CONCLUSIONS AND PERSPECTIVES

Recently reported crystal structures of GCPII provide structural insight into the organization of the substrate binding cavity and highlight residues implicated in substrate / inhibitor binding in the S1' site of the enzyme. To complement and extend the structural studies, we constructed a QM/MM model of GCPII in complex with its substrate, N-acetyl-aspartyl-glutamate, which enabled us to predict additional amino acid residues interacting with the bound substrate, and used site-directed mutagenesis to assess the contribution of individual residues for substrate / inhibitor binding and enzymatic activity of GCPII. We prepared and characterized 12 GCPII mutants targeting the amino acids in the vicinity of substrate/inhibitor binding pockets. The experimental results suggest that residues (especially Arg210) in the S1' site are critical for substrate / inhibitor binding, whereas the residues forming the S1 pocket might be more important for the 'fine-tuning' of GCPII substrate specificity and appear to be relevant for substrate turnover and may play a role in the enzyme's mechanism of action.

Even though the QM/MM calculations of the NAAG binding mode in the GCPII active site enabled us to predict the structure and enzyme–substrate interactions in the S1 binding site, the complete description of reaction mechanism of GCPII is beyond the scope of our study. We would like to look more closely into the catalytic mechanism of glutamate carboxypeptidase II. An interesting approach is a study of a putative proton shuttle Glu424, located near zinc ions in the active site of GCPII. The mutation of this residue might show us its role in enzyme catalysis; moreover, we might obtain a crystal structure of GCPII with bound unsplit substrate N-acetyl-aspartyl-glutamate.

Human GCPII consists of 750 amino acids, and six individual domains were predicted to constitute the protein structure. We reported the analysis of the contribution of these putative domains to the structure and function of recombinant human GCPII. We cloned 13 mutants of human GCPII that are truncated or extended at one or both the N- and C-termini of the GCPII sequence. The clones were used to generate stably transfected Drosophila Schneider's cells, and the expression and carboxypeptidase activities of the individual protein products were determined. The results clearly show that the amino acids at the extreme C-terminus of GCPII are crucial for the hydrolytic activity of the enzyme and, furthermore, that no more than 60 amino acids can be deleted from the N-terminus without compromising the carboxypeptidase activity of GCPII.

We created a molecular model of GCPIII and provided interpretation of the distinct substrate specificity of both enzymes, and examine the amino acid residues responsible for the differences by site-directed mutagenesis. These results may help to design potent and selective inhibitors of both enzymes. Such inhibitors would be helpful to evaluate and distinguish biological roles of the two individual enzymes.

We believe that GCPIII activity is significant enough to account for the NAAG-hydrolyzing activity observed in the tissues of GCPII knock-out mice and that GCPIII might thus represent a valid pharmaceutical target.

Why would brain harbour two similar enzymes with the same enzymatic activity? The honest answer is: we do not know. The possible explanation might be that GCPII and GCPIII possess different biological roles in the brain. One of our goals is to find GCPIII molecular partner and to clarify the function of GCPIII.

Only very limited and controversial data on the expression and localization of GCPII in human brain are available. Therefore, we set out the first systematic analysis of the expression of GCPII in human brain using immunochemical detection. We used a novel monoclonal antibody GCP-04, which recognizes an epitope on the extracellular part of the enzyme and is more sensitive to GCPII than to the homologous protein GCPIII. We also showed that this antibody is more sensitive in immunoblots than the widely used antibody 7E11. Immunohistochemical analysis revealed GCPII expression in all parts of the human brain. GCPII seems to be expressed exclusively in astrocytes, especially in those localized in the white matter. Our published results are only starting point in further studies on the role of GCPII in the human brain.

It is generally known that GCPII is expressed in prostate and overexpressed during the prostate cancer. Analogically, we showed GCPII expression in astrocytes and we would like to investigate further the GCPII expression in brain tumors, especially in the astrocytomas.

In the benign prostate PSM' mRNA is overexpressed over GCPII. Interestingly enough, in the case of prostate cancer this expression pattern is reversed. Very few information is known about protein designated PSM', a truncated form of GCPII. We investigated the origin of PSM' and its trafficking in the cells.

Our experiments revealed that PSM' is a proteolytically active N-linked glycoprotein. Surprisingly, it is not a product of alternatively spliced GCPII mRNA, which is generally accepted fact. We hypothesize that it might be a product of proteolytic processing of the full

We undertook this study before the first crystal structure of GCPII was determined. X-ray structures provided evidence that the ectodomain of GCPII is composed of three domains. All these domains form active site of the enzyme and are indispensable for the GCPII enzymatic activity, which explains why changes on both N- and C- terminus are so detrimental to protein stability and activity.

We report crystal structures of the human GCPII complexed with three glutamate mimetics/derivatives, 2-(phosphonomethyl)pentanedioic acid, quisqualic acid, and L-serine *O*-sulfate. Despite the structural differences between the distal parts of the inhibitors, all three compounds share similar binding modes in the S1' site (pharmacophore pocket) of GCPII, where they are stabilized by a combination of polar and van der Waals interactions. The structural variety of the distal parts of the inhibitors leads to rearrangements of the S1' site that are necessary for efficient interactions between the enzyme and an inhibitor.

The set of structures presented here, in connection with the available biochemical data, illustrates a flexibility of the GCPII pharmacophore pocket and underlines the structural features required for potent GCPII inhibition.

Our data could be used for the development of the new GCPII inhibitors using the rational structure-based drug design approach and could draw attention to the modification in the inhibitor structure, which can improve the pharmacokinetic profile and potency towards GCPII.

Experiments with GCPII knock-out mice showed that GCPII is not the only one NAAG-hydrolyzing enzyme in the brain. We presumed that glutamate carboxypeptidase III (GCPIII), a close homolog of GCPII, might complement for GCPII activity in these knock-out mice.

While human GCPII is an important pharmacological target in the neurotransmission and degenerative diseases, no biochemical study of human GCPIII is available at present. We cloned, expressed and characterized a recombinant human GCPIII.

We show that GCPIII lacks dipeptidylpeptidase IV-like activity, its activity is dependent on N-glycosylation, and is sensitive to several known inhibitors of GCPII effectively inhibit it. In comparison to GCPII, GCPIII has lower N-acetyl-aspartyl-glutamate-hydrolyzing activity, different pH and salt concentration dependence, and distinct substrate specificity.

length GCPII upon internalization and endosomal trafficking, but our data suggest it is also not the case. We can only speculate that this species might be produced by a proteolytic processing event inside the Golgi apparatus and then translocated by an unknown mechanism into the cytosol.

Insights into GCPII processing and the origin of its truncated form PSM' might improve our understanding of the behavior of GCPII, a therapeutic target for prostate cancer, as well as our general understanding of N-glycosylation and the trafficking of cytosolic proteins.