Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 10821–10824, December 1991
Developmental Biology

An extraovarian protein accumulated in mosquito oocytes is a carboxypeptidase activated in embryos

(insect/yolk protein/vitellogenesis/*Aedes aegypti*/processing protease)

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Communicated by Wendell L. Roelofs, September 9, 1991 (received for review July 16, 1991)

ABSTRACT **We report a phenomenon previously unknown for oviparous animals; in *Aedes aegypti* mosquitoes a serine carboxypeptidase is synthesized extraovarially and then internalized by oocytes. The cDNA encoding mosquito vitellogenic carboxypeptidase (VCP) was cloned and sequenced. The VCP cDNA hybridizes to a 1.5-kilobase mRNA present only in the fat body of vitellogenic females. The deduced amino acid sequence of VCP shares significant homology with members of the serine carboxypeptidase family. Binding assays using a serine protease inhibitor, [^{3}H]diisopropyl fluorophosphate, showed that VCP is activated in eggs at the onset of embryonic development. Activation of VCP is associated with the reduction in its size from 53 kDa (inactive proenzyme) to 48 kDa (active enzyme). The active, 48-kDa, form of VCP is maximally present at the middle of embryonic development and disappears by the end.**

In oviparous animals, development of an embryo depends upon utilization of yolk proteins accumulated by oocytes during vitellogenesis. In most oviparous animals—e.g., nematodes, arthropods, and vertebrates—the task of producing massive amounts of yolk protein precursors is performed by extraovarian tissues (1–5). The developing oocytes of these animals are highly specialized for the specific accumulation of extraovarian yolk protein precursors. The process of receptor-mediated endocytosis by which yolk protein precursors are accumulated in oocyte yolk bodies is well characterized for insects and vertebrates (6, 7). In contrast, the mechanisms of degradation of yolk proteins have received considerably less attention. Several enzymes that participate in hydrolysis of yolk proteins in insect embryos have been recently described (8–11). However, the origin of these enzymes and the time of their delivery into yolk bodies are unknown.

In this paper, we report a biological phenomenon previously unknown for insects or other oviparous animals. In vitellogenic female mosquitoes, an extraovarian tissue, the fat body, synthesizes not only the yolk protein precursor vitellogenin but also a proenzyme of a serine carboxypeptidase. We have named it vitellogenic carboxypeptidase (VCP).† This enzyme is internalized by developing oocytes, accumulated in yolk bodies, and activated during embryonic development.

MATERIALS AND METHODS

Animals. *Aedes aegypti* mosquitoes were reared as described by Hays and Raikhel (12). The embryonic stages of *A. aegypti* were determined according to Raminani and Cupp (13).

Cloning and Sequencing of cDNA. A cDNA clone of VCP was isolated by immunoscreening of a λgt11 library generated from fat bodies of vitellogenic female mosquitoes. This cDNA clone, pVCP-1, was used to isolate another cDNA clone, pVCP-2, encoding the 5′-end 1020 base pairs (bp) of VCP from the same library. The pVCP-2 cDNA clone was sequenced in both directions with the dideoxy chain termination method (ref. 14, pp. 13.65–13.69).

The missing part of the 3′-end sequence was obtained by the polymerase chain reaction, using 20 nucleotides at the 3′ end of pVCP-1 and $(dT)_{17}$ as primers and including the *Xba* I digestion site at the 5′ ends of both primers (15). Amplification was achieved by using cDNA reverse transcribed from 20 μg of total RNA prepared from whole bodies of mosquitoes 24 hr after a blood meal as templates. The specific amplified band, identified by Northern blot hybridization to 1.5-kilobase (kb) mRNA, was subcloned in pUC119 for sequencing. Like the pVCP-2 cDNA clone, the 3′-end VCP cloned was sequenced in both directions.

Protein Purification for Partial Sequencing. Mosquito ovaries, 48 hr after a blood meal, were homogenized in 20 mM Tris·HCl, pH 7.5/150 mM NaCl/0.02% sodium azide. The VCP was isolated from the protein extract by anion-exchange, affinity, and hydrophobic chromatographic steps using DEAE-Sepharose CL-4B, concanavalin A, and phenyl-Sepharose columns, respectively. A fraction with nearly pure protein was resolved by SDS/PAGE and subjected to peptide sequencing using the method and facilities described by Cho *et al.* (16).

Purification and Analysis of RNA. Isolation of total and polyadenylylated RNA [poly(A)$^+$ RNA] from mosquito tissues was performed as previously described (17).

In vitro translation of mosquito poly(A)$^+$ RNA was performed as described by Bose and Raikhel (17), using rabbit reticulocyte lysate (Promega). The VCP precursor was identified in *in vitro* translation products of poly(A)$^+$ RNA by immunoprecipitation with anti-VCP polyclonal antibodies (12).

The 840-bp insert from pVCP-1, a putative VCP cDNA clone, was used for hybrid selection of VCP mRNA from the fat body poly(A)$^+$ RNA according to Sambrook *et al.* (ref. 14, pp. 8.50–8.51). The hybrid-selected mRNA was translated *in vitro* in rabbit reticulocyte lysate and the protein product was resolved by SDS/PAGE. The pUC119 plasmid without an insert was used for hybrid selection of mRNA as control.

For the Northern analysis, total RNA was separated by 1.2% formaldehyde/agarose gel electrophoresis, transferred to nitrocellulose paper, and hybridized first to a ^{32}P-labeled 840-bp VCP cDNA insert from pVCP-1 under high-stringency conditions. RNA hybridized to the VCP cDNA

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Abbreviations: DFP, diisopropyl fluorophosphate; VCP, vitellogenic carboxypeptidase; WCP, wheat carboxypeptidase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M79452).

probe was visualized by autoradiography of the blot. The probe was then stripped from the blot (ref. 14, p. 9.58) and the blot was rehybridized with a ^{32}P-labeled 1.8-kb fragment of vitellogenin gene A1 (gift of H. H. Hagedorn, University of Arizona) under high-stringency conditions.

Immunoblot Analysis. Preparation of proteins, SDS/PAGE, and immunoblotting were performed as described by Hays and Raikhel (12).

Binding to Protein Inhibitor. Protein extracts, prepared as for immunoblotting but without any protease inhibitors, were incubated with [^{3}H]diisopropyl fluorophosphate ([^{3}H]DFP; DuPont) at 0.1 μCi/μl (1 μCi = 1 kBq) for 1 hr at room temperature. These mixtures were then separated by SDS/PAGE under reducing conditions and processed for fluorography.

Analysis of Deduced Amino Acid Sequence. The analysis of deduced amino acid sequence was performed by using the FASTA program (University of Wisconsin Genetics Computer Group software) according to the algorithm of Lipman and Pearson (18). The deduced amino acid sequence of VCP was compared with amino acid sequences from the National Biomedical Research Foundation Protein Data Base (release 21.0) and with deduced amino acid sequences translated from GenBank (release 66.0) and European Molecular Biology Laboratory (release 19.0) data bases. The initial score was calculated from the best subsequence alignment of two sequences. The optimized score, calculated by considering insertions or deletions, gives a better indication about functional relation between proteins. The statistical significance of the scores was evaluated by Z value [(similarity score − mean of random scores)/(standard deviation of random scores); $Z > 10$ indicates statistical significance]. The similarities between VCP and other sequences were calculated as the percentage of amino acid number having identical residues or functional substitutions relative to the total number of amino acids in VCP (441).

RESULTS AND DISCUSSION

Recently, a female-specific protein (M_r = 53,000), initially called 53KP, has been found in *A. aegypti*. Like vitellogenin, this protein is synthesized by the fat body of vitellogenic females under the control of 20-hydroxyecdysone. The kinetics of the 53KP production by the vitellogenic fat body is also similar to that of vitellogenin: it is produced as early as 4 hr and reaches its peak near 24 hr after the initiation of vitellogenesis. Synthesis then drops to low levels by 36 hr and declines to background levels by 48 hr. This protein is secreted to the hemolymph and is selectively accumulated in yolk bodies of developing oocytes (12). Data presented here show that this protein (VCP) is a serine carboxypeptidase.

The cDNA encoding VCP was cloned by a combination of immunoscreening of a λgt11 cDNA library and the polymerase chain reaction. The identity of the cDNA was confirmed by direct sequencing of the N terminus of the purified VCP and by *in vitro* translation of hybrid selected mRNA (data not shown).

Northern blot analysis demonstrated that transcription of the VCP mRNA is limited to female fat bodies and is initiated after a blood meal. Similar to vitellogenin mRNA, the VCP mRNA in the fat body is maximal at the peak of the protein production, 24 hr after a blood meal, then it declines to background levels by 48 hr after a blood meal (Figs. 1 and 2).

The sequence of full-length cDNA encoding VCP, confirmed by the sequencing of cDNA clones in both directions, is presented in Fig. 3. The size of VCP mRNA of 1.5 kb, estimated by Northern blot analysis, is in agreement with the 1511-bp mRNA estimated from the cDNA sequence. The VCP cDNA has a single open reading frame that encodes a protein of 441 amino acids with a deduced molecular mass of

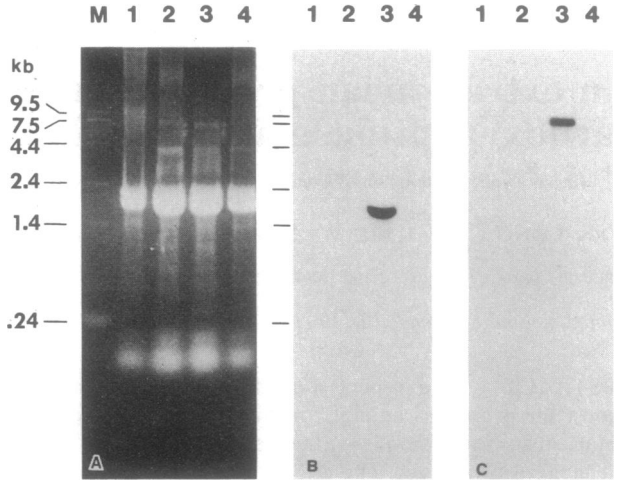

FIG. 1. Northern blot analysis of sex- and stage-specific expression of the VCP mRNA transcript. Total RNA was extracted from whole bodies of male mosquitoes (lane 1) and fat bodies of female mosquitoes before a blood meal (lane 2) or 24 and 48 hr after a blood meal (lanes 3 and 4, respectively). An RNA ladder (BRL) was resolved in lane M. (*A*) Agarose gel stained with ethidium bromide. (*B*) Hybridization with 0.84-kb fragment of VCP cDNA. (*C*) Hybridization with 1.8-kb fragment of mosquito vitellogenin A1 gene.

50,153 Da. Cell-free translation of VCP mRNA revealed that the VCP precursor is a 50-kDa polypeptide (data not shown). Hydropathy analysis (19, 20) of the deduced amino acid sequence has shown that VCP exhibits properties typical of a secretory protein. The amino acid sequence of VCP has only one potential glycosylation site, at position Asn-135. Glycosylation of VCP by mannose polysaccharide, which accounts for about 2 kDa of the molecular mass, was demonstrated previously (12).

In other insects, small extraovarian proteins are also involved in formation of yolk protein reserves. Microvitellogenin (31 kDa) of *Manduca sexta* and *Hyalophora cecropia* and the 30-kDa protein of *Bombyx mori* are produced by the fat body and deposited in oocytes (21–23). The nucleotide sequence of *Manduca* microvitellogenin cDNA has 70% similarity to the cDNA sequence coding for the *Bombyx*

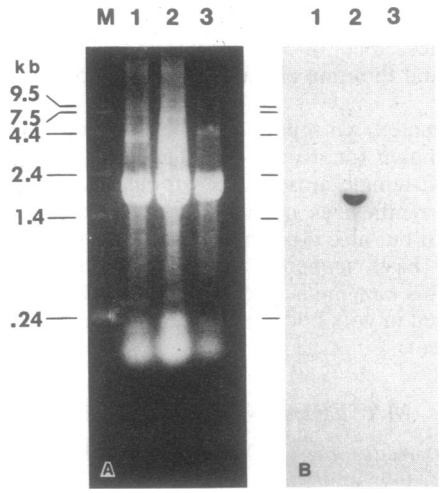

FIG. 2. Northern blot analysis of tissue-specific expression of the VCP mRNA transcript. Total RNA was extracted from ovaries (lane 1), fat bodies (lane 2), and midgut (lane 3) of female mosquitoes 24 hr after a blood meal. An RNA ladder (BRL) was resolved in lane M. (*A*) Agarose gel stained with ethidium bromide. (*B*) Hybridization with 0.84-kb fragment of VCP cDNA.

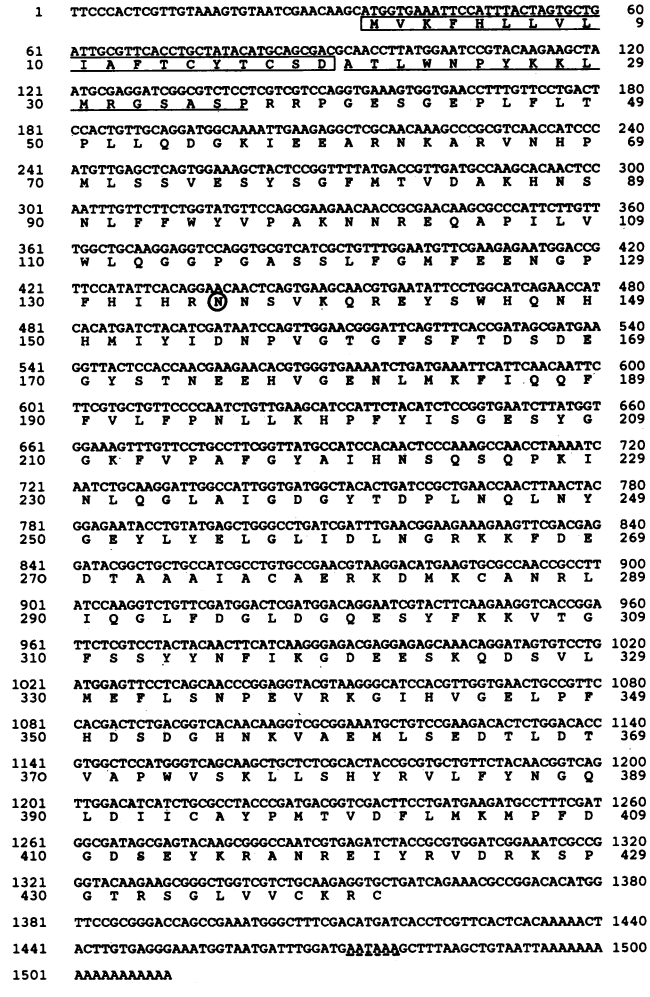

FIG. 3. Nucleotide and deduced amino acid sequences of mosquito VCP. The amino acid sequence matching the N terminus determined from purified VCP is underlined by a solid line. The signal peptide is boxed. The circled amino acid (N) is a potential glycosylation site. A putative polyadenylylation signal, AATAAA, is underlined by a broken line.

30-kDa protein, indicating a close evolutionary relationship between these proteins (24–26).

Comparison of amino acid sequences has not revealed any similarity between mosquito VCP and either *Manduca* microvitellogenin or *Bombyx* 30-kDa protein. Unexpectedly, the VCP sequence exhibited significant similarity with members of a family of serine carboxypeptidases (Table 1). The similarity between the amino acid sequences of mosquito VCP and these carboxypeptidases is highest at the N-terminal portion, which includes two conserved domains (27–32). The highest identity (28.1%) was found in a stretch of 392

Table 1. Homology of mosquito VCP to serine carboxypeptidases

Source	Protein	Score	*Z* value	Similarity, %
Wheat	Carboxypeptidase Y homolog	523	81.1	62.3
Mouse	Protective protein	434	54.8	45.6
Human	Protective protein	422	59.2	48.2
Barley	Serine carboxypeptidase I	394	42.9	56.7
Yeast	Carboxypeptidase Y	383	43.2	31.4
Barley	Serine carboxypeptidase II	380	49.1	35.4
Yeast	KEX1 carboxypeptidase	339	40.0	42.8

Only optimized scores are presented in this table; *Z* value is a statistical significance of optimized scores ($Z > 10$ indicates statistical significance).

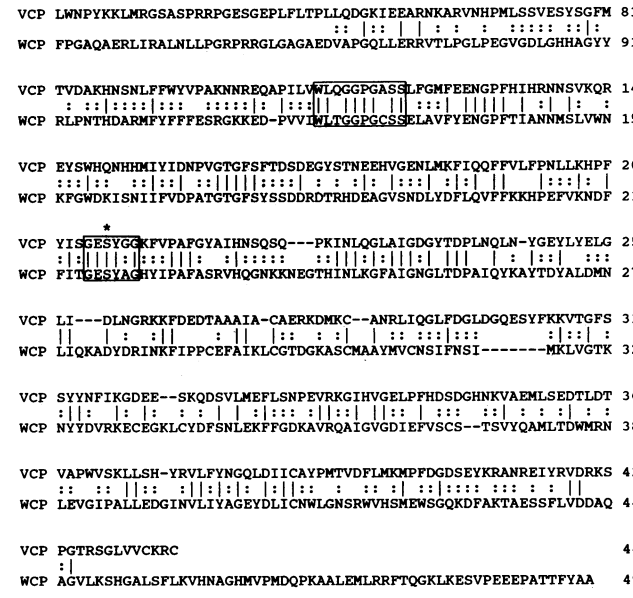

FIG. 4. Alignment of amino acid sequences of mosquito VCP and WCP. Amino acid sequences deduced from corresponding cDNAs were aligned by FASTA computer program. Vertical lines indicate identical residues and colons denote functional substitutions. Only portions of both amino acid sequences, flanking the overlapping regions, are presented. Two conserved domains found in sequences of serine carboxypeptidases are boxed. The serine protease catalytic center is marked by an asterisk.

amino acids of VCP and wheat carboxypeptidase Y homolog (WCP) (Fig. 4). However, considering both identical and conservative replacements, similarity between VCP and WCP is 62.3%. In mosquito VCP the conserved domains are at positions Trp-110 to Ser-119 and Gly-205 to Gly-210 (Fig. 4). The Ser-207 of the VCP second domain corresponds to Ser-257 of yeast carboxypeptidase Y, in a sequence that was shown to be the catalytic center of serine carboxypeptidases (33). In contrast to mosquito VCP and WCP (Fig. 4), all other members of the serine carboxypeptidase family have three conserved domains (27–32). Functional implications of these differences are not clear. Unlike the situation for serine endopeptidases, the importance of three domains for carboxypeptidase activity has not been established (34). Similar to all other serine carboxypeptidases in the family (27–32), the mosquito VCP does not have any significant homology with any known sequences of trypsin-like serine endopeptidases or esterases.

Internalization of VCP in mosquito oocytes occurs without any changes in its size (12). The molecular mass of VCP, however, decreases by 0.5–1.0 kDa at the onset of embryonic development (Fig. 5). As embryogenesis progresses, VCP undergoes further reduction in its size and another, immunologically related 48-kDa band appears (Fig. 5). By the end of embryonic development, VCP degrades into smaller peptides, which disappear once the first-instar larva hatches (Fig. 5).

Radiolabeled serine protease inhibitor, [^{3}H]DFP, which binds to the active center of serine proteases (35), binds weakly to VCP in oocytes (Fig. 5, lanes 1). The intensity of binding to VCP, however, increases at the onset and reaches maximum in the middle of embryogenesis, when the inhibitor binds to a VCP band of 48 kDa (Fig. 5). The binding of DFP to VCP can be inhibited by prior treatment of protein extracts with other serine protease inhibitors, phenylmethylsulfonyl fluoride or leupeptin (data not shown). On the basis of the above results, we conclude that VCP is synthesized by the fat body and internalized by oocytes as a proenzyme, which is

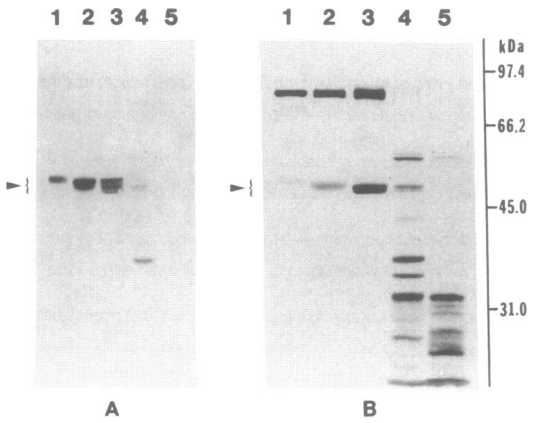

FIG. 5. Immunoblot (*A*) and [^{3}H]DFP binding (*B*) analyses of VCP during mosquito embryonic development. Both panels contain protein extracts from the following: lane 1, mosquito ovaries removed 24 hr after a blood meal during peak yolk accumulation; lane 2, 0- to 3-hr postoviposition eggs at the onset of embryogenesis; lane 3, 44- to 47-hr postoviposition eggs during midembryogenesis; lane 4, 94- to 97-hr postoviposition eggs at the end of embryogenesis; lane 5, first-instar larva. The mosquito VCP bands are shown by arrows. The high molecular mass polypeptide bound to DFP (*B*) is not immunologically related to VCP. In both panels, proteins were resolved by SDS/PAGE on 9% gels under reducing conditions. The molecular mass standards in order of decreasing mass are phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase (Bio-Rad).

then activated in eggs at the onset of embryogenesis. The activation of VCP is associated with the increase in its electrophoretic mobility. It is not clear, however, whether this increase in VCP mobility is due to its proteolytic cleavage or deglycosylation. The mouse carboxypeptidase (protective protein) is activated as a result of cleavage and DFP binds only to the activated carboxypeptidase subunit that contains the serine catalytic center (31, 32).

Some of the serine carboxypeptidases with which VCP shares significant homology, such as human and mouse protective proteins and yeast KEX1 carboxypeptidase, are implicated in proteolytic activation of a number of enzymes or other biologically active molecules (29–32). The mosquito VCP could play a similar role by activating hydrolytic enzymes that are involved in degradation of yolk proteins in developing embryos. Alternatively, it could function as an exopeptidase in sequential degradation of vitellogenin.

The mosquito VCP is different from proteases known to hydrolyze yolk proteins in insect embryos: an acidic cathepsin B-like protease of *Drosophila* and a thiol protease or cathepsin L-like protease of *Bombyx* (8–11). Similar to mosquito VCP, all these proteases are deposited as proenzymes in yolk bodies of eggs and activated in embryos. *Drosophila* cathepsin B-like protease is activated as a result of proteolysis by a serine protease (25 kDa) which is also present in egg yolk bodies (9). It is not known, however, whether any of these enzymes are of extraovarian origin or whether they are synthesized by developing oocytes themselves. The mosquito VCP, therefore, is, to our knowledge, the first example among oviparous animals of a proteolytic enzyme shown to be produced by an extraovarian tissue and accumulated by oocytes for use in embryonic development.

We thank Dr. T. S. Dhadialla and Mr. A. R. Hays for their advice in protein purification, Dr. H. H. Hagedorn for his generous gift of vitellogenin gene probe, and Drs. T. S. Dhadialla, T. B. Friedman, J. R. Miller, and N. V. Raikhel for their critical reading of the manuscript. This work was supported by grants from the National Institutes of Health and from the Biotechnology Center of the Michigan State University to A.S.R.

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