RESEARCH ARTICLE

Differential roles played by the native cysteine residues of the yeast glutathione transporter, Hgt1p

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Abstract

Hgt1p, a high-affinity glutathione transporter from the yeast *Saccharomyces cerevisiae*, belongs to the structurally uncharacterized oligopeptide transporter (OPT) family. To initiate structural studies on Hgt1p, a cysteine-free (cys-free) Hgt1p was generated. This cys-free Hgt1p was nonfunctional and pointed to a critical role being played by the native cysteine residues of Hgt1p. To investigate their role, genetic and biochemical approaches were undertaken. Functional suppressors of the cys-free Hgt1p were isolated, and yielded double revertants bearing C622 and C632. Subsequent biochemical characterization of the individual C622S/A or C632S/A mutations revealed that both these cysteine residues were, in fact, individually indispensable for Hgt1p function and were required for trafficking to the plasma membrane. However, despite their essentiality, the presence of only these two native cysteines in Hgt1p generated a very weak glutathione transporter with minimal functional activity. Hence, the remaining 10 cysteines were also contributing towards Hgt1p activity, although they were not found to be singly responsible or crucial for Hgt1p functional activity. These residues, however, contributed cumulatively towards the stability and the functionality of Hgt1p, without affecting the trafficking to the cell surface. The study reveals differential roles for the cysteines of Hgt1p and provides first insights into the structural features of an OPT family member.

Introduction

Hgt1p or ScOpt1p, a polytopic membrane protein, from the yeast *Saccharomyces cerevisiae*, was the first high-affinity glutathione transporter to be identified in any system (Bourbouloux *et al.*, 2000). Hgt1p belongs to a relatively novel and largely uncharacterized oligopeptide transporter (OPT) family, which is completely distinct from the previously characterized peptide transporter family and ATP-binding cassette (ABC) superfamily (Lubkowitz *et al.*, 1998; Lubkowitz, 2006). The members of this family are restricted to fungi, plants and prokaryotes (Yen *et al.*, 2001). Phylogenetic analyses of the OPT family reveal two distinct clades, namely the peptide transporting (PT) clade and the yellow stripe (YS) clade, which also differ with respect to their substrate specificity (Lubkowitz, 2006). While the PT homologues transport oligopeptides, glutathione, glutathione conjugates or glutathione derivatives, the YS clade mediates uptake of metal-chelating secondary amino acids such as iron–mugineic acid complexes. Despite the existence of multiple homologues in different organisms, the biochemical characterization and physiological significance of only a few of the homologues such as AtOpt3, AtOpt4, AtYsl1, AtYsl2, CaOpt1, Isp4, Hgt1p, OsYsl2, Pgt1, TsYsl3 and ZmYs1 have been reported so far (Lubkowitz, 2006; Osawa *et al.*, 2006; Stacey *et al.*, 2008; Thakur *et al.*, 2008). A large number of members need to be defined with respect to their substrate specificity and physiological role, especially the bacterial homologues, of which none have been studied so far. Further, no information is available on the mechanistic or the structural aspects of any member of the OPT family. Elucidation of the structural features that govern the substrate specificity and affinity of the known members of the OPT family can provide a complementary approach in our understanding of this novel family.

Hgt1p is one of the best-characterized members of the OPT family in terms of its substrate specificity

(Bourbouloux *et al.*, 2000; Hauser *et al.*, 2000; Srikanth *et al.*, 2005; Osawa *et al.*, 2006), and its native host *S. cerevisiae* is a well-established model system and easily amendable for mutagenesis-based structure–function studies. To initiate a systematic study of structurally and functionally important residues and regions of Hgt1p, we have initially focused on elucidating the functional (or structural) roles played by the cysteine residues of Hgt1p. Hgt1p has 12 native cysteines, which are distributed in different regions of the protein according to a computationally predicted topology model for Hgt1p (Fig. 1; Wiles *et al.*, 2006). The initial focus on the cysteine residues was for the following reasons: (1) Hgt1p, while being a part of the sulphur assimilatory pathway (Srikanth *et al.*, 2005), has an unusually high percentage of sulphur amino acid residues (as compared with the other proteins of the sulphur assimilation pathway that include even the transporter proteins such as the yeast cysteine transporter, Yct1p) (Kaur & Bachhawat, 2007). As proteins of this pathway are otherwise known to have lower levels of these amino acids (Baudouin-Cornu *et al.*, 2001), it suggests an important role for the native cysteine residues. (2) Among the 12 cysteines of Hgt1p, two are partially conserved among the other OPT members (Wiles *et al.*, 2006), while the others are not conserved and six of the latter are in the predicted transmembrane domains, suggesting that these residues might have some functional significance. (3) With a future goal of undertaking cysteine scanning mutagenesis-based structure–function analysis of Hgt1p, the importance of the cysteine residues in the functionality of Hgt1p would need to be clearly understood, especially because the substrate, glutathione, itself contains a cysteine moiety with a reactive sulphydryl group.

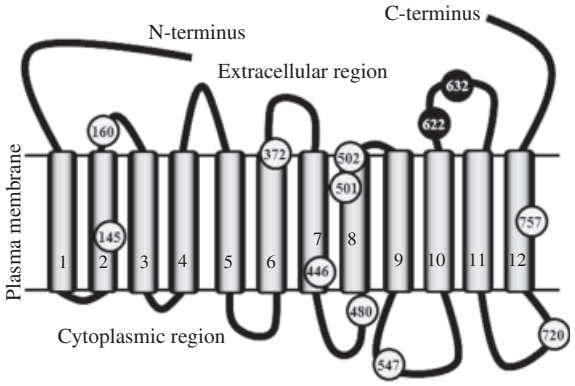

Fig. 1. Pictorial representation of putative Hgt1p topology showing the location of the native cysteine residues. The topology model is based on the prediction of Becker and coworkers (Wiles *et al.*, 2006). The 12 transmembrane domains are shown as rectangular bars, and the cysteine residues are marked as solid circles. The partially conserved cysteines are shaded dark grey, while the remaining 10 nonconserved cysteines are shaded lighter grey.

The present study describes our efforts in these directions, where we have been able to delineate the roles played by cysteines in the functionality of Hgt1p. The approach adopted to investigate the cysteines was through creation of a cys-free Hgt1p. As this was found to be nonfunctional, we devised a 'dual complementation-cum-toxicity' genetic screen to genetically isolate suppressors and this has enabled us to define roles for the cysteine residues of Hgt1p. Our results indicate that the two cysteines, C622 and C632, which are also evolutionarily coconserved among the OPT clade to which Hgt1p belongs, are essential for the functionality of Hgt1p. The role of these two cysteines was found to be in enabling the proper protein trafficking to the plasma membrane. The remaining 10 cysteines were individually not important for the functionality of Hgt1p, but cumulatively contributed to the stability and eventual functionality of Hgt1p without playing any role in the trafficking to the cell surface. The results provide the first insights into the residues important for the functionality of a member of the OPT family.

Materials and methods

Chemicals and reagents

All the chemicals used in this study were obtained from commercial sources and were of analytical grade. Media components, fine chemicals and reagents were purchased from Sigma-Aldrich (St. Louis), HiMedia (Mumbai, India), Merck India Ltd (Mumbai, India), USB Corporation (Ohio) or Difco. Oligonucleotides were purchased from Biobasic Inc. (Markham, ON, Canada). Restriction enzymes, Vent DNA polymerase and *Taq* DNA polymerase and other DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA). The DNA-sequencing kit (ABI PRISM 3130 XL with dye termination cycle sequencing ready reaction kit) was obtained from Perkin-Elmer (Norwalk, CT). Gel-extraction kits and plasmid miniprep columns were obtained from Qiagen (Valencia, CA) or Sigma-Aldrich. [^{35}S]-GSH (specific activity 1000 Ci mmol^{-1}) was purchased from Bhabha Atomic Research Centre, Mumbai, India. HA-Tag (6E2) mouse monoclonal antibody and horse anti-mouse horseradish peroxidase-linked antibody were bought from Cell Signaling (Danvers, MA). Alexa Flour® 488-conjugated goat anti-mouse antibody was obtained from Molecular probes (Eugene, OR). Hybond ECL (nitrocellulose) membrane and ECL plus Western blotting detection reagents were purchased from Amersham Biosciences (UK).

Strains, media and growth conditions

The *Eschericha coli* strain DH5α was used as a cloning host. The *S. cerevisiae* strain ABC817, bearing an *HGT1*

disruption in the BY4741background (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hgt1Δ::LEU2*) is deficient in glutathione uptake ability and was used in the experimental studies (Bourbouloux *et al.*, 2000). The other yeast strains used were ABC 733 (BY4741: *MAT*a *his3Δ1 leu2Δ0 met15Δ ura3Δ0*) (Brachmann *et al.*, 1998), ABC 1082 (*MAT*α *his 3 leu2 ura3 ade2 trp1 met 28::LEU2*) (Blaiseau & Thomas, 1998) and ABC 2032 (*MAT*a *his3Δ1 leu2Δ0 met15Δ ura3Δ0 YJL212c::GFPHIS3MX*) [Invitrogen (Huh *et al.*, 2003)]. Growth, handling of bacteria and yeast and all the molecular techniques used in the study were according to standard protocols (Guthrie & Fink, 1991; Sambrook *et al.*, 1989).

Epitope tagging of *HGT1*

Epitope tagging of *HGT1* under the TEF promoter

The plasmid pTEF-HGT1, which has *HGT1* cloned downstream of the TEF promoter at BamHI and EcoRI sites (Bourbouloux *et al.*, 2000), was used as a template to introduce a hexa-histidine epitope at the N-terminus and the hemagglutinin (HA) epitope tag at the C-terminus in a PCR-based reaction. The epitope tagging was carried out in two steps. In the first step, primer 212-N-HISBAMf containing the sequence encoding the hexa-histidine epitope and the 212-Sma1r reverse primer (Table 1) was used to amplify the N-terminal His-tagged 500-bp *HGT1* fragment, which was subcloned at BamHI and SmaI of pTEF-HGT1, to generate the pTEF-His-HGT1 plasmid. In the second tag, the primers Hpa1_For and reverse primer HA_TAG_REV, which contains the hemagglutinin epitope sequence just upstream of stop codon (Table 1), was used to amplify a C-terminus hemagglutinin-tagged 600-bp HGT1 fragment. This fragment was subcloned at the HpaI and EcoRI sites of the pTEF-His-HGT1 plasmid to generate the pTEF-His-HGT1-HA plasmid. The construct was confirmed by sequencing and all the cys-free mutants were made in this double-tagged recombinant *HGT1*.

Table 1. List of oligonucleotides and their sequences in this study

Oligomer name	Sequence (5′ to 3′)
212-N-HISBAMf	ACC ACA GGA TCC ATG CAT CAC CAT CAC CAT CACATG
212-Sma1r	TAA ATG GGC CCGGGT TTA AAT CG
Hpa1_For	CAG GCA TGG TTA ACG TTG GTG
HA_TAG_REV	CTC CGA ATT CTT ATG CAT AAT CAG GAA CAT CAT ATG GAT AAT ACC ACC ATT TAT CAT AAC CAA ATG TC
HGTI EcoRVSnaBI	TCT CCT GAT ATC TAC GTA CCT CCG TCT TCA CTC ATC C
HGTIBamHIb	ACT CAT GGA TCC TGT GAC GTT ATA TAA TTG TTG CG
C446Sf	CGC CGT TTT TGT CCA CTC CAT CTT ATA CCA CGG
C446Sr	CCG TGG TAT AAG ATG GAG TGG ACA AAA ACG GCG
C480Sf	CCA AGA ACT ATA AGG ATA GTC CCG ATT GGT GGT ATT TAC
C480Sr	GTA AAT ACC ACC AAT CGG GAC TAT CCT TAT AGT TCT TGG
C547Sf	GAA TAT TAT CAC AGA ATT GAT CTC CGG TTA TAT GCT GCC
C547Sr	GGC AGC ATA TAA CCG GAG ATC AAT TCT GTG ATA ATA TTC
C622A_F	CAT AAT ATC GAT GGC TTA GCT ACC ACC GAT CAA C
C622A_R	GTT GAT CGG TGG TAG CTA AGC CAT CGA TAT TAT G
C622SF	CAT AAT ATC GAT GGC TTA TCT ACC ACC GAT CAA CCA AAT G
C622SR	CAT TTG GTT GAT CGG TGG TAG ATA AGC CAT CGA TAT T
C632A_F	CCA AAT GGC TTC ACT GCT GCT AAT GGT CGC ACG G
C632A-R	CCG TGC GAC CAT TAG CAG CAG TGA AGC CAT TTG G
C632SF	CCA AAT GGC TTC ACT TCT GCT AAT GGT CGC ACG G
C632SR	CCG TGC GAC CAT TAG CAG AAG TGA AGC CAT TTGG
C757Sf	CGT CAT CAT CTT CTT GTC TGT ACA GTA CCC AGG TGG
C757Sr	CCA CCT GGG TAC TGT ACA GAC AAG AAG ATG ATG A

Epitope tagging of *HGT1* under native promoter

For the construction of the plasmid pNP-His-HGT1 bearing *HGT1* under its native promoter, 600-bp upstream activating sequences of *HGT1* were PCR amplified from the yeast genomic DNA using the primer pairs HGTIEcoRVSnaBIf and HGTIBamHIb (Table 1) and digested with EcoRV and BamHI restriction enzymes. The resulting fragment was subcloned into the plasmid pTEF-His-HGT1 so as to replace the TEF promoter. The plasmid pTEF-His-HGT1was digested with SacI, blunted by Klenow filling and then digested with BamHI to release the TEF promoter, and the 600-bp native promoter fragment generated above was cloned into this vector backbone to give the pNP-His-HGT1 plasmid. Subsequently, the 600-bp HpaI/EcoRI fragment from the plasmid pTEF-His-HGT1-HA was subcloned at the same sites into the plasmid pNP-His-HGT1 to generate pNP-His-HGT1-HA.

Construction of cys-free mutants

The cysteine to serine/alanine site-directed mutations were performed using the QuikChange® Site-Directed

Mutagenesis kit. The double-tagged HGT1 (His-HGT1-HA) was subcloned into the pBSK vector and used as a template together with different primer pairs (Table 1), in mutagenic PCR, set up as per the manufacturer's instruction. The resulting mutants were sequenced to confirm the presence of the desired nucleotide changes and rule out any undesired mutations introduced during the mutagenic procedure. The fragments containing the mutations were subcloned back into the TEF vector background (pTEF-His-HGT1-HA) using appropriate restriction sites, for subsequent analyses.

The 12-cys-free-HGT1 was constructed in a two-phase strategy. In the first phase, a 6-cys-free molecule bearing six cysteine to serine mutations (C145S, C160S, C372S C501S, C502S and C720S) was contracted out to Bangalore Genei, India. In the second phase, the 6-cys-free-Hgt1p cloned in the pBSK vector was used as a parent molecule to sequentially create the other cysteine to serine substitutions. In this manner, a 9-cys-free-Hgt1p (bearing nine C → S mutations: C145S, C160S, C372S C501S, C502S, C720S, C446S, C547S and C622S), 10-cys-free-Hgt1p (bearing C145S, C160S, C372S C501S, C502S, C720S, C446S, C547S, C622S and C757S), 11-cys-free-Hgt1p (bearing C145S, C160S, C372S C501S, C502S, C720S, C446S, C547S, C622S, C632S and C757S) and the 12-cys-free-Hgt1p (devoid of all the native cysteines) were created.

In order to explore the role of native cysteine residues, the 12-cys-free-HGT1 was fragmented by subcloning different regions of the mutant into the pTEF-His-HGT1-HA plasmid, thereby generating a series of cys-free mutants bearing different numbers and combinations of cysteine to serine mutations (Table 2).

Hydroxylamine mutagenesis of the pTEF-12-cys-free-HGT1

The random *in vitro* mutagenesis of the 12-cys-free-HGT1 was performed using hydroxylamine, by a previously described protocol (Rose & Fink, 1987). Briefly, 10 μg plasmid DNA was incubated in 0.5 mL of hydroxylamine solution (90 mg NaOH, 350 mg hydroxylamine-HCl in 5 mL water, pH around 6.5, freshly prepared before use). This mixture was incubated at 37 °C for 20 h and the reaction was terminated by adding 10 μL NaCl, 50 μL bovine serum albumin (1 mg mL^{-1}) and 1 mL of ethanol. The DNA was precipitated by incubating this mixture at −70 °C. This precipitation step was repeated three times. Finally, the pool of mutagenized plasmid was used directly for yeast transformation.

Dual complementation-cum-toxicity assay

The yeast strain ABC 817 (*met15*Δ *hgt1*Δ) was transformed with a single copy, centromere vector expressing wild type or different *HGT1* mutants expressed downstream of the TEF promoter. Transformants were grown in minimal media containing methionine and other supplements, without uracil, overnight. These cultures were reinoculated in the same media and allowed to grow till they reached the exponential phase. Equal numbers of cells were harvested, washed with water and resuspended in sterile water to an $OD_{600\,nm}$ of 0.2. These were serially diluted 1 : 10, 1 : 100 and 1 : 1000. Ten microlitres of these cell resuspensions were spotted on minimal medium containing different concentrations of glutathione (15, 30, 50, 100, 150 and 200 μM) or methionine (200 μM) as the sole organic sulphur source.

Table 2. List of cys-free mutants constructed in this study

Mutant name	Cysteine mutations
C446S	C446S
C480S	C480S
C547S	C547S
C622S	C622S
C632S	C622S
C622A	C622A
C632A	C632A
2-Cys-free-A (2CF-A)	C145S, C160S
2-Cys-free-B (2CF-B)	C720S, C757S
3-Cys-free (3CF)	C372S, C501S, C502S
4-Cys-free (4CF)	C145S, C160S, C720S, C757S
5-Cys-free (5CF)	C372S, C446S, C501S, C502S, C547S
6-Cys-free (6CF)	C372S, C446S, C480S, C501S, C502S, C547S
8-Cys-free-A (8CF-A)	C145S, C160S, C372S, C446S, C480S, C501S, C502S, C547S
8-Cys-free-B (8CF-B)	C372S, C446S, C480S, C501S, C502S, C547S, C727S, C750S
10-Cys-free (10CF) (12-cys-free revertant)	C145S, C160S, C372S, C446S, C480S, C501S, C502S, C547S, C720S, C757S
12-Cys-free (12CF)	C145S, C160S, C372S, C446S, C480S, C501S, C502S, C547S, C622S, C632S, C720S, C757S

The plates were incubated at 30 °C for 2–3 days and photographs were taken.

Glutathione transport assay

ABC 817 (*met15*Δ *hgt1*Δ) strain was transformed with different plasmid constructs bearing wild type or *HGT1* mutants under the TEF promoter and were grown in minimal media containing methionine and other supplements, without uracil, overnight. These cultures were reinoculated in the same media and allowed to grow till they reached the exponential phase. Cells were harvested and washed and placed on ice in an MES-buffered medium, until the transport was initiated. Transport experiments were carried out with [^{35}S]-GSH as described earlier (Bourbouloux *et al.*, 2000).

Preparation of cell extract and immunoblot analysis

Total crude cell extracts were prepared as described previously, with certain modifications (Aggarwal & Mondal, 2006). The protein concentration was estimated using the Bradford reagent (Sigma-Aldrich) with bovine serum albumin as a standard. Western blot analysis was performed using a modified version of the standard Western blot (Kaur & Bachhawat, 2009). Densitometry analysis of the unsaturated band signals was performed using the SCION IMAGE software to quantify the protein expression levels in different mutants. The resulting signal intensity was normalized with respect to the band surface area (in square pixels) and expressed in arbitrary units. The relative protein expression levels in the mutant Hgt1p were represented as percentage expression relative to wild-type Hgt1p.

Cellular localization of the mutants by confocal microscopy

To localize Hgt1p and its different cys-free mutants, indirect immunofluorescence was performed using a published protocol (Severance *et al.*, 2004). Images were obtained with an inverted LSM510 META laser scanning confocal microscope (Carl Zeiss) fitted with a Plan-Apochromat × 100 (numerical aperture, 1.4) oil immersion objective. The 488-nm line of an argon ion laser was directed over an HFT UV/488 beam splitter, and fluorescence was detected using an NFT 490 beam splitter in combination with a BP 505–530 band pass filter. The images obtained were processed using ADOBE PHOTOSHOP version 5.5.

Sequence analysis

The *HGT1* ORF and Hgt1p sequences were retrieved from the *Saccharomyces* genome database.

The Hgt1p sequence was used as a query sequence in the PSI-BLAST search of the nonredundant protein database from Entrez at the NCBI website (http://www.ncbi.nlm.nih.gov/) to retrieve a current list of homologues of Hgt1p. The iterations were repeated a total of seven times to obtain 249 sequences in all. The sequences that were a repetition of another protein such as truncations or those with a single mismatch (most likely allelic variants) and the distant homologues belonging to the YS clade were manually removed to yield a final list of 205 unique sequences. In the case of the one OPT member from *Aspergillus terreus* (gi|115399078), which appeared to lack the two conserved cysteines corresponding to C622 and C632 in Hgt1p, the genomic contig sequence was retrieved. The sequence was translated in the three different frames and the translated products were examined for potential exonic status by BLAST analysis against the *S. cerevisiae* genome.

For the phylogenetic analysis, multiple sequence alignment of the protein sequences was generated using CLUSTALX program using default parameters (Thompson *et al.*, 1997), and the TREEVIEW software was used to visualize the phylogenetic tree of the family.

Results

A dual complementation-cum-toxicity plate assay to functionally evaluate mutants of *HGT1*

*met15*Δ strains are organic sulphur auxotrophs that can grow on methionine, cysteine or glutathione as the sole source of organic sulphur. By providing glutathione at different concentrations as the sole source of organic sulphur, a sensitive plate-based growth assay was devised for rapid evaluation of *HGT1* mutants and for genetic selection strategies. The assay was based on the dual behaviour of *HGT1* expressed under the TEF promoter in a *met15*Δ*hgt1*Δ strain. While at low glutathione concentrations (15 μM or lower), *HGT1* expressed under the TEF promoter complements the growth defect in a *met15*Δ*hgt1*Δ strain very well (Bourbouloux *et al.*, 2000), the same construct results in toxicity when cells are grown in a medium containing 30 μM or higher glutathione concentrations (Fig. 2a). The cellular toxicity and eventual cessation of growth results from accumulation of high levels of glutathione in the cells as reported previously (Srikanth *et al.*, 2005). Thus, using this plate assay, the ability of the various mutant transporters expressed under the TEF promoter could be tested in two different ways for their ability to transport reduced glutathione (GSH): firstly, the ability to complement the GSH transport defect of the strain *met15*Δ*hgt1*Δ at low concentrations of GSH, and secondly, the ability to cause toxicity in media containing higher concentrations of GSH.

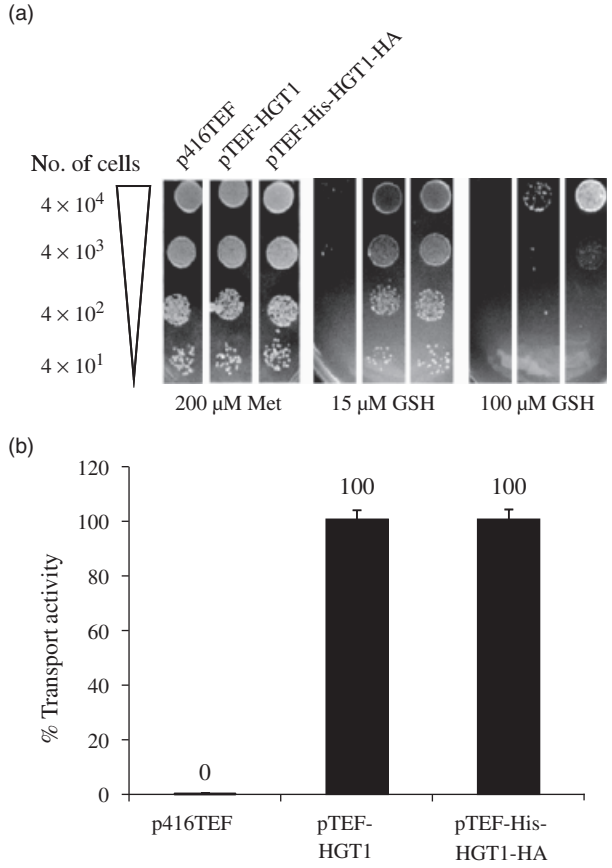

Fig. 2. A dual complementation-cum-toxicity plate assay for screening the functionality of Hgt1p mutants. The plasmid bearing *HGT1* and His-HGT1-HA double-tagged version of HGT1 under the TEF promoter and the corresponding vector (p416TEF) were transformed into ABC 817 (*met15*Δ*hgt1*Δ). The transformants were used for the functional characterization of hemagglutinin-tagged Hgt1p as follows: (a) a growth-based dual complementation-cum-toxicity assay: the transformants were grown to the exponential phase in minimal ammonia medium containing methionine, harvested, washed and resuspended in water and serially diluted to yield 0.2, 0.02, 0.002 and 0.0002 $OD_{600\,nm}$ of cells. Ten microlitres of these dilutions were spotted on minimal ammonia medium containing different concentrations of glutathione (GSH). The photographs were taken after 2–3 days of incubation at 30 °C. (b) Measurement of the rate of uptake of radiolabelled [^{35}S]-GSH uptake: the transformants were grown in minimal medium and used for the transport assay as described in Materials and methods. Log-phase cells were incubated with 100 μM ^{35}[S]-GSH for different time intervals and the initial rate of cysteine uptake was calculated by measuring the radiolabelled GSH accumulated in the cells at 1- and 3-min time intervals. Data are represented as the percentage of rate of uptake of GSH by His-Hgt1p-HA relative to His-Hgt1p. The rate of GSH uptake in His-Hgt1p was 55 ± 3.30 nmol min^{-1} mg^{-1} protein. The experiment was repeated twice and representative data are shown as % mean ± SD.

Hence, this assay was termed as the dual complementation-cum-toxicity assay.

For detection of Hgt1p, the protein was tagged at its N-terminus with the hexa-histidine tag and at its C-terminus with an hemagglutinin epitope tag (designated as His-Hgt1p-HA). To establish the dual complementation-cum-toxicity plate assay as a screen for the mutants, we evaluated the behaviour of the tagged Hgt1p in the plate assay and compared with the measurements of radiolabelled GSH uptake in the *met15*Δ*hgt1*Δ strain complemented with the control empty vector (pTEF416), wild-type *HGT1* (pTEF-HGT1) and double-tagged *HGT1* (pTEF-His-HGT1-HA) expressed under the TEF promoter. We observed that the ^{35}[S]-glutathione uptake activity of the tagged protein did not differ as compared with the wild-type protein (Fig. 2b). This was also seen in the complementation assay at 15 μM glutathione, where we observed comparable growth between untagged and tagged Hgt1p (Fig. 2a). Thus, the relative performance of the tagged Hgt1p compared with the untagged Hgt1p in the plate assay correlated well with the quantitative assay based on the uptake of radiolabelled glutathione by the transformants. As the epitope tagging did not bear a significant effect on the functionality of the transporter, it was used as the template to create all the cysteine mutants and considered as the wild type in this study. Of the two tags, only the C-terminal hemagglutinin-epitope tag was detectable on Western blots, and even in the case of detection of the hemagglutinin-epitope tag, we had to devise a modified protocol for consistent detection of the signal (Materials and methods; Kaur & Bachhawat, 2009).

However, when we examined the functionality through the toxicity assay, we observed a slightly compromised functional activity of tagged Hgt1p, as evident by marginal growth of the pTEF-His-HGT1-HA-transformed strain at 100 μM glutathione vs. no growth for the pTEF-HGT1-transformed strain (Fig. 2a). This observation suggested that the plate assay was more sensitive as compared with the radiolabelled uptake assay. A likely reason is that the plate assay is carried out over a 2-day incubation period and is thus able to detect even subtle differences in the functionality of the mutants that were often missed by the short-incubation-based transport assay.

Hgt1p is a plasma membrane-localized protein

The hemagglutinin-tagged Hgt1p construct was used to study the cellular localization of the transporter. Although genetic and biochemical data from our lab suggested a plasma membrane localization for Hgt1p (Bourbouloux *et al.*, 2000), localization studies were never carried out on Hgt1p. Furthermore, a previous report on genome-wide analysis of protein localization in *S. cerevisiae*, using C-terminus green fluorescent protein (GFP)-tagged proteins expressed from their native promoter in the chromosome itself, has predicted that Hgt1p is localized in the endoplasmic reticulum (ER) (Huh *et al.*, 2003). To determine whether the GFP-tagged Hgt1p was really functional in such

a strain, we obtained a strain bearing a C-terminus GFP-tagged chromosomal copy of *HGT1* in a *met15*Δ background. The strain was examined for growth on glutathione. We observed that this strain failed to grow on glutathione as the sole source of organic sulphur, but it could grow on methionine, suggesting a severe defect in the functionality of the GFP-tagged Hgt1p (Supporting Information, Fig. S1). Thus, no conclusion should be drawn regarding the localization of Hgt1p from such a nonfunctional construct.

We thus sought to study the *in vivo* localization of Hgt1p using the nine amino acid epitope (hemagglutinin)-tagged Hgt1p that retained function as described above (Fig. 2). The *met15*Δ*hgt1*Δ strain (ABC 817) was transformed with hemagglutinin-tagged *HGT1* expressed under the TEF promoter (pTEF-His-HGT1-HA) and the corresponding vector (p416TEF) and subjected to immunostaining using anti-hemagglutinin antibody as the primary antibody, followed by the Alexa-488-conjugated secondary antibody. Confocal fluorescent images of the pTEF-His-HGT1-HA transformed cells showed a clear cell surface labelling, implying plasma membrane localization for the protein, in contrast to the vector-transformed cells, in which no signal was observed (Fig. 3). Although majority of the cells showed a bright signal at the cellular periphery, some intracellular signal was also seen in a few of the cells, which might result from the use of an overexpression system (TEF promoter). To rule out an aberrant targeting of the protein due to its overexpression, the cellular localization of Hgt1p was also analysed under its native promoter.

The plasmid construct pNP-His-HGT1-HA bearing the C-terminus hemagglutinin-tagged *HGT1* gene cloned under the native promoter in a single copy, centromeric vector (pRS416 derived) by PCR amplification was transformed into the *met15*Δ*hgt1*Δ strain (ABC 817) and used for confocal study after growing the cells under nonrepressive sulphur conditions (20 μM methionine) to induce the gene. However, no signal could be observed for the protein. As the construct showed functional complementation in the *met15*Δ*hgt1*Δ strain (ABC 817) (data not shown), it was possible that the inability to detect the signal in the cells might be due to poor protein expression levels under the native promoter. Yeast strains deleted for *MET28* (a sulphur regulatory transcription factor) are derepressed for *HGT1* (Srikanth *et al.*, 2005). Hence, localization of Hgt1p under the native promoter was examined in a *met28*Δ strain (ABC 1082). As shown in Fig. 3, a clear signal for the protein was observed on the cellular periphery for the majority of the cells.

Although the Hgt1p signal, when expressed from the native promoter, became apparent in a *met28*Δ strain, the expression levels of the transporter were significantly lower than that expressed from the TEF promoter because no glutathione toxicity was observed in the *met28*Δ background (data not shown). As the fluorescent signal under both the TEF and the native promoters was predominantly observed at the cellular periphery, the wild-type Hgt1p appears to be exclusively localized to the plasma membrane. As the staining pattern in the *met28*Δ background was essentially similar to the one observed for Hgt1p expressed from the TEF promoter, all the subsequent localization studies of the mutants of Hgt1p have been carried under the TEF promoter.

A cys-free Hgt1p is nonfunctional. Suppressor analysis of cys-free Hgt1p reveals that both residues C622 and C632 are needed for a functional Hgt1p

To generate a cys-free Hgt1p, the 12 native cysteines in Hgt1p were replaced with serine by site-directed

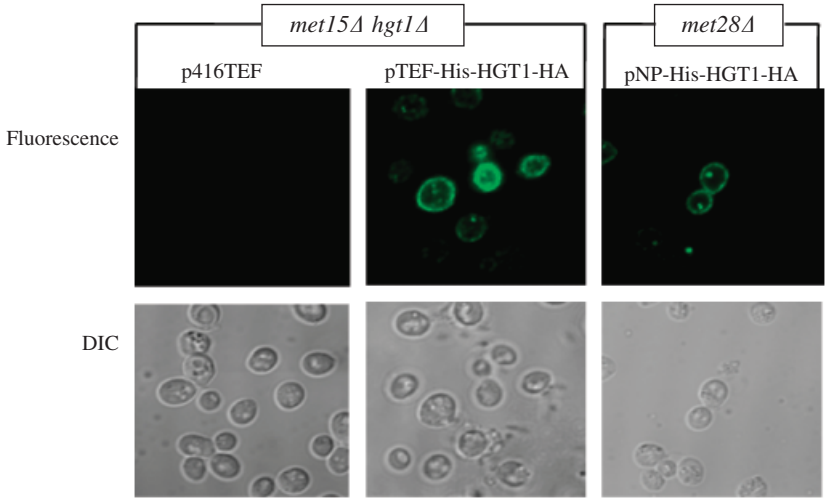

Fig. 3. Hgt1p is a plasma membrane-localized protein. Confocal analysis of hemagglutinin-tagged Hgt1p-*met15*Δ*hgt1*Δ (ABC 817) transformed with plasmids bearing double epitope-tagged Hgt1p expressed under the TEF promoter (pTEF-His-HGT1-HA) and the corresponding vector (p416TEF) and *met28*Δ (ABC 1082) transformed with double epitope-tagged Hgt1p expressed under the native promoter (pNP-His-HGT1-HA) and were grown to the log phase, fixed, labelled by indirect immunofluorescence using mouse anti-HA (Cell Signaling) primary antibody (at 1 : 100 dilution) and goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Cell Signaling) secondary antibody (at 1 : 500 dilution), and visualized using a confocal microscope, as described in Materials and methods. DIC, differential interference contrast.

mutagenesis (see Materials and methods). The serine residue was chosen as it represents the most conservative change possible in terms of the physico-chemical properties of the amino acids. The cys-free Hgt1p was cloned in a TEF-based single-copy vector and transformed into the *met15*Δ*hgt1*Δ strain. The cys-free Hgt1p failed to complement the *met15*Δ*hgt1*Δ, with no growth observed at low as well as high glutathione concentrations like the vector control strain (Fig. 4a). Further, no significant glutathione uptake could be detected in the *met15*Δ*hgt1*Δ cells transformed with cys-free Hgt1p as compared with the vector control (Fig. 4b), even with transport experiments carried out with hourly time points (instead of minutes, data not shown). Hence, the mutation of all the native cysteine residues to serine resulted in complete loss in the transport capacity of Hgt1p, thereby suggesting that one or more of the native cysteines were essential for the function of Hgt1p. Alternatively, it could be that serine is not an acceptable substitution at the position of one or more native cysteines, as has been reported for certain other membrane proteins (Jung *et al.*, 1994; Weissborn *et al.*, 1997; Stitham *et al.*, 2006; Arendt *et al.*, 2007). Hence, to identify such critical cysteine residues that, upon substitution, lead to a nonfunctional cys-free Hgt1p, a genetic strategy based on random mutagenesis and intragenic suppressor analysis was adopted.

A randomly mutagenized library of the nonfunctional cys-free Hgt1 was generated *in vitro* using hydroxylamine and directly transformed into the yeast strain ABC817 (*met15*Δ*hgt1*Δ). The resulting transformants were screened for a functional revertant by direct selection in minimal media containing 15, 30 or 70 μM GSH. A total of 23 000 colonies were screened and 21 transformants were obtained (five in the plates bearing 15 μM, 11 in 30 μM and five in 70 μM plates). Plasmids were retrieved from these yeast clones and were retransformed into the *met15*Δ*hgt1*Δ strain and re-examined for their functional activity using the growth-based dual complementation-cum-toxicity assay. These plasmids could complement the glutathione transport defect of the *met15*Δ*hgt1*Δ strain even at 15 and 30 μM GSH (data not shown and Fig. S2), as opposed to the cys-free Hgt1p, which did not support the growth even at 100 μM GSH concentration (Fig. 4a).

Eleven of these clones, selected on the basis of marginal differences in their ability to complement the *met15*Δ*hgt1*Δ strain, were sequenced to identify the site of mutation in them. Interestingly, in each of the clones sequenced, the resulting suppressor mutation was a double revertant with S622C and S632C mutation, bearing a TCT (Ser) to TGT (Cys) codon change in each of the cases. Complete sequencing of one of the clones showed that there was no other mutation in this clone apart from the reversion of these two cysteines (designated as 10-cys-free in the subsequent sections). Although hydroxylamine normally generates mutants with a GC to AT bias, extended treatment with hydroxylamine has been reported to create additional lesions including C to G transversion and deletions, which might explain the appearance of these unusual base changes in our genetic screen (Busby *et al.*, 1982a, b, N. Kasturia & A.K. Bachhawat, unpublished data). To reconfirm these findings, we carried out an independent round of the intragenic suppressor screen. The second screen also yielded the same results (data not shown).

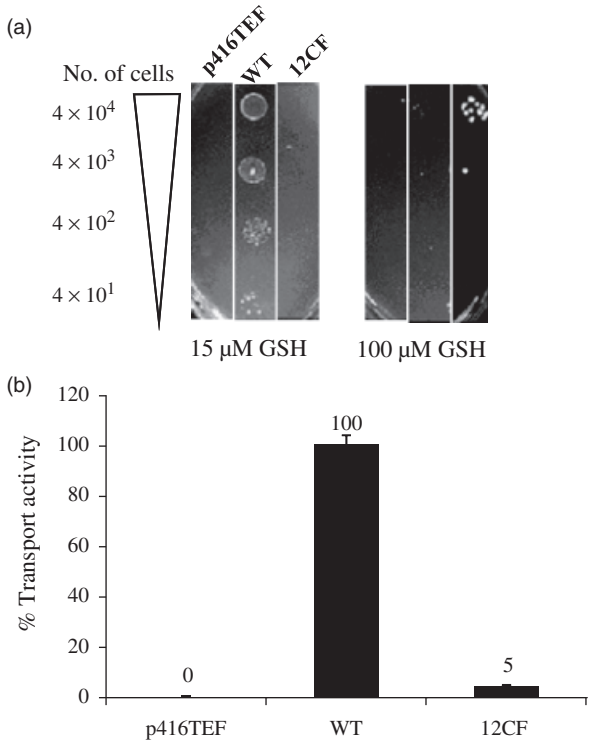

Fig. 4. The cys-free-Hgt1p lacking the 12 native cysteine residues is nonfunctional. The plasmid bearing wild-type Hgt1p (WT) and the 12CF under the TEF promoter and the corresponding vector (p416TEF) were transformed into ABC 817 (*met15*Δ*hgt1*Δ). The transformants were used for the functional characterization of hemagglutinin-tagged 12-cys-free-Hgt1p by (a) a growth-based dual complementation-cum-toxicity assay and (b) percentage rate of uptake of radiolabelled [^{35}S]-GSH uptake, as described in Materials and methods and in the legend to Fig. 2. The rate of GSH uptake in wild-type Hgt1p was 39.4 ± 1.6 nmol min^{-1} mg^{-1} protein. The experiment was repeated twice, in duplicate, and representative data are shown as mean ± SD.

The repeated isolation of the mutants bearing C622 and C632 as a double revertant in two independent genetic screens that exhibited enhanced functional activity over the original 12-cys-free Hgt1p mutant strongly suggests that the cysteines C622 and C632 are important for the function of Hgt1p and are the minimum number of native cysteines needed for a functional Hgt1p.

C622 and C632 are indispensable for the functional activity of Hgt1p

To examine the importance of the C622 and C632 residues in the function of Hgt1p in greater detail, C622 and C632 were individually mutated to serine to generate single mutants for these residues (Hgt1p-C622S and Hgt1p-C632S). The single C622S and C632S mutants of Hgt1p were transformed into the *met15*Δ*hgt1*Δ strain (ABC 817) and compared for their functional activity with the wild-type Hgt1p (His-Hgt1p-HA) and the hemagglutinin-tagged 12-cys-free revertant with C622 and C632 (10-cys-free, 10CF) using the plate assay (Fig. 5a) and the glutathione uptake assay (Fig. 5b).

For the evaluation of the mutants by a dual complementation-cum-toxicity assay, the transformants were subjected to serial dilution spotting on minimal media, containing either methionine or a range of glutathione concentrations as the sole source of organic sulphur. The single mutants of Hgt1p, C622S and C632S, showed a very significant loss in activity in terms of their ability to support the growth of the *met15*Δ*hgt1*Δ strain (ABC 817) at low glutathione concentrations and to be able to mediate toxicity to the strain at higher concentrations (Fig. 5a). Unlike the single cysteine mutants, C622S and C632S, which completely failed to support the growth of the *hgt1*Δ*met15*Δ strain (ABC 817) at low glutathione concentrations, the 10-cys-free Hgt1p, which had 10 of the 12 native cysteine mutated to serine, but

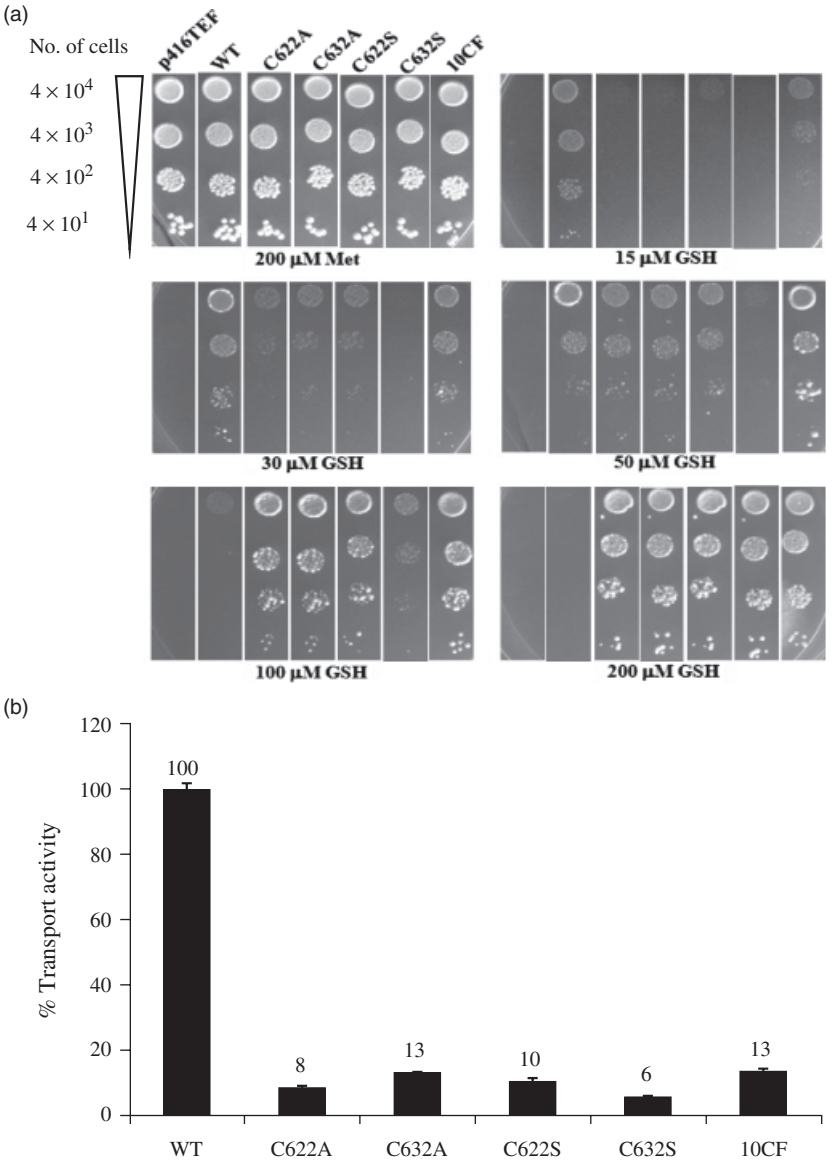

Fig. 5. Functional analysis of C622 and C632 mutants of Hgt1p. The C622A-HGT1, C632A-HGT1, C622S-HGT1, C632S-HGT1, 10CF-HGT1, and wild-type *HGT1* (WT), along with the control vector (p416TEF), were transformed into the *met15*Δ*hgt1*Δ yeast strain (ABC 817) and used for (a) a plate-based dual complementation-cum-toxicity assay by dilution spotting on minimal media containing different concentrations of glutathione and (b) measurement of rate of radiolabelled glutathione uptake. Data are represented as a percentage of the rate of uptake of GSH by the mutants relative to the wild-type Hgt1p. The rate of GSH uptake in wild-type Hgt1p was 42.4 ± 0.7 nmol min^{-1} mg^{-1} protein. The experiment was repeated twice, in duplicate, and representative data are shown as mean ± SD.

the C622 and C632 retained, showed a partial complementation of the *hgt1*Δ defect at a low glutathione concentration (15 μM GSH plate, Fig. 5a). None of the mutants resulted in toxicity in the strain, even up to 200 μM glutathione, implying a severely compromised ability to mediate glutathione uptake by the mutants.

To directly assess the glutathione transport capability of the single cysteine Hgt1p mutants, the rate of radiolabelled glutathione uptake was measured in the *met15*Δ*hgt1*Δ strain (ABC 817) transformed with the respective plasmids. As expected, and in agreement with the plate assay, the drastic effect of the single C622S and C632S mutations on the functionality of Hgt1p was also observed in the transport assay, with Hgt1p-C622S and Hgt1p-C632S showing 10% and 6% functional activity relative to the wild-type Hgt1p. Interestingly, the functional activity of the 10-cys-free mutant was observed to be nearly 13%. However, this was despite the fact that the 10-cys-free Hgt1p contained C622 and C632 and did retain the partial ability to complement the *hgt1*Δ defect on the plate. Thus, the transport assay could not differentiate between the weak functionality of the 10-cys-free mutant (as seen in the plate assay) compared with the virtually null activity of the C622S and C632S mutants (Fig. 5). However, the significantly reduced activity of 10-cys-free Hgt1p suggests that one or more of the other 10 native cysteines might also contribute in generating a fully functional transporter (investigated in the subsequent section).

To evaluate whether the complete loss in activity of the single mutants, C622S and C632S, was due to introduction of a serine residue specifically as has been reported for some of the other transporters (Jung *et al.*, 1994; Weissborn *et al.*, 1997; Stitham *et al.*, 2006), the cysteines were also mutated to alanine to generate C622A and C632A mutants of Hgt1p. The C622A and C632A mutants of Hgt1p were also found to be nonfunctional (Fig. 5). Hence, the complete loss in activity of the single mutants of C622 and C632 was observed irrespective of whether these residues were replaced by a small hydrophobic residue (alanine) or an isosteric polar residue (serine), suggesting that the importance of these two cysteine residues was due to the presence of the thiol group.

We also carried out a deletion of an 11 amino acid stretch from C622 to C632, including the two cysteine residues, in Hgt1p [(C622-C632)Δ mutan], to see whether the deletion of this stretch might be tolerated in terms of the Hgt1p functionality. However, the deletion led to a complete loss in the activity of the protein as reflected by the inability of the mutant to support growth of the *met15*Δ*hgt1*Δ strain (ABC 817) at even a high (200 μM) glutathione concentration (data not shown).

These observations together suggest that the presence of the two partially conserved cysteines *per se* is obligatory for the functional activity of Hgt1p. The importance of these two residues is additionally highlighted by the fact that they both appeared together in the functional revertant of the nonfunctional 12-cys-free Hgt1p molecule and by the weak activity of the 10-cys-free Hgt1p containing C622 and C632 as observed in the sensitive plate assay.

Functional evaluation into the role of the remaining 10 native cysteine reveals a cumulative contributory effect towards the functionality of Hgt1p

While the presence of C622 and C632 is essential for restoring the functional activity of the cys-free Hgt1p, the two cysteine residues together could restore only up to 15–20% of the wild-type activity. This suggests that the one or more of the 10 native cysteine residues were also needed for generating a fully functional transporter. To dissect the role of the other native cysteine residues, a series of mutants with different combinations and number of cysteine residues replaced with serine were generated, by subcloning fragments from the existing cys-free Hgt1p molecules into the hemagglutinin-tagged wild-type Hgt1p background (Table 2). The different 'cys-free' mutants of Hgt1p were transformed into the *met15*Δ*hgt1*Δ strain (ABC 817) and evaluated for their functional activity in comparison with the wild-type Hgt1p (His-Hgt1p-HA) using the growth-based dual-complementation-cum-toxicity assay (Fig. 6a). A careful analysis of the growth pattern of the mutants revealed an interesting correlation between the glutathione uptake ability and the number of cysteine residues mutated to serine in Hgt1p. While the 2-cys-free, 3-cys-free and 4-cys-free mutants of Hgt1p showed growth comparable to the wild-type Hgt1p at 15 μM glutathione (complementation), which decreased with increasing glutathione concentration (toxicity effect), mutants with five or more native cysteine to serine mutations showed weaker complementation of the cells at a low glutathione concentration, and growth became better with increasing glutathione concentrations. Unlike the wild-type Hgt1p, no toxicity was observed for this latter set of mutants. The most extreme case was 12-cys-free Hgt1p, which failed to complement the *hgt1*Δ even at 200 μM glutathione as observed previously (Fig. 3a). Taken together, these plate assays reveal a gradual loss in transport activity with increasing number of cysteine to serine mutations.

To generate a quantitative picture of the observation made in the plate assay, the rate of glutathione uptake was measured in the *met15*Δ*hgt1*Δ strain (ABC 817) transformed with different cys-free constructs. As shown in Fig. 6b, the results of the biochemical assay were in agreement with the plate assay and a gradual decrease in the percentage transport activity was observed as one moved

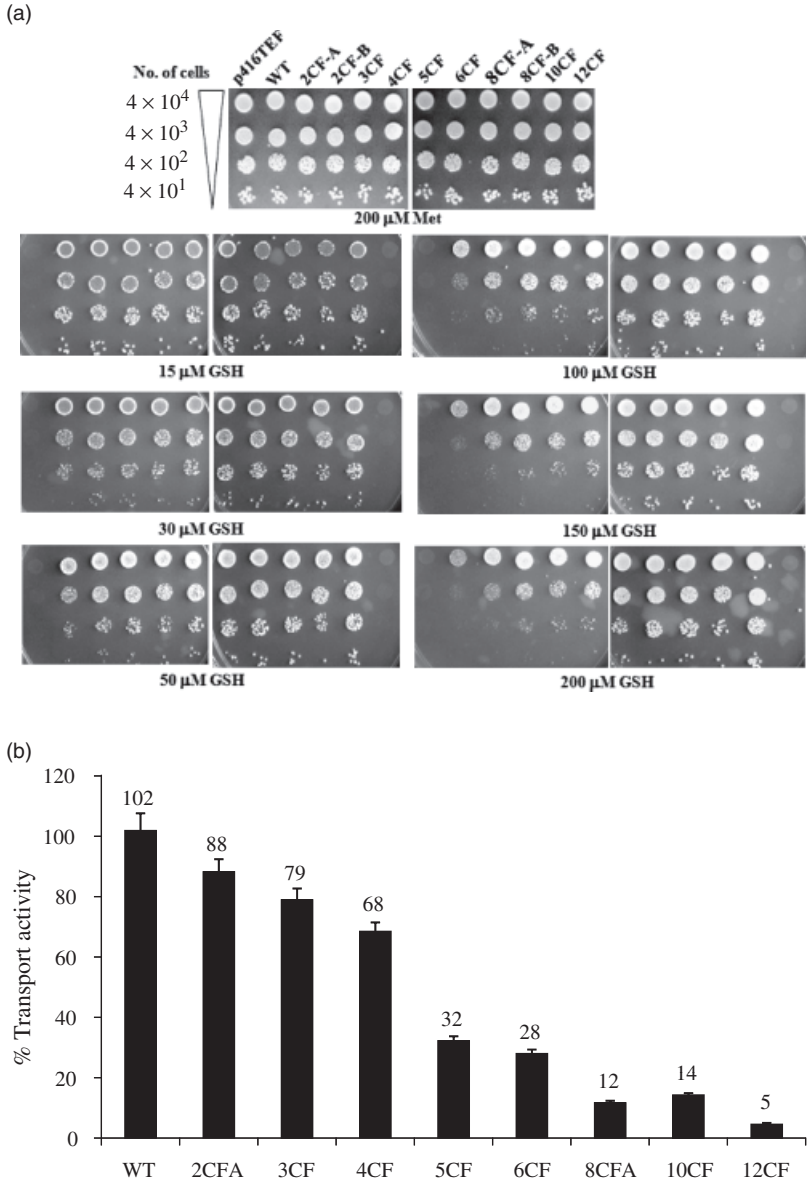

Fig. 6. Functional analysis of different cys-free mutants of Hgt1p. The *met15*Δ*hgt1*Δ strain (ABC 817) was transformed with wild-type Hgt1p and the different cys-free Hgt1p mutants (2CF-A, 2CF-B, 3CF, 4CF, 5CF, 6CF, 8CF-A, 8CF-B, 10CF and 12CF) expressed under TEF promoter, along with the control vector (p416TEF), and used for (a) a plate-based dual complementation-cum-toxicity assay by dilution spotting on minimal media containing different concentrations of glutathione and (b) measurement of the rate of radiolabelled glutathione uptake. Data are represented as a percentage of the rate of uptake by the mutants relative to wild-type Hgt1p (WT). The rate of GSH uptake in wild-type Hgt1p was 41.7 ± 6.0 nmol min^{-1} mg^{-1}-protein. The experiment was repeated twice, in triplicate, and representative data are shown as mean ± SD.

from wild-type Hgt1p to 12-cys-free Hgt1p, the latter exhibiting virtually no activity.

The 2-cys-free-A (2CF-A), 3-cys-free (3CF) and 4-cys-free (4CF) showed 88%, 79% and 68% transport activity, respectively, compared with the wild-type Hgt1p. This implies that the mutation of C145, C160, C372, C501, C502, C720 and C757 did not have any significant impact on the functional activity of Hgt1p individually. However, cumulatively, they were clearly contributing to the Hgt1p functional activity. The 5-cys-free (5CF) and 6-cys-free (6CF) Hgt1p mutants showed a more drastic decline in activity compared with the 3-cys-free Hgt1p, with the activity decreasing by nearly 50%. The 5-cys-free and 6-cys-free Hgt1p had C446S, C480S and C547S mutations in addition to C372S, C501S and C502S in the 3-cys-free mutants. To evaluate the contribution of these residues, the individual C446S, C480S and C547S mutants were also constructed and evaluated for functionality using the dual-plate assay and the measurement of glutathione uptake. However, these single mutations did not have any significant effect on the activity of Hgt1p both in terms of their ability to complement the glutathione defect in the *met15*Δ*hgt1*Δ strain (ABC 817) and in terms of causing toxicity to the cells (data not shown). The percentage transport activity was also found to be comparable to the wild-type Hgt1p, with C446S, C480S and C547S mutants exhibiting 85%, 87% and 88% glutathione uptake activity relative to the wild-type Hgt1p.

The functional analysis of the different nonconserved cys-free mutants of Hgt1p thus revealed that none of these cysteines are essential for the Hgt1p activity individually, although each seemed to be contributing in generating a fully functional transporter, a cumulative effect of which could be observed in a 10-cys-free Hgt1p molecule.

The C622A/S and C632A/S mutations do not cause a significant alteration in the protein expression. Substitution of the remaining cysteine with serine residues cumulatively results in decreased protein expression levels

The loss of functional activity of the individual cysteine mutants (C622S and C632S) and the other 'cys-free' mutants could be due to decreased expression levels, impaired trafficking of these mutants to the plasma membrane or an intrinsic loss in the activity of the protein. Hence, to assess the contribution of the cysteine residues in the stability or the expression of Hgt1p, the crude cell lysate prepared from wild type or different cys-free mutants, transformed *met15*Δ*hgt1*Δ yeast strains were subjected to immunoblot analysis using anti-hemagglutinin monoclonal antibody. As shown in Fig. 7, a band around 85 kDa was visualized in the crude lysate prepared from the cells transformed with the wild-type Hgt1p, which was absent in the vector-transformed cells. A signal for a band corresponding to Hgt1p could be visualized in the crude cell lysates prepared from the C622A/S and C632A/S mutants as well (Fig. 7a), suggesting that replacement of these individual cysteines did not significantly affect the protein synthesis or stability. Densitometry analysis of the unsaturated band signals was performed to quantify the protein expression levels for the mutants, relative to the wild-type protein. While for the alanine mutants the total protein expression levels were comparable to the wild-type protein levels (91% and 79% for C622A and C632A, respectively), the serine mutants showed nearly a twofold decrease in the protein expression levels (51% and 47% for C622S and C632S, respectively). Although a slight decline in the protein levels was seen, significant levels of proteins were still detected in these single cysteine mutants. These findings suggested that mutation of

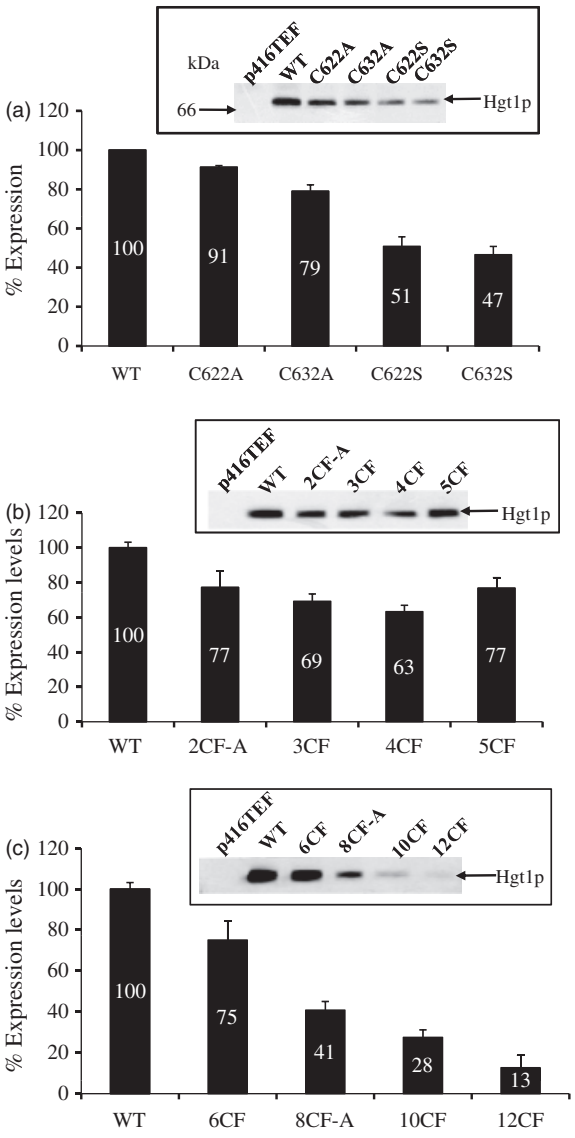

Fig. 7. Quantification of the total protein expression levels of the different cys-free mutants of Hgt1p. The crude extracts prepared from the *met15*Δ*hgt1*Δ strain (ABC 817) transformed with plasmids bearing the different cys-free mutants of Hgt1p were prepared and c. 20 μg total protein was resolved using 9% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, electroblotted to a nitrocellulose membrane. After the transfer, the blot was incubated in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 55 °C for 15 min with mild agitation, followed by two washes with TBST at room temperature and probed with mouse anti-hemagglutinin (Cell Signaling) at 1 : 1000 dilution as the primary antibody, and goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Cell Signaling, at 1 : 2500 dilution) as the secondary antibody. The signal was detected using the ECL kit (Amersham Bioscience). Molecular weight markers (SM0431, MBI fermentas) were used to estimate the protein molecular weight. The total protein was quantified by densitometry analysis of protein bands. Data are expressed as percentage protein expression normalized to the wild-type expression level, and are the mean of the protein expression levels obtained in three independent experiments. A representative blot is shown in the inset. Equal loading of the proteins (20 μg) in each well of the gel was visually monitored by Coomassie staining of a duplicate gel and Ponceau S staining of the membrane after transfer (data not shown). (a) % Protein expression for C622A, C622S, C632A, C632S Hgt1p mutants; (b) % protein expression for 2CF-A, 3CF, 4CF and 5CF; (c) % protein expression for 6CF, 8CF-A, 10CF and 12CF.

the conserved cysteine residues did not exert a significant effect on the synthesis or the stability of the protein. Furthermore, even the twofold decrease in the protein expression levels of the serine mutants could not explain the complete loss in the functional activity of these mutants.

In contrast, the densitometry analysis of Western blots of Hgt1p molecules with multiple cysteine mutations revealed that there was a gradual decrease in the expression levels of the proteins (Fig. 7b and c), which correlated with the loss in activity of the mutants (Fig. 6b), both of which appeared to be a function of the number of remaining cysteines in the mutant. Thus, the 8-cys-free, 10-cys-free and 12-cys-free Hgt1p, which showed very little transport activity, had low protein expression levels as well. These results suggest that while replacement of individual cysteines with serine might result in only a minor perturbation in the local conformation of the protein, the multiple cysteine replacements in the same protein molecule tend to magnify these minor faults. This eventually leads to a molecule with a significant structural instability and hence decreased protein levels, which is responsible for the observed loss in functional activity.

Replacement of single C622 and C632 residues to serine in Hgt1p affects the trafficking of the transporter to the cell surface

As Hgt1p is a plasma membrane transporter, the loss in functional activity upon mutation of C622 and C632 could be a result of defective trafficking of the mutants to the cell surface. This possibility needed to be examined. Thus, the cell surface expression levels of the mutants were compared with the wild-type Hgt1p by immunostaining, using anti-hemagglutinin antibody as the primary antibody, followed by the Alexa-488-conjugated secondary antibody. As the substitution of C622 and C632 with either serine or alanine yielded a similar functional phenotype, we performed the confocal studies only with C622S and C632S mutants. The confocal studies of the transformants showed a bright, cell surface signal for the wild-type Hgt1p, as expected (Fig. 8a). However, in sharp contrast to the wild-type protein, the signal for the mutants was barely detectable on the cell surface, with the majority of the protein being retained intracellularly (Fig. 8a). The pattern of staining for the mutants was typical for ER localization, with a distinct perinuclear ring and additional staining near the cell periphery (Preuss *et al.*, 1991). This suggested that the mutants were becoming trapped in the ER.

The examination of the cell surface protein expression level of the 5CF, 6CF, 8-cys-free (8CF), 10CF and 12-cys-free (12CF) revealed a very intense cell surface signal on the cellular periphery for the 5CF and the 6CF mutants (Fig. 8b), suggesting that these mutants did not have any obvious defect in protein trafficking to the cell surface. For the 8CF and 10CF, a cell surface signal was observed in the majority of the cells (shown with a white arrow, Fig. 8b). This poor signal intensity was likely a consequence of the decreased protein expression levels observed for these mutants. In contrast, for the 12CF mutant, no cell surface signal could be visualized in almost all the cells observed. The signal intensity was also very weak, and was found to be diffused throughout the cytoplasm.

Thus, the confocal analysis of the cys-free mutants suggests that although the C622S and C632S mutants are synthesized and stable in the cell, they are inefficiently trafficked to the cell surface, resulting in a poor cell surface expression of the transporter and hence nonfunctionality of the mutants. In contrast, the mutation of the nonconserved cysteine residues did not lead to a defect in the trafficking of the protein to the cell surface, although they had an effect on the protein expression levels as shown in the previous section.

Phylogenetic analysis of the OPT family in the light of the essential role of C622 and C632

The above findings highlight the essentiality of having both C622 and C632 for the functional activity of Hgt1p. To see whether this information could help understand the other members of the OPT family and reveal some new insights into this relatively uncharacterized family of transporters (and might also assist in further classifying the members), we performed a multiple sequence alignment of the members of the OPT family. A previous study undertook a multiple alignment of the 103 homologue members of the PT clade in the OPT family and found that these two cysteine residues fell into the 75–89% conservation group (Wiles *et al.*, 2006). Since then, several new genomes have been sequenced and new sequences have been deposited in the genome database. We therefore retrieved the current list of the Hgt1p homologues as described in Materials and methods. A total of 205 unique members of the PT clade were obtained, and subjected to multiple sequence alignment analyses using the CLUSTALX software. The alignment revealed an interesting insight into the conservation pattern of C622 and C632 in the family. While 37 members did not contain cysteine residues corresponding to either C622 or C632 in Hgt1p, the remaining members always had both the cysteines conserved together, showing 82% conservation across the PT clade. Moreover, all the members that contained the two cysteines clustered, including one member that lacks the two conserved cysteine residues. Thus, all the 169 members (with one exception) in this cluster contained both the conserved cysteine residues corresponding to C622 and C632 in Hgt1p. Multiple sequence alignment using a few of the randomly selected PT members has been used to

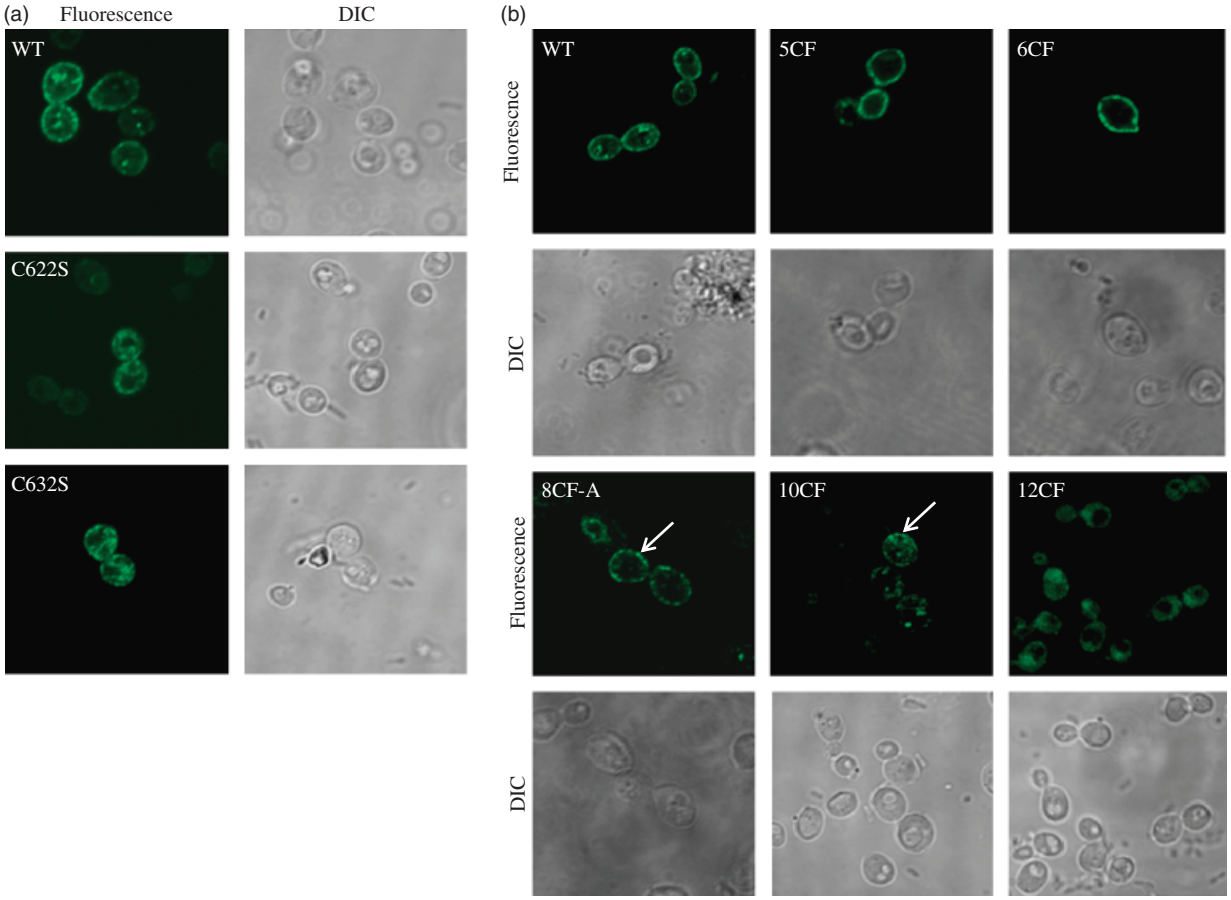

Fig. 8. Cell surface expression analysis of the C622S, C632S and the different cys-free mutants of Hgt1p by indirect immunofluorescence and confocal analysis. The *met15*Δ*hgt1*Δ strain (ABC 817) was transformed with plasmids bearing wild-type *HGT1,* C622S-HGT1 and C632S-HGT1 and the different cys-free mutants of Hgt1p and labelled by indirect immunofluorescence using mouse anti-hemagglutinin (Cell Signaling) primary antibody (at 1 : 100 dilution), and goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Cell Signaling) secondary antibody (at 1 : 500 dilution), and visualized using a confocal microscope, as described in Materials and methods. Cell surface localizations in 8CF and 10CF are shown by the white arrow. DIC, differential interference contrast.

create an unrooted phylogenetic tree to represent this observation, shown in Fig. 9. The one homologue lacking the two conserved cysteine residues (Ate2, Fig. 9a), yet clustering with the other 169 members (Ate2, Fig. 9a), is a hypothetical protein from *A. terreus* (gi|115399078|). The sequence alignment of the protein with Hgt1p revealed that although the protein has a high overall similarity to Hgt1p (44% identity and 62% similarity), there was a gap of about 50 amino acids in a region corresponding to the two conserved cysteine residues, C622 and C632, in Hgt1p. On a closer examination of the sequence, we were able to discover that this was likely a result of misannotation of the genomic sequence. One of the exons appears to have been missed and a translation product of this missing exon shows significant similarity to Hgt1p (34% identity and 55% similarity, *E*-value = $2e^{-06}$ on a 66 amino acid stretch) and also contained the two, similarly spaced, conserved cysteine residues (data not shown). Thus, the presence of the two conserved cysteines in the PT clade seems to define a new cluster within the family (Cluster I, Fig. 9b), while those lacking the conserved cysteine residues form a separate cluster (Cluster II, Fig. 9b).

An extension of this analysis to the YS clade of the OPT family reveals a similar subclustering, based on the conservation of the cysteine residues (Fig. 9b). A large cluster comprising of 90 plant YS members contains four absolutely conserved cysteines (Cluster III), although none of these align with the C622 and C632 in Hgt1p. Instead, these conserved cysteine residues correspond to amino acid positions 604, 654, 745 and 767 in Hgt1p. In contrast, members of the YS cluster in fungi and bacteria do not have any conserved cysteine residues (Cluster IV).

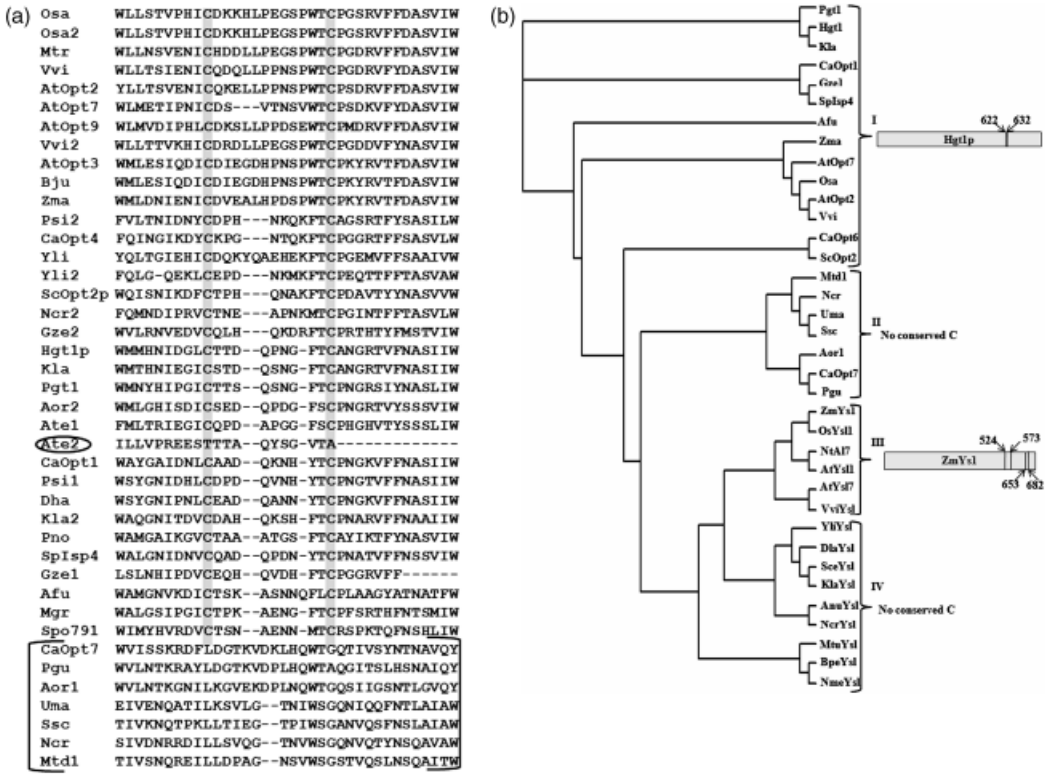

Fig. 9. Phylogenetic analysis of the OPT member reveals coconservation of C622 and C632 across the majority of members among the PT clade and four conserved cysteine residues among the plant homologues of the YS clade. (a) Multiple sequence alignment of the representative members from the PT clade of the OPT family (enlisted in Table S1) showing the coconservation of C622 and C632 (Shaded grey). The members lacking C622 and C632 formed a distinct cluster from the members containing both the cysteine residues and have been clubbed by a bracket. One notable exception, Ate2 (eclipsed), represents the *Aspergillus terreus* protein (gi|115399078) that appeared to lack the two conserved cysteines corresponding to C622 and C632 in Hgt1p, and yet clustered with the other members of the PT clade that have the conserved cysteine residues. (b) Schematic representation of the pattern of cysteine conservation in the OPT family. a rectangular cladogram of representative members of the OPT family (enlisted in Table S2), revealing the conservation pattern of cysteine residues across the family. Hgt1p with conserved cysteine residues, C622 and C632, is shown as a representative for the PT clade and ZmYs1 with conserved cysteine residues at 524, 573, 653 and 682 is shown as a representative of plant homologues in the YS clade. Cluster I, members of the PT clade containing the two conserved cysteine residues. Cluster II, members of PT clade lacking the two conserved cysteine residues. Cluster III, the plant homologue of YS clade containing the four conserved cysteine residues. Cluster IV, fungal and bacterial members of the the YS clade, which lack any conserved cysteine residues.

Discussion

Cysteine, with its nucleophilic thiol side chain, has been shown to play versatile roles in the functional activity of the membrane protein, depending on their topological location in the protein. While the cysteine residues in the transmembrane domains frequently line the translocation channel of the transporter (Jung *et al.*, 1994; Yao *et al.*, 2001; Jimenez-Vidal *et al.*, 2004), the cysteine residues in the extracellular loops and the intracellular loops often undergo various covalent modifications, such as disulphide bond formation in the former case and lipid conjugation for the latter set of residues, which have shown to be critical for the protein stability and expression and/or trafficking of the membrane proteins to their destination. In such a scenario, tampering with the native cysteine residues can lead to either loss in the functional activity or the structural stability or both for a membrane protein (Karnik & Khorana, 1990; Cho *et al.*, 2000; Ennion & Evans, 2002; Henriksen *et al.*, 2005; Stitham *et al.*, 2006). Despite this, for some membrane proteins, it has been possible to generate a cys-free derivative without a significant effect on the functional activity of the protein (Weissborn *et al.*, 1997; Valdes *et al.*, 2004; Kafasla *et al.*, 2007). However, considering that in some previous reports, wherein individual cysteines were found not to be essential for the functional activity of the protein and yet simultaneous replacement of all the native cysteine residues led to a nonfunctional protein molecule (Petrov & Slayman, 1995; Pajor *et al.*, 1999; Tanaka *et al.*, 2004), a slightly different strategy was adopted to explore the role of native cysteines in Hgt1p function. Rather than concentrating on individual cysteines, a cys-free Hgt1p molecule was generated as a first

step itself and evaluated for the functional activity. Although the cys-free Hgt1p was found to be nonfunctional, the random mutagenesis and intragenic suppressor of the cys-free Hgt1p enabled a rapid dissection of the differential contribution of the partially conserved cysteine (C622 and C632) and nonconserved cysteine residues in Hgt1p functional activity. While the presence of both C622 and C632 was found to be indispensable for trafficking of Hgt1p to the cell surface and its functionality, none of the remaining 10 native cysteines could be identified to be individually important for the functional activity of Hgt1p. However, introduction of multiple cysteine to serine substitutions in the protein led to a gradual loss in the functional activity of the protein. This gradual loss in activity correlated reasonably well with the gradual decrease in the protein expression levels of the corresponding mutants, and suggested that, cumulatively, the cysteines contributed in the maintenance of the native configuration of the transporter.

The essential role played by the C622 and C632 residues for Hgt1p function, the defective cell surface trafficking seen when either one of the residues was mutated to serine or alanine, the coconservation of the two cysteines among other members of the PT clade of the OPT family members and the genetic suppressor data where both C622 and C632 reverted together to yield a functional protein from the nonfunctional 12-cys-free Hgt1p all point to the interdependence of these residues. These observations, in combination with the putative extracellular location of these two cysteine residues (Fig. 1), strongly support the likelihood of the two cysteine residues to be covalently linked through a disulphide bond in Hgt1p. However, direct evidence for the existence of a disulphide bond in Hgt1p could not be provided, as attempts to purify the protein from its native host for MS analysis have proved futile so far. The existence of a disulphide bond in Hgt1p was explored by monitoring the mobility of the protein in a denaturing gel in the presence or absence of a reducing agent. No significant difference was observed in the mobility of the wild-type protein under the reducing and nonreducing conditions (data not shown). However, as the two conserved cysteines are only nine amino acids apart in the primary sequence of the protein, the breaking of the putative disulphide bond by the reducing agent might not generate a sufficient difference in the hydrodynamic radius of the protein, to be observed on the gel.

In the light of the putative disulphide bond between C622 and C632, two likely explanations can be envisioned for the observed trafficking defect in the C622A/S or C632A/S. In the first explanation, the proper tertiary conformation adopted by the disulphide-bonded Hgt1p might be needed to ensure the trafficking of the protein to the cell surface, making the presence of the disulphide in the protein critical for trafficking to the cell surface (Ai & Liao, 2002). An alternative explanation is that the mutation of either of the cysteine residues in the disulphide-bonded pair to serine (or alanine) might lead to a non-native disulphide bond in Hgt1p involving the unpaired cysteine, thereby leading to gross misfolding of the protein (Chen *et al.*, 1997; Henriksen *et al.*, 2005), or may promote heterotypic aggregation of newly synthesized protein chains (Sawyer *et al.*, 1994). In either case, the presence of non-native conformations in the ER would activate the highly stringent quality control in the ER, resulting in the retention of the protein in the ER, as observed with the single cysteine mutants of Hgt1p (Ellgaard & Helenius, 2003). While it is not easy to differentiate between the two possibilities, the genetic suppressor data where the functional revertants of the nonfunctional cys-free Hgt1p had both the C622S and the C632S reverting back as cysteine favour the first explanation that the presence of the disulphide bond in Hgt1p is in fact critical for its efficient trafficking to the cell surface.

Although the defective trafficking of the C622S and C632S mutants suggests a structural role for the native disulphide bond, it might also be required for the functional activity of the transporter activity. A functional role in ligand binding has been proposed for the serotonin transporter (Chen *et al.*, 1997) and the GPCR family (Stitham *et al.*, 2006). However, in the case of Hgt1p, the glutathione uptake ability of Hgt1p was not affected by the presence of even high levels of reducing agents (data not shown). The lack of effect might arise from the inaccessibility of the disulphide in a fully folded protein (Cho *et al.*, 2000). However, it could also be an indicator of the lack of a functional role for the disulphide bond in GSH uptake by Hgt1p. More detailed biophysical experiments with a purified protein in the future might be needed to examine these possibilities more conclusively.

Being the first investigation of this nature in this large and important OPT family, the findings that we have obtained with Hgt1p have important implications for other members of the OPT family. The conservation of cysteines was found to be a useful aid for further classification of the OPT family into clusters, and hence, four clusters could be distinguished within the previously defined two clades of the OPT family. Furthermore, the high degree of conservation of the cysteine residues corresponding to the C622 and C632 in the other homologues of PT clade and four cysteines in the plant members of YS clade suggests that in these members also, the cysteine might be cross-linked via a disulphide bond, and that a common structural architecture exists for these diverse members. However, the fact that bacterial and fungal YS members as well as 36 homologues from the PT clade lack these conserved cysteine residues suggests that these proteins have evolved an alternative mechanism that enables the trafficking of the protein to the cell surface in the absence of the disulphide bond present in the other members of the

family. Finally, the lack of functionality of cys-free Hgt1p, and the inability to obtain functional suppressors devoid of cysteine residues, suggests the need to develop more innovative strategies to explore the structural features of Hgt1p and the other OPT homologues.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. C-terminus GFP-tagged Hgt1p is nonfunctional.
Fig S2. Screening of the revertants obtained in the genetic suppressor screen.
Fig. S3. Unrooted phylogenetic tree for the representative members of the PT clade reveal that members lacking the two cysteines (corresponding to C622 and C632 in Hgt1p) form a separate subcluster (eclipsed).
Table S1. List of the representative members of the PT clade used to construct the phylogenetic tree in Fig. 9a and Supporting Information, Fig. S3, to depict the conservation pattern of the cysteine residues corresponding to C622 and C632 in Hgt1p.
Table S2. List of the representative members of the OPT clade used to construct the phylogenetic tree in Fig. 9b to depict the conservation pattern of the cysteine residues across the OPT family.

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