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Storage and secretion of the peritrophic matrix protein Ag-Aper1 and trypsin in the midgut of *Anopheles gambiae*

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Abstract

The gene *Ag-Aper1* encodes a peritrophic matrix (PM) protein from the mosquito *Anopheles gambiae*. *Ag-Aper1* gene expression and protein localization in the mosquito midgut were studied during the course of a blood meal. Ag-Aper1 mRNA abundance does not change appreciably during the course of blood ingestion and digestion. Prior to a blood meal, the protein is stored in secretory vesicles of midgut epithelial cells. Moreover, Ag-Aper1 colocalizes to the same secretory vesicles as trypsin, indicating that these proteins use a common secretory pathway. Blood feeding triggers the secretion of vesicle contents into the midgut lumen, after which Ag-Aper1 is incorporated into the PM. Newly synthesized Ag-Aper1 protein was again detected within the midgut epithelial cells at 60 h after blood ingestion.

Keywords: mosquito, *Anopheles gambiae*, peritrophic matrix, trypsin, midgut.

Introduction

The peritrophic matrix (PM) is an acellular, semipermeable layer that lines the digestive tract of most insects (Peters, 1992). The PM defines an inner endoperitrophic space, which contains the ingested food, and an outer ectoperitrophic space which is the space between the PM and the midgut epithelium. There are two types of PM in insects (Wigglesworth, 1930; Peters, 1992). Type 1 PM is thick (typically 1–20 μm) and is produced by epithelial cells of the midgut. Most adult haematophagous insects, including mosquitoes, produce a type 1 PM that is secreted in response to distension of the midgut by an ingested blood meal (Shao *et al*., 2001). The PM forms a bag-like structure that separates the ingested blood from the surrounding epithelium. Type 2 PM is thin (typically 0.1–2 μm) and is produced by a specialized organ, the cardia, located at the junction of the foregut and midgut. Type 2 PM is constitutively produced, forming an open-ended sleeve-like structure along the length of the digestive tract.

The PM is composed mainly of proteins, including glycoproteins and proteoglycans (Richards & Richards, 1977; Peters, 1992). In addition, chitin is also thought to be an important structural component of the PM (Peters, 1992) by providing strength as well as a framework on to which the proteins attach (Schorderet *et al*., 1998; Wang & Granados, 2001). During recent years, a number of PM proteins have been isolated from a variety of insects (reviewed by Tellam *et al*., 1999). A common feature of these PM proteins is the occurrence of chitin-binding domains that may crosslink chitin fibrils thus creating a three-dimensional molecular structure (Tellam *et al*., 1999; Wang & Granados, 2001). Another possibility is that the chitin-binding domains bind to the GlcNAc-containing oligosaccharide moieties of other PM proteins, thus providing an alternative means for establishing a three-dimensional network (Tellam *et al*., 1999).

A number of functions have been attributed to the PM, including protection against pathogens and abrasion and, in case of the larval type 2 PM, compartmentalization of digestion (Billingsley, 1990; Tellam, 1996; Lehane, 1997; Terra, 2001). In adult mosquitoes the PM may delay digestion (Villalon *et al*., 2003). The mosquito *Anopheles gambiae* is the principal vector of the malaria parasite *Plasmodium falciparum* in sub-Saharan Africa. In order to complete development within its mosquito vector the parasite, which is ingested with a blood meal from its vertebrate host, must traverse the PM and invade the midgut epithelium (Ghosh *et al*., 2000). Therefore, the PM represents a barrier to malaria transmission (Sieber *et al*., 1991; Shahabuddin *et al*., 1993; Shahabuddin *et al*., 1995). To traverse this barrier, the malaria parasite secretes a chitinase

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to disrupt the PM locally (Huber *et al.*, 1991; Tsai *et al.*, 2001).

Very little is known about the molecular mechanisms involved in the synthesis of the PM. Understanding the molecular composition of the PM is a prerequisite for understanding its structure and assembly (Wang & Granados, 2001). Shen & Jacobs-Lorena (1998) had previously partially characterized a putative PM protein, Ag-Aper1, from the mosquito *An. gambiae*. The full length *An. gambiae Ag-Aper1* cDNA encodes a protein with a secretory signal peptide, followed by two chitin-binding domains separated by a short peptide linker. Furthermore, Ag-Aper1 was shown to bind chitin *in vitro*. Here we use antiserum raised against the recombinant Ag-Aper1 protein to examine its intracellular localization prior to blood feeding, its incorporation into the PM after blood feeding and its synthesis in preparation for the following blood meal. We provide the first direct evidence for storage of a PM protein in the midgut epithelium of anopheline mosquitoes and its secretion and incorporation into the PM following a blood meal.

Results

Expression of Ag-Aper1 *mRNA*

The *Ag-Aper1* gene is expressed only in adults and only in the gut (Shen & Jacobs-Lorena, 1998). As the adult mosquito PM forms only after ingestion of a blood meal, Northern analysis was used to investigate any changes of *Ag-Aper1* mRNA abundance in the midgut as a function of time after ingestion of a blood meal. As shown in Fig. 1, *Ag-Aper1* mRNA abundance changes little after ingestion of a blood meal.

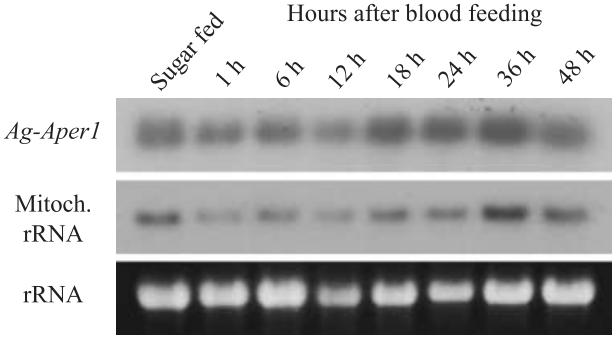

Figure 1. *Ag-Aper1* gene expression during a blood meal. Northern blot of total RNA isolated from adult female mosquito midguts before a blood meal (sugar-fed) and at different times after blood feeding as indicated. Upper panel: the blot was hybridized with a ^{32}P-labelled Ag-Aper1 probe. Middle panel: the same blot rehybridized with a ^{32}P-labelled mitochondrial rRNA probe used as a loading control. Lower panel: ethidium bromide-stained gel showing the ribosomal RNA before transfer to the membrane.

Expression of Ag-Aper1 and trypsin proteins

Rabbit polyclonal antiserum was raised against a recombinant Ag-Aper1-GST fusion protein expressed in *E. coli*. The specificity of the antiserum was tested by Western blot analysis. Ag-Aper1 protein is detected in the midgut of both sugar-fed and blood-fed mosquitoes but not in carcasses (Fig. 2A), as expected from its mRNA expression profile. Moreover, preimmune serum from the same rabbit failed to detect any protein in midguts from blood-fed mosquitoes (Fig. 2B), further confirming the specificity of the antibody. The antiserum detects a band of approximately 24 kDa, which is larger than the 15 kDa size predicted from its cDNA. Similarly, when a recombinant Ag-Aper1 protein was expressed from recombinant baculovirus in insect cells and detected using an antibody to total peritrophic matrix proteins, a band of approximately 30 kDa was detected (Shen & Jacobs-Lorena, 1998). The difference in apparent and predicted protein sizes of Ag-Aper1 may be due to glycosylation of the native protein.

Previous attribution of Ag-Aper1 as a PM protein was based only on circumstantial evidence, namely on tissue specificity of gene expression, on predicted domain function

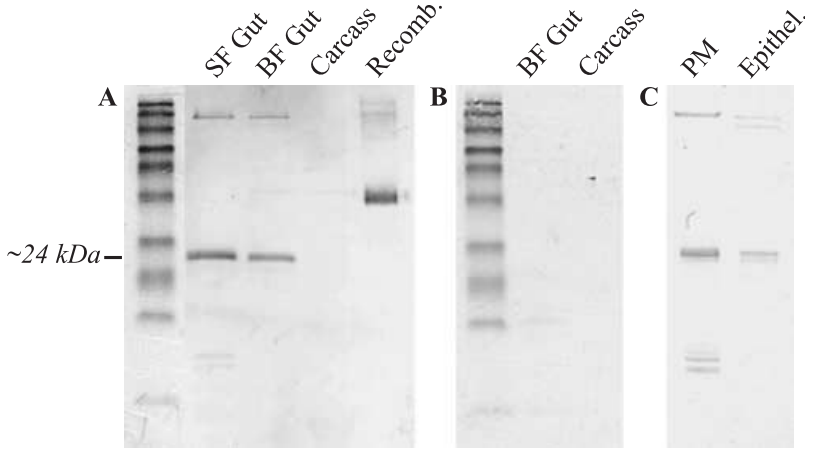

Figure 2. Specificity of the anti-(Ag-Aper1) antibody. (A) Western blot of proteins from the gut of a sugar-fed female (SF gut), a midgut from a female 12 h after a blood meal (BF gut), a carcass (all tissues except midgut) from a female 12 h after a blood meal and recombinant GST-fusion protein (Recomb.), detected with anti-(Ag-Aper1) immune serum. The recombinant is larger than the native protein because of the GST tag. (B) Western blot of midgut and carcass proteins from a female 12 h after a blood meal detected with preimmune serum. (C) Western blot of proteins from isolated PM and the corresponding gut epithelium (Epithel.), detected with anti-(Ag-Aper1) immune serum.

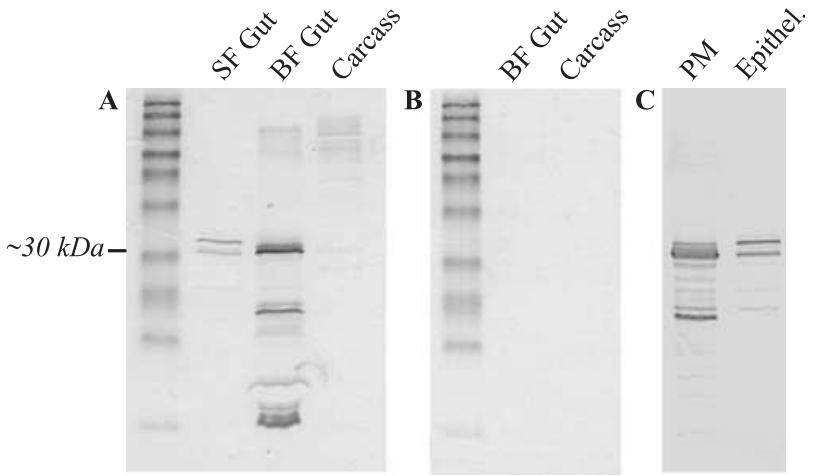

Figure 3. Specificity of the anti-Trypsin1 antibody. (A) Western blot of proteins from the gut of a sugar-fed female (SF gut), a midgut from a female 12 h after a blood meal (BF gut), and a carcass (all tissues except midgut) from a female 12 h after a blood meal, detected with anti-Trypsin1 immune serum. (B) Western blot of midgut and carcass proteins from a female 12 h after a blood meal detected with preimmune serum. (C) Western blot of proteins from isolated PM and enclosed endoperitrophic material and the corresponding gut epithelium (Epithel.) detected with anti-Trypsin1 immune serum.

(chitin binding) and the ability of the protein to bind chitin *in vitro*. To obtain direct evidence that Ag-Aper1 is a PM protein, dissected midguts and PMs were analysed with an anti-(Ag-Aper1) antibody. Mosquitoes were fed on a protein-free meal and their guts were dissected 12 h later. A protein-free meal allows the PM proteins to be studied in the absence of large amounts of blood proteins that may interfere with the analysis. The intact PM plus the endoperitrophic material contained within were dissected away from the midgut epithelium and analysed separately on Western blots. The majority of the Ag-Aper1 protein was associated with the PM fraction, while some Ag-Aper1 was also detectable in the midgut epithelium (Fig. 2C). The latter may represent PM fragments that remained attached to the midguts during dissection and/or newly synthesized protein located within the epithelial cells.

The expression of another abundantly secreted midgut protein, the trypsin Antryp1 (Müller *et al.*, 1993), was compared with that of Ag-Aper1 (Fig. 3). Bands of ~30 and ~33 kDa were detected on Western blots (Fig. 3A). Prior to a blood meal, the larger form predominates (Fig. 3A, left lane) while at 12 h after a blood meal the smaller form predominates (Fig. 3A, middle lane). The larger form is presumed to correspond to the proenzyme and the smaller form to the processed enzyme. The lower bands detected in the guts of blood-fed mosquitoes presumably represent protein that is degraded in the proteolytic enzyme-rich midgut lumen. As expected, no trypsin was detected in carcass tissues and preimmune sera did not detect any proteins in unfed or blood-fed midguts (Fig. 3B). At 12 h after a protein-free meal, the majority of the enzyme had been secreted into the endoperitrophic space, which is present within the PM (see above; Fig. 3C). There are seven *An. gambiae* trypsin genes and their protein sequences share significant homology. Consequently, it is possible that the antibodies produced against recombinant Antryp1 cross-react with the other trypsins. However, the amount

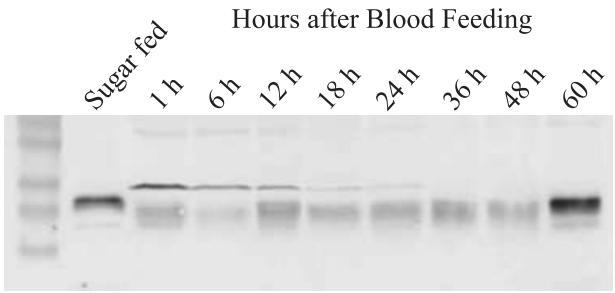

Figure 4. Expression pattern of Ag-Aper1 protein as a function of time after a blood meal. Western blot of proteins isolated from adult mosquito midguts before a blood meal (sugar-fed) and at different times after blood feeding as indicated, detected with the anti-(Ag-Aper1) immune serum.

of protein detected by the antibodies increases after blood feeding and this is only characteristic of Antryp1 and Antryp2 (Müller *et al.*, 1995). Furthermore, Antryp2 was not detected in unfed guts by Western blot by Müller *et al.* (1995). As the antibodies were raised against Antryp1 it is likely that they have the strongest reactivity against this protein.

Temporal pattern of Ag-Aper1 expression after intake of a blood meal

The temporal pattern of Ag-Aper1 protein expression was investigated by Western analysis of whole midguts dissected before and at different times after a blood meal (Fig. 4). The amount of Ag-Aper1 protein is fairly constant during the course of a blood meal indicating that the protein is stable within the digestive environment of the midgut. As the PM must maintain its integrity within the midgut lumen, the proteins associated with the PM have probably evolved to be resistant to proteolytic cleavage. After blood feeding, the protein bands appear diffuse and initially separated. This is due to the large amount of blood protein present in the samples. At 60 h Ag-Aper1 abundance increases in preparation for the next blood meal (see below).

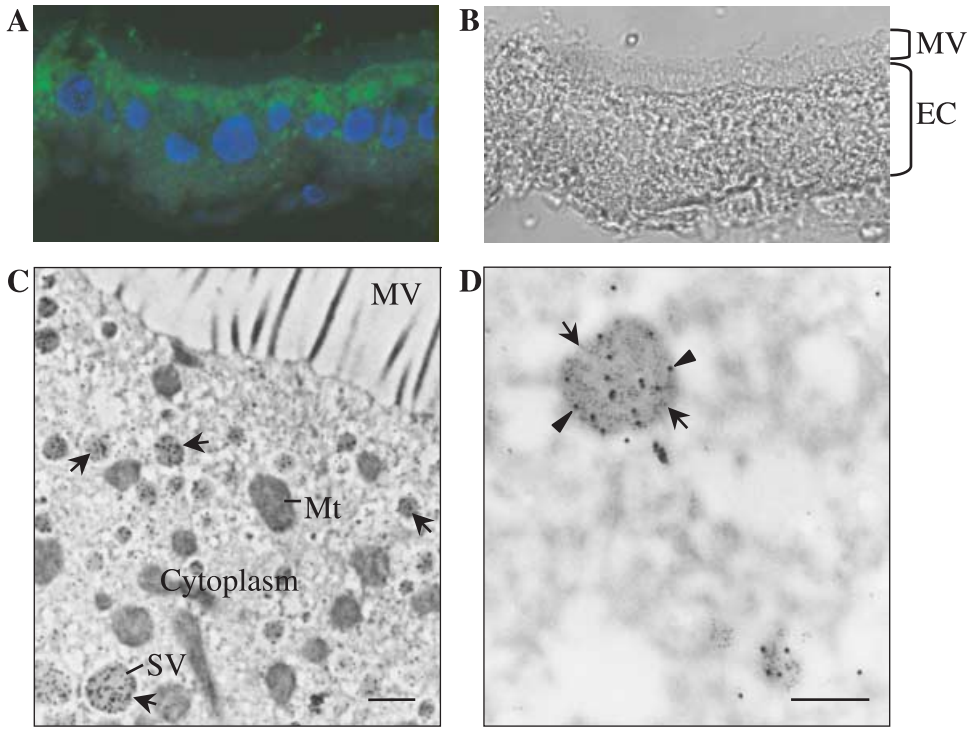

Figure 5. Subcellular localization of Ag-Aper1 and trypsin in midguts of sugar-fed females. (A) Confocal image of a midgut section from a sugar-fed adult female mosquito exposed to the anti-(Ag-Aper1) antibody and detected by immunofluorescence using a FITC-conjugated secondary antibody. Cell nuclei are stained with DAPI and appear blue. The midgut lumen is toward the top and the basal cell surface is toward the bottom. (B) The same section as the one to the left viewed with bright field optics. The microvillar brush border (MV) faces the midgut lumen. EC, epithelial cell layer. (C) Ultrathin midgut section exposed to anti-(Ag-Aper1) serum followed by gold-conjugated secondary antibody, examined by electron microscopy. The arrows point to gold particles within secretory vesicles (SV) below the lumenal surface of the epithelial cell. MV, microvilli; Mt, mitochondrion. (D) Ultrathin midgut section double labelled with anti-(Ag-Aper1) and anti-Trypsin1 sera. The specimen was incubated with secondary antibodies tagged either with 5 nm gold particles (Ag-Aper1; arrows) or with 15 nm gold particles (Trypsin1; arrow heads) and examined by electron microscopy. Scale bars = 1 μm.

Subcellular localization of Ag-Aper1 and trypsin

The midgut epithelial cells from anopheline mosquitoes contain a large number of apical secretion vesicles prior to blood feeding. Upon distension of the epithelium by ingestion of a blood meal, these apical vesicles disappear and their contents are presumably released into the midgut lumen. These apical secretion vesicles have been hypothesized to contain digestive enzymes and PM proteins but no direct evidence is available to support this assertion. The occurrence of a predicted signal peptide in the *Ag-Aper1* gene and the presence of the protein in the midgut of sugar-fed mosquitoes (Fig. 3A) are consistent with this hypothesis.

The subcellular localization of Ag-Aper1 was investigated using immunofluorescence- and electron-microscopy on cross sections of the posterior midgut. In sugar-fed mosquitoes, the Ag-Aper1 protein is distributed preferentially to the lumenal side of the midgut epithelial cells (Fig. 5A), coinciding with the location of the secretory vesicles. Similar sections exposed to preimmune serum failed to produce any fluorescent signal (data not shown). Immunoelectron microscopy further revealed that Ag-Aper1 is stored in apical vesicles (Fig. 5C). Interestingly, Ag-Aper1 and trypsin colocalize to the same vesicles (Fig. 5D), indicating that PM proteins and digestive enzymes use a common pathway of regulated secretion. Preimmune sera for both proteins failed to give any signal in similar sections (data not shown).

Changes in the distribution of Ag-Aper1 and trypsin as a function of time after a blood meal

The distribution of Ag-Aper1 in midguts dissected at different times after a blood meal was examined by immunofluorescence microscopy of cross sections of the posterior midgut using the anti-(Ag-Aper1) antibody (Fig. 6). Ag-Aper1 begins to be released into the midgut lumen soon (10 min) after blood ingestion, quickly forms a diffuse layer around the blood meal (1 h) and after 6 h the protein forms a more compact layer coincident with the PM, which is maintained until around 60 h. At this time, digestion is complete, the PM starts to break down and newly synthesized Ag-Aper1 can be again detected within the epithelial cells in anticipation of a new blood meal.

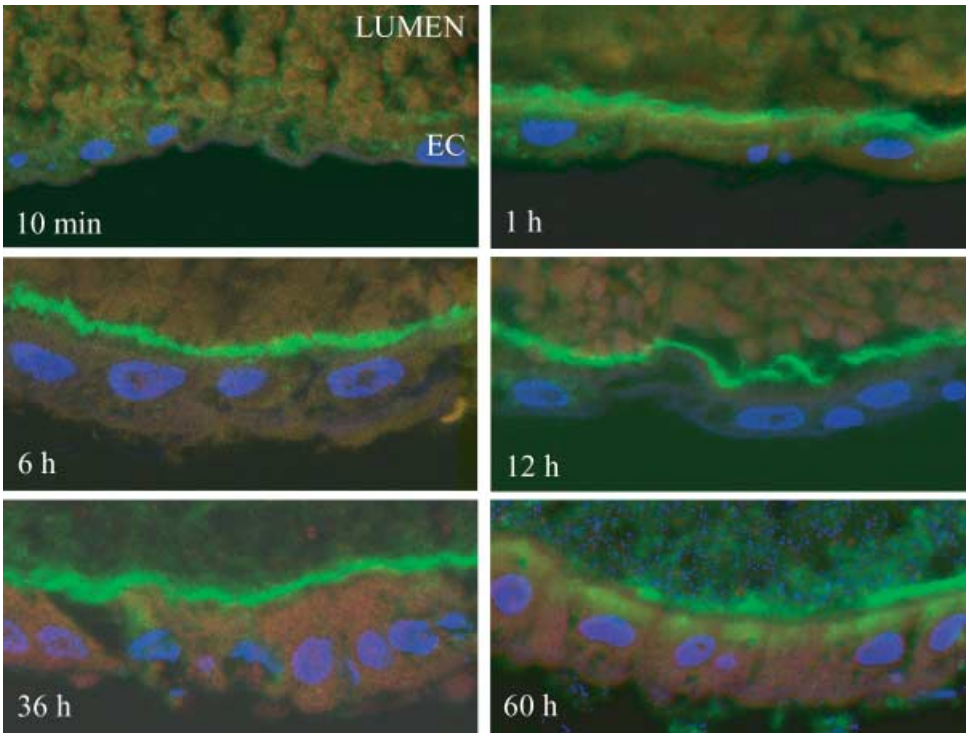

Figure 6. Localization of Ag-Aper1 in midguts at different times after a blood meal. Confocal images of midgut sections from blood-fed adult female mosquitoes dissected at various times after blood feeding. The sections were exposed to the anti-(Ag-Aper1) antibody and detected by immunofluorescence using an FITC-conjugated secondary antibody. In each case, the midgut lumen containing the blood meal is at the top and the epithelial cell layer (EC) is toward the bottom. Cell nuclei were stained with DAPI and appear blue.

The localization of Ag-Aper1 and trypsin in midguts of blood-fed mosquitoes was examined at higher resolution by immuno-electron microscopy (Fig. 7). At 6 h, Ag-Aper1 is found associated with the PM and over the microvilli (Fig. 7A). At 12 h Ag-Aper1 is seen accumulating at the PM layer, although some protein is also seen over the microvilli (Fig. 7B). The gold particles associated with the microvilli may represent protein that is associated with chitin fibres that are polymerizing within the microvillar layer. Nothing is known about the site of chitin synthesis in the mosquito midgut, although chitin fibres have been shown to assemble within the microvillar layer in other insect midguts (Harper & Hopkins, 1997; Harper & Granados, 1999). In contrast to Ag-Aper1, trypsin distribution at 12 h is more diffuse, extending from the microvilli into the blood meal (Fig. 7C). By 24 h Ag-Aper1 is almost exclusively localized to the PM forming a very distinct layer (Fig. 7D). Ag-Aper1 labelling within the endoperitrophic space is very sparse highlighting its tight association with the PM. These data are in agreement with the observations of Freyvogel & Staubli (1965) in which a mature PM was seen no earlier than 13 h post blood feeding in *An. gambiae*. Little Ag-Aper1 or trypsin is found in the epithelial cells after blood feeding, which is consistent with their rapid secretion after blood ingestion.

The relative distribution of Ag-Aper1 and trypsin was investigated by double immunofluorescence microscopy of midguts dissected at 24 h after a blood meal (Fig. 8). Antryp1 (green) is abundant and diffuses into the blood bolus, while Ag-Aper1 (red fluorescence but yellow in the photo, due to overlap with the green trypsin signal) is tightly associated with the PM, as expected. When sections from midguts dissected at increasing times after a blood meal were observed, the trypsin signal penetrated further into the blood meal (data not shown). These results are consistent with those for *Aedes aegypti* (Staubli *et al*., 1966; Graf *et al*., 1986) and correlate well with the massive increase in Antryp1 expression as digestion proceeds (Müller *et al*., 1995; Lemos *et al*., 1996).

Discussion

PM formation in mosquitoes is triggered by physical distension of the midgut epithelium, perhaps mediated by changes of the cellular cytoskeleton (Freyvogel & Jaquet, 1965; Berner *et al*., 1983; Billingsley & Rudin, 1992; Lemos *et al*., 1996). Yet, there are fundamental differences in how the PM forms in anopheline and culicine mosquitoes. The epithelial cells of anopheline mosquitoes contain a large number of apical vesicles prior to blood ingestion (Staubli *et al*., 1966; Hecker, 1977; Berner *et al*., 1983; Billingsley & Rudin, 1992). Upon blood feeding, the vesicles disappear and their contents are presumably released into the lumen

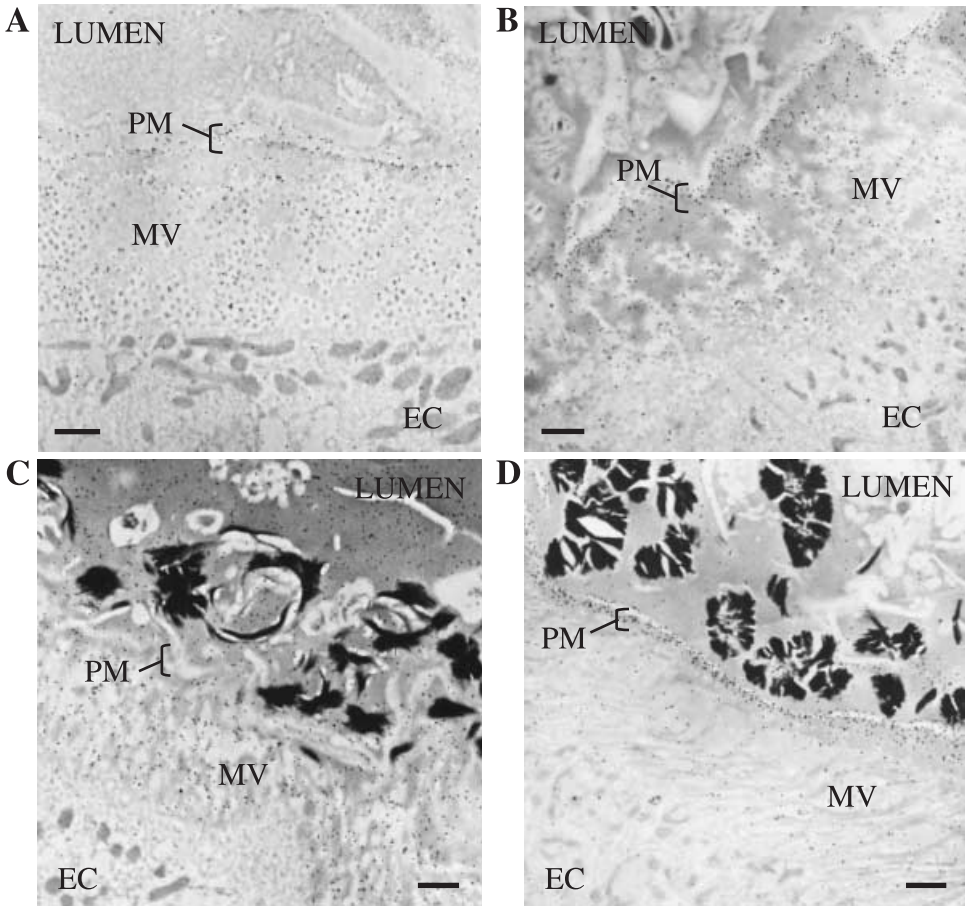

Figure 7. Localization of Ag-Aper1 and trypsin in midguts from blood-fed mosquitoes. Immuno-electron microscopy of ultrathin midgut sections from adult female mosquitoes, 6 (A), 12 (B) and 24 (D) hours after blood feeding. The sections were exposed to anti-(Ag-Aper1) immune serum followed by 15 nm gold-conjugated secondary antibody. (C) Midgut section from an adult female mosquito 12 h after blood feeding, exposed to anti-Trypsin1 serum. The PM, epithelial cell cytoplasm (EC), microvilli (MV) and midgut lumen are indicated. The dark aggregates in B, C and D are iron-containing byproducts of digestion. Scale bars = 1 μm.

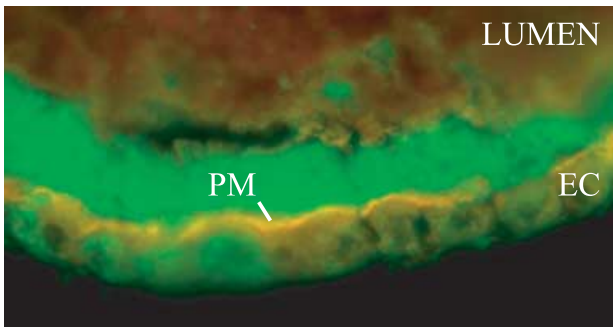

Figure 8. Simultaneous localization of Ag-Aper1 and Trypsin1 in the midguts from a blood-fed female. Section of an adult female midgut 24 h after blood feeding exposed to the anti-(Ag-Aper1) and anti-Trypsin1 sera and detected by immunofluorescence using Rhodamine- and FITC-conjugated secondary antibodies, respectively. The midgut epithelial cells (EC) are toward the bottom and the blood meal in the midgut lumen is toward the top.

by a process of regulated secretion (Staubli *et al.*, 1966; Berner *et al.*, 1983; Graf *et al.*, 1986). Here we provide the first direct evidence that a PM protein, Ag-Aper1, is produced prior to blood feeding and stored in apical secretory vesicles. Furthermore, Ag-Aper1 colocalizes to the same secretory vesicles as trypsin indicating a common path for the secretion of proteins with widely different functions.

It was observed that when the midgut of *An. stephensi* was experimentally distended *in vivo* by ingestion of latex beads or by an enema, followed about 1 day later by a blood meal, no PM formed (Berner *et al.*, 1983; Billingsley & Rudin, 1992). Presumably, the stored PM components were depleted upon the latex meal and not enough time had elapsed to replenish the PM components when the blood meal was taken. This interpretation is consistent with our results, since readily detectable intracellular Ag-Aper1 protein was first observed at 60 h and not at 24 h after a blood meal (Fig. 7). Berner *et al.* (1983) found that no PM forms in the presence of a mRNA-polymerase inhibitor, raising the possibility that not all PM components are stored as proteins.

In contrast with anophelines, culicine mosquitoes have few, if any, secretory vesicles (Staubli *et al.*, 1966; Hecker, 1977; Billingsley, 1990; Billingsley & Rudin, 1992) and the RER is often assembled into characteristic whorls resembling fingerprints (Bertram & Bird, 1961; Staubli *et al.*, 1966;

Hecker, 1977). After a blood meal, the RER whorls unfold, a process that correlates with the activation of translation of mRNAs encoding PM proteins and digestive enzymes (Staubli *et al*., 1966). Thus, mRNAs encoding PM proteins (rather than the proteins themselves) are thought to be stored in the guts of sugar-fed culicine mosquitoes (Staubli *et al*., 1966; Graf *et al*., 1986). Consistent with this view, mRNA encoding the *Ae. aegypti* PM protein AEIMUC1 is present before and after blood feeding, while the corresponding protein can only be detected after blood feeding (M. Devenport, P.H. Alvarenga, L. Shao, H. Fujioka, A. Ghosh, P.L. Oliveira & M. Jocobs-Lorena, unpublished results). However, the mRNA for a second *Ae. aegypti* PM protein, Aa-Aper50, only appears (and is translated) after blood feeding (L. Shao, M. Davenport, H. Fujioka & M. Jacobs-Lorena, unpublished results), suggesting transcriptional regulation.

Bolognesi *et al*. (2001) reported a peritrophic matrix protein from the larvae of the lepidopteran *Spodoptera frugiperda* which, similar to adult mosquitoes, produces a type 1 PM This protein is secreted by anterior midgut cells by microapocrine secretion (vesicles pinched off from the microvilli). A membrane-bound trypsin is secreted by the same route (Jordao *et al*., 1999). There is no evidence for microapocrine secretion of PM proteins or digestive enzymes in either the anterior or posterior midgut of mosquitoes (Hecker, 1977). This probably reflects distinct differences in the structure and function of the type 1 PM in these two groups of insects. *S. frugiperda* also produces a soluble trypsin in the posterior midgut cells that appears to be released by exocytosis (Jordao *et al*., 1999). This second trypsin more closely resembles trypsin secretion in anophelines, which also occurs by exocytosis (Berner *et al*., 1983).The abundance of the Ag-Aper1 mRNA and protein does not change appreciably following a blood meal (Figs 1 and 4). These observations suggest that the mRNA is stable and that synthesis of the protein may be translationally regulated. After secretion, the protein is re-synthesized (Fig. 6) and once enough protein has accumulated in the cell, synthesis must stop. The alternate view that Ag-Aper1 synthesis is constitutive and that the protein constantly turns over in the storage vesicles is considered to be less likely.

By Western analysis, we found that Antryp1 pro-enzyme is stored in midgut epithelial cells prior to a blood meal, in agreement with earlier results of Müller *et al*. (1995) and Lemos *et al*. (1996) obtained by use of enzymatic assays. We found that trypsin is stored in vesicles (Fig. 5) that are secreted after blood ingestion (Fig. 7). Trypsin secretion coincides with activation of the pro-enzyme (Fig. 3A) and autoactivation of recombinant Antryp1 has been observed *in vitro* (Müller *et al*., 1995). However, the mechanism for this activation in the mosquito gut remains to be elucidated. Expression of the *Antryp1* gene is strongly induced only after feeding on a protein-containing meal (Müller *et al*., 1995; Lemos *et al*., 1996). We hypothesize that the stored trypsin serves the function of 'tester enzyme', similar to the early trypsin of *Ae. aegypti* (Noriega & Wells, 1999). If the stored enzyme encounters protein when secreted, the digestion products will activate expression of its own gene in a positive loop.

The PM of adult *An. gambiae* mosquitoes may contain as many as forty different proteins (Moskalyk *et al*., 1996). The completion of the *An. gambiae* genome sequencing project and its ongoing annotation has led to the identification of a number of other putative *An. gambiae* PM proteins (Holt *et al*., 2002). It will be interesting to determine how many of these proteins are produced and stored prior to blood feeding, and what their roles are in PM assembly, structure and function. It may also be possible to identify conserved regulatory elements controlling the expression and secretion of the PM proteins. These may have uses for generating transgenic mosquitoes expressing exogenous antipathogenic factors that are stored in the midgut epithelium and secreted soon after blood ingestion (Ito *et al*., 2002; Moreira *et al*., 2002; E.G. Abraham, M. Donnelly-Doman, H. Fujioka, A. Ghosh, L. Moreira & M. Jacobs-Lorena, unpublished results).

Experimental procedures

Mosquitoes

An. gambiae larvae (strain 4arr) were reared on dry cat food. Adults were maintained on a 14 h:10 h light-dark period at 27 °C and 80% relative humidity. The adults were offered 20% sucrose solution *ad libitum*. Three to four day-old females were blood-fed on anaesthetized mice. Protein-free meals consisted of latex beads (LB-5, Sigma; Billingsley and Rudin, 1992) suspended in 150 mM NaCl, 10 mM $NaHCO_3$ pH 7.0 (Moskalyk *et al*., 1996) with 1 mM ATP added as a phagostimulant (Galun *et al*., 1963).

Northern blot analysis

Guts were dissected from either sugar-fed or blood-fed adult female mosquitoes at different times after a blood meal. All tissues were immediately frozen in an ethanol/dry ice bath and stored at –80 °C. Total RNA was extracted with TRI-Reagent (Molecular Research Center). Around 3.0 μg of total RNA was separated in each lane of a 1.5% agarose/formaldehyde gel and transferred to a nylon membrane (Gene Screen, New England). Hybridization was performed first with the full-length *Ag-Aper1* cDNA probe (Shen & Jacobs-Lorena, 1998) and then with a mitochondrial rRNA probe, C2 (Lemos *et al*., 1996), to indicate loading.

Preparation of the anti-(Ag-Aper1) and anti-Antryp1 antibodies

The full-length *Ag-Aper1* coding sequence (GenBank accession no. AF030431) was amplified by PCR and cloned into the pGEX-4T-1 expression vector (Pharmacia Biotech). Full-length Ag-Aper1 was expressed as a GST fusion protein in *E. coli* BL21-CodonPlus™ (DE3)-RP cells (Stratagene). Polyclonal anti-(Ag-Aper1) antibodies were generated in a rabbit after three intradermal protein injections at one-month intervals. For the initial immunization the fusion protein was purified as an inclusion body from the expressing cells

and approximately 550 μg of the sample was size fractionated by electrophoresis on a 12% polyacrylamide/SDS gel. Full-length fusion protein was excised from the gel, homogenized in 500 μl PBS and mixed with an equal volume of Freund's complete adjuvant. For the two subsequent boosts, the full-length protein was affinity purified using Glutathione Sepharose 4B beads (Pharmacia Biotech), excised from a 10% polyacrylamide/SDS gel, homogenized in 500 μl PBS and mixed with an equal volume of Freund's incomplete adjuvant. Immune serum was collected one month after the second boost and tested for activity by ELISA using the fusion protein as the antigen.

Polyclonal anti-Antryp1 antibodies were generated in the same way as described above except that the full-length protein (Müller *et al.*, 1993; GenBank accession no. Z18889) was expressed in a pET vector with a His tag and purified as an inclusion body for immunizations.

Immunoblot analysis

The following materials were collected and analysed by electrophoresis on 15% polyacrylamide/SDS gels: (1) guts from sugar-fed adult females (0.5 gut/lane) (2) guts from adult females 12 h after a blood meal (0.5 gut/lane) (3) carcasses (all tissues minus gut) from these females (0.5 carcass/lane) (4) intact PM (including the enclosed endoperitrophic material) and separately, the corresponding gut epithelium, from adult females 12 h after a latex meal (see above) dissected in 50% ethanol in PBS (1 PM or gut/lane), and (5) guts from females dissected at different times after a blood meal (0.5 gut/lane). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore), incubated with rabbit anti-(Ag-Aper1) (1 : 5000 dilution) or anti-Antryp1 (1 : 2000 dilution) polyclonal antibodies and the bound antibody was detected with an alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Promega, 1 : 7500 dilution) followed by detection with BCIP/NBT substrates. Pre-immune sera at the same concentrations were used as controls.

Immunofluorescence of midgut sections

Guts from blood-fed mosquitoes were dissected in 4% paraformaldehyde in PBS, fixed for 2 h at room temperature, and washed three times in PBS. After dehydration in a graded ethanol/PBS series, the guts were treated with xylenes, embedded in Paraplast (Oxford Labware) and 7–14 μm sections were mounted onto glass slides. The slides were washed twice in xylenes at room temperature followed by rehydration in a graded ethanol/PBS series. In order to reduce autofluorescence, slides were dehydrated and rehydrated in a graded methanol/PBS series. The sections were blocked in 10% non-fat milk and 0.1% Triton X-100 in PBS for 2 h at room temperature and incubated overnight at room temperature with the anti-(Ag-Aper1) or preimmune sera (1: 2000 dilution in blocking solution). Slides were washed three times for 30 min with blocking solution and incubated for 2 h at room temperature with FITC-conjugated anti-rabbit antibodies (Sigma, 1 : 600 dilution) followed by final washes in blocking solution. Nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) diluted with Slowfade Antifade solution (Molecular Probes). Immunostaining images were acquired by fluorescence microscopy using a Leica DMLB microscope and Spot™ RT Color camera model 2.2.0 with v.3.0 software (Diagnostic Instruments, Inc.) at 400× magnification. Confocal images were acquired using a 63× N.A. 1.4 oil immersion planapochromat objective with a Zeiss LSM 510 system.

Double labelling immunofluorescence of midgut sections

The above protocol was followed and slides were incubated sequentially with the following antibodies diluted in blocking solution: anti-(Ag-Aper1) primary serum (1: 2000 dilution) overnight at room temperature; Rhodamine-conjugated anti-rabbit Fab fragments (Jackson labs; 7.5 μg/ml) for 3 h at room temperature; an excess of unconjugated anti-rabbit Fab fragments (Jackson labs; 10 μg/ml) for 3 h at room temperature to block any unbound primary antibody; anti-Antryp1 (1 : 500 dilution) overnight at room temperature; and FITC-conjugated anti-rabbit antibody (Sigma, 1 : 600 dilution) for 2 h at room temperature. Slides were washed three times for 30 min with blocking solution after all antibody incubations. Red (rhodamine) and green (FITC) fluorescence were detected simultaneously using a double cube filter on the Leica DMLB microscope as described above.

Immuno-electron microscopy (IEM)

Adult female mosquito midguts, dissected before and at different times after blood feeding, were fixed for 1 h at 4 °C with 2% formaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Fixed samples were washed, dehydrated and embedded in LR White resin (Polysciences). Thin sections were blocked in PBS containing 5% w/v non-fat dry milk and 0.01% v/v Tween 20 (PBTM) for 30 min at room temperature (RT). Grids were then incubated with anti-(Ag-Aper1) or anti-Antryp1 primary antibodies (diluted 1 : 10–100) in PBTM for 2 h at RT. Control grids were incubated with preimmune rabbit sera in PBTM at the same dilution. After washing, grids were incubated for 1 h in 15 nm gold-conjugated goat anti-rabbit IgG (Amersham Life Sciences) diluted 1 : 20 in PBS containing 1% w/v bovine serum albumin (BSA) and 0.01% v/v Tween 20 (PBTB), rinsed with PBTB, and fixed with 2.5% glutaraldehyde to stabilize the gold particles. Samples were stained with uranyl acetate and lead citrate, and then examined in a Zeiss CEM902 electron microscope (Oberkochen, Germany).

IEM double-labelling

The first antibody/gold marker pair was applied to one surface of the section, which had been placed on a naked grid without a supporting film. Subsequently, that surface of the labelled side was dried and the other surface was labelled with a second antibody marker pair. Grids were fixed with glutaraldehyde to stabilize the gold particles, and then stained with uranyl acetate and lead citrate.

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