SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

EXAMINATION OF PROTEIN MODIFYING ENZYMES IN *DROSOPHILA* SPECIES

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The Examination takes place at the Library of the Department of Physiology, Faculty of Medicine, University of Debrecen at 11:00 am, 22nd October, 2015

Head of the Defense Committee:	Prof. László Csernoch, PhD, DSc
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1. INTRODUCTION

1.1. Posttranslational modification of proteins

Most of the proteins of the cells undergo chemical modifications after translation, in a process termed posttranslational modification (PTM). PTM allows proteins to gain their functions, to form interactions, and to reach their cellular localization. Due to PTM, there are much more functionally different proteins in a eukaryotic cell than the number of protein coding genes in the genome. PTMs can be reversible or irreversible, depending on the type of the chemical modification. The most common reversible modification is phosphorylation, while proteolysis is a frequent irreversible PTM.

1.2. Phosphorylation-dephosphorylation of proteins

During phosphorylation the gamma phosphate of ATP is transferred to serine, threonine, or tyrosine amino acid residues of proteins. Around 99 percent of the phosphate groups are attached to serine or threonine side chains, the rest is bound to tyrosine. The process is catalyzed by protein kinases, and the opposite reaction, i.e. the dephosphorylation is promoted by phosphatases. Through the action of these two enzymes, the modification becomes reversible. According to a modest estimation about one third of the eukaryotic proteins can be phosphorylated. Phosphorylation is implicated in many physiological functions including regulation of the cell division cycle, apoptosis, signal transduction, energy metabolism, and differentiation.

1.2.1. Protein phosphatases

Phosphoprotein phosphatases (PPP) are important members of the serine/threonine specific group; they represent a significant proportion of the

phosphatase activity in the eukaryotic cells. This enzyme family contains the classical PP1, PP2A, and PP2B enzymes as well as the novel protein phosphatases PP4, PP5, PP6, and PP7. The distinction between the PPP members is based on the effect of specific inhibitors. For instance, Inhibitor 1 and 2 proteins are specific inhibitors of PP1, and okadaic acid inhibits PP2A. The catalytic subunit of PP1 (PP1c) is a 35 kDa molecular mass protein. Its core region has been well conserved during evolution, while the C- and N-terminal segments are more variable. A large number of regulatory subunits can interact with PP1c, forming a variety of different holoenzymes. The regulatory/targeting subunits modulate substrate specificity, direct the holoenzyme to different cell compartments, and can bind to other interacting partners. For example G_M and G_L are the best known mammalian glycogen binding regulatory subunits that target the phosphatase to the polysaccharide.

1.2.2. The role of protein phosphatase 1 in the glycogen metabolism

The regulatory role of PP1 in glycogen metabolism is well known. The key enzymes of the synthesis and breakdown of glycogen are controlled by phosphorylation, and the PP1 holoenzyme catalyzes the dephosphorylation of these proteins. The glycogen binding regulatory subunits of human PP1c are the PPP1R3 (G_M), PPP1R3B (G_L), R5 and R6 proteins. In a complex with PP1c, they facilitate the dephosphorylation of glycogen phosphorylase and synthase enzymes, so they activate the synthesis and inhibit the degradation of glycogen.

1.3. Regulation of glycogen metabolism in *Drosophila melanogaster*

Glycogen metabolism and the regulation of the process are similar in *Drosophila* and mammalians. The key enzymes of the glycogen metabolism, glycogen phosphorylase and glycogen synthase are dephosphorylated by PP1. In *D. melanogaster* there are four PP1c isoforms, PP1-87B, PP1-13C, PP1-96A, and PP1-9C. The role of these isoforms in glycogen metabolism has not been clarified yet. The

deletion of the predominant isoform, PP1-87B, is lethal, so the examination of the role of PP1-87B in glycogen metabolism is impossible in null mutants. The investigation of the glycogen binding subunits could circumvent this problem and seems to be a suitable strategy to explore the functioning of the phosphatase. However, the glycogen binding regulatory subunits of *Drosophila* PP1c have not been characterized yet either. Based on protein sequence analysis, we identified two potential *Drosophila* proteins, CG9238/Gbs-70E and CG9619/Gbs-76A, which can be considered as homologs of human glycogen binding subunits. Both of them contain conserved glycogen- and PP1c binding regions. It has been known from the literature, that Gbs-76A can bind all PP1c isoforms, while Gbs-70E can interact with the PP1-87B and PP1-96A isoforms. On the other hand, the interaction of these proteins with glycogen or their effect on PP1 activity has not been investigated. Based on the information in the literature, the Gbs-70E seems to play a dominant role in the fruit flies, so we selected this protein for our study.

1.4. Proteolysis of proteins

The proteolysis of proteins is an irreversible posttranslational modification, in which the peptide bond between the amino acid residues of the proteins is cleaved. The process is catalyzed by the protease enzymes. They are classified according to the reactive group in their active site as metallo-, serine, aspartate, or cysteine proteases. Proteolysis is implicated in coagulation, activation of peptide hormones and digestive enzymes, or remodeling of the extracellular matrix. Calpains belong to the calcium dependent cysteine protease family that are present in most organisms and have a broad tissue distribution. They are implicated in a variety of functions including myoblastfusion, cell migration, or regulation of the diameter of blood vessels. There are 16 genes encoding calpains in the human genome. The deletion of CAPNS1 and CAPN2 is embryonic lethal. In *D. melanogaster* there are four calpain genes (*CalpA, CalpB, CalpC* and *CalpD*). The canonical and active CalpA and CalpB are considered

to be the homologs of human CAPN2. Previously we have investigated the physiological functions of *CalpA* and *CalpB*. In the present study we applied the same approach to characterize the role of a putative PP1 glycogen binding subunit in *Drosophila* species.

2. AIMS

For the functional analysis of the putative *Drosophila* PP1 glycogen binding subunit CG9238/Gbs-70E we designed the following experiments:

- Biochemical examination of the *in vitro* functioning of the glycogen- and PP1c binding regions in the Gbs-70E protein. Investigation of the effect of Gbs-70E on PP1c activity.

- Determination of the expression pattern of the *Gbs-70E* gene in different developmental stages and body parts of *Drosophila melanogaster*. Comparison of the gene expression pattern of Gbs-70E orthologs in other *Drosophila* species.

- Generation of mutant strains with classical genetic and RNA interference methods in order to manipulate the expression of the *Gbs-70E* gene. Determination of the physiological role(s) of the Gbs-70E protein by the investigation of the mutant strains.

3. MATERIALS AND METHODS

3.1. *Drosophila* strains

We used w^{1118} and Ore-R control strains, P-element insertion strains, deletion mutants which were generated by our collaborating partners, and RNA interference strains of *D. melanogaster*. RNA interference was activated by crossing with driver strains. For the examination of Gbs-70E orthologs, the *D. eugracilis, D. yakuba, D. virilis, D. willistoni,* and *D. biarmipes* species were also studied.

3.2. Nucleic acid studying methods

Genomic DNA was isolated with phenol-chloroform method. Total RNA was isolated with TRIzol reagent and its DNA contamination was eliminated with DNase treatment. cDNA synthesis was performed with reverse transcriptase enzyme and oligo(dT). PCR reactions were carried out with Taq DNA polymerase, the PCR products were analyzed with agarose gel electrophoresis.

3.3. Protein expression

The cDNA of *Gbs-70E RA* was cloned into pGEX-5X-1 or pET-28a plasmids. Constructs were transformed into DH5α *E. coli* competent cells. Plasmids were isolated and tested with restriction analysis and DNA sequencing. For the expression of recombinant protein, the chosen construct was transformed into BLR *E. coli* competent cells. A small overnight culture was prepared. Next day the culture was grown in bigger volume, and the expression of the recombinant protein was induced. The cells were centrifuged and the recombinant proteins were purified with affinity chromatography.

3.4. Immunological methods

Production of antibodies: α-GST and α-Gbs-70E antibodies were generated by our co-workers. For producing α-Gbs-70E antibody, recombinant Gbs-70E protein was repeatedly injected into rabbits. Antisera were prepared from rabbit blood and were tested with recombinant Gbs-70E protein in dot blot and Western blot experiments.

Western blot: Protein samples were separated in SDS polyacrylamide gels, and were electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked and incubated with a primary antibody overnight. The following day the membranes were washed, incubated with the secondary antibody, and washed again. The protein bands were visualized with a chemiluminescene kit.

Pull down: *D. melanogaster* embryo extract was prepared. The lysate was sonicated on ice, centrifuged, and the pellet was discarded. Meanwhile recombinant 6xHis/Gbs-70E protein was incubated with Ni-agarose beads and buffer, and the sample was centrifuged. Then the recombinant protein on Ni-agarose was incubated with the embryo lysate, and was collected by centrifugation. Non-treated Ni-agarose beads were used in negative control experiment. Beads were collected, washed, and the samples were analyzed by Western blotting with α-PP1c antibody.

Immunoprecipitation: *D. melanogaster* embryo extract was made and cleared by centrifugation. The pellet was discarded; the supernatant was pre-cleared on Protein-A-agarose beads, and centrifuged. Meanwhile Gbs-70E antibody was bound to Protein-A-agarose. The sample was washed with lysis buffer and centrifuged. Pre-cleared lysate and beads with Gbs-70E antibody were incubated overnight. Next day the sample was centrifuged, washed, and analyzed by Western blotting with α-PP1β antibody.

3.5. Biochemical assays

Sedimentation experiment: Glycogen was incubated with GST/Gbs-70E recombinant protein, and layered over sucrose solution. After ultracentrifugation the pellet and supernatant fractions were separated. GST/TIMAP recombinant protein was used in a parallel negative control experiment. Samples from the supernatant and pellet were analyzed by Western blotting with α-GST antibody.

Phosphatase activity assay: Purified rabbit skeletal muscle PP1c activity was examined in the absence or presence of recombinant GST/Gbs-70E protein either with ^{32}P-phosphorylase-a or with ^{32}P-myosin light chain substrate.

The protein concentration was measured according to Bradford, with BSA reference solution. *The glycogen content* was measured by the phenol-sulfuric acid assay.

3.6. Biological tests

Yeast two hybrid assay: The cDNA of *Gbs-70E RA* was cloned into pGBKT7 plasmid to generate the bait construct. The four isoforms of *Drosophila* PP1c cDNA that were cloned into the pACT prey vector were kindly provided by Dainmark Bennett. The bait construct was cotransformed with different prey constructs into yeast cells, and tested on synthetic media. For negative control experiments PPY-pGBKT7 and MYPT-75D-pACT constructs, for positive control experiments PPYR1-pGADT7 and PPY-pGBKT7 constructs were co-transformed.

For lifespan determination Drosophila strains were maintained at a density of 20 flies per vial. Flies were transferred to new vials three times per week and deaths were scored every other day. *In the fertility test* females and males were maintained in vials at constant humidity, and the number of eggs laid was scored after 4 days. *For the testing of viability* eggs were placed in a vial, and the number of adults emerging was scored.

4. RESULTS AND DISCUSSION

4.1. Study of the *Drosophila* protein phosphatase 1 glycogen binding subunits

Based on protein sequence analysis we identified two potential *Drosophila* PP1 interacting proteins, *CG9238/Gbs-70E* and *CG9619/Gbs-76A.* By comparing the amino acid sequences of the putative *Drosophila* PP1 subunits with the human and *C. elegans* glycogen binding subunits, we noted a high degree of sequence similarity in the conserved core region of the proteins, first of all in the PP1c binding motive and in the glycogen binding domain. Based on the data from the literature we concluded that Gbs-70E was the dominant isoform, so we have chosen Gbs-70E for detailed examination. From the databases we learnt that three mRNAs (the shortest form is *RA*, and the two longer transcripts are *RB* and *RC*) can be transcribed from the *Gbs-70E* gene, and two proteins (a shorter isoform is PA and a longer protein PB/PC) can be translated from the mRNAs. The conserved core region with the PP1c binding motive and the glycogen binding domain is the same in both isoforms of Gbs-70E proteins.

In the first part of our work we demonstrated with biochemical experiments the functioning of the conserved PP1c binding motive and glycogen binding domain. Based on our yeast two hybrid results, Gbs-70E can interact with all of the four isoforms of *Drosophila* PP1c. The interaction was confirmed by independent methods including pull down assay and co-immunoprecipitation. Although there is an amino acid substitution in the canonical PP1c binding region, Gbs-70E can be considered as a PP1 subunit. In addition, with sedimentation analysis we demonstrated the interaction between Gbs-70E and glycogen *in vitro*, so we proved that the protein is indeed a glycogen binding subunit of *Drosophila* PP1c.

Next we investigated the effect of Gbs-70E on protein phosphatase activity. We found that recombinant Gbs-70E moderately decreased the activity of PP1c with both phosphorylase-a, and myosin light chain substrates alike. From the lack of

substrate specificity we concluded that Gbs-70E is an ancient regulatory subunit of PP1, which does not influence enzyme activity significantly under physiological conditions, rather it is tethering the phosphatase to glycogen and to other interacting partners. Based on the large number of interacting partners reported in the literature, Gbs-70E may act as a scaffolding protein. The intrinsically disordered nature of the N-terminal region of Gbs-70E supports our assumption, because this region contains many potential protein binding sites.

4.2. The examination of the expression of the *Gbs-70E* gene

The expression pattern of *Gbs-70E* was analyzed by RT-PCR in *D. melanogaster*. Based on our results, the two longer mRNAs, *RB* and *RC* are present in all developmental stages and sexes, while the short mRNA *RA* is expressed only in embryos and in the ovary of females. These findings are in agreement with the DNA chip results, RNA sequencing data, *in situ* hybridization results, and information on the EST sequences of Gbs-70E in the Flybase *Drosophila* database. Thus we concluded that the shorter protein isoform of Gbs-70E has a maternal origin and may have a role in embryogenesis.

For the investigation of the evolutionary aspects of the glycogen binding subunit first we compared the amino acid sequences of the Gbs-70E orthologs in different *Drosophila* species. We noted that the structural changes of these proteins reflect the phylogenetic relationships of the examined strains. Then we tested the preservation of the sex specific expression pattern of *Drosophila melanogaster Gbs-70E RA*. We found that the expression pattern was similar to that of *D. melanogaster* in the closely related strains, but the presence of the short *RA* mRNA was not conserved in distant relatives. Therefore, it is likely that the shorter *Gbs-70E RA* variant has no indispensable physiological functions.

4.3. Functional analysis of *Drosophila* Gbs-70E

During our previous work on *Drosophila* calpains we worked out a research strategy, what was applied in the examination of the Gbs-70E phosphatase regulatory subunit as well. In both studies, which were carried out in a national collaboration, we reduced the gene expression in *D. melanogaster* strains with molecular genetic methods, and used them to determine the function of the deleted gene. We obtained two P-element insertion mutants, termed GT1 and EP and generated new deletion mutants (del81, del277) from the EP insertion strain. By RT-PCR and Western blotting we found that the P-element insertions already reduced the gene expression, but the effect of the deletions was more significant; the del81 deletion strain was considered to be a null-mutant. Since the deletions did not cause lethality, the *Gbs-70E* gene is not essential. In agreement with our initial hypothesis, we found that the glycogen content was significantly reduced in the del81 deletion mutant. So we proved that the Gbs-70E is a functionally relevant glycogen binding subunit of PP1.

Besides we found that Gbs-70E influences longevity of the *Drosophila*. With RNA interference strains we confirmed the previously published fact that the moderately reduced expression of Gbs-70E extends lifespan, probably because of the slowing-down of glycogen metabolism reduces of the metabolic load. On the other hand, the drastic loss of Gbs-70E in the del81 deletion mutant results in the significant decrease of lifespan. The lower penetrance of the RNA interference method resulted only a slight reduction of gene expression, and lead to a somewhat misleading conclusion regarding the function of *Gbs-70E* gene.

Based on its expression pattern, we assumed that Gbs-70E was important in embryogenesis. We compared the fertility of the females and found that the number of eggs laid was significantly reduced by the del81 mutation. In this way we demonstrated that Gbs-70E has a role in the early stages of egg development inside the ovary of the females.

In conclusion, our experiments show that according to its structural and biochemical properties the Gbs-70E glycogen binding subunit of *D. melanogaster* PP1, is an ancient PP1c binding protein, which is less similar to the well-known human G_M and G_L subunits. It has many interacting partners and may act as a scaffolding protein. The deletion of Gbs-70E does not cause lethality, so the gene and gene product of Gbs-70E is not essential. We demonstrated that Gbs-70E plays an important role in the glycogen metabolism, besides it influences longevity and embryogenesis in *Drosophila* as well.

When comparing the results described above with our previous functional studies of *Drosophila* calpains we can find several similarities. We applied the same research approach in both projects: after the generation and characterization of strains with reduced gene expression, we determined the role of the deleted gene with the functional analysis of the mutants. In both examinations we manipulated the gene expression with classical genetic methods (deletion mutants) and RNA interference. Compared to the number of the homologous human genes, there were less *Drosophila* orthologs in both cases: there are 4 *Drosophila* vs. 16 human calpains, and 2 *Drosophila* vs. at least 4 human glycogen binding subunits. There was one dominant *Drosophila* paralog in both examinations. Previously, with our co-workers we identified the central role of the *Drosophila CalpB* in the cell migration, while in the present work we proved that Gbs-70E the glycogen binding subunit of *Drosophila* PP1 controls glycogen level.

5. SUMMARY

In our previous study we introduced a molecular genetics based experimental strategy with our collaborators, which was used for the revealing the role of a calcium dependent protease, CalpB in the migration of the border cells in *Drosophila melanogaster*. As a continuation of this collaboration we adopted a similar approach to investigate the physiological function of a putative glycogen binding regulatory subunits of the protein phosphatase 1 (PP1) holoenzyme, in *Drosophila*. The regulation of glycogen metabolism by phosphorylation-dephosphorylation reactions is well-known process in which PP1 plays an important role. However the glycogen binding subunits of PP1 that target the phosphatase to glycogen have not been characterized yet in the fruit flies. Based on gene and protein sequence analysis we identified two putative *D. melanogaster* PP1 glycogen binding subunits, and in our present work we studied one of them, the Gbs-70E gene and its gene product. We noted that the glycogen and PP1 catalytic subunit binding structural elements are well conserved in the Gbs-70E protein. First we tested with biochemical experiments the role of these two regions, and demonstrated that Gbs-70E was able to bind to all of the PP1 catalytic subunit isoforms and to glycogen. Thus we proved that Gbs-70E was indeed a functional glycogen binding subunit of PP1. Next we investigated the gene expression patterns of the shortest and the two longer transcripts during ontogeny and in different body parts. The shortest mRNA is present only in embryos and in the ovary of females, suggesting a maternal origin and the role of the shorter protein in embryogenesis. After the deletion of the gene with molecular genetic methods we found that the robust reduction of Gbs-70E expression resulted in the decrease of glycogen content and the lifespan of the imagos, and in the decline of the fertility of females. In conclusion, we found that the Gbs-70E glycogen binding regulatory subunit of PP1 has a role in the regulation of glycogen metabolism, determination of lifespan, and the embryogenesis in *Drosophila*.

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List of publications related to the dissertation

1. **Kerekes, É.**, Kókai, E., Páldy, F.S., Dombrádi, V.: Functional analysis of the glycogen binding subunit CG9238/Gbs-70E of protein phosphatase 1 in Drosophila melanogaster. *Insect Biochem. Mol. Biol. 49*, 70-79, 2014.
   DOI: http://dx.doi.org/10.1016/j.ibmb.2014.04.002
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2. Kókai, E., Páldy, F.S., Somogyi, K., Chougule, A., Pál, M., **Kerekes, É.**, Deák, P., Friedrich, P., Dombrádi, V., Ádám, G.: CalpB modulates border cell migration in Drosophila egg chambers. *BMC Dev. Biol. 12* (1), 1-26, 2012.
   DOI: http://dx.doi.org/10.1186/1471-213X-12-20
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Poster presentations related to the dissertation

Éva Kerekes, Endre Kókai, Ferenc Páldy, János Gausz, Péter Friedrich, Viktor Dombrádi: Searching for the function(s) of the CG9238 gene in *Drosophila*. Europhosphatase 2013: Protein Phosphatases in Health and Disease, Rehovot, Israel. 2013.

Éva Kerekes, Ferenc Pop, Géza Ádám, János Gausz, Péter Friedrich, Viktor Dombrádi, Endre Kókai: Functional analysis of *Drosophila* CG9238 gene. Conference of Hungarian Biochemical Society, 2011, Pécs, Hungary. Biochemistry, XXXV/3, 34.

Éva Kerekes, Ferenc Pop, Géza Ádám, János Gausz, Péter Friedrich, Viktor Dombrádi, Endre Kókai: Functional analysis of *Drosophila* CG9238 gene. Conference of Hungarian Biochemical Society, 2010, Budapest, Hungary. XXXIV/3, 36.

Oral presentations related to the dissertation

Éva Kerekes, Endre Kókai, Ferencz Páldy, Viktor Dombrádi: Functional analysis of the glycogen binding subunit CG9238/Gbs-70E of protein phosphatase 1 in *Drosophila melanogaster*. Signaling pathways in cancer biology, Mátraháza. 2014.

Éva Kerekes, Endre Kókai, Ferencz Páldy, Viktor Dombrádi: Investigation of the human R5 homolog *Drosophila* CG9238. Signaling pathways in cancer biology, Egerszalók. 2013.

Éva Kerekes, Endre Kókai, Ferencz Páldy, Viktor Dombrádi: Searching for the function(s) of the CG9238 gene in *Drosophila*. Annual Symposium of the Doctoral School of Molecular Medicine, University of Debrecen, Debrecen. 2012. and Symposium of the Doctoral School of Molecular Medicine, University of Debrecen, Debrecen. 2013.

Éva Kerekes, Endre Kókai, Ferencz Páldy, Viktor Dombrádi: Characterization of R5 glycogen binding subunit homolog in *Drosophila*. Signal transduction and skin biology: a training course, Galyatető. 2010. and Annual Symposium of the Doctoral School of Molecular Medicine, University of Debrecen, Debrecen. 2011.

Endre Kókai, Éva Kerekes, Ferenc Pop, Géza Ádám, János Gausz, Péter Friedrich, Viktor Dombrádi: Examination of the functions of *Drosophila* protein phosphatase 1 enzyme interacting CG9238 geneproduct. VIII. Conference of Hungarian Genetics and XV. Days of Cell- and Developmental Biology. Nyíregyháza. 2009.