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Biochemistry and Molecular Biology of Protein Phosphatase 1 in *Drosophila melanogaster*

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Protein phosphatase 1 (PP1) activity was detected with exogenous and endogenous substrates in *Drosophila* extract. The catalytic subunit of PP1 was purified to apparent homogeneity. The physicochemical and biochemical properties of the preparation were very similar to those of rabbit PP1. One PP1 catalytic subunit was cloned from a *Drosophila* cDNA library with the aid of a rabbit PP1 cDNA and oligonucleotide probes. Subsequently, three additional genes encoding for highly similar PP1 isoforms in *Drosophila* were identified and cloned. It was found that the primary structure of PP1 had been very well conserved during evolution.
One of the isoforms, called PP1(87B), was predominantly expressed during all developmental stages of the insect. Several mutations affecting the PP1(87B) gene were characterized. Based on the phenotype of different mutant alleles, it was concluded that PP1(87B) was involved in viability, mitosis, interphase chromatin condensation and learning. Our results demonstrate that PP1(87B) has several functions which cannot be complemented by other PP1 isoforms of *Drosophila*.

INTRODUCTION

Phosphorylation is a frequent postsynthetic protein modification. According to a modest estimation, about 10 percent of the protein content in an eukaryotic cell undergoes this modification. Although not all of the protein phosphorylation reactions have a well defined physiological role numerous examples indicate that it is a general regulatory mechanism.

The phosphorylation of proteins is catalyzed by protein kinases (Figure 1). They incorporate the γ-phosphate of ATP (sometimes of GTP, too) into Ser,

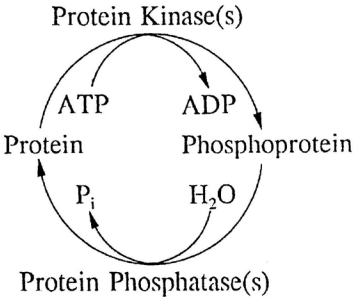

Figure 1. Protein phosphorylation-dephosphorylation cycle.

Thr or Tyr residues, and frequently alter the function of the target protein(s). In order to create a complete regulatory cycle it is imperative to have another reaction which converts the phosphoprotein back to the dephosphorylated form. Under physiological conditions, this is achieved by the hydrolysis of the phosphoester bond. The latter reaction is catalyzed by protein phosphatases (Figure 1). In his review paper Tony Hunter estimated the number of protein kinases to be 1001.[1] In reply, two recent reviews stated that there were 1002 protein phosphatases.[2,3] Through the exact number of the members of the two great enzyme families is not known yet, it seems reasonable to speculate that the final count will result in as many phosphatases as kinases, since they perform complementary and equally important roles.

In our studies, we selected protein phosphatase 1 (PP1) for detailed investigation. This enzyme dephosphorylates the Ser and Thr residues of a large number of substrate proteins. By definition, it is inhibited by two heat stable proteins called inhibitor-1 and -2, and is relatively specific for the ß-subunit of phosphorylase kinase.[4] PP1 was one of the first phosphatases whose catalytic subunit was purified to homogeneity[5,6] and thoroughly characterized (for a recent review see Ref. 7). PP1 was found to be associated with subcellular organelles, and the localization of its activity suggested several important functions for this enzyme (Table I). Originally, we were interested in the regulation of glycogen metabolism, while later on we became more involved in the cell cycle research and in the studies of neuronal function. Our goal was to obtain direct evidence for the role of PP1 in this processes.

Our model animal was *Drosophila melanogaster*. The small flies were not ideal for conventional biochemical work; however, this problem was solved by establishing mass culturing conditions. On the other hand, the relative ease of genetic manipulations and the extensive genetic background complied during the last decades were an advantage of our selection. We planned a multidisciplinary approach and designed an experimental strategy that involved intensive international co-operation (Table II).

TABLE I

Localization of protein phosphatase 1 activity within eukaryotic cells and its suggested physiological roles

Compartment	Role
Glycogen particles	Glycogen metabolism
Myosin filament	Muscle contraction
Sarcoplasmic reticulum	Muscle contraction
Microsomes	Cholesterol synthesis
Ribosomes	Protein synthesis
Nucleus	Cell cycle
Postsynaptic densities	Modulation of nervous activity

TABLE II

Identification of physiological role(s) for protein phosphatase 1 (Experimental strategy)

1. Detection of protein phosphatase 1 activity in *Drosophila* extract.
2. Purification of the catalytic subunit of protein phosphatase 1 (PP1) from *Drosophila*.
3. Characterization of *Drosophila* PP1 and comparison with rabbit PP1.
4. Screening of a *Drosophila* cDNA library with rabbit PP1 cDNA and oligonucleotide probes (based on homology).
5. Isolation of positive clones.
6. DNA purification and sequencing.
7. Prediction of protein sequence and comparison with that of rabbit PP1.
8. Localization of PP1 gene in *Drosophila* salivary gland polytene chromosomes.
9. Search for the existing mutants assigned to the PP1 gene's cytological position (or induction of new mutations in the same region).
10. Characterization of the PP1 mutant phenotype(s) and deduction of the physiological function(s) for the wild type gene.
11. Rescue of the mutant phenotype(s) by transformation of the wild type PP1 gene into the mutant allele(s).
12. Identification of the mutation(s) in the gene of PP1.

RESULTS AND DISCUSSION

In a joined project with Peter Friedrich's group (Institute of Enzymology, Biological Research Center of the Academy of Sciences, Budapest, Hungary) we proved that protein phosphatases regulated glycogen degradation in *D.*

TABLE III

Comparison of protein phosphatase 1 catalytic subunits

Property	*Drosophila*	Rabbit
Molecular mass by		
native gel filtration	33,000	37,000
SDS PAGE	33,000	37,000
Amino acid composition	very similar	very similar
N-terminal residue	blocked	blocked
Phosphorylase phosphatase activity	high	high
Metal ion requirement	no	no
Effect of inhibitor-1 and -2	inhibition	inhibition
Specificity for phosphorylase kinase subunits	ß	ß
Effect of polycations	inhibition*	inhibition
Effect of heparin	inhibition	inhibition
Interaction with heparin-Sepharose	retention	retention

*at $I = 0.05$

melanogaster.[8] Using endogenous and exogenous substrates, we demonstrated that both PP1 and protein phosphatase 2A (PP2A) activities were present in the crude extract of *Drosophila*.[9,10] In an independent work, Orgad *et al.*[11] obtained similar results.

As a next step, we generated the catalytic subunit of PP1 from its holoenzyme form(s) by $(NH_4)_2SO_4$-ethanol treatment and purified it to apparent homogeneity by heparin-Sepharose affinity chromatography and MonoQ FPLC.[12] The preparation was characterized and compared to the well known rabbit muscle counterpart (Table III). Except for two minor differences (slightly smaller molecular mass, and the fact that polycation inhibition was observed only at physiological ionic strength), the examined physicochemical and biochemical properties of the two preparations were very similar if not identical. Our results suggest a high level of conservation of the structure and function of PP1 during evolution.

The molecular biology part of the project was completed in Philip Cohen's laboratory (Department of Biochemistry, The University, Dundee, Scotland, UK) with Patricia T.W. Cohen and co-workers. The project was soon joined by David M. Glover's group (Department of Biochemistry, Imperial College of Science, Technology and Medicine, London and later Department of Biochemistry, The University, Dundee, UK) who did the molecular genetics. The starting point of the work was the screening of *Drosophila*

cDNA libraries with a rabbit muscle PP1 catalytic subunit cDNA fragment,[13] and with a corresponding oligonucleotide.[14] We were able to find a large number of positive clones, with the heterologous probes. After isolation of some strongly positive clones we purified and sequenced their cDNA.[15,16] One cDNA contained a single open reading frame encoding 302 amino acids. The predicted amino acid sequence of the clone was very much similar (92% identity) to the previously published rabbit muscle PP1α sequence.[13] The differences were clustered first of all at the extreme *N*-terminal and *C*-terminal segments, the *Drosophila* protein being 26 amino acids shorter at the *C*-terminus. The significance of this observation was several-fold. (i) It demonstrated that our experimental strategy had worked, screening with heterologous probes resulted in the right PP1 clone. (ii) Our biochemical results were substantiated by primary structure data. (iii) It turned out that the amino acid sequence of PP1 catalytic subunits had been extremely well conserved during evolution. Only histones and calmodulin have a better conserved structure, PP1 is one of the "most conservative" enzymes described till now.[17]

The chromosomal localization of the corresponding gene was done by J. Myles Axton in London. He performed several *in situ* hybridizations to *Drosophila* salivary gland polytene chromosomes and found that the cloned cDNA recognized four distinct sites. The strongest labelling was in the third chromosome at 87B6-12 position, but strong hybridization was also observed at 97A2-5 (in the third chromosome), 9C1-2 and 13C1-2 (both in the X chromosome) positions.[15] This observation suggested that the *Drosophila* genom could encompass as many as four PP1 genes. Our hypothesis was confirmed by subsequent cloning of the four PP1 catalytic subunits.[16–19] They were named after the localization of their genes (Table IV). These genes encode for a group of highly similar proteins. PP1(9C) is somewhat different from the others, it contains some signature amino acid substitutions characteristic of the PP1ß subclass of animal PP1, while the rest are more similar to the PP1α subclass.[18] Other differences are found at the two ends of the sequence, the *C*-terminus of PP1(13C) and PP1(87B) is shorter (so the predicted molecular mass of the encoded proteins is smaller) than that of the other two isoforms. The pattern of potential phosphorylation sites is characteristicly distinct between the isoforms; however, the significance of these differences is not yet clear (Table IV). Specific mRNAs for the four isoforms were detected (Table V), indicating that all the four genes were active.[15,18,19]

The discovery of the isoenzyme structure of PP1 was of considerable interest. Our papers[15,16] and that of Ohkura *et al.*[20] were the first reports indicating that the catalytic subunit of PP1 had several genes in *Drosophila melanogaster* as well as *Schizosaccharomyces pombe*. By now it turned out to be a general rule in most organisms.[7] This finding forced us to reformulate our original question. Since then, we were asking if the isoforms had

TABLE IV

Comparison of *Drosophila* protein phosphatase 1 catalytic subunit isoforms

Isoform	PP1(9C)	PP1(13C)	PP1(87B)	PP1(96A)
Localization of the gene	9C1-2	13C1-2	87B6-12	96A2-5
Number of amino acids	330	302	302	325
Predicted molecular mass (kDa)	37.7	34.5	34.5	37.2
Amino acid identity (%)* with				
PP1(9C)	100	85	85	86
PP1(13C)	85	100	94	92
PP1(87B)	85	94	100	96
PP1(96A)	86	92	96	100
Potential phosphorylation sites				
A-kinase	–	Thr 143	Ser 143	Thr 143
src^{60} kinase	Tyr 304	–	–	Tyr 304
				Tyr 306

*in 302 amino acids

identical or different functions and, if yes, what was the function of each PP1 catalytic subunit. Our original experimental plan was nearly compromised by the high sequence similarity of PP1 isoforms in *Drosophila* (Table IV) because it suggested overlapping functions. If this had been the case, we could have had a difficult time finding mutants with an easily recognizable phenotype. Fortunately, the expression of the four isoforms was significantly different and was also dependent on the developmental stage of the flies

TABLE V

mRNA levels for the isoforms of protein phosphatase 1 catalytic subunit in *Drosophila*

Isoform	Stage of development			
	Embryo	Larvae	Pupae	Imago
PP1(9C)	+	+	++	++
PP1(13C)	–	–	–	(+)
PP1(87B)	+++	+++	+++	+++
PP1(96A)	+	(+)	(+)	(+)

(Table V). In all stages, the mRNA of PP1(87B) was predominant. In fact, it was so constant that it could be used as standard in developmental Northern blotts (*cf.* 21). The abundance of PP1(87B) mRNA suggested that if this gene was mutated, the other isoforms were probably unable to complement the mutation. Thus, we concentrated our efforts on the PP1(87B) gene.

The 87B region of the 3rd chromosome had been extensively mapped by János Gausz and co-workers (Institute of Genetics, Biological Research Center of the Academy of Science, Szeged, Hungary) using saturation mutagenesis.[22] With the help of large deletion mutations (deficiencies) generated by this group,[22,23] we were able to narrow down the position of the PP1(87B) gene to two closely spaced bands (87B 8-9) in the physical map of the *Drosophila* genom. Importantly, members of the lethal complementation group ck19[22] as well as allelic position effect variegation suppressor mutations[23,24] had the same localization. The name and phenotype of these mutants are listed in Table VI. The homozygous (and hemizygous) mutants die at the 3rd instar larvae/puparium boundary, but heterozygotes are viable. All mutations suppress position effect variegation,[23,24] which reflects suppression of interphase chromatin condensation.[25] Four of the mutants are affected in mitosis,[23,26] abnormalities can be seen in the actively dividing brain and imaginal discs of the third instar larvae, and include hypercondensation of the chromosomes, collapsed structure of the mitotic spindle, as well as polyploid or hyperploid cells with multiple spindles.[26] The mutants enter mitosis in a normal way but are unable to complete anaphase and exit mitosis properly.

The fact that the above phenotypes were due to the mutation of the PP1(87B) gene was proven by rescue experiments.[23,26] All the phenotypes were cured by transformation of mutant embryos with the intact wild type PP1(87B) gene (Table VI). The rescue was unsuccessful when the coding region of the wild type PP1(87B) gene was interrupted by stop codons.[26] The

TABLE VI

Phenotypes and rescue of *Drosophila* PP1(87B) mutants

Name	Lethality	Mitosis	Position effect variegation*	Rescue by PP1(87B) gene
e211	+	abnormal	weakly suppressed	+
hs46	+	abnormal	weakly suppressed	+
e078	+	normal	suppressed	+
Su-var(3)6^{01}	(+)	normal	suppressed	+
1311	+	abnormal	suppressed	+
1455	+	abnormal	suppressed	+

*Related to interphase chromatin condensation

genetic experiments summarized in Table VI were completed in the laboratories of David M. Glover, János Gausz and Günter Reuter (Department of Genetics, Martin Luther University, Halle, Germany).

The results suggest that the PP1(87B) isoform has several functions. Some of these must be essential since the mutations are lethal or semilethal, *e.g.* Su-var(3)6^{01}. The other three PP1 genes were not affected, still they were not able to complement the mutation of the predominant PP1(87B) isoform. One can conclude that either the PP1(87B) gene has some specific functions not shared by the other isoforms or, alternatively, the level of expression of the other three genes is not sufficient to compensate for the mutation in the PP1(87B). One of these specialized functions of PP1(87B) is related to the condensation of chromosomes. There are two points of action during the cell cycle. One of them occurs during the interphase and is expressed in the suppression of position effect variegation, the other is in the mitosis. However, the mechanism of these two actions must be different, since DNA condensation is decreased (position effect variegation is suppressed) during interphase while it is increased (hypercondensation) during mitosis. Clearly, different target proteins are involved in the two phenomena. Another feature of the mutations is that the two processes are affected differentially (see Table VI).

In order to get a better understanding of the nature of mutations, we carried out a combined biochemical and molecular biology study. We found that PP1 activity was reduced in the third instar larvae of all mutants (23,27 and Table VII); the same mutations did not influence PP2A activity[27] and a mutant from the neighboring complementation group ck18 had a wild type level of PP1 activity.[27] The structural basis of the decreased activity was determined by cloning the wild type and mutated genes.[27,28] The gene structure of the wild strains (Oregon R and Canton S) as well as that of four mutants is shown in Figure 2. The mRNA size and abundance data and the summary of the sequencing work are given in Table VII. It is seen that three mutants do not synthesize a detectable level of PP1(87B) mRNA (in the case of e211 it can be explained by a large deletion eliminating upstream regulatory elements) and one mutant (hs46) produces mRNAs of a reduced size and concentration (because of a shorter deletion). The shorter mRNAs do not contain the initiation codon and, thus, can be considered nonfunctional. In conclusion, four mutations prevent the synthesis of the PP1(87B) protein. It is also seen that even these null mutants contain 16–20% of the wild strain's PP1 activity. We believe that this residual activity can be attributed to the other isoforms of PP1, which are not influenced by the mutation of the PP1(87B) gene. This means that the residual activity is not sufficient to sustain viability.

In two mutants, the codon of the same essential Gly 220 residue is altered, giving rise to mRNAs of normal size but encoding mutant proteins.

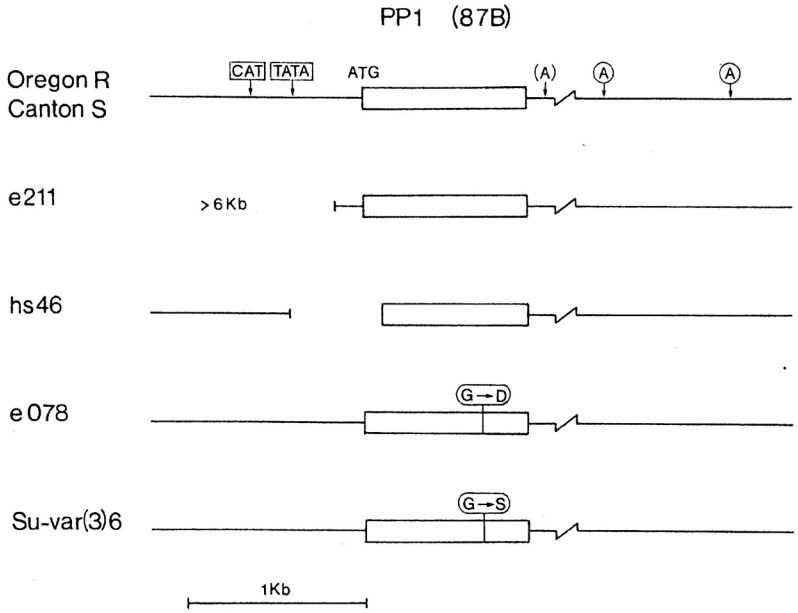

Figure 2. The structures of wild type and mutant PP1(87B) genes. The open bar represents the coding region, the zig-zag line shows the position of an intron. CAT=CAT box, TATA=TATA box, ATG=initiation methionine codon, A=polyadenylation signals (the one in the parentheses is probably not functional). The positions of deletions and pointmutations in the mutants are shown.

TABLE VII

Properties of *Drosophila* PP1(87B) mutants as compared to the wild type Oregon R strain

Name	PP1 activity* (% of wild type)	PP1(87B)	
		mRNA	Gene structure
Oregon R	100	1.6 and 2.5 kb	wild type
e211	20	absent	promoter deleted
hs46	21	(1.1 and 2.0 kb)	start deleted
e078	35	1.6 and 2.5 kb	point mutation G220D
Su-var(3)6^{01}	21	1.6 and 2.5 kb	point mutation G220S
1311	16	absent	?
1455	17	absent	?

*Measured in 3rd instar larvae
? Not analyzed

It is probable that the point mutants retain a low level of PP1(87B) activity which is enough to complete mitosis normally, though it is less than that required for the unsuppressed position effect variegation. From the combination of the data, one can conclude that null mutants affect mitosis, and that mutants with a large 5'-end deletion are weaker suppressors of position effect variegation than the rest (Tables VI and VII).

The fact that PP1(87B) mutations become detrimental only in the third instar larval stage can also be explained. It is known that *Drosophila* eggs contain a deposit of proteins and mRNA of maternal origin. We suppose that the maternal PP1(87B) runs out just at the end of the third larval stage and PP1 activity drops below a certain threshold level, required for essential cellular functions. The importance of the maternal PP1(87B) was demonstrated by pole cell transplantation into maternally lethal mutant embryos.[23]

Our threshold hypothesis has been supported by the studies of conditional PP1 mutants in lower eukaryotes. The temperature sensitive BimG11 mutant of *Aspergillus nidulans* is affected in the PP1 catalytic subunit gene and becomes blocked in mitosis as temperature reaches 40 °C[29] and, concomitantly, the PP1 activity decreases to 15% of the starting value.[30] The *Schizosaccharomyces pombe* mutant, called dis2-11, is cold sensitive.[31] When it is cooled down to 20 °C, a block in mitosis develops in the mutant.[20] It was found that a point mutation in one of the PP1 catalytic subunit genes is responsible for the nearly complete loss of PP1 activity and abnormal mitosis.[32] These results, together with our observations, indicate that the requirement of a certain level of PP1 activity for the exit from mitosis is a general phenomenon.

The semilethality of the Su-var(3)6^{01} mutants made it possible to investigate the role of PP1 not only in the larvae but also in the fully developed imago of *D. melanogaster*. In collaboration with Peter Friedrich, Hans-Christof Spatz (Institute of Biology, University of Freiburg, Freiburg, Germany) and co-workers, we found that this mutation seriously impaired associative learning and conditioning, while nonassociative learning was improved.[33] Although the precise mechanism is not known, one can conclude that PP1(87B) is the part of a regulatory network controlling neuromodulation in *Drosophila*.

CONCLUSION

In our work, we identified several functions of one PP1 isoform in *Drosophila*. Maternally deposited PP1(87B) is essential in the early embryonic development, and de novo synthesized PP1(87B) is required for the metamorphosis of the larvae into puparium. PP1(87B) influences elementary processes, like DNA condensation during the interphase and mitosis, and is

also involved is such a complex mechanism as modulation of neuronal function. The list is still not complete, we believe that PP1(87B) has pleiotropic roles some of which still await discovery. We also demonstrated that the other isoforms cannot substitute PP1(87B) in the aforementioned processes; however, their physiological role(s) remains an enigma. Clearly, further studies are required to unravel all of the functions of PP1 isoforms in *Drosophila*.

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SAŽETAK

Biokemija i molekularna biologija proteina fosfataze 1 u *Drosophila melanogaster*

Viktor Dombrádi

Aktivnost proteina fosfataze 1 (PP1) dokazana je egzogenim i endogenim supstratima u ekstraktu *Drosophila-e*. Katalitička podjedinica *Drosophila-e* pročišćena je do prividne homogenosti. Fizikalno-kemijska i biokemijska svojstva preparata bila su vrlo slična onima kunića PP1. Jedna PP1 katalitička podjedinica je klonirana iz cDNA biblioteke *Drosophila-e* s pomoću PP1 cDNA kunića i oligonukleotidnih sondi. Zatim su identificirana i klonirana tri dodatna gena koja determiniraju vrlo slične *izo*-oblike PP1 *Drosophila-e*. Utvrđeno je da je primarna struktura PP1 vrlo dobro konzervirana tijekom evolucije.

Jedan od *izo*-oblika, nazvan PP1 (87B), bio je dominantno eksprimiran tijekom svih razvojnih stadija insekta. Karakterizirano je nekoliko mutacija gena PP1 (87B). Na temelju fenotipa različitih mutantnih alela zaključeno je da je PP1 (87B) važan za vijabilnost, mitozu, kondenzaciju interfaznog kromatina i učenje. Naši rezultati pokazuju da PP1 (87B) ima nekoliko funkcija koje se ne mogu komplementirati drugim *izo*-oblicima *Drosophila-e*.