

**THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)**

**EXAMINATION OF CALPAIN AND PROTEIN KINASE/PHOSPHATASE  
SYSTEMS**

Interaction between the post-synthetic protein modifying systems

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# 1. Introduction

## 1.1 Post-translational modification of protein

During post-translational modifications (PTMs) the chemical properties of amino acid residues of the proteins are changing. These changes occur after the DNA has been transcribed into RNA and translated into proteins, and contribute to the diversity of protein structure. Following their synthesis most of the proteins undergo chemical changes before they gain their final functions. The PTMs increase the number of proteins' forms and regulate their function by modifying their activity and cellular localization. The PTMs can be divided into two major groups. The first category includes any enzyme-catalyzed covalent modification, in which some chemical group is added to one or more amino acid residues in a protein. For example, the functional group can be alkyl, phosphate or glycosyl group, but larger units (lipids, carbohydrates) and other prosthetic groups can be linked to the proteins, too. The second category of PTMs includes those mechanisms, in which irreversible hydrolytic cleavage of the peptide bonds of the proteins occur.

## 1.2 Protein phosphorylation and dephosphorylation

The reversible phosphorylation of proteins is one of the most common PTMs, which take place occurs in both prokaryotic and eukaryotic organisms. Phosphorylation reactions are carried out by protein kinases, so that they transfer the gamma phosphate group of ATP, less often of GTP, to the serine, threonine, and tyrosine side chains of proteins. Approximately 20 % of ATP produced by the cells is consumed in the course of their reaction. A large proportion (about 99%) of phosphate is connected to the amino acid residues of Ser or Thr and only a small part binds to Tyr side chains. The protein conformation can be altered during the phosphorylation due to the rearrangement of electrostatic interactions, whereby the function of a protein can be modified. Reversible protein phosphorylation plays essential roles in cell energy metabolism, proliferation, growth and differentiation, motility and regulation of metabolic processes. This PTM can only become an effective regulatory tool, if it is possible to remove the phosphate group of a protein, consequently, the process should be reversible. The hydrolytic removal of a phosphate group is catalyzed by protein phosphatases. The regulation is achieved by the harmonical co-operation of protein kinases and protein phosphatases. It is not surprising that the phosphorylation state of the substrate proteins is determined by the activity and interplay of these two major enzyme groups.

### **1.2.1 Classification of protein phosphatases**

Protein kinases constitute a large group, of which all members fall into the same enzyme family. In contrast, the number of protein phosphatases is considerably less, nevertheless they can be divided into several groups as a consequence of convergent evolution. The phosphatases can be classified on the basis of their substrate specificity, reaction mechanisms and structure. One part of them dephosphorylates the Ser and Thr side chains of proteins, while others are specific to the Tyr side chains. In addition, there are some so called “dual-specificity” phosphatases (DSP), which are capable of hydrolyzing the phosphoryl groups from the Ser/Thr and Tyr side chains alike. The Ser/Thr specific phosphatases can be further divided into subfamilies such as phosphoprotein phosphatases (PPP), metal ion dependent protein phosphatases (PPM), haloacid dehalogenase (HAD) like phosphatases as well as the RNA polymerase II C-terminal domain specific protein phosphatases (SCP). PPP and PPM enzymes contain two metal ions in their active centre, which promote the hydrolysis of phosphate groups from Ser or Thr side chains of proteins. The PPP subfamily includes PP1, PP2A and PP2B separated initially on the basis of substrate specificity and sensitivity to heat-stable inhibitor proteins. The more recently discovered “novel” members of the PPP family show a transition between the former three groups according to their structures and properties.

### **1.2.2 Characterization of protein phosphatase Z (PPZ)**

Two phosphatases have been identified among the novel enzymes, which occur only in fungi (PPZ and PPG). It is known that the genes *PPZ1* and *PPZ2* in *S. cerevisiae*, *pzh1* in *S. pombe*, *Pz11* in *N. crassa* and *CaPPZ1* in *C. albicans* encode PPZ proteins. All of them consist of three distinct parts. The C-terminal catalytic domain (which is responsible for enzyme activity) and the approximately 50-amino-acid-long N-terminal part (which is required for the membrane binding) show a high degree of sequence similarity in each protein. Since the catalytic domain is similar to the catalytic subunit of PP1, the PPZ enzymes are classified as type 1 Ser/Thr phosphatases. The rest of the N-terminal domain and the segment connecting the two domains are extremely variable. This intrinsically unstructured N-terminal region has a regulatory role, as it can fold on the catalytic domain and consequently can inhibit enzyme activity. A detailed characterization of PPZ protein phosphatases in *S. cerevisiae* has been already published. It was shown that PPZ plays an important role in the maintenance of osmotic stability, cell size and integrity of yeast cells. PPZ interacts with the PKC-activated mitogen-activated protein kinase (MAPK) pathway that modulates cell wall

synthesis and affects cell integrity. The latest results suggest that PPZ acts through the regulation of the Trk1/2 mediated  $K^+$  transport. The functions of the *C. albicans* *CaPPZ1* gene have not been determined yet, we only know that the disruption of either one or two alleles in the diploid organism is not lethal. That is why we initiated the study of the protein phosphatase Z1 gene in *C. albicans*.

### **1.3 Limited proteolysis of proteins**

The second large group of post-translational covalent modifications is the irreversible proteolysis of proteins. The process is catalyzed by proteases. If the protein is completely broken down into amino acids, we speak about total proteolysis or degradation, however if the cleavage takes place only at well defined place/places, it is called limited proteolysis. The hydrolytic cleavage of the peptide bonds in proteins is an essential element of maintaining homeostasis by regulating the dynamics of protein turnover. Since the operation of proteases is an irreversible process, their activity is under strict control. The autocatalytic cleavage can also be classified as limited proteolysis. It splits one or more peptide bonds and leads to the activation of a protein precursor. Calpains, which are in the centre of our attention, belong to the papain superfamily. They are activated by this mechanism, namely they eliminate their N-terminal part in response to a  $Ca^{2+}$  signal.

#### **1.3.1 Characterization of calpains**

Calpains are  $Ca^{2+}$  activated neutral, cytoplasmic cysteine proteases, which perform limited proteolysis of their substrate proteins. Calpains exist in a number of species. In mammals 16, in *Drosophila* 4 calpain genes can be found. Some calpains have a heterodimeric structure, the large and small subunits form dimers, however, most of the calpains are composed of only one large subunit. The large subunit consists of four domains in typical calpains, while these domains are lacking or have other structure in the atypical calpains. The best characterized members of the mammalian calpains are the  $\mu$ - and  $m$ -calpains. They were named on the basis of micromolar and millimolar  $Ca^{2+}$  concentrations required for their activation. These enzymes are heterodimers consisting of a large 80 kDa and a small 30 kDa subunit. The regulation of these essential mammalian proteases is an enigma, because the non-physiological  $Ca^{2+}$  concentration required for activation suggests that other factors also play a role in the activation mechanism. It is known that phosphorylation (as a post-translational event) or regulatory substances (such as phospholipids) can alter the activity and  $Ca^{2+}$  sensitivity of calpains. In addition calpastatin (which is an inhibitor protein) and

autolysis can also influence proteolytic activity. The physiological function of the calpain system is not fully understood in normal cells, however, we know that it plays important roles in signal transduction, cytoskeletal remodeling, cell cycle, apoptosis and cell motility. A number of pathological conditions are known which are caused by a genetic disorder of calpains or by modified  $\text{Ca}^{2+}$  homeostasis resulting in abnormal calpain activity. The calpains are involved in gastric cancer, type 2 diabetes mellitus, Alzheimer's disease, cataract, muscular dystrophies, multiple sclerosis and stroke.

### 1.3.2 Characterization of calpain B

Four calpain genes were identified in the *Drosophila melanogaster* genome (A, B, C and D), from which calpain A and B encode an active protease, while the gene products of calpain C and D are proteolytically inactive. The *Drosophila* calpains are very similar to the 80 kDa large subunit of mammalian m-calpain. Their common feature is that they consist of a single polypeptide chain and do not contain the small subunit. Calpain B has a molecular mass of 104 kDa. It has an extremely long N-terminal domain of 224 amino acid. It is a  $\text{Ca}^{2+}$ -dependent protease that can be activated at millimolar  $[\text{Ca}^{2+}]$ . Upon  $\text{Ca}^{2+}$ -dependent activation the N-terminal region is clipped off in an autoproteolytic process. It has been found that  $\text{Ca}^{2+}$  binding induces a series of conformational changes ranging from the calmodulin-like domain IV to the acidic region of domain III that leads to the closing of catalytic subdomains IIa and IIb and the activation of the protein. So the increase of cytoplasmic  $[\text{Ca}^{2+}]$  is essential for the activation of calpain B. However, the  $\text{Ca}^{2+}$  concentration required for the *in vitro* half-maximal activation is far from the physiological range. It is known that phospholipids (PIP<sub>2</sub>, PIP, PI) slightly increase both the activation rate and the maximal activity of calpain B. However,  $\text{Ca}^{2+}$  sensitivity of the protein was hardly reduced. Phosphorylation, as another possible factor in reducing the need for  $\text{Ca}^{2+}$ , has not been reported yet in the case of *Drosophila* calpains. Since phosphorylation of mammalian m-calpain has already been verified and it was demonstrated that this post-synthetic modification affected the activity of the enzyme, we investigated if calpain B can be regulated by phosphorylation.

## 2. Aims of the study

In our work we attempted to answer two questions and formulated the following aims:

### **Phosphorylation of *Drosophila* calpain B**

Question: Can *Drosophila* calpain B be phosphorylated, and if so, what is its effect on the activity?

Aims:

1. *In vitro* phosphorylation of recombinant calpain B by protein kinase A (PKA) as well as by extracellular signal-regulated protein kinases 1 and 2 (ERK1 and ERK2)
2. Determination of phosphorylation stoichiometry
3. Identification of phosphorylation site/sites by mass spectrometric methods
4. Investigation of the effect of *in vitro* phosphorylation on the functions of the enzyme
5. Demonstration of the *in vivo* phosphorylation of calpain B

### **The polymorphism of protein phosphatase Z1 gene in *Candida albicans***

Question: What is the structure of the *C. albicans* PPZ1 gene? Can the polymorphism of this gene be proven, and if so, could it be used for diagnostic purposes?

Aims:

1. Identification of the *C. albicans* PPZ1 (CaPPZ1) gene
2. Sequencing the CaPPZ1 gene from various *C. albicans* strains
3. Analysis of genetic polymorphism and its structural aspects
4. Utilization of the polymorphism to characterize clinical *C. albicans* samples

### 3. Materials and methods

#### 3.1 Materials

The majority of chemicals used in our experiments were obtained from Sigma-Aldrich. The pSP72 vector containing the cDNA of *S. cerevisiae* PPZ1 was received from Prof. Joaquin Ariño (University of Barcelona, Barcelona, Spain); the recombinant wild-type, inactive and the mutant calpain B enzymes as well as the MAP2c protein were provided by Dr. Anita Alexa (HAS, BRC, Institute of Enzymology, Budapest); the rabbit immune serum against calpain B was obtained from Prof. Anna Erdei (University of Loránd Eötvös, Budapest). The vector-specific primers (T7 and SP6) were provided by DNA sequencing labs (HAS BRC and BIOMI Ltd); the *CaPPZ1* gene-specific oligonucleotide primers were purchased from Integrated DNA Technologies, Inc. *C. albicans* clinical isolates were originated from the Department of Medical Microbiology of the Medical and Health Science Center of the University of the Debrecen. *C. albicans* reference strains were obtained from ATCC, the *Escherichia coli* DH5 $\alpha$  strain was purchased from Novagen, the *Drosophila* Schneider S2 cell line was from Invitrogen.

#### 3.2 Methods

##### 3.2.1 Cultivation procedures

The reference and clinical strains of *Candida albicans* were propagated in Sabouraud dextrose liquid medium. Fungal cells were isolated from the diluted culture of the ATCC 10231 reference strain and subcultures were grown from a single cell. The *C. albicans* strains were identified using the CHROMagar Candida test, the API ID32C panel, and the Micronaut-Candida system. *Drosophila* S2 cells were maintained in Schneider's insect medium supplemented with FBS, penicillin or streptomycin.

##### 3.2.2 Nucleic acid analyzing methods

###### *Isolation of genomic DNA*

Genomic DNA was isolated from *C. albicans* by the phenol-chloroform method. The concentration of DNA was determined on the basis of absorption at 260 nm. The purity of DNA was estimated from the ratio of OD<sub>260</sub>/OD<sub>280</sub> and was verified by gel electrophoresis in 1 % agarose gels.

### *Polymerase chain reaction and DNA cloning*

Based on the NCBI DNA database *C. albicans* protein phosphatase Z gene-specific oligonucleotide primers were designed. An approximately 2.7 kbp fragment of the gene was amplified by Long PCR polymerase enzyme mix, while the hypervariable region (0.4 kbp) of the *CaPPZI* gene was amplified by Pfu polymerase. The PCR products were verified by gel electrophoresis.

The PCR products were purified on Microcon ultracell YM-100 columns for direct sequencing. The introduction of an A-tail with Taq polymerase was necessary for cloning the Pfu polymerase generated PCR products. The “tailed” DNA was ligated into the pGEM-T Easy vector with the aid of T4 DNA ligase. Competent *E. coli* DH5 $\alpha$  cells were transformed and plasmid DNA was isolated with a plasmid isolation kit. The size of the inserted DNA was determined by agarose gel electrophoresis after digestion with *EcoRI*.

### *DNA sequencing*

DNA was sequenced in the Sequencing Laboratory of Biological Research Center of the Hungarian Academy of Sciences in Szeged and at the BIOMI Ltd. in Gödöllő using either vector-specific T7 and SP6 primers or phosphatase-specific primers.

### *RFLP analysis*

PCR products produced by the amplification of the hypervariable region of the *CaPPZI* gene were digested with the *AccI*, *AluI*, and *DdeI* enzymes. The size of the restriction fragments was analyzed by electrophoresis in a 5 % polyacrylamide gel.

### 3.2.3 Protein analyzing methods

The molecular mass and the purity of protein samples were determined by SDS-PAGE using standard molecular-weight protein mixtures for comparison. The concentration of the proteins was determined by the Bradford method.

### *Protein phosphorylation*

Recombinant wild type, inactive and mutated calpain B proteins were phosphorylated *in vitro* by PKA, ERK1, and ERK2 enzymes. In case of radioactive phosphorylation, the reaction mixture also contained [ $\gamma^{32}\text{P}$ ] ATP. The incorporated radioactivity was determined by the measurement of Cherenkov radiation and was also detected after SDS-PAGE by

autoradiography. After non-radioactive phosphorylation reactions the samples were sent for MS analysis.

S2 cells were grown to 100 % confluency in the course of *in vivo* phosphorylation experiments. In order to stimulate the PKA pathway, cells were treated by forskolin. To activate the ERK pathway, cells were treated by EGF. In both cases protein dephosphorylation was inhibited with calyculin-A. Treated and untreated cells were lysated, then the lysate was centrifuged and the supernatant was used for immunoprecipitation.

### *Immunoprecipitation*

The *in vivo* phosphorylation of calpain B was tested by immunoprecipitation. To avoid nonspecific binding, supernatants obtained from the treated and lysated S2 cells was pre-cleared with Protein A-Sepharose. In parallel, protein A-Sepharose was incubated with anti-calpain B antibody in lysis buffer. Then the pre-cleared lysate was mixed with the anti-calpain B antibody-coupled Protein A-Sepharose. The resin was washed with lysis buffer and the bound proteins were released by boiling in SDS sample buffer. One part of the sample was analyzed by Western blotting, another part was tested by MS analysis.

### *Mass spectrometry*

Samples received from phosphorylation or immunoprecipitation experiments were separated by SDS-PAGE and the 104 kDa calpain bands were cut out. The MS analysis of samples was carried out by the Proteomics Lab of the Medical and Health Science Center of the University of Debrecen and by the Mass Spectrometry Laboratory of Biological Research Center of the Hungarian Academy of Sciences. The phosphopeptide fragments were identified by LC-MS/MS.

### *Calpain B activity assays*

For the activity measurements, calpain B proteins were phosphorylated by PKA, ERK1 and ERK2 enzymes. In order to produce non-phosphorylated (control) samples the proteins were incubated under identical conditions without the addition of kinase. After gel filtration of samples, the activation and proteolytic activity of calpain preparations was determined using three different methods: (i) fluorimetric assays with the aid of a fluorescent peptide substrate, (ii) SDS-PAGE based method with the MAP2c substrate or (iii) autolysis.

#### 3.2.4 Analysis and evaluation of the data

Primers used for DNA amplification and sequencing were designed by the pDraw32 software. Restriction sites were predicted by the same software. DNA and protein sequences were compared with the ClustalW2 software. The phylogenetic analysis and sequence comparisons were performed with the MEGA 4.1 software. Phosphorylation sites of *Drosophila* calpain B were analyzed using the Motif Scan software, the structure of the protein was analyzed with the IUPred server. The mathematical modeling of enzyme kinetic data was performed by Dr. Gábor Takács (Department of Theoretical Physics, University of Eötvös Loránd, Budapest, Hungary). The homologous model of the catalytic domain of *C. albicans* PPZ1 protein was constructed by Dr. Péter Bagossi (Department of Biochemistry and Molecular Biology, Medical and Health Science Center of the University of the Debrecen); the homologous model of *Drosophila* calpain B was constructed by Dr. Zoltán Bozóky (HAS BRC Institute of Enzymology). The mean and standard deviation of the results was determined with the Excel software of the Microsoft Corporation.

## 4. Results and discussion

### 4.1 Regulation of calpain B by phosphorylation in *Drosophila melanogaster*

#### 4.1.1 *In vitro* phosphorylation of recombinant calpain B

Phosphorylation of any of the *Drosophila* calpains had not been reported earlier. Therefore, at the beginning of our work a bioinformatic analysis of the primary structure of *Drosophila* calpain B was performed. It revealed five putative PKA and several ERK phosphorylation sites. In order to prove the structural predictions, recombinant calpain B was phosphorylated with PKA as well as ERK1 and ERK2 kinases in the presence of [ $\gamma^{32}\text{P}$ ]ATP *in vitro*. We found PKA incorporated  $0.20 \pm 0.09$  (n=5), ERK1  $0.62 \pm 0.27$  (n=6) and ERK2  $0.73 \pm 0.17$  (n=7) mole P/mole protein. For the identification of the phosphorylated amino acid residues, non-radioactive ATP was used instead of [ $\gamma^{32}\text{P}$ ]ATP in the course of calpain B phosphorylation. Samples were analyzed by LC-MS/MS. The analysis of phosphopeptide fragments proved that the wild type calpain B was phosphorylated at Ser240 and Ser845 by PKA, whereas the ERK1/ERK2 enzymes phosphorylated the same Thr747 residue.

#### 4.1.2 *The localization of the phosphorylation sites in calpain B*

The three dimensional structure of *Drosophila* calpain B has not been determined yet. Thus, the human m-calpain was used for homology modeling and for the prediction of the steric localization of phosphorylation sites. According to the model Ser845 lies in domain IV within the second EF-hand motif. The nearby amino acid residues create a favorable environment for PKA recognition. The second PKA site at Ser240 is at the end of domain I, close to the activating scission site. As its environment is less preferred by PKA, we consider Ser240 as a secondary site of PKA phosphorylation. Thr747, the phosphorylation site of ERK1/ERK2, is situated at the so-called “transducer region” between domains III and IV, which plays a key role in the activation of the enzyme, because this region transmits the  $\text{Ca}^{2+}$  signal from the  $\text{Ca}^{2+}$ -binding domain IV to the catalytic domain II. Thus, according to homologous modeling the amino acid residues phosphorylated by PKA and ERK1/ERK2 enzymes are situated in important regions of the molecule, which may regulate the enzyme activity and activation.

#### 4.1.3 Effects of phosphorylation on the activity and activation of calpain B

Three different methods were used to find out how phosphorylation affected the activity and the activation of the enzyme. First, the activity was determined at high  $\text{Ca}^{2+}$  concentration with a fluorescent dipeptide substrate (LY-AMC). The analysis of kinetic curves suggested that the phosphorylated forms were activated faster and had a greater activity than the non-phosphorylated enzyme. ERK2 exerted the greatest effect, which is probably explained by the higher level of phosphorylation. It is known that the microtubule-associated protein 2c (MAP2c) is a good substrate for calpains, therefore MAP2c was digested at lower  $[\text{Ca}^{2+}]$ . We monitored the disappearance of the 62 kDa MAP2c band with the aid of SDS-PAGE. According to the results, the phosphorylated enzyme digested the protein substrate faster than the non-phosphorylated form. We also found that the effect of PKA was larger at lower  $\text{Ca}^{2+}$  concentrations. However, this method was not suitable for the assay of the rapid activation of calpain B. That is why we used an autolysis assay where we monitored the disappearance of the 104 kDa native calpain B band as a function of time. Although the phosphorylation had only a minor effect on enzyme activation, it was visible that the phosphorylated calpain B was activated faster than the non-phosphorylated enzyme.

To sum it up, we proved that the phosphorylation enhanced both the rate of activation of calpain B and the maximum activity of the enzyme.

#### 4.1.4 Effects of phosphorylation mimicking mutations on the activity and activation of calpain B

We generated two phosphorylation site mutants (T747E and S845E) in which the negative Glu side-chain introduced by site directed mutagenesis mimicked the effect of phosphorylation. Fluorimetric activity assays revealed that the mutations affected the operation of protein in two ways. On the one hand, they increased the rate of reaction and  $\text{Ca}^{2+}$ -sensitivity of calpain B. The activity enhancement was greater at low  $[\text{Ca}^{2+}]$ . We conclude that the mutants are activated at lower  $\text{Ca}^{2+}$  concentrations, but are less sensitive to changes in  $\text{Ca}^{2+}$  concentration than the wild-type enzyme. On the other hand, the mutations increased the activation of calpain B in comparison with the wild-type enzyme. Although phosphorylation and the point mutations are not fully equivalent, the analysis of mutants confirmed the results of the phosphorylation experiments.

#### 4.1.5 *In vivo* phosphorylation of calpain B

Phosphorylation of calpain B was also examined in *Drosophila* S2 cell lines to explore the possible physiological relevance of our *in vitro* results. Cells were treated with kinase

activators, then the calpain B protein was isolated by immunoprecipitation and SDS-PAGE. Mass spectrometry revealed that Thr747 (which can be found in the transducer region of the protein) and Ser240 (which is situated at the end of domain I) were phosphorylated in the EGF treated cells. Since EGF activated the MAP kinase/ERK pathway phosphorylation of Ser240 was not expected, especially because the PKA signaling pathway activating forskolin treatment had no detectable effect on this site. It is possible that, due to the EGF induced Ca<sup>2+</sup> influx, CaMKII kinase was activated and it phosphorylated the Ser240 residue.

In summary, our experiments demonstrated that Thr747 side chain of calpain B is phosphorylated *in vitro* and *in vivo* by ERK1/2 that can elevate the activation and activity of enzyme.

## **4.2 Examination of the polymorphism of protein phosphatase Z1 gene in *Candida albicans***

### *4.2.1 Identification of C. albicans PPZ gene by Southern hybridization*

In accordance with data in the literature and in the NCBI DNA databases, *C. albicans* has only one PPZ gene. With Southern experiments we confirmed the prediction and termed the gene CaPPZ1.

### *4.2.2 The polymorphism of the CaPPZ1 gene*

An approximately 2.7 kbp fragment of the CaPPZ1 gene was amplified by PCR using specific oligonucleotide primers and genomic DNA isolated from different *C. albicans* strains. The amplicons were cloned and sequenced. In the course of analysis of the DNA sequences we observed that the sequences differed from each other and from the DNA sequences found in the NCBI database. We observed these differences even if we analyzed clones originated from the same strain. Therefore, *C. albicans* subcultures were propagated from a single cell. After cloning and sequencing we demonstrated that ATCC 10231 was heterozygous for CaPPZ1 and contained two novel alleles which were called CaPPZ1-2 and CaPPZ1-3. We termed the CaO19.726 sequence from the NCBI database the CaPPZ1-1 allele. Later on we identified one more allele of the gene in a *C. albicans* clinical isolate which we called CaPPZ1-4. Our experiments also showed that significant sequence variations can be detected in the samples originated from the clinical practice. Sequence differences (nucleotide replacements, insertion, deletion) can be found along the coding and noncoding regions of the gene. The 3'-noncoding region showed the greatest variability.

#### 4.2.3 Structural implications of the genetic polymorphism

Nucleotide differences in the coding region resulted in nine amino acid exchanges in the primary structure of the CaPPZ1 protein. The N-terminal regulatory domain of the protein is intrinsically unstructured, thus the effects of polymorphisms in this region cannot be forecasted. However, the 3D structure of the C-terminal catalytic domain of CaPPZ1 can be predicted from the atomic coordinates of rabbit muscle PP1 catalytic subunit by homology modeling. From the model we concluded that the Asp261Asn exchange (that takes place close to the catalytic center) may influence the activity, the Cys337Arg conversion (which is situated in central  $\beta$ -folds) may modify enzyme stability, while the Gly333Glu replacement (at the surface of the protein) probably has no effect on catalysis. More pronounced polymorphism was found in the noncoding regions of the gene, however, the significance of these variations is not known.

We concluded that the heterozygosity of *C. albicans* strains, allelic variation as well as sporadic point mutations causing microvariability enhance the genetic diversity of the opportunistic pathogenic fungus.

#### 4.2.4 Characterization of clinical *C. albicans* isolates

Based on the examination of the polymorphism of *CaPPZ1* gene we deemed that the analysis of the characteristic sequence differences in 3'-noncoding hypervariable region would be suitable for the genotyping of *C. albicans* strains cultivated from clinical samples. As a proof of principle, we randomly selected 14 *C. albicans* isolates from the *Candida* Collection of the Department of Medical Microbiology, University of Debrecen. The samples were obtained from patients who suffered from systemic candidiasis.

DNA sequencing results were confirmed by RFLP analysis. Based on the typical sequence motifs, restriction enzymes were selected which are specific for each allele. By DNA sequencing and RFLP we found no more than five *CaPPZ1* allele combinations in different *C. albicans* isolates. In two instances the genotypes of *C. albicans* isolates were different in the bloodstream and in the nonsterile site of the body, which is most likely explained by nosocomial infection.

According to our results, the polymorphism of the hypervariable region of the *CaPPZ1* gene may be a useful tool for genotyping clinical isolates and for the identification of the origin of the infection.

## 5. Summary

The intracellular signaling pathways are regulated by complex networks of enzyme systems. There is a remarkable interplay between the proteolytic and the protein kinase/phosphatase systems. Calpains regulate their target proteins via irreversible limited proteolysis, while protein kinases and phosphatases alter proteins by reversible phosphorylation, turning them into various conformations and functional stages.

In the first part of our work we studied the phosphorylation of calpain B in *Drosophila melanogaster*. We confirmed the presence of the predicted hypothetical phosphorylation sites with *in vitro* experiments. We found that recombinant calpain B can be phosphorylated by PKA as well as by ERK1 and ERK2 kinases. Mass spectrometric analysis revealed that PKA phosphorylates Ser240 and Ser845, in addition, Thr747 was identified as the site of phosphorylation of either ERK1 or ERK2. We employed three independent methods to investigate the effects of phosphorylation on enzyme functions. We demonstrated that phosphorylation increased both of the autoproteolytic activation and the activity of calpain B. Moreover, the kinase treatment enhanced the Ca<sup>2+</sup> sensitivity of the enzyme. Experiments with phosphorylation mimicking mutants (Thr747Glu and Ser845Glu) supported our previous conclusions. The *in vivo* phosphorylation of Thr747 was detected in EGF stimulated *Drosophila* S2 cells. Our results indicate that ERK signaling pathway activated by extracellular signals results in the phosphorylation and activation of fruit fly calpain B. We also demonstrated the *in vivo* phosphorylation of Ser240 that is presumably not caused by PKA, but by CaMKII. Collectively, our data support the notion that the proteolytic activity of calpain B is regulated by protein phosphorylation/dephosphorylation.

In the second part of our work we investigated the gene of protein phosphatases Z, an important regulatory enzyme in *Candida albicans*. As the first step of the study we proved with Southern blot experiments that, in accordance with data in the literature, *C. albicans* has only one PPZ gene termed *CaPPZI*. The cloning and sequencing of the *CaPPZI* of the ATCC 10231 reference strain revealed the polymorphism of the gene and the heterozygosity of the strain. By the analysis of the gene in clinical samples and reference strains we discovered the existence of four distinct *CaPPZI* alleles. Characteristic nucleotide replacements and insertions/deletions were detected at the coding and noncoding regions. The 3'-noncoding regions of the *CaPPZI* alleles showed an exceptionally high degree of variability. The sequencing of this area showed that the hypervariable regions from clinical and reference strains fall into one of the previously identified four alleles. The DNA sequencing data were confirmed by RFLP analysis. Altogether we found no more than five *CaPPZI* allele combinations in different *C. albicans* strains. In addition we detected individual point mutations, which elevated the genetic heterogeneity of this pathogenic yeast. According to our results, the hypervariable region of the *CaPPZI* gene may be suitable for genotyping *C. albicans* isolated from clinical samples. By comparing the haplotypes of the strains one can reveal the origin of the infection.

## 6. Publications

**This thesis is based on the following publications:**

**László Kovács\***, Anita Alexa\*, Eva Klement, Endre Kókai, Ágnes Tantos, Gergő Gógl, Tamás Sperka, Katalin F. Medzihradzky, József Tözsér, Viktor Dombrádi and Péter Friedrich (2009). Regulation of Calpain B from *Drosophila melanogaster* by Phosphorylation. *FEBS J* 276 (17):4959-72. IF: 3.139

\* shared first authorship

**László Kovács**, Ilona Farkas, László Majoros, Márton Miskei, István Pócsi, Viktor Dombrádi (2010) The polymorphism of protein phosphatase Z1 gene in *Candida albicans*. Journal of Basic Microbiology. *J Basic Microbiol* Published Online: 14 May 2010. IF: 1. 319

*Presentations related to the thesis*

Kovács L, Ádám Cs, Berényi E, Horváth A, Majoros L, Aradi J, Dombrádi V (2006). 4-Tiodezoxiuridin *Candida-ellenes* hatása. *MTA Antibiotikum Munkabizottság és MTA Nukleotidkémia Munkabizottság Tudományos Ülése*, Debrecen

Alexa A, Kovács L, Sperka T, Tözsér J, Dombrádi V, Friedrich P (2007). A foszforiláció növeli a *Drosophila* kalpain B aktivitását. *A Magyar Proteomikai Társaság 2007. évi Vándorgyűlése*, Debrecen

*Posters related to the thesis*

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