DMEM, according to the manufacturer’s instructions (Invitrogen). 24 h following transfection, the cells were lysed by sonication in 25 mM Hepes, pH 7.1, 400 mM KCl, 1 mM DTT, and 1% Triton X-100 containing a mammitinase C–galactosidase inhibitor mixture (Sigma). The soluble fraction was obtained by centrifugation at 20,000 x g for 10 min, and the protein concentration was determined using the Coomassie Plus reagent (Pierce). β-Galactosidase activity was measured using o-nitrophenyl-b-galactopiranoside as a

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2. K. Swoboda, M. Korson, and M. Natowicz, manuscript in preparation.

3. J. Mauthe and S. Pind, manuscript in preparation.
substrate (24). PHGDH was assayed in the direction of NADH oxidation by monitoring the decrease in absorbance at 340 nm. Assays were performed at 30 °C in 25 mM Hepes, pH 7.1, 400 mM KCl, 1 mM DTT, 100 μM NADH, and 50 μM phosphohydroxyxypyruvate in a final volume of 1 ml (1, 21). Absorbance measurements were taken every 5 s for 5–10 min at 340 nm (model 3000 spectrophotometer, 3000 series), and the initial slopes of the decrease in absorbance were determined. One unit of enzyme activity is defined as the amount that oxidizes 1 μmol of NADH/min under these assay conditions. Corrections were made for transfection efficiency by dividing the specific activity of PHGDH in a dish of cells by a factor determined by dividing the activity of β-galactosidase in that same dish by the average β-galactosidase activity in all dishes on that day.

Expression and Purification of PHGDH—An NcoI restriction site was introduced into the pcDNA-PHGDH construct by site-specific mutagenesis (QuickChange™ kit; Stratagene) to change the A at position −1 of the PHGDH cDNA to a C. The forward primer used for mutagenesis was 5′-CCCAAGGCGCATTGACAAGCCTGGTTCGAAATCTCCGG-3′, with the nucleotide change highlighted in italics and the resulting NcoI site underlined. The reverse primer for mutagenesis was complementary to the forward primer. A 212-bp XbaI/BatEII fragment that encompassed this modified region was removed from the mutagenesis product and used to replace this region in another aliquot of pcDNA-PHGDH that had not undergone mutagenesis. It was sequenced to ensure that only the desired mutation had been introduced. The entire PHGDH coding region was then removed from this vector by digestion with NcoI and BanII and inserted into the same sites of the pBR322 plasmid (Amersham Biosciences). The resulting plasmid was introduced into competent BL-21 CodonPlus™ (modified from Ref. 27). Membranes were blocked for 1 h in phosphate-buffered saline containing 0.1% Tween 20 and 5% skim milk powder. The membranes were washed twice with ice-cold phosphate-buffered saline and lysed with 1 ml of lysis buffer (25 mM Hepes, 150 mM NaCl, 0.2% Triton X-100, 0.2% digitonin, 1 mM DTT, pH 7.5, containing a mixture of proteinase inhibitors). After scraping from the plates, insoluble material was removed from the lysates by centrifugation at 17,000 × g for 10 min. Supernatants were incubated overnight at 4 °C with the 1D35 antibody preadsorbed onto protein G-agarose (Sigma). Immune complexes were washed six times with lysis buffer (25 mM Hepes, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS, pH 7.5) and eluted and denatured with Laemmli sample buffer (28). Samples were separated by SDS-PAGE using 10% gels, fixed, dried, and exposed to film or a phosphorimaging screen (Bio-Rad). Images were analyzed using a Personal Molecular Imager FX and quantified using Quantity One software (Bio-Rad).

RESULTS

Characterization of the PHGDH cDNA—Based upon their similarity to the sequence of rat liver PHGDH (21), a series of overlapping expressed sequence tags was identified that predicted the entire cDNA encoding human PHGDH. Primers were designed to amplify the coding region and 47 nucleotides of the 5′-untranslated region of this cDNA from human HeLa cells using reverse transcriptase-PCR. A product of the expected size (~1.6 kb) was obtained and cloned into the pcDNA 3.1 expression vector. The sequence obtained for the coding region of the HeLa cell PHGDH was identical to that predicted from the overlapping expressed sequence tags (not shown). It was 88% identical to the rat sequence through the coding region and predicted a 553-amino acid protein that was 94% identical to the rat enzyme. With the exception of a single base substitution (G → T) at position 75, our sequence was also identical to the sequence of PHGDH that was subsequently deposited into GenBank™ (accession number AF006043; Ref. 18). This substitution changes amino acid 25 to Asp rather than Glu as reported by Cho et al. (18). The same substitution is also present in our family members and in all of the expressed sequence tags we examined as well as the sequence recently reported by Klomp et al. (4).

Identification of the 1468G → A Mutation—cDNAs encoding PHGDH were also cloned and sequenced from skin fibroblasts derived from the probands, using the strategy described in the preceding paragraph. The only difference noted in these samples was a 1468G → A transition, resulting in a substitution of the Val at amino acid 490 with Met (V490M). This change also introduced an Hsp92II restriction enzyme site into the DNA, providing a rapid method to screen for this mutation. A 238-bp fragment that included one Hsp92II site in control samples and two sites if the 1468G → A mutation was present, was amplified from genomic DNA (Fig. 1). Restriction digests of control DNA yielded bands of 193 and 45 bp (Fig. 1, lanes 1 and 2). In samples containing the 1468G → A mutation, the 193-bp product was cleaved in two, producing 136- and 57-bp fragments. Lanes 4 and 5 show that the probands were homozygous for the 1468G → A mutation, with only the 136-, 57-, and 45-bp fragments remaining following digestion. Samples from the parents (lanes 3 and 6) were heterozygous, since all four fragments were present following digestion. These results are consistent with an autosomal recessive mode of inheritance for this mutation.

Our results provide an independent identification of the V490M mutation and support its assignment as a disease-causing mutation (4). Since eight of the first nine patients reported with this enzyme deficiency carry this mutation and come from differing ethnic backgrounds, it suggests that the 1468G → A mutation is a common cause of this deficiency. To
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Effect of the V490M Mutation on Enzyme Activity—Transient transfection studies were employed to characterize the effect of the V490M substitution on the enzymatic activity of the mu-

Effect of the V490M Mutation on Steady-state Concentration of the Enzyme—Theoretically, the V490M mutation could reduce the yield of PHGDH activity through a direct effect on reaction kinetics or through an indirect effect, by reducing the steady-state concentration of enzyme in the cells. Recent results from in vitro translation studies suggest that the mutation does not alter the $K_m$ of the mutant enzyme but rather decreases its $V_{\text{max}}$ (4). However, since accurate $V_{\text{max}}$ estimates are dependent upon knowing the exact amount of native enzyme assayed, confirmation of these results awaits further kinetic measurements using purified wild-type and mutant PHGDH. To evaluate the effect of the V490M mutation on the steady-state concentration of PHGDH in our transfected cells, equal amounts of protein from the cell lysates described in Fig. 2A were separated by electrophoresis and analyzed by immuno blotting with a monoclonal antibody specific for PHGDH (characterized further in Fig. 3B). Fig. 2B shows that the cells transfected with V490M PHGDH (lanes 6–10) had significantly lower levels of immunoreactive PHGDH than the cells transfected with the wild-type enzyme (lanes 1–5). Similar results were observed in five other transfection experiments, indicating that the V490M mutation decreases the activity of PHGDH by reducing its steady-state concentration in the cell.

To confirm that the results observed following transient transfection reflected the fates of wild-type and V490M PHGDH when expressed at physiological levels, similar experiments were performed on skin fibroblasts obtained from a control individual and from the probands’ family. Once again, lower levels of PHGDH activity were obtained in samples homozygous for the V490M mutation when compared with heterozygotes or the control fibroblasts (Fig. 3A, compare lanes 4 and 5 with lanes 1–3). Fig. 3B demonstrates that there was a correspondingly lower level of immunoreactive protein in the fibroblasts homozygous for the V490M mutation, when compared with the control fibroblasts or those from the parents (Fig. 3B, compare lanes 6 and 7 with lanes 3–5). The specificity

FIG. 1. Analysis of the 1468G→A mutation. A, schematic diagram illustrating the approach taken to detect the mutation. A 238-bp fragment including 115 bp of intronic and 123 bp of exonic sequence was amplified from genomic DNA using the primers designated f and r. Digestion with Hsp92II at position 193 would produce 45- and 193-bp fragments. In the presence of the 1468G→A mutation, the 193-bp fragment would be cleaved into 57- and 136-bp fragments. B, analysis of samples from a control subject and the affected family. Samples in all lanes except lane 1 were digested with Hsp92II. Lanes 1 and 2, a control subject; lanes 3 and 6, the parents; lanes 4 and 5, the affected siblings. DNA markers (M) of 298, 220, 201, 154, and 134 bp are visible in the left-hand lane.

FIG. 2. Transient expression of wild-type and V490M PHGDH in BHK cells. BHK cells were transfected with plasmids encoding wild-type or V490M PHGDH and harvested following a 24-h incubation. A, the graph shows the specific activities of PHGDH detected in lysates from cells transfected with the wild-type or V490M constructs. Values are the mean ± S.D. of measurements made from five independently transfected plates of cells. Three plates of untransfected control cells were also assayed. Results were normalized for transfection efficiency as described under “Experimental Procedures.” Typically, the efficiency of transfection was very similar between individual plates, and the normalization had a minimal effect on the results. B, 2 μg of total protein from each transfected cell lysate in A was separated by SDS-PAGE, transferred to nitrocellulose, and visualized by immunoblotting with 0.1 μg/ml 13D5. Lanes 1–5, plates transfected with wild-type PHGDH; lanes 6–10, plates transfected with V490M PHGDH.
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of this antibody for PHGDH was confirmed by its reaction with purified PHGDH (lane 1) and a single, identically sized protein of ~55 kDa in the HeLa and control fibroblast samples (lanes 2 and 3). This is the first report of the PHGDH protein levels in patients’ cells and provides additional support for the hypothesis that the V490M mutation decreases the steady-state concentration of PHGDH in cells.

By visual inspection, it appears that the levels of immunoreactive V490M PHGDH in Figs. 2 and 3 were reduced to a similar extent as the reductions in activity observed for the mutant enzyme. However, it is difficult to accurately quantify the chemiluminescence blots to verify this speculation. An alternative method of comparing these data is presented in Fig. 4.

Five replicate plates were transfected with wild-type or V490M mutant PHGDH and assayed as described in Fig. 2. The immunoblot shown in Fig. 4 was prepared by loading equivalent units of enzyme activity per lane on the gel rather than equivalent total protein. The signals obtained from each of the lanes were very similar to one another; specifically, the signal from the wild-type samples (lanes 1–5) was indistinguishable from the mutant samples (lanes 6–10). This shows that when equal units of enzyme activity were loaded on the gel, equal levels of immunoreactive PHGDH were observed. Taken together, these experiments indicate that the V490M mutation reduces the steady-state concentration of PHGDH in cells but has a much smaller effect on the initial rate kinetics of the mutant.

Effect of the V490M Mutation on Enzyme Stability and Turnover—Since the cellular level of a protein is governed by its rates of synthesis and degradation, we wished to determine whether the V490M mutation affected this balance. Previous work reported that the 1468G→A substitution does not alter the rate of synthesis or stability of the mRNA encoding PHGDH (4), suggesting that the rate of protein synthesis is also not affected. In Fig. 5 we demonstrate, through pulse-chase and immunoprecipitation analyses, that the V490M substitution increased the degradation of this mutant. Replicate plates of BHK cells transfected with constructs encoding wild-type or V490M PHGDH were labeled by a 20-min pulse of [35S]Met/Cys and then chased for 0–6 h in complete medium. PHGDH was immunoprecipitated from these cells, and quantified by phosphorimaging analysis following SDS-PAGE. Inspection of the autoradiograph indicated that a significant band representing PHGDH was present in all lanes transfection with the constructs (Fig. 5A, lanes 1–5 and 7–11) and was absent from the control (lane 6). It was also apparent that the labeling of the mutant protein (lanes 7–11) decreased much greater during the chase period than did the wild-type protein (lanes 1–5). There was a significant decrease in the labeling of both proteins during the first hour of the chase, after which the rate of decrease was much slower (Fig. 5B). Approximately 25% of the mutant protein remained following 6 h of chase, compared with ~70% of the wild-type protein.

Further experiments probed whether the V490M mutation decreased the stability of PHGDH, which could account for the increased rate of degradation. As an initial attempt to investigate this problem, the thermal inactivation profiles of wild-type and V490M PHGDH were compared. Cell lysates from the transfection experiments described in Fig. 4 were incubated at increasing temperatures for 5 min (Fig. 6A) or for increasing times at 45 °C (Fig. 6B), and then the initial rates of enzyme activity remaining in the extracts were determined. The graphs show that the V490M PHGDH was inactivated to a similar extent as the wild-type PHGDH under all of the test conditions, with the inactivation curves following pseudo-first-order kinetics as a function of time. These results indicate that the active forms of V490M and wild-type PHGDH display a similar stability to thermal inactivation. These data further suggest that the increased degradation of the mutant enzyme observed in Fig. 5 did not arise due to stability differences between the “functional” forms of the wild-type versus the mutant enzyme.

DISCUSSION

The results presented in this report represent an independent confirmation of the 1468G→A (V490M) mutation in PHGDH and affirms its role in the pathogenesis of enzyme deficiency (4). Our identification of the same mutation in the Ashkenazi Jewish population that was first described in the Turkish and European families suggests that this is a common cause of this deficiency. Thus, there is a strong rationale to screen for this mutation in any patients suspected to have a serine biosynthesis deficiency. The diagnostic test described in our report using genomic DNA offers a rapid and accurate means to this end.
Individual plates of BHK cells were labeled by a 20-min pulse of $[\text{35S}]\text{Met/Cys}$ and then chased for 0–6 h in complete medium. Total cell lysates were immunoprecipitated using the 13D5 monoclonal antibody, separated by SDS-PAGE, and subjected to autoradiography and phosphorimaging analysis. A, autoradiograph obtained following a 6-h exposure of the dried gel to film. B, quantification of the PHGDH bands by phosphorimaging analysis, with the amount of wild-type (closed circles) or mutant (open circles) PHGDH at 0 h of chase normalized to 100%. This experiment was repeated two other times with similar results.

Possible mechanisms for how the V490M mutation reduces PHGDH activity can be proposed from our knowledge of this enzyme from other organisms. PHGDH from Escherichia coli is a 410-amino acid protein (29) whose three-dimensional structure has been solved (30). It is a homotetramer, with each monomer being made up of three distinct domains: a nucleotide-binding domain (residues 108–294), a substrate-binding domain (residues 7–107 and 295–336), and a regulatory domain that binds serine (residues 337–340). The main contact points between the subunits are at the level of the nucleotide-binding domains and the regulatory domains. Sequence alignments comparing the enzyme from E. coli to other organisms indicate that the highest homology is found among the various nucleotide- and substrate-binding domains, whereas the regulatory domains are less conserved (4, 21, 31). Indeed, the regulatory domains from most other organisms sequenced to date are ∼100–140 amino acids longer than that of the enzyme from E. coli (the human enzyme is 533 amino acids in length). The V490M mutation appears unlikely to influence the nucleotide- or substrate-binding sites of the human enzyme directly, due to its location 43 amino acids from the carboxyl terminus. However, in E. coli, serine acts as an allosteric inhibitor of PHGDH by binding to the carboxyl-terminal regulatory domain and decreasing the $V_{\text{max}}$ of the active site, some 30 Å distant (30, 32). A flexible Gly-Gly hinge region between the regulatory and substrate-binding domains has been proposed to undergo a conformational change that influences the enzyme $V_{\text{max}}$; there is experimental evidence to support this hypothesis (33–35). Although the rat and human enzymes are not regulated by serine (21), it is conceivable that the V490M mutation could cause a conformational change to decrease $V_{\text{max}}$ as was observed following in vitro translation (4). However, our results, obtained following in vivo expression of the enzymes, do not support this model. We found that the principal effect of the V490M mutation was to decrease its steady-state concentration in cells. With the possible caveat that we examined soluble cell lysates and not purified protein, our results also indicate that when the levels of immunoreactive wild-type and mutant PHGDH were normalized, the initial rate kinetics of the mutant enzyme were very similar to the wild-type enzyme. In addition, the active forms of both enzymes displayed similar stability to thermal denaturation, suggesting that the V490M PHGDH was not less stable than the wild-type enzyme once it achieved a mature conformation. Thus, by all criteria that we measured, the mature, functional forms of the two enzymes were very similar. The differences in the conclusions reached in this report and by other investigators (4) are probably due to differences in the expression systems utilized.

In vitro translations are performed at temperatures below 37 °C, which can result in more efficient protein folding, and they are designed

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4 S. Pind, unpublished results.
to prevent rapid proteolysis, which could lead to accumulation of misfolded, inactive proteins.

Rather than affecting the stability or the activity of the PHGDH, several lines of evidence suggest that the V490M mutation impairs the folding and/or assembly of the enzyme. Misfolding mutations are common and often result in degradation of the affected protein, reducing its steady-state concentration within the cell (for recent reviews, see Refs. 36–38). The regulatory domain of E. coli PHGDH forms an extensive interface (−1000 Å) for formation of the tetramers (30). Achouri et al. (21) have shown that removal of the carboxyl-terminal 209 amino acids of the rat enzyme lowers but does not abolish enzyme activity but does block the ability of enzyme dimers to form tetramers. The increased rate of degradation of the V490M mutant enzyme in the first hour following synthesis suggests that a lower percentage of the newly synthesized mutant enzyme achieved a “proteinase-resistant” or mature conformation, thus resulting in its degradation. This lower efficiency of folding results in the lower steady-state concentration that we noted in both the transfected cells and the fibroblasts. We also observed that the mutant PHGDH could not be recovered from the soluble fraction when expressed in bacteria under any conditions that were tried. In contrast, more than 75% of the wild-type enzyme could be recovered in the soluble fraction using this expression system (results not shown). Taken together, our results suggest that the V490M mutation lowers the efficiency of folding of the mutant enzyme into its mature, enzymatically active conformation but does not reduce the activity of any mutant enzyme that does fold correctly. Precedents for this type of mutation include the cystic fibrosis transmembrane conductance regulator, where the ΔF508 deletion prevents the folding of this mutant, but following purification and reconstitution in vitro the mutant CI−channel functions very similarly to the wild type (39). In addition, the R147W mutation of short-chain acyl-CoA dehydrogenase has been reported to inhibit folding of the enzyme but not affect the stability or the activity of that portion of the enzyme that does fold properly (37).

Clinical Implications—It is particularly striking that the neurodevelopmental phenotype of the PHGDH deficiency is so severe, given that the two known amino acid substitutions in that enzyme are relatively conservative and the mutant proteins characterized to date retain significant residual activity. The central nervous system appears to be particularly sensitive to the low concentration of serine that results from a deficiency in its production through the PHGDH pathway. The severity of the neurological and developmental impairments in children with this deficiency indicates that the production of serine through alternate pathways, such as proteolysis or through the glycine-cleavage complex and the reverse reaction of serine hydroxymethyltransferase, is not adequate to meet the requirements of the brain. Whether this is due to a lower capacity of the alternative pathways, low expression in this organ, or an inadequate supply of the glycine precursor is not known. In addition, although serine could be provided from other tissues in the body or from the diet, studies in rats have shown that it is transported across the blood-brain barrier relatively inefficiently (40). Serine transport by the neutral amino acid carrier is limited at normal plasma concentrations of amino acids, since the transporter prefers the larger, more hydrophobic amino acids (40–42).

The importance of, if not requirement for, serine in specific cell types in the central nervous system has been documented using several model systems. For example, neurons may be dependent upon an exogenous supply of serine. Serine promotes the morphological differentiation of chicken dorsal root ganglion neurons in vitro (43). Hippocampal neurons cultured in the absence of exogenous serine or glycine showed a greatly diminished capacity to synthesize phosphatidylserine and sphingolipids (44). Further studies have shown that serine is released from rat astrogial-rich cultures and is a trophic factor for the survival and growth of cultured neurons (45–47). These in vitro studies are supported by recent immunolocalization results, showing that PHGDH is not expressed in Purkinje neurons in the rat cerebellar cortex but is highly expressed in the Bergman glia, a native astroglia in this region (47). More extensive results have been reported for mouse brain, where PHGDH is expressed highest in the radial glia/astrocyte lineage and in the olfactory ensheathing glia (48).

Conclusions—We have characterized a common, panethonic mutation in PHGDH that reduces its activity in cells. In contrast to the work of others, we noted that the reduction of enzyme activity associated with the V490M mutation was due to an increased turnover of the mutant PHGDH, most likely as a consequence of impaired protein folding and/or assembly. These results provide a genetic and biochemical explanation for the origin of the disease in our probands and verify that the low levels of serine observed are the result of a deficiency of this enzyme. It remains to be determined how low serine concentrations impair function in the central nervous system. Possible mechanisms include global limitations on protein and/or lipid synthesis, insufficient levels of the neurotransmitters glycine and β-serine, and the absence of serine as a trophic factor for neuronal growth, migration, and survival. In addition, the roles of radial glial cells and excitatory amino acids in neuronal development (49, 50) may well be impaired in a state of serine insufficiency. While resolution of these possibilities requires more study, it remains important to identify patients with deficiencies in serine biosynthesis, since they may benefit from replacement therapy.

Acknowledgments—We are indebted to the family and the anonymous donors described in this paper. We thank Dr. Barbara Triggs-Raine for numerous helpful discussions; Glenn Palomaki for advice on statistics; and Orest Pilipowicz and Anjali Gandhi for technical assistance.

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