Insect Biochemistry and Molecular Biology 42 (2012) 776–789

Contents lists available at SciVerse ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Grasshopper Lazarillo, a GPI-anchored Lipocalin, increases *Drosophila* longevity and stress resistance, and functionally replaces its secreted homolog NLaz

Mario Ruiz [a], Claude Wicker-Thomas [b], Diego Sanchez [a,1], Maria D. Ganfornina [a,*,1]

[a] Instituto de Biología y Genética Molecular, Departamento de Bioquímica y Biología Molecular y Fisiología, Universidad de Valladolid-CSIC, c/Sanz y Forés 3, 47003 Valladolid, Spain
[b] CNRS, UPR9034, LEGS and University Paris Sud, 91198 Gif sur Yvette Cedex, France

ARTICLE INFO

Article history:
Received 29 May 2012
Received in revised form
11 July 2012
Accepted 17 July 2012

Keywords:
Lipocalin
Oxidative stress
NLaz
Aging
Lipid storage

ABSTRACT

Lazarillo (Laz) is a glycosyl-phosphatidylinositol (GPI)-linked glycoprotein first characterized in the developing nervous system of the grasshopper *Schistocerca americana*. It belongs to the Lipocalins, a functionally diverse family of mostly secreted proteins. In this work we test whether the protective capacity known for Laz homologs in flies and vertebrates (NLaz, GLaz and ApoD) is evolutionarily conserved in grasshopper Laz, and can be exerted from the plasma membrane in a cell-autonomous manner. First we demonstrate that extracellular forms of Laz have autocrine and paracrine protecting effects for oxidative stress-challenged *Drosophila* S2 cells. Then we assay the effects of overexpressing GPI-linked Laz in adult *Drosophila* and whether it rescues both known and novel phenotypes of NLaz null mutants. Local effects of GPI-linked Laz inside and outside the nervous system promote survival upon different stress forms, and extend lifespan and healthspan of the flies in a cell-type dependent manner. Outside the nervous system, expression in fat body cells but not in hemocytes results in protection. Within the nervous system, glial cell expression is more effective than neuronal expression. Laz actions are sexually dimorphic in some expression domains. Fat storage promotion and not modifications in hydrocarbon profiles or quantities explain the starvation–desiccation resistance caused by Laz over-expression. This effect is exerted when Laz is expressed ubiquitously or in dopaminergic cells, but not in hemocytes. Grasshopper Laz functionally restores the loss of NLaz, rescuing stress-sensitivity as well as premature accumulation of aging-related damage, monitored by advanced glycation end products (AGEs). However Laz does not rescue NLaz courtship behavioral defects. Finally, the presence of two new Lipocalins with predicted GPI-anchors in mosquitoes shows that the functional advantages of GPI-linkage have been commonly exploited by Lipocalins in the arthropodan lineage.

1. Introduction

Lazarillo (Laz) is a glycoprotein linked to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) tail, and first characterized in the developing nervous system of the grasshopper *Schistocerca americana* (Ganfornina et al., 1995; Sanchez et al., 1995, 2000a). Laz is expressed in a specific subset of neuroblasts, neurons and ganglion mother cells of the central nervous system (CNS). In the peripheral nervous system (PNS) Laz is detected in all sensory neurons and in a group of enteric nervous system neurons (Ganfornina et al., 1996; Sanchez et al., 1995). Its expression is neither limited to the embryonic stage, since it continues throughout adulthood, nor to the nervous system. Outside the nervous system Laz is associated mainly with the excretory system: malpighian tubules and subesophageal body. During nervous system development, Laz is involved in the growth and guidance of developing pioneer axons. Grasshopper embryos treated with a monoclonal antibody against Laz show axonal aberrant growth and misdirection (Sanchez et al., 1995). Lazarillo has been used as a marker of subsets of pioneer neurons also in other orthopterans, as in the economically important desert locust *Schistocerca gregaria* (Boyan et al., 2002, 2004; Boyan and Williams, 2004; Graf et al., 2000). However a test for grasshopper Laz functions during adulthood has not been performed yet.

Lazarillo belongs to the Lipocalin protein family, an ancient and functionally diverse family of secreted proteins that share a similar structural fold, often in the presence of low sequence similarity

* Corresponding author. Tel.: +34 983 184814; fax: +34 983 184800.
E-mail address: opabinia@ibgm.uva.es (M.D. Ganfornina).
[1] MDG and DS contributed equally to this work.

0965-1748/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibmb.2012.07.005

(Ganfornina et al., 2000). Their structure is defined by an eight-stranded antiparallel β-barrel that forms a binding pocket, usually hydrophobic (Flower, 1996; Flower et al., 2000; Ganfornina et al., 2006b). Molecular phylogenetic studies have shown that arthropodan Lipocalins have diversified in five main groups (Ganfornina et al., 2006a): a Laz-related clade of insect Lipocalins, a clade of Lipocalins found in Crustacea, a clade of biliproteins and related insect Lipocalins, and two large family expansions occurring in blood-feeding insects and arachnids. Grasshopper Laz has a basal position in the arthropodan Lipocalins phylogenetic tree, and the Laz-related clade is the closest relative to the chordate Lipocalin Apolipoprotein D (ApoD) (Ganfornina et al., 2000; Sanchez et al., 2003).

Lipocalins play a great number of different roles: bacteriostatic effect, retinoids transport, prostaglandins synthesis, control of reproductive behavior, and arthropod coloration among others (reviewed by Akerström et al. (2006)). The function of a given Lipocalin must be intimately related both with its hydrophobic ligands and with its site of expression. The nature of Laz physiological ligand(s) is still unknown, but a set of candidates has been studied *in vitro*. Lazarillo is able to bind retinoic acid and fatty acids (Sanchez et al., 2008).

The domain of expression of all insect Lipocalins analyzed so far commonly includes, although is not restricted to, ectodermal derivatives, with nervous system and epidermal tissues been recurrent sites of expression (reviewed by Ganfornina et al. (2006a)). Lepidopteran Lipocalins like Bilin binding protein from *Pieris brasssicae* and *Samia cynthia* or Insecticyanin from *Manduca sexta* are expressed in epidermis, and some of them have been demonstrated to play roles in cuticle coloration (Riddiford et al., 1990; Saito, 1998; Sehringer and Kayser, 2006) and prevention of oxidative damage (Schmidt and Skerra, 1994). Brain Lipocalins have been identified both in Diptera and Lepidoptera. The lepidopteran protein Hyphantrin from *Hyphantria cunea* is up-regulated in the brain in response to injury (Kim et al., 2005). Also, Bombyrin and Gallerin were isolated from pupal brain extracts of *Bombyx mori* and *Galleria mellonella* (Filippov et al., 1995; Sakai et al., 2001), but their function remains unknown.

In *Drosophila melanogaster*, two Lipocalins expressed in the nervous system are the Lazarillo homologs Neural-Lazarillo (NLaz) and Glial-Lazarillo (GLaz) (Ganfornina et al., 2000; Sanchez et al., 2003, 2000b). NLaz and GLaz have a key role in the regulation of longevity and defense against oxidative stress (Hull-Thompson et al., 2009; Ruiz et al., 2011; Sanchez et al., 2006b), and over-expressing NLaz or GLaz increases lifespan and survival against different forms of oxidative stress (Hull-Thompson et al., 2009; Walker et al., 2006).

The function of ApoD, the closest chordate Laz homolog, has also been studied extensively in diverse model organisms as well as in humans. Its expression in the brain is the most consistently up-regulated upon aging in different mammalian species (de Magalhaes et al., 2009; Loerch et al., 2008), and it is also up-regulated in many human neurodegenerative diseases (reviewed by Van Dijk et al. (2006)). Moreover, ApoD have demonstrated protective effects in the mouse brain upon oxidative stress (Bajo-Graneras et al., 2011a, 2011b; Ganfornina et al., 2008). That this protective function of ApoD is a conserved trait is supported by the results with transgenic flies expressing human ApoD. Like NLaz and GLaz, over-expressing human ApoD increases fly longevity and resistance against different stresses (Muffat et al., 2008).

So far the Lazarillo GPI-anchor is a unique feature within metazoan Lipocalins, which are mainly secreted proteins. Lipocalins linked to membranes through other mechanisms have been reported in bacteria and plants (Bishop et al., 2006; Charron and Sarhan, 2006), and indirect interactions of Lipocalins with membranes have been described for α1-acid-glicoprotein (Nishi et al., 2002), β-Lactoglobulin (Martins et al., 2008) and Tear Lipocalin (Saaren-Seppala et al., 2005). These data point to the existence of membrane receptors for Lipocalins, some of which have been already characterized, mostly for vertebrate Lipocalins (Hvidberg et al., 2005; Kawaguchi et al., 2007; Wojnar et al., 2001), but also for Insecticyanin in *M. sexta* (Kang et al., 1997).

In this work we test the protective potential of grasshopper Lazarillo in a cell culture system using the MT promoter system, and in adult fruit flies expressing grasshopper Laz with the UAS/GAL4 system. We test whether the GPI-linked Lazarillo, locally acting within and outside the nervous system, is able to promote survival upon different stress forms and extend the lifespan and healthspan of flies. Fat storage and hydrocarbon profiles are also explored as variables potentially controlled by the Lazarillo-related Lipocalins and contributing to their mechanism of regulating stress resistance and longevity. We also test whether the membrane-anchored Lazarillo is able to functionally restore the loss of NLaz by exploring stress resistance, biomarkers of aging-related damage, and behavioral phenotypes. Our purpose is to assay whether the protective capacity of NLaz, GLaz and ApoD is evolutionarily conserved in the grasshopper Lazarillo protein, and if this role can be exerted linked to the plasma membrane by a GPI-tail in a cell-autonomous manner. Finally, we found two new Lipocalins with predicted GPI-anchors in mosquitoes that point to the functional advantages of GPI-linkage as being commonly exploited by Lipocalins in the arthropodan lineage.

2. Material and methods

2.1. Cell culture

Drosophila S2 cells were maintained as semi-adherent cultures at 27 °C in Express-Five medium (Gibco) supplemented with 10% L-Glutamine, 50 U/ml Penicillin, and 50 μg/ml Streptomycin. The culture medium was replaced twice a week.

2.2. Cloning and purification of the Lazarillo protein

A fragment of the Lazarillo cDNA, translating into residues 1–192 of the precursor (Uniprot reference P49291), and thus missing the GPI signal peptide, was subcloned using the EcoRI and NotI sites into the pRmHa3 vector (Bunch et al., 1988; Sanchez et al., 2008). This system expresses the cloned sequence under the control of the inducible Drosophila metallothionein promoter, and the presence of a C-terminal His-tag sequence allows for protein purification from the culture medium.

The Laz-pRmHa3 plasmid was co-transfected with the selection vector pCoBlast (conferring blasticidin resistance) into Drosophila S2 cells using FuGENE6 (Roche) at 3:1 ratio according to the manufacturer instructions. Transfected cells were selected with 25 μg/ml blasticidin-S for 3 weeks (Invitrogen).

The recombinant protein was expressed in spinner flasks with blasticidin free medium, upon induction with 1 mM $CuSO_4$ for 5–7 days. The secreted Lazarillo protein was purified from the cell medium by immobilized metal affinity chromatography using nickel - nitrilotriacetic acid (Ni-NTA) resin (5-Prime). The Laz protein was further purified by size exclusion chromatography (Superdex prep grade 75, Amersham Biosciences) in PNEA buffer (25 mM PIPES, 150 Mm NaCl, 1 mM EDTA, 0.02% NaN_3). The purified protein was deglycosylated by treatment with peptide-N-glycosidase F (PNGase-F) from *Flavobacterium meningosepticum* (New England Biolabs) after denaturation following the protocol supplied by the manufacturer.