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 hgt1Δ disruptants were non-viable in a glutathione biosynthetic mutant (gsh1Δ) background. The glutathione repressor transport activity observed in wild type cells was also absent in the hgt1Δ strains. The transporter was cloned and kinetic studies indicated that Hgt1p had a high affinity for glutathione (Km = 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 associated with glutathione biosynthesis, glutathione may also be taken up from 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 Km 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 Toy 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 transformants were carried out using the lithium acetate method (19).
Cloning of HGT1—HGT1 was cloned by PCR. The following primers were used for amplifying HGT1 from chromosomal DNA of a wild type strain (YPH499); Y6L-Bam, 5′-CCGCGGATCCATGATTCACACCTATTATCAAAACC-3′ and Y6L-Eco, 5′-CAAGGAGCTGATCCAGGGATTATGATAAGTTACATG-3′. The 2.4-kilobase PCR product obtained was digested with EcoRI and BamHI and cloned into a single copy, URA3-based yeast expression vector downstream of the TEF promoter (20).

Tetrad Analysis—To check the viability of gh1Δ and gh1Δ double mutants, a diploid heterozygous for both markers was constructed by crossing ABC698 (gh1Δ::LEU2) with ABC828 (gh1Δ::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 uracil, minus GSH, minus leucine, and minus tryptophan plates.

Diploids heterozygous for ptr2Δ and gh1Δ, and diploids heterozygous for ypr194Δ and gh1Δ were constructed in a similar way. The diploids were sporulated and dissected as described above. The disruption at the GH1 locus was followed by glutathione auxotrophy, while disruption at either the YPR194Δ locus or the PTR2Δ locus was followed either by uracil 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 KanMX marker for PTR2 disruption, the URA3 marker for YPR194Δ 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, YPR194Δ 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 hgt1Δ::LEU2 and the ypr194Δ::URA3 disruptions in BY 4741 were then PCR amplified and yielded larger flanking regions and introduced into YPH 499. The disruptions were confirmed by PCR.

The primers used for the disruption of YPR194Δ were: 194-URA-F, 5′-ATGCAAGAATATTTAAGATGGAAGTAGATCCACACCGTTTTCAATCTCAAT-3′ and 194-URA-R, 5′-GGGTCATATGATCAGGTTTCTGTTTACAAATGTTATG-3′. The primers used for YPR194Δ disruption were: 194-URA-F, 5′-ATGCCAATGGAATATTTAAGATGGAAGTAGATCCACACCGTTTTCAATCTCAAT-3′ and 194-REV, 5′-GGGTCATATGATCAGGTTTCTGTTTACAAATGTTATG-3′. The used in the 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 A600 0.6, washed with a large volume of sterile water (4 °C), and 20 ml MES/ROH, 5 ml CaCl2, 2.5 mM MgCl2, 2% glucose (pH 5.5) (unless otherwise stated). They were then resuspended in the buffered medium (culture medium +0.6, washed with a large volume of sterile water (4 °C), and 20 ml MES/ROH, 5 ml CaCl2, 2.5 mM MgCl2, 2% glucose (pH 5.5) (unless otherwise stated). They were finally resuspended in the buffered medium (culture medium +0.6, washed with a large volume of sterile water (4 °C), and 20 ml MES/ROH, 5 ml CaCl2, 2.5 mM MgCl2, 2% glucose (pH 5.5) (unless otherwise stated). They were finally resuspended in the buffered medium (culture medium (26) using bovine serum albumin as a control.

RESULTS

The Yeast Plasma Membrane Peptide Transporter, Ptp2p, Is Unable to Transport Glutathione—S. cerevisiae has been reported to have a single peptide transporter, Ptp2p, that can transport dipeptides and tripeptides (27). Due to the wide substrate specificity reported for this transporter, the possibility that Ptp2p might be the transporter for the tripeptide glu-
Transporter—Studies with the yeast have revealed a new peptide transporter family that was able to transport glutathione. Data are the mean ± S.E. of 8 samples from two independent experiments.

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 concerns 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 hgt1Δ strains cultivated in the presence or absence of glutathione was studied. The data (Fig. 6) clearly indicated...
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 hgt1Δ and gsh1Δ was constructed. The hgt1Δ/HGT1 gsh1Δ/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 gsh1Δ spores were subsequently analyzed to see if any of the gsh1Δ spores carried the hgt1Δ disrupted gene. This analysis was done by PCR since

both gsh1Δ and the hgt1Δ deletions were marked by the LEU2 marker. All the gsh1Δ spores were analyzed and none of the gsh1Δ spores were found to carry the hgt1Δ disrupted gene. Furthermore, the missing spores in the 2-spore and 3-spore tetrad corresponded to the gsh1Δ hgt1Δ 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 gsh1Δ hgt1Δ spore could be isolated under these conditions. The non-viability of the gsh1Δ hgt1Δ 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 met15Δ hgt1Δ 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
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 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 glutathione transport activity upon gene disruption, and by restoration of transport activity by complementation of a hgt1Δ 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 hgt1Δ 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...
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 hgt1Δ 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 hgt1Δ and gsh1Δ 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 hgt1Δ gsh1Δ spores and the inability of hgt1Δ met15Δ 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 gsh1Δ (38). It is possible, therefore, that genes such as isp4Δ 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 isp4Δ 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 Caec-
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 ($K_m = 0.3 \text{ mM}$; 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 ($K_m = 90 \mu\text{M}$), 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 model 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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REFERENCES

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