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Drosophila Chitinase 2 is expressed in chitin producing organs for cuticle formation

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ABSTRACT

The architecture of the outer body wall cuticle is fundamental to protect arthropods against invading pathogens and numerous other harmful stresses. Such robust cuticles are formed by parallel running chitin microfibrils. Molting and also local wounding leads to dynamic assembly and disassembly of the chitin-matrix throughout development. However, the underlying molecular mechanisms that organize proper chitin-matrix formation are poorly known. Recently we identified a key region for cuticle thickening at the apical cell surface, the cuticle assembly zone, where Obstructor-A (Obst-A) coordinates the formation of the chitin-matrix. Obst-A binds chitin and the deacetylase Serpentine (Serp) in a core complex, which is required for chitin-matrix maturation and preservation. Here we present evidence that Chitinase 2 (Cht2) could be essential for this molecular machinery. We show that Cht2 is expressed in the chitin-matrix of epidermis, trachea, and the digestive system. There, Cht2 is enriched at the apical cell surface and the dense chitin-matrix. We further show that in *Cht2* knockdown larvae the assembly zone is rudimentary, preventing normal cuticle formation and pore canal organization. As sequence similarities of Cht2 and the core complex proteins indicate evolutionarily conserved molecular mechanisms, our findings suggest that Cht2 is involved in chitin formation also in other insects.

1. Introduction

The epidermal and tracheal cuticles are the outermost physical barriers that protect insects against a harmful environment and are required to maintain homeostasis of body fluids. However, numerous events, such as metamorphosis, tissue remodeling and wounding lead to dynamic changes in the cuticle structure (Galko and Krasnow, 2004; Moussian and Uv, 2005; Matsuda et al., 2007; Jaspers et al., 2014; Pesch et al., 2016). Any impairment of cuticle assembly and degradation processes is fatal to the protection system resulting in insects' death (Petkau et al., 2012; Pesch et al., 2015).

The insect cuticle comprises three main layers. The outermost epicuticle and the envelope may both play roles to provide a water barrier (Gibbs, 1998; Shaik et al., 2011; Curtis et al., 2013), protecting insects against swelling and dehydration (Jaspers et al., 2014). The largest and most prominent part is formed by the lamellate procuticle (protein-chitin-matrix) that dramatically thickens during late larval development. In *Drosophila* final larval stage the procuticle increases from approximately six to more than fifty lamellae, with almost one lamella forming per hour (Kaznowski et al., 1985; Pesch et al., 2015). Underlying molecular mechanisms that regulate chitin-matrix organization at the assembly zone and thickening of the procuticle, finally control cuticle properties, such as stability and resistance against mechanical stresses and invading pathogens (Petkau et al., 2012; Pesch et al., 2015).

New cuticle material is deposited at the apical cell surface (Merzendorfer, 2006; Moussian et al., 2015), where the chitin-matrix becomes organized into a compact and lamellar structure (Moussian, 2013). Newly synthesized chitin-polymers, consisting of β-1,4-linked N-acetylglucosamines variable in length and diameter, undergo self-assembly into chitin-nanofibers that may serve as founding structure for chitin-matrices (Merzendorfer, 2006; Chandran et al., 2016). Local cuticular transpiration may affect the fine-tuning of cuticle properties (Klocke and Schmitz, 2011). However, resembling morphologies of chitin-matrices and the

Abbreviations: asp, anterior spiracles; Cht, Chitinase; ep, epidermis; fg, foregut; hi, hindgut; Idgf, Imaginal disc growth factor; Knk, Knickkopf; mg, midgut; Obst-A, Obstructor-A; psp, posterior spiracles; pv, proventriculus; Serp, Serpentine; sg, salivary glands; ts, tracheal system; vm, ventral midline.

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correlating mechanical and physical properties (Vincent and Wegst, 2004; Chandran et al., 2016) indicate the requirement of conserved genetic mechanisms among arthropod species that control extracellular matrix formation.

Nearly thirty years ago it has been discussed that the assembly zone (synonyms are deposition zone/adhesion zone) is a morphological stable and permeable matrix at the cell surface, through which all chitin precursors pass (Wolfgang et al., 1987). Recently, it was shown in *Drosophila* larvae that assembly and formation of cuticular layers occurs in the assembly zone at the apical cell surface (Pesch et al., 2015). There chitin is bound by a core complex of proteins that coordinate chitin-matrix assembly, packaging and finally cuticle stability (Petkau et al., 2012). The *obstructor-A* (*obst-A*) gene, a member of the *obstructor* multigene family, encodes a small protein with three Chitin-Binding-Domains type 2 (CBD2) (Behr and Hoch, 2005; Petkau et al., 2012) and belongs to the conserved cuticular proteins analogous to peritrophins (CPAP) (Jasrapuria et al., 2010, 2012; Willis, 2010; Dittmer et al., 2015; Tetreau et al., 2015). The *obst-A* gene is expressed in cells of cuticle forming organs (Behr and Hoch, 2005) and its gene product is secreted towards the extracellular matrix. There it binds chitin to form a scaffold that recruits and localizes the deacetylase Serpentine (Serp) (Luschnig et al., 2006; Wang et al., 2006) in the matrix, which matures and improves properties of the forming matrix in the tracheal lumina (Petkau et al., 2012) as well as in the epidermis (Pesch et al., 2015). In addition, the GPI-linked Knickkopf (Knk) protein is required at the Obst-A scaffold (Petkau et al., 2012; Pesch et al., 2015), where Knk organizes cuticle differentiation (Moussian et al., 2006) and probably protects the newly synthesized cuticles against chitinolytic attack (Chaudhari et al., 2011). Finally, loss of Obst-A function causes severe cuticle defects in larvae, such as absence of the assembly zone and a deformed procuticle structure leading to an impaired cuticle barrier, which is fragile against environmental stresses (Petkau et al., 2012; Pesch et al., 2015).

Altogether, the new cuticular material contains chitin nanofibrils in an amorphous matrix of proteins (Zhu et al., 2016), which require the Obst-A mediated core complex for chitin-matrix formation. Here we discuss a novel and unexpected molecular mechanism of chitin-matrix formation that involves the Chitinase 2 (Cht2) protein. Chitinases belong to the glycosylhydrolases which are widely spread in all kingdoms, including bacteria, plants and animals (Adrangi and Faramarzi, 2013). In insects the class II glycosylhydrolase (Glyco 18) domain is characteristic for the large gene family consisting of *Chitinases (Cht)* and *Chitinase-like imaginal disc growth factors (idgf)*. The insect Chts may act like endochitinases which digest the β-1,4-linkages of polymeric chitin. Their main function is probably the turnover of chitin-containing extracellular matrices in the cuticle and peritrophic matrix during molting of insects (Arakane and Muthukrishnan, 2010). The Glyco 18 domain sequence is well-conserved among insects and its chitinolytic activity has been chemically characterized for a number of Chitinases in *Drosophila* and *Tribolium* (Zhu et al., 2008a, 2008b; Arakane and Muthukrishnan, 2010). Thus, it is believed that Chts may be key enzymes for chitin degradation but, since there are many members in insects with diverse roles during development, their precise function is not understood.

Ten *Cht* and six *idgf* genes have been identified in the *Drosophila* genome (Zhu et al., 2008a; Zhang et al., 2011a; Pesch et al., 2016). A first systematic RNAi-based knockdown screen of individual *Drosophila Cht* and *idgf* genes showed high mortality rates and cuticle molting defects in larvae and pupae upon *Cht2* RNA reduction. In addition, during larval intermolt stages the *Cht2* knockdown led to a destabilized and fragile epidermal cuticle that failed to resist mechanical stresses and did not protect larvae from invading pathogens (Pesch et al., 2016). In this study we systematically investigated the Cht2 protein expression and localization pattern. Cht2 is expressed in ectodermal cuticle forming organs, such as epidermis, trachea and the digestive system, already from late embryonic stages onwards. At the subcellular level we identified the Cht2 protein to be enriched in the assembly zone of the body wall cuticle and scattering along the lamellate chitin-matrix of the procuticle. Reduction of the Cht2 protein upon RNAi-knockdown results in the absence of the characteristic assembly zone structure preventing the thickening of the lamellate procuticle and the formation of normal pore-canal like structures in *Drosophila* larvae.

2. Material and methods

2.1. Fly work

Vienna (Austria)/Bloomington (USA) stock centers (http://stockcenter.vdrc.at/control/main and http://flystocks.bio.indiana.

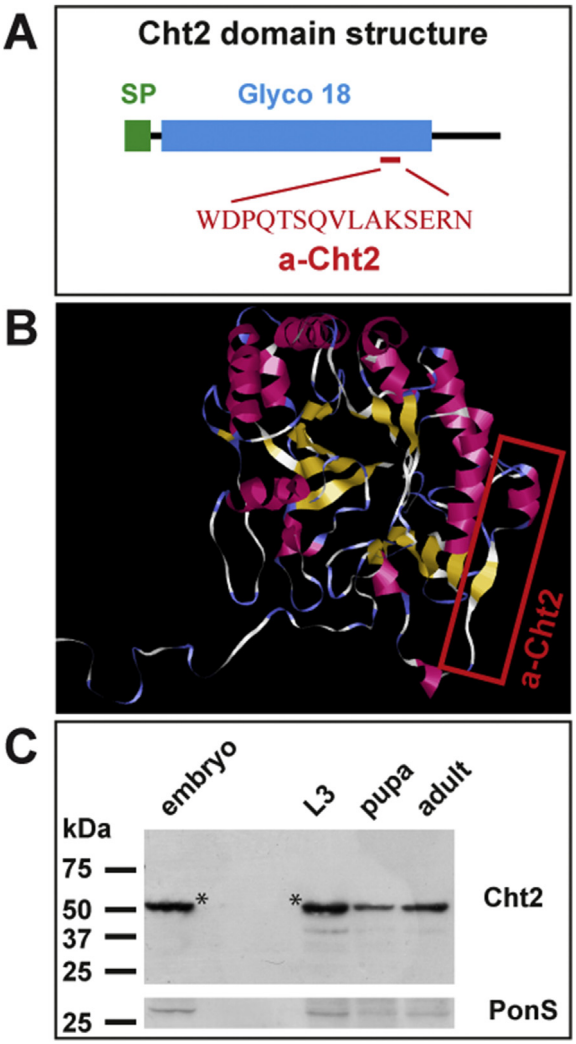

Fig. 1. Cht2 is a Glyco18 domain protein expressed throughout *Drosophila* lifetime. (A) The cartoon illustrates Cht2 domains predicted by the SMART tool (EMBL, Heidelberg). The signal peptide is indicated in green, the Glyco 18 domain (amino acid 41–389) is shown in blue. The red line marks the epitope region recognized by the Cht2 antibody (sequence is indicated, amino acids 335–349). (B) The predicted Cht2 topology is visualized with RasMol (2.7.5.2; openrasmol.org) indicating the exposed epitope peptide recognized by the Cht2 antibody. (C) Immunoblots using the Cht2 antibody show a predominant signal (asterisks) correlating well with the predicted Cht2 mass (54 kDa) of total protein extracts of embryos, third instar larvae, pupae and adults. The PonceauS (PonS) control confirms presence of protein extracts in the different lanes.

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