Mosquito Cathepsin B-like Protease Involved in Embryonic Degradation of Vitellin Is Produced as a Latent Extravascular Precursor*

(Received for publication, January 20, 1999, and in revised form, February 12, 1999)

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Here we report identification of a novel member of the thiol protease superfamily in the yellow fever mosquito, Aedes aegypti. It is synthesized and secreted as a latent proenzyme in a sex-, stage-, and tissue-specific manner by the fat body, an insect metabolic tissue, of female mosquitoes during vitellogenesis in response to blood feeding. The secreted, hemolymph form of the enzyme is a large molecule, likely a hexamer, consisting of 44-kDa subunits. The deduced amino acid sequence of this 44-kDa precursor shares high similarity with cathepsin B but not with other mammalian cathepsins. We have named this mosquito enzyme vitellogenic cathepsin B (VCB). VCB decreases to 42 kDa after internalization by oocytes. In mature yolk bodies, VCB is located in the matrix surrounding the crystalline yolk protein, vitellin. At the onset of embryogenesis, VCB is further processed to 33 kDa. The embryo extract containing the 33-kDa VCB is active toward benzoyloxycarbonyl-Arg-para-nitroanilide, a cathepsin B-specific substrate, and degrades vitellinogen, the vitellin precursor. Both of these enzymatic activities are prevented by trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), a thiol protease inhibitor. Furthermore, addition of the anti-VCB antibody to the embryonic extract prevented cleavage of vitellinogen, strongly indicating that the activated VCB is involved in embryonic degradation of vitellin.

Cathepsin B is a thiol (cysteine) protease with both endopeptidase and peptidylpeptidase activities. Due to its broad specificity, cathepsin B plays a key role in intracellular protein catabolism in the lysosomal system (1). Cathepsin B has been well characterized both enzymatically and molecularly (2–10). The mammalian cathepsin B has been implicated in tumor invasion, progression, and metastasis (11–15). Tumor-specific cathepsin B is secreted by malignant cells as a latent high molecular weight precursor, presumably activated at cell contacts (16, 17).

In addition, cathepsins B, as well as the related cathepsins L, have been identified in numerous parasitic protozoa and helminthes, including prevalent pathogens of human and domestic animals (18–26). In the blood-sucking bug, Rhodnius prolixus, cathepsin B is the major gut proteolytic enzyme (27). In these organisms, cathepsins B and L are presumably involved in the degradation of host hemoglobin.

In insects and other arthropods, cathepsins B and L also participate in key developmental processes. In the flesh fly, Sarcophaga peregrina, hemocytes produce the extracellular form of a cathepsin B-like enzyme that participates in decomposition of the larval fat body during metamorphosis (28–30). Moreover, cathepsins B and L have been implicated in degradation of yolk proteins during embryonic development (31–41).

The elucidation of developmental mechanisms in the mosquito is important because this insect transmits the most devastating of vector-borne human diseases, including malaria, lymphatic filariasis, Dengue fever, and many others. Little is known, however, about the process of yolk protein degradation in the mosquito embryo. Previously, we have found that during vitellogenesis, the female fat body, a metabolic tissue analogous to the vertebrate liver, synthesizes and secretes a latent proenzyme of a serine carboxypeptidase which is homologous to yeast carboxypeptidase Y (42, 43). This 53-kDa proenzyme, which we named vitellogenic carboxypeptidase (VCP), is specifically accumulated by developing oocytes and deposited in yolk bodies. Although we have demonstrated that VCP is activated during embryogenesis, its role remains unknown.

In this paper we report the discovery of an unusual cathepsin-B-like thiol protease from the mosquito, Aedes aegypti. Similar to VCP, this enzyme is produced by the fat body of vitellogenic female mosquitoes in response to blood feeding. The cDNA encoding this unique mosquito enzyme, which we named vitellogenic cathepsin B (VCB), exhibits high similarity to vertebrate cathepsin B. VCB is secreted by the fat body as a latent proenzyme, similar in size to the latent tumor cathepsin B

*This work was supported by National Institutes of Health Grants AI-24716 and AI-32154 (to A. S. R.) and by National Science Council (Taiwan) 86-2314-B-010-095 (to W.-L. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF127592.

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Received for publication, January 20, 1999, and in revised form, February 12, 1999

Printed in U.S.A.
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(44-kDa); it is accumulated by developing oocytes, where it is stored in yolk bodies. For the embryogenesis, VCB is processed to contain the appropriate concentration of [35S]methionine (pulse labeling) for 24-h post-blood fed female mosquitoes. cDNA was synthesized from the ovaries (18 h post-blood meal) into 100 μl of TES-buffered culture media modified (49, 50) for ovaries (Aedes aegypti, Aedes aegypti). The ovaries were removed after 2.5 h, rinsed 3 times in APS and frozen at −20 °C.

In Vitro Protein Labeling—In vitro culture of fat body and ovaries was as described previously (42). Synthesized and secreted fat body proteins were radiolabeled by incubating 3 female or 6 male fat bodies at 27 °C in 50 μl of TES-buffered culture media modified (49, 50) to contain the appropriate concentration of [35S]methionine (pulse media). If only pulse media was needed, fat bodies were incubated for 3 h. Ovary-synthesized proteins were labeled in vitro by placing five pairs of ovaries (18 h post-blood meal) into 100 μl of TES-buffered culture media (51, 52) with [35S]methionine replacing unlabeled methionine. The ovaries were removed after 2.5 h, rinsed 3 times in APS and frozen at −80 °C.

Amino Acid Sequence Analysis—The proteins, prepared as described above for antibody production, were electroblotted onto polyvinylidene difluoride membrane (0.2 μm pore size; Bio-Rad). The 44- and 42-kDa bands were excised from the blots of fat body-secreted and ovarian protein, respectively. The blots were sent to Harvard Microchem (Harvard Medical School, Cambridge, MA) for sequencing. The probe used for screening a cDNA library for VCB was generated by PCR. DNA was isolated from fat bodies of 24-h post-blood fed female mosquitoes. cDNA was synthesized from the fat body mRNA and used as the template for PCR. An antisense degenerate primer was constructed from a portion of an internal peptide sequence, MADVDEL, obtained from microsequencing a tryptic fragment of the 44-kDa fat body-secreted VCB. The sequence of the first primer was: ATGGCAGA/TGGAGA/CAGA/TTCG/ATGAGAGA/GAGA/GA/TGGAT/T/T. The second primer was a 17-mer (dT). The resulting PCR product was subcloned into a pGEM-T vector for sequencing. The confirmed VCB fragment was used to screen a mosquito vitellogenic fat body cDNA library which was constructed at the EcoRI site of the EcoRI B fragment (containing the vitellogenin gene). The confirmed amino acid sequence was analyzed by FASTA, Motifs, and Gap programs of Genetics Computer Group software (University of Wisconsin, WI).

DNA Analysis and Alignment—The putative signal peptide in the deduced amino acid sequence was determined according to Kyte and Doolittle (43). The deduced amino acid sequence was searched in FASTA database (44) and was aligned to other members by Clustal W (45) and MUSCLE (46). The database search for homologous members was performed with FASTA, Motifs, and Gap programs of Genetics Computer Group software (University of Wisconsin, WI).

Isolation of RNA and Northern Blot Hybridization—Total RNA was prepared using the guanidine isothiocyanate method (57–59). For Northern blot analyses, total RNA and RNA markers (Life Technologies) were separated by electrophoresis in 1.2% agarose formaldehyde gels. The RNA was transferred to a nitrocellulose membrane and hybridized to [32P]-labeled probes. The conditions of hybridization at high stringency were used according to Cho et al. (60). A 255-bp EcoRI-HincII fragment located at the 5'-end of the VCB cDNA was used as a probe.

As a control for RNA loading, the cytoplasmic actin 5C gene of A. aegypti was used to probe the RNA in the actin mRNA. Cloning of mosquito actin cDNA was described previously (61).

In Vitro Transcription and Translation—In vitro transcription and translation, the entire VCB cDNA was subcloned into pGEM 7Z (+) (Promega) under control of the SP6 promoter. Plasmid DNA was purified by the Wizard Miniprep DNA Purification System (Promega). Two micrograms of purified plasmid DNA were used for transcription and translation in the SP6 T7T Coupled Biotinylate Lysate system (Promega). [35S]Methionine was used to label the translated product. All protocols were followed as described by the manufacturer.

Antibody Production—Ovaries dissected from A. aegypti 28 h after blood feeding were processed and subjected to DEAE-Sepharose anion-exchange chromatography (51). Proteins in the Vg-free unbound fraction were separated by preparative SDS-PAGE. A band containing VCB (42 kDa) was excised and electroeluted using an ISCO model 1750 Electrophoretic Concentrator according to recommendations of the manufacturer. The elute provided the source of antigen to produce antibodies to this peptide (VCB-Ab1). Rabbits were injected with antigen/adjuvant (TiterMax, CytoRx) two times at a 1-month interval. Serum proteins from the immunized rabbit were precipitated with ammonium sulfate at 55% of saturation and reconstituted at 5 mg/ml.

To avoid possible cross-hybridization with other cathepsins B, an Escherichia coli-expressed fusion protein, containing the VCB 20-kDa N-terminal region was used for production of another polyclonal antibody (VCB-Ab2). This VCB region contained the signal peptide, the VCP and the Vg large subunit as well as monoclonal antibodies against Vg small subunit as well as monoclonal antibodies against Vg small subunit were already available (42, 62). To avoid possible cross-hybridization with other cathepsins B, an Escherichia coli-expressed fusion protein, containing the VCB 20-kDa N-terminal region was used for production of another polyclonal antibody (VCB-Ab2). This VCB region contained the signal peptide, the entire prepeptide, and an N-terminal portion of the mature VCB, which was less conserved that the rest of the protein. The antibody production was done as described previously (46–48). The titration of antibodies against VCP and the Vg large subunit as well as monoclonal antibodies against Vg small subunit were already available (42, 62).

Immunoblots and Immunoprecipitation—Proteins from SDS-PAGE and native PAGE gels were transferred to BA-S 83 supported nitrocellulose (Schleicher and Schuell) as described previously (48). Immunoprecipitive bands were detected with the ECL Western blotting detection system (Amersham). Prior to immunoprecipitation, Vn or Vg were partially removed from ovarian extracts or fat body secretions, respectively, using a suspension of DEAE-Sepharose CL-6B as described previously (42). After removal of Vn or Vg, VCB was immunoprecipitated with anti-VCB antibodies. Protein A-Sepharose was added as a precipitating agent (42). After immunoprecipitation, the resulting pellet was washed and used in radioimmunoprecipitation or SDS-PAGE and fluorography experiments (42).

Immunolabeling—For immunocytochemical examination, tissues were fixed with 2% formaldehyde and 0.1% glutaraldehyde in 0.1 m sodium phosphate buffer, pH 7.4, for 45–60 min at room temperature. The specimens were then blocked with 100 μg/ml glycine in the same buffer for 1 h at room temperature. For light immunocytochemistry, 3–8 μm sections were treated with poly-L-lysine-coated slides and processed as described by Raikhel and Lea (63). The results were visualized by using phase-contrast and epifluorescence microscopy.

For electron microscopy, the tissues were dehydrated in ethanol and embedded in LR-White resin. The ultra-thin sections were prepared using an Ultracut E microtome (Reichert-Jung) and mounted on formvar-coated or uncoated nickel grids. The labeling was carried out on...
Enzymatic assay—For enzymatic assay, embryos, 12–48 h after egg deposition, were extracted in B3 buffer containing 1 mM PMSF, 1 mM AEBSF, and 5 μM pepstatin. Each enzymatic reaction received 38 μg of total protein of embryonic extract. All enzymatic reactions were performed in duplicate using the MES buffer, pH 5.5, containing 100 mM MES, 1 mM PMSF, 1 mM AEBSF, 5 μM pepstatin, and 2.5 mM EDTA. Enzymatic assays were initiated by the addition of 81.1 mM Z-Arg-Arg-pNA (Bachem Biosciences) in 100 mM methanol to the reaction mixture, yielding a final concentration of 1.6 mM substrate and 2% methanol. To study the effect of specific inhibitors, either E-64 or chymostatin were added to the protein mixtures in the following concentrations: 0.3, 0.6, 1.2, or 2.5 mM; the mixture was incubated for 20 min at 37 °C prior to the addition of the enzymatic substrate. The control reaction was pre-incubated without any inhibitor. Data were collected by measuring the absorbance at 405 nm with a spectrophotometer after 2.5 h. A standard curve of pNA was plotted and regressed by the linear least-squares method. This curve was used to calculate the amount of pNA liberated by enzymatic activity. In experiments to determine the optimum pH of the enzyme, a range of pH from 3.5 to 7.5 was used.

RESULTS

Identification of a Novel Yolk Protein Precursor from the Mosquito Fat Body—Previous analysis of proteins synthesized and secreted by the vitellogenic mosquito fat body has revealed a polypeptide of 44 kDa, in addition to Vg and VCP; furthermore, the presence of a polypeptide of 42 kDa has been observed among proteins extracted from vitellogenic ovaries or newly laid eggs (42). Here, polyclonal antibodies were produced against the 42-kDa polypeptide that was gel-purified from the ovarian source (VCB-Ah1). Immunoblot analysis showed that these antibodies recognized not only the 42-kDa ovarian polypeptide but also the single polypeptide of 44 kDa from vitellogenic fat bodies and their secretions, thus confirming the immunological identity of these two polypeptides. The immunoreactive polypeptides were present in neither previtellogenic female fat bodies nor males (Fig. 1).

Amino acid sequences were obtained by microsequencing tryptic fragments of the 42-kDa ovarian polypeptide and the 44-kDa polypeptide from vitellogenic fat body secretions. These sequences were analyzed for protein similarity. The results indicated that microsequenced fragments from both 42- and 44-kDa polypeptides shared similarity with human and other vertebrate cathepsins B (data not shown).

Cloning and Analysis of the cDNA Encoding the 44-kDa Yolk Protein Precursor, Vitellogenic Cathepsin B—A 863-bp cDNA fragment was obtained from the amplification of vitellogenic fat body RNA by the PCR technique using the degenerate primer derived from the 44-kDa polypeptide sequence and an oligo(dT) primer. Both ends of the fragment matched the PCR primer sequences, and an open reading frame showed high similarity to vertebrate cathepsin B (data not shown). This PCR fragment was subcloned into a pGEM-T vector and was subsequently used as a probe to screen the cDNA library prepared from the fat bodies of vitellogenic female mosquitoes.

Several positive clones were isolated from the cDNA library, all of which measured approximately 1.3 kb and shared identical restriction maps. Partial sequencing of these clones showed they contained a sequence identical to that of the PCR fragment. The longest clone was sequenced from both strands. This cDNA clone of 1,239 bp contained an open reading frame of 1,158 nucleotides. It had an unusual putative polyadenylation signal, ATTAAA, reported previously (67, 68), located 20 bp upstream of the poly(A) tail (Fig. 2). The cDNA clone encoded a pre-protein of 386 amino acid residues with a predicted size of 43,069 Da. The two sequences determined by direct microsequencing of the ovarian 42-kDa VCB and the fat body 44-kDa VCB were both found in its deduced amino acid sequence (Fig. 2). Hydropathy analysis (56) revealed that the protein was hydrophilic, possessing a strongly hydrophobic putative signal peptide of 16 residues (Fig. 2); moreover, the protein was expected to be positively charged with an isoelectric point (pI) of 7.95. However, in light of two potential N-linked glycosylation sites (69) and 10 potential phosphorylation sites (70) in the deduced VCB amino acid sequence, the fully processed protein could differ in its net charge (Fig. 2).

To verify its identity, the 1.3-kb cDNA clone was expressed using a coupled in vitro transcription/translation system. A single polypeptide of the predicted size, 43 kDa, was immunoprecipitated from the translation reaction by the VCB-Ah1 antibodies, prepared against ovarian 42-kDa polypeptide (Fig. 3). Furthermore, in the presence of canine microsomal membranes, the expressed polypeptide increased to 44 kDa, identical in size to the 44-kDa VCB secreted by the fat body (Fig. 3). The 1.3-kb cDNA clone was also used as a probe for Northern hybridization. It hybridized strongly only to 1.3-kb mRNA from the fat body of vitellogenic female mosquitoes (see below). Taken together, these data confirm that the 1.3-kb cDNA encodes the 44-kDa fat body precursor of the ovarian 42-kDa VCB.
Mosquito 44-kDa Yolk Protein Precursor (VCB) Is a Proenzyme Homologous to Cathepsin B—The deduced amino acid sequence of VCB exhibited high similarity to the family of eukaryotic thiol proteases, particularly the mammalian cathepsins B and cathepsin B-like proteases of invertebrates (Fig. 4). However, it shared only limited similarity to other cathepsins and related proteases, such as papain (not shown). The multiple alignment of homologous thiol proteases predicted that a putative start of the mature VCB occupied position Leu-125 (Figs. 2 and 4). Furthermore, three active sites characteristic for cathepsin B were conserved in VCB at positions Cys-150, His-315, and Asn-335 (Figs. 2 and 4). In addition, from six disulfate bonds typical for thiol proteases (4), the cysteine positions of five putative bonds were conserved in VCB.

In contrast to mature enzymes (Fig. 4), the pro-peptide portion of VCB exhibited no significant similarity to those of cathepsins B (data not shown). Interestingly, however, similar to mammalian cathepsin B, the mosquito VCB pro-peptide has one putative glycosylation site, while the cathepsin B-like proteases of other invertebrates have none (Fig. 2).

Mosquito VCB Is a Fat Body-specific Protein Produced in Vitellogenic Females in Response to a Blood Meal—Use of the entire VCB cDNA clone as a probe in the Northern blot analyses revealed a 1.3-kb transcript specific to vitellogenic fat bodies; additionally, weak hybridization to a slightly smaller mRNA band was observed in all other tested tissues (data not shown). This trace hybridization was likely due to partial similarity shared by VCB and other thiol proteases possibly of lysosomal origin. However, the use of a 208-bp of 5′-end of VCB fragment including the 5′-end noncoding region and the pro-portion of VCB as a probe resulted in hybridization only to the 1.3-kb transcript from vitellogenic fat bodies (Fig. 5). Thus, Northern blot analyses confirmed the data we obtained at the protein level and demonstrated that the VCB gene was expressed exclusively in the fat body of vitellogenic female mosquitoes (Fig. 5). The 1.3-kb VCB transcript appeared in the fat body of vitellogenic female mosquitoes.
body only after blood feeding, indicating that similarly to yolk protein precursors, it was initiated in response to a blood meal. The levels of the 1.3-kb VCB transcript reached a peak between 18 and 24 h PBM and then gradually declined. The message was not detected in the fat bodies at the end of vitellogenic cycle, 42 and 48 h PBM (Fig. 6).

The secretory activity of the fat body with respect to production of yolk protein precursors was evaluated by pulse labeling using [35S]methionine for 1 h and collecting the chase media secretions for analysis. VCB secretion was monitored by VCB-Ab1 antibodies. As a control, anti-VCP antibodies were used to trace secretion of this yolk protein precursor. The data from these analyses showed that the kinetics of VCB secretion by the fat body were similar to those of VCP (Fig. 7). Both yolk protein precursors were detected at 4 h after initiation of vitellogenesis by a blood meal. Their synthesis and secretion increased rapidly to a maximum at 24-h post-blood meal, declined to a very low level by 36 h, and reached almost background level by 48 h (Fig. 7).

Western blot analyses of the VCB protein in the fat body, utilizing either VCB-Ab1 or VCB-Ab2 antibodies, revealed a 44-kDa protein profile similar to that of secreted VCB, indicating that like yolk protein precursors, Vg and VCP, VCB was not stored in the fat body after its synthesis and was immediately secreted into the hemolymph (not shown). Both VCB-Ab1 and VCB-Ab2 antibodies recognized the same 44-kDa protein band in fat body preparations at all stages of vitellogenesis when the VCP gene was expressed (not shown).

**VCB Is Localized in Fat Body Cell's Secretory and Oocyte's Endocytic Organelles**—Previously (66) we utilized the VCB-Ab1 antibodies (designated there as anti-44KP antibodies) for localization of VCB. In this work we compared the localization of VCB by VCB-Ab2, produced against the recombinant VCB, with that by VCB-Ab1, produced against purified ovarian 42-kDa peptide. Fluorescent immunohistochemical analyses using either VCB-Ab1 or VCB-Ab2 antibodies demonstrated that VCB was localized only in the vitellogenic fat body and ovary after a blood meal (Figs. 8 and 9). In both tissues, the distribution of VCB was similar to that of VCP (42). Of particular interest was localization of VCB in the ovary, where it was seen surrounding the yolk granules (Fig. 9B).

The subcellular distribution of VCB, immunocytochemistry at the electron microscopic level, was used to localize VCB in the vitellogenic fat body and ovary. In the fat body cells, the trophocytes, VCB was present in the organelles of the secretory pathway: the Golgi complex and secretory granules. When double immunolabeling was performed utilizing antibodies for Vg, VCB, and protein A-colloidal gold particles of two different sizes, VCB was co-localized with Vg in these organelles, indicating the simultaneous processing of these proteins in the trophocyte’s secretory system (Fig. 10).

In vitellogenic ovarian follicles, which consist of the oocyte and nurse cells surrounded by follicle cells, VCB was present only in the oocyte’s vesicles, endosomes, and yolk bodies (66). Simultaneous labeling for VCB and Vg demonstrated that these proteins were mixed in endosomes (66). However, localization of VCB differed dramatically in mature yolk bodies, where VCB was distributed as a narrow layer on the surface of the crystalline Vn, a storage form of Vg (Fig. 11). Morphologically, this area of the yolk body was visible as a non-crystalline matrix separating the crystalline yolk from the yolk body membrane. Double immunolocalization showed that in mature yolk bodies, this non-crystalline matrix was free of Vn, while, in contrast, the crystalline yolk was always free of VCB (66). The distribution of VCB in mature yolk bodies was similar in oocytes at the peak of endocytosis (24 h post-blood meal) and in those after termination of yolk accumulation (48 h post-blood meal).

**Correlation of Changes in the Native and Subunit Composition of VCB in the Ovaries and Eggs with Embryonic Development**—The processing of the 44-kDa hemolymph form of VCB in oocytes and eggs was monitored by immunoblot analysis and SDS-PAGE. In the oocyte, the internalized VCB reduced to 42 kDa; it remained unchanged for most of oocyte development; at 24 h PBM (Fig. 12, lane 1) in oocytes at the peak of protein uptake (51). However, in fully grown oocytes with completed protein yolk accumulation and nearly completed choriogenesis (87) at 48 h PBM, a 37-kDa band appeared which was recognized by either VCP-Ab1 or VCB-Ab2 antibodies (Fig. 12, lane 2). VCB further diminished to 33 kDa at the onset of embryogenesis in newly laid eggs (Fig. 12, lane 3). Immunoblot analyses utilizing either VCB-Ab1 or VCB-Ab2 showed that VCB was maintained as a 33-kDa polypeptide until the end of embryogenesis (Figs. 12 and 13). No immunopositive VCB bands were detected in extracts of newly hatched first instar larvae or later stages (Fig. 13). Native PAGE and immunoblot analyses revealed that in the hemolymph, VCB existed as a high molecular weight molecule: its apparent size varied slightly depending on gel conditions within an average value of 236 ± 7 kDa (Fig. 15). The VCB native size decreased to 132 ± 4 kDa at the onset of embryonic development in the egg, when VCB was processed to its 33-kDa form; there was also a minor immunopositive band of 66 kDa present in the same preparation (Fig. 14).

**Mosquito VCB Is Activated in Embryos and Is Involved in Degradation of the Major Yolk Protein, Vitellin**—The processing of VCB to its 33-kDa form is reminiscent of the proteolytic activation of mammalian cathepsin B (71). To evaluate whether this VCB processing results in its enzymatic activity, we used Z-Arg-Arg-pNA, a substrate exhibiting specificity to cathepsin B (72). Secretions from vitellogenic fat bodies, containing 44-kDa VCB, did not exhibit appreciable cathepsin B enzymatic activity (data not shown). In contrast, protein extracts from embryos containing 33-kDa VCB showed significant cathepsin B activity (Fig. 15). Enzymatic activity was
optimal at pH 5.5 (data not shown). This enzymatic activity, associated with embryonic extracts containing the 33-kDa VCB was highly sensitive to E-64. It showed partial sensitivity to chymostatin (Fig. 15).

Next, we determined whether the processing of VCB to its 33-kDa form coincided with the beginning of vitellin (Vn) degradation. We applied immunoblot analysis using monoclonal antibodies against the Vg/Vn small 66-kDa subunit (Fig. 16A) and polyclonal antibodies against the Vg/Vn large 200-kDa subunit (Fig. 16B). The immunoblot analysis of Vn in the ovary and embryos of the same stages as in Fig. 12 showed that clear signs of Vn cleavage were evident only in embryos, coinciding with the processing of VCB to the 33-kDa form. Interestingly that the large Vn subunit exhibited a degradation pattern earlier than the small Vn subunit (Fig. 16, A and B, lanes 3 and 4).

**Fig. 4.** Alignment of the deduced amino acid sequence of mosquito VCB with other thiol proteases. Asterisks mark the consensus residues of enzyme active sites (*). Conserved amino acids are marked by black boxes. Paired numbers under the sequences denote the locations of cysteine residues that form a disulfide bond. Abbreviations are: AaVCB, *A. aegypti* VCB; HsCtB, human cathepsin B, *Homo sapiens* (3); RnCtB, rat cathepsin B, *Rattus norvegicus* (3); BtCtB, bovine cathepsin B, *Bos taurus* (87); MmCtB, mouse cathepsin B, *Mus musculus* (3); Sp29K, 29-kDa protease of the blowfly, *S. peregrina* (30); SjCtB, trematode cathepsin B, *Schistosoma japonicum* (25); SmCyP, trematode cysteine protease, *S. mansoni* (20); HcCy1, nematode gut thiol protease 1, *Hemonchus contortus* (21); HcCy2, nematode thiol protease 2, *H. contortus* (23); CeCy1, nematode gut-specific cysteine protease, *Caenorhabditis elegans* (88). The descent rank of similarity to mosquito VCB was determined by Inhib index of FastA (Genetics Computer Group software).

**Fig. 5.** Northern blot analysis of gene expression of mosquito VCB. Twenty micrograms of total RNA from different sexes, tissues, and stages were separated by a 1.2% agarose gel and stained with ethidium bromide (A). After photography, the gel was transferred to a nitrocellulose membrane and hybridized with a 32P-labeled 208-bp 5’-end fragment of the VCB cDNA, including the noncoding region and the prepro-portion of VCB (B). Lane 1, whole male; lane 2, vitellogenic female midgut, 24 h PBM; lane 3, vitellogenic female ovary, 24 h PBM; lane 4, pre-vitellogenic female fat body, 24 h PBM; lane 5, post-vitellogenic female fat body, 48 h PBM. RNA markers (Bio-Lab) are shown to the left in A.

**Fig. 6.** VCB RNA kinetics in the mosquito fat body. For each time point, total RNA (20 μg) extracted from the fat body was resolved in a 1.2% formaldehyde gel. A, the ethidium bromide stained gel. B, Northern hybridization with a 32P-labeled 208-bp VCB specific probe. Lane 1, pre-vitellogenic female fat body; lanes 2–9, vitellogenic female fat bodies: lane 2, 6 h PBM; lane 3, 12 h PBM; lane 4, 18 h PBM; lane 5, 24 h PBM; lane 6, 30 h PBM; lane 7, 36 h PBM; lane 8, 42 h PBM; lane 9, 48 h PBM. RNA markers (Bio-Lab) are shown to the left in A.
To test whether or not pro-VCB is processed and activated under acidic conditions, as are mammalian cathepsins B (7, 73), the secretory 44-kDa and ovarian 42-kDa forms of VCB were incubated under different acidic pH conditions (4.0 and 5.5). After treatment with acidic pH, the 44-kDa pro-VCB neither catalyzed the substrate Z-Arg-Arg-pNA (Fig. 19A, line 1) nor degraded [35S]methionine-labeled Vg (not shown). Immunoblot analysis showed that the 44-kDa pro-VCB was processed to a 35-kDa peptide but not to a 33-kDa one (Fig. 19B, lanes 3 and 4). Unlike the 44-kDa pro-VCB, the ovarian extract, containing the 42-kDa VCB, exhibited cathepsin B activity after treatment with acidic pH (Fig. 19A). The immunoblot revealed that VCB was processed to a 33-kDa form (Fig. 19B, lane 5). VCB processing was equally efficient at pH 4.0 and 5.5 and unaffected by the presence of protease inhibitors specific to serine, aspartic, and metalloproteinases (Fig. 19B).

**DISCUSSION**

In this paper, we report an unusual form of cathepsin B-like thiol protease from the mosquito, *A. aegypti*. Cloning and analysis of the cDNA encoding VCB demonstrated its high similarity to mammalian cathepsins B and invertebrate cathepsin B-like proteases, but not to other related cathepsins. The structural analysis of the deduced amino acid VCB sequence suggests that the folding and activity of this enzyme are likely similar to cathepsins B: the predicted start of mature VCB at Leu-125 was similar to mammalian cathepsins B; moreover, the active sites and cysteine positions of five putative disulfide bonds were conserved in VCB (Fig. 4).

The mosquito VCB was also observed to resemble mammalian cathepsins B in its size. It was secreted as a proenzyme of 44 kDa similar in size to the latent pro-cathepsin B secreted by malignant cells (71). Moreover, the 33-kDa embryonic form of VCB exhibited the same size as the active single chain cathepsin B (71).

The presence of the 33-kDa embryonic form of VCB was shown to correlate with activity characteristic of cathepsin B (72). This activity was highly expressed with the cathepsin-B-specific substrate, Z-Arg-Arg-pNA; it was sensitive to E-64, but considerably less to chymostatin.

The unique feature that we observed for this mosquito cathepsin-B-like enzyme was its exclusive synthesis and secretion by the fat body of vitellogenic female mosquitoes as a latent, high molecular size precursor consisting of five or six subunits with a molecular mass of 44 kDa. Its synthesis in the female fat body is initiated by blood feeding, and the kinetics of its secretion by the vitellogenic fat body are similar to those of the yolk protein precursors, Vg (59) and VCP (42, 43).

We established the link between the 44-kDa fat body-secreted hemolymph VCB and the 42-kDa ovarian VCB by using the anti-42-kDa VCB (VCB-Ab1) in immunoblotting analyses. The anti-VCB-specific antibodies, generated against the recombinant protein (VCB-Ab2), verified the identity of 44 and 42 kDa, and other processed peptides as well. Despite the large amounts of VCB present in the ovary, neither its mRNA nor synthesis were detected there. Thus, the fat body is the only source of the 44-kDa polypeptide as a precursor of the ovarian 42-kDa polypeptide, which suggests that its accumulation in the ovary occurs by endocytosis, similar to other yolk protein precursors (73). VCB immunolocalization in the ovary confirmed that this yolk protein precursor is internalized in developing oocytes via the endocytotic pathway. Interestingly, VCB is segregated from crystalline Vn in mature yolk bodies, the accumulative endocytic organelle of the oocyte, being present only in the non-crystalline matrix surrounding Vn. In this matrix of mature yolk bodies, VCB is mixed with VCP (66). It is, therefore, likely that the presence of both these proenzymes...
in the matrix surrounding crystalline Vn enhances their rapid activation at the onset of embryonic development, the point at which yolk bodies undergo acidification. The latter event has been documented for both insects and vertebrates (74, 75).

The fat body-secreted hemolymph form of VCB is stable latent proenzyme which unlike mammalian cathepsins B (7, 76), cannot be activated by acidic pH alone. Here, the reduction of the hemolymph pro-VCB to 35 kDa (not 33-kDa) after treatment with acidic pH was not sufficient for enzyme activation. Clearly, an additional step or steps are required for the activation of the latent hemolymph pro-VCB. In our experiments, pro-VCB did not activate to the 33-kDa peptide itself in an in vitro transcription/translation system (Fig. 3), as has been reported for Schistosoma mansoni cathepsin-B-like cysteine protease (77). This stability of the hemolymph pro-VCB as a latent proenzyme prior to its internalization by developing oocytes is physiologically important. It may be additionally enhanced by the presence of the glycosylation site in its proenzyme portion.

After treatment with acidic pH, the ovarian extract containing the 42-kDa VCB exhibited enhanced cathepsin B activity. Significantly, under these conditions, most of the ovarian 42-kDa VCB was processed to the 33-kDa polypeptide. Although
the nature of this processing event is unclear, the removal of 2 kDa from each VCB subunit apparently renders pro-VCB capable of activation by acidic pH after the onset of embryonic development. Although the activation reactions were performed using crude extracts, it is unlikely that the proteolytic processing of VCB by treatment with acidic pH was a function of another protease, as both the 44-kDa hemolymph and the 42-kDa ovarian VCB were processed to 35 and 33 kDa in the presence of protease inhibitors. Autocatalytic cleavage under acidic conditions, which has been reported for mammalian cathepsins B (7, 76), may be a possible mechanism for the activation of the 42-kDa VCB in the mosquito embryo.

The estimation of native molecular mass suggests that in the hemolymph, VCB exists as a hexamer or a pentamer of 44-kDa subunits (Fig. 14). A hexameric structure is characteristic for some insect hemolymph proteins (78). The feature of VCB may also increase its stability in the hemolymph; alternatively, it may be important for the recognition of VCB by oocyte receptors. Here, we found that at the onset of embryonic development, when VCB was processed to the active 33-kDa form, its native size was reduced to 132 kDa, which likely corresponded to a tetramer of 33-kDa subunits. The presence of a minor 66-kDa band indicated that at least some of the 33-kDa VCB were in dimer form (Fig. 14).

Our experiments utilizing 35S-labeled Vg as a substrate suggest that VCB likely plays a key role in the degradation of Vn, the major yolk protein in mosquito embryos. The embryonic extract containing the 33-kDa VCB displayed high activity to Vg digestion, and this degradation was blocked by E-64. Moreover, the specific anti-VCB antibodies almost entirely inhibited cleavage of Vg by the embryonic extract containing the 33-kDa VCB, strongly suggesting that VCB is the key enzyme in embryonic development. A, immunoblot analysis using monoclonal antibodies against the Vg/Vn small 66-kDa subunit; B, using polyclonal antibodies against the Vg/Vn large 200-kDa subunit. Lanes 1, ovaries at the peak of Vg uptake, 24 h PBM; lanes 2, ovaries with eggs nearly complete in their development, 48 h PBM; lanes 3, 0–3 h post-oviposition eggs at the onset of embryonic development; lanes 4, 48-h post-oviposition eggs during mid-embryogenesis; lanes 5, 96-h post-oviposition eggs at the end of embryogenesis; lanes 6, newly hatched first instar larvae. The molecular standards in order of decreasing molecular mass (in kDa) were myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor (Bio-Rad).
Proteases similar to cathepsins B and L have been shown to be active during embryonic development in a number of insects and other arthropods (31–40). Best studied is the cathepsin L-like cysteine protease from the silkworm, Bombyx mori (35–38): it is produced as a latent proenzyme of 47 kDa and processed under acidic conditions to a 39-kDa form, the active enzyme present in embryos. In addition to exhibiting enzymatic properties characteristic of cathepsin L-like cysteine proteases, this Bombyx protease shows high sequence similarity to mammalian cathepsin L. Importantly, the direct action of Bombyx cathepsin L-like cysteine protease on vitellin has been demonstrated. The cDNA encoding the proenzyme of this Bombyx cathepsin L-like cysteine protease has been cloned from the ovarian cDNA library. Furthermore, immunological analyses have shown that it is produced in the ovary by follicular cells and is then deposited in developing oocytes (35–38).

In Drosophila, cathepsin B-like protease is presumed to be maternally produced; however, its precise origin is not known (32). Previously, we demonstrated that mosquito VCP, one of the enzymes present in the yolk bodies, is secreted by the fat body (42; 43). Furthermore, immunocytochemical study has suggested that the fat body and follicular epithelium are the sources of the vitellogenin-processing protease (79). In this study, however, we provided solid molecular and biochemical proofs that the mosquito embryonic cathepsin B-like protease that we describe here is produced exclusively by the fat body as a yolk protein precursor.

In oviparous animals, extraovarian tissues play an important role in egg maturation by producing yolk protein precursors, which serve as a major nutritional source for developing embryos. The liver of oviparous vertebrates and the fat body of invertebrates, which serve as a major nutritional source for developing embryos, contain the enzymes present in the yolk bodies, is secreted by the fat body (42; 43). Furthermore, immunocytochemical study has suggested that the fat body and follicular epithelium are the sources of the vitellogenin-processing protease (79). In this study, however, we provided solid molecular and biochemical proofs that the mosquito embryonic cathepsin B-like protease that we describe here is produced exclusively by the fat body as a yolk protein precursor.
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insects both produce large quantities of yolk protein precursors, which are subsequently internalized by developing oocytes and deposited in yolk bodies. The most abundant of these yolk protein precursors is Vg, a large glycoprophopholipid consisting of both invertebrate and vertebrate oviparous animals (80–83). In Lepidopteran insects, the fat body additionally produces a smaller yolk protein precursor, either microvitellinogen or the 30-kDa yolk protein (84–86). In the mosquito, however, the proenzymes VCB and VCP are produced by the fat body as yolk protein precursors. The discovery that enzymes involved in the embryonic degradation of yolk proteins are produced as precursors by an extracellular organelle that enzymes involved in the embryonic degradation of yolk proteins are produced as precursors by an extracellular organelle that enzymes involved in the embryonic degradation of yolk proteins are produced as precursors by an extracellular organelle.

Acknowledgments—We thank Drs. T. Sappington, K. Deitsch, and A. Biran for critical reading of the manuscript.

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