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SUMMARY OF THE THESIS

Glutathione (GSH), γ-glutamyl-cysteinyl-glycine, is the most abundant non-protein thiol compound present in almost all eukaryotic and several prokaryotic organisms. Owing to its low redox potential (-240 mV) and highly reactive thiol group, it plays a significant role in the maintenance of the cellular reducing milieu and the detoxification of various toxic compounds. Thus, glutathione homeostasis in the cell and its organelles is critical for the cell survival under normal physiological and stress conditions. Apart from the *de novo* synthesis of glutathione from its constituent amino acids, the cellular GSH reservoirs are also maintained by uptake from the extracellular medium by specific transporters.

Hgt1p, a polytopic membrane protein, from the yeast *Saccharomyces cerevisiae*, was the first high-affinity glutathione transporter to be identified in any system. Interestingly, Hgt1p belongs to a relatively novel and largely uncharacterized Oligopeptide Transporter (OPT) family, which is completely distinct from the previously characterized Peptide transporter (PTR) family and ATP-binding cassette (ABC) superfamily. The members of this family are restricted to fungi, plants and prokaryotes. The preliminary functional characterization of a few of the members of the OPT family have revealed the physiological significance of this family in uptake and distribution of oligopeptides of three to five residues in fungi and plants and in maintenance of metal homeostasis in plants. However, our understanding of this important family of transporters is greatly handicapped by the lack of structural information. As Hgt1p is the best characterized member of the OPT family in terms of its substrate specificity, and furthermore, its native host, *S. cerevisiae*, is a well established model system, the primary objective of this study was to obtain insights into the substrate-binding motifs and other signature sequences that are crucial for functional activity of the protein. Two independent approaches, substituted cysteine accessibility method (SCAM) and alanine scanning mutagenesis of the polar/charged residues in the transmembrane domains of Hgt1p were adopted for this study.

To initiate cysteine-scanning mutagenesis, a cysteine-free Hgt1p molecule was previously generated in the lab to use it as a parent molecule for the structure-function study of Hgt1p. The cysteine-free Hgt1p was, however, non-functional. A genetic approach to isolate functional revertants of cysteine-free Hgt1p always picked up

functional molecules with double reversions that regained two partially conserved cysteines at positions 622 and 632 of Hgt1p, which retained 10-15% of the functional activity. This lack of functional activity of the cysteine-free Hgt1p molecule and limited restoration of the activity by the reversion of partially conserved C622 and C632 was very intriguing, but was not investigated in the earlier study.

In the present study, therefore, a detailed investigation on the role of 12 native cysteine residues in the functional activity of Hgt1p was carried out using a series of "cysteine-free" Hgt1p mutants. This study revealed differential roles for the conserved versus non-conserved native cysteine residues in Hgt1p.

The functional characterization of the single C622S/A and C632 S/A mutants using the plate based dual complementation-cum-toxicity assay and the radiolabelled glutathione uptake assay revealed that the substitution of either C622 or C632 with a polar, isosteric serine residue or a smaller, hydrophobic alanine residue resulted in complete loss in the functional activity of the transporter. This loss in activity was found to be a consequence of a complete loss in cell surface protein expression levels, though the total protein expression levels remained comparable to the wild-type protein expression levels. Further, a re-evaluation of the conservation pattern of the two cysteine residues, C622 and C632, across the Hgt1p homologue members revealed a co-evolutionary pattern of appearance of the two cysteines as they were either present together or absent together in a total of 205 Hgt1p homologues. The reversion to the two cysteines in the *in vitro* genetic screen, the co-existence of both cysteine in the natural sequences, and the identical functional phenotypes seen when either one of the residues was mutated to serine or alanine, all point to the interdependence of these residues, and strongly suggest the existence of a disulphide bridge between the two cysteine residues that is absolutely essential for the trafficking of the transporter to the cell surface.

In contrast to C622 and C632, none of the remaining 10 native cysteines were found to be individually important for the functional activity of Hgt1p. However, it was observed that introduction of an increasing number of cysteine to serine substitutions in the protein, led to a gradual loss in the protein stability and a corresponding decline in the functional activity of Hgt1p. No defects were seen, however, in the trafficking of these mutants to the cell surface.

The complete loss in functional activity in the 12 cys-free Hgt1p and the essential role of the native cysteines in the functional activity of Hgt1p, hindered the application of SCAM analysis for the structure-function characterization of Hgt1p. However, preliminary standardization for the SCAM analysis revealed that the wild-type Hgt1p was not sensitive to methanethiosulfonates (MTS) derivatives, but was inhibited by pCMBS. A glutathione analogue with alkylated thiol group (GS-NEM) was able to confer protection upon Hgt1p against inhibition by pCMBS, when co-incubated with the inhibitor in the assay medium. These initial results suggest that the wild-type could be used in SCAM analysis, though results need to be carefully interpreted considering the presence of native cysteines in the parent molecule.

In a second approach, alanine scanning of polar or charged residues in the putative transmembrane domains of Hgt1p was undertaken to obtain insights into the structural features of Hgt1p that govern its substrate specificity. In the absence of any previous information or hints on the important domains in Hgt1p (or any other member of the OPT family), a comprehensive strategy was adopted to select the charged and polar residues within the transmembrane domains of Hgt1p for the mutational analysis. The transmembrane domains within Hgt1p were predicted using multiple topology prediction softwares. By applying a majority-vote approach among the predictions of these softwares, a consensus topology model was generated, which was in agreement with a previously proposed topology model by the Becker's group. The topology model comprised of 12 transmembrane domains in Hgt1p. Helix-wheel projections of the 12 transmembrane domains identified 9 of the transmembrane domains that were considerably rich in polar/ charged residues. As glutathione is a hydrophilic substrate, such polar/ charged residues could potentially line the translocation channel of Hgt1p. From these 9 transmembrane domains and two intracellular loops, a total of 22 charged and polar residues were selected for site-directed mutagenesis. The two intracellular loops, regions 537-568 and 707-724, were included in our analysis because they were also predicted by some of the softwares as being potential transmembrane domains, but were omitted from the topology model as these regions are proline-rich, hence theoretically unlikely to form a helix. The effect of each of these 22 different mutants on the functional activity of Hgt1p was analyzed using the plate-based dual complementation assay and radiolabelled glutathione uptake assay. This analysis

identified 11 mutants, which exhibited severe to moderate loss in functional activity of the transporter. These 11 mutants were investigated for their effect on protein expression levels, trafficking to the cell surface followed by detailed kinetic studies. The detailed biochemical characterization of these set of mutants revealed their differential contribution in the functional activity of the protein. Based upon these analyses, four of the transmembrane domains (TMD1, TMD4, TMD9 and TMD12) and the intracellular loops in the region 537-568 containing the highly conserved proline rich motif were found to be essential for the transporter activity of the protein. We could further define N124 in TMD1, Q526 in TMD9 and K562 in the intracellular loop to be directly participating in glutathione transport by Hgt1p. While N124 and Q526 residues seem to be essential for the substrate binding either directly or indirectly, no role could be assigned to K562 residue. We were also able to isolate second-site suppressors for Q526A mutant in two different hydrophilic loops of Hgt1p, which might hint at structural/functional interactions between the TMD9 and the respective loops of Hgt1p, which harbor the second-site suppressors for Q526A.

Together, the results of these two independent mutational approaches provide the first insights into the mechanism of substrate translocation by Hgt1p, a member of a novel and important transporter family (OPT family), which is very poorly defined both in terms of its functional and structural features.

In addition to the structural characterization of the yeast glutathione transporter, a study was also initiated to identify the existence of a possible cysteine-specific transporter in *S. cerevisiae.* Cysteine transport in the yeast *S. cerevisiae* has been shown to be mediated by at least eight different permeases, none of which are specific for cysteine. However considering the importance of cysteine homeostasis in the cell, we anticipated existence of a high-affinity cysteine specific transporter, under sulphur regulation. Data-mining of the existing genome-wide data was carried out to identify membrane transporters that were derepressed under conditions of increased cysteine requirements in the cell. A candidate transporter, Yll055wp of unassigned function, belonging to the Dal5p transporter family was identified. Null mutants of *YLL055w*, but not of the other genes encoding for transporters that mediate cysteine uptake such as *GAP1*, *GNP1*, *MUP1* or *AGP1*, in a *met15Δ* background, resulted in a growth defect

when cysteine, at low concentrations, was provided as the sole sulphur source. Transport experiments further revealed that Yll055wp was the major contributor to cysteine transport under these conditions. The biochemical characterization analysis of the transporter revealed it was plasma membrane localized transporter that exhibited high specificity and affinity for cysteine (K_m = 55 μM). The results reveal that *YLL055w* encodes the principal cysteine transporter in *S. cerevisiae*, which we have named as *YCT1* (Yeast Cysteine Transporter). Further, a tight sulphur regulation of *YLL055w* expression mediated by the Met4p-dependent sulphur regulatory network suggests that the transporter forms an integral part of sulphur biosynthesis pathway and is crucial in maintenance of cysteine homeostasis in the yeast *S. cerevisiae*. Interestingly Yct1p belongs to the Dal5p family of transporters rather than the amino acid permease family to which all the known amino acid transporters belong.