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Expression of human inositol monophosphatase suppresses galactose toxicity in *Saccharomyces cerevisiae*: possible implications in galactosemia

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Abstract

A suppressor of galactose toxicity in a *gal7* yeast strain (lacking galactose 1-phosphate uridyl transferase) has been isolated from a HeLa cell cDNA library. Analysis of the plasmid clone indicated that the insert has an ORF identical to that of hIMPase (human *myo*-inositol monophosphatase). The ability of hIMPase to suppress galactose toxicity is sensitive to the presence of Li^+ in the medium. A *gal7* yeast strain harboring a plasmid containing cloned hIMPase grows on galactose as a sole carbon source. hIMPase mediated galactose metabolism is dependent on the functionality of *GAL1* as well as *GAL10* encoded galactokinase and epimerase respectively. These results predicted that the UDP-glucose/galactose pyrophosphorylase mediated pathway may be responsible for the relief of galactose toxicity. Experiments conducted to test this prediction revealed that expression of *UGP1* encoded UDP-glucose pyrophosphorylase can indeed overcome the relief of galactose toxicity. Moreover, expression of *UGP1* allows a *gal7* strain to grow on galactose as a sole carbon source. Unlike the hIMPase mediated relief of galactose toxicity, *UGP1* mediated relief of galactose toxicity is lithium insensitive. Based on our results and on the basis of available information on galactose toxicity, we suggest an alternative explanation for the molecular mechanism of galactose toxicity.

Keywords: Galactosemia; Galactose toxicity; Inositol monophosphatase; UDP-glucose pyrophosphorylase; *Saccharomyces cerevisiae*

1. Introduction

Conversion of galactose to glucose is a evolutionarily conserved process. This metabolic process is mediated by the Leloir enzymes and deficiency in any of these enzymes gives rise to an inherited autosomal recessive disorder called galactosemia [1]. It occurs at a frequency of 1:30 000 to 1:60 000 in the general population [1,2]. Galactosemia has been categorized into three types, of which classical galactosemia is the most common form which is due to the deficiency of galactose 1-phosphate uridyl transferase encoded by *GALT* [1,2].

Classical galactosemia, a potentially lethal disorder, is well studied and yet not well understood in terms of the metabolic and biochemical basis of its diverse phenotypes [3]. Available data strongly sug-

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gest that accumulation of galactose 1-phosphate (Gal-1-P) is the primary cause of classical galactosemia [4]. While the most common treatment of dietary restriction of galactose relieves acute symptoms, long term defects such as developmental deficiencies, ovarian failure, mental disabilities, speech defects, etc. still persist [1–3]. Although the biochemical basis of Gal-1-P mediated toxicity is not clearly understood, it is becoming increasingly clear that the long term defects observed in treated patients are due to the endogenous production of Gal-1-P [4].

Studies in model systems only serve to emphasize the need to develop an animal model, such as a transferase deficient mouse, to decipher the underlying biochemical mechanisms of classical galactosemia [4]. Since the *GALT* deficient mouse does not manifest any of the symptoms shown by the patients suffering from galactosemia [5], a better defined genetic model is needed to understand the biochemical and metabolic basis of galactosemia.

The yeast *Saccharomyces cerevisiae* has played a significant role in the cloning, expression and the study of structure-function relationship of Leloir enzymes from humans [6–10]. Analogous to humans, a yeast strain defective in *GAL7* (corresponding to *GALT* in humans) exhibits galactose toxicity in the presence of galactose and is unable to grow on alternative carbon sources such as glycerol due to the accumulation of Gal-1-P [11]. This feature, and given that yeast is a genetically and biochemically more amenable system, prompted us to examine some facets of galactosemia.

Genetic suppression of galactose toxicity due to the reduction in the expression of *GAL1* promoter mediated function has been used to isolate genes that encode regulatory proteins [12,13]. We reasoned that it may be possible to isolate genes from humans that can suppress the above phenotype, by reducing the formation or accumulation of Gal-1-P below the threshold required to manifest galactose toxicity. Characterization of the suppressors that sequester, hydrolyze or metabolize to reduce Gal-1-P accumulation was expected to provide valuable information towards understanding the biochemical basis of classical galactosemia. This attempt resulted in the isolation of human *myo*-inositol monophosphatase (hIMPase) as the suppressor of galactose toxicity in yeast. Biochemical and genetic studies were carried out to define the mechanism of this suppression. These studies suggest that hIMPase might be an important target for the Gal-1-P mediated toxicity syndrome in classical galactosemia.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli strains DH5α and XL1-Blue MRF′ were used for routine recombinant DNA work. Yeast strains YM147 (α *ade 2-101 ura 3-52 trp 1-289 gal1)* [14], Sc289 (α *ade2-101 ura3-52 trp 1-289 gal7Δ102*) and Sc652 (*a ade 2-101 ile ura3-52 leu 2-3,112 trp 1-HIII, his3Δ-1 MEL1*) [15] were gifts from James E. Hopper. Yeast strain PJB5 (*a ade 2-101 ile ura3-52 leu 2-3,112 trp 1-HIII, his3Δ-1 MEL1-gal10::LEU2*) is derived from Sc652 by disrupting the *GAL10* locus as described [14]. Plasmids pUC19 and YEp*lac*181 [16] were used for general cloning purposes. pWJ220 carries a cassette to disrupt the genomic locus of *GAL10* [14]. Plasmid pYEJBGAL1 carries the yeast *GAL1* gene under the *CYC1* promoter in YEp24 [17]. The *GAL7* gene in YEp24 (*GAL7*) isolated from yeast genomic library was a gift from James E. Hopper. Yeast expression vectors p416TEF (p416) and p426TEF (p426) were gifts from Martin Funk [18]. These expression vectors are pBLUESCRIPT based plasmids carrying TEF promoter for expression of homologous/heterologous proteins in yeast. Plasmids p416 and p426 carry *URA3* as the selectable marker for yeast. All of them carry an ampicillin resistance marker for propagation in *E. coli*. Plasmid p416 is a low-copy expression vector whereas p426 is multi-copy expression vector. pNV11, a human cDNA expression library, was a gift from Kuniharo Matsumoto [19]. It is a 2 μm based multi-copy shuttle vector consisting of *URA3* as the selectable marker for yeast and ampicillin resistance marker for *E. coli*. The cDNAs were expressed from yeast *TDH* promoter. Plasmid pJMGal2μ (gift from J. François) carries *UGP1* under the control of the *GAL10*/*CYC1* promoter. It has *URA3* and ampicillin resistance as the selectable marker for yeast and *E. coli*, respectively [20].

2.2. Chemicals

X-gal (5-gromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-α-D-thiogalactopyranoside) were purchased from Genei (Bangalore, India). T4 DNA ligase, DNA polymerase (Klenow fragment), CIAP (calf intestinal alkaline phosphatase), restriction enzymes and dNTPs were purchased from Amersham (Arlington Heights, IL, USA). Yeast extract, Bacto-peptone, nitrogen base without amino acids were bought from Difco (Detroit, MI, USA). Agar, Tris-HCl, EDTA, BSA, galactose, galactose 1-phosphate, glucose, amino acids, ATP, *p*-nitrophenyl phosphate (*p*NPP), $MgCl_2$ were purchased from Sigma (St. Louis, MO, USA) and other chemicals used in this study were of the highest grade commercially available. ^{14}C-Labelled galactose 1-phosphate was purchased from DuPont NEN (catalog number NEC579) and had a specific activity of 352.7 mCi/mmol and a concentration of 0.02 mCi/ml.

2.3. Media and growth conditions

Yeast strains were propagated and maintained as described [21]. Yeast strains were propagated either in YEPD (0.5% yeast extract, 1% Bacto-peptone and 2% glucose) or synthetic media. Synthetic media were prepared by mixing either complete amino acids mixture (Comp) or amino acids mixture lacking either uracil (Ura^-) or leucine (Leu^-) with yeast nitrogenous base without amino acids. Final concentrations of amino acid mixture and yeast nitrogen base were 700 mg/l and 6.7 g/l, respectively. Carbon sources were added to the synthetic media to final concentrations of 2% (w/v) glucose (Glu), 3% (v/v) glycerol (Gly), 1.5% (w/v) galactose (Gal) or Gly+0.05% (w/v) galactose (Gly/Gal). *E. coli* strains were grown in LB (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) medium for general propagation and ampicillin at a final concentration of 100 μg/ml was supplemented when required for plasmid maintenance. Yeast strains were transformed by the lithium acetate method while *E. coli* competent cells were made using $CaCl_2$ [22].

2.4. Isolation of pDKJ132 as a suppressor of galactose toxicity

Galactose is toxic to Sc289 and hence it cannot utilize alternative carbon sources such as glycerol in the presence of galactose. This is because of Gal-1-P accumulation in the absence of *GAL7* encoded galactose 1-phosphate uridyl transferase [11]. It was transformed with pNV11 (cDNA library) and transformants capable of growing in Ura^- Glu medium were selected. These transformants were then replicated onto Ura^- plates containing 0.05% galactose and 3.0% glycerol (Ura^- Gly/Gal). Transformants capable of growing were picked up from approximately 300 000 transformants and subjected to a plasmid-loss experiment. This method differentiates between spontaneous genomic mutations and plasmid linked suppression of galactose toxicity. Only one transformant showed plasmid linked suppression of galactose toxicity. The plasmid from the above transformant was isolated and was named pDKJ132. Retransformation of Sc289 with pDKJ132 confirmed that suppression of galactose toxicity is a plasmid-borne trait.

2.5. Recombinant DNA work

All recombinant DNA manipulations were done as described [22]. pDKJ132-181 was constructed by cloning the 2.2 kb *Xho*I fragment, containing the full length cDNA insert of pDKJ132, at the *Sal*I site of YEp*lac*181. pDKJ132Δ*Xho*I is a derivative of pDKJ132 lacking the 2.2 kb cDNA insert. It was constructed by digesting pDKJ132 with *Xho*I and religating the vector backbone. The cDNA insert was cloned at the unique *Sal*I site of p416TEF and p426TEF to give p416-132 and p426-132, respectively.

2.6. DNA sequencing

Plasmid pDKJ132-181 was sequenced at the automated DNA sequencing facility at the Indian Institute of Science, Bangalore, India. M-13 forward and reverse primers as well as internal primers PJB1p (5′-CCA GAG ACT GTG AGA ATG G-3′), PJB4p (5′-CCA TTC TCA CAG TCT CTG G-3′), PJB9p (5′-GGA TGT ATG ATA TGC CTG G-3′), PJB10p

(5′-CCA GGC ATA TCA TAC ATC C-3′), PJB11p (5′-CAG AAT CCA GCG GCA ATG-3′), PJB12p (5′-CAT TGC CGC TGG ATT CTG-3′) were used to sequence pDKJ132-181.

2.7. Enzyme assays

IMPase assays were performed with cell extracts made from yeast transformants harboring appropriate plasmids, using *p*NPP as the substrate [23]. Mid log phase cells of Sc289 transformed with yeast expression plasmids were used to prepare cell extracts using the glass bead method. Since hIMPase has been shown to be heat stable [24], heat shock was given for 15 min at 75°C and the protein precipitate was removed by centrifugation at 10 000 rpm at 4°C for 20 min. The supernatant was used for the enzyme assays. The above protocol had to be used to reduce the non-specific hydrolytic activity present in the cell extract. Different volumes of cell extracts were added to a 0.5 ml reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 0.1 mM EGTA. *p*NPP was used as the substrate at a final concentration of 5 mM. The reaction was terminated by adding 0.9 ml of a 0.1 M KOH solution to 0.1 ml of reaction mixture. LiCl at 100 mM was used in the reaction to measure the lithium sensitive activity. The reaction was found to progress at a linear rate for at least 5 h. The reaction was carried out for 3 h at 37°C. Absorbance at 405 nm was recorded as a measure of enzymatic activity, to determine the amount of *p*-nitrophenol released.

The assay for UDP-galactose pyrophosphorylase was done according to the protocol developed by Abraham and Howell [25]. Sc289 cells harboring appropriate plasmids were grown in medium containing Ura^- Gly until mid log phase. Galactose was added to cells harboring cloned IMPase or *UGP1* to a final concentration of 0.1% and were subsequently allowed to grow for a further 2 h. Cells were harvested and cell extracts were prepared using glass beads. The extracts obtained were used for enzyme assays. The reaction mixture contained 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 5 mM DTT, 4 mM UTP and 19 μM Gal-1-P. The Gal-1-P solution was made by mixing 100 μl of ^{14}C-labelled Gal-1-P (specific activity 352.7 mCi/mmol and concentration 0.02 mCi/ml) to 900 μl of 15 μM 'cold' Gal-1-P. As IMPase has been shown to hydrolyze Gal-1-P, LiCl at a concentration of 10 mM was added, to inhibit this reaction. The reaction was initiated by the addition of 10 μl of cell extract to 10 μl of pre-mix solution. The activity was found to be linear for at least 1 h. All reactions were carried out at 30°C, for 30 min and in quadruplicate. The reaction was stopped by heat inactivation. One unit of alkaline phosphatase suspended in 2 μl buffer was added to each tube and incubated at 37°C for 30 min to hydrolyze unused Gal-1-P remaining in the solution. An independent experiment had shown that 1 U of alkaline phosphatase was sufficient to hydrolyze the remaining Gal-1-P. The phosphatase reaction was stopped by heat inactivation. An 11 μl sample from the reaction volume was applied to a DEAE-cellulose disc (DE-81, 2.4 cm in diameter, Whatman). The disc was dried at 37°C for about 2 h and then washed with double distilled water thrice to remove unbound sugar. The disc was dried again before measuring the radioactivity in the DE-81 paper containing UDP-galactose formed during the reaction. The radioactivity incorporated was counted using a LKB liquid scintillation counter. Upon repeating the experiments in quadruplicate for three times a deviation of 10% was observed. Protein was estimated by the Bradford assay [26] with appropriately diluted protein samples. The OD was measured at 595 nm with BSA as standard.

3. Results

3.1. hIMPase is the suppressor of galactose toxicity

Preliminary restriction enzyme analysis of the plasmid clone pDKJ132 revealed that the cDNA insert can be liberated as a 2.2 kb *Xho*I fragment. Plasmid pDKJ132-181 was used as a template to sequence both the strands of the ORF present in the cDNA insert. The DNA sequence was used to search for homologous sequences in GenBank using the BLAST. The sequence was found to be identical to the reported hIMPase sequence [24]. Since the size of the cDNA present in pDKJ132 was found to agree with the size of hIMPase mRNA [24], we inferred that the cloned gene represents the full length cDNA of hIMPase. To examine the dosage effect,

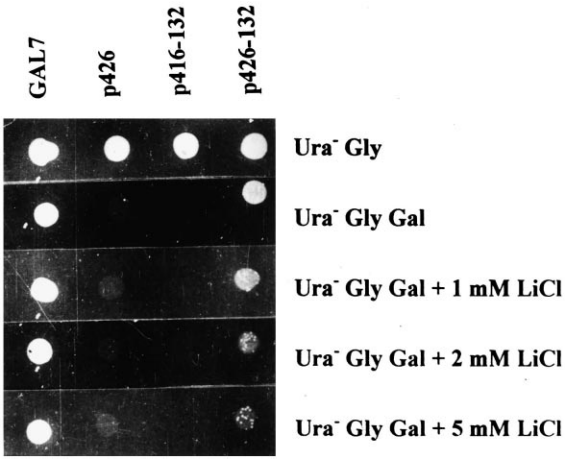

Fig. 1. Relief of galactose toxicity in Sc289 harboring hIMPase and effect of lithium. 10^5 cells of Sc289 transformants bearing plasmids GAL7 (GAL7 gene in YEp24), p426 (control), p416-132 (low-copy) and p426-132 (multi-copy) were spotted on to Ura$^-$ Gly or Ura$^-$ Gly/Gal plates with different concentrations of LiCl as shown.

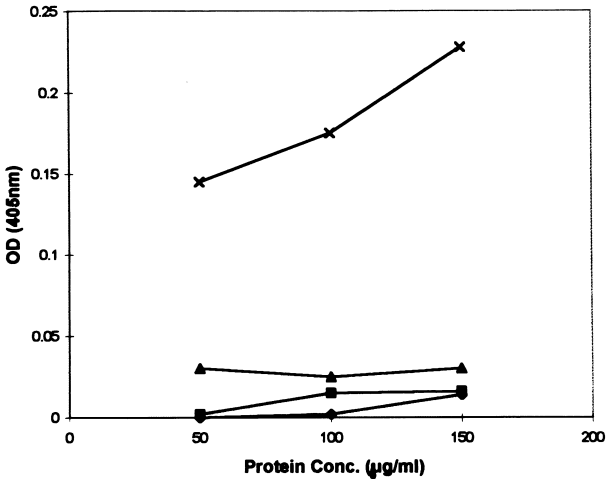

Fig. 2. Determination of hIMPase activity. *p*NPP hydrolytic activity of cell extracts from cells bearing pDKJ132, in the absence (×) and presence of 100 mM LiCl (△). □ and ◇ represent hydrolytic activity from cells bearing pDKJ132Δ*Xho*I with and without LiCl respectively.

the full length cDNA was sub-cloned into low-copy as well as multi-copy yeast expression vectors. It was found that expression from a multi-copy expression vector is necessary to relieve galactose toxicity (Fig. 1, compare p416-132 with p426-132).

Lithium salts are known to inhibit IMPase activity in vitro [27]. In order to test whether the ability of hIMPase to suppress galactose toxicity was sensitive to Li salts, various concentrations of LiCl were added to the media (Fig. 1). It was observed that Li^+, even at a concentration of 2 mM, restores galactose toxicity. Suppression of galactose toxicity by hIMPase was found to be sensitive to LiCl at concentrations close to its IC_{50} (1.6 mM) [28]. These results suggest that IMPase activity per se is required for the relief of galactose toxicity.

To confirm that hIMPase is expressed in yeast, enzyme assays were performed on extracts obtained from Sc289 cells bearing either the plasmid pDKJ132 or pDKJ132Δ*Xho*I (vector control). The hydrolytic activity in extracts obtained from cells bearing hIMPase was found to increase as a function of protein concentration (Fig. 2). The activity was also found to be inhibited by Li^+ confirming that the hydrolytic activity is due to the expression of hIMPase.

It is clear from our studies that overexpression of hIMPase relieves galactose toxicity in a *gal7* yeast strain through one or more of the following mechanisms: (a) IMPase facilitates metabolism of galactose, thereby reducing Gal-1-P accumulation, (b) IMPase reduces the accumulation of Gal-1-P. This could be due to interference with galactose uptake, expression of galactokinase, etc. (c) A combination of the above two possibilities.

3.2. *hIMPase bypasses a gal7 block in galactose metabolism*

If relief of galactose toxicity in Sc289 bearing

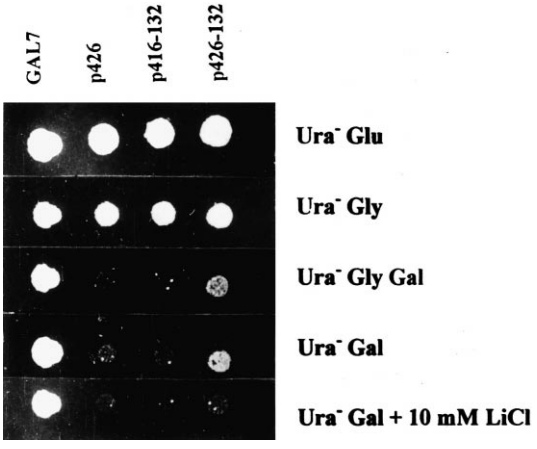

Fig. 3. Utilization of galactose as the sole carbon source by Sc289 transformants harboring hIMPase. Sc289 transformants bearing indicated plasmids grown in Ura$^-$ Glu were replicated onto different plates as indicated.

hIMPase is due to galactose metabolism, it is expected that Sc289 bearing hIMPase would grow in galactose as a sole carbon source. Sc289 bearing hIMPase (p426-132) was indeed able to grow on galactose as the sole carbon source (Fig. 3), and its growth on galactose was sensitive to lithium (Fig. 3), indicating involvement of hIMPase in this process. This result clearly suggests that IMPase does not interfere in the uptake of galactose. The fact that Sc289 bearing hIMPase can utilize galactose as the sole carbon source indicates that either galactose or Gal-1-P is being metabolized. In either case galactose toxicity is expected to be abolished.

3.3. *hIMPase cannot bypass a gal1 or gal10 block in galactose metabolism*

To determine whether galactose or Gal-1-P is being metabolized, YM147, a *gal1* yeast strain (lacking galactokinase), was transformed with either vector alone or with a plasmid bearing hIMPase. If the transformant bearing hIMPase grows in galactose, then it can be inferred that galactose and not galactose 1-phosphate is metabolized. On the other hand, if it does not grow in galactose, it can be inferred that Gal-1-P is metabolized. It was found that YM147 bearing hIMPase expression plasmid, p426-132, could not grow on galactose as the sole carbon source (Fig. 4). This result suggested that YM147 bearing hIMPase metabolizes Gal-1-P rather than galactose.

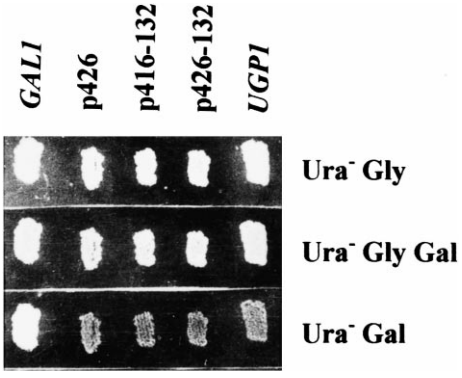

Fig. 4. Effect of expression of hIMPase and *UGP1* in YM147 (*gal1*). Strain YM147 transformants bearing *GAL1* (pYEJB-GAL1), p426 (control), p416-132 (low-copy) and p426-132 (multi-copy) and UGP1 (pJMGal2μ) were replicated onto different plates as indicated.

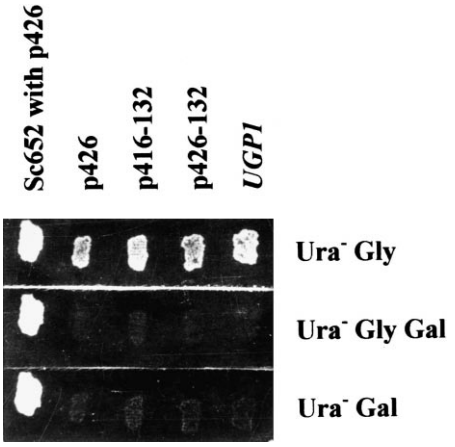

Fig. 5. Effect of hIMPase and *UGP1* expression in PJB5 (*gal10*). Strain PJB5 transformants bearing plasmids p426 (control), p416-132 (low-copy), p426-132 (multi-copy) and UGP1 (pJMGal2μ) were replicated onto different plates as indicated. Wild-type strain Sc652 (parent strain of PJB5), transformed with p426 (control plasmid), was taken as the positive control.

If hIMPase metabolizes Gal-1-P independent of *GAL10* encoded epimerase, it is expected that a *gal10* yeast strain harboring hIMPase would grow on galactose, similar to a *gal7* yeast strain. However, PJB5, a *gal10* yeast strain, harboring hIMPase was unable to grow in the presence of galactose (Fig. 5). This result demonstrates that *GAL10* encoded epimerase function is necessary for IMPase mediated metabolism of Gal-1-P in a *gal7* yeast strain. The relief of toxicity in Sc289 by hIMPase could be because of the reduction of Gal-1-P due to its metabolism. Alternatively, in Sc289 bearing hIMPase, galactose metabolism occurs due to the relief of galactose toxicity and galactose metabolism per se may not be responsible for the relief of galactose toxicity.

3.4. *Expression of hIMPase modulates UDP-galactose pyrophosphorylase activity*

Regardless of the cause and effect relationship between Gal-1-P toxicity and Gal-1-P metabolism, it was necessary to determine the pathway of Gal-1-P metabolism in Sc289 harboring multiple copies of hIMPase. The only possible route for the metabolism of Gal-1-P other than *GAL7* but involving epimerase reaction is mediated by UDP-galactose pyrophosphorylase. The reaction catalyzed by UDP-galactose

pyrophosphorylase (EC 2.7.7.10) is:

$$\text{Gal-1-P} + \text{UTP} \leftrightarrow \text{UDP-Gal} + \text{PPi}$$

Although the activity of this enzyme in yeast was reported in 1953 [29], to date the gene encoding this enzyme has not been identified. It is assumed that the UDP-glucose pyrophosphorylase has a broader substrate specificity and can also catalyze the reaction of UDP-galactose pyrophosphorylase [30,31]. Thus, in a *gal7* background, Gal-1-P can be metabolized by a combination of two enzyme activities, epimerase encoded by *GAL10* and UDP-glucose/galactose pyrophosphorylase encoded by *UGP1*, as shown below.

$$\text{Gal-1-P} \underset{UGP1}{\leftrightarrow} \text{UDP-Gal} \underset{GAL10}{\leftrightarrow} \text{UDP-Glu} \underset{UGP1}{\leftrightarrow} \text{Glu-1-P}$$

If such a scheme is operational in a *gal7* yeast strain harboring IMPase, then neither a *gal10* nor *a gal1* yeast strain harboring IMPase is predicted to grow on galactose. Our results are consistent with these predictions (see Section 3.3). A *gal7* yeast strain harboring IMPase in the presence of galactose would have sufficient galactokinase (*GAL1* gene product) to convert galactose to Gal-1-P as well as epimerase (*GAL10* gene product) to convert UDP-Gal to UDP-Glu. However, a *gal7* strain, otherwise wild-type, is not able to grow on galactose unless IMPase is overexpressed. It is possible that the endogenous UDP-glucose/galactose pyrophosphorylase may not be sufficient or active to metabolize Gal-1-P in a *gal7* yeast strain.

Table 1
Determination of UDP-galactose pyrophosphorylase activity in vitro

Plasmid	Carbon source	Activity[a]
p426 (control)	Glycerol	25 (100%)
p426 (control)	Glycerol+galactose	34 (139%)
p426-132 (hIMPase)	Glycerol	33 (135%)
p426-132 (hIMPase)	Glycerol+galactose	44 (180%)
pJMGal2μ (UGP1)	Glycerol	37 (100%)
pJMGal2μ (UGP1)	Glycerol+galactose	183 (489%)

Enzyme assays were carried out using cell extracts obtained from Sc289 transformants bearing indicated plasmids as mentioned in Section 2. Numbers in parentheses indicate relative activity.

[a]Activity is expressed as pmol of UDP-galactose formed per mg protein per 30 min.

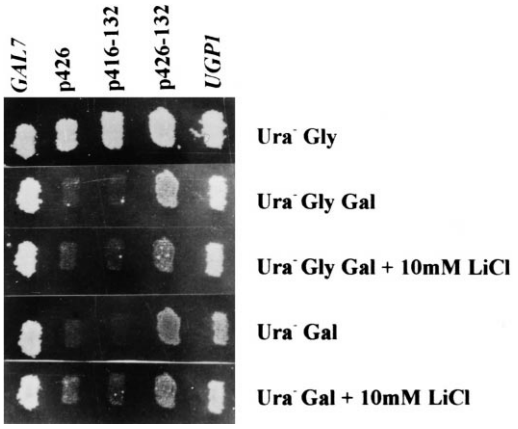

Fig. 6. Effect of *UGP1* and hIMPase expression in Sc289. Sc289 transformants bearing GAL7 (*GAL7* in YEp24), p426 (control), p416-132 (low-copy), p426-132 (multi-copy) and UGP1 (pJMGal2μ) were replicated onto different plates as indicated.

Therefore, we wanted to determine the role of *UGP1*, encoding UDP-glucose pyrophosphorylase, in the alternative pathway of galactose metabolism. For this purpose, yeast strains Sc289 (*gal7*), YM147 (*gal1*) and PJB5 (*gal10*) were transformed with plasmid pJMGal2μ expressing *UGP1* under a *GAL10/CYC1* promoter and the effect on galactose toxicity was determined. It was found that overexpression of *UGP1*, like hIMPase, relieves galactose toxicity in Sc289 (Fig. 6). Consistent with our hypothesis, *UGP1* expression in a *gal1* or *gal10* background did not confer growth on galactose (Figs. 4 and 5).

If *UGP1* relieves galactose toxicity by metabolizing galactose 1-phosphate, then it is expected that a *gal7* mutant strain bearing only *UGP1* should grow on galactose as a sole carbon source. However, it has been shown that overexpression of *UGP1* under the *GAL10* promoter causes retarded growth in the presence of 2% galactose as the sole carbon source [20]. Therefore, we determined whether a *gal7* strain bearing pJMGal2μ could grow on $<2\%$ galactose. *gal7* strain bearing *UGP1* was found to grow well on 0.25% galactose as a sole carbon source (Fig. 6). The above pathway has been proposed to be responsible for the metabolism of galactose in a galactosemic patient [32] and our results represent a parallel extension in yeast.

We also considered the possibility that overexpres-

sion of IMPase relieves galactose toxicity by modulating UDP-glucose/galactose pyrophosphorylase activity. To test this, we examined the level of UDP-galactose pyrophosphorylase activity from yeast cells harboring IMPase. UDP-galactose pyrophosphorylase activity was found to be elevated in cells harboring hIMPase (Table 1) as compared to the vector control. However, enzyme activity was also higher in cells grown in galactose as compared to glycerol alone. The activity was substantially higher in cells bearing hIMPase compared to its absence and also grown in presence of galactose. These results indicate that both hIMPase and galactose positively modulate activity of UDP-galactose pyrophosphorylase independently. As a positive control, UDP-galactose pyrophosphorylase activity was determined in cells harboring *UGP1* in the presence of glycerol and galactose (Table 1). Our results are consistent with the earlier observation that UDP-glucose pyrophosphorylase also has UDP-galactose pyrophosphorylase activity [30,31].

4. Discussion

To date, the biochemical basis underlying galactose toxicity in classical galactosemia remains an enigma. This is largely due to the lack of proper experimental models to understand the biochemical and metabolic basis of galactose toxicity. Based on cyclical phosphorylation and dephosphorylation of galactose in uridyl transferase deficient human fibroblasts [33], red blood cells [34,35] and chicken brain [36], it has been suggested that the toxicity is due to depletion of energy. Another proposal is that the toxicity is due to the inhibition of the enzymes of carbohydrate metabolism by Gal-1-P. This view is based mainly on observations in vitro (reviewed in [4]). Impairment in the galactosylation of glycolipids and glycoproteins of various tissues suggested that it could be another causative factor for the long term neurological defects observed in galactosemic patients. Results of GALT expression in rats during the embryonic and postnatal periods are consistent with the above proposal ([37], see review [3]).

To understand the molecular basis of galactosemia, mice deficient in GALT have been generated [5]. These mice do not have symptoms that are reminiscent of galactosemia despite the accumulation of comparable amounts of Gal-1-P in the tissues. Based on this, it has been suggested that *GALT* deficient mice could serve as a better model, if the reasons for the lack of symptoms can be determined. It seems unlikely that the toxicity in galactosemic patients is only due to Gal-1-P accumulation. If this were the case, *GAL7* deficient mice that accumulate comparable amounts of Gal-1-P as humans should also have been affected.

Based on our observations, we suggest that IMPase is one of the key elements of Gal-1-P mediated toxicity in galactosemic patients. Our observation is further supported by the following facts. (a) It has been observed that inositol is depleted in galactosemic patients (reviewed in [38]). (b) Recently, based on the observation that Gal-1-P is as good a substrate as inositol monophosphate for IMPase from human, rat and bovine brains, it has been suggested that the function of IMPase must include a possible role for IMPase in the regulation of galactose metabolism in vivo [39]. (c) Our observation that overexpression of hIMPase alleviates galactose toxicity in a lithium sensitive manner suggests that IMPase function is important for the modulation of intracellular levels of Gal-1-P.

In light of our observations and available data, we suggest a novel biochemical mechanism for the toxic effects of Gal-1-P in galactosemic patients. In galactosemic patients, accumulated Gal-1-P could act as a substrate for IMPase, thereby interfering with its normal function. The phosphoinositide mediated cell signalling plays a crucial role in the regulation of a variety of biological processes. The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C generates D-1,4,5-inositoltriphosphate (1,4,5-IP_3) and diacylglycerol which act as second messengers in the target cell. 1,4,5-IP_3 finally gives rise to inositol 4-phosphate, inositol 1-phosphate and inositol 3-phosphate [40]. IMPase plays a crucial role in the hydrolysis of these inositol monophosphates to free inositol which is then reused for the synthesis of PIP_2. This recycling is particularly important in the brain, since (a) free inositol does not enter the brain due to the blood-brain barrier and (b) the brain has a limited capacity for de novo synthesis of inositol (reviewed in [41]). Excess Gal-1-P accumulation in galactosemic patients could interfere

with the normal function of IMPase thereby causing a disturbance in the phosphoinositide mediated signal transduction process. In consonance with this function of IMPase, galactosemic patients are found to suffer from neurological abnormalities such as mental retardation, learning disabilities, etc.

The alleviation of toxicity in Sc289 by *UGP1* overexpression may be due to the utilization of Gal-1-P in the presence of UDP-glucose pyrophosphorylase so that Gal-1-P may not accumulate above the threshold required to interfere with the endogenous IMPase function. As a corollary, in a *gal7* strain otherwise normal with respect to IMPase, endogenous UDP-glucose pyrophosphorylase may not be sufficient to reduce the Gal-1-P concentration below the threshold. However, when hIMPase is overexpressed in Sc289, Gal-1-P toxicity is relieved, and endogenous UDP-glucose pyrophosphorylase is sufficient to provide growth on galactose as the sole carbon source. Therefore, we suggest that it is the relative concentrations of Gal-1-P, IMPase and UDP-glucose pyrophosphorylase that determine the status of toxicity. It has been proposed that UDP-galactose pyrophosphorylase, which constitutes only 5% of galactose 1-phosphate uridyl transferase in adult liver, is engaged in the production of Gal-1-P while in galactosemic patients it might be responsible for the metabolism of Gal-1-P [42]. However, the amount of UDP-galactose pyrophosphorylase in fetal blood was almost 50% of uridyl transferase [42]. The fact that galactosemic patients do have the ability to metabolize galactose, albeit at a slower rate [43], is consistent with the proposal that UDP-galactose pyrophosphorylase may be involved in the utilization of Gal-1-P. The observation that *GALT* knockout mice are normal despite the accumulation of Gal-1-P suggests that mechanisms other than galactose metabolism could exist for the relief of galactose toxicity in mice. It is conceivable that in mice accumulation of Gal-1-P may not interfere with the normal function of IMPase in signal transduction pathway. Therefore, to understand the biochemical mechanism of galactose toxicity better, it is important to dissociate galactose utilization from relief of Gal-1-P toxicity.

How does Gal-1-P interfere with the normal functioning of IMPase to cause toxicity in yeast? Like mammalian cells, yeast cells posses phosphoinositide signalling mechanism and this pathway has been shown to be essential for cell proliferation [44]. Inhibition of IMPase using Li^+ has been shown to affect biological processes such as development and/or differentiation in amphibia, zebra fish, sea urchin and slime mold [45]. In *E. coli*, it has been shown that a mutation in the *suhB* gene product which encodes inositol monophosphatase affects protein export, stress response, and DNA replication [46]. If IMPase also has similar functions in yeast, interference with this is expected to cause toxicity. Therefore, IMPase function in yeast for Gal-1-P mediated toxicity warrants further investigation.

Our data provide new insights into the reasons why Gal-1-P exerts toxic effects in galactosemic patients. On the basis of our experiments we suggest that in addition to the primary defects that may result in Gal-1-P accumulation, impairment in other enzymes such as IMPase UDP-galactose pyrophosphorylase and other Gal-1-P hydrolyzing enzymes singly or in combination could exacerbate the spectrum of toxic effects. Further investigations in this direction are under way.

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