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Divergent roles of the *Drosophila melanogaster* globins

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ABSTRACT

In contrast to long-held assumptions, the gene repertoire of most insects includes hemoglobins. Analyses of the genome of the fruitfly *Drosophila melanogaster* identified three distinct hemoglobin genes (*glob1*, *glob2*, and *glob3*). While *glob1* is predominantly associated with the tracheal system and fat body, *glob2* and *glob3* are almost exclusively expressed in the testis. The physiological role of globins in *Drosophila* is uncertain. Here, we studied the functions of the three globins in a cell culture system. *Drosophila* Schneider 2 (S2) cells were stably transfected with each of the three globins and the empty vector as control. Under hypoxia (1% atmospheric O_2), only *glob1* overexpression enhanced the activity of mitochondrial oxidases and the ATP content. However, the positive effect of *glob1 expression* disappeared after 24 h hypoxia, suggesting metabolic adaptations of the S2 cells. *glob2* and *glob3* had no positive effect on hypoxia-survival. After application of oxidative stress by H_2O_2, *glob2* dramatically enhanced the viability of S2 cells. Evaluation of the intracellular localization of the globins using specific antibodies and green fluorescent protein-fusion constructs suggested that glob1 and glob2 most likely reside in the cytoplasm, while glob3 is associated with structures that may represent parts of the intracellular transport machinery. *In silico* analyses of public RNA-Seq data from different developmental stages provided that *glob1* is co-expressed with genes of the aerobic energy metabolism, while *glob2* and *glob3* expression can be related to spermatogenesis and reproduction. Together, the results indicate divergent functions of the *Drosophila* globins: glob1 may play a role in the O_2-dependent metabolism while glob2 may protect spermatogenesis from reactive oxygen species.

1. Introduction

In most hexapods (insects and allies), gas exchange is mediated by the tracheal system, which connects the inner organs with ambient air. The tracheal O_2 supply, which essentially takes place by diffusion, was long thought to be sufficient for the support of oxidative metabolism in insects. Only recently it has become evident that hexapods in fact have additional respiratory proteins that may enhance O_2 supply (for review, see: Burmester, 2015; Burmester and Hankeln, 2007). Firstly, copper (Cu^+)-containing hemocyanin transports O_2 in many ametabolous and hemimetabolous hexapod orders (Burmester, 2015; Burmester and Hankeln, 2007; Hagner-Holler et al., 2004). Secondly, apparently all insects also possess (hemo-)globins (Hbs).

In most vertebrate and many invertebrate species, globins serve for the transport of O_2 in the blood and also enhance O_2 supply to muscle and other tissues (Burmester and Hankeln, 2014; Dickerson and Geis, 1983; Weber and Vinogradov, 2001). Some globins, however, may also have other or additional functions: For example, globins may detoxify reactive oxygen species (ROS) (Flögel et al., 2004; Koch and Burmester, 2016), mediate the turnover of nitric oxide (NO) (Flögel et al., 2001; Hendgen-Cotta et al., 2008) or may be involved in cellular signaling processes (De Henau et al., 2015). The standard globin structure (globin fold) comprises about 140–150 amino acids with eight α-helical segments (named A-H) that form a characteristic 3-over-3 α-helical sandwich structure, which embeds the O_2-binding heme group. Some globins diverge from this standard protein structure by having N- and/or C-terminal protein extensions or by forming multimeric globin chains.

Among insects, Hbs were initially thought to be restricted to the aquatic larvae of chironomid midges, the parasitic larvae of the horse botfly *Gasterophilus intestinalis* and some backswimmers of the genera *Anisops* and *Buenoa* (Notonectidae) (Burmester and Hankeln, 2007; Wawrowski et al., 2012; Weber and Vinogradov, 2001). While the

Abbreviations: GFP, green fluorescent protein; GO, gene ontology; Hb, hemoglobin; qRT-PCR, quantitative real-time RT-PCR; ROS, reactive oxygen species; RPKM, Reads Per Kilobase per Million mapped reads; S2 cell, *D. melanogaster* Schneider 2 cells

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Chironomidae secrete fat body-produced hemoglobins into their hemolymph, the botfly and the backswimmers harbor high concentrations of intracellular Hb in specialized fat body-derived organs. The accumulation of Hb is most likely an evolutionary response to hypoxic environments where it enhances the ability to deliver or to store O_2 (Burmester, 2015). However, within the past years, it has become evident that intracellular Hbs are actually present in many different insects that live in normoxic environments. A typical globin gene was first discovered in the fruitfly *D. melanogaster* (Burmester and Hankeln, 1999). With the advent of insect genome projects, Hb genes have been identified in many insects, for example the honeybee *Apis mellifera* (Hankeln et al., 2006), the lepidopterans *Bombyx mori* and *Samia cynthia* (Kawaoka et al., 2009), and the mosquitoes *Anopheles gambiae* and *Aedes aegypti* (Burmester et al., 2007), These data led to the conclusion that globins belong to the standard gene repertoire of insects and have an essential role in the physiology of insect (Burmester, 2015; Burmester and Hankeln, 2007).

The fruitfly *Drosophila melanogaster* harbors three distinct globin genes (*glob1*, *glob2* and *glob3*) (Burmester and Hankeln, 1999; Burmester et al., 2006). Little is known about *glob2* and *glob3*, two related gene duplicates, which are essentially male-specific and restricted in their expression to the testis (Gleixner et al., 2012). Their physiological role is still uncertain, although it has been speculated that they protect spermatogenesis from reactive oxygen species (ROS) (Gleixner et al., 2012). *Glob1* is expressed at high concentrations mainly in the tracheal system and fat body of *Drosophila* embryos, larvae, and adults (Burmester, 2015; Hankeln et al., 2002). The glob1 protein displays a typical globin fold, but shows a hexacoordinate binding scheme of the heme iron atom in its deoxygenated state (de Sanctis et al., 2005). Kinetic studies showed an O_2 affinity with a half-saturation pressure of $P50 = 0.12$ Torr, which is in the range of other insect globins (Hankeln et al., 2002). Recent studies using P-element (Yadav et al., 2015) or RNAi-mediated (Gleixner et al., 2016) *glob1*-knockdown gave partly conflicting results, either suggesting a role of glob1 in early development and ROS protection (Yadav et al., 2015) or supporting a more conventional role of glob1 in O_2 homeostasis (Gleixner et al., 2016).

To better understand the functions of the globins in *Drosophila*, we evaluated their ability to convey cellular tolerance to hypoxia and ROS in a cell culture system, studied their intracellular localization, and employed publicly available transcriptomic data to identify co-expressing genes.

2. Materials and methods

2.1. Culturing and transfection of S2 cells

D. melanogaster Schneider 2 (S2) cells were grown in 25 cm^2, 75 cm^2, or 175 cm^2 flasks (Sarstedt, Nümbrecht, Germany) in sterile modified Schneider's Drosophila Medium with L-glutamine (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France) in a humidified atmosphere at 27 °C.

The S2 cells were stably transfected with the pIB/V5-His vector that contained the cDNAs of one of the three *D. melanogaster* globin genes (*glob1*, *glob2* or *glob3*) using the Nanofection kit (PAA, Pasching, Austria). Cells carrying an empty vector were employed as mock control. After transfection and selection with blasticidin (PAA), RNA was extracted and expression of the globins was verified with quantitative real-time RT-PCR (qRT-PCR) as described below. For visualization of intracellular localization of the proteins, fusion constructs were generated that carried a green fluorescent protein (GFP) at the C-termini of the globins (glob1-GFP, glob2-GFP, glob3-GFP). The constructs were ligated into the pIB/V5-His or pAc5.1/V5-His/*lacZ* vectors and transfected into S2 cells as described.

2.2. RNA extraction and expression analysis

For qRT-PCR analyses, cells were lysed in a QIAshredder homogenizer, and total RNA was extracted from 10^7 cells with the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The quality and integrity of the RNA were evaluated by agarose gel electrophoresis. The RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Bonn, Germany) was used for cDNA synthesis. 1 µg total RNA was applied to each reaction.qRT-PCR analyses were performed on the ABI Prism 7000 or 7500 Real-Time PCR Systems (Applied Biosystems, Darmstadt, Germany). The reactions were run in triplicates in 25 µl including the Power SYBR-Green PCR Master Mix, 1 µl of cDNA and primers (final concentration 0.2 mM). Amplifications were carried out using a standard PCR protocol (step 1: 95 °C for 10 min, step 2–4: 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s; 40 cycles step 2–4). The specificity of the amplifications was evaluated by dissociation curve analyses. mRNA copy numbers were calculated with the ABI Prism System Sequence Detection Software (Applied Biosystems) using the standard curve method and further evaluated with Microsoft Excel 2013.

2.3. Hypoxia and oxidative stress experiments

For hypoxia experiments, 5×10^6 S2 cells were transferred into a 25 cm^2 flask and were kept at 1% O_2 in a CB150 incubator (Binder, Tuttlingen, Germany) for 3, 24 or 48 h at 27 °C. All experiments were carried out with three technical and at least three biological replicates.

Oxidative stress was induced with a freshly prepared 10 mM H_2O_2 stock solution. The cells (passage number < 35) were stressed with final concentrations of H_2O_2 of 200 µM, 350 µM, and 500 µM for 24 h. All experiments were carried out with at least three replicates with 10^5 cells per well in a 96-flat bottom well plate.

2.4. Cell viability tests

The viability of the cells was assayed by the WST-1 test (Roche, Mannheim, Germany), which measures the activity of the mitochondrial dehydrogenases, and by the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Mannheim, Germany), which measures the ATP content of the cells. Both tests were carried out according to the manufacturers' instructions. Absorbance at 450 nm (WST-1) and luminescence (CellTiter-Glo®) were determined with a DTX-880 Multimode Plate Reader (Beckman Coulter, Krefeld). Statistical analysis was done with the means of the technical replicates using the IBM SPSS Statistics 20 software and GraphPad Prism 5. Comparisons of the means of the transfected cells with each other and with mock controls were performed employing two-way ANOVA and Bonferroni correction. *p*-Values < 0.05 were considered as significant.

2.5. Immunofluorescence and intracellular localization

S2 cells were stably transfected as described and transferred onto polylysine-coated Culture Slides (BD Falcons, Heidelberg). The cells were for 10 min fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS: 140 mM NaCl, 27 mM KCl, 10 mM phosphate buffer, pH 7.4), washed three times with PBS, and blocked 1 h in 1% bovine serum albumin (BSA) in PBS. Glob1 was detected using a specific antibody (Hankeln et al., 2002) diluted 1:200 in PBS/1% BSA. In addition, antibodies against GFP (Abcam, Cambridge) and the Golgi marker GM130 (Abcam, Cambridge) were employed. After incubation with the appropriate secondary antibody, the slides were washed, and the nuclei were stained with Hoechst dye 33258 (0.3 µg/ml, Calbiochem, Darmstadt, Germany) in the dark. The slides were embedded in Mowiol (Calbiochem, Darmstadt, Germany) and analyzed using an Olympus BX51 research microscope equipped with a digital camera. Images were combined using Adobe Photoshop CS6.