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Retroactive, a Membrane-Anchored Extracellular Protein Related to Vertebrate Snake Neurotoxin-Like Proteins, Is Required for Cuticle Organization in the Larva of *Drosophila melanogaster*

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Mutations in the *rtv* gene cause disarrangement of chitin fibers in the cuticle of the *Drosophila* larva, and occasionally the cuticle detaches from the epidermis. We have identified the *rtv* gene, and using the new HHpred homology detection method, we show that the Rtv protein defines a new family of disulfide-rich proteins in insects that are related to vertebrate snake neurotoxin-like proteins, including CD59 and transforming growth factor-β type II receptors. Rtv is an extracellular membrane-anchored protein exposing six aromatic residues that may mediate binding to chitin. We propose that this binding function of Rtv may assist the organization of chitin fibers at the epidermal cell surface during cuticle assembly. ***Developmental Dynamics 233:1056–1063, 2005.*** **© 2005 Wiley-Liss, Inc.**

Key words: cuticle; chitin; *Drosophila*; *retroactive*; neurotoxin-like proteins

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INTRODUCTION

The cuticle of *Drosophila* larva is a stratified extracellular matrix produced by epidermal cells (Retnakaran and Binnington, 1991). One can distinguish the outermost lipid and wax containing envelope, the middle protein-rich epicuticle, and the basal procuticle that harbors the polysaccharide chitin associated with proteins. Differentiation of the cuticle requires the temporal and spatial coordination of synthesis and secretion of the cuticular components. The stability of the cuticle arises from anti-parallel packing of the chitin fibers (α-chitin). These fibers form microfibrils aligned in horizontal layers (laminae) that are often rotated with respect to each other (Bouligand, 1965; Neville, 1975; Merzendorfer and Zimoch, 2003). Additional hardening of the cuticle involves crosslinking of proteins and chitin with catecholamines and phenols, a process called sclerotization (Wright, 1987; Sugumaran, 1998).

Although many of the components present in the cuticle have been characterized, little is known about the factors that direct cuticle assembly. Phenoloxidases, for instance, mediate sclerotization of the epicuticle by the conversion of polyphenols to quinones that crosslink proteins (Locke and Krishnan, 1971; Binnington and Barrett, 1988). Furthermore, it has been proposed that cuticular proteins in the epicuticle have the capacity to self-assemble (Locke, 2001). Self-assembly is believed to occur during microfibril formation and helicoidal arrangement of chitin laminae in the procuticle as well (Neville, 1975). However, an instructive role for fiber orientation is assigned to the so-called plaques at the apical plasma membrane that are thought to harbor enzymes required for chitin synthesis (Locke, 1976). On the molecular level, it has been proposed that β-sheet conformation in the conserved domains of cuticular

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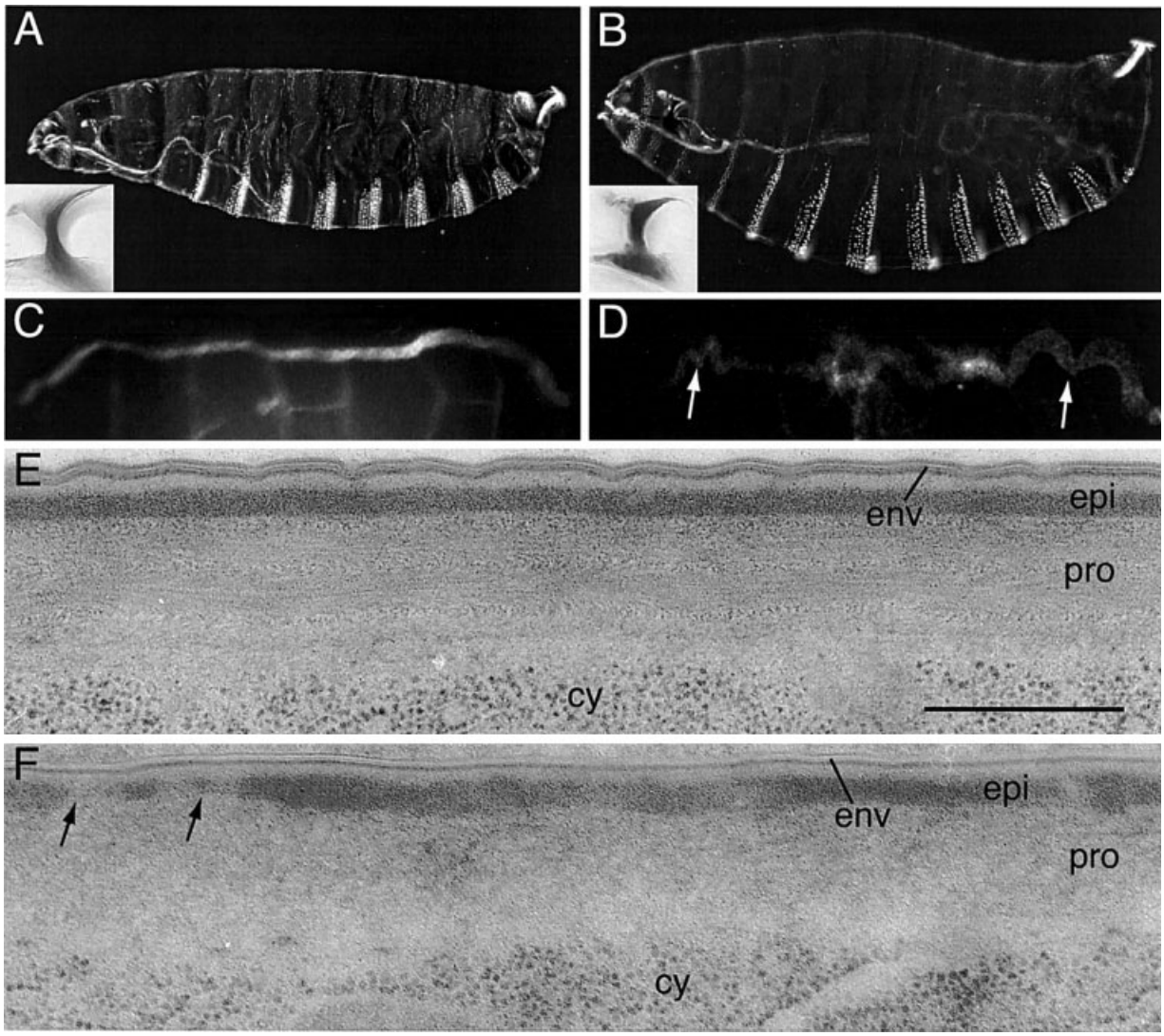

Fig. 1. Establishment of larval body shape requires *rtv* that is involved in chitin fiber organization. **A:** In cuticle preparations, the wild-type larva has a spindle like shape. **B:** By contrast, the *rtv*^{11} mutant larva appears bloated. The inserts in A and B highlight the head skeleton that, in *rtv* mutant larvae, appears to be more condensed and deformed. **C,D:** Chitin (white signal) is present in stage 17 wild-type (C) and *rtv*^{11} mutant embryos (D), as shown for the tracheae. Compared with the wild-type embryo, the tracheae of *rtv*^{11} mutant embryos are undulated (arrows). **E:** By transmission electron microscopy, the cuticle of the wild-type larva consists of three layers: the outermost envelope (env), the middle electron dense epicuticle (epi), and the inner procuticle containing laminae (pro). **F:** The layers of the larval *rtv*^{11} cuticle are present, but the epicuticle disintegrates (arrows) and the procuticle is devoid of laminae. Cy, cytoplasm. Animals in A–D are oriented anterior to the left. Scale bar = 500 nm.

proteins such as the R&R chitin binding motif and the tachycitin-like chitin binding domain may be crucial for chitin binding (Suetake et al., 2000; Rebers and Willis, 2001; Hamodrakas et al., 2002; Togawa et al., 2004) and that this association may be responsible for the characteristic organization of the laminae (Fraenkel and Rudall, 1947; Iconomidou et al., 1999). Commonly, aromatic amino acids have been demonstrated to be implicated in the interaction between chitin binding proteins and their substrate (Asensio et al., 1998; Rebers and Willis, 2001; Hardt and Laine, 2004; Katouno et al., 2004).

Using the *Drosophila* larva as a model system, we chose a forward genetic approach to uncover the mechanisms of cuticle assembly in insects. Mutations affecting cuticle differentiation in *Drosophila* have been identified in various screens (Jürgens et al., 1984; Wieschaus et al., 1984; Ostrowski et al., 2002; Luschnig et al., 2004). Here, we present the analysis of the effects of mutations in the *retroactive* (*rtv*) gene (Wieschaus et al., 1984) on cuticle development in the embryo. In addition, we have isolated the *rtv* gene, and based on data obtained with bioinformatic tools including the new HHpred homology detection method, we propose a model for Rtv function during cuticle assembly.

RESULTS AND DISCUSSION

Phenotype of *rtv* Larvae

The cuticle of wild-type larvae is attached to the epidermis and reflects the characteristic larval body shape (Fig. 1A). By contrast, the cuticle of *rtv* larvae detaches from the epidermis

and expands conferring a bloated phenotype (Fig. 1B). The head skeleton of *rtv* larvae is strongly sclerotized, and its characteristic fibrous structure is missing. In addition, their tracheae fail to form air-filled tubes and are undulated (Fig. 1C,D). In summary, the tissues affected have in common that they produce a covering cuticle. As a consequence of their defects, *rtv* larvae cannot hatch and die. Of interest, they are hyperactive and occasionally turn around in the egg case.

The phenotypic features of *rtv* larvae are shared by larvae that are homozygously mutant for two other genes, *knickkopf* (*knk*) and *krotzkopf verkehrt* (*kkv*; Jürgens et al., 1984; Wieschaus et al., 1984; Ostrowski et al., 2002), suggesting that these genes may be involved in the same process. The *knk* gene encodes an unknown protein, and the *kkv* gene codes for the Chitin Synthase-1 (CS-1/Kkv) that produces all chitin in the epidermis, including the head skeleton and the tracheae (Ostrowski et al., 2002; Merzendorfer and Zimoch, 2003). To test for the presence of chitin in *rtv* larvae in comparison to wild-type larvae, we performed a histochemical analysis to visualize chitin using Fluostain (Fig. 1C,D). Wild-type larvae produce chitin in the epidermis, the head skeleton, and the tracheae. In *rtv* mutant larvae, chitin is present, albeit its texture is not normal.

To study the organization of chitin fibers in the cuticle of *rtv* larvae, we analyzed the ultrastructure of the cuticle by electron microscopy (Fig. 1E,F). The larval wild-type cuticle consists of three layers, the outer bipartite envelope, the protein-rich epicuticle, and the lower chitinous procuticle. The cuticle from *rtv* larvae is stratified but disorganized insofar as the epicuticle is discontinuous and frayed and chitin laminae are not visible in the procuticle. By contrast, the envelope appears to be unaffected. Eventually, the aberrant cuticle detaches from the underlying epidermis. These observations suggest that Rtv may organize the chitin fibers in the procuticle that in turn supports the stability of the upper epicuticle. Alternatively, Rtv may function as a stabilizing factor within the epicuticle that, in turn, stabilizes the procuticle. In either case, an interaction between the epi- and the procuticle is crucial for cuticle integrity and requires Rtv function.

Molecular Identification of *rtv*

To understand the molecular function of Rtv, we identified the gene by genetic mapping. Based on available data on its location on the X chromosome (Wieschaus et al., 1984), the region harboring the gene was narrowed down by deficiency mapping to an interval that contains 25 predicted genes (Fig. 2A). To identify the mutations in genomic DNA from two mutant alleles, rtv^{11} and rtv^{BNd}, candidate genes from this region were sequenced. A nonsense mutation ($TTA^{Leu6} \rightarrow TAA^{stop}$, rtv^{11}) and a missense mutation ($TGT^{Cys100} \rightarrow TAT^{Tyr}$, rtv^{BNd}) were detected within CG1397 (Fig. 2B), whereas only silent mutations were found in other genes. We therefore conclude that CG1397 represents *rtv*. Note that the rtv^{11} mutation probably causes a complete loss of Rtv function.

rtv Is Expressed in the Head Skeleton, the Tracheae, and the Epidermis of the Embryo

To learn more about *rtv* biology, we monitored the expression pattern of the *rtv* transcript during embryogenesis (Fig. 3). Transcription of *rtv* starts at stage 16 within the developing head skeleton, the pharynx, and the tracheae. Later, at stage 17, it occurs additionally in the epidermal cells. Hence, *rtv* is expressed in those tissues that are affected in *rtv* mutant larvae. As expected, the course of *rtv* expression coincides with the formation of the procuticle (Hillman and Lesnik, 1970).

Rtv Is a Small Membrane-Anchored Extracellular Protein

The *rtv* gene codes for an unknown protein with 151 residues that has a predicted N-terminal signal peptide cleaved between glycine[21] and leucine[22] to target the protein to the extracellular space (Fig. 2B). Rtv is predicted to be linked to the membrane by a transmembrane helix at the C-terminus (residues 129–148). A search for a putative GPI-anchor signal that can be difficult to distinguish from transmembrane helices gave negative results with two different methods (Eisenhaber et al., 1999; Kronegg and Buloz, 1999).

A BLAST search on the NCBI Web site with the 151 amino acid sequence of Rtv yielded putative orthologues in other insects: *Anopheles gambiae* XP317897 (70% sequence identity) and *Apis mellifera* hmm3029 (50%; Fig. 4A). In Rtv and its orthologous sequences, *N*-glycosylation is predicted to occur at an asparagine (asparagine[45] in Rtv) and a tyrosine (tyrosine[80] in Rtv) presumably is *O*-sulfated. *O*-Sulfation has been demonstrated to be involved in protein–protein interaction (Moore, 2003).

Rtv Is Homologous to a Diverse Superfamily of Extracellular Domains of Cell Surface Receptors and Snake Neurotoxins

For remote homology detection, we first constructed an alignment of 27 homologues of Rtv using the HMMER package (Eddy, 1998) and by manual inspection of sequence matches. We started the HHpred server (Soding, 2004) for remote homology detection with this alignment. HHpred has been shown previously to be several times more sensitive than BLAST, PSI-BLAST, or HHMER. We searched the SCOP database (Murzin et al., 1995), which contains all domains of known structure. The top 13 matches all belong to the neurotoxin-like SCOP superfamily that harbors a family of proteins containing extracellular domains such as CD59 (Kieffer et al., 1994), the transforming growth factor-beta (TGF-β) type II receptors (TBRII) including the bone morphogenetic protein Ia receptor (BRIA; Kirsch et al., 2000), and the type II Activin receptor (ActRII; Boesen et al., 2002), as well as a family of snake neurotoxins (Segalas et al., 1995; Torres et al., 2001; Nirthanan et al., 2002). The similarity to Rtv is only detectable when comparing entire alignments of homologues with HHpred and is not evident on the sequence level (Fig. 4A). It is important to note that Rtv does not contain any known chitin binding do-

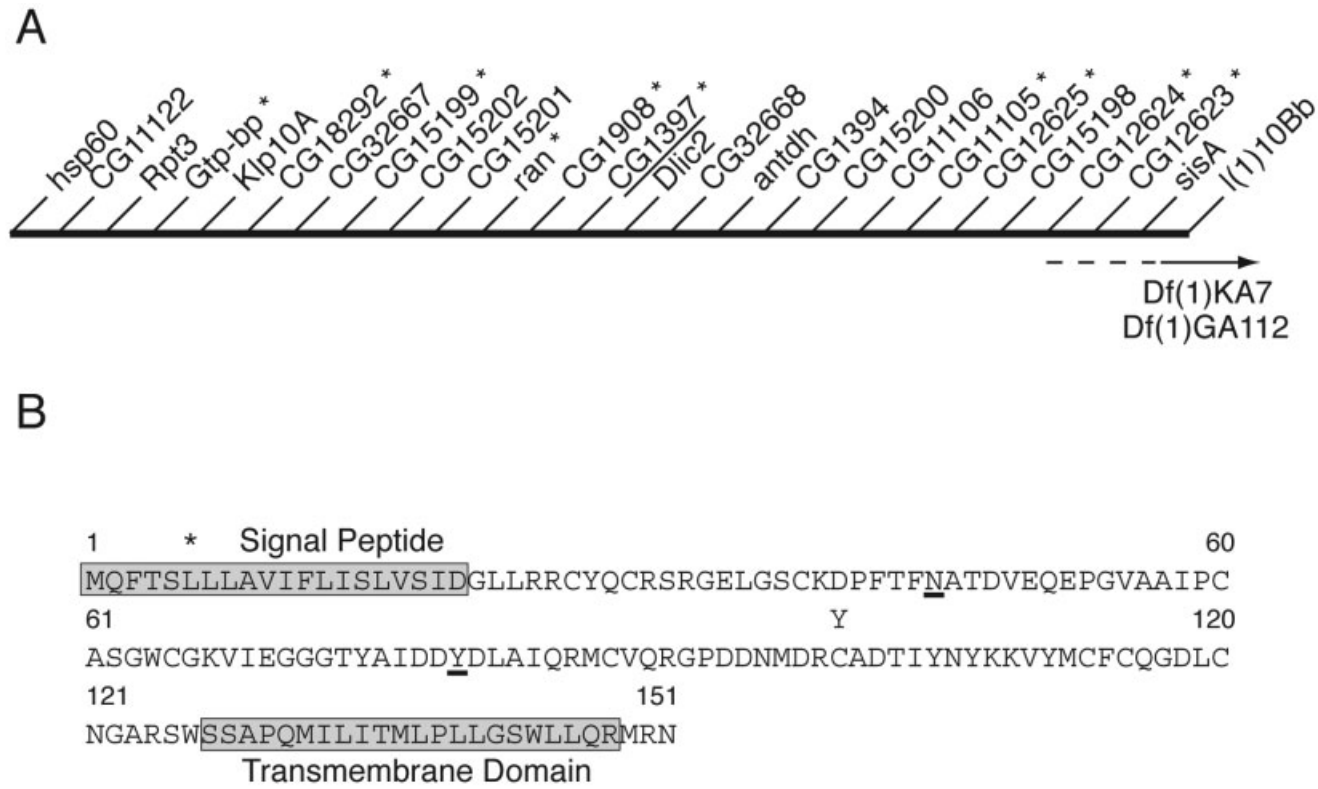

Fig. 2. Cloning of the *rtv* gene. **A:** The *rtv* locus was mapped genetically to a region harboring 25 genes. Previously, *rtv* was found to be proximal to *hsp60* (Zhimulev et al., 1981a,b). The proximal limit of the *rtv* region is represented by *l(1)10Bb* that fails to complement the deficiencies Df(1)KA7 and Df(1)GA112 (arrow), which do not uncover *rtv*. Genes from this region coding for proteins predicted to be secreted or to be involved in the secretion pathway were designated as candidate genes (asterisks). They were amplified using genomic DNA from the alleles rtv^{11} and rtv^{BNd} and sequenced. Note that the distances between the loci are not to scale. **B:** Point mutations were identified that either dramatically alter the protein sequence of CG1397 (C^{100} to Y, rtv^{BNd}) or result in a truncated protein (stop after S^5, rtv^{11}), demonstrating that CG1397 is *rtv*. The Rtv protein with 151 amino acids has a predicted molecular weight of 16842.55 Daltons and a pI of 5.33. It harbors an N-terminal signal peptide and a C-terminal transmembrane domain (gray boxes). The protein is predicted to be modified at N^{45} (*N*-glycosylation, underlined) and at Y^{80} (*O*-sulfation, underlined).

main or any other motif found in cuticle proteins (Andersen et al., 1995; Shen and Jacobs-Lorena, 1999; Suetake et al., 2000, 2002) and, therefore, defines a new class of cuticle proteins.

The structures of the domains of the neurotoxin-like SCOP superfamily are characterized by a sandwich of two β-sheets, composed of two and three strands, respectively, that are connected by three conserved disulfide bonds (Fig. 4A). Rtv and the homologous cell surface receptor domains contain additional disulfide bonds that may restrain the flexibility of the loops between successive β-strands. The loops on one side are short, but on the other side, they are longer and reach out like fingers, which is why this fold is also called three-finger neurotoxin fold. The finger loops, numbered I–III in Figure 4A, have been implicated by site-directed mutagenesis and structural studies in mediating binding of neurotoxins and cell surface receptors to their cognate partners (Bodian et al., 1997; Kirsch et al., 2000; Tsetlin and Hucho, 2004).

The importance of the conserved cysteines has been shown repeatedly in many members of this superfamily and is confirmed by the mutation rtv^{BNd} (cysteine^{100}→tyrosine) that leads to the abolishment of one of the disulfide bonds and presumably results in a partially unfolded protein. The highly conserved tyrosine[27] and the C-terminal DxCN motif (positions 118 to 121 in Rtv) occupy positions in the protein core and, therefore, may be crucial for structural stability. Among sequences with the highest score of structural similarity to Rtv, and indeed the most prominent members of this superfamily of proteins, are CD59 and the ectodomain of TGF-β type II receptors.

In mammals, the membrane-anchored CD59 is a key factor that protects host cells from lysis mediated by the complement membrane attack complex (MAC) during a host defense reaction (Miwa and Song, 2001). It assumes its role by binding to the terminal complement proteins C8 and C9, thereby inhibiting the membrane insertion of the MAC. Finger loop residues (aspartate[24], tryptophan[40], arginine[53], and glutamate[56]) have been shown in mutagenesis experiments to be important for human CD59 function without contributing to protein folding, but these residues are not conserved in Rtv (Bodian et al., 1997). To date, no CD59 homologue has been reported in *Drosophila*.

TGF-β type II receptors are inserted into the plasma membrane with a single transmembrane domain, and are composed of the N-terminal ligand binding ectodomain facing the extracellular space, and a C-terminal intracellular kinase domain (ten Dijke and Hill, 2004). Upon binding of the ligand, the receptor associates with a

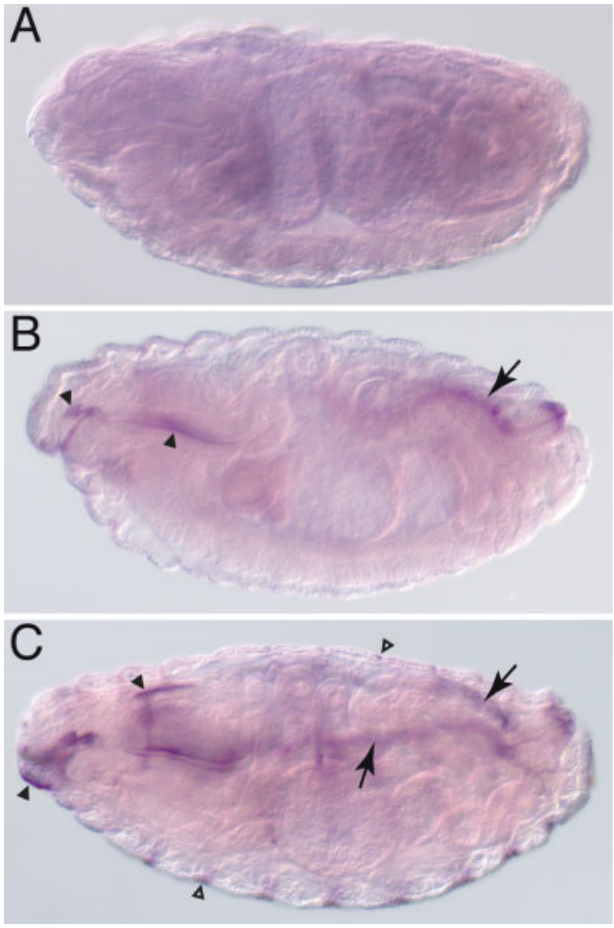

Fig. 3. Expression of *rtv* during embryogenesis. **A,B:** Expression of *rtv* is not detected at stage 15 or earlier (A), but starts at stage 16 in the developing head skeleton including the pharynx (solid arrowheads) and the tracheae (arrow, B). **C:** At stage 17, the epidermal cells also begin to express *rtv* (open arrowheads). Note that, in all panels, the tracheae are out of focal plane. Embryos are oriented anterior to the left.

type I receptor molecule, thereby activating downstream signalling through the intracellular kinase domain. In *Drosophila*, three TGF-β type II receptor homologous proteins exist, and they have been characterized genetically and biochemically. Punt (Pnt) is implicated in embryonic dorsoventral as well as wing patterning (Podos and Ferguson, 1999). Wishful thinking (Wit) plays a central role in regulation of neuromuscular synapse formation (Aberle et al., 2002; Marques et al., 2002). Finally, Baboon (Babo) regulates cell proliferation during larval development (Brummel et al., 1999). Overall, there is no evidence that these proteins may be involved in cuticle differentiation.

To the other members of the family, the three-finger neurotoxins such as muscarinic toxin (Segalas et al., 1995) and candoxin (Nirthanan et al., 2002) found in the venoms of different snakes, the structural similarity of Rtv is significant but less pronounced. These free extracellular neurotoxins bind acetylcholine receptors in nerve and muscle cells, thereby competitively inhibiting acetylcholine binding and depolarization of postsynaptic membranes. The conserved arginine in the second loop of neurotoxins (ar-

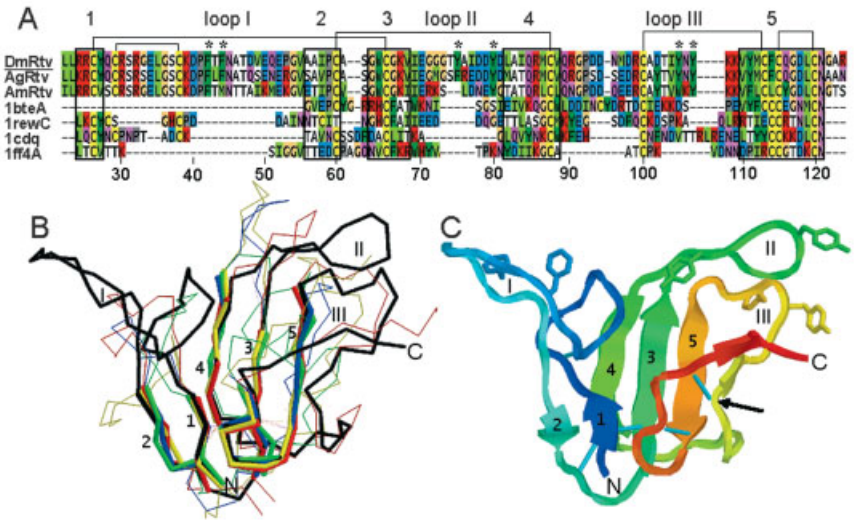

Fig. 4.

Fig. 4. Rtv adopts a three-finger neurotoxin fold with long fingers carrying exposed aromatic residues. **A:** Sequence alignment of three Rtv orthologues from *Drosophila melanogaster*, *Anopheles gambiae*, and *Apis mellifera* with four proteins adopting the three finger neurotoxin fold: Type II activin receptor (1bteA), bone morphogenetic protein Ia receptor ectodomain (1rewC), CD59 (1cdq), and the snake neurotoxin muscarinic toxin (1ff4A). **B:** Structural superposition of a model of DmRtv (black), with 1bteA (red), 1rewC (orange), 1cdq (green), and 1ff4A (blue). **C:** Structural model of DmRtv as a cartoon. There are five structurally conserved blocks, shown as boxed residues in A and as thick tubes in B, that largely overlap with the five conserved β-strands shown as cartoons in C. Rtv has three particularly long fingers (loop I–III) that point upward in B and C and that each carry two aromatic residues (marked by asterisks in A). These fingers vary grossly between homologues and cannot be modeled precisely. Disulfide bonds are shown as square brackets in A and as cyan-colored sticks in C. The rtv^{BNd} mutation (C^{100} to Y) is marked by an arrow in C. In A, residues at the ends that are not alignable to DmRtv are omitted. The DmRtv sequence starts at the likely cutting site for the signal peptide. The Rtv membrane helix and linker residues at the C-terminus are also omitted.

ginine[36] in bungarotoxin) that interacts with tryptophan residues in acetylcholine receptors (Samson et al., 2002) is not present in Rtv.

Modeling of the Structure of Rtv

We have built a multiple alignment of Rtv and four of its closest homologues with known structure and used it to model the structure of Rtv (Fig. 4A,B). Of interest, Rtv has longer finger loops than any of its homologues of known structure. As a consequence, these loops may be relatively floppy and able to move around in the solvent, especially because loops I–III have a strongly hydrophilic character with many charged or polar residues. A notable exception are six aromatic residues, two phenylalanines and four tyrosines, that are marked by asterisks in Figure 4A. The sequence of the loops is well-conserved between Rtv and its orthologues and may mediate binding to a cuticular factor common for insects. Indeed, aromatic residues have been shown by various site-directed mutagenesis studies and by nuclear magnetic resonance spectroscopy to be the key sites for binding of chitin (Asensio et al., 1998; Rebers and Willis, 2001; Hardt and Laine, 2004; Katouno et al., 2004). For instance, binding of prokaryotic chitinases to chitin depends on exposed phenylalanines, tyrosines, and tryptophans (Hardt and Laine, 2004; Katouno et al., 2004). Moreover, it has been shown that the chitin binding activity of insect cuticle proteins harboring the R&R motif derives from conserved tyrosines and phenylalanines (Rebers and Willis, 2001).

Thus, it is possible that the aromatic residues may be used by Rtv and its orthologues to tether the emerging chitin chains to the plasma membrane. Alternatively, however, Rtv may function through the interaction with other proteins, including Knk, the lack of which cause identical phenotypes. In any case, we hypothesize that Rtv may contribute to the function of plaques at the apical plasma membrane of epidermal cells that have been proposed to play a central role in chitin fiber orientation (Locke, 1976, 1991). In the future, we will test our predictions in biochemical and genetic analyses of the Rtv protein to shed light on the complex process of insect cuticle organization.

EXPERIMENTAL PROCEDURES

Genetic Analysis

The rtv^{BNd} mutation was isolated in a screen for ethyl-methyl-sulfonate (EMS) -induced zygotic mutations on the X chromosome aimed at identifying loci involved in body morphogenesis (B. M., Nina Vogt, Iris Koch, unpublished data). The rtv^{11} mutation was isolated in an EMS screen for mutations affecting the pattern of the larval cuticle (Wieschaus et al., 1984). To identify homozygous *rtv* embryos and larvae, mutant stocks were balanced with FM7c chromosome harboring UAS-GFP under the control of the *krüppel*-promoter.

Microscopy

Figure 1A,B and their inserts were obtained by Darkfield and Nomarski microscopy on a Zeiss Axiophot microscope, respectively. For histochemistry, embryos were staged and fixed using standard methods (Wieschaus and Nüsslein-Volhard, 1986). Fixed embryos were incubated overnight at 4°C with 2 μg/ml Fluostain I (Sigma), a Calcofluor White derivate that emits blue light upon association with polysaccharides when exited with ultraviolet light (Maeda and Ishida, 1967). For in situ hybridization, a Digoxigenin (Dig) -labeled antisense probe was generated using the CG1397 cDNA LD10746 obtained from the Berkeley Drosophila Genome Project (BDGP; http://www.fruitfly.org/about/materials/cDNA-list.html). The probe was detected with an AP-conjugated anti-Dig antibody, and visualized through NBT/BCIP precipitation (Tautz and Pfeifle, 1989). Following the same procedure, embryos hybridized with the control sense probe were blank (unpublished data). For transmission electron microscopy, larvae were cryoimmobilized and treated as described in (McDonald and Morphew, 1993; McDonald and Müller-Reichert, 2002).

Molecular Analysis

DNA isolation, polymerase chain reaction, and sequencing were performed using standard protocols (Ashburner, 1989; Sambrook et al., 1989; Ausubel et al., 1994).

Bioinformatic Analysis

For transmembrane helix and signal peptide prediction, the Rtv sequence was submitted to various servers (see links at http://www.hgmp.mrc.ac.uk/GenomeWeb/prot-transmembrane.htm, and in particular the Phobius [Kall et al., 2004] and SignalP servers [Bendtsen et al., 2004]). The DGPI (Kronegg and Buloz, 1999) and big-PI (Eisenhaber et al., 1999) servers were used for the prediction of GPI-anchors. The *N*-glycosylation and *O*-sulfation sites were predicted using software at the ExPASy Proteomics Server (www.expasy.org).

The HHpred server at the Max-Planck Institute for Developmental Biology (http://protevo.eb.tuebingen.mpg.de/toolkit/index.php?view=hhpred) was used to find remotely homologous proteins with known structure. We used the pairwise alignments generated by HHpred and the structural alignment of the four homologues to construct a consensus alignment for homology modeling. The Rtv structure was generated with Modeller7v7 (Sali and Blundell, 1993).

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