Identification of Human Gene Products Containing Pro-Pro-x-Tyr (PY) Motifs that Enhance Glutathione and Endocytotic Marker Uptake in Yeast

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PY-motif • Yeast • Membrane • Transport • Endocytosis • Glutathione

Abstract
In an attempt to identify genes involved in glutathione (GSH) transport, a human mammary gland cDNA library was screened for clones capable of complementing a defect in GSH uptake in yeast cells that lack Hgt1p, the primary yeast GSH uptake transporter. Five genes capable of rescuing growth on sulfur-deficient GSH-containing medium were identified: prostate transmembrane protein, androgen induced 1 (PMEPA1); lysosomal-associated protein transmembrane 4 alpha (LAPTM4α); solute carrier family 25, member 1 (SLC25A1); lipopolysaccharide-induced TNF factor (LITAF); and cysteine/tyrosine-rich-1 (CYYR1). All of these genes encode small integral membrane proteins of unknown function, although none appear to encode prototypical GSH transporters. Nevertheless, they all increased both intracellular glutathione levels and [3H]GSH uptake rates. [3H]GSH uptake was uniformly inhibited by high concentrations of unlabeled GSH, GSSG, and ophthalmic acid. Interestingly, each protein is predicted to contain Pro-Pro-x-Tyr (PY) motifs, which are thought to be important for regulating protein cell surface expression. Uptake of the endocytotic markers lucifer yellow and FM4-64 was also enhanced by each of the five genes. Mutations of the PY motifs in LITAF largely abolished all of its effects. In summary, although the results do not reveal novel GSH transporters, they identify five PY-containing human gene products that may influence plasma membrane transport activity.

Introduction
Glutathione (GSH) is a major intracellular thiol, and is present in virtually all eukaryotic and most prokaryotic cells at concentrations ranging from 0.5 to 10 mM. Maintenance of GSH levels is required for many critical cell processes, and disturbances in GSH homeostasis have been implicated in the etiology and/or progression of a number of human diseases, including cancer, diseases of aging, and cardiovascular, inflammatory, immune, liver and...
neurodegenerative diseases [1]. Intracellular and extracellular GSH concentrations are normally tightly regulated to maintain normal physiological functions, and the two major mechanisms involved in this regulation are GSH biosynthesis and GSH efflux from cells [2]. Despite the increasing evidence that some of the multidrug resistance-associated proteins (MRP/ABCC) mediate GSH export, it is unknown whether the MRPs are the sole or even the major GSH exporters in human cells. Given the important functions of GSH in multiple cellular processes, it is possible that additional GSH transporters are present in the human genome, but have not yet been identified at molecular level [3, 4].

With the aim of identifying additional GSH transporters, we screened a human mammary gland cDNA library using an expression cloning strategy in yeast. Unlike mammalian cells, S. cerevisiae are able to take up GSH from the extracellular medium, and this uptake is mediated primarily by a high affinity GSH uptake transporter, Hgt1p [5]. In addition to Hgt1p, a low-affinity GSH uptake mechanism is present in yeast cells; however, the gene or genes responsible for this alternate GSH uptake process have not yet been identified [5, 6]. Orthologues of HGT1 are not present in higher eukaryotic organisms. The deletion of the HGT1 gene in S. cerevisiae leads to a marked impairment in the ability to grow in media in which the growth deficiency. Because the present screen tested GSH accumulation by these cells and therefore rescue the growth deficiency. Because the present screen tested for genes that lead to enhanced GSH uptake in yeast, it is unable to identify unidirectional GSH exporters, but should theoretically be able to identify bidirectional GSH transporters (e.g., secondary active transporters) or unidirectional GSH uptake transporters.

Five genes capable of complementing the HGT1 deletion were recovered, providing clues into the potential functions of human PMEPA1 (prostate transmembrane protein, androgen induced 1), LAPTM4α (lysosomal-associated protein transmembrane 4 alpha), SLC25A1 (solute carrier family 25, member 1), LITAF (lipopolysaccharide-induced TNF factor), and CYYR1 (cysteine/tyrosine-rich1). Because each of the encoded proteins is predicted to have PY motifs, which are thought to serve as binding sites for the ubiquitin ligases yeast Rsp5 (reverses Spt-phenotype 5) or human NEDD4 (neural precursor cell expressed, developmentally down-regulated 4), these genes may play a role in modulating protein cell surface expression.

### Materials and Methods

#### Materials

Culture media components were obtained from Clontech Laboratories, Inc. (Mountain View, CA), Difco (Sparks, MD), Invitrogen (Carlsbad, CA) and Sigma-Aldrich (St. Louis, MO). \(^{3}H\)Glutathione was purchased from Perkin Elmer (Boston, MA). Acivicin, glycine, glutamic acid, glutathione reductase, GSH, GSGS, Latrunculin A, lucifer yellow, lycytase, 0.1% poly-lysine, and yeast protease inhibitor cocktail (P8215) were obtained from Sigma (St. Louis, MO), N-(3-triethylammoniumpropyl)-4- (6-4-(diethylamino)phenyl) hexatrienylpyridinium dibromide (FM4-64) was obtained from Molecular Probes (Carlsbad, CA). Oligonucleotides were purchased from IDT (Coralville, IA). Ophthalmic acid was purchased from Bachem Bioscience Inc. (Philadelphia, PA). Zymolyase 100T was purchased from Seikagaku Corp. (Tokyo, Japan). The anti-FLAG M2 monoclonal antibody (F165) and anti-mouse IgG peroxidase conjugate antibody (A9044) were purchased from Sigma-Aldrich (St. Louis, MO); anti-Pep12p (A-21273) and anti-Dpm1p (A-6429) were obtained from Molecular Probe (Eugene, OR); anti-Pma1p (sc-57978) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All chemicals used were of the highest analytical grade possible.

#### Yeast strains and culture condition

The hgt1-Δ yeast strain (ABC822: MATa ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 lys2-801 ade2-101 hgt1A::LEU2) and its parental yeast strain (ABC154: MATa ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 lys2-801 ade2-101)) were kindly provided by Dr. A.K. Bachhawat (Institute of Microbial Technology, Chandigarh, India). In the mutant strain, the HGT1 gene was replaced with the LEU2 gene [5]. Rich YPAD medium, (1% yeast extract, 2% Bacto peptone, 2% glucose, 0.002% adenine) was used for routine yeast culture. Yeast transformants were selectively grown on minimal selective medium (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% glucose, supplemented with the required amino acids and bases). Sulfur-deficient medium was prepared based on minimal selective medium with the modification that all sulfur-containing reagents were substituted with equal amounts of the corresponding chloride salt (i.e., CuCl₂, MgCl₂, ZnCl₂, MnCl₂, and NH₄Cl) and free adenine base [5]. For solid media, 2% agar was added.

#### Yeast transformation and selection

The human mammary gland cDNA library was built in the low copy yeast expression vector pMETPGK3 was kindly provided by Dr. S.G Oliver, University of Manchester, Manchester UK. This cDNA library contains approximately 330,000 independent colonies, and protein expression is driven by the MET3 promoter [7]. The hgt1-Δ cells were transformed with plasmid DNA from the human mammary gland cDNA library or with the empty vector using the lithium acetate method [8]. Transformants were selected directly on solid sulfur-deficient medium supplemented with 200 µM or 500 µM GSH. Colonies exhibiting robust growth in comparison to the control colonies bearing the empty vector were chosen for subsequent...
selection in liquid sulfur-deficient medium supplemented with either 500 μM or 750 μM GSH. Growth was monitored over time by measuring the optical density expressed as OD₆₀₀.

**DNA preparation, sequencing and analysis**

Plasmid DNA was extracted from individual positive yeast colonies and was used as the template to amplify the cDNA inserts with Promega Pfx DNA polymerase (Madison, WI). Primers used in PCR reactions were designed based on the sequences of MET3 promoter (CTC TCT GTC GTA ACA GTT GT) and of PGK1 terminator (GGC AAT TCC TTA CCT TCC AA). PCR products were purified using the Qiagen PCR purification kit (Valencia, CA) and sequenced by the Mount Desert Island Biological Laboratory DNA Sequencing Facility (Salisbury Cove, ME) with the MET3 primer. A representative of each class of cDNA insert, as determined by the alignment analysis using the Vector NTI program (Frederick, MD), was amplified by *E.coli* and transformed back into yeast. Once it was confirmed that the retransformed cells were able to rescue growth in liquid sulfur-deficient medium supplemented with either 500 or 750 μM GSH as the sole sulfur source repeatedly, nucleotide sequence comparisons were performed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) network service against the NCBI databases. For each identified cDNA, protein topology was analyzed based on the NCBI protein reference sequence. Transmembrane domains were predicted using the HMTOP program (Prediction of transmembrane helices and topology of proteins, http://www.enzim.hu/hmmtop/index.html), and PY motifs were predicted by ELM (The Eukaryotic Linear Motif resource for Functional Sites in protein, http://elm.edu.org/).

Ndfip2 (Nedd4 family interacting protein 2) cDNA clone was obtained from the Mouse MGC and IMAGE Clones collection of Thermo Scientific (CAT# 8360772, Huntsville, AL). The insert was excised from pCR4-TOPO vector using EcoRI, and was subsequently subcloned into the pESC-URA vector. The pESC-Ndfip2 clone was confirmed by nucleotide sequencing. Wild type yeast strain and the hgt1-Δ strain were transformed with pESC-URA vector or each of the four LITAF constructs. Wild type yeast cells bearing the empty pESC-URA vector or each of the four LITAF constructs. Wild type yeast cells were then transformed with the empty pESC-URA vector or with each of the four LITAF constructs. Wild type yeast cells bearing the empty pESC-URA vector or each of these four LITAF constructs were grown to mid-log phase, and proteins were isolated by vigorously vortexing with the same volume of glass beads in Celllytic Y Yeast Cell Lysis/Extraction Reagent (Sigma-Aldrich, St. Louis, MO) freshly supplemented with protease inhibitor cocktail (Sigma) and 10 mM dithiothreitol. Whole cell lysates (40 μg) were separated on 4-15% (w/v) SDS-PAGE gels (Bio-Rad). The separated polypeptides were electrotransferred on to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for 120
min at 95 V using a wet-transfer apparatus (Bio-Rad). The membranes were blocked overnight in 5% (w/v) dried milk in TBST (20 mM Tris, 140 mM NaCl, 0.05% Tween-20, pH 7.4) at 4°C. The blots were probed with anti-FLAG at 1:4000 or with anti-Actin at 1:500 for 2 h at room temperature, and then with respective secondary antibodies at 1:3000 for 1 h at room temperature. Antibody binding was detected using Perkin-Elmer enhanced-chemiluminescence technique (Waltham, Massachusetts).

Subcellular fractionation

Subcellular fractions were isolated essentially as described by Rieder et al. [12]. Yeast cells transformed with LITAF-FLAG were grown to minimal selective medium containing raffinose as carbon source to an early log phase, and 2% galactose was added for 6 h. Collected cells were converted to spheroplasts with Zymolyase 100T (25 µg/ OD600). Spheroplasts were then lysed in HEPES/KAc buffer (20 mM HEPES/KOH pH 6.8, 50 mM potassium acetate, 200 mM sorbitol, 1 mM EDTA, and freshly added 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol and 1X yeast protease inhibitor cocktail) using a Dounce homogenizer (15 strokes with a tight-fitting pestle). The lysate was cleared by centrifuging at 300 × g for 5 min. A portion of the resulting supernatant was used as the S300 fraction, and the rest was centrifuged at 13,000 × g for 10 min. The resulting pellet (P13000) was stored on ice. A portion of this supernatant was saved as the S13000 fraction, and the rest was centrifuged at 100,000 × g for 60 min to generate S100000 and P100000 fractions. Proteins were precipitated from each fraction using 10% TCA and 20 µg of each fraction were separated on BioRad 12% SDS-PAGE gels. Membranes were probed with primary antibodies (mouse anti-FLAG monoclonal antibody 1:4000, mouse anti-Pma1p antibody 1:200, mouse anti-Pep12p antibody 1:4000, and mouse anti-Dpm1p antibody 1:2000) overnight at 4°C, and then with secondary antibody for 1 h at room temperature. Antibody binding was visualized using Perkin Elmer chemiluminescence reagents.

Indirect immunofluorescence microscopy

The cellular localization of LITAF was determined essentially as described by Pringle et al. [13]. After being induced by galactose for 6 h, yeast cells were fixed with 4% formaldehyde and converted to spheroplasts with lyticase. Spheroplast suspension was applied to the multiple-well polylsine-coated microscope slide (PolySciences, Warrington, PA). After being blotted with anti-FLAG antibody for overnight at 4°C, the spheroplasts were stained with Rhodamine Red-X-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. The samples were viewed using an FV1000 Olympus Laser Scanning Confocal Microscope (Olympus America, Melville, NY) at 60× magnification. Cell morphology was viewed by differential interference contrast (DIC, Nomarski), and cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Images were processed using FV1000 software (Olympus America, Melville, NY) and Adobe Photoshop (Adobe Systems, San Jose, CA).

Data analysis

Data are given as mean ± SE. Mean values were considered to be significantly different when P<0.05 by use of a two-way ANOVA or a one-way ANOVA followed by Bonferroni’s multiple comparison test. Software used for statistical analysis was GraphPad Prism (version 5 for windows; GraphPad Software, San Diego CA).
Results

PMEPA1, LAPT4α, SLC25A1, LITAF, and CYYR1 rescue the impaired growth of hgt1-Δ in medium in which GSH is the only sulfur source. hgt1-Δ cells were transformed with a human mammary cDNA library which contains about 300,000 individual clones, and the transformants were subjected to selection on sulfur-deficient medium supplemented with either 500 or 750 µM GSH. Approximately 300 colonies grew faster on this medium than colonies from cells transformed with the empty pMETtPGK3 vector, giving rise to colonies that were at least two times larger than the cells containing only vector. The growth of these larger colonies was subsequently assayed quantitatively in liquid sulfur-deficient medium supplemented with either 500 or 750 µM GSH. Cell growth was monitored over a period of 108 h. Values are given as means ± SEM, N=3. *Significantly different from hgt1-Δ cells transformed with the empty vector pMETtPGK3 (p<0.05).

Table 1. Summary of subcellular localization, possible cellular functions and predicted associations with human diseases. Charcot-Marie-Tooth disease type 1C (CMT1C); Extramammary Paget disease (EMPD); Crohn’s Disease (CD); Ulcerative colitis (UC); DiGeorge syndrome (DGS); Velo-cardio-facial syndrome (VCFS); conotruncal anomaly face syndrome (CTAF).

Fig. 2. Expression of five human genes rescues the growth impairment of hgt1-Δ cells in sulfur-deficient medium in the presence of 500 µM or 750 µM GSH as the sole sulfur source. Cells were grown overnight at 30°C in minimal selective medium. Subsequently, they were washed and inoculated at a cell density (expressed as OD600) of 0.01 in sulfur-deficient medium supplemented with either 500 or 750 µM GSH. Cell growth was monitored over a period of 108 h. Values are given as means ± SEM, N=3. *Significantly different from hgt1-Δ cells transformed with the empty vector pMETtPGK3 (p<0.05).
SLC25A1 are predicted to have 4 and 5 transmembrane domains, respectively. Remarkably, all five genes are predicted to encode proteins containing PY motifs (xPPxY, P = Pro, Y = Tyr, x = any amino acid; Fig. 1).

As illustrated in Fig. 2, these five human genes were able to rescue the impaired growth of hgt1-Δ cells in sulfur-deficient medium supplemented with 500 µM GSH (Fig. 2B) or 750 µM GSH (Fig. 2D), restoring cell growth to nearly the same level as that of wild type cells (Figs. 2A, 2C). To confirm that the growth recovery was due to an increase in intracellular glutathione concentration, hgt1-Δ transformants and wild type cells transformed with empty vector were cultured in sulfur-deficient medium supplemented with 750 µM GSH. After 48 h, the total intracellular glutathione concentration in wild type cells was about 700 pmol/10⁶ cells (~10 mM), which represents the normal physiological glutathione concentration in yeast cells, whereas glutathione concentration in hgt1-Δ cells was below the detection limit of the current method (25 pmole) (Fig. 3). Glutathione levels in hgt1-Δ cells expressing each of the five genes were about 10% of those of wild type cells (~1 mM)(Fig. 3). Nevertheless, these modest increases in intracellular glutathione concentration may be sufficient to restore their growth (Figs. 2B, 2D).

*The expression of these five human genes in hgt1-Δ cells leads to enhanced [3H]GSH uptake, which can be inhibited by high concentrations of GSH, GSSG and ophthalmic acid*

To determine whether the higher intracellular glutathione levels seen in hgt1-Δ cells transformed with the five human genes were due to increased GSH uptake, [3H]GSH transport activities of the wild type and hgt1-Δ transformants were measured after being cultured overnight in sulfur-deficient medium. Fig. 4A demonstrates that while hgt1-Δ cells are impaired in [3H]GSH uptake compared to the WT cells, these cells are able to accumulate some GSH by other mechanisms. hgt1-Δ cells transformed with LAPTMs4α, SLC25A1, LITAF or CYYR1 all showed increased uptake of [3H]GSH (Fig. 4B). The uptake of [3H]GSH in hgt1-Δ cells transformed with PMEPA1 was also increased, however to a smaller extent (Fig. 4B). Note that the initial [3H]GSH uptake rate (1 min uptake) in hgt1-Δ cells transformed with LAPTMs4α, SLC25A1, LITAF or CYYR1 was even higher than that in wild type cells (Fig. 5).

To gain insight into the mechanism for the enhanced [3H]GSH uptake by these transformants, initial uptake

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**Fig. 3.** Expression of five human genes increases the intracellular total glutathione concentration in hgt1-Δ cells. Cells were grown overnight at 30°C in minimal selective medium. Subsequently they were washed and inoculated at a cell density (expressed as OD₆₀₀) of 0.01 in sulfur-deficient medium supplemented with 750 µM GSH at 30°C for 48 h. Collected cells were lysed and intracellular total glutathione content was measured. Values are given as means ± SEM, N=3. BD = below detection limit.

**Fig. 4.** [3H]GSH uptake is enhanced in hgt1-Δ cells transformed with the five human genes. After growth at 30°C in minimal selective medium to a cell density (expressed as OD₆₀₀) of approximately 0.5, cells were washed and grown for another 11-12 h at 30°C in the sulfur-deficient medium in the absence of GSH. For the uptake experiments, cells were incubated with 500 µM [3H]GSH in MES buffer. Values are given as means ± SEM, N=3-7 individual experiments. *Significantly different from hgt1-Δ cells transformed with the empty pMETtPGK3 vector (p<0.05).
rates were measured at 1 min in the presence and absence of various unlabeled potential competitive inhibitors. As shown in Fig. 5A, addition of 20 mM GSH, GSSG, or ophthalmic acid (L-γ-glutamyl-L-α-aminobutyryl-glycine, a GSH analog) resulted in the inhibition of the initial uptake rate of [3H]GSH in the wild type cells transformed with the empty pMETtPGK3 vector. However, the amino acids glutamic acid and glycine had no effect (Fig. 5A), consistent with the known substrate specificity of Hgt1p (5). In contrast, no inhibitory effect of any of these compounds was observed in the hgt1-Δ cells transformed with the empty pMETtPGK3 vector (Fig. 5B). Addition of these compounds to hgt1-Δ cells transformed with PMEPA1 (Fig. 5C), LAPTM4α (Fig. 5D), SCL25A1 (Fig. 5E), LITAF (Fig. 5F) and CYYR1 (Fig. 5G) resulted in a uniform pattern of inhibition. These results suggest that the enhanced GSH uptake in the hgt1-Δ cells transformed with these five human genes is occurring by a common transport mechanism that is inhibitable by the compounds GSH, GSSG, and ophthalmic acid that also inhibit Hgt1p. However, because the inhibition was evident only at high mM concentrations of these compounds, the
relative affinity for GSH uptake by this as yet undefined transport mechanism appears to be relatively low.

hgt1-Δ cells transformed with the five human genes show enhanced uptake of endocytic markers

As illustrated in Fig. 1, all five identified proteins are predicted to contain PY motifs, which have been implicated to play a role in protein-protein interactions involving binding to WW domains in target proteins, including NEDD4 and its yeast homolog Rsp5. NEDD4/Rsp5 E3 ligases mediate ubiquitin-dependent endocytosis, which is a key component of the regulation of plasma membrane protein abundance [14]. To examine whether these five human genes affect endocytic processes in yeast, the uptake of lucifer yellow (a commonly used marker for fluid phase endocytosis) and FM4-64 (a marker for membrane labeling and membrane turnover) was measured in wild type and hgt1-Δ transformants after overnight culture in sulfur-deficient medium. hgt1-Δ cells transformed with LAPTM4α, SLC25A1, LITAF or CYYR1 showed a significant increase in FM4-64 uptake (Fig. 6A) and in lucifer yellow uptake (Fig. 6B) when
compared to the wild type cells or to hgt1-Δ cells, consistent with the hypothesis that these genes are stimulating membrane turnover in hgt1-Δ cells. PMEPA1 expressing cells also exhibited slightly increased accumulation of these two endocytotic markers, although the difference was not statistically significant (Fig. 6).

The enhanced [3H]GSH uptake in hgt1-Δ cells transformed with PMEPA1, LAPTM4α, SLC25A1, LITAF or CYYR1 is not inhibited by latrunculin A

To test for possible connections between the enhanced GSH uptake and the increased endocytotic marker uptake, latrunculin A (LAT-A) treatment was applied to hgt1-Δ transformants prior to measuring the uptake of FM4-64 and of [3H]GSH in these cells. LAT-A disrupts the actin cytoskeleton by sequestering actin monomers, and therefore has been widely used as an endocytotic inhibitor in yeasts [15]. hgt1-Δ transformants were pre-treated with 20 µM LAT-A or with 1% DMSO (vehicle control) for 60 minutes. As shown in Fig. 7A, LAT-A pretreatment resulted in decreased FM4-64 uptake in each tested strain, suggesting that endocytosis in these cells was inhibited by LAT-A treatment. In agreement with the results of figure 6A, cells expressing each of the five genes had higher rates of FM4-64 uptake than that of hgt1-Δ cells; however, LAT-A did not appear to be effective at inhibiting the FM4-64 uptake that was stimulated by each of the five genes (Fig. 7A). Note for example, that whereas LAT-A decreased FM4-64 uptake by about 65% in the control hgt1-Δ cells, it only decreased FM4-64 uptake by 38% in cells expressing LITAF (Fig. 7A).

LAT-A had no effect on either the basal [3H]GSH uptake or the enhanced [3H]GSH uptake observed in cells transformed with the five genes (Fig. 7B). These results suggest that neither the increased GSH uptake, nor the increased uptake of general endocytotic markers in hgt1-Δ cells transformed with PMEPA1, LAPTM4α, SLC25A1, LITAF or CYYR1 is directly due to an increase in conventional endocytotic processes. Although the mechanism of the enhanced [3H]GSH uptake is not known, the present results suggest that it is due to increased activity or membrane delivery of the as yet unidentified endogenous GSH uptake mechanism.

Subcellular localization of LITAF

To further characterize the potential mechanism by which these proteins stimulate GSH uptake, additional studies examined the intracellular localization of LITAF in yeast cells. When expressed in yeast, FLAG tagged LITAF was recognized as a protein of about 25 kDa, which is similar to the size of LITAF detected in

Fig. 9. PY motif mutations largely abolish the effect of LITAF on intracellular GSH levels, and on [3H]GSH and FM4-64 uptake. PPSY motifs in LITAF were changed to PPSA by site-directed mutagenesis. Wild type strain ABC154 was transformed with empty vector, LITAF, or LITAF with PY mutations and whole cell lysate of each transformant was subjected to SDS-PAGE gel. The blot was probed with anti-FLAG antibody for LITAF expression, and with anti-actin for loading control (9A). Values are given as means ± SEM, N=3-6. *Significantly different intracellular glutathione concentration (9B), [3H]GSH uptake (9C) and FM4-64 uptake (9D) when compared to wild type cells transformed with the empty pESC-URA vector (p<0.05).
mammalian cells [16]. Upon subcellular fractionation, LITAF was mainly present in the P_{13000} fraction, but was also detected in the P_{100000} fraction, albeit at much lower levels (Fig. 8A). Dpm1p (Dol-P-Man Synthase, an endoplasmic reticulum membrane marker protein) and Pma1p (Plasma Membrane H^+-ATPase, a plasma membrane marker protein) also showed a predominant distribution in the P_{13000} fraction with low levels in the P_{100000} fraction, whereas Pep12p (carboxyPEPtidase Y-deficient, an endosome/Golgi membrane marker protein) was detected in the P_{13000} and P_{100000} fractions at roughly comparable levels (Fig. 8A). Essentially no LITAF was detected in the P_{100000} fraction, and to stimulate [3H]GSH and FM4-64 uptake (Fig. 9B). The results demonstrate that LITAF is membrane protein. The distribution of LITAF between P_{13000} and P_{100000} indicates that LITAF is likely associated with large organelles such as the endoplasmic reticulum and the plasma membrane.

Intracellular localization of LITAF was also examined using indirect immunostaining and confocal fluorescence microscopy (Fig. 8B). The results demonstrate that LITAF was expressed diffusely throughout the cell, and was found around the periphery of the cell. Taken together, the results in Fig. 8 indicate that LITAF is a membrane protein, and is associated with the membrane of intracellular organelles and with the plasma membrane (Fig. 8B).

**Mutation of the two predicted PY motifs of LITAF largely abolishes the ability of this protein to increase intracellular glutathione levels, and to stimulate [3H]GSH and FM4-64 uptake**

To examine the functional significance of the PY motifs in these genes, LITAF was selected for mutational analysis. The two predicted PY motifs of LITAF were mutated from PPSY to PPSA, and wild type yeast cells were transformed with both wild type LITAF and three LITAF mutants. Mutation of these two PY motifs had only minimal effects on the expression level of LITAF protein (Fig. 9A); however, they essentially abolished the ability of LITAF to increase intracellular glutathione levels, and to stimulate [3H]GSH and FM4-64 uptake (Fig. 9B-D). As illustrated in figure 9, wild type cells transformed with LITAF had higher intracellular glutathione levels, and enhanced [3H]GSH and FM4-64 uptake, compared with untransformed cells, consistent with results obtained from hgt1-Δ cells transformed with LITAF (Figs. 3, 4 and 6) suggesting that the functions of LITAF are not specifically to complement the HGT1 deficiency, but are more likely to regulate membrane turnover by an common mechanism. Intracellular glutathione levels, [3H]GSH uptake rates and FM4-64 uptake rates of wild type cells transformed with LITAF mutants were generally comparable to those of control cells (wild type cells transformed with empty vector pESC-URA), indicating that these effects of LITAF in yeast depend on its PY motifs. The subcellular localization of these mutants in yeasts was also examined, however no differences were detected using indirect immunofluorescence (data not shown).

Expression of mouse Ndfip2 (N4WBP5) has no effect on [3H]GSH uptake in either the wild type or the hgt1-Δ yeast strain

To examine whether another PY-containing protein has similar effects on [3H]GSH uptake in yeast cells, mouse Ndfip2 (Nedd4 family interacting protein 2) was expressed in both the wild type yeast strain and the hgt1-Δ strain. [3H]GSH uptake was measured for 30 min in yeast cells transformed with the pESC-URA vector or Ndfip2 after being grown overnight in a sulfur-deficient medium that contained galactose. However, the expression of Ndfip2 had no effect on [3H]GSH uptake in either strain (data not shown), suggesting that the transport activity of the endogenous yeast low affinity GSH uptake mechanism is not modulated by Ndfip2.

**Discussion**

Bourbouloux et al. [5] previously demonstrated that Hgt1p is quantitatively the major GSH uptake transporter in *S. cerevisiae*. Without this transporter, the cells have minimal ability to take up GSH from the culture medium and show marked growth retardation when GSH is the only sulfur source. However, hgt1-Δ cells do retain some ability to take up GSH at a slow rate (e.g., see Fig. 4A), and to grow slowly using GSH as a sulfur source (Fig. 2). Thus, yeast cells have an alternate mechanism for taking up GSH, although this alternative mechanism has not been identified at the molecular level and has not been characterized biochemically.

The present study identifies five human genes, PMEPA1, LAPTM4α, SLC25A1, LITAF, and CYYR1 that can functionally complement the growth defect of hgt1-Δ cells cultured in a medium in which GSH is the only sulfur source. Each of these genes encodes a protein containing PY motifs that are thought to be important for regulating protein cell surface expression via interacting with the ubiquitin ligases NEDD4/Rsp5p [14, 17]. The first PY motif was identified through a functional cDNA library screen for putative ligands of the WW domain of
the Yes-associated protein [18]. The presence of these motifs allows the PY motif-containing proteins to bind to WW domains in target proteins, including the E3 ubiquitin ligase NEDD4 [19]. NEDD4 protein belongs to the Hect-domain family of E3 ubiquitin-protein ligase. A major function of such proteins is to down-regulate the cell surface expression of membrane proteins via ubiquitin-dependent endocytosis, followed by degradation in both proteasomes and lysosomes [14, 20]. Mutations in the dependent endocytosis, followed by degradation in both surface expression of membrane proteins via ubiquitin-motifs allows the PY motif-containing proteins to bind to the Yes-associated protein [18]. The presence of these amiloride-sensitive epithelial sodium channel (ENaC) by interfering with the Nedd4-mediated regulation of ENaC [28]. Ndfip2 contains three transmembrane domains and two amino terminal PPxY motifs, which physically interact with the WW domains of Nedd4 family proteins. However, this stimulatory effect of Ndfip2 on ENaC activity appears to be relatively selective, as Ndfip1, a highly related protein that also contains PY motifs, has no effect on ENaC activity [28]. In addition, Ndfip2 did not increase the transport activity of CFTR or Kir1.1a, indicating selectivity for the target proteins. In the present study, expression of Ndfip2 in yeast cells did not produce stimulatory effects in GSH uptake, but this negative result does not necessarily exclude the proposed model, as there are many possible reasons for the lack of effect, including differential specificity, sensitivity, and affinity for possible binding sites in yeast proteins.

Expression of the five genes also led to greater accumulation of the endocytotic markers lucifer yellow and FM4-64 in yeast. Whereas the basal accumulation of FM4-64 was inhibited by LAT-A, neither the higher rates of FM4-64 uptake nor the enhanced GSH uptake observed in cells expressing the five human genes was affected by LAT-A, indicating that the enhanced GSH uptake associated with expression of the human genes is not mediated by a LAT-A-sensitive endocytotic mechanism. Upon subcellular fractionation, LITAF was found in the P_{13000} fraction which primarily contains large organelles and the plasma membrane. Indirect immunostaining of LITAF resulted in a staining pattern characteristic of localization to the plasma membrane and intracellular membranes. Mutation of the two PY motifs of LITAF resulted in loss of the observed effects, indicating that this motif plays an essential role in the effects of LITAF expression in yeast.

To date, little is known about the biological functions of the five genes identified in the present study. SLC25A1 is mapped to chromosomal region 22q11, and microdeletions in this region are associated with DiGeorge syndrome, velo-cardio-facial syndrome and conotruncal anomaly face syndrome [29]. Although it is not known whether SLC25A1 plays a role in GSH homeostasis, some members of SLC25A family may contribute to mitochondrial GSH uptake. In particular, the dicarboxylate (SLC25A10) and 2-oxoglutarate (SLC25A11) carriers of the inner mitochondrial membrane are believed to catalyze uptake of GSH into the matrix through an exchange mechanism [30]. The three members of the lysosomal-associated protein transmembrane (LAPTM) family, LAPTM4α, LAPTM4β and LAPTM5 are thought to function as transporters or channels, although their precise
function is unknown. When expressed in a drug-sensitive strain of *S. cerevisiae*, mouse LAPTM4α regulates intracellular compartmentalization of amphipathic solutes and confers cellular resistance or hypersensitivity to a range of drugs [31]. Both CYYR1 and PMEPA1 have been implicated in human diseases, although their functions are also unknown. CYYR1 is expressed in a broad range of human tissues, and the central cysteine-tyrosine-rich domain is strongly conserved from lower vertebrates to humans [32]. Alternative splicing and expression of the CYYR1 transcript has been reported in human neuroendocrine tumors [33]. PMEPA1 was first described as an androgen-induced gene. It is widely expressed in normal and solid malignant tissues, with the highest expression in prostate tissue [34]. The expression of PMEPA1 is increased in primary and metastasized colon tumors and several other solid tumors [35, 36], whereas it is decreased in prostate tumor tissues [34]. In addition, PMEPA1 is also induced by tumor growth factor β [36]. LITAF is present in most human tissues and its expression can be induced by multiple pathways, including TNFα- and p53-activated pathways [16, 37, 38]. Elevated mRNA and protein expression of LITAF has been found in intestinal tissues from patients with Crohn’s disease and ulcerative colitis [39, 40]. In addition, mutations of LITAF cause Charcot-Marie-Tooth disease type 1C (CMT1C), an autosomal dominant demyelinating peripheral neuropathy [41]. Although the etiology of CMT1C is not understood yet, a role of LITAF in modulating protein trafficking and turnover has been proposed to be involved [26, 42].

In summary, the present study identifies five human genes that rescue growth of a HGT1-deficient yeast strain, and provides clues into the potential function of these genes. Currently, the functions of the five genes are unknown, although four of them have been implicated in human diseases [35, 39, 41, 43-45]. A better understanding of their physiological functions may be expected to clarify the etiology and pathogenesis of these diseases.

**Abbreviations**

CYYR1 (cysteine/tyrosine-rich1); FM4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl)hexatrienyl) pyridinium dibromide); GSH (reduced glutathione); LAPTM4α (lysosomal-associated protein transmembrane 4 alpha); LITAF (lipopolysaccharide-induced TNF factor); Ndfip2 (Nedd4 family interacting protein 2); NEDD4 (neural precursor cell expressed, developmentally down-regulated 4); PY (xPPxY, P = Pro, Y = Tyr, x = any amino acid); RSP5 (reverses Spt-phenotype); SLC25A1 (solute carrier family 25, member 1); PMEPA1 (prostate transmembrane protein, androgen induced 1).

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