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Functional analysis of the glycogen binding subunit CG9238/Gbs-70E of protein phosphatase 1 in *Drosophila melanogaster*

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ABSTRACT

The product of the *CG9238* gene that we termed glycogen binding subunit 70E (*Gbs-70E*) was characterized by biochemical and molecular genetics methods. The interaction between Gbs-70E and all catalytic subunits of protein phosphatase 1 (Pp1-87B, Pp1-9C, Pp1-96A and Pp1-13C) of *Drosophila melanogaster* was confirmed by pairwise yeast two-hybrid tests, co-immunoprecipitation and pull down experiments. The binding of Gbs-70E to glycogen was demonstrated by sedimentation analysis. With RT-PCR we found that the mRNAs coding for the longer Gbs-70E PB/PC protein were expressed in all developmental stages of the fruit flies while the mRNA for the shorter Gbs-70E PA was restricted to the eggs and the ovaries of the adult females. The development specific expression of the shorter splice variant was not conserved in different *Drosophila* species. The expression level of the gene was manipulated by P-element insertions and gene deletion to analyze the functions of the gene product. A small or moderate reduction in the gene expression resulted in no significant changes, however, a deletion mutant expressing very low level of the transcript lived shorter and exhibited reduced glycogen content in the imagos. In addition, the gene deletion decreased the fertility of the fruit flies. Our results prove that Gbs-70E functions as the glycogen binding subunit of protein phosphatase 1 that regulates glycogen content and plays a role in the development of eggs in *D. melanogaster*.

1. Introduction

The regulation of glycogen degradation by phosphorylation and dephosphorylation is a classical example for the controlling of enzyme activity by reversible post-translational modifications. From the fundamental works of Krebs and Fischer we know that glycogen phosphorylase, the key enzyme of glycogen breakdown, is phosphorylated and activated by phosphorylase kinase; in turn it is dephosphorylated and inactivated by protein phosphatases (Fischer, 2013). According to *in vitro* assays, both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) can catalyze the hydrolytic removal of phosphate from phosphorylase a (Ingebritsen et al., 1983). However, only PP1 is considered as a physiological regulator since its catalytic subunit (PP1c) is directed to the glycogen bound substrates by specific regulatory subunits. In skeletal muscle, the GM subunit (termed PPP1R3 in accordance with the human genome project) targets PP1c to glycogen as well as to the sarcoplasmic reticulum and mediates the hormonal control of phosphatase binding via the reversible phosphorylation of its N-terminal segment (Hubbard and Cohen, 1993). In liver cells, the glycogen binding GL (PPP1R4) subunit ensures the sequential dephosphorylation of phosphorylase a, and glycogen synthase b. It binds to the allosteric regulator, phosphorylase a, that prevents the dephosphorylation of synthase b until all of the phosphorylase a is converted to phosphorylase b and thus coordinates the inactivation of glycogen degradation and the activation of glycogen synthesis (Alemany and Cohen, 1986). The PPP1R5 (Doherty et al., 1996) and PPP1R6 (Armstrong et al., 1997) glycogen binding subunits have a broad tissue distribution and promote the dephosphorylation reactions mainly by localizing PP1c close to its substrates. In all of the above regulatory proteins the essential structural features, i.e. the PP1c binding RVxF motif (Ceulemans and Bollen, 2006) and the

Abbreviations: PP1c, protein phosphatase 1 catalytic subunit; Gbs-70E, glycogen binding subunit 70E.

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polysaccharide binding domain (Doherty et al., 1996) are conserved (Fig. 1).

In insects, octopamine and adipokinetic hormones activate the mobilization of glycogen that supplies the energy source for locomotion and other essential biological processes (Lorenz and Gade, 2009). Upon the action of these hormones the key enzyme of glycogen degradation, glycogen phosphorylase is activated in the fat body and trehalose is synthesized from the released glucosyl units (Arrese and Soulages, 2010). Traditionally our group has used *Drosophila melanogaster* as a model organism to study glycogen metabolism in insects because of its well established genetics and the availability of advanced molecular tools for precise genetic manipulations. Previously we reported on the isolation and biochemical properties of glycogen phosphorylase (GlyP or DGPH) from *D. melanogaster* (Dombrádi et al., 1985). The regulation of this enzyme by phosphorylation and dephosphorylation was demonstrated (Dombrádi et al., 1986), and its interconverting enzymes; phosphorylase kinase (Dombrádi et al., 1987a) and protein phosphatase 1 (Dombrádi et al., 1987b) were also characterized. It was found that the activation of phosphorylase kinase by Ca^{2+} was the main mechanism triggering glycogen degradation, while the elevation of cAMP acting via the PKA kinase had only a slight effect (Dombrádi et al., 1987a). By imprecise P-element excision we generated a phosphorylase mutant, containing a truncated P-element upstream of the *GlyP* gene that exhibited reduced gene expression, low enzyme activity, high glycogen content, and weak viability (Tick et al., 1999). According to subsequent molecular genetic studies homozygous *GlyP* knockouts are lethal, and hypomorphic alleles with reduced phosphorylase activity survive but show declined flight performance (Eanes et al., 2006). The genetic analysis of the activating phosphorylase kinase is in agreement with the above data. The complete loss of the maternal and zygotic components of the catalytic subunit of phosphorylase kinase (DPhK-γ or PhKγ) causes embryonic lethality while the genetic elimination of the zygotic component alone results in reduced viability and impaired leg muscle development (Bahri and Chia, 1994). The deletion of the dominant allele of the catalytic subunit of protein phosphatase 1 (Pp1-87B) reduces the phosphorylase phosphatase activity by 80% in the third instar larva (Dombrádi et al., 1990) that die at the end of the last larval stage of development (Axton et al., 1990). In addition to controlling glycogen degradation by the dephosphorylation of active phosphorylase, protein phosphatase 1 can also activate glycogen synthesis by dephosphorylating and activating glycogen synthase termed GlyS in *D. melanogaster*. The genetic knockdown of the *GlyS* gene proved to be lethal (http://flybase.org/reports/FBrf0200327.html; Dietzl et al., 2007), providing an additional proof for the significance of glycogen metabolism in insects. Thus all of the enzyme components of glycogen metabolism have been isolated, cloned, and investigated by biochemical and molecular biology methods in *D. melanogaster* (cf. Graphical Abstract). However, it has been difficult to assess the significance of Pp1-87B from the genetic experiments, since protein phosphatase 1 has broad substrate specificity; in fact the lethal phenotype of the mutants was attributed to sever mitotic defects (Axton et al., 1990). The question is further complicated by the fact that there are four protein phosphatase 1 genes in *D. melanogaster* that were identified according to their chromosomal localization as *Pp1-9C, Pp1-13C, Pp1-87B*, and *Pp1-96A* (Dombrádi et al., 1989).

In order to circumvent the technical difficulties we decided to tackle the problem by investigating the glycogen binding subunits of the phosphatase. Based on primary structure comparisons of proteins we suggested that *D. melanogaster* contained two genes in a putative orthologous group of the human G subunits termed CG9238 and CG9619 according to their Celera Genome codes (Kókai et al., 2001). Our predictions were confirmed when Giot et al. (2003) reported that CG9238 interacted with Pp1-87B and Pp1-96A, and when Bennett et al. (2006) found that CG9619 interacted with all of the known *Drosophila* PP1 isoforms in yeast two-hybrid experiments. Bennett et al. (2006) proposed that *CG9619* codes the glycogen binding subunit of protein phosphatase 1 in the fruit flies and termed it as *Gbs-76A*. In our studies we focused on the investigation of the other ortholog *CG9238*, that we renamed *Gbs-70E*. From the latter gene three mRNAs are transcribed (see Fig. 3) due to the alternative splicing of exon 2 and to alternative polyadenylations. From the transcripts two proteins are translated, the shorter Gbs-70E PA and the longer Gbs-70E PB/PC. The typical phosphatase binding motif and glycogen binding domain is preserved in all of the *Drosophila* paralogs (Fig. 1). We selected *Gbs-70E* since according to high throughput genetic screens it was involved in experimental tauopathy (Shulman and Feany, 2003; Chen et al., 2007), in olfactory avoidance (Sambandan et al., 2006), in tolerance against alcohol (Kong et al., 2010) and in the lifespan determination of *Drosophila* (Magwire et al., 2010). From large scale gene expression studies it was concluded that this gene was up-regulated by the addition of glucose but not by starvation (Zinke et al., 2002), by exposure to alcohol (Morozova et al., 2006), by paraquat stress (Girardot et al., 2004), and was down-regulated by pentylenetetrazole drug treatment for 12 or 48 h (Mohammad et al., 2009). Furthermore, the *Caenorhabditis elegans* ortholog of *Gbs-70E* (H18N 23.2) has important functions because its knocking down resulted in embryonic lethality and morphological disorders (Rual et al., 2004; http://www.wormbase.org/species/c_elegans/rnai/WBRNAi00033721#50413–10). In comparison, the silencing or the mutation of the *Gbs-76A* gene did not reveal any obvious phenotypes as all of the available alleles are viable and fertile according to the FlyBase database (http://flybase.org/reports/FBgn0036862.html). An OrthoB search of whole genome sequences of fifty insect species revealed that all of the investigated insects had a conserved *Gbs-70E* ortholog (http://cegg.unige.ch/OrthoDB7/results?searchtext=EOG71PDB0), while the *Gbs-76A* orthologous gene (http://cegg.unige.ch/OrthoDB7/results?searchtext=EOG74NHG7) was missing from four species, indicating that the latter was probably less important. From the information in the literature and in the databases it was hypothesized that *Gbs-70E* is the paralog that plays the dominant physiological

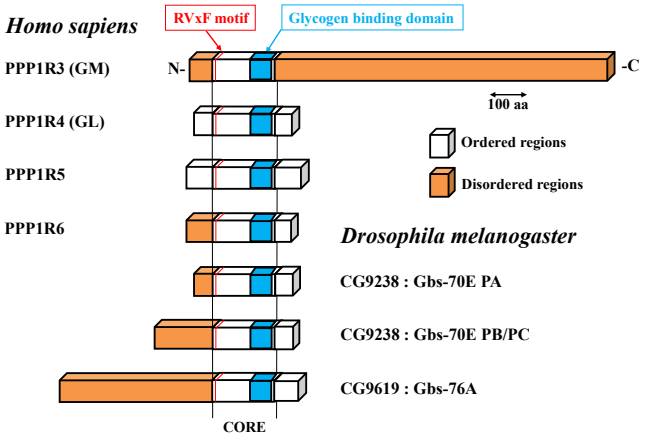

Fig. 1. The amino acid sequences of human PPP1R3-to-PPP1R6 and *Drosophila* Gbs-70E (splice variants PA and PB/PC) as well as Gbs-76A were aligned (see Supplementary Fig. 1A). The traditional abbreviations for GM and GL are also shown for convenient identifications. Ordered (globular) and disordered segments of the polypeptides (see Supplementary Fig. 1B) are indicated by white and orange bars. The phosphatase binding RVxF motif and the glycogen binding domain inside the common core region are shown in red and blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)