CHARACTERIZATION OF THE INSECT CUTICLE
SCLEROTIZATION HORMONE BURSICON AND
BURSICON-REGULATED GENES IN THE HOUSE FLY

*MUSCA DOMESTICA*

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by
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CHARACTERIZATION OF THE INSECT CUTICLE SCLEROTIZATION HORMONE BURSICON AND BURSICON-REGULATED GENES IN THE HOUSE FLY

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Songjie Wang

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ABSTRACT

Bursicon is a neurohormone that regulates cuticle sclerotization (tanning and hardening) and the wing expansion processes in insects. Bursicon was discovered over forty years ago from the blowfly *Calliphora erythrocephala* in a neck-ligated fly assay. However due to the difficulties in hormone protein purification and the lack of molecular techniques, the genes encoding bursicon were not identified until in the years of 2004-2005. Studies in *Drosophila melanogaster* have indicated that bursicon is actually a heterodimeric cystine knot family protein containing two subunits, bursicon α (bur α or burs) and bursicon β (bur β or pburs), which are encoded by two individual genes *burs α* and *burs β* (or *burs* and *pburs*). Although bursicon genes have been cloned from several insect species or predicted from insect genomes, little is known about its mechanisms in the cuticle sclerotization and wing expansion processes, especially the signal transduction pathway(s) and the genes regulated by bursicon.
In the current study, the house fly, *Musca domestica*, was selected as the research model, because of its relatively larger body size for easy dissection and microscopic manipulation when compared to *Drosophila*, its distinguished cuticle sclerotization phenotype for easy detection, and a house fly bioassay system we developed in our laboratory for the detection of bursicon activity. The *burs α* and *burs β* genes were cloned from *M. domestica* using 5’ and 3’ rapid amplification of cDNA ends (RACEs). The *M. domestica burs α* gene has an open reading frame (ORF) of 531 bps, encoding a 176 amino acid (a.a.) polypeptide with a predicted molecular weight of 19.5 kDa, while the *burs β* gene has an ORF of 444 bps, encoding a 147 a.a. polypeptide with a predicted molecular weight of 17 kDa. The *M. domestica* *burs α* and *burs β* both share 79% sequence identity with their *Drosophila* counterparts, and the identities with the bursicon sequences from other insect species range from 47% to 61%. A developmental study revealed that both *M. domestica burs α* and *burs β* transcripts were present in larval and pupal stages, maximally expressed in pharate adults, and declined sharply after adult emergence, suggesting the release of the hormone at adult emergence. Recombinant *Musca* and *Drosophila* bursicon heterodimer (*r-bursicon*) was expressed in mammalian 293 cells and insect Highfive™ cells. *R*-bursicon expressed in both systems showed a strong bursicon activity in the neck-ligated house fly assay, and also showed cross species activities between *Musca* and *Drosophila*. Fluorescence in situ hybridization (FISH) studies of bursicon distribution in the central nervous system of *M. domestica* and *D. melanogaster* indicated that in *M. domestica*, both *burs α* and *burs β* transcripts were expressed in a set of neurosecretory cells (NSCs) in the subesosophagael ganglia (SEG) and abdominal ganglia (AG) in larvae and pupae, but only in the fused...
thoracic-abdominal ganglia (TAG) in the adults. This is similar to the expression pattern as detected in *D. melanogaster* although some differences do exist.

By using r-bursicon with DNA microarray analysis, a series of genes were identified that are very likely involved in the bursicon-regulated cuticle sclerotization and wing expansion processes. Among these identified genes, two genes were cloned and sequenced for further study in the house fly *M. domestica*. One of them, *mdSu(H)*, is a homolog of the *D. melanogaster* Suppressor of Hairless (*Su(H)*) gene. *Su(H)* is a neurogenic transcriptional factor and has been demonstrated to be involved in regulating several insect neurogenesis processes and many other physiological events in insects. Real-time RT-PCR analysis indicated that the level of *mdSu(H)* transcript was up-regulated by ~2.5-3 folds 1 h after r-bursicon injection, which correlated well with the cuticle sclerotization process observed in the r-bursicon injected neck-ligated flies. Developmental studies showed that in normal (non-ligated) flies, this gene was also highly expressed after ecdysis, indicating its participation in post-ecdysis events, possibly cuticle sclerotization and/or the wing expansion process. *Su(H)* is a component of the Notch signaling pathway. After analyzing our DNA microarray data, we found that 11 other genes, which are either directly involved in or associated with the Notch signaling pathway, were also up-regulated upon r-bursicon stimulation in the neck-ligated fly assay. This indicates that the Notch pathway might be an important signal transduction pathway involved in the bursicon-mediated sclerotization and wing expansion processes. A second gene of interest to us, *mdPH*, which is a homolog of *D. melanogaster* pleckstrin homology (*PH*) gene encoding a pleckstrin homology (PH) protein, was also cloned and sequenced in *M. domestica*. Analysis of the *mdPH* gene showed that it is down-regulated by ~2-3
fold upon r-bursicon stimulation in the neck-ligated flies, starting from 40 minutes after injection. Developmental analysis of mdPH transcripts in the non-ligated house flies also showed that it is down-regulated after adult emergence. PH domains are present in a number of proteins. Many of their functions are related to signal transduction, e.g. in the G protein coupled receptor signaling pathway, which is now widely accepted as a downstream transduction pathway during bursicon-mediated cuticle sclerotization and wing expansion. In this pathway, PH has an inhibitory effect on G protein coupled receptor (GPCR) by activating G protein coupled receptor kinase-2 (GRK-2) and then phosphorylating the GPCR, thus repressing the activation of GPCR and blocking the transduction of signal. Rapid down-regulation of the repressor PH upon r-bursicon administration allows activation of GPCR and subsequent signal transduction, leading to cuticle sclerotization and wing expansion. This result indicates that PH is also very likely an important element involved in the bursicon signal transduction pathway.
CHAPTER 1

LITERATURE REVIEW

1.1 Insect cuticle

1.1.1 Insect integument

The insect integument is a vital organ for insect survival. The amazing success of the insects on the planet earth can be attributed in no small measure to the incredible mixture of flexibility and strength of the insect integument. The combination of flexibility and hardness of the insect integument allows insects to move freely without loss of defense and protection.

A typical insect integument is mainly composed of three parts (Fig. 1-1). The outer layer, the cuticle, is most visible together with the attendant bristles and hairs. Below this are the epidermis, which is a cell layer responsible for synthesis and release of various materials for the cuticle, and the basement membrane, which is a non-cellular layer that serves as a barrier between the integument and the inside tissues. The outer layer, the cuticle, gives the insect integument most of its color and hardness (Nation, 2002).
Fig. 1-1. The general structure of the insect integument (Image is modified from http://www.nazarian.ir/biodb/en/insects_anatomy.asp).
1.1.2 The insect cuticle

The insect cuticle is a relatively thin layer of non-cellular material. It constitutes the external surface of the body, the tracheae, the anterior and posterior parts of the alimentary canal and the reproductive system. The insect cuticle is a hard layer of exoskeleton. The hardened, or sclerotized, cuticle has several important functions: to provide protection against desiccation (or from imbibing excessive water in case of aquatic insects), external physical injuries and microorganisms and to serve as muscle attachment for insect movement and flight. Yet, these functions are inherently dependent upon the occurrence of sclerotization of the cuticle during molting. Insects are especially vulnerable between molts, when the cuticle is soft and flexible. A better understanding of the physiological events involved in molting, specifically sclerotization of the cuticle, could possibly provide ways to exploit these vulnerabilities in controlling pest populations.

1.1.3 The cuticular components

The insect cuticle is divided into two different types: the hard and stiff regions, which are called the sclerites; and the more flexible and pliable regions, called the arthrodial membranes, which connect among the sclerites and make the various forms of locomotion possible (Andersen, 2005). The properties of the individual regions of the cuticle correspond to their different functions. The regions corresponding to flight are characterized by near-optimal balance between stiffness and flexibility, while those regions corresponding with protection are mostly covered by hard and rigid sclerites.
A typical insect cuticle consists of three layers: the endocuticle, the exocuticle, and the epicuticle. The endocuticle and the exocuticle are mostly composed of a large number of layers of protein and chitin fibres laying down in a laminated pattern such that the individual strands in each layer cross each other, creating an extremely tough and flexible substance. The epicuticle contains no chitin and is highly resistant to water and other solvents. The relative amounts of chitin and proteins, chitin architecture, protein composition, water content, intracuticular pH and the degree of sclerotization are the main factors affecting the hardness and rigidity of the insect cuticle.

1.1.4 Chemical components of cuticle

Chemically, insect cuticle contains: chitin, a polysaccharide polymer of N-acetyl glucosamine; proteins; lipids that function in waterproofing; and phenols and quinines that are involved in the insect cuticle sclerotization process (Nation, 2002).

Chitin:

Chitin is an important constituent of the insect cuticle. Chitin is a polymer of N-acetyl-β-D-glucosamine residues linked together by β-(1,4)-glycosidic linkages (Nation, 2002). Chitin occurs in long chains in nature and is packed into a crystallite structure that is embedded in a protein matrix. The content of chitin can constitute up to 40% of the dry mass of insect exuviate depending on different species and different types of the cuticle (Kramer et al., 1995). Chitin is found in the exo- and endocuticle, but not in the epicuticle, the outermost layer of the cuticle (Andersen, 1979a, b). It functions as a light but mechanically strong scaffold material and is always associated with several cuticle proteins that mainly determine the mechanical properties of the cuticle. Insect growth and
development is strictly dependent on the capability to remodel chitinous structures (Merzendorfer, 2003).

**Proteins:**

Proteins are another major and important cuticular component. Cuticular proteins are synthesized in the epidermal cells and are then secreted into the cuticle from the apical surface of these cells (Nation, 2002). Cuticular proteins are generally classified into two classes: nonstructural proteins and structural proteins (Willis *et al.*, 2005).

*Nonstructural proteins:*

Pigments

So far proteins from three classes of pigments have been identified and sequenced in insects: insecticyanins, which are blue pigments, and two anonymous yellow proteins (Willis *et al.*, 2005). In the insect cuticle, these proteins, in cooperation with carotene, confer the green coloration. One of the yellow proteins, encoded by the gene *yellow*, has been reported to be responsible for the melanization of the cuticle (Kornezos and Chia, 1992), and cis- and trans- regulation of the *yellow* gene is responsible for the color differences in different species and different patterns within a species (Wittkopp *et al.*, 2002). The other yellow pigment protein has putative carotene and/or JH binding activity and was proposed to be involved in transporting carotene into the cuticle (Wybrandt and Andersen, 2001).

Enzymes

A lot of types of enzymes are located in the insect cuticle having different functions during cuticular events, such as molting and sclerotization. Several of the
enzymes involved in sclerotization, such as prophenoloxidase, diphenoloxidase, laccases, peroxidases, isomerases, and tautomerases, have been identified in insect cuticle (Andersen, 1979a, 1979b).

Defense proteins

Defense proteins, such as cecropin (Lee and Brey, 1994) and scolexin (Molnar et al., 2001), are involved in insect immune response to pathogens like bacteria and viruses.

Arylphorins

Arylphorins are proteins with a high content of aromatic amino acids and some lipid; the function of arylphorin proteins is not clear.

Structural proteins:

There are a lot of structural proteins present in insect cuticle. Over one hundred structural proteins have been sequenced, and in addition, almost 200 more proteins were predicted based on the available genomic sequences in the fruit fly *D. melanogaster* and the mosquito *Anopheles gambiae* (Nation, 2002). Many of these structural proteins have common consensus sequences, such as Rebers and Riddiford Consensus (R&R Consensus) and Pfam00379 consensus (Andersen et al., 1995a, 2000; Finn et al., 2008), so their presence in the cuticle can sometimes be predicted simply based on sequence analysis.

1.2 Insect molting and cuticle sclerotization

The final step of insect development and maturation is a process called metamorphosis. Insect metamorphosis is composed of two steps: molting and sclerotization.
1.2.1 Molting

Insects’ success on the earth is partly due to the hard exoskeleton or cuticle. The hard cuticle provides insects muscle attachment for movement and protects insects from water loss, external physical injury and pathogens. However, the cuticle also prevents insects from growth due to lack of the ability to expand. To grow bigger, insects must periodically shed the old cuticle and replace the old one with a new bigger one. The shedding of the old cuticle and replacement of a new one is called molting.

Molting in insects begins with the separation of the cuticle from the underlying epidermal cells (apolysis) and ends with the shedding of the old cuticle (ecdysis). During apolysis, the old cuticle (including the endocuticle, exocuticle and epicuticle) is detached from the underneath newly synthesized endocuticle and the epidermis. Then after apolysis, molting fluid is secreted into the space between the old cuticle and the epidermis (the exuvial space). The fluid contains inactive enzymes that are activated when the new epicuticle is secreted. This prevents the enzymes from digesting the new procuticle as it is laid down. After the synthesis of the new epicuticle, the lower region of the old cuticle - the endocuticle - is then digested by the enzymes and subsequently absorbed. The exocuticle and epicuticle resist digestion and are hence shed at ecdysis (Nation, 2002).

1.2.2 Sclerotization

Right after molting, since the newly synthesized cuticle is very soft, insects are extremely vulnerable: they have limited mobility which makes them easy prey for predators; the cuticle is unable to provide enough protection from desiccation (or from
imbibing excessive water in the case of aquatic insects) and pathogens. Thus, for the survival of insects, a cuticle hardening and darkening process, the sclerotization (or tanning), must occur. Insect cuticle sclerotization is a cuticle hardening and darkening process that occurs after insect molting. Fig 1-2 shows a typical sclerotization process after adult emergence in the house fly *M. domestica*. Sclerotization is a chemical process by which certain regions of the insect cuticle undergo an irreversible change from a soft layer into a stiffer and harder structure. Sclerotization mainly occurs in the exocuticle and epicuticle, which are the two outer most layers in insect cuticle; while in the endocuticle, although a high amount of chitin and proteins are present, very little sclerotization occurs and this layer of cuticle always remains soft and flexible. Sclerotized cuticle has decreased deformability, extractability of matrix proteins, and increased resistance towards dissolvants and digestive enzymes (Nation, 2002). And thus due to the extreme stability of sclerotized insect cuticle, studies on the chemical changes occurring during the sclerotization process have encountered a great deal of difficulties. Until today, many aspects of the sclerotization process are still poorly understood.

### 1.2.3 Mechanism of sclerotization

Fig 1-2. A typical example of the cuticle sclerotization process occurring after adult emergence in the house fly *M. domestica*. 
There are two currently proposed models as to how catecholamines are transformed during the final sclerotization process. The first and most widely accepted model is the quinone tanning hypothesis. This model was first proposed by Pryor (1940 a, b), modified by many researchers and well reviewed by Andersen (2005). The quinone-tanning hypothesis proposes that N-acylcatecholamines are oxidized by a phenoloxidase to quinones and quinone methides, which serve as electrophilic cross-linking agents to form covalent cross-links between cuticular proteins. As shown in Fig. 1-3, the amino acid tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine (DOPA) through the action of tyrosine dehydrogenase. Then DOPA is transformed into dopamine by decarboxylation under dopa decarboxylase activity. Dopamine is the key intermediate compound for the formation of sclerotization and another very likely related agent called melanin, a compound of central importance for insect coagulation and wound response processes. The synthesized dopamine is then N-acylated to either N-acetyldopamine (NADA) or N-β-alanyldopamine (NBAD), both of which can serve as precursors in the sclerotization process. As described in Fig. 1-4, NADA (or NBAD) can be oxidized to o-quinone, which in turn reacts with nucleophilic groups, whereby catecholic structure is regained and the nucleophile is linked to the aromatic ring. The o-quinones of NADA and NBAD can also be enzymatically isomerized to p-quinone methide. The p-quinone methides are then enzymatically isomerized to unsaturated catechol derivatives, dehydro-NADA and dehydro-NBAD, which after oxidation to unsaturated quinones can react with catechols to form dihydroxyphenyl-dihydrobenzodioxine derivatives and with other nucleophilic groups to form other unidentified compounds. These derivatives, after
Fig. 1-3. Biosynthesis of the sclerotization precursors NADA and NBAD from tyrosine. The enzyme tyrosine dehydrogenase hydrolases tyrosine to 3,4-dihydroxyphenylanaline (DOPA), which is then decarboxylated to dopamine by the enzyme dopa-decarboxylase. Dopamine can then be acylated to either N-acetyldopamine (NADA) or N-β-alanyldopamine (NBAD). (Model taken from Anderson, 2005)
Fig. 1-4. Cuticular oxidation of NADA and NBAD to various quinone derivatives. NADA and NBAD are oxidated to various quinone isomers (o-quinone, p-quinone), which then react with catechols to form various known and/or unidentified derivatives. These derivatives, after further linking with proteins and possibly chitin, form the final color and stiffness of the cuticle. (Model taken from Anderson, 2005)
further linked with other proteins and perhaps chitin, to provide the final color and stiffness of the sclerotized cuticle. The appearance and properties of cuticle from different body regions of the same animal can vary widely, and a considerable part of this variation, such as the different coloration and mechanical properties, is probably due to quantitative or qualitative differences in the sclerotization process. The proposed sclerotization reactions reflect studies in only a few insect species, such as the cuticle of *C. erythrocephala* larvae, the pupae of the tobacco hornworm *Manduca sexta*, and the *Locusta migratoria* femurs (Andersen, 2005). The exact mechanism of the chemical reactions taking place during cuticle sclerotization is still far from being clearly elucidated and will need more studies.

The second hypothesis for the mechanism of sclerotization is that incorporation of large quantities of oxidized N-acylcatechols displaces water and dehydrates the cuticle to such an extent that cuticular proteins become trapped in a hydrophobic matrix of polymerized N-acylcatechols (Vincent and Hillerton, 1979). However due to lack of evidence and experimental data, fewer scientists now support this hypothesis.

The proposed hypotheses for insect cuticle sclerotization indicate the possible chemical reactions taking place to make the final rigid cuticle. However, these are very likely the processes that take place at the final level within the whole sclerotization pathway. Much research is still in progress to find out how these reactions are controlled and regulated from the initial level, that is how enzymes are activated and deactivated, and how signals are initiated and transmitted to the target tissue. So far, it has been proposed and believed that the cuticle sclerotization process is under control of a series of hormones.
1.3 The control of the sclerotization process: hormones

At the end of the insect molting process, insects shed their old cuticle by performing the ecdysis sequence, an innate behavior consisting of three steps: pre-ecdysis, ecdysis, and postecdysis. The coordination of these processes is regulated by at least six hormones, prothoracicotropic hormone (PTTH), ecdysteroid 20-hydroxyecdysone (20-E), eclosion hormone (EH), ecdisis triggering hormone (ETH), crustacean cardioactive peptide (CCAP), and bursicon (Ewer et al., 2002).

The possible sequential control was proposed and summarized by Davis et al. (2007), as cited in Fig. 1-5. The very first hormone, PTTH, initiates the whole process and is responsible for controlling the release of 20-E. The change in the titre of 20-E stimulates the release of ETH and EH, and gives the signal for the initiation of ecdysis and the synthesis of a new cuticle (Truman, 1981). ETH and EH act in a positive feedback loop to cause further releases of each other (Ewer et al., 1997). ETH and EH together are responsible for the first step of molting -- pre-ecdysis (Baker et al., 1999). It was recently reported that a subset of bursicon containing neurons in the thoracic ganglia in the larvae of the hawkmoth M. sexta express ETH subtype A receptors (ETHR-A), indicating that these cells are the targets of ETH, and ETH may directly regulate the expression and/or release of bursicon (Dai et al., 2008).

At the last step after ecdysis, the last hormone in this signal cascade, bursicon, is released and causes the sclerotization of the newly synthesized cuticle and the expansion of insect wings (Dewey et al., 2004; Huang et al., 2007; Luo et al., 2005; Mendive et al., 2005). Another hormone, crustacean cardioactive peptide (CCAP), which behaves as a cardioaccelerator, is co-localized in all neurosecretory cells that produce bursicon.
(Kostron et al., 1996; Luan et al., 2005). The major function of CCAP hormone in molting and sclerotization process is not well understood, but it was proposed that CCAP might be responsible for activating the PKA signaling pathway (Luo et al., 2005) and initiating tyrosine hydroxylase (TH) translation, which is thought to be a critical enzyme involved in the synthesis of tanning materials following eclosion (Davis et al., 2007).

Although it is widely accepted that the bursicon hormone alone controls insect sclerotization, the exact signal transduction pathway that it acts through is yet not well characterized.

**Sclerotization in larvae and pupae**

Sclerotization occurs not only in insects after the pupa to adult molting, but also takes place in early developmental stages. In insect that undergo larval to larval and/or larval to pupal molting, the sclerotization process also occurs in various types of tissues, such as mandibles and puparium. The control of these types of sclerotization has not been very well characterized, but it is very likely that they are different from the sclerotization that occurs after pupal to adult molting.

In higher holometabolous orders, hardening of insect cuticle during puparium is controlled by a set of hormones called the pupariation factors. In these orders, insects discard their whole thoracic and abdominal epidermis during metamorphosis and build a new adult body wall from imaginal discs and abdominal histoblasts. At the end of the last larval stage, the cuticle contracts to a smooth barrel shape puparium, which sclerotizes and provides a safe place where adult development takes place. Sclerotization of the puparium is controlled by a hormone called puparium tanning factor (PTF). Two
PTTH → Ecdysone

ETH +

EH +

CCAP +

Bursicon

Behaviour: pre-ecdysis ecdysis cuticle sclerotization

Fig. 1-5. The neuropeptide signaling pathway at eclosion. The first hormone, PTTH, controls the release of ecdysone (20-E). A decrease in the level of 20-E triggers the release of ecdysis-triggering hormone (ETH) and eclosion hormone (EH), which further stimulate the release of each other in a manner of positive feedback. ETH and EH together regulate pre-ecdysis behavior. EH causes the release of crustacean cardioactive peptide (CCAP), which shuts off pre-ecdysis and causes release of the hormone bursicon for the final cuticle sclerotization process (Modified from Davis et al., 2007).
additional hormones, anterior segment retraction factor (ARF) and puparium-immobilizing factor (PIF), are also likely involved in puparium formation (Zdarek, 1985). Anterior segment retraction factor causes anterior segments of the larva to retract and form the larval integuments into the barrel-shaped puparium. Puparium-immobilizing factor causes the larvae to become immobile for a period of time. The three hormones seem to be secreted simultaneously to cause the hardened puparium (Denlinger and Zdarek, 1994). Recently a group of peptides, which all belong to the pyrokinin hormone family, were identified to be potential pupariation factors in several insect species, including the grey flesh fly Neobellieria bullata (formerly called Sarcophaga bullata), the coackroach Leucophaea maderae, and the locust, L. migratoria (Zdarek et al., 1997, 1998). One of these factors, a SVQFKPRLaamide designated as Neb-pyrokinin-2 (Neb-PK-2), is believed to be the primary pupariation factor (Verleyen et al., 2004). These factors are similar in sequences and are homologous to the products of two Drosophila genes, capa and hugin, which are two closely related FXPRLaamide encoding genes (Verleyen et al., 2004).

1.4 Bursicon - the insect cuticle sclerotization hormone

1.4.1 Discovery

The initial discoveries in the physiology of cuticle sclerotization, or ‘tanning’ involved the development of a bioassay utilizing newly-emerged, decapitated blowflies, Calliphora erythrocephala, with unsclerotized cuticle and hemolymph from blowflies shortly after emergence (Cottrell, 1962a; Fraenkel and Hsiao, 1962, 1965). The bioassay established that some hormonal control factor was involved in tanning because the
decapitated flies would not undergo cuticle sclerotization until they were injected with hemolymph from newly emerged flies. Based on this finding, researchers determined that the sclerotization results from a factor released into the hemolymph from the fly head, and named the factor ‘bursicon’ (Fraenkel and Hsiao, 1965). Bursicon’s presence has since been established in several other orders besides Diptera, including Orthoptera, Lepidoptera, Hemiptera, and Coleoptera (Cottrell, 1962b; Fraenkel and Hsiao, 1965; Kostron et al., 1996; Mills, 1965, 1966; Mills et al., 1965; Padgham, 1976a, b; Post, 1972; Post and De Jong, 1973; Reynolds, 1977; Reynolds et al., 1979; Srivastava and Hopkins, 1975; Taghert and Truman, 1982a, b; Truman, 1973; Vincent, 1971, 1972).

### 1.4.2 Biochemical and molecular properties

Results from several studies using different insect species have established the polypeptide nature of bursicon, including evidence indicating that the tanning factor is non-dialysable, relatively insoluble in organic solvents, able to be precipitated with loss of activity by reagents such as alcohol and acetone, and able to be inactivated by trypsin or pronase treatments (Cottrell, 1962b; Fraenkel and Hsiao, 1965; Taghert and Truman, 1982a).

Since the discovery of the bursicon hormone and the demonstration of its protein nature, scientists have been trying to determine its biochemical and molecular properties. However this process has been very difficult because of the lack of information of its gene and protein sequences.

For a long time the determination of bursicon’s molecular mass was accomplished by SDS-PAGE and/or gel chromatography of nervous system homogenates,
subsequent division of the gel into slices, protein elution from these slices, and a test for bursicon activity of the eluted proteins in the ligated fly bioassay. Bursicon’s molecular size has been reported differently in various insect species based on many studies. Fraenkel et al. (1966) determined the molecular weight of bursicon in blowfly hemolymph to be about 40 kDa through the use of size-exclusive chromatography, with subsequent chromatography studies estimating a size of >30 kDa (Reynolds, 1976; Seligman and Doy, 1973). Kostron et al. (1996) compared bursicon’s size in several insect species, including C. erythrocephala, Periplaneta americana, Gryllus bimaculatus, L. migratoria, and Tenebrio molitor, and estimated it to be about 30 kDa. The molecular size of bursicon in M. sexta has been reported as >60 kDa by Reynolds (1977) and ~20-30 kDa by Taghert and Truman (1982a).

The synthesis and release of bursicon has long been considered to be from the central nervous system. Bursicon activity has been detected in most parts of the central nervous system, including the brain, corpora cardiaca/corpora allata complex, thoracic and abdominal ganglia, but the distribution varies in different insect species. Fraenkel and Hsiao (1963, 1965) showed that bursicon was most active in the fused thoracic-abdominal ganglia (TAG) of the blowfly C. erythrocephala, with the concurrent decrease in this activity as hemolymph activity increased. In the American cockroach, P. americana, Mills et al. (1965) attributed bursicon release to the abdominal ganglia. In the tobacco hornworm, M. sexta, bursicon was also acknowledged to be released from the abdominal ganglia (Truman, 1973; Taghert and Truman, 1982a, b).

In the recent several years, the generation and availability of bursicon antibodies have helped the progress in the study of bursicon. Honegger et al. (2002) partially
purified bursicon in the American cockroach, *P. americana*, using high performance liquid chromatography technology and two-dimensional gel electrophoresis. Antibodies raised against this partially purified protein labeled different patterns of bursicon-containing neurons in the central nervous systems of *P. americana*, the crickets *Teleogryllus commodus* and *G. bimaculatus*, the moth *M. sexta*, and the fruit fly *D. melanogaster*, indicating the high conservation of this hormone among insect species.

The variability in the reported size and release sites suggests that bursicon may exist in different forms between various insect species, but its activity, with an initial post-emergence surge and subsequent steady decline, was common in most species studied.

The partially purified bursicon from nerve cords of the cockroach *P. americana* also made it possible for exploring the genomic information of this hormone. By comparing the partial sequences with available genomic data, Dewey et al. (2004) identified the *D. melanogaster* gene CG13419 (*burs*, or *burs α*) as a candidate bursicon gene.

The *burs α* gene encodes a peptide with a predicted final molecular weight of 15 kDa. This predicted bursicon protein belongs to the cystine knot family, which includes vertebrate transforming growth factor-β (TGF-β) and various glycoprotein hormones. The link between this *Drosophila* gene and the bursicon hormone was established from the defective post-ecdysial behavior of flies, such as the cuticle sclerotization and wing expansion, with loss-of-function mutations in this gene, and the corresponding decrease in bursicon bioactivity in mutant extracts. Accordingly based on the previous finding of the putative molecular mass of the mature bursicon hormone in several insect species, it was
proposed that bursicon would be a homodimeric 30 kDa protein made of two 15 kDa burs
α subunits (Dewey et al., 2004). However, later results showed that the recombinant burs
protein had no bioactivity, and it was then hypothesized that the burs α gene product need
to heterodimerize with another cystine knot protein encoded by the D. melanogaster gene
CG15284 (pburs or burs β) (Luo et al., 2005; Mendive et al., 2005), which encodes a
protein of approximately 14 kDa, for a valid bursicon activity. R-bursicon α+β
heterodimer was shown to be capable of inducing tanning in the neck-ligated fly bioassay
in vivo.

1.4.3 Burs α and burs β: Evolutionary conservation

Based on the bursicon sequences discovered in D. melanogaster and now the
available genomic information, genes encoding bursicon were identified in several other
insect species, including the African malaria mosquito A. gambiae (GenBank Accession
nos. AY735442, AY735443), the honey bee Apis mellifera (GenBank Accession
nos. AM420631, AM420632), the silkworm Bombyx mori (GenBank Accession nos.
NM_001098375, NM_001043824), the tobacco hornworm M. sexta (GenBank Accession
nos. DQ094149, DQ291147), and the red flour beetle Tribolium castaneum (GenBank
Accession nos. DQ156996, DQ156997). Bursicon sequences in some of these species
were aligned to analyze the conservation of the sequences, as shown in Fig. 1-6.
Recently, by screening of the protostomian and deuterostomian genome and
complementary DNA (cDNA) databases, bursicon genes (burs α or burs β or both) have
been either identified or predicted in several other species, including the water flea
Daphnia arenata (GenBank Accession nos. EU139431, EU139430), the green crab
Carcinus maenas (GenBank Accession nos. EU139428, EU1394289) (Wilcockson et al., 2008), the echinoderm purple sea urchin Strongylocentrotus purpuratus (GenBank Accession nos. NM_001110249, NM_001110247) (Van Loy et al., 2007), and the American dog tick Dermacentor variabilis (partial sequences) (GenBank Accession nos. EU574002, EU616824) (Van Loy et al., 2007). The finding of bursicon in these species indicates that bursicon is present in a wide range of crustaceans and some other closely related organisms and that bursicon might play a common role in these organisms. Protein sequence alignments also indicated that bursicon in some of these species is also highly conserved with bursicon sequences in insect species, especially the same signature of 11 conserved cysteine residues (Van Loy et al., 2007). However in nematodes, such as Caenorhabditis elegans, which are believed to be phylogenetically related with other molting animals, sequences related to bursicon have not been identified, suggesting the loss of bursicon activity in these species. In higher vertebrate animal species, potential bursicon homologous sequences were also screened. Results showed that many growth factors, such as bone morphogenetic proteins (BMPs) and their antagonists, transforming growth factor beta (TGF-β) family members, glycoprotein hormone subunits and extracellular matrix proteins such as mucins, share similar cystine knot structure with bursicon. However none of these predicted cystine knot proteins displays sufficient overall amino acid similarity to be convincingly identified as bursicon orthologues (Vanloy et al., 2007).
Fig. 1-6. Alignment of bursicon protein sequences of different insect species. Burs α and burs β from mosquito (ag: *A. gambiae*), silkworm (bm: *B. mori*), the honey bee (am: *A. mellifera*), the fruit fly (dm: *D. melanogaster*) and the tryptic peptides identified in bursicon preparations from the cockroach (pa: *P. americana*) were aligned. Conserved burs α and burs β sequences are shaded in grey and yellow, respectively. Identical residues are dark shaded (Mendive et al., 2005).
1.4.4 Bursicon protein and gene structures

Bursicon is the first heterodimer cystine knot hormone protein found in insects (Dewey et al., 2004; Luo et al., 2005; Mendive et al., 2005). Based on the available cystine knot protein structures, the structure of bursicon can be predicted. Both burs α and burs β subunits belong to the cystine knot protein family, with an uneven number of cystine residues containing five intramolecular disulfide bonds between C2-C8, C3-C9, C4-C10, and C5-C11, and a possible formation of an intermolecular bridge by C6, as shown in Fig. 1-7 (Luo et al., 2005). This structure ensures a covalent association between the burs α and burs β subunits and possible ligand binding ability. However it is not known why these two subunits with similar structure have to bind together for a valid bursicon hormone function.

The gene structures of bursicon have been studied in detail. In D. melanogaster, sequence analysis of the genomic clone and its corresponding cDNA indicated that the burs α gene contains three short exons (43, 42 and 88 amino acids, respectively) and two introns (Dewey et al., 2004). Genetic analysis in several insect species, including D. melanogaster, A. melifera, B. mori, and A. gambiae, indicated that the burs β gene contains 2-3 exons (The number depends on species) (Luo et al., 2005). By comparison with all available insect and related arthropod genomes, Robertson et al. (2007) provided evidence that the ancestral insect burs α gene has four coding exons. These exons are located in the same locus and are spliced in a conventional manner in almost all insect species except mosquitoes, including A. gambiae, A. aegypti and C. pipiens. In these mosquito species, bursicon is translated from an mRNA molecular derived from four
Fig. 1-7. Predicted cystine knot structure of burs α or burs β subunit. Structure shows the cystine residues that are responsible for the formation of disulfide bonds, the five possible intramolecular disulfide bonds between C2-C8, C3-C9, C4-C10, and C5-C11, and a possible formation of an intermolecular bridge by C6 (Luo et al., 2005).
Fig. 1-8. Intron-exon arrangement of *burs* α and *burs* β genes in different insects. Analyses of the *burs* α and *burs* β gene structures from the six insects, *M. domestica*, *D. melanogaster*, *T. castaneum*, *A. mellifera*, *B. mori*, and *A. gambiae*, indicated that *burs* α has three to four exons, while *burs* β consists of two or three exons. Exons are represented by boxes. The numbers indicate amino acid residues for each exon and nucleotides for each intron (An et al., 2008b).
exons (one 5' UTR and three coding sequences); however, the third exon on the mRNA is on a different chromosome arm (2R) from the first, second, and fourth exons, which are together on chromosome arm 2L. The assembly of the final mature burs α mRNA is due to a special splicing process called trans-splicing, which splices the number 3 exon out and inserts it between exon 2 and 4. An et al. (2008) has analyzed the intron-exon arrangement of both burs α and burs β in the house fly *M. domestica* and compared the arrangement in several insect species, including *M. domestica*, *D. melanogaster*, *T. castaneum*, *A. mellifera*, *B. mori*, and *A. gambiae*, as shown in Fig. 1-8.

### 1.4.5 Subcellular distribution and release of bursicon

In the last several years, researchers were able to partial purify the bursicon hormone protein and raise polyclonal antibodies against bursicon. By using antibodies raised against a bursicon partial sequence from the American cockroach *P. americana*, the distribution of bursicon hormone proteins in several insect species has been studied (Dewey et al., 2004; Honegger et al., 2002). In *P. americana*, a polyclonal antibody against bursicon raised in a rabbit exclusively labeled two pairs of cells that also express CCAP (CCAP-ir cells) in the three thoracic and the five abdominal ganglia. Also a cluster of approximately 10 cells on each side of the brain in the ventroanterior region possibly contained bursicon, which might contribute to bursicon released and/or stored in the corpora cardiac (CC), because in the CC bursicon was also labeled in the outer cortical layer. However this was uncertain because the staining in the CC could come from the neurons in the ventral nerve cord.

Using the same antibodies in two cricket species, *G. bimaculatus* and *T.
*commodus*, similar staining patterns in the ventral nervous systems, but not in the brain, were found (Honegger *et al.*, 2002). In *M. sexta*, four pairs of bilateral neurons in each ganglion of the ventral nerve cord in larvae, but only three pairs in pharate adults, could be detected (Honegger *et al.*, 2002). However recent studies using antibody developed specifically against *M. sexta* showed that both bursicon subunits are present in each thoracic and abdominal ganglia in larvae and pupae, but are only in abdominal ganglia in the adults, with the number of the cells restricted to only 1-2 pairs in each ganglion (Dai *et al.*, 2008). This suggests that although antibodies against the highly conserved bursicon protein served cross species, they may also show additional false signals during localization of the bursicon containing neurons. In *D. melanogaster*, in the brain of the third instar larvae, the pupal and the adult stages, a group of three to four neurons on each side of the brain were labeled. Axons of these neurons were followed into the thoracic neuromeres, and additional fibers projected toward the CC, indicating the possible bursicon release pathway (Honegger *et al.*, 2002). Other reports also showed that bursicon-immunoreactive processes were also present in the nerves exiting the abdominal ganglion. The abundance of immune-reactivity in these nerves suggests that bursicon may be destined for release into the hemolymph from abdominal ganglia (Luan *et al.*, 2006).

In *D. melanogaster* pharate adults, it has been found that bursα and bursβ co-localized to about seven pairs of neurons in the fused thoracic-abdominal ganglia, and bursα is expressed in two pairs of neurons in the subesophageal ganglion while bursβ is only expressed in one pair of these cells. And occasionally both bursα and bursβ subunits can be found in one pair of cells in the brain (Luan *et al.*, 2006). In *M. sexta*, bursα and bursβ are both detected in a group of intrinsic cells in the corpora cardiaca (CC), suggesting the
release of bursicon hormone may likely go through the CC into the hemolymph (Dai et al., 2008).

With the recent discovery of burs \( \alpha \) and burs \( \beta \) gene sequences, scientists are able to investigate the transcriptional level expression pattern of bursicon. In *D. melanogaster*, Dewey et al., (2004) demonstrated that *burs \( \alpha \)* is expressed in some, if not all, CCAP containing neurosecretory cells (NSCs) in the abdominal ganglia (AG) of third instar larvae, but nothing in either the brain or the subesophageal ganglia (SEG). The transcripts of *burs \( \beta \)* were only located in 4 pairs of neurosecretory cells in the abdominal ganglia (Luo et al., 2005). However in *M. sexta*, both burs \( \alpha \) and burs \( \beta \) transcripts were co-localized in 1-2 pairs of NSCs in some but not all ganglia including SEG, all thoracic ganglia (TG) 1-3 and AG1 in 3\textsuperscript{rd} instar larvae; and in pupae and pharate adults, both transcripts could be detected in 1-2 pairs of NSCs in every ganglia including SEG, all TG and AG (Dai et al., 2008).

To summarize, the localization of bursicon containing neurosecretary cells has showed a great deal of variation regarding to different insect species and study of bursicon transcripts or proteins. Due to these differences and discrepancies in the bursicon staining patterns, more detailed studies will be required to determine the expression pattern of bursicon, the origin of bursicon in the abdominal nerves, and the timing and site(s) of its release.

**1.4.6 Bursicon signal transduction pathway**

Although bursicon’s discovery was made a long time ago, it has not been elucidated as to how it plays its role in the insect cuticle sclerotization process. Currently
great efforts are being made to study the signal transduction pathway(s) that bursicon regulates.

In recent years, a subfamily of G protein-coupled receptors, named LGRs, was identified, which have a large ecto-domain containing leucine-rich repeats. Genes in this family are conserved in invertebrates and vertebrates (Nishi et al., 2000). LGRs can be divided into three groups: group A, vertebrate glycoprotein hormone receptors; group B, *D. melanogaster* LGR2 (DLGR2) (Eriksen et al., 2000) and vertebrate orphan receptors LGR4, 5, 6 (Hsu, 2003); and group C, mammalian relaxin_INSL3 receptors (Hsu et al., 2002; Kumagai et al., 2002).

Based on molecular genetic analyses in *D. melanogaster*, bursicon was proposed to act through the G protein-coupled receptor DLGR2, which is encoded by the gene *rickets*. Baker and Truman (2002) reported that a loss-of-function mutation in the gene *rickets* resulted in failure in initiating the behavioral and tanning process following ecdysis, and this gene was confirmed to encode the glycoprotein hormone receptor DLGR2, which belongs to the group DLGRs. In mutant flies, although the hormone bursicon was produced and released, insects were not able to perform cuticle sclerotization. Mutation in the *rickets* gene also blocked the behavioral program for wing expansion, a process that occurs after the eclosion and accompanies cuticle sclerotization. So DLGR2 is likely one of the downstream elements for bursicon function in the cuticle sclerotization and wing expansion process. An *in vitro* binding assays using cells over-expressing DLGR2 and r-bursicon heterodimer also indicated that r-bursicon is capable of high-affinity binding to the DLGR2 (Luo et al., 2005; Mendive et al., 2005). Although it has not yet been identified which tissue of the insect express DLGR2, it has
been reported that this receptor is only expressed in embryo and pupal stages of Drosophila, but not in the larval and adult stages (Eriksen et al., 2000). Fig. 1-9 shows a generalized G protein coupled receptor mediated signal transduction pathway in animals.  

Another component that is possibly involved in the bursicon-mediated cuticle sclerotization process is cyclic AMP (cAMP). cAMP is an important secondary messenger for many signal transduction pathways. Earlier studies on blowflies have implicated an increase in cAMP upon the action of bursicon (Reynolds, 1980; Seligman and Doy, 1972, 1973; Von Knorre et al., 1972). Recent studies using an \textit{in vitro} binding assay also indicated that r-bursicon stimulated cAMP production in a dose-dependent manner, Fig. 1-10 (Luo et al., 2005). Injection of the rickets mutated flies with a cAMP analog resulted in melanization of the abdominal tergites, showing that at least this part of the response machinery is functional in the mutants and cAMP might be a critical element downstream of the receptor DLGR2 (Baker et al., 2002). Although these findings imply the role of cAMP in mediating the bursicon-mediated signal transduction pathway, so far little is known about what kind of downstream elements are regulated by cAMP and how cAMP is involved in this process. Also it should be noted that cAMP injections do not have any effects on the components of the wing expansion program in either rickets mutated or wild-type flies. So if cAMP is involved in the bursicon-mediated signal pathway, it is probably only associated with cuticle sclerotization (Baker et al., 2002).
Fig. 1-9. Generalized G-protein-coupled receptor mediated signal transduction pathways in animals. Various ligands bind to the transmembrane G-protein-coupled receptor (GPCRs) and stimulate downstream signal transduction elements in the cell membrane, cytoplasm, and nucleus. Binding of ligand leads to a conformational change in the G protein, resulting in the dissociation of Gα from Gβγ subunits. The α subunits of G proteins are divided into four subfamilies: Gαs, Gαi, Gαq and Gα12, and each of these G protein activates several downstream effectors (Image is after Dorsam and Gutkind, 2007).
Using RNAi knock down of bursicon expression in *B. mori*, Huang *et al.* (2007) reported three genes that are regulated by bursicon. One gene shows a similarity with a thioesterase superfamily member 2 in *D. melanogaster*, and the second gene is the silkworm trehalase gene, while the third gene is an unknown gene. Since it has long been proposed that several enzymes are possibly involved in the final synthesis of the sclerotization agents, Davis *et al.* (2007) further investigated two enzymes, tyrosine hydroxylase (TH, encoded by *ple*) and dopa decarboxylase (DDC, encoded by *Ddc*), which are responsible for the synthesis of dopamine (DA), in *D. melanogaster*. Data showed that both *ple* and *Ddc* transcripts begin to accumulate before eclosion, and the activity of DDC is high before eclosion, indicating DDC is not regulated in the sclerotization process. However, TH activity is present before eclosion, disappears at eclosion, then re-accumulates rapidly within one hour of eclosion, and is transiently activated by phosphorylation during sclerotization, indicating TH might be the final regulatory switch to control cuticle sclerotization.
Fig. 1-10. *In vitro* study of the effects of r-bursicon $\alpha + \beta$ heterodimer or single subunit only (burs $\alpha$ or burs $\beta$) on the titre of cAMP in DLGR2 expressing cells. The level of cAMP production is induced in a sigmoid concentration-action manner when and only when both burs $\alpha$ and burs $\beta$ are present in the conditioned cell medium. When only burs $\alpha$ or burs $\beta$ only was added, there is no increase in cAMP level (Image is after Luo *et al.*, 2005).
1.4.7 Bursicon, wing expansion and programmed cell death

In addition to inducing the insect cuticle sclerotization process, bursicon is also involved in the insect wing expansion. The insect wing expansion process is another physiological event occurring after insect ecdysis and accompanying cuticle sclerotization. These processes occur at the same time, but the mechanisms under which they are controlled and regulated are still not clear. Recent research showed that mutation in the bursicon genes causes the failure of initiation of the behavioral program for wing expansion and results in defects in wing expansion in both the fruit fly \textit{D. melanogaster} and the silkworm \textit{B. mori} (Dewey et al., 2004; Huang et al., 2007), which indicates that bursicon is the hormone required to initiate both of these processes in insects. It was also shown that mutation of the \textit{rickets} gene, which encodes the DLGR2 receptor as a putative bursicon receptor, inhibited wing expansion in \textit{Drosophila} (Kimura et al., 2004).

Although it is not clear how wing expansion in insects is controlled, it has been demonstrated that wing development and expansion is strongly associated with programmed cell death and removal of cell debris from the wing tissue (Johnson and Milner, 1987; Kiger et al., 2007; Kimura et al., 2004; Natzle et al., 2008; Seligman et al., 1972, 1975). Shortly after eclosion, hemolymph pressure forces the expansion of the folded wing blade. At the same time, the dorsal and ventral epithelial cell layers delaminate from the outside cuticle, undergo a process called epithelial-mesenchymal transition (EMT), and then exit the wing accompanied by the initiation of a programmed cell death under the control of a yet to be identified signaling pathway (Kiger et al., 2007; Natzle et al., 2008).

Programmed cell death (apoptosis) is crucial for the development and
maintenance of multicellular organisms, to control cell number, to remove infected, mutated or damaged cells, and to eliminate cells that are no longer required at a given stage of development (Jacobson et al., 1997; Vaux and Korsmeyer, 1999). During development, apoptotic, autophagic and nonlysosomal cell death have been widely reported (Clarke, 1990; Schweichel and Merker, 1973). The components and mechanisms for apoptosis are conserved in a wide variety of organisms, from worms and flies to humans. They involve the activity of cysteine proteases (caspases) (Kumar and Doumanis, 2000) and their negative regulators, such as Inhibitor of Apoptosis Proteins (IAPs) (Hay, 2000), and the pro-apoptotic activators, such as reaper (rpr) (White et al., 1994), head involution defective (hid; Wrinkled, W - FlyBase) (Grether et al., 1995), grim (Chen et al., 1996) and sickle (skl) (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002a), which promote cell death by inhibiting the function of the IAPs (Holley et al., 2002; Ryoo et al., 2002; Wang et al., 1999; Wing et al., 2002b; Yoo et al., 2002). There should be multiple cell death and survival signals that regulate the turning on and shutting off of the apoptotic genes. However the nature of these signal transduction pathways is nearly unknown. At the last step of metamorphosis, newly emerged adults undergo extensive cell death. Many of the abdominal muscles and associated neurons die within a day of adult emergence (Finlayson, 1975; Truman, 1983; Kimura and Truman, 1990). In addition, the wing epidermis is also removed by cell death at the time of wing spreading in the large fly Lucilia cuprina (Seligman et al., 1975) and in D. melanogaster (Johnson and Milner, 1987). At the last step of metamorphosis in Drosophila, the wing epidermal cells are removed by programmed cell death during the wing spreading behavior after eclosion. Using a TUNEL assay and transmission electron microscopy, Kimura et al.
(2004) demonstrated that cell death was accompanied by DNA fragmentation and that this cell death exhibited extensive vacuoles, implying possible autophagy. Ectopic expression of an anti-apoptotic gene, p35, inhibited the cell death, indicating the involvement of caspases.

It was reported that both cell death and the removal process is controlled by changes in the levels of the steroid hormone 20-hydroxyecdysone (20E) through regulation of a series of early response and late response genes, including the expression of the cell death activator genes, rpr and hid, which then repress the Drosophila inhibitor of apoptosis proteins (IAPs) Diap2 (Jiang et al., 1997; Lee et al., 2002a; Lee et al., 2002b). Changes in 20-E hormone titre, in combination with the fine-scale expression of the ecdysone receptor, control the timing and spacing of cell death during metamorphosis. Though ecdysone-dependent regulation is the possible pathway controlling cell death, it does not exclude the possibility of the involvement of other hormones in wing expansion and programmed cell death. It is very likely that bursicon co-regulates the programmed cell death with other factors during the posteclosion wing maturation process. It has been shown that stimulation of components downstream of bursicon, such as a membrane permeant analog of cAMP, or ectopic expression of constitutively active forms of G proteins or PKA, induced precocious cell death; and conversely, cell death was inhibited in wing clones lacking G protein or PKA function. Therefore, activation of the cAMP/PKA signaling pathway is likely required for transduction of the hormonal signal that induces wing epidermal cell death after eclosion (Kimura et al., 2004).
CHAPTER 2

MOLECULAR CHARACTERIZATION OF BURSICON AND STUDY OF NOVEL BURSICON-REGULATED GENES IN THE HOUSE FLY *MUSCA DOMESTICA*

A portion of this work was published in the following:


* These authors contributed equally.

**Note**: In this part of research, Dr. Shiheng An contributed to the expression and purification of recombinant bursicon heterodimer proteins in both insect cells and mammalian cells (An *et al.*, 2008b), and major experiments and analyses of bursicon regulated genes using DNA microarray (An *et al.*, 2008a) (see figure 2-5 and appendix 1).
2.1 Chapter summary

Bursicon is a neuropeptide that regulates cuticle sclerotization (hardening and tanning) via a G-protein coupled receptor. It consists of two subunits, an alpha and a beta. In the present study, burs α and burs β genes were cloned in the house fly *M. domestica* using 3’ and 5’ RACEs. Sequence analysis showed that bursicon is conserved in all insect species, especially in the cystine knot region. Then *burs α* and *burs β* genes were analyzed for their transcript levels and distribution in the central nervous systems of the house fly *M. domestica*. RT-PCR analysis revealed that both bursicon subunits were present in the central nervous system of larval and pupal stages, reached maximal level in pharate adults, and declined sharply after adult emergence, suggesting the release of the hormone upon adult emergence. In situ localization of bursicon transcripts showed that both burs α and burs β transcripts were expressed in a set of neurosecretory cells (NSCs) in the suboesophageal ganglia (SOG) and abdominal ganglia (AG) in *M. domestica* larvae, and the highly fused thoracic-abdominal ganglia (TAG) in *M. domestica* adults, which are similar to the patterns in *D. melanogaster* with some differences. Using the r-bursicon heterodimer expressed in mammalian 293 cells and insect Highfive™ cells, a series of genes were identified, which are regulated by bursicon in the neck-ligated house flies. Two genes, *CG7985hh* and *CG30287hh*, which are homologous to two *D. melanogaster* genes *CG7985* and *CG30287*, were further studied and confirmed for their up-regulation by using real-time RT-PCR, indicating their likely involvement in the cuticle sclerotization process.
2.2 Introduction

To accommodate growth, insects must periodically shed their old exoskeleton and replace it with a new one (molting). After each molt, the newly formed exoskeleton must go through a sclerotization (hardening and tanning) process, which is vital for insect survival because the newly formed exocuticle is soft, flexible, and unable to protect the insect from water loss, external physical injuries or pathogens. The primary factor responsible for initiating the sclerotization process is a neuropeptide known as “bursicon”.

Bursicon was first discovered over four decades ago in the blowfly, *C. erythrocephala* (Cottrell, 1962a; Fraenkel and Hsiao, 1962). When blowfly adults were neck-ligated immediately after emergence, the neck-ligation prevented the cuticle from being sclerotized. When the neck-ligated adults were injected with hemolymph collected shortly after adult emergence (containing the hardening and tanning factor) or with brain extract from newly emerged flies, the neck-ligated flies became sclerotized. Using this simple but elegant bioassay, Fraenkel and Hsiao (1965) determined that cuticle sclerotization in the newly emerged blowfly was under the control of a hemolymph factor, which was released from the fly head shortly after emergence, and named the factor ‘bursicon’.

Using the blowfly bioassay, bursicon activity was subsequently reported in several other dipteran species including: *Sarcophaga bullata* (Cottrell, 1962b; Fogal and Fraenkel, 1969; Fraenkel and Hsiao, 1962, 1965), *Phormia regina* (Fraenkel and Hsiao, 1962, 1965), *Lucilia spp* (Cottrell, 1962b; Seligman and Doy, 1972, 1973), and *D.*
Bursicon’s presence has since been established in several other insect orders as well, including Orthoptera (Fraenkel and Hsiao, 1965; Honegger et al., 2002; Kostron et al., 1995, 1996, 1999; Mills, 1965; Mills et al., 1965; Srivastava and Hopkins, 1975; Vincent, 1971, 1972), Hemiptera (Fraenkel and Hsiao, 1965), Coleoptera (Fraenkel and Hsiao, 1965; Kaltenhauser et al., 1995), and Lepidoptera (Fraenkel and Hsiao, 1965; Post, 1972; Post and De Jong, 1973; Reynolds, 1977; Reynolds et al., 1979; Taghert and Truman, 1982a, b; Truman, 1973).

For a long time since its discovery, bursicon was thought to be a single protein with a molecular size between 30 and 60 kDa, which varies greatly in different insect species. However, a recent discovery in *D. melanogaster* showed that functional bursicon is actually a heterodimer protein consisting of two subunits, bursicon α (AJ862523) and burs β (AJ862524) with molecular weights of 16kDa and 14kDa respectively (Luo et al., 2005; Mendive et al., 2005). It is the first heterodimeric cystine knot hormone discovered in insects. Based on available gene sequences in the NCBI data bank, both *Drosophila* burs α and burs β subunits have similar sequence matches in several insect species including the malaria mosquito *A. gambiae*, the honey bee *A. mellifera*, the silkworm *B. mori*, the tobacco hornworm *M. sexta*, and the red flour beetle *T. castaneum*.

Genetic analyses in *D. melanogaster* showed that bursicon mediates cuticle sclerotization and the wing expansion process via a specific G protein-coupled receptor DLGR2, encoded by the *rickets* gene (Baker and Truman, 2002). DLGR2, once activated, is hypothesized to activate a cAMP/PKA signaling pathway (Kimura et al., 2004). It has now been shown that r-bursicon binds to and activates DLGR2, which in turn leads to a dose-dependent intracellular increase in adenyl cyclase activity and cAMP production in
the mammalian 293T cells and COS-7 cells that over-express DLGR2 (Luo et al., 2005; Mendive et al., 2005). A gene silencing study reveals that injection of the double-stranded bursicon α RNA into B. mori pupae significantly reduces the level of bursicon α mRNA in pupae, resulting in a deficit in wing expansion in adults (Huang et al., 2007).

Although there is much research going on for bursicon study, and great progress has been made during the last few years, many aspects of this hormone still remain unclear. Additionally, a lot of discrepancies remain, e.g. the timing and location of bursicon expression, the release pathway of bursicon, and particularly the signaling pathway downstream of bursicon and its receptor DLGR2. Our long term goal is to investigate the molecular mechanisms of the bursicon signaling pathway that leads to cuticle sclerotization. This particular study reports the molecular cloning of burs α and burs β genes in the house fly M. domestica, expression of bursicon heterodimer proteins and their functional analyses, the transcriptional expression profile and in situ localization of burs α and burs β genes in the central nerve system (CNS), and the identification of several bursicon-regulated genes in the house fly M. domestica.

2.3 Materials and Methods

Experimental insects

House fly larvae were reared on artificial diet (Carolina Biological Supply, Burlington, NC) at 30°C under constant darkness and adults were fed on a 1:1 mixture of granulated sugar and powdered milk at 30°C under 16h light: 8h dark. D. melanogaster (wild type^ore) were reared on artificial diet (Fisher Scientific, Pittsburgh, PA) at 24°C
under constant darkness.

**Cloning of burs \( \alpha \) and burs \( \beta \) genes using 5’ and 3’ RACE**

To obtain the full-length house fly \( burs \alpha \) and \( burs \beta \) cDNAs, rapid amplification of cDNA ends (RACE) was performed using a 5’ and 3’RACE system (Invitrogen, Carlsbad, CA). The primers used in RACE (Table 2-1) were designed based on the conserved sequences of \( D. \) melanogaster (\( burs \alpha \): AJ862523, \( burs \beta \): AJ862524), \( T. \) castaneum (\( burs \alpha \): DQ138189, \( burs \beta \): DQ138190), \( A. \) gambiae (\( burs \alpha \): AY735442, \( burs \beta \): AY823259), \( B. \) mori (\( burs \alpha \): BN000691, \( burs \beta \): BN000690), and \( A. \) mellifera (\( burs \alpha \): AM420631, \( burs \beta \): AM420632).

Central nervous systems (CNS) from the house fly were dissected under Ringers’ solution (3.6 mM NaCl, 54.3 mM KCl, 8.0 mM CaCl\(_2\), and 28.3 mM MgCl\(_2\)) from pharate adults, which contain high levels of \( burs \alpha \) and \( burs \beta \) transcripts. Total RNA was extracted from the CNS using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For the amplification of 3’ ends of cDNA, the first-strand cDNA was synthesized using a manufacturer-supplied adapter primer. The supplied abridged universal amplification primer, the bursicon gene specific primer and the nested primer were used in subsequent amplification. For the amplification of 5’ ends of cDNA, the bursicon gene specific primer was used for the first-strand cDNA synthesis. Two nested primers were used for subsequent amplification. The PCR products from 3’ and 5’ RACEs were purified, sequenced at the MU DNA Core Facility, and used as reference for designing gene specific primers. When the full length house fly \( burs \alpha \) and \( burs \beta \) sequences were obtained, the open reading frames were amplified using the forward primers with an \( XhoI \) restriction site (CTCGAG) and reverse primers with a \( BamHI \)
### Burs α

**3'-RACE**

- Primer 1: 5'-AAGATCTGGCAAATGGACCG-3' (GSP)
- Primer 2: 5'-CCTGCATGTGCTGCCAGGA-3' (Nested GSP)

**5'-RACE**

- Primer 1: 5'-GCATGGCCGACACATGCAC-3' (GSP)
- Primer 2: 5'-TCCTGGCAGCACATGCAGG-3' (Nested GSP)
- Primer 3: 5'-CGGTCCATTTGCCAGATCTT-3' (Nested GSP)

### Burs β

**3'-RACE**

- Primer 1: 5'-TGCAACAGTCAGGTGAACC-3' (GSP)
- Primer 2: 5'-GAAAGACTGCTACTGCTGCCG-3' (Nested GSP)

**5'-RACE**

- Primer 1: 5'-ATCGCCACATTTGAAGCACT-3' (GSP)
- Primer 2: 5'-CGGCAGCAGTAGCAGTCTTTC-3' (Nested GSP)
- Primer 3: 5'-GGTTGCACCTGACTGTTGCA-3' (Nested GSP)

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**Table 2-1.** Gene specific primers (GSPs) and nested GSPs designed according to burs α and burs β protein alignment in other insects
restriction site (GGATCC) (Table 2-2), cloned into the pGEM-T-Easy vector (Promega, Madison, WI), and sequenced again for confirmation of the correct insertion.

**Protein sequence alignments**

Analyses of the deduced amino acid sequences of the house fly burs $\alpha$ and burs $\beta$ were carried out using the ExPASy server (www.expasy.ch) for prediction of domains, motifs, and signal peptides. Homology searches of the house fly burs $\alpha$ and burs $\beta$ proteins were made on the NCBI platform (www.ncbi.nih.gov). Alignments of bursicon sequences from *M. domestica* with sequences from other insect species were carried out using ClustalW (www.ebi.ac.uk) and edited with Genedoc software (http://www.genedoc.us/).

**RT-PCR analysis of the temporal expression profiles of burs $\alpha$ and burs $\beta$ genes**

The CNSs were dissected from the indicated developmental stages of the house fly under Ringers’ solution at $4^\circ$C and stored at -80$^\circ$C for RNA extraction. Total RNA was extracted from the CNS using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions and quantified spectrophotometrically (at 260 nm).

Prior to cDNA synthesis, total RNA was treated with DNase I (Promega, Madison, WI) to remove genomic DNA from the samples. The first-strand cDNA was synthesized using 5 $\mu$g total RNA from each developmental stage and primed using oligo (dT) based on the SuperScript™ First-Strand synthesis kit (Invitrogen, Carlsbad, CA). The following primers were designed for gene amplification: bursicon $\alpha$ 5’-ATG GAA GTT TCA GTT TTT CG-3’ (forward) and 5’-TTA CTG CAA TGC TAT CCT TC-3’ (reverse); burs $\beta$ 5’-ATG CTT AAA TTG AAA TT-3’ (forward) and 5’-TTA TCT
Table 2-2. Primers for PCR amplification of full length *burs α* and *burs β* cDNA. The underlined indicates the inserted *XhoI* restriction site (CTCGAG) for forward primers and the *BamHI* restriction site (GGATCC) for reverse primers.
CGT GAA ATC ACC AC-3’ (reverse). To normalize the cDNA, the house-keeping ribosomal protein 49 gene (rp49) primers 5’-TAC AGG CCC AAG ATC GTG AA-3’ (forward) and 5’-GAC AAT CTC CTT GCG CT T CT-3’ (reverse) were used to amplify cDNA from the same samples as for bursicon analysis. The PCR products were subjected to electrophoresis in a 1.0% agarose gel and the gel image was analyzed using the AlphaImager software, version 5.5 (Alpha-imager Primate Ltd., Bangalore, ID).

**Fluorescence In Situ Hybridization (FISH) localization of bursicon transcripts in *Musca* and *Drosophila***

FISH experiments were carried out to localize burs α and burs β transcripts in the CNS of *M. domestica*. To construct FISH probes, *burs α* and *burs β* cDNAs were amplified again by PCR using designed primers with *BamH*I and *Xho*I restriction sites as indicated by the underline: *burs α* 5’-GGA TTC ATG GAA GTT TCA GTT TTT CG-3’ (forward) and 5’-CTC GAG TTA CTG CAA TGC TAT CCT TC-3’ (reverse); *burs β* 5’-GGA TTC ATG CTT AAA TTG TGG AAA TT-3’ (forward) and 5’-CTC GAG TTA TCT CGT GAA ATC ACC AC-3’ (reverse). The PCR products of *burs α* and *burs β* were individually inserted into pGEM-T-Easy vectors (Promega, Madison, WI) following the manufacturer’s instructions. The pGEM-T-Easy vectors containing either the *burs α* or *burs β* gene in the desired orientation were then linearized with appropriate enzymes and used for transcription with SP6 and T7 RNA polymerases (Invitrogen, Carlsbad, CA) to generate anti-sense and sense probes, respectively. The anti-sense and sense (as negative control) probes for both *burs α* and *burs β* genes were labeled using the FISH Tag™ RNA Muticolor Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. *Burs α* probes were labeled in red and *burs β* probes were labeled in green.
CNSs were dissected from house flies at different developmental stages (including 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} instar larvae; prepupae; pharate adults; 0, 1, 24 and 48 hour adults) and fixed in 4\% paraformaldehyde at 4\textdegree{}C overnight. Subsequent treatments were carried out following the FISH Tag\textsuperscript{TM} instruction manual. After fixation, tissues were washed by a methanol dehydration series and then stored in methanol at -20\textdegree{}C to clear background staining. All the remaining steps were performed at room-temperature unless indicated otherwise. Tissues were rehydrated from methanol through a series of methanol/PBST washes as follows: 1X5 min. 75\% MeOH / 25\% PBST, 1X5 min. 50\% MeOH / 50\% PBST, 1X5 min. 30\% MeOH / 70\% PBST, and then 2X5 min. PBST. Samples were then fixed for 1 hour with 4\% paraformaldehyde. Next, samples were permeabilized to increase probe penetration with: 3X5 min. PBST, 5 min. 1X Proteinase K in PBST (10\textmu{}g/ml), and 2X5 min. PBST. The samples were post-fixed in 5\% formaldehyde for 20 min, washed with PBST 3 times, pre-hybridized in hybridization buffer (50\% formamide, 5X SSC, 100 \textmu{}g/\textmu{}l fragmented salmon testes DNA, 50 \textmu{}g/ml heparin, 0.1\% Tween-20) and rocked gently at 55\textdegree{}C for at least two hours to block non-specific RNA binding. Following this blocking step, antisense probe RNA (pre-warmed at 80\textdegree{}C for 2 mins to dissociate any secondary structure) was added at a dilution of 1:100 in hybridization buffer, and samples hybridized with gentle rocking for 12-16 hours at 55\textdegree{}C. Control samples were hybridized with the same amount of sense probe under identical conditions. After hybridization, samples were washed with hybridization buffer 2X 1 hour at 55\textdegree{}C, 1X 1 hour 50\% hybridization buffer / 50\% PBST, and then 3X 10 min. PBST at room temperature. The CNS samples were mounted in a SlowFade Gold antifade reagent and visualized with a confocal microscope (BioRad...
Radiance 2000 Confocal System) at the MU Cytology Core. For the purpose of comparison, the CNSs of Drosophila were also subjected to FISH staining. The neurosecretory properties of the bursicon containing cells were determined by the large size of the cell nuclei, distinguishable axon endings, and the secretion granules inside the cells.

Expression of recombinant burs $\alpha$ and burs $\beta$ proteins in mammalian and insect cells

To express recombinant house fly burs $\alpha$ and burs $\beta$ heterodimer protein, the open reading frames of burs $\alpha$ and burs $\beta$ were first amplified using the forward primers with $XhoI$ restriction site (CTCGAG) and reverse primers with $BamH1$ restriction site (GGATCC) (Table 2), cloned into the pGEM-T-Easy vector (Promega, Madison, WI), and sequenced again for confirmation of correct insertion.

To express the recombinant house fly bursicon in mammalian 293 cells, burs $\alpha$ and burs $\beta$ cDNAs were retrieved from the pGEM-T-Easy vector using $XhoI$ and $BamH1$ and ligated respectively into pcDNA3.1 expression vector predigested with $XhoI$ and $BamH1$. The pcDNA3.1 vector containing burs $\alpha$ or burs $\beta$ cDNA was further sequenced for confirmation of correct insertion.

The pcDNA3.1 plasmid (2 mg) containing burs $\alpha$ and burs $\beta$ was used to transfect mammalian 293 cells either individually or simultaneously using SatisFection™ Transfection Reagent (Stratagene, La Jolla, CA). After 16 h initial transfection, the serum-free DMEM cell culture medium was replaced with the same medium containing 10% fetal bovine serum and the transfected cells were incubated for additional 24 h. The cell medium was then replaced with serum-free DMEM medium and cultured for 48 h. At the end of incubation, the medium was collected, centrifuged at 2000g to remove cell
debris and stored at -80°C for bursicon bioassay. Burs α and burs β recombinant proteins were also expressed in insect High Five™ cells using the Bac-to-Bac baculovirus expression system according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). In brief, burs α and burs β cDNAs, retrieved from the pGEM-T-Easy vector using XhoI and BamH1, were ligated into pFastBac™ donor plasmids respectively. The purified pFastBacTM constructs were used to transform DH10Bac™ E. coli cells to generate recombinant bacmid. The recombinant bacmid DNA was analyzed using PCR for confirmation of recombination and used to transfect insect High Five™ cells to produce recombinant baculovirus. The recombinant baculoviruses were used to infect insect High Five™ cells to express the rbursicon protein. The expressed r-bursicon protein was analyzed using western blot before performing the bursicon bioassay to ensure the r-bursicon expression.

Bioassay of Recombinant Bursicon

The rbursicon was assessed for its hormonal activity in the house fly adults neck-ligated with dental floss immediately after emergence. After 1 h, 0.5 ml (120 ng/ml) of the recombinant protein containing burs α or burs β (control) or the burs α+β heterodimer was injected into the thorax-abdomens of untanned ligated flies using a glass needle mounted on a microinjection system. The medium, which had been passed over the Ni-NTA His bind resin (Qiagen, Valencia, CA) from mammalian 293 cells transfected with blank pcDNA3.1 plasmid or from insect High FiveTM cells transfected with blank bacmid, was used as a sham control. The purified rbursicon protein was quantified using a protein quantification kit (Bio-Rad, Hercules, CA). The CNS extract of newly emerged flies was used as a positive control (0.5 CNS equivalent/fly). Cuticle sclerotization was
evaluated at 3 h after injection and photographed using a Leica MZ16 microscope with a
Q-imaging digital camera and MicroPublisher 5.0 software.

**Real-time RT-PCR analysis of bursicon-regulated genes**

To analyze bursicon-regulated genes, newly emerged house fly adults were
neck-ligated immediately after emergence. After a one hour waiting period, the
neck-ligated flies that did not show any cuticle tanning were injected with the r-bursicon
using a microinjection system. Bursicon α dissolved in the same injection buffer was used
as a negative control. Brain homogenate in 0.01M PBS was used as a positive control (0.5
brain equivalent/fly). Injected flies were collected at indicated time periods after injection
(20 min, 40 min, 1 h, 2 h, 12 h and 24 h) and placed at -80°C for subsequent experiments.

Total RNA was isolated from the flies using Trizol reagent and treated with
RNase-free DNase I. The RNA concentration was determined by measuring the
absorbance at 260 nm on a spectrophotometer. Total RNA (2 μg) from each sample was
used for the first-strand cDNA synthesis as previously described. The synthesized cDNA
was used as template for analysis of gene transcription by real-time RT-PCR. Primers
designed for *CG7985hh* were: 5’-GAC TAC AAT GTT CGG CAC AA-3’ (forward) and
5’-GGC AGT GGT CTC TGT GGC CA-3’ (reverse); and primers for *CG30287hh* were:
5’-ACG TTC TGA CGG CTG CCC AC-3’ (forward) and 5’- GAG CAG AGC TAT GTC
ATT CT-3’ (reverse). *rp49* gene was used for normalization using the same primers as
described for RT-PCR. Real-time RT-PCR amplification and analysis were carried out on
Applied Biosystems 7300 Fast Real-time RT-PCR System (ABI). The final reaction
volume was 20 μL using ABI SYBR Green Supermix (ABI). The thermal cycle
conditions used in real time PCR were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. The specificity of the SYBR green PCR signal was confirmed by melting curve analysis and agarose gel electrophoresis. The transcript level was quantified using the comparative CT (Cross Threshold, the PCR cycle number that crosses the signal threshold) method (Livark and Schmittgen, 2001). The CT of the gene \( rp49 \) was subtracted from CT of the target gene to obtain \( \Delta CT \). The normalized fold changes of the target gene transcript level were expressed as \( 2^{-\Delta\Delta CT} \), where \( \Delta\Delta CT \) is equal to \( \Delta CT_{\text{treated sample}} - \Delta CT_{\text{control}} \).

**Statistical analysis**

Experiments of the real-time RT-PCR were carried out three times, and results were expressed as mean +/- SD. Differences between different time-points were evaluated with t-test.

### 2.4 Results

**Cloning and sequence analysis of burs \( \alpha \) and burs \( \beta \) genes**

Full length sequences of the house fly burs \( \alpha \) and burs \( \beta \) genes were obtained using 3’ and 5’ RACEs. As shown in Fig. 2-1, the house fly bursicon \( \alpha \) gene has an ORF of 531 bps, encoding a 176 a.a. polypeptide with a predicted molecular weight of 19.5 kDa and a theoretical pI of 6.86. The predicted molecular weight of the matured burs \( \alpha \) protein is 15.3 kDa. The polyadenylation signal sequence (AATAAA) is localized 99 nts downstream of the stop codon (TAA) (Fig. 2-1). The burs \( \beta \) gene has an ORF of 444 bps with 2 exons and 1 intron, encoding a 147 a.a. polypeptide with a predicted molecular...
weight of 17 kDa and a theoretical pI of 5.45. The predicted molecular weight of the matured burs β is 13.8 kDa. The polyadenylation signal sequence is localized 75 nts downstream of the stop codon (Fig. 2-2).

**Multiple protein sequence alignments**

To identify any similarities of the house fly bursicon α and β subunits to potential biologically significant domains or motifs in existing protein families, searches were carried out using the ExPASy server. The search results revealed a C-terminal cystine knot domain (a.a. 55-145) and a cell attachment sequence (a.a. 36-38) in the house fly burs α sequence (Fig. 2-3). The burs β has a N-glycosylation site (a.a. 22-25), two N-myristoylation sites (a.a. 73-78 and a.a. 116-121), and a C-terminal cystine knot domain (a.a. 45-143) (Fig. 2-4). Amino acid sequence alignment reveals that the house fly bursicon α shares 79% sequence identity with *D. melanogaster*, 49% with *M. sexta*, 49% with *B. mori*, 55% with *A. gambiae*, 60% with *T. castaneum*, 55% with *A. mellifera*, 46% with *D. arenata*, 42% with *C. maenas* and 22% with *S. purpuratus* respectively (Fig. 2-3). Similarly, the house fly burs β has 79% sequence identity with *D. melanogaster*, 50% with *M. sexta*, 51% with *B. mori*, 47% with *A. gambiae*, 55% with *T. castaneum*, 50% with *A. mellifera*, 44% with *D. arenata*, 46% with *C. maenas* and 27% with *S. purpuratus* respectively (Fig. 2-4). Significant variation occurs at the N-terminal regions of burs α and burs β among 10 species analyzed. However, if the signal peptide at the N-terminal is excluded from the sequences of the 10 species (signal peptide cleavage site is indicated by the arrow), the mature house fly burs α and burs β subunits share much higher sequence identity with other species, up to 92% and 93% with their *Drosophila* counterparts, respectively.
Fig. 2-1. The house fly burrs a nucleic acid and predicted amino acid sequences. The red
letters indicate the start and stop codons. The polyadenylation signal sequence (AATAAA)
is indicated by the red underline.

```
1  CTCTAGTTTGGCTGGCCCTTGGTAGGGATCGGCTGAGCT
61  ATTAGGAATAATATTTGCTACATAGATATAGTTGATCTACTATTAGTGCTTTGG
121  TAAAATACAAATCTCGTGTGGAGAATATTAAGTTATGCTATGCTTTTGAGTTATG
9  NQNLRCGILSGMILIMIL
181  TCAGTTCCTGATGATACAGGTCGATGTAATGCGATTTATAGGACTGATAATG
29  QFDGDLAVGDNVIGTDNI
241  TTCCCCATATGGTGTATGGCACTAGAATATCTACATCCTGAGG
49  SHTGDQCUTPVTUVLYQYG
301  CTGTGTACCTAAAAACTACCTCTCTCTCTGTGGCTGGCGATGTCGTTATTATCC
69  CVPKPIPSFACVGRCASYIQ
361  GGTATCTGGGAGTAATTTGCAATGACCTGGTGGCTATGTGCTGTACAGAATCGG
89  VSQSKIQMPRSMMCQESCQ
421  TGAACGGGCTGGCTGGCTGTGCTATGGGAAAAGTGGAGGCTGAAATTT
109  TEREAASLVFCPKVKHGERKF
481  TAAAGAAAGGCTTCTCAACAAAGGCACCCCTGGGAATGTATGTGCAGCACTCCTGAGT
129  KTKLVAPECMCRECTSIDE
541  AGAATTCTGGGATTGACCACAAGAAATTTGCCGCTTCCGATGAGAGGCACTCAAATA
149  ESGIVFQEIAGYSDEGCPLN
601  TCATTTCAGAAGGATAGCAGTACAAAATGCACGCAAAATACATTCTATTGGTT
169  HFRRIALQ
661  TAATTATATAGAAAACTGACACAACACACACACCAGCAGCAAAATACATCAATAATTATTCTGATG
721  TAGTAAATATATATATTTGGGACCTGAAAACGGAACAAACAAAAAAAAAAAAAAAAAAAA
781  AAAAAAAAAAAAAAAA
```
Fig. 2-2. The house fly burs β nucleic acid and predicted amino acid sequences. The red letters indicate the start and stop codons. The polyadenylation signal sequence (AATAAA) is indicated by the red underline.
Fig. 2-3. Alignment of the house fly burs α amino acid sequence with the sequences from other species. M.d.: M. domestica; D.m.: D. melanogaster; M.s.: M. sexta; B.m: B. mori; A.g.: A. gambiae; T.c.: T. castaneum; A.m.: A. mellifera; D.a.: D. arenata; C.m.: C. maenas; S.p.: S. purpuratus. The dark shaded residues indicate 100% identity and the gray shading represents 80% identity between analyzed species. The black box indicates the cystine knot domain. The asterisks show the conserved cysteine residues. The arrow indicates the signal peptide cleavage site.
Fig. 2-4. Alignment of the house fly burs β amino acid sequence with the sequences from other species. M.d.: *M. domestica*; D.m.: *D. melanogaster*; M.s.: *M. sexta*; B.m.: *B. mori*; A.g.: *A. gambiae*; T.c.: *T. castaneum*; A.m.: *A. mellifera*; D.a.: *D. arenata*; C.m.: *C. maenas*; S.p.: *S. purpuratus*. The dark shaded residues indicate 100% identity and the gray shading represents 80% identity between analyzed species. The black box indicates the cystine knot domain. The asterisks show the conserved cysteine residues. The arrow indicates the signal peptide cleavage site.
The cystine knot sequence in burs α and burs β subunits is highly conserved. The house fly bursicon α shares 98% identity with the *Drosophila* sequence in this region and 71-95% identity with sequences of the remaining insect species (Fig. 2-3). The same is true for burs β. Most importantly, the 11 cysteine residues in both burs α and burs β subunits are completely conserved in all 10 analyzed species (Fig. 2-3 and 2-4).

**Expression of recombinant burs α and burs β in mammalian and insect cells**

Burs α, burs β and burs α+β heterodimer proteins were expressed in mammalian 293 cells and insect High Five™ cells. To facilitate purification, *M. domestica* burs α and burs β constructs were tagged with His-6- tagged epitopes at the N terminus by replacing the endogenous signal peptide with the epitope tags. Conditioned media were further purified by using metal chelating Sepharose (Amersham Pharmacia) against His-6-burs α and His-6-burs β. Protein purity and biochemical characteristics were analyzed after electrophoresis by using a 12% SDS polyacrylamide gel and then western blot analysis (Fig. 2-5).

**Functional analysis of the r-bursicon in neck-ligated flies**

The r-bursicon protein, expressed in mammalian 293 cells or insect Highfive™ cells, was assayed for bursicon activity using the neck-ligated fly assay. As shown in Fig. 2-6, no sign of cuticle tanning was observed in the neck-ligated flies injected with 1 μl of the supernatant from cell culture transfected with blank vector only (Fig. 2-6a) or vector with burs α (Fig. 2-6b) or vector with burs β (Fig. 2-6c). Cuticle tanning was detected only in the neck-ligated flies injected with the CNS homogenate (0.5 CNS equivalent) (Fig. 2-6d), with the supernatant of insect Highfive™ cells (Fig. 2-6e) or the supernatant
Fig. 2-5. Western blot analysis of recombinant bursicon heterodimer proteins expressed from mammalian 293 cells. Lane 1-3 refers to burs $\alpha$ subunit only, burs $\beta$ subunit only, and burs $\alpha+\beta$ expressed together, respectively.
of mammalian 293 cells (Fig. 2-6f) co-transfected with bursicon α and β vectors. An identical result was obtained when the neck-ligated *D. melanogaster* flies were injected with the recombinant *M. domestica* bursicon heterodimer expressed in mammalian 293 cells and insect Highfive™ cells (data not shown).

**RT-PCR analysis of the developmental profiles of burs α and burs β transcripts**

To assess the developmental transcript level of *burs* α and *burs* β in the CNS of the house fly, semi-quantitative RT-PCR was performed using RNA prepared from the CNS of different developmental stages of the house fly. RT-PCR analysis revealed a similar pattern for both *burs* α and *burs* β transcripts. As shown in Fig. 2-7, both *burs* α and *burs* β transcripts were detected in the CNS of 48h larvae, increased gradually from late larvae to pupal stages, and reached the maximum level in pharate adults. Both transcripts declined sharply after adult emergence and became barely detectable 24h later, suggesting the release of bursicon proteins after translation.
Fig. 2-6. Functional assay of the r-bursicon heterodimer in neck-ligated house flies. Newly emerged flies were neck-ligated right at emergence and the neck-ligated flies with the unsclerotized cuticle 1 h after neck-ligation were individually injected with 0.5μl of cell culture media transfected with blank pcDNA 3.1 vector as a negative control (a) or the vector with bursicon α (b) or the vector with burs β (c) or the bursicon α vector and the burs β vector expressed in insect High Five™ cells (e) or in mammalian 293 cells (f). CNS homogenate (0.5 CNS equivalent) from newly emerged flies was used as a positive control (d).
Fig. 2-7. RT-PCR analysis of \textit{burs} \textit{\alpha} and \textit{burs} \textit{\beta} transcripts in the CNS of \textit{M. domestica} at different developmental stages. The CNSs were dissected from the indicated developmental stages of the house fly and total RNA was extracted for RT-PCR amplification of \textit{burs} \textit{\alpha} (a) and \textit{burs} \textit{\beta} (b) transcripts. c: \textit{rp49} gene was used as control. d, e: Relative quantification of both transcripts in different developmental stages. M: DNA marker; 48L: 48 h larvae (late second instar); 72L: 72 h larvae (early third instar); 96L: 96 h larvae (late third instar); PP: Pre-pupae; PA: Pharate adults; 0A: 0 h adults; 24A: 24 h adults. Relative bursicon transcript levels were calculated as percentage to their levels in pharate adult stage, which were treated as 100%. The data (d and e) represent the mean ± SE of three biological replicates.
FISH localization of \textit{burs} $\alpha$ and \textit{burs} $\beta$ transcripts

FISH was performed to localize bursicon-containing neurosecretory cells within the CNS of the house fly on a developmental basis. Identical experiments were also performed in \textit{D. melanogaster} for the purpose of comparison. In \textit{Musca} larvae, both \textit{burs} $\alpha$ and \textit{burs} $\beta$ transcripts were detected mainly in 5-6 pairs of neurosecretory cells in the ventral nervous system (VNS) of the house fly at all three larval stages (Fig. 2-8). In addition, a weak signal of \textit{burs} $\alpha$ transcript was also detected in a pair of bilateral cells located at the base of suboesophageal ganglion. Both \textit{Musca} \textit{burs} $\alpha$ and \textit{Drosophila} \textit{burs} $\alpha$ probes stained an additional 10 pairs of bilateral neurosecretory cells (NSCs) in the VNS. In \textit{Drosophila} larvae, \textit{burs} $\beta$ transcripts were detected only in 4 pairs of NSCs in the abdominal ganglia while \textit{burs} $\alpha$ transcripts were detected in an additional set of bilateral cells (Fig. 2-9).

In \textit{Musca} adults as shown in Fig. 2-10, both \textit{burs} $\alpha$ and \textit{burs} $\beta$ transcripts were detected exclusively in 5-7 pairs of NSCs in the highly fused thoracico-abdominal ganglia (TAG). All of these cells were located in the third thoracic ganglion and the abdominal ganglion. In \textit{Drosophila} adults (Fig. 2-11), similar staining patterns of both \textit{burs} $\alpha$ and \textit{burs} $\beta$ transcripts were detected in about 5-7 pairs of NSCs in the third thoracic ganglion and the abdominal ganglion. Similarly, both \textit{Musca} and \textit{Drosophila} \textit{burs} $\alpha$ and \textit{burs} $\beta$ transcript levels were high in pharate adult, newly emerged adult and 1 h old adult, decreased dramatically at 24 h after emergence, and disappeared 48 h after emergence.
Fig. 2-8. Fluorescence in situ hybridization of burs $\alpha$ and burs $\beta$ transcripts in the CNS of *M. domestica* larvae and prepupae. The CNSs were dissected from the indicated larval and prepupal stages, fixed and hybridized with antisense *burs* $\alpha$ (red) and *burs* $\beta$ (green) probes. The CNS samples were visualized with a confocal microscope and photographed. Yellow in the merged pictures indicates the NSCs with the co-localized burs $\alpha$ and burs $\beta$ signals. Transparent pictures show the images of the CNS. The far right panel shows the partial enlarged image showing the co-localized *burs* $\alpha$ and *burs* $\beta$ NSCs (yellow) and the adjacent bilateral NSCs with *burs* $\alpha$ signal only (red). L1: first instar larvae; L2: second instar larvae; L3: third instar larvae; PP: prepupae. Each image represents a typical pattern of a minimum of 10 house fly CNS samples. (Scale bars indicate 300$\mu$m.)
Fig. 2-9. Fluorescence in situ hybridization of $burs\alpha$ and $burs\beta$ transcripts in the CNS of *D. melanogaster* larvae and prepupae. The CNSs were dissected from the indicated larval and prepupal stages, and fixed and hybridized with antisense $burs\alpha$ (red) and $burs\beta$ (green) probes. The CNS samples were visualized with a confocal microscope and photographed. Yellow color in the merged pictures indicates the NSCs with the co-localized $burs\alpha$ and $burs\beta$ signals. Transparent pictures show the images of the CNS. L1: first instar larvae; L2: second instar larvae; L3: third instar larvae; PP: prepupae. Each image represents a typical pattern of a minimum of 10 *Drosophila* CNS samples. (Scale bars indicate 100\(\mu\)m.)
Fig. 2-10. Fluorescence in situ hybridization of burs α and burs β transcripts in the CNS of M. domestica adults. The CNSs were dissected from the indicated adult stages, and fixed and hybridized with antisense burs α (red) and burs β (green) probes. The CNS samples were visualized with a confocal microscope and photographed. Yellow in the merged pictures indicates the NSCs with the co-localized burs α and burs β signals. Transparent pictures show the images of the CNS. Each image represents a typical pattern of a minimum of 10 house fly CNSs. The far right panel shows the typical image of the CNS of M. domestica adult as a reference. PA: pharate adult; 0A: 0 h adult; 1A: 1 h adult; 24A: 24 h adult; 48A: 48 h adult. (Scale bars indicate 300μm.)
Fig. 2-11. Fluorescence in situ hybridization of \textit{burs} \textit{\alpha} and \textit{burs} \textit{\beta} transcripts in the CNS of \textit{D. melanogaster} adults. The CNSs were dissected from the indicated adult stages, fixed and hybridized with anti-sense \textit{burs} \textit{\alpha} (red) and \textit{burs} \textit{\beta} (green) probes. The CNS samples were visualized under confocal microscope and photographed. Yellow in the merged pictures indicates the NSCs with the co-localized \textit{burs} \textit{\alpha} and \textit{burs} \textit{\beta} signals. Transparent pictures show the images of the CNS. The far right panel shows the typical image of the CNS of \textit{D. melanogaster} adult as reference. Each image represents a typical of a minimum 10 fly CNSs. PA: pharate adult; 0A: 0 h adult; 1A: 1 h adult; 24A: 24 h adult; 48A: 48 h adult. (Scale bars indicate 100\(\mu\)m.)
Real Time PCR analysis of two bursicon-regulated genes

To identify bursicon-regulated genes in the house fly, total RNA was isolated from the neck-ligated flies injected with either r-bursicon $\alpha+\beta$ heterodimer or bursicon $\alpha$ only (control). DNA microarray analysis was performed to identify bursicon-regulated genes using the GeneChip® Genome 2.0 Array (Affymetrix, Santa Clara, CA) covering over 500,000 data points to measure the expression of 18,500 transcripts and variants. The DNA microarray identified 42 bursicon-regulated genes with $\geq1.5$ fold up-regulation when compared with the control (data not shown). Two *M. domestica* bursicon up-regulated genes identified via DNA microarray were verified by real time PCR. The two genes are homologs of *D. melanogaster* CG7985 and CG30287. Due to the lack of available genomic information for *M. domestica*, they were annotated CG7985hh and CG30287hh (hh stands for house fly homolog). As shown in Fig. 2-12 (a), the level of CG7985hh mRNA in the r-bursicon injected flies is up-regulated by 2.6 fold at 40 min, and dropped slowly at 1 h and 2 h after injection. The transcript level decreased dramatically at 12 h and reached the lowest level at 24 h. For CG30287hh, a similar pattern of its transcript levels was also observed, although induction time and rate were slightly behind and lower than those of CG7985hh respectively (Fig. 2-12 b).
Fig. 2-12. Real-time RT-PCR analysis of the transcript levels of \textit{CG7985hh} and \textit{CG30287hh} genes in \textit{M. domestica}. The newly emerged flies were neck-lighted immediately after emergence and injected with 1 \( \mu l \) of r-bursicon heterodimer (60 ng/\( \mu l \)). The control received r-bursicon \( \alpha \) only (60 ng/\( \mu l \)). The RNA was isolated for real-time RT-PCR analysis from the flies at the indicated time periods after r-bursicon injection. (a) The transcript level of \textit{CG7985hh} mRNA in the injected flies reaches the maximum of 3.6-fold at 40 min, and drops slowly at 1 and 2 h after injection. The transcript level decreases dramatically at 12 h and reaches the lowest level at 24 h. (b) Similarly for \textit{CG30287hh}, the transcript level increases at 20 min and 40 min while the highest level appears at 1 h post-injection. Then the transcript returns to normal level after 12 h. The induction time and rate are slightly behind and lower than those of \textit{CG7985hh} respectively. The data represent the mean ± SE of three biological replicates. * indicates p-value statistically significant (p ≤ 0.05).
2.5 Discussion

Using 3’ and 5’ RACEs, *burs α* and *burs β* genes were cloned and sequenced from the house fly *M. domestica*. Sequence alignment of the house fly *burs α* with the sequences from 6 different insect species reveals that the house fly *burs α* shares the highest sequence identity (79%) with the *Drosophila* counterpart and the lowest (22%) with the *S. purpuratus* sequence (Fig. 2-3). Similarly, the house fly *burs β* shares the highest sequence identity (79%) with the *Drosophila* and the lowest (27%) with the *S. purpuratus* sequence (Fig. 2-4). High sequence identities of *burs α* and *burs β* between *M. domestica* and *D. melanogaster* suggest that bursicon might have cross-species activity between these two species. However, significant sequence variation occurs between the flies and non-fly species. But the key 11 cysteine amino acid residues required for forming the cystine knot in both *burs α* and *burs β* subunits are completely conserved among the 10 analyzed species (Fig. 2-3 and 2-4), suggesting that these cysteine residues may play a vital role in maintaining bursicon structure and function.

After cloning the house fly *burs α* and *burs β* genes, recombinant house fly bursicon protein was expressed in both mammalian 293 and insect Highfive™ cells and the r-bursicon demonstrated strong biological activity in neck-ligated fly assay (An *et al.*, 2008b) (Fig. 2-6). These results confirm the previous report that functional bursicon is indeed a heterodimer of *burs α* and *burs β* subunits (Luo *et al.*, 2005; Mendive *et al.*, 2005). Sequence alignment reveals that house fly *burs α* and *burs β* subunits share 79% sequence identity with the *Drosophila* counterparts. The sequence identity goes up to 92-93% when the signal peptides from the N-terminal are excluded from both species. This prompted us to test whether bursicon from *M. domestica* has cross-species activity in
the *D. melanogaster* fly assay. As expected, the recombinant *Musca* bursicon exhibited a strong tanning activity in the fruit fly (An *et al.*, 2008b). This is not surprising since the brain extract or hemolymph from several dipteran species including *S. bullata* (Cottrell, 1962b; Fogal and Fraenkel, 1969; Fraenkel and Hsiao, 1962), *Phormia regina* (Fraenkel and Hsiao, 1965), and *Lucilia spp* (Cottrell, 1962b; Seligman and Doy, 1972) is able to induce cuticle sclerotization in the blowfly assay.

RT-PCR analysis of the house fly *burs α* and *burs β* genes in the CNS reveals that both *burs α* and *burs β* genes were transcribed as early as in the first instar larvae, reached the maximum levels in pharate adult, and declined sharply after adult emergence. The sharp decline of the *burs α* and *burs β* transcripts after adult emergence observed in the house fly CNS is in agreement with the reports in *Drosophila* (Luo *et al.*, 2005; Mendive *et al.*, 2005) and in *B. mori* (Huang *et al.*, 2007), indicating active translation and release of bursicon into hemolymph for cuticle sclerotization of the newly emerged fly. The developmental profiles of *burs α* and *burs β* transcripts in larval and pupal stages of the house fly show a relatively steady increase with a little fluctuation; while a big fluctuation of *burs α* and *burs β* transcripts was reported in the larval and pupal stages of *Drosophila*, with bursicon totally missing from several developmental stages (Luo *et al.*, 2005; Mendive *et al.*, 2005). It is unlikely that the fluctuation of *burs α* and *burs β* transcripts in larval and pupal stages of *Drosophila* is due to the periodic synthesis and release of the hormone for the purpose of cuticle sclerotization. Fraenkel (1975) showed that puparium tanning factor (PTF) of the larval blowfly did not exhibit tanning activity in the adult blowfly bursicon assay although bursicon and PTF are both involved in cuticle sclerotization. The bursicon identified in *Musca* and *Drosophila* is likely to be the
hormone regulating the cuticle sclerotization in newly emerged adults, not in larvae and pupae. Therefore, it is unlikely to have periodical releases of bursicon in larval and pupal stages unless other unidentified functions are associated with bursicon. The discrepancy in the levels of $burs\alpha$ and $burs\beta$ transcripts in larval and pupal stages between *Musca* and *Drosophila* appears to be the source of RNA used in RT-PCR analysis of $burs\alpha$ and $burs\beta$ transcripts. In *Drosophila*, RNA used for RT-PCR was extracted from whole fly body (Luo *et al.*, 2005; Mendive *et al.*, 2005) while in *Musca* RNA was from the CNS (Fig. 2-7).

In situ hybridization results showed that in *M. domestica*, both bursicon transcripts appeared as early as in the first larval instar, increased steadily in the rest of the larval and pupal stages, reached the maximum level in pharate adults and newly emerged adults, and then decreased over time until barely detectable in the 48h adults (Fig. 2-8 and 2-10). These results confirm the developmental profiles of bursicon transcripts observed in RT-PCR (Fig. 2-7). Interestingly, $burs\alpha$ and $burs\beta$ transcripts in *Musca* larvae are co-expressed in 5-6 pairs of NSCs (Fig.2-8) in the VNS; while in *Drosophila*, $burs\alpha$ and $burs\beta$ are co-expressed only in 4 pairs of NSCs (Fig. 2-9). Besides the NSCs that co-express $burs\alpha$ and $burs\beta$, *Drosophila* $burs\alpha$ probes stained an additional ~10 pairs of bilateral NSCs in the VNS. Although house fly $burs\alpha$ probes also stained the bilateral NSCs, it was weak staining compared to the main 5-6 pairs of NSCs. The pair of NSCs located at the base of the sub-eosophageal ganglion in *Musca* larvae was not stained in *Drosophila*, which is in accordance with the data reported by Luo (Luo *et al.*, 2005) and Dewey (Dewey *et al.*, 2004). The difference in the number and signal strength of the bursicon expressing cells between *Musca* and *Drosophila* may reflect a
species difference, which results in a novel distribution of bursicon-containing cells or unidentified roles the extra cells play in the house fly.

This result also is consistent with the discrepancy of the reported localizations of bursicon in different insect species. Early studies showed that bursicon is produced by only four pairs of neurons in each of the abdominal ganglia of *M. sexta* (Taghert and Truman, 1982b) and in *Drosophila* (Honegger et al., 2002; Luo et al., 2005); however, bursicon’s presence can be detected in homogenates of all ganglia of the ventral nerve cord of a variety of different insects (Fraenkel and Hsiao, 1965; Grillot et al., 1976; Taghert and Truman, 1982a; Vincent, 1972).

In adult stages, the patterns of *burs α* and *burs β* transcripts in *Musca* and *Drosophila* are similar (Fig. 2-10 and 2-11). Both *burs α* and *burs β* transcripts are detected in the same number and pattern of NSCs. In situ staining patterns in early *Musca* adults are consistent with previous work reported in *Drosophila* pharate adults using bursicon specific antibodies (Luan et al., 2006), suggesting that these NSCs are responsible for transcriptional and translational expression of bursicon heterodimers.

Our experiments did not include investigation on any possible differences between male and female flies. Since our FISH experiments involved using a large number of fly individuals (minimum of ten for each replicate) to investigate the expression profile of the bursicon neurohormone, both male and female flies were very likely investigated simultaneously. This excludes the possibility of gender specific patterns. Plus the fact that insect tanning activity is a common physiological event in both male and female insects, it is very likely that male and female flies have similar staining patterns.

Real time PCR revealed that the two genes *CG7985hh* and *CG30287hh* are
up-regulated upon bursicon injection. In *Drosophila*, *CG7985* gene has not yet been functionally studied. However, this gene has a glycoside hydrolase catalytic domain and is believed to encode a member of the O-glycosyl hydrolase family, which is a widespread group of enzymes that hydrolyzes the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. Glycoside hydrolases are typically named after the substrate that they act upon and they include two important insect related enzymes: chitinase and trehalase. In insects, chitinases are a group of important chitinolytic enzymes that hydrolyse the cuticle and peritrophic membrane and are vital to molting in insects. Potential involvement of these enzymes in insect molting and the cuticle sclerotization process is in agreement with the dissolving of chitin and detachment of the newly formed cuticle from the old one during these processes. Trehalases are another group of enzymes that function in insect flight muscle. It was reported that trehalase is regulated by cAMP-dependent phosphorylation in yeast species (Ortiz *et al.*, 1983), indicating the potential involvement of trehalase in the cAMP mediated tanning process in insects. It was also reported that trehalase might be involved in the bursicon-regulated tanning process (Huang *et al.*, 2007). Another bursicon up-regulated gene, *CG30287hh*, encodes one type of serine protease responsible for the digestion of proteins in insects. Insect cuticle has a high concentration of protein cross-linked with chitin. This serine protease very likely plays a role in digestion and re-formation of cuticle proteins for a cross-linking purpose in the cuticle sclerotization.
2.6 Conclusion

In this study, bursicon genes were cloned and analyzed in the house fly *M. domestica*, and their transcriptional profiles and localization in the central nervous system of the house fly were also studied. More importantly, by using fly neck-ligation assay, r-bursicon injection and DNA microarray, a series of genes that are up-/down- regulated by bursicon were identified in *D. melanogaster* and *M. domestica*. With the aim of elucidating bursicon signal transduction pathway, I selected two very interesting genes, *suppressor of hairless* and *pleckstrin homology* for further study. These two genes will also be cloned and analyzed in the house fly, and their potential functions and involvement in the bursicon-regulated signal transduction pathway will be discussed in chapter 3 and chapter 4, respectively.
CHAPTER 3

CLONING AND CHARACTERIZATION OF A BURSICON-REGULATED GENE $MDSU(H)$ AND ANALYSIS OF ITS RELATED NOTCH SIGNAL PATHWAY IN THE HOUSE FLY $MUSCA DOMESTICA$

A portion of this work has resulted in the following manuscript:

3.1 Chapter summary

Bursicon is a neuropeptide that regulates cuticle sclerotization (hardening and darkening) via a G-protein coupled receptor. The downstream signal transduction pathway during the insect cuticle sclerotization process is currently not well elucidated. In previous studies, a panel of genes that are regulated by bursicon were identified by using r-bursicon and DNA microarray. One of the genes, a homolog of *D. melanogaster* Suppressor of Hairless, or *Su(H)* gene, has drawn our attention. In the present study, the homolog of *Su(H)* in the house fly *M. domestica*, the *mdSu(H)*, was cloned by 3’ and 5’ RACEs. Real-time RT-PCR analysis indicated that the level of *mdSu(H)* transcript is up-regulated by ~2.5-3 fold 1 h after r-bursicon injection, which correlates well with the cuticle sclerotization process observed in r-bursicon injected flies. Su(H) is a transcription factor and is mainly involved in the Notch signaling pathway, which is an important signal transduction pathway that regulates cell differentiation during neurogenesis and controls cell death in insect wings. This correlates well with the bursicon-regulated cuticle sclerotization and wing expansion processes, which involve programmed cell death in insect wings and possible cell fate determination during cuticle and wing development. So it is very likely that *Su(H)* and its related Notch signaling pathway are essential components involved in the bursicon-regulated cuticle sclerotization and wing expansion process.
3.2 Introduction

In *Drosophila*, the proposed receptor of bursicon has been identified as a G protein-coupled receptor DLGR2 encoded by the *rickets* gene (Baker and Truman, 2002). *In vitro* study has shown that mutation of the receptor *rickets* gene causes defects in cuticle sclerotization and wing expansion (Baker and Truman, 2002). Upon activation, DLGR2 is hypothesized to activate the cAMP/PKA signaling pathway which probably finally leads to cuticle sclerotization (Kimura *et al.*, 2004; Luo *et al.*, 2005; Mendive *et al.*, 2005).

Since the identification of the bursicon genes and then protein sequences, researchers have been trying to find out what signal transduction pathways these neuropeptides use and what components are involved in these pathways. Recently a gene silencing study revealed that injection of double-stranded bursicon α RNA into silkworm *B. mori* pupae significantly reduced the level of bursicon α mRNA in pupae, resulting in a decrease in wing expansion and increase in the expression of three genes, including a thioesterase gene, a trehalase gene, and an unknown gene, suggesting their potential involvement in the cuticle sclerotization process in *B. mori* (Huang *et al.*, 2007). By using r-bursicon and DNA microarray analysis, a series of bursicon-regulated genes have recently been identified in the fruit fly *D. melanogaster* (An *et al.*, 2008 a and b) and the house fly *M. domestica* (my unpublished data). The genes identified were either up-regulated or down-regulated by r-bursicon either 1 h or 3 h after the injection of r-bursicon.

Despite the recent advances in bursicon research, the mechanism of bursicon activity and the signaling pathway downstream of the release of hormone is still far from
known. Therefore, more studies are needed to identify the signal transduction components downstream of bursicon and its receptor DLGR2.

In chapter 2, a set of genes that are regulated upon bursicon activity were identified in the house fly *M. domestica*. One of them, a gene homologous to *Drosophila Suppressor of Hairless (Su(H))*, has drawn our further attention. Suppressor of hairless, Su(H), is a transcriptional factor mainly involved in the Notch signaling pathway and in neurogenesis. The *Su(H)* gene has been identified in many organisms including several insect species and mammalian species. It has been reported to participate in a number of physiological events and control the expression of downstream genes.

The present study reports the molecular cloning and developmental expression of *mdSu(H)* gene in the house fly *M. domestica*. By using the recombinant house fly bursicon, it was shown that *mdSu(H)* is up-regulated by r-bursicon in the neck-ligated house fly assay and its expression profile correlates well with the cuticle sclerotization process, indicating its involvement in this process in insects.

### 3.3 Materials and Methods

**Experimental insects**

House fly larvae were reared on artificial diet (Carolina Biological Supply, Burlington, NC) at 30°C under constant darkness and adults were fed on a 1:1 mixture of granulated sugar and powder milk at 30°C under 16h light: 8h darkness.
Amplification of the conserved fragment of the \textit{mdSu(H)} gene

Two pharate adults of the house fly \textit{M. domestica} were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated and transcribed into cDNA using the SuperScript\textsuperscript{TM} III First-Strand synthesis system from Invitrogen. PCR primers (as shown in Table 3-1) were designed based on conserved sequences of the \textit{Su(H)} gene found by alignment in other insect species including \textit{D. melanogaster}, \textit{C. pipiens}, \textit{A. gambiae}, \textit{A. aegypti}, \textit{T. castaneum}, and \textit{A. mellifera}. After agarose gel electrophoresis, PCR bands were cut out from the gel, eluted using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), cloned into the pGEM-T-Easy Vector (Promega, Madison, WI) and sent for sequencing at the DNA Core Facility of University of Missouri.

Cloning of \textit{mdSu(H)} gene using 3’ and 5’ RACEs

To obtain a full-length \textit{mdSu(H)} cDNA, rapid amplification of cDNA ends (RACEs) was performed using a 5’RACE and 3’RACE system (Invitrogen, Carlsbad, CA). The gene specific primers (GSPs) and the nested gene-specific primers (nested GSPs) used in the RACE protocol were designed based on the amplified \textit{mdSu(H)} gene fragment in \textit{M. domestica} (Table 3-2).

Total RNA was extracted from the newly emerged house fly (whole body) using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For amplification of the 3’ end of the cDNA, the first-strand cDNA was synthesized using the manufacturer-supplied adapter primer. The supplied abridged universal amplification primer, the gene specific primer and the nested primers were used in a subsequent amplifications. For the amplification of the 5’ end of the cDNA, the gene
specific primer was used for the first-strand cDNA synthesis. Two nested primers were used for subsequent amplification. The PCR products of 3’ and 5’ RACEs were gel purified, cloned into the pGEM-T-Easy Vector (Promega, Madison, WI) and sequenced at the DNA Core Facility of the University of Missouri.

**Amino acid sequence alignments and analysis**

The mdSu(H) amino acid sequence was deduced based on the cloned cDNA sequence, and sequence analysis was performed on the ExPASy server (www.expasy.ch) for prediction of functional domains, motifs, and signal peptides. Protein tertiary structure prediction was performed on the Swiss Model server using First Approach Mode (http://swissmodel.expasy.org/). Homology searches of the house fly mdSu(H) protein were made on the NCBI platform (www.ncbi.nih.gov). Alignments of multiple sequences were carried out using the EBI ClustalW2 platform (www.ebi.ac.uk) and edited manually with GeneDoc software (http://www.ncbi.nlm.nih.gov/). A phylogenetic tree was developed using Mega 4 software (http://www.megasoftware.net).

**Analysis of mdSu(H) transcript after r-bursicon injection**

To analyze the effect of r-bursicon on the transcription of the mdSu(H) gene, newly emerged house fly adults were neck-ligated immediately after emergence. After a one hour waiting period, the neck-ligated flies that did not show any cuticle tanning were injected with r-bursicon protein (100 ng/fly) using a microinjection system. Burs α subunit dissolved in the same injection buffer (100 ng/fly) was used as a negative control. Injected flies were collected at different time periods after injection (20 min, 40 min, 1 h, 2 h, 3 h and 12 h) and immediately placed at -80°C.
Total RNA was isolated from the collected flies using Trizol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I. RNA concentration was determined by measuring the absorbance at 260 nm on a spectrophotometer. Total RNA (2 μg) from each sample was used for the first-strand cDNA synthesis as previously described. The synthesized cDNA was used as a template for analysis of gene transcription by real-time RT-PCR (qPCR). The primers designed for qPCR analysis of \textit{mdSu(H)} were: 5’-TTTATGCTGTCCGTGAAGATGTT-3’ (forward) and 5’-TAGACGATGCATGGAAATGG-3’ (reverse). The ribosomal protein 49 (\textit{rp49}) gene (forward primer: 5’-TACAGGCCCCAGATCGTGAA-3’; reverse primer: 5’-GACAATCTCCTTGCCTTCT-3’) was used for normalization. The qPCR amplification and analysis were carried out on Applied Biosystem 7300 Fast Real-time RT-PCR System using an ABI SYBR Green Supermix buffer system. The final reaction volume was 20 μl. The thermal cycle conditions used in real time PCR were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min.

**Developmental analysis of the \textit{mdSu(H)} transcript**

To analyze the transcript level of the \textit{mdSu(H)} gene in different developmental stages of the house fly, the flies were collected at different life stages (1\textsuperscript{st} instar larvae, 2\textsuperscript{nd} instar larvae, 3\textsuperscript{rd} instar larvae, early stage pupae (2 h after pupation), pharate adults, 0 hour adults, 20 minute adults, 40 minute adults, 1 hour adults, 2 hour adults, and 3 hour adults) and immediately placed at -80°C. Total RNA was extracted, quantified and samples were subjected to qPCR following the same procedures as described above in section 2.4. The house fly \textit{rp49} gene was used for normalization.
**Statistical analysis**

Experiments of the real-time RT-PCR were carried out three times, and results were expressed as mean +/- SD. Differences between different time-points were evaluated with t-test.

**3.4 Results**

**Amplification of a conserved fragment of mdSu(H) gene in M. domestica**

Using a pair of primers CG3497F2 and CG3497R1 (Table 3-1), a 450bp fragment was amplified from the house fly cDNA sample. The other 4 primers did not work in *M. domestica*. After subsequent cloning and sequencing, a conserved fragment of the *mdSu(H)* gene containing 448 bps was obtained from the house fly *M. domestica* (Fig. 3-1). A homology search of the NCBI database indicated that this fragment is a *M. domestica* homolog of the *D. melanogaster Su(H)* gene with 80% nucleotide identity.

**Cloning and sequence analysis of the house fly mdSu(H) gene using 3’ and 5’ RACEs**

Based on the identified fragment of the *mdSu(H)* gene, RACE GSPs and nested GSPs were designed using primer3 (V. 0.4.0) software. All designed primers are listed in Table 3-2 and their relative positions are marked by arrows (Fig. 3-1), with green letters indicating the 3’ RACE primers and red letters representing the 5’ RACE primers.
Forward primers:
- CG3497 F1  5’-ATGGAGAAGTACATGCGCGAGC-3’
- CG3497 F2  5’-TTTATGCTGTCGGTGAAGATGTT-3’
- CG3497 F3  5’-AAGGTAGACAAGCAGATGGC-3’

Reverse primers:
- CG3497 R1  5’-AAGGCGCATTTGTGCAGCTG-3’
- CG3497 R2  5’-CCGTCATTGATCATCTCCTTGTT-3’
- CG3497 R3  5’-GGTTCAGGTGTATACGTGAA-3’

**Table 3-1.** PCR primers designed according to the conserved sequences of the Su(H) gene in several insect species, including *D. melanogaster, A. gambiae, A. aegypti, C. pipiens, T. castaneum* and *A. mellifera.*
Fig. 3-1. Amplified cDNA fragment of *M. domestica* mdSu(H) gene using the 3497F2 and 3497R1 primers. Primers for amplification of the *mdSu(H)* fragment are indicated with an underline. Designed 3’ and 5’ RACE GSP and nested GSP primers are indicated in red and green letters, respectively.
3’-RACE primers:

- Primer 1: 5’- TTGGGGTCTTCATTCGAAAC -3’  
  (GSP)
- Primer 2: 5’- CACAATGGGGTGCTTACAA -3’  
  (Nested GSP)
- Primer 3: 5’- GGATGGCCTACCACGTTTA-3’  
  (Nested GSP)

5’-RACE primers:

- Primer 1: 5’- ACAAGGCCATTTGTTTGTCC -3’  
  (GSP)
- Primer 2: 5’- TAGACGATGCATGGAAATGG -3’  
  (Nested GSP)
- Primer 3: 5’- CCGCTGGCTATACAAAGGTC -3’  
  (Nested GSP)

---

**Table 3-2.** Gene specific primers (GSPs) and nested gene-specific primers (nested GSPs) designed according amplified mdSu(H) fragment in *M. domestica* (Fig. 3-1).
A full length sequence of the house fly *mdSu(H)* gene was obtained using 3’ and 5’ RACEs and the mdSu(H) amino acid sequence was deduced. As shown in Fig. 3-2, a full length *mdSu(H)* cDNA is 1824 nucleotides (nts) long, with an open reading frame (ORF) of 1647 bps. There is an 8-bp 5’ untranslated region and a 177-bp 3’ untranslated region following the ORF. The 3’ untranslated region contains two polyadenylation signal sequences (AATAAA), which are located 2 nts and 123 nts downstream of the stop codon (TAA). The 1647-bp ORF encodes a 548-amino acid peptide, the house fly mdSu(H).

Analysis of the house fly mdSu(H) reveals 3 predicted domains: a LAG1-DNA binding domain (amino acid 126-257, blue box in Fig. 3-3), a Beta-trefoil domain (amino acid 258-407, green box in Fig. 3-3), and an IPT/TIG domain (amino acid 434-523, red box in Fig. 3-3). The LAG1 domain family is found mainly in DNA-binding proteins. It has a beta sandwich structure with nine strands in two beta-sheets and allows for DNA binding (Kovall *et al.*, 2004). The beta-trefoil domain family adopts a capped beta-barrel with internal pseudo three-fold symmetry, and is the site of mutually exclusive interactions with the intracellular domain of the Notch receptor (NotchIC) and co-repressors (Kovall *et al.*, 2004). The IPT/TIG domain is an integrase domain, which is homologous to a mammalian gene responsible for DNA site-specific integration in the generation of immunoglobulin diversity. The function of this domain is so far not very well understood. These domains together indicate that mdSu(H) has the function of DNA binding and transcription factor activity.
Fig. 3-2. House fly mdSu(H) full length cDNA and deduced amino acid sequences. Red letters indicate the start codon and stop codon, and green letters indicate the polyadenylation signal (AATAAA) sequences.
Fig. 3-3. Multiple alignments of the mdSu(H) amino acid sequence from *M. domestica* with its homologous proteins from other insect species. Mus, *Musca domestica*; Dro, *Drosophila melanogaster*; Ano, *Anopheles gambiae*; Cul, *Culex pipiens*; Aed, *Aedes aegypti*; Tri, *Tribolium castaneum*; Api, *Apis mellifera*; Nas, *Nasonia vitripennis*; Acy, *Acyrthosiphon pisum*. Numbers on the right side indicate the amino acid position of different sequences. Grey and black shading indicate the conservations of amino acids. Black shaded amino acids represent 100% conservation, while grey shaded amino acids represent 70-90% conservation. Alignments were performed using the ClustalW2 platform on www.ebi.ac.uk. Amino acids composing three predicted functional domains of the mdSu(H) protein are also shown by colored boxes. LAG1-DNA binding domain: amino acid 126-257, blue box; Beta-trefoil domain: amino acids 258-407, green box; and IPT/TIG domain: amino acids 434-523, red box.
Fig. 3-4. Predicted tertiary structures of the mdSu(H) protein from *M. domestica* (a) and its homologous protein Su(H) from *D. melanogaster* (b). Relative positions of the three functional domains are indicated by arrows. 1. LAG1-DNA binding domain; 2. Beta-trefoil domain; 3. IPT/TIG domain.
Tertiary structure prediction of the mdSu(H) protein showed the relative positions and shapes of the three domains (Fig. 3-4). Comparison of the predicted structure between *M. domestica* and *D. melanogaster* showed no significant difference; indicating that the functional domains are conserved between the two species.

**Multiple sequence alignments and phylogenetic analysis of mdSu(H) with its homologs in other insect species**

The house fly mdSu(H) sequence is aligned with its homologs in other insect species in which the Su(H) homologous sequences have been identified, including *D. melanogaster*, *A. gambiae*, *A. aegypti*, *C. pipiens*, *T. castaneum*, *A. mellifera*, *Nasonia vitripennis*, and *Acyrthosiphon pisum*. Multiple sequence alignments show that this gene is highly conserved among different species, especially the sequences spanning the three functional domains (amino acids 126-523) (Fig. 3-3), indicating the conserved function of the mdSu(H) protein. As summarized in Table 3-3, the identities of the whole sequence range from 57-74%, and similarities are slightly higher ranging between 63-79%.

A phylogenetic tree was generated among species in which Su(H) or its homolog has been identified, including insect species and other organisms ranging from arthropods to mammals. As shown in Fig. 3-5, the phylogenetic analysis reveals that the *M. domestica* mdSu(H) is most closely related to its *D. melanogaster* counterpart, which is in accordance with the evolitional relationship between these two species compared to other organisms.
Table 3-3. Summary of identities and similarities of the house fly mdSu(H) and its homologs in other insect species.

<table>
<thead>
<tr>
<th></th>
<th>Musca</th>
<th>Drosophila</th>
<th>Anopheles</th>
<th>Culex</th>
<th>Aedes</th>
<th>Tribolium</th>
<th>Apis</th>
<th>Nasonia</th>
<th>Acyrthosiphon</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Identity</strong></td>
<td>548a.a.</td>
<td>74%</td>
<td>72%</td>
<td>57%</td>
<td>67%</td>
<td>69%</td>
<td>68%</td>
<td>65%</td>
<td>64%</td>
</tr>
<tr>
<td><strong>Similarity</strong></td>
<td>0</td>
<td>76%</td>
<td>79%</td>
<td>63%</td>
<td>73%</td>
<td>75%</td>
<td>74%</td>
<td>72%</td>
<td>71%</td>
</tr>
<tr>
<td><strong>Gap</strong></td>
<td>0</td>
<td>10%</td>
<td>8%</td>
<td>21%</td>
<td>15%</td>
<td>15%</td>
<td>16%</td>
<td>12%</td>
<td>14%</td>
</tr>
</tbody>
</table>
Real-time RT-PCR analysis of *M. domestica* *mdSu(H)* transcripts after bursicon stimulation

After injection of the r-bursicon heterodimer, the expression level of the house fly *mdSu(H)* transcript increased gradually, reached about 2.5 fold higher after one hour, and declined back to a basal level thereafter (Fig. 3-6). The enhanced expression of the house fly *mdSu(H)* transcript by r-bursicon injection is consistent with the cuticle sclerotization process induced by r-bursicon administration in neck-ligated flies (Fig. 3-7), in which cuticle tanning started to be visible at 40 min to 1 h in both female and male flies.

Developmental analysis of *mdSu(H)* transcript at different developmental stages of the house fly

The expression of *mdSu(H)* transcript was examined at different life stages of the house fly. As shown in Fig. 3-8, the expression of *mdSu(H)* transcript is detected as early as in the first larval instar. The expression level increases gradually with the development of the fly from larvae to pupae. A significant increase was noticed in the newly emerged adults. At 1 hour after emergence, the level of *mdSu(H)* transcript increases to 3.5 fold as compared to a newly emerged fly before its decline to the level as seen in the newly emerged fly. The temporal expression profile of the *mdSu(H)* transcript again correlated well with the cuticle sclerotization process induced by r-bursicon injection (Fig. 3-7).
Fig. 3-6. Real-time RT-PCR analysis of the temporal response of \textit{mdSu(H)} to r-bursicon activity. Newly emerged flies were neck-lighted immediately after emergence and then injected with 1 \( \mu \)l/fly of r-bursicon heterodimer (100ng/\( \mu \)l). The control flies received 1\( \mu \)l/fly burs \( \alpha \) only in the same injection buffer (100ng/\( \mu \)l). Total RNA was extracted from flies at different time periods (0 min, 20 min, 40 min, 1 h, 2 h, 3 h, and 12 h) after bursicon injection. Expression levels were normalized to the expression of the house fly \textit{rp49} gene. The data represent the mean ± SE of three biological samples. * indicates p-value statistically significant (p≤0.05) as compared to control (0 min).
Fig. 3-7. Temporal response of neck-ligated *M. domestica* flies to r-bursicon injection. *M. domestica* flies were neck-ligated immediately upon adult emergence and placed at room temperature for 1 hour. Then flies that did not show sclerotization activity were injected through the dorsal thorax into body cavity with r-bursicon α+β heterodimer or α subunit only in the same injection buffer as a control. Pictures showing sclerotization activity were taken at different time points after injection (0 min, 20 min, 40 min, 1 h, 2 h, and 3 h). ♀ indicates female flies and ♂ represents male flies.
Fig. 3-8. Relative quantification of the mdSu(H) transcript level in different developmental stages in the house fly, *M. domestica*. To analyze the transcriptional expression of the mdSu(H) gene in different house fly developmental stages, house fly whole bodies were collected at different life stages (1L, 1\textsuperscript{st} instar larvae; 2L, 2\textsuperscript{nd} instar larvae; 3L, 3\textsuperscript{rd} instar larvae; EP, 2 hour early stage pupae; PA, pharate adults; 0A, 0 hour adults; 20mA, 20 minute adults; 40mA, 40 minute adults; 1hA, 1 hour adults; 2hA, 2 hour adults; 3hA, 3 hour adults). Total RNA was extracted, quantified, reverse transcribed into cDNA, and then subjected to real-time RT-PCR analysis. Expression levels were normalized to the expression of the house fly *rp49* gene. The data represent the mean ± SE of three biological samples. * indicates p-value statistically significant (p≤0.05) as compared to control (0 A).
3.5 Discussion

This study reports the cloning and sequencing of the house fly *mdSu(H)* gene using 3’ and 5’ RACEs. Most importantly, we showed that the transcript level of the house fly *mdSu(H)* gene is up-regulated by r-bursicon in the neck-ligated house fly assay. Sequence analysis revealed that mdSu(H) is highly conserved in insects and other organisms. Upon r-bursicon injection, the transcript level of the *mdSu(H)* gene in the ligated flies is up-regulated 2.5-3 fold at 1 h after r-bursicon injection (Fig. 3-6). In the normal (non-ligated) flies, the transcript of *mdSu(H)* also increases shortly after fly emergence, reaching its maximum level at 1 h after adult emergence. These results demonstrate that the *mdSu(H)* gene is indeed regulated by bursicon and may play an important role in the cuticle sclerotization process.

Su(H) is a neurogenic transcription factor and has been demonstrated to be involved in regulating the insect neurogenesis process. Although Su(H) has not yet been reported to be directly related to the bursicon-induced cuticle sclerotization process, it has been shown to be involved in many other physiological events in insects. For example, Su(H) has been widely studied and accepted as one of the integral components involved in the Notch signaling pathway, which is a cell signaling system in most multi-cellular organisms (Fortini *et al.*, 1994).

Notch proteins are single-pass transmembrane receptors that often interact with transmembrane ligands associated with nearby cells. Su(H) is generally associated with the cytosolic fraction of cells, where it interacts with the intracellular end of Notch. Su(H) acts as a down-stream effector of Notch signaling following Notch interactions with its ligands. These interactions lead to translocation of Su(H) to the nucleus. Within the
nucleus, Su(H) forms a transcription factor complex with several other elements, and regulates expression of downstream genes (Fiúza and Arias, 2007). Hence, Notch regulates nuclear transcriptional events by controlling the cellular location of DNA binding proteins. This regulatory mechanism allows Su(H) to serve in a wide range of cellular processes.

Insect cuticle is not evenly sclerotized. Many different patterns, as well as different degrees of stiffness, can be observed during sclerotization with regard to insect species, body parts and sexes. For example, Fig. 3-7 shows the different patterns that are formed in the house fly, both regionally and sexually. It has been suggested that the different patterns and degrees of sclerotization are under the control of local mechanisms. And such control is very likely provided by the underneath epidermal cells, from which certain sclerotization precursors are secreted into the cuticle and oxidized by enzymes to form covalent linkages to proteins and chitin (Andersen, 2005). So far there is nothing known of how the epidermal cells are regulated to give different signals for different patterns and degrees of sclerotization. By analyzing our Drosophila microarray data, we have identified 11 additional Notch signaling related genes whose transcription was up-regulated by r-bursicon by 1.5-1.9 fold at 1 h, but not at 3 h after r-bursicon injection in the neck-ligated fly assay. These genes include CG4722-RA encoding big brain, CG2534-RA encoding canoe, CG3619-RA encoding delta, CG3929-RA encoding deltex, CG15112-RC encoding enabled, CG8118-RA encoding mastermind, CG17077-RB encoding pointed, CG4244-RD encoding suppressor of deltex, CG7807-RB encoding AP-2, CG10605-RA encoding for caupolican, and CG12052-RN encoding longitudinals lacking. These results indicate that the mdSu(H) and very likely the Notch signaling
pathway are involved in the cuticle sclerotization process and suggest a possible mechanism responsible for the control of the epidermal cells. During sclerotization, Su(H) and Notch signal pathway determine different fates of the epidermal cells by regulating expression of certain target genes, and then the differently differentiated cells in turn control the patterns and degrees of sclerotization.

Notch signaling pathway is involved in several biological processes that might be related to the insect sclerotization process. During insect development, Su(H) and the Notch signaling pathway control alternative cell fate in adult epidermis and pupal notum cells of *Drosophila* (Schweisguth, 1995; Schweisguth and Posakony, 1994), contribute to imaginal disc-derived wing morphogenesis and wing vein formation (Crozatier *et al.*, 2003; Curtiss *et al.*, 2002; Johannes *et al.*, 2002; Klein *et al.*, 2000), regulate expression of several cuticle patterning genes, such as *Dfrizzled 2*, *hairy*, *shaggy* and *patched*, in *Drosophila* (Wesley, 1999), and participate at least partly in controlling cell proliferation and cell death in development of insect wings and other tissues (Giraldez *et al.*, 2003). The development and expansion of insect wings is to a great extent related to the programmed cell death of wing hypodermis and to the removal of cell debris (Seligman *et al.*, 1972, 1975; Johnson and Milner, 1987; Kimura *et al.*, 2004; Kiger *et al.*, 2007).

Recent studies have linked the insect wing expansion and maturation to bursicon activity and the cuticle sclerotization process. Loss of function of bursicon blocked cuticle sclerotization as well as wing expansion in both *D. melanogaster* and *B. mori* adults (Huang *et al.*, 2007; Luan *et al.*, 2006). The factor that triggers wing epidermal cell death is probably bursicon (Kimura *et al.*, 2004). These results, together with the involvement of Su(H) and the Notch signaling pathway in controlling wing cell proliferation and death,
suggest the likely crosstalk between the Su(H)/Notch pathway and the bursicon-mediated cuticle sclerotization and wing development processes.

Genetic analysis also showed that Su(H) is strongly related to another factor named friend-of-echinoid (fred), which is involved in adult chitin-based cuticle pattern formation process (Chandra et al., 2003). Loss of function of fred in Drosophila results in loss of epithelium, which in turn results in a smaller notum and scutellum, loss or duplication of sensory bristles, and even deformity of cuticle while over-expression of Su(H) suppresses these phenotypes (Chandra et al., 2003). The relationship between these two factors strongly indicates their involvement in insect cuticle development, and a possible mechanism of post-eclosion cuticle modification which requires the participation of chitin.

Su(H) can undergo a functional switch between a repressor and an activator under control of binding and detachment of co-repressors (Morel et al., 2000, 2001), with its major function as repressing neurogenesis. The up-regulation of the Su(H) gene via bursicon could also lead us to speculate that, after insect eclosion and during the final step of insect development, the sclerotization process, the final maturation of insect tissues and organs, including neural systems especially peripheral nervous system such as cuticle, hairs and bristles, results in the termination of further tissue and organ development. This in turn might require the down-regulation of various suppressors, including the important neurogenic suppressor, Su(H). Although it may not directly contribute to insect cuticle sclerotization and the wing expansion process, the up-regulation of the Su(H) factor by bursicon inspires our curiosity about how these events are correlated and how the timing of them is so accurately connected and well controlled.
CHAPTER 4

CLONING AND CHARACTERIZATION OF A BURSICON-REGULATED GENE PLECKSTRIN HOMOLOGY IN THE HOUSE FLY MUSCA DOMESTICA
4.1 Chapter summary

In chapter 2, a series of genes were identified that are regulated by bursicon using r-bursicon and DNA microarray. We selected two particularly interesting genes for further analyses. One gene, \textit{mdSu(H)}, was previously characterized and discussed in the last chapter. The other gene, which encodes a protein homologous to the \textit{D. melanogaster} pleckstrin homology (PH) domain encoded by CG32982, was down-regulated after injection of r-bursicon in the neck-ligated fly assay and is very likely involved in bursicon-mediated cuticle sclerotization and wing expansion processes. The PH domain is a protein domain that occurs in a wide range of proteins and plays an important role in intracellular signaling. In studies reported in this chapter, we cloned its homologous gene, \textit{mdPH}, from the house fly \textit{M. domestica} by using 3’ and 5’ RACEs. Real-time RT-PCR analysis indicated that the level of \textit{mdPH} transcript is down-regulated \( \sim 2.5-3 \) fold 1 h after r-bursicon injection into neck-ligated house flies. Developmental analysis of the \textit{mdPH} transcript in normal (non-ligated) house flies showed that the \textit{mdPH} transcript level increases in larval and pupal stages, reaches its highest level in pharate adults and newly emerged adults, and then decreases dramatically \( \sim 2.5-3 \) fold in adults at 40 minutes after emergence and older. It has been reported that one of the major functions of the PH domain is to activate a G protein coupled receptor kinase (GRK), which then phosphorylates and deactivates the G protein coupled receptor (GPCR) and therefore blocks the GPCR signal transduction pathway. The increase of the \textit{mdPH} transcript levels before adult emergence suggests the need for expression of the mdPH protein and thus the repression of the GPCR to prevent activation of downstream elements to permit bursicon-regulated cuticle sclerotization and the wing expansion processes to occur; while
the down-regulation of \textit{mdPH} expression after adult emergence indicates the requirement to unblock GPCR signal transduction pathway, which then allows the activation of GPCR pathway for the downstream signal transduction to induce normal cuticle sclerotization and wing expansion processes.

4.2 Introduction

Pleckstrin homology (PH) is a protein domain of approximately 120 amino acids that occurs in a wide variety of proteins involved in intracellular signaling or in ones that are constituents of the cytoskeleton. The PH domain was first reported in 1993 in a number of signal transducing proteins, which suggests that this domain is widely present (Haslam \textit{et al.}, 1993; Mayer \textit{et al.}, 1993). Since its first discovery, nearly 100 different eukaryotic proteins with pleckstrin homology domains have been identified. Many of these proteins are involved in receiving and transmitting signals at the interface between the membrane and cytosol. The first reported function for the pleckstrin homology domain was that it is the major substrate of protein kinase C (PKC) in platelets (Tyers \textit{et al.}, 1988). It was reported that PH domain binds to phosphatidylinositol in cellular membranes, such as phosphatidylinositol (3,4,5)-triphosphate and phosphatidylinositol (4,5)-bisphosphate, (Harlan \textit{et al.}, 1994) and to proteins, such as the \( \beta\gamma \) subunit of G proteins and to PKC, to activate various enzymes, e.g. phospholipases C\( \beta_1 \) (PLC\( \beta_1 \)). Activation of PLC\( \beta_1 \)PH results in hydrolysis of phosphoinositide to release two substrates, inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol (Berridge, 1993; Nishizuka, 1995), which are very important secondary messangers involved in signaling pathways leading to cell growth and transformation (Carpenter \textit{et al.}, 1996). Up to now,
several molecules, including phosphatidylinositol phosphates, G protein βγ subunits, G protein α subunits, RACK1, PKC and F-actin have been identified as ligands of PH domains.

G protein coupled receptors (GPCRs) are membrane located receptors with important roles in mediating cell signals. GPCRs are deactivated upon the phosphorylation and activation of G protein coupled receptor kinase-2 (GRKs). This desensitization is mediated by activation of protein kinase C (PKC) (Yang et al., 2003). Also PKC could possibly directly phosphorylate GPR and initiate desensitization of the GPCR receptors (Garcia-Sainz et al., 2000). The PH domains located in GRKs are the main domain that recognizes the G protein substrate and plays a pivotal role in mediating the phosphorylation and desensitization of the GPCR receptors. PKC is a family of serine/threonine protein kinases, which plays significant roles in numerous cellular responses, including cell proliferation, differentiation, growth control, tumor progression and apoptosis etc. (Nishizuka, 2001). However, the detail mechanism of how PKC interacts with and activates GRK still remains unclear (Yang et al., 2003).

In Drosophila, the proposed receptor for bursicon has been identified as a G protein-coupled receptor DLGR2 encoded by the rickets gene, which when mutated caused defects in cuticle sclerotization and wing expansion (Baker and Truman, 2002). Previously we identified a gene, CG32982 (PH), which is down-regulated by bursicon. PH gene encodes a protein with a pleckstrin homology domain that is possibly involved in bursicon-mediated insect cuticle sclerotization and wing expansion processes.

In this study, the PH homologous gene, mdPH, was cloned from the house fly M. domestica, and its transcript level was analyzed by using r-bursicon and a neck-ligated
house fly assay. It is shown that the *mdPH* gene is down-regulated upon r-busicon activity in vivo, suggesting that mdPH is possibly involved in the bursicon-mediated cuticle sclerotization and wing expansion processes.

### 4.3 Materials and Methods

**Experimental insects**

House fly larvae were reared on artificial diet (Carolina Biological Supply, Burlington, NC) at 30°C under constant darkness and adults were fed on a 1:1 mixture of granulated sugar and powder milk at 30°C under 16h light: 8h darkness.

**Amplification of the conserved fragment of mdPH gene**

Two pharate adults of the house fly *M. domestica* were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated and retrotranscribed into cDNA using SuperScript™ III First-Strand synthesis system from Invitrogen. PCR primers (as shown in Table 4-1) were designed based on conserved sequences of the *PH* domain gene alignment in other insect species including *D. melanogaster*, *A. gambiae* and *A. aegypti*. After agarose gel electrophoresis, PCR bands were cut out from the gel, eluted using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), cloned into the pGEM-T-Easy Vector (Promega, Madison, WI) and sent for sequencing.

**Cloning of the mdPH gene using 3’ and 5’ RACEs**

To obtain a full-length *mdPH* cDNA, rapid amplification of cDNA ends (RACE) was performed using both a 5’ and a 3’ RACE system (Invitrogen, Carlsbad, CA). The
Forward primers:

CG32982 F1  5’- CGCTGGAAGGAGCGATACTT -3’
CG32982 F2  5’- CTGTTCTCCGATTCGGTGCC -3’
CG32982 F3  5’- AAACGCGGACTGCTCTGGCAGCA -3’

Reverse primers:

CG32982 R1  5’- AGCAGACCAATGGCGCTGTA -3’
CG32982 R2  5’- TGCGGCGTCGAGTGGTTCGT -3’
CG32982 R3  5’- GGCGTGTCCAGGCCACTATCA -3’

Table 4-1. PCR primers designed according to the conserved sequences of the PH gene in other insect species, including D. melanogaster, A. gambiae, A. aegypti, C. pipiens, T. castaneum and A. mellifera.
gene specific primers (GSPs) and the nested gene-specific primers (nested GSPs) used in the RACE protocol were designed based on the amplified *mdPH* gene fragment in *M. domestica*.

Total RNA was extracted from the newly emerged house fly (whole body) using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For the amplification of the 3’ end of the cDNA, the first-strand cDNA was synthesized using the manufacturer-supplied adapter primer. The supplied abridged universal amplification primer, the gene specific primer and the nested primers were used for subsequent amplification. For the amplification of the 5’ end of the cDNA, the gene specific primer was used for the first-strand cDNA synthesis. Two nested primers were used for subsequent amplification. The PCR products of 3’ and 5’ RACEs were gel purified, cloned into the pGEM-T-Easy Vector (Promega, Madison, WI) and sequenced at the DNA Core Facility of the University of Missouri.

**Amino acid sequence alignments and analysis**

The house fly *mdPH* amino acid sequence was deduced based on the cloned cDNA sequence, and sequence analysis was performed on the ExPASy server (www.expasy.ch) for prediction of functional domains, motifs, and signal peptides. Homology searches of the house fly *mdPH* protein were made on the NCBI platform (www.ncbi.nih.gov). Alignments of multiple sequences were carried out using the EBI ClustalW2 platform (www.ebi.ac.uk) and edited manually with GeneDoc software (http://www.nrbsc.org/downloads/gd322700.exe).
Analysis of the *mdPH* transcript after r-bursicon injection

To analyze the effect of r-bursicon on the transcription of the *mdPH* gene, newly emerged house fly adults were neck-ligated immediately after emergence. After a one hour waiting period, the neck-ligated flies that did not show any cuticle tanning were injected with r-bursicon protein (100 ng/fly) using a microinjection system. The burs α subunit alone dissolved in the same injection buffer (100 ng/fly) was used as a negative control. Injected flies were collected at different time periods after injection (20 min, 40 min, 1 h, 2 h, 3 h and 12 h) and immediately placed at -80°C.

Total RNA was isolated from the collected flies using the Trizol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I. The RNA concentration was determined by measuring the absorbance at 260 nm on a spectrophotometer. Total RNA (2 μg) from each sample was used for the first-strand cDNA synthesis as previously described. The synthesized cDNA was used as a template for analysis of gene transcription by real-time RT-PCR (qPCR). The primers designed for qPCR analysis of *mdPH* were: 5’-ATGCAATCTCTGTGAGCACGCCAGGACG-3’ (forward) and 5’-AGCAGCTGTAGGAGCGACGAT-3’ (reverse). The *rp49* gene (forward primer: 5’TACAGGCCCAAGATCGTGAA-3’; reverse primer: 5’-GACAATCTCCTTGCAGCTCTCT-3’) was used for normalization. The qPCR amplification and analysis were carried out on an Applied Biosystem 7300 Fast Real-time RT-PCR System using ABI SYBR Green Supermix buffer system. The final reaction volume was 20 μl. The thermal cycle conditions used in real time PCR were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min.
Developmental analysis of the \textit{mdPH} transcript

To analyze the transcription of the \textit{mdPH} gene in different developmental stages of the house fly, normal (non-ligated) flies were collected at different life stages (1\textsuperscript{st} instar larvae, 2\textsuperscript{nd} instar larvae, 3\textsuperscript{rd} instar larvae, early stage pupae (2 h after pupation), pharate adults, 0 h adults, 20 min adult, 40 min adult, 1 h adults, 2 h adults, and 3 h adults) and immediately placed at -80°C. Total RNA was extracted, quantified and samples were subjected to qPCR following the same procedures as previously described. The house fly \textit{rp49} gene was used for normalization.

Statistical analysis

Experiments of the real-time RT-PCR were carried out three times, and results were expressed as mean +/- SD. Differences between different time-points were evaluated with t-test.

4.4 Results

Amplification of a conserved fragment of the \textit{mdPH} gene in \textit{M. domestica}

Using the pair of primers CG32982 F3 and CG32982 R3 (Table 4-1), a 1 kb fragment was amplified from the house fly cDNA sample. After subsequent cloning and sequencing, a conserved fragment of the \textit{mdPH} gene containing 1013 bps was obtained from the house fly \textit{M. domestica} (Fig. 4-1). A homology search of the NCBI database indicated that this fragment is a \textit{M. domestica} homolog of the \textit{D. melanogaster pleckstrin homology} gene, with 82\% nucleotide identity.
Fig. 4. Amplified cDNA fragment of the *M. domestica* *mdPH* gene using 32982 F3 and 32982 R3 primers. Based on the amplified *mdPH* cDNA fragment, 3' and 5' RACE gene specific primers (GSP) and nested GSPs were designed, as indicated in green and red letters, respectively.

```plaintext
32982 F3
5'-AAACCGCGACTGCTCTGGCAGCAACGTCAGTGGTTTCAAGGTTAGATGAAAGATATTT
TTTGGTTTGGACACGTGAGACACCTCAGCTGTTTATTAAAGAGCAGCTACGGTCCAGCAATGAACGT
GCTCCCGACCTGAGCAATTATATTAGGTCAAATTGTTGATGTTGGAAAAAGTGGAAATGG
TTAAATCGTCGCTCCTACAGTGTATTGTTTACCTCTTGAGCTGGGATAGAGTTTTTATAC
GATGCAGAAGGGCCTCGAAGATTTGGTTCGAACCTGTGGGAGGAAATGTAACATTACCTCCAAG
GAACGTCTCAGCTGAGCCTGAAAAATAGCCCAACAGCTCCAGATCCCAGACCTCCTGGGCTCACC
CGTCTCAACATGCCTCACTGCAATGACCATTCTCGGTTAGTTAGTTAACCTATTCCAGTGCTTGG
GACGATTGGTTGATGACCAAGGCCAGTGTGATTGGTGTTGGACGTTGGCCTAAAACAGGGGA
CAAAACGATATGTTGTTTATGTTGTTAACAATTCATTCCTCCTCTCTCGAAGACTCTGTACCCGATCT
GAGTGCCTAAATATGCAAATACATAATCAATCATTAGTGGTTATGTCACCAATCATTAATCACCAGGC
AGAAGATACCCCTATTGCGCTCGGAGCCGCTGCAAACGCGATAACATACTGGTTACTCGAATGGTT
ACTCGTCGCAAGATTATCATGTTTCGAAATTGGCAGGTTTACGGTTACCCAGGCGGTATT
CATCAACAGCAAGTTTCGCTTGACCATCATTGTCAGCGAGGAGGCTGAGGAT-3'
32982 R3
```
Cloning and sequence analysis of the house fly \textit{mdPH} gene using 3’ and 5’ RACEs

Based on the identified fragment of the \textit{mdPH} gene, RACE GSPs and nested GSPs were designed using primer3 (V. 0.4.0) software. All designed primers are listed in Table 4-2, and their relative positions are indicated by colors and arrows (Fig. 4-1), with green letters indicating the 3’ RACE primers and red letters representing the 5’ RACE primers.

A full length sequence of the house fly \textit{mdPH} gene was obtained using 3’ and 5’ RACEs and the \textit{mdPH} amino acid sequence was deduced. As shown in Fig. 4-2, a full length \textit{mdPH} cDNA is 1790 nucleotides long, with an open reading frame (ORF) of 1623 bps. There are a 37-bp 5’ untranslated region and a 130-bp 3’ untranslated region following the ORF. The 3’ untranslated region contains two potential polyadenylation signal sequences (AAATAA, and AACAAA), which are located 16 nts and 42 nts downstream of the stop codon (TAA), respectively. The 1623-bp ORF encodes a 541-amino acid protein, the house fly \textit{mdPH}. Sequence analysis revealed that this protein has one predicted domain: the pleckstrin homology (PH) domain, spanning from a.a. 17 to a.a. 121.
3’-RACE

Primer 1: 5’- GCCAGCGGTAACAATAGTCG-3’ (GSP)
Primer 2: 5’- TCGAATTGCAATGGCTTGTA-3’ (Nested GSP)
Primer 3: 5’- CAGTTTCGGTTCGACTCACA-3’ (Nested GSP)

5’-RACE

Primer 1: 5’- AGCACTGTAGGAGCGACGAT-3’ (GSP)
Primer 2: 5’- GCTGGACCTGATGCTCTTTT-3’ (Nested GSP)
Primer 3: 5’- ACAACGATCAGGTGCTTG-3’ (Nested GSP)

Table 4-2. Gene specific primers (GSPs) and nested GSPs for RACEs designed according to the amplified mdPH fragment of *M. domestica* (Fig. 4-1).
Fig. 4-2. Full length cDNA and deduced amino acid sequences of the house fly mdPH. The red letters indicate the start codon and stop codon, and green letters indicate potential polyadenylation signal (AAATAA, AACAAA) sequences.
Multiple sequence alignments of mdPH with its homologs in other insect species

The house fly mdPH sequence is aligned with its homologs in several other insect species in which the PH homologous sequences have been identified, including *D. melanogaster*, *A. gambiae*, and *A. aegypti*. Alignment results showed that the house fly *M. domestica* mdPH has a 75% identity with *D. melanogaster*, 41% with *A. gambiae*, and 42% with its *A. aegypti* counterparts. Sequence similarity is 80%, 50% and 52%, respectively. The most conserved fragment is located in the PH domain, which is predicted critical for the function of this protein, as shown in Fig. 4-3.

**Real-time RT-PCR analysis of *M. domestica* mdPH transcript after r-bursicon injection**

After injection of the the r-bursicon heterodimer, the expression of the house fly mdPH transcript was decreased about 2-2.5 fold from as early as 40 min after injection, and this decrease was maintained until at least at 12 h after injection with little fluctuations (Fig. 4-4).

**Real-time RT-PCR analysis of mdPH transcript level at different developmental stages of the house fly**

The levels of the *mdPH* transcripts were examined in normal (non-ligated) house flies at different life stages using real-time RT-PCR. As shown in Fig. 4-5, the *mdPH* transcript is detected as early as in the first larval instar. The levels increase gradually with the development of the fly from larvae to pupae. A significant increase was noticed in the pharate adults and the newly emerged adults. Right after emergence, the level of the *mdPH* transcript decreases. Starting from 40 min, the level of *mdPH* is decreased ~2.5-3 fold and remains at about the same level thereafter.
Fig. 4-3. Multiple alignments of the mdPH amino acid sequence from *M. domestica* with its homologous proteins from other insect species. Mus, *M. domestica*; Dro, *D. melanogaster*; Ano, *A. gambiae*; Aed, *A. aegypti*. Numbers on the right side indicate the amino acid position of different sequences. Grey and black shading indicate the conservation of amino acids. Black shaded amino acids represent 100% conservation, while grey shaded amino acids represent 70-90% conservation. Alignments were performed using the ClustalW2 platform on www.ebi.ac.uk. Amino acids composing the predicted functional domain of the mdPH protein are from a.a. 17 to a.a. 121, as indicated by the red box.
Fig. 4-4. Real-time RT-PCR analysis of the temporal response of *mdPH* to r-bursicon injection. Newly emerged flies were neck-lighted immediately after emergence and then injected with 1 μl/fly of the r-bursicon heterodimer (100ng/μl). The control flies received 1μl/fly bursα only in the same injection buffer (100ng/ul). Total RNA was extracted from flies at different time periods (0 min, 20 min, 40 min, 1 h, 2 h, 3 h, and 12 h) after bursicon injection. *mdPH* mRNA levels were normalized to the expression of the house fly *rp49* gene. The data represent the mean ± SE of three biological samples. * indicates p-value statistically significant (p≤0.05) as compared to control (0 min).
Fig. 4-5. Quantification of the *mdPH* transcript levels in different developmental stages in the house fly, *M. domestica*. To analyze the transcript level of the *mdPH* gene in different house fly developmental stages, house fly whole bodies were collected at different life stages (1L, 1<sup>st</sup> instar larvae; 2L, 2<sup>nd</sup> instar larvae; 3L, 3<sup>rd</sup> instar larvae; EP, 2 hour early stage pupae; PA, pharate adults; 0A, 0 hour adults; 20mA, 20 minute adults; 40mA, 40 minute adults; 1hA, 1 hour adults; 2hA, 2 hour adults; 12hA, 12 hour adults). Total RNA was extracted, quantified, reversed transcribed into cDNA, and then subjected to real-time RT-PCR analysis. *mdPH* levels were normalized to the levels of the house fly *rp49* gene. The data represent the mean ± SE of three biological samples. * indicates p-value statistically significant (p≤0.05) as compared to control (0 A).
4.5 Discussion

This chapter reports the cloning and sequencing of the house fly *M. domestica* pleckstrin homology gene, *mdPH*, using 3’ and 5’RACE. Real time PCR analysis of *mdPH* in the neck-ligated house flies that received r-bursicon revealed that the level of the *mdPH* gene was down-regulated by ~2.5 fold starting from approximately 40 minutes after injection of r-bursicon into the flies. The repression was not reversed for the rest of the time points. Developmental study of *mdPH* in normal house flies showed that the level of *mdPH* transcripts is very low in larvae, increases in subsequent developmental stages, and reaches the maximal level right after emergence. The expression level of *mdPH* decreases by 2.5~3 fold 40 min after adult emergence. These results indicate that *mdPH* is regulated by bursicon during the bursicon-mediated cuticle sclerotization and/or the wing expansion process. The pleckstrin homology domain is known to be involved in signal transduction pathways such as G protein coupled receptor (GPCRs) signaling pathway by activating a G protein coupled receptor kinase-2 and repressing the activation of GRCR. It is possible that the down-regulation of the *mdPH* transcript levels unblocks the repression of G protein by *mdPH*, which then activate G protein coupled receptor pathway allowing a path of downstream signal transduction leading to normal sclerotization of cuticle and wing expansion processes. The increase of the *mdPH* transcripts level before adult emergence could indicate a need of mdPH and thus the need to repress the GPCR to prevent activation of downstream elements for the induction of bursicon-regulated cuticle sclerotization and wing expansion processes, because during that time period, cuticle sclerotization and wing expansion are not yet supposed to take place. Starting from 40 min after adult emergence, the level of *mdPH* starts to decrease.
dramatically, suggesting that the block of GPCR signal transduction pathway is no longer needed and bursicon-regulated cuticle sclerotization and wing expansion processes need to be initiated. The time difference between the adult emergence and the down-regulation of *mdPH* gene could be due to the time needed for bursicon release and for the action of bursicon on the mdPH protein to turn on the GPCR signaling pathway.

Although data suggest that *mdPH* is down-regulated by bursicon activity and mdPH is very possibly involved in regulating the signal transduction pathway downstream of bursicon, we are currently only speculating that it helps to regulate the activation/deactivation of the GPCR signaling pathway. More research is needed to further investigate the exact role of mdPH during the bursicon-regulated cuticle sclerotization and the wing expansion process.
CONCLUSIONS AND PERSPECTIVES

The advances in molecular biology in the recent years have made it possible for researchers to solve many problems that many scientists have been working on for a long time. This is also true for insect physiology. With the available genomic sequence in the fruit fly *D. melanogaster* (Adams *et al.*, 2000), the last uncharacterized important insect neurohormone, bursicon, was recently characterized and its sequences identified. Upon finding of bursicon genes, scientists are now able to either predict or clone bursicon’s homolog genes/sequences in other insect species. However finding of its genes and protein sequences is far from enough. The most important and valuable thing is to understand the mechanisms of action of the bursicon hormone. Now more and more researchers are interested in working on the signal transduction process of bursicon-mediated cuticle sclerotization and wing expansion, with the aim of elucidating bursicon’s mechanisms of action.

In the current study, bursicon genes were cloned in the house fly *M. domestica*, and analyzed for their transcriptional profiles by using RT-PCR and fluorescence in situ hybridization. More importantly, by expressing a functional house fly r-bursicon and using a well developed house fly neck-ligation assay, a series of genes were identified, which are up-/down-regulated by r-bursicon and are very likely involved in the bursicon-regulated cuticle sclerotization and wing expansion process. After analyses of these genes, two of them, *Su(H)* and *PH*, were selected for further study. By cloning and characterizing the *mdSu(H)* and *mdPH* genes of the house fly *M. domestica*, we
demonstrated two excellent signal transduction components that are very likely involved in the bursicon-regulated cuticle sclerotization and wing expansion process. The identification of these components may help future bursicon research with the aim of elucidating bursicon’s mechanisms of action.

As an extension of the current study, future study will probably involve one or several of the following directions: (1) further investigation of the two signal transduction components, mdSu(H) and mdPH, to find out their exact function during insect cuticle sclerotization and wing expansion using various techniques, including gene expression and functional analysis, and RNAi knock down, etc., (2) a proteomic study to identify the proteins that are up-/down-regulated by bursicon using r-bursicon and the fly neck-ligation assay, (3) since the signal transduction process usually involves protein phosphorylation, it might be possible to identify protein phosphorylation upon bursicon stimulation, (4) the recent identification of bursicon homologous sequences in non-insect species, especially the echinoderm, the purple sea urchin may indicate that along with being a hormone regulating insect cuticle sclerotization and wing expansion, bursicon may also have other functions. It might be very interesting to find out the additional functions that bursicon has in other organisms, and (5) although bursicon transcripts and proteins have been localized in the insect neurosystem, it is still not clear where mature bursicon proteins are assembled, stored and released, and how these are controlled. Detailed studies are needed to address these questions.
REFERENCES


Schweisguth, F. (1995). Suppressor of Hairless is required for signal reception during...


APPENDIX 1: Bursicon-regulated genes identified by using DNA microarray in the fruit fly *D. melanogaster*.

(From An, S., Wang, S., Gilbert, L., Beersntsen, B., Ellersieck, M., Song, Q., 2008a).

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<th>Fold 3h</th>
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</tbody>
</table>

The genes in bold were detected at both 1 and 3 h post-r-bursicon injection. The name in parenthesis indicates a functional domain.

Ng: gene with no known function associated with it.
APPENDIX 2: RNAi of bursicon in the house fly *M. domestica*

Recently, it was shown that the injection of bursicon double-strand RNA (dsRNA) into the pupae of the silkworm, *B. mori*, caused the RNA interference (RNAi) and subsequent bursicon gene silencing, and resulted in a deficit in the wing expansion process (Huang et al., 2007). In an attempt to identify bursicon regulated genes, RNAi was tried in the house fly *M. domestica*.

**Experimental procedure**

**Synthesis of dsRNA**

CNSs were dissected from the house fly under Ringers’ solution at 4°C and stored at -80°C for RNA extraction. Total RNA was extracted from the CNSs using the Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was treated with DNase I (Promega, Madison, WI) to remove genomic DNA. First-strand cDNA was synthesized using the SuperScript™ First-Strand synthesis kit (Invitrogen, Carlsbad, CA). Full length *burs α* and *burs β* cDNAs were amplified by PCR using designed primers with the *BamH*1 and the *Xho*1 restriction sites as indicated by an underline: *burs α* 5’-*GGA TTC ATG GAA GTT TCA GTT TTT CG-3’* (forward) and 5’-*CTC GAG TTA CTG CAA TGC TAT CCT TC-3’* (reverse); *burs β* 5’-*GGA TTC ATG CTT AAA TTG TGG AAA TT-3’* (forward) and 5’-*CTC GAG TTA TCT CGT GAA ATC ACC AC-3’* (reverse). The PCR products of *burs α* and *burs β* were individually inserted into the pGEM-T-Easy vectors (Promega, Madison, WI). The pGEM-T-Easy vectors containing either *burs α* or *burs β* gene with desired orientation were then linearized with appropriate enzymes and used for
transcription with SP6 and T7 RNA polymerases (Invitrogen, Carlsbad, CA) to synthesize
double strand RNA (dsRNA). The synthesized product was then treated with DNase I and
RNase for 1 h at 37 °C. The dsRNA was added to 200 ul PBS buffer and checked on a
1.5 % agarose gel to ensure successful formation of double strand and to quantify the
concentration of the synthesized dsRNA. No control dsRNA was synthesized.

**Injection of dsRNA**

Two microlitres (about 5 ug) of dsRNA were injected into house fly third instar
larvae, prepupae (white immobilized pupae that just started pupation process) and early
stage pupae (pupae showed red sclerotization in puparium) using a micro-injector. In total,
120 flies (40 in each of the 3 stages) were injected with *burs α* dsRNA, and 120 flies (40
in each of the 3 stages) were injected with *burs β* dsRNA. 120 control flies (40 in each of
the 3 stages) received PBS buffer only. Flies were then allowed to mature into adults and
observed for adult cuticle sclerotization and wing expansion.

**Results**

All flies that received either *burs α* or *burs β* dsRNA showed normal
post-eclosion cuticle sclerotization and wing expansion activity. The time needed to
complete the cuticle sclerotization and wing expansion in the treatment house flies has no
difference when compared to the control house flies.

**Discussion**

Systemic RNAi so far has not been reported to work in either *D. melanogaster* or
*M. domestica*, so injection of bursicon dsRNA into larvae or pupae of the house fly is probably not going to induce bursicon gene silencing and subsequent defects in cuticle sclerotization and wing expansion.
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