CHARACTERIZATION OF A HAEMOLYMPH PROTEIN HP19
AND ITS POSSIBLE ROLE IN NONGENOMIC ACTIONS OF
20-HYDROXYECYDYSONE DURING THE POSTEMBRYONIC
DEVELOPMENT OF RICE MOTH, CORCYRA CEPHALONICA

Synopsis of the thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

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Layout of the thesis-

The thesis deals with the appearance of a stage and tissue specifically regulated haemolymph protein HP19, identified and characterized during this study. The thesis is written with a general introduction and methods used followed by results. The results section is divided into five chapters, indicating the stepwise approach adopted to carry out the study. Finally a comprehensive discussion and few hypothetical models on the basis of the findings of the study are discussed.

The results section which is divided into five chapters is as follows:

Chapter I- Identification, isolation and characterization of HP19 in Corcyra cephalonica

Chapter II- Cloning, sequencing and molecular characterization of HP19

Chapter III- Role of HP19 during the postembryonic development

Chapter IV- Effect of HP19 on 20-hydroxyecdysone dependent actions

Chapter V- Mechanism of 20-hydroxyecdysone regulated actions that is mediated by HP19

Introduction-

Postembryonic development in insects involves growth, molting and metamorphosis. These events are controlled by the hormones that are mainly secreted by the brain, corpora cardiaca, corpora allata and prothoracic glands. Further, the roles of these hormones have been widely studied during the postembryonic development especially in the holometabolous insect resulting in vast literature. However, knowledge regarding the regulation of various hormone mediated actions during the postembryonic development of insects is limited and the field remains largely unexplored.

According to the basic model of the endocrine control, the neurosecretory cells in brain in response to appropriate stimuli synthesize and secrete various neuropeptides such as allatotrophic and prothoracicotropic hormone, which in turn stimulate the corpora allata and prothoracic glands to synthesize and secrete the morphogenetic hormones namely juvenile
hormones (JHs- sesquiterpenes) and ecdysteroids (a group of steroid hormones) respectively. The relative titre and interplay of these hormones orchestrates the progression of one developmental stage to the next. The ecdysteroids regulate the onset and the timing of the molt, whereas JHs regulate the quality of the molt (Gilbert et al., 1996).

The haemolymph ecdysteroid titre at any given stage is reflection of several metabolic processes. The alteration in haemolymph ecdysteroid titre is responsible for eliciting the change in commitment. Hence, the ecdysteroids are necessary for metamorphosis as well as for the regulation of critical sequence of behavioral, physiological and biochemical changes which takes place in insects. Extensive studies have been carried out on the regulation of ecdysteroid titre at the synthesis level. However, studies on other regulatory mechanisms are very limited and one of the major thrust area open for research. In lepidopterans like in most holometabolous insects, the ecdysteroid that can elicit molting and metamorphosis is 20-hydroxyecdysone (20E), the active form of prohormone ecdysone (Karlson, 1996).

Steroid hormones mostly propose to act through its interaction with specific intracellular receptor. These receptors are DNA-binding proteins of the nuclear receptor superfamily. The steroid-receptor complex binds to hormone-responsive elements on the chromatin and regulates gene transcription and translation. This mode of cellular action is generally referred to as a genomic action (Beato and Klug, 2000). Ashburner et al., (1974) proposed similar genomic mode of action for 20E. Extensive studies based on this model, over the past few decades have provided insight into the molecular mechanism of 20E action. From these studies has emerged, a clearer understanding of the mechanism by which a systemic hormonal signal is refined into stage and tissue specific developmental responses.

Apart from the traditional genomic mode of action of steroid hormones there are several reports that suggest rapid nongenomic effect of steroids and which acts independently of gene transcription or translation. Unlike genomic effect the nongenomic effect is rapid and the response is apparent within few seconds to 10 minutes. Further, this response is unaffected by the transcriptional or translational inhibitors (Losel and Wehling, 2003).
A major area with regard to understanding the regulation of 20E action is that of tissue specificity. The hormone has different effects on the same tissue at different developmental stages. Thus, studies focused on molecular basis of differentiation during development and built-in regulatory mechanisms at the tissue level largely aid in understanding the regulation of 20E action and such studies call for immediate attention. The fat body in insects is an important metabolic centre and can be compared to the vertebrate liver, in view of the complex functions performed by it. The tissue is structurally organized to provide maximal exposure to the haemolymph and because of its changing metabolic role & integral position in maintaining metabolic homeostasis, the fat body serves with increasing frequency as a model for examining endocrine regulation (Kunkel, 1981; Riddiford et al., 2001; Hansen et al., 2002).

Extensive studies have revealed that 20E stimulates synthesis of various proteins in different tissues during postembryonic development of holometabolous insects. In lepidopteran insects the uptake of hexamerins by fat body (Kirankumar et al., 1997, 1998) as well as the male accessory reproductive gland (Ismail and Dutta-Gupta, 1990, 1991; Dutta-Gupta and Ismail, 1992; Ismail et al., 1993) is also shown to be regulated by ecdysteroids. Detailed studies have shown that the lysosomal activity in the whole body as well in the fat body exhibits a definite pattern during postembryonic and adult development (Ashok and Dutta-Gupta, 1988).

As insects do not feed during pupal stage, they depend on nutrients that have previously accumulated in the larval period. In all holometabolous insects investigated so far, amino acids and energy are supplied by proteins that have been selectively taken up by the fat body from the haemolymph (Haunerland, 1996; Burmester and Scheller, 1999). Most of the incorporated proteins belong to the family of hexamerins named according to their composition of six identical or closely related subunits (Telfer and Kunkel, 1991). The hexamerins are synthesized mainly by the fat body of actively feeding larval stage and are sequestered back into the fat bodies of prepupal and non-feeding pupal stage. This sequestration or uptake of hexamerins is a receptor mediated endocytotic process. The uptake is unique relative to the endocytosis process of eukaryotes because the hexamerin receptor does not belong to the low density lipoprotein (LDL) superfamily (Burmester and Scheller, 1999).
The hexamerin receptors have been recognized in dipteran as well as lepidopteran insects and ecdysteroids have been suggested to activate the receptor for hexamerin uptake (Ueno and Natori, 1984; Wang and Haunerland, 1993, 1994; Chung et al., 1995; Burmester and Scheller, 1997, 1999). Using ligand binding studies, our laboratory has earlier demonstrated the presence of 120 kDa hexamerin receptor in the fat body membrane of rice moth, *Corcyra cephalonica* (Kirankumar et al., 1997). The receptor was found to be present in the last larval instar, and - at maximal concentration - in the prepupal stage. The sequestration of hexamerin in *C. cephalonica*, like in other lepidopteran insects, was not observed during the larval stage (Ismail and Dutta-Gupta, 1990). However, 20E treatment induced a precocious uptake of hexamers in the late-last instar (LLI) larval fat body (Ismail and Dutta-Gupta, 1990). These studies suggest that ecdysteroid hormone activates the hexamerin receptor prior to hexamerin uptake.

As mentioned, metamorphosis in insects is the transition from the larval to the adult stage and the events are controlled by ecdysteroid hormones (Riddiford et al., 2001; Trumann and Riddiford, 2002; Gilbert et al., 2002). It involves the breakdown of larval structures and the formation of new tissues. The former occurs either by the apoptosis of individual cells or autophagy of group of cells (Thummel, 2001). As a part of cell remodeling during metamorphosis, acidic autophagic vacuoles accumulate in the fat body cell and the activity of several lysosomal enzymes such as acid phosphatases increase and cause the lysis of larval tissues (Sass and Kovacs, 1980; Verkuil, 1980; Thummel, 2001; Lee and Baehriecke, 2001). The fat body that fills a large fraction of the insect body shows high activity of lysosomal enzymes (Hansen et al., 2002). The lysosomal enzymes play an important role in histolysis of larval organs, cellular destruction, tissue remodeling, and reorganization. Acid phosphatase (ACP) (EC 3.1.3.2) is one of the commonly used marker enzyme to study the lysosomal activity in insect and is found in every organism studied to date. The ACP exists in multiple forms and different isoymes. The stimulation of the lysosomal activity by ecdysteroids is well demonstrated in several insects including *C. cephalonica* and the increase in the lysosomal activity is governed by the elevation of 20E levels (Verkuil, et al., 1979a; Verkuil, 1980; Ashok and Dutta-Gupta, 1988; Sass et al., 1989; Kutuzova et al., 1991). Some data also provide evidence that the induction in lysosomal activity by ecdysteroids is governed at a nongenonomic level (Verkuil, 1979b). However, no clear evidence has been provided to support the hypothesis. In the present study an attempt has been made to understand the activation of acid phosphatase by 20E in the fat body cells of the rice moth, *C. cephalonica*. Apart from the
lysosomal acid phosphatases there exists another acid phosphatase, the phosphatidic acid phosphatases or PAPs (3.1.3.4). The PAPs are of type-1 PAP (PAP1) and type-2 PAP (PAP2). The PAP1 (38-43 kDa) is the cytosolic or membrane bound acid phosphatase and is involved in the supply of diacylglycerols in the classical pathway of glycerolipid biosynthesis by dephosphorylating phosphatidic acid phosphatase whereas the PAP2 (35-50 kDa) is membrane bound and is involved in signal transduction that is mediated by phospholipase D (Kai et al., 1996).

Literature search suggests that 20E not only exerts its action through the modulation of transcription but also by its action at the post-translational level e.g., by protein phosphorylation (Itoh et al., 1985; Sass, 1988). Based on the earlier studies from our laboratory on the effects of 20E, CaM kinase II, hexamerin receptor and tyrosine kinase were identified as the marker proteins for the present study. These markers offered a system to find out if their phosphorylation/activity was regulated by 20E and was there an alteration in the phosphorylation status/activity of these proteins in the presence of haemolymph protein HP19.

**Why present study?**

The present interest to characterize haemolymph protein (HP) in order to understand the regulation of ecdysteroid action in insects rose from the repeated interesting observation that injection of exogenous 20E stimulated the lysosomal activity in the fat body of the thorax-ligated larvae of *C. cephalonica* (Ashok and Dutta-Gupta, 1988). However, addition of 20E to larval fat body cultures failed to stimulate the enzyme activity. Studies conducted earlier in *Manduca sexta* also revealed that the ACP activity was not induced in response to exogenous 20E alone but the reason was elusive (Caglayan, 1990). However, Ashok and Dutta-Gupta (1991) for the first time reported a significant stimulation in the fat body acid phosphatase activity, when haemolymph from larval stage of *C. cephalonica* was added to the fat body cultures in the presence of 20E. This suggested that the hormone might require the presence of some additional factors under *in vitro* condition, which are present in the *in vivo* system to mediate the action, on lysosomal activity. Therefore an attempt was made in the present study to analyze the possible role of haemolymph factor(s) if any, in mediating 20E action on selected marker proteins. Thus, a protein in the haemolymph named **HP19 i.e. haemolymph protein of mass 19 kDa** was identified as a possible molecule in regulating few of the 20E mediated actions in some of the lepidopteran insect species.
Objectives of the present study-

Postembryonic development in insects involves growth, molting and metamorphosis. These events are controlled mainly by two morphogenetic hormones namely ecdysteroids and juvenile hormones. There is vast information about the regulation of these hormones at synthesis as well as at its titer level. The information also exists about the interaction of these hormones among themselves to regulate metamorphosis. However, knowledge regarding the regulation of hormone dependent actions is limited and the field remains largely unexplored. In the present study, an attempt has been made to understand this important aspect of the molting hormone, ecdysteroids. The study discusses the appearance of a stage and tissue specifically regulated protein HP19. This protein is identified in the present study and is found to be responsible for the regulation of few of the 20-hydroxyecdysone dependent actions.

The specific objectives of the dissertation work are given below:

- Confirmation of the presence of factor in haemolymph required for 20E stimulation of acid phosphatase.
- Understand the nature of the haemolymph factor, its isolation, characterization and developmental regulation.
- Molecular characterization of HP19.
- Role of HP19 in postembryonic development of Corcyra cephalonica.
- Effect of HP19 on 20-hydroxyecdysone dependent actions.
- Mechanism of 20-hydroxyecdysone regulated actions that is mediated by HP19.
Methodologies-

Some of the methods adopted to achieve the above mentioned objectives are given below-

- Denaturing PAGE - Laemmli (1970)
- Non denaturing PAGE - Burmester et al., (1999)
- In vitro phosphorylation - Combest & Gilbert (1986); Shanavas et al., (1998)
- Back phosphorylation - Forn and Greengard (1975)
- Purification of IgG - Protein A-affinity column
- Immunoprecipitation - Protein A-Sepharose beads
- Histochemical studies - Standard procedure
- Immunohistochemical studies - Meltzer et al., (1997)
- In vivo immobilization of proteins- Del pine et al., (1998)
- Western Hybridization - Towbin et al., (1979) and BCIP/NBT detection
- Southern Hybridization - Sambrook et al., (1989)
- Northern Hybridization - Sambrook et al., (1989)
- cDNA library construction - Clontech and Stratagene manual
- Immunoscreening - Clontech manual
- Hybridization screening - Clontech manual
- Acid phosphatase assay - Henrickson and Clever (1972)
- Tyrosine kinase assay - Casnellie et al., (1982)
- CaM kinase II assay - Fukunaga et al., (1989)

Results and Discussion-

Chapter I - Identification, isolation and characterization of HP19 in Corcyra cephalonica

Earlier studies from our laboratory revealed that 20-hydroxyecdysone (20E) under in vivo condition stimulates the fat body acid phosphatase (ACP) activity in Spodoptera litura (Sridevi et al., 1987) and Corcyra cephalonica (Ashok and Dutta-Gupta, 1988), but fat body cultures require factor or factors from haemolymph to show the stimulatory effect on enzyme activity (Ashok and Dutta-Gupta, 1991). In the first part of my doctoral study, I confirmed the
presence of factor required by the 20E to stimulate the fat body ACP activity of rice moth, *Corcyra cephalonica*. Different pretreatments of haemolymph prior to its addition to the fat body culture rendered the haemolymph impotent in mediating the 20E regulated ACP activity, suggesting the factor to be proteinaceous in nature.

In the attempts to purify the protein, the total haemolymph protein was fractionated on Sephadex G-50 and thus the active fraction was found to be a protein of ~22 kDa calculated from the elution profile, or 19 kDa, calculated from the mobility on SDS-PAGE. On the basis of these results the purification of the active haemolymph protein was carried out, first by fractionating the total haemolymph protein using 30 kDa cut-off membrane filters followed by gel filtration chromatography. Thus, a contaminant free pure polypeptide band of 19 kDa was identified that mediated the 20E regulated ACP activity and was named as **HP19**. Starting with 50 mg total haemolymph protein, we obtained a 98.5-fold purification with 0.05% yield.

Although nanogram of the protein was found to be sufficient for mediation even in crude or partially purified fractions, the yield of the purified protein was very low. Other limitations in purification were the requirement of large amounts of haemolymph of a specific developmental stage (LLI) and the removal of the major contaminating protein, hexamerin, that constitutes 75-80% of total haemolymph proteins (Haunerland, 1996). Therefore, an antibody against HP19 was raised by electro-eluting HP19 and was confirmed to be specific against HP19. When this antibody was added together with haemolymph to cultures of LLI fat body, the haemolymph failed to mediate the 20E dependent action. Similarly, when the haemolymph was first immunoprecipitated and the resulting complex and the supernatant was added to the culture, HP19 action was suppressed.

Western analysis of denatured as well as of non-denatured PAGE demonstrated that HP19 is a monomeric protein without any subunits in *C. cephalonica* and is probably the product of a single copy gene. Four independent methods, co-culturing, western analysis, immuno-histochemistry, and *in situ* immuno-staining, revealed that HP19 is synthesized by the hind gut associated lobular fat body (HGLFB) from where it is released into the haemolymph. This is further confirmed by the tissue specific gene expression only in HGLFB. Western analysis evidences a difference of ~5 kDa in the mass of HP19 in HGLFB (i.e 24 kDa) and in haemolymph (19 kDa). The predicted mass of the unmodified translated HP19 cDNA was close to the HP19 synthesized in HGLFB.

The results indicate that the biosynthesis of HP19 takes place in the HGLFB during the total last larval instar *i.e* the early-last (ELI), mid-last (MLI) and late-last instar (LLI) stage.
The protein is rapidly released into the haemolymph. The maximal HP19 concentration in the tissue as well as in the haemolymph could be observed in LLI. It is notable that only haemolymph from this LLI developmental stage is capable of mediating the 20E effect on ACP activity. Hence, the activity of HP19 is developmentally regulated, although the molecular mechanism of activation of ACP by ecdysteroid hormone is unclear at this point.

Apart from *C. cephalonica*, similar HP19 like protein was found in few other lepidopteran insects investigated but could not be detected in a diptera, *Calliphora vicina*. However, western analysis revealed that the mass of the protein in these lepidopteran species was slightly different and some of them had additional subunits. The studies, clearly suggest that HP19 is not species specific at least for lepidopteran insects but is definitely stage specific in its activity.

**Chapter II- Cloning, sequencing and molecular characterization of HP19**

To get more insight into the nature and function of HP19, we produced and characterized *C. cephalonica* HP19 cDNA (CcHP19). For the identification of the cDNA encoding the HP19 protein, a cDNA expression library, prepared from the RNA of HGLFB of LLI larva was immunoscreened using polyclonal HP19 antibody. This HP19 cDNA was 634 nucleotides long, with an open reading frame of 585 bp, which encodes a protein of 195 amino acids. The calculated molecular mass of the translated unmodified protein was 22.95 kDa, which is close to the mass of HP19 detected in HGLFB, a tissue that synthesizes the protein. The polypeptide comprises 12.3% basic (9 arg, 1 his and 14 lys) and 13.3% the acidic residues (10 asp, 16 glu). The estimated isoelectric point (pI) is 5.36. There is no cys residue in the sequence. The cDNA sequence begins with the methionine start codon at position 1 and translation stop codon at 586. A 3’ untranslated sequence containing a polyadenylation signal AATAAA is located at 588 nucleotide followed by a poly (A) tract. The polyadenylation signal overlapped the translation stop codon TAA by one base.

The amino acid sequence deduced from HP19 cDNA did not show a typical signal peptide necessary for transmembrane transport (Von Heijne, 1986.), probably due to the lack of any hydrophobic sequence (Feng *et al.*, 1999). Therefore, we conclude that might be a cleavage occurs before the release into the haemolymph. The presence of two putative N-glycosylation sites (Asn51-Arg52-Thr53-Leu54 and Asn116-Glu117-Thr118-Ala119) indicates that the protein is secreted from the synthesizing cells.
The comparison of the *C. cephalonica* HP19 (CcHP19) cDNA with the sequences in GenBank, showed 67% identity with *Choristoneura fumiferana* GST (CfGST) (Feng et al., 1999). Similarities with other invertebrate GST were found to be less than 38%. Although the CcHP19 cDNA sequence revealed 67% identity with CfGST, affinity purified GST from *C. cephalonica* had no enhancing effect on the 20E dependent ACP activity when compared with purified HP19 or recombinant HP19. Further, the haemolymph as well as the purified HP19 had negligible GST activity. It was also found while purification of GST by affinity chromatography, the protein fractions that did not bind to glutathione affinity matrix enhanced the 20E dependent ACP activity, again suggesting that it is not a GST molecule.

**Chapter III- Role of HP19 during the postembryonic development**

In order to understand the role of HP19 in insect growth and development during the postembryonic life of *Corcyra cephalonica*, the protein was *in vivo* immuno-complexed and rendered unavailable for mediation of 20E dependent actions. This was done by injecting HP19 antibody to final (Vth) instar larvae, an approach frequently used to understand the physiological processes (Hiraoka and Hayakawa, 1990; Del Pino et al., 1998). This injection of HP19 antibody interfered with the proteins physiological action and led to the growth of either nonviable larval-pupal or pupal-adult intermediates as compared to controls where the larvae grew normally and gave a normal healthy adult. Further analysis on various parameters suggested that compared to controls the antibody injected larvae had several alterations. Although the difference in % mortality was not significant when compared with controls but other changes were significant. The antibody injected larvae, showed reduced salivation, delayed reduction in body length and head capsule size. The time of pupation though was normal but most of the pupae that developed were abnormal, non-viable larval-pupal intermediates and some of the larvae which could metamorphose into pupae finally gave rise to non-viable pupal-adult intermediates. The ACP activity profile demonstrated gradual increase in the activity of control sets of fat body collected after different days of post-injection corroborating with the reported developmental profile of ACP (Dutta-Gupta and Ashok, 1988). However antibody injected insects had almost static fat body ACP activity after different days of post injection. This suggested that the acid phosphatase has role in insect growth and development by possibly regulating the histolysis of larval organs and HP19 plays role in the regulation of ACP activity.
The protein profile of fat body and haemolymph revealed that HP19 not only regulates ACP activity but also has a role in regulating hexamerin sequestration. Hexamerins are high molecular weight multimeric proteins synthesized by actively feeding larval fat body cells and released into haemolymph. These hexamerins are later sequestered back via a receptor mediated endocytosis process by the non-feeding pupal fat body cells to meet its energy requirement (Haunerland, 1996). In the present study, HP19, although did not interfere with the synthesis of hexamerins but as compared to control the antibody injected larvae showed no sequestration of hexamerins after 14 days of post injection. Immunohistochemical staining of the fat body tissue sections collected after different days of post-injection further confirmed that HP19 antibody injected insects had improper sequestration confirming the hypothesis that HP19 not only has effect on ACP activity but also regulates hexamerin sequestration. The macroscopic view of the whole mount fat body demonstrated that in HP19 antibody injected sets, there was a distinct change in the destruction and tissue remodeling as compared to controls further confirming the hypothesis of a possible role of HP19 in regulating insect growth and development.

Chapter IV- Effect of HP19 on 20-hydroxyecdysone regulated actions

Studies from our laboratory have revealed that in addition to the increase in lysosomal activity in the whole body as well as in the fat body by 20E, it also stimulates the synthesis of various proteins in different tissues during the postembryonic development of the lepidopteran insects (Ray et al., 1987 a,b; Sridevi et al., 1988, 1989; Ismail and Dutta-Gupta 1990; Shanavas et al., 1996). The uptake or sequestration of storage proteins (hexamerins) by the fat body (KiranKumar et al., 1997) as well as the male accessory reproductive gland (Ismail and Dutta-Gupta, 1990, 1991; Dutta-Gupta and Ismail, 1992; Ismail et al., 1993) was also shown to be regulated by ecdysteroids. Earlier studies in dipteran insects suggested a post-translational activation of hexamerin receptor by 20E. Studies from our laboratory suggested that hexamerin receptor undergoes protein phosphorylation that is regulated by 20E.

In the present study an attempt was made to understand some of the 20E regulated actions and effect of HP19 on them. For this three proteins namely hexamerin receptor, tyrosine kinase and CaM kinase II were identified as the potential markers. In addition to this attempt was also made to understand the protein kinase C (PKC) regulation.
A. Effect on hexamerin receptor phosphorylation- Previous studies from our laboratory suggests that the 120 kDa hexamerin binding protein in the fat body cell membranes of *C. cephalonica* is activated by ecdysteroid hormone (Kirankumar *et al.*, 1997). The study further suggested the post-translational modification of the receptor by protein phosphorylation (Kirankumar, 1998). In the present study attempt was made to understand the role of 20E in the phosphorylation of 120 kDa hexamerin receptor. The results suggested that 20E stimulates the phosphorylation of 120 kDa hexamerin binding protein that has been demonstrated to represent the receptor. The 20E stimulated phosphorylation is mediated partly by a tyrosine kinase as monoclonal phosphotyrosine antibodies cross-react with the receptor and phosphorylation is blocked partly by genistein. The receptor phosphorylation is developmentally regulated. Back phosphorylation study provides additional evidence for 20E regulation of hexamerin receptor phosphorylation in intact fat body. Phosphorylation of the receptor was found to be essential prerequisite for hexamerin uptake. Since this 20E stimulated phosphorylation of hexamerin receptor occurs in intact tissue, membrane as well as in the fat body homogenates and the uptake of hexamerin by the phosphorylated receptor is unaffected by the inhibitors of transcription and translation, suggest that the activation of hexamerin receptor for the uptake occurs at a nongenomic level and is unaccompanied with the increase of transcript or protein synthesis.

In addition to this the hexamerin receptor was also identified by ligand binding studies in another hexamerin sequestering tissue *i.e.*, male accessory reproductive glands which was found to undergo phosphorylation like the receptor present in the fat body. The phosphorylation of the hexamerin receptor was found to be inhibited by HP19.

B. Effect on tyrosine kinase activity- As discussed above that the phosphorylation of 120 kDa hexamerin receptor is mediated partly by a tyrosine kinase and the phosphorylated hexamerin receptor was found to be essential prerequisite for activation followed by hexamerin uptake. Since ATP is cell impermeable hence the phosphorylation of hexamerin receptor in cultured fat body followed by increased uptake of $[^{35}\text{S}]$ methionine labeled hexamerin suggested that the hexamerin receptor is probably a cell surface receptor with intrinsic kinase activity. Hence we checked the *in vitro* uptake of hexamerins by carrying the phosphorylation of hexamerin receptor with $[\gamma^{32}\text{P}]$ ATP. The results revealed 20E induced phosphorylation of 120 kDa hexamerin receptor in cultured fat body. The immunohistochemical staining of the 20E supplemented cultured fat body using monoclonal anti-phosphotyrosine antibody indicated the
presence of the phosphorylated tyrosine residue in the fat body cells. We extended the study to check if the hexamerin receptor phosphorylation is mediated by receptor tyrosine kinase (RTK). For this we used a series of RTKs inhibitor tyrphostins (AG9, AG490, AG94, AG528 & AG879) but only one of the inhibitor AG879 at high concentration (100 µM) inhibited the phosphorylation of hexamerin receptor. This suggests that probably a RTK mediates the phosphorylation of hexamerin receptor. However, the result is inconclusive to understand if the hexamerin receptor is a cell surface receptor with intrinsic kinase activity.

The study indicated that tyrosine kinase is present in the fat body, which is developmentally regulated. Fat body tyrosine kinase activity study with a synthetic peptide as substrate indicated that the LLI stage has highest tyrosine kinase activity and ligation reduces the activity indicating the hormonal dependence. The 20E was found to induce the fat body tyrosine kinase activity both in vivo and cultured fat body. The fat body tyrosine kinase activity was inhibited by the presence of HP19 both in cultured fat body as well as in the homogenate. In addition to this, the study also revealed a tyrosine kinase mediated phosphorylation of 48 kDa protein. The effect of HP19 on the phosphorylation of 48 kDa phosphorylation was not carried out as it was found to be independent of 20E and dependent on juvenile hormone (JH).

C. Effect on CaM kinase II activity and autophosphorylation- Earlier studies from our laboratory revealed that the CaM kinase II activity and its autophosphorylation in the CNS and fat body is developmentally regulated (Shanavas et al., 1998; Vasanthi, 1999). In the present study, the results indicate that CaM Kinase II is developmentally regulated with highest activity at LLI stage. The activity as well as autophosphorylation is 20E dependent. Addition of 20E to ELI and LLI ligated larva stimulates the CaM kinase II activity which is inhibited by HP19. Similarly 20E induces the autophosphorylation of CaM Kinase II, but is inhibited by HP19.

D. Effect on protein kinase C (PKC)- In order to understand the effect of HP19 on other 20E regulated actions the study was extended on PKC regulation in the fat body of C. cephalonica. The results indicated that although thorax-ligation shows reduction in PKC activity but the activity was highest during ELI stage and gradually decreased at the LLI and pre-pupal stage. This indicate that PKC activity is possibly JH dependent and not on 20E. This was confirmed with the in vivo injection of 20E to the thorax-ligated larvae that showed no stimulation in the
fat body PKC activity. Since the PKC activity was unaffected by 20E, it was not considered worthwhile to check the effect of HP19 on the fat body PKC activity.

These studies on the effect of HP19 on 20E mediated action such as stimulation of ACP activity, phosphorylation of hexamerin receptor, tyrosine kinase activity and CaM kinase II activity and its autophosphorylation in the fat body of *C. cephalonica* suggested that HP19 most likely has either a kinase inhibitory/phosphatase activating effect. This however remains to be studied. Present study suggests that HP19 has a regulatory role on the 20E stimulated ACP activity, phosphorylation of hexamerin receptor and the kinases (tyrosine kinase and CaM kinase II).

**Chapter V- Mechanism of 20-hydroxyecdysone regulated actions that is mediated by HP19**

A wealth of data on the molecular mechanism of ecdysteroid action shows that the transcriptional cascade leading to molting and metamorphosis is initiated when 20E binds to its nuclear receptor (Beato and Klug, 2000). For about four decades evidence has accumulated that some of the hormonally induced effects seemed to be too rapid for the classical model (Falkenstein *et al.*, 2000; Losel and Wehling, 2003). This evidence casts doubt on the so called genomic pathway as the sole mode of steroid action. To date, several modes for nongenomic steroid actions are being examined. Most of them are thought to continuously modulate the long-term program allowing cells or organs to adapt rapidly to environmental changes. Numerous experiments with a huge number of different species display that insect metamorphosis in general is under the genetic control of ecdysteroids. Studies also indicate that some events, necessary for and accompanying metamorphosis, are controlled by 20E at a nongenomic level. However, studies on these mechanisms are restricted to a small number of experimental systems e.g. the activation of lysosomal enzymes and the hexamerin receptor.

**A. Nongenomic regulation of acid phosphatase activity**-

To learn more about the molecular mechanisms of ecdysteroid in regulating the ACP activity assisted by HP19, the fat bodies were incubated for several time periods with HP19 and 20E. A minimum incubation of 4 h was essential for the stimulation in the enzyme activity by 20E; time enough for a genomic hormone action. However, the measured
stimulation was unaffected by transcriptional or translational inhibitors, indicating the independence of gene activation. Furthermore, the in vitro study with fat body homogenate showed a rapid stimulation (within seconds to 1 min) of the enzyme activity. Since the homogenate preparation is essentially a cell- or nucleus disintegrated fraction, this suggests that cell/nuclear integrity is not an essential requirement for the effect of 20E on ACP activity. This possible nongenomic regulation was further strengthened by the results that in the presence of 20E, fat body cultures showed a higher incorporation rate of $[^{35}\text{S}]$ methionine which was inhibited by transcriptional or translational inhibitors. This, however, had no effect on the ACP activity and the presence of HP19 in the tissue culture rendered 20E to stimulate the enzyme activity even in the presence of inhibitors of transcription and translation.

After confirming the regulation of ACP activity by 20E in presence of HP19 at protein level, the study was extended to understand the regulation at RNA level. For this, the approach was taken to clone the ACP of C. cephalonica. Incidentally while screening for the hexamerin cDNA from the fat body expression library using polyclonal antibody against C. cephalonica hexamers, one of the false positive showed sequence homology with phosphatidic acid phosphatase (PAP). This clone was in vitro transcribed to get RNA probe which was used for hybridization screening of the library and thus one clean positive was picked and used for second round of screening. In this way two identical positive clones were picked and were sequenced after restriction analysis. The partial cDNA sequence of C. cephalonica PAP showed ~38% sequence homology with Drosophila PAP (wunen gene). When this clone was used for southern hybridization, it showed that PAP is product of multiple copy gene. Activity profile showed highest ACP activity in visceral fat body and this matched with the northern profile from the RNA of different tissues of C. cephalonica.

To understand the nongenomic regulation of ecdysteroid mediated by HP19 at molecular level, the RNA from fat body which was cultured with 20E and HP19 along with the controls were subjected to northern analysis. The results indicated that the presence of HP19 that mediates the 20E stimulation of ACP activity did not show increase in the transcript of the PAP. This suggests the nongenomic regulation of ACP. These studies however gives no clue if the ACPs that are regulated by HP19 is lysosomal and required for autophagy during metamorphosis or it is a membrane bound protein required for the phospholipase D activation in signal transduction.
B. Nongenomic regulation of hexamerin receptor phosphorylation, tyrosine kinase activity and CaM kinase II activity and autophosphorylation - The inhibitory effect of HP19 on the 20E induced fat body tyrosine kinase activity in cultured fat body, although occurs at longer time incubation but is unaffected by the inhibitors of transcription and translation. This further confirms the nongenomic regulation of 20E mediated by HP19. The nongenomic regulation of fat body tyrosine kinase activity occurs even in the homogenate preparation. Similar inhibition was also seen with the 20E induced autophosphorylation and activity of CaM kinase II indicated that 20E nongenomically regulates the CaM kinase II activity in the fat body of *C. cephalonica*.

Regulation of HP19 -

Present study indicated a multiple role of HP19 on some of the 20E dependent actions. The HP19 mediated the 20E stimulation of ACP activity and inhibited the 20E induced phosphorylation of hexamerin receptor, activity of tyrosine kinase and CaM kinase II activity and autophosphorylation. Present study suggests that HP19 either is a kinase inhibitor or a phosphatase activator. However, it is not clear at present and further studies are required to confirm this. The results also indicate that HP19 might be a protease like molecule to regulate the 20E dependent actions. We also compare this relatively stable HP19 (good shelf life) with other ubiquitously distributed regulatory molecules that have multiple roles in cellular functions. However, HP19 did not show any similarity at western level with proteins like 14-3-3, stathmin and ubiquitin. In order to understand if HP19 was itself phosphorylated, to regulate the selected 20E dependent actions. The results indicated that there is no phosphorylation of HP19 in absence or presence of calcium either in HGLFB, a tissue that synthesizes this protein or in the haemolymph where it is released.

Summary and conclusion -

In short a haemolymph protein HP19, identified and characterized in the present study appears to be an important regulatory protein in the postembryonic life of *C. cephalonica*. It mediates the 20E stimulated fat body ACP activity. Further, 20E induced activity and autophosphorylation of CaM kinase II are inhibited by HP19. In the absence of HP19, the hormone (20E) induces the tyrosine kinase activity which in turn stimulates the phosphorylation of hexamerin receptor which is followed by increased hexamerin uptake.
When HP19 is present, there is an inhibition of tyrosine kinase activity, which results in reduction of phosphorylation of hexamerin receptor. Hexamerins are synthesized by actively feeding larval fat body cells and released into haemolymph. The hexamerin synthesis is regulated by the morphogenetic hormones (juvenile hormone and ecdysteroids) through their interaction with nuclear receptors (Wang et al., 1995). The presence of active HP19 at late-last larval instar stage possibly inhibits the fat body tyrosine kinase activity, hence there is no phosphorylation of hexamerin receptor, and hexamerins are not sequestered by larval fat body cells. At pupal stage, the HP19 is inactive, hence there is stimulation of tyrosine kinase by 20E (endogenous titer at this stage is high) which in turn mediates the phosphorylation of hexamerin receptor and the uptake of hexamerins occurs.

Unanswered questions-

The present study addresses a very important aspect in the hormone regulated actions by a haemolymph protein, HP19 during the postembryonic life of insects. However, this study fails to answer many questions and opens up a new avenue for further study to understand several aspect (i) Is there an interaction of 20-hydroxyecdysone (20E) with HP19? (ii) Does the hexamerin receptor possess intrinsic tyrosine kinase activity that autophosphorylates the receptor for hexamerin uptake? (iii) Is HP19 a kinase inhibitor or a phosphatase activator? and (iv) What are the mechanisms or pathways by which the HP19 mediates the nongenomic effect of ecdysteroids?
References-


