Analysis of functions of the chitin deacetylase gene family in Tribolium castaneum

Yasuyuki Arakane\textsuperscript{a}, Radhika Dixit\textsuperscript{a}, Khurshida Begum\textsuperscript{b}, Yoonseong Park\textsuperscript{b}, Charles A. Specht\textsuperscript{c}, Hans Merzendorfer\textsuperscript{d}, Karl J. Kramer\textsuperscript{a,e}, Subbaratnam Muthukrishnan\textsuperscript{a}, Richard W. Beeman\textsuperscript{c,*}

\textsuperscript{a} Department of Biochemistry, Kansas State University, 141 Chalmers Hall, Manhattan, KS 66506, USA
\textsuperscript{b} Department of Entomology, Kansas State University, 123 Waters Hall, Manhattan, KS 66506, USA
\textsuperscript{c} Department of Medicine, University of Massachusetts, Worcester, MA 01605, USA
\textsuperscript{d} Department of Biology, Chemistry and Animal Physiology, University of Osnabrück, Osnabrück D-49069, Germany
\textsuperscript{e} Grain Marketing and Production Research Center, ARS-USDA, 1515 College Avenue, Manhattan, KS 66502, USA

\textbf{ABSTRACT}

The expression profiles of nine genes encoding chitin deacetylase (CDA)-like proteins were studied during development and in various tissues of the red flour beetle, Tribolium castaneum, by RT-PCR. TcCDA1, TcCDA2 and TcCDA3 were expressed throughout all stages of development, while TcCDA6–9 were expressed predominantly during larval feeding stages. In situ hybridization experiments revealed that both TcCDA1 and TcCDA2 were expressed in epidermal cells. Polyclonal antibody to TcCDA1 detected an immunoreactive protein in larval tracheae. TcCDA6 through TcCDA9, which belong to a distinct subgroup of gut-specific CDAs, were transcribed in the cells lining the midgut, including epithelial cells. TcCDA3 was expressed in the thoracic muscles, whereas TcCDA4 was expressed in early imaginal appendages. To study the function(s) of individual TcCDA genes, double-stranded RNAs (dsRNA) specific for each gene were injected into insects at different developmental stages and the phenotypes were monitored. No visible phenotypic changes were observed after injection of dsRNAs for TcCDA3 to 9, whereas injection of dsRNAs for TcCDA1 or TcCDA2 affected all types of molts, including larval–larval, larval–pupal and pupal–adult. Insects treated with these dsRNAs could not shed the old cuticle and were trapped in their exuviae. Interestingly, unique and very dissimilar adult phenotypes were observed after injection of dsRNAs that specifically down-regulated either of the two alternatively spliced transcripts of TcCDA2, namely TcCDA2a or TcCDA2b. These results reveal functional specialization among T. castaneum CDA genes and splice variants.

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1. Introduction

Chitin, the \(\beta\)-(1,4)-linked homopolymer of \(N\)-acetylglucosamine, is one of the major components of the exoskeleton of insects and other arthropods. The extracellular matrix (ECM) of the exoskeleton is modified in different ways to make the cuticle either rigid and thick, or thin and flexible, or to generate specialized structures such as mandibles with sharp cutting edges (Kramer and Muthukrishnan, 2005). Chitin deacetylases (CDAs, EC 3.5.1.41) are secreted proteins belonging to a family of extracellular chitin-modifying enzymes that catalyze the \(N\)-deacetylation of chitin to form chitosan, a polymer of \(\beta\)-(1,4)-linked \(\alpha\)-glucosamine residues (Tsigos et al., 2000). The extent of chitin deacetylation may vary a great deal among different arthropods and sometimes among different parts of the same animal. This modification might contribute to the affinity of chitosan for a variety of proteins distinct from those that bind specifically to chitin.

CDAs are metalloenzymes that are members of a family of carbohydrate esterases (carbohydrate active enzymes family CE4; http://www.cazy.org/fam/CE4.html) and have been well characterized in pathogenic fungi and bacteria (Caufrier et al., 2003). CE4 esterases catalyze the deacetylation of different carbohydrate substrates including chitin, xylan and peptidoglycan. However, to date there has been only one published report demonstrating chitin deacetylase activity of an insect CDA protein (Toprak et al., 2008). A cDNA encoding an insect CDA-like protein was first characterized by Guo et al. (2005) from a midgut cDNA expression library of the cabbage looper, Trichoplusia ni. This protein was associated with the peritrophic membrane and had strong chitin-binding activity. Three \emph{Drosophila melanogaster} genes encoding putative chitin deacetylase-like proteins were identified by Luschnig et al. (2006) and Wang et al. (2006). Two of these genes,
serpentine (CG32209) and vermiform (CG8756), play critical roles in shaping the tracheal tubes, as well as regulating the structural properties of epidermal cuticle. By searching BeetleBase (http://bioinformatics.ksu.edu/BeetleBase/), which contains annotated genes and predicted proteins of the red flour beetle, Tribolium castaneum, we identified nine genes encoding CDA-like proteins with sequence similarity to DmSerp and DmVerm (Dixit et al., 2008). A comparative analysis of CDA families in other beetle species with fully sequenced genomes, including D. melanogaster, Anopheles gamiacheae and Apis mellifera, revealed variation in the number of CDA genes, with T. castaneum having the greatest number. These include some recently duplicated genes found clustered in its genome. Based on amino acid sequence similarity, we have classified insect CDAs into five groups, I–V (Dixit et al., 2008).

In our phylogenetic classification the DmSerp and DmVerm genes belong to group I of the family of CDAs. The discovery of additional members and groups in CDA families from several insect species raises interesting questions about the need for such a large family and about the functions of individual CDAs. The ease of carrying out dsRNA-mediated down-regulation of transcripts in T. castaneum at any desired stage of development prompted us to carry out a functional analysis of all members of the CDA family of proteins in this beetle. Gene-specific dsRNAs for all of the nine TcCDAs, including dsRNAs for the alternatively spliced transcripts of TcCDA2 and TcCDA5, were injected into insects at appropriate stages of development based on their expression patterns, and the resulting phenotypes were observed. The results indicate unique functions for some of the CDA genes. Specific ratios of chitosan-to-chitin may be critical for specific functions of particular chitin-containing body parts and for insect survival.

2. Materials and methods

2.1. Beetle cultures

The GA-1 strain (Haliscak and Beeman, 1983) of T. castaneum was used in all experiments. Beetles were reared on whole flour containing 5% yeast dried brewers’ yeast at 30 °C under standard conditions (Beeman and Stuart, 1990).

2.2. Developmental and tissue-specific expression profiles of the T. castaneum CDA gene family

The developmental expression patterns and tissue specificities of expression of the nine putative T. castaneum CDAs were analyzed by RT-PCR. Total RNA was isolated from whole insects at various stages of growth including embryos, young larvae (instars 4–5), last-instar larvae, prepupa, pupae and adults by using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. To examine gene expression in specific tissues, 15 larvae were dissected to obtain separate pools of foregut, anterior midgut, middle midgut, posterior midgut, hindgut and remaining carcass. Dissected to obtain separate pools of foregut, anterior midgut, middle midgut, posterior midgut, hindgut and remaining carcass. These were stained with polyclonal antibodies raised in rabbits against albumin and 0.1% Tween 20. Then the sections were treated for 1 h, indolyl phosphate (NBT/BCIP). Color development was monitored using a Leica CTR6500 inverse microscope with a dissecting microscope and arrested by repeated washes with PBS. The image was observed using a Nikon E-800 microscope with differential interference contrast optics, captured by a digital camera (CoolSnap ES). The captured images were enhanced in Photoshop 7.0 by adjusting only the global brightness/contrast.

2.3. In situ hybridization

Specific expression patterns of TcCDAs were investigated by in situ hybridization to an isolated larval alimentary canal or to whole animal. The samples were fixed in Bouin or Carnoy's solution, dehydrated in a series of increasing ethanol concentrations (70, 90, 100%) and in chloroform, and then embedded in Paraplast®. Paraffin sections 7–10 μm in thickness were arrayed on slide glasses, then paraffin was removed with xylene, and rehydrated. The samples were treated with proteinase K (10 μg/ml) for 10 min and fixed with 4% paraformaldehyde for 15 min. Between each step, at least two washes with PBST (PBS with 0.2% Triton-X 100) were performed. Hybridization was performed in the hybridization solution (HS, 50% formamide, 5 × SSC, 50 μg/ml heparin, 0.1% Tween 20, and 100 μg/ml salmon sperm DNA) at 48 °C for 20–30 h. Gene-specific digoxigenin (DIG)-labeled DNA probes were prepared with the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, Mannheim, Germany). Single-stranded anti-sense and sense probes (the latter as negative controls) were prepared by asymmetric PCR (see Supplementary Table 2 for probe sequences). The sample after the hybridization was washed with HS alone for 2–4 h at 48 °C. Subsequent washes were serially with HS, HS in 50% PBST, and PBST for 5 min each at room temperature. The DIG-alkaline phosphatase signal was developed by a colorimetric method using nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). Color development was monitored using a Leica CTR6500 inverse microscope with differential interference contrast optics, captured by a digital camera (CoolSnap ES). The captured images were enhanced in Photoshop 7.0 by adjusting only the global brightness/contrast.

2.4. Immunostaining of tissue sections

Serial cryosectioning was performed as described previously (Klein et al., 1991). Transverse sections of late T. castaneum larvae were stained with polyclonal antibodies raised in rabbits against recombinant TcCDA1 (complete coding sequence), which had been expressed in the Hi-5 (Trichoplusia ni) insect cell line and purified by Ni-NTA chromatography as described previously (Zhu et al., 2008). All incubations for immunofluorescence staining were performed at room temperature. The sections were rinsed three times for 5 min in PBS buffer, then incubated for 5 min in PBS buffer containing 50 mM ammonium chloride, and rinsed again three times in PBS buffer. Blocking was performed for 1 h, incubating the sections in Block-buffer (PBS buffer containing 2% bovine serum albumin and 0.1% Tween 20). Then the sections were treated for 1 h, either with anti-TcCDA1 antibodies or with the corresponding pre-immune serum (each diluted 1:1000 in Block-buffer). After rinsing the sections again three times for 5 min in PBS buffer and performing an additional blocking step, Alexa Fluor® 488 goat anti-rabbit IgG antibodies (Invitrogen) were applied for 1 h at a 1:500 dilution with PBS. After this treatment the sections were rinsed again with PBS buffer as described above and a nuclear staining was performed with 4',6-diamidine-2'-phenylindole-dihydrochloride (DAPI, 1 μg/ml in PBS buffer). After a renewed washing with PBS buffer the sections were covered with Fluoromount (Sigma). Fluorescence was viewed with a Leica CTR6500 inverse microscope using a Leica EL6000 light source, appropriate filter cubes (A4, L5) and the LAS AF software.

2.5. Double-stranded RNA synthesis and injection

Double-stranded RNA (dsRNA) syntheses and injections were carried out as described by Arakane et al. (2005a). Unique regions...
of each CDA gene were chosen as templates for synthesis of dsRNAs in order to avoid off-target effects on other CDAs that share similar nucleotide sequences. In most cases, RNA interference (RNAi) experiments were replicated by choosing two non-overlapping regions from each gene for dsRNA design. The primers used for dsRNA syntheses, lengths of the dsRNAs and the nucleotide positions are shown in Supplementary Table 3.

Double-stranded RNA (200 ng per insect) corresponding to each target-gene region was injected into pupal and last-instar larvae or prepupae. The dsRNA for *T. castaneum vermilion* (dsTcVer) was used as both a negative control for non-specific effects of dsRNA and as a positive control for monitoring effectiveness of RNAi by following eye color. Groups of 20 insects were used for each treatment.

Parental RNAi was also conducted as described in Arakane et al. (2008) in order to observe any adverse effects on fecundity, oviposition behavior, or in the development of the resulting progeny. dsRNAs for TcCDAs 1 or TcCDAs 2 along with the control dsRNA for TcVer were injected into adult females that were subsequently allowed to mate with equal numbers of untreated males. Eggs were collected every four days for ~1 month, for observation of progeny phenotypes.

### 2.6. Real-time RT-PCR analyses of TcCDAs transcript levels after dsRNA injections

The transcript depletion of specific TcCDAs after injection of a mixture of the four dsRNAs for TcCDAs 4, TcCDAs 7, TcCDAs 8 and TcCDAs 9 was quantified by real-time RT-PCR as described in Arakane et al. (2008). dsRNA for TcVer (200 ng) was injected as a negative control for specific functional knockdown (of eye pigmentation). RNA was isolated from a pool of three to five animals for each treatment. The transcript levels of the internal control gene, TcRps6, were also measured to normalize for differences in the concentrations of cDNA templates. The primer sets used are shown in Supplementary Table 4. Real-time PCR was conducted using the Takara SYBR Ex Taq® premix reagent with the “shuttle PCR” program. At the end of the PCR reaction, a melt curve was generated to rule out the possibility of undesirable side-products. The Ct values were determined and used for comparative quantitative analysis.

### 3. Results

#### 3.1. Developmental expression profiles of *T. castaneum* CDA gene family

The developmental expression patterns as well as the tissue specificities of gene expression of all nine putative *T. castaneum* CDAs, including the alternatively spliced transcripts of TcCDAs 2 and TcCDAs 5, were determined by RT-PCR (Fig. 1). The cDNAs were prepared from RNAs isolated at different stages of development, ranging from embryo to adult. The group I CDA gene transcripts TcCDAs 1, TcCDAs 2a and TcCDAs 2b were expressed at all stages of development. TcCDAs 3, which belongs to group II, revealed a unique pattern of expression, with the highest level of transcript accumulation occurring in the pupal stage. Group III TcCDAs 4 transcripts were observed in all stages except for the pupa, a pattern almost perfectly complementary to that of the group II gene. Both of the alternatively spliced group IV transcripts, TcCDAs 5a and TcCDAs 5b, were expressed at all stages. The group V CDA genes TcCDAs 6, 7, 8 and 9, were expressed mainly in larval stages.

qPCR results indicated that TcCDAs 1, TcCDAs 2a and TcCDAs 2b transcripts were elevated immediately after both the larval–pupal molt and the pupal–adult molt. Titer values reached peak levels on the day of either molt but then declined rapidly, suggesting that CDAs have critical functions immediately after these molts (Fig. 2).

#### 3.2. Tissue and cell specificity of expression of *T. castaneum* CDAs

In order to study the tissue specificity of expression of the nine TcCDAs, RT-PCR analyses were performed using RNAs isolated from foregut, anterior midgut, middle midgut, posterior midgut, hindgut, or carcass (which includes whole body minus head, gut, and posterior tip) of insects consisting of a mixture of pupal and last-instar feeding stage larvae (Fig. 3). The group V CDA genes TcCDAs 6–9 were expressed predominantly in the larval feeding stages, specifically in the gut but not in the carcass. With the exception of TcDAs 5b, whose transcripts were detected in larval midgut, all of the other CDA genes belonging to groups I, II, III and IV are expressed only in the carcass and not gut, suggesting their expression in epidermal and/or tracheal cells. As expected, TcCDAs 3 transcripts were not detected in any larval tissue, consistent with the previous observation that this gene is not highly expressed in whole larvae.

#### 3.3. In situ hybridization

The results from *in situ* hybridization were generally in agreement with the RT-PCR data and enabled higher-resolution analysis of transcript localization (Figs. 4 and 5). The group I TcCDAs 1 gene was expressed in epithelial cells of the rectum (Fig. 4A) and subsets of exoskeletal epidermis (Fig. 4B and C). In particular, in insects at the molting stage (pharate pupa) with two layers of cuticle, the epidermal cells were strongly stained, while those epidermal cells with only one layer of cuticle did not show staining, indicating that TcCDAs 1 is expressed after starting the synthesis of new cuticle during ecdysis. With the TcCDAs 2 probe, entire epidermal cells including the cells in the appendages (leg, elytra and head in Fig. 4E–G, respectively) were strongly stained in the pharate adult stage. In pharate adults, a positive signal for the TcCDAs 3 probe was found only in a structure that appeared to represent thoracic muscle that was in the remodeling stage of metamorphosis (Fig. 4I–K). The TcCDAs 4 probe stained the epidermal cells in the imaginal appendages (Fig. 4L). We were unable to identify positive signals with the TcCDAs 5 probe in pharate pupae or pharate adults.

All of the CDAs in group V, TcCDAs 6–9, were expressed in gut tissue in the late larval stage (Fig. 5). TcCDAs 6 expression was found in the anterior and posterior midgut. Specifically, positive signals were found in regenerative cells and endocrine cells, the latter being narrow cells sandwiched between columnar cells scattered over the length of midgut (Fig. 5A). The absence of an RT-PCR band in the mid-midgut (Fig. 3) could be consistent with a low level of TcCDAs 6 expression in a small set of cells, resulting in difficulty in PCR detection. TcCDAs 7 signal occurred only in the columnar cells of the posterior region of the midgut (Fig. 5B). TcCDAs 8 was highly expressed in the whole midgut (Fig. 5C) and to a lesser extent in the hindgut epithelial cells (Fig. 5D). Both regenerative and columnar cells of the midgut and hindgut epithelium were stained with the probe. The positively staining hindgut epithelial cells were within the Malpighian tubule-covered cryptonephridial complex (Fig. 5D). Interestingly, the positive signals in the columnar cells were located in the apical portion. TcCDAs 9 expression was found in the columnar cells of the anterior- and mid-midgut (Fig. 5E) but not in the posterior midgut near the junction with the hindgut. No corresponding signals were identified in negative controls using sense-strand probes for TcCDAs 1, 2, 3, 4, 8, or 9 (e.g., Fig. 5F). Table 1 summarizes the expression patterns of members of the CDA gene family in the gut of *T. castaneum*.
3.4. Immunostaining of tissue sections

We were unable to find evidence of CDA transcription in tracheal cells using any of the above-mentioned anti-sense probes, even though CDA1 and CDA2 expressions have been demonstrated in embryonic tracheal cells of D. melanogaster (Luschnig et al., 2006; Wang et al., 2006). We therefore decided to attempt immunodetection of CDA proteins. Immunofluorescence staining of serial cryosections from the abdomen of a last-instar larva using anti-TcCDA1 antibodies indicated strong expression of an immunoreactive protein in epidermal cuticle (not shown) and tracheae, while control sections treated with pre-immune serum did not yield any fluorescence signals (Fig. 6).

In immunoblots of crude extracts from T. castaneum tissues the anti-TcCDA1 antibody revealed a single major band at 64 kDa (data not shown), consistent with the observed size of the recombinant protein. The absence of detectable CDA transcripts in tracheal tissue with abundant CDA protein could be explained by precise timing and rapid clearing of CDA mRNAs in combination with a slow turnover rate for the corresponding proteins in the extracellular matrix.

3.5. Phenotypes of insects injected with dsRNA for CDAs

3.5.1. TcCDA1 and TcCDA2

To determine the roles, if any, of TcCDA1 and TcCDA2 in insect development, dsRNAs for TcCDA1 and TcCDA2 were injected into penultimate-instar larvae, last-instar larvae and pharate pupae. Selective knockdown of each transcript was confirmed by RT-PCR. The animals injected with either dsRNA exhibited very similar lethal phenotypes and severe developmental blocks at the larval–larval, larval–pupal and pupal–adult molts. The pronotum was split open, but the animals failed to shed their old cuticles (Fig. 7). TcCDA2 has two isoforms as a result of alternative splicing of exon 3 (Dixit et al., 2008). To investigate the functions of individual isoforms, exon-specific RNAi was done by injecting dsRNAs for either one of the two alternatively spliced exons of TcCDA2 into penultimate-instar larvae. Injection of dsRNAs for TcCDA2a or TcCDA2b did not affect any molt, including larval–larval, larval–pupal and pupal–adult molts, but did result in abnormal adult behavior or phenotype. Adults developing from larvae injected with dsRNA for TcCDA2a suffered locomotor impairment as a result of their inability to articulate the femoral–tibial joints. They could...
neither bend nor unbend these joints, depending on the pre-established leg position. They eventually died, about 2 wk after molting, probably due to the stress associated with locomotor impairment. In contrast, animals injected with dsRNA for TcCDA2b had normal control of leg joints, but had an alligator-like crinkling of the general cuticle, primarily in the elytra (Fig. 8).

Parental RNAi was carried out to observe the effects of down-regulation of specific CDA transcripts on embryonic and early larval morphology and development. Adult females (1-month-old) were injected with dsRNA for TcCDA1, TcCDA2a or TcCDA2b or a mixture of the dsRNAs for TcCDA2a and TcCDA2b. Female fecundity and viability of embryos were followed over a period of several weeks. Normal oviposition rates were observed (5–10 eggs per day per female) after injection of dsRNAs for TcCDA1, TcCDA2a or TcCDA2b, but egg-hatch was reduced. In the first week, there was a reduction in egg-hatch rates following all these dsRNA treatments (>80%) with the exception of dsRNA for TcCDA2b. However, after one week, insects injected with TcCDA1 showed a rapid recovery from the parental RNAi effect, and hatching efficiency returned to normal levels. On the contrary, most eggs (>90%) laid by females injected with dsRNA for TcCDA2a or a mixture of dsRNAs for TcCDA2a and TcCDA2b failed to hatch for up to several weeks after dsRNA injections. Unhatched eggs appeared to contain fully developed larvae. Even those hatchlings that did manage to escape from the eggshell died without molting to the second instar (Fig. 9). On the other hand, all eggs laid by females injected with dsRNA for TcCDA2b hatched, and the hatchlings successfully molted to the second larval instar. This result suggests a splice-variant-specific requirement for TcCDA2a during embryonic development.

3.6. TcCDA3 through TcCDA9

Preliminary experiments indicated that injections of dsRNAs for TcCDA3–9 into larvae (up to 400 ng per insect) did not result in any observable morphological or developmental abnormalities. Therefore, PCR was carried out to monitor the effects of dsRNA injections on transcript depletion. Total RNA was isolated from larvae three to
four days after dsRNA injections. The transcript levels for most of the target TcCDA3–9 genes were significantly decreased (data not shown).

TcCDA6 through TcCDA9 are all expressed specifically in the gut. We considered the possibility that our failure to see a phenotypic effect upon knockdown of at least some individual TcCDA transcripts might have been due to functional redundancy among the gut TcCDAs. Therefore, we injected a mixture of these four dsRNAs into larvae and then monitored transcript levels by real-time RT-PCR. The transcript levels of individual CDAs were decreased by about 50–100-fold (Fig. 10), yet we could detect no adverse effects on the appearance, behavior or survival of dsRNA-treated insects. The function, if any, of these larval gut-specific group V CDAs thus remains a mystery.

4. Discussion

We have studied the expression patterns of the nine members of the CDA gene family in T. castaneum. Each of these nine genes clearly belongs in either of two distinct categories, based on tissue specificity. TcCDA1–5 are expressed predominantly in non-gut tissues, whereas TcCDA6–9 are expressed primarily in the midgut. The non-gut CDAs all encode proteins with chitin-binding domains (ChBDs), while the midgut-specific ones all lack this domain.

4.1. Midgut-specific CDAs

Like TcCDA6–9 described in this work, the CDA-like protein TnPM-P42, found associated with the midgut peritrophic membrane (PM) in Trichoplusia ni (Guo et al., 2005), falls into group V of the insect CDAs, all known members of which lack not only the ChBD, but also the low density lipoprotein receptor a (LDLa)-like domain (Dixit et al., 2008). The TnPM-P42 gene is expressed only in gut tissue and not in integument or fat body, and only during feeding stages. Thus, group V CDAs appear to comprise a gut-specific subgroup. The gut-specificity of CDA group V expression, as well as our finding that expression of these genes is confined to the larval feeding periods, suggests a possible role for these proteins in the modification of PM-associated chitin. TnPM-P42 was not shown to have catalytic activity, but McCDA1, a midgut-specific, group V chitin deacetylase from the Noctuid moth, Mamestra configurata, was recently expressed in Escherichia coli and shown to have chitin deacetylase activity, the first such demonstration for any insect CDA (Toprak et al., 2008).

There were some interesting differences in the expression profiles of group V genes along the length of the gut. TcCDA6 transcripts were found in the anterior and posterior midgut, whereas transcripts for TcCDA7 were found mostly in the posterior midgut. On the other hand, transcripts for TcCDA8 and 9 were found throughout the midgut and even in the hindgut. The latter two
transcripts were less abundant in the posterior midgut compared to the anterior and middle midgut.

There were also differences in the types of cells in which the CDA genes were expressed. TcCDA8 transcripts were found in regenerative and columnar cells of the midgut and in hindgut epithelial cells. TcCDA9 was expressed in midgut columnar cells and in hindgut epithelial cells. In the case of TcCDA7, the transcripts were found exclusively in columnar cells. The significance of these subtle differences in distribution of transcripts for these CDA in the gut is unclear. One possible explanation is that each of the encoded CDAs may be designed to function in different parts of the gut, leading to differences in the properties of the PM such as the chitin/chitosan ratio, type of PM-associated proteins, or PM permeability. The differences in PM permeability along the length of the midgut may allow enzymes to equilibrate at different rates with those in the ectoperitrophic space. A gradient of protein concentrations, as well as recirculation of digestive enzymes along the ectoperitrophic space, have been proposed (Terra and Ferreira, 1994; Bolognesi et al., 2008). Alternatively, the CDAs may exhibit differences in their enzymatic properties such as pH optima or substrate specificities.

Most of the CDA transcripts have an exclusively apical localization within the cells. It is possible that the polysomes that are translating these transcripts are transported to the endoplasmic reticulum close to the apical sides of these cells. A similarly asymmetric distribution of transcripts has been described for chitin synthase B (CHS-B) in Manduca sexta gut cells (Zimoch and Merzendorfer, 2002).

Sequence differences among genes were sufficient to enable us to design dsRNAs that were expected to be target-gene-selective in their action, and to not reduce the levels of transcripts of other CDA-like genes or splice variants. Injection of dsRNAs for TcCDA6 through TcCDA9 did not yield any observable phenotypes, even though the extent of knockdown was quite substantial. Injection of higher concentrations of dsRNAs or mixtures of dsRNAs also failed to generate any visible phenotypic defects. dsRNAs for the gut-specific TcCDAs6, 7, 8 and 9 were co-injected during the young larval stage and transcript levels were measured 3 days after injection, revealing that the extent of knockdown was nearly 50--100-fold in most cases. Nevertheless, no adverse phenotypic effects were observed, precluding new insight into the possible functions of these genes. Possible roles for the gut CDAs (in addition to modification of PM-associated chitin) include modification of chitin present in the diet or modification of chitooligosaccharides that are inhibitory to the action of chitinases. Yet another explanation is that some of the chitin deacetylases could have a role in defense against microbial pathogens that contain chitin. Removal of acetyl groups of chitin may alter properties of cell walls of fungi. Absence of RNAi effects suggests that the gut CDAs may not be essential for survival under laboratory rearing conditions, but they still could contribute to the robustness of the insects, or even be essential for survival, under less ideal environmental conditions or pathogenic stresses.

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**Fig. 5.** In situ hybridization analysis of cell type and tissues expressing TcCDA6, 7, 8, and 9 in the gut of T. castaneum. All pictures are arranged to show the hemolymph side above and the lumen side below. Arrowheads indicate positive signal. Stage, tissue and cell type are further described in Table 1. Scale bar indicates 50 μm. Indicated cell types include regenerative cells (A and C, upper arrows), endocrine cell (A, lower arrow) and columnar cell (C, lower arrow).

**Table 1** Summary of the results of in situ hybridization experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Stage</th>
<th>Tissue and cell type</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcCDA1</td>
<td>Pharate pupa</td>
<td>Hindgut epithelial cells and subsets of exoskeletal epidermis</td>
<td>4A-D</td>
</tr>
<tr>
<td>TcCDA2</td>
<td>Pharate adult</td>
<td>Entire exoskeletal epidermis</td>
<td>4E-H</td>
</tr>
<tr>
<td>TcCDA3</td>
<td>Pharate adult</td>
<td>Thoracic muscle</td>
<td>4I-K</td>
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<td>TcCDA4</td>
<td>Pharate pupa</td>
<td>Early imaginal appendages</td>
<td>4L</td>
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<td>TcCDA6</td>
<td>Late larva</td>
<td>Midgut regenerative cells and endocrine type of cells</td>
<td>5A</td>
</tr>
<tr>
<td>TcCDA7</td>
<td>Late larva</td>
<td>Midgut columnar cells</td>
<td>5B</td>
</tr>
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<td>TcCDA8</td>
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<td>5C and D</td>
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<td>TcCDA9</td>
<td>Late larva</td>
<td>Midgut columnar cells and hindgut epithelial cells</td>
<td>5E and F</td>
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* TcCDA5 not determined.
4.2. Non-gut CDAs

The biological necessity for having multiple CDA-like proteins in insects is unclear. However, the differences in tissue specificity and developmental patterns of expression of some CDAs provide clues concerning their possible functions. The *T. castaneum* group I CDAs, TcCDA1 and TcCDA2, are orthologs of proteins encoded by the *D. melanogaster* serpentine and vermiform genes, which have been

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**Fig. 6.** Immunofluorescence staining of larval tracheae with anti-CDA1 antibodies. Serial cryosections (transverse, 20 μm) from the abdomen of a late larva were incubated with anti-CDA1 antibodies that were detected by Alexa Fluor® 488 conjugated anti-rabbit IgG antibodies. To visualize cell nuclei, the sections were additionally stained with DAPI. (A) Tracheal staining by anti-CDA1 antibodies (green) and nuclear staining by DAPI (blue). (B) Control staining of the immediately proximal section treated with pre-immune serum and stained with DAPI. (C, D) Transmitted light micrographs corresponding to the fluorescence images shown in (A) and (B), respectively, showing tracheal branches in the abdominal region of the cryosections (encircled). A similar pattern of staining was observed when CDA2 antibodies were used (data not shown).

**Fig. 7.** Phenotypes of animals injected with dsRNA for TcCDA1 and TcCDA2. dsRNAs for TcCDA1 and TcCDA2 (200 ng per insect; n = 20) were injected into young larvae, last-instar larvae and pharate pupae. dsRNA for EGFP was injected as the control. Injection of dsRNAs for TcCDA1 or TcCDA2 affected all molts, including the larval–larval, larval–pupal and pupal–adult. Insects treated with these dsRNAs did not shed their old cuticles and were trapped in the exuviae.
shown to be expressed in tracheal lining cells and epidermis, and to have a role in tracheal tube morphogenesis and in determining body shape during embryogenesis (Luschnig et al., 2006; Wang et al., 2006). The presence of transcripts for TcCDA1 and TcCDA2 in the carcass, but not in the gut, is consistent with a similar role for these genes in T. castaneum cuticle maturation. TcCDA1 and TcCDA2 are also expressed at all developmental stages, reflecting a continuous requirement for cuticle synthesis in this species. Functional conservation between D. melanogaster and T. castaneum CDAs is further supported by our finding that CDA1-like immunoreactivity is strongly expressed and in the tracheal system of larvae.

The persistence of a representative member of each of the five groups of CDAs and the alternatively spliced transcripts of TcCDA2 and TcCDA5 in the four insect species that we have analyzed, D. melanogaster, A. gambiae, A. mellifera and T. castaneum (Dixit et al., 2008), suggests that each of these CDA proteins serves an important and conserved function. Groups II, III and IV are represented by only one gene in each group, although there are two TcCDA5 isoforms arising from alternatively spliced transcripts. Each of these transcripts has a distinctly different developmental pattern and/or tissue specificity of expression. TcCDA3 is the only gene with a peak of expression at the pupal stage. This gene is expressed in a tissue presumed to be the developing adult thoracic muscle, where there is no evidence for the presence of chitin. It is possible that TcCDA3 acts on substrates other than chitin, such as glycoproteins, proteoglycans or mucopolysaccharides. TcCDA4 is expressed in early imaginal appendages. The expression profile of this gene indicates that it is unlikely to be involved in development beyond the larval stages. TcCDA5a and b are both expressed primarily in the carcass, but the former seems to be carcass-specific, while the latter shows additional expression in the larval midgut.

No adverse consequences were detected after RNAi for the non-gut genes TcCDA3, 4 or 5, but lethal phenotypes were observed in beetles treated with dsRNA for either TcCDA1 or TcCDA2 at every molt (Fig. 7). Even though the dorsal split was observed during molting, treated insects failed to shed their old cuticles. Presumably, in the absence of a functional TcCDA1 or TcCDA2, the newly synthesized cuticle lacks mechanical strength as a result of alterations in the degree of cross-linking to cuticular proteins. Such cross-links would normally confer sufficient strength, elasticity or...
other physical properties to enable the insect to emerge from the old cuticle. Even though the developmental expression patterns of TcCDA1 and TcCDA2 are similar and involve the same cell types, these genes apparently perform some non-redundant functions, because suppression of either transcript results in lethality at every molt. There do appear to be subtle differences in the types of epidermal cells that express these two CDA genes. Whether this is the basis of the requirement of both genes for normal molting remains to be determined. TcCDA2 but not TcCDA1 influences egg-hatch, as shown by our parental RNAi experiments. However, this effect was predominantly due to a lack of the TcCDA2a isoform, because selective knockdown of the alternatively spliced TcCDA2b transcript did not bring about this effect.

Alternative exon sequences were sufficiently unique to enable design of splice-variant-selective dsRNAs. Differences in the phenotypes seen in insects injected with dsRNAs for the alternate exons of TcCDA2 at the larval stage suggest that there might be tissue specificity in alternative splicing (Fig. 8). While knockdown of both of the alternatively spliced TcCDA2 transcripts using a dsRNA for the common exons results in a lethal phenotype at every molt, dsRNAs specific for either one of the alternate exons did not result in molting failure or immediate lethality, suggesting that the TcCDA2 isoforms can complement each other’s vital functions, with the exception of egg-hatch. When larvae were injected with either one of these two exon-specific dsRNAs, the insects developed into adults, but they exhibited an abnormal appearance or behavior. TcCDA2a RNAi affected only the movement of the femoral–tibial joint, whereas down-regulation of TcCDA2b transcripts resulted in elytra with crinkled, “alligator-like” dorsal surfaces. Although the presence of chitin in the femoral–tibial joint has not been established, we speculate that the TcCDA2a but not the TcCDA2b isoform is expressed in this region. Similarly, we would predict that the TcCDA2b isoform is expressed in the elytra. These enzymes might differ in their activity or regulation to account for these observed differences. The knockdown of CDA transcripts in specific tissues may result in an altered ratio of chitosan-to-chitin, thus affecting the physical properties of the joints either directly, or indirectly by influencing the nature of the proteins that selectively bind to either chitin or chitosan.

The phenotypes observed after treatment with dsRNAs for TcCDA1 and TcCDA2 are similar to those we have reported previously with dsRNA for TcCHS-A. The chitin content of animals injected with the latter was drastically reduced (Arakane et al., 2005b). Luschnig et al. (2006) and Wang et al. (2006) have shown that in D. melanogaster mutants lacking either CDA1 and CDA2, chitin content was apparently not affected, but that the fibrous structure of the tracheal chitin cylinder as well as the shape and dimensions of the tracheal tube and body were altered. Our results indicate that down-regulation of transcripts for TcCDA1 or TcCDA2 results in a phenotype similar to that observed in the absence/reduction of chitin due to loss of CHS. Even though we have not analyzed the structure of the cuticle or tracheal tubes in animals injected with dsRNA for TcCDA1 or TcCDA2, we expect that there will be a loss of structural integrity of the chitin laminae overlying the epidermal cells or lining the tracheal cylinder. It is likely that there are specialized proteins that bind to deacetylated parts of chitin, whereas other proteins bind to fully acetylated stretches of the polysaccharide. These proteins may help to organize the chitin filaments of the epidermis and tracheal system and to provide the needed rigidity/elasticity to shape the underlying cells or tubes. Possible candidates include the membrane-bound proteins, retro-active (RTV) and knickkopf (KNK), which have been shown to affect cuticular integrity in D. melanogaster (Moussian et al., 2006).

In summary, we have demonstrated that multiple genes encoding CDA-like proteins are present and expressed in T. castaneum with differing developmental and tissue-specific patterns of expression. CDAs from several insects belonging to dipteran and coleopteran lineages fall into five groups by phylogenetic analysis, which suggests that these proteins have evolved over a long period of time. Results from RNAi studies reported here demonstrate that group I CDAs are the only ones with established biological functions. These enzymes are involved in molting, possibly by modulating cuticular and tracheal chitin in the embryonic stages as demonstrated in D. melanogaster (Luschnig et al., 2006; Wang et al., 2006). Our studies have extended the understanding of the role of CDAs in insect development by demonstrating that there are specific requirements for CDA1 and CDA2 at every molt and even during egg-hatch. We have also found that the alternatively spliced transcripts of TcCDA2 have specialized functions. TcCDA2a is required for movement of the femoral–tibial joint and TcCDA2b is required for proper formation of adult elytra. So far we have not been able to assign specific roles to CDAs belonging to groups II through V, because RNAi-induced transcript depletion for these genes does not result in any observable phenotypic or developmental defects. An investigation of the properties of the individual enzymes may reveal additional information regarding their physiological functions.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ibmb.2009.02.002.
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