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Identification and Characterization of a Mutation, in the Human UDP-Galactose-4-Epimerase Gene, Associated with Generalized Epimerase-Deficiency Galactosemia

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Summary

Epimerase-deficiency galactosemia results from impairment of the human enzyme UDP-galactose-4-epimerase (hGALE). We and others have identified substitution mutations in the hGALE alleles of patients with the clinically mild, peripheral form of epimerase deficiency. We report here the first identification of an hGALE mutation in a patient with the clinically severe, generalized form of epimerase deficiency. The mutation, V94M, was found on both GALE alleles of this patient. This same mutation also was found in the homozygous state in two additional patients with generalized epimerase deficiency. The specific activity of the V94M-hGALE protein expressed in yeast was severely reduced with regard to UDP-galactose and partially reduced with regard to UDP-N-acetylgalactosamine. In contrast, two GALE-variant proteins associated with peripheral epimerase deficiency, L313M-hGALE and D103G-hGALE, demonstrated near-normal levels of activity with regard to both substrates, but a third allele, G90E-hGALE, demonstrated little, if any, detectable activity, despite near-normal abundance. G90E originally was identified in a heterozygous patient whose other allele remains uncharacterized. Thermal lability and protease-sensitivity studies demonstrated compromised stability in all of the partially active mutant enzymes.

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Introduction

Epimerase-deficiency galactosemia (MIM 230350) results from impairment of the human enzyme UDP-galactose-4-epimerase (hGALE; E.C.5.1.3.2), which normally catalyzes the third step in the Leloir pathway of galactose metabolism (Segal and Berry 1995), as well as the interconversion of UDP-N-acetylgalactosamine (UDP-GalNAc) and UDP-N-acetylglucosamine (UDP-GlcNAc) (Maley and Maley 1959; Piller et al. 1983; Kingsley et al. 1986*a*). Historically, two forms of this autosomal recessive disorder have been distinguished: the first is termed "peripheral," because patients exhibit enzyme deficiency that is restricted to the circulating blood cells, and the second is termed "generalized," because patients exhibit epimerase deficiency in all tissues tested. The peripheral form of epimerase deficiency is considered to be clinically benign (Segal and Berry 1995) and can be quite common in some ethnic groups (Alano et al. 1997). In contrast, only five patients with generalized epimerase deficiency have been reported; all have shown poor growth and learning difficulties, among other complications, despite a galactose-restricted diet (Walter et al., in press). Finally, a unique patient, identified by newborn screening, who remained clinically well in infancy, despite a normal diet, but who later demonstrated significant developmental delays and was shown to have only partial epimerase activity in his cells was described recently (Quimby et al. 1997; Alano et al. 1998). The molecular basis of the distinction between these various forms of epimerase deficiency remains unknown and is addressed by the experiments reported here.

The first clues to a potential explanation were revealed >20 years ago when Mitchell et al. (1975) reported the reversal of hGALE deficiency in cultures of leukocytes and lymphocytes derived from patients with the peripheral form of the disorder. These authors noted that stimulation of patient cells, with phytohemagglutinin, resulted in epimerase-activity levels close to those observed in controls. Transformation of the patient cells to create

long-term lymphoblast lines also resulted in essentially normal epimerase activity. A comparison of the properties of hGALE derived from patient lymphoblasts versus that derived from controls revealed similar Michaelis kinetic (Km) values and electrophoretic mobilities but increased heat sensitivity in the patient-derived samples. These authors concluded that either an increased rate of synthesis of a mutant enzyme or derepression of an additional epimerase locus in the cultured cells might account for their observations. The results reported in this article strongly favor the first possibility.

The human cDNA and gene encoding hGALE were cloned and sequenced recently by Daude et al. (1995) and by Maceratesi et al. (1998). The availability of these sequences has enabled the characterization, by us and others, of hGALE alleles derived from patients with epimerase-deficiency galactosemia. Seven such mutations—N34S, G90E, D103G, L183P, K257R, L313M, and G319E—all derived from patients with peripheral (Maceratesi et al. 1998) or near-peripheral epimerase-deficiency galactosemia (Quimby et al. 1997; Alano et al. 1998), have been reported to date.

We report here the first identification of an hGALE mutation, V94M, derived from a patient with the severe, generalized form of epimerase-deficiency galactosemia. Subsequent testing of samples from two additional generalized epimerase-deficiency patients, who are believed to be unrelated to the first proband, revealed that both patients also were homozygous for V94M. We used a yeast expression system for the human enzyme to characterize both this mutant allele and others derived from patients with peripheral epimerase deficiency, to address the question of whether there is a molecular/biochemical distinction between the hGALE alleles associated with the two reported forms of epimerase-deficiency galactosemia. Our results, combined with those reported elsewhere, suggest that some naturally occurring hGALE substitution mutations result in severe catalytic impairment, whereas others have little effect on enzyme function. The functionally mild mutations, however, result in partial enzyme destabilization that may cause GALE activity to fall below the threshold of detection in quiescent cells, such as those in the circulating blood, leading to the observed absence of GALE activity in these cells. The three patients with generalized epimerase-deficiency described here are the only individuals genotyped to date who each carry two mutant alleles of GALE that demonstrate severe catalytic impairment.

Patients, Material, and Methods

Patients

The proband originally studied was the product of a consanguineous marriage and has been described elsewhere (Holton et al. 1981; Henderson and Holton 1983; Walter et al., in press). She presented at age 5 d with symptoms of classic galactosemia, including vomiting, hypotonia, jaundice, galactosuria, and hepatomegaly. Enzyme activities of galactose-1-phosphate uridylyltransferase and galactokinase were found to be within the normal range. However, hGALE activity was deficient both in erythrocytes and in cultured skin fibroblasts. Clinical follow-up at age 19 mo confirmed that, even while on a galactose-restricted diet, the child remained hypotonic, with an enlarged spleen and developmental delay (Henderson and Holton 1983). Later in life she was noted to have nerve deafness and moderate learning difficulties, but there was no evidence of ovarian dysfunction (Holton 1990; Walter et al., in press). The other two patients tested (patients 4 and 5 in the report by Walter et al. [in press]) were first cousins from an extended consanguineous family not known to be related to the first patient. One of these two additional patients is the sibling of another generalized epimerase-deficiency patient, described elsewhere (Sardharwalla et al. 1988).

Reverse Transcription–PCR Amplification and Analysis of hGALE cDNA and Genomic DNA

Total RNA was isolated from cultured fibroblast cells from the proband and her parents, as described elsewhere (Fridovich-Keil et al. 1991). First-strand cDNA synthesis was performed by use of GIBCO Superscript II RNase H− reverse transcriptase, according to the manufacturer's recommendations. Then, the cDNA template was amplified by PCR with three sets of primers: HEPIMF2 (5′-GCCGGAATTCATAAAAATGGCAGAGAAGGTGCTGG-3′) and HEPIM5R (5′-CGATGAAGAACTTGGACTTGC-3′), HEPIM6F (5′-GGAACGTAGTGCTGCTGCGC-3′) and HEPIM11R2 (5′-CTGCTTTTCCTGGTCCTTGGTAG-3′), and HEPIM4F (5′-GGACCATCCAGCTTCTGG-3′) and HEPIM9R (5′-CCAGAGGCCTTCTCCATAGCC-3′). Independently amplified samples were sequenced directly by use of the Big Dye kit (Perkin Elmer), as recommended by the manufacturer. Genomic confirmation of the identified mutation was accomplished as described in Results, by use of primers HEPIMF3 (5′-GCGGCGGGTCCAGGAGCTGAC-3′) and HEPIMR6 (5′-TCCTCACCAATGCAGCCAGAGGC-3′).

Yeast Strains, Plasmids, and Expression Studies

All recombinant DNA manipulations were performed according to standard techniques (Sambrook et al. 1989) and by use of *Escherichia coli* strain XL1-Blue (Stratagene). The yeast strain used was yBBQ1 (MATa gal80 gal10-120 ura3-52 his3-200 ade2-101 lys2-801 tyr1-501) (Quimby et al. 1997). All mutant hGALE alleles

expressed in yeast were recreated from the wild-type sequence, by use of the Stratagene QuickChange site-directed mutagenesis kit, and the resultant clones were confirmed by dideoxy sequencing. All hGALE alleles to be transformed into yeast were subcloned into the low-copy-number expression plasmids pBQy1 and pBQy4 (Quimby et al. 1997). All yeast transformations, culture manipulations, and extract preparations were performed according to standard protocols, as described elsewhere (Fridovich-Keil 1995*a,* 1995*b;* Elsevier et al. 1996; Quimby et al. 1997).

GALE-Activity Assays

GALE activity was evaluated in yeast extracts by determination of the conversion of UDP-galactose (UDP-gal) to UDP-glucose (UDP-glc) or of UDP-GalNAc to UDP-GlcNAc. With regard to the first substrate, assays were performed essentially as described elsewhere (Quimby et al. 1997). In brief, each 25-μl reaction contained, except where otherwise noted, 5 μl premix (0.06 μM [0.1 μCi] ^{14}C-UDP-gal; Amersham CFB129, 2 mM [unlabeled] UDP-gal, 0.2 M glycine buffer [pH 8.7]), 5 μl 20-mM NAD, and 15 μl yeast extract diluted in lysis buffer (20 mM HEPES/KOH [pH 7.5], 1 mM DTT, and 0.3 mg BSA/ml). With regard to the second substrate, assays were performed as above, except that each 25-μl reaction contained 8.75 μl premix (209 μM [0.1 μCi] ^{14}C-UDP-GalNAc, 28.6 mM pyruvate, and 286 mM glycine [pH 8.7]), 5 μl 20-mM NAD, and 11.25 μl yeast extract diluted in lysis buffer. All reactions were incubated at 37°C for 30 min, were stopped by boiling for 10 min, and then were centrifuged in a microfuge on high speed for 10 min, at room temperature, to pellet insoluble material. Finally, 10 μl of each supernatant was spotted onto a prewashed, predried PEI-cellulose thin-layer chromatography (TLC) plate (Baker) and was dried in an incubator at 37°C for 30 min. The chromatogram was run in a single dimension, for 16–24 h, with a solvent of 1.5 mM $Na_2B_4O_7$, 5 mM H_3BO_3, and 25% ethylene glycol. Paper toweling was clipped at the top of each TLC plate, to extend the effective run time of each assay. After chromatography, each plate was removed from the solvent and air dried. Areas containing radioactive material were visualized and quantitated by use of a BioRad phosphorimager. Because a low level of background signal that did not increase with load and that was not visible as a spot was nonetheless detected, by the phosphorimager, in the "product" range of each negative control lane, this signal was subtracted from all other values, prior to calculation of the activity levels plotted.

Trypsin Cleavage Assay

Each reaction included 80 μg soluble yeast lysate and 40 ng trypsin (GIBCO 1 ×), incubated in a total volume of 100 μl at 30°C for 0, 2.5, 5, 10, 15, 20, 25, or 30 min. Reactions were stopped by boiling (5 min) in 1 × sample buffer (2% SDS, 10% sucrose, 62.5 mM Tris [pH 6.8], 15.5 mg DTT/ml, and bromophenol blue) and then were subjected to PAGE followed by western blot analysis with the anti-hGALE polyclonal antiserum EU69, as described below. Comparison of two independent analyses confirmed the reproducibility of the results obtained.

Western Blot Analysis

Western blot analyses were performed essentially as described elsewhere (Fridovich-Keil et al. 1995*b*); hGALE was detected by use of a rabbit polyclonal antiserum (EU69) raised against purified hexahistidine-tagged human epimerase protein. EU69 was used at a dilution of 1:40,000. To control for loading of lanes, a rabbit polyclonal antiserum generated against yeast cyclophilin A (Zydowsky et al. 1992) also was included, at a dilution of 1:30,000. The secondary antibody used was a donkey anti–rabbit Ig coupled to horseradish peroxidase (Amersham). Signals were visualized by use of the enhanced chemiluminescence (ECL) kit (Amersham) and were quantitated by use of a Molecular Dynamics Personal Densitometer SI scanning laser densitometer.

Results

Pedigree Analysis and Identification of the V94M Substitution

Direct sequencing of cDNA samples PCR amplified from the proband and both parents revealed a G→A transition at position 269 (downstream of the start codon), resulting in the predicted amino acid substitution of methionine for valine at residue 94 (V94M). The mutation was detected in the homozygous state in the child and in the heterozygous state in both parents, which is consistent with the consanguineous history of the family. Because this substitution fortuitously created a *Nco*I restriction site, it was easily confirmed in genomic DNA, as illustrated in figure 1. In brief, a 1.6-kb fragment that spanned the relevant region and included a second, constitutive *Nco*I site was PCR amplified from genomic DNA of the child, both parents, and two unrelated controls. *Nco*I digestion of the wild-type sequence, followed by agarose-gel electrophoresis, yielded bands at 1.3 kb and 0.3 kb (fig. 1, *unblackened arrows*), whereas digestion of the mutant sequence yielded bands at 0.8 kb and 0.5 kb (fig. 1, *blackened arrows*) and at 0.3 kb. Results

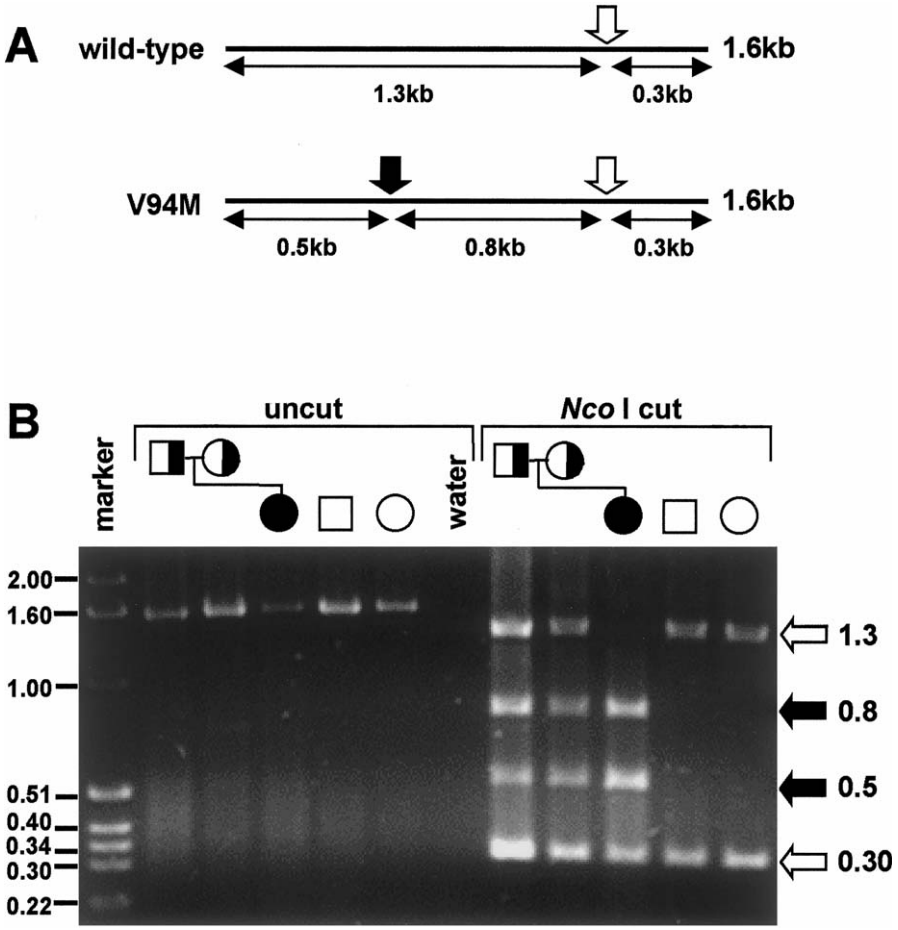

Figure 1 Confirmation of the G269A mutation in genomic DNA. *A*, Diagram indicating positions of constitutive (*unblackened arrows*) and mutation-specific (*blackened arrow*) *Nco*I restriction sites within a PCR-amplified fragment. *B*, Ethidium bromide–stained agarose gel demonstrating fragment sizes obtained by PCR amplification and *Nco*I digestion of genomic DNA from both parents, the patient, and two unrelated controls. Marker band sizes, in kilobases, are indicated to the left. Unblackened arrows to the right indicate wild-type bands, and blackened arrows to the right indicate mutation-specific bands.

of the genomic analysis were fully consistent with the cDNA-sequencing results.

Two additional samples of genomic DNA, representing two other patients with generalized epimerase deficiency (patients 4 and 5 in the report by Walter et al. [in press]), also were tested as described above (data not shown). Both demonstrated *Nco*I cleavage patterns that were indistinguishable from those of the original proband. Direct sequencing of PCR products, representing the appropriate regions from both of these patients, further confirmed the presence of the G269A mutation (encoding the V94M substitution) in the homozygous state in both patients (data not shown).

Expression of Wild-Type and Mutant Alleles of hGALE in the Homozygous State in Yeast

To probe the functional impact of V94M on hGALE, we recreated this substitution by site-directed mutagenesis of the otherwise wild-type sequence (see Patients, Material, and Methods) and expressed the encoded protein from a low-copy-number centromeric (CEN) plasmid in a null-background strain of yeast, yBBQ1 (Quimby et al. 1997). To facilitate a comparison of the biochemical properties of this mutant protein with those of others representing patients with peripheral epimerase deficiency, we also recreated and expressed three additional mutant alleles, G90E-, D103G-, and L313M-hGALE (Maceratesi et al. 1998).

As a first test of the ability of the mutant hGALE proteins to function in yeast, strains of yBBQ1 expressing each protein were assayed for growth on synthetic medium containing galactose as the sole carbon source. As illustrated in figure 2, cultures of cells expressing L313M-hGALE or D103G-hGALE grew indistinguishably from cells expressing wild-type hGALE. In contrast, cells expressing V94M-hGALE grew poorly, and cells

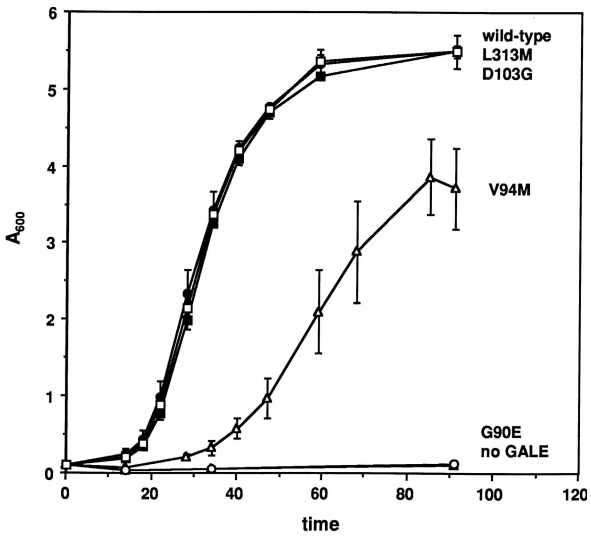

Figure 2 Growth curves of yBBQ1 yeast expressing the indicated alleles of hGALE cultured in medium containing galactose as the sole carbon source. Each point plotted represents the average ± SD of three cultures.

expressing G90E-hGALE did not grow any better than those from the negative control, which expressed no hGALE. All cultures grew well on medium containing glucose as the carbon source (data not shown).

As a further test of the functional capacity of the substituted hGALE proteins, crude lysates of cells expressing each protein were assayed, relative to positive and negative controls, for activity with respect to UDP-gal, as described in Patients, Material, and Methods. As illustrated in figure 3 (*top panel*), both D103G-hGALE and L313M-hGALE extracts demonstrated near-normal activity, whereas both G90E-hGALE and V94M-hGALE extracts demonstrated little, if any, detectable activity. These results were fully consistent with the growth curves described above. Western blot analyses of each extract, with a rabbit polyclonal antiserum against hGALE, further demonstrated that all the mutant proteins exhibited essentially normal levels of abundance. A second antiserum, which detected an endogenous yeast protein, cyclophilin, was included to control for loading of lanes. Estimates of activity, adjusted for small differences in abundance (fig. 3, *middle panel*), again indicated that both D103G-hGALE and L313M-hGALE remained largely active, whereas both G90E-hGALE and V94M-hGALE did not.

Impact of NAD on Mutant hGALE Proteins Expressed in Yeast

Previous studies of another patient allele, N34S-hGALE, which exhibited ~70% wild-type levels of activity under standard in vitro conditions (Quimby et al. 1997), demonstrated that activity was restored to wild-type levels when increased exogenous NAD was added. We therefore tested activity in duplicate extracts representing wild-type, D103G-hGALE, and L313M-hGALE proteins, under a range of exogenous NAD concentrations; all six samples demonstrated indistinguishable profiles as a function of NAD (data not shown). Therefore, excess exogenous NAD did not rescue full activity from these substituted hGALE proteins, indicating allele specificity of the N34S-hGALE result.

Activity of Wild-type and Mutant hGALE Proteins, with Respect to UDP-GalNAc

As an additional test of the functional capacity of each substituted hGALE enzyme, assays were performed by use of UDP-GalNAc as substrate. Extracts of cells expressing wild-type hGALE converted UDP-GalNAc to UDP-GlcNAc, at a rate of 4.83 nmoles/mg protein/min (table 1). As had been seen for the UDP-gal studies, extracts of cells expressing both D103G-hGALE and L313M-hGALE demonstrated near-normal activity,

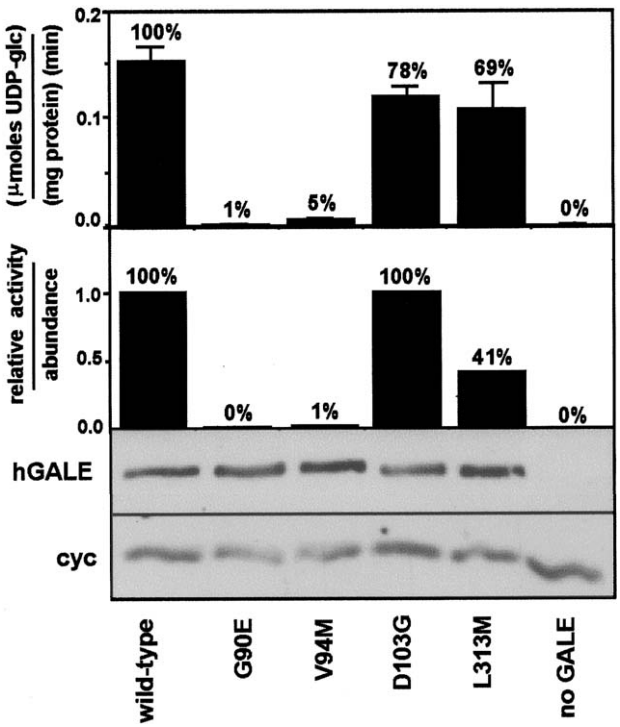

Figure 3 Activity and abundance of wild-type and mutant hGALE enzymes expressed in yeast. *Top,* Conversion of UDP-gal to UDP-glc detected in crude lysates of yeast expressing each of the indicated hGALE alleles in the homozygous state ($n = 3$). *Middle,* Average activity values from the top panel divided by average abundance (estimated by densitometric analysis of three independent western blots), normalized to wild-type. *Bottom,* Representative western blot analyses of lysates probed with antisera that detect hGALE and yeast cyclophilin (an endogenous control for loading).

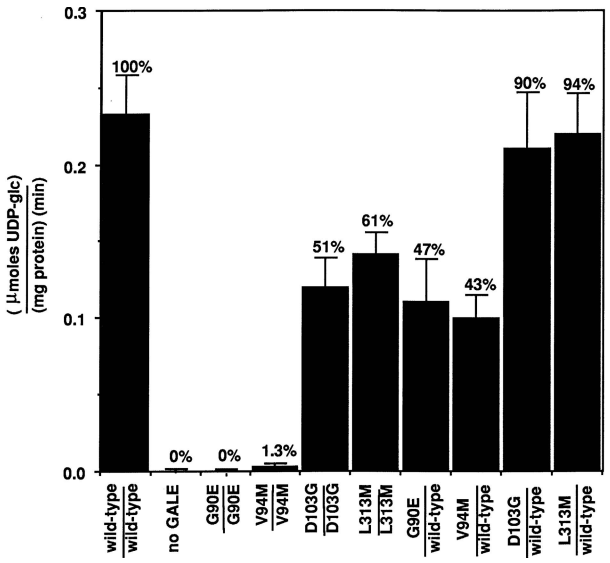

Figure 4 Conversion of UDP-gal to UDP-glc detected in crude lysates of yeast expressing each of the indicated combinations of hGALE alleles ($n = 3$).

whereas extracts of cells expressing G90E-hGALE exhibited no activity. In contrast, however, extracts of cells expressing V94M-hGALE, which exhibited only 5% activity with respect to UDP-gal, exhibited 24% activity when challenged with UDP-GalNAc. Clearly, not all mutations impacted both activities equally.

Expression of Mutant Alleles of hGALE in the Heterozygous State in Yeast

In an effort to uncover potential allelic interactions, such as those reported elsewhere (Quimby et al. 1997), each hGALE mutant allele characterized above also was expressed in yeast in the heterozygous state, together with the wild-type sequence. As illustrated in figure 4, extracts both of D103G-hGALE/wild-type cells and of L313M-hGALE/wild-type cells demonstrated near-normal levels of activity (90% and 94% wild type, respectively), whereas extracts both of G90E-hGALE/wild-type cells and of V94M-hGALE/wild-type cells demonstrated approximately half-normal levels of activity (47% and 43% wild type, respectively). Therefore, no clear evidence of either partial dominant negative or partial dominant positive interaction involving these alleles was observed.

Studies of Enzyme Stability

We addressed the question of hGALE enzyme stability by using two approaches: the first measured wild-type and mutant enzyme activities as a function of temperature, and the second measured wild-type and mutant protein sensitivities to trypsin digestion as a function of time. As illustrated in figure 5, extracts of cells expressing both D103G-hGALE and L313M-hGALE were markedly more sensitive than wild-type cells to temperatures >50°C. Indeed, at 53°C both mutant enzymes were almost completely inactive, whereas the wild-type enzyme still demonstrated near-maximal activity. With regard to trypsin digestion, one mutant protein (L313M-hGALE) exhibited essentially wild-type resistance, one (V94M-hGALE) exhibited more than wild-type resistance, and two (G90E-hGALE and D103G-hGALE) exhibited markedly compromised resistance (figure 6). Although neither test mimics the actual conditions in a circulating red blood cell, these data together suggest that the proteins encoded by all the tested patient-derived hGALE alleles were either severely impaired (e.g., V94M and G90E) or, by at least some measure, less stable than the wild-type protein.

Discussion

The results reported here demonstrate two major points. First, structural substitution mutations in hGALE can associate with generalized epimerase-deficiency galactosemia as well as with the peripheral form of the disease. This observation is in contrast with the suggestion of Maceratesi et al. (1998), who identified five substitution mutations in the DNA of patients with peripheral epimerase deficiency and who hypothesized that mutations outside the coding region of hGALE that impact gene expression might explain the difference between the peripheral and generalized forms of the disease. Although as-yet-unidentified regulatory mutations

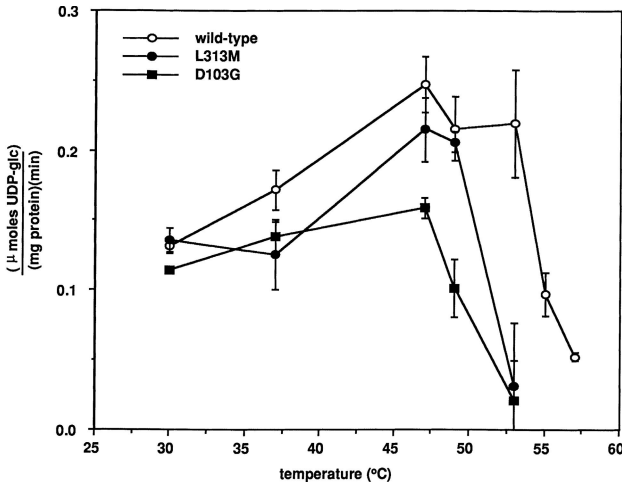

Figure 5 Impact of temperature on activity of wild-type and mutant hGALE enzymes. Assays were performed on crude lysates of yeast expressing each of the hGALE alleles indicated. Values plotted represent averages ± SD ($n = 3$).

Table 1

Activity of Wild-Type and Substituted hGALE Proteins, with Respect to UDP-GalNAc

hGALE Allele	Activity Level (%)[a] [nmoles UDP-GlcNAc/ mg protein/min]
Wild-type	4.83 ± .24 (100)
G90E	.00 ± .05 (0)
V94M	1.14 ± .12 (24)
D103G	3.36 ± .12 (70)
L313M	5.63 ± .56 (117)
No GALE	.00 ± .02 (0)

[a] Values are average ± SD ($n = 3$).

may exist in some patients, the data reported here demonstrate that regulatory mutations are not required to explain the distinction between the two recognized forms of epimerase-deficiency galactosemia.

The second major point reported here concerns the "other" epimerase reaction normally catalyzed by hGALE, namely, the interconversion of UDP-GalNAc and UDP-GlcNAc. In view of the key roles played by these compounds in the assembly of complex polysaccharides and other glycosylated macromolecules, especially in the brain, the impact of patient mutations on this reaction may be as, if not more, relevant for the pathogenesis of epimerase-deficiency galactosemia as is the interruption of the Leloir pathway. Our results (fig. 3 and table 1) clearly demonstrate that some patient mutations (e.g., G90E and D103G) disrupt hGALE activity equally, with respect to both substrates, whereas others do not. For example, in our study, V94M-hGALE exhibited only 5% activity with respect to UDP-gal but 24% activity with respect to UDP-GalNAc. Interestingly, in studies of fibroblasts from the original proband, Kingsley et al. (1986*b*) detected 1.8% wild-type activity by using UDP-gal as substrate and 11.6% activity by using UDP-GalNAc as substrate. The clinical outcome for this patient might have been even more severe if she had not retained this significant level of activity with respect to UDP-GalNAc. Although all the substituted proteins tested here exhibited activity toward UDP-GalNAc that was equal to or greater than the activity that they exhibited toward UDP-gal, it seems reasonable to assume that this may not be a general rule. Other naturally occurring patient alleles may encode substituted proteins that function well in the Leloir pathway but that are impaired in their interconversion of UDP-GalNAc and UDP-GlcNAc. Since many clinical labs currently test only the conversion of UDP-gal to UDP-glc, these patients might not be detected.

Allelic Interaction

Previously, we studied a compound heterozygous patient with epimerase deficiency whose alleles demonstrated partial dominant negative interactions both with wild-type hGALE and with each other (Quimby et al. 1997). We saw no evidence of such allelic interaction with the four alleles in this study, suggesting that these interactions are allele specific and may represent the exception rather than the rule for naturally occurring mutations in hGALE.

Structural Studies

In an effort to understand the structural distinction between hGALE substitutions resulting in severe versus mild enzymatic impairment, we homology mapped each of the mutations studied to the *E. coli* GALE (eGALE) crystal structure (coordinate set 1KVQ, Brookhaven Protein Data Bank [Thoden et al. 1997]). Our results suggested potential explanations for at least some of the experimental data obtained. For example, hGALE residue V94 corresponds with eGALE residue V86, which is predicted to lie in a loop near the core of the enzyme, ~5 Å from the bound-substrate sugar ring. Although substitution of methionine for valine at that position caused no obvious steric problems, the longer side chain on the methionine may disrupt either interactions with the substrate or the local protein structure needed for efficient catalysis. hGALE residue G90 corresponds to eGALE residue G82, which forms part of a β-strand, near the core of the protein, that is surrounded by NAD on three sides. The substitution of a glutamic acid for a neutral glycine would put a negative charge in the middle of the protein, perhaps disrupting the structural integrity of the protein, if not it's ability to interact with NAD. hGALE residue D103 corresponds to eGALE E95, which is predicted to make a salt bridge with lysine 92 (K92).

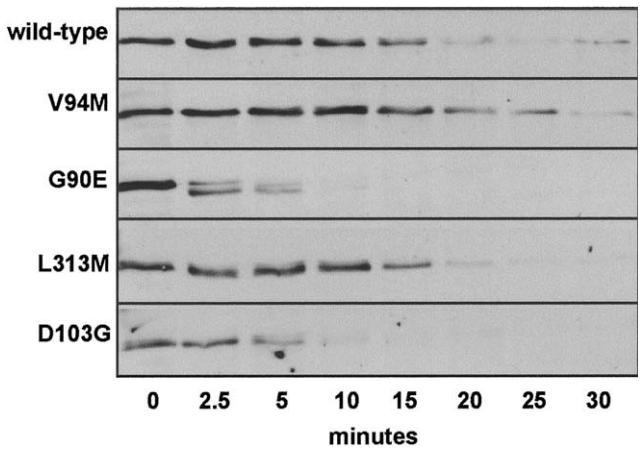

Figure 6 Trypsin cleavage of wild-type and mutant hGALE proteins. Yeast lysates prepared from cells expressing the indicated alleles of hGALE were incubated with trypsin for the times indicated and then were subjected to western blot analysis as described in Patients, Material, and Methods.

Since the hGALE residue corresponding to eGALE K92 is also a lysine (K100), one might predict that aspartic acid 103 in hGALE also normally participates in a salt bridge; clearly, substitution of a glycine (D103G) for the natural acidic residue would be predicted to disrupt this interaction. Finally, hGALE residue L313 corresponds to eGALE residue K305, which is predicted to lie in a short α-helix near the outside surface of the protein. The structural impact of the substitution of a methionine at this position in the human protein remains unclear.

Genotypes and Phenotypes

The data reported here demonstrate that all three patients with generalized epimerase deficiency who were tested are homozygotes for the same mutation, V94M, despite the fact that they currently are believed to originate from two unrelated families. Furthermore, two of these three are the siblings of the only other two patients with generalized epimerase deficiency reported to date (Walter et al., in press). By extrapolation, we therefore can conclude that all five reported patients with generalized epimerase-deficiency galactosemia must be homozygotes for the same mutation. Whether future cases of unrelated patients with this disorder also will carry V94M remains to be seen.

Perhaps the two most clinically relevant questions raised by this study, as well as by our previous report (Quimby et al. 1997), are (1) whether epimerase-deficiency galactosemia is clinically a binary disorder or a continuum and (2) whether a genotype-phenotype pattern is emerging. In view of the small number of patients genotyped and the lack of long-term follow-up data for the overwhelming majority of patients currently categorized as having the peripheral form of the disorder, these questions cannot be answered at present. However, even from the small number of patient alleles now biochemically characterized, it is clear that some mutations appear to be mild and others severe and that these mutations do not assort neatly along clinical lines. The most severe allele modeled to date is G90E, which was identified originally in a heterozygous patient ostensibly with peripheral epimerase deficiency (Maceratesi et al. 1998). We can only hypothesize that the other allele in this patient must retain significant activity. Since most patients with peripheral epimerase deficiency either are never diagnosed or are lost to follow-up very early in life, we also cannot rule out the possibility that even these patients may not be truly asymptomatic. For example, at least some of these patients may be at increased risk for cataracts (Endres and Shin 1990; Jakobs et al. 1990; Schulpis et al. 1993). Furthermore, the possibility of other later-onset complications is reinforced by the observation that clinical symptoms were not apparent in the ostensibly peripheral compound heterozygous patient, reported elsewhere (Quimby et al. 1997; Alano et al. 1998), until he was >1 year old. Clearly, further studies will be required, to reveal the true genetic, biochemical, and clinical spectrum of epimerase-deficiency galactosemia.

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Electronic-Database Information

Accession number and URL for data in this article are as follows:

Brookhaven Protein Data Bank, http://www.pdb.bnl.gov
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for epimerase-deficiency galactosemia [MIM 230350])

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