Glutathione (GSH), L-γ-glutamyl-L-cysteinyl-glycine, is the major low molecular weight thiol compound present in almost all eukaryotic cells and is present at intracellular concentrations ranging from 0.1 mM-10 mM. GSH acts as the principal redox buffer, plays an important role in oxidative stress response, and influences several essential processes such as gene expression, cell proliferation and apoptosis. GSH biosynthesis and metabolism proceeds through the γ-glutamyl cycle. γ-Glutamyl transpeptidase (γ-GT), catalyzes the first step in the degradation of glutathione which involves either the cleavage and transfer of the γ-glutamyl moiety to an acceptor amino acid (or hydrolysis to release glutamate) and release of cysteinylglycine. Despite exhaustive studies in mammalian cells many questions remain unanswered. In yeasts, the role of γ-GT in GSH homeostasis is still poorly understood. The present study was undertaken to understand more clearly the role of γ-GT in GSH degradation and GSH homeostasis in yeasts.

Studies on γ-GT were initiated by first attempting to determine the levels of γ-GT activity in the widely used lab yeast strain YPH499 (A *S. cerevisiae* strain 'congenic' to S288C) under different growth conditions. Despite several attempts, under a variety of growth conditions, to determine the levels of activity of this enzyme, γ-GT activity could not be detected in this strain. However, in another wild type strain that was also an S288C derivative, BY4742, significant activity could be detected. The gene encoding γ-GT in yeast, *ECM38*, was cloned and sequenced from both YPH499 and BY4742 to understand the basis for the differences in γ-GT activity seen in the two S288C strains. The sequence of the *ECM38* gene from BY4742 completely matched the published sequence, but the sequence from YPH499 showed 11 mismatches. Out of 11 mismatches, 2 of the mismatches led to alterations in the encoded amino acids (histidine to arginine at amino acid position 171 and glycine to aspartic acid at position 494). One of the two changes (G→D) corresponded to a change in an amino acid that was a highly conserved residue present in γ-GT of a wide ranging number of organisms and was probably responsible for the lack of activity in YPH499. The *ECM38* genes were also expressed downstream of a strong promoter and it was observed that the overexpressed γ-GT from YPH499 was still non-functional, while the same from BY4742 was functional. The promoter region of ECM38 from YPH499 also had several polymorphisms but retained functionality. To trace the origin of the

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polymorphisms in YPH499, γ-GT activity was measured in different YPH strains constructed at the same time as YPH499. YPH1 and YPH2, the progenitor strains of YPH499 were also evaluated. γ-GT activity was checked and *ECM38* gene sequenced in these two progenitor strains in an attempt to trace the origin of the polymorphisms. γ-GT activity was seen in YPH2 but no activity was seen in YPH1. The sequencing of relevant regions of *ECM38* gene from these two strains revealed that YPH1 showed identical polymorphisms to YPH499, including the critical G→D change responsible for loss of activity. YPH2 on the other hand showed only two variations in total, but lacked the G →D variation responsible for lack of activity in γ-GT of YPH499. These results thus demonstrate that among the two parent strains, YPH1 was the bearer of the polymorphisms responsible for the consequent lack of γ-GT activity seen in strains carrying this polymorphism. Attempts to trace the polymorphisms further back were unsuccessful. In the final construction of YPH499, one of the parents of the parent diploid was YPH1, explaining the polymorphisms observed in YPH499.

To obtain insights on the role of γ-GT in GSH turnover and homeostasis, studies were carried out to investigate the regulation of γ-GT at the transcriptional level by nitrogen sources, sulphur sources and GSH. Studies on the regulation of *ECM38* gene were carried out using promoter-β-gal fusions and indicate that *ECM38* was primarily under nitrogen regulation that is mediated by the GATA family of transcription factors, Gln3p and Ure2p. The *ECM38* gene was, however not found to be regulated transcriptionally by glutathione.

γ-GT is the only enzyme known to be involved in the degradation and turnover of glutathione in all living systems and the possibility that in addition to γ-GT, a second degradation pathway might exist in living cells is an aspect of glutathione homeostasis that has not been addressed. To investigate whether glutathione turnover and degradation can occur even in the absence of γ-GT, studies were carried out by examining how the presence or absence of γ-GT affects growth of yeasts and total GSH levels in strain backgrounds where endogenous synthesis of glutathione does not occur. The studies revealed that GSH levels were higher in *ecm38Δ* cells in comparison to wild type strain. The over-expression of *ECM38* restored glutathione levels in *ecm38Δ* strains, but did not lead to increased GSH depletion. To examine whether γ-GT is the

sole enzyme responsible for GSH degradation, a genetic strategy was employed where a *met15Δ* strain requiring glutathione (or other organic sulphur sources) as a sole source of exogenous sulphur was used. An *ecm38Δ* was constructed in a *met15Δ* background and the growth of these cells in glutathione and methionine was examined. Surprisingly it was observed that the utilization of glutathione as an exogenous sulphur source in *met15Δ* strains is independent of the presence or absence of a functional γ-GT enzyme. This result clearly indicates that an alternative pathway for glutathione degradation and utilization - independent of γ-GT- exists in yeast cells. Prior to this report, the only enzyme known to be involved in the degradation of glutathione in all living cells had been γ-GT. Further studies revealed that the alternative γ-GT independent pathway for GSH degradation is quite distinct in terms of its regulation as it was not regulated by the nature of the nitrogen source, unlike γ-GT. The alternative pathway for GSH degradation was found to proceed through the formation of cysteine, since cysteine utilization deficient mutants (*str2Δ*) were also deficient in GSH utilization. In addition to utilization of GSH as a sole sulphur source, yeast cells could also utilize GSH as a source of glycine, but not as a source of glutamate or nitrogen.

Efforts were made to identify the precise components of this alternative pathway of GSH degradation. The yeast genome sequence is known, and well annotated into defined functional categories, and deletion strains having individual genes deleted are available commercially (from EUROSCARF) in the desired background (*met15Δ*). A strategy was therefore taken to procure strains deleted in different peptidases, and other pathways, in the *met15Δ* strain background. The growth of these strains was examined on SD plates having GSH, cysteine or methionine as the sole source of exogenous sulphur. The studies indicated that the known aminopeptidases, carboxypeptidases and dipeptidases of *S. cerevisiae* did not play a role in the utilization of GSH. Yeast strains multiply deficient in the 4 known aminopeptidases or 4 known carboxypeptidases were also created to rule out possible redundancy in function. However the strains containing these multiple disruptions in carboxypeptidases and aminopeptidases were still unaffected in the GSH utilization pathway. The role of vacuole and vacuolar processes in GSH degradation through the alternative pathway was also examined by looking at the role of vacuolar GSH-conjugate pumps, autophagy mutants and vacuolar biogenesis mutants. The observation

that GSH utilization is unaffected in mutants unable to undergo autophagy as well as in vacuolar biogenesis mutants strongly indicates that the vacuole was not involved in GSH turnover by the alternative pathway. The role of Peptidylglutamyl-peptide hydrolyzing activity (PGPH) activity of the proteasome in GSH utilization was investigated. GSH utilization as a sole source of sulphur was found to be unaffected in proteasomal mutants lacking PGPH activity, which excludes the involvement of this proteasomal activity in GSH degradation.

A genome-wide expression approach was also employed to identify the possible components of the pathway using cDNA microarray analysis. The global response profile of *S. cerevisiae*, when it is exposed to levels of GSH that are toxic for the cells and will presumably have increased degradation were examined. The results reveal that GSH toxicity affects several cellular pathways and about 2% (78) of 2940 genes analysed show a change in expression. Out of these 36 genes were found to be up regulated or induced while 42 genes were down regulated or repressed. However, these genome-wide expression studies of *S. cerevisiae* failed to reveal any known or unknown peptidases that could be directly implicated in GSH catabolism.

As the genome-wide approaches failed to provide any clue or insights about the genes involved in the alternative pathway for GSH degradation, a classical genetic approach to look for mutants unable to utilize GSH as a sole source of sulphur was taken. A total of 33 mutants were isolated by EMS mutagenesis of a *S. cerevisiae* *met15Δecm38Δ* strain that were unable to utilize GSH as the sole source of exogenous sulphur. The 33 mutants could be placed in four complementation groups and were named as *dug1*, *dug2*, *dug3* and *dug4* (defective in utilization of GSH). The *dug4* group mutants were found to be identical with GSH transporter gene (*HGT1*) and most mutants (23) fell in this group. The three novel complementation groups *dug1*, *dug2* and *dug3* had 2, 3 and 5 mutants respectively. The gene for one of these complementation groups (*dug1*) was identified as *YFR044c* by genomic DNA library complementation of the *dug1* mutant. The *met15Δyfr044cΔ* was unable to utilize GSH as a sole source of exogenous sulphur like the *met15Δdug1* mutant and this defect could be complemented by *YFR044c* cloned under a strong constitutive promoter as well as on its native promoter. *YFR044c* was so far known to code for a hypothetical

protein of unknown biological and molecular functions. The sequence comparison of Yfr044cp reveals homology to several proteins that belong to the M20 family of metallohydrolases. The growth of *dug1* mutant on γ-glutamylcysteine as a sole source of sulphur was also examined. The failure of *dug1* mutants (but not *dug2* or *dug3*) to utilize γ-glutamylcysteine indicates that *YFR044c* functions as a peptidase *in vivo* like other peptidases of M20 family and probably mediates the first step in GSH utilization by cleaving the γ-glutamyl bond. One of the other two complementation group mutants, *dug2* or *dug3* might be mediating the cleavage of cysteine-glycine, which is produced after the cleavage or transfer of γ-glutamyl group from GSH. The presence of another complementation group indicates that other steps might be involved in the utilization of GSH. Our results with these GSH utilization defective mutants have shown for the first time that γ-GT is not the only enzyme mediating GSH turnover. The isolation of these GSH utilization defective mutants in yeasts opens up many new possibilities with regard to GSH homeostasis and their further characterization will significantly increase our understanding of the exact mechanism of GSH metabolism and homeostasis in cells.

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