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Embryonic expression of *Drosophila* ceramide synthase *schlank* in developing gut, CNS and PNS

André Voelzmann, Reinhard Bauer *

LIMES-Institute, Program Unit Development & Genetics, Laboratory for Molecular Developmental Biology, University of Bonn, Carl-Troll-Str. 31, D-53115 Bonn, Germany

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ABSTRACT

Schlank is a member of the highly conserved ceramide synthase family and controls growth and body fat in *Drosophila*. Ceramide synthases are key enzymes in the sphingolipid *de novo* synthesis pathway. Ceramide synthase proteins and the (dihydro)ceramide produced are involved in a variety of biological processes among them apoptosis and neurodegeneration. The full extent of their involvement in these processes will require a precise analysis of the distribution and expression pattern of ceramide synthases. Paralogs of the ceramide synthase family have been found in all eukaryotes studied, however the mRNA and protein expression patterns have not yet been analysed systematically. In this study, we use antibodies that specifically recognize Schlank, a *schlank* mRNA probe and an endogenous *schlank* promoter driven LacZ reporter line to reveal the expression pattern of *Schlank* throughout embryogenesis. We found that *Schlank* is expressed in all embryonic epithelia during embryogenesis including the developing epidermis and the gastrointestinal tract. In addition, *Schlank* is upregulated in the developing central (CNS) and peripheral nervous system (PNS). Co-staining experiments with neuronal and glial markers revealed specific expression of Schlank in glial and neuronal cells of the CNS and PNS.

1. Results and discussion

The ceramide synthase (CerS) protein family comprises a group of highly conserved transmembrane proteins, which are found in all eukaryotes studied so far (Levy and Futerman, 2010). CerS proteins share common structural features. All of them contain a conserved Lag1 motif that is required for dihydroceramide synthesis (Spassieva et al., 2006; Kageyama-Yahara and Riezman, 2006). In addition, in higher eukaryotes, most paralogs in a given species include a homeodomain-like amino acid stretch. Its function is not yet understood. The main feature of all CerS identified so far is that they mediate the acylation of sphinganine yielding dihydroceramide, which is largely reduced to ceramide, the key intermediate of sphingolipid metabolism (Merrill, 2002). Ceramide signaling is important for various cellular processes, e.g. growth, differentiation, stress resistance, apoptosis, cell senescence, neurodegeneration, insulin function, and lipid homeostasis (Cutler and Mattson, 2001; Jazwinski and Conzelmann, 2002; Obeid and Hannun, 2003; Stratford et al., 2004; Bauer et al., 2009).

Most organisms, except for some species like various insects, e.g. bees and flies (Voelzmann and Bauer, 2010), contain more than one CerS gene. In mammals six CerS family members (CerS1–CerS6) were identified. Although they carry out the same chemical reaction each CerS protein shows a preference for a defined subset of fatty acyl-CoAs used for *N*-acylation (Pewzner-Jung et al., 2006). Analysis of CerS mRNA expression shows complex and specific expression patterns for all six CerS genes.

All CerS but CerS 3 are expressed in the central nervous system (CNS), although at lower levels than CerS1 (Levy and Futerman, 2010 for review). Whereas CerS1 expression is mainly confined to tissues of the nervous system (Laviad et al., 2008; Becker et al., 2008) and CerS3 expression to the testis (Mizutani et al., 2006), CerS2, CerS4, CerS5, and CerS6 display a much wider tissue distribution (Laviad et al., 2008; Mizutani et al., 2005, 2006). Consistently, deficiency of CerS1, causes cerebellar Purkinje cell neurodegeneration in mice (Zhao et al., 2009). Disruption of the CerS2 caused myelin degeneration, which is in agreement with the restricted expression of this gene within the brain to oligodendrocytes as shown by *lacZ* reporter gene expression (Imgrund et al., 2009).

Nonetheless, the lack of specific antibodies allowed CerS tissue distribution studies only by transcript expression analysis on homogenized organs or by *in situ* hybridizations in organ sections. The precise distribution and the expression pattern of all the CerS is not yet mapped in detail.

* Corresponding author. Tel.: +49 228 73 627 44; fax: +49 228 73 627 37.
E-mail address: r.bauer@uni-bonn.de (R. Bauer).

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In *Drosophila melanogaster* only one gene encoding a Lag1-motif containing CerS protein (*schlank*) could be identified to date. Schlank was shown to be a *bona fide* ceramide synthase and contains a Lag1-motif (Bauer et al., 2009) as well as a homeodomain (Voelzmann and Bauer, 2010). In contrast to mammalian CerS, Schlank appears to be involved in the synthesis of a rather broad spectrum of ceramides. Mutant analysis showed that *Drosophila* Schlank is not only involved in ceramide synthesis. Rather, it is the first *in vivo* model providing strong evidence for a close link between body fat regulation and CerS proteins (Bauer et al., 2009; Bauer, 2010; Kraut, 2011). This is in agreement with the strong expression of Schlank protein in the larval fat body where it is functionally required (Bauer et al., 2009).

In the present study we describe the temporal and spatial expression of *schlank* during *Drosophila* embryogenesis.

1.1. Specificity of the Schlank antibodies and the mRNA probe

To examine the temporal and spatial *schlank* expression we have performed P-element *lacZ* reporter gene expression analysis, whole mount *in situ* hybridizations, and antibody stainings. P element enhancer-trap lines, which reflect the expression of neighboring genes, are a widely applied tool to study the expression of genes close to the insertion site. Thus, activity in specific tissues and developmental periods can easily be observed. For reporter gene expression analysis we used the *schlank*^{G0349} P{lacW} fly line (Fig. 1A; Bauer et al., 2009; Peter et al., 2002). The P-element inserted in the 5′ UTR of the first exon of the *schlank* gene locus (Bauer et al., 2009) contains a *lacZ* reporter gene under control of the *schlank* promoter. *In situ* hybridization experiments were done with a specific *schlank* antisense mRNA probe (Fig. 1A and I–K). Specificity of the *schlank* probe was demonstrated by *in situ* hybridization analysis in *schlank*^{G0349} mutant embryos using riboprobes for *schlank* and *orthopedia*, a gene that is expressed in the hindgut, the anal pads, and the CNS (Simeone et al., 1994) and which serves as an internal staining control (Fig. 1I).

In order to study the protein expression pattern, we used two polyclonal anti-Schlank guinea pig antibodies, Schlank CT1 (sCT1) and Schlank CT2 (sCT2). Anti-Schlank CT1 was raised against a peptide containing the very C-terminal amino acids 385–400 (Fig. 1B; Bauer et al., 2009) bound to Keyhole limpet hemocyanin (KLH). Anti-Schlank CT2 was generated by immunisation of guinea pigs with a GST fusion protein containing Schlank amino acids 326–400 (Fig. 1B). Specificity of sCT1 was already shown in a prior publication (Bauer et al., 2009) and specificity of the sCT2 antibody is demonstrated by gain and loss of function experiments: In transgenic flies expressing a C-terminally HA-tagged full length Schlank protein (UAS*schlankHA*) via an *engrailed* (*en*) driver line (*en*GAL4), sCT2 and HA signals are both seen in an overlapping Engrailed pattern (Fig. 1C–E). Stainings of sCT1 (Fig. 1F) show the same pattern as stainings of sCT2 (Fig. 1G). Furthermore, the sCT2 staining is strongly reduced in hemizygous *schlank*^{G0349} mutant embryos compared to the staining of heterozygous balancer siblings (Fig. 1G and H). Together, our data show that our antibodies specifically recognize Schlank protein in *Drosophila*.

1.2. Schlank expression in Drosophila embryos

Recently, we described that *schlank* mRNA is found already from earliest stages of embryogenesis onwards. We could detect ubiquitous expression of *schlank* both on mRNA and protein expression level (Bauer et al., 2009, supplement), which is in agreement with the fact that *schlank* has a maternal supply.

Here we demonstrate that the basic ubiquitous expression pattern is maintained throughout embryonic development. However, a strong upregulation of *schlank* in specific cells and organs can be demonstrated by LacZ- as well as *in situ* staining for mRNA and by antibody staining for protein levels (Fig. 2).

Schlank is strongly expressed in a segmentally reiterated pattern in the epidermis (Fig. 2A–A″ and B–B″), in the ventral nerve cord (Fig. 2B–B″ and C–C″), and in the developing brain (Fig. 2D–D″ and E–E″). It is also found in the ectodermal fore- and hindgut, the endodermal anterior and posterior midgut, and in the posterior spiracles (Fig. 2C–C″, D–D″, and E–E″). Additionally, Schlank is seen in the sensory organs (Fig. 2E–E″).

In summary, comparison of P element reporter gene expression, *schlank* mRNA as well as antibody stainings show a very high degree of consistency. This underlines the specificity of the antibodies used for Schlank protein stainings. Schlank shows a highly distinct expression in the developing gastrointestinal tract and the developing nervous system.

1.2.1. Expression in the gastrointestinal tract

The morphological development of the gastrointestinal tract and its underlying signaling events are very similar in insects and vertebrates (Fuss and Hoch, 2002; Hoch and Pankratz, 1996; Stainier, 2005). Initially, a primitive tube is formed and subdivided along its anterior-posterior axis into fore-, mid-, and hindgut. Further subdivision then gives rise to organ appendages such as lungs, liver and pancreas in mammals and malpighian tubes and proventriculus in *Drosophila* (Baumann, 1993; Stainier, 2005; Strasburger, 1932).

To specify the region where *schlank* expression is upregulated during gut development, we used the markers Crumbs (Crb) to visualize ectodermal epithelial cells of the foregut (Fig. 3A and B, yellow arrow) and Myosin heavy chain (MHC, Fig. 3C yellow arrows), which stains visceral mesoderm surrounding the cells of the developing fore- and midgut (Pankratz and Hoch, 1995). Schlank is strongly expressed at the ectodermal foregut/endodermal midgut boundary (Fig. 3A and B, white arrows) at the site of a ball-like evagination in the mesoderm-free (MHC-free) region (Fig. 3C). The ball-like evagination initiates the first step of proventriculus formation, which later functions as a valve to regulate food passage from the foregut into the midgut in *Drosophila* larvae (Strasburger, 1932).

The hindgut is subdivided into three regions: small intestine, large intestine and rectum (Hoch and Pankratz, 1996; Iwaki et al., 2001; Takashima and Murakami, 2001). Schlank is strongly expressed in the large intestine in a single cell layer (Fig. 3D–F) and especially at the border between large (li) intestine and rectum (rec; hindgut apical surface marked by Crb, cells visualized via FAS-II; Fig. 3D–F) as well as the border between large and small intestine (Fig. 3G). In contrast, rectum and small intestine show only basal expression (Fig. 3D and G).

The hindgut subdivision is achieved by a number of signaling processes involving Wingless (Wg), Hedgehog (Hh), Engrailed (En) and Decapentaplegic (Dpp) (Takashima and Murakami, 2001).

Ceramides or its metabolites such as Sphingosine-1-phosphate and Ceramide-1-phosphate have been implicated in signaling processes (Adachi-Yamada et al., 1999; Hannun and Obeid, 2008). It is thus quite attractive to speculate on a function of sphingolipid signaling and its potential mediation by Schlank during regionalization processes at fore- and hindgut.

1.2.2. Expression in the embryonic central nervous system

The nervous system is known to be enriched with a variety of lipids, especially glycolipids and sphingolipids (Matsuda et al., 2007). Alterations of sphingolipids have been reported in many neurodegenerative disorders, but how these changes contribute to disease pathogenesis is vague (Adibhatla and Hatcher, 2008; Ben-David and Futerman, 2010).