Mediators of Galactose Sensitivity in UDP-Galactose 4'-Epimerase-impaired Mammalian Cells*

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UDP-galactose 4'-epimerase (GALE) catalyzes the final step in the Leloir pathway of galactose metabolism, interconverting UDP-galactose and UDP-glucose. Unlike its Escherichia coli counterpart, mammalian GALE also interconverts UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine. Considering the key roles played by all four of these UDP-sugars in glycosylation, human GALE therefore not only contributes to the Leloir pathway, but also functions as a gatekeeper overseeing the ratios of important substrate pools required for the synthesis of glycosylated macromolecules. Defects in human GALE result in the disorder epimerase-deficiency galactosemia. To explore the relationship among GALE activity, substrate specificity, metabolic balance, and galactose sensitivity in mammalian cells, we employed a previously described GALE-null line of Chinese hamster ovary cells, ldlD. Using a transfection protocol, we generated ldlD derivative cell lines that expressed different levels of wild-type human GALE or E. coli GALE and compared the phenotypes and metabolic profiles of these lines cultured in the presence versus absence of galactose. We found that GALE-null cells accumulated abnormally high levels of Gal-1-P and UDP-Gal and abnormally low levels of UDP-Glc and UDP-GlcNAc in the presence of galactose and that human GALE expression corrected each of these defects. Comparing the human GALE- and E. coli GALE-expressing cells, we found that although GALE activity toward both substrates was required to restore metabolic balance, UDP-GalNAc activity was not required for cell proliferation in the presence of otherwise cytostatic concentrations of galactose. Finally, we found that uridine supplementation, which essentially corrected UDP-Glc and, to a lesser extent UDP-GlcNAc depletion, enabled ldlD cells to proliferate in the presence of galactose despite the continued accumulation of Gal-1-P and UDP-Gal. These data offer important insights into the mechanism of galactose sensitivity in epimerase-impaired cells and suggest a potential novel therapy for patients with epimerase-deficiency galactosemia.

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UDP-galactose 4'-epimerase (GALE, EC 5.1.3.2) catalyzes the third step in the highly conserved Leloir pathway of galactose metabolism in species ranging from bacteria to humans (1). As illustrated in Fig. 1, GALE is a reversible enzyme, interconverting UDP-galactose (UDP-Gal) and UDP-glucose (UDP-Glc, reaction 1). Although Escherichia coli GALE catalyzes only the interconversion of UDP-Gal and UDP-Glc, mammalian GALE enzymes also interconvert UDP-N-acetylgalactosamine (UDP-GalNAc) and UDP-N-acetylgalactosamine (UDP-GlcNAc, reaction 2) (2–5). UDP-GalNAc is the obligate first sugar donor for all mucin-type O-linked glycosylation reactions in mammalian cells (6), and all four UDP-sugars serve as activated substrate donors for both N- and O-linked glycosylation reactions. GALE therefore not only catalyzes an essential step in the Leloir pathway, but also serves as a gatekeeper overseeing the ratios of key substrate pools required for the synthesis of glycogen, glycosaminoglycans, proteoglycans, glycoproteins, and glycolipids in mammals.

Defects in human GALE (hGALE) result in the inherited metabolic disorder epimerase-deficiency galactosemia (OMIM 230350) (7). The majority of patients with epimerase deficiency demonstrate an apparently benign form of the disorder in which enzyme impairment is restricted to the circulating red and white blood cells (7). These patients are said to have peripheral epimerase deficiency. A small number of patients have also been identified who demonstrate a more generalized enzyme impairment that extends to other tissues, resulting in potentially significant clinical disease (8–10). Finally, several reports suggest that intermediate forms of epimerase deficiency may also exist (11–13). Although the basis for this biochemical and clinical spectrum of epimerase deficiency remains unknown, studies of patient GALE sequences have demonstrated significant variability (11, 14–17), suggesting that allelic heterogeneity may be one contributing factor. Nonetheless, the precise role of human GALE in mediating both galactose metabolism and the pathophysiology of galactose sensitivity in epimerase deficiency remains unclear.

As one approach toward addressing this question, both we and others have developed and applied yeast model systems in which GALE (GAL10) is either deleted or impaired (11, 14, 15, 18, 19). GALE-deficient yeast are not only unable to grow on media containing galactose as the sole carbon source; they also fail to grow on media containing alternate carbon sources, such as carbon source.
as glycerol/ethanol or raffinose, if even trace amounts of galactose are added (e.g. 0.0025%) (19). These results demonstrate that GALE-null yeast are not only Gal- or unable to metabolize galactose fully, they are also galactose-sensitive (15, 18, 19). Prior and ongoing studies have helped to identify potential factors mediating galactose sensitivity in GALE-null yeast (15, 18, 19); nonetheless, how these results relate to galactose metabolism and sensitivity in GALE-deficient mammalian cells has remained unknown.

With the experiments presented here we have begun to address the mechanism of galactose sensitivity in GALE-impaired mammalian cells utilizing a strain of Chinese hamster ovary cells called idld. idld cells were originally isolated more than 20 years ago on the basis of impaired low density lipoprotein receptor function (20), a phenotype later revealed as secondary to a complete absence of GALE activity, which resulted in the aberrant processing of both N- and O-linked glycoproteins, including the low density lipoprotein receptor (21). It is important to note that although idld cells are viable in standard glucose-containing media despite their lack of GALE, no other truly GALE-null mammalian cells have been reported. In particular, cells derived from even the most severely affected epimerase-deficient patients are not fully GALE-null (14, 15, 22). This observation stands in stark contrast to transferase deficiency, in which patients demonstrating no detectable activity, and even large genomic deletions, have been reported (23).

Although viable, idld cells clearly exist in a precarious balance. Because of their lack of GALE, they cannot synthesize endogenous galactose or GalNAc and therefore depend on exogenous sources of these sugars for normal glycosylation (21). These same cells, however, exhibit impaired growth when exposed to greater than 0.125 mM galactose (24). The mechanism behind this impaired growth phenotype has remained unclear and is addressed in part by the experiments presented here. In particular, we have asked three questions: 1) What are the metabolic changes associated with galactose sensitivity in idld cells? 2) How much hGALE activity is required to relieve the apparent galactose sensitivity of idld cells? 3) What is the relationship among GALE substrate specificity, metabolic imbalance, and galactose sensitivity in idld-derived cells?

To address these questions, we first characterized both idld and wild-type CHO cells cultured in the presence or absence of 0.25 mM galactose. As expected, we observed a number of galactose-specific abnormalities in the idld cells, including impaired cell growth, elevated Gal-1-P and UDP-Gal, and depressed UDP-Glc, UDP-GlcNAc, and UDP-GalNAc. To define the level of wild-type human GALE activity required to complement the idld cell growth impairment and metabolic abnormalities, we generated transfectants stably expressing either low (5–10%) or near normal levels of hGALE activity. The near normal hGALE-expressing cells (ldld[hGALE-wt]) demonstrated full complementation of some abnormalities, including the growth, Gal-1-P, and UDP-Gal phenotypes and significant complementation of others, including the UDP-Glc, UDP-GlcNAc, and UDP-GalNAc phenotypes. In contrast, the 5–10% hGALE-expressing cells (ldld[hGALE-low]) exhibited only marginal complementation of each of these abnormalities.

To probe the role of GALE substrate specificity in galactose sensitivity, we generated idld transfectants expressing E. coli GALE, which completely lacks UDP-GalNAc activity. As expected, both galactose consumption and internal metabolite levels, including Gal-1-P, UDP-Gal, UDP-Glc, and UDP-GlcNAc, were complemented as well in these idld[eGALE] cells as in the ldld[hGALE-wt] cells, although UDP-GalNAc remained undetectable. Nonetheless, the idld[eGALE] cells grew as well in the presence of 0.25 mM galactose as did their hGALE-expressing counterparts, demonstrating that the apparent growth impairment of GALE-null cells exposed to galactose was independent of UDP-GalNAc deficiency and therefore likely independent of O-linked glycosylation defects.

Finally, as a first step toward addressing the issue of mechanism, we tested the impact of nucleoside supplementation and found that uridine alone was sufficient to relieve the galactose-specific growth impairment of idld cells. It is particularly interesting to note that although uridine supplementation did correct the UDP-Glc and, to a lesser extent, UDP-GlcNAc depletion of galactose-exposed idld cells, both the Gal-1-P and UDP-Gal levels in these cells remained abnormally high. These results suggest that it is not the dramatic accumulation of Gal-1-P or UDP-Gal, but rather the depletion of uridine that is cytostatic to idld cells exposed to galactose. Combined, these results provide a first step toward understanding the mechanism of galactose sensitivity in GALE-impaired mammalian cells and suggest a potential novel therapy for epimerase-deficiency galactosemia.

MATERIALS AND METHODS

Plasmids—HA-tagged forms of both the human GALE (hGALE, JF4169–4170) and E. coli GALE (eGALE, JF4387) enzymes were expressed in idld cells using a derivative of the pCDNA3 (Invitrogen) vector in which the cytomegalovirus promoter had been removed and replaced with the mouse galactose-1-phosphate uridylyltransferase (mGALT) promoter. The mGALT promoter sequence (25) was generated through PCR amplification of wild-type mouse genomic DNA (kind gift from Dr. David Weinschenker, Emory University) using the following primers, which contained the restriction sites MluI and HindIII for ease of subcloning: mGALTproMluI5′-GGGCCGGCGCCGTATCCGTGGCGGGACGTAAGCGACGACGACCAAC-3′; and mGALTproHindIII15′-GGCGAAGCTTATCCGCTCACTTTATGAGCGACGGG-3′. The wild-type E. coli GALE sequence was obtained via PCR amplification from genomic E. coli DNA using the following primers, which engineered an HA tag onto the 3′ end of the open reading frame: eGALEF2: 5′-GGCGCGATATTTTCTAAGGTTGCTAGAGTTGCTTGTTAACCGGTTGGG-3′; and eGALERH15′-GGCGGCGCCGACCTAGATATCTTTGAGCAGCGCTCGTGGTTAATCGGTGATATTTTCTAAGGTTGCTAGAGTTGCTTGTTAACCGGTTGGG-3′. All amplified, subcloned alleles were confirmed by dye-exosequencing.

Cell Culture and Generation of Transfected Cell Lines—All mammalian cell lines used in these studies are listed in Table I. The control cell line, CHO-K1 (kind gift from Dr. Curt Hagedorn, Emory University), and mutant idld cell line were maintained in Ham’s F-12 medium containing 10% FBS. All cells were maintained at 37 °C in a humidified 5% CO2 incubator (NuAire) and harvested for splitting or analysis by standard protocols using trypsin/EDTA.

Cells were transfected in 6-well dishes using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. 24 h after transfection, cultures were trypsinized and replated at >1:10 dilution into Ham’s F-12 medium supplemented with 10% FBS. G418 selection was initiated 24 h later by the addition of appropriate levels of drug (200 μg/ml G418, Cellgro). After ~14 days of drug selection, individual clones were isolated by trypsinization within cloning rings.

FIG. 1. Pathways of galactose metabolism. The enzymes of the Leloir pathway are boxed. Dashed lines indicate low level side reactions.
Galactose Sensitivity of GALE-impaired Cells

Enzyme activities in lldD cells stably expressing human GALE (hGALE) or E. coli GALE (eGALE)

| Strain (comments) | GALE activity (UDP-Gal) pmol/μg/min | GALE activity (UDP-GalNAc) pmol/μg/min |
|-------------------|----------------------------------|--------------------------------------|
| CHO-K1 (wild-type GALE) | 2.08 ± 0.21 | 2.27 ± 0.54 |
| lldD (GALE-null, from CHO-K1) | Undetectable | Undetectable |
| JFm342 (lldD(hGALE-wt)) | 1.64 ± 0.13 | 2.54 ± 0.49 |
| JFm348 (lldD(hGALE-low)) | 0.10 ± 0.05 | 0.28 ± 0.03 |
| JFm361 (lldD(eGALE)) | 0.82 ± 0.06 | Undetectable |

and purified by further subculture in medium containing G418. Expression of GALE in each clone was confirmed by Western blot analysis against the HA tag and by GALE activity assays. Clonal lines of transfecants were maintained in Ham’s F-12 medium containing glutamine and 10 mM glucose, supplemented with 10% FBS and 200 μg/ml G418.

Generation of Lipoprotein-deficient Serum (LPDS)—Newborn calf LPDS was prepared according to methods described by Goldstein et al. (26) and modified by Krieger (27). Briefly, whole newborn calf serum (In vitrogen) was adjusted to a final density of 1.215 g/ml with solid (Sigma). The serum was separated by ultracentrifugation at 59,000 rpm for 36 h at 4 °C in a Beckman 70 Ti rotor. The top, lipoprotein-rich fraction was removed through aspiration. The lipoprotein-deficient fraction was then subjected to five sequential rounds of dialysis against 0.25 mM galactose. After 72 h, cells were harvested and tested to a protein concentration of 60 mg/ml by dilution with sterile 150 mM NaCl.

Cell Viability Assays—lldD and wild-type CHO cells were plated in triplicate in Ham’s F-12 medium containing glutamine, 10 mM glucose, and 2% LPDS. After cells reached 90% confluence (48 h), the medium was removed and replaced by fresh medium either with or without 0.25 mM galactose. After 24 h, cells were harvested and tested for viability by exclusion of the vital dye, trypan blue (Sigma), according to the manufacturer’s instructions. Briefly, a 0.2-ml cell suspension in phosphate-buffered saline (PBS) was added to 0.5 ml of 0.4% trypan blue and 0.3 ml of PBS. The suspension was incubated for 5 min before microscopic examination, in which both stained and unstained cells were counted using a hemacytometer. A minimum of 100 cells were counted.

Uridine Rescue—lldD cells were plated into the wells of a 24-well plate at 10,000 cells/well in Ham’s F-12 medium containing 10 mM glucose and supplemented with 100 μl/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 3% LPDS. After 1 day of culture the medium from each well was removed and replaced with fresh medium containing 0, 0.125, or 0.25 mM galactose with or without the addition of 10 μM uridine, thymidine, guanosine, adenosine, or cytidine. After an additional 5 days of culture, monolayers were fixed and stained with crystal violet, as described previously (28). Metabolite studies on lldD cells grown in the presence and absence of 100 μM uridine were performed as described below.

Preparation of Protein Lysates and Enzyme Assays—Proteins were extracted by repeated freeze/thaw in 100 mM glycine buffer 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 μM leupeptin). Crude protein extracts were fractionated over F-30 Bio-Spin columns (Bio-Rad) to remove small metabolites prior to further analysis. Protein determinations were made using the Bio-Rad protein assay reagent, as recommended by the manufacturer, and quantified using a standard curve of bovine serum albumin.

Individual Leloir pathway enzyme activities were measured using standard enzymatic assay procedures with quantification of reactants and products by HPLC. In brief, all enzymatic reactions were carried out at 37 °C for 30 min, essentially as described previously (19) with appropriate levels of protein included to remain within the linear range of the assay. Enzyme activity in each assay is defined as pmol of product formed per μg of protein/min.

The following mobile phase buffers were used for separation of carbohydrate substrates and products: Buffer A, 15 mM NaOH; Buffer B, 50 mM NaOH, 1 mM sodium acetate; and Buffer C, 15 mM NaOH, 1 mM Na2B4O7,10H2O. The following procedures were used for HPLC separation and quantitation of enzymatic activities and products. For galactokinase: 98% A and 2% B (5 to 3 min), a linear increase of B to 25% (3 to 25 min), hold at 75% A and 25% B (25 to 27 min), and a linear decrease of B to 2% (30 to 60 min). For GALT: 85% A and 15% B (10 to 15 min), a linear increase of B to 25% (5 to 10 min), a linear increase of B to 70% (10 to 15 min), hold at 30% A and 70% B (15 to 30 min), and a linear decrease of B to 15% (30 to 32 min). For GALE, UDP-Gal: 35% C and 65% B for 20 min. For GALE, UDP-GalNAc: 45% C and 55% B for 30 min. The flow rate was maintained at 1 ml/min for all separation procedures.

Incorporation Assay—The colorimetric Cell Proliferation ELISA kit (Roche Applied Science), which monitors incorporation of BrdUrd into newly synthesized DNA, was used according to the manufacturer’s recommendations to quantify the proliferation of cells grown in the presence versus absence of galactose. Briefly, cells were plated at 100 cells/200 μl of medium into each well of a 96-well plate, using medium containing 2% LPDS, and grown for 72 h. As described under “Results,” some wells also contained small amounts of galactose or mannose. At the end of the 72-h growth period, cells were exposed to BrdUrd for 8 h and then fixed for 30 min with the provided solution. Next, cells were incubated for 1.5 h with a monoclonal peroxidase-labeled antibody directed against BrdUrd, provided by the manufacturer. After removal, cells were washed three times with 200 μl of 1× washing solution. Finally, the substrate was allowed to develop for 10 min before addition of tetramethylbenzidine (TMB) to the time points specified in each figure. A 250-μl sample of medium from each plate was also collected at each time point for analysis of external galactose consumption. Trypsinized cells were harvested by centrifugation at 3,500 rpm for 5 min, after which the supernatant was aspirated, and the cells were washed once with 5 ml of cold PBS before further manipulation.

Analysis of Internal Metabolites—Metabolite samples from cells were prepared using a modified form of the procedure originally described by Smits et al. (29). Briefly, each cell pellet was resuspended in 11 ml of cold PBS, of which 1 ml was used for protein determination via the Bio-Rad DC protein assay, as recommended by the manufacturer. The remaining 10 ml of cell suspension was quenched in 20 ml of 60% MeOH (−20 °C), after which cells were collected by 20-min centrifugation at 2,000 rpm, 4 °C in an Eppendorf 5810R centrifuge. Intracellular metabolites were extracted by vigorous agitation for 45 min at 4 °C in a 4:2:1 mixture of CHCl3/MeOH/water, with a final volume of 875 μl. The aqueous layer was collected after high speed centrifugation for 10 min at 4 °C. The remaining organic phase was extracted a second time with 125 μl of MeOH and 125 μl of water. Aqueous layers were combined and dried under vacuum without heat (10 h). Finally, dried metabolites were rehydrated with sterile Milli-Q water to a concentration corresponding to 1.5–2.75 μg of protein/μl, using protein values determined in parallel samples as described above. Rehydrated samples were filtered through 0.2-μm nylon filters (Alltech) before HPLC fractionation.

Analysis of External Galactose Consumption—Each 250-μl sample of culture medium was added directly into 500 μl of 60% MeOH (−20 °C). External metabolites were extracted as described above from a 375-μl fraction of the medium/MeOH mixture. After drying, metabolite pellets were rehydrated and analyzed by HPLC as described above. Rehydrated samples were filtered through 0.2-μm nylon filters (Alltech) before HPLC fractionation.
were rehydrated in 390 \( \mu \)l of sterile Milli-Q water and filtered before HPLC fractionation.

**Small Metabolite Analysis by HPLC**—HPLC analysis was performed using a DX600 HPLC system (Dionex) consisting of a Dionex AS50 autosampler, a Dionex GP50 gradient pump, and a Dionex ED50 electrochemical detector, as described previously (19). In brief, carbohydrates were separated on a CarboPac PA10 column (250 \( \times \) 4 mm) with an amino trap (50 \( \times \) 4 mm) placed before the analysis column and a borate trap (50 \( \times \) 4 mm) placed before the injector port to remove trace amounts of borate from the mobile phase buffers. The borate trap was removed for high salt protocols, identifying UDP-sugars, to allow for better separation of these metabolites. For all samples, 15 \( \mu \)l was injected into a 25-\( \mu \)l injection loop. Samples were maintained at 4 °C in the autosampler tray, and the chromatography was performed at room temperature.

Hexoses and hexose phosphates were separated using a low salt gradient procedure with a flow rate of 1 ml/min. Gradient 1 was 98% A and 2% B (10 to 8 min), a linear increase of B to 30% (8 to 15 min), a linear increase of B to 50% (15 to 25 min), hold 50% A and 50% B (25 to 30 min), a linear decrease of B to 2% (30 to 35 min). External galactose was determined using a low salt gradient procedure. Gradient 2 was 99.5% A and 0.5% B at a 1 ml/min flow rate (15 to 0 min), 99.5% A and 0.5% B at a 0.5 ml/min flow rate (0 to 25 min), a linear increase of B to 50% (25 to 35 min), hold 50% A and 50% B at a 1 ml/min flow rate (35 to 40 min), and a linear decrease of B to 0% (40 to 45 min). UDP-hexoses were separated using a high salt gradient procedure with a flow rate of 0.8 ml/min. Gradient 3 was 50% C and 50% B for (−5 to 1 min), a linear increase of B to 70% (1 to 22 min), hold 30% C and 70% (22 to 27 min), and a linear decrease of B to 50%. Injections were made at 1 ml/min. UDP was separated isocratically with a flow rate of 1.0 ml/min, holding B at 70% and A at 30% for 25 min. Metabolites were detected and quantified as described previously (19).

**RESULTS**

**Galactose-sensitive Growth of GALE-null IdID Cells**—Prior to initiating studies of galactose metabolism in IdID cells, we first sought to compare these cells with control CHO cells with regard to both the extent and specificity of their Leloir enzyme impairment. Both the CHO and IdID cells exhibited statistically indistinguishable activity levels of galactokinase (15.21 ± 4.74 versus 20.14 ± 3.57 pmol/\( \mu \)g/min, respectively, \( p > 0.05 \)) and GALT (24.38 ± 9.18 versus 9.78 ± 4.77 pmol/\( \mu \)g/min, respectively, \( p > 0.05 \)), and only the CHO cells demonstrated detectable GALE activity (Table 1).

In an effort both to confirm and to quantitate the apparently galactose-sensitive growth of IdID cells reported previously (24), we monitored levels of BrdUrd incorporated into cultures of both IdID and CHO cells grown in the absence versus presence of 0.25 mM galactose. To limit the ability of the cells to scavenge galactose and GalNAc from glycolipids and glycoproteins present in the extracellular medium, the culture medium in these experiments was supplemented with very low levels (2% v/v) of LPDS (see “Materials and Methods”) in place of normal FBS. In brief, both lines were plated directly into either normal medium, which contained 10 mM glucose, or into normal medium supplemented with 0.25 mM galactose. As a control, cells were also plated into medium supplemented with 0.25 mM mannose. After 72 h in culture, BrdUrd was added to each plate, with incorporation monitored by ELISA, as described under “Materials and Methods.” To facilitate comparison, the level of BrdUrd incorporated into each cell line cultured in glucose alone was set to 100%, with BrdUrd incorporation by the same cells cultured in the presence of galactose or mannose scaled accordingly.

As illustrated in Fig. 2A, wild-type CHO cells incorporated more BrdUrd in the presence of galactose than in its absence. In contrast, IdID cells incorporated only about 25% as much BrdUrd in the presence of galactose as in its absence. This inhibition was galactose-specific, as demonstrated by the mannose control. Finally, cell viability studies conducted in parallel demonstrated that both cultures remained more than 90% viable after 72 h in the presence of galactose (Fig. 2B), confirming that galactose exposure was cytostatic to the IdID cells, but not cytotoxic during the time course of the experiment.

**Impaired Metabolism of Galactose in IdID Cells**—To explore the impact of GALE deficiency on galactose metabolism in IdID cells, both the CHO and IdID cells were cultured in medium either with or without the addition of 0.25 mM galactose. As described above, normal FBS was replaced in these media with 2% LPDS. All cultures were incubated for up to 72 h, with media and cells harvested at 0-, 1-, 6-, 12-, or 24-h intervals for the measurement of both external galactose and internal metabolites. As illustrated in Fig. 3A, external galactose was depleted rapidly from the culture medium of CHO cells, presumably as a consequence of the cellular uptake of galactose and its sequential conversion via the Leloir pathway to Gal-1-P, UDP-Gal, and UDP-Glc (Fig. 1), which was then metabolized extensively via known routes of intermediary metabolism. In contrast, there was little if any consumption of external galactose by the IdID cells, presumably reflecting the GALE block, which prevented conversion of UDP-Gal to UDP-Glc and thereby “backed up the system,” causing intracellular accumulation of galactose and the proximal metabolites Gal-1-P and...
UDP-Gal and limiting the uptake of further galactose.

Analysis of internal metabolites confirmed these expectations. As presented in Fig. 3, B–F, both Gal-1-P and UDP-Gal rose steadily in galactose-treated ldlD cells, peaking at about 24 h, although neither metabolite achieved detectable levels in ldlD cells in the absence of galactose or in wild-type cells incubated with or without galactose (Fig. 3, B and C). UDP-GalNAc levels (Fig. 3D) fluctuated between about 0.5 and 1.5 pmol/μg of protein in the wild-type CHO cells independently of galactose exposure, although they remained essentially undetectable in ldlD cells. Finally, UDP-Glc (Fig. 3E) and UDP-GlcNAc (Fig. 3F) presented similar profiles in wild-type cells regardless of galactose exposure and in ldlD cells in the absence of galactose, although both metabolites were markedly depressed in ldlD cells exposed to galactose. In ldlD cells the absence of UDP-GalNAc, and the galactose-induced increases in Gal-1-P and UDP-Gal were expected because of the deficiency of GALE and its place in the Leloir and associated pathways (Fig. 1). The striking galactose-induced reduction in UDP-Glc and UDP-GlcNAc in these cells will be discussed below. The dip in UDP-Glc, UDP-GlcNAc, and UDP-GalNAc levels seen at 6 and 12 h was highly reproducible and may reflect the effect of refeeding at t = 0.

It is particularly interesting to note the UDP-Glc/UDP-Gal and UDP-GlcNAc/UDP-GalNAc ratios in both cell lines. As presented in Table III, these ratios ranged from about 2 to about 7 in CHO cells and remained stable regardless of the medium. In contrast, in ldlD cells these ratios fluctuated wildly or were incalculable because of a 0 denominator.

Expression of Low versus Near Normal Levels of Wild-type Human GALE in ldlD Cells—To test the ability of wild-type human GALE to complement the impaired galactose metabolism and galactose sensitivity of ldlD cells, we generated transfectants stably expressing either 5–10% wild-type (ldlD[hGALE-low]) or near normal levels (ldlD[hGALE-wt]) of hGALE activity (Table I). To enable appropriate levels of epimerase expression, the strong viral cytomegalovirus promoter in the transfecting plasmid was replaced by the mouse GALT promoter (see “Materials and Methods”). The purpose of these experiments was 2-fold. First, because ldlD cells were generated by extensive mutagenesis of wild-type CHO cells and thus most likely carry mutations in loci other than the structural gene for GALE (20), it was important to define what phenotypic abnormalities could be complemented by the introduction of near normal levels of GALE activity. Second, considering that yeast studies have demonstrated a galactose hypersensitivity in GALE-null compared with GALT-null cells (19) and considering that patients with very low residual GALT have been demonstrated to metabolize galactose indistinguishably from normal controls (30), it was of interest to see how ldlD cells would respond to restoration of only marginal levels of mammalian GALE.

With regard to cell proliferation, we found that the ldlD-[hGALE-wt] cells incorporated normal levels of BrdUr in the presence of galactose, whereas the ldlD[hGALE-low] cells demonstrated partial restoration of growth under these conditions (Fig. 4). Furthermore, galactose consumption was enhanced by expression of either near wild-type [hGALE-wt] or low [hGALE-low] levels of hGALE activity, although it did not reach the same levels seen in CHO cultures (Fig. 5A). This difference may reflect the mutated genetic background of ldlD cells.

With regard to internal metabolites, near normal levels of hGALE were sufficient to eliminate the abnormal accumulation of both Gal-1-P and UDP-Gal in galactose-exposed ldlD-[hGALE-wt] cells (Fig. 5, B and C) and to restore nearly normal levels of UDP-GalNAc (Fig. 5D). Low level hGALE expression provided partial correction of the metabolite levels. Furthermore, the galactose sensitivity of the UDP-Glc and UDP-GlcNAc levels were substantially reduced in ldlD[hGALE-wt] cells, whereas there was much less correction in the ldlD-[hGALE-low] cells (Fig. 5, E and F).
Galactose Sensitivity of GALE-impaired Cells

Probing the Impact of GALE Substrate Specificity on Galactose Metabolism and Sensitivity in Mammalian Cells—As described previously, wild-type mammalian GALE readily interconverts both UDP-Gal/UDP-Glc and UDP-GalNac/UDP-GlcNAc (2–5). To address the overlapping or potentially distinct roles of these two activities in defining galactose metabolism and sensitivity in mammalian cells, we generated an ldID-derivative line expressing wild-type E. coli GALE that exhibits only one of these activities, UDP-Gal/UDP-Glc interconversion. Transfection was performed as described under “Materials and Methods,” and the clone selected for further study (ldID[eGALE]) demonstrated levels of GALE activity which were within a factor of 2 of ldID[hGALE-wt] with regard to UDP-Gal, but as expected, demonstrated no activity with regard to UDP-GalNac (Table 1).

As illustrated in Figs. 6 and 7, E. coli GALE was able to complement most ldID abnormalities as well as the human enzyme. The ldID[eGALE] cells grew as well in the presence of galactose as did the ldID[hGALE-wt] cells (Fig. 6), and galactose consumption between the two transfected lines was indistinguishable (Fig. 7A). With regard to internal metabolites, like their hGALE-expressing counterparts, the galactose-treated ldID[eGALE] cells did not accumulate Gal-1-P or UDP-Gal (Fig. 7, B and C), and the relative levels of UDP-Glc and UDP-GlcNAc were less sensitive to galactose in these cells than in untransfected ldID cells (Fig. 7, E and F). Finally, as expected, UDP-GalNac levels remained essentially undetectable in both ldID and ldID[eGALE] cells (Fig. 7D). These results demonstrate that the galactose-specific growth inhibition of ldID cells was not dependent on UDP-GalNac depletion.

Uridine Rescue of IdID Cell Growth in the Presence of Galactose—The observation that GALE-impaired cells exposed to galactose accumulate high levels of UDP-Gal and become deficient in UDP-Glc and UDP-GlcNAc, coupled with the body of literature describing selective depletion of UTP pools in mammalian tissues exposed to D-galactosamine (e.g. Ref. 31), led us to suspect that UTP depletion might contribute to the galactose sensitivity of ldID cells. As a first test of this hypothesis, we asked whether nucleoside supplementation could relieve the galactose-specific growth impairment of ldID cells. ldID cells were plated in the presence or absence of galactose, with or
without a variety of different nucleosides (uridine, thymidine, guanosine, adenosine, or cytidine) (see “Materials and Methods”). After 5 days, the cultures were fixed, stained with crystal violet, and inspected for evidence of cell growth. As presented in Table II, the results clearly demonstrated that uridine supplementation was sufficient to enable nearly normal growth of the ldlD cells despite the presence of galactose, whereas none of the other nucleosides had any detectable effect. These results suggest that uridine depletion contributes to the galactose sensitivity of growth in GALE-null cells.

As a further test of this possibility, we assessed UTP and other relevant metabolite levels in ldlD cells grown in the presence and absence of both galactose and uridine. As presented in Fig. 8, the results were fully consistent with the hypothesis; UTP, UDP-Glc, and UDP-GlcNAc were all notably depleted in ldlD cells exposed to galactose, and uridine supplementation either fully, or at least partially, reversed all three abnormalities. In contrast, uridine supplementation failed to inhibit the abnormal accumulation of either Gal-1-P or UDP-Gal in these cells, although there was a small drop in the level of Gal-1-P and a small increase in the level of UDP-Gal. These small fluctuations likely reflect the activities of GALT and UDP-glucose pyrophosphorylase functioning in the presence of restored levels of UTP and UDP-Glc. Finally, consistent with the results in Fig. 3, UDP-GalNAc levels remained near base line in all of the ldlD cultures, and uridine supplementation had no notable impact on any of the metabolites measured in ldlD cells cultured in the absence of galactose. Together these data clearly implicate uridine/UTP depletion as a key mediator of galactose sensitivity in GALE-impaired mammalian cells.

**DISCUSSION**

Although epimerase-deficiency galactosemia has been recognized for decades as a clinical disorder (7), this is, to our knowledge, the first report of studies directly testing the role of epimerase impairment in galactose metabolism and sensitivity in mammalian cells. Using as a model system derivatives of the epimerase-null CHO cell line ldlD (20, 21), we have asked three questions. 1) What metabolic changes are associated with galactose impairment in both the presence and absence of environmental galactose? 2) How much wild-type hGALE is required to relieve the galactose-specific abnormalities seen in ldlD cells? 3) What is the relationship, if any, among epimerase activity, substrate specificity, metabolites, and galactose sensitivity in mammalian cells?

Galactose Metabolism and Sensitivity in GALE-null Mammalian Cells—With regard to the first question posed, we assessed UTP and other relevant metabolite levels in ldlD cells grown in the presence and absence of both galactose and uridine. As presented in Fig. 8, the results were fully consistent with the hypothesis; UTP, UDP-Glc, and UDP-GlcNAc were all notably depleted in ldlD cells exposed to galactose, and uridine supplementation either fully, or at least partially, reversed all three abnormalities. In contrast, uridine supplementation failed to inhibit the abnormal accumulation of either Gal-1-P or UDP-Gal in these cells, although there was a small drop in the level of Gal-1-P and a small increase in the level of UDP-Gal. These small fluctuations likely reflect the activities of GALT and UDP-glucose pyrophosphorylase functioning in the presence of restored levels of UTP and UDP-Glc. Finally, consistent with the results in Fig. 3, UDP-GalNAc levels remained near base line in all of the ldlD cultures, and uridine supplementation had no notable impact on any of the metabolites measured in ldlD cells cultured in the absence of galactose. Together these data clearly implicate uridine/UTP depletion as a key mediator of galactose sensitivity in GALE-impaired mammalian cells.
clearly imply that no such backdoor exists for the interconversion of UDP-Gal and UDP-Glc in epimerase-null eukaryotes.

With regard to intracellular metabolites in GALE-null cells exposed to galactose, perhaps our most striking finding is not the abnormal accumulation of Gal-1-P and UDP-Gal, as these results recapitulate observations reported for both GALE-impaired patients (10) and GALE-null yeast (19), or the expected inability of ldlD cells to maintain normal levels of UDP-GalNAc in either the presence or absence of environmental galactose, but rather the inability of GALE-null cells to maintain normal levels of UDP-Glc and UDP-GlcNAc in the presence of high extracellular galactose. As a result, the GALE-null ldlD cells were unable to maintain normal UDP-GlcNAc/UDP-GalNAc ratios in the absence of galactose, or normal UDP-Glc/UDP-Gal and UDP-GlcNAc/UDP-GalNAc ratios in the presence of galactose (Table III). These data underscore the essential role of GALE, in both the presence and absence of environmental galactose, as a gatekeeper maintaining normal ratios of four key glycosylation substrates in mammals. These data are further consistent with the conclusion that UDP-GalNAc cannot be generated in ldlD cells from free galactose or other sources in the absence of GALE, as inferred previously (24).

As a measure of the in vivo response of GALE-null mammalian cells to galactose exposure, we monitored the incorporation of BrdUrd into wild-type and ldlD cells cultured in the presence versus absence of 0.25 mM galactose. Although this level of galactose is much higher than would be seen in the serum of normal infants, it is low compared with levels seen in the serum of galactosemic infants on milk formula, which can reach 10–20 mM (35). As illustrated in Fig. 2, unlike wild-type CHO cells, ldlD cells cultured under these conditions incorporated only about 25% as much BrdUrd in the presence of galactose as in its absence, and this impairment was galactose-specific. This result is important because it represents a quantifiable phenotypic abnormality in the ldlD cells and demonstrates that galactose exposure not only altered the metabolic profile of these cells; it made them sick.

Complementation of ldlD Cell Phenotypes by Normal Human GALE—The observation that normal levels of wild-type hGALE could bring ldlD cell galactose consumption and metabolites close, but not identical, to those seen in wild-type CHO cells is fully expected, given that ldlD cells were isolated after extensive mutagenesis of wild-type CHO cells. This result is also fundamental because it confirms that the galactose-specific defect in these cells is both GALE-specific and recessive.

GALE Substrate Specificity and Galactose Sensitivity in Mammalian Cells—The fact that wild-type mammalian GALE enzymes catalyze two reactions, the interconversion of UDP-Gal/UDP-Glc and UDP-GalNAc/UDP-GlcNAc, left open the question of how impairment of each of these two activities...
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CHO and ldlD cells were cultured in the presence versus the absence of galactose and assessed at different times for the concentration of internal metabolites, as described under “Materials and Methods.” *Ratios derived from separate experiments are listed; average ± S.E. could not be calculated because n < 3 (at least one ratio had a 0 denominator). ***All ratios could not be calculated because of 0 denominators.

| Strain | Ratio of UDP-Glc/UDP-Gal (n) | 0 h | 24 h | 72 h | 0 h | 24 h | 72 h |
|--------|-------------------------------|-----|-----|-----|-----|-----|-----|
| CHO    | 7.8 ± 4.6 (3)                | 5.9 ± 3.3 (3) | 1.75* | 7.8 ± 4.6 (3) | 7.4 ± 4.9 (3) | 2.0 ± 1.0* |
| ldlD   | 0.9*                         | 20.0, 4.0*     | 4.0*  | 0.9*                      | 0.0 ± 0.0 (3) | 0.0 ± 0.0 (3) |

| Strain | Ratio of UDP-GlcNAc/UDP-GalNAc (n) | 0 h | 24 h | 72 h | 0 h | 24 h | 72 h |
|--------|-----------------------------------|-----|-----|-----|-----|-----|-----|
| CHO    | 2.8 ± 0.2 (3)                     | 2.9 ± 0.0 (3) | 1.9 ± 0.9 (3) | 2.8 ± 0.2 (3) | 2.9 ± 0.0 (3) | 2.1 ± 0.4 (3) |
| ldlD   | ***                              | *** | *** | *** | *** | *** | *** |

Contributes to the phenotype of galactose sensitivity in GALE-deficient mammalian cells and patients. This point is especially pressing given that all five patients reported to date with severe (generalized) epimerase-deficiency galactosemia are homozygous for the V94M-hGALE allele, associated with 5-fold greater impairment of GALE activity toward UDP-Gal than toward UDP-GalNAc (14, 15).

Our results for cells expressing near normal levels of E. coli GALE activity demonstrated that UDP-GalNAc does not mediate the galactose-specific growth impairment of GALE-null cells in culture. It is important to note, however, that this result does not imply that UDP-GalNAc is irrelevant to the pathophysiology of epimerase-deficiency galactosemia.

**Roles of Specific Metabolites in Galactose Sensitivity**—The intracellular metabolites followed in this study, two (Gal-1-P, UDP-Gal) remain almost undetectable in wild-type cells regardless of the presence or absence of elevated environmental galactose, and three (UDP-Glc, UDP-GlcNAc, UDP-GlcNac) normally accumulate to significant levels in both the presence and absence of galactose (Fig. 3). We observed that GALE-null ldlD cells exposed to galactose accumulated high levels of Gal-1-P, and hGALE-underexpressing ldlD cells accumulated intermediate levels; none of the other lines accumulated detectable Gal-1-P. These data are consistent with prior patient reports (10) as well as with related studies from yeast (18, 19, 33, 36) that all either demonstrated or inferred a relationship between the degree of GALE impairment and the degree of abnormal Gal-1-P accumulation in the presence of exogenous galactose. Similarly, stable accumulation of very high levels of UDP-Gal was observed only in ldlD cells exposed to galactose and to a lesser extent also in ldlD[hGALE-low] cells. None of the other cell lines demonstrated any significant accumulation of UDP-Gal despite galactose exposure. As with Gal-1-P, accumulation of abnormally high levels of UDP-Gal therefore also correlated fully with galactose-compromised growth of GALE-impaired cells. As explained below, this large pool of UDP-Gal may represent a UDP-sink, leading to uridine depletion and a potential spectrum of negative downstream effects in the GALE-impaired cells exposed to galactose.

Levels of UDP-Glc and UDP-GlcNac were predominantly independent of the presence versus absence of exogenous galactose in wild-type CHO cells, ldlD[hGALE-wt] and ldlD[eGALE] cells, and were galactose-dependent in ldlD and ldlD[hGALE-low] cells. Galactose dependence of these metabolites (UDP-Glc and UDP-GlcNac) therefore correlated with galactose-specific growth impairment in these studies. In contrast, UDP-GalNac levels did not correlate with galactose impairment of cell growth in GALE-null cells. Nonetheless, the tight regulation of UDP-Glc/UDP-Gal ratios in patients and mammalian cells (38) and the tight ratios of UDP-GlcNac/UDP-GalNac demonstrated here for wild-type cells (Table III) suggest that these are important parameters that are carefully regulated.

**Impact of GALE Impairment on the Leloir Pathway: the Uridine Connection**—Among the metabolic abnormalities detected here which fully correlated with galactose-specific growth impairment of ldlD cells and their derivatives were elevated levels of two metabolites, Gal-1-P and UDP-Gal, and depleted levels of two others, UDP-Glc and UDP-GlcNac. Although a number of distinct hypotheses can be invoked to explain the relationship between these abnormalities, one seems particularly likely, namely that the Leloir pathway block at GALE prevented the “recycling” of UDP-Gal into UDP-Glc (Fig. 1) and that the accumulation of extraordinarily high levels of UDP-Gal further resulted in a UDP-sink, effectively depleting the cell of UTP/uridine. Because UTP is required for the de novo synthesis of UDP-Glc via UDP-glucose pyrophosphorylase and for the de novo synthesis of UDP-GlcNac, via UDP-N-acetylgalactosamine pyrophosphorylase, these cells were limited further in their ability to synthesize UDP-Glc and UDP-GlcNac. Substrate (UDP-Glc) limitation and product (UDP-Gal) inhibition in turn hampered the activity of GALT, thereby resulting in abnormal accumulation of Gal-1-P (Fig. 1). Finally, high Gal-1-P, acting as an inhibitor, further limited the abilities of UDP-glucose pyrophosphorylase and UDP-N-acetylgalactosamine pyrophosphorylase to generate UDP-Glc and UDP-GlcNac, respectively, as described previously (39). At very low rates each of these enzymes can utilize Gal-1-P or GalNac-1-P, coupled with UTP, to produce more UDP-Gal or UDP-GlcNac, respectively, although these reactions would compound the existing metabolic imbalance rather than relieve it. This proposed scenario not only explains the data presented here, but also is consistent with the existing body of literature describing selective depletion of UTP pools in mammalian tissues exposed to α-galactosamine (e.g. Ref. 31).

Our result, demonstrating that uridine supplementation relieves the galactose-specific growth inhibition of ldlD cells, is important because it supports the hypothesis that uridine depletion contributes to the galactose sensitivity of mammalian GALE deficiency. In addition, although we report here two relevant metabolic consequences of uridine depletion, namely UDP-Glc and UDP-GlcNac depletion, undoubtedly there are others, the most obvious of which may involve RNA synthesis. Studies are currently under way to explore this possibility.

The data reported here provide an important first step toward explaining, at least in part, the mechanism of pathophysiology in epimerase-deficiency galactosemia. Furthermore, these data offer the possibility of a novel intervention: dietary uridine supplementation. Considering that altered uridine me-
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tabolites, including abnormal ratios of UDP-Glc/UDP-Gal, are also characteristic of GALT-impaired cells and patients (7, 19, 35, 37), these results may prove significant with regard to the mechanism of pathophysiology in classic transferase-deficiency galactosemia as well.

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