Group I chitin deacetylases are essential for higher order organization of chitin fibers in beetle cuticle

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This article contains Table S1, Figs. S1–S5, and Videos S1–S3.

Roles in the organization of the cuticle (exoskeleton) of two chitin deacetylases (CDAs) belonging to group I, TcCDA1 and TcCDA2, as well as two alternatively spliced forms of the latter, TcCDA2a and TcCDA2b, from the red flour beetle, Tribolium castaneum, were examined in different body parts using transmission EM and RNAi. Even though all TcCDAs are co-expressed in cuticle-forming cells from the hardened forewing (elytron) and ventral abdomen, as well as in the softer hindwing and dorsal abdomen, there are significant differences in the tissue specificity of expression of the alternatively spliced transcripts. Loss of either TcCDA1 or TcCDA2 protein by RNAi causes abnormalities in organization of chitinous horizontal laminae and vertical pore canals in all regions of the procuticle of both the hard and soft cuticles. Simultaneous RNAi for TcCDA1 and TcCDA2 produces the most serious abnormalities. RNAi of either TcCDA2a or TcCDA2b affects cuticle integrity to some extent. Following RNAi, there is accumulation of smaller disorganized fibers in both the horizontal laminae and pore canals, indicating that TcCDAs play a critical role in elongation/organization of smaller nanofibers into longer fibers, which is essential for structural integrity of both hard/thick and soft/thin cuticles. Immunolocalization of TcCDA1 and TcCDA2 proteins and effects of RNAi on their accumulation indicate that these two proteins function in concert exclusively in the assembly zone in a step involving the higher order organization of the procuticle.

Chitin deacetylases (CDAs, EC 3.5.1.41)3 belong to family 4 of carbohydrate esterases (CE-4) that can deacetylate a variety of polysaccharides including chitin and peptidoglycans (1). They are found widely in fungi, nematodes, insects, and other arthropods (2–7). In insects, genes encoding CDA-like proteins have been identified and classified into five groups (8–11). Group I CDAs (CDA1s and CDA2s) have a peritrophin-A type chitin-binding domain (CBM14) and a low-density lipoprotein receptor A-type domain in addition to the CE-4 domain. They influence development and molting by acting on extracellular matrix-associated chitin in cuticle, trachea, and pharyngeal lining (5, 12–16). In Drosophila melanogaster embryos, disruption of DmCDA1 (serpentine) and DmCDA2 (vermiform) genes resulted in an abnormal chitin core in the tracheal lumen and elongated tracheal tubes (12, 13). These two proteins along with two other cuticular proteins, Obstructor A and Knickkopf, have been proposed to interact in the assembly zone of the cuticle to provide epidermal cuticle integrity of D. melanogaster larval cuticle (17, 18). In Tribolium castaneum, RNAi of TcCDA1, TcCDA2, and the two splice isoforms of the latter, TcCDA2a and TcCDA2b, led to molting failure, abnormal limb movements, and wrinkled elytra, as well as a failure of embryonic development (16). In the brown plant hopper, Nilaparvata lugenes, RNAi of NICDA1, NICDA2, and NICDA4 caused mortality, growth reduction, and molting failure (14). Recently, Yu et al. (15) reported that RNAi of LmCDA2 caused a less compacted and larger abdominal cuticle with less well-defined chitinous laminae in the migratory locust, Locusta migratoria. The authors reported that in LmCDA2-deficient insects, the chitinous laminae were not helicoidally arranged based on the trajectory of the pore canals, which followed a straight path through the cuticle rather than the helicoidal path observed in abdominal cuticles of control insects.

In this study, we have carried out RNAi for TcCDA1 and TcCDA2 of T. castaneum, either alone or in combinations including two TcCDA2 isoforms, TcCDA2a and TcCDA2b, and analyzed the ultrastructure of the normally laminar cuticles from different cuticle-forming tissues (hard and soft), as well as the nonlamellar tracheal taenidial cuticle. We report that the TcCDAs work cooperatively in the assembly zone to determine the overall shape and structural integrity of the horizontally oriented laminar cuticle and the vertically oriented pore canals, suggesting that group I CDAs are important for the higher ordered organization of chitin bundles in T. castaneum cuticle.

Results

Loss of function phenotypes produced by RNAi for TcCDAs

We previously reported that RNAi for TcCDA1 or TcCDA2 at the larval stage led to the arrest of growth at the subsequent...
larval–larval or larval–pupal molts and mortality (16). Injection of either dsTcCDA1 or dsTcCDA2 into 0–1-day-old pupae leads to a substantial depletion of the targeted mRNA at the pharate adult stage (5-day-old pupae) with no significant change in the levels of transcripts for the other TcCDA (top two panels in Fig. 1A). In addition, administration of dsTcCDA1/2 significantly suppressed both TcCDA1 and TcCDA2 transcripts. All of those dsRNA-treated pupae failed to eclose into adults (top panels in Fig. 1A).

Injections of dsRNA corresponding to alternative exon 3 of the TcCDA2 gene, which codes for the chitin-binding domain, at the late larval stages does not lead to growth arrest and the adults eclosed at the normal time (16). However, the resulting dsTcCDA2a (with alternate exon 3a)-treated adults failed to articulate the limbs and had impaired locomotion (middle left panel in Fig. 1B and see Videos S1–S3). In contrast, RNAi of TcCDA2b (with alternate exon 3b) did not affect limb movement but resulted in rough and visible surface wrinkles in the pronotum and dorsal elytra (middle and bottom panels in Fig. 1B). The selectivity and efficiency of the alternate exon-specific dsRNAs on depletion of the targeted exon-containing transcripts is shown in Fig. 1A (bottom two panels).

Spatial expression patterns of TcCDAs

The transcript abundance of TcCDA1, TcCDA2a, and TcCDA2b in soft hindwing tissue and in the hardened elytron (forewing tissue), both of which rely on the same wing gene network for their patterning (19), was analyzed by real-time PCR. TcCDA1 and TcCDA2a were expressed at nearly similar levels in both tissues, but the proportion of the TcCDA2b transcript was significantly lower in the hindwing tissue (Fig. 2A, right panel). A similar lower level of expression of TcCDA2b was observed in the dorsal abdomen relative to the ventral abdomen (Fig. 2B, right panel). Thus, there appears to be a correlation between the high ratio of TcCDA2a/TcCDA2b
transcripts and the softness/hardness of the cuticle elaborated by these epidermal tissues.

**Ultrastructure of elytral dorsal cuticle from TcCDA1- and TcCDA2-deficient insects**

To determine the effect of depletion of TcCDA1 or TcCDA2 transcripts on cuticle integrity, transmission electron microscopic (TEM) observations of the rigid elytral dorsal cuticle isolated at the pharate adult stage (5-day-old pupae) were carried out. Elytra from dsTcVer-treated insects exhibited regularly spaced, alternating electron-dense and electron-lucent chitin/protein laminae parallel to the epidermal cell surface, as well as numerous vertically oriented pore canals (relative to the epidermal cell surface) with a long central chitin core of pore canal fibers (PCFs) (Fig. 3A). In contrast, the elytra from insects treated with dsTcCDAl or dsTcCDAb exhibited varying degrees of abnormal cuticular organization involving both the horizontal laminae and the vertical pore canals. In the elytral dorsal cuticle, the laminae were not easily discernible following RNAi for TcCDA1 (Fig. 3B) and were grossly affected after TcCDA2 RNAi, exhibiting electron-lucent pore canals that were bent and did not transverse vertically through the procuticle (Fig. 3C). The laminae were completely missing after RNAi for both TcCDA1 and TcCDA2 (Fig. 3D). In addition, the interface between the apical epidermal cell surface and the procuticle is ill-defined and not flattened as in the dsTcVer controls. The apical plasma membrane protrusions (APMPs) appeared to extend further into the disorganized procuticle. PCFs that assemble inside the pore canals were quite abnormal, exhibiting amorphous fibrous material compared with those in the control (Fig. 3D). These abnormalities were more severe in dsTcCDA2-treated elytra compared with dsTcCDA1-treated ones and were the most severe in insects treated with both of the dsRNAs.

To determine whether the severity of the structural disorder in the cuticle varies along its length, we examined the sections in proximal, middle, and distal parts of the cuticle relative to the epidermal cell surface using higher magnification TEM analyses. There were no differences in the relative disorganization along the length of the entire procuticle with all of the TcCDA dsRNA treatments (Fig. S1, A–J). In contrast, the outermost envelope layer and the epicuticle that lies immediately below it appeared to be unaffected by these treatments (Fig. S1, D, G, and J). Similar ultrastructural defects were observed after injection of dsTcCDAl, dsTcCDA2, or dsTcCDA1/2 in other body regions associated with rigid cuticles such as the ventral abdomen and leg of T. castaneum adults (Fig. S2).

**Ultrastructure of dorsal elytral cuticle after alternative exon-specific RNAi for TcCDA2**

Because injection of dsRNA for TcCDA2a or TcCDA2b did not affect adult eclosion (Fig. 1B), it was also of interest to study the integrity of the rigid dorsal elytral cuticle after these treatments. RNAi for TcCDA2a, which affects limb articulation and gait but not the elytral surface, yielded no obvious differences in morphology of either the horizontal laminae or vertical pore canals in this tissue compared with the dsTcVer-treated control (Fig. 3E and Fig. S1, M–O). In contrast, the wrinkled elytra from dsTcCDA2b-treated insects exhibited a disruption of horizontal laminae and irregularly formed pore canals that contain less compact and unorganized PCFs throughout the entire length of the procuticle (Fig. 3F and Fig. S1, P–R). These ultrastructural
defects resembled those present in TcCDA1-deficient insects (Fig. 3B and Fig. S1, D–F). Comparable ultrastructural abnormalities produced by RNAi for TcCDA2b were observed in other body regions covered with rigid cuticles such as the ventral abdomen and leg (Fig. S2).

Morphology of pore canals and PCFs after RNAi for TcCDAs

The above-described results were obtained with vertical TEM sections of the cuticle, that is, normal to the epidermal cell surface. To reveal the finer details of pore canal and PCF organization, cross-sections of the elytral dorsal cuticle were made parallel to the epidermal cell surface. In the dsTcVer-treated control cuticle, the pore canals appeared as near circular columns, representing the outermost chitin fiber columns with a thick supporting axis in the middle. In cross-sections, they had the appearance of rods radiating from the center (Fig. 4A) (20–22). The pore canals were probably filled with a dark-colored protein matrix with a circular outer wall of chitin fibers and a central vertical-supporting thicker chitin fiber bundle.

RNAi for TcCDA1 had a moderate effect on the structure of the pore canals (Fig. 4B), but there was a severe disruption of these pore canals after RNAi of TcCDA2 (Fig. 4C). The circular column of chitinous PCFs had collapsed, and there was a diffuse matrix of electron-poor material accumulating in the middle of the presumptive pore canals. We interpret these bright electron-poor areas in the middle of the disrupted canals as representing disorganized chitin nanofibrils that failed to form higher order bundles or longer fibers. The disruptive effect is most severe when both of the TcCDA1 and TcCDA2 transcripts are depleted (Fig. 4D). RNAi of TcCDA2a did not appear to alter the pore canal/PCF organization (Fig. 4E), but depletion of TcCDA2b transcripts caused a discernible loss of pore canal structural integrity (Fig. 4F), which was similar to the result obtained with dsTcCDA1-treated insects (Fig. 4B).

Ultrastructure of hindwing cuticle from TcCDA1- and TcCDA2-deficient insects

Because TcCDA1 and TcCDA2 transcripts were also detected in tissues secreting soft cuticle (e.g. hindwing and dorsal abdomen), we analyzed the ultrastructure of the cuticles secreted by these tissues by TEM after RNAi for these TcCDA genes. As shown in Fig. 5A, the hindwing from dsTcVer-treated control insects exhibited a helicoidal procuticle (inferred from the crescent-shaped pore canal progression and fiber bundles in oblique sections) with considerably fewer horizontal laminae than the elytron. RNAi of either TcCDA1 or TcCDA2 resulted in a disorganized procuticle in which the pore canals were not visible (Fig. 5, B and C). Injection of dsTcCDA1 plus dsTcCDA2 led to a near complete loss of procuticle organization in the hindwing (Fig. 5D).

The results of RNAi of the two alternatively spliced forms of TcCDA2 transcripts were consistent with the expression levels of these forms in the hindwing. RNAi for TcCDA2b, which was expressed at a relatively lower level when compared with that in the elytra, did not produce visible cuticular defects (Fig. 5F). On the other hand, RNAi for TcCDA2a resulted in a disordered cuticle and irregular pore canals (Fig. 5E). The results of RNAi of the dorsal abdominal cuticle, which closely resembles the hindwing cuticle in its organization, follow a similar pattern. Both TcCDA1 and TcCDA2 are important for normal soft cuticle organization, and the simultaneous depletion of both TcCDA transcripts has the most deleterious consequences (Fig. S3).

Cooperation between TcCDA1 and TcCDA2

Both TcCDA1 and TcCDA2 were expressed in the same tissues (e.g. the elytron, hindwing, abdomen, and leg), and yet depletion of either CDA alone results in cuticular defects. This
finding was puzzling because we assumed that either enzyme alone would be able to deacetylate chitin or carry out another common essential function independent of the other. We considered the possibility that the two proteins may cooperate with each other in the deacetylation step and that individually they may not be as active. We have not been successful so far in demonstrating that highly purified TcCDA1, TcCDA2a, and TcCDA2b preparations from an insect cell line expression system deacetylate, either alone or in combination, any of the several potential substrates that we have tested including colloidal chitin, ethylene glycol chitin, or chitooligosaccharides in vitro. We then investigated the possibility that depletion of one TcCDA may have an influence on the other TcCDAs stability or secretion when some type of heterocomplex formation is required for these processes.

We raised antibodies against TcCDA1 and TcCDA2 recombinant proteins to localize TcCDA1 and TcCDA2 proteins in the cell/cuticle by immunofluorescent and immunogold-labeling TEM analyses. Both TcCDA1 and TcCDA2 were found predominantly in the region that lies between the plasma mem-
brane of the epidermal cell and the procuticle and just outside of the apical plasma membrane protrusions (Fig. 6 and Figs. S4 and S5). Because both proteins clearly have a cleavable signal peptide and are indeed secreted after expression in insect cells, we assumed that these proteins accumulate in the assembly zone where chitin fibers are assembled into higher ordered structures. Interestingly, RNAi of either TcCDA1 or TcCDA2 resulted in almost complete loss of signal for both TcCDA1 and TcCDA2 from these regions (Fig. 6 and Fig. S5). These results suggested that both of the TcCDA proteins are transported to the outside together, presumably in the form of a complex. Western blotting analysis (in the presence of SDS) supported the hypothesis that RNAi of each CDA affects at least partially the steady-state level of the other CDA (Fig. 7A). In addition, under native-PAGE conditions, there was evidence for the presence of a complex with an apparent size expected for a dimer of TcCDA1 and TcCDA2 (Fig. 7B). RNAi of either TcCDA1 or TcCDA2 resulted in the depletion of this putative complex, further supporting the possibility that these two proteins physically interact with each other.

**Discussion**

Insect CDAs and the genes encoding them have been studied for over 10 years and shown to be essential for development, survival, and proper cuticular morphology. However, until now we had very few clues regarding their precise function and the steps in cuticle assembly in which they participate. Although fungal and nematode CDAs clearly possess the ability to deacetylate soluble chitin derivatives and partially deacetylated chitin and chitooligosaccharides in vivo and in vitro (2, 4, 23, 24), there are only a couple of studies that have reported such activity for purified preparations of CDAs from insect tissue extracts or from expression systems involving insect CDA genes (25, 26). On the other hand, several studies including ours could not detect chitin deacetylase activity in vitro from comparable preparations (8, 27, 28). Nevertheless, the finding that naturally occurring chitin isolated from insects does indeed have up to about 20% deacetylated sugars (glucosamine residues) (29) indicates that deacetylation must occur in vivo. It is likely that in insect cuticle, deacetylation occurs on nascent chitin chains and that in vitro studies failed to reproduce those conditions/substrates/presence of co-factors required for activity. Furthermore, the lethality associated with mutations of CDA genes, as well as the developmental defects and cuticular abnormalities exhibited by insects following RNAi of the CDA1 and CDA2 genes, further suggests that these proteins are essential for insect survival and normal cuticular development (16).

In the present study, we have extended our earlier observations to include other hard cuticles including the ventral abdominal and leg cuticles, as well as soft cuticles including those of the dorsal abdomen and hindwing. TEM analyses of the ultrastructure of the cuticles reported here have shown that the structural integrity is compromised upon depletion of TcCDA1 and TcCDA2. In particular, the horizontally stacked laminae and the vertically oriented pore canals become unorganized and lack rigidity with evidence of accumulation of disordered nanofibrils throughout the procuticle. Even the two alternatively spliced isoforms of TcCDA2, TcCDA2a and TcCDA2b, appear to have critical functions in specific tissues such as the elytron and leg joints. TcCDA1, TcCDA2a, and TcCDA2b are co-expressed in several (perhaps all) cuticle-forming tissues at the same developmental time. However, there is no functional compensation for the requirement of either of these proteins by other closely related TcCDAs, as evidenced from the finding that abnormal phenotypes are observed when any one of them is down-regulated by RNAi. Although TcCDA1 and TcCDA2 are required at all developmental stages, the requirement for TcCDA2a and TcCDA2b indicates tissue specificity. The tissues affected by depletion of TcCDAs include soft and thin cuticles, as well as the hard and thick cuticles, the common factor being that they all form laminated cuticles. In contrast, the taenidia of the tracheal system lack the parallel laminae characteristic of other cuticles (Fig. 8). In *D. melanogaster*, studies of tracheal tubule formation during embryogenesis have shown that processing of the luminal chitin cylinder is affected in *DmCDA1* (serpentine) and *DmCDA2* (vermiform) mutants (12, 13). However, RNAi for TcCDAs at the late larval or pupal stages of *T. castaneum* did not result in any changes in the taenidial cuticle including the accumulation of electron dense material at their apices (Fig. 8).

TEM analyses have provided clues regarding the step(s) in cuticle assembly that may require deacetylated chitin. The loss of the laminar arrangement of procuticular layers in both soft and hard cuticles and the accumulation of smaller fibrils that follows RNAi of either TcCDA indicates that, without deacetylation or any other function fulfilled by these proteins, native chitin nanofibrils (presumed to be the ~2.8 nm chitin crystals) are randomly distributed in the cuticle and do not form...
higher order assemblies of chitin, which ultimately result in formation of neatly stacked chitin laminae or long pore canal fibers. Based on the model of cellulose synthesis in plants and bacteria, polymeric assemblies of chitin synthase subunits are thought to lead to the production of coaxial bundles of chitin crystallites containing 18–25 chitin chains with a diameter of \( \sim 3 \) nm, which then associate with cuticular proteins to form larger composites. Neville \textit{et al.} (30) reported that a common arrangement of chitin crystallites embedded in the matrix of proteins in the procuticle of arthropods including insects is a hexagonal or pseudo-hexagonal lattice with a size range of \( \sim 4.5–9.0 \) nm for each side. Nanofibers with diameters ranging from 20 to 100 nm in cicada cuticles (31), 25 nm in the claval furrow of \( L. \) \textit{migratoria} (32), and 160–300 nm in the jaws of the Australian plague locust (33) have been reported. Nanofibers with a diameter of 50–250 nm and a length of 300 nm have been proposed as the building blocks of horizontally stacked laminae (34). In the horizontal laminae of \( T. \) \textit{castaneum}, we see fibers that are much longer than 300 nm, and in the pore canals, fibers with a thickness in the range of 40–80 nm are observed. Presumably, the elongation of the basic chitin crystallites of 3 nm described by Neville \textit{et al.} (30) occurs via side to side stacking and end to end joining facilitated by specific proteins to reach (or exceed) the dimensions of the epidermal cell (\( \sim 10 \) \( \mu \)m). Subsequent arrangement of long linear composites into parallel two-dimensional laminae by side-by-side alignment and the vertical stacking of these laminae may require specialized proteins. In the ds\textit{TcVer}-treated controls, the laminae appear as long fibers with sizes measured \( \sim 2.5 \) \( \mu \)m or longer, especially where the pore canals are not obscuring fiber visualization. Even though it is somewhat difficult to precisely measure the dimensions of the fibers that accumulate upon RNAi of
TcCDAl or TcCDAl2, a visual inspection suggests that the fibers are of a similar thickness as in the control, but significantly shorter with many in the ~500-nm range. After RNAi for both TcCDAs, these fibers are rarely longer than 300 nm, suggesting that the elongation of the basic nanofibrils is disturbed in the absence of TcCDAs. In addition, the fibers assume random crisscross orientations. Even inside the pore canals, a similar loss of accumulation of long orderly fibers can be seen. In cross-sections of the pore canals, the loss of fiber organization/orientation is mildly evident after RNAi for TcCDAl, more pronounced after RNAi for TcCDAl2, and most obvious when both TcCDAs are down-regulated together. These results suggest that cuticle integrity is lost because of a failure to lengthen chitin nanofibrils and organize them into higher order structures that include multilayered horizontal laminae, as well as thick and long bundles of chitin fibers that structurally reinforce the pore canals in the vertical direction.

All parts of the cuticle including proximal, middle, and distal parts moving away from the epidermal cell surface and the pore canals are affected equally by the loss of either TcCDAl or TcCDAl2 proteins. This result could have led to the expectation that these two TcCDAs are distributed evenly throughout the procuticle and thus affect chitin nanofibril organization. However, the results of immunolocalization of either TcCDAl or TcCDAl2 indicate that this is not the case. Both proteins are confined to what we believe to be the assembly zone that lies between the epidermal cell surface and the innermost part of the forming procuticle (35). How could a protein that is localized exclusively in the assembly zone influence the structure of distal layers of the cuticle? We propose that these TcCDAs function as enzymes presumably deacetylating nascent chitin partially in the assembly zone and not as just binding proteins that influence the assembly of nanofibrils at distal locations.

Our results with T. castaneum are in agreement with the reported predominant localization of Serpentine (CDA1) and Vermiform (CDA2) proteins (both have chitin deacetylase domains) in the assembly zone of D. melanogaster larval cuticle, where they presumably interact with other cuticular proteins, chitinases and chitin (17, 18) but differ from those of Yu et al. (15), who localized LmCDA2 from L. migratoria to the region of the procuticle just below the epicuticle and concluded that this CDA is responsible for the helicoidal organization of the chitin laminae reaching all the way to the base of the procuticle. They proposed that in the absence of LmCDA2 accumulation at this location, the underlying procuticle was forced to assume the configuration of a parallel stack of laminae instead of the normal helicoidal stack. The pore canals also did not traverse the procuticle in a helicoidal path as in the controls. In contrast, in T. castaneum, both TcCDAl and TcCDAl2 proteins are found in the assembly zone and just outside the APMPs that give rise to the pore canals. In addition, depletion of either one of the TcCDAs results in a disorganized cuticle filled with smaller fibers without the normally stacked laminae. It should be pointed out that the exocuticle in rigid cuticles of the T. castaneum adult is nonhelicoidal (20, 22). Even in the hindwing, where the cuticle is much thinner and helicoidal, depletion of TcCDAl or TcCDAl plus TcCDAl2 results in a nearly complete loss of the laminar organization. There was no evidence of alteration of the thickness of the T. castaneum cuticle as was observed in the abdominal cuticle of L. migratoria (15).

We obtained additional insights into why the two secreted TcCDAs do not functionally compensate for each other by following their accumulation in the assembly zone after administration of either one of the two dsRNAs. RNAi of TcCDAl resulted in loss of accumulation not only of TcCDAl protein as expected but also led to the absence of TcCDAl2 in the assembly

Figure 8. Neither TcCDAl nor TcCDAl2 affects organization of nonlaminar tracheal taenidial cuticle. Morphology of tracheal cuticle from pharate adults (5-day-old pupae) treated with dsTcCDAl, dsTcCDAl2, dsTcCDAl2a, dsTcCDAl2b, or dsTcVer was analyzed by TEM. The taenidia of the tracheal system lacks the laminae characteristic of body wall or wing tissues. RNAi of these TcCDAs did not result in any obvious changes in the taenidial cuticle structure including the accumulation of electron dense material at their apices (arrows). TAE, taenidia; PT, pupal trachea. Scale bar, 500 nm.

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zone and vice versa. We interpret these data as suggesting that the transport/secretion of these two proteins may involve formation of a complex of these two proteins in the endoplasmic reticulum or at some point in the vesicular transport pathway. Western blotting analyses of protein extracts from elytra after RNAi of either TcCDA1 or TcCDA2 showed almost complete depletion of the targeted protein, as well as a partial depletion of the other TcCDA (Fig. 7). Furthermore, native-PAGE revealed that, in addition to the 60–65-kDa TcCDA1 and TcCDA2 proteins, larger immunoreactive protein complexes with apparent molecular masses of ~120–130 kDa were detected in the protein extract of dsTvEr-treated control insects in addition to the monomers (Fig. 7B). Because this larger immunoreactive protein (black arrow) (and the targeted TcCDA protein) was absent/reduced in both TcCDA1- and TcCDA2-deficient insects, it appears that a TcCDA1–TcCDA2 heterodimeric complex is present. There is some evidence for the formation of homodimeric TcCDA1 or TcCDA2 complexes when RNAi was performed with a single dsRNA for TcCDA1 or TcCDA2 (red or blue arrows). These results suggest the possibility that after synthesis, the two TcCDA proteins associate with each other to promote their stability/transport into the assembly zone. However, the detection of immunoreactive material in Western blots after depletion of either TcCDA indicates the accumulation of the nontargeted TcCDA in some intracellular location (not detected by the immunogold labeling). We propose that the two TcCDAs may function cooperatively in the deacetylation step and/or influence each other’s stability/accumulation in the assembly zone. We are currently pursuing this possibility by performing some direct biochemical experiments.

Conclusions

We conclude that TcCDA1 and TcCDA2 help to determine the overall three-dimensional architecture of the procuticle by affecting not only the laminar arrangement of chitin-protein layers but also the structure of the vertically oriented pore canals. However, the envelope or epicuticle and nonlaminar mature adult tracheal taenidial cuticle are unaffected. We further postulate that the deacetylated parts of chitin serve as attachment points for other proteins involved in end-to-end aggregation and/or further assembly of chitin nanofibrils into larger assemblies such as larger microfibrils, laminae, or pore canal fibers. The α-chitin crystallites that form after the chitin chains emerge from the active sites of extrusion helices of chitin synthases are unsuitable substrates for CDAs unless they are partially disassociated by the activity of some proteins. Presumably, CDA1 and CDA2 work in concert with chitinase 7, an enzyme implicated in releasing the nascent chitin chains (40). Such ends that are not involved in hydrogen bond formation with other chitin chains may be more easily accessible to CDAs and solvent. All three of these proteins have peritrophin A–type chitin-binding domains that may help them to bind to crystalline chitin and provide ligand-binding free energy that may aid in decrystallization of the substrate (36). It is interesting to point out that chitinase 7 and chitin synthases have transmembrane or membrane-anchoring domains, whereas CDAs do not. Why CDA1 and CDA2 do not diffuse away from the assembly zone into the rest of the procuticle remains an open question. Finally, how these multiple deacetylases work together with multiple chitinases, multiple chitin synthases, and multiple chitin-binding proteins to synthesize, bind with, deacetylase and hydrolyze chitin, and partially deacetylase chitin in cuticles exhibiting different mechanical properties are major unanswered questions in insect science.

Experimental procedures

Insects

T. castaneum GA-1 strain (37) was used to study adult cuticle development. Insects were reared at 30 °C at 50% relative humidity in wheat flour containing 5% (v/v) brewer’s yeast. Under this rearing condition, adult eclosion occurs 5 days after pupation.

Real-time PCR

Total RNA was isolated from elytra, hindwings, and ventral and dorsal abdomens dissected from 0-day-old adults (n = 10) by using the RNAeasy mini kit and then treated with DNase I (Qiagen). 1 μg of total RNA was used to prepare cDNA using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions. Total RNA was independently isolated for each of the three replicates. Real-time PCR was performed using the Thermal Cycler Dice real-time PCR system (Takara) in a 50-μl reaction mixture containing 1 μl of template cDNA, 25 μl of SYBR Premix Ex Taq (Takara), and 0.2 μM of each primer with the program: initial denaturation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. At the end of the PCR, a melt curve was generated to evaluate the possibility of undesirable side products. The threshold cycle number (Ct) was determined and used for comparative quantitative analyses. The transcription levels of the T. castaneum ribosomal protein S6 (TcRpS6) were measured to normalize for differences between the concentrations of cDNA templates. See Table S1 for the primer sequences used for real-time PCR experiments and their amplification efficiencies.

RNAi

dsRNA for TcCDA1 (dsTcCDA1), TcCDA2 (dsTcCDA2), TcCDA2a (dsTcCDA2a), and TcCDA2b (dsTcCDA2b) were synthesized as described previously (16) and then injected into 0–1-day-old pupae (150 ng/insect). Insects injected with dsRNA for the T. castaneum Vermilion gene (dsTvEr) served as a negative control (38). The primer sequences used for amplification of the templates for synthesizing dsRNAs are shown in Table S1. To analyze the knockdown levels of TcCDA transcripts, total RNA was isolated from whole insects of 5-day-old pupae (n = 3). Total RNA was independently isolated for each of three replicates, and significant differences were analyzed using a Student’s t test.

Western blotting

TcCDA1 antibody was generated in rabbits against the purified recombinant TcCDA1 protein expressed in insect cells as the antigen (16). Similarly, to generate TcCDA2 antibody, recombinant TcCDA2 protein was expressed in the High-Five
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cells using the baculovirus expression system (BD Biosciences) and purified from the culture medium by nickel–nitrilotriacetic acid affinity column chromatography as described previously (39). See Table S1 for the primer sequences used for expression of the recombinant TcCDA2 protein. Purified TcCDA2 was used as the antigen to generate rabbit antiserum (Cocalico Biologicals, Inc.). To evaluate the specificity of the anti-TcCDA1 and anti-TcCDA2 antisera, Western blotting analysis was performed. Protein extracts prepared from the elytra dissected from the 5-day-old pupae (n = 5) that had been previously injected with dsRNA for the TcCDA1 or TcCDAs at the 0–1-day-old pupal stage were analyzed by 10% SDS-PAGE followed by Coomassie Blue staining or Western blotting using the anti-TcCDA1 or anti-TcCDA2 polyclonal antibodies as described previously (22). To investigate the formation of TcCDA protein complex(es), the protein extracts were also analyzed by the 4–16% NativePAGE™ Bis-Tris gel system (Invitrogen) according to the manufacturer’s protocol.

Transmission EM (TEM)

TEM was performed as described previously (21). Pharate adults (5-day-old pupae) that had been injected at the 0–1-day-old pupal stage with dsTcCDA1, dsTcCDA2, dsTcCDA1/2 (coinjection), dsTcCDA2a, dsTcCDA2b, or dsTcVer were collected and fixed as described previously (21, 22). Ultra-thin sections (~90 nm) of different tissues were stained with 4% uranyl acetate, and then cuticle ultrastructure was observed using a transmission electron microscope (JEOL, JEM-14000). Immunogold-labeling TEM was carried out as described previously (21, 22) using anti-TcCDA1 and anti-TcCDA2. Gold-labeled (10 nm) wheat germ agglutinin (EY Laboratories) was used to detect cuticular chitin.

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References

1. Blair, D. E., and van Aalten, D. M. (2004) Structures of Bacillus subtilis PdaA, a family 4 carbohydrate esterase, and a complex with N-acetylgalcosamine. FEBS Lett. 570, 13–19 CrossRef Medline
2. Tsigos, I., Martiínotou, A., Kaftazopoulos, D., and Bouriotis, V. (2000) Chitin deacetylases: new, versatile tools in biotechnology. Trends Biotechnol. 18, 429–435 CrossRef Medline
3. Vollmer, W., and Tomass, A. (2000) The pgdA gene encodes for a peptidoglycan N-acetylgalactosaminidase of Streptococcus pneumoniae. J. Biol. Chem. 275, 20496–20505 CrossRef Medline
4. Zhao, Y., Park, R. D., and Muzzarelli, R. A. (2010) Chitin deacetylases: properties and applications. Mar. Drugs 8, 24–46 CrossRef Medline
5. Heustis, R. J., Ng, H. K., Brand, K. J., Rogers, M. C., Le, L. T., Specht, C. A., and Fuhrman, J. A. (2012) Pharyngeal polysaccharide deacetylases affect development in the nematode C. elegans and deacetylate chitin in vitro. PLoS One 7, e40826 CrossRef Medline
6. Aruchami, M., Gowri, N., and Sundara-Rajulu, (1986) Chitin deacetylases in invertebrates. In Chitin in Nature and Technology (Muzzarelli, R., Jeuniaux, C., and Gooday, G. W., eds), pp. 263–265, Plenum Press, New York
7. Zhao, Y., Ju, W. T., Jo, G. H., Jung, W. J., and Park, R. D. (2011) Perspectives of chitin deacetylation research. In Biotechnology of Biopolymers (El-Nashar, M., ed) pp 131–144, InTech, Shanghai
8. Guo, W., Li, G., Pang, Y., and Wang, P. (2005) A novel chitin-binding protein identified from the peritrophic membrane of the cabbage looper, Trichoplusia ni. Insect Biochem. Mol. Biol. 35, 1224–1234 CrossRef Medline
9. Dixit, R., Arakane, Y., Specht, C. A., Richard, C., Kramer, K. J., Beeman, R. W., and Muthukrishnan, S. (2008) Domain organization and phylogenetic analysis of proteins from the chitin deacetylase gene family of Tribolium castaneum and three other species of insects. Insect Biochem. Mol. Biol. 38, 440–451 CrossRef Medline
10. Tetreau, G., Cao, X., Chen, Y. R., Muthukrishnan, S., Jiang, H., Blissard, G. W., Kanost, M. R., and Wang, P. (2015) Overview of chitin metabolism enzymes in Manduca sexta: identification, domain organization, phylogenetic analysis and gene expression. Insect Biochem. Mol. Biol. 62, 114–126 CrossRef Medline
11. Muthukrishnan, S., Merzendorfer, H., Arakane, Y., and Yang, Q. (2016) Chitin metabolic pathways in insects and their regulation. In Extracellular Composite Matrices in Arthropods (Cohen, E., and Moussian, B., eds), pp. 31–65, Springer, Cham, Switzerland
12. Luschnig, S., Bätz, T., Armbruster, K., and Krasnow, M. A. (2006) Serpentine and vermiform encode matrix proteins with chitin binding and deacetylation domains that limit tracheal tube length in Drosophila. Curr. Biol. 16, 186–194 CrossRef Medline
13. Wang, S., Jayaram, S. A., Hemphälä, J., Senti, K. A., Tsarouhas, V., Jin, H., and Samakovlis, C. (2006) Septate-junction-dependent luminal deposition of chitin deacetylases restricts tube elongation in the Drosophila trachea. Curr. Biol. 16, 180–185 CrossRef Medline
14. Xi, Y., Pan, P. L., Ye, Y. X., Yu, B., and Zhang, C. X. (2014) Chitin deacetylase family genes in the brown planthopper, Nilaparvata lugens (Hemiptera: Delphaciidae). Insect Mol. Biol. 23, 695–705 CrossRef Medline
15. Yu, R., Liu, W., Li, D., Zhao, X., Ding, G., Zhang, M., Ma, E., Zhu, K., Li, S., Moussian, B., and Zhang, J. (2016) Helicoidal organization of chitin in the cuticle of the migratory locust requires the function of the chitin deacetylase2 enzyme (LmCDA2). J. Biol. Chem. 291, 24352–24363 CrossRef Medline
16. Arakane, Y., Dixit, R., Begum, K., Park, Y., Specht, C. A., Merzendorfer, H., Kramer, K. J., Muthukrishnan, S., and Beeman, R. W. (2009) Analysis of functions of the chitin deacetylase gene family in Tribolium castaneum. Insect Biochem. Mol. Biol. 39, 355–365 CrossRef Medline
17. Pesch, Y. Y., Riedel, D., and Behr, M. (2015) Obstructor A organizes matrix assembly at the apical cell surface to promote enzymatic cuticle maturation in Drosophila. J. Biol. Chem. 290, 10071–10082 CrossRef Medline
18. Pesch, Y. Y., Riedel, D., Patil, K. R., Loch, G., and Behr, M. (2016) Chitinases and Imaginal disc growth factors organize the extracellular matrix formation at barrier tissues in insects. Sci. Rep. 6, 18340 CrossRef Medline
19. Tomoyasu, Y., Arakane, Y., Kramer, K. J., and Denell, R. E. (2009) Repeated co-options of exoskeleton formation during wing-to-elytron evolution in beetles. Curr. Biol. 19, 2057–2065 CrossRef Medline
20. Noh, M. Y., Muthukrishnan, S., Kramer, K. J., and Arakane, Y. (2017) Development and ultrastructure of the rigid dorsal and flexible ventral cuticles of the elytron of the red flour beetle, Tribolium castaneum. Insect Biochem. Mol. Biol. 91, 21–33 CrossRef Medline
21. Noh, M. Y., Kramer, K. J., Muthukrishnan, S., Kanost, M. R., Beeman, R. W., and Arakane, Y. (2014) Two major cuticular proteins are required for assembly of horizontal laminae and vertical pore canals in rigid cuticle of Tribolium castaneum. Insect Biochem. Mol. Biol. 53, 22–29 CrossRef Medline
22. Noh, M. Y., Muthukrishnan, S., Kramer, K. J., and Arakane, Y. (2015) Tribolium castaneum RR-1 cuticular protein TcCPRA is required for formation of pore canals in rigid cuticle. PLoS Genet. 11, e1004963 CrossRef Medline
23. Tokuyasu, K., Ono, H., Ohnishi-Kameyama, M., Hayashi, K., and Morii, Y. (1997) Deacetylation of chitin oligosaccharides of dp 2–4 by chitin deacetylase from Colletotrichum lindemuthianum. Carbohydr. Res. 303, 353–358 CrossRef Medline
Group I chitin deacetylases and insect cuticle structure

24. Kang, L., Chen, X., Zhai, C., and Ma, L. (2012) Synthesis and high expression of chitin deacetylase from Colletotrichum lindemuthianum in Pichia pastoris GS115. *J. Microbiol. Biotechnol.* 22, 1202–1207. CrossRef Medline

25. Toprak, U., Baldwin, D., Erlandson, M., Gillott, C., Hou, X., Coutu, C., and Hegedus, D. D. (2008) A chitin deacetylase and putative insect intestinal lipases are components of the *Mamestra configurata* (Lepidoptera: Noc-tuidae) peritrophic matrix. *Insect Mol. Biol.* 17, 573–585. CrossRef Medline

26. Zhong, X. W., Wang, X. H., Tan, X., Xia, Q. Y., Xiang, Z. H., and Zhao, P. (2014) Identification and molecular characterization of a chitin deacetylase from *Bombyx mori* peritrophic membrane. *Int. J. Mol. Sci.* 15, 1946–1961. CrossRef Medline

27. Jakubowska, A. K., Caccia, S., Gordon, K. H., Ferré, J., and Herrero, S. (2010) Downregulation of a chitin deacetylase-like protein in response to baculovirus infection and its application for improving baculovirus infectivity. *J. Virol.* 84, 2547–2555. CrossRef Medline

28. Qu, M., Ma, L., Chen, P., and Yang, Q. (2014) Proteomic analysis of insect molting fluid with a focus on enzymes involved in chitin degradation. *J. Proteome Res.* 13, 2931–2940. CrossRef Medline

29. Kaya, M., Mujtaba, M., Ehrlich, H., Salaberria, A. M., Baran, T., Amemiya, C. T., Galli, R., Akyuz, L., Sargin, I., and Labidi, J. (2017) On chemistry of gamma-chitin. *Carbohydr. Polym.* 176, 177–186. CrossRef Medline

30. Neville, A. C., Parry, D. A., and Woodhead-Galloway, J. (1976) The chitin crystallite in arthropod cuticle. *J. Cell Sci.* 21, 73–82. Medline

31. Chandran, R., Williams, L., Hung, A., Nowlin, K., and LaJeunesse, D. (2016) SEM characterization of anatomical variation in chitin organization in insect and arthropod cuticles. *Micron* 82, 74–85. CrossRef Medline

32. Banerjee, S. (1988) Organization of wing cuticle in *Locusta migratoria Linnaeus, Tropoidacris cristata Linnaeus and Romalea microptera Beau-vais* (Orthoptera: Acrididae). *Int. J. Insect Morphol. Embryol.* 17, 313–326. CrossRef

33. Gardiner, B. G., and Khan, M. F. (1979) A new form of insect cuticle. *Zoo J. Linn. Soc* 66, 91–94. CrossRef

34. Fabritius, H. O., Sachs, C., Triguero, P. R., and Roobe, D. (2009) Influence of structural principles on the mechanics of a biological fiber-based composite material with hierarchical organization: the exoskeleton of the lobster *Homarus americanus*. *Adv. Mater.* 21, 391–400. CrossRef

35. Locke, M., and Huie, P. (1979) Apolysis and the turnover of plasma membrane plaques during cuticle formation in an insect. *Tissue Cell* 11, 277–291. CrossRef Medline

36. Beckham, G. T., and Crowley, M. F. (2011) Examination of the alpha-chitin structure and decrystallization thermodynamics at the nanoscale. *J. Phys. Chem. B* 115, 4516–4522. CrossRef Medline

37. Haliscak, J. P., and Beeman, R. W. (1983) Status of malathion resistance in five genera of beetles infesting farm-stored corn, wheat, and oats in the United States. *J. Econ. Entomol.* 76, 717–722. CrossRef

38. Arakane, Y., Lomakin, J., Beeman, R. W., Muthukrishnan, S., Gehrke, S. H., Kanost, M. R., and Kramer, K. J. (2009) Molecular and functional analyses of amino acid decarboxylases involved in cuticle tanning in *Tribolium castaneum*. *J. Biol. Chem.* 284, 16584–16594. CrossRef Medline

39. Arakane, Y., Zhu, Q., Matsumiya, M., Muthukrishnan, S., and Kramer, K. J. (2003) Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect Biochem. Mol. Biol.* 33, 631–648. CrossRef Medline

40. Noh, M. Y., Muthukrishnan, S., Kramer, K. J., and Arakane, Y. (2018) A chitinase with two catalytic domains is required for organization of the cuticular extracellular matrix of a beetle. *PLoS Genet.* 14, e1007307. CrossRef Medline