Characterization of a carboxypeptidase A gene from the mosquito, *Aedes aegypti*

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Abstract

A gut-specific carboxypeptidase A gene (*AeCP A*) from the mosquito, *Aedes aegypti*, was cloned and characterized. The gene has an open reading frame that predicts a protein of 427 amino acids, 61% of which are identical to an *Anopheles gambiae* carboxypeptidase A sequence. *AeCP A* messenger RNA (mRNA) was not detected during larval and pupal development. In situ hybridization experiments indicated that *AeCP A* mRNA is expressed by posterior midgut epithelial cells. In sharp contrast to *An. gambiae* carboxypeptidase A gene expression, *AeCP A* mRNA accumulates to high levels only late (≈16–24 h) after ingestion of a blood meal. The temporal profile of *AeCP A* gene induction is similar to that of *Ae. aegypti* late trypsin, suggesting the existence of common regulatory elements.

Keywords: carboxypeptidase A, mosquito, *Aedes aegypti*.

Introduction

The first site of interaction between mosquito vectors of human disease and the pathogens they transmit is the gut. Blood ingestion also initiates the reproductive cycle of anautogenous mosquitoes. Consequently, digestion of the blood meal in *Aedes aegypti*, the yellow fever mosquito, has been studied extensively at the molecular level (Barillas-Mury & Wells, 1995; Noriega & Wells, 1999). Ingestion of a blood meal induces two phases of trypsin synthesis in the adult female midgut. The first phase encompasses the initial (4–6 h) following a blood meal. It is characterized by low levels of trypsin, termed early trypsin, in the gut lumen. The second phase, between 8 and 36 h after blood feeding, is characterized by high levels of late trypsin activity.

The enzymatic activity of early trypsin plays a unique and critical role in the regulation of late trypsin synthesis. Early trypsin somehow acts as a ‘sensor’ of blood in the gut lumen. It carries out limited proteolysis of the ingested proteins, and the product polypeptides induce the synthesis of late trypsin. Late trypsin activity hydrolyses the majority of protein in the blood meal. The regulation of early and late trypsin is significantly different. Early trypsin synthesis is regulated at the translational level, while late trypsin synthesis is regulated at the transcriptional level.

To complete proteolysis and yield amino acid products from a blood meal, the endo- and exopeptidase activity of trypsin must be complemented by exopeptidases. Aliphatic amino acid residues are sequentially cleaved from the C termini of proteins by carboxypeptidase A (EC 3.4.17.1). Carboxypeptidase A genes have been previously cloned from two haematophagous insects: the blackfly, *Simulium vittatum* (Ramos et al., 1993), and *Anopheles gambiae* (Edwards et al., 1997). In both insects, carboxypeptidase A messenger RNA (mRNA) expression is induced by a blood meal. However, the temporal dynamics of this induction differ remarkably between the two insects. Within the context of a comparative approach to investigating mosquito gut-specific gene regulation, this paper describes the cloning and expression of an *Ae. aegypti* carboxypeptidase A (*AeCP A*) gene.

Results and Discussion

Isolation of an *Aedes aegypti* carboxypeptidase A gene

When *Ae. aegypti* midgut cDNA was used as a template for polymerase chain reaction (PCR) with primers based on conserved sequence, a 321-bp amplification product with sequence similarity to carboxypeptidases was obtained. It was used as a probe to screen an *Ae. aegypti* genomic library and a HindIII restriction fragment of a positive clone was subsequently subcloned and partially sequenced (Fig. 1). A total of 2159 bp were sequenced, including a
1281-bp open reading frame, 689 bp of upstream sequence and 186 bp of downstream sequence (Fig. 2). The continuous open reading frame suggests that there are no introns interrupting the protein coding sequence. A putative TATA box is present 57 bp upstream from the putative start codon and a consensus polyadenylation site (AATAAA) is present 23 bp downstream of the termination codon. An arthropod initiator consensus sequence (Cherbas et al., 1988) is present 363 bp downstream (bold underline) of the putative start codon (Fig. 1).

**Figure 1.** Restriction map of an AeCP A genomic Hind III subclone. B, Bam HI; E, Eco RI; H, Hind III; S, Sma I. Numbers above the horizontal line represent approximate distance (in nucleotide number) from the left Hind III site. The open rectangle represents the open reading frame. The bar above the sequence indicates the location of the AeCPA RT-PCR product.

**Figure 2.** Nucleotide and predicted amino acid sequence of the Ae. aegypti carboxypeptidase A genomic clone. The putative TATA box and polyadenylation signal are underlined. A valine at position 363 (bold underline) is predicted to confer a preference for cleavage of uncharged amino acids with an aromatic or aliphatic side chain (Gardell et al., 1988). Nucleotide and amino acid numbers are presented to the right.
& Cherbas, 1993) was not found between the putative TATA box and the start codon.

**Comparative sequence analysis**

The predicted 427-amino acid (≈48 kDa) AeCP A sequence is most similar to the carboxypeptidase A sequences of three other dipteran species, An. gambiae (Edwards et al., 1997), S. vittatum (Ramos et al., 1993) and D. heteroneura with 61, 53 and 51% identity, respectively. The most similar vertebrate carboxypeptidase A sequence is from Rattus norvegicus (Gardell et al., 1988) at 38% identity. In contrast, AeCP A appears to be more closely related (84% identity) to an Ae. aegypti fat-body specific vitellogenic carboxypeptidase gene (AeVCP) (Cho et al., 1991; Deitsch & Raikhel, 1993). AeVCP belongs to the serine class of carboxypeptidases which is distinct from the family of metalloproteases that includes carboxypeptidase A.

Those residues critical for structure and function for a rat carboxypeptidase A gene (Gardell et al., 1988) are also conserved in the AeCP A sequence (Fig. 3). A hallmark of the carboxypeptidase A class of enzymes is the presence of an aliphatic residue at the active site of the enzyme. In the AeCP A sequence, this residue is predicted to be Val 363. Similarly, by comparison with the bovine carboxypeptidase A sequence, activation of the Aedes pro-carboxypeptidase A is likely to occur by cleavage at Arg 91 or 93 (Avilés et al., 1993).

Comparative analysis of the 689-bp upstream region of AeCP A with the same region of other gut-specific genes, including Ae. aegypti late trypsin and other dipteran carboxypeptidase A genes, did not reveal any obvious motifs. However, these genes are similarly regulated (e.g. gut specificity and induction by a blood meal) and they could share some common regulatory elements. Indeed, some gut-specific regulatory sequences can be conserved between insects as black fly carboxypeptidase A and An. gambiae trypsin upstream sequences drive the gut-specific expression of reporter genes in transgenic Drosophila (Xiong et al., 1995a; Skavdils et al., 1996). Furthermore, D. melanogaster nuclear proteins have been shown to bind potential regulatory DNA elements of a gut-specific An. gambiae trypsin gene (Shen & Jacobs-Lorena, 1998). The elucidation of mosquito gut-specific regulatory elements remains an important challenge for future research.

**Messenger RNA expression profiles**

The developmental- and tissue-specificity of AeCP A mRNA expression was assessed by Northern blot analysis of RNA extracted from larval, pupal and adult tissues (Fig. 4). AeCP A mRNA was undetectable in larval gut or non-gut (carcass) tissues, in pupae and in the carcasses of adult mosquitoes. A single band at ≈1.3 kb was detectable in samples of gut RNA from sugar-fed mosquitoes. The strength of the hybridization signal with RNAs from sugar-fed mosquitoes was somewhat variable. However, AeCP A mRNA abundance was always considerably higher in guts of blood-fed mosquitoes, suggesting that AeCP A transcription is enhanced by blood ingestion. No other bands were apparent at any other place on the blot, indicating that the probe and hybridization conditions were specific to the AeCP A transcript.

In situ hybridization experiments showed that AeCP A is expressed all along the epithelium of the posterior (expansible part) midgut (Fig. 5). This is the location of ingested blood and the compartment where digestion occurs. The time course of AeCP A mRNA induction by a blood meal was determined by Northern blots (Fig. 6). After a lag of approximately 4 h, AeCP A transcript abundance increases sharply to reach a peak at ≈16–20 h and then drops quickly after 24 h.

This pattern of AeCP A mRNA expression following a blood meal is similar to the expression profile of carboxypeptidase A mRNA in S. vittatum (Ramos et al., 1993). It is in sharp contrast to that of a carboxypeptidase A gene in An. gambiae (AgCP A), where the titre of AgCP A transcripts increase markedly (≈10-fold) and rapidly (3–4 h) following a blood meal. This peak of early expression occurs about 1 day before the peak of trypsin expression (Edwards et al., 1997), but is transient, and by 24 h after the blood meal, AgCP A mRNA abundance decreases to levels comparable to those prior to a blood meal. Trypsin mRNA, on the other hand, peaks at late times (≈20–24 h) in S. vittatum (Xiong & Jacobs-Lorena, 1995b), An. gambiae (Lemos et al., 1996; Edwards et al., 1997) and Ae. aegypti (Fig. 5). The different temporal pattern of carboxypeptidase A mRNA expression in the two mosquito species suggests that the AeCP A and AgCP A genes serve different functions.

Early carboxypeptidase A expression in An. gambiae may be required for the activation of trypsin gene expression perhaps by the release of free amino acids from peptides generated soon after blood ingestion (Edwards et al., 1997). In Ae. aegypti, the similarity between the patterns of AeCP A and late trypsin expression during the final stages of blood digestion suggests that AeCP A serves a single role in late digestion by hydrolysis of the products of endopeptidases. An identical, single role was also suggested for S. vittatum carboxypeptidase A following a blood meal (Ramos et al., 1993). The concomitant expression profiles of AeCP A and Aedes late trypsin (Fig. 6) raises the possibility that these genes may share common regulatory elements.

Methods to analyse Ae. aegypti promoter activity in transgenic Ae. aegypti have become available using the transposable elements Mariner and Hermes (Coates et al., 1998, 1999; Jasinskiene et al., 1998). These substantial innovations provide invaluable new tools for the investigation of carboxypeptidase A function and gene regulation with experiments conducted in vivo.
Figure 3. Alignment of predicted amino acid sequences of AeCP A and the four most similar carboxypeptidase A sequences, as determined by an NCBI BLAST search. Predicted sequences were analysed using CLUSTAL W (1.74) multiple sequence alignment and the MacVector Software 6.0 package (Oxford Molecular Group). The accession numbers for each of the sequences are as follows: Ae. aegypti (AF165923), An. gambiae (AF000953), S. vittatum (U22453), D. heteroneura (AF049231) and Rattus norvegicus (M23714).
Experimental procedures

Mosquitoes and feeding protocols
Aedes aegypti mosquitoes were reared at 26°C and 80% relative humidity according to the methods of Munstermann (1997). Adults were used between 3 and 5 days posteclosion. Mosquitoes were denied access to sugar but had access to water for 12 h before feeding. Mosquitoes were fed with blood on anaesthetized mice or on a glass feeder (Lillie) heated to 37°C by a circulating water bath.

Polymerase chain reaction amplification, cloning and sequencing
Degenerate oligonucleotide primers (shown below) corresponding to regions of homology between S. vittatum, An. gambiae and mouse carboxypeptidase A genes, were used for reverse tr anscription PCR (RT-PCR).

Forward: 5¢-A TTCACGC(C/G)CG(A/C)GAA TGGA T-3¢
Reverse: 5¢-C(G/T)GGTCTC(A/G)ACCTCGGA(A/G)AA-3¢

PCR conditions were as follows: 94°C, 1 min; 58°C, 1 min; 72°C, 1 min 30 s, with forty cycles. cDNA generated from 0.2 µg of total Ae. aegypti midgut RNA was used as template. The RT-PCR product was cloned into the vector pGEM T-Easy (Promega). Approximately 600,000 plaques of a λDashII Ae. aegypti genomic library (kindly provided by Dr F. Collins, Notre Dame University, IN), were screened using the radiolabelled cloned RT-PCR fragment as a probe according to the methods of Sambrook et al. (1989). A 4.2-kb HindIII restriction fragment of a single genomic clone that contained the RT-PCR sequence (as identified by Southern analysis) was subcloned into the plasmid pBluescript IIKS+ vector (Stratagene) (see Fig. 1). Sequencing was performed with an automated ABI Prism Dye Terminator Cycle sequencer and custom synthetic oligonucleotide primers (Integrated DNA Tech.).

Northern analysis and in situ hybridization
Midguts were dissected from twenty mosquitoes at each time point analysed, frozen in liquid nitrogen immediately after dissection, and stored at −80°C. Total RNA was extracted using Tri- Reagent (Molecular Research Center). Total RNA (1 µg/lane) was separated on a 1.5% agarose gel containing 10% formaldehyde in MOPS buffer and transferred to a nylon membrane (GeneScreen, New England Nuclear). RNA was crosslinked to the nylon membrane using a UV crosslinker (Stratagene). The blots were hybridized sequentially with a radiolabelled 2.6 kb Eco RI fragment of the AeCP A genomic clone, with a Ae. aegypti late trypsin probe (Barillas-Mury et al., 1991), and with a 18S ribosomal RNA probe from Ae. albopictus (Baldridge & Fallon, 1991) as a loading control. After hybridization, blots were washed three times for 30 min each in 0.1% sodium dodecyl sulphate (SDS) and 0.2·SSC (1·SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C.

AeCP A was localized in the gut by in situ hybridization using an antisense AeCP A and sense AeCP A (control) RNA. These probes were generated by in vitro transcription with T7 RNA polymerase.

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polymerase in a reaction containing digoxigenin-labelled uridine triphosphate (UTP) (Boehringer-Mannheim). Midguts were dissected from mosquitoes 24 h after feeding on a meal of albumin (100 mg/ml in 0.15 M sodium bicarbonate) and fixed in 4% paraformaldehyde + 0.2% glutaraldehyde. Paraffin sections of guts were permeabilized with Triton X-100 and Proteinase K. Hybridization and detection were carried out as described by Kimmonth (1996). The GenBank accession number is AF165923.

Acknowledgements

This investigation received financial support from the National Institute of Allergy and Infectious Diseases and the John D. and Catherine T. MacArthur Foundation to M.J.-L. A.M. received a postgraduate scholarship from Queen’s University and V.K.W. acknowledges support from NSERC (Canada).

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