Determinants of Function and Substrate Specificity in Human UDP-galactose 4\'\'-Epimerase*

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UDP-galactose 4\'\'-epimerase (GALE) interconverts UDP-galactose and UDP-glucose in the final step of the Leloir pathway. Unlike the Escherichia coli enzyme, human GALE (hGALE) also efficiently interconverts a larger pair of substrates: UDP-N-acetylglactosamine and UDP-N-acetylglucosamine. The basis of this differential substrate specificity has remained obscure. Recently, however, x-ray crystallographic data have both predicted essential active site residues and suggested that differential active site cleft volume may be a key factor in determining GALE substrate selectivity. We report here a direct test of this hypothesis. In brief, we have created four substituted alleles: S132A, Y157F, S132A/Y157F, and C307Y-hGALE. While the first three substitutions were predicted to disrupt catalytic activity, the fourth was predicted to reduce active site cleft volume, thereby limiting entry or rotation of the larger but not the smaller substrate. All four alleles were expressed in a null-background strain of Saccharomyces cerevisiae and characterized in terms of activity with regard to both UDP-galactose and UDP-N-acetylglactosamine. The S132A/Y157F and C307Y-hGALE proteins were also expressed in Pichia pastoris and purified for analysis. In all forms tested, the Y157F, S132A, and Y157F/S132A-hGALE proteins each demonstrated a complete loss of activity with respect to both substrates. In contrast, the C307Y-hGALE demonstrated normal activity with respect to UDP-galactose but complete loss of activity with respect to UDP-N-acetylglactosamine. Together, these results serve to validate the wild-type hGALE crystal structure and fully support the hypothesis that residue 307 acts as a gatekeeper mediating substrate access to the hGALE active site.

UDP-galactose 4\'\'-epimerase (GALE); EC 5.1.3.2 catalyzes the third step in the Leloir pathway of galactose metabolism (Fig. 1), interconverting UDP-galactose (UDP-Gal) and UDP-glucose (UDP-Glc) (1). Impairment of human GALE (hGALE) results in the inherited metabolic disorder, epimerase-deficiency galactosemia (OMIM (Online Mendelian Inheritance in Man) number 230350) (2). Clinical phenotypes range from ostensibly benign to severe, although conclusions about outcome and prevalence are confounded by issues of limited ascertainment and a lack of long term patient follow-up in most cases. The biochemical bases of pathophysiology and clinical variability in epimerase deficiency remain unclear, although allelic heterogeneity at the hGALE locus may represent one contributing factor (2).

Unlike Escherichia coli GALE (eGALE), which interconverts only UDP-Gal and UDP-Glc, human GALE also interconverts UDP-N-acetylglactosamine (UDP-GalNAc) and UDP-N-acetylglucosamine (UDP-GlcNAc) (2–8). Considering that all four of these UDP-hexoses serve as sugar donors for glycosyltransferases and enzymes, and that UDP-GalNAc is the obligate first sugar donor for most mucin-type O-linked glycosylation in humans (9), hGALE therefore not only catalyzes an essential step in the Leloir pathway but also serves as a substrate control point in human cells, influencing the biosynthesis of glycoproteins and glycolipids.

Previous studies from our group and others (10–14) have demonstrated that different patient mutations exhibit dramatically different levels of impact on hGALE function. Furthermore, while some mutations (e.g. G90E) impact interconversion of UDP-Gal/UDP-Glc and UDP-GalNAc/UDP-GlcNAc equally, others do not. For example, as compared with the wild-type enzyme, V94M-hGALE, associated with severe epimerase-deficiency galactosemia, retains only 5% activity with regard to UDP-Gal/UDP-Glc but 25% activity with regard to UDP-GalNAc/UDP-GlcNAc (13, 14). The role of impaired activity with regard to each substrate in defining patient outcome remains unknown.

Within the last 8 years, crystal structures for both the human and E. coli GALE enzymes have been solved at high resolution (8, 15–20), offering not only insight into mechanism but also a powerful tool for predicting structure/function relationships in these enzymes. In brief, these structures, combined with biochemical analyses (22), suggest that catalysis begins with abstraction of the 4\'\'-hydroxyl hydrogen of the sugar substrate by a conserved tyrosine base (Tyr157) eGALE/ Trypt157 hGALE) and transfer of a hydride from C4 of the sugar to C4 of the NAD\(^+\) cofactor (Fig. 2A). The result is formation of a 4\'\'-ketopyranose intermediate and NADH. The 4\'\'-ketopyranose then rotates within the active site by \(-180^\circ\), enabling subsequent return of the hydride from NADH back to the opposite face of the sugar, with return of the proton from the tyrosine to the 4\'\'-hydroxyl oxygen, generating epimerized product (1). In addition to the conserved tyrosine, a second residue in the
active site, Ser^{124} eGALE/Ser^{132} hGALE, appears to form a low barrier hydrogen bond with the sugar substrate (Fig. 2A), facilitating both removal of the 4′-hydroxyl hydrogen by the tyrosine and transfer of the hydride from the sugar to the NAD⁺ ring. While mutations testing the roles of these residues in eGALE have been reported previously (18, 22), the human and E. coli GALE sequences differ by at least 48% at the amino acid level, leaving open the question of just how conserved the roles of these specific residues might be. We report here the first test and confirmation of both the individual and combined roles of Ser^{132} and Tyr^{157} in human GALE.

One of the most intriguing questions with regard to GALE, however, addresses the issue of substrate specificity; in particular, why both the E. coli and human enzymes interconvert UDP-GalNAc/UDP-GlcNAc with equal efficiency, but only the human enzyme, which despite its naturally large cleft is nonetheless devoid of endogenous GALE due to a genomic deletion of his 3-11, leu 2-3, 112 ura 3-1 trp1-1 can 1-100 rad 5-1, produces an active site cleft of an epimerase will skew substrate selection toward smaller substrates. These data further raise concern that each naturally occurring residue at this position in WbpP and hGALE may play some previously unappreciated but key role in catalysis rather than simply acting as a gatekeeper limiting access to the active site.

We report here a third and definitive test of the GALE cleft size hypothesis, namely a direct demonstration of the role of residue Cys^{307} as a determinant of substrate specificity in hGALE. Although the C307Y substituted human GALE protein proved insufficiently stable for an x-ray crystallographic analysis, kinetic studies demonstrated fully wild-type activity with regard to the smaller substrate (UDP-Gal) but an almost 42-fold loss of activity with regard to the larger substrate (UDP-GalNAc). This result clearly supports the cleft size hypothesis of substrate specificity in GALE. What is more, unlike previous studies, it also underscores the point that while a small cleft size may preclude access to large substrates, a large cleft size does not necessitate inefficiency with regard to smaller substrates. Contrary to the WbpP report (24), the data presented here further demonstrate that the cysteine at position 307 in hGALE is not essential for catalytic activity.

**Experimental Procedures**

**Construction of Expression Plasmids**—All hGALE mutations were created by site-directed mutagenesis of the otherwise wild-type sequence using the QuickChange system (Stratagene) as described by the manufacturer. Primers used to generate alleles were the following: C307Y, forward (Y157FEPIMF: 5′-GGTTGTACCAACCCTTTCGGCAAGTCC-3′); reverse (Y157FEPIMR: 5′-TTGGTGATGTGGCAGCCTATTAC-3′); S132A/Y157F, forward (S132AEPIMF: 5′-GGTTGTACCAACCCTTTCGGCAAGTCC-3′), reverse (S132AEPIMR: 5′-GGTTGTACCAACCCTTTCGGCAAGTCC-3′); Y157F, forward (Y157FEPIMF: 5′-GTACACAGTGGCTGCGCTGCTGAACACCAG-3′), reverse (Y157FEPIMR: 5′-GGTTGTACCAACCCTTTCGGCAAGTCC-3′).

To create the S132AT157F double mutation, an existing hGALE Y157F sequence was mutagenized a second time with primers S132AEPIMF and S132AEPIMR. All alleles were confirmed by dideoxy sequencing.

**Expression of hGALE Alleles in Saccharomyces cerevisiae**—All plasmids used in this study are listed in Table I. Each hGALE allele was expressed in S. cerevisiae from the yeast glyceraldehyde-3-phosphate dehydrogenase promoter on a low copy number (centromeric) plasmid, pMM33, derived from the yeast-bacterial shuttle vector YCPlac33 (25). pMM33 was derived from the yeast-bacterial shuttle vector YCPlac33 (25) and the expression plasmid pYPEGAP.BX (kind gift of Dr. Warren Kruger, Fox Chase Cancer Center). All yeast manipulations were carried out according to standard techniques as described previously (26).

All yeast strains used in this study are listed in Table II. Strain JF3c6 was generated from the haploid parent W303 (MATa, ade 2-1 his 3-11,15 leu 2-3,112 uro 3-1 trp1-1 can 1-100 Rad 5-1), knockdown in S. cerevisiae strains expressing hGALE were grown at 30 °C to A600 ≈ 1.0 in synthetic medium deficient in uracil. Cells were harvested from 5-ml cultures and soluble cell lysates prepared essentially as described previously (28, 29). Briefly, cell pellets were resuspended in lysis buffer (20 mM HEPES/KOH, pH 7.5, 1 mM dithiothreitol, and 0.3 mg of bovine serum albumin/ml) containing...
protease inhibitors (0.3 mM aprotinin, 0.63 μg/ml pepstatin, 2 mM antipain, 1 mM phosphoramidon, 0.2 μg/ml chymostatin, 8 mM E64, 1 mM phenylmethylsulfonyl fluoride, and 0.50 μg/ml leupeptin) and disrupted by vigorous agitation for 15 min at 4°C in the presence of acid-washed 0.5-mm glass beads. Lysates were clarified by centrifugation for 15 min at 14,000 rpm in an Eppendorf microcentrifuge at 4°C. Both crude and purified samples were assessed for protein concentration using the Bio-Rad protein assay reagent, as recommended by the manufacturer, using bovine serum albumin as a standard.

Western Blot Analysis—Western blot analyses for protein expression were performed essentially as described previously (13, 30). Briefly, hGALE was detected by incubation with a 1:80,000 dilution of a rabbit polyclonal antiserum (EU69) raised against purified hexahistidine-tagged human epimerase. A second rabbit polyclonal antiserum, generated against endogenous yeast cyclophilin A (31), was included at a 1:120,000 dilution to control for loading of lanes. The secondary antibody was horseradish peroxidase-conjugated sheep anti-rabbit IgG (Chemicon), used at a 1:5,000 dilution. Signals were detected by enhanced chemiluminescence. Immediately before use, 1.5 ml of 30% (w/w) H2O2 were added to 10 ml of a working solution (1.25 mM luminol, 0.2 mM p-coumaric acid, and 100 mM Tris-HCl, pH 8.5). The resultant solution was added to the nitrocellulose blot and incubated for 2 min before exposure to film.

Expression of hGALE Alleles in Pichia pastoris—For overexpression in the methylotrophic yeast P. pastoris, hGALE alleles were subcloned into the high copy number Pichia expression plasmid pPIC3.5K (Invitrogen). Cloning was facilitated by a gap-repair procedure (32) utilizing EcoRI/SalI fragments of the hGALE sequence that contained the desired mutations cotransformed with gapped pPIC3.5K backbone that already contained the wild-type hGALE sequence (linearized AflII). The
resultant plasmids were confirmed by restriction enzyme digestion and dideoxy sequencing of relevant regions.

Each of the mutated hGALE plasmids was then linearized using Sall and integrated into the genome of P. pastoris strain GS115 in multiple copy using a spheroplasting protocol, as recommended by Invitrogen. Desired transformants were selected as described previously (20) and expanded in a New Brunswick Scientific Bioflo 3000 fermenter. Cultures were harvested and pellets resuspended in breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 5% glycerol). Cells were lysed either by agitation with glass beads using a Beadbeater (Biospec) or by at least three passages at 7–10 p.s.i. through a French pressure cell (Thermo Scientific). Lysates were clarified by repeated centrifugation (two times) at 12,000 rpm for 10 min in a Sorvall GS-3 rotor at 4°C. Human GALE protein was then purified from extracts as described previously (20).

**In Vitro Analysis of UDP-Gal/UDP-Glc Interconversion.**—Allots of each purified enzyme were stored in 50% glycerol with 4 mM NAD⁺ in liquid nitrogen, while crude extracts were stored in lysis buffer at −85°C until use. All crude extracts were passed through Micro BioSpin 30 columns (Bio-Rad) to remove small metabolites before further analysis. Assays to determine the level of GALE activity with respect to UDP-Gal were performed essentially as described previously (20). Enzymatic conversion from substrate to product was detected either by radioactive assay, as described previously (13), or by nonradioactive assay, with substrates and products separated and quantitated by HPLC. The results from both forms of analysis were indistinguishable (data not shown). For radioactive assays, conversion of UDP-Gal to UDP-Glc was measured in an 12.5-ml reaction containing 2.5 μl of premix (0.05 μM of UDP-[14C]Gal (Amersham Biosciences), 2 mM cold UDP-Gal, 0.2 mM glycine buffer, pH 8.7), 2.5 μl of 20 μM NAD⁺, and 7.5 μl of protein diluted in lysis buffer. The amount of protein used per reaction was adjusted to stay within the linear range of the assay. Reactions were incubated at 37°C for 30 min and stopped by boiling at 100°C for 10 min. Following centrifugation at 14,000 rpm in an Eppendorf microcentrifuge for 15 min, 10 μl of each sample were spotted onto a prewashed polyethyleneimine-cellulose TLC plate (Baker). After thorough drying, the plate was developed for 16 h in a solvent containing 1.5 mM Na₂B₄O₇, 5 mM H₂BO₃, and 25% ethylene glycol. Finally, plates were air-dried before overnight exposure to phosphor screens (Amersham Biosciences). Images were visualized using a Typhoon 9200 variable mode imager and quantified using ImageQuant software (both from Amersham Biosciences).

For nonradioactive analysis of GALE activity, the same assay protocol described above was used, with minor modifications. In particular, UDP-[14C]galactose was removed from the premix and the volume replaced by water. The assay proceeded through the 30-min incubation described above and was then stopped by addition of 2.5 volumes of ice-cold 100% methanol. After brief vortex agitation, samples were spun at 14,000 rpm for 10 min in an Eppendorf microcentrifuge at 4°C. Supernatants were collected and dried under vacuum with low heat. Resultant pellets were then resuspended in 250 μl of double distilled H₂O and filtered by centrifugation for 5 min at 4,000 × g through a 0.2-μm nylon micro-spin filter tube (Altech). Finally, a 15-μl aliquot of each sample was applied to a Dionex HPLC (Dionex, Sunnyvale, CA) for separation and quantitation of UDP-Gal and UDP-Glc, as described below.

**In Vitro Analysis of UDP-GalNAc/UDP-GlcNAc Interconversion—** The radioactivity method for detecting conversion of UDP-GalNAc to UDP-GlcNAc was performed essentially as described above for UDP-Gal, with the following assay components per 25-μl reaction: 8.75 μl of premix (0.04 μM of UDP-[14C]GalNAc (ICN), 1.89 mM cold UDP-GalNAc, 28.6 mM pyruvate, 286 mM glycine, pH 8.7), 5 μl of 20 mM NAD⁺ and 11.25 μl of protein diluted in 100 mM glycine buffer containing protease inhibitors. Assays were performed as for UDP-Gal, with a TLC run time of 10 h, and quantified as described for UDP-Gal.

For nonradioactive analysis, protein samples were diluted to a final volume of 7.5 μl. For each reaction, 2.5 μl of 20 mM NAD⁺ and 2.5 μl of premix (3.3 mM UDP-GalNAc, 0.5 mM glycine, pH 8.7) were added, for a final reaction volume of 12.5 μl. Assay mixtures were incubated at 37°C for 30 min before stopping by addition of 2.5 volumes of ice-cold 100% methanol. Samples were mixed by vortex agitation, spun, dried, resuspended, and filtered as described above for the corresponding UDP-Gal assays, except that these samples were resuspended in 750 μl of double distilled H₂O. Finally, an aliquot of 20 μl was applied to a Dionex HPLC for separation and quantitation of UDP-GalNAc and UDP-GlcNAc.

**Analysis of Small Metabolites—** Identification and quantitation of small carbohydrate metabolites was carried out on a DX600 HPLC system (Dionex) consisting of a Dionex AS50 autosampler, a Dionex GP50 gradient pump, and a Dionex ED50 electrochemical detector, essentially as described previously (33, 34). Carbohydrates were separated on a CarboPac PA10 column, 250 × 4 mm, with a CarboPac PA10 guard column, 50 × 4 mm, placed before the analysis column and a borate trap placed after. Because exclusion of the borate trap resulted in greater separation of the UDP-GalNAc and UDP-GlcNAc, the trap was removed for analysis of the substrates and products from UDP-GalNAc epimerase assays.

The following mobile phase buffers were used for HPLC analysis:

![Diagram of the active site of human GALE](image-url)
buffer A, 15 mM NaOH, and buffer B, 50 mM NaOH, 1 mM NaAc. UDP-Gal and UDP-Glc were separated using a high salt isocratic procedure: 30% buffer A and 70% buffer B for 20 min with a flow rate of 1 ml/min. UDP-GalNAc and UDP-GlcNAc were separated using an isocratic procedure: 45% buffer A and 55% buffer B for 40 min with a flow rate of 0.75 ml/min.

Carbohydrates were quantified using PeakNet software version 6.4 (Dionex) and based on integration of peak areas with comparison to standards. For evaluation of UDP-hexoses, the following standard solutions (1×) was used: 10 μM UDP-GalNAc, 10 μM UDP-GlcNAc, 100 μM UDP-Gal, 100 μM UDP-Glc, and 66 μM NAD⁺.

RESULTS

Expression of Wild-type and Substituted Human GALE Enzymes in S. cerevisiae—To probe the functional roles of residues Ser¹³², Tyr¹⁵⁷, and Cys³⁰⁷ in human GALE, an otherwise wild-type hGALE allele was mutated at all three positions, and each of the resultant S132A, Y157F, S132A/Y157F, and C307Y substituted GALE proteins expressed in a null-background strain of S. cerevisiae. All four alleles were expressed initially from the yeast glyceraldehyde-3-phosphate dehydrogenase promoter on centromeric (low copy number) plasmids (Tables I and II) and the corresponding proteins characterized biochemically in the context of soluble cell lysates. Lysates of yeast expressing wild-type hGALE, and no hGALE, served as positive and negative controls, respectively.

As illustrated in Fig. 4, each substituted hGALE protein remained soluble and was present in lysates at levels comparable with that of the wild-type protein. As expected, the negative control strain demonstrated no hGALE signal, although a faint cross-reacting band migrating just below the position of hGALE was visible. As a control for loading, the filter was also probed with a rabbit polyclonal antiserum directed against the endogenous yeast protein cyclophilin, as described previously (31). As expected, no cyclophilin signal was observed in the control lane (Fig. 4, lane 1) representing purified wild-type hGALE.

Expression and Purification of Wild-type and Substituted Human GALE Enzymes Expressed in P. pastoris—To enable more definitive kinetic analyses of the S132A/Y157F and C307Y-hGALE proteins, both were overexpressed in P. pastoris and purified to apparent homogeneity (data not shown), as described previously for wild-type hGALE (20). Both substituted proteins remained soluble, enabling direct tests of catalytic activity and substrate specificity. Unfortunately, however, neither substituted protein remained sufficiently stable to produce crystals adequate for x-ray diffraction, preventing high resolution structural studies. The cause of the instability remains unknown.

Functional Roles of Ser¹³² and Tyr¹⁵⁷ in Human GALE—To ascertain the impact of both the S132A and Y157F substitutions on hGALE activity, lysates of null-background yeast expressing each substituted allele were subjected to standard GALE activity assays, as described under “Experimental Procedures.” With regard to both potential substrates, UDP-Gal and UDP-GalNAc, both the S132A and Y157F singly substituted proteins, as well as the S132A/Y157F doubly substituted protein, were completely inactive (Table III).

To confirm further this observation in a more defined system, activity assays were repeated using both potential substrates and both the wild-type and S132A/Y157F-hGALE purified proteins (Table IV). Again, the substituted protein demonstrated no detectable activity with regard to either substrate, while the wild-type protein demonstrated the expected levels of activity (23). These results confirm the key catalytic roles predicted for both the Ser¹³² and Tyr¹⁵⁷ residues of human GALE.

Role of Cys³⁰⁷ in Defining Substrate Specificity of Human GALE—To test the predicted role of cysteine 307 as a determinant of substrate specificity in human GALE, the relevant codon in an otherwise wild-type hGALE cDNA was mutated to encode tyrosine. The resultant C307Y-hGALE allele was expressed in a null-background strain of yeast, as described above (Fig. 4). Finally, in vitro GALE activity assays were performed using soluble lysates of these cells, exposed either to UDP-Gal or to UDP-GalNAc. As shown in Table V, lysates containing the C307Y-hGALE protein displayed completely normal activity with regard to UDP-Gal but no activity above background with regard to UDP-GalNAc. The fact that near-normal levels of activity were retained with regard to one substrate but not the other clearly distinguishes C307Y from the S132A and Y157F substitutions and confirms that the Cys³⁰⁷ residue is not essential for catalysis.

As a more definitive test of C307Y-hGALE function, activity assays were repeated using both the wild-type and C307Y hGALE purified proteins (Table VI). Consistent with earlier assays (Table V), no loss of activity was seen for the C307Y protein with regard to the smaller substrate (UDP-Gal), although an almost 42-fold loss of activity was seen with regard to the larger substrate (UDP-GalNAc).

**Table II**

| Strain numbers | Comments | Human GALE expressed |
|----------------|----------|---------------------|
| JFY3682        |          | gal10-null, no plasmids |
| JFY3763–3765   |          | JFY3682 carrying plasmid JF2812 |
| JFY4604–4606   |          | JFY3682 carrying plasmid JF2900 |
| JFY3769–3771   |          | JFY3682 carrying plasmid JF3744 |
| JFY3766–3768   |          | JFY3682 carrying plasmid JF3735 |
| JFY4030–4040   |          | JFY3682 carrying plasmid JF3941 |
| JFY4067–4069   |          | JFY3682 carrying plasmid JF4023 |

**Fig. 4. Abundance of wild-type and mutant hGALE enzymes expressed in gal10-null yeast.** Representative Western blot analyses of lysates probed with antisera detecting hGALE and the endogenous yeast protein cyclophilin (as a control for loading) are shown. Lane 1 was loaded with 100 ng of purified wild-type hGALE protein. Lanes 2–7 were each loaded with 5 μg of soluble extract from yeast expressing the following alleles of hGALE: no hGALE (lane 2), wild-type hGALE (lane 3), C307Y-hGALE (lane 4), S132A-hGALE (lane 5), Y157F-hGALE (lane 6), and S132A/Y157F-hGALE (lane 7).
**Active Site of Human GALE**

**TABLE III**

*Impact of amino acid substitutions S132A and Y157F on the function of human GALE expressed in S. cerevisiae*

*n*, the number of independent yeast colonies examined.

| Substrate | Expressed GALE allele | Specific activity | S.D. | n | Relative activity |
|-----------|-----------------------|------------------|------|---|------------------|
| UDP-Gal   | WT-hGALE              | 1.08 × 10⁻²       | 3.0 × 10⁻³ | 3 | 1.00 ± 0.28     |
|           | no hGALE              | 6.3 × 10⁻⁴       | 5.2 × 10⁻⁴ | 3 | 0.06 ± 0.05     |
|           | S132A-hGALE           | 1.4 × 10⁻⁴       | 1.4 × 10⁻⁴ | 3 | 0.01 ± 0.01     |
|           | Y157F-hGALE           | 4.1 × 10⁻⁴       | 3.1 × 10⁻⁴ | 3 | 0.04 ± 0.03     |
|           | S132A/Y157F-hGALE     | 1.3 × 10⁻⁴       | 2.2 × 10⁻⁴ | 3 | 0.01 ± 0.02     |
| UDP-GalNAc| WT-hGALE              | 6.4 × 10⁻²       | 1.2 × 10⁻² | 3 | 1.00 ± 0.19     |
|           | no hGALE              | 7.4 × 10⁻³       | 1.3 × 10⁻⁴ | 3 | 0.00 ± 0.00     |
|           | S132A-hGALE           | 1.2 × 10⁻⁵       | 2.0 × 10⁻⁵ | 3 | 0.00 ± 0.00     |
|           | Y157F-hGALE           | 1.7 × 10⁻⁴       | 2.9 × 10⁻⁴ | 3 | 0.00 ± 0.00     |
|           | S132A/Y157F-hGALE     | 2.2 × 10⁻⁴       | 1.3 × 10⁻³ | 3 | 0.00 ± 0.02     |

* Indistinguishable from the negative control in pairwise t tests (p > 0.05).

**TABLE IV**

*Impact of amino acid substitutions S132A / Y157F on the function of purified human GALE*

*n*, the number of separate analyses performed with aliquots of the indicated enzymes.

| Substrate | Enzyme            | Specific activity | S.D. | n | Relative activity |
|-----------|-------------------|------------------|------|---|------------------|
| UDP-Gal   | WT hGALE          | 49.48            | 19.5 | 32| 1.00 ± 0.39      |
|           | S132A/Y157F-hGALE | 9.66 × 10⁻⁵      | 3.71 × 10⁻⁵ | 9 | 0.00 ± 0.00     |
| UDP-GalNAc| WT hGALE          | 36.45            | 13.25 | 18| 1.00 ± 0.36     |
|           | S132A/Y157F-hGALE | 7.15 × 10⁻⁷      | 1.75 × 10⁻⁶ | 6 | 0.00 ± 0.00     |

**TABLE V**

*Impact of amino acid substitution C307Y on the function of human GALE expressed in S. cerevisiae*

*n*, the number of independent yeast colonies examined.

| Substrate | Expressed GALE allele | Specific activity | S.D. | n | Relative activity |
|-----------|-----------------------|------------------|------|---|------------------|
| UDP-Gal   | WT hGALE              | 1.08 × 10⁻²       | 3.0 × 10⁻² | 3 | 1.00 ± 0.28     |
|           | no hGALE              | 6.3 × 10⁻⁴       | 5.2 × 10⁻⁴ | 3 | 0.06 ± 0.05     |
|           | C307Y-hGALE           | 5.8 × 10⁻³       | 6.3 × 10⁻³ | 3 | 0.54 ± 0.06     |
| UDP-GalNAc| WT hGALE              | 6.4 × 10⁻²       | 1.2 × 10⁻² | 3 | 1.00 ± 0.19     |
|           | no hGALE              | 7.4 × 10⁻⁵       | 1.3 × 10⁻⁴ | 3 | 0.00 ± 0.00     |
|           | C307Y-hGALE           | 4.1 × 10⁻⁴       | 2.0 × 10⁻⁴ | 3 | 0.01 ± 0.00     |

* Indistinguishable from the wild-type protein in pairwise t tests (p > 0.05).

**TABLE VI**

*Impact of amino acid substitution C307Y on the function of human GALE*

*n*, the number of separate analyses performed with aliquots of the indicated enzymes.

| Substrate | Enzyme            | Specific activity | S.D. | n | Relative activity |
|-----------|-------------------|------------------|------|---|------------------|
| UDP-Gal   | WT hGALE          | 49.48            | 19.5 | 32| 1.00 ± 0.39      |
|           | C307Y-hGALE       | 59.78            | 23.31 | 21| 1.21 ± 0.47     |
| UDP-GalNAc| WT hGALE          | 36.45            | 13.25 | 18| 1.00 ± 0.36     |
|           | C307Y-hGALE       | 0.87             | 0.65 | 18| 0.02 ± 0.02     |

* Indistinguishable from the wild-type protein in pairwise t tests (p > 0.05).

**DISCUSSION**

One of the key unresolved questions regarding human GALE has stemmed from the observation that while some GALE enzymes are efficient at epimerizing only smaller or only larger substrates, the human enzyme is efficient at epimerizing both. Prior crystallographic and mutational approaches in other species have yielded conflicting results regarding the role of active site cleft volume as a determinant of GALE substrate specificity and in particular the role of a single residue, Tyr209 in eGALE, or Ser216 in WbpP, in defining that volume (23, 24). We report here the results of mutational analyses of three residues in hGALE, Ser132, Tyr157, and Cys307. Our data demonstrate that, as predicted by the wild-type hGALE crystal structure (8, 20), the first two residues are essential for catalysis of any substrate, and the third, as predicted by the “cleft size hypothesis,” normally functions to enable activity with regard to larger substrates. In addition to demonstrating that a bulky residue can prevent activity against larger substrates without compromising activity against smaller substrates, these data also serve as a first functional test and validation of the previously reported crystal structure of wild-type human GALE (8, 20).

*Roles of Ser132 and Tyr157 in hGALE—*Residues corresponding to positions Ser132 and Tyr157 in hGALE are conserved if not identical in all GALE enzymes sequenced to date (representative genes listed in Table VII). As illustrated in Fig. 2A,
these residues are predicted from the wild-type hGALE crystal structure (8, 20) to form hydrogen bonds with both the sugar substrate and NAD$^+$ cofactor that not only help to position the substrate for catalysis but also help to initiate the first catalytic step. Previous mutational analyses of these residues in E. coli GALE demonstrated their catalytic significance in that protein (22), and the results presented here (Tables III and IV) demonstrate that those roles have been conserved in the human enzyme. The substitutions made, S132A and Y157F, were selected to mimic as closely as possible the natural residues while preventing the formation of the predicted hydrogen bonds (see Fig. 2B). Recent structural analysis of the Wbp epimerase from P. aeruginosa suggests that the roles of the corresponding residues in that protein have been conserved as well (24).

**Role of Cys$^{307}$ in hGALE**—The issue of substrate specificity of hGALE, and the impact of naturally occurring mutations on that specificity, have been questioned by both basic science and clinical interest for many years, in part because it remains unknown which activity of hGALE mediates severity of outcome for patients with epimerase-deficiency galactosemia (13, 14). Structural and/or mutational studies from E. coli (20, 23), human (8, 23), *Yersinia enterocolitica* (35), and *P. aeruginosa* (24) all suggest that too small an active site cleft volume can prevent an otherwise functional epimerase from acting upon larger substrates (UDP-GalNAc/UDP-GlcNAc), although these same reports also demonstrate that a large active site cleft may or may not pose challenges to the epimerization of smaller substrates (UDP-Gal/UDP-Glc). All of these studies (8, 23, 24, 35) further suggest that the residue corresponding to Cys$^{307}$ in hGALE acts as a form of “gatekeeper,” controlling the size of the active site cleft (Fig. 3). The results presented here are fully consistent with those conclusions and further suggest that some as yet unappreciated mechanism must enable wild-type human GALE to function efficiently against both large and small substrates, presumably by limiting the ability of smaller substrates to assume unproductive conformations within the large active site space.

**GALE and GNE Genes**—Cross-species comparisons of GALE-related sequences from different sources demonstrate that a small number of amino acids, Leu, Tyr, Cys, Ser, Phe, and Val, can occupy the gatekeeper position (Table VII). Of the corresponding enzymes, only those encoding Cys or Ser at the gatekeeper position have demonstrated activity against UDP-GalNAc/UDP-GlcNAc (2, 6, 24, 35, 36). Enzymes encoding Tyr or Leu at the gatekeeper position exhibit essentially no activity against UDP-GalNAc, and those encoding Val or Phe remain either untested or unclear at this time (37–39). As a test of the substrate specificity of the *S. cerevisiae* GALE, which encodes Leu at the gatekeeper position, we performed activity assays using both UDP-Gal and UDP-GalNAc with soluble lysates from both wild-type and GALE-null (gal10-null) yeast. The wild-type lysates demonstrated a specific activity of 468.82 ± 123.02 pmol/μg/min (n = 3) when challenged with UDP-Gal, but only 1.16 ± 0.27 pmol/μg/min (n = 3) when challenged with UDP-GalNAc. Lysates prepared from gal10-null yeast demonstrated no detectable activity against either substrate.

In species that utilize GalNAc in their glycoproteins and/or glycolipids but whose GALE enzyme is not active against UDP-GalNAc/UDP-GlcNAc, a second locus is often found encoding an enzyme capable of epimerizing UDP-GalNAc/UDP-GlcNAc but not necessarily UDP-Gal/UDP-Glc. Examples include the Wbp gene product of *P. aeruginosa* (24), the GalE2 gene product of *Streptococcus gordonii* (36), and the GNE gene product of *E. coli* (Ref. 37 and Table VII). The existence of these additional genes presents an evolutionary paradox, namely that some species maintain a two-gene system to accomplish what appears to be the same job performed in other species by a single gene. Clearly, issues of differential gene expression, distinct subcellular protein localization, or differential regulation may explain the two-gene necessity in some species. The possibility also remains that one or both enzymes in these species may act in vivo upon additional substrates not appreciated at this time.

Considering the essential roles of the GALE/GNE enzymes in maintaining viability and/or virulence of major pathogens (e.g. Refs. 40 and 41), together with the role of human GALE in epimerase-deficiency galactosemia, unveiling key similarities and differences in the structure/function relationships of these enzymes is an issue not only of basic science interest but of clinical significance as well. The results presented here represent an important step toward that goal.

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