Studies on Glutathione Degradation in Yeast Saccharomyces Cerevisiae.

Summary:

Glutathione (GSH or γ-glutamyl-cysteinyl-glycine) is the most abundant low molecular weight cellular thiol compound found in almost all eukaryotes and some prokaryotes. GSH plays a key role not only in the maintenance of the intra-cellular reducing environment but also in response to the oxidative, metal and xenobiotic stress. It is also an important storage molecule for sulphur and nitrogen. GSH metabolism essentially proceeds though the γ-glutamyl cycle. GSH degradation is catalyzed by the γ-glutamyl transpeptidase enzyme (γ-GT) encoded by *ECM38* gene in *S. cerevisiae*. Although γ-GT has been considered to be the only enzyme involved in GSH degradation, previous studies from this lab with *S. cerevisiae met15Δ* strain (organic sulphur auxotrophs) and a *S. cerevisiae met15Δecm38Δ* strain (in which γ-GT gene has also been knocked out) have revealed that the yeast *S. cerevisiae* can utilize GSH as a sole source of sulphur by a degradation pathway, independent of γ-GT. To elucidate this "alternative pathway of GSH degradation", EMS mutagenesis of a *S. cerevisiae met15Δecm38Δ* strain was carried out by previous worker. Analysis of these mutants revealed the presence of four complementation groups, *dug1*, *dug2*, *dug3* and *dug4*. The *dug4* mutant was functionally complemented by *HGT1*, the yeast GSH transporter. The *DUG1* gene was also cloned and found to correspond to *YFR044c*, an ORF of unknown function but a putative di-peptidase of the M20 peptidase family. Little else was known about the pathway.

In the present work, efforts were made to decipher the genes corresponding to *dug2* and *dug3* mutant complementation group and to decipher the role of the Dug1p, Dug2p and Dug3p proteins in the alternative pathway of GSH degradation.

The *DUG2* gene was cloned by complementation of the *dug2* mutant by a yeast genomic library and subsequent analysis of the complementing fragment revealed that the ORF *YBR281c* corresponded to *DUG2*. Sequence analyses showed that Dug2p contained a putative WD40 domain at the N-terminus (2-370 amino acid) and a putative M20 peptidase domain at the C-terminus (441-878 amino acid). The peptidase domain of Dug1p and Dug2p were homologous. Multiple sequence alignment of Dug1p, Dug2p along with the *Lactobacillus delbrueckii* PepV di-peptidase belonging to the M20 family of peptidase (whose crystal structure has been solved) have revealed that the Zn^{2+} metal

binding amino acid residues and catalytic residues were conserved in both Dug1p and Dug2p. The proteins did not play redundant functions since over-expression of Dug1p or Dug2p could not functionally complement *dug2* or *dug1* phenotype respectively.

The *DUG3* gene was cloned by complementation of the *dug3* mutant by a yeast genomic library and subsequent analysis of the complementing fragment revealed that the ORF *YNL191w* corresponded to *DUG3*. Sequence analyses of Dug3p protein have shown the presence at the N-terminus (45-254 amino acids) of a putative Glutamine_Amidotranseferase-classII (Gln_AT-II) domain. Sequence alignment studies with known and well-characterized members of Gln_AT-II proteins have revealed that the key residues, like the nucleophilic cysteine (C2), are conserved in Dug3p as well.

Database searches using the Dug1p, Dug2p and Dug3p protein sequences have revealed that while the orthologues of Dug1p di-peptidase are found in almost all organisms, the true orthologues of Dug2p and Dug3p are only present in some fungi. It seems likely, therefore, that the alternative pathway might be restricted only to certain fungi.

To obtain insights into the roles of the Dug proteins, the utilization of various di- and tri-peptides was investigated. It was found that Dug1p, but not Dug2p nor Dug3p was required for the cleavage and utilization of cysteinyl-glycine di-peptide and α-glutamyl-cysteinyl-glycine tri-peptide. On the other hand, all the Dug proteins were found indispensable for the cleavage and utilization of γ-glutamyl-cysteine as the sole source of sulphur. This indicated that the unusual γ-glutamyl-cysteine peptide bond resists cleavage by Dug1p and its cleavage additionally requires participation of Dug2p and Dug3p.

Additional growth experiments were carried out to see how glutathione and α-glu-cys-gly could be used as a source of either glutamate or glycine. These experiments were carried out using an *aco1Δ* strain (deficient in aconitase), which is a glutamate auxotroph, and a *gly1Δ* strain (deficient in Threonine aldolase), which is a glycine auxotroph. However, no new insights were revealed on the functioning of *DUG1, DUG2* or *DUG3* from these studies.

In order to further understand the functioning of Dug proteins, we addressed the question whether these proteins functioned sequentially. Detailed analysis of yeast extracts of various *dug* mutant strains for the presence of small molecular weight thiols

did not reveal the accumulation of any GSH degradation intermediates as seen from HPLC analyses of fluorescently labeled thiols using monobromobimane. The failure to detect accumulation of any novel or known thiol intermediates in *dug* mutants, suggested that either the Dug proteins did not participate sequentially or the intermediates formed might be very unstable. There was the additional possibility that these proteins might act together temporally and spatially as a complex, to catalyze a single step in the degradation of glutathione.

To explore the latter possibility, detailed experiments were carried out to determine protein-protein interactions between the Dug proteins.

The Dug protein interactions were analyzed using yeast-two-hybrid and Co-Immunoprecipitation assays. We observed that Dug1p interacted with Dug2p, and Dug2p also interacted with Dug3p. Dug1p was found to interact with itself. Formation of a possible ternary complex was also further analyzed and was also demonstrated by CoIP assay. These detailed interaction studies show that the Dug proteins form a multi-protein complex, to carry out GSH degradation.

Localization studies revealed that this pathway seemed to operate in the cytoplasm since Dug1pGFP, Dug2pGFP and Dug3pGFP were observed using confocal microscopy to localize in the cytoplasm.

Detailed studies on regulation of expression of *DUG* genes using 600bp promoter β-gal fusion constructs have led us to conclude that expression of *DUG1* and *DUG2* gene are not under the control of sulphur or nitrogen sources in the medium. The expression of *DUG3* gene was, however, found to be regulated by the sulphur sources. *DUG3* expression is de-repressed maximally under sulphur starvation condition. The *DUG3* promoter sequence alignment studies with orthologous promoters from other *Sachharomyces spp.* revealed the presence of a classical sulphur regulatory motif 5'-AAACTGTG-3' at -174 to -165 bp position upstream of ATG that was conserved in all the *Saccharomyces spp.* and suggests that the regulation of *DUG3* may be under the classical sulphur regulatory pathway.

Based on all the results, we have proposed a model for the alternative pathway of GSH degradation. Under sulphur limiting condition, where GSH is present as the sole source of sulphur, the expression of *DUG3* is de-repressed, and Dug3p recruits Dug2p to

form a complex. Dug2p interacts with Dug1p and thereby the three Dug proteins form a ternary complex in the cytoplasm. GSH and γ-glutamyl-cysteine are recognized for degradation by the ternary complex. The presence of Dug3p in the complex having a putative Glutamine_amidotransferase (Gln_AT-II) domain, suggests that it functions to amidates the γ-glutamic acid residue. The GSH, so modified, is recognized by the hetero-dimeric Dug1p/Dug2p di-peptidase that is part of the complex to degrade glutathione into its constituent amino acids. Dug1p can also exist independently as a functional homodimer, which can cleave normal di- or tri-peptides like cys-gly or α-glu-cys-gly respectively.