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Functional characterization of the K257R and G319E-hGALE alleles found in patients with ostensibly peripheral epimerase deficiency galactosemia

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Abstract

Epimerase deficiency galactosemia is an autosomal recessive condition resulting from the impairment of UDP-galactose 4′-epimerase (hGALE). Although a small number of clinically severe patients have been reported who exhibit "generalized" GALE deficiency, the vast majority exhibit an apparently benign "peripheral" form of the disorder in which enzyme impairment is restricted to the circulating red and white blood cells. Previously, preliminary data were reported suggesting that GALE deficiency is 10-fold more common among African-Americans than among non-African-Americans, and that two missense mutations, K257R and G319E, are found in at least some of these patients. We report here functional studies of these alleles involving expression of the substituted human enzymes in a null-background strain of yeast. Although under normal assay conditions both substituted proteins demonstrate enzyme activities indistinguishable from the wild-type, one (G319E) demonstrates mild impairment under conditions of substrate limitation. No impairments are evident under conditions of cofactor (NAD) limitation. These results are consistent with the apparently benign status of peripheral epimerase deficiency galactosemia, but leave open the question of why patients with these substitutions demonstrate GALE deficiency in their red blood cells. While the possibility remains that K257R and G319E may cause tissue-specific impairments not recapitulated in vitro or in yeast, an equally if not more plausible explanation suggested by interspecies sequence alignments is that both substitutions may be polymorphisms that exist in linkage disequilibrium with other, as yet unidentified causal mutations.

Keywords: Galactosemia; GALE; UDP-galactose 4′-epimerase; Yeast; K257R; G319E

Introduction

Human UDP-galactose 4′-epimerase (hGALE; E.C.5.1.3.2) catalyzes the third step of the Leloir pathway of galactose metabolism (Fig. 1), as well as the interconversion of UDP-*N*-acetylgalactosamine (UDP-GalNAc) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) [1–3]. Impairment of this enzyme results in a variant form of galactosemia known as epimerase deficiency galactosemia (MIM 230350).

Historically, patients with epimerase deficiency galactosemia have been subdivided into two groups according to the extent of their enzyme impairment [1]. The predominant group has included clinically well individuals with GALE deficiency restricted to their circulating red and white blood cells. These patients were said to have a

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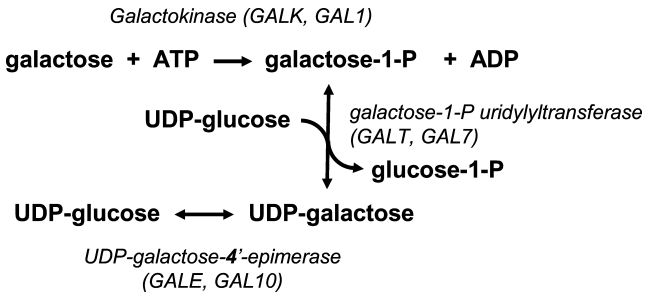

Fig. 1. The Leloir pathway of galactose metabolism.

peripheral, ostensibly benign form of the disorder [4]. In contrast, a small number of clinically severe patients were also reported with GALE deficiency documented in tissues beyond their circulating blood; these patients were said to have a generalized form of the disorder [5]. Finally, what appears to be an intermediate form of GALE deficiency has also been reported [6–8], including the case of one child who demonstrated intermediate levels of hGALE impairment in non-peripheral cells, and a delayed onset of symptoms [6,7]. Unfortunately, due to limited screening and the loss of most clinically well infants to follow-up, the majority of intermediate cases are likely to go undiagnosed and therefore untreated.

In the past 10 years, both the cDNA and gene encoding human GALE have been cloned and sequenced [9,10], enabling the identification of candidate mutations in patient alleles. Indeed, a large and growing number of candidate mutations have now been identified in hGALE patient alleles [6,7,10–13], offering the possibility that allelic heterogeneity may underlie some if not much of the observed biochemical and phenotypic heterogeneity. As with other disease genes, however, the relationship between candidate mutations and biochemical or patient outcome can be complex, especially for missense or non-coding changes. As an approach to address this issue with regard to missense mutations in hGALE, we previously developed a null-background yeast expression system for the human enzyme [7]. Using this system, we have demonstrated that different patient mutations can exhibit dramatically different impacts on hGALE function, both in vitro [7,12] and in vivo [14].

We report here functional studies of two previously reported but functionally uncharacterized hGALE substitutions: K257R and G319E. Earlier work [10,15] reported the identification of both of these mutations in African-American patients with ostensibly peripheral GALE deficiency. Here, we have performed both in vitro and in vivo studies of these hGALE alleles expressed in yeast, and found little detectable impairment relative to the wild-type enzyme. While the possibility remains that these substitutions may exhibit some tissue-specific impairment in human blood that is not recapitulated in vitro or in yeast, the data reported here, combined with the results of interspecies sequence comparisons, suggest an alternative explanation—namely that one or both substitutions may be polymorphisms that exist in linkage disequilibrium with other, as yet unidentified causal mutations.

Materials and methods

Expression of K257R and G319E-hGALE enzymes in yeast

To enable expression of the K257R and G319E-hGALE alleles in a null-background strain of yeast, both were recreated from the wild-type sequence by site directed mutagenesis as described elsewhere [12], and confirmed by DNA sequencing of the entire allele. Each wild-type or substituted hGALE allele was then subcloned into each of the low-copy number (*CEN*) expression plasmids pBQy1 and pBQy4, both of which have been described previously [7]. The resulting plasmid constructs, together with the appropriate negative controls (no hGALE), are listed in Table 1. Finally, pairs of plasmids representing the desired hGALE allele combinations were cotransfected into JFy3835, a null-background haploid strain of *Saccharomyces cerevisiae* derived from W303 (MATa ade 2–1 his 3–11,15 leu 2–3,112 ura 3–1 trp1–1 can 1–100 RAD 5+, the kind gift of Rodney Rothstein, Columbia University, New York, NY) by sequential one-step disruption of each of the *gal10* and *gal80* loci [16]. The resulting yeast strains are listed in Table 2. As a control, each plasmid was introduced and confirmed in single-transfectants prior to the cotransfection procedure.

For analyses of the proteins expressed, cultures were grown to an OD_{600} of about one in synthetic glycerol/

Table 1
Plasmids used in this study

Strain code	Plasmid backbone (comments)	hGALE allele
JF1183	BQy1 (CEN, *HIS4*)	None
JF1607	BQy1.hGALE (CEN, *HIS4*)	Wild-type
JF3395	BQy1.hGALE.K257R (CEN, *HIS4*)	K257R
JF2703	BQy1.hGALE.G319E (CEN, *HIS4*)	G319E
JF1725	BQy4 (CEN, *URA3*)	None
JF1800	BQy4.hGALE (CEN, *URA3*)	Wild-type
JF3385	BQy4.hGALE.K257R (CEN, *URA3*)	K257R
JF3348	BQy4.hGALE.G319E (CEN, *URA3*)	G319E

Table 2
Yeast strains used in this study

Strain code(s)	Plasmids	hGALE alleles
JFy4331, 4332, 4333	JF1183, JF1725	None
JFy4711, 4712, 4714	JF1607, JF1800	Wild-type/wild-type
JFy4322, 4323, 4324	JF3395, JF3385	K257R/K257R
JFy4325, 4326, 4327	JF2703, JF3348	G319E/G319E
JFy4628, 4629, 4630	JF3395, JF3348	K257R/G319E
JFy4328, 4329, 4630	JF2703, JF3385	G319E/K257R

ethanol medium lacking both uracil and histidine, then harvested by centrifugation, washed once with sterile water, and lysed at 4 °C by vigorous vortex agitation with acid washed 0.5 mm glass beads in the presence of lysis buffer (see below) plus protease inhibitors (0.625 μg/ml pepstatin A, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1.25 μg/ml antipain, 0.44 μg/ml phosphoramidon, 2.88 μg/ml *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane, 0.2 μg/ml chymostatin, and 10 nM phenylmethanesulfonyl fluoride, all from Sigma). Lysis buffers used were 20 mM Hepes/KOH [pH 7.5], 1 mM DTT, and 0.3 mg BSA/ml for UDP-Gal assays and some UDP-GalNAc assays, and 100 mM glycine [pH 8.7] for other UDP-GalNAc assays. Finally, lysates were clarified by centrifugation for 10 min at 4 °C in an Eppendorf microfuge at high speed (14000 rpm), after which each supernatant was transferred to a fresh tube and assessed for protein concentration using the Bio-Rad protein reagent, as recommended by the manufacturer with BSA as a standard. Samples were stored at −85 °C until use.

Western blot analysis

Western blot analyses were performed essentially as described elsewhere [17]. hGALE was detected using a 1:80,000 dilution of rabbit polyclonal antiserum (EU69) raised against purified hexahistidine-tagged human epimerase protein. As a control for loading, a second antiserum directed against endogenous yeast cyclophilin A [18] was also included at a dilution of 1:120,000. Signals were visualized by enhanced chemiluminescence (ECL, Amersham–Pharmacia Biotech), using a 1:5000 dilution of a horseradish peroxidase conjugated secondary antibody directed against rabbit Ig (Amersham–Pharmacia Biotech), as recommended by the manufacturer.

hGALE activity assays using soluble yeast lysates

GALE activity was evaluated in yeast extracts by monitoring the conversion of UDP-galactose (UDP-Gal) to UDP-glucose (UDP-Glc), and the conversion of UDP-*N*-acetylgalactosamine (UDP-GalNAc) to UDP-*N*-acetylglucosamine (UDP-GlcNAc). Except where otherwise noted, each 12.5 μl reaction contained 2.5 μl premix (2 mM UDP-Gal or UDP-GalNAc, and 0.2 M glycine buffer [pH 8.7]), 2.5 μl 20 mM NAD, and 7.5 μl yeast extract diluted in lysis buffer. To avoid the potential confusion of endogenous metabolites with substrates or products from the reactions, extracts were passed over Bio-spin 30 columns (Bio-Rad) prior to analysis to remove all small metabolites. All reactions were incubated at 37 °C for 30 min, then stopped by the addition of 35 μl ice-cold 100% methanol, vortexed at high speed, and centrifuged at 4 °C in a microfuge at high speed for 10 min to pellet insolubles. Each supernatant was transferred to a clean tube and dried under vacuum. Pellets were resuspended in either 250 μl distilled water (for UDP-Gal assays) or 750 μl distilled water (for UDP-GalNAc assays) and filtered through 0.2 μm nylon micro-spin columns (Alltech, #2494). Finally, substrates (UDP-Gal or UDP-GalNAc) and products (UDP-Glc and UDP-GlcNAc) were separated and quantitated using a DX600 HPLC system (Dionex) as described elsewhere [19].

K_m values for the wild-type, K257R, and G319E-hGALE proteins were calculated from the results of multiple assays performed under conditions of variable substrate (UDP-Gal) concentration. The concentrations tested ranged from 0.1 to 2.0 mM UDP-Gal. Data were fit to the Michaelis–Menton equation using a least squares regression, and respective K_m values calculated using the ORIGINS 7 program (OriginLab, Northampton, MA). A minimum of nine points was used for each analysis, and each point represented the average of at least three separate assays.

The cofactor dependence of each protein was also monitored, as described above, by varying the concentration of NAD added to each reaction, holding the UDP-Gal substrate level constant at 0.4 mM. The concentrations of NAD tested ranged from 0 to 4 mM NAD. As above, data were analyzed using the ORIGINS 7 program.

Galactose sensitivity of yeast expressing wild-type vs. substituted hGALE

Growth curves were performed using a micro-plate reader (Bio-Tek Instruments; Model EL808) with a 600 nm filter. The cover of each 96-well plate (NUNC) was treated with Viz scuba mask defog (S & S Fun Stuff, Atlanta, GA) prior to incubation to prevent condensation. Just prior to the initiation of each growth curve, log-phase yeast were diluted into the appropriate medium (100 μl/well) at a starting $OD_{600} = 0.05$. Plates were incubated at 30 °C with constant shaking for the indicated length of time, with OD_{600} measurements read on each well every 30 min.

Results and discussion

Expression of the K257R and G319E-hGALE enzymes in a null-background strain of S. cerevisiae

As a direct approach to test the impact of the K257R and G319E substitutions on hGALE function, we recreated each substitution within the context of an otherwise wild-type hGALE sequence (Table 1, see Materials and methods), and expressed each in JFy3835, a null-background strain of yeast (Table 2). To reflect the patient population more accurately, we generated strains of yeast each coexpressing two alleles of hGALE, so that

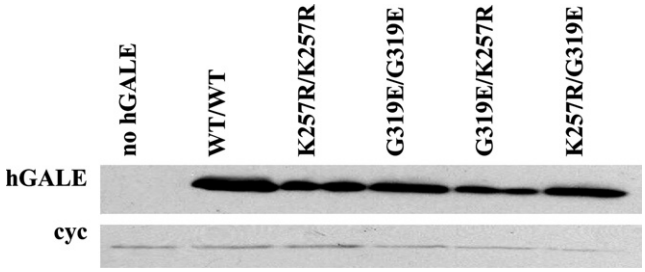

Fig. 2. Expression of wild-type and substituted alleles of human GALE in *S. cerevisiae*. Soluble lysates from yeast expressing the indicated alleles of human GALE were subjected to Western blot analysis as described under Materials and methods. Abundance of the endogenous yeast protein cyclophilin was monitored in each lane as a control for loading.

both the homozygous and compound-heterozygous states could be modeled. As demonstrated by Western blot analysis of soluble lysates from these strains (Fig. 2), both the K257R and G319E substituted hGALE proteins were expressed at levels indistinguishable from that of the wild-type protein. Staining the filter with an antiserum directed against an endogenous yeast protein, cyclophilin [18], provided a convenient internal control for the loading of lanes in this experiment. Although the results of a single Western blot analysis are presented, this procedure was repeated at least three times on independent samples with indistinguishable results.

Impact of the K257R and G319E substitutions on activity and stability of hGALE

To test the impact of the K257R and G319E substitutions on hGALE activity, soluble lysates from yeast expressing each of the allele combinations presented in Fig. 2 were also subjected to standard hGALE activity assays, first using UDP-Gal as the substrate, and then using UDP-GalNAc as the substrate (see Materials and methods). As illustrated in Fig. 3A, no loss of activity with regard to UDP-Gal was detected for any of the alleles or allele combinations tested under normal assay conditions. Indeed, one substituted allele (G319E) in the homozygous state appeared slightly more active than did wild-type hGALE (Fig. 3A). Similar results were obtained with regard to UDP-GalNAc (data not shown).

To explore the possibility of a subtle or cryptic impact on hGALE function, activity assays were repeated using sub-optimal concentrations of either substrate or cofactor (NAD). Such manipulations have revealed clear impairments in other GALE alleles (e.g., N34S-hGALE [7] and V94M-hGALE [14]). Although these studies did identify a mild K_m increase for the G319E-hGALE protein (Fig. 3B), no impairment was detected for the K257R protein. Furthermore, parallel studies performed under conditions of limiting NAD detected no differences for any of the three proteins tested (data not shown). Although these assays were performed using soluble cell lysates rather than purified proteins, it is reassuring to see that the K_m value calculated for wild-type hGALE here (0.12 ± 0.04 mM UDP-Gal) is remarkably similar to that reported previously (0.15 ± 0.02 mM UDP-Gal) from studies of the purified protein [14]. While it is tempting to speculate that the altered K_m of G319E-hGALE may result in impaired activity in vivo, where substrate concentrations may be limiting, this K_m change cannot account for the RBC hGALE impairment reported for these patients, as those activities were measured in vitro under conditions of excess substrate.

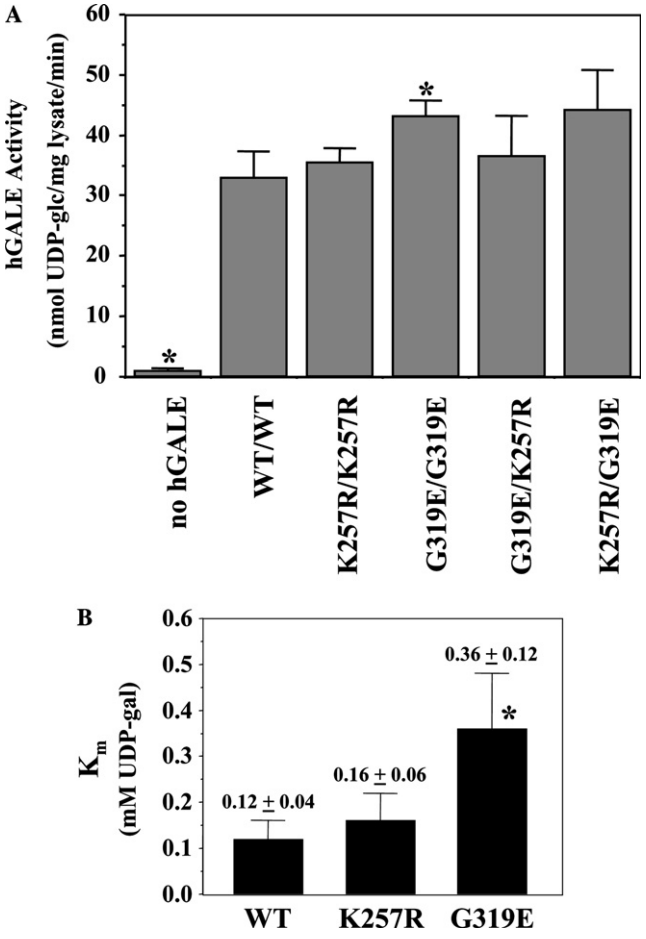

Fig. 3. Activity of the K257R and G319E human GALE proteins with regard to UDP-Gal. (A) Soluble lysates of yeast expressing the indicated alleles of hGALE were subjected to standard activity assays as described under Materials and methods. Values plotted represent averages ± SD of three independent assays. Bars that are statistically distinct from the wild-type value are indicated with an asterisk (*). (B) K_m values calculated for the wild-type, K257R, and G319E hGALE proteins expressed in yeast (see Materials and methods). The asterisk (*) indicates statistical significance.

Finally, we explored the possibility that one or both substituted hGALEs might be less stable than the wild-type protein by challenging each with partial trypsin digestion and/or activity assay at elevated temperatures. Again, each of these manipulations has revealed clear if

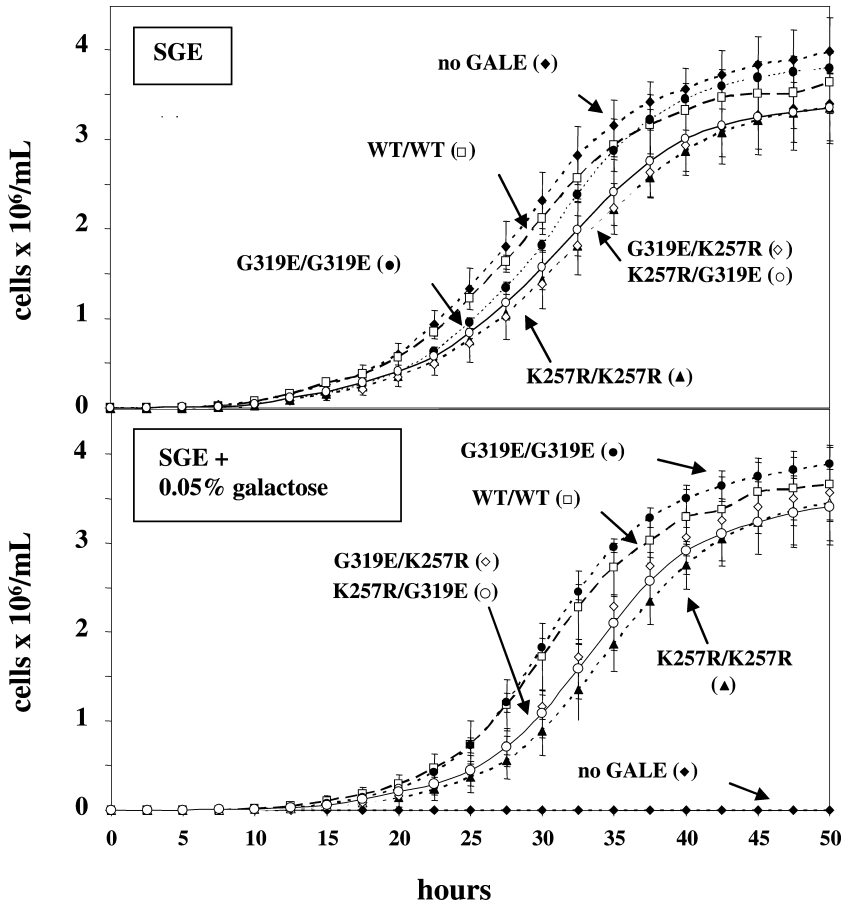

Fig. 4. Function of the K257R and G319E human GALE enzymes in vivo. As an in vivo test of the functional capacity of K257R and G319E hGALE, yeast cultures expressing these substituted proteins as their only GALE were monitored for growth in the presence and absence of galactose, as described under Materials and methods.

subtle impairments in other patient alleles [12,14]. Nonetheless, by every manipulation tested, both the K257R and G319E-hGALE proteins appeared to be at least as stable, if not more stable, than wild-type hGALE (data not shown).

Sensitivity of yeast expressing K257R or G319E-hGALE enzymes to galactose

As a final approach to test the possibility of functional impairment of K257R or G319E-hGALE, we investigated the growth rates of yeast expressing these alleles in the presence of low levels of galactose. Previously, we have demonstrated that yeast expressing impaired alleles of hGALE do not grow as well as their wild-type counterparts in medium containing even very low levels of galactose (e.g., 0.05%) despite the presence of other carbon sources (e.g., glycerol and ethanol) that can be fully metabolized [14]. As illustrated in Fig. 4, this was not the case for yeast expressing either K257R, G319E, or both alleles of hGALE. Indeed, all strains tested, except for the negative control, which expressed no hGALE, grew indistinguishably in both the absence and the presence of galactose (Fig. 4).

Combined, these data suggest that both K257R and G319E hGALE are nearly, if not fully functional enzymes, leading to the hypothesis that one or both substitutions may be neutral polymorphisms. Consistent with this conclusion, inspection of the hGALE X-ray crystal structure [20] further suggests that neither substitution impacts regions of the protein that are close to the active site or to the subunit interface (Fig. 5). Considering that both mutations have been identified predominantly if not exclusively in African-American patients [10,15], it is tempting to speculate that both sequence variations may be of African origin, although this point remains to be confirmed.

While the formal possibility remains that one or both substitutions may result in some form of RBC tissue-specific impairment not recapitulated in vitro or in yeast, the fact that interspecies sequence alignments (Table 3) demonstrate both positions to be tolerant of change, with R rather than K appearing as the normal position 257 residue in at least two of the species queried, argues against this possibility. The most likely conclusion, therefore, may be that K257R is a neutral polymorphism, and G319E an almost neutral polymorphism, both existing in the human population in

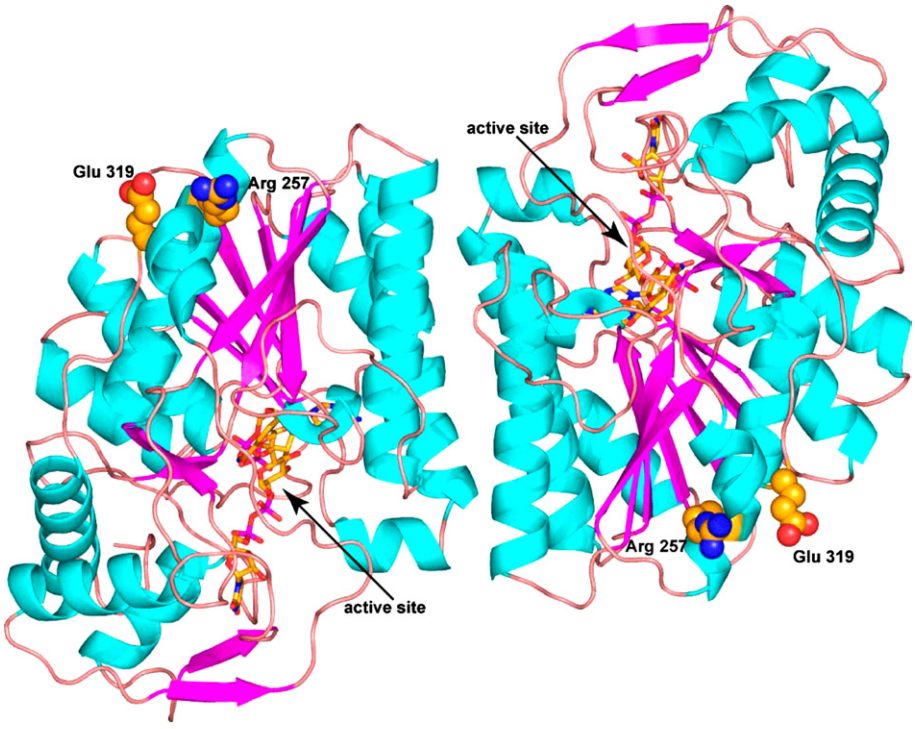

Fig. 5. The K257R and G319E substitutions in human GALE. The K257R and G319E substitutions were modeled against the wild-type human GALE structure (PDB coordinates 1EK6) using the graphics program TURBO [21,22]. Helixes are displayed in aqua, strands of sheet in magenta, and regions of random coil in salmon. Both the NAD and UDP-glucose ligands are depicted in stick representations, and the two sites of substitution are shown in a space filling representation. The figure was produced with PyMOL (http://www.pymol.org [23]).

Table 3
Sequence variability at residues corresponding to K257 and G319 in hGALE

Accession No.	Species	Sequence around residue 297	Sequence around residue 319
Q14376	*Homo sapiens*	IAALRKLKEQC	AQEELGWTAAL
Q8R059	*Mus musculus*	IAALKKLKEQC	AHEELGWTAAL
P18645	*Rattus norvegicus*	IAALKKLKEQC	AHEELGWTAAL
CK461833*	*Sus scrofa*	IAALRKLKEQC	ALKELGWTAAL
Q9CNY5	*Pasteurella multocida*	LKALDRHEGDA	AKTELNWTAAR
P24325	*Haemophilus influenzae*	LKALQRHENDA	AAKELGWVAER
P35673	*Erwinia amylovora*	LKALDHLSAIE	ADKELNWRVSR
Q57301	*Yersinia enterocolitica*	LSTLINLT–S	AHLELGWYAKR
P09147	*Escherichia coli*	VVAMEKLANKP	ADRELNWRVTR
P04397	*Saccharomyces cerevisiae*	IAALQYLEAYN	AKRELKWQTEL
Q7YZA8	*Trypanosoma cruzi*	ILALDYLAGLD	AKKALGWELKY

All sequence alignments were performed using the NCBI BLAST program [24] using tools at expasy.org. Residues corresponding to positions 257 and 319 in hGALE are shaded. Accession numbers are SwisProt/TrEMBL.

* The EST number (for *Sus scrofa*) is from NCBI.

linkage disequilibrium with other, as yet unidentified causal mutations. Considering that the alleles in which these substitutions were identified were sequenced predominantly with regard to their exons and open reading frames, non-coding variations could easily have been overlooked. Indeed, given the apparent tissue-specificity of the enzyme impairment observed in patients with peripheral GALE deficiency, regulatory rather than structural GALE mutations may be the more likely. Future studies will be directed at identifying and characterizing these predicted mutations.

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enzymes, thereby revealing the K_m alteration in the G319E protein. This work was supported by award DK46403 from the National Institutes of Health (to J.L.F.K.).

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