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Hgt1p, a High Affinity Glutathione Transporter from the Yeast *Saccharomyces cerevisiae**

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A high affinity glutathione transporter has been identified, cloned, and characterized from the yeast *Saccharomyces cerevisiae.* This transporter, Hgt1p, represents the first high affinity glutathione transporter to be described from any system so far. The strategy for the identification involved investigating candidate glutathione transporters from the yeast genome sequence project followed by genetic and physiological investigations. This approach revealed *HGT1* (open reading frame *YJL212c*) as encoding a high affinity glutathione transporter. Yeast strains deleted in *HGT1* did not show any detectable plasma membrane glutathione transport, and *hgt1*Δ disruptants were non-viable in a glutathione biosynthetic mutant (*gsh1*Δ) background. The glutathione repressible transport activity observed in wild type cells was also absent in the *hgt1*Δ strains. The transporter was cloned and kinetic studies indicated that Hgt1p had a high affinity for glutathione (K_m = 54 μM)) and was not sensitive to competition by amino acids, dipeptides, or other tripeptides. Significant inhibition was observed, however, with oxidized glutathione and glutathione conjugates. The transporter reveals a novel class of transporters that has homologues in other yeasts and plants but with no apparent homologues in either *Escherichia coli* or in higher eukaryotes other than plants.

Glutathione is the most abundant non-protein thiol compound present in almost all prokaryotic and eukaryotic cells. It plays numerous roles including control of redox potential, protection against oxidative stress, detoxification of endogenous and exogenously derived toxins, protein folding, storage, and transport of organic sulfur (1, 2). In humans, several diseases have been strongly correlated with altered intracellular glutathione levels (3, 4). The biosynthesis of glutathione is mediated by two cytoplasmic enzymes, GSH1 (γ-glutamylcysteine synthase) and GSH2 (glutathione synthetase). Glutathione may be either utilized in the cytosol or transported by specific transporters to the endoplasmic reticulum (5, 6), the mitochondria (7), and the extracellular milieu (8). In addition to endogenous biosynthesis, glutathione may also be taken up from the extracellular environment. Biochemical evidence for specific transporters mediating glutathione uptake has been obtained in bacteria (9), yeasts (10), plants (11), and mammalian cells (12). Despite these various reports describing glutathione transport into the cell and into the different organelles, and the importance of this process in maintaining glutathione homeostasis, no gene encoding a glutathione transporter has been cloned so far from any organism. The initial report of the cloning of the rat sinusoidal (13) and canalicular glutathione hepatic transporters (14) now appears to be artifactual as the nucleotide sequences of these genes are almost identical to *Escherichia coli* open reading frames, and the results could not be reproduced in other laboratories (15, 16). The mammalian multidrug resistance associated protein (*MRP1*) and its yeast homologue, *YCF1*, which belong to the ABC transporter family are able to transport glutathione out of the cytoplasm. However, they do so with very low affinity, having a K_m for glutathione in the range of about 15 mM (17, 18), and their primary function is really in the efflux of glutathione conjugates.

In the present report we describe, for the first time, the identification, cloning, and characterization of a high affinity plasma membrane transporter mediating glutathione uptake in the yeast *Saccharomyces cerevisiae*. This identification reveals a novel family of transport proteins that have homologues in other yeasts and plants, but no homologues as yet discovered in either prokaryotes or other higher eukaryotes.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All chemicals used were of analytical reagent grade. Media components were either purchased from Hi Media (India), from Sigma or from Difco. Bacteriological agar used in France was from Biokar Diagnostics, Beauvais (France). Vent DNA polymerase was from New England Biolabs while *Taq* Polymerase was from Promega. Oligonucleotides were purchased from Ransom Hill Biosciences and Gemini Biotech. Radioactivity was from NEN Life Science Products.

Yeast Strains and Growth—The list of yeast strains used in this study is shown in Table I. Yeasts were routinely maintained on YPD medium. The minimal medium contained YNB, glucose, and ammonium sulfate supplemented with the required amino acids and bases. Glutathione was added wherever necessary at concentrations of 250 μM. Sulfur-limited medium was made by substituting ammonium sulfate with ammonium chloride. Sporulation plates were prepared as described by Kaiser *et al.* (19).

Yeast DNA Isolation and Yeast Transformation—Yeast chromosomal DNA was isolated by the glass bead lysis method and yeast transformations were carried out using the lithium acetate method (19).

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TABLE I
List of strains used in this study

Strain	Genotype	Source
YPH 499 (ABC 154)	*MATa ura3–52 leu2-Δ1 lys2–801 his3-Δ200 trp1-Δ63 ade2–101*	K. Kuchler
ABC 579	*MATα ura3–52 leu2-Δ1 lys2–801 his3-Δ200 trpl-Δ63 ade2–101*	Lab Strain
ABC 738	*MATa ura3–52 leu2-Δ1 lys2–801 his3-Δ200 trpl-Δ63 ade2–101 ptr2Δ::KanMX2*	This study
ABC 733 (BY 4741)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	J. Boeke
ABC 797	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ypr194Δ::URA3*	This study
ABC 817	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hgt1Δ::LEU2*	This study
ABC 818	*MATa ura3–52 leu2-Δ1 lys2–801 his3-Δ200 trp1-Δ63 ade2–101 ypr194Δ::URA3*	This study
ABC 819	*MATa ura3–52 leu2-Δ1 lys2–801 his3-Δ200 trp1-Δ63 ade2–101 ptr2Δ::KanMX2 ypr194Δ::URA3*	This study
ABC 822	*MATa ura3–52 leu2-Δ1 lys2–801 his3-Δ200 trp1-Δ63 ade2–101 hgt1Δ::LEU2*	This study
ABC 823	*MATa ura3–52 leu2-Δ1 lys2–801 his3-Δ200 trp1-Δ63 ade2–101 ypr194Δ::URA3 hgt1Δ::LEU2*	This study
ABC 835	*MATa ura3–52 leu2-Δ1 lys2–801 his3-Δ200 trp1-Δ63 ade2–101 ptr2Δ::KanMX2 hgt1Δ::LEU*	This study
ABC 842	*MATa ura3–52 leu2-Δ1 lys2–801 his3-Δ200 trp1-Δ63 ade2–101 ptr2Δ::KanMX2 ypr194Δ::URA3 hgt1Δ::LEU2*	This study
ABC 869	*MATα ura3–52 leu2-Δ1 lys2–80 his3-Δ200 trp1-Δ63 ade2–101 gsh1Δ::LEU2*	This study

Cloning of HGT1—HGT1 was cloned by PCR.[1] The following primers were used for amplifying *HGT1* from chromosomal DNA of a wild type strain (YPH499); YJL-Bam, 5′-CCGCGAATTCATGATTACCACCATTTATCATAACC-3′ and YJL-Eco, 5′-CACAGGATCCATGAGTACCATTTATAGGGAGAGC-3′. The 2.4-kilobase PCR product obtained was digested with *Eco*RI and *Bam*HI and cloned into a single copy, *URA3*-based yeast expression vector downstream of the TEF promoter (20).

Tetrad Analysis—To check the viability of *gsh1*Δ and *hgt1*Δ double deletes, a diploid heterozygous for both markers was constructed by crossing ABC869 (*gsh1Δ::LEU2*) with ABC822 (*hgt1Δ::LEU2*). The diploid was sporulated on minimal sporulation plates (19) and tetrads were dissected. The spores were dropped on either YPD or YPD supplemented with excess glutathione (1 mM). After initial patching on YPD plates, spores were replica plated on SD minus uracile, minus GSH, minus leucine, and minus tryptophane plates.

Diploids heterozygous for *ptr2*Δ and *gsh1*Δ, and diploids heterozygous for *ypr194*Δ and *gsh1*Δ were constructed in a similar way. The diploids were sporulated and dissected as described above. The disruption at the *GSH1* locus was followed by glutathione auxotrophy, while disruption at either the *YPR194c* locus or the *PTR2* locus was followed either by uracile prototrophy or resistance to G418, respectively.

Construction of Strains—The genotypes of the strains used in all the experiments are listed in Table I and their construction is described below.

YPH 499 was used as wild type strain in all these experiments. Disruption of the different genes was carried out by one-step PCR-mediated gene disruption (21). Strains triply disrupted in the different peptide and putative peptide transporters were constructed by using the *KanMX2* marker for *PTR2* disruption, the *URA3* marker for *YPR194c* disruption, and the *LEU2* marker for *HGT1* disruption. These disruptions were to be eventually constructed in YPH 499. However, as this strain is not completely disrupted in either the *URA3* allele or the *LEU2* allele, PCR-mediated gene disruption using the *URA3*/*LEU2* alleles would not occur efficiently. Therefore, *YPR194c* and *HGT1* were first disrupted through PCR-mediated gene disruption in the strain BY 4741 which carries null alleles of both *URA3* and *LEU2* (22). The *hgt1* Δ*::LEU2* and the *ypr194c*Δ*::URA3* disruptions in BY 4741 were then PCR amplified to yield larger flanking regions and introduced into YPH 499. The disruptions were confirmed by PCR.

The primers used for the disruption of *YPR194c* were: 194-URA-F, 5′-AGTCAAAGATAAAGTTATAATTGATGAGAAGGTATCCACAGCTTTTCAATTCAAT-3′ and 194-URA-R, 5′-GGGCTCATATTAAGCATTCCACCTACAAATAACATTGGGTAATAACTGATATAATTA-3′.

The primers used to confirm *YPR194c* disruption were: 194-FOR, 5′-ATTAGAAATTATGAGTGAAAC-3′ and 194-REV, 5′-GTTCTAGTCATGGATAGTGTC-3′.

The primers used to disrupt *HGT1* were: 212-LEU-F, 5′-CCATTTATAGGGAGAGCGACTCGTTGGAGTCGGAGCCCTCGACTACGTCGTAAGGCCG-3, and 212-LEU-R, 5′-CAAGCCTTGTCTCATGACAATAAATCCGTATAGCTTGAATGGAATCCCAACAATTACA-3′.

The primers used for the confirmation of *HGT1* disruptions were: 212-FOR, 5′-GATTACCACCATTTATCATAACC-3′ and 212-REV, 5′-CGTCACAGAACACATGAGTACC-3′.

For the disruption of *PTR2*, the *KanMX2* selection marker was used and the transformants were selected on G418 containing plates (23). The primers used for *PTR2* disruption were: PTR-DEL1, 5′-ATCCCAGCCAAGGCTCAGATGCTCAGGACGAAAAGCAGCTGAAGCTTCGTACGC-3′ and PTR-DEL2, 5′-CTGGTCGGCAATCAACACGGAAAGGTTAGCTTTAATCATAGGCCACTAGTGGATCTG-3′.

The primers used to confirm *PTR2* disruption were: PTR2-FOR, 5′-ATAAACGGATCCAATGCTCAACCATCCCAGCC-3′ and PTR2-REV, 5′-ATGCACAAAAGCTTGCAGAACCAAAGGCGTCGTTAGTC-3′. Each of the disruptions was made in an YPH 499 background. The double and triple disruptions were carried out sequentially. Disruptions in GSH1 were constructed using a GSH1::LEU2 plasmid (24).

Transport Experiments—Cells were grown on minimal liquid medium YNB complemented with ammonium sulfate and 2% glucose. When several strains differing in auxotrophy were compared, the substances required by the most deleted yeast were added to the growth medium of all strains. Cells were incubated at 28 °C for growth for 12 h, rotary shaken at 200 rpm. The cells were harvested at A_{600} 0.6, washed with a large volume of sterile water (4 °C), and 20 mM MES/KOH, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 2% glucose (pH 5.5) (unless otherwise stated). They were finally resuspended in the buffered medium (culture medium volume/25), aliquoted in 100-μl samples, and kept on ice.

After a 10-min incubation of the cells at 28 °C, [^{3}H]GSH (1657.6 GBq $mmol^{-1}$) was added to the buffered medium to a final concentration of 0.5 mM (final specific activity 12.33 Mbq $mmol^{-1}$). At selected times, uptake was stopped by diluting the medium with a 20-fold volume of water (4 °C) and filtering the cells through a glass fiber filter (Sartorius AG, 37070 Goettingen, Germany). The cells trapped on the filter were washed twice with the same volume of cold water. The filter was dried and placed in a scintillation vial containing 4 ml of Ecolite (ICN, Orsay, France). The radioactivity was counted after correction for background and quenching (Packard Instruments, Les Ulis, France).

Synthesis of GS-NEM—The *N*-ethylmaleimide *S*-conjugate of glutathione was synthesized by mixing NEM dissolved in 20 mM Tris-HCl with the same amount of GSH dissolved in the same buffer. After 1 h a room temperature, dithiothreitol was added to neutralize NEM in excess (25). The purity of the conjugate was checked by mass spectrometry.

Measurement of Protein—Protein content was measured by the method of Lowry *et al.* (26) using bovine serum albumin as a control.

RESULTS

The Yeast Plasma Membrane Peptide Transporter, Ptr2p, Is Unable to Transport Glutathione—*S. cerevisiae* has been reported to have a single peptide transporter, Ptr2p, that can transport dipeptides and tripeptides (27). Due to the wide substrate specificity reported for this transporter, the possibility that Ptr2p might be the transporter for the tripeptide glu-

[1] The abbreviations used are: PCR, polymerase chain reaction; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; GSH, reduced glutathione; GSSG, oxidized glutathione, GS-NEM, *N*-ethylmaleimide *S*-conjugate of GSH; NEM, *N*-ethylmaleimide; HGT, high affinity glutathione transporter; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid.

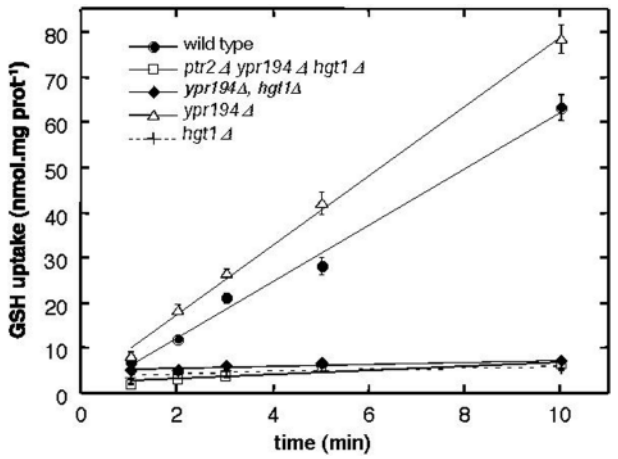

FIG. 1. **GSH uptake in wild type YPH499 and various yeast strains disrupted in different putative transporters.** The strains used were YPH499 (●), *ptr2*Δ *ypr194*Δ *hgt1*Δ (□), *ypr194*Δ *hgt1*Δ (◆), *ypr194*Δ (△), and *hgt1*Δ (+). The cells were incubated in 0.5 mM [^{3}H]glutathione. Data are the mean ± S.E. of 8 samples from two independent experiments.

tathione was initially examined. Yeast strains defective in glutathione biosynthesis (*gsh1*Δ) depend on exogenous glutathione for growth (28, 29). Should Ptr2p be the transporter mediating glutathione uptake, then *ptr2*Δ*gsh1*Δ spores would not be viable. Tetrad dissections of diploids heterozygous for *ptr2*Δ and *gsh1*Δ were carried out. However, among the several tetrads that were dissected, all 4 spores were viable and several *ptr2*Δ *gsh1*Δ spores were isolated that did not appear to have any visible growth defect. This suggested that even if Ptr2p mediated glutathione transport, it was not the sole transporter. Potential glutathione transport activity of Ptr2p was determined by comparing glutathione uptake in a wild type and *ptr2*Δ strains. The data clearly indicate that Ptr2p does not mediate GSH transport (data not shown).

HGT1 Encodes a Yeast Plasma Membrane Glutathione Transporter—Studies with the yeast *Candida albicans* earlier have revealed a new peptide transporter family that was able to transport tetra- and pentapeptides (30). The gene *OPT1* has homologues in *Schizosaccharomyces pombe* (Isp4$^+$) and *S. cerevisiae* (open reading frame YJL212c and YPR194c). Disruption of YPR194c in *S. cerevisiae* did not cause any difference in sensitivity to toxic oligopeptides. Overexpression revealed a very mild phenotype. However, no phenotype was found after either disruption or overexpression of YJL212c (31). A possible role of these proteins in glutathione transport was tested. Strains disrupted in both *YJL212c* and *YPR194c* genes were constructed. When the double disrupted strains were examined for transport activity, virtually no GSH transport could be observed (Fig. 1). Examination of the transport capacity of single disruptants showed a total lack of transport in the *yjl212*Δ strain, whereas the *ypr194*Δ strain took up GSH at the same rate as the wild type (Fig. 1). The transport activity of the triple disruptant of *yjl212*Δ *ypr194*Δ *ptr2*Δ was comparable to the transport activity of *yjl212*Δ single disruptants (Fig. 1). Altogether, the data strongly suggest that the protein encoded by YJL212c (from now on referred to as HGT1, high affinity glutathione transporter 1) is probably the glutathione transporter in the plasma membrane of these cells. To further confirm the function of HGT1, the transport activity of *hgt1*Δ disruptants complemented by the *HGT1* gene was studied. The *HGT1* gene was cloned and expressed in a single copy expression vector downstream of the TEF promoter. Transformation of the *hgt1*Δ disruptants with this construct restored GSH transport (data not shown), which definitively established that Hgt1p is a glutathione transporter. The deduced amino acid sequence indicates that *HGT1* gene encodes a 799-amino acid polypeptide with a predicted molecular mass of 91627 Da and a pI of 9.00. Analysis of the hydropathy profile (32) suggests the presence of 12–14 putative transmembrane domains (31), which is typical for many transporter proteins. The N and C termini are hydrophilic, and the N terminus is particularly long, with a stretch of about 100 amino acids (Fig. 2).

Functional Characterization of Hgt1p—Transport activity of *HGT1* was further characterized with the wild type as well as with the *hgt1*Δ/TEF-*HGT1* strain. pH dependence studies indicated that the initial rate of GSH uptake into the wild type and *hgt1*Δ/TEF-*HGT1* strain was maximal between pH 5.0 and 5.5 (data not shown). Further studies were run at pH 5.5. Hardly any transport activity was detected at 4 °C, indicating that uptake was an active process (Fig. 3). The transport of GSH was also significantly inhibited by the protonophore CCCP (Fig. 3), suggesting that transport activity depended on the transmembrane pH gradient. Kinetic studies yielded a K_m of 53.9 ± 5.5 μM (mean of four measurements) and a V_{max} of 10.0 ± 0.6 nmol of GSH·mg protein^{-1} min^{-1} (Fig. 4). Substrate specificity was studied by measuring uptake of labeled GSH in the presence of a 100-fold excess of various unlabeled potential competitors. A 100-fold excess of unlabeled GSH was used as a control to compare the different potential competitors. Amino acids like L-Pro, Gly, and L-Glu had little effect on GSH transport (Fig. 3). In addition, the *hgt1*Δ strain was able to transport L-Pro at rates similar to the wild type strain (data not shown), which suggests that amino acid transport is not the primary function of Hgt1p. Marginal inhibition was observed with both L- and D-Cys, as well as with glutamine. However, various di- or tripeptides such as Gly-Gly, Gly-Gly-Gly, and Gly-Glu as well as the dipeptides γ-Glu-Cys and Cys-Gly were poor inhibitors (Fig. 3). In contrast, oxidized glutathione (GSSG) and the glutathione conjugate GS-NEM were almost as inhibitory as GSH itself. Because the synthesis of the GS-NEM conjugate requires the use of small amounts of DTT to neutralize excess NEM, additional controls were run to test the effects of DTT on the uptake of GSH in the presence and absence of GS-NEM. These controls showed that DTT does not affect the results, and therefore that GS-NEM does indeed inhibit GSH uptake (Fig. 3). That glutathione *S*-conjugates may be transported by Hgt1p was further checked by uptake studies with labeled GS-NEM (Fig. 5). Labeled GS-NEM could indeed be taken up by the *hgt1*Δ/TEF-*HGT1* strain, but not by the *hgt1*Δ mutant, which clearly shows that Hgt1p transports both GSH as well as GS conjugates.

Hgt1p Is the Primary Glutathione Transporter in the Plasma Membrane of S. cerevisiae—Although virtually no GSH transport was observed in strains disrupted solely in *HGT1*, the gene has been predicted to belong to an oligopeptide permease transporter family (31). The presence of other glutathione transporters that would be induced under some conditions, therefore, could not be ruled out. This possibility was also suggested by an earlier report characterizing glutathione transport in *S. cerevisiae*, which concerned strains defective in sulfur metabolism (10). This report indicated the existence of two saturable glutathione uptake systems with K_m values of 0.45 μM and 2 mM, respectively. The low affinity system was due to the activity of a constitutive transporter, and the high affinity system corresponded to a sulfur repressible transporter. The high affinity transport activity was inducible and maximally induced by the absence of sulfur in the medium. The transport activity of the wild type and of *hgt1*Δ strains cultivated in the presence or absence of sulfur, as well as in the presence or absence of glutathione was studied. The data (Fig. 6) clearly indicated

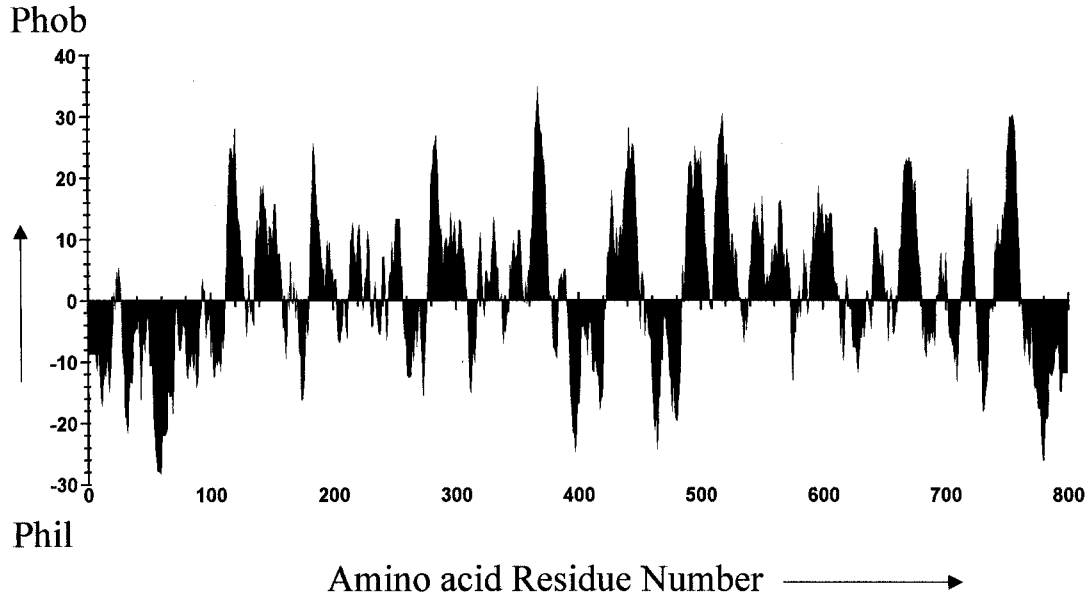

FIG. 2. **Hydropathy plot of Hgt1p.** The peptide sequence derived from *HGT1* was plotted according to Kyte and Doolittle with a window of 9 amino acids. Hydrophobic regions are given a positive hydropathy index.

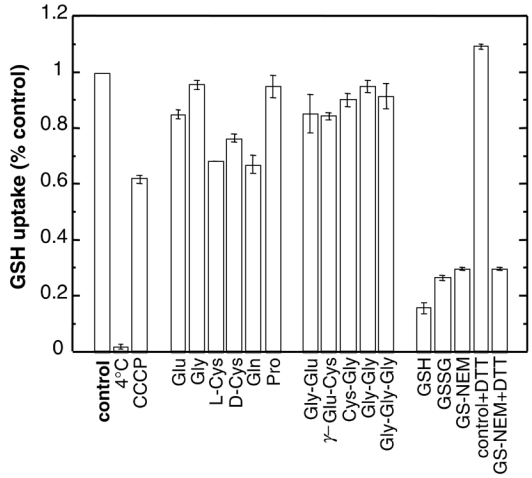

FIG. 3. **Effect of low temperature and of various compounds on GSH uptake in *hgt1*Δ, TEF-*HGT1* yeast.** The initial rates of uptake from a 0.5 mM GSH solution were determined at 4 °C, or in the presence of various compounds as indicated. Data were plotted as % of control (net initial rate of GSH uptake). The control initial rate of GSH uptake was 12.3 ± 0.3 nmol mg protein^{-1} min^{-1}. CCCP was used at 10 μM, amino acids, dipeptides, tripeptides, GSH, GSSG, and GS-NEM were all present at 5 mM. When preparing the GS-NEM conjugate solution, excess NEM was neutralized with DTT (final concentration 1.67 mM). Corresponding controls were carried out in the same conditions. Results are the mean ± S.E. of 8 to 12 samples from two or three independent experiments.

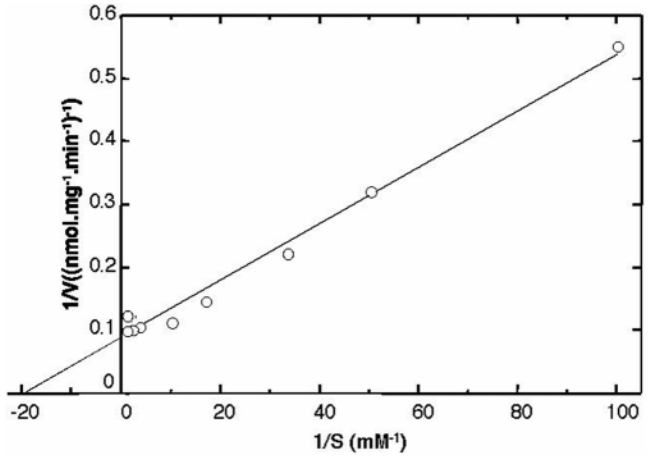

FIG. 4. **Double reciprocal plots of GSH uptake kinetics in *hgt1*Δ strains transformed with TEF-*HGT1*.** Initial rates of uptake were measured at GSH concentrations ranging from 10 μM to 1 mM, between 1 and 3 min. Data are representative from three experiments (4 replicates per experiment).

that the absence of glutathione was a strong inducer (or derepressor) of GSH transport activity in the wild type strain, whereas sulfur deficiency *per se* had no effect. The specific repression of the transport activity by glutathione argues strongly for Hgt1p being primarily a glutathione transporter.

The possible existence of other glutathione transporters was also examined by a genetic approach. A diploid heterozygous for *hgt1*Δ and *gsh1*Δ was constructed. The *hgt1*Δ/*HGT1* *gsh1*Δ/*GSH1* diploid was sporulated and dissected. Among 15 analyzed tetrads, one was 4-spore, 8 were 3-spore, and 6 were 2-spore. The presence of a wild type or of a disrupted *HGT1* gene was confirmed by PCR. All the *gsh1*Δ spores were subsequently analyzed to see if any of the *gsh1*Δ spores carried the *hgt1*Δ disrupted gene. This analysis was done by PCR since both *gsh1*Δ and the *hgt1*Δ deletions were marked by the *LEU2* marker. All the *gsh1*Δ spores were analyzed and none of the *gsh1*Δ spores were found to carry the *hgt1*Δ disrupted gene. Furthermore, the missing spores in the 2-spore and 3-spore tetrad corresponded to the *gsh1*Δ *hgt1*Δ double deletes. No spore appeared even after prolonged incubation of the plates. Tetrad dissections were also carried out by dropping the spores on media containing higher (1 mM) concentrations of glutathione to determine if there may still be a low affinity transporter. Again, no *gsh1*Δ *hgt1*Δ spore could be isolated under these conditions. The non-viability of the *gsh1*Δ *hgt1*Δ spores further confirmed that the *HGT1* gene encoded the primary glutathione transporter in the plasma membrane of this yeast. Yeast strains bearing a deletion in the *MET15* (*MET17*) gene are unable to utilize inorganic sources of sulfur for growth but can use organic sulfur sources such as methionine, cysteine, and glutathione (10, 33). We therefore constructed a *met15*Δ *hgt1*Δ double deletion and examined its growth on different sources of organic sulfur. These strains could grow on methionine as a source of organic sulfur but were specifically unable to utilize glutathione as a source of organic sulfur (Fig. 7). The results

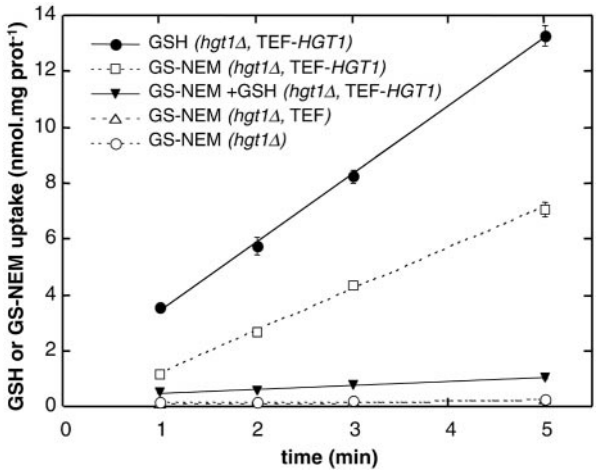

FIG. 5. **Hgt1p-mediated uptake of [^{3}H]glutathione-*N*-ethylmaleimide conjugate (GS-NEM).** *hgt1*Δ, (○), *hgt1*Δ, TEF (△), and *hgt1*Δ, TEF-HGT1 (□) strains were incubated in 0.1 mM [^{3}H]GS-NEM for various times. To ensure the total elimination of GSH in the GS-NEM solution, NEM was added in excess before conjugation and afterward neutralized with DTT (final concentration of 5 μM). Competitive inhibition of GS-NEM uptake was carried out with added GSH at a final concentration of 1 mM (▼). For comparison, GSH uptake was also measured in *hgt1*Δ, TEF-*HGT1* (●) yeasts from a 0.1 mM solution. Each point is the mean ± S.E. of four samples.

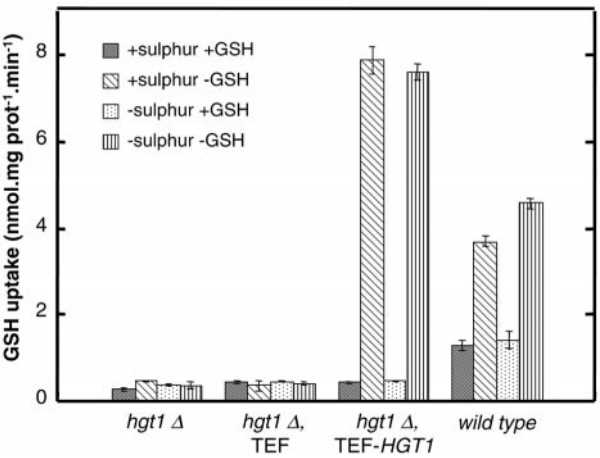

FIG. 6. **Inductibility of GSH uptake.** Various yeasts (*hgt1*Δ; *hgt1*Δ, TEF; *hgt1*Δ, TEF- *HGT1*; wild type YPH499) were grown either in minimum medium YNB supplemented with amino acids/bases (+ sulfur), or in sulfur-limited medium (minimum medium YNB supplemented with amino acids/bases where ammonium sulfate was replaced by ammonium chloride; − sulfur) for 12 h. Each set was separately grown in its respective medium supplemented (+ 250 μM GSH) or not (− GSH) for about 12 h till A_{600} = 0.6. GSH uptake time course was run as described earlier. The initial rate of GSH uptake was then calculated from the time course data. Results are the mean ± S.E. of 8 samples from two independent experiments.

are in agreement with the function of Hgt1p being described as a glutathione transporter, and are also in agreement with Hgt1p being the primary glutathione transporter in the plasma membrane of this yeast.

Homologues of Hgt1p in Other Organisms—Data base searches for Hgt1p homologues in other organisms using different BLAST/BLAST-PSI programs (34, 35) yielded several homologues in yeasts and plants. The proteins displayed between 38 and 51% identity and between 57 and 68% similarity over the entire stretch to Hgt1p. The genome of the yeast *C. albicans* contains a single homologue identified so far, Opt1p, while three homologues were found in *S. pombe* (Isp4p and accession numbers AL023590.1 and Z99164.1) (Fig. 8). The Isp4p and Opt1p proteins have been putatively identified as

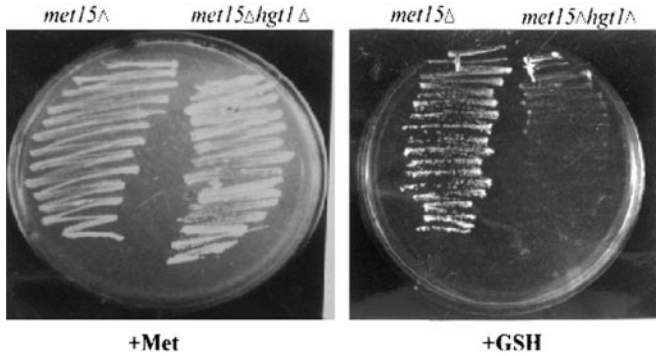

FIG. 7. **Growth of *met15*Δ and *met15*Δ*hgt1*Δ strains on minimal medium containing either methionine (+ Met) or glutathione (+GSH) as a source of organic sulfur.**

oligopeptide permeases in these yeasts (31), but in the light of our findings and the close homology to Hgt1p, it is possible that their primary function may be as glutathione transporters. The genome of *S. cerevisiae* also contains an homologue to Hgt1p (Ypr194cp). Although the function of Ypr194cp is unclear, studies with strains carrying a deletion in *YPR194c* did not contribute in any way to plasma membrane glutathione transport (Fig. 1). One possibility is that it might be localized to a different organelle. Several homologues were also found in plants (*Arabidopsis thaliana*) and ESTs in cotton and in *Neurospora crassa*. Surprisingly, no homologue was found in either *E. coli* or any other prokaryote or in any other multicellular eukaryotes other than plants. A BLAST search using different domains of this protein also failed to pick up any homologue in systems apart from plants and yeasts.

DISCUSSION

The present paper describes the cloning and characterization of Hgt1p, a high affinity plasma membrane glutathione transporter from the yeast *S. cerevisiae*. Hgt1p is the first high affinity glutathione transporter described from any system so far. The strategy employed involved the identification of candidate glutathione transporters from the yeast genome sequence, followed by a combination of genetic and physiological (growth properties, uptake studies) approaches. Evidence that *HGT1* encodes a glutathione transporter was obtained by loss of GSH transport activity upon gene disruption, and by restoration of transport activity by complementation of a *hgt1*Δ mutant with a plasmid bearing the *HGT1* gene. Several lines of evidence suggest that glutathione uptake is the primary function of the protein encoded by *HGT1*. The *HGT1* product mediates a specific uptake of GSH, GSSG, and GS-NEM. Neither an excess of L-amino acids nor various di- and tripeptides affects this uptake to any significant extent, and the *hgt1*Δ strain takes up amino acids such as proline at a normal rate. Derepression of GSH transport activity by glutathione deficiency is a further indication that Hgt1p is primarily a glutathione transporter and not a nonspecific oligopeptide permease as it was thought previously (31). Although Hgt1p clearly functions at the plasma membrane, the possibility that it might also be localized to a different membrane has not been examined.

The 40% inhibition by CCCP was similar to the CCCP inhibition levels seen in proton-coupled glutathione transport observed in plant protoplasts (11). Inhibition by CCCP is never complete in proton-coupled systems, because there is a residual passive electrical component of the proton-motive force, even if the pH gradient would be completely collapsed.

Hgt1p represents a novel class of transporter proteins. Sequence analysis revealed virtually no homology of the Hgt1p

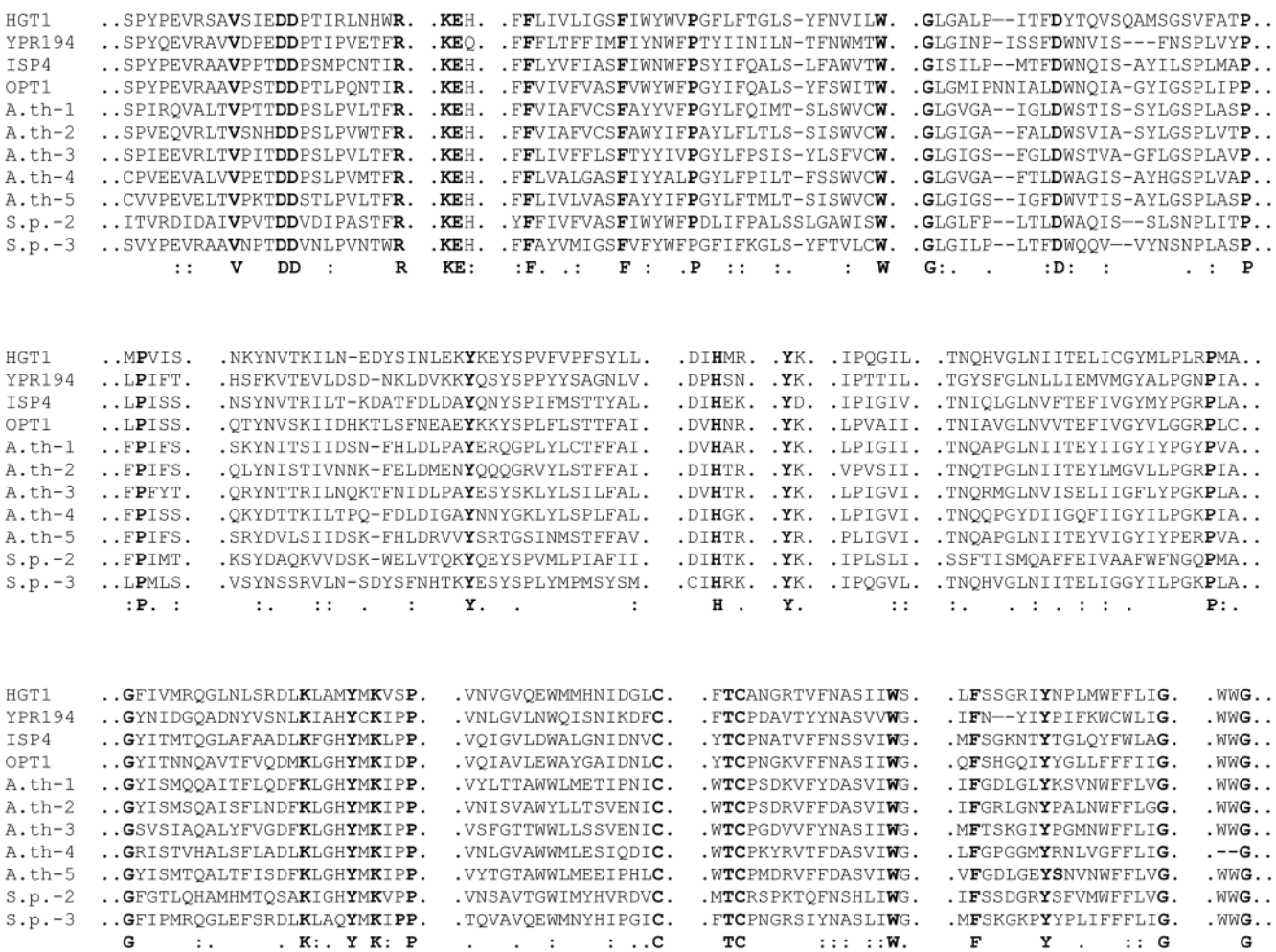

FIG. 8. **Multiple sequence alignment of the different yeast and plant homologues of Hgt1p.** The alignment indicates the more conserved regions and the amino acid residues that display complete identity among all 10 proteins of the family. The alignment was carried out using Clustal W with default parameters (39). *A.th-1*, accession number AAC35527; *A.th-2*, accession number AC000132; *A.th-3*, accession number S45495; *A.th-4*, accession number, z97341; *A.th-5*, accession number Al03062; *S. pom-2*, accession number Al023590.1; *S.pom-3*, accession number Z99164.1.

with the glutathione-conjugate pumps, YCF1 or MRP, that are able to mediate transport of glutathione with low affinity (17, 18). Hgt1p also appeared to be distinct from the yeast peptide transporters, Ptr2p, as well as other amino acid, dicarboxylic acid, and tricarboxylic transporters.

Our attempts to identify other secondary plasma membrane glutathione transporters in *S. cerevisiae* were unsuccessful. The *hgt1*Δ strain did not display any detectable glutathione uptake activity, and transport activity in this strain could not be induced by deficiency of glutathione or other sulfur compounds. Furthermore, double mutants in *hgt1*Δ and *gsh1*Δ were non-viable and could not be rescued even in the presence of high glutathione concentrations. Ptr2p which displays a wide substrate specificity to number of di- and tripeptides was also completely unable to mediate uptake of external glutathione. Even if a second glutathione transporter exists, its contribution to glutathione uptake must, for these reasons, be considered very minimal.

The *S. cerevisiae* genome contains a close homologue of Hgt1p, Ypr194cp, but disruption of this gene did not affect glutathione uptake to any discernable extent. In addition, the lethality of the *hgt1*Δ *gsh1*Δ spores and the inability of *hgt1*Δ *met15*Δ spores to grow on glutathione further suggest that Ypr194cp makes no significant contribution to plasma membrane glutathione uptake. Overexpression of Ypr194cp, but not Hgt1p, results in mild toxicity of certain tetra- and pentapeptides (31). Therefore, either *YPR194c* indeed encodes an oligopeptide transporter protein that is very closely related to *HGT1*, or, more likely, it encodes a glutathione transporter localized into a different organelle.

The *S. pombe* Isp4p gene is a close homologue of Hgt1p (38% identity and 57% similarity over the entire stretch). This gene was initially identified as a gene induced during sporulation (37) and its product displays oligopeptide transport activity for tetra- and pentapeptides (31). *S. pombe* diploids homozygous for defects in glutathione biosynthesis fail to sporulate, indicating an increased glutathione requirement during the sporulation process (28). A similar observation has been made earlier with *S. cerevisiae* diploids homozygous for *gsh1*Δ (38). It is possible, therefore, that genes such as $isp4^+$ are induced during sporulation to meet the increased requirement for glutathione. The strong homology to *HGT1* does indeed suggest that the primary function of the protein encoded by $isp4^+$ is glutathione uptake, but this remains to be demonstrated. Two other homologues of *S. pombe* as well as five homologues from the *A. thaliana* genome have been revealed. The functional characterization of each of these putative glutathione transporters needs further investigations.

It is surprising that *HGT1* did not display any homologues in eukaryotes other than yeasts and plants. This includes *Cae-*

norhabditis elegans for which the complete genome sequence is now available. However, extensive studies have been carried out on glutathione transport in mammalian liver cells, where glutathione plays a particularly important role. In these cells, the transporters have a much lower affinity for glutathione (K_m = 0.3 mM; Ref. 8) than that measured for Hgt1p. However, other studies with human small intestinal epithelial cells (12) have indicated the presence of high affinity glutathione transporters (K_m = 90 μM), an affinity comparable to that of Hgt1p. Therefore, high affinity glutathione transporters for glutathione also probably exist in mammalian systems, but they have yet to be identified. It is also possible that there may be a second class of glutathione transporters in these systems. If indeed glutathione uptake is mediated by different proteins in plants and animals, then the plant protein, easily accessible from the free space, and absent in animals would be a good target for herbicides not toxic to animals. The description of *HGT1* and the existence of several yeast and plant homologues should greatly facilitate the cloning, analysis, and our understanding of these transporters.

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