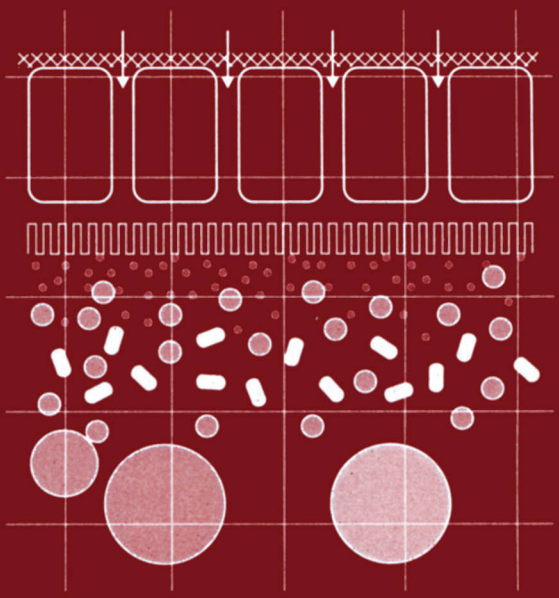
Reproductive Biology of Invertebrates

VOLUME XII • PART B

Progress in Vitellogenesis



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REPRODUCTIVE BIOLOGY OF INVERTEBRATES

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REPRODUCTIVE BIOLOGY OF INVERTEBRATES

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VOLUME XII, PART B
Progress in Vitellogenesis

Science Publishers, Inc.
Enfield (NH), USA Plymouth, UK

CIP data will be provided on request

SCIENCE PUBLISHERS, INC.
Post Office Box 699
Enfield, New Hampshire 03748
United States of America

Internet site: *http://www.scipub.net*

sales@scipub.net (marketing department)
editor@scipub.net (editorial department)
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ISBN 13: 978-1-57808-299-5 (hbk) (Vol. XII, Part B)

Volumes I-XI published by John Wiley & Sons.

Published by Science Publishers Inc., Enfield, NH, USA.

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PREFACE TO THE PROGRESS SERIES

With the release of Volume VI, Part B in 1994, we completed all the projected six volumes (eight books) in the series, "Reproductive Biology of Invertebrates." Going by the reviews, the series has been, on the whole, extremely well received: as one reviewer put it, "it is difficult to imagine any institution with a serious research or teaching involvement in invertebrate reproductive biology finding the series anything but indispensable."

Strengths apart, we find (and this has been pointed out by us in the Series Preface appearing in Volumes I-VI) serious gaps exist in the series as chapters could not be commissioned on some of the phyla and groups, for various reasons, a task we were forced to leave to the future. Another drawback of the series, we think, is that contributions analysing sexuality, reproduction and development from a strictly comparative and evolutionary point of view do not form a part of it as treatment has been phyletic within a thematic framework, though some observations from a comparative point of view do find a place here and there in some contributions. It may also be pointed out this is a difficult class of contributions to commission in as much as comparative gametologists, physiologists, biochemists, endocrinologists, sexologists, and developmental biologists with on-hand experience in different phyletic groups and an overall view of the animal kingdom are becoming rarer and rarer, most investigators confining their observations and comparisons to species or to the phyletic group in question at the most. Third, because of delays in production schedule some contributions were dated even as they appeared in print. The volume of new material that has come to light since Volume I was published in 1983 is formidably large, calling for updating on an extensive scale to ensure the topicality, encyclopaedic nature, and continued utility of this well-established series.

This task is by no means easy, as in some areas such as developmental biology, because of its association with the use of techniques and procedures of molecular biology, genetics and cell physiology, advances at the cutting edge are being made so fast parts of contributions become dated even as they are being hurriedly printed. The scenario is one of the scientists who are hotly chasing the truth in turn being chased even more hotly by the editors as well as the publisher, and all becoming helpless before the great master and leveller, time, with all its philosophical dimensions and manifestations!

Each volume in the "Progress Series" in "Reproductive Biology of Invertebrates" is being organized and guest-edited by a scientist of international repute who is active, has substantial work in the area to his credit, and can impart the necessary degree of authoritativeness and incisiveness to the volume. This has brought to each volume in the series contributions from a wider range of leading authors and laboratories actively pursuing research in the field. Furthermore, as all freedom was given to the guest-editors to organize the volumes departing from the usual phyletic format followed in Volumes I-VI, we ended up refreshingly with bouquets of some of the choicest flowers which we are happy to pass on to the readers.

K.G. ADIYODI
R.G. ADIYODI

PREFACE TO VOLUME XII, PART B

ALEXANDER S. RAIKHEL

Welcome to part B of "Progress in Vitellogenesis". This two-part publication has been designed as an overview of the key process of egg maturation, vitellogenesis, an area in which considerable progress has been achieved in recent years. This progress is mostly due to the adaptation of modern biological techniques to research in vitellogenesis. The first part of "Progress in Vitellogenesis," edited by A.S. Raikhel and T.W. Sapping ton, was published in 2002, Volume XII, Part A of a series, "Progress in Invertebrate Reproduction." It includes reviews on the biochemical properties of yolk proteins and the vitellogenesis process in many groups of invertebrates. Several chapters are devoted to comparative analyses of yolk protein sequences gained by cloning and sequencing of their genes or cDNAs. Thus, the first part of "Progress in Vitellogenesis" has a strong molecular-evolutionary emphasis (Bownes and Pathirana, 2002; Sappington *et al.*, 2002; Taylor and Chinzei, 2002; Telfer, 2002; Wilder *et al.*, 2002; Winter, 2002; Yokota and Sappington, 2002).

The focus of Part B of "Progress in Vitellogenesis" is to review major, recent accomplishments made in elucidating vitellogenic events at the cellular, biochemical, and molecular biological levels. This volume focuses on insects because vitellogenesis in this group of invertebrates has been thoroughly researched. The events reviewed in twelve chapters of this Volume span from analysis of cellular and biochemical events of yolk protein precursor (YPP) biosynthesis to hormonal and tissue-specific regulation of *YPP* gene expression (Chapters 1-6). Chapters 7-12 review later stages of vitellogenesis from cellular events of YPP receptor-mediated endocytosis in developing occytes and molecular analysis of occyte YPP receptors, to cellular and biochemical events YPP proteolytic degradation in developing embryos.

Vitellogenins (Vgs) represent evolutionarily-conserved major YPP's in most oviparous animals (Sappinton and Raikhel, 2002). In Chapter 1, entitled "Biosynthesis and Processing of Insect Vitellogenins" (Tufail *et al.*, current volume), the authors reviewed recent data on this topic. In insects, Vgs are synthesized as

large precursors and are processed along the secretary pathway to yield post-translationally modified polypeptides of the mature oligomeric Vg molecules, which are then secreted into the haemolymph. The extent to which Vgs are processed in the fat body varies greatly among various insect groups. Recent biochemical and molecular studies have added immensely to our understanding of Vg biosynthesis. Overall, picture which has emerged is that the post-transcriptional cleavage Vg precursors differs significantly in two major groups of insects. Namely, the cleavage in Vgs in hemimetabolous insects is more complicated (cleaved into multiple polypeptides) than in holometabolus insects, where Vgs are cleaved only into two polypetides (one large and one small).

Production of YPPs for developing oocytes requires a major investment on the part of a vitellogenic female. In most animals, this process is achieved in a highly dedicated, tissue-specific manner. In insects, the fat body is the major site of YPP production. Chapter 2, "The Cell Biology of Yolk Protein Precursor Synthesis and Secretion," (Giorgi *et al.,* current volume) examines the cellular apects of YPP synthesis and secretion, concentrating mainly on the insect fat body. The additional production of YPP by ovarian follicular epithelial cells is also reviewed for higher Diptera in Chapter 5 (Bownes, this volume).

Endocrine control of vitellogenesis and yolk protein gene expression is governed by several hormones. The two major classes hormonal regulators of these events are juvenile hormones (JHs) and ecdysteroids, namely 20-hydoxyecdysone (20E). Major events of reproduction in Hemimetabola insect orders with incomplete metamorphosis are governed by juvenile hormone (JH). With the switch to a complete metamorphosis, control strategies evolved differently. In beetles, JH remains the major regulatory hormone of reproductive events. In Hymenoptera, the reproductive role of JH is complicated by the evolution of social organization. In Lepideoptera, female reproduction is regulated either by JH or by ecdysteroids. In dipteran insects, mosquitoes and flies, ecdysteroids take the leading role as hormonal regulators. However, JH remains an important regulator in dipteran female insects in preparing reproductive tissues for ecdysteroid-mediated events, such as vitellogenesis. The hormonal regulation of *YPP* gene expression in major insect groups is reviewed in Chapter 3 (Wang *et al.*), 4 (Belles) and 5 (Bownes) of this volume.

One of the key questions in understanding hormonal regulation is how a generalized hormonal signal affects expression of a specific gene in a particular tissue in a precise manner. In insects, the hormones JH and 20E play major roles in determining the timing and levels of *YPP* gene expression. However, organs and cells of a multicellular organism can respond differently to the same hormonal stimulus. Moreover, the same hormore could have dramatically different effects on expression of genes in the same tissue or cell. Thus, the strict control of developmental gene expression cannot be achieved by the action of hormone alone. Investigation of the tissue-, sex-, and stage-restricted expression of hormonally regulated genes clearly indicates that correct gene expression is achieved by coordination of the

hormonal gene-regulatory hierarchies with other transcription factors expressed in a spatially and temporally restricted manner. In Chapter 6, "Molecular Mechanisams of Tissue-Specific Gene Expression in Insects," Martin *et al.* (current volume) discuss basic mechanisms of tissue specificity using well-characterized genes from the fruitfly *Drosophila melanogaster* and the mosquito *Aedes aegypti* as examples. The authors also reviewed the role of transcription factors commonly associated with tissue-specific gene expression.

Oocytes of oviparous animals specifically internalize their YPP in a process of receptor-mediated endocytosis (RME). To accomplish efficient uptake of enormous amounts of YPP required for the future needs of the developing embryo, oocytes are extraordinarily specialized for RME. These unique specialization of insect oocytes of RME, which represent the best stuided examples among invertebrates, are reviewed in Chapter 7, "Receptor-Mediated Endocytosis of Yolk Proteins in Insects Oocytes" (Snigirevskaya and Raikhel, current volume).

Receptors for some insect yolk proteins have been cloned and molecularly characterized. Insect vitellogenin receptors are a unique group of the low-density lipoprotein receptor (LDLR) family, which have two ligand-binding extracellular domains containing clusters of five and eight cysteine-rich repeats, respectively. Chapter 8, entitled "Insect Vitellogenin/Yolk Protein receptors" (Sappington and Raikhel, current volume), reviews the current state of our knowledge concerning insect YPP oocyte receptors.

Lipids are a critical source of energy during insect embryogenesis. Chapter 9, "Accumulation of Lipids in Insect Oocytes", Antwerpen *et al.* (current volume) review current information on the uptake and storage of lipids by insect oocytes. The interactions of lipid transport proteins, Vg and lipophorin, with their oocyte receptors, and the cellular uptake of lipids are addressed. The intracellular lipid transport pathway is discussed and a working model for uptake and storage of lipids by insect oocytes is presented.

Vitellin (Vn), the Vg form deposited in oocytes, accounts for 60 to 90% of the total protein content of mature oocytes. Depending on the insect species, other yolk proteins unrelated to Vn can consitute from 1 to 40% oocyte total protein (Telfer, 2002). The additional YPs provide the developing embryo with bilivedin, carotenoids, iron, calcium, haem, additional amino acids, phosphates, lipids, and carbohydrates. The purpose of Chapter 10, "Non-Vitellogenin Yolk Proteins" (Masuda *et al.* current volume) is to review the current knowledge about these non-vitellin protein components of insect oocytes.

In insect eggs, embryogenesis is accompanied by a gradual degradation of yolk granules into various components, and the sequential cleavage of YPs into smaller molecules via a limited hydrolysis. Recently, significant advances have been made in our understanding of the biochemistry of this process. Chapter 11, entitled "Protein Degradation during Insect Embryogenesis" (Yamahama *et al.*, current volume) describes biochemical aspects of YP degradation in insect eggs

during embryogenesis. These mechanisms are mainly elucidated in three insects, the silkmoth *Bombyx mori,* the cockroach, *Blattella germanica,* and the yellow fever mosquito, *Aedes aegypti.* Detailed analysis of these three insects forms the foundation of this chapter.

Finally, in Chapter 12, "Biochemical and Ultrastructural Aspects of Vitellin Utilization during Embryogenesis", Giorgi and Nordin continue exploring insect emryogenesis at the biochemical and cellular levels. Of particular importance is the analysis of mechanisms leading to the activation of egg proteolytic enzymes, such as acidification of yolk bodies. New light is shed on the role of cellular events taking place in early embryos, such as the utilization of yolk proteins by vitellophages, specialized embryonic cells.

In summary, the authors of the current volume, all experts in the field of insect vitellogenesis, present reviews on cellular, biochemical and molecular aspects which accompany this key event of egg maturation. Although this volume focuses on insects, the wide knowledge base derived from the comprehensive treatment of insect vitellogenesis should serve as a valuable resource for researches and students interested in reproduction of oviparous animals in general and of invertebrates in particular.

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1. BIOSYNTHESIS AND PROCESSING OF INSECT VITELLOGENINS

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I. INTRODUCTION

Insects are the most adapted group of animals on earth. Their success appears partly due to extreme levels of fertility and highly specialized modes of reproduction. An extreme example of such productivity is a queen bee, which every day generates a quantity of eggs roughly equal to her body weight.

In 1954, Telfer showed a sex-linked "female-protein" that participates in yolk formation in the haemolymph of the silkmoth, *Hyalophora cecropia*. The fat body was identified as the source of this protein. It was named "vitellogenin" by Pan *et al.*, (1969) as the precursor of vitellin (Vn) or yolk protein. Presently, it has been recognized that in insects the female fat body is the major site of Vg synthesis (Engelmann, 1983; Wyatt, 1991; Wyatt and Davey, 1996). However, it has become clear that the fat body is not the only vitellogenic tissue, and that in some insects ovarian follicular epithelium produces yolk polypeptides (Yps) (Wyatt, 1991; Belles, 1998; Telfer, 2002; Giorgi *et al.*, (chapter 2, this volume).

The production of eggs depends, among other factors, upon biosynthesis of the major yolk protein precursor, vitellogenin (Vg), which is produced extra-ovarially by the fat body, secreted into the haemolymph, and then taken up specifically by the developing oocytes. During this process, Vg undergoes proteolytic cleavage, glycosylation, lipidation, and phosphorylation. After incorporation into developing oocytes, Vg is converted into the major storage protein of future eggs, vitellin (Vn),

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which serves as a source of amino acids, carbohydrates, lipids, and phosphates for the developing embryo.

The general consensus today is that proteins destined for secretion pass away from the site of synthesis in the rough endoplasmic reticulum to the Golgi apparatus following an anterograde flow of vesicles directed to the cell surface (Pfeffer and Rothman, 1987). Nascent proteins must first be translocated across the membrane enclosing the endoplasmic reticulum cisternae (Hedge and Lingappa, 1999) and then must be post-translationally modified into functional products by a stepwise displacement through cisternae of the Golgi apparatus and the emerging secretory granules (Brion *et al.*, 1992).

Vitellogenins are synthesized as large precursors and are variously processed along the secretory pathway to yield the post-translationally modified polypeptides of the mature oligomeric Vg protein secreted into the haemolymph. The extent to which Vgs are processed in the fat body varies greatly among various insect groups (Telfer, 2002; Sappington *et al.*, 2002). However, recent cloning of Vg cDNA and genes from several insects indicates a high level of conservation in the first steps of their processing.

Recent biochemical and molecular studies have added immensely to our understanding of Vg biosynthesis. Overall, the picture that has emerged is that the post-transcriptional cleavage of Vg precursors differs significantly in two major groups of insects. Namely, the cleavage in Vgs from hemimetabolous insects is more complicated (cleaved into several polypeptides) than in those from holometabolous insects, where Vgs are cleaved only into two polypeptides (one large and one small). Exceptions are Vgs of higher Hymenoptera (suborder Apocrita), in which a primary Vg gene product of about 180 kDa is secreted without processing.

II. VITELLOGENINS

Vitellogenins, or Vgs, are proteins that serve as precursors of Vns, the major egg yolk proteins in insects and other oviparous animals. Insect Vgs are encoded by mRNAs of 6-7 kb and translated as primary products of ~200 kDa, which are cleaved into subunits (apoproteins) ranging from 50 to 180 kDa. Following extensive co- and post-translational modifications, Vg subunits form high-molecular-weight oligomeric phospholipoglycoproteins (400-600 kDa) that are secreted into the haemolymph of vitellogenic females (Trewitt *et al.*, 1992; Chen *et al.*, 1994; Yano *et al.*, 1994; Kageyama *et al.*, 1994; Hiremath and Lehtoma, 1997; Hirai *et al.*, 1998; Lee *et al.*, 2000a, b; Comas *et al.*, 2000; Tufail *et al.*, 2000, 2001; Tufail and Takeda, 2002). Vgs generally exist as oligomers, but monomeric molecules of about 300 kDa are known from *Nauphoeta cinereae* (Imboden *et al.*, 1987).

The Vgs of most insects are members of a conserved family of proteins that are present in organisms as diverse as nematodes and vertebrates (Blumenthal and

Zucker-Aprison, 1987; Spieth *et al.,* 1991; Sappington *et al.,* 2002). The Vg primary product has been characterized from 12 insect species, six of them belonging to Hemimetabola [*Riptortus clavatus* (Heteroptera) (Hirai *et al.,* 1998), *Graptopsaltria nigrofuscata* (Homoptera) (Lee *et al.,* 2000b), *Plautia stali* (Heteroptera) (Lee *et al.,* 2000a), *Blattella germanica* (Dictyoptera) (Comas *et al.,* 2000), *Periplaneta americana* (Dictyoptera) (Tufail *et al.,* 2000, 2001), *Leucophaea maderae* (Dictyoptera) (Tufail and Takeda, 2002)], and six belonging to Holometabola [*Anthonomus grandis* (Coleoptera) (Trewitt *et al.,* 1992), *Aedes aegypti* (Diptera) (Chen *et al.,* 1994), *Bombyx mori* (Lepidoptera) (Yano *et al.,* 1994), *Athalia rosae* (Hymenoptera) (Kageyama *et al.,* 1994, Nose *et al.,* 1997), *Pimpla nipponica* (Hymenoptera) (Nose *et al.,* 1997), *Lymantria dispar* (Lepidoptera) (Hiremath and Lehtoma, 1997)]. The comparison of the protein primary structures of Vgs from different insect species has shown that they are highly conserved and form a gene superfamily (Chen *et al.,* 1997; Lee *et al.,* 2000b; Sappington *et al.,* 2002). The insect Vgs have been shown to be homologous with the Vgs of other invertebrates such as nematodes and those of oviparous vertebrates such as amphibians and birds (Spieth *et al.,* 1985; Blumenthal and Zucker-Aprison, 1987; Nardelli *et al.,* 1987; Wahli, 1988; Byrne *et al.,* 1989; Trewitt *et al.,* 1992; Chen *et al.,* 1994). Similarly, insect Vgs share homology with Vgs of other arthopods, such as millipedes (Prasath and Subramoniam, 1991), crustaceans (Chen and Chen, 1993), and ticks (Chinzei *et al.,* 1983; Rosell and Coons, 1991).

In higher Diptera, yolk proteins are different from those of other insect Vgs. They are small relative to those of other insects and consist of several polypeptides (~ 45 kDa). In *Drosophila. melanogaster,* for example, three major yolk polypeptides (YPs) of 46, 45, and 44 kDa exist, each encoded by a single-copy gene located on the X-chromosome (Bownes *et al.,* 1993). The products of these *yp* genes are secreted without processing (Bownes and Pathirana, 2002). Homologous YPs are found in other higher Diptera such as the fruit fly, *Ceratitis capitata* (Rina and Savakis, 1991), and the blowfly, *Calliphora erythrocephala* (Martinez and Bownes, 1994). Surprisingly, the YPs of higher Diptera are structurally similar to mammalian triacylglycerol lipase but without an enzymatic centre (Baker, 1988; Terpstra and AB, 1988).

III. VITELLOGENIN GENES IN INSECTS

The genes or cDNAs encoding pre-Vgs have been identified and cloned from several hemimetabolous insects (Hirai *et al.,* 1998; Comas *et al.,* 2000; Lee *et al.,* 2000a, b; Tufail *et al.,* 2000, 2001; Tufail and Takeda, 2002). The number of genes varies (from one to several) in different species. Two Vg genes (*Vg1* and *Vg2*) are present in *P. americana,* and the complete cDNAs have been recently cloned and

characterized (Tufail *et al.*, 2000, 2001). The Vg1 and Vg2 cDNAs encode mature proteins of 1,880 and 1,860 amino acid residues in length, respectively, with approximately 30% identity in their structures. Multiple Vg genes have also been reported in *Locusta migratoria* (Wyatt *et al.*, 1984), *R. clavatus* (Hirai *et al.*, 1998), and *P. stali* (Lee *et al.*, 2000a).

In *L. migratoria,* two Vg genes, both located on the X chromosome (Bradfield and Wyatt, 1983; Wyatt *et al.*, 1984), have been cloned and partially sequenced (Locke *et al.*, 1987). *Vg* genes A and B are 12 and 10.5 kb long, respectively, each encoding a 6,300-nucleotide mRNA (Chinzei *et al.*, 1982). Both genes are transcribed in the fat body of adult females in response to a juvenile hormone (JH) (Dhadialla and Wyatt, 1983; Chinzei and Wyatt, 1985) and are expressed coordinately (Dhadialla *et al.*, 1987). In the bean bug, *R. clavatus*, Hirai *et al.*, (1998) identified two *Vg* genes and reported the complete cDNA for the *Vg-1* gene, which is 5,736 nucleotides long. In *P. stali*, three complete cDNAs for three Vg genes (*Vg-1*, *Vg-2* and *Vg-3*) have been characterized (Lee *et al.*, 2000a). The Vg-1, -2, and –3 cDNAs produced mature proteins of 1,890, 1,839, and 1885 amino acid residues in length, respectively. The overall amino acid identity ratio was 52% between Vg-1 and Vg-2, 44% between Vg-1 and Vg-3, and 45% between Vg-2 and Vg-3 (Lee *et al.*, 2000a). The mRNAs for these genes were detected only in the female fat body cells and were about 6-7 kb in length (Lee *et al.*, 2000a).

In *G. nigrofuscata* and *B. germanica,* only one cDNA for Vg has been reported (Lee *et al.*, 2000b; Comas *et al.*, 2000). The complete cDNA for *G. nigrofuscata* Vg is 6,205 bp residues long, which encodes a mature protein of 1971 amino acid residues (Lee *et al.*, 2000b), whereas the complete cDNA for *B. germanica* Vg is 5,749 bp long, and the protein it encodes is 1,845 amino acid residues long (Comas *et al.*, 2000). Recently, the complete cDNA for Vg of *L. maderae* has also been cloned (Tufail and Takeda, 2002), and a female-specific Vg mRNA of 6.6 kb has been observed only in the fat body cells. Although there is no evidence for multiple *Vg* genes from *L. maderae,* cloning and sequencing of the other Vg cDNA from this species has been accomplished (Tufail, 2003) and has revealed stretches of amino acid sequences (four in number) different from the one reported previously (Tufail and Takeda, 2002).

There are five Vg genes in *A. aegypti* (Romans *et al.*, 1995). In contrast, in the silkworm *Bombyx mori* Vg is encoded by a single gene.

It remains unclear why multiple Vg genes exist in some species and not in others. It has been proposed that multiple Vg genes may be necessary to provide a large amount of Vn within a short egg maturation period (Tufail *et al.*, 2001). There is also a possibility that multiple genes, apparently derived from a common ancestor, might have acquired different functions. For instance, the plasma clotting protein in the crayfish shares common characteristics with Vgs but has unique functions (Hall *et al.*, 1999). Cloning of Vg cDNAs and of genomic DNAs from many more species should prove to be of importance for elucidating these questions.

IV. BIOSYNTHESIS AND PROTEOLYTIC PROCESSING OF VITELLOGENINS IN HEMIMETABOLOUS INSECTS

The most thorough studies of the Vg biosynthesis in hemimetabolous insects and of the post-translational behaviour of this molecule have been carried out on the cockroaches *P. americana, L. madeare,* and *B. germanica.* In *P. americana*, the expression of two *Vg* genes (*Vg1* and *Vg2*) and the post-translational processing gives rise to four Vg polypeptides—three major [170, 100 (multi-subunits), and 50 kDa] and a minor (150 kDa) (Fig. 1) (Tufail *et al.,* 2000, 2001). Two multi-subunit precursor polypeptide with M_r of about 260 kDa comprise a dimer of the oligomeric Vg molecule of about 520 kDa (Storella *et al.*, 1985). The *Vg1* gene in this species starts being expressed in two days after emergence in the female fat body cells, whereas Vg is first detected in the haemolymph by immunobloting in four-day-old adult females, two days after the mRNA of the Vg gene first appears (Fig. 2) (Tufail *et al.,* 2000). A study on the Vg content in the haemolymph of *P. americana*

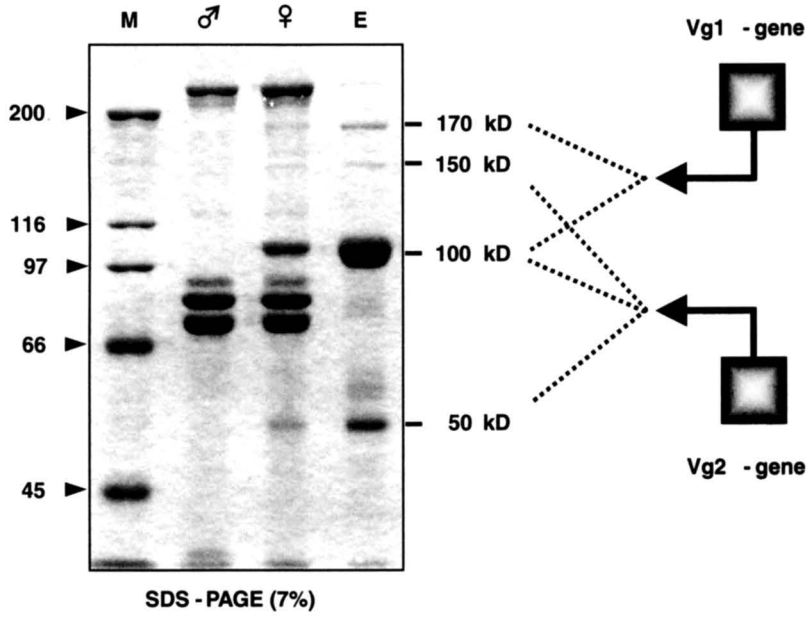

Fig. 1. Biosynthesis of *P. americana* Vgs. The synthesis of Vgs in *P. americana* is controlled by two Vg genes. The primary translation products are cleaved soon after their synthesis into four polypeptide "subunits," which are secreted as four Vg polypeptides (170, 150, 100, and 50 kDa) (lane-3, female haemolymph) and are deposited in the egg (E) as four respective Vn polypeptides (lane-4). The gene-specific origin of Vg/Vn polypeptides is shown on the right. The 150-kDa polypeptide is a minor Vg/Vn polypeptide. The 100-kDa band is a multi-subunit polypeptide that originates from both Vg genes (*Vg1* and *Vg2*). M indicates the molecular weight markers (kDa). (Based on Tufail *et al.,* 2000, 2001).

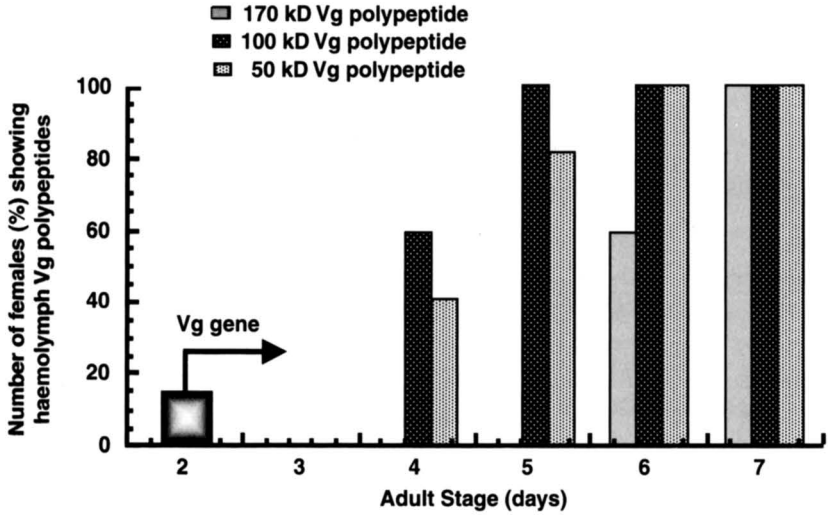

Fig. 2. A graphic presentation of Vg gene expression, and the stage-specific synthesis of haemolymph Vgs in the female of *P. americana.* The Vg gene starts being expressed (the expression of only Vg1 gene has been detected) in two-day-old adult female fat body cells. The primary translation products are cleaved and were detected by Western blot analysis first in the haemolymph of four-day-old adult females, two days after the Vg gene expression. The 100- and 50-kDa Vg polypeptides, being the more visible ones, appear earlier as compared with the 170-kDa Vg polypeptide. The stage-specific synthesis of only major haemolymph Vg polypeptides (see Fig. 1) is presented here (based on Tufail, 2000; Tufail *et al.,* 2000, 2001).

showed that its level in the haemolymph increased continuously until day 8 and then decreased, being sequestered by developing oocytes (Fig. 3) (Tufail, 2000).

The Vg of the *L. maderae* is made up of four polypeptides with molecular weights of 112, 100, 92, and 50 kDa (Tufail and Takeda, 2002). Biosynthesis of these polypeptides in the female fat body requires translation of a 220-kDa pre-pro-Vg (Don-Wheeler and Engelmann, 1997) encoded by a female-specific 6.6-kb transcript (Tufail and Takeda, 2002). These Vg polypeptides are assembled into the protein backbone of the secreted Vg dimer (della-Cioppa and Engelmann, 1987). The Vg of the related cockroach species *B. germanica* consists of two major polypeptides of 160 kDa and 102 kDa, respectively, which originate from the precursor polypeptide of 250 kDa before being secreted into the haemolymph of the vitellogenic female. The 160-kDa Vg polypeptide has been reported as being processed further post-endocytically in the oocyte just prior to chorionation, resulting in the production of two additional polypeptides of 95 and 50 kDa (Wojchowski *et al.,* 1986). However, in order to draw a more conclusive picture with regard to the diversity of proteolytic processing of insect Vgs, some of the earlier studies will require re-investigation using more powerful protease inhibitors.

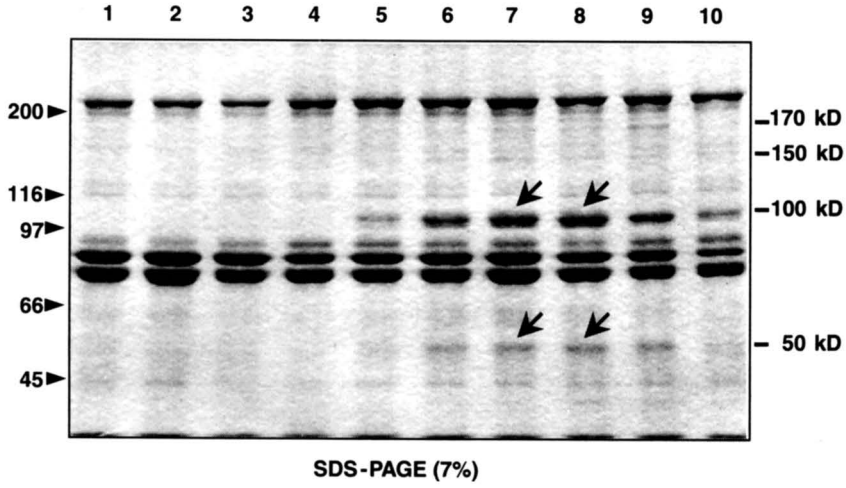

Fig. 3. The content fluctuations of *P. americana* Vgs. The content fluctuations of *P. americana* Vgs were determined through SDS-PAGE by comparing the haemolymphs from 1- to 10-day-old adult females. The same amount of haemolymph samples (0.05 µl equivalent per lane) was loaded on each lane. Arrowheads on the left indicate the molecular weight markers (kDa). The content level of the Vg polypeptides (indicated on the right) after first appearing in the haemolymph increased continuously until day 7/8 (shown with arrrows) and then decreased as it was taken up by the developing oocytes. The 170- and 150-kDa Vg polypeptide bands were barely visible on the SDS-PAGE gels (From Tufail, 2000).

V. PROTEOLYTIC PROCESSING OF VITELLOGENINS IN HEMIMETABOLOUS INSECTS

The Vg primary translation product so far cloned from hemimetabolous insects is cleaved soon after its synthesis (Fig. 4). The pre-pro-Vg cleavage is accomplished by the subtilisin-like endoproteases, proprotein convertases (Barr, 1991; Rouille et al., 1995), which recognize a consensus (R/K)XX(R/K) sequence motif proceeding immediately to the cleavage site. These sequence motifs are found in Vgs of all known hemimetabolous insects, with their positions particularly conserved near the N-terminus (Fig. 4). In *L. maderae* Vg, there are 11 such sequences, with 3 actually used for post-translational cleavage (Tufail and Takeda, 2002). The analysis of the deduced amino acid sequence of Vg from *R. clavatus* has revealed four putative enzymatic cleavage sites in the Vg precursor molecule, two of which matched the consensus sequence for dibasic processing endoproteases (Hirai *et al.,* 1998).

In hemimetabolous insects such as *L. maderae*, *P. americana,* and *R. clavatus*, the pro-Vg is cleaved into several polypeptides, including polypeptides of ~80-110 kDa (medium polypeptides), in addition to large and small polypeptides (Hirai *et al.,* 1998; Tufail *et al.,* 2000, 2001; Tufail and Takeda, 2002). It has also been reported that Vgs from some hemimetabolous insects, such as *L. maderae* and *R.*

clavatus, are not completely processed in the fat body and that a further processing occurs in the oocyte after the Vgs have been deposited in the latter (Hirai *et al.*, 1998; Tufail and Takeda, 2002).

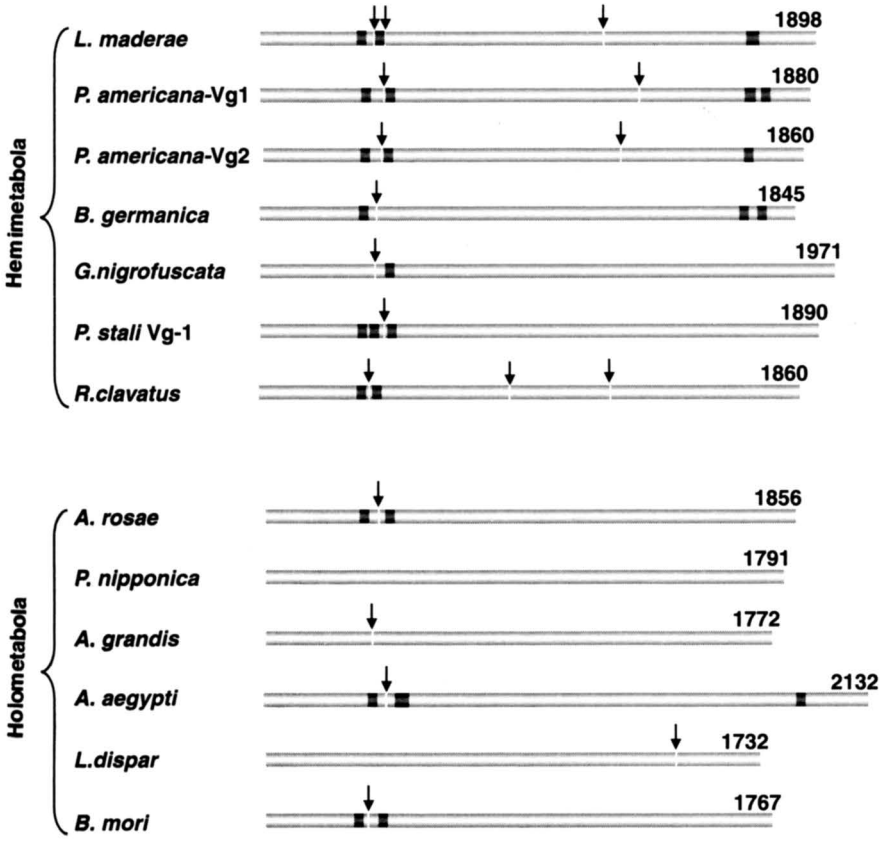

Fig. 4. Schematic representation of the cleavage sites and polyserine domains in Vgs from 12 insect species (see the text for references). The arrows and white lines indicate the putative or determined cleavage sites following the consensus RXXR cleavage site sequence. The black segments show the polyserine stretches. Numbers indicate the amino acid residues deduced from the N-terminal (excluding the signal peptides). In the case of *P. stali*, isoform 1 has been shown.

A. Proteolytic Processing of *L. maderae* Vg.

The Vg of *L. maderae* is composed of four polypeptides with molecular weights of 112, 100, 92, and 55 kDa that have been shown to be the cleaved products originating from a single precursor in the fat body (Tufail and Takeda, 2002). The precursor polypeptide is 220 kDa (Don-Wheeler and Engelmann, 1997), which is translated from a fat body-specific Vg mRNA of 6.6 kb (Tufail and Takeda, 2002). There is

evidence that the 92-kDa Vg polypeptide, after incorporation into the oocyte, is further processed, which results in the production of an additional 90-kDa polypeptide in the egg extracts (Tufail and Takeda, 2002). The processing steps in *L. maderae* Vg are shown in Fig. 5 (Tufail and Takeda, 2002).

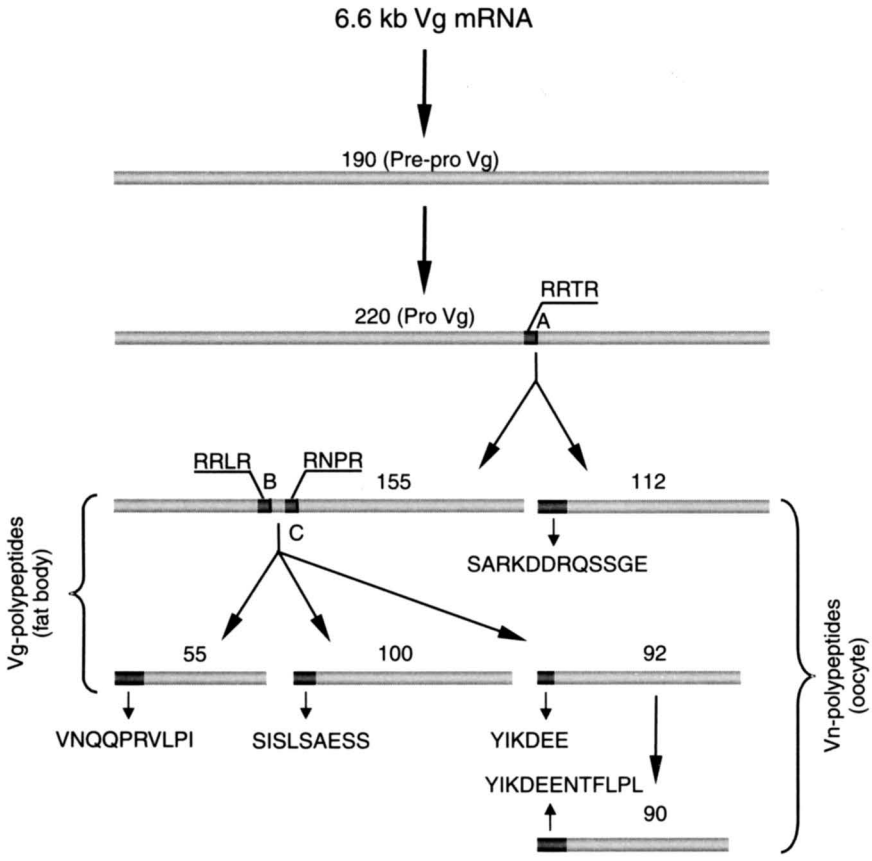

Fig. 5. The synthesis of *L. maderae* Vg from 6.6 kb Vg mRNA, and its processing in the fat body and oocyte. Numbers show the molecular sizes (kDa) of the polypeptides and letters indicate the cleavage sites on the amino acid sequence. The precursor polypeptide is cleaved into four polypeptides (112, 55, 100, and 92 kDa) in the fat body before being secreted into the haemolymph. The Vn in the oocyte is further processed and results in the production of an additional 90-kDa Vn polypeptide. The determined N-terminal amino acid sequences of the Vn polypeptides are shown with black segments and arrows. The amino acid sequences (raised and underlined) are the determined cleavage site sequences and are shown with black segments just before the cleavage sites. (Reproduced from Tufail and Takeda, 2002, with permission).

The pro-Vg of *L. maderae* is first cleaved completely at site A, following the RRTR cleavage site sequence, into 155- and 112-kDa polypeptides. The amino-terminal sequence of the 112-kDa polypeptide matched the deduced amino acid

sequence of the precursor just following the cleavage site sequence RRTR (Tufail and Takeda, 2002). The 155-kDa polypeptide is then further processed at sites B (following RRLR) and C (following RNPR), resulting in the production of 55-, 100-, and 92-kDa polypeptides (Fig. 5) in the fat body before being secreted into the haemolymph. The 155-kDa polypeptide, however, was not observed on the SDS-PAGE gels. The authors speculate that the latter would be a short-lived polypeptide and would be processed soon after its appearance (Tufail and Takeda, 2002). Tufail and Takeda (2002) demonstrated that the complete cleavage at site B first produces a 55-kDa polypeptide, and then incomplete cleavage at site C gives two more polypeptides (100 and 92 kDa). The 92-kDa Vn polypeptide is then further processed to a 90-kDa Vn polypeptide after incorporation into the oocyte. The matching of N-terminal amino acid sequences of both (92- and 90-kDa) Vn polypeptides (Fig. 5) (Tufail and Takeda, 2002) demonstrates that the 90-kDa fragment must have come from the cleavage of the 92-kDa polypeptide at the C-terminus. Tufail and Takeda (2002) have suggested, however, that an RXXR motif is not in the appropriate vicinity of the C-terminal cleavage site, suggesting that an oocyte enzyme different from the dibasic proteases is involved in cleavage. Moreover, the authors have speculated that the 100-kDa fragment may be incompletely processed at cleavage site C in the egg and that the 92-kDa fragment may be incompletely processed to 90-kDa at the C-terminal site. This would account for the fact that the 100- and 92-kDa Vn polypeptides are minor components of the egg extracts (Tufail and Takeda, 2002).

B. Proteolytic Processing of *P. americana* Vgs.

P. americana has two Vg genes (Tufail *et al.*, 2000, 2001) that encode precursor polypeptides of ~275 and ~266 kDa, respectively (Storella *et al.*, 1985), which undergo proteolytic processing and produce subunits of 170, 150, 100 (a multi-subunit polypeptide) and 50 kDa (Fig. 1) (Tufail *et al.*, 2000, 2001). On the basis of deduced amino acid sequence of both Vgs (Vg1 and Vg2), Tufail *et al.*, (2000, 2001) have shown that the 170-kDa polypeptide and one of the 100-kDa subunits originate from the *Vg1* gene product. On the other hand, the 150-kDa polypeptide, a second 100-kDa polipeptide, and the 50-kDa polypeptide originate from the *Vg2* gene. The pathway of post-translational processing from precursor polypeptides is summarized in Fig. 6.

The Vg1 precursor polypeptide is first processed completely at site A (following the RTRR cleavage site sequence), resulting in the production of 100- and 170-kDa polypeptides (Tufail *et al.*, 2001). The N-terminal amino acid sequence of the 170-kDa polypeptide matched the deduced amino acid sequence of Vg1 following the RTRR cleavage site sequence and thus formed the carboxy terminal region of the precursor (Fig. 6) (Tufail *et al.*, 2000, 2001). The 170-kDa polypeptide is then cleaved at site B to produce 100- and 70-kDa polypeptides (Storella *et al.*, 1985).

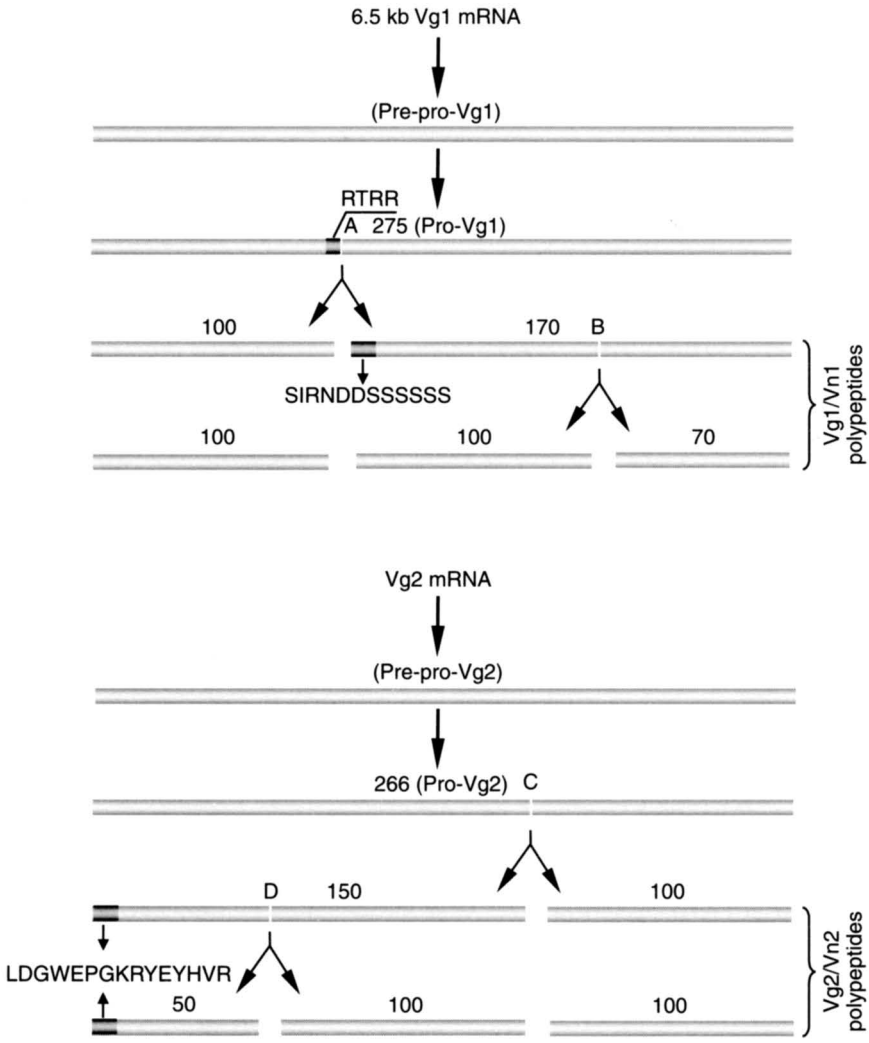

Fig. 6. The synthesis and processing patterns of *P. americana* Vgs (Vg1 and Vg2). Pro-Vg is synthesized and then processed in the fat body before being secreted into the haemolymph (see text). Numbers indicate the molecular sizes (kDa) of the polypeptides and letters indicate the cleavage sites. The determined N-terminal amino acid sequences are shown with shaded boxes and arrows. The determined cleavage site sequence is indicated with a dark area just before the cleavage site and is raised and underlined. Reproduced from Tufail *et al.*, (2000, 2001).

In the case of the Vg2 polypeptide, the main processing site is C, resulting in production of the 150- and 100-kDa polypeptides. The 150-kDa Vg2 polypeptide is then further processed at site D, resulting in the production of 50-and 100-kDa polypeptides in the fat body. The determined N-terminal amino acid sequences of

the 150- and 50-kDa polypeptides share the same sequence, suggesting that they are thus derived from the N-terminus of the Vg2 (Fig. 6) (Tufail *et al.*, 2001). The cleavage at site D is incomplete, so that the 150-kDa polypeptides remain as a minor component in the haemolymph and egg extracts (see Fig. 1) (Tufail *et al.*, 2001).

C. Proteolytic Processing of *R. clavatus* Vg-1

In *R. clavatus*, two Vgs (Vg1 and Vg2) have been identified, and the complete cDNA for one of them (Vg1) has been cloned and characterized (Hirai *et al.*, 1998). The Vg1 of this species is composed of seven polypeptides [four major (170, 120, 63, and 54 kDa) and three minor (210, 105, and 82 kDa)], whereas the Vn1 is composed of eight polypeptides (170, 120, 105, 82, 63, 54, 52, and 37 kDa) (Shinoda *et al.*, 1996). All the Vg1/Vn1 polypeptides were the processed products originated from a single precursor of ~210 kDa (Hirai *et al.*, 1998). The processing patterns as demonstrated by Hirai *et al.* (1998) are summarized in Fig. 7.

The authors have demonstrated that the Vg1 precursor polypeptide (Pro-Vg1) is first cleaved completely at site A, following the RIRR cleavage site sequence, into 54- and 170-kDa polypeptides, in which the N-terminal amino acid sequence of the latter matches the amino acid sequence deduced from the clone. The 170-kDa Vg1 polypeptide is then cleaved at site C-1, following the RWSR cleavage site sequence, into 63- and 120-kDa polypeptides, and at site D-1, following KFKKAN, into 105- and 82-kDa polypeptides. Although KFKKAN is not a consensus sequence for the protease, Hirai *et al.* (1998) have proposed that the dibasic endoprotease might cut next to KFKK (which fits the consensus) and that two additional residues (Ala and Asn) are then removed by another protease. They have also demonstrated that the cleavage at both sites is not a complete one, because if the proteases cleaved at sites C-1 and D-1 completely no 170-kDa polypeptide, which is indeed a major component of Vn-1, would remain. After incorporation into the ovaries, the 54-, 105-, and 120-kDa Vn1 polypeptides are further processed at sites B, C-2 (following RWSR), and D-2 (following KFKKAN), respectively, which results in the production of two additional polypeptides of 52 and 37 kDa (Fig. 7) (Hirai *et al.*, 1998). The precise position of site B is, however, unknown, and in addition no consensus site for the dibasic endoprotease is found in the expected region. The authors have demonstrated that the 54-kDa polypeptide is cleaved at the C-terminus and that the cleavage at this site is supposed to be accomplished by another type of protease (Hirai *et al.*, 1998).

It has also been reported that during processing some minor components such as the 105 and 82 kDa of Vg1 become major components in Vn1 after its incorporation into the ovaries. This indicates that cleavage occurs at the D-1 site, mainly in the ovaries (Hirai *et al.*, 1998). Thus, a further processing of Vn-polypeptides in *R. clavatus* at sites D-1, D-2, and C-2 is of particular importance and points to the presence of a dibasic endoprotease-like enzyme not only in the fat

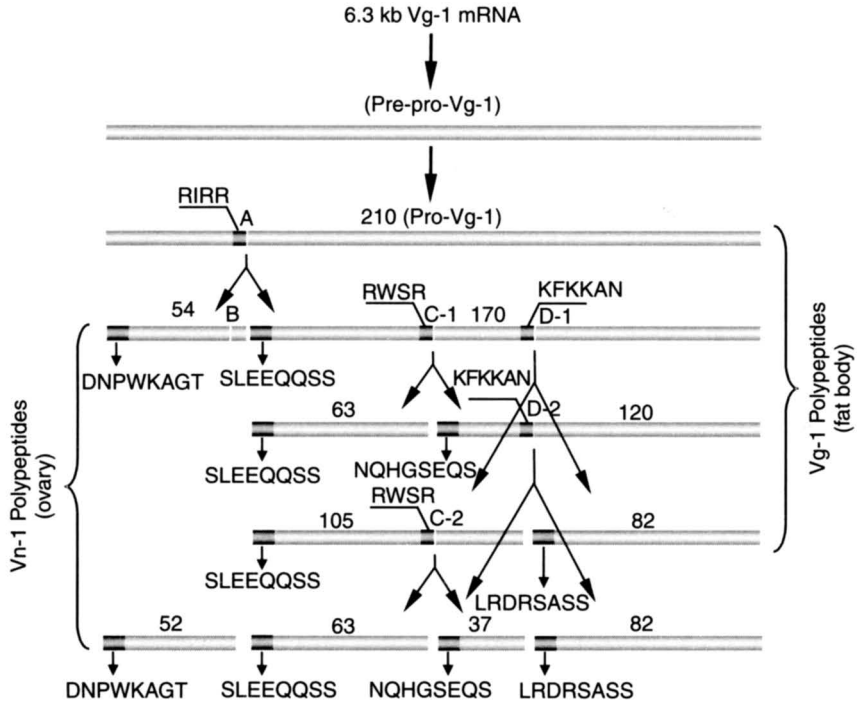

Fig. 7. The synthesis of pro-vitellogenin-1 (Pro-Vg1) of *R. clavatus,* and its processing in the fat body and ovary. Numbers indicate the molecular masses (kDa) of the polypeptides and letters indicate the cleavage sites. Letters with numbers (e.g., C-1, C-2) indicate the same cleavage position on the amino acid sequence. Seven polypeptides are generated in the fat bodies, but three of them (210, 105, and 82 kDa) are very minor components of Vg1. All of the polypeptides except 210 kDa exist as Vn1 components in the ovary. The Vn1, after incorporation into the ovaries, is further processed, resulting in the production of two additional polypeptides (52 and 37 kDa). The determined N-terminal amino acid sequences of the Vn1 polypeptides are shown with dark areas and arrows, whereas the determined cleavage sites showing the RXXR consensus sequence are indicated with dark areas immediately before the cleavage sites and are raised and underlined. See text for KFKKAN. Reproduced with permission from Hirai *et al.,* (1998).

body but also in the ovaries. Moreover, the authors have demonstrated that the cleavage is incomplete at all processing sites except A in the 210-kDa polypeptide (Hirai *et al.,* 1998).

The incomplete cleavage of Vg molecules seems to be common in hemimetabolous insects, as has been observed in the Vgs of *L. maderae*, *P. americana,* and *R. clavatus,* which accounts for the production of several polypeptides. In insects such as *A. aegypti* (Dhadialla and Raikhel, 1990), *B. mori* (Yano *et al.,* 1994), and *G. nigrofuscata* (Lee *et al.,* 2000b), where Vg precursors are cleaved at a single site and just split into one large and one small polypeptide, the sum of the molecular weights of the component polypeptides is equal to that of

the precursor. On the other hand, for species such as *L. maderae*, *P. americana,* and *R. clavatus* that have Vgs with incomplete cleavage the total molecular weight is much larger than that of the precursor. This increase in molecular weight is supposed to be due to the existence of uncleaved intermediate polypeptides, which are actually not the end products of proteolytic processing (Hirai *et al.,* 1998; Tufail, *et al.,* 2000, 2001; Tufail and Takeda, 2002).

VI. PROTEOLYTIC PROCESSING OF THE VG PRECURSOR IN HOLOMETABOLOUS INSECTS

In contrast to the Vgs of hemimetabolous insects, the Vg precursor in holometabolous insects is cleaved only into two polypeptides (one large and one small) (Chen *et al.,* 1994; Chen *et al.,* 1997; Sappington *et al.,* 2002) (Fig. 4). As with other apocritan Hymenoptera, the parasitic wasp *Pimpla nipponica* has a Vg that is not cleaved (Nose *et al.,* 1997) (Fig. 4). In the fall armyworm moth (*Spodoptera frugiperda*), only a single Vg apoprotein could be detected by SDS-PAGE of haemolymph and ovarian extracts, although other lepidopteran Vgs normally consist of two subunits (Sorge *et al.,* 2000). The Vg for the moth *L. dispar* is cleaved once to form two subunits, but the cleavage site is in a short variable region of subdomain IV in the VWD module (Hiremath and Lehtoma, 1997). The region of typical insect Vg cleavage between subdomains I and II is missing entirely in *L. dispar* (Sappington and Raikhel, 1998). Positional correspondence among all Vg sequences across subdomains indicates that cleavage sites arise and are disabled by local changes in sequence within regions tolerant of evolutionary change, rather than through exon shuffling.

The Vg biosynthesis and processing have been studied in great detail in the mosquito *A. aegypti* (Fig. 8). Native Vg in this insect has a molecular weight of 337,000 and an isoelectric point of 6.3. It consists of two polypeptide subunits with M_r = 200, 000 and 65,000, respectively and both Vg subunits derive from a common precursor of 220 kDa and thus originate from the same gene. The observation that the 220-kDa polypeptide could be immuno-precipitated by Vg polypeptide-specific monoclonal antibodies proved definitely that this translation product is indeed the precursor of both Vg subunits (Fig. 9). *In vitro* pulse-chase experiments with radioactive amino acids revealed that the 220-kDa Vg precursor is cleaved in a rate-limiting and regulated manner to give rise to two Vg polypeptides of 190,000 and 62,000, respectively. In turn, these Vg polypeptides undergo post-translational modifications leading to mature Vg subunits of 200,000 and 66,000, respectively (Fig. 10) (Dhadialla and Raikhel, 1990).

The cDNA for Vg has been cloned and sequenced (Chen *et al.,* 1994). Using monoclonal Vg subunit-specific antibodies, the small polypeptide was shown to be located at the N-terminal of the Vg precursor, while the large polypeptide coincides with the carboxy terminal of the precursor (Fig. 8B). The cleavage site between the

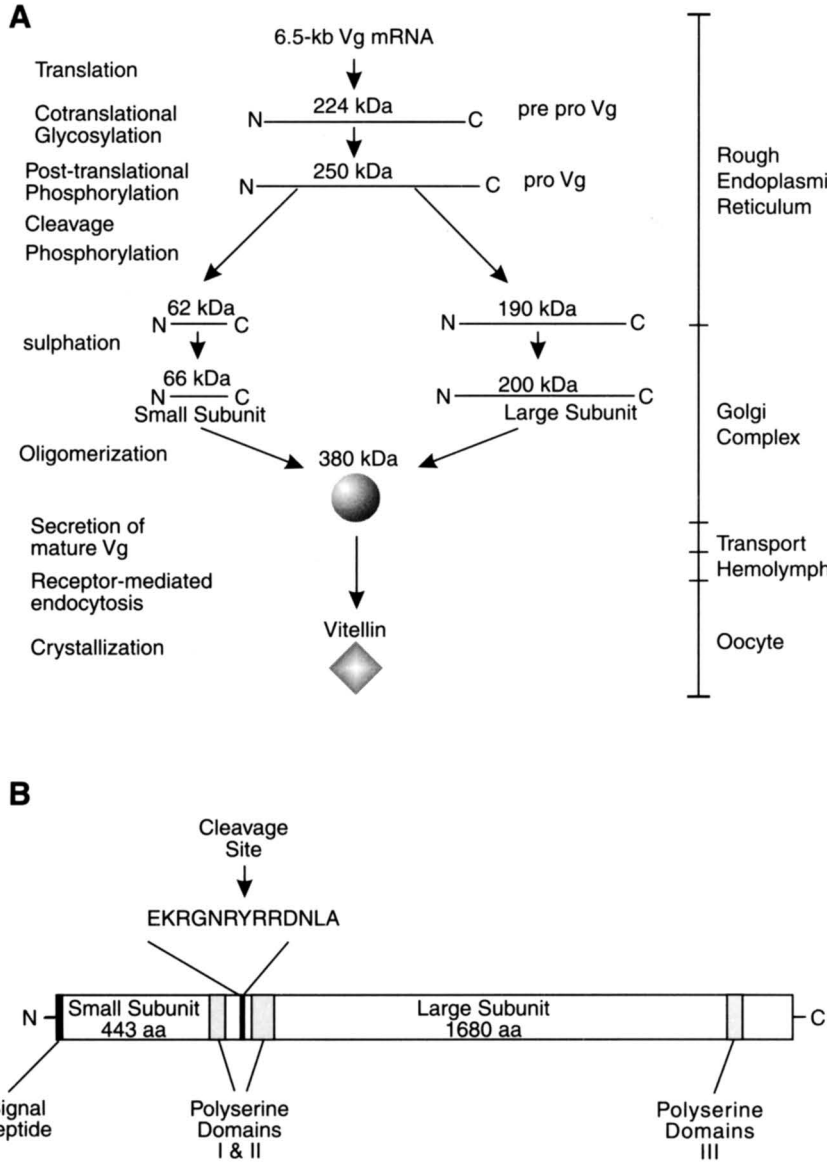

Fig. 8. Co- and post-translational processing (A) of the *Aedes aegypti* pre-pro-vitellogenin (B) from synthesis in the fat body to crystallization as vitellin in the oocyte. The cleavage site is indicated by an arrow in B and amino acid sequence, which is recognized by the cleavage enzyme, vitellogenin convertase, is presented at the base of the arrow (From Sappington and Raikhel, 1998a, with permission)

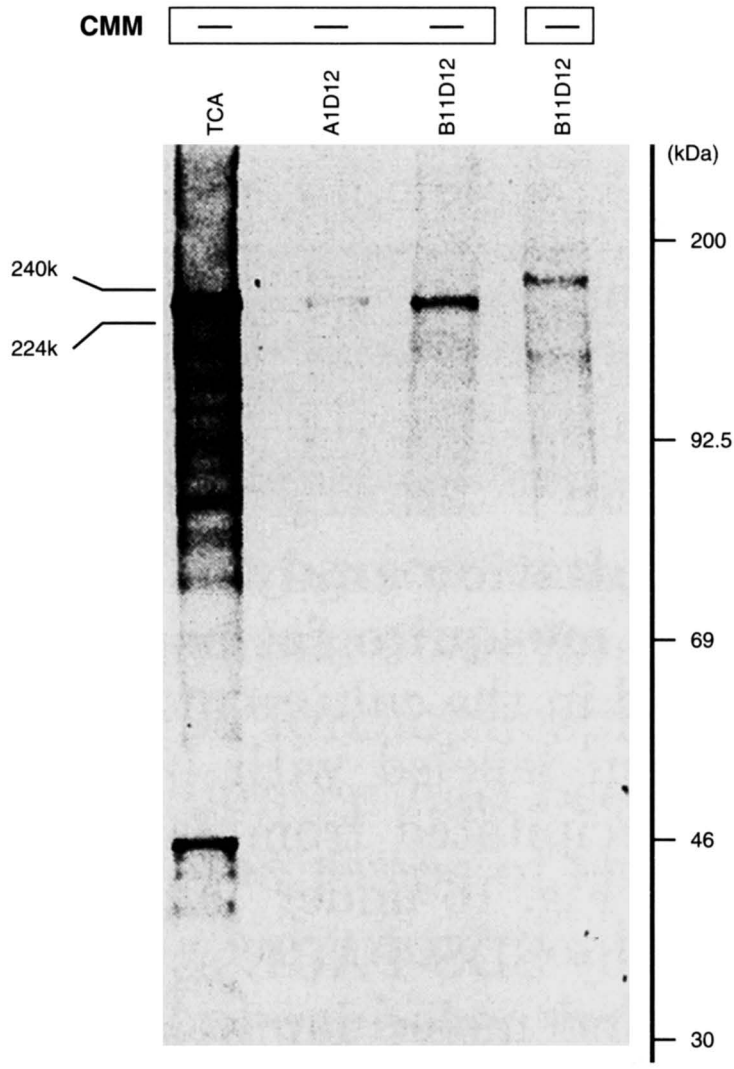

Fig. 9. Immunoprecipitation analysis of cell-free translation products of Poly(A)$^+$ RNA from the vitellogenic mosquito fat body. Poly(A)$^+$ RNA from fat bodies of vitellogenic female mosquitoes was isolated and translated in cell-free translation system in the presence (+) or absence (-) of canine pancreatic microsomal membranes (*CMM*). Products of translation were either immunoprecipitated with individual mAB (A1D12 specific to the large Vg subunit or B11D12 specific to the small Vg subunit) or precipitated with trichloroacetic acid (*TCA*) and analyzed by SDS-PAGE on 5-10% gradient gels under reducing conditions. The gels were fluorographed to visualize the radiolabelled polypeptides. The molecular weights of translation products recognized by Vg mABs are indicated on the left and the molecular weight standards on the right. The standards in order of decreasing *M*, were myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase (Amersham Corp.) (From Dhadialla and Raikhel, 1990, with permission)

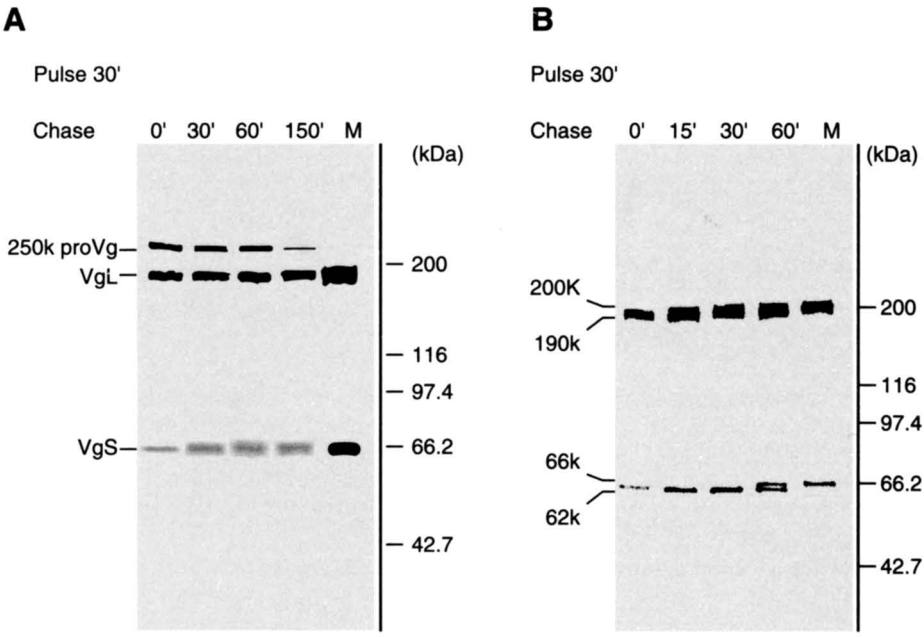

Fig. 10. Time course of [^{35}S]methionine labelling of the Vg precursor (**A**) and Vg subunits (**B**) in mosquito fat bodies analyzed by SDS-PAGE. **A**, previtellogenic fat bodies, stimulated with 20-hydroxyecdysone, were pulse labelled with [^{35}S]methionine for 30 min and chased. **B**, fat bodies from vitellogenic mosquitoes (18-22 h post-blood feeding) were pulse labelled with [^{35}S]methionine for 30 min and chased for the indicated times. Fat body extracts were prepared at the indicated times and the extracts immunoprecipitated with a mixture of mAB to Vg. The immunoprecipitates were resolved by SDS-PAGE and analyzed as described in the legend to Fig. 9. Proteins secreted into the 120-min chase medium (M) were also analyzed. The molecular weights of the Vg polypeptides are indicated on the left and the standards on the right. The standards in order of decreasing *M*, are myosin ß-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalubmin (Bio-Rad). (From Dhadialla and Raikhel, 1990, with permission)

two polypeptide subunits in the Vg precursor molecule consists of a consensus sequence specific for subtilisin-processing endoproteases conserved among insects (Chen *et al.,* 1994). This enzyme, referred to as vitellogenin convertase (VC), has been characterized, and its cDNA has been cloned from a vitellogenic fat body of *A. aegypti* (Chen and Raikhel, 1996). This mosquito vitellogenin convertase is similar in sequence to human and *Drosophila* furins and a *Drosophila* convertase (Barr *et al.,* 1991; Roebroek *et al.*1991, 1992; Hayflick *et al.* 1992). The deduced amino acid sequence of VC has a strong similarlity to a domain structure characteristic of furin-like convertases (Fig. 11). Many biologically important proteins are processed from their respective precursors by proteolytic cleavage. There is remarkable conservation of this event among eukaryotic organisms: precursor cleavage occurs at a paired-basic site with the concensus Arg-X-Arg/Lys-Arg and is accomplished

by convertases, subtilisin-like serine endoproteases. Convertases have been shown to proteolytically cleave and activate precursors of neuropeptides, peptide hormones, membrane receptors, growth factors, and viral proteins (Mizuno and Matsue, 1994; Seidah and Chretien, 1997; Gensberg *et al.,* 1998).

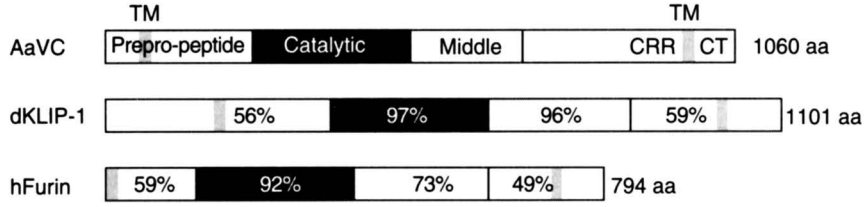

Fig. 11. Schematic representation of the convertases : mosquito vitellogenic convertase (AaVC), *Drosophila* (dKLIP-1), and human furin (hFurin) convertases. Amino acid sequences were aligned on the basis of maximal amino acid similarity. The percentage shown in each domain of dKLIP-1 and hFurin indicates the similarity of that domain to the mosquito VC. CRR, cysteine-rich region; CT, cytoplasmic domain; TM, transmembrane domain. (From Chen and Raikhel, 1996, with permission)

The observation that the co-expression of this enzyme cDNA with the mosquito Vg cDNA leads to a correct processing of the pro-Vg precursor into two cleavage polypeptide products has definitely proved the functional role played by this fat body-specific enzyme in Vg processing (Fig. 12) (Chen and Raikhel, 1996). Essentially, VC is expressed only in the fat body of vitellogenic female mosquitoes. Interestingly, the expression of VC mRNA coincides with that of Vg mRNA in the early stages of vitellogenesis, but it reaches its peak between 12 and 18 hr post-blood meal, while Vg is at 24 hr (Chen and Raikhel, 1996). Cloning of a fat body-specific convertase responsible for the cleavage of mosquito pro-Vg has filled an important gap in our understanding of the biosynthetic pathway of insect Vgs.

VII. POST-TRANSLATIONAL PROCESSING OF VITELLOGENINS

A. Glycosylation

To date, the insect Vgs that have been characterized are glycosylated (Dhadialla and Raikhel, 1990; Kunkel and Nordin, 1985; Osir *et al.,* 1986a; della-Cioppa and Engelmann, 1987; Don-Wheeler and Engelmann, 1997; Giorgi *et al.,* 1998). Glycosylation is a key event in the post-translational processing of Vg precursors to yield the mature yolk polypeptides. An extensive study of the carbohydrate composition of insect Vg was carried out in the cockroach *B. germanica* (Kunkel *et al.,* 1980). A glycopeptide fraction resolved by gel filtration and pronase digestion consisted of a single oligosaccharide with several mannosyl units linked to the asparagine residues of the Vg polypeptides. Using concanavalin A and anti-*Blattella* antibodies, a number of lectin-binding sites were demonstrated in the Vn molecule

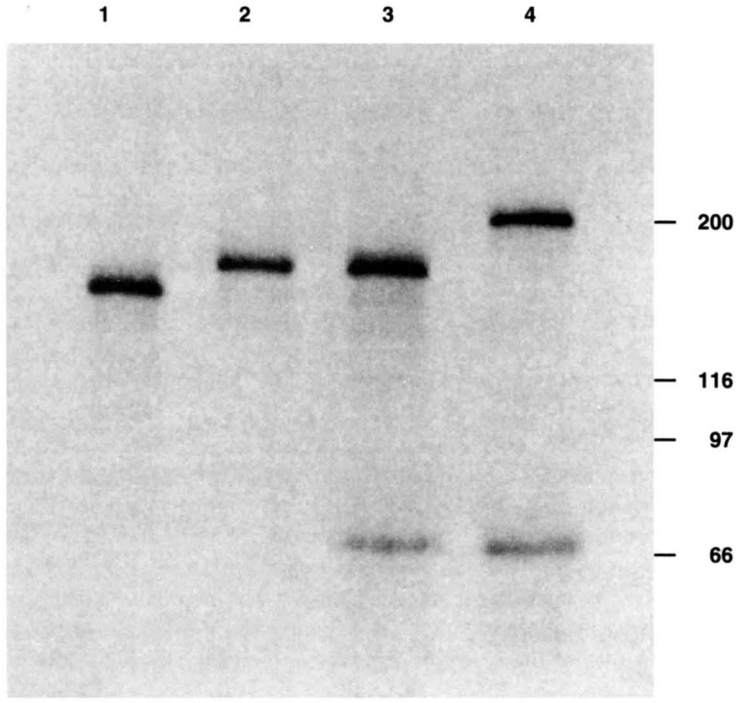

Fig. 12. *In vitro* proteolytic processing of pro-Vg by VC. Truncated Vg cDNA containing the cleavage site (pTVg) was expressed in a coupled TNT system. To demonstrate the proteolytic processing activity of VC, pTVg was coexpressed with pVC. Expression products were immunoprecipitated by monoclonal antibodies specific to the small subunit of mosquito Vg and analyzed by SDS/PAGE: Lane 1, pTVg cDNA; lane 2, pTVg cDNA with canine microsomal membranes; lane 3, pTVg and pVC cDNAs with canine microsomal membranes; lane 4, Vg secreted by cultured fat body. Note appearance of two subunits after corexpression of pTVg with pVC. Markers used are as Fig. 2. (Form Chen and Raikhel, 1996, with permission)

(Kunkel *et al.*, 1980). In the same species, deglycosylation with tunicamycin resulted in Vg accumulation within the fat body, suggesting that glycosylation is an important step in the post-translational modification to yield the mature yolk polypeptides capable of secretion (Wojchowski *et al.*, 1986). A complex role for covalent-bonded carbohydrates in cockroach Vg was reported by Konig *et al.* (1988). Here, glycopeptide prepared by protease digestion of Vg inhibited binding of the intact protein, but after α-mannosidase treatment, which removes most of the mannose residues, these glycopeptides stimulated binding of the intact protein. The authors concluded that high-mannose oligosaccharides were necessary, but not sufficient, for binding of Vg to the receptor. In contrast, however, Osir *et al.* (1986a, b) have reported that the moth *M. sexta* Vg deglycosylated with endoglycosidase-H competed effectively with the native Vg protein and was taken up by the developing oocytes.

In *L. maderae*, all mature Vg polypeptides are glycosylated (Don-Wheeler and Engelmann, 1997), and carbohydrates attached are exclusively composed of *N*-linked mannose oligosaccharides (Konig *et al.*, 1988). These findings have been confirmed by the existence of 13 putative asparagine-linked glycosylation sites (having consensus sequence NXS/T) in a recently cloned Vg cDNA of *L. maderae* (Tufail and Takeda, 2002). Such putative asparagine-linked glycosylation sites were also found in both Vgs (Vg1 and Vg2) of *P. americana* (Tufail *et al.*, 2000; 2001), the numbers of which were 20 and 17, respectively. In *L. maderae*, it was shown that the primary translation product of 190 kDa increased its molecular size to 203 kDa (della-Cioppa and Engelmann, 1987). This increase has been attributed to co-translational glycosylation (della-Cioppa and Engelmann, 1987). Studying the role of glycosylation in Vg synthesis, processing, and secretion from *L. maderae*, della-Cioppa and Engelmann (1987) demonstrated that the blocking of glycosylation with tunicamycin produced a deglycosylated Vg precursor of 190 kDa, which was accumulated within the fat body, suggesting that glycosylation is an important step in subsequent secretion of Vg by the fat body. This study led to the conclusion that although glycosylation does not play any role in Vg synthesis its involvement in the subsequent processing steps (such as phosphorylation, proteolysis) is important.

In vitro inhibition of glycosylation with tunicamycin and carbohydrate extraction by endoglycosidase H have demonstrated that Vg polypeptides in the stick insect *Carausius morosus* are already fully glycosylated prior to secretion from the fat body and that no further modification occurrs upon transfer to the oocyte (Giorgi *et al.*, 1998).

In the tobacco hornworm *M. sexta*, a combination of several chromatographic techniques and proton NMR spectroscopy made it possible to prove that fat body glycosylation involves the transfer of an oligosaccharide unit composed of Glu_3-Man_9-$GlcNAc_2$ to the asparagine residue of the Vg precursor in the moth *M. sexta* (Osir *et al.*, 1986b).

In *A. aegypti*, glycosylation of the Vg precursor was shown to occur co-translationally prior to the proteolytic cleavage. Treatment of female fat bodies with tunicamycin, the drug preventing glycosylation, resulted in the Vg precursor being 226 kDa compared to 250 kDa of untreated pro-vitellogenin (Fig. 13). A similar reduction was also obtained by digesting the two Vg polypeptides separately with endoglycosidase H (Dhadialla and Raikhel, 1990). Scanning densitometry revealed that pro-Vg is heavily glycosylated suggesting that this even occurs co-translationally (Fig. 14). Labelling with radioactive carbohydrate precursors revealed that the Vg subunits of *A. aegypti* were both composed of mannose and glucosamine and that they accounted for up 10,000 in the large Vg polypeptide and for 15,000 in the small Vg polypeptide (Raikhel and Bose, 1988). The amino acid sequence deduced from the mosquito Vg cDNA indicates that up to 17 putative N-linked glycosylation sites may be contained in both Vg polypeptides (Chen et al., 1994). Thus, glycosylation of insect Vg seems to fit the general model common to all eukaryotic glycoproteins whereby oligosaccharides are pre-assembled by progressive addition of their respective nucleotidyl donors and co-translationally transferred to the nascent proteins.

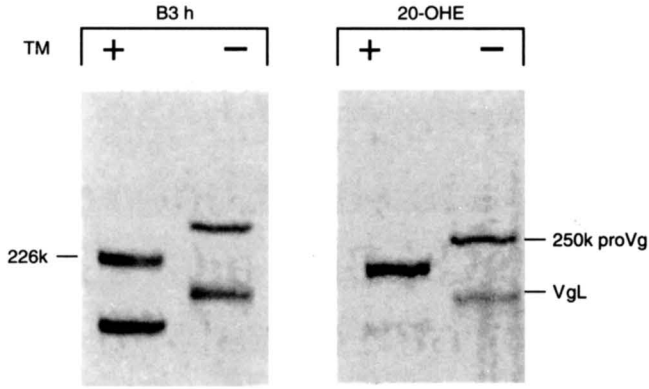

Fig. 13. Effect of tunicamycin on processing of the mosquito Vg precursor. Fat bodies, stimulated either by a blood meal *in vivo* (B3h) or by 20-hydroxyecdysone (20-OHE) *in vitro*, were incubated in the presence (+) or the absence (-) of tunicamycin for 1 h and then labelled with [^{35}S]methionine. Proteins were analyzed by immunoprecipitation and SDS-PAGE as described in the legend to Figure 10. 226k is the unprocessed Vg precursor produced in the presence of tunicamycin. Other abbreviations are as listed in the legend to Figure 10. (From Dhadialla and Raikhel, 1990, with permission).

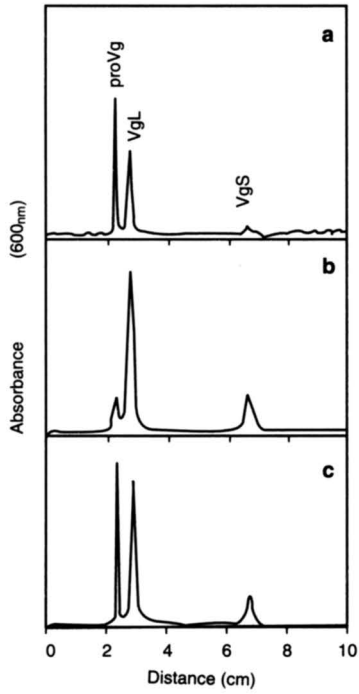

Fig. 14. Analysis of the ratio of the mosquito Vg precursor to its subunits, labelled with different radioactive precursors, by scanning densitometry. Fat bodies, stimulated with 20-OHE as described in the legend to Figure 10, were pulse labelled with **(a)** a mixture of D-[2-^{3}H]mannose and [^{3}H]*N*-acetylglucosamine, **(b)** [^{32}P]orthophosphate, or **(c)** [^{35}S]methionine. Proteins were immunoprecipitated and subjected to SDS-PAGE. Radiograms were scanned at 600 nm using a Gilson spectrophotometer. pro-Vg, 250 kDa Vg precursor, VgL, large Vg subunit; VgS, small Vg subunit. (From Dhadialla and Raikhel, 1990, with permission)

Further processing of the glycosylated Vg entails removal of the distal glucose residues and addition of several monosaccharides to yield the high-mannose oligosaccharides attached of each polypeptide subunit (Wojchowski *et al.*, 1986). Previously, a similar conclusion was achieved by Kunkel *et al.*, (1980), who observed that the elution profile of Vg oligosaccharides does not change during the time intervals between secretion from the fat body and uptake by the oocyte. Both the large and small Vg subunits of *A. aegypti* have also been shown to retain their carbohydrate moieties in unmodified form after internalization and post-endocytic processing in the oocyte (Raikhel and Bose, 1988).

As for the potential role that oligosaccharides may play in the processing and secretion of insect Vgs, Kunkel and Nordin (1985) advanced the hypotheses that they may either (1) confer certain structural features on the glycosylated Vg polypeptides that direct them toward vesicle-mediated exocytosis, or (2) provide a certain stability to the protein subunits such as preventing improper folding prior to secretion. The functional role played by glycosylation in processing the Vg precursor was clearly demonstrated by the observation that treating female fat bodies with tunicamycin prevented not only glycosylation of Vg subunits but also their phosphorylation and sulphation resulting in unprocessed 190,000 and 50,000 polypeptides. In addition, unprocessed Vg was accumulated intracellularly (Fig. 15) (Dhadialla and Raikhel, 1990).

B. Phosphorylation

Insect Vgs are heavily phosphorylated. In the cockroach *L. maderae*, the Vg phosphorylation and its major subunits were studied by [^{32}P]*ortho* phosphate labelling and subsequent SDS-PAGE autoradiography (della-Cioppa and Engelmann, 1987). Following co-translational glycosylation, the 203-kDa Vg precursor is post-translationally phosphorylated to form a precursor polypeptide of 220 kDa in the fat-body endoplasmic reticulum. The analysis of the deduced amino acid sequence of *L. maderae* Vg has also suggested a high level of phosphorylation (Tufail and Takeda, 2002). The predicted phosphorylation sites were serine (S), threonine (T), and tyrosine (Y) residues, and once again the phosphorylation at S-residues among them was particularly high, due primarily to the fact that the polyserine domains exist at both of the termini (Fig. 4) (Tufail and Takeda, 2002). These polyserine domains also existed in both Vgs (Vg1 and Vg2) deduced from cDNAs of *P. americana* and were found to be highly phosphorylated (Tufail *et al.*, 2000, 2001).

In vertebrates, a component of Vg, phosvitin, also contains a polyserine region, and most of the serine residues are phosphorylated. There is evidence that dephosphorylation of Vg reduces its uptake by oocytes (Miller *et al.*, 1982; Dhadialla *et al.*, 1992), suggesting that phosphorylated residues may contribute to the interaction between Vg and its receptor on the oocyte surface.

The existence of polyserine domains is the characteristic of all hemimetabolous insect Vgs, which are at least conserved at the N-terminus of all sequenced Vgs (Fig. 4). The cockroach Vgs are, however, unique in harbouring these domains at the carboxy terminal as well.

The Vgs of holometabolous insects are also heavily phosphorylated. Phosphorylation in *B. mori* was initially studied by *in vivo* injection of [^{32}P]-orthophosphate and *in vitro* incubation with [^{32}P]-ATP. Under these experimental conditions, exposure to the radiotracer resulted in the addition of [^{32}P]-phosphate groups to the serine residues of Vn polypeptides, clearly indicating that the silkworm Vn is a phosphoprotein (Takahashi, 1983). Protein kinase activities capable of phosphorylating Vn were identified in various silkworm tissues, including the ovary, and were shown to accomplish phosphorylation in a cAMP-dependent manner (Takahashi, 1987).

In the mosquito *A. aegypti*, Vg polypeptides have been shown to be phosphorylated by radiolabelling with [^{32}P]-orthophosphate and immunoprecipitation with specific monoclonal antibodies (Raikhel and Bose, 1988). Scanning densitometry revealed that pro-Vg was minimally phosphorylated, while both subunits were heavily phosphorylated. (Fig. 14) (Dhadialla and Raikhel, 1990). In a time-course study, Dhadialla and Raikhel (1990) have demonstrated that phosphorylation of Vg in *A. aegypti* is a post-translational modification that occurs in two steps: first, affecting the Vg precursor prior to its proteolytical cleavage, and, second, affecting the two immature polypeptide subunits. Cloning and sequencing of the Vg cDNA has helped to demonstrate that the mosquito Vg contains three extensive polyserine domains that are similar to but not homologous with those of the vertebrate phosvitins (Chen *et al.*, 1994). However, the extent of polyserine stretches has not been observed in Vgs of other holometabolous insects.

C. Sulphation and Oligomerization

In addition to being glycosylated and phosphorylated, insect Vgs are post-translationally modified by sulpho-conjugation. Protein sulphation was first demonstrated in *D. melanogaster*. Following *in vivo* radiolabelling with inorganic [^{35}S]-sulphate, radioactive label associated with all three yolk polypeptides (Baeuerle and Huttner, 1985). Pulse-labelling experiments with [^{35}S]-sulphate of mouse fibroblasts expressing *Drosophila* YP2 demonstrated that the intracellular site of tyrosine sulphation is the trans-Golgi network (Friederich *et al.*, 1988). This observation is in line with the finding that tyrosylprotein sulphotransferase, the enzyme that catalyzes protein sulphation at tyrosine residues, is specifically oriented toward the lumen in the cisternae of the trans-Golgi region (Baeuerle and Huttner, 1985, 1987; Huttner, 1988).

Sulphation has been demontrated for the mosquito Vg, in which both polypeptide subunits are sulphated (Fig. 16) (Dhadialla and Raikhel, 1990). Using the Golgi-

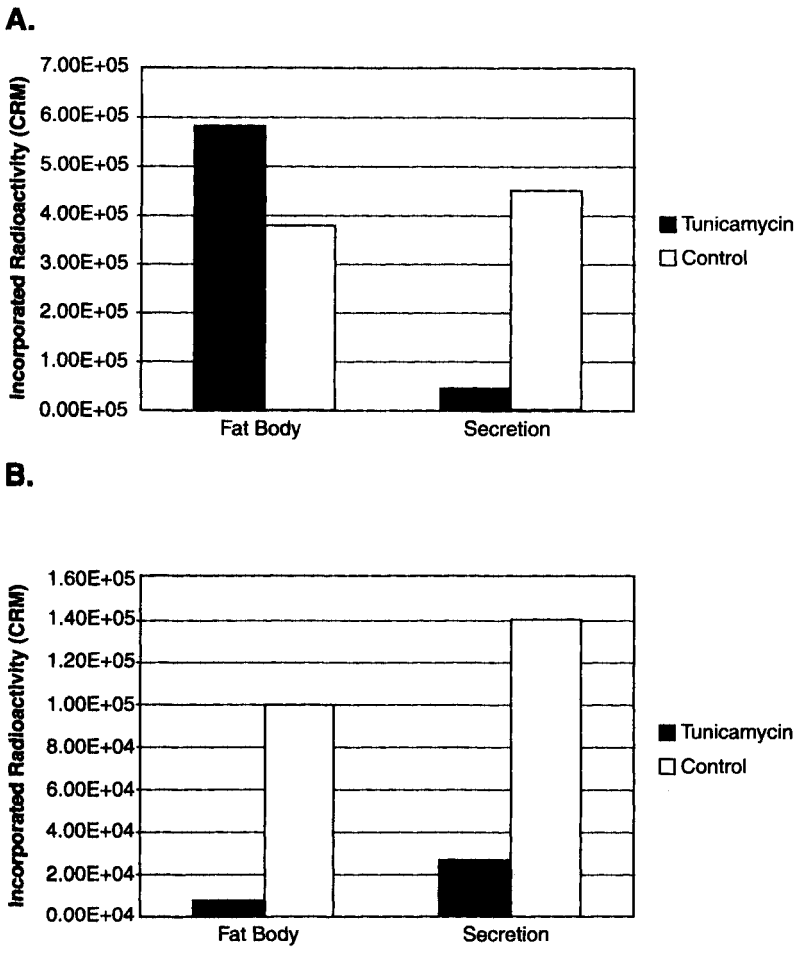

Fig. 15. Effect of tunicamycin and secretion of vitellogenin in the mosquito fat body. Vitellogenic fat bodies (18-22 h post-blood feeding) were preincubated in mosquito Ringer's solution buffered with 25 mN TES, pH 7.5 for 1 h. The fat bodies were pulse labelled for 1 h in incomplete media with [^{35}S]methionine (A), D-[2-^{3}H]mannose (not shown) or [^{32}P]orthophosphate acid (B). Another set of pulse-labelled fat bodies were chased in complete media for another hour. Preincubation and pulse-chase labelling was done in the continued presence or absence of tunicamycin. Vg was immunoprecipated from fat body extracts or chase media and radioactivity incorporated into Vg was determined. Each value is the mean + S.E. from three replicas of 3 fat bodies each. (based on Dhadialla and Raikhel, 1990)

specific inhibitor monensin, it has been established that this step in the post-translational modification of mosquito Vg subunits occurs in the Golgi apparatus as the last step prior to their oligomerization and secretion to yield the mature Vg sub units of 200,000 and 65,000 (Fig. 17) (Dhadialla and Raikhel, 1990). The observation

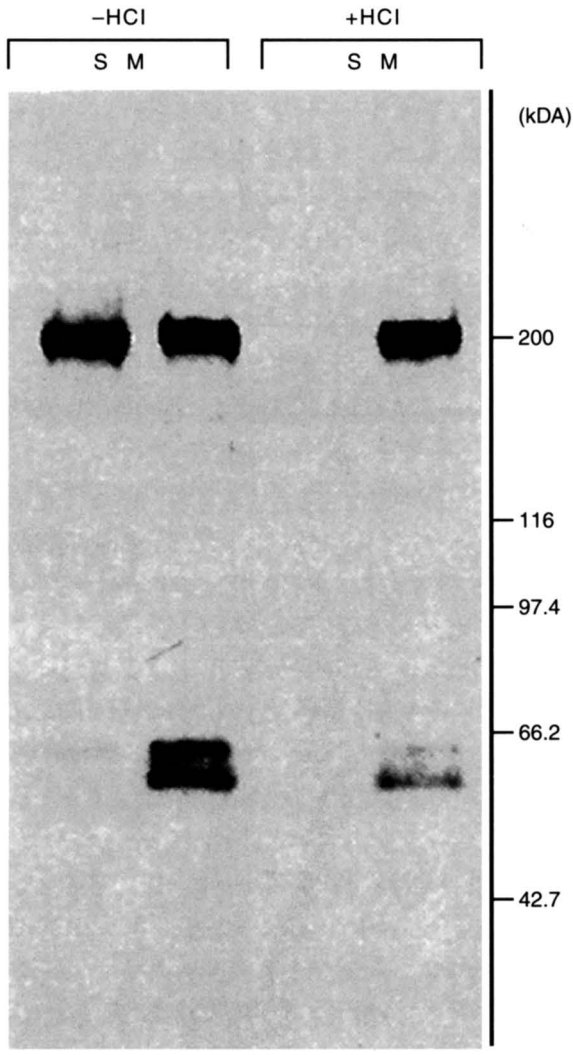

Fig. 16. Sulphation of mosquito Vg. Vitellogenic fat bodies were preincubated in sulphation medium for 30 min before pulse labelling with sodium [^{35}S]sulphate for 2 h in the same medium. Proteins secreted into the medium were used for analysis. Secreted proteins labelled with [^{35}S]methionine were obtained as described in legend to Fig. 2. Labelled Vg was immunoprecipitated from both the samples and analyzed in duplicate by SDS-PAGE as described in legend to Fig. 2. After staining the gels for proteins, half of the gel was subjected to HCl treatment to confirm sulphation of Vg. Gel portions containing sulphated or methionine-labelled Vg are indicated by *S* and *M*, respectively. The molecular weights on the right are of the same standards as mentioned in the legend to Figure 10. (From Dhadialla and Raikhel, 1990, with permission)

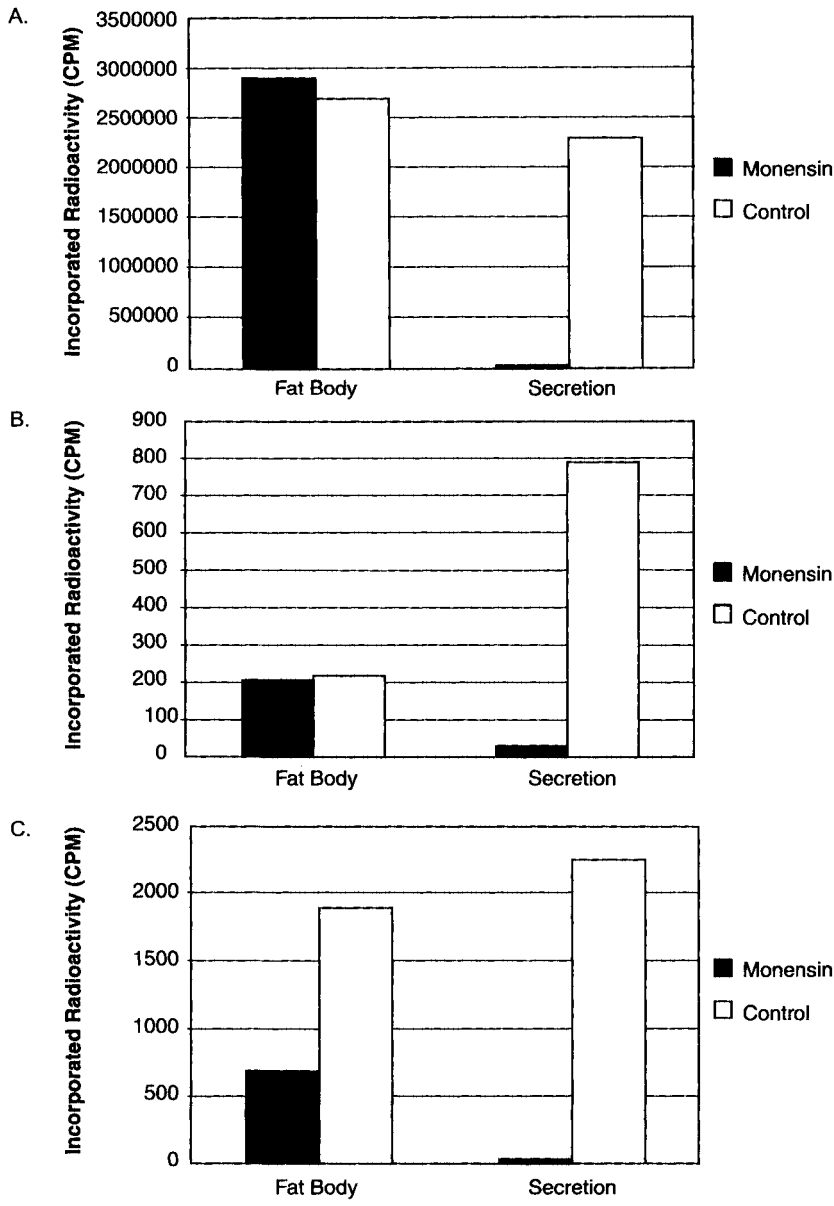

Fig. 17. Effect of monensin on phosphorylation and sulphation of mosquito vitellogenin. After preincubation in the presence or absence of monensin for 30 min, fat bodies were pulsed labelled for 1 h with [^{35}S]methionine (A) or sodium [^{35}S]sulphate (C) and the label chased for 2 h in the continued presence or absence of monensin. In the case of [^{32}P]orthophosphate acid labelling (B), fat bodies were preincubated and pulse labelled with the radiolabel for 30 min each in the presence or absence of monensin. The radiolabel was not chased in this case. After the pulse-chase or pulse periods, proteins in the fat body and the media were immunoprecipitated to detemine the amount of radioactivity incorporated. Each value is a mean of two [^{35}S] or three [^{32}P] determinations. (based on Dhadialla and Raikhel, 1990)