Hardeep Kaur (2010). ***Studies on the Dug 1p, Dug 2p and Dug 3p Proteins involved in the alternative pathway of glutathione degradation in S. cerevisae.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
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SUMMARY OF THE THESIS

Glutathione is the most abundant low molecular weight thiol compound found in most all eukaryotes and few prokaryotes. Its relatively low redox potential, ability to exist in both oxidised (GSSG) and reduced (GSH) forms, stability towards cellular peptidases owing to its γ-glutamyl linkage, together with its high intracellular concentration (upto 10mM) make glutathione a major redox buffer of the cell. Other functions of glutathione include scavenging of free radicals and metal ions, detoxification of xenobiotics and regulation of cellular processes like signal transduction, proliferation and apoptosis. Glutathione also acts as a source of sulphur and nitrogen in yeast cells.

Glutathione metabolism is carried out by the γ-glutamyl cycle where γ–glutamyl transpeptidase (or γGT), catalyzes the first step of glutathione degradation by removing the γ-glutamyl moiety. γGT is conserved from bacteria to mammals and was for long considered to be the only enzyme having this activity.

Previous studies from our lab had identified a novel pathway of glutathione degradation in yeast working independent to γGT and requiring the participation of three novel ORFs, *DUG1(YFR044c), DUG2(YBR281c)* and *DUG3(YNL191w).* This pathway appeared to be fungal specific as homologues of *DUG2* and *DUG3* were absent in other kingdoms. *DUG1* encoded for protein having homology to M20A peptidases domain. *DUG2* encoded for protein having a WD40 domain at N-terminus and a M20A peptidase at C-terminus. *DUG3* encoded for protein having glutamine amidotransferase class II domain. Based on the results from Co-IP and yeast two hybrid studies interactions were shown among Dug1p-Dug1p, Dug1p-Dug2p and Dug2p-Dug3p. Based on these findings, Dug1p, Dug2p and Dug3p proteins were proposed to functions as a *DUG* complex in glutathione degradation. However as genetic studies had indicated that *DUG1* gene product was also required for degradation of normal di-peptides and tri-peptides, Dug1p was predicted to have two independent functions, first as a part of *DUG* complex in glutathione degradation, and second as a di and tri-peptidase independent of Dug2p and Dug3p. The *DUG* proteins were also localized to the cytosol and only *DUG3* expression was shown to be induced under sulphur limitation conditions.

With this background, in the present study we endeavoured to further enlarge our knowledge about the *DUG* proteins and the putative *DUG* complex.

Recombinant Dug1p was expressed and purified from *E.coli.* Dug1p was shown to exist as an homodimer in solution and as a metallopeptidase requiring either Mn^{++} or Zn^{++} for activity. Mn^{++} appeared a better activator than Zn^{++}. In contrast to the *in vivo* genetic results, purified Dug1p did not cleave tri-peptides and tetra- peptides used in the study including Glu-Cys-Gly which was shown earlier to be cleaved by Dug1p *in vivo*. Substrate specificity analysis of purified Dug1p showed that it cleaves Cys-Gly preferentially over all the substrates tested in the study. Mutational analysis of the active site residue revealed that Dug1p is a true M20A peptidase. From the *in vitro* data together with the observation that Dug1p was the only peptidase responsible for cleavage of Cys-Gly *in vivo*, we could conclude that Dug1p is the Cys-Gly peptidase of the γ-glutamyl cycle responsible for cleaving Cys-Gly released by the action of γGT on glutathione. The gene encoding the Cys-Gly peptidase activity in *S.cerevisiae* had hitherto not been identified.

The presence of Dug1p homologues in other fungi and in metazoan prompted us to investigate if these were also functioning as Cys-Gly peptidases. Prior to our demonstration of Dug1p as a Cys-Gly peptidase, all the known members of the Cys-Gly peptidase were shown to belong to M1, M19 and M17 family of metallopepetidase unlike Dug1p which belonged to M20 family. Dug1p homologue in *S.pombe* (SpDug1p) was also demonstrated in this study to be the major Cys-Gly peptidase of *S.pombe* although it had homologues of the M1, M19 and M17 families. Two Homologues of Dug1p were also present in humans as CNDP1 (carnosinase 1) and CNDP2 (carnosinase related nonspecific dipeptidase or carnosinase II). Among these, CNDP2 was shown to complement the *dug1Δ* in *S.cerevisiae* for utilization of Cys-Gly as sole source of sulphur. We purified recombinant CNDP2 and showed it functions as a Mn^{++} dependent Cys-Gly specific dipeptidase with substrate specificity similar to Dug1p. We have thus proposed that the true function of CNDP2 is as a major Cys-Gly peptidase in humans. Together these studies suggested that Dug1p represents a novel family of Cys-Gly peptidase belonging to M20A peptidase family

The Dug2p protein was also characterized in detail. To characterize Dug2p, it was purified from *E.coli*. The M20A peptidase domain of Dug2p shows 32% identity and 50% similarity to Dug1p but purified Dug2p lacked Cys-Gly peptidase activity. It was shown to primarily exist as a homodimer in solution where dimerization was mediated by the M20A peptidase domain located at the C-terminus of Dug2p. Dug1p homodimerization was also confirmed *in vivo* by yeast two hybrid studies. Dug2p M20A peptidase domain has a

nserved active site residue (E586). However mutational analysis of Dug2p (E586A) ealed that Dug2p does not have any catalytic activity *in vivo* in glutathione degradation. is is supported by the observation that isolated M20A peptidase domain of Dug2p when pressed in yeast failed to complement *dug1Δ* for glutathione degradation.

The Dug3p protein was also investigated and characterized. The importance of the N-rminal GATaseII domain of Dug3p in glutathione degradation was investigated by site-irected mutagenesis and genetic approaches. Several predicted active site mutants of the GATase domain of Dug3p failed to complement *dug3Δ* deletion for utilization of glutathione. The length of the GATase II domain in most of the proteins has shown to be approximately 200 amino acids. As the length of the Dug3p is 357aa, the role of the C-terminus of Dug3p was also investigated and shown to be important for its function in glutathione degradation. Full length Dug3p was also required for its interaction with Dug2p. Recombinant Dug3p could be purified from *E.coli* only after the introduction of a GGGGGGIP linked at the C-terminus of Dug3p before the His-tag. Recombinant Dug3p primarily exists as a monomer unlike other members of GATaseII family which are mainly oligomers.

To investigate the *DUG* complex, we investigated the interactions between the different *DUG* proteins. This involved the heterodimerizations as well as possible homodimerizations. Most of the members of M20A peptidase family are homodimers where dimerization is shown to be essential for catalysis. The monomers in the M20A homodimers are catalytically active but each monomer requires a histidine from the other monomer for polarization of the substrate and hence catalysis at its active site, a phenomenon known as transactivation. Comparison with known M20A peptidases undergoing transactivation revealed the corresponding histidine residues were conserved in Dug1p and Dug2p as H233 and H648 respectively. In the Dug1p-Dug2p heterodimer (expected to form in a *DUG* complex), it was possible that although Dug2p was not catalytically active *per se* it may transactivate Dug1p during catalysis by providing H648 at the active site of Dug1p. However H648A or H648N mutants of *DUG2* failed to show any defect. In contrast H233N mutant of *DUG1* showed a growth defect on glutathione plates suggesting that Dug1p homodimer was required for glutathione degradation. Dug1p dimerization was also shown to be essential for Cys-Gly peptidase activity as H233A Dug1p shows negligible Cys-Gly activity compared to wild type.

The requirement of Dug1p dimerization was further supported by the observation that Dug1p 171A (active site mutation) and Dug1p H233N (transactivation mutation) although inactive, could complement each other when co-expressed together. The ability of human CNDP2 to complement *dug1Δ* for glutathione utilization was also observed. Put together these results suggests that Dug1p functions as Cys-Gly peptidase as a homodimer independent of Dug2p and Dug3p in the γ-glutamyl cycle and in the *DUG* pathway of glutathione degradation.

The biochemical and mutational studies described in this thesis thus suggest a different model of glutathione degradation by the *DUG* proteins than proposed earlier. As Dug2p can interact with itself via M20A dimerization domain and with Dug3p via WD40 domain it will provide the Dug3p with the oligomeric status normally seen in GATase proteins. Dug2p-Dug3p are likely to form a $(Dug2p\text{-}Dug3p)_2$ complex. This oligomerization may be required for the substrate binding or modulation of Dug3p activity or both. As Dug3p is a member of the NTN family of hydrolases (to which γGT belongs) it is likely to be responsible for the cleavage of the γ-glutamyl linkage of glutathione. We thus propose that activity of the $(Dug2p\text{-}Dug3p)_2$ complex removes the glutamate moiety from glutathione and releases Cys-Gly which is acted upon by Dug1p. Dug1p thus functions independently in glutathione degradation in both the classical, γ-glutamyl transpeptidase dependent and the alternative '*DUG*' pathway of glutathione degradation. Although the model that we have proposed is consistent with all the biochemical and mutational studies carried out in this thesis, a final validation would be achieved after $(Dug2p\text{-}Dug3p)_2$ complex is purified and the γ-glutamyl cleavage activity of the complex demonstrated *in vitro*. We are currently working in this direction