Title:	Characterization of ChaC2 Proteins and Their Role in Glutathione Degradation
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Abstract: The work carried out in this thesis towards the above objectives is summarized in the following sections: The ChaC2 proteins are glutathione degrading enzymes: Comparative analysis of human ChaC1 and human ChaC2 proteins and crystal structure of the yeast GCG1 (yeast ChaC2) protein. ChaC family proteins are known to be present from bacteria to humans. Mammalian ChaC1 is a glutathione degrading enzyme localized in the cytosol and degrades cytosolic pools of glutathione (Kumar et al. 2012). Levels of ChaC1 were shown to be upregulated during ER stress (Mungrue et al. 2009). A single ChaC enzyme is present in lower organisms. However in the higher multicellular organisms there are two ChaC homologues that have been named as ChaC1 and ChaC2. Although mouse ChaC1 was characterized (Kumar et al. 2012), the ChaC2 proteins have not been investigated. In this thesis we have shown (using in vivo assays) that human ChaC2 protein (HsChaC2) functions in glutathione degradation and degrades glutathione with a lower catalytic efficiency than human ChaC1 (HsChaC1). The HsChaC1 and HsChaC2 proteins were purified by recombinant expression in E. coli and kinetic parameters were determined by in vitro biochemical assays. The kinetic comparison of HsChaC1 and HsChaC2 proteins indicated that although both proteins have similar Km values (Km of 2.2 ± 0.4 mM and Km of 3.7 ± 0.4 mM for human ChaC1 and human ChaC2 respectively) towards glutathione they differed in their catalytic efficiencies (kcat of 225.2 ± 1 min-1 and kcat of 15.9 ± 1.0 min-1 for HsChaC1 and HsChaC2 respectively). The HsChaC2 protein is fourteen fold less catalytically efficient than HsChaC1 proteins. The expression studies in human cell lines under ER stress and sulphur starvation conditions indicated that the HsChaC2 protein maintains a basal level expression in contrast to the human ChaC1 protein, which is induced under ER stress. The ChaC protein in lower eukaryotes such as ChaC of E. coli was also evaluated. Functional evaluation suggested that the single ChaC members in the lower organisms are functionally orthologous to the ChaC2 proteins (and not the ChaC1 proteins). Our studies suggested that the ChaC2 protein which is involved in constitutive low level turnover of glutathione is the ancestral enzyme, while ChaC1 proteins present exclusively in higher eukaryotes might have evolved with higher catalytic efficiency for carrying out acute glutathione turnover required under conditions of stress. To identify the crystal structure of ChaC family proteins we attempted to crystallize HsChaC1 and HsChaC2 proteins. However, it was difficult to get highly purified fractions of these proteins suitable for crystallization studies. Hence we crystallized the ChaC2 homologue of S. cerevisiae GCG1 (in collaboration with Dr. S. Karthikeyan, Institute of Microbial Technology Chandigarh, India). The crystal structure of GCG1 protein was solved at a resolution of 1.48 Å. The crystal structure of GCG1 protein represents the first structure of the ChaC family. The ChaC2 proteins of Arabidopsis thaliana function in glutathione degradation: Absence of the classical γ-glutamylcyclotransferase enzymes in plants. In plants the enzyme γ-glutamyl transpeptidase (γ-GT) has been the only enzyme known to initiate glutathione degradation. The plant Arabidopsis thaliana has three γ-GTs: γ-GT1, γ-GT2 and γ-GT4. These are membrane bound proteins with γ-GT1 and γ-GT2 present at the plasma membrane and γ-GT4 at the vacuolar membrane (Ohkama-Ohtsu et al. 2007). The γ-GTs in plants act on non-cytosolic pools of glutathione. Cytosolic glutathione degrading enzymes were not identified or characterized in plants at the time this work commenced. A search of the Arabidopsis thaliana sequence database (TAIR, The Arabidopsis Information Resource) yielded three members of the ChaC family proteins: GGCT2;1, GGCT2;2 and GGCT2;3. These genes were cloned both for in vivo functional studies in yeast and for recombinant expression in E. coli. The characterization of these proteins by expression studies in yeast indicated that these proteins function as glutathione degrading enzymes. The GGCT2;1 enzyme, during the course of our studies, was investigated by a different group (Paulose et al. 2013) and was shown to be a γ-glutamylcyclotransferase involved in recycling of glutamate during Cadmium and Arsenic stress and was also shown to be capable of glutathione degradation in vitro. The GGCT2;2 and GGCT2;3 proteins were purified and characterized by in vitro assays. The in vitro studies indicate that GGCT2;2 and GGCT2;3 are functional orthologues of ChaC2 family proteins and degrade glutathione with low catalytic efficiencies (kcat of GGCT2;2 and GGCT2;3 are 38.6 ± 0.7 min-1 and 6.8 ± 0.5 min-1 respectively). The Km of these proteins are however physiologically relevant (Km of GGCT2;2 and GGCT2;3 were 1.7 ± 0.1 mM and 4.9 ± 1.1 mM respectively) and within the range of the glutathione levels in the cells (up to 10 mM). We have also carried out an investigation for the presence or absence of γ-glutamylcyclotransferases in A. thaliana. The γ-glutamylcyclotransferases have been reported in mammals as enzymes that act on γ-glutamylamino acids (one of the products formed during the degradation of glutathione by γ-GT enzymes). The sequence homologues of human γ-glutamylcyclotransferase are absent in plants. Since human γ-glutamylcyclotransferase enzyme is a γ-glutamylcyclotransferase fold (γ-GCT/BtrG fold) protein, we have analyzed all the possible γ-glutamylcyclotransferase fold proteins (structural homologues) in A. thaliana. The analysis revealed fourteen such proteins in A. thaliana; of these two are the γ-glutamylamine cyclotransferases (γ-GACT), three are the ChaC proteins and nine

	belongs to a novel family of γ- glutamylcyclotransferases with unknown function and exclusively found in plants. Evaluating γ-glutamylcyclotransferase activity towards γ-glutamyl amino acids in each group indicated that none of these proteins exhibit activity towards γ-glutamyl-methionine which is a potential substrate for human γ-glutamylcyclotransferase. These findings suggested the absence of classical γ-glutamylcyclotransferase in plants and have led to a better understanding of glutathione homeostasis and γ-glutamyl cycle in plants.
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