7 Chitin Metabolism in Insects

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7.1. Introduction

"Chitin Metabolism in Insects" was the title of chapters in both the original edition of the *Comprehensive Insect Physiology, Biochemistry and Pharmacology* series published in 1985 and the follow-up *Comprehensive Molecular Insect Science* series in 2005 (Kramer *et al.*, 1985; Kramer and Muthukrishnan, 2005). Since 2005 substantial progress in gaining additional understanding of this topic has continued to take place, primarily through the application of the techniques of molecular genetics, functional genomics, proteomics, transcriptomics, metabolomics, and biotechnology to an assortment of studies focused on insect chitin metabolism. Several other reviews have also been published that have reported on some of the advances that have taken place (Dahiya *et al.*, 2006; Merzendorfer, 2006, 2009; Arakane and Muthukrishnan, 2010). Most interestingly, the list of genes and gene products found to be involved in insect chitin metabolism has been lengthened significantly. In this chapter we will highlight some of the more recent and important findings, with emphasis on results obtained from studies conducted on the synthesis, structure, physical state, modification, organization, and degradation of chitin in insect tissues, as well as the interplay of chitin with chitin-binding proteins, the regulation of genes responsible for chitin metabolism, and, finally, the targeting of chitin metabolism for insect-control purposes.

7.2. Chitin Structure and Occurrence

Chitin is the major polysaccharide present in insects and many other invertebrates as well as in several microbes, including fungi. Structurally, it is the simplest of the glycosaminoglycans, being a β(1→4) linked linear homopolymer of N-acetylglucosamine (GlcNAc, $[C_8H_{13}O_5N]_n$, where $n >> 1$). It serves as the skeletal polysaccharide of several animal phyla, such as the Arthropoda, Annelida, Molluska,

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and Coelenterata. In several groups of fungi, chitin replaces cellulose as the structural polysaccharide. In insects, it is found in the body wall or cuticle, gut lining or peritrophic matrix (PM), salivary gland, trachea, eggshells, and muscle attachment points. In the course of evolution, insects have made excellent use of the rigidity and chemical stability of the polymeric chitin to assemble both hard and soft extracellular structures such as the cuticle (exoskeleton) and PM respectively, both of which enable insects to be protected from the environment while allowing for growth, mobility, respiration, and communication. All of these structures are primarily composites of chitin fibers and proteins with varying degrees of hydration and trace materials distributed along the structures. The insolubility and structural complexity of the cuticle has limited its study. However, sclerotized cuticle can be modeled as an interpenetrating network of chitin fibers with embedded cross-linked protein and pigments. Both synthesis and degradation of chitin take place at multiple developmental stages in the cuticle and the PM. It is usually synthesized as portions of the old endocuticle and PM and trachaea are resorbed, and the digested materials are recycled. Although primarily composed of poly-GlcNAc, chitin also can contain a small percentage of unsubstituted (or *N*-deacetylated) glucosamine (GlcNAc) residues, making it a GlcNAc-GlcN heteropolymer (Muzzarelli, 1973; Fukamizo *et al.*, 1986). When the epidermal and gut cells synthesize and secrete a particular form of chitin consisting of antiparallel chains or alpha-chitin, the chains are assembled into microfibrils and then into sheets. As layers of chitin are added, the sheets are cross-oriented relative to one another at a constant angle to form a helicoidal bundle (known as the Bouligand structure), which can contribute to the formation of an extremely strong, plywood-like material.

Although there is no doubt that there are strong noncovalent interactions between chitin and chitin-binding proteins, there is only weak indirect evidence that there are covalent interactions between them. The evidence so far for direct involvement of chitin in cross-links to proteins has been inconclusive. Results of solid state NMR and chemical analyses have indicated the presence of trace levels of aromatic amino acids in chitin preparations, suggesting that those amino acids were there because they were involved in protein cross-links with chitin (Schaefer *et al.*, 1987). Additional spectroscopic evidence for glucosamine–catecholamine adducts derived from chitin–protein cross-links in cuticle was obtained using electrospray mass spectrometry and tandem mass spectrometry (Kerwin *et al.*, 1999). However, those observations have not been investigated further. More direct evidence for chitin–protein cross-links from studies of intact cuticle instead of degraded or digested samples is needed before the precise nature of the covalent interactions of cuticular proteins with chitin fibers can be resolved (Demolliens *et al.*, 2008).

Alpha-chitin fibers, because of their hydrophilic nature, are generally highly hydrated. Chitin dehydration via impregnation of hydrophobic proteins probably contributes to tissue stiffening and deplasticization (Vincent, 2009). In addition, the formation of a cross-linked and interpenetrating protein network in the dehydrated composite leads to additional hardening (Andersen, 2010); thus, chemical bonds surely play a crucial role in cuticle mechanics by increasing the load carried by the proteins and by providing a hydrophobic "coating" around the chitin nanofibers, thus preventing softening of the latter by water adsorption. Chitin nanofibrils probably form the initial template, similar to glass or carbon fiber mats in composite processing. Filler proteins and catechols are then secreted through the chitinous procuticle. Once oxidation of catechols to quinones and quinone methides has occurred, cross-linking and hardening of the extracellular matrix ensues. As sclerotization proceeds, water is progressively expelled. The precise role of water removal on the structural properties of the cuticle is not fully understood, in part because the effect of water on individual components of the composite is poorly understood, but some progress is starting to take place. Also, the individual contributions of chitin and protein to the mechanical properties are unknown. In the hydrated state, there is considerable variation in moduli reported for chitosan/chitin scaffolds (Wu *et al.*, 2006). There is a difference of several orders of magnitude in the stiffness of chitin/chitosan between the fully hydrated state, where it is present as a porous, water-saturated scaffold, and the dry state. To mimic the action of catechols to stiffen chitosan scaffolds, Wu *et al.* (2005) achieved a two-fold increase in stiffness after treatment of chitosan films with oxidized catechols. Although there was a significant increase in stiffness, it was less than the increase observed from insect cuticle tanning. Recently, dynamic mechanical analysis of insect cuticle during maturation revealed that while the water content has an important role in determining cuticle mechanical properties, the tanning reactions themselves contribute substantially to these properties beyond simply inducing dehydration (Lomakin *et al.*, 2011). Cuticle, whether tanned or untanned, increases in hardness while drying, but the increase is generally less than that observed from tanning alone.

7.3. Chitin Synthesis

Although extensive knowledge on the precise molecular mechanism of chitin synthesis is lacking, substantial progress has been made regarding the function and regulation of several genes involved in the chitin biosynthetic pathway. In the past 10 years, many genes coding for key enzymes of this pathway have been isolated and sequenced from various insect species. Analyses of their expression in different tissues during development have provided the first clues about their function. The availability of *Drosophila melanogaster*

(fruit fly) mutants defective in some of these genes, together with the ability to specifically silence their expression by RNAi in the fly and other species, has boosted our understanding of this process. Most progress has been made on chitin synthases (CHSs), which have been identified in a variety of organisms, including fungi, nematodes, mollusks, and insects. Amino acid sequence similarities have been the principal tools used for identifying CHSs, which form a subfamily within a larger group of the glycosyltransferases (family GT2) that catalyze the transfer of a sugar moiety from an activated sugar donor onto saccharide or non-saccharide acceptors (Coutinho *et al.*, 2003; Cantarel *et al.*, 2009). CHS has not been an easy enzyme to assay, which has made its study rather difficult. Traditionally, CHS activity is measured by a radioactive assay using [^{14}C]- or [^{3}H]-labeled UDP-GlcNAc as the precursor followed by quantification of insoluble radiolabeled chitin after acid precipitation. Alternately, a high-throughput non-radioactive assay is available, which involves binding of synthesized chitin to a wheat germ agglutinin (WGA)-coated surface, followed by detection of the polymer with a horseradish peroxidase–WGA conjugate (Lucero *et al.*, 2002). Also, the direct incorporation of fluorescently labeled substrates, such as certain dansyl-UDP-GlcNAc analogs, may prove to be useful for developing fluorescence-based enzyme assays (Yeager and Finney, 2005). The paucity of information concerning the enzyme's biochemical and kinetic properties was mainly due to the inability to obtain active soluble CHS preparations. Recently, however, a purification and solubilization protocol has been developed, which allowed purifying CHS-B from the midgut of *Manduca sexta* (tobacco hornworm) as an active, oligomeric complex (Maue *et al.*, 2009). In addition, first attempts to heterologously express CHSs from protists and fungi in yeast systems turned out to be successful (Van Dellen *et al.*, 2006; Martinez-Rucobo *et al.*, 2009; Barreto *et al.*, 2010). These purification and expression protocols should facilitate greater progress in insect CHS studies in the future.

7.3.1. Sites of Chitin Biosynthesis

The epidermis and the midgut are two major tissues where chitin synthesis occurs in insects. Epidermal cells are responsible for the deposition of new cuticle during each molt, and the midgut cells are generally associated with the formation of the PM during feeding. Both the cuticle and the PM contain chitin microfibrils, which function as a matrix that binds numerous cuticle and PM proteins. However, chitin is associated with other tissues as well, including the head skeleton, foregut, hindgut, trachea, wing hinges, salivary glands, and mouthparts of adults and/or larvae. In early development, chitin is additionally found in the cuticle of the developing larva within the embryo, as well as in the extra-embryonic serosal cuticle and the eggshells (Wilson and Cryan, 1997; Moreira *et al.*, 2007; Rezende *et al.* 2008). In general, it is assumed that the cells closest to the site where chitin is found are responsible for its biosynthesis. However, this interpretation is somewhat complicated by the fact that assembly of chitin microfibrils occurs in the extracellular space and is influenced by proteins that organize their deposition (see section 7.6).

7.3.1.1. Chitin synthesis in the epidermis and tracheal system Chitin is a major constituent of the cuticle, the outermost layer of insects, which serves as an exoskeleton and protects against various harming agents. Within the cuticle, chitin is mainly found in the procuticle, with higher amounts in the endocuticle than in the exocuticle, but is absent from the epicuticle (Sass *et al.*, 1994). Chitin deposition in the cuticle was recently reinvestigated in *Drosophila* embryos in an ultrastructural study using electron microscopy and gold-conjugated wheat germ agglutinin (gold-WGA), which binds to GlcNAc residues in chitin and glycoproteins (Schwarz and Moussian, 2007). In agreement with previous findings, gold particles could only be detected in the procuticle but not in the epicuticle. The gross architecture of the procuticle is established mainly by consecutive layers of chitin bundles of microfibrils embedded in a matrix of cuticle proteins. The orientation of a single lamina of chitin microfibrils can be twisted in relation to the neighboring layers above and below it by different angles in different insect species, giving rise to helicoidal or pseudo-orthogonal textures. Much of what we know on cuticle differentiation derives from ultrastructural studies of cuticle renewal during insect molting (Locke, 2001; Moussian, 2010). The classical concept of cuticle formation is based on three sequential phases. First, the envelope is laid down at the plasma membrane surface, usually above electron-dense plaques at the tips of microvilli, which were postulated to carry the chitin-synthesizing machinery (Locke, 1991). Then, the epicuticle is assembled beneath the envelope. Finally, the procuticle, which is considerably thicker than the other two layers, is assembled and oriented at the cell surface. However, a recent ultrastructural study of cuticle differentiation in *Drosophila* embryos revealed a slightly different picture, as envelope, epicuticle, and procuticle are partially formed in parallel in the first phase, then the cuticle thickens in the second phase, and in a third phase the chitin laminae acquire their final orientation (reviewed in Moussian *et al.*, 2006). Interestingly, the apical membrane of the embryonic epidermis does not form microvilli-like protrusions. Instead, it exhibits longitudinal microtubule-stabilized furrows, which were called apical undulae and are oriented perpendicular to the first layers of chitin microfibrils (Schwarz and Moussian, 2007). These apical undulae may have a crucial role in determining the orientation of chitin microfibrils, at least in the embryonic cuticle. Factors that affect the shape of the apical membrane, such as syntaxin 1A, indirectly affect chitin orientation, presumably by

interfering with the transport of proteins involved in cuticle or chitin assembly (Moussian *et al.*, 2007).

During embryogenesis, chitin synthesis also plays a role for tracheal morphogenesis. Chitin is also found in the tracheal cuticle, which has been thought to have a composition similar to that of the epidermal cuticle. This point needs clarification by direct chemical analysis of tracheal cuticle. However, it came as a surprise when two research groups reported independently that chitin forms a transient lumenal matrix during tracheal development in *Drosophila* embryos (Devine *et al.*, 2005; Tonning *et al.*, 2005). The lumenal chitin appears to be necessary to control tube size, diameter, and shape by orchestrating the function of surrounding tracheal cells. *Drosophila* genetics, in combination with different microscopic techniques, have proven most valuable in dissecting cuticle differentiation, and yielded a number of factors that are involved in controlling this process. Some of these factors will be discussed in more detail later in this chapter (see section 7.6.).

In addition to the histochemical detection of chitin with colored or fluorescent compounds that bind to chitin with different specificities, the expression of CHS genes has been used to identify chitin-synthesizing tissues. *CHS* gene expression was analyzed in various insects by RT-PCR, Northern blots, and *in situ* hybridization. These studies clearly demonstrated that epidermal and tracheal cells express *CHS* genes, and hence confirmed that these epithelia are sites of chitin biosynthesis. The first cDNA encoding an insect chitin synthase was identified by Tellam and colleagues (2000) in *Lucilia cuprina* (sheep blow fly), and termed *LcCHS1*. RT-PCR using total RNA preparations from the carcass and trachea indicated expression of *LcCHS1* in these tissues. *In situ* hybridization revealed a strong signal for the *LcCHS1* mRNA in a single layer of epidermal cells immediately underneath the procuticle. Similar results were obtained for the expression of homologous *CHS* (also referred to as *CHS-A*) genes from other insect sources, including *D. melanogaster*, *M. sexta*, *Spodoptera frugiperda* (fall armyworm), and *T. castaneum* (red flour beetle) (Ibrahim *et al.*, 2000; Gagou *et al.*, 2002; Zhu *et al.*, 2002; Arakane *et al.*, 2004; Bolognesi *et al.*, 2005; Hogenkamp *et al.*, 2005; Zimoch *et al.*, 2005). In agreement with the detection of chitin in eggs, *CHS* gene expression was reported during embryogenesis by RT-PCR using RNA from *Lucilia sericata* and *Aedes aegypti* eggs (Moreira *et al.*, 2007; Tarone *et al.*, 2007; Rezende *et al.*, 2008).

7.3.1.2. Chitin synthesis in the midgut Chitin is a component of the insect PM, and accounts for about 3–13% (w/w) of its dry weight. There are two patterns of PM production in insects. Type I PMs are synthesized and delaminated throughout the entire midgut epithelium. Type II PMs are formed as a continuous lining of the gut, which is produced by a specialized region of the anterior midgut called the cardia (Lehane, 1997). The most detailed picture of chitin synthesis and its association with PM proteins has emerged from observations using transmission, scanning electron, light, and fluorescence microscopy (TEM, SEM, LM, and FM, respectively) in three lepidopteran species; namely, *Ostrinia nubilalis* (European corn borer), *Trichoplusia ni* (cabbage looper), and *M. sexta* (Harper and Hopkins, 1997; Harper *et al.*, 1998; Harper and Granados, 1999; Wang and Granados, 2000; Hopkins and Harper, 2001; Zimoch and Merzendorfer, 2002). TEM in combination with gold-WGA staining demonstrated that the PM of *O. nubilalis* contains a fibrous, chitin-containing matrix that appears first at the tips of the microvilli of the midgut epithelial cells just past the stomadeal valves, and is rapidly assimilated into a thin PM surrounding the food bolus (Harper and Hopkins, 1997). The PM becomes thicker and multilayered in the middle and posterior regions of the midgut. The orthogonal lattice of chitin meshwork is slightly larger than the diameter of the microvilli. SEM and LM studies revealed that the PM delaminates from the tips of the microvilli. This observation suggests that microvilli serve as sites (and possibly as templates) for the organization of the PM by laying down a matrix of chitin microfibrils, which associate with PM proteins. A similar pattern of delamination of the PM containing both chitin and intestinal mucins was demonstrated in larvae of *T. ni* (Harper and Granados, 1999; Wang and Granados, 2000). Incorporating WGA into the diet can interrupt formation of the PM. WGA-fed *O. nubilalis* larvae exhibited an unorganized PM, which was multilayered and thicker than the normal PM (Hopkins and Harper, 2001). WGA was actually associated with the PM as well as with the microvillar surface, as revealed by immunostaining with antibodies specific for WGA. Because there was very little WGA within the epithelial cells, the interaction of WGA appears to be extracellular. Presumably, WGA interferes with the formation of the organized chitin network and/or the association of PM proteins with the chitin network, leading to a reduced protein association with the PM (Harper *et al.*, 1998). There was also extensive disintegration of the microvilli, and the appearance of dark inclusion bodies, as well as apparent microvillar fragments within the thickened multilayered PM. Species such as *M. sexta*, which secrete multiple and thickened PMs that are somewhat randomly organized, tolerated WGA better, and sequestered larger amounts of WGA within the multilayered PM (Hopkins and Harper, 2001).

As in the case of epidermal chitin synthesis, RT-PCR, Northern blots and *in situ* hybridization demonstrated the expression of a gene encoding a midgut specific CHS form. This gene was originally identified in *D. melanogaster*, but its expression and function were characterized in *Aedes aegypti*, *M. sexta*, and *T. castaneum* (Ibrahim *et al.*, 2000; Zimoch and Merzendorfer, 2002; Arakane *et al.*, 2004), as well as more recently in *S. exigua*, *S. frugiperda*, and *O. nubilalis* (Bolognesi *et al.*, 2005; Kumar *et al.*, 2008; Khajuria *et al.*, 2010).

The first evidence that midgut cells express a *CHS* gene was provided by Ibrahim *et al.* (2000) for female *Ae. aegypti* mosquitoes dissected several hours after a blood meal. *In situ* hybridizations with an antisense RNA probe for *AeCHS2* (*CHS-B*) in blood-fed mosquitoes localized the mRNA at the apical site of midgut epithelial cells. Likewise, *in situ* hybridization with an antisense RNA probe to *MsCHS2* (*CHS-B*) from *M. sexta* revealed that high levels of transcripts for this gene are present in apical regions of the columnar cells of the anterior midgut but completely absent in the epidermis or tracheal system of *M. sexta* larvae (Zimoch and Merzendorfer, 2002). An antibody to the catalytic domain of the *M. sexta*, CHS was used to detect the enzyme in midgut brush border membranes at the extreme apical ends of microvilli, a result suggestive of some special compartment or possibly apical membrane-associated vesicles. In line with its assumed role in PM formation during feeding stages, *MsCHS2* mRNA was detected in the midgut of feeding but not of starving or molting larvae (Zimoch *et al.*, 2005). Similar expression patterns were reported for *S. exigua*, *S. frugiperda*, and *O. nubilalis* by RT-PCR (Bolognesi *et al.*, 2005; Kumar *et al.*, 2008; Khajuria *et al.*, 2010). In *S. frugiperda*, chitin could be stained in the PM only when *SfCHS2* (*CHS-B*) expression was detectable (Bolognesi *et al.*, 2005). From the finding that *TcCHS2* (encoding *CHS-B*) expression was observed in *T. castaneum* only in late larvae and adults, but not in pupal stages, where chitin is synthesized during cuticle formation, it was concluded that *TcCHS-B* functions in the course of PM formation in the midgut (Arakane *et al.*, 2004), a hypothesis further substantiated by RNAi experiments (Arakane *et al.*, 2005; see also section 7.3.4.3).

7.3.2. Chitin Biosynthetic Pathway

It has been assumed that most parts of the chitin biosynthetic pathway of insects would be similar or identical to the Leloir pathway, which has been worked out extensively in fungi and other microbes (**Figure 1**). This appears to be the case except for some minor details (Palli and Retnakaran, 1999). The source of the sugar residues for chitin synthesis can be traced to fat body glycogen, which is acted upon by glycogen phosphorylase. Glucose-1-P produced by this reaction is converted to trehalose, which is released into the hemolymph. Trehalose, the extracellular source of sugar in many insect species, is acted upon by a trehalase, which is widely distributed in insect tissues, including the epidermis and gut, to yield intracellular glucose (Becker *et al.*, 1996). This view was recently substantiated by Chen *et al.* (2010), who showed that the RNAi-induced knockdown of the expression of two trehalase-encoding genes, *SeTre1* and *SeTre2*, caused downregulation of the CHS-encoding genes *SeCHS1* and *SeCHS2*, respectively, and led to reduced chitin levels in the cuticle and the PM. The conversion of glucose to fructose-6-P needed for chitin synthesis involves two glycolytic enzymes present in the cytosol. These enzymes are hexokinase and glucose-6-P isomerase, which convert glucose to fructose-6-P. From the latter, the chitin biosynthetic pathway branches off, with the first enzyme catalyzing this branch being glutamine fructose-6-phosphate amidotransferase (GFAT, E. C. 2.6.1.16), which might be thought of as the first committed step in amino sugar biosynthesis. The conversion of fructose-6-P to GlcNAc phosphate involves amination, acetyl transfer, and an isomerization step, which moves the phosphate from C-6 to C-1 (catalyzed by a phospho-N-acetylglucosamine mutase). The conversion of this compound to the nucleotide sugar derivative follows the standard pathway and leads to the formation of an UDP-derivative of GlcNAc, which serves as the substrate for CHS. The entire chitin biosynthetic pathway is outlined in **Figure 1**. The involvement of dolichol-linked GlcNAc as a precursor for chitin was proposed quite some time ago (Horst, 1983), but that hypothesis has received very limited experimental support (Quesada-Allue, 1982). At this point, this possibility remains unproven. Similarly, the requirement for a primer to which the GlcNAc residues can be transferred also remains speculative. Based on the model for glycogen biosynthesis, which requires glycogenin as the primer (Gibbons *et al.*, 2002), CHS or an associated protein may fulfill this priming function. Because each sugar residue in chitin is rotated 180° relative to the preceding sugar, which requires CHS to accommodate a alternating "up/down" configuration, another precursor, UDP-chitobiose, has been proposed to be a disaccharide donor during biosynthesis (Chang *et al.*, 2003). Evaluation of radiolabeled UDP-chitobiose as a CHS substrate in yeast, however, revealed that it was not incorporated into chitin. Nevertheless, by testing monomeric and dimeric uridine-derived nucleoside inhibitors as mechanistic probes Yeager and Finney (2004) found a 10-fold greater inhibition for the dimeric inhibitor than the corresponding monomeric inhibitor. However, both inhibitors bound with low affinities in the millimolar range. The stereochemical problem in chitin synthesis of adding GlcNAc to the growing chain in two opposite orientations resembles the situation with hyaluronan synthases (HAS), which produce the hyaluronan polymer from two different monosaccharides, UDP-GlcNAc and UDP-glucuronic acid. HASs are "dual action" glycosyltransferases that accomplish hyaluronan biosynthesis by two substrate-binding and active sites (Weigel and DeAngelis, 2007). As class I HASs are related to chitin synthase, two binding sites for alternating GlcNAc orientations may also occur in CHSs.

7.3.2.1. Key enzymes The biosynthetic pathway of chitin can be thought of as consisting of three subreactions. The first set leads to the formation of the amino sugar, GlcNAc, the second to its activated form UDP-GlcNAc, and the last yields the polymeric chitin

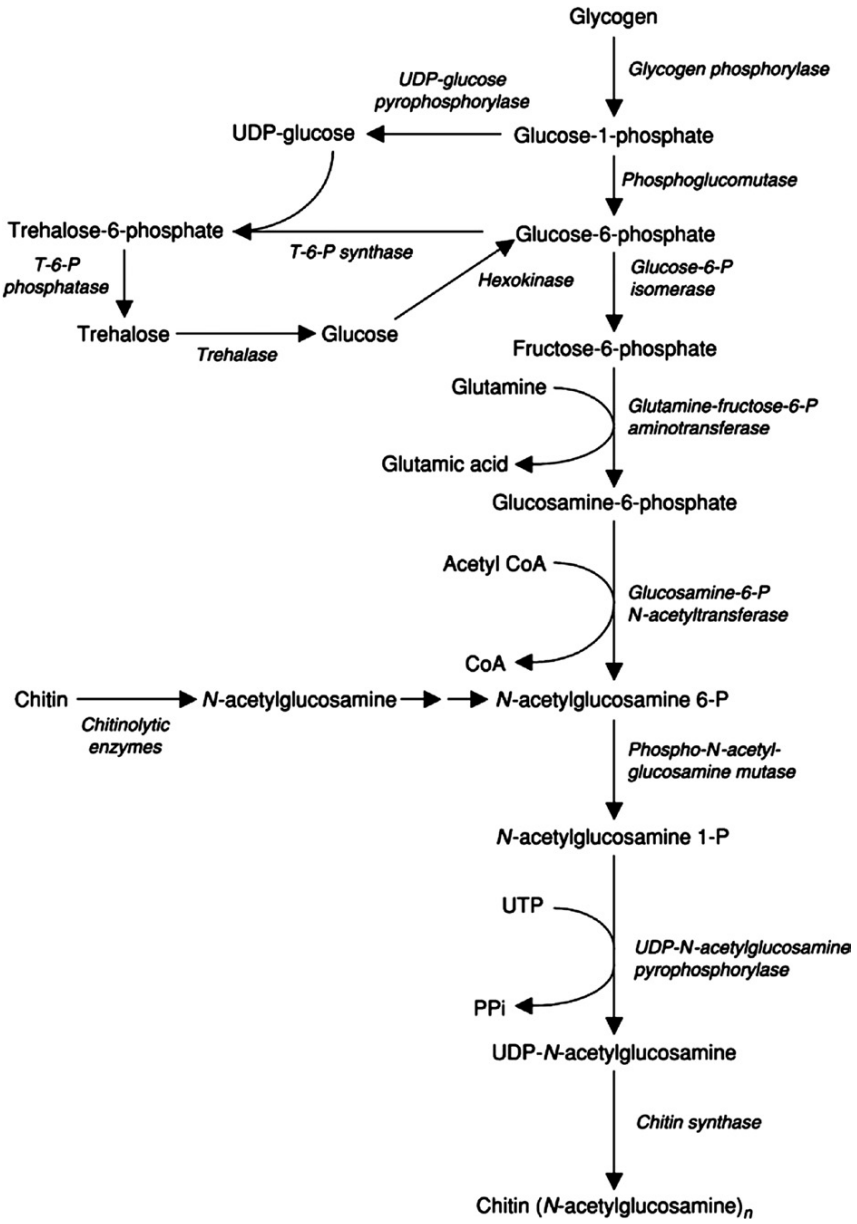

Figure 1 Biosynthetic pathway for chitin in insects starting from glycogen, trehalose, and recycled chitin.

from the amino sugar. The rate-limiting enzyme in the first subreaction appears to be glutamine-fructose-6-phosphate aminotransferase (GFAT, EC 2.6.1.16), which is found in the cytosol. The critical enzyme in the second subreaction is UDP-N-acetylglucosamine pyrophosphorylase (UAP, EC 2.7.7.23), which is also found in the cytosol, and that in the last subreaction is CHS (EC 2.4.1.16), which is localized in its active form at the plasma membrane. Not surprisingly, these three enzymes appear to be major sites of regulation of chitin synthesis.

7.3.2.2. Function and regulation of GFATs In *Drosophila*, two genes encoding GFAT (*Gfat1* and *Gfat2*) have been identified (Adams *et al.*, 2000; Graack *et al.*, 2001). Both of these genes are on chromosome 3, but they are present at different locations. Their intron–exon organizations are different, as are the amino acid sequences of the encoded proteins. GFAT consists of two separate domains: an N-terminal domain that has both glutamine-binding and aminotransferase motifs identified in GFATs from other sources; and a C-terminal domain with both fructose-6-phosphate binding and isomerase motifs. *Gfat1* is expressed in embryos in the developing tracheal system, cuticle-forming tissues, and corpora cells of larval salivary glands (Graack *et al.*, 2001). The major regulation of GFAT1 appears to be post-translational. When *Gfat1* was expressed in yeast cells, the resulting enzyme was feedback-inhibited by UDP-GlcNAc, and

was stimulated by protein kinase A (PKA). Even though it has not been demonstrated that there is a phosphorylated form of GFAT1 which is susceptible to feedback inhibition by UDP-GlcNAc, this possibility remains viable. However, the situation may be complicated by overlapping kinase activities, as recently a novel, highly conserved phosphorylation site was identified, which accounts for *in vivo* phosphorylation of human GFAT1 overexpressed in insect cells by protein kinases other than PKA (Li *et al.*, 2007). Examination of a mutant that mimics phosphorylation at this site demonstrated that the modification stimulates glucosamine-6-phosphate-synthesizing activity, but has no effect on UDP-GlcNAc inhibition.

Another insect species in which *Gfat1* function and regulation has been analyzed in more detail is *Aedes aegypti*. The mosquito gene has no introns, and the promoter appears to contain sequence elements related to ecdysteroid response elements (EcRE) as well as E74 and Broad complex Z4 elements. E74 and Broad complex Z4 proteins are transcription factors known to be upregulated by ecdysone (Thummel, 1996). Two Gfat1 transcripts with different sizes were observed in Northern blot analyses of RNA from adult females, and their levels increased further after blood feeding (Kato *et al.*, 2002). Since ecdysteroid titers increase following blood feeding, it is possible that this gene is under the control of ecdysteroids, either directly or indirectly. Silencing of gene expression by dsRNA injection additionally revealed that GFAT1 is necessary for chitin synthesis in the course of PM formation in the midgut, which occurs in female mosquitoes in response to a blood meal (Kato *et al.*, 2006). Feedback inhibition of chitin synthesis by UDP-GlcNAc has also been reported in this study, indicating that the mosquito enzyme is likely to be regulated in a manner similar to the *Drosophila* enzyme.

7.3.2.3. Function and regulation of UAPs Insect genomes usually possess only one gene encoding UDP-GlcNAc pyrophosphorylase (UAP). The known exception is *T. castaneum*, which has two *UAP* genes. The first phenotypes for defects in the *UAP* gene were described in *Drosophila*, where this gene was alternately termed *mummy*, *cabrio*, or *cystic*, according to three phenotypes that were identified in independent genetic screens for genes involved in tracheal, epidermal, and CNS development (Nüsslein-Vollhard *et al.*, 1984; Hummel *et al.*, 1999; Beitel and Krasnow, 2000). While *cystic* was originally recognized to be important for tracheal morphogenesis and tube size control, *mummy* and *cabrio* mutants were reported to exhibit severe defects in cuticle formation and CNS development of the embryo. Eventually, all of these genes were shown to be allelic by Araújo *et al.* (2005) and Schimmepfeng *et al.* (2006), and the gene encoding UAP is now consistently named *mummy* (*mmy*). Interestingly, the *mmy* mutant phenotype is similar to that of the so-called "halloween" mutants, which fail to produce the morphogenetic hormone 20-hydroxyecdysone (Gilbert, 2004). UAP functions in apical extracellular matrix formation by producing UDP-GlcNAc needed for chitin synthesis and for protein glycosylation. Consequently, deletion or defects in *mmy* can lead to the complete absence of chitin in the cuticle and tracheal lumen, as evidenced by a lack of WGA staining in mutant embryos or larvae carrying a single nucleotide substitution leading to the exchange of glycine to valine at position 261 (Tonning *et al.*, 2006). Moreover, the epithelial organization is affected in *mmy* mutants, as adherens junctions between epidermal cells appear wider than in wild type embryos, and the characteristic ladder-like structure of the septate junctions is missing. Additionally, a membrane-integral septate junction component (Fas3) is delocalized in the mutant, indicating that *mmy* may have an additional function in proper localization of membrane-bound septate junction components (Tonning *et al.*, 2006). Expression of *mmy* is hormonally regulated in apical extracellular matrix-differentiating tissues, and selectively upregulated when chitinous material is deposited during development. It is possible that the enzyme is also regulated at the post-translational level by uridine, as this nucleic acid base was shown to be an effective inhibitor for the yeast enzyme (Yamamoto *et al.*, 1980). In *Ae. aegypti* the gene encoding UAP is constitutively expressed throughout all life stages, and blood feeding does not significantly alter mRNA levels (Kato *et al.*, 2005). The cDNA was cloned and the enzyme expressed as a recombinant enzyme, allowing determination of substrate specificity. The enzyme uses GlcNAc-1-P as a substrate, but it also exhibited low activity when incubated with Glc-1-P. In *T. castaneum* two UAP isoforms were identified, which share 60% identical amino acids but differ significantly in their developmental and tissue-specific expression patterns, as well as in function, as revealed by RNAi studies (Arakane *et al.*, 2010). While the knockdown of *TcUAP1* transcripts caused arrested development at the larval–larval, larval–pupal, and pupal–adult molts, knockdown of *TcUAP2* retarded larval growth or resulted in pupal paralysis. Results of chitin-staining experiments in cuticle and PM indicated that chitin deposition is prevented only when *TcUAP1*, but not when *TcUAP2*, expression was blocked. However, both genes are essential for beetle development and survival. TcUAP1 obviously is required for chitin synthesis in the course of cuticle and PM formation, whereas TcUAP2 appears to have other critical roles, presumably in glycosylation of proteins.

7.3.3. Chitin Synthases: Organization of Genes and Biochemical Properties

7.3.3.1. Number and organization of *CHS*-encoding genes *CHS* genes from numerous unicellular and filamentous species of fungi have been isolated and

characterized (reviewed in Roncero, 2002; Horiuchi, 2009). Genome sequencing revealed three to nine *CHS* genes per individual fungal species, which were categorized into seven gene classes. In contrast, nematode, mollusk, crustacean, and insect genomes contain only one or two *CHS* genes per species (**Figure 2A**). Since Tellam *et al.* (2000) published the first cDNA sequence for a CHS from *Lucilia cuprina* (sheep blowfly), cDNA sequences for CHSs have been reported from numerous invertebrates, and the availability of an increasing number of genome sequences has provided additional information on *CHS* genes. Nematode *CHS*s were from two filarial pathogens, *Brugia malayi*, and *Dirofilaria immitis*, the plant parasite *Meloidogyne artiellia* and *Caenorhabditis elegans* (Harris *et al.*, 2000; Harris and Fuhrman, 2002; Veronico *et al.*, 2001). In both *D. immitis* and *M. artiella*, there is currently only evidence for a single gene, but in *B. malyai* and *C. elegans*, two genes were identified. *CeCHS1* is required for eggshell formation, whereas *CeCHS2* is needed to form the grinder in the ectodermal pharynx (Zhang *et al.*, 2005). CHS sequences from crustaceans and chelicerates were deduced from the *Daphnia pulex* and *Ixodes scapularis* genome projects, both of which have two *CHS* genes. Likewise, all insect genomes available so far harbor two *CHS* genes, which have been divided into class A and class B genes, with the latter appearing to be the more ancient form (**Figure 2A**). The insect species from which complete cDNAs for CHSs have been isolated are *L. cuprina* (Tellam *et al.*, 2000), *D. melanogaster* (Gagou *et al.*, 2002), *Ae. aegypti* (Ibrahim *et al.*, 2000), *Anopheles quadrimaculatus* (Zhang and Zhu, 2006), *M. sexta* (Zhu *et al.*, 2002), *S. frugiperda* (Bolognesi *et al.*, 2005), *Spodoptera exigua* (Chen at al., 2007; Kumar *et al.*, 2008) and *T. castaneum* (Arakane *et al.*, 2004). Genomic sequences from *Anopheles gambiae*, *T. castaneum*, *D. melanogaster* and *M. sexta*, which were deduced from available genome projects or obtained by individual nucleotide sequencing, were used to determine the organization of *CHS* genes in these species (**Figure 3**).

The overall structure of *CHS* genes varies among different insect species and gene classes. The numbers of exons range from 8 to 24, with lengths from 46 bp to more than 3000 bp. While most genes contain at least some exons that contribute longer ORFs, the lepidopteran *CHS* genes appear more fragmentized, because they contain a higher number of shorter exons (Zhu *et al.*, 2002; Kumar *et al.*, 2008). Insect *CHS-A* genes have two mutually exclusive exons, resulting in two mRNA splice variants. Both exons code for 59 amino acids comprising extracellular, transmembrane and intracellular domains, the latter being located near the carboxyl terminus of the protein. One major difference between the two exons that are alternately spliced is that all of the *b* forms code for segments that have a site for *N*-linked glycosylation just before the transmembrane helix,

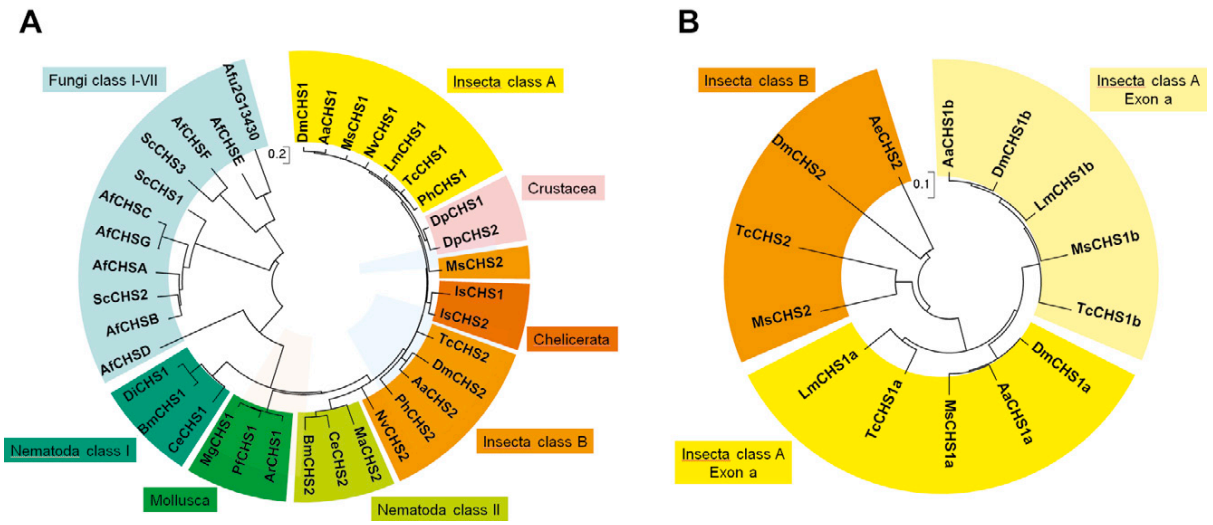

Figure 2 Phylogenetic trees of CHS proteins and conserved exons. The trees are based on ClustalW alignments and were performed with the neighbor joining method. Bootstrap tests of phylogeny were performed with 10,000 replications., (A) Bootstrap consensus tree of CHS proteins from fungi, nematodes, mollusks and arthropods., (B) Bootstrap consensus tree of exons a and b found in class A CHS genes, and the corresponding region of class B CHS genes. *Aa*, *Aedes aegypti* (XP_001662200.1, XP_001651163.1); *Af*, *Aspergillus fumigatus* (XP_749322.1, XP_746604.1, XP_748263.1, XP_752630.1, CAA70736.1, XP_747364.1, XP_754184.1, XP_755676.1); *Ar*, *Atrina rigida* (AAY86556.1); *Bm*, *Brugia malayi* (XP_001898491.1, AAS77206.1); *Ce*, *Caenorhabditis elegans* (NP_492113.2, NP_493682.2); *Dm*, *Drosophila melanogaster* (AAG22215.3, AAF51798.2); *Di*, *Dirofilaria immitis* (AAG39382.1); *Dp*, *Daphnia pulex* (NCBI_GNO_134384, NCBI_GNO_326244); *Is*, *Ixodes scapularis* (XP_002405234.1; XP_002405231.1); *Lm*, *Locusta migratoria* (ACY38589.1); *Ms*, *Manduca sexta* (AAL38051.2, AAX20091.1); *Ma*, *Meloidogyne artiellia* (AAG40111.1); *Mg*, *Mytilus galloprovincialis* (ABQ08059.1); *Nv*, *Nasonia vitripennis* (XP_001602290.1, XP_001602181.1); *Pf*, *Pinctada fucata* (BAF73720.1); *Ph*, *Pediculus humanus corporis* (XP_002423597.1, XP_002423604.1); *Sc*, *Saccharomyces cerevisiae* (NP_014207.1, NP_009594.1, NP_009579.1); *Tc*, *Tribolium castaneum* (AAQ55059.1, AAQ55061.1).

whereas none of the *a* forms do. The precise physiological significance of alternate exon usage and potential glycosylation in CHS expression and function is still unknown, even though it is clear that there is developmental regulation of alternate exon usage (see section 7.3.4.2.).

7.3.3.2. Modular structure of chitin synthases CHSs are members of family GT2 of the glycosyltransferases (Coutinho *et al.*, 2003), which generally utilize a mechanism where inversion of the anomeric configuration of the sugar donor occurs. The protein fold (termed GT-A) for this family is considered to be two associated β/α/β domains that form a continuous central sheet of at least eight β-strands. The GT-A enzymes share a common ribose/metal ion-coordinating motif (termed DxD motif) as well as another carboxylate residue that acts as a catalytic base. The general organization of CHSs has been deduced from a comparison of amino acid sequences of these enzymes from several species of insects, nematodes, and yeasts (Merzendorfer, 2006). These enzymes have three distinguishable domains: an N-terminal domain with moderate sequence conservation among different species and containing several transmembrane segments; a central catalytic domain that is believed to be orientated toward the cytoplasm; and a C-terminal domain with multiple transmembrane segments (**Figure 4**). The catalytic domain contains several highly conserved stretches including GT2 consensus sequences, which have been suggested to be involved in binding of UDP, the donor and acceptor saccharides, and the product. They include sequences similar to the Walker A and B motifs for binding of the nucleotide moiety (Walker *et al.*, 1982), sequences similar to the DXD and G(X)4(Y/F)R motifs likely involved in substrate binding, the GEDRxx(T/S) motif at the acceptor binding site, and the (Q/R)XXRW motif involved in product binding. The latter motif is present only in processive GTs. While the transmembrane segments in the N-terminal domain show different patterns among different insect species, the transmembrane segments in the C-terminal domain are remarkably conserved both with respect to their location and the spacing between adjacent transmembrane segments. Particularly striking is the fact that five such transmembrane segments are found in a cluster immediately following the catalytic domain, and two more segments are located closer to the C-terminus. The cluster of five transmembrane helices spanning the membrane, known as 5-TMS (5-transmembrane spans), has been suggested to be involved in the extrusion of the polymerized chitin chains across the plasma membrane to the exterior of the cell, as has been proposed for the extrusion of cellulose (Richmond, 2000). Following the last transmembrane helix of the 5-TMS, a sequence similar to the (S/T)WGT(R/K) motif found in fungal chitin synthases is located at the extracellular site. The CHSs derived from class A genes were predicted to have a coiled-coil region following the 5-TMS region (Zhu *et al.*, 2002; Arakane *et al.*, 2004). Also, all of the genes encoding the class A CHSs have two alternate exons (corresponding to alternate exon 7 of *D. melanogaster*, exon 8 of *T. castaneum*, exon 6 of *A. gambiae*, and the exon 20 homolog of *M. sexta*). The alternate exons are located on the C-terminal side of the 5-TMS region, and encode the next transmembrane segment and flanking

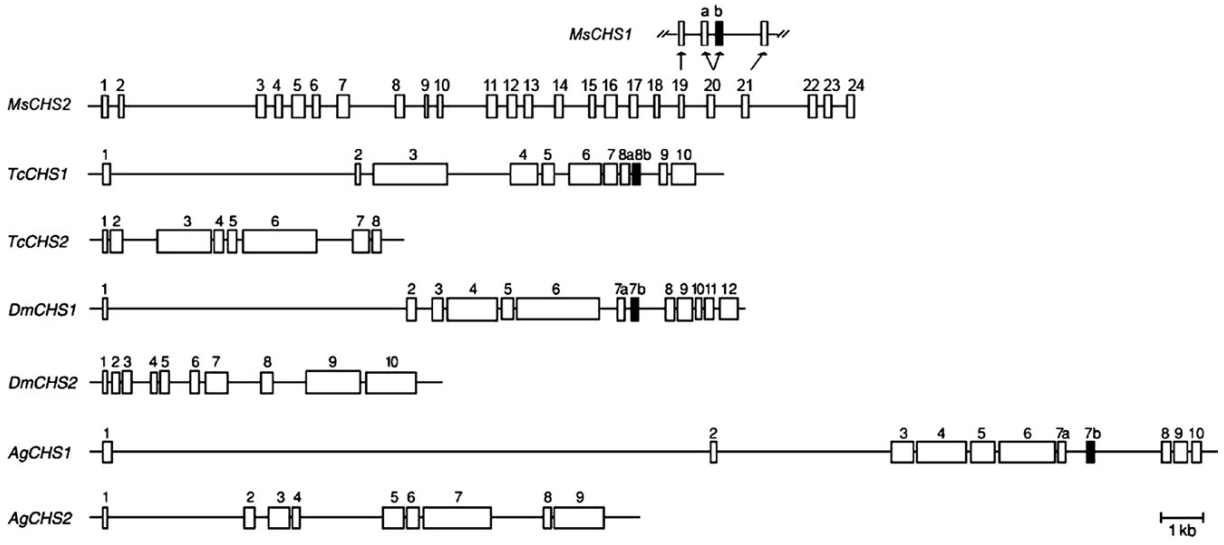

Figure 3 Schematic diagram of the organization of insect *CHS-A* and *CHS-B* genes. The exon–intron organization was deduced from comparisons of available cDNA and genomic sequences. Boxes indicate exons; lines indicate introns. The second of the two alternative exons (8b) of *TcCHS1*, *DmCHS1 (7b)*, *AgCHS2 (7b)*, and *MsCHS1* (homolog of exon 20 from *MsCHS2*, *20b*) are indicated as closed boxes (modified according to Arakane *et al.*, 2004, and Hogenkamp *et al.*, 2005). *Ag*, *Anopheles gambiae*; *Dm*, *Drosophila melanogaster*; *Ms*, *Manduca sexta*; *Tc*, *Tribolium castaneum*.

sequences (**Figure 4**). The alternate exon-encoded regions of the CHS proteins differ in sequence by as much as 30%, and most of these differences are in the regions flanking the transmembrane segment. This finding suggests that the proteins may differ in their ability to interact with cytosolic or extracellular proteins, which might regulate chitin synthesis, transport, and/or organization. An attractive hypothesis is that these flanking sequences may influence the plasma membrane location of a CHS by interacting with cytoskeletal elements, or perhaps by generation of extracellular vesicles involved in chitin assembly.

7.3.3.3. Zymogenic properties of chitin synthase In numerous fungal and insect systems, chitin synthesis is activated by trypsin and other serine proteases, suggesting that CHS is produced as a zymogen (reviewed in Merzendorfer, 2006). However, there is very little knowledge on the significance of this phenomenon in arthropods. In yeast, which has three *CHS* genes, proteolytic activation by trypsin has been reported for Chs1 and Chs2 (Cabib and Farkas, 1971; Sburlati and Cabib, 1986). With Chs3, the situation is more complicated, as the zymogenic properties appear to depend on UDP-GlcNAc and additional proteins, such as the regulatory subunit Chs4 (Choi *et al.*, 1994; Ono *et al.*, 2000). However, no endogenous proteinase has been identified that would cleave the CHS zymogen. The zymogenic properties of yeast Chs2 and Chs3 have been reinvestigated recently. For Chs2 it was demonstrated that trypsin acts on a soluble protease that, once activated, stimulates Chs2 activity (Martínez-Rucobo *et al.*, 2009). Another study reports a role of the CaaX proteinase Ste24 in chitin synthesis (Meissner *et al.*, 2010). Ste24 is a membrane-integral protease of the endoplasmic reticulum, which is known to be involved in proteolytic maturation of the yeast mating factor **a**. Yeast two-hybrid studies have indicated, however, that Ste24 interacts with Chs3. The interacting domain was mapped to a cytosolic region that immediately precedes the catalytic domain of Chs3. Deletion of *ste24* led to Calcofluor white (CFW) resistance and decreased chitin levels, whereas overexpression led to CWF hypersensitivity and increased chitin levels. The CFW phenotype of wild type cells could be rescued by expressing the homologous gene from *T. castaneum* in *ste24*Δ cells, indicating orthologous functions. Although Ste24 directly binds to Chs3, it appears not to be a substrate of the protease. Instead, genetic experiments indicate that Chs4 is cleaved by Ste24 in a prenylation-dependent manner at its C-terminal CaaX motif, and that this processing is required for intracellular transport of Chs3 to the plasma membrane (Meissner *et al.*, 2010). Addition of trypsin to cell-free extracts obtained from different insect species such as *Diaprepes abbreviatus*, *M. sexta*, *T. castaneum*, and S*tomoxys calcitrans* leads to the stimulation of chitin synthesis by

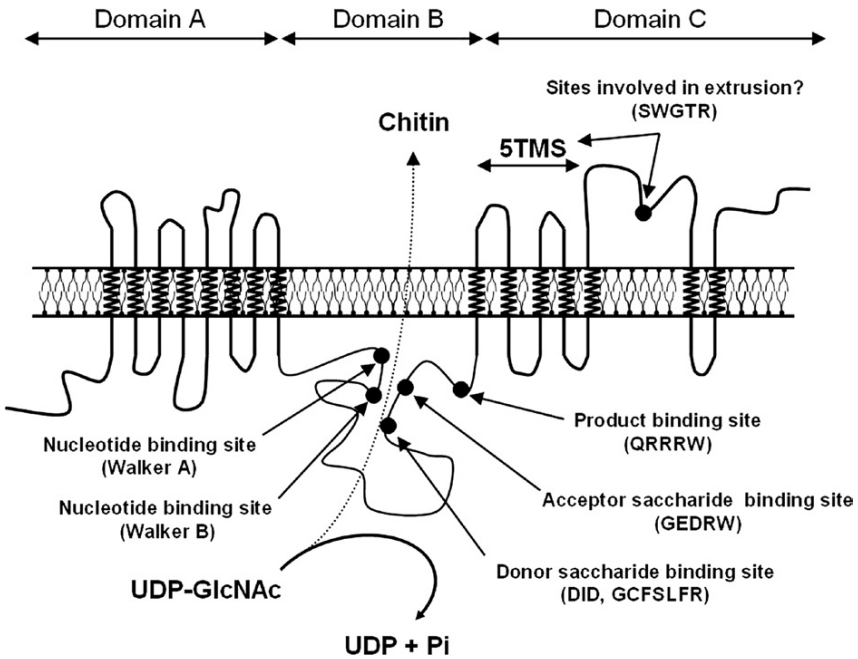

Figure 4 Structural model of the tripartite domain organization of *Drosophila* DmCHS1. The N-terminal domain A of *Drosophila* contains 8 transmembrane helices (TMHs), but this number varies, between different insect species, from 7 to 10. The central domain B is facing the cytoplasm, and forms the catalytic site. The ensuing domain C contains 5+2 TMHs, and the C-terminus is located at the extraplasmatic site. Generally, all TMHs are highly conserved in insects. Putative motifs involved in nucleotide, donor, acceptor, and product binding are indicated. The polymer is synthesized in the cytosol and the chitin chain needs to be translocated across the membrane, a process that might require the 5TMS cluster and the extrusion motif SWGTR.

30–50% (Cohen and Casida 1980b; Mayer *et al.*, 1980; Ward *et al.*, 1991; Zimoch *et al.*, 2005). In *Manduca*, trypsin-dependent stimulation of chitin synthesis was observed in crude midgut extracts, but not in membrane fractions of the midgut. However, it could be restored by re-adding a soluble fraction, suggesting that trypsin does not directly act on CHS but on a soluble protein that in turn stimulates chitin synthesis, which is similar to the recent finding of a soluble factor that activates yeast Chs2 (Zimoch *et al.* 2005; Martínez-Rucobo *et al.*, 2009). Attempts to directly purify and identify the soluble factor from *M. sexta* have failed. However, a chympotrypsin-like peptidase (MsCTLP-1) was identified in the midgut, which binds to the extracellular C-terminal domain of MsCHS2 (Broehan *et al.*, 2007). MsCTLP-1 is secreted into the gut lumen when the larvae start to feed, and it stimulates chitin synthesis after being proteolytically activated by trypsin (Broehan *et al.*, 2008). In line with the assumption that CHS enzymes are produced as zymogens, denaturing gel electrophoresis and immunoblotting of oligomeric CHS complexes purified from the midgut of *M. sexta* yielded a distinct pattern of CHS fragments, which is consistent with the assumption that the CHS monomer is cleaved twice during maturation, and that the resulting three fragments are part of the active enzyme (Maue *et al.*, 2009).

7.3.4. Chitin Synthases: Regulation and Function

7.3.4.1. Regulation of chitin synthase gene expression Insect class *CHS1*and *CHS2* genes encoding CHS-A and CHS-B enzymes, respectively, are expressed in different tissues and exhibit different patterns of expression during development. Although technical difficulties associated with the isolation of specific tissues free of other contaminating tissues (mainly trachea) initially hampered their unambiguous assignment, some general conclusions can be drawn from studies investigating *CHS* gene expression in different species and stages of development. *CHS* genes are expressed at all stages of growth, including embryonic, larval, pupal, and adult stages. *CHS1* genes are expressed over a wider range of developmental stages (Tellam *et al.*, 2000; Gagou *et al.*, 2002; Zhu *et al.*, 2002). *CHS2* genes are not expressed in the embryonic or pupal stages but are expressed in the larval stages, especially during feeding in the last instar and in adults, including blood-fed mosquitoes (Ibrahim *et al.*, 2000; Zimoch and Merzendorfer, 2002; Arakane *et al.*, 2004). These developmental differences in *CHS1* and *CHS2* expression prompted the assumption that insect *CHS* genes have specialized functions in different tissues or at different developmental stages. Accordingly, *LcCHS1* is expressed only in the carcass (larva minus internal tissues) and trachea of *L. cuprina*, but not in salivary gland, crop, cardia, midgut, or hindgut (Tellam *et al.*, 2000). In blood-fed female mosquitoes, a gene encoding a CHS-B enzyme is expressed in epithelial cells of the midgut (Ibrahim *et al.*, 2000). In *T. castaneum*, *TcCHS1* is expressed in embryos, larvae, pupae, and young adults, but not in mature adults (more than a month old), while *TcCHS2* is expressed at early and late larval stages as well as in adult stages, but not in embryos and pupae (Arakane *et al.*, 2004). Similar expression profiles were reported in two lepidopteran insects for the *CHS* genes *SeCHS1* in *S. exigua* and *SfCHS2* in *S. frugiperda* (Bolognesi *et al.*, 2005; Chen *et al.*, 2007). Tissue-specific expression was also investigated systematically in *M. sexta* (Zhu *et al.*, 2002; Hogenkamp *et al.*, 2005; Zimoch *et al.*, 2005). These studies demonstrated that *MsCHS1* is expressed in epidermal cells and in the tracheal system of larvae and pupae, whereas *MsCHS2* is expressed only in midgut tissue. Transcriptional regulation of *CHS* expression has been suggested to be mediated by ecdysone-responsive elements in the upstream regions of both *Drosophila* genes (Merzendorfer and Zimoch, 2003; also see section 7.7). For *krotzkopf verkehrt* (*kkv*), the gene encoding DmCHS1, another mode of transcriptional control appears to exist, as it is strongly upregulated in epidermal cells surrounding wounds caused by microinjection needles (Pearson *et al.*, 2009). What is remarkable, however, is that *kkv* uses a fundamentally different signaling pathway for wound activation than other genes involved in wound healing, such as *ddc* and *ple* coding for dopa decarboxylase and tyrosine hydroxylase, respectively. While the latter two genes require the JUN/FOS and grainy head (GRH) transcription factors to induce the wound response, transcriptional activities of the identified wound enhancer in the *kkv* upstream region was not affected by these transcription factors (Pearson *et al.*, 2009).

A more recent finding is that a chitinous serosal cuticle containing chitin is produced very early in development of *Aedes aegypti* (Rezende *et al.*, 2008). The serosal cuticle was shown to contain chitin and to be responsible for the development of desiccation tolerance of mosquito eggs. The serosal chitin is apparently the product of a class A CHS derived from the *CHS1* gene. This burst of chitin synthesis occurs long before organogenesis and before formation of the larval cuticle. Chitin has also been detected in eggs, eggshells, and ovaries of *Aedes aegypti* (Moreira *et al.*, 2007). In ovaries and eggs of *T. castaneum*, we have detected transcripts *of TcCHS-A* (our unpublished data).

To summarize, the analysis of expression patterns of the two *CHS* genes in different tissues and periods of development of several insects suggests that class A CHS enzymes are synthesized by epidermal cells when cuticle deposition occurs in embryos, larvae, pupae, and young adults, whereas class B enzymes are produced by the midgut epithelial cells in the course of PM formation in the larval and adult stages and is probably limited to these feeding stages.

7.3.4.2. Tissue-specific expression of alternate exons The genes encoding class A CHSs from *D. melanogaster*, *A. gambiae*, *Ae. aegypti*, *T. castaneum*, and *M. sexta*, but not the genes encoding class B CHSs, exhibit two alternately spliced exons, which are highly conserved between different insect species (**Figure 2B**). Each exon encodes a 59-amino acid segment following the 5-TMS region. This segment contains a 20-aa transmembrane region and flanking sequences. In addition, the presence of a predicted coiled–coil region immediately following the 5-TMS region in the CHSs encoded by those genes that have the alternate exons suggests a link between these two structural features, and the possibility of regulation of alternate exon usage. In agreement with this idea, transcripts containing either one of these exons have been detected in *T. castaneum*, *M. sexta*, and, more recently, in *Ae. aegypti* (Arakane *et al.*, 2004; Hogenkamp *et al.*, 2005; Zimoch *et al.*, 2005; Chen *et al.*, 2007; Rezende *et al.*, 2008). In *T. castaneum* embryos, transcripts with either exon 8a or 8b were detected, whereas in last instar larvae and prepupae, only exon 8a transcripts were present. In the pupal stage, however, transcripts with exon 8a or exon 8b were abundant, along with trace amounts of a transcript with both exons. In mature adults none of these transcripts were detected, whereas *TcCHS2* transcripts were easily detected (Arakane *et al.*, 2004). Injection of dsRNA specific to either one of both alternately spliced mRNAs revealed that splice variant 8a of *TcCHS1* is required for both the larval–pupal and pupal–adult molts, whereas splice variant 8b is required only for the latter. This finding, together with the relative amounts of these mRNAs, suggested that the splice variant with exon 8a contributes mostly to pupal cuticular chitin synthesis. Nevertheless, the variant with exon 8b appears to have a vital role in the emergence of the adult from the pupal cuticle, which obviously cannot be fulfilled by the exon 8a isoform alone. With regard to relative amounts of both splice variants, similar results were observed in fifth instar larvae of *M. sexta* (Hogenkamp *et al.*, 2005; Zimoch *et al.*, 2005). RT-PCR based detection of the alternately spliced transcripts at different developmental stages in the epidermis revealed that the ratio of mRNA levels for both splice variants varies during development, with *MsCHS1* exon 20a being more predominant generally than that with exon 20b (Hogenkamp *et al.*, 2005). Tracheal cells also express both variants of *MsCHS1*, but, in this tissue, *MsCHS1* with exon 20b is more abundant (Zimoch *et al.*, 2005). The latter finding was confirmed also in *L. migratoria* (Zhang *et al.*, 2010a). When *LmCHS1* expression was silenced by dsRNA injection into second instar nymphs, the locusts developed three distinct phenotypes exhibiting severe molting defects and eventually died. While the knockdown of *LmCHS1a* expression revealed phenotypes similar to those for *LmCHS1*, the knockdown of *LmCHS1* transcripts with alternate exon b, which is more abundant than the one with alternate exon a in tracheal tissue, led to crimped cuticles. The major finding of that study, however, was that the function of insect CHSs and their alternate exons are conserved in both holo- and hemimetabolous insects.

As discussed above, AaCHS1 accounts for chitin synthesis in the course of serosal cuticle formation in *Ae. aegypti* embryos, and two splice variants containing either exon 6a or exon 6b have been identified. Quantitative PCR showed that at the moment of serosal cuticle formation, splice variant 6a is predominantly expressed. The biochemical basis for a specific function, however, remains unknown.

7.3.4.3. Knockout mutants and RNAi reveal differential functions of *CHS* genes *Drosophila* mutants and RNAi experiments were extremely helpful in analyzing the differential functions of the two *CHS* genes. EMS mutagenesis and screening of the resultant mutant embryos for defects in epidermal differentiation and cuticular patterning helped to identify genes involved in controlling cuticle morphology (Jüergens *et al.*, 1984; Nüsslein-Volhard *et al.*, 1984; Wieschaus *et al.*, 1984; Ostrowski *et al.*, 2002). These genes include *kkv*, *knickkopf* (*knk*), *grainy head* (*grh*), retroactive (*rtv*), and zepellin (*zep*), some of which will be discussed later, in section 7.6. Mutations in these genes resulted in poor cuticle integrity and reversal of embryonic orientation in the egg to varying degrees. Generally, homozygous mutant embryos failed to hatch. When these mutant embryos were mechanically devitellinized, the cuticles became grossly enlarged, yielding the "blimp" phenotype. Interestingly, embryos derived from wild type females treated with diflubenzuron or lufenuron displayed a similar "blimp-like" phenotype when devitellinized, indicating that either genetic or chemical disruption of chitin deposition leads to this phenotype (Ostrowski *et al.*, 2002; Gangishetti *et al.*, 2009). Also, inhibition of chitin synthesis in *D. melanogaster* embryos induced by the CHS-specific inhibitor nikkomycine Z leads to cuticle defects, as they are similar to those observed in *Drosophila kkv* mutants (Tonning *et al.*, 2006; Gangishetti *et al.*, 2009). Ostrowski *et al.* (2002) characterized the *kkv* gene and identified it as a *CHS*-like gene, and Moussian *et al.* (2005a) finally showed that this class A CHS is essential for chitin synthesis in epidermal and tracheal cuticles. Careful analysis of the ultrastructure of the embryonic cuticle of *kkv* mutants confirmed that chitin synthesis by a class A CHS is essential for procuticle formation. Another interesting finding was that in *kkv* mutants the cuticle frequently detaches from underlying epidermal or tracheal cells, suggesting that chitin is also required for anchoring the cuticle (Moussian *et al.*, 2005a). In addition, the head skeleton of *kkv* mutant embryos is undersized and deformed, and sclerotization and pigmentation

are impaired. An unexpected finding was that *kkv* is required for tracheal tube expansion, which starts before chitin is actually deposited in the tracheal cuticle during embryogenesis (Devine *et al.*, 2005; Tonning *et al.*, 2005). This finding suggested that chitin has an additional function in early tracheal morphogenesis. Histological stainings with Congo red, WGA, or a fluorescence-labeled chitin-binding domain revealed that the tracheal lumen contains chitin cables before the tracheal cuticle is formed. The loss of lumenal chitin evidently affects subapical cytoskeletal organization of tracheal cells. Therefore, it was hypothesized that the chitinous lumenal matrix is sensed by tracheal cells to coordinate cytoskeletal organization, which controls the diameter size of the tracheae.

RNAi experiments to interfere with *CHS* expression and investigate CHS function have been performed in *T. castaneum*, *A. aegypti*, *A. gambiae*, *S. exigua*, *O. nubilalis*, and *Locusta migratoria*. In *T. castaneum*, injection of dsRNA for either *TcCHS1* or *TcCHS2* into young larvae, penultimate instar larvae, and prepupae resulted in a substantial knockdown of *TcCHS1* and *TcCHS2* mRNA levels. *TcCHS1*-specific RNAi disrupted larval–larval, larval–pupal, and pupal–adult molts, and caused a significant reduction in total chitin content (Arakane *et al.*, 2005). Interestingly, the phenotypes differed significantly depending on whether the insects were injected in the penultimate larval, last larval, or prepupal instar. The first of these groups failed to pupate and died without any splitting of the old larval cuticle, while the second group initiated the larval–pupal molt, but the pupae died without shedding their larval exuviae, although splitting of the old cuticle had occurred. The third group failed to carry out the pupal–adult molt, and died as pharate adults trapped in their pupal exuviae. In contrast, *TcCHS2* dsRNA injection into last instar larvae or prepupae had no effect on pupal or adult development, but when injected into penultimate instar, the larvae shrank in size and died without molting to the last instar. As the knockdown affected only immature or penultimate larvae, it was suggested that *TcCHS2* knockdown impairs chitin synthesis necessary for PM formation. Indeed, when midguts prepared from last instar larvae treated with dsRNA to *TcCHS1* and *TcCHS2* were stained with a fluorescein-conjugated chitin-binding domain, a fluorescent PM was detected in larvae treated with dsRNA for *TcCHS1*, but not following RNAi for *TcCHS2* (Arakane *et al.*, 2005). These experiments provided strong evidence that the *Tribolium* chitin synthase genes, *TcCHS1* and *TcCHS2*, have different functions, as they are involved in the synthesis of chitin in epidermal/tracheal cuticles and midgut PM, respectively. In a succeeding study, dsRNA for either one of the two *CHS* genes was injected into young and old female adults to investigate effects on egg-laying and embryogenesis (Arakane *et al.*, 2008). When dsRNA for *TcCHS1* was injected into young female adults (less than 10 days old), the beetles died without laying any eggs. When older female adults were injected, the beetles developed normally and laid eggs that were drastically reduced in chitin content and failed to hatch. The embryos had a twisted and enlarged blimp-like phenotype (**Figure 5**). Hence, *TcCHS1* appears to have roles in the development of embryos and adults, in addition to its role in cuticle formation. Interestingly, injection of dsRNA for *TcCHS2* into adults led to a significant reduction in chitin content of the PM, and caused death after 2 weeks. The female beetles treated with dsRNA for *TcCHS2* also failed to lay eggs, presumably due to starvation, because the fat body was significantly depleted due to autophagy (Arakane *et al.*, 2008). Similar to the situation in the beetle, RNAi experiments to knock down the *AaCHS2* transcripts of *Ae. aegypti* showed that it is required in female mosquitoes for the *de novo* synthesis of the PM after a blood meal (Kato *et al.*, 2006).

As the function of CHSs is vital for insect development and survival, RNAi-mediated knockdown of *CHS* genes could be a powerful approach in pest control. Based on the observation that chitin synthesis can be blocked by dsRNA injection in mosquitoes, Zhang *et al.* (2010b) developed a method to generate a systemic knockdown of *CHS* gene expression in *A. gambiae* larvae by feeding nanoparticles consisting of chitosan and dsRNA specific for the target gene. In line with the presumed function of both CHSs in cuticle and PM syntheses, the larvae became more susceptible to diflubenzuron and to Calcofluor White (CFW), when *AgCHS1* or *AgCHS2* expression, respectively, was inhibited. Another promising approach would be to feed bacteria expressing dsRNA to target genes, as was originally performed with *C. elegans* (Timmons and Fire, 1998). Indeed, when *E. coli* bacteria expressing dsRNA to *SeCHS1* were fed to larvae of the lepidopteran pest *S. exigua*, the survival rate was decreased as they advanced in development (Tian *et al.*, 2009).

7.4. Chitin Degradation and Modification

Insects must periodically replace their old cuticle with a new one because it is too rigid to allow for growth. Key to this process is the elaboration of the molting fluid with an assortment of chtitinases and proteases. Chitinases are among a group of proteins that insects use to digest the structural polysaccharide in their exoskeletons and gut linings during the molting process (Kramer *et al.*, 1985; Kramer and Koga, 1986; Kramer and Muthukrishnan, 1997; Fukamizo, 2000). Precise regulation of chitin metabolism is a complex and intricate process that is critical for insect growth, metamorphosis, organogenesis, and survival (Arakane and Muthukrishnan, 2010). Chitin content, which fluctuates throughout the life cycle of the insect, is directly influenced not only by chitin synthases

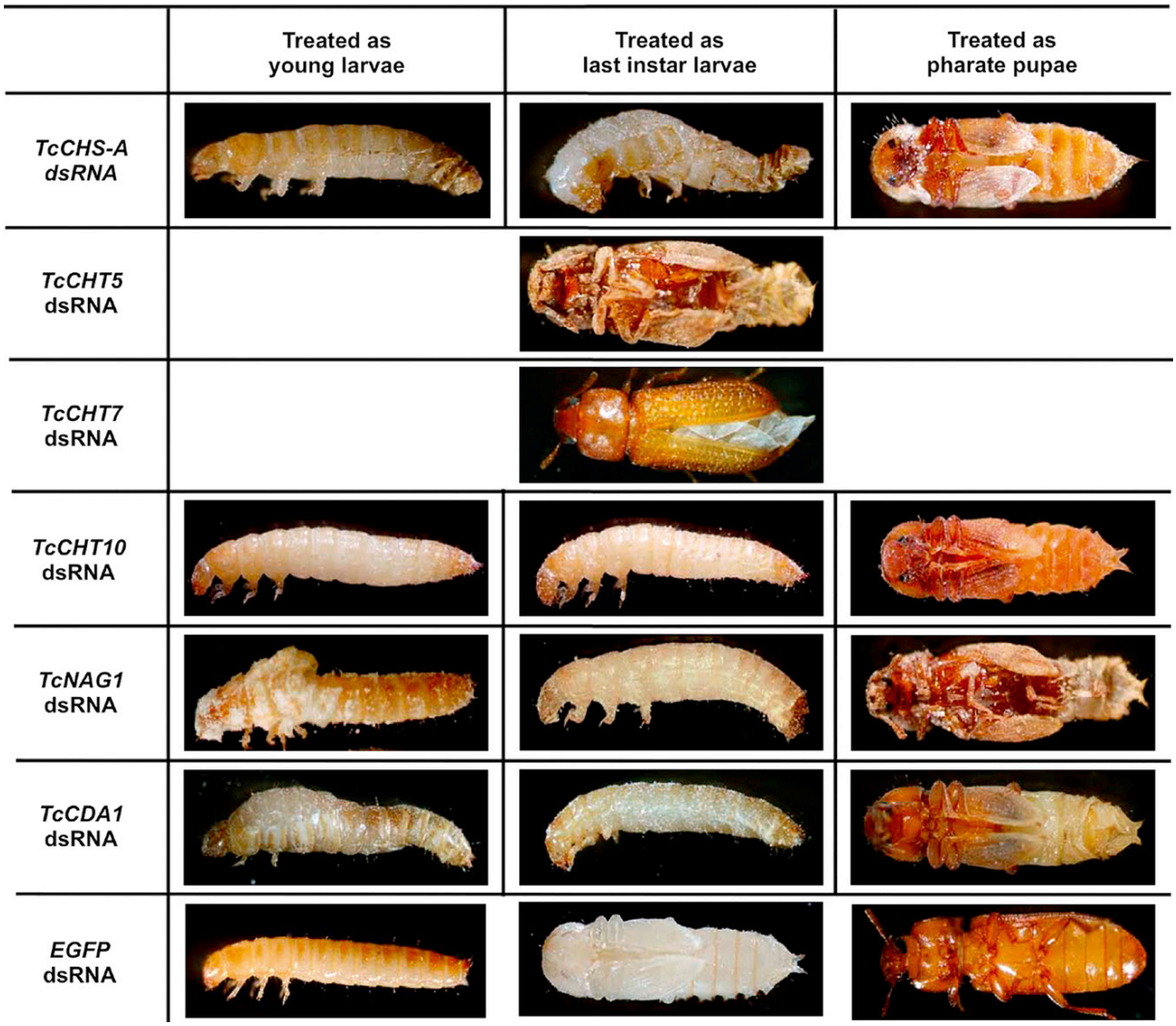

Figure 5 Phenotypes of *T. castaneum* larvae after RNAi for genes of chitin metabolism. dsRNAs for the indicated genes (200 ng per insect, $n = 20$) were injected into penultimate instar larvae (young larvae), last instar larvae, pharate pupae as indicated above each panel. All animals injected with dsRNA for *CHS-A*, *TcCHT10*, *TcNAG1*, and *TcCDA1* died at the ensuing molt. Unlike RNAi of *TcCHT10*, injection of dsRNA (200 ng per insect) for *TcCHT5* into penultimate instar and last instar larvae as well as pharate pupae prevented only adult molt. When dsRNA for *TcCHT7* (200 ng per insect) was injected into pharate pupae, normal phenotypes were observed in the pupal stage. However, unlike buffer-injected controls, *TcCHT7* dsRNA-treated insects failed to expand their adult elytra and their wings did not fold properly (modified from Zhu *et al.*, 2008c). Animals injected with control dsRNA for *EGFP* developed in a normal fashion, and had no mortality or abnormal phenotype.

(CHSs), but also by chitinases (CHTs, EC 3.2.1.14) and β-*N*-acetylglucosaminidases (NAGs, EC 3.2.1.52). Chitin is digested in the cuticle and PM to GlcNAc by a binary enzyme system composed of CHT and NAG (Fukamizo and Kramer, 1985a, 1985b; Filho *et al.*, 2002). The former enzyme from molting fluid hydrolyzes chitin into oligosaccharides, whereas the latter, which is also found in the molting fluid, further degrades the oligomers to the monomer from the non-reducing end. In some cases, additional unrelated proteins that possess one or more chitin-binding domains (CBD), but are devoid of chitinolytic activity, enhance degradation of chitin (Vaaje-Kolstad *et al.*, 2005). This system also probably operates in the gut during degradation of PM, and increases the porosity of the PM. It may also help in the digestion of chitin-containing prey (Bolognesi *et al.*, 2005; Khajuria *et al.*, 2010).

The precise control of chitin content is critical not only for the survival of the insect, but also for optimal function of individual anatomical structures such as wings and other appendages. In addition, modulation of the physical properties of chitin-containing structures of insects is accomplished, in part, by the deacetylation of the polysaccharide by chitin deacetylases (CDAs, EC 3.5.1.41). Partially deacetylated chitin may have different protein-binding and physical properties than those of chitin. The process of partially deacetylating chitin and the importance of this modification for insect growth

and development have emerged as new areas of research in insect molecular science (Luschnig *et al.*, 2006; Wang *et al.*, 2006; Arakane *et al.*, 2009).

7.4.1. Insect Chitinases

7.4.1.1. Cloning of genes encoding insect chitinases and chitinase-like proteins Since the first report of an insect chitinase, its cDNA and its corresponding gene from *M. sexta* (*MsCHT5*) (Koga *et al.*, 1987; Kramer *et al.*, 1993; Choi *et al.*, 1997; Kramer and Muthukrishnan, 1997), numerous insect *CHT* genes and cDNAs have been cloned and characterized from several insect species belonging to different orders, including dipterans, lepidopterans, coleopterans, hemipterans, and hymenopterans (Kramer and Muthukrishnan, 2005). The organization of most of these genes is very similar to that of *MsCHT5*, and most of the proteins display a domain architecture consisting of catalytic, linker, and/or chitin-binding domains (CBD) similar to MsCHT5. These genes/enzymes include epidermal chitinases from the silkworm *Bombyx mori* (Kim *et al.*, 1998; Abdel-Banat and Koga, 2001), the fall webworm *Hyphantria cunea* (Kim *et al.*, 1998), wasp venom from *Chelonus* sp. (Krishnan *et al.*, 1994), the common cutworm *Spodoptera litura* (Shinoda *et al.*, 2001), the fall armyworm *Spodoptera frugiperda* (Bolognesi *et al.*, 2005), a molt-associated chitinase from the spruce budworm *Choristoneura fumiferana* (Zheng *et al.*, 2002), and midgut-associated chitinases from the malaria mosquito *A. gambiae* (Shen and Jacobs-Lorena, 1997), yellow fever mosquito *Ae. aegypti* (de la Vega *et al.*, 1998; Khajuria *et al.*, 2010), the beetle *Phaedon cochleariae* (Girard and Jouanin, 1999), and the sand fly *Lutzomyia longipalpis* (Ramalho-Ortigao and Traub-Cseko, 2003), as well as several deduced from *Drosophila* genome data. A smaller linkerless fat body-specific chitinase from the tsetse fly *Glossina morsitans* (Yan *et al.*, 2002), and a very large epidermal chitinase with five copies of the catalytic domain and multiple chitin-binding domain from the yellow mealworm *Tenebrio molitor* (Royer *et al.*, 2002), have also been described.

Daimon *et al.* (2003) described a gene encoding another type of chitinase from the silkworm, BmCHT-h. The encoded chitinase shared extensive similarities with microbial and baculoviral chitinases (73% amino acid sequence identity to *Serratia marcescens* chitinase, and 63% identity to *Autographa californica* nuclear polyhedrosis virus chitinase). Even though this enzyme had the signature sequence characteristic of a family 18 chitinase, it had a rather low percentage of sequence identity with the family of insect chitinases. It was suggested that an ancestral species of *B. mori* acquired this chitinase gene via horizontal gene transfer from *Serratia* or a baculovirus. A gene encoding a CHT-like protein that is highly related to BmCHT-h was also found in the pea aphid, *Acyrthosiphon pisum* (Nakabachi *et al.*, 2010).

Only after the complete genome sequences became available was it recognized that insect genomes contain a large number of genes encoding CHT-like proteins widely divergent not only in their DNA and amino acid sequences, but also in the organization of their domains (Zhu *et al.*, 2004, 2008a; Arakane and Muthukrishnan, 2010). The number of *CHT* genes per insect genome is in the range of 7 to 24 for *D. melanogaster*, *A. gambiae*, *Ae. aegypti*, *B. mori*, *A. pisum*, and *T. castaneum*. This range excludes genes encoding CHT-like proteins whose consensus sequences are poorly conserved (see section 7.1.2.2; Khajuria *et al.*, 2010; Nakabachi *et al.*, 2010; Zhu *et al.*, 2004, 2008a). The 22 genes that encode CHTs or chitinase-like proteins (CHLPs) in *T. castaneum* have been divided into eight subgroups, based on sequence similarity and domain organization (**Figure 6**) (Arakane

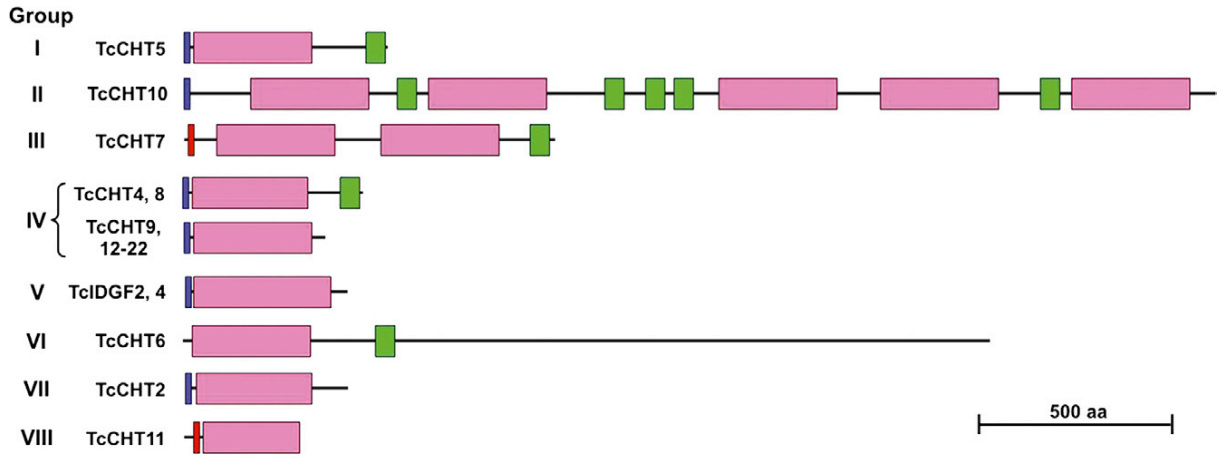

Figure 6 Domain organization of *T. castaneum* chitinase gene family. The program SMART was used to analyze the identified domains. TcCHT7 and TcCHT11 have a single transmembrane span at the N-terminal region. Blue boxes, signal peptide; pink boxes, catalytic domain; green boxes, chitin binding domain; red boxes, transmembrane span; lines, linker regions.

and Muthukrishnan, 2010). The chitinases in all insect species can be similarly classified into multiple groups (**Figure 7**). There is only one copy of the gene encoding a group I chitinase (CHT5) in all species except for *A. gambiae*, *Ae. aegypti*, and the human body louse *Pediculus humanus corporis*, in which obvious gene duplications have occurred, resulting in one to four additional copies (Khajuria *et al.*, 2010). To date, only one gene representing each of the groups II, III, VI, VII, and VIII (CHT10, 7, 6, and 11, respectively) has been found in various insect species. Interestingly, in addition to the group III *CHT* genes (CHT7s with two catalytic domains) identified in fully sequenced insect genomes such as *T. castaneum*, *D. melanogaster*, *A. gambiae*, *Ae. aegypti*, *C. pipiens*, *A. mellifera*, *N. vitripennis*, *A. pisum*, and *P. corporis*, orthologs have also been found in non-insect arthropod genomes, including those of the crustacean water flea *Daphnia pulex*, and the arachnid deer tick *Ixodes scapularis*, indicating an ancient origin of CHT7 that predates separation of the class Chelicerata more than five million years ago. Group IV appears to be the largest group in all insect species studied, containing 5, 8, 10, and 14 genes in *D. melanogaster*, *A. gambiae*, *Ae. aegypti*, and *T. castaneum*, respectively. The sole exception so far is *A. pisum*. No chitinase gene encoding a protein that belongs to this group was identified in *A. pisum* (Nakabachi *et al.*, 2010). In *T. castaneum*, most group IV *CHT* genes form a large cluster within a small region of the genome, suggesting the occurrence of a recent gene duplication event. Group V is composed of the genes encoding CHLPs such as imaginal disc growth factors (IDGFs). The number of genes for this group ranges from one in *B. mori* and *A. pisum* to as many as six in *D. melanogaster* (Arakane and Muthukrishnan, 2010; Nakabachi *et al.*, 2010).

7.4.1.2. Domain organization of insect chitinases Insect CHTs belong to family-18 glycosylhydrolases (the GH-18 super family) and function in hydrolysis of chitin in the exoskeleton and PM-associated chitin in the midgut, utilizing an endo-type cleavage mechanism during the molting process (Kramer and Muthukrishnan, 1997, 2005). Members of the CHT family contain a multidomain structural organization that includes a leader peptide and/or a transmembrane span, one to five catalytic domains (GH-18), multiple Ser/Thr-rich linker regions that are usually heavily glycosylated, and zero to seven six-cysteine-containing chitin-binding domains (CBDs) related to the peritrophin A domain (**Figure 6**; Royer *et al.*, 2002; Arakane *et al.*, 2003; Zhu *et al.*, 2008b). The catalytic domains of all insect CHTs, which are comprised of about 370 amino acids, assume a $\beta 8/\alpha 8$-barrel structure and possess signature motifs of family 18 glycosylhydrolases (Kramer *et al.*, 1993; Perrakis *et al.*, 1994, Terwisscha van Scheltinga *et al.*, 1994; de la Vega *et al.*, 1998; Fusetti *et al.*, 2002; Varela *et al.*, 2002; Tsai *et al.*, 2004; Arakane and Muthukrishnan, 2010). The consensus sequence for conserved motif I is KXX(V/L/I)A(V/L)GGW in the $\beta 3$-strand, where X is a non-conserved amino acid. The conserved motif II is FDG(L/F)DLDWE(Y/F)P, which is known to be located in or near the catalytic site ($\beta 4$-strand) of the enzyme, with a glutamate residue (E) being the most critical residue in this motif as the putative proton donor in the catalytic mechanism (Watanabe *et al.*, 1993; Lu *et al.*, 2002; Zhang *et al.*, 2002). Conserved motifs III and IV are MXYDL(R/H)G in the $\beta 6$-strand and GAM(T/V)WA(I/L)DMDD in the $\beta 8$-strand.

CBDs found in insect CHTs all belong to carbohydrate-binding module 14 (CBM-14, pfam 01607; ChtBD2 family = SMART family 00494, Boraston *et al.*, 2004). Insect CBDs are only about 60 amino acids long and have less conserved amino acid sequences, with the exception of the six cysteines and several aromatic residues whose relative locations are highly conserved (Jasrapuria *et al.*, 2010). The proposed function(s) of the CBD is to help anchor the enzyme onto the insoluble chitin to enhance chitin degradation efficiency (Linder *et al.*, 1996; Arakane *et al.*, 2003). As described in section 7.4.1.1, based on the amino acid sequence similarity and domain architecture, insect CHTs can be classified into eight groups (**Figures 6** and **7**). Group I CHTs (CHT5s) represent the prototypical and enzymatically characterized CHTs purified from molting fluid and/or integument of *M. sexta* and *B. mori* (Koga *et al.*, 1983, 1997). All of these group members contain a signal peptide, one catalytic domain, a Ser/Thr-rich linker region, and one CBD. Group II CHTs (CHT10s) are rather diverse in their domain architecture, and have four or five catalytic domains, together with four to seven CBDs. Dipterans and *A. pisum* (hemiptera) appear to be unique in having only four catalytic domains and four CBDs. The domain corresponding to the most N-terminal catalytic domain and one CBD found in group II chitinases from other species appear to be missing in the dipteran CHT10s (Zhu *et al.*, 2008b; Arakane and Muthukrishnan, 2010; Nakabachi *et al.*, 2010). The second catalytic unit of all CHT10s (the first catalytic unit in the case of the dipteran and *A. pisum* proteins) is predicted to lack chitinolytic activity due to a substitution of the most critical amino acid residue glutamate (E) with asparagine (N) in conserved motif II. Group III CHTs (CHT7s) possess two catalytic domains and one C-terminal CBD. The first catalytic domains of the group III proteins from all insect species studied share greater sequence similarity with each other than they do to the second catalytic domain, suggesting a unique function and/or evolutionary origin for each of the catalytic domains. Unlike most insect CHTs, CHT7s are predicted to have an N-terminal transmembrane segment, and are likely to be membrane-bound proteins. Indeed, recombinant *T. castaneum* CHT7 (TcCHT7) that was expressed in Hi-5 insect

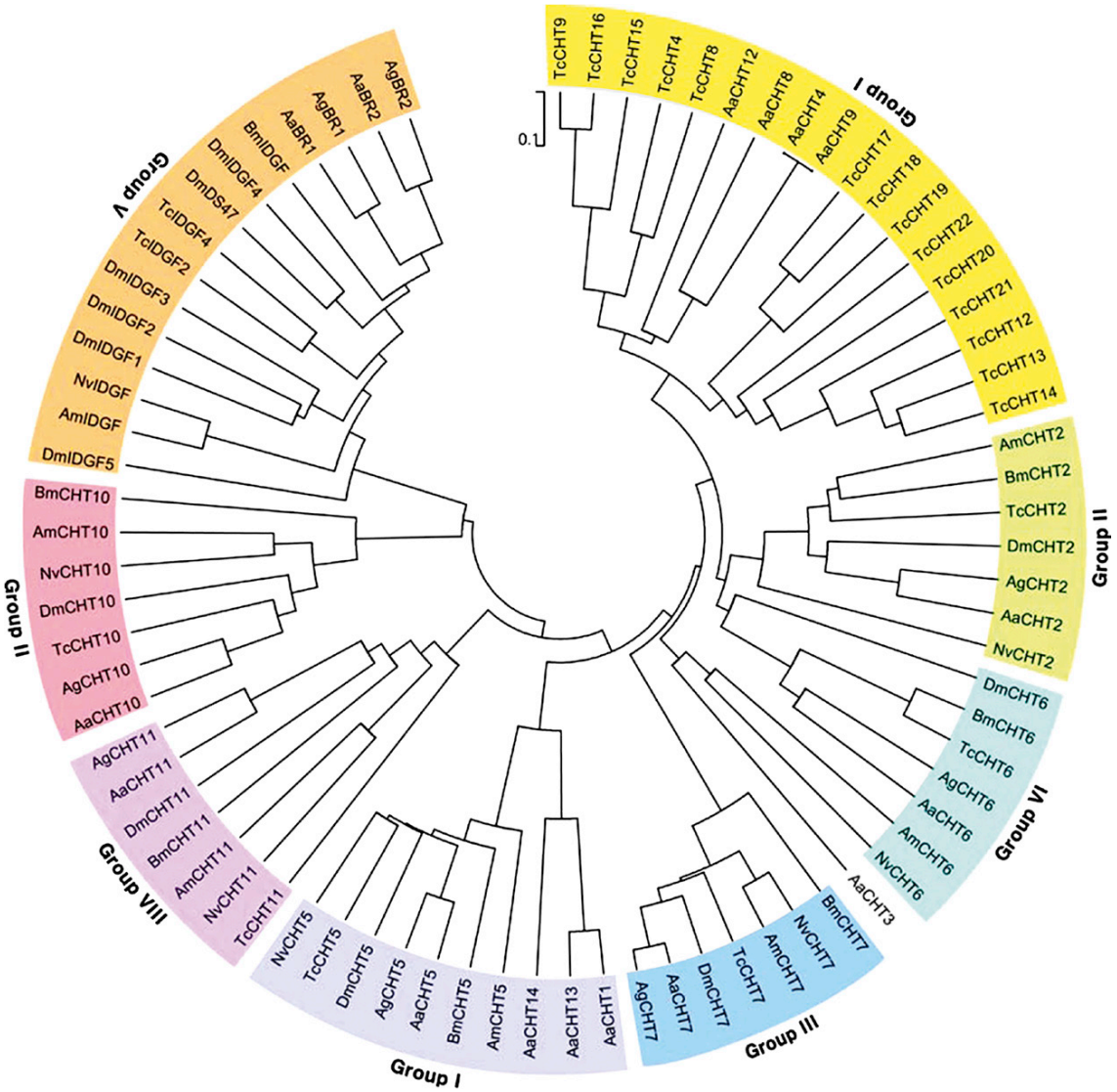

Figure 7 Phylogenetic analysis of putative chitinases and chitinase-like proteins (IDGFs) in insects. ClustalW software was used to perform multiple sequence alignments prior to phylogenetic analysis. The phylogenetic tree was constructed by MEGA 4.0 software using UPGMA (Tamura *et al.*, 2007). Protein sequences obtained from GenBank as follows: *Tribolium castaneum*, TcCHT2 (AY873913); TcCHT4 (EF125543); TcCHT5 (AY675073); TcCHT6 (AY873916); TcCHT7 (DQ659247); TcCHT8 (DQ659248); TcCHT9 (DQ659249); TcCHT10 (DQ659250); TcCHT11 (DQ659251);TcCHT12 (XM_967709); TcCHT13 (DQ659252); TcCHT14 (XM_967912); TcCHT15 (XM_967984); TcCHT16 (AY873915); TcCHT17 (XP_972719); TcCHT18 (XP_973161); TcCHT19 (XP_973119); TcCHT20 (NP_001034516); TcCHT21 (NP_001034517); TcCHT22 (NP_001038095); TcIDGF2 (DQ659253); TcIDGF4 (DQ659254); *Aedes aegypti*, AaCHT1 (XP_001656232); AaCHT2 (XP_001662520); AaCHT3 (XP_001663568); AaCHT4 (XP_001663099); AaCHT5 (XP_001656234); AaCHT6 (XP_001662588); AaCHT7 (XP_001650020); AaCHT8 (XP_001663098); AaCHT9 (XP_001663099); AaCHT10 (XP_001655973); AaCHT11 (XP_001654045); AaCHT12 (XP_001658836); AaCHT13 (XP_001656231); AaCHT14 (XP_001656233); AaBR1 (XP_001660745); AaBR2 (XP_001660748); *Apis mellifera*, AmCHT2 (XP_623744); AmCHT5 (XP_623995); AmCHT6 (XP_393252); AmCHT7 (XP_396925); AmCHT10 (XP_395734); AmCHT11 (XP_395707); AmIDGF (XP_396769); *Drosophila melanogaster*, DmCHT2 (NP_477298); DmCHT5 (NP_650314); DmCHT6 (NP_572598); DmCHT7 (NP_647768); DmCHT10 (NP_001036422); DmCHT11 (NP_572361); DmIDGF1 (NP_477258); DmIDGF2 (NP_477257); DmIDGF3 (NP_723967); DmIDGF4 (NP_727374); DmIDGF5 (NP_611321); DmDS47 (NM_057733); *Bombyx mori*, BmCHT2 (BGIBMGA009695); BmCHT5 (BGIBMGA010240); BmCHT6 (BGIBMGA009890); BmCHT7 (BGIBMGA005539); BmCHT10 (BGIBMGA006874); BmCHT11 (BGIBMGA005859); BmIDGF (BGIBMGA000648); *Anopheles gambiae*, AgCHT2 (XP_315650); AgCHT5 (XP_001237469); AgCHT6 (AGAP000198); AgCHT7 (XP_308858); AgCHT10 (XP_001238192); AgCHT11 (XP_310662); AgBR1 (AAS80137); AgBR2 (AY496421); *Nasonia vitripennis*, NvCHT2 (XP_001601416); NvCHT5 (NP_001155084); NvCHT6 (); NvCHT7 (XP_001604515); NvCHT10 (XR_036825); NvCHT11 (XP_001604954); NvIDGF (XP_001599305.

cells using the baculovirus protein expression system was found to be in the cell pellet rather than in the medium, as expected for secreted proteins. The washed cell pellet containing recombinant TcCHT7 could hydrolyze chitin added to the culture medium, suggesting that the catalytic domains of this putative membrane-bound protein face the extracellular space (Arakane, unpublished data). Group IV CHTs comprise the largest and most divergent group of proteins. CHTs in this group have a signal peptide and one catalytic domain. Most (but not all) of the members lack a CBD (**Figure 6**). Group V chitinase-like proteins (CHLPs) include the imaginal disc growth factors (IDGFs) and the hemocyte aggregation inhibitor protein (HAIP, Kanost *et al.*, 1994; Pan *et al.*, 2010). CHLPs have a signal peptide, one catalytic domain, and no CBDs. Like other family-18 proteins, the crystal structure of *D. melanogaster* IDGF2 and homology modeling of all proteins in this group revealed the $\beta_8\alpha_8$-TIM barrel structure (Varela *et al.*, 2002). However, members of this group have an additional loop sequence located between the β4-strand and the α4-helix immediately after conserved region II. Although these proteins possess all four of the family-18 conserved motifs, the glutamate residue in conserved motif II is substituted by a glutamine in all members of the group, with the exception of two *T. castaneum* IDGFs (TcIDGF2 and TcIDGF4; Zhu *et al.*, 2008b). TcIDGF2 and TcIDGF4 retain the glutamate residue in conserved region II but lack chitinase activity, either due to a D to A substitution in the conserved motif II, or to an extra loop stretching between the β4-strand and the α4-helix that possibly interferes with a productive substrate–enzyme interaction (Zhu *et al.*, 2008a), or both. Group VI CHTs (CHT6s) exhibit a domain architecture similar to that of group I (a signal peptide, one catalytic domain, and one CBD), but they have a very long C-terminal stretch (e.g., 1819 amino acids in length after the CBD in TcCHT6) that has no predicted conserved domain (**Figure 6**) except for the *A. pisum* enzyme, which possesses an additional CBD at the C-terminal region (Nakabachi *et al.*, 2010). Group VII CHTs (CHT2s) possess a domain architecture similar to that of group IV CHTs, which have a signal peptide, one catalytic domain, and no CBDs. They are classified as a separate group because phylogenetic analysis clearly indicates that these CHTs form a different clade near group II CHT10s. Group VIII CHTs (CHT11s) have one catalytic domain and no CBD. Interestingly, they have a predicted transmembrane segment instead of a signal peptide at the N-terminus, and they fall into a branch next to group III (CHT7s), all of which are predicted to be membrane-bound proteins.

7.4.1.3. Gene expression and functions of insect chitinases The redundancy of genes for CHTs raises important questions about their functions. Several insect CHT cDNAs have been obtained from epidermis, gut, and fat body, and extensively characterized (Kramer and Muthukrishnan, 2005). The epidermal endochitinases presumably function in turnover of the old cuticle, as these enzymes are found in the molting fluid along with *N*-acetylglucosaminidases, whereas the gut CHTs are thought to participate in the breakdown of chitin in the PM. In *T. castaneum*, tissue specificity and developmental patterns of expression of the 22 *TcCHT* and *TcCHLP* genes were analyzed by RT-PCR using cDNAs prepared from RNAs isolated at different developmental stages, such as embryo, larva, pharate pupa, pupa, and adult (Zhu *et al.*, 2008c; Arakane and Muthukrishnan, 2010). The group I gene *TcCHT5*, group II gene *TcCHT10*, group III gene *TcCHT7*, group V genes *TcIDGF2* and *TcIDGF4*, group VI gene *TcCHT6*, group VII gene *TcCHT2*, and group VIII gene *TcCHT11* are expressed at all stages analyzed, with some variation, whereas all group IV genes (*TcCHT*s *2*, *4*, *8*, *9*, and *12 to 22*) were predominantly expressed in the feeding stages (larva and adult). In addition, all chitinase genes belonging to group IV were expressed in larval gut tissue but not in the carcass (whole body minus gut), suggesting a possible function of these TcCHTs in PM-associated chitin turnover or digestion of dietary chitin (Zhu *et al.*, 2008c). Khajuria *et al.* (2010) recently reported that orally feeding dsRNA for a midgut-specific chitinase gene (encoding a group IV CHT) from larvae of *O. nubilalis* (*OnCHT*) significantly reduced the transcript levels of this gene and led to a significant increase of chitin content in the PM. The body weight of dsRNA *OnCHT*-fed larvae was decreased by 54% as compared with that of control dsRNA *GFP*-fed larvae, suggesting that some group IV CHTs are critical for regulating PM-chitin content, insect growth, and development. Interestingly, *A. pisum* appears to have no group IV *CHT* genes (Nakabachi *et al.*, 2010). *A. pisum* (hemipteran) possesses a perimicrovillar membrane (PMM) that is devoid of chitin, suggesting that group IV CHTs may not play a role in the PM turnover. Instead, one *CHT* gene, *ApCHT6* (encoding a group VIII CHT), was highly expressed in the midgut of *A. pisum*. Similarly, *TcCHT11* (encoding a group VIII CHT) was expressed in larval midgut, but not in the carcass (Arakane and Muthukrishnan, 2010). Group VIII CHTs, as well as group VI CHTs, may play critical roles in PM/PMM chitin degradation and turnover.

RNAi for group IV chitinases in *T. castaneum* for individual chitinases (and some combinations of chitinases) failed to produce any visible phenotypes, perhaps reflecting the redundant functions of this large group of chitinolytic enzymes. In contrast, injection of dsRNA for all chitinases belonging to groups I, II, III, and V resulted in unique lethal phenotypes. The most severe molting defect was observed after injection of dsRNA for *TcCHT10* (encoding a group II CHT). Injections of dsRNA for *TcCHT10*

prevented the embryo from hatching and also averted all types of molts, including larval–larval, larval–pupal, and pupal–adult, depending on the timing of administration of the dsRNA (**Figure 5**; Zhu *et al.*, 2008c). These results suggest a critical role for group II CHTs at every molt and developmental stage. Other CHTs (e.g., CHT5, also expressed in the epidermis) could not compensate for the loss of function of a group II CHT.

Unlike RNAi for *TcCHT10*, injection of ds*TcCHT5* (encoding a group I CHT) prevented only the pupal–adult molt (**Figure 5**). Although the gene encoding this prototypical CHT was expressed throughout all developmental stages, and the corresponding enzymes from several other insect species have been found in larval molting fluid, the failure to obtain a larval–larval or larval–pupal molting arrest probably indicates that one or more of the other CHTs (e.g., group II CHT, TcCHT10) could compensate for TcCHT5 at all molts except during adult eclosion. Group III CHTs, which appear to encode membrane-bound enzymes with two catalytic domains and one CBD at the C-terminus, appear to be critical for tissue differentiation, rather than chitin degradation associated with molting. Indeed, in *D. melanogaster*, expression of the *DmCHT7* (CG1869) gene increased more than 40-fold in the wing during the 32- to 40-h pupal wing differentiation period (Ren *et al.*, 2005). In *T. castaneum*, injection of dsRNA for *CHT7* resulted in a defective elytral and hindwing expansion without affecting molting (**Figure 5**; Zhu *et al.*, 2008c). Group V is composed of IDGFs that are known to be involved in cell proliferation and differentiation (Kawamura *et al.*, 1999; Zhang *et al.*, 2006). It is worthy of note that although group V CHTs have no chitinolytic activity (Zhu *et al.*, 2008b), they appear to be important for the adult molt. Injection of dsRNA for one of these *CHLP*s in *T. castaneum*, *TcIDGF4*, prevented adult eclosion (Zhu *et al.*, 2008c). It is possible that TcIDGF4 may be required for tracheal proliferation during adult metamorphosis. Two *A. gambiae* proteins, AgBR1 and AgBR2, which belong to this group, were induced specifically in the hemolymph by bacterial challenge (Shi and Paskewitz, 2004), suggesting that some members of the CHLP group (and/or members of other CHT groups) may have a role in the immune response.

7.4.2. Insect *N*-Acetylglucosaminidases

7.4.2.1. Phylogenetic analysis of insect *N*-acetylglucosaminidases Beta-*N*-acetylglucosaminidases (NAGs; EC 3.2.1.30) have been defined as enzymes that release –acetylglucosamine residues from the non-reducing end of chitooligosaccharides and from glycoproteins with terminal *N*-acetylglucosamines. Insect NAGs are members of the family-20 hexosaminidase super-family of the glycosylhydrolases of the Carbohydrate Active Enzymes database, CAZY (Coutinho and Henrissat, 1999; Cantarel., *et al.*, 2009). These enzymes have been detected in the molting fluid, hemolymph, integument, and gut tissues of several species of insects (Kramer and Koga, 1986; Hogenkamp *et al.*, 2008), and cooperate with CHTs to hydrolyze chitin to generate monomers of N-acetylglucosamine (Fukamizo and Kramer, 1985a, 1985b). Insect CHTs are unable to convert the chitin substrate completely to GlcNAc monomers. Therefore, NAG is the enzyme primarily responsible for the production of the monomer from chitooligosaccharides for recycling. Kinetic studies with *M. sexta* CHT (MsCHT5, group I CHT) have revealed that this enzyme is subject to substrate and/or product inhibition when chitooligosaccharides and/or colloidal chitin are utilized as substrates (Koga *et al.*, 1982, 1983; Arakane *et al.*, 2003). Therefore, one of the potential functions of NAGs may be to prevent the accumulation of chitooligosaccharides at concentrations that are high enough to interfere with efficient degradation of chitin by CHT (Kramer and Muthukrishnan, 2005).

cDNAs for epidermal *β-N*-acetylglucosaminidases of *B. mori*, *B. mandarina*, *T. ni*, and *M. sexta* have been isolated and characterized (Nagamatsu *et al.*, 1995; Zen *et al.*, 1996; Goo *et al.*, 1999; Hogenkamp *et al.*, 2008). A NAG also has been detected in the gut of *Ae. aegypti*, where its activity increased dramatically upon blood feeding (Filho *et al.*, 2002). A search of the *D. melanogaster*, *A. gambiae*, *Ae. aegypti*, *Culex pipiens*, *A. mellifera*, *N. vitripennis*, *B. mori*, and *T. castaneum* genome databases revealed the presence of multiple *NAG* genes, as well as the genes encoding *β-N*-acetylhexosaminidases (*HEX*s) in these species (Hogenkamp *et al.*, 2008). Phylogenetic analysis of NAGs from these insects indicates that NAGs can be classified into four distinct groups – NAG group I (NAG1), NAG group II (NAG2), *N*-glycan processing NAGs (FDL) (group III, Leonard *et al.*, 2006), and HEX group IV – according to their amino acid sequences (**Figure 8**). To date, only a single gene representing each of the groups I, II, and III has been found in the various insect species, with the exception of *C. pipiens*, which appears to have three genes encoding NAG-like proteins closely related to group I NAGs. Group I is composed of the enzymatically well-characterized NAGs, including NAGs from *M. sexta* (MsNAG1) and *B. mori* (BmNAG1). DmHEXO2, which has been shown to have NAG activity (Mark *et al.*, 2003; Leonard *et al.*, 2006), was placed in group II. Group III is composed of the *D. melanogaster* fused lobes protein (DmFDL), along with the fused lobes (fdl) homologs of other insect species (Leonard *et al.*, 2006). All of the proteins belonging to this group possess a predicted transmembrane anchor and a signal anchor, except for a signal peptide that can be found in NAGs belonging to groups I, II, and IV. In *T. castaneum*, TcNAG3 could not be unambiguously assigned to any of

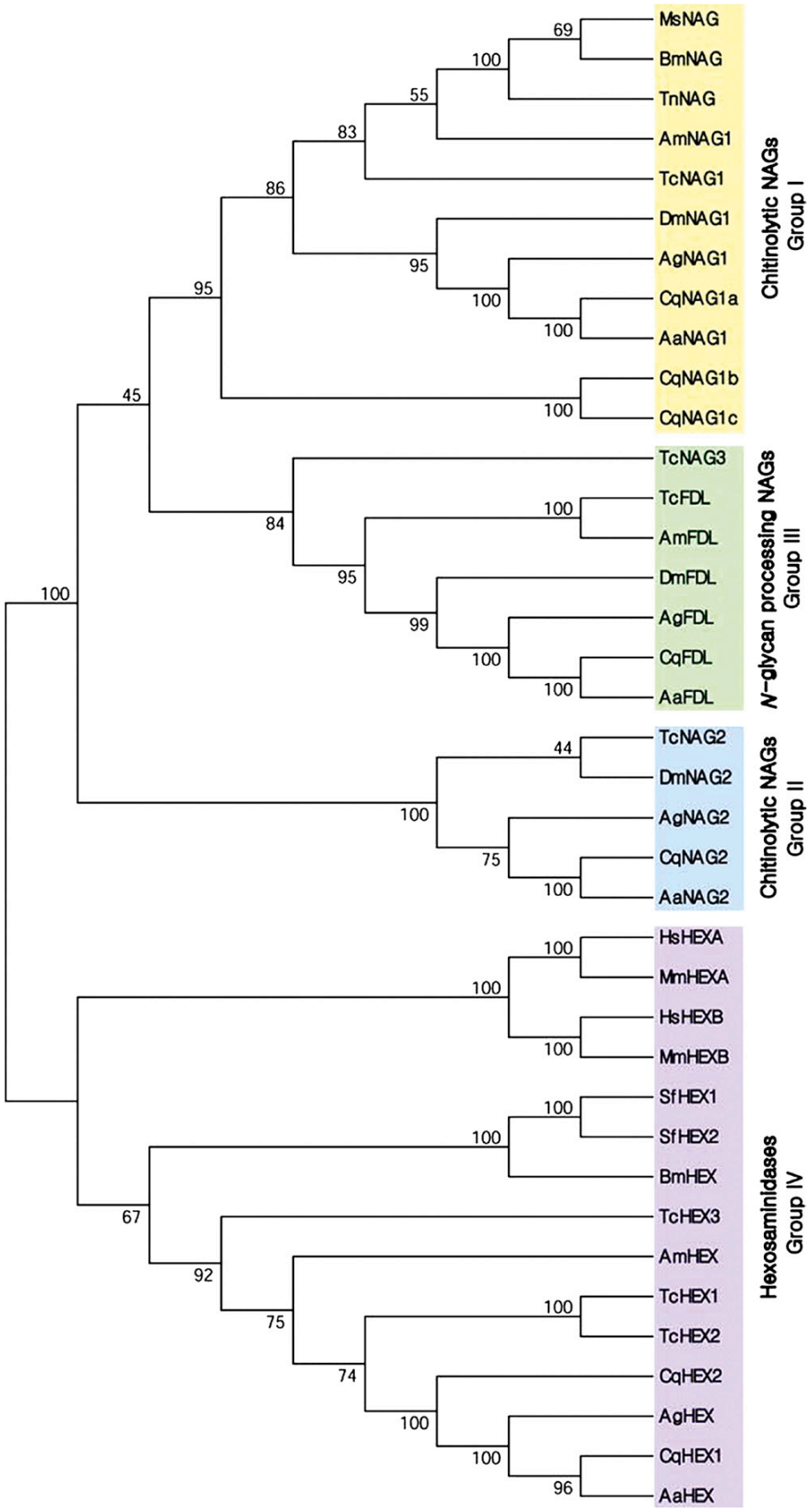
MsNAG
BmNAG
TnNAG
AmNAG1
TcNAG1
DmNAG1
AgNAG1
CqNAG1a
AaNAG1
CqNAG1b
CqNAG1c
Chitinolytic NAGs
Group I
TcNAG3
TcFDL
AmFDL
DmFDL
AgFDL
CqFDL
AaFDL
N-glycan processing NAGs
Group III
TcNAG2
DmNAG2
AgNAG2
CqNAG2
AaNAG2
Chitinolytic NAGs
Group II
HsHEXA
MmHEXA
HsHEXB
MmHEXB
SfHEX1
SfHEX2
BmHEX
TcHEX3
AmHEX
TcHEX1
TcHEX2
CqHEX2
AgHEX
CqHEX1
AaHEX
Hexosaminidases
Group IV
69
100
55
83
86
95
100
100
95
45
100
84
100
95
99
100
100
100
44
100
75
100
100
100
100
100
100
100
67
92
75
74
100
100
96

Figure 8 Phylogenetic analysis of NAGs and hexoaminidases in *Tribolium*, other insects and metazoans. MEGA4.0 (Tamura *et al.*, 2007) was used to construct the consensus phylogenetic tree using UPGMA. Bootstrap analyses of 1000 replications are shown. Protein sequences extracted from GenBank include: MsNAG, *Manduca sexta* (AY368703); BmNAG, *Bombyx mori* (genbank: AF326597); TnNAG, *Trichoplusia ni* (AY078172); AmNAG1, *Apis mellifera* (XM_624790); TcNAG1, *Tribolium castaneum* (EF592536); DmNAG1 (DmHEXO1), *Drosophila melanogaster* (NM_079200); AgNAG1, *Anopheles gambiae* (XP_315391); CqNAG1a, *Culex quinquefasciatus* (XP_001864406); AaNAG1, *Aedes aegypti* (EAT43909); CqNAG1b, *Culex quinquefasciatus* (XP_001864407); CqNAG1c, *Culex quinquefasciatus* (XP_001866097); TcNAG3, *Tribolium castaneum* (EF592538); TcFDL, *Tribolium castaneum* (EF592539); AmFDL, *Apis mellifera* (XP_394963); DmFDL, *Drosophila melanogaster* (NP_725178); AgFDL, *Anopheles gambiae* XP_308677); CqFDL, *Culex quinquefasciatus* (XP_001850423); AaFDL, *Aedes aegypti* (EAT36388); TcNAG2, *Tribolium castaneum* (EF592537); DmNAG2 (DmHEXO2), *Drosophila melanogaster* (NM_080342); AgNAG2, *Anopheles gambiae* (XM_307483); CqNAG2, *Culex quinquefasciatus* (XP_001842710); AaNAG2, *Aedes aegypti* (EAT40440); HsHEXA, *Homo sapiens* (NM_000520); HsHEXB, *Homo sapiens* (NM_000521); MsHEXA, *Mus musculus* (NM_010421); MsHEXB, *Mus musculus* (NM_010422); SfHEX1, *Spodoptera frugiperda* (DQ183187); SfHEX2, *Spodoptera frugiperda* (DQ249307); BmHEX, *Bombyx mori* (AY601817); TcHEX3, *Tribolium castaneum* (XM_970565); AmHEX, *Apis mellifera* (XM_001122538); TcHEX1, *Tribolium castaneum* (XM_970563); TcHEX2, *Tribolium castaneum* (XM_970567); CqHEX2, *Culex quinquefasciatus* (XP_001867058); AgHEX, *Anopheles gambiae* (XM_319210); CqFEX1, *Culex quinquefasciatus* (XP_001867057); and AaHEX, *Aedes aegypti* (EAT43655.

the three subgroups. TcNAG3 is more closely related to TcFDL than to TcNAG1 and TcNAG2, but the TcFDL and TcNAG3 genes are present on different linkage groups (**Figure 8**, Hogenkamp *et al.*, 2008).

7.4.2.2. Expression and functional analysis of insect *N*-acetylglucosaminidases Hogenkamp and colleagues (2008) performed dsRNA-mediated post-transcriptional downregulation (RNAi) of transcripts for all four *NAG* genes from a single insect species (*T. castaneum*) to study the functions of insect NAGs. Injection of a dsRNA corresponding to any one *TcNAG* gene resulted in substantial downregulation of the target transcript without significantly affecting the levels of the other *TcNAG* transcripts. Depletion of transcripts for any one of the targeted genes produced lethal molting arrest phenotypes. However, some of the injected insects did succeed in completing each type of molt (larval–larval, larval–pupal, and pupal–adult). TcNAG1 appeared to be most critical in chitin catabolism during molting. Administration of dsRNA for *TcNAG1* resulted in developmental arrest, and more than 80% of the insects died at the time of the next molt (**Figure 5**). During each type of molt, larval–larval, larval–pupal, and pupal–adult, the insects were unable to completely shed their exoskeleton. The pupa–adult molting phenotype produced by injection of dsRNA for *TcNAG1* is strikingly similar to that obtained in RNAi studies with ds*TcCHT5* (**Figure 5**; see section 7.4.1.3). Insects injected with dsRNA for *TcCHT5* also failed to shed their old cuticle, and the new cuticle was visible underneath the old cuticle (Zhu *et al.*, 2008c; Arakane and Muthukrishnan, 2010). It has been shown that in *M. sexta*, CHT is susceptible to oligosaccharide inhibition (Koga *et al.*, 1982, 1983; Arakane *et al.*, 2003). Injection of dsRNA for *TcNAG1* may result in the accumulation of chitiooligosaccharides in the molting fluid, and therefore it may cause inhibition of TcCHT5 activity, resulting in a phenotype similar to that observed in dsRNA for *TcCHT5*-treated insects. The high level of expression of *TcNAG1*, its phylogenetic relationship to other well-characterized molting-associated insect NAGs (**Figure 8**), and the phenotypic effect of knocking down *TcNAG1* transcripts suggest that, among all of the TcNAGs, TcNAG1 (group I NAG) is the enzyme primarily responsible for the efficient degradation of cuticular chitin, in concert with TcCHT5 (group I CHT), in *T. castaneum*, and that this may be the case in other insect species as well.

Although TcNAG1 is most likely to be the principal NAG for catabolism of cuticle-associated chitin, the other three NAGs identified in *T. castaneum* also appear to play important and perhaps indispensable roles in cuticle turnover and development. Injection of dsRNA for *TcNAG2* (encoding a group II NAG orthologous to DmHEXO2) prevents all types of molts, especially the pupal–adult molt. Like the phenotype produced by injection of dsRNA for *TcNAG1* (**Figure 5**), more than 75% of the animals treated with dsRNA for *TcNAG2* were unable to fully shed the old pupal cuticle. Since injection of dsRNA for *TcNAG2* did not change the level of *TcNAG1* transcripts, TcNAG1 could not compensate for the lack of TcNAG2 in adult eclosion in *T. castaneum*. In addition, *TcNAG2* transcript level in the midgut is relatively higher than that in the carcass (whole body minus midgut), suggesting *TcNAG2* as well as *TcNAG1*, which are highly expressed in both tissues, also play critical roles in the PM-associated chitin turnover.

Group III consists of the insect orthologs of the *D. melanogaster* fused lobes gene, *DmFDL*. The FDL proteins are predicted to be membrane-bound, with a single transmembrane helix located near the N-terminus. Furthermore, ultracentrifugation experiments on a lepidopteran protein from the culture media of *Sf*9 and *Sf*21 cells indicated that a major portion of the NAG activity

resided in the membrane fraction (Altmann *et al.*, 1995; Tomiya *et al.*, 2006). This lepidopteran NAG was capable of effectively hydrolyzing chitotriose-PA (pyridylamino), while the recombinant DmFDL was unable to digest chitotriose (Leonard *et al.*, 2006). The latter hydrolyzed only the GlcNAc residue attached to the α-1,3-linked mannose of the core pentasaccharide of *N*-glycans. No cleavage activity of any other GlcNAc residues was observed, including the GlcNAc residue attached to the α-1,6-linked mannose of the core pentasaccharide. Furthermore, DmFDL did not catalyze the endo-type hydrolysis of the N,N′-diacetylchitobiosyl unit in the high-mannose pentasaccharide core. A similar *N*-glycan substrate specificity for the terminal GlcNAc attached to the α-1,3-linked mannose was observed in membrane-bound β-*N*-acetylhexosaminidases from several lepidopteran insect cell lines, including Sf21, Bm-N, and Mb-0503 (Altmann *et al.*, 1995; Tomiya *et al.*, 2006). Taken together, FDLs may play a critical role in *N*-glycan processing.

Unlike RNAi for *TcNAG1* (group I NAG), injection of dsRNA for *TcFDL* exhibits a small percentage (10–20%) of lethal molting defect phenotypes at the larval–larval and larval–pupal molts (Hogenkamp *et al.*, 2008). Much higher mortality (80%), however, was observed at the pupal–adult molting stage, indicating that TcFDL plays an essential role for adult eclosion. The transcript level of *TcFDL* in the midgut was relatively low compared to that of the carcass. Therefore, the observed lethal phenotype at the pharate adult stage may be a direct result of the knockdown of this transcript in the cuticular epidermal cells, rather than in the gut lining cells. If TcFDL does in fact play a role in chitin turnover in the cuticle, then this protein may be secreted and not membrane-bound. Indeed, Leonard and colleagues (2006) have observed that DmFDL is, to a large extent, secreted into the extracellular space. Whether there is another point of regulation at the level of release of membrane-bound FDLs is an interesting possibility.

Another *T. castaneum NAG*, *TcNAG3*, has not been unambiguously assigned to any of the three NAG groups (**Figure 8**). Similar to *TcNAG2* (group II NAG), *TcNAG3* is also expressed at a significantly higher level in the larval midgut than in the carcass (Hogenkamp *et al.*, 2008). Furthermore, an analysis of the developmental pattern of expression of *TcNAG3* indicated that it is primarily expressed during the larval stages. Unlike RNAi for the other three *TcNAGs*, injection of ds*TcNAG3* did not consistently result in lethal phenotypes, and the majority of dsRNA-injected insects survived to adults with no visible phenotypic changes. However, a small number of individuals (approximately 20%) did exhibit a lethal larval phenotype similar to that of *TcNAG1* RNAi (**Figure 5**). In addition, a few insects (approximately 10%) exhibited a lethal pharate adult molting phenotype after dsRNA *TcNAG3* injection. These insects were unable to fully shed their old pupal cuticle, similar to the phenotypes observed after dsRNA *TcNAG1* and dsRNA *TcNAG2* injections. The *TcNAG3* gene is expressed predominantly in the larval stages, with only trace levels of expression in the pupal and adult stages (Hogenkamp *et al.*, 2008). In other insect species analyzed, only genes that can be classified into groups *NAG1*, *NAG2*, and *FDL* have been identified (**Figure 8**). Therefore, *TcNAG3* appears to be unique, and its relatively high expression in the midgut compared to the carcass suggests that it may be specialized for the turnover of PM-associated chitin rather than cuticular chitin during larval stages.

7.4.3. Insect Chitin Deacetylases

7.4.3.1. Phylogenetic analysis and domain organization of chitin deacetylases The extracellular matrix (ECM) of the insect exoskeleton is modified in different ways to give the cuticle its proper physiological and mechanical properties – namely, rigidity and thickness, or flexibility and thinness (Kramer and Muthukrishnan, 2005). Chitin deacetylases (CDAs, EC 3.5.1.41) are secreted metalloproteins that belong to a family of extracellular chitin-modifying enzymes that catalyze the *N*-deacetylation of chitin to form chitosan, a polymer of *β*-1,4-linked D-glucosamine residues with electrostatic properties very different from chitin. This modification might contribute to the affinity of chitosan for a variety of cuticular proteins distinct from those that bind specifically to chitin. CDAs have been well characterized in various fungi and bacteria (Caufrier *et al.*, 2003), and belong to the carbohydrate esterase family 4 (CE4) of the CAZY database (www.cazy.org; Cantarel *et al.*, 2009). CE4 esterases catalyze deacetylation of different carbohydrate substrates, such as chitin, acetylxylan, and bacterial peptidoglycan. Chitooligosaccharide deacetylases and NodB, a nodulation protein from *Rhizobium*, belong to this family, and possess a similar catalytic domain (John *et al.*, 1993).

The first cDNA encoding an insect CDA-like protein (TnPM-P42, also referred to as TnCDA9) was characterized from the PM in the cabbage looper, *Trichoplusia ni*, only 5 years ago (Guo *et al.*, 2005). Since then, several genes/cDNAs encoding insect CDAs have been identified from different species (Luschnig *et al.*, 2006; Wang *et al.*, 2006; Campbell *et al.*, 2008; Dixit *et al.*, 2008; Toprak *et al.*, 2008; Jakubowska *et al.*, 2010). A comparative analysis of *CDA* gene families in several insect species with fully sequenced genomes, including Diptera, Coleoptera, Hymenoptera, and Lepidoptera, revealed that the number of *CDA* genes varies with species. Based on amino acid sequence similarity, insect CDAs are classified into five groups, I to V (**Figure 9**; Dixit *et al.*, 2008; Jakubowska *et al.*, 2010).

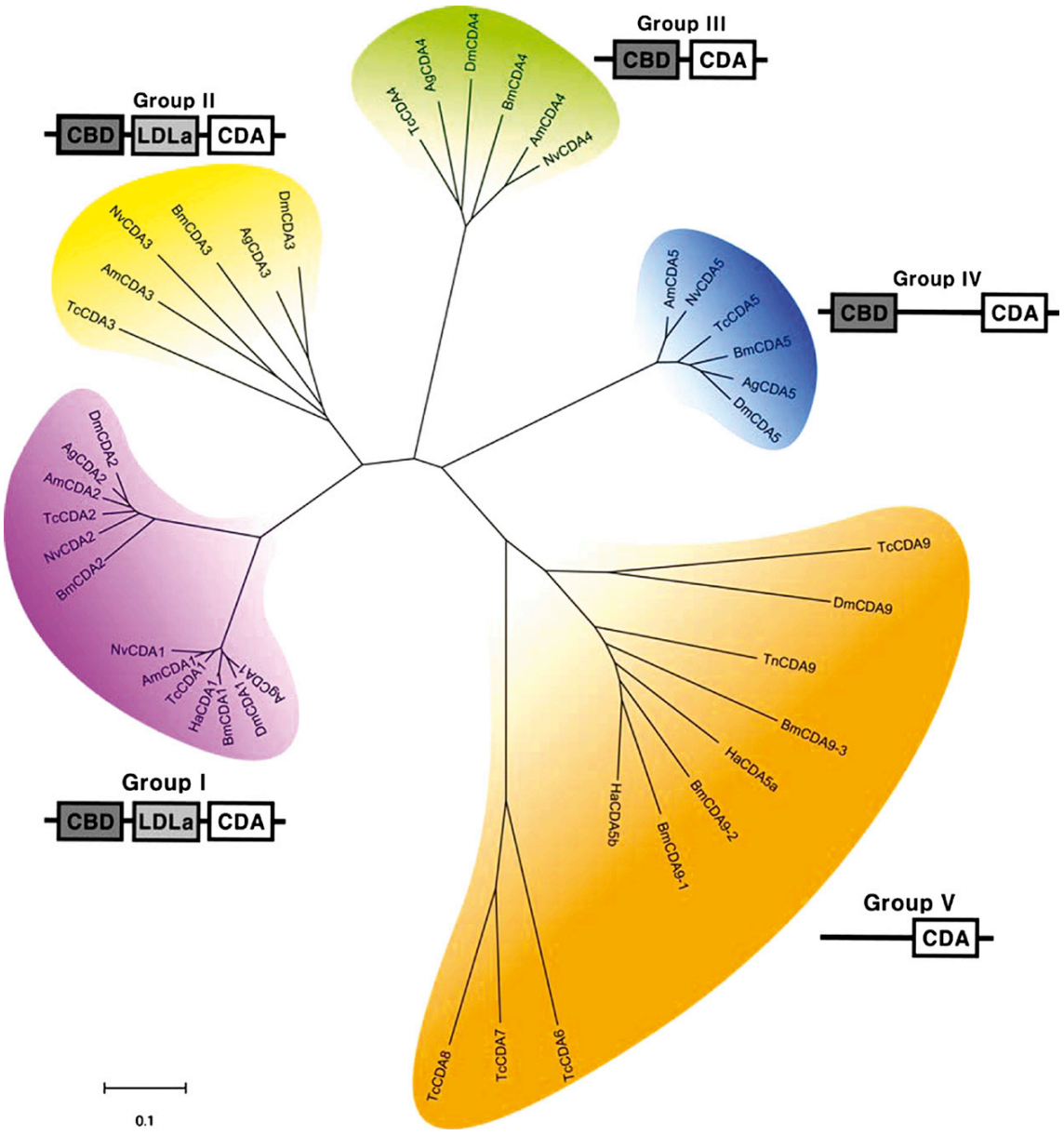

Figure 9 A phylogenetic tree of putative CDAs from different insects. A consensus phylogenetic tree was constructed using neighbor-joining method in the software MEGA 4.0 (Tamura *et al.*, 2007). Protein sequences obtained from GenBank as follows; NvCDA1, *Nasonia vitripennis* (XP_001604765); AmCDA1, *Apis mellifera* (XP_391915); TcCDA1, *Tribolium castaneum* (ABU2522); HaCDA1, *Helicoverpa armigera* (ADB43610); BmCDA1, *Bombyx mori* (BGIBMGA006213); DmCDA1, *Drosophila melanogaster* (NP_730444); AgCDA1, *Anopheles gambiae* (XP_320597); DmCDA2, *Drosophila melanogaster* (NP_001163469); AgCDA2, *Anopheles gambiae* (XP_320596); AmCDA2, *Apis mellifera* (XP_623723); TcCDA2, *Tribolium castaneum* (ABU25224); NvCDA2, *Nasonia vitripennis* (XP_001604838); BmCDA2, *Bombyx mori* (BGIBMGA006214); DmCDA3, *Drosophila melanogaster* (NP_609806); AgCDA3, *Anopheles gambiae* (XP_317336); BmCDA3, *Bombyx mori* (BGIBMGA008988); NvCDA3, *Nasonia vitripennis* (XP_001606617); AmCDA3, *Apis mellifera* (XP_001121246); TcCDA3, *Tribolium castaneum* (ABW74145); TcCDA4, *Tribolium castaneum* (ABW74146); AgCDA4, *Anopheles gambiae* (XP_310753); DmCDA4, *Drosophila melanogaster* (NP_728468); BmCDA4, *Bombyx mori* (BGIBMGA010573); AmCDA4, *Apis mellifera* (XP_001120478); NvCDA4, *Nasonia vitripennis* (XP_001607989); AmCDA5, *Apis mellifera* (XP_624655); NvCDA5, *Nasonia vitripennis* (XP_001603918); TcCDA5, *Tribolium castaneum* (ABW74147); BmCDA5, *Bombyx mori* (BGIBMGA002696); AgCDA5, *Anopheles gambiae* (XP_316929); DmCDA5, *Drosophila melanogaster* (NP_001097044); TcCDA6, *Tribolium castaneum* (ABW74149); TcCDA7, *Tribolium castaneum* (ABW74150); TcCDA8, *Tribolium castaneum* (ABW74151); TcCDA9, *Tribolium castaneum* (ABW74152); DmCDA9, *Drosophila melanogaster* (NP_611192); TnCDA9, *Trichoplusia ni* (AAY46199); BmCDA9-3, *Bombyx mori* (BGIBMGA013758); HaCDA5a, *Helicoverpa armigera* (ADB43611); HaCDA5b, *Helicoverpa armigera* (ADB43612); BmCDA9-1, *Bombyx mori* (BGIBMGA013756); BmCDA9-2, *Bombyx mori* (BGIBMGA013757).

Group I CDAs (CDA1s and CDA2s) consist of *D. melanogaster Serpentine* (*DmSerp*) and *Vermiform* (*DmVerm*) (referred to as *DmCDA1* and *DmCDA2*, respectively) and their orthologs (*CDAs 1* and *2*) from each species. All group I CDAs have a chitin-binding peritrophin-A domain (CBD), a low-density lipoprotein receptor class A domain (LDLa), and a CDA catalytic domain. There are two to four transcript variants produced by alternative splicing and/or exon skipping from the *CDA2* pre-mRNAs (Dixit *et al.*, 2008). Group II, III, and IV families are represented by only one CDA in each species, namely CDA3, CDA4, and CDA5, respectively. Although, like group I CDAs, CDA3s also possess a single copy of each of the three domains, the overall amino acid sequence identity is only about 38% with CDA1s and CDA2s (amino acid sequence identity between CDA1s and CDA2s is about 60%). Group III enzymes (CDA4s) have a single copy of the CBD and the CDA catalytic domain, but lack an LDLa domain. Group IV CDAs (CDA5s), like CDA4s, each possess a single CBD and a single CDA catalytic domain. These two domains, however, are connected by a long Ser/Thr/Pro/Gln-rich linker (e.g., about 2400 amino acids in AgCDA5), which results in CDA5s being the largest CDA proteins. At least three insect species, *D. melanogaster*, *A. mellifera*, and *T. castaneum*, have more than one isoform of CDA5 due to alternative splicing and/or exon skipping during the processing of pre-mRNA for these genes. Group V consists of two subgroups. One subgroup includes the CDA9s. Two CDAs (HaCDA5a and HaCDA5b), identified recently by proteomic analysis and EST sequence analysis of the PM of the cotton bollworm *Helicoverpa armigera* (Campbell *et al.*, 2008; Jakubowska *et al.*, 2010), also belong to this CDA9 subgroup of group V (**Figure 9**). Interestingly, two lepidopterans, *B. mori* and *H. armigera*, appear to have multiple genes related to CDA9. The other subgroup of group V consists of paralogs from *T. castaneum* only (TcCDAs 6, 7, and 8), and not from other insect species. All the proteins belonging to this group have only a CDA catalytic domain, and no CBD or LDLa domains.

7.4.3.2. Functional analysis of insect chitin deacetylases Developmental patterns and tissue-specific expression of different *CDA* genes in the same species suggest that the chitin deacetylases may have specific functions. In *D. melanogaster*, the two group I genes, *DmSerp* (*DmCDA1*) and *DmVerm* (*DmCDA2*), are required for normal tracheal tube development and morphology (Lusching *et al.*, 2006; Wang *et al.*, 2006). *D. melanogaster* mutants lacking either *serp* or *verm* exhibited excessively long and tortuous embryonic tracheal tubes. In *T. castaneum*, injection of dsRNA for *TcCDA1* or *TcCDA2*, which are predominantly expressed in epidermis and tracheae, prevented all types of molts, including larval–larval, larval–pupal, and pupal–adult (**Figure 5**; Arakane *et al.*, 2009). Furthermore, alternative exon-specific RNAi for *TcCDA2* (*TcCDA2a* and *TcCDA2b*) revealed functional specialization of the isoforms for this CDA. Unlike exon non-specific RNAi for *TcCDA2*, injection of dsRNAs specific for either one of alternative exons did not prevent any molts, suggesting that the proteins TcCDA2a and TcCDA2b could compensate for each other. However, the resulting adults exhibited different abnormal phenotypes. RNAi for *TcCDA2a* affected only femoral–tibial joint movement, while dsRNA for *TcCDA2b* resulted in elytra with crinkled and rough dorsal surfaces (Arakane *et al.*, 2009). These results suggest that group I CDAs play critical roles in maintaining the structural integrity of the cuticlular chitin laminae and chitin fibers of the tracheal tube. It is possible that there are unique cuticular proteins that preferentially bind to deacetylated portions of chitin, whereas others preferentially bind to fully acetylated chitin. These proteins may help to organize the chitinous cuticular layers and provide the proper rigidity and/or flexibility in different regions of the cuticle.

Injection of a mixture of dsRNAs for *T. castaneum* group V CDAs, *TcCDAs 6*, *7*, *8*, and *9*, which are all predominantly expressed in the gut, significantly reduced the transcript levels of individual *CDA*s. However, no adverse effects on the appearance, behavior, or survival of these dsRNA-treated insects were observed (Arakane *et al.*, 2009). Interestingly, Jakubowska *et al.* (2010) observed that one of the group V (CDA9 subgroup) *CDA* genes from *H. armigera* (*HaCDA5a*) was downregulated by baculovirus infection in larvae. Like TnCDA9, HaCDA5a had a strong binding affinity for chitin, although it lacks any predicted chitin-binding domain. Incubation of the PM from *S. frugiperda* with recombinant HaCDA5a increased PM permeability in a concentration-dependent manner. Infection of insects with a recombinant baculovirus carrying this gene significantly increased the speed of kill for *S. frugiperda* and *S. exigua*. Together, these observations indicate that the group V CDA, HaCDA5a, may have a role in determining PM structure/morphology or permeability. For instance, downregulation of transcripts for this gene after pathogen attack resulted in reduced PM permeability, presumably to avoid pathogen infection. Additional studies in the future may reveal the physiological functions of the many CDAs belonging to groups II, III, and IV.

7.5. Chitin-Binding Proteins

Chitin is almost always found in association with numerous proteins that influence the overall mechanical and physicochemical properties of the chitin–protein matrix, which can range from very rigid (e.g., head capsule and mouth parts) to fully flexible (e.g., larval body and wing cuticle). Since chitin is an extracellular matrix polysaccharide, the proteins that have an affinity for chitin are

expected to be extracellularly secreted proteins. This is generally true, with the constraint that some CBPs can be in vesicles or storage granules between the time they are synthesized and when they are secreted or released into the extracellular space by exocytosis.

There are three broad groups of insect proteins containing sequence motifs that have been associated with chitin-binding ability. The first group consists of a very large number of insect cuticular proteins, belonging to the CPR family, containing a consensus sequence(s) known as the extended Rebers & Riddiford Consensus (R&R Consensus) of a stretch of about 70 amino acids that defines pfam 00379 (Willis, 2010; see also Chapter 5). The second group of proteins contains an amino acid sequence motif known as the "peritrophin A" motif (Tellam *et al.*, 1999). To avoid confusion about its biological role(s), this motif will be referred to as the ChtBD2 domain in this chapter, because it is found not only in the group of proteins extracted from the peritrophic matrix, but also in proteins extracted from (or expressed in) cuticle-forming tissues. Proteins with the ChtBD2 motif are further subdivided intro three groups: peritrophic matrix proteins (with 1–19 ChtBD2 domains, determined to date); cuticular proteins analogous to peritrophins-3 (with 3 ChtBD2 domains); and cuticular proteins analogous to peritrophins-1 (with 1 ChtBD2 domain) (Jasrapuria *et al.*, 2010). This domain consists of a linear sequence of about 60 amino acids with 6 cysteines and conserved spacings between successive cysteine residues. The ChtBD2 domain defines family 14 of carbohydrate-binding proteins with chitin-binding ability (CBM14; pfam01607; SMART 00494). The second group also includes enzymes of chitin metabolism (chitinases, chitin deacetylases, and a protease) that have one or more ChtBD2 domains in addition to their catalytic domains. The third group of chitin-binding proteins consists of the family of antimicrobial peptides related to tachystatins from horseshoe crab (denoted as A1, A2, B1, B2, and C subfamilies), as well as the calcium channel antagonists, agatoxins from spider venom. Tachystatins are expressed in hemocytes, where they are stored in the form of small granules and are released into the hemolymph upon an immune stimulus. This group of proteins with six cysteines and a high affinity for chitin has a triple-stranded β-sheet structure with an inhibitory cysteine knot motif (Fujitani *et al.*, 2007). This structure is quite different from the peritrophin A motif and tachystatin (see below), and belongs to pfam 11478. They are not associated with cuticle or the PM, but they do play a major role in immune defense against bacteria, fungi, and other pathogens.

Representative members of each of the three groups of chitin-binding proteins have been extracted from the cuticle or the PM, or isolated from hemocytes. They have also been expressed in bacterial or other hosts, and some of the purified proteins have been shown to have chitin-binding ability. Several proteins belonging to the first and second groups of chitin-binding proteins are only predicted from known cDNA or genomic sequences and have not been biochemically characterized, largely as a result of difficulties associated with extracting them from highly sclerotized cuticular preparations or exuviae. The following sections will focus on the proteins of the second group of proteins with ChtBD2 motifs, and also include a limited discussion of group 3 chitin-binding proteins. A discussion on the first group of cuticular proteins with the R&R or other consensus motifs is kept to a minimum, because it is the subject of Chapter 5 in this book (Willis, 2010).

7.5.1. Chitin-Binding Proteins with the R&R Consensus

The CPR family of cuticular proteins is generally rich in histidines and devoid of cysteines. The absence of cysteines has been regarded as a defining characteristic of this group of proteins, with rare exceptions. The number of cuticular proteins belonging to the CPR subfamily in different insects varies widely, ranging from 32 in *A. mellifera* to >150 in *A. gambiae* (see Chapter 5), indicating a genus-specific expansion of specific families of cuticular proteins. Among the many families of cuticular proteins in insects, only some members of the CPR family with the R&R Consensus have been unequivocally shown to bind to chitin (summarized in Chapter 5). A member each of the Tweedle family from *B. mori* (Tang *et al.*, 2010) and one protein of the CPAP family (see below) have also been shown to possess chitin-binding ability. Modeling studies using the 65-aa long R&R Consensus have led to the notion that this region assumes a half-barrel structure into which a liner chain of *N*-acetylglucosamines can be fitted using van der Waals interactions between the sugar oligomer and the hydrophobic rings of conserved aromatic amino acids in this consensus (Iconomidou *et al.*, 2005). In an interesting study, Rebers and Willis (2001) demonstrated that the addition of this consensus sequence alone to glutathione-S-transferase resulted in acquisition of an affinity for chitin by this chimeric protein.

7.5.2. Peritrophic Matrix Proteins

The second group of proteins with the ChtBD2 motif is the family of proteins known as "peritrophins" that can be extracted from the PM using strong denaturing/chaotropic reagents, such as 6-M urea or 6-M guanidine hydrochloride (Tellam *et al.*, 1999). The extracted PMPs or recombinantly expressed PMPs have chitin-binding activity (Elvin *et al.*, 1996; Wijffels *et al.*, 2001; Wang *et al.*, 2004). This motif was shown to be responsible for binding to chitin by expressing a single ChtBD2 domain of *Trichoplusia ni* peritrophin, CBP1, in an insect cell line, and demonstrating its chitin-binding ability (Wang

et al., 2004). Proteins with multiple ChtBD2 domains are commonly found strongly associated with the PM. Not all of them are actually extractable, even with strong chaotropic agents. Some require extraction with strong organic solvents, such as anhydrous trifluoromethanesulfonic acid, which also deglycosylates O-linked glycoproteins (Campbell *et al.*, 2008).

The number of ChtBD2 domains in insect PMPs varies from 1 to as many as 19 in the bertha armyworm *Mamestra configurata* (Shi *et al.*, 2004; Dinglasan *et al.*, 2009; Venancio *et al.*, 2009; Jasrapuria *et al.*, 2010; Toprak *et al.*, 2010). Some PMPs have multiple ChtBD2 repeats in a tandem arrangement with short spacers rich in P, S, and T residues. Some of these linkers are potential sites of O-glycosylation. Other PMPs have mucin domains interspersed between ChtBD2 domains in various patterns of alternating ChtBD2 and mucin domains (Wang *et al.*, 2004; Venancio *et al.*, 2009). PMPs with only one or two ChtBD2 domains have also been reported (Jasrapuria *et al.*, 2010; Toprak *et al.*, 2010). The number of PMPs in different species is variable. Both *Ae. aegypti* and *D. melanogaster* have been predicted to have about 65 PMPs, though many of these may not be components of the PM (Venancio *et al.*, 2009). Detailed expression studies of all proteins with ChtBD2 domains in *T. castnaeum* have demonstrated that there are only 11 *bona fide* PMPs in this beetle (Jasrapuria *et al.*, 2010). Direct proteomic analysis of >200 proteins extracted from PMs dissected from adult *A. gambiae* females fed a protein-free diet has revealed the presence of only 12 PMPs, with the number of ChtBD2 repeats ranging from 1 to 4. It is likely that the total number of PMPs in insects is in the range of 10–20, although it can't be ruled out that additional *PMP* genes are expressed in the gut. However, their conceptual protein products were not detected in proteomic analyses because they were still in the insoluble pellet after extraction with detergents used in an extensive study (Dinglasan *et al.*, 2009). Interestingly, different *PMP* genes of *T. castaneum* were not expressed uniformly through the length of the midgut, with some *PMP*s being expressed in the anterior midgut, whereas others coding for proteins with multiple ChtBD2 domains were expressed in the posterior midgut (Jasrapuria *et al.*, 2010). Whether this differential spatial expression results in altered permeability of the PM along the length of the midgut remains to be investigated.

7.5.3. Cuticular Proteins Analogous to Peritrophins (CPAPs)

In addition to the *PMP* genes, which are expressed exclusively in the midgut lining cells, there are other genes encoding proteins with ChtBD2 domains, which are expressed in tissues other than the midgut. All of these proteins are predicted to have a cleavable signal peptide, and are expected to be capable of interacting with extracellular chitin. These genes are expressed predominantly in epidermal tissue as well as in other cuticle-forming tissues, including tracheae, elytra, hindwings, and hindgut. These genes have been subdivided into two groups, *CPAP1* and *CPAP3*, to reflect the fact that they encode proteins with one or three ChtBD2 domains, respectively (Jasrapuria *et al.*, 2010). *CPAP3* is the new name given to the orthologs of the previously characterized *D. melanogaster* "*obstructor*" or "gasp" gene family.

Mutants of the *D. melanogaster CPAP3-C* gene are embryo-lethal, and have been reported to exhibit cuticular defects (Barry *et al.*, 1999; Behr and Hoch, 2005). In *D. melanogaster* there are 10 genes encoding CPAP3 proteins, which can be further subdivided into two groups of 5 genes each. Only orthologs for the first group (*CPAP3-A*, *CPAP3-B*, *CPAP3-C*, *CPAP3-D*, and *CPAP-E*) are present in insects other than *Drosophila* species. There are significant variations in the expression profiles of these genes in different cuticle-forming tissues and/or developmental stages, suggesting functional differences among the CPAP3 proteins. RNA interference studies carried out in *T. castaneum* are consistent with such specialized functions of individual CPAP3 proteins (Jasrapuria *et al.*, unpublished data).

While it is expected that the CPAP3 proteins with three ChtBD2s will bind to chitin strongly, this has been demonstrated for only one recombinant protein from the spruce budworm *Choristoneura fumiferana*, which was expressed in *E. coli* (Nisole *et al.*, 2010). However, only a minor percentage of the His-tagged protein bound to the chitin, with the major portion appearing in the flow-through fraction, perhaps indicating that not all molecules of this recombinant protein had folded properly to exhibit strong chitin-binding activity. So far, there is no report of expression of this class of proteins in an insect cell system that may overcome the problem of misfolding as demonstrated for two PMP proteins with 10 and 12 repeats of ChtBD2 domains (Wang *et al.*, 2004).

A second group of genes encoding proteins with one ChtBD2 domain, referred to as the *CPAP1* family proteins, has been characterized extensively using a bioinformatics analysis of the *T. castaneum* genome (Jasrapuria *et al.*, 2010). These proteins vary extensively in size, and in the location of the ChtBD2 domain. Like CPAP3, they are also expressed in cuticle-forming tissues and have putative cleavable signal sequences consistent with a role involving interactions with chitin. So far, there are no reports on the chitin-binding ability of these proteins. Only some of these proteins have orthologs in *D. melanogaster*, casting doubt on whether these proteins are ubiquitous in insects. However, RNAi studies have produced lethal phenotypes when transcripts for 3 of the 10 genes encoding CPAP1 proteins were depleted in *T. castaneum* (Jasrapuria, unpublished data).

7.5.4. Enzymes of Chitin Metabolism

Enzymes of chitin metabolism, including some members of the chitinase and chitin deacetylase families, have the ChtBD2 motif (Kramer *et al.*, 1993; Campbell *et al.*, 2008; Dixit *et al.*, 2008; Zhu *et al.*, 2008a). The presence of one or more copies of this ChtBD2 motif has been suggested to increase the affinity of enzymes of chitin metabolism for the insoluble substrate, chitin, and to increase the processivity of these extracellular enzymes. Support for this idea comes from the drastic loss of ability to bind to insoluble chitin upon removal of the region containing the ChtBD2 motif from the C-terminal region of an *M. sexta* chitinase, which follows the catalytic domain. A C-terminal fragment of only 58 amino acids with this domain did bind to colloidal chitin, and addition of one or two copies of this domain to the chitinase catalytic domain progressively increased the affinity of the chitinase to colloidal chitin (Arakane *et al.*, 2003). While most of these enzymes have only one ChtBD2 motif, one class of insect chitinases (group II) has four or five ChtBD2 motifs dispersed among multiple catalytic domains (Royer *et al.*, 2002; Zhu *et al.* 2008a). A role for these multiple ChtBD2s in facilitating the depolymerizing chitin crystallites has been suggested (Arakane and Muthukrishnan, 2010).

7.5.5. Role of Secondary Structure of ChtBD2 Motif in Binding to Chitin

Tertiary structures based on 2D-NMR studies in solution are available for only two insect proteins with ChtBD2 domains with high affinity for chitin; namely, tachycitin and scarabacin. The antimicrobial peptide tachycitin from the horseshoe crab, which has a structure different from the tachystatins, is 76 aa long, and has a higher K_m for chitin binding than tachystatin – 19.5 μM versus 4.3 μM, respectively (Kawabata *et al.*, 2003). The second chitin-binding antimicrobial peptide for which an NMR-deduced structure is available is scarabacin from the coconut rhinocerous beetle *Oryctes rhinoceros*, which is 36 aa long and has a $K_d = 1.3$ μM (Hemmi *et al.*, 2003). A comparison of these two structures with that of another chitin-binding minimal fragment called hevein-32, from the rubber latex protein hevein, has provided some interesting insights about the role of a part of the ChtBD2 motif in chitin binding.

Tachycitin has 10 cysteines in the form of 5 disulfide bonds, and has significant similarity to several peritrophins from a wide spectrum of insects, including PMP3 of *T. castaneum*, with which it shares 51% amino acid sequence identity. Of these 10 cysteines, 5 are in perfect register with the linear arrangement of the cysteines in PMP3 ChtBD2 domains, without introducing gaps in either sequence, except for the first cysteine in the motif. More importantly, the amino acid sequence from positions 40 to 60 of tachycitin, which includes one disulfide bond, shares significant similarities to those of several other peritrophins from a wide range of insect species (Suetake *et al.*, 2000). Furthermore, the three-dimensional structure of this stretch of 21 amino acids is nearly identical to that of a hevein-32 from positions 20 to 32 (Aboitiz *et al.*, 2004). Both proteins have two anti-parallel β-sheets followed by a short α-helix in this region, which also includes a disulfide bond. Several aromatic amino acids that have been shown to contact the oligosaccharide ligands $(GlcNAc)_{3–6}$ are also conserved in the two sequences.

The 3D structure of scarabacin reveals the presence of only one disulfide bond between cys18 and cys29. The C-terminal half of this peptide from cys18 to ser 36 also has a secondary structure consisting of two anti-parallel β-sheets and a short α-helical turn super-imposable on hevein-32 or tachycitin (Hemmi *et al.*, 2003). These data suggest that only the C-terminal half of the ChtBD2 domain may be critical for chitin binding. Consistent with this interpretation is the finding that the N-terminal half of tachycitin has a completely different 3D structure, consisting of a three-stranded β-sheet while retaining the hevein/scarabacin-like chitin-binding motif on the C-terminal domain (Suetake *et al.*, 2000; Hemmi *et al.*, 2003). These data suggest that all three chitin-binding proteins (hevein, scarabacin, and tachycitin) share a common chitin-binding secondary/tertiary structure, even though they do not have extensive amino acid sequence identity. By extrapolation, we expect that all of the proteins with ChtBD2 domains will also have this structural motif consisting of two anti-parallel β-sheets and a short α-helical turn.

A protein from the vestimentiferan *Riftia pachyptila* has been shown to bind specifically to β-chitin, but not to α-chitin or cellulose (Chamoy *et al.*, 2001). The sequence of this protein includes a cysteine-rich region that resembles the C-terminal region of many mammalian chitinases, and is likely to be a chitin-biding motif. However, it does not have the consensus sequence or the characteristic spacing between adjacent cysteines of the ChtBD2 motif, and may represent yet another type of chitin-binding domain.

7.6. Chitin-Organizing Proteins

In addition to the CPR proteins with the R&R Consensus and the CPAP proteins with the ChtBD2 motif, which are expected to interact with chitin, some additional proteins may be associated with chitin, and help to organize it into bundles and the laminae that are characteristic of a mature procuticle. Two proteins encoded by *Knickkopf* (*Knk*) and *Retroactive* (*Rtv*) genes are known to be involved in this process in *D. melanogaster* (Ostrowski

et al., 2002; Moussian *et al.*, 2005b; Tonning *et al.*, 2005). Mutations in these two genes result in a dilated cuticle and loss of the fibrillar organization of tracheal chitin, and death of the developing embryo. Transmission electron microscopic analyses of the developing embryonic cuticle in these mutants revealed loss of the laminar architecture of chitin and the accumulation of electron-dense material in the procuticle.

How do these two proteins function to organize the cuticle-associated chitin? The domain organization of these proteins and their predicted properties offer some hints. The 75-kDa KNK is a GPI-anchored membrane protein with a multidomain architecture consisting of two DM13 domains, a dopamine monooxygenase N-terminal domain (DOMON domain), and a unique C-terminal region that has not been associated with any well-characterized domain. However, this sequence has some similarities to plastocyanin (Moussian, 2010). Interestingly, this region also has some sequence similarity to several plant proteins that possess DM13 and DOMON domains as well as a cytochrome b561 domain. It is possible that Knickkopf and its orthologs are extracellular proteins that may have a role in oxidation–reduction reactions perhaps involving dopamine. *T. castaneum* KNK expressed in a baculovirus-insect cell expression system does bind to colloidal chitin (Chaudhari, unpublished data). RNAi of this *KNK* gene results in loss of chitin, and this loss appears to be due to the protective effect of KNK on chitin against degradation by chitinolytic enzymes. The distribution of this protein between the procuticle and plasma membrane is consistent with such a chitin-protective role.

RTV is also a membrane-bound protein with a single C-terminal transmembrane domain, which localizes this protein to the apical surface of the plasma membrane (Schwarz and Moussian, 2007). RTV mutants have a spindle-shaped body, and often the cuticle separates from the epidermal layer underneath. This protein, which is about 150 amino acids long, has 10 cysteines, belongs to the neurotoxin-like SCOP superfamily of proteins, and has a β-sandwich structure with 2 and 3 β-strands in the 2 β–sheets. Its ability to bind to chitin has not been demonstrated, but the six aromatic amino acids present in the loops indicate such a possibility.

7.7. Hormonal Regulation of Chitin Metabolism

Chitinolytic activity in the molting fluid rises just prior to each molt and falls shortly thereafter. These changes parallel the increasing and falling ecdysteroid titers prior to ecdysis, as observed initially by Kimura (1976). A direct role for ecdysteroids in inducing chitinase expression was demonstrated using *M. sexta* larval abdomens that were precluded from receiving hormonal signals from the brain by a ligature below the second thoracic segment. Injection of 20-hydroxyecdysone (20HE) into these ligated abdomens resulted in a sharp and rapid increase in transcripts for chitinase. This increase was abolished by a simultaneous injection of a juvenile hormone mimic (Fukamizo & Kramer, 1987). Koga *et al.* (1992) reported a similar induction of chitinase by ecdysteroid, utilizing isolated *Bombyx* abdomens. Zheng *et al.* (2003) observed that injection of an ecdysteroid agonist resulted in induction of expression of a chitinase gene in epidermal tissue of *C. afumiferana*, and demonstrated the accumulation of chitinase in molting fluid. It appears that hormonal regulation of chitinase genes occurs in a broad range of insect species. However, the presence of multiple genes encoding chitinases was not appreciated when these early studies were done, and it was not apparent which class of chitinases was induced by the ecdysteroid treatment. Based on our present knowledge about the tissue specificity of expression of different groups of chitinases, it is likely that these early studies were only focused on the expression of group I chitinases.

A group II chitinase gene with five catalytic domains from the beetle *Tenebrio molitor* has also been shown to be hormonally regulated (Royer *et al.*, 2002). During pupal–adult metamorphosis, the abundance of transcripts for this gene paralleled the changes in ecdysteroid (20HE) titers during metamorphosis. Interestingly, even topical application of the JH analog, methoprene, induced transcripts for this chitinase within 8 hours after treatment. These results are somewhat contradictory to the studies on *M. sexta* chitinase, in which JH had no inductive effect on chitinase transcript levels (Kramer *et al.*, 1993). In *B. mori*, another chitinase gene, *BmChiR1*, required 20HE for induction, and was suppressed by the simultaneous application of a JH analog (Takahashi *et al.*, 2002). Even though this chitinase was reported to have only two inactive catalytic domains and one CBD, our bioinformatics analysis (Merzendorfer, unpublished data) indicated that this gene actually encodes a protein with five catalytic domains and seven CBDs, and appears to be a group II chitinase. A recent study on the regulation of chitinase gene expression in a shrimp species demonstrated that it is induced by ecdysteroids. Hence, ecdysteroids may be required for induction of chitinases in most arthropods (Priya *et al.*, 2009). While it is clear that the expression of more than one chitinase gene is controlled by ecdysteroid and possibly by JH, it is likely that these effects are mediated through one or more transcription factors induced by ecdysteroids (Riddiford *et al.*, 2003). However, there are no published reports on the identification of hormone response elements in the promoters of any of the insect chitinase genes.

There is little evidence to support the idea that hormones play a direct role in the control of chitin synthesis. Instead, chitin synthesis is initiated at about the time of (or prior to) apolysis, when new cuticle is being deposited.

In general, chitin synthesis reaches peak levels in between molts when new cuticle is being synthesized at the maximal rate. In larval stages, this is also the period when PM-associated chitin is synthesized. Thus, both CHS-A and CHS-B levels are high during feeding periods in larval stages. In the pupal stage *CHS-B* transcripts are undetectable, whereas levels of transcripts for *CHS-A* have multiple peaks roughly corresponding to periods of synthesis of pupal cuticle, adult epidermal cuticle, and tracheal chitin (Hogenkamp *et al.*, 2005; Arakane *et al.*, 2008).

7.8. Chitin Metabolism and Insect Control

7.8.1. Inhibition of Chitin Synthesis

The absence of chitin in animals and plants has led to the development of insect control strategies that target enzymes involved in the synthesis, modification, and degradation of chitin. Several membrane proteins that are likely to be involved in the assembly of chitin in the procuticle, or regulation of chitin metabolism, may also be attractive targets. Compounds that directly or indirectly interfere with chitin biosynthesis include peptidyl nucleosides, acylureas, thiadiazines, and different kinds of chitin-binding molecules. The peptidyl nucleosides were isolated originally from different *Streptomyces* species, and include polyoxins and nikkomycins (Hori *et al.*, 1971; Dahn *et al.*, 1976). They are substrate analogs resembling the structure of UDP-GlcNAc, and competitively inhibit chitin synthases of fungal and insect sources, with nikkomycin being the most potent inhibitor (Cohen, 2001). As peptidyl nucleosides that exhibit low permeability across the hydrophobic epicuticle are easily degraded in the intestine and show toxic side effects in vertebrates, they have not been developed further to control insect pests, but some of them are in use as fungicides in agriculture (Zhang and Miller, 1999; Cohen, 2001; Ruiz-Herrera and San-Blas, 2003). In contrast, since the discovery of the high insecticidal potential of diflubenzuron in the early 1970s by Dutch scientists, various acylurea derivatives, such as lufenuron, novaluron, and hexaflumuron, have been developed commercially for controlling agricultural pests (Palli and Retnakaran, 1999). They have been shown to inhibit chitin synthesis and to disturb cuticle formation, causing abortive molting. Ultrastructural analysis revealed defects in chitin synthesis, abnormal deposition of endocuticular layers, and impaired PM formation. Studies with these "chitin synthesis inhibitors" have provided some insights concerning the role of chitin in development, and its biological function. In particular, the use of the acylurea compound lufenuron has provided substantial information on chitin synthesis during *Drosophila* development (Wilson and Cryan, 1997). The effects of this insect growth regulator were complex and variable, depending on the developmental stage and dose at which the insects were exposed to this agent. When newly hatched larvae were reared on a diet containing very low concentrations of lufenuron, the larvae did not die until the second or third instar, and some pupariated even though the pupae were abnormally compressed. Pharate adults either failed to eclose or died shortly after emergence, and had deformed legs. The flight ability of the emerged adults was also affected when the larvae were exposed to very low concentrations of lufenuron. First and second instar larvae fed higher concentrations of lufenuron had normal growth and physical activity for several hours, but the insects died at about the time of the next ecdysis. Third instar larvae fed high concentrations of lufenuron underwent pupariation, but the puparia had an abnormal appearance, and the anterior spiracles failed to evert. Strikingly, adults showed no mortality and had no flight disability even when fed high levels of lufenuron, indicating that once all chitin-containing structures had been formed, this "chitin inhibitor" had very little effect on morphology and function. Thus, insect development is affected by lufenuron at all stages when chitin synthesis occurs. Another phase of insect development affected by this compound was egg hatching, even though oviposition was normal. The embryos completed development, but failed to rupture the vitelline membrane. In an ultrastructural study of acylurea effects on *Drosophila* embryogenesis, Gangishetti and colleagues have shown recently that egg hatching is completely abolished after treating female flies with a high dose of lufenuron and mating them with untreated males (Gangishetti *et al.*, 2009). In line with its lower insecticidal activity, the same treatment performed with diflubenzuron resulted in a constant rate of larval survival. Overall, the hatching rates depended on the dosage of the insecticides. The embryonic phenotypes were grouped into five classes: (1) hatching wild type larvae; (2) non-hatching larvae that appeared slightly bloated after being released manually from the eggshells; (3) non-hatching larvae with a strongly melanized head skeleton and a cuticle detached from the epidermis, which is similar to *knk* and *rtv* phenotypes (see section 7.6); (4) non-hatching larvae with a crumbled head skeleton and detached cuticle, which is similar to the *kkv* phenotype (see section 7.3.4.3. and **Figure 5**); and (5) non-hatching larvae with strong segmentation and morphological defects. The latter phenotypes were indistinguishable from the effects of the nucleoside peptide antibiotic nikkomycin, which is a competitive inhibitor of chitin synthase. Electron microscopy revealed that the treatment with lower doses of the insecticides affected cuticle thickness and orientation of microfibrils, while higher doses disrupted chitin synthesis completely, as evidenced by the lack of Calcofluor white fluorescence in the cuticle (Gangishetti *et al.*, 2009). Interestingly, no changes in *kkv* and

mummy gene expression were observed, but the expression of certain genes encoding cytochrome P450 enzymes was substantially upregulated, indicating that the respective enzymes are involved in diflubenzuron and lufenuron detoxification. Similar results were also observed in *Tribolium*, where diflubenzuron fed to larvae did not significantly influence *TcCHS1* or *TcCHS2* expression, but did affect mRNA levels for certain cytochrome P450 enzymes (merzendorfer, unpublished data). In contrast to *Drosophila* and *Tribolium*, RT-PCR and Northern blot analyses carried out with *A. quadrimaculatus* revealed a two-fold upregulation of *AqCHS1* mRNA levels in response to a high dose of diflubenzuron, while the chitin content in surviving larvae decreased in a dose-dependent manner (Zhang and Zhu, 2006). The observed increase in *AqCHS1* mRNA levels associated with a decrease in chitin content corroborates the common view that acylurea insecticides affect chitin synthesis at a post-transcriptional level. Hence, diflubenzuron-induced *AqCHS1* expression may serve as a mechanism to compensate for chitin deficiency.

Several studies have aimed to elucidate the underlying mechanism of the insecticidal activity of diflubenzuron. Diflubenzuron efficiently blocks chitin synthesis, as the incorporation of radiolabeled sugars into the growing chitin chain is inhibited (Post and Vincent, 1973; Hajjar and Casida, 1978; Mayer *et al.*, 1980; Clarke and Jewess, 1990). However, in contrast to peptidyl nucleosides that block chitin polymerization, diflubenzuron obviously does not affect the catalytic step, because chitin synthesis is not impaired in cell-free systems (Cohen and Casida 1980a; Mayer *et al.*, 1980; Kitahara *et al.*, 1983; Zimoch *et al.*, 2005). It also does not interfere with any of the metabolic reactions yielding UDP-N-acetylglucosamine, and neither does it affect chitin synthesis in fungi (Verloop and Ferrel, 1977; Cohen, 1987). Based on these and other findings, it was suggested that diflubenzuron acts at a post-catalytic step of chitin synthesis (Cohen, 2001). Many other mechanisms for the action of diflubenzuron have been suggested, including effects on glycolytic enzymes, chitinases, phenoloxidases, hormonal sites, and microsomal oxidases (Ishaaya and Cohen, 1974; Ishaaya and Ascher, 1977; Mitlin *et al.*, 1977; DeLoach *et al.*, 1981; Soltani, 1984). Studies using imaginal discs and cell-free systems indicated that benzoylphenylureas inhibit ecdysteroid-dependent GlcNAc incorporation into chitin (Mikolajczyk *et al.*, 1994; Oberlander and Silhacek, 1998). These results indicated that acylurea compounds target ecdysone-dependent sites, which eventually leads to inhibition of chitin formation. However, direct proof for this hypothesis is lacking. On the basis of competitive binding assays performed with glibenclamide, a more recent study suggested that a sulfonylurea receptor might be the target for diflubenzuron (Abo-Elghar *et al.*, 2004). As the sulfonylurea receptors (SURs) may also act as regulatory subunits of inward rectifying potassium channels in insects (Akasaka *et al.*, 2006), inhibition of a SUR could alter the membrane potential in such a way that Ca^{2+} homeostasis and eventually protein secretion required for cuticle and PM formation is affected. In line with this assumption, glibenclamide as well as diflubenzuron were found to affect Ca^{2+} uptake by isolated cuticular vesicles from the German cockroach *Blatella germanica* (Abo-Elghar *et al.*, 2004). Although the significance of this finding remains uncertain, future research following up on this hypothesis may elucidate the target site of acylureas.

Another chemical group of "chitin synthesis inhibitors" comprises thiadiazine derivatives, such as buprofezin (Applaud), which is used as an insecticide that specifically acts on sucking insects such as homopterans and hemipterans (Kanno, 1981). Although quite different in chemical structure, the effect of buprofezin resembles that of acylureas, as it blocks incorporation of radiolabeled chitin precursors and interferes with insect development. However, buprofezin may have a different target site in insects, as it also blocks acetylcholinesterase (AChE) activity. The activity of AChE in crude homogenates from the whitefly *Bemisia tabaci* was significantly inhibited by buprofezin at a concentration of 0.5 μM (Cottage and Gunning, 2006). Strikingly, inhibition was not observed in buprofezin-resistant flies.

Chitin-binding molecules interfere with the microfibril assembly, and hence block chitin deposition at its final step. There are polysaccharide-binding dyes, such as Calcofluor White (CFW), Congo red or primuline, which interfere with chitin crystallization by disrupting hydrogen bond formation and hence perturbing microfibril assembly (Vermeulen and Wessels, 1986). Accordingly, these dyes were reported to impair fungal cell wall morphogenesis (Selitrennikoff, 1984; Roncero and Duran, 1985). In insects, the process of PM formation appears to be particularly susceptible to CFW, and its effects were studied in flies, mosquitoes and caterpillars. Injection of as little as 0.05 μg CFW into *Calliphora erythrocephala* flies led to perturbations of PM formation and increased permeabilities for FITC-labeled dextrans with molecular masses ranging between 17 and 32 kDa (Zimmermann and Peters, 1987). However, in contrast to other PM-disrupting agents such as dithiothreitol or chitinase, changes in PM permeabilities for FITC-labeled dextrans with a molecular mass of 2 MDa were not observed when mosquito larvae were treated with CFW or Congo red (Edwards and Jacobs-Lorena, 2000). In *L. cuprina* the PM structure was not affected, although the larvae showed growth retardation and a reduction in lifespan (Tellam and Eisenmann, 2000). In the mite *Acarus siro*, combinations of diflubenzuron and CFW were most effective in reducing chitin content of the PM (Sobotnik *et al.*, 2008). Hence, combinations of CFW with other insecticidal compounds affecting chitin synthesis may

prove to be a useful strategy for insect control. Disruption of the PM structure was consistently reported in various lepidopteran species (Wang and Granados, 2000; Bolognesi *et al.*, 2001; Zhu *et al.*, 2007). When larvae of *T. ni* and *S. exigua* were fed with a CFW-containing diet, an increase in PM permeability was observed and the larvae became more susceptible to baculoviral infections. Interestingly, a significant amount of proteins was released upon CFW treatment, which may explain altered permeabilities (Wang and Granados, 2000; Zhu *et al.*, 2007). Next to chitin-binding dyes, numerous sugar-binding proteins (lectins) from animals and plants such as galectins, WGA, and chitinase-like lectins bind chitin or chitosan because of their high preference for GlcNAc. Like CFW, they disrupt PM formation in numerous cases, and therefore have been investigated for their insecticidal potential (Cohen, 2010). The effects of WGA on PM formation are summarized in section 7.3.1.2. However, these types of proteins also bind to glycoproteins and proteoglycans present in the PM, and hence their particular mode of action is difficult to asseess *in vivo*.

7.8.2. Exploiting Chitinases for Insect Control

Chitinases have been used in a variety of ways for insect control and other purposes (Kramer and Muthukrishnan, 1997; Gooday, 1999). Several chitinase inhibitors with biological activity have been identified based on natural products chemistry (Spindler and Spindler-Barth, 1999), such as allosamidin, which mimics the carbohydrate substrate (Rao *et al.*, 2003), and cyclic peptides (Houston *et al.*, 2002). Although useful for biochemical studies, none of these chitin catabolic inhibitors have been developed for commercial use, primarily because of the high cost of production and potential side effects. As we learn more details about chitinase catalysis, it might become more economically feasible to develop and optimize chitinase inhibitors for insect pest management.

Fungi and plants use chitinases for establishing infection and as a defense against invading pathogens, respectively. Entomopathogens secrete a plethora of extracellular proteins with potential activity in insect hosts. One of these proteins is chitinase, which is used by fungi such as *Metarhizium anisopliae* to help penetrate the host cuticle and render host tissues suitable for consumption (St Leger *et al.*, 1996; Krieger de Moraes *et al.*, 2003). Among the 10 most frequent transcripts in a strain of *M. anisopliae* are 3 encoding chitinases and a chitosanase (Freimoser *et al.*, 2003a). However, when *M. anisopliae* was transformed to overexpress its native chitinase, the pathogenicity towards the tobacco hornworm was unaltered, suggesting that wild type levels of chitinase are not limiting for cuticle penetration (Screen *et al.*, 2001). Another fungal species, *Conidiobolus coronatus*, also produces both endo- and exo-acting chitinolytic enzymes during growth on insect cuticle (Freimoser *et al.*, 2003b). Apparently, both *M. anisopliae* and *C. coronatus* produce a chitinolytic enzyme system to degrade cuticular components.

Both microbial and insect chitinases have been shown to enhance the toxicity of the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) (Regev *et al.*, 1996; Tantimavanich *et al.*, 1997; Ding *et al.*, 1998; Sampson and Gooday, 1998; Wiwat *et al.*, 2000). For example, when the chitinolytic activities of several strains of *B. thuringiensis* were compared with their insecticidal activity, it was determined that the enzyme could enhance the toxicity of Bt to *S. exigua* larvae by more than two-fold (Liu *et al.*, 2002). Microbial chitinases have been used in mixing experiments to increase the potency of entomopathogenic microorganisms (Kramer and Muthukrishnan 1997). Synergistic effects between chitinolytic enzymes and microbial insecticides were reported as early as the 1970s. Bacterial chitinolytic enzymes were first used to enhance the activity of Bt and a baculovirus. Larvae of *C. fumiferana* died more rapidly when exposed to chitinase–Bt mixtures than when exposed to the enzyme or bacterium alone (Smirnoff and Valero, 1972; Lysenko, 1976; Morris, 1976). Mortality of gypsy moth, *Lymantria dispar*, larvae was enhanced when chitinase was mixed with Bt, relative to treatment with Bt alone, in laboratory experiments (Dubois, 1977). The toxic effect was correlated positively with enzyme levels (Gunner *et al.*, 1985). The larvicidal activity of a nuclear polyhedrosis virus toward *L. dispar* larvae was increased about five-fold when it was administered with a bacterial chitinase (Shapiro *et al.*, 1987).

Inducible chitinolytic enzymes from bacteria cause insect mortality under certain conditions. These enzymes may compromise the structural integrity of the PM barrier and improve the effectiveness of a Bt toxin by enhancing contact of the toxin molecules with their epithelial membrane receptors. For example, five chitinolytic bacterial strains isolated from midguts of *Spodoptera littoralis* induced a synergistic increase in larval mortality when combined with Bt spore-crystal suspensions relative to either an individual bacterial strain or a Bt suspension alone (Sneh *et al.*, 1983). An enhanced toxic effect toward *S. littoralis* also resulted when a combination of low levels of a truncated recombinant Bt toxin and a bacterial endochitinase was incorporated into a semisynthetic insect diet (Regev *et al.*, 1996). Crude chitinase preparations from *B. circulans* enhanced the toxicity of Bt *kurstaki* toward diamondback moth larvae (Wiwat *et al.*, 1996). Liu *et al.* (2002) reported that several strains of Bt produced their own chitinases, which had synergistic larvicidal activity with the endotoxins.

A family-18 insect chitinase has been used as an enhancer of baculovirus toxicity and as a host plant resistance factor in transgenic plants. Introduction of an *M. sexta* chitinase cDNA into *Autographa californica* multiple nuclear polyhedrosis viral (AcMNPV) DNA

accelerated the rate of killing of fall armyworm compared to the wild type virus (Gopalakrishnan *et al.*, 1995). Baculoviral chitinases themselves play a role in liquefaction of insect hosts (Hawtin *et al.*, 1997; Thomas *et al.*, 2000). A constitutively expressed exochitinase from *B. thuringiensis* potentiated the insecticidal effect of the vegetative insecticidal protein Vip when they were fed to neonate larvae of *S. litura* (Arora *et al.*, 2003). Mutagenesis of the AcMNPV chitinase gene resulted in cessation of liquefaction of infected *T. ni* larvae, supporting a role of chitinase in viral spreading (Thomas *et al.*, 2000). When diet containing AcMNPV chitinase expressed in *E. coli* was fed to *B. mori* larvae, a dose-dependent increase in loss of integrity of the PM was observed. Even at a dose of 1 mg/g of larvae, there was 100% mortality (Rao *et al.*, 2004).

Tobacco budworms were killed when reared on transgenic tobacco expressing a truncated, enzymatically active form of *M. sexta* class I chitinase (Ding *et al.*, 1998). A synergistic interaction between insect chitinase expressed in transgenic tobacco plants and Bt applied as a spray at sublethal levels occurred when using the tobacco hornworm as the test insect. In contrast to results obtained with the tobacco budworm, studies with the hornworm revealed no consistent differences in larval growth or foliar damage when the insects were reared on first-generation transgenic chitinase-positive tobacco plants as compared to chitinase-negative control plants. When Bt toxin was applied at levels where no growth inhibition was observed on control plants, chitinase-positive plants had significantly less foliar damage and lower larval biomass production. These results indicated that the insect chitinase transgene did potentiate the effect of sublethal doses of Bt toxin, and *vice versa* (Ding *et al.*, 1998), but chitinase was not very effective on its own as a biocontrol agent. Tomato plants have been transformed with fungal chitinase genes with concomitant enhancements in resistance to insect pests (Gongora *et al.*, 2001). Effects observed include reduced growth rates and increased mortality, as well as a decrease in plant height and flowering time, with an increase in the number of flowers and fruits (Gongora and Broadway, 2002). Chitinase-secreting bacteria have been used to suppress herbivorous insect pests. A strain of *Enterobacter cloacae* transformed with a chitinase gene digested the chitinous membranes of phytophagous ladybird beetles, *Epilachna vigintioctopunctata*, and also suppressed leaf feeding and oviposition when the beetles ingested transformed bacteria entrapped in alginate microbeads sprayed on tomato seedlings (Otsu *et al.*, 2003). When pure chitinase from tomato moth larvae was injected into larvae, decreased cuticle thickness and 100% mortality was observed even at a low dose (2.5 µg/g). Insects fed this protein exhibited reductions in growth and food consumption (Fitches *et al.*, 2004). Acaricidal activity of a purified chitinase from a hard tick, *Haemophysalis longicornis*, has also been demonstrated (Assenga *et al.*, 2006). Immunization with this chitinase as the antigen protected mice from tick infections (You *et al.*, 2009).

Several GlcNAc-specific lectins from plants have been evaluated for insect toxicity (Harper *et al.*, 1998; Macedo *et al.*, 2003). These proteins appear to disrupt the integrity of the PM by binding to chitin or glycan receptors on the surface of cells lining the insect gut. Moreover, they may bind to glycosylated digestive enzymes and inhibit their activity. Another type of plant chitin-binding protein is the seed storage protein vicilin, which is actually a family of oligomeric proteins with variable degrees of glycosylation (Macedo *et al.*, 1993; Shutov *et al.*, 1995). Some vicilins are insecticidal to bruchid beetles and stalk borers (Sales *et al.*, 2001; Mota *et al.*, 2003). Apparently, these proteins bind to the PM, causing developmental abnormalities and reduced survival rates. To date, no non-enzymatic carbohydrate-binding protein derived from an insect has been evaluated for biocidal activity. A novel approach has been proposed to develop strategies for insect control by utilizing chitin-binding molecules to specifically target formation of the PM. CFW, a chemical whitener with chitin-binding properties, was used as a model compound in the diet to inhibit PM formation in *T. ni*, and to increase larval susceptibility to baculovirus infection (Wang and Granados, 2000). It was also effective in suppressing PM formation in *S. frugiperda*, and at the same time in preventing the establishment of a decreasing gradient of proteinases along the midgut tissue (Bolognesi *et al.*, 2001).

A protease from *A. gambiae* with a chitin-binding domain has been described, which may be involved in insect defense (Danielli *et al.*, 2000). This 147-kDa protein, sp22D, is expressed in a variety of tissues, most strongly in hemocytes, and is secreted into the hemolymph. Upon bacterial infection, the transcripts for this protein increase by about two-fold, suggesting a role in insect defense. This protein has a multidomain organization that includes two copies of an N-terminal ChtBD2 domain, a C-terminal protease domain, and several receptor domains. It binds strongly to chitin, and undergoes complex proteolytic processing during pupal to adult metamorphosis. It has been proposed that exposure of this protease to chitin may regulate its activity during tissue remodeling or wounding.

Two synthetic peptides were found to inhibit *A. gambiae* midgut chitinase, and also to block sporogonic development of the human malaria parasite *Plasmodium falciparum* and avian malaria parasite *P. gallinaceum*, when the peptides were fed to infected mosquitoes (Bhatnagar *et al.*, 2003). The design of these peptides was based on the putative proregion sequence of mosquito midgut chitinase. The results indicated that expression of chitinase inhibitory peptides in transgenic mosquitoes might alter the vectorial capacity of mosquitoes to transmit malaria.

7.9. Future Studies and Concluding Remarks

Although substantial progress in studies of insect chitin metabolism has occurred since the initial edition of *Comprehensive Insect Physiology, Biochemistry and Pharmacology* was published in 1985, we still do not know much about how chitin is produced and transported across the cell membrane so that it can interact perfectly with other components for assembly of supramolecular extracellular matrices such as the exoskeleton and PM. These structures are still very much biochemical puzzles in which we do not understand well how the various components come together during morphogenesis, or are digested during the molting process. Hopefully, this chapter will stimulate more effort to gain an understanding of how insects utilize chitin metabolism for growth and development, and also to facilitate development of materials that may perturb insect chitin metabolism for pest management purposes.

Since 2005, many questions have been answered about the biosynthesis of insect chitin, including: why do insects have two genes for CHS, and at what developmental stages are the various CHSs produced? However, we know little about the unique properties and functions of each CHS. Of particular interest is the role of alternate splicing in generating different isoforms of CHSs from the same gene. The developmental cues that control alternate splicing and how they affect chitin synthesis and/or deposition will be the subject of future studies. Attempts to express full-length *CHS* genes in heterologous systems for the production of active recombinant enzymes or subdomains has met with very limited success, probably because CHSs are membrane-bound proteins. The recent finding that proteolytic processing may be necessary for CHS activation may also have contributed to this lack of success (Broehan *et al.*, 2007, 2008). In the future, the availability of pure proteins and molecular probes for specific CHSs would facilitate a better understanding of chitin biosynthesis and its regulation.

Two other significant questions about the regulation of insect chitin biosynthesis are: what is the mechanism of the initiation phase, and is there an autocatalytic initiator molecule? Like glycogen synthesis, chitin synthesis may involve both initiation and elongation phases. As the initiator of glycogen synthesis, glycogenin transfers glucose from UDP-glucose to itself to generate an oligosaccharide–protein primer for elongation (Gibbons *et al.*, 2002). Like chitin synthase, glycogenin is a glycosyltransferase, which raises the question of whether chitin synthase has an autocatalytic function similar to glycogenin, and whether there is a separate "chitinogenin"-like protein. Another possibility is the participation of a lipid primer for chitin synthesis. Cellulose synthesis in plants involves the transfer of lipid-linked cellulodextrins to a growing glucan chain (Read and Bacic, 2002). The lipid in this case is sitosterol-β-glucoside. No lipid primer has been identified to date for insect chitin synthesis.

Little is known about the catalytic mechanism of any insect CHS. Once active insect CHS-related recombinant proteins can be produced in a cell line, site-directed mutagenesis can be used to probe for essential residues in the catalytic and regulatory domains. It is likely that acidic amino acids play critical roles in CHS catalysis in a manner comparable to those identified in other glycosyltransferases (Hefner *et al.*, 2002) and in yeast chitin synthases (Nagahashi *et al.*, 1995).

One of the major unanswered questions about insect chitinolytic enzymes and chitin deaceylases is: why are there so many of these enzymes? Some species have more than 20 chitinase or chitinase-like genes, and we only know the function of a few of them. Chitinolytic enzymes are gaining importance for their biotechnological applications in agriculture and healthcare (Dahiya *et al.*, 2006). Additional success in using chitinases from both insects and other organisms for different applications depends on a better understanding of their biochemistry and regulation so that their useful properties can be optimized through genetic and biochemical engineering. Reasons for the rather high number of chitinolytic and chitin deacetylase enzymes with various domain structures are not fully understood.

So far there has been little success in using chitinase in pest-control applications, but it may prove more useful as an enhancer protein in a cocktail with other biopesticides targeted at the cuticle or gut (Fiandra *et al.*, 2010; Di Maro *et al.*, 2010). Also, only a few catalytic domains or chitin-binding domains, or various combinations thereof (domain shuffling and/or swapping), have been evaluated for biocidal activity, and thus further toxicological experimentation after recombinations is warranted (Zakariassen *et al.*, 2009; Li and Greene, 2010; Neeraja *et al.*, 2010). With good progress occurring in regard to functional analysis from RNAi studies, the ability to choose an appropriate target gene or protein associated with insect chitin metabolism that can be exploited to achieve targeted and selective control of pest insects has improved.

A hypothetical model for chitin-containing extracellular matrices in insects is the following: a fiber-reinforced composite structure whereby chitin fibers form the initial scaffold that is subsequently impregnated with a blend of proteins into which some components of lower abundance, such as water, catechols, lipids, pigments, and minerals, are interspersed. For a soft hydrated material such as the PM and trachea, chitin/chitosan and protein are the major components that associate primarily non-covalently via hydrogen bonding, as well as through hydrophobic and electrostatic interactions with relatively little protein cross-linking. The chitin-organizing proteins may have a role in the precise arrangement of the individual laminar layers of chitin, as well as their relative orientation with

the layers above and below it. For matrices that become sclerotized, such as tanned cuticle, catechols are incorporated and oxidized to quinones and quinone methides, which subsequently cross-link the proteins, and perhaps chitin/chitosan as well. Future studies are needed to characterize more fully the covalent and non-covalent interactions and reactions of chitin, protein, lipid, mineral salts, and oxidized catechols (chitin–water, chitin–protein, chitin–catechol, chitin–lipid, chitin–pigment, chitin–mineral interactions) from appropriate secretory tissues. Results from such studies will provide critical insights into the anabolic and catabolic pathways by which the chitin–protein composite is formed and recycled, as well as into the bioinspired fabrication of environmentally sustainable load-bearing materials whose formulation is based, at least in part, on insect chitin chemistry and metabolism.

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