An ovarian follicular epithelium protein of the silkworm (*Bombyx mori*) that associates with the vitelline membrane and contributes to the structural integrity of the follicle

Frederic Kendirgi[a,1], Luc Swevers[b], Kostas Iatrou[a,b,*]

[a] *Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive N.W., Calgary, AB, Canada T2N 4N1*
[b] *Institute of Biology, National Centre for Scientific Research 'Demokritos', P.O. Box 60228, 15310 Aghia Paraskevi (Athens), Greece*

Received 29 April 2002; revised 19 June 2002; accepted 20 June 2002

First published online 2 July 2002

Edited by Ned Mantei

Abstract **We have cloned and functionally characterized a novel protein, BmVMP30, which is synthesized by the cells of the follicular epithelium of the ovarian follicles of the domesticated silkworm *Bombyx mori*, secreted from them and associated with the vitelline membrane. BmVMP30 is a 30 kDa protein that bears limited structural features reminiscent of other insect vitelline membrane proteins. Although BmVMP30 does not share pronounced similarities or signature motifs with other reported proteins, its temporal and spatial expression and its behavior throughout oogenesis suggest that it is a novel member of the insect vitelline membrane protein family. The protein is expressed exclusively in the cells of the follicular epithelium during stages −15 to −1 of vitellogenesis, secreted from them and, ultimately, localized at the junction between the oocyte and the eggshell, where the vitelline membrane is located. Treatment of follicles with an antisense oligonucleotide that encompasses the translation initiation codon results in the production of an N-terminally truncated protein and disruption of the integrity of the follicular epithelium. Antisense oligonucleotide treatment, however, has no effect on the implementation of the developmental program that directs the autonomous progression of ovarian follicles through the last stages of vitellogenesis and choriogenesis.**

Key words: *Bombyx mori*; Silkmoth; Oogenesis; Vitelline membrane; Follicular cell; Antisense DNA

1. Introduction

Oogenesis in the domesticated silkworm (*Bombyx mori*) is characterized by the asynchronous development of follicles resulting in the formation of linear arrays (ovarioles) of progressively maturing follicles [1]. Each follicle is a functional developing unit consisting of three cell types: one oocyte, an anterior cap-forming cluster of seven nurse cells of germ cell origin, and a surrounding monolayer of approximately 5000 epithelial (follicular) cells of mesodermal origin [2]. The follicular epithelium can be viewed as an exocrine gland programmed to secrete unidirectionally (toward the oocyte) the eggshell components, vitelline membrane (VM) and chorion [1,3]. As follicles mature, they move through the ovarioles and undergo successively vitellogenesis (yolk uptake) and choriogenesis (eggshell formation). During the last five days of metamorphosis, one follicle in each of the four ovarioles terminates protein uptake from the hemolymph every 2 to 2.4 h [4], an event that can be reproduced faithfully by in vitro culturing of isolated follicles or strings of follicles inside their ovarioles [5]. This fundamental characteristic enables the isolation of follicles at every stage of follicle development.

Ovarian development is triggered by the steroid hormone 20-hydroxy-ecdysone (20E; [6]). Previously, it has been hypothesized that 20E helps to bring the follicles to a critical developmental stage, where the establishment and implementation of an autonomous developmental program takes place [5]. Indeed, a restricted number of pre-choriogenic follicles cultured in vitro are capable of completing oogenesis autonomously [5]. This critical point of autonomous program implementation is currently estimated at vitellogenic stage −35 (±5) (I. Linström-Dinnètz, L. Swevers and K. Iatrou, unpublished results). Thus, follicles more mature than stage −35 (±5) can complete vitellogenesis and initiate and complete choriogenesis in the absence of factors extrinsic to the follicle, and this suggests that stage-specific regulators must be expressed in a temporal fashion in order for the follicular cells to undergo terminal differentiation.

One of the products of follicular cell differentiation is the VM that lies directly over the oocyte plasma membrane. In contrast to vertebrates in which the VM is secreted by the oocyte [7], in insects such as *Drosophila* and *Bombyx*, VM proteins (VMPs) are synthesized by and secreted from the cells of the follicular epithelium [1,8]. In the silkworm, the secretion of electron dense material at the follicular cell–oocyte interface at day 3 of pharate-adult development marks the initial stage of VM formation [2]. During vitellogenesis, this membrane thickens as electron dense material is added. When the VM approaches its final thickness, layers of overlapping protein plates (chorion) stack on top of it forming a water barrier that shields the oocyte from dehydration [1].

Thus far, no components of the silkworm VM have been analyzed. The present study involves the cloning and functional characterization of a novel protein, BmVMP30, that appears to be involved in the maintenance of the structural integrity of the VM and the follicular epithelium. BmVMP30 is expressed exclusively in the epithelium of middle/late vitel-

*Corresponding author. Fax: (30)-10-651 1767.
E-mail address: iatrou@mail.demokritos.gr (K. Iatrou).

[1] Present address: Department of Cell Biology and Physiology, Box 8228, Washington University School of Medicine, 660 South Euclid Av., St-Louis, MO 63110, USA.

0014-5793/02/$22.00
PII: S0014-5793(02)03003-X

logenic follicles and its expression requires a decreased titer of 20E in the hemolymph. The protein accumulates at the follicular epithelium–oocyte interface as well as at the intercellular junctions between follicular cells. Antisense oligonucleotide-mediated inhibition of gene function [9] reveals that BmVMP30 is involved in the maintenance of the structural integrity of the follicular epithelium and the adjacent VM during middle/late vitellogenesis in vitro, but does not play a regulatory role in the execution of the autonomous program of follicular cell differentiation (completion of vitellogenesis and choriogenesis).

2. Materials and methods

2.1. Animals

The silkworm strain used was a hybrid provided by the Forest Pest Management Institute, Sault Ste-Marie, Canada. Rearing was carried out on artificial diet (Yakuroto Co., Japan) as previously described [10].

2.2. Mid-late vitellogenic (M/LV)-RNA-enriched cDNA library synthesis

Ovarian follicles were lysed in Trizol™ (Gibco-BRL) and total RNA was extracted according to the manufacturer's recommendations. Poly $(A)^+$ RNA was prepared and used to generate double stranded (ds) cDNA as previously described [11]. cDNA fragments ranging between 100 and 500 bp were prepared by digestion with *Alu*I or *Alu*I+*Rsa*I.

To amplify specific ds cDNA populations after a round of subtractive hybridization (SH; below), the ds cDNA pool derived from pre-/early vitellogenic follicles (hereafter referred to as 'driver' cDNA) was ligated to a ds *Eco*RI adaptor, while a ds *Eco*RV adaptor was ligated to the digested middle/late vitellogenic ds cDNA (hereafter referred to as 'tracer' cDNA) [11].

2.3. Subtractive hybridization

Photobiotinylation of the driver cDNA was carried out as previously described [12]. Fifty μg of biotinylated driver was hybridized with 2.5 μg of polymerase chain reaction (PCR)-amplified tracer in 20 μl of hybridization buffer [0.75 M NaCl; 25 mM HEPES, pH 7.3; 5 mM EDTA; 0.1% sodium dodecyl sulfate (SDS)] at 68°C for 20 h. After hybridization, 130 μl of HEPES buffer was added, and the reaction was incubated for an additional 5 min at 68°C prior to cooling down to room temperature. One hundred and fifty μl of streptavidin-coated magnetic beads (Magnespheres™; Promega) in 2× binding buffer (2 M NaCl; 10 mM Tris–HCl, pH 7.5; 1 mM EDTA) were added to remove the biotinylated nucleic acid. After several rounds of addition and removal of Magnespheres, the tracer cDNA underwent another short hybridization to 50 μg of biotinylated driver, incubation with Magnespheres™ and removal of the beads. One set of long (20 h) and short (2 h) hybridizations was considered as one round of SH [11].

To prepare for the next round of SH, 10% of the subtracted tracer cDNA was amplified by PCR. PCR-amplified tracer recovered after three rounds of SH was digested with *Eco*RV and subsequently cloned into the corresponding site of the pBluescript/SK^+ vector (Stratagene) to yield the M/LV-RNA-enriched cDNA library.

2.4. Differential screening of the M/LV-RNA-enriched cDNA library

After plating of the M/LV-RNA-enriched cDNA library, double bacterial lifts were prepared [13] and hybridizations were carried out as described [14] using ^{32}P-labeled tracer or driver cDNA as probes. Autoradiograms of membranes probed with the tracer DNA were superimposed over those probed with driver cDNA. Colonies with tracer-specific signals were reamplified from the template membranes and inserts were isolated by PCR using T7 and T3 primers (Invitrogen).

2.5. Cloning of BmVMP30 cDNA

From a bacterial clone isolated from the M/LV-enriched cDNA library, a ^{32}P-labeled PCR probe was prepared using T7 and T3 pBluescript™ primers [15]. This radio-labeled probe was used to screen a total vitellogenic λgt11 phage cDNA library from *B. mori* follicular cells as described [10]. The inserts of two overlapping phage clones that, in combination, reconstitute the full BmVMP30 open reading frame (ORF), were cloned as *Not*I fragments into pBluescript/SK^+.

2.6. DNA sequencing

PCR dideoxynucleotide sequencing was carried out using ds DNA and the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit™ (Perkin-Elmer Corp.). Sequences were analyzed using DNASIS™ sequence analysis software (Hitachi Software Engineering Co., Ltd, 1993).

2.7. Inverse PCR

Ten μg of *B. mori* genomic DNA, isolated from silk glands as previously described [16], was digested with *Hin*dIII. One hundred ng of *Hin*dIII digested DNA was self-ligated and 10% of the reaction was subsequently amplified by PCR using two divergently oriented BmVMP30 cDNA-specific primers:

RP1 (5′-GTCGGAAACTGAGCCATGACCGGG-3′) and

FP1 (5′-CGTCACTGTTTGGTGGTTGTGGAG-3′ for 35 cycles (1 min at 94°C, 1 min at 60°C and 4 min at 72°C). The PCR product was cloned into the *Eco*RV site of pBluescript/SK^+.

2.8. Northern blot analysis

Northern blot analysis was carried out as described [14]. Hybridization probes were a 0.8 kbp PCR fragment corresponding to the BmVMP30 ORF, a 0.8 kbp *Pst*I/*Xho*I digest of A3 actin gene encompassing the C-terminal part of the ORF and the 3′UTR portion of its mRNA [17], and a fragment corresponding to the 28S rRNA gene prepared from plasmid p*Bm*R161 as described [18].

2.9. Whole mount in situ hybridization

Whole mount in situ hybridization of silkmoth ovarioles using digoxigenin-labeled RNA probes was carried out as described [6]. After hybridization and incubation with anti-digoxigenin HRP-coupled antibodies, digoxigenin-labeled RNA duplexes were detected by diaminobenzidine staining. After color development, the ovarioles were washed in phosphate-buffered saline (PBS), dehydrated in successive ethanol baths, mounted in Permount (Fisher Scientific) and examined using a Zeiss IM35 inverted microscope.

To generate digoxigenin-labeled riboprobes, plasmid DNA containing a PCR fragment representing 980 bp of BmVMP30 cDNA cloned into the *Eco*RV site of its polylinker was linearized with either *Afl*II (for antisense probe) or *Bgl*II (for sense probe) and used as template for in vitro transcription reactions in the presence of digoxigenin-11-UTP (Boehringer Mannheim) using T7 or T3 RNA polymerase (Pharmacia) according to the manufacturer's recommendations (Boehringer Mannheim). After synthesis, the sizes of the riboprobes were reduced to approximately 100 nt by alkaline hydrolysis [19].

2.10. Antibody generation and affinity purification

A portion of BmVMP30 cDNA flanked by the restriction enzymes *Bst*BI and *Eco*RI (from AA 39 until 146) was cloned in frame with the bacterial glutathione-*S*-transferase (GST) ORF in pGEX 5X-3 (Pharmacia) and overexpressed in *E. coli* XL-1 Blue cells. The GST-BmVMP30/*Bst*BI-*Eco*RI fusion protein was subsequently purified on glutathione-*S*-sepharose beads (Pharmacia) and ∼300 μg were used to immunize rabbits (New Zealand White females, Riemans). The collected polyclonal antiserum was pre-adsorbed twice for 2 h at 4°C over an Affi-gel 10™-GST slurry to remove anti-GST antibodies and the supernatant was subsequently passed over an Affi-gel 10™-GST-BmVMP30 column to isolate BmVMP30 specific antibodies. The column was extensively washed and specific antibodies were eluted in acidic elution buffer (0.5% acetic acid, 0.15 M NaCl) prior to neutralization with 0.75 volume of 1 M Tris–HCl, pH 9.

To prepare antibody affinity purification columns, purified GST and GST fusion proteins were dialyzed against coupling buffer (0.1 M MOPS pH 7.5) and 20–30 mg of protein were mixed with 1 ml of Affi-Gel 10™ (BioRad) in a maximum volume of 4.5 ml of coupling buffer. The coupling reaction was carried out for 4 h at room temperature and the Affi-gel 10™ was collected by low speed centrifugation. Two hundred μl of 1 M ethanolamine pH 8 was added to block active esters prior to washing with 10 volumes of Tris-buffered saline (TBS: 20 mM Tris pH 7.5, 0.5 M NaCl). One ml Affi-gel™ columns were stored at 4°C in TBS containing 0.02% w/v Na-azide.

2.11. Tissue fractionation and Western blot analysis

Follicular cell protein extracts were generated from follicles resuspended in 5 μl/follicle of cracking buffer [125 mM Tris (pH 6.8), 5% β-mercaptoethanol, 2% SDS and 4 M urea]. After gentle rocking to prevent oocyte lysis, the supernatant was removed and processed for SDS–polyacrylamide gel electrophoresis (PAGE). The residual oocyte fraction was also pelleted and homogenized to obtain the oocyte fraction of the follicles. Other tissues were homogenized in ice cold PBS prior to being processed for SDS–PAGE.

To isolate eggshells, choriogenic follicles or oviposited eggs were cut in two and washed extensively in PBS to remove the oocyte content. To separate the VMs from the chorion, egg shells were suspended in 95% ethanol and vortexed for 30 s to 1 min. VMs (flocculent material remaining in suspension while the chorion sediments at the bottom of the tube) were recovered manually.

Chorion solubilization was performed in 50 mM Tris–HCl (pH 9), 8 M Urea, 0.07 M dithiothreitol (DTT), 1.5 mM EDTA and 1.5 mM lysine for 3 h at room temperature followed by addition of iodoacetamide and Tris–HCl, pH 8.4, to final concentrations of 0.33 and 0.4 M, respectively. After 15 min of incubation in the dark, the reaction was terminated by addition of β-mercaptoethanol to a final concentration of 1.43 M and the samples were prepared for PAGE.

Proteins were processed for ECL™ Western blotting as described by the manufacturer (Amersham). In Western blots, the BmVMP30 antibodies were used at a 1:5000 dilution while the chorion antibodies [20] were used at 1:1000.

2.12. Immunoprecipitation

Follicles were freshly dissected out from day 5 pupae in ice-cold homogenizing buffer (25 mM HEPES; 0.14 M NaCl; 5.7 M KCl, pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and 250 mM sucrose. The follicles were resuspended in lysis buffer [50 mM Tris, pH 8; 150 mM NaCl; 2 mM PMSF and 10 μg/ml aprotinin (Sigma)] and homogenized on ice. The insoluble fraction was pelleted at 14 000 rpm and resuspended in lysis buffer before the addition of Triton X-100 to a final concentration of 0.2%. After mixing for 15 min at 4°C, the second insoluble fraction was pelleted by centrifugation at 14 000 rpm for 15 min. The supernatant was pre-cleared using 10 μl of rabbit pre-immune serum and 100 μl of 50% protein A agarose bead slurry (Upstate Biotechnology Inc.) pre-washed in 50 mM Tris, pH 8. Immunoprecipitation was carried out using 1 μl (1/500 final dilution) of affinity purified anti-BmVMP30 antibody in the presence of 100 μl of 50% protein A agarose bead slurry. After incubation at 4°C overnight under constant rotation, the beads were removed by low speed centrifugation and washed three times for 10 min in lysis buffer containing 0.2% v/v Triton X-100. The beads were subsequently resuspended in 50 μl of electrophoresis buffer and directly loaded on a SDS–PAGE.

2.13. Whole mount immunocytochemistry

Whole mount immunocytochemistry of silkmoth ovarioles was carried out as described [21]. To detect BmVMP30, samples were first incubated overnight with a 1:1000 dilution of BmVMP30 antibody at 4°C. Incubations with secondary antibody (1:1000 dilution of Cy3-labeled donkey anti-rabbit antibody (Jackson Immuno-Research Lab., Inc.) were also carried out overnight at 4°C. Tubulin staining was carried out using mouse anti-*Drosophila* tubulin antibodies at 1:1000 in combination with fluorescein-labeled anti-mouse antibodies (at 1:1000; Jackson Immuno-Research Lab., Inc.) in similar fashion as for BmVMP30. Whole follicles were analyzed under a confocal Leica DMRE fluorescent microscope at 526 nm and the digital images were recorded and processed using the Princeton Image acquisition software and Adobe Photoshop 5.0, respectively.

2.14. Antisense oligonucleotide inhibition assays

Vitellogenic follicles (stage −20) were dissected from a day 5 pupa and incubated in 14 μl/follicle of Grace's medium (Gibco-BRL) containing 0.5% fetal calf serum and 50 μg/ml gentamycin (Gibco-BRL) under 100% oxygen atmosphere as described [5], with constant amounts of oligonucleotide. After three days, follicles were collected and processed for light or fluorescence microscopy and/or Western blot analysis.

Fully phosphorothioated oligonucleotides with the sequences 5′-CCTTACAGAGCAGTTCCATT-3′ (AS-Met1), 5′-TTACCTTGACGAGACATTCC-3′ (inverse) and 5′-GGCATAGCGTTTGAATTTGTGTTGGG-3′ (AS-Met4) were synthesized by the Core DNA Service, University of Calgary, Canada. Oligonucleotides AS-Met1 and AS-Met4 encompass and extend 15 bases downstream of the first and fourth ATG codon, respectively, of the BmVMP30 ORF. The inverse oligonucleotide corresponds to the inverted sequence of AS-Met1 and was used as a control for specificity.

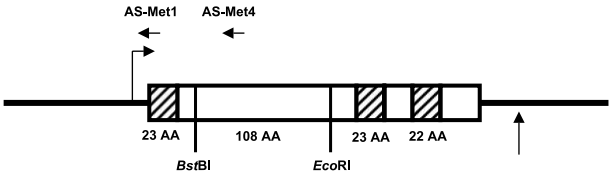


Fig. 1. Structure of *BmVMP30* gene. The structure presented is compiled from the cDNA clones isolated from the vitellogenic cDNA library and the genomic fragment obtained by inverse PCR (GenBank accession number AF 294885; 1456 nt). The transcription start and polyadenylation sites are indicated by a bended arrow and an arrow above and below the map, respectively. In the transcribed sequences, the ORF is displayed as a box while the 5′ and 3′UTR sequences are shown by a black line. Note that the BmVMP30 gene is intronless. The 5′- and 3′-flanking gene sequences (upstream of transcription start site and downstream of polyadenylation site) that were cloned by inverse PCR are also shown by the black line. In the ORF, two C-terminal hydrophobic domains (22–23 AA) and a 23 AA N-terminal region believed to have a signal peptide-like function are highlighted as cross-hatched boxes. Indicated are also the *Bst*BI and *Eco*RI restriction sites that flank the most hydrophilic region (108 AA) of the BmVMP30 ORF used to generate anti-BmVMP30 antibodies. The position of the sequences complementary to the phosphorothioated antisense oligonucleotides AS-Met1 and AS-Met4 used in the antisense inhibition studies is also indicated.

3. Results

3.1. Cloning and characterization of BmVMP30

To isolate cDNAs that are expressed specifically during middle/late vitellogenesis, the M/LV-enriched cDNA library was differentially screened using ^{32}P-labeled cDNA probes obtained from early and middle/late vitellogenic follicles (stages earlier than −40 and −30 to −1, respectively). Colonies hybridizing only to the middle/late vitellogenic probe were isolated and their inserts analyzed by sequencing. After confirmation of the stage specificity and removal of clones containing cDNA sequences that encode the yolk protein produced by the follicular cells (ESP protein; [22]) [23], 74 sequences were obtained of which three corresponded to a novel protein (BmVMP30) which is described here.

To obtain full-length BmVMP30 cDNA, a phage cDNA library generated from silkmoth follicular cell mRNA [10] was screened using as probe the partial cDNA sequences obtained from the subtractive library. Two overlapping cDNA clones were isolated, the longest of which contained a 819 bp complete ORF. This cDNA also contained 119 nucleotides (nt) of 3′ untranslated (UTR) and a 5′ UTR of 11 nt harboring two in-frame stop codons (GenBank accession number AF 294885; Fig. 1).

To obtain extended 5′ and 3′ UTR and flanking sequences of BmVMP30 mRNA, inverse PCR experiments were carried out using as template self-ligated genomic DNA. Using this strategy, a 2.5 kb amplification product was isolated whose sequences contained the 5′ and 3′ ends of the cloned cDNA sequences and adjacent sequences in genomic DNA. Parallel PCR amplification of genomic DNA using primers that were derived from the two ends of the available cDNA clone re-

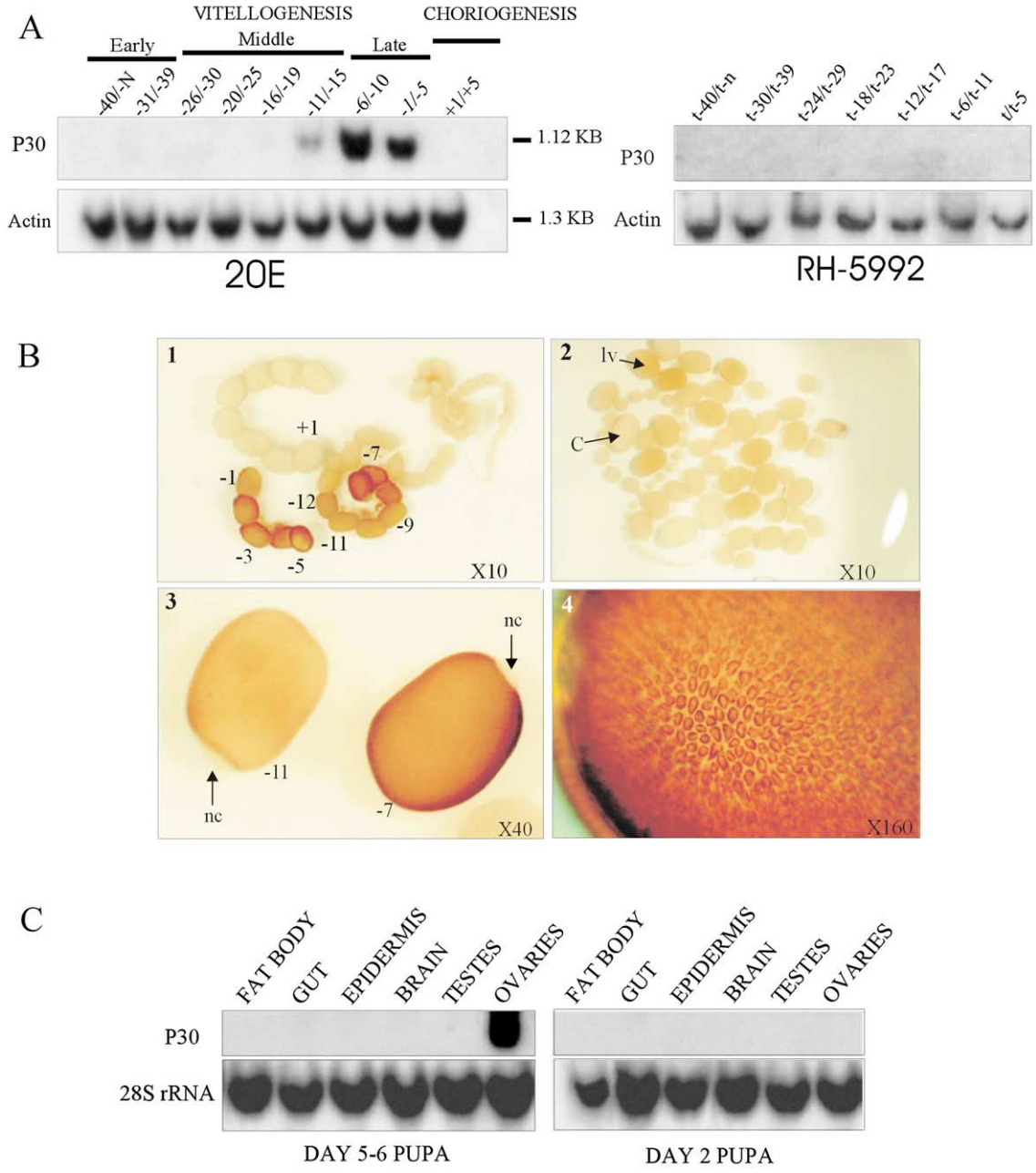


Fig. 2. Developmental profiles of BmVMP30 mRNA. A: Northern blot analysis of BmVMP30 (P30) mRNA expression in staged ovarian follicles during normal development (left) or derived from RH-5992-treated pupae (right). Follicular stages are numbered according to the beginning of choriogenesis (+1; Swevers and Iatrou [5]) in the left panel or numbered according to the terminal (arrested) follicle (t; Swevers and Iatrou [6]) in the right panel. Developmental periods of vitellogenesis (early-middle-late) and choriogenesis as well as the MW of the hybridizing mRNA species are indicated. B: Localization of BmVMP30 mRNA in whole follicles by in situ hybridization. Follicles were hybridized to antisense (panels 1, 3 and 4) or sense (panel 2) digoxigenin-labeled BmVMP30 RNA probes and stained for hybridization signals. Numbers indicate the developmental stages of the relevant follicles: (−) refers to vitellogenic follicles; (+) refers to choriogenic ones; nc, nurse cells; lv, late vitellogenic follicle; c, choriogenic follicle. C: Tissue specificity of BmVMP30 (P30) mRNA expression as determined by Northern blot analysis. Control hybridizations were carried out using a probe specific to 28S rRNA.

vealed that the *BmVMP30* gene does not contain any intronic sequences interrupting the cloned cDNA portion (data not shown). Therefore, the additional sequences acquired by inverse PCR represent additional 5′ UTR and promoter sequences of the *BmVMP30* gene at the 5′ end as well as additional 3′ UTR and 3′ gene flanking at the 3′ end (GenBank accession number AF 294885; Fig. 1).

The conceptual protein encoded by the BmVMP30 ORF has a predicted mass of 30.1 kDa. Two C-terminal hydrophobic domains of 23 and 22 amino acids, separated by a stretch of 22 hydrophilic residues, are prominent features of the protein. A third hydrophobic region was also found at the N-terminus of the conceptually translated protein and likely represents a signal peptide sequence (Fig. 1).

3.2. Developmental profiles of BmVMP30 mRNA

The accumulation pattern of BmVMP30 mRNA in ovarian follicles was analyzed by Northern hybridization using as

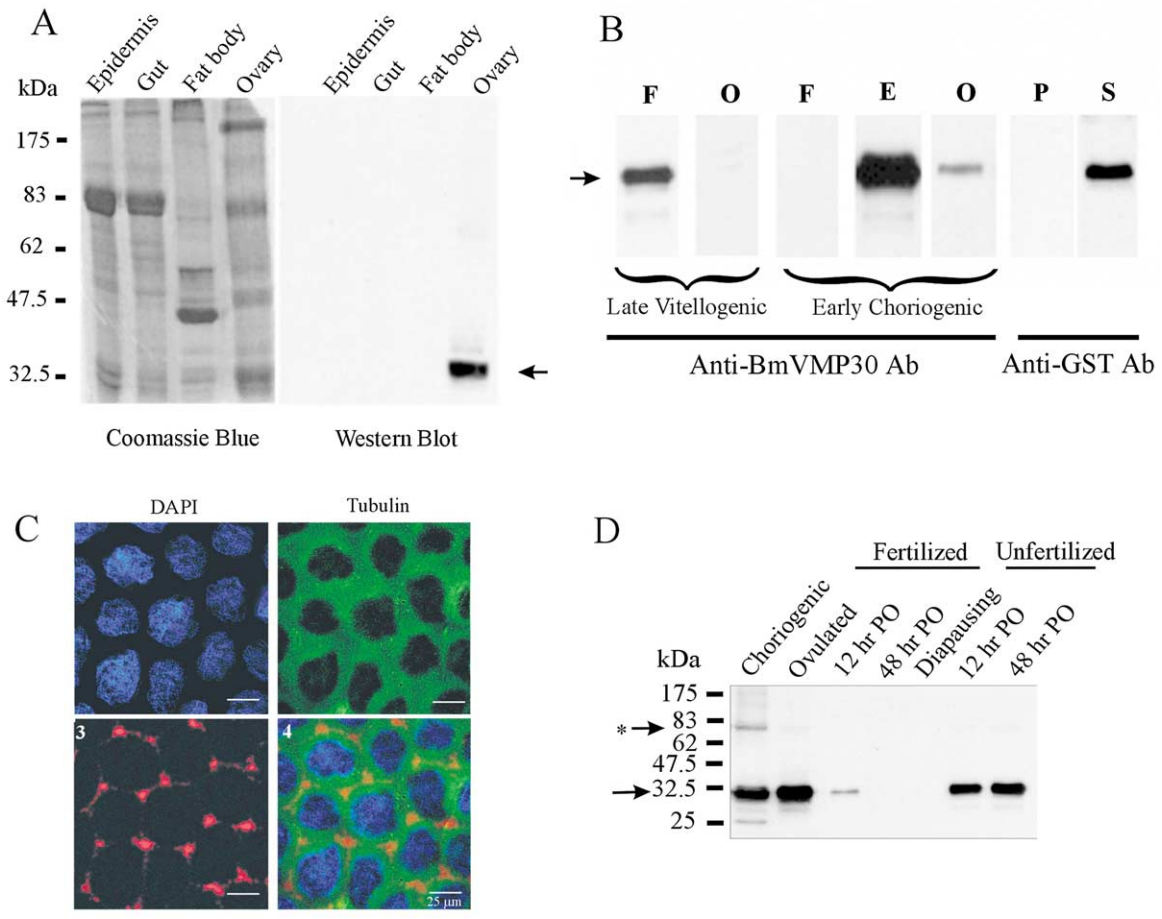


Fig. 3. Developmental profiles of BmVMP30 protein. A: Western blot analysis using BmVMP30 antibodies showing ovary-specific expression of BmVMP30 protein (arrow). Tissues are derived from day 5–6 pupae. MW markers are indicated on the left. B: Western blot analysis of the subfollicular distribution of BmVMP30 protein (arrow) during oogenesis using as probe anti-BmVMP30 antibodies. Follicular cell extracts derived from late vitellogenic, early choriogenic or mixtures of late vitellogenic and early choriogenic follicles (lanes P and S) were immunoprecipitated using anti-BmVMP30 or anti-GST antibodies, as indicated, followed by Western blot analysis using anti-BmVMP30 antibodies. Abbreviations: F, follicular cell extracts; O, oocyte extracts; E, solubilized eggshell; P and S, immunoprecipitate and supernatant, respectively, obtained by GST-antibodies. C: Localization of BmVMP30 protein in the inter-follicular cell spaces of a late vitellogenic follicle (top view). DAPI-staining (1) is shown in blue, while anti-tubulin (2) and anti-BmVMP30 (3) immunofluorescence is in green and red, respectively. Panel 4 shows an overlay of all the fluorescent signals. D: Western blot analysis of BmVMP30 protein (arrow) expression in solubilized eggshells of choriogenic follicles and mature eggs after ovulation and oviposition. Oviposited eggs were obtained from mated ('fertilized') or non-mated ('unfertilized') females. The 70 kDa BmVMP30 complex that exists in the extract from choriogenic follicles is also indicated by an arrow and asterisk. Abbreviation: PO = post-oviposition. MW markers are indicated on the left.

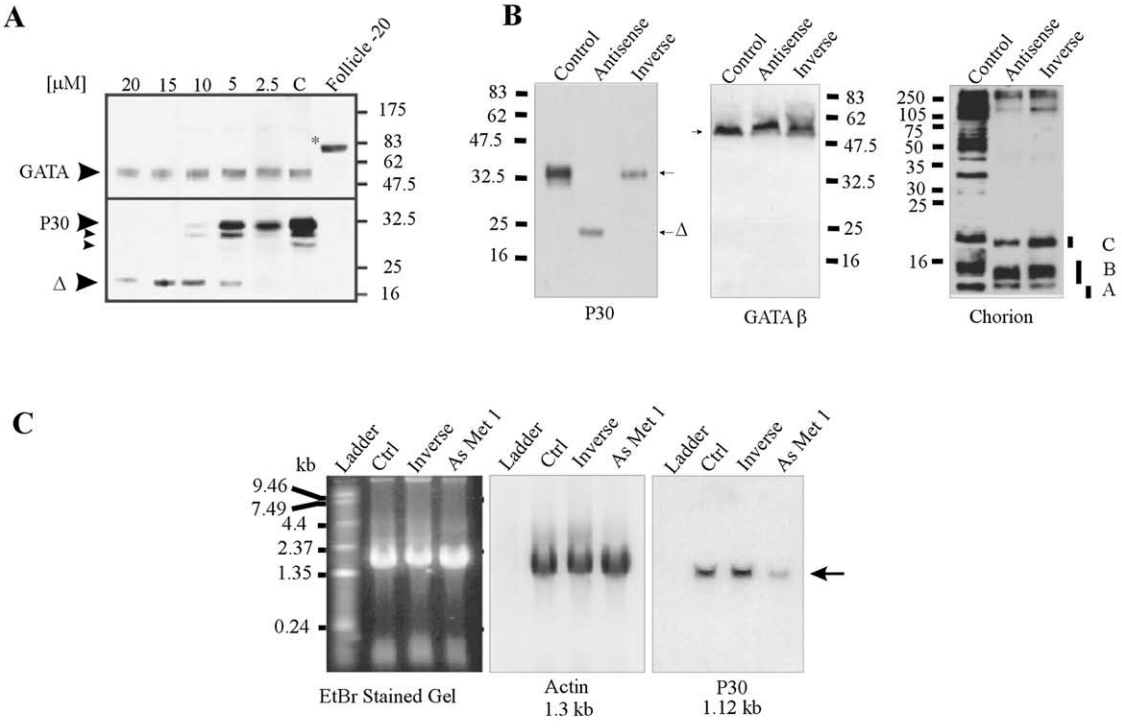
probes RNA isolated from pools of 5–10 follicles along the developing ovariole (Fig. 2A, left panel). The developmental blot revealed that BmVMP30 mRNA is not present in the follicles during early and middle vitellogenesis but appears during the late vitellogenic period (follicle stages −15 to −1). The peak of accumulation occurs at stages −6/−10, and the mRNA levels decline at the end of vitellogenesis (stages −1/−5). During choriogenesis, BmVMP30 mRNA is undetectable.

In situ hybridization experiments were carried out to identify the ovarian cell type in which *BmVMP30* gene expression occurs. As shown in Fig. 2B, BmVMP30 mRNA is present only in the cells of the follicular epithelium but not in the nurse cells. Interestingly, BmVMP30 mRNA appears to be confined to a relatively narrow cytoplasmic ring surrounding the nuclei of the follicular cells (Fig. 2B, panel 4) rather than being uniformly distributed throughout the cytoplasm, as observed for other follicular cell-specific mRNAs [6]. The in situ hybridization experiments also confirmed the developmental profile that was revealed through Northern blot analysis. During vitellogenic period −12 to −9, BmVMP30 mRNA levels are low but they increase sharply during stages −8 to −6. BmVMP30 mRNA declines during the last stages of vitellogenesis and is completely absent from choriogenic follicles (Fig. 2B, panel 1). No specific signals were obtained with the BmVMP30 sense RNA probe (Fig. 2B, panel 2).

To deduce the tissue distribution of BmVMP30 mRNA, Northern hybridizations were carried out using RNA obtained from tissues dissected from day 2 and day 5–6 male and female pharate adults, which have high and low ecdysteroid titers, respectively, in their hemolymph [21] (Fig. 2C). BmVMP30 mRNA is undetectable in all examined tissues of day 2 pupae, including ovaries (whose follicles have not yet attained middle vitellogenesis). This contrasts the situation with ovaries of day 5–6 females where a strong 1.1 kb hybridizing mRNA band is detected in ovarian tissue. During the same period, no BmVMP30 mRNA is detectable in any of the other tissues examined, including brain, testes, epidermis, fat

body and gut (Fig. 2C). Thus, BmVMP30 mRNA is expressed exclusively in ovarian tissue during pharate adult development when ecdysteroid titers are declining in the hemolymph.

Ovarian development in the silkmoth is induced by 20E at the beginning of pupation [24]. To investigate in more detail the hormonal control of *BmVMP30* gene expression during oogenesis, the accumulation pattern of BmVMP30 mRNA in ovarioles-treated with the non-steroidal 20E-agonist RH-5992 was examined (Fig. 2A, right panel). Although RH-5992 induces the early phases of the ecdysone response in a similar fashion as 20E, the RH-5992-induced response is not sustained. Because of this, the expression of genes requiring a decline in hormone titer for induction is suppressed and a developmental arrest ensues [25]. Injection of RH-5992 into ligated silkmoth pupal abdomens induces ovarian follicle development (initiation of vitellogenesis) but is followed by a



developmental arrest at mid-vitellogenesis (around stage −20; [24]). As shown in Fig. 2A (right panel), no BmVMP30 mRNA accumulates in ovarioles induced by RH-5992, suggesting that the expression of BmVMP30 during late vitellogenesis requires the absence of active 20E signaling in the follicles.

3.3. Developmental profiles of BmVMP30 protein

To examine the tissue distribution and expression patterns of BmVMP30, polyclonal antibodies raised against it were used in Western blot and immunocytochemistry studies. In Western blots of extracts prepared from various tissues of female pharate adults (day 5–6 pupae), a single protein band of an approximate mass of 32 kDa was detected in ovarian extracts, while no signals were detectable in the extracts obtained from the epidermis, gut or fat body (Fig. 3A). When extracts prepared from developing follicles were examined, the 32 kDa polypeptide was found to be present in late vitellogenic follicular cell extracts (stages −17 to −1; Fig. 3B) but absent from follicular cells of early choriogenic follicles (stages +1 to +10). The presence of BmVMP30 in follicular cell extracts of late vitellogenic follicles and its absence from the extracts obtained from the follicular cells of choriogenic follicles is in agreement with the accumulation patterns of BmVMP30 mRNA (Fig. 2A).

Despite the absence of BmVMP30 protein (and mRNA) from the cells of the follicular epithelium during choriogenesis (mild conditions of protein extraction), the presence of the 32 kDa protein was evident in the solubilized eggshells (harsh conditions of protein extraction) of choriogenic and ovulated follicles as well as in those of unfertilized eggs (Fig. 3B,D). By contrast, only minor BmVMP30 immunoreactivity was found in oocyte extracts, probably resulting from contaminating eggshell fragments (Fig. 3B, lane 5). Thus, it appears that, as oogenesis progresses, BmVMP30 is secreted by the follicular epithelium towards the oocyte and forms part of the eggshell structure which includes the VM and the chorion. When VMs were mechanically dissociated from the chorion of ovulated eggs and subjected to Western blot analysis, both the VM and the chorion fractions were found to contain BmVMP30 protein (data not shown).

In the chorion fraction, new immunoprecipitable proteins, of approximate sizes 70 and 150 kDa, were also detected (Fig. 3D). Attempts to remove the latter by increasing the concentration of reducing agent and incubation time were unsuccessful (data not shown). Thus, the slow-migrating complexes may result from the cross-linking of BmVMP30 to eggshell components, presumably chorion proteins. In contrast, two-dimensional SDS–PAGE analysis of follicular cell proteins of middle/late vitellogenic follicles, in which the first dimension was run in the absence of β-mercaptoethanol and the second one after reduction, has shown that BmVMP30 can form higher order complexes that are completely dissociated to monomer sizes in the second dimension (data not shown). Presumably, these complexes involve disulfide bridge formation and BmVMP30 becomes more permanently cross-linked during chorion formation.

Upon fertilization, a drastic decrease was observed in the amount of eggshell BmVMP30 at 12 h post-oviposition (PO) with BmVMP30 becoming undetectable by 48 h PO and in diapausing eggs (Fig. 3D). By contrast, this decrease in BmVMP30 protein was not observed in unfertilized eggs.

Finally, immunocytochemistry localization studies showed that BmVMP30 is localized in middle/late vitellogenic follicles at the junction between the follicular epithelium and the oocyte (Fig. 4E, panel 3). Confocal microscopy using Cy3-labeled secondary antibodies enabled the localization of this protein in the intercellular spaces between neighboring follicular cells and at the follicular cell–oocyte interface (Figs. 3C and 4E, panel 3). When the follicular epithelium is viewed perpendicular to the follicle surface and the optical section is at the middle of the columnar follicular cells, a punctate pattern of BmVMP30 staining is observed (Fig. 3C). Thus, BmVMP30 appears to be secreted at the lateral membranes of the follicular cells but not on the entire lateral surface. It is known that the follicular cells are attached to each other through cell–cell adhesion junctions but that spaces exist, through which proteins can reach the oocyte surface from the hemolymph (e.g. vitellogenin) [26]. The punctate pattern at this optical section can therefore be explained by assuming that BmVMP30 is secreted in the discrete lateral spaces between the follicular cells. At the apical side of the follicular cells and when viewed tangentially to the oocyte surface, on the other hand, a uniform staining pattern is observed (Fig.

←

Fig. 4. Effects of antisense oligonucleotides on BmVMP30 expression and function in vitro. A: Western blot demonstrating the effects of different concentrations (2.5 to 20 μM) of BmVMP30 antisense oligonucleotide (AS-Met1) on BmVMP30 (P30) protein expression after in vitro culture of middle/late vitellogenic follicles (stage −20) for 48 h. No BmVMP30 was initially detected in mid-vitellogenic follicle −20 (rightmost lane). Arrows point to truncated BmVMP30 proteins presumed to originate from the use of alternative translation start sites; 'Δ' indicates the presence of 21 kDa ΔBmVMP30 protein. The presence of BmGATAβ (55 kDa) was also assessed on the same blot using BmGATAβ N-terminal antibodies [38]. Note that this antiserum recognizes an additional unknown ∼70 kDa protein in −20 follicles (star). Abbreviation: C, control −20 follicles cultured in vitro for 48 h in the absence of antisense oligonucleotide. Molecular weight markers are indicated at right. B: Western blot demonstrating the effects of 15 μM of antisense or inverse oligonucleotide on BmVMP30 (P30) (left), BmGATAβ (middle) or chorion protein (right) expression after in vitro culture of middle/late vitellogenic follicles (stage −20) for 48 h. The presence of BmVMP30, ΔBmVMP30 (Δ) and BmGATAβ proteins are indicated by arrowheads while the different chorion protein families (A, B, C) are indicated by bars. Numbers refer to molecular weights (kDa) of protein markers. C: Northern blot analysis demonstrating the effects of 15 μM of antisense (AS-Met1) or inverse oligonucleotide on BmVMP30 (P30) and actin mRNA expression after in vitro culture of middle/late vitellogenic follicles (stage −20) for 48 h. The specific reduction of BmVMP30 mRNA by antisense oligonucleotide treatment is indicated by an arrow. RNA MW markers are indicated on the left. D: Light microscopy observations of the integrity of the follicular epithelium and the oocyte of developing follicles following treatment with 15 μM of antisense oligonucleotide (1–3) or inverse oligonucleotide (4 and 5) or without treatment (6 and 7) for 48 h in vitro. Note the abnormal distribution of the yolk within the antisense oligonucleotide-treated follicles (white arrow in 3). E: Fluorescence microscopy observations of the integrity of the follicular epithelium and the oocyte of developing follicles following treatment with 15 μM of antisense oligonucleotide (2, 4, 6) or inverse oligonucleotide (1, 3, 5) for 48 h in vitro. Follicles were stained with DAPI (1 and 2) or stained with fluorescent anti-BmVMP30 (3 and 4) or anti-tubulin (5 and 6) antibody complexes. Note the disruption of the follicular epithelium characterized by gaps in the nuclei staining (2), diffuse localization of BmVMP30 epitopes within the follicular cells (4) and a non-homogeneous tubulin staining (6). The presumed location of BmVMP30 and the vitelline membrane is marked by a white arrow in 3 (bar = 50 μm).

4E, panel 3). Co-localization studies using anti-tubulin antibodies (to stain the cytoplasm of the follicular cells) and DAPI (to stain the nuclei) confirm that BmVMP30 is concentrated at the intercellular junction between three or four follicular cells and not throughout the cytoplasm of the epithelial cells (Fig. 3C).

3.4. Analysis of *BmVMP30* function during oogenesis

To analyze the function of BmVMP30 during oogenesis, the approach of antisense oligodeoxynucleotide-mediated inhibition of gene expression was employed [9]. Antisense (AS-Met1) and control inverse phosphorothioated oligonucleotides were designed that encompassed the mRNA region containing the translation initiation codon for BmVMP30 (Fig. 1). In an attempt to block the translation of endogenous BmVMP30, follicles at vitellogenic stage −20 were incubated for 48 h in vitro (i.e. a time period that allows the follicles to enter choriogenesis) with various concentrations of the AS-Met1 oligonucleotide. The viability of the follicles was assessed by analyzing the incorporation of ^{35}S-labeled methionine into protein. Concentrations of 20 μM were found to be toxic, while at 15 μM there was significant incorporation of radioactivity into protein that was comparable to control follicles.

The impact of increasing amounts of phosphorothioated antisense oligonucleotide on BmVMP30 protein expression was assessed by Western blot. When the concentration of AS-Met1 increased from 2.5 to 20 μM, the amounts of full-length BmVMP30 protein diminished and reached undetectable levels at 15 μM (Fig. 4A,B). Concomitantly, a 21 kDa protein that is recognized by the antibodies appeared (ΔBmVMP30) whose accumulation was proportional to the increase in the concentration of AS-Met1.

The accumulation of ΔBmVMP30 (Fig. 4A,B) may be explained by the usage of an internal ATG start codon, downstream of the first site that is putatively blocked by the antisense oligonucleotide. In contrast to the disruption of BmVMP30 protein accumulation, the expression of the chorion gene regulator BmGATAβ (M. Lunke and K. I., unpublished results; [14]) was not adversely affected by the presence of oligonucleotide in the culture medium (Fig. 4A,B).

Attempts to reproduce these results in vivo were unsuccessful (data not shown), because the daily injection of antisense oligonucleotides at the optimal concentration mentioned above yielded no inhibition of BmVMP30 expression and no noticeable phenotype (see also further below). Presumably in vivo injected oligonucleotides are rapidly cleared from the hemolymph by non-ovarian tissues such as fat body and Malpighian tubules and only limited amounts may be taken up by the developing follicles.

When the inverse oligonucleotide was used at similar concentrations as AS-Met1, no truncated BmVMP30 protein was detected in Western blot analysis (Fig. 4B, left). We noted, however, that the amount of full-length BmVMP30 was reduced upon treatment with inverse oligonucleotide at high concentration (15 μM; Fig. 4B, left) and therefore cannot rule out a slightly toxic effect of phosphorothioated oligonucleotides on in vitro cultured follicles. It is possible that the addition of phosphorothioated oligonucleotide in the medium causes a slight delay in development that is reflected in a reduced accumulation of BmVMP30 protein. Interestingly, while incubation with the AS-Met1 oligonucleotide has a pronounced effect on the expression of BmVMP30 in follicular cells and, as described below, causes the disorganization of the follicular epithelium, developmental progression of the follicular cells through the end of vitellogenesis and into choriogenesis was not affected; Western blot analysis showed both BmGATAβ and chorion protein expression in follicular cells of antisense and inverse oligonucleotide-treated follicles to remain largely unaffected relative to untreated follicles (Fig. 4B, middle and right panels).

Because we hypothesized that the 21 kDa protein detected by the BmVMP30 antibody in follicles that were subjected to antisense AS-Met1 oligonucleotide treatment may have reflected the use of the fourth methionine codon in the BmVMP30 ORF, a second antisense oligonucleotide was designed against this ATG codon (AS-Met4; Fig. 1) in an attempt to knock out completely the expression of *BmVMP30*. However, we found that the use of AS-Met4 did not affect the translation of the protein, while in experiments in which AS-Met1 and AS-Met4 were combined, no additional effects on BmVMP30 expression could be observed compared to the use of AS-Met1 alone (data not shown).

To further clarify the mechanism by which AS-Met1 blocks the expression of BmVMP30 protein, Northern blot analysis was carried out. As shown in Fig. 4C, the amount of BmVMP30 mRNA was drastically reduced following treatment with AS-Met1 oligonucleotide, while the levels of a control mRNA (actin) were unaffected. The presence of inverse oligonucleotide, on the other hand, did not result in a reduction of BmVMP30 mRNA (Fig. 4C). These results indicate that the AS-Met1 oligonucleotide also reduces the levels of BmVMP30 mRNA in the follicles, possibly by targeting the mRNA for degradation by RNaseH [27].

In contrast to follicles incubated either with inverse oligonucleotide or with culture medium alone, follicles cultured in vitro in the presence of 15 μM antisense oligonucleotide show a distinct phenotype. As seen in panels 1–3 of Fig. 4D, the follicular epithelium of follicles incubated with the antisense oligonucleotide is hardly distinguishable, and the follicle is held together by the outer basal lamina. Moreover, the oocyte integrity is compromised, as illustrated by the internal 'bleeding' of the yolk within the boundaries of the follicle (panels 1–3 of Fig. 4D). DAPI-staining of the follicles shows that gaps exist in the follicular epithelium suggesting that the follicular cells are no longer arranged in an orderly manner (panel 2 of Fig. 4E). Immunofluorescent experiments using BmVMP30 antibodies show that unlike the wild-type protein, the truncated BmVMP30 protein that is induced following antisense nucleotide treatment (Fig. 4A,B) is unable to localize at the junction between the follicular cells and the oocyte (compare panel 4 with panel 3 in Fig. 4E). Rather, the fluorescent signal derived from truncated BmVMP30 protein remains diffused throughout the cytoplasm of the follicular cells (panel 4 of Fig. 4E). The accumulation into the cytoplasm can be explained by the lack of an N-terminal signal peptide sequence in ΔBmVMP30 resulting from the blockage of the first translation initiation codon of the BmVMP30 ORF by the antisense oligonucleotide AS-Met1. Finally, staining with tubulin antibodies confirms the loss of the follicular epithelium's structural integrity in follicles-treated with the antisense oligonucleotide (panel 6 of Fig. 4E). By contrast, no distinguishable phenotype could be discerned in follicles cultured in the presence of the inverse oligonucleotide (panels 4 and 5 of Fig. 4D) relative to untreated follicles (panels 6 and 7 of Fig. 4D).

DAPI-staining as well as imunofluorescent detection of BmVMP30 and tubulin in inverse oligonucleotide-treated follicles also did not show any irregularities in the organization of the follicular epithelium (panels 1, 3 and 5 of Fig. 4E).

4. Discussion

To understand the developmental program that is initiated at mid-vitellogenesis and results in the autonomous implementation of choriogenesis in the follicular epithelium [5], a M/LV-RNA-enriched cDNA library was differentially screened for the presence of cDNAs whose mRNAs become expressed during stages −30 and later of follicular development. Our screen resulted in the isolation of the cDNA encoding a novel protein of 30 kDa, BmVMP30.

Our data show that BmVMP30 becomes incorporated into the silkmoth eggshell, most likely as a component of the VM. The expression pattern of BmVMP30 and its subfollicular localization during oogenesis and after egg-laying agrees very well with the predicted behavior of VMPs. BmVMP30 mRNA levels are high during the middle/late stages of vitellogenesis but become subsequently undetectable in choriogenic follicles (Fig. 2A,B) such that the entire pool of BmVMP30 must be synthesized by the end of vitellogenesis. This behavior is reminiscent of the *Drosophila* VMPs, which are expressed during a narrow window of oogenesis, S9-S10 (prior to eggshell synthesis) and constitute the entire pool of VMPs for the rest of development [7,28,29]. After translation, BmVMP30 protein is secreted outside the epithelial cells toward the oocyte because it remains associated with the egg after ovulation, i.e. after removal of the follicular epithelium that synthesizes BmVMP30. Immunolocalization studies point to the concentration of BmVMP30 in the intercellular junction between follicular cells as well as at the oocyte–follicular epithelium interface (Figs. 3C and 4E, panel 3) and, in Western blot analysis, BmVMP30 is found in total eggshell extracts as well as isolated VMs from follicles and mature eggs (Fig. 3B,D; data not shown). Most notably, chorion extracts contain BmVMP30 immunoreactive proteins of higher molecular weight, which probably reflect the cross-linking of BmVMP30 either to itself or to other proteins that are incorporated in the eggshell (Fig. 3D).

During vitellogenesis, a functional VM is not formed in the follicles as it would not allow transport of the yolk proteins to the oocyte. In *Drosophila*, VM components accumulate initially between oocyte and follicular epithelium as small vesicles, the vitelline bodies, and formation of a functional VM is initiated just prior to choriogenesis through vesicle fusion and hardening [7]. Thus, the presence of BmVMP30 in the intercellular spaces between the follicular cells and the oocyte in vitellogenic follicles (Figs. 3C and 4E, panel 3) likely reflects its secretion by the follicular cells and its subsequent accumulation in similar vitelline bodies before the formation of a functional membrane at the termination of vitellogenesis.

The finding that BmVMP30 becomes undetectable upon fertilization is in agreement with its role as a VM component. It is well known that immediately after fertilization, an extensive reorganization occurs at the oocyte surface and that the 'fertilization membrane' is formed [30]. Electron microscopy studies have shown that an extensive rearrangement of the VM also takes place following sperm entry in *Bombyx* egg, culminating in the formation of the fertilization membrane [31]. Besides secretion of new material by the oocyte, changes in the VM following fertilization seem to involve also proteolytic processing or degradation of its components. Proteolytic processing of BmVMP30 may result in loss of the hydrophilic region at the N-terminus and loss of the epitopes recognized by the antibody used in our studies.

In dipteran insects, the characterized VMPs are proline, serine, glycine and alanine rich [8]. They possess an N-terminal hydrophobic region believed to act as a signal peptide and contain a highly conserved 38 AA hydrophobic core domain (VM domain) towards the C-terminus [32]. This region is so highly conserved that cross-hybridization between the genes can be achieved [32,33]. The small genes encoding these proteins also lack introns [7]. Although BmVMP30 does not have significant sequence identity with dipteran VMPs, including the VM domain that is conserved among Diptera (data not shown), it does share with them a distinct overall proline distribution, a signal peptide, a similar general hydropathy profile and the lack of introns in the gene structure (Fig. 1).

BmVMP30 does not become expressed in ovaries that are induced by the ecdysone agonist RH-5992 (Fig. 2A, right panel). Because treatment with RH-5992 causes persistent ecdysone signaling in the follicles, it induces a developmental arrest at stages requiring a decline in ecdysone signaling for their further development. Thus, BmVMP30 belongs to the class of genes in the ecdysone regulatory hierarchy that are expressed following a decline of ecdysone titer in the hemolymph. It is possible that BmVMP30 expression is induced by BmFTZ-F1, a nuclear receptor that is known to be induced during falling ecdysone titers and is expressed in ovarian follicles concomitantly with BmVMP30 [24].

When the hormonal regulation of BmVMP30 is compared with that of the dipteran VMs, it appears that, in contrast to the situation in *Bombyx*, the mosquito VM 15a genes are induced by ecdysteroids in vivo and in vitro [33,34]. This probably reflects differences between the mosquito and the silkmoth regarding the general control of ovarian development by 20E [24,35].

Antisense oligonucleotide inhibition of gene function [9] shows that BmVMP30 is required for the maintenance of follicle integrity. The disruption of the integrity of the follicular epithelium and oocyte by inhibition of BmVMP30 function (Fig. 4D,E) can be explained by the concomitant disruption of the VM which provides a skeletal support for both cell types during the assembly of the eggshell at choriogenesis. However, the basal lamina remained intact and kept the follicle from disintegrating. This result reinforces the hypothesis that BmVMP30 is associated with the oocyte membrane and the follicular epithelium and that it is essential for the connection between follicular epithelium and oocyte.

The phenotype observed by treating *Bombyx* follicles with antisense oligonucleotide directed against BmVMP30 differs from the phenotype observed for VMP mutants in *Drosophila*. In null mutants for the VMP sV23, no effects on the integrity of the follicle were reported [7,8] although the mutant eggs developed collapsed eggshells that were infertile [36]. These observations indicate that a functional VM is not required in *Drosophila* for maintaining the structure of the follicle but rather that it provides an initial scaffold for chorion assembly and micropyle formation for fertilization. However, *Bombyx* follicles are much larger than those of *Drosophila* and may need additional support to maintain their integrity.

More specifically, in *Bombyx* there is a large uptake of water by the oocyte in a short period just prior to choriogenesis [1] and this may require the formation of a robust VM by the follicular epithelium.

Antisense oligonucleotide inhibition of BmVMP30 function appears to result in the production of a truncated protein, ΔBmVMP30 (Fig. 4A,B). The truncated protein may have arisen from the usage of an internal ATG start codon after the first methionine codon is blocked by the antisense oligonucleotide and therefore is predicted to lack the N-terminal signal peptide (Fig. 1). BmVMP30 immunoreactive material accumulates in the cytoplasm of cells of the follicular epithelium of follicles treated with antisense oligonucleotide, therefore, the protein does not seem to be secreted in this case (Fig. 4E, panel 4). Besides inhibition of translation, antisense oligonucleotides also have an effect on the amount of BmVMP30 mRNA present in follicular cells (Fig. 4C). Thus, oligonucleotide-mediated inhibition of gene function is carried out in silkmoth ovarian follicles (in vitro) according in two ways, i.e. inhibition of translation initiation and targeted RNA degradation.

Although structurally compromised, follicles expressing truncated BmVMP30 are able to complete their vitellogenic development and enter choriogenesis, as seen by the expression of the late vitellogenic marker BmGATAβ and early chorion proteins (Fig. 4B). This was not totally unexpected as silkworm egg development mutants generally complete oogenesis [37]. Thus, the integrity of the follicle does not affect the function of the follicular epithelium, as it progresses normally through the developmental program in vitro. Experiments could not be pursued for longer than 48 h because of the fragility of the BmVMP30 antisense treated follicles. Therefore, the long-term effects on the development of follicles and eggs (e.g. completion of choriogenesis) could not be assessed.

The isolation and functional characterization of BmVMP30 reflects our efforts to isolate and analyze genes expressed after the commitment stage for follicular cells and required for the initiation and completion of the long-term developmental program of choriogenesis. While BmVMP30 was shown not to be involved in the implementation of this autonomous program, efforts are currently underway to characterize its gene promoter (Fig. 1), a first step toward the isolation of regulatory factors expressed during the late stages of vitellogenesis.

References

[1] Kafatos, F.C., Regier, J.C., Mazur, G.D., Nadel, M.R., Blau, H.M., Petri, W.H., Wyman, A.R., Gelinas, R.E., Moore, P.B., Paul, M., Efstradiatis, A., Vournakis, J.N., Goldsmith, M.R., Hunsley, J.R., Baker, B., Nardi, J. and Koehler, M. (1977) in: Biochemical Differentiation in Insect Glands (Beermann, W., Ed.), Vol. 8, pp. 45–145, Springer, Berlin.
[2] Yamauchi, H. and Yoshitake, N. (1984) J. Morphol. 179, 21–31.
[3] Nadel, M.R., Goldsmith, M.R., Goplerud, J. and Kafatos, F.C. (1980) Dev. Biol. 75, 41–58.
[4] Bock, S.C., Campo, K. and Goldsmith, M.R. (1986) Dev. Biol. 117, 215–225.
[5] Swevers, L. and Iatrou, K. (1992) Dev. Biol. 150, 12–22.
[6] Swevers, L. and Iatrou, K. (1998) Mech. Dev. 72, 3–13.
[7] Spradling, A.C. (1993) in: The Development of *Drosophila melanogaster* (Bate, M. and Martinez-Arias, A., Eds.), pp. 365–386, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[8] Waring, G.L. (2000) Int. Rev. Cytol. 198, 67–108.
[9] Scanlon, K.J., Ohta, Y., Ishida, H., Kijima, H., Ohkawa, T., Kaminski, A., Tsai, J., Horng, G. and Kashani-Sabet, M. (1995) FASEB J. 13, 1288–1296.
[10] Swevers, L., Drevet, J.R., Lunke, M.D. and Iatrou, K. (1995) Mol. Biol. 25, 857–866.
[11] Patel, M. and Sive, H. (1996) in: Current Protocols in Molecular Biology, Vol. 1, Unit 5.9, Wiley.
[12] Sive, L.H. and St.John, T. (1988) Nucleic Acids Res. 18, 10937.
[13] Hanahan, D. and Meselson, M. (1980) Gene 10, 63–67.
[14] Drevet, J.R., Swevers, L. and Iatrou, K. (1995) J. Mol. Biol. 246, 43–53.
[15] Schowalter, D.B. and Sommer, S.S. (1989) Anal. Biochem. 177, 90–94.
[16] Drevet, J.R., Skeiky, Y.A.W. and Iatrou, K. (1994) J. Biol. Chem. 269, 10660–10667.
[17] Mounier, N. and Prudhomme, J.-C. (1986) Biochimie 68, 1053–1061.
[18] Fujiwara, H., Oruga, T., Takada, N., Miyajima, N., Ishizawa, H. and Maekawa, H. (1984) Nucleic Acids Res. 12, 6861–6869.
[19] Brahic, M. and Haase, A.T. (1978) Proc. Natl. Acad. Sci. USA 75, 6125–6129.
[20] Iatrou, K., Meidinger, R.G. and Goldsmith, M.R. (1989) Proc. Natl. Acad. Sci. USA 86, 9129–9133.
[21] Eystathioy, T., Swevers, L. and Iatrou, K. (2001) Mech. Dev. 103, 107–115.
[22] Sato, Y. and Yamashita, O. (1991) Insect Biochem. 21, 495–505.
[23] Kendirgi, F. (2000) Ph.D. Thesis, The University of Calgary, Calgary, Alberta, Canada.
[24] Swevers, L. and Iatrou, K. (1999) Insect Biochem. Mol. Biol. 29, 955–963.
[25] Retnakaran, A., Hiruma, K., Palli, S.R. and Riddiford, L.M. (1995) Insect Biochem. Mol. Biol. 25, 109–117.
[26] Telfer, W.H. and Anderson, L.M. (1968) Dev. Biol. 17, 512–535.
[27] Walder, R.Y. and Walder, J.A. (1988) Proc. Natl. Acad. Sci. USA 85, 5011–5015.
[28] Higgins, M.J., Walker, V.K., Holden, J.J. and White, B.N. (1984) Dev. Biol. 105, 155–165.
[29] Pascucci, T., Perrino, J., Mahowal, A.P. and Waring, G.L. (1996) Dev. Biol. 177, 590–598.
[30] Sardet, C., Prodon, F., Dumollard, R., Chang, P. and Chênevert, J. (2002) Dev. Biol. 241, 1–23.
[31] Miya, K. (1984) in: Insect Ultrastructure (King, R.C. and Akai, H., Eds.), Vol. 1, pp. 49–73, Plenum Press, New York.
[32] Scherer, L.J., Harris, D.H. and Petri, W.H. (1988) Dev. Biol. 130, 786–788.
[33] Lin, Y., Hamblin, M.T., Edwards, M.J., Barillas-Mury, C., Kanost, M.R., Knipple, D.C., Wolfner, M.F. and Hagedorn, H.H. (1993) Dev. Biol. 155, 558–568.
[34] Edwards, M.J., Severson, D.W. and Hagedorn, H.H. (1998) Insect Biochem. Mol. Biol. 28, 915–925.
[35] Raikhel, A.S., Miura, K. and Segraves, W.A. (1999) Am. Zool. 39, 722–735.
[36] Savant, S.S. and Waring, G.L. (1989) Dev. Biol. 135, 43–52.
[37] Goldsmith, M.R. (1995) in: Molecular Model Systems in the Lepidoptera (Goldsmith, M.R. and Wilkins, A.S., Eds.), pp. 21–76, Cambridge University press, Cambridge.
[38] Lunke, M.D. (2000) Ph.D. Thesis, The University of Calgary, Calgary, Alberta, Canada.