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The Peritrophic Matrix of Hematophagous Insects

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The peritrophic matrix (PM) is an extracellular envelope that lines the digestive tract of most insects. It is thought to play key roles in protecting insects from pathogens and facilitating digestion. Until recently, little information was available on the molecular composition of the PM. This review summarizes recent progress in the study of the PM from hematophagous insects, with emphasis on molecular and physiological aspects. Topics discussed include the presence of chitin and protein diversity in the PM, cloning and characterization of genes encoding PM proteins, PM permeability, and the role of the PM as a barrier for pathogens. Arch. Insect Biochem. Physiol. 47:119–125, 2001. © 2001 Wiley-Liss, Inc.

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INTRODUCTION

The peritrophic matrix (PM) is an acellular sheath that separates the ingested food from the absorptive/secretory intestinal epithelium. The peritrophic matrix, also known as the peritrophic membrane, can be classified into two distinct types based on the site of its synthesis (Peters, 1992). Type 1 PM is produced from all midgut epithelial cells. Typically, type 1 PMs of hematophagous insects are produced in direct response to blood feeding, forming a thick bag-like structure that completely surrounds the ingested meal (Jacobs-Lorena and Oo, 1996; Tellam et al., 1999). In contrast, most type 1 PMs of non-hematophagous insects (e.g., lepidopteran larvae) are constitutively synthesized (Peters, 1992). Type 2 PM is produced by a small group of highly specialized cells in the cardia, an organ located at the junction between the foregut and the midgut (Peters, 1992; Jacobs-Lorena and Oo, 1996; Tellam et al., 1999). This type of PM is a thin open-ended tube-like structure lining the entire midgut and hindgut. Despite the morphological differences between type 1 and 2 PMs, the differences in their molecular constitution are poorly understood. While both types of PM contain chitin, proteins, and proteoglycans, little is known about the individual components. Indeed, the first gene encoding a PM protein was identified only 5 years ago (Elvin et al., 1996). Among the difficulties encountered in the study of the PM are the small amount of material available and the frequent contamination by food remnants or enzymes secreted by the midgut epithelium. Several review articles have summarized the diverse aspects of PM regarding its nomenclature, components, synthesis, and proposed functions (Richards and Richards, 1977; Peters, 1992; Eisemann and Binnington, 1994; Jacobs-Lorena

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and Oo, 1996; Lehane, 1997; Tellam et al., 1999). Here we attempt to highlight current advances in the study of the PM of hematophagous insects and discuss future research prospects. Features of PM from non-hematophagous insects are also mentioned and discussed in more detail elsewhere in this issue.

CHITIN

Chitin, a linear polymer of N-acetylglucosamine (GlcNAc), is thought to be an important structural component of the PM, providing strength and a framework for assembly. GlcNAc was shown to be present in the type 1 PM from *Aedes aegypti* (Huber et al., 1991) but apparently not in the PM from *Anopheles stephensi* (Berner et al., 1983). However, chitinases can dramatically alter the structure of type 1 PM from both mosquito species in vitro and in vivo (Huber et al., 1991; Shahabuddin et al., 1993), providing strong evidence that chitin plays a major role in maintaining type 1 PM structure of both mosquitoes. Recently, Tellam et al. (1999) suggested that the evidence for the presence of chitin in type 2 PM needs to be reexamined. Their argument was due largely to the consideration that the histochemical techniques used in previous studies to detect chitin in the PM also detect glycosylated proteins and most PM proteins identified so far are glycosylated. More recently, using a combination of biochemical, histochemical, and molecular biological analyses, they reinvestigated the chitin content of the type 2 larval PM from *Lucilia cuprina* (blowfly) (Tellam and Eisemann, 2000). Although no single test is completely conclusive, two lines of evidence indicate that chitin constitutes a small but significant proportion of the mass of *L. cuprina* type 2 PM: (1) chitin accounts for approximately 5% of the dry weight of the PM; (2) incubation of *L. cuprina* type 2 larval PM with a chitinase did not noticeably change its structure. In a separate study, Edwards and Jacobs-Lorena (2000) also showed that incubation of dissected *Ae. aegypti* or *An. gambiae* type 2 larval PM in 1 U/ml chitinase did not change on PM structure when observed by light microscopy. However, using a newly developed in vivo assay for estimating mosquito larval PM permeability (see "PERMEABILITY AND DIGESTION"), chitinase treatment was found to have a limited but significant effect on permeability. All available data are consistent with the hypothesis that chitin is an essential structural component of type 2 PMs, even if its abundance is relatively low. Resistance to chitinase treatment can be attributed to steric hindrance by other tightly linked PM components (proteins, proteoglycans) that may block access of the chitinase to its substrate. However, the data do not exclude the alternate hypothesis that chitin plays no major structural role. Further research is needed to clarify these issues.

PM PROTEINS

In a range of insects, proteins have been reported to account for 22–55% of the total mass of the PM (Lehane, 1997). However, information about the PM protein composition is limited. Two-dimensional gel electrophoresis of a type 2 PM from adult *Glossina morsitans morsitans* (tsetse fly) suggested approximately 40 proteins (Lehane et al., 1996). Similar 2D-gel analyses indicated that the type 1 PMs of *Ae. aegypti* and *An. gambiae* also have about 20–40 major proteins, whereas type 1 PM from black flies contains only 2 major proteins (Ramos et al., 1994; Moskalyk et al., 1996). The number of proteins may be underestimated since harsher treatments of PM preparations with denaturing agents may extract additional proteins (East et al., 1993).

Further understanding of PM proteins has been achieved since the application of molecular biology techniques to this field. In 1996, Elvin et al. cloned and characterized a type 2 PM protein (peritrophin-44) from the larvae of *L. cuprina* (Elvin et al., 1996). Two further type 2 PM proteins (peritrophin-48 and peritrophin-95) have also been isolated from *L. cuprina* larvae (Casu et al., 1997; Schorderet et al., 1998). All three peritrophins have similar organization in that they possess an amino-terminal secretory signal sequence followed by five domains, each 65 amino acids in length, containing 6 conserved cysteines. These cysteine-rich domains are similar to the chitin-binding domains of chitinases from several animals and microorganisms (Kramer and Muthukrishnan, 1997). This, along with the fact that peritrophin-44 binds to reacetylated chitosan in vitro, strongly suggested that these domains in-

teract with the chitin and possibly maintain PM structure by crosslinking chitin fibrils. The apparent differences between peritrophin-44/-48 and peritrophin-95 are the presence of a proline-rich (mucin-like) carboxyl-terminal domain and extensive glycosylation of the latter. Wang et al. identified a novel invertebrate intestinal mucin, designated insect intestinal mucin (IIM), from the larvae of the lepidopteran *Trichoplusia ni* (Wang and Granados, 1997b). Sequence analysis of IIM also indicated putative chitin binding domains.

In our laboratory, immunoscreening of a midgut cDNA expression library with an anti-PM antibody led to the cloning of a putative type 1 PM protein gene, *Ag-Aper1,* from the mosquito *Anopheles gambiae* (Shen and Jacobs-Lorena, 1998). The predicted protein is comprised of two tandem chitin-binding domains separated by a short proline-rich linker. The presumed capability of *Ag-Aper1* to bind chitin was verified by a functional assay using baculovirus-expressed recombinant protein. Therefore, this protein is proposed to act as a molecular linker that connects PM chitin fibrils into a three-dimensional network (Shen and Jacobs-Lorena, 1998). Immunological localization of *Ag-Aper1* in sections of the midgut indicated that before the blood meal the protein is associated with the apical region of the epithelial cells, while after the blood meal the protein was depleted from the cells and was associated with the PM (Devenport and Jacobs-Lorena, unpublished observations). A second cDNA was cloned from the same library that encodes a predicted mucin-like protein, *Ag-Muc1* (Shen et al., 1999). *Ag-Muc1* consists of a central core of seven repeated threonine-rich motifs flanked by hydrophobic amino- and carboxy-terminal domains. When *Ag-Muc1* was tagged with a green fluorescent protein (GFP) and expressed in cultured insect cells using a baculovirus vector, the protein localized to the surface of the cells. Interestingly, surface localization was dependent on both the amino-terminal (a secretory signal sequence) and the carboxy-terminal (a putative GPI anchor domain) hydrophobic domains (Shen et al., 1999). These observations suggest that *Ag-Muc1* lines the lumenal surface of the mosquito gut and is, therefore, not an integral part of the PM. Note that in other insects, mucin-like proteins were found associated with the PM, rather than the cell surface (Casu et al., 1997; Wang and Granados, 1997b). Also, Western blot analysis indicated that *Ag-Muc1* protein is absent prior to but present after blood feeding (Devenport and Jacobs-Lorena, unpublished observations) whereas the mRNA is present in unfed guts (Shen et al., 1999) suggesting translational regulation of *Ag-Muc1* expression.

Recently, Rayms-Keller et al. (2000) isolated a cDNA from *Ae. aegypti* larvae that encodes a metal-inducible protein, *AEIMUC*. The predicted protein contains three chitin-binding domains and one mucin-like domain. Interestingly, the mRNA is also expressed in adult guts after a blood meal or exposure to heavy metals, suggesting that the same protein may be part of the larval type 2 and adult type 1 PM. However, association of this protein with the PM remains to be demonstrated directly. In our laboratory, screening of an *Ae. aegypti* adult midgut cDNA expression library with an anti-PM antibody led to the isolation of 3 genes encoding putative PM proteins (Shao, unpublished observations). One of these appears to be *AEIMUC1*, suggesting that this protein is, indeed, a component of adult PMs. A second cDNA encodes a protein with several putative chitin-binding and mucin domains. The third protein did not show any homology to sequences in databases.

Type 2 PM of mosquito larvae is synthesized in the cardia. Using a subtractive hybridization procedure (cardia minus gut), we have recently identified a cDNA, termed *Ag-Lper1*, that encodes a secretory signal sequence followed by a 183 amino acid glutamine rich (11%) protein (Donelly-Doman, unpublished observations). An antibody made against the recombinant *Ag-Lper1* protein was used to further characterize this gene. On Western blots, the antibody recognizes cardia and PM but not carcass proteins. On tissue sections, the antibody reacted with cytoplasmic vesicles of cells of the cardia's outer layer. Interestingly, *Ag-Lper1* has significant sequence similarity with the mammalian involucrin protein. Involucrins and other structural proteins make up a tough extracellular protein envelope of the cornified epidermal layer. Note that both structures (the PM and the epidermis) form barriers separating two compartments. The amide group of the glutamine side chain has extensive hydrogen-bonding capacity and may function in the interaction with other PM components. It might also be cross-linked by

transglutamidases (Aeschlimann and Thomazy, 2000). We have also isolated a putative PM protein gene from adult *Anopheles* mosquitoes, which also has high glutamine content. Glutamine-rich proteins may constitute a new family of PM structural proteins.

PERMEABILITY AND DIGESTION

While considerable progress has been made toward the characterization of PM components, much less has been learned about PM function. Because the PM completely separates the food bolus from the secretory/absorptive epithelium, intense molecular traffic must occur through it. Digestive enzymes must cross the PM to reach the food bolus and the hydrolytic products of digestion must traverse the PM in the opposite direction to be absorbed by the epithelial cells. It follows that the PM must have sufficient porosity to allow this molecular transit. Several approaches have been used to study PM permeability in non-hematophagous insects (Peters and Wiese, 1985; Santos and Terra, 1986; Terra, 1990; Barbehenn and Martin, 1995). Recently, we devised a non-invasive in vivo assay for estimating mosquito type 2 larval PM permeability (Edwards and Jacobs-Lorena, 2000). Permeability was determined by feeding the larvae with fluorescein isothiocyanate (FITC)-dextrans (size range 4.4–2,000 kDa) and measuring their appearance in the gastric caeca (tracer dextrans must traverse the PM to reach the caeca). The results indicated that the larval PM was permeable to dextrans of up to 148 kDa. Additionally, the assay was used to devise a treatment for the disruption of the PM. Disruption was assayed by measuring the passage of labeled dextrans of 2 million Daltons, a size similar to that of virus particles. Dithiothreitol (a reducing agent) and, to a lesser extent, chitinase were effective in disrupting the PM while polyoxin D (an inhibitor of chitin synthesis), pronase (a non-specific protease), and calcofluor (a chitin-binding compound) did not alter the permeability significantly. Dithiothreitol is presumed to disrupt protein folding and protein-protein interactions by breaking disulfide bonds. That calcofluor disrupts type 1 PM of lepidopterans (Wang and Granados, 2000) but not type 2 PM of mosquitoes (Edwards and Jacobs-Lorena, 2000) points to fundamental differences in the organization of the larval PMs of these two organisms. In agreement with the results with mosquitoes, calcofluor and polyoxin D had no apparent effect on PM structure of *L. cuprina* (Tellam and Eisemann, 2000). However, these compounds caused larval mortality in *L. cuprina* but not in mosquitoes. Unfortunately, no comparable experimental data on PM permeability are available for the type 1 PM of hematophagous insects. The completely different mode of their PM formation (it is induced by food ingestion) poses great technical challenges for such measurements.

In order to examine whether the type I PM of adult mosquitoes influences the rate of digestion, we recently compared the time course of digestion when adult mosquitoes were fed with blood alone or with blood plus chitinase (chitinase prevents PM formation). Digestion was measured by assaying total protein content of guts dissected at different times after the meal. We found that digestion proceeds significantly faster in the absence of the PM (Villalón, unpublished observations). In separate experiments, we compared rates of digestion in mosquitoes fed with blood containing anti-PM antibody (experimental) and with blood containing pre-immune serum (control). Likewise, experimental mosquitoes digested blood faster than controls. Therefore, treatments that prevent PM formation (chitinase) or alter its structure (antibodies) increase the rate of digestion. The observation that the mosquito midgut secretes a chitinase in response to a blood meal (Shen and Jacobs-Lorena, 1997) can be interpreted in light of these results. It is possible that the mosquito chitinase plays a role in controlling PM porosity and thickness. Final PM thickness during digestion would result from a balance between PM synthesis by the midgut epithelium and degradation by the secreted chitinase. It seems counterintuitive that the mosquito produces a structure (the PM) that delays digestion and thus might reduce fitness. The answer to this paradox might be that protection plays an important evolutionary role. Recall that most organisms have a structure protecting their digestive tract, be it the PM or perimicrovillar membrane in insects, or the mucous layer in vertebrates. The perimicrovillar membrane is a second plasma

membrane that covers the microvilli of certain insects (e.g., *Rhodnius*) (Ferreira et al. 1988).

PROTECTION FROM PATHOGENS

Circumstantial evidence suggests that the PM plays a role in protecting the midgut from pathogens (Shahabuddin et al., 1993, 1996; Regev et al., 1996; Wang and Granados, 1997a, 2000). For instance, the PM is impermeable to virus-sized particles (see preceding text) and thus can protect against viral infections. In order to infect the midgut of lepidopteran larvae, baculoviruses use a proteolytic enzyme (enhancin) that degrades a PM mucin (Wang and Granados, 1997a). Furthermore, disruption of the PM greatly enhances the effectiveness of bacterial toxins (Regev et al., 1996) or viral infections (Wang and Granados, 2000).

To complete its life cycle in the mosquito, *Plasmodium* (the causative agent of malaria) proceeds through a complex developmental program that includes mating, differentiation into a motile ookinete, and invasion of the midgut epithelium (Ghosh et al., 2000). By the time that the ookinete moves out of the blood meal, the PM has fully matured and, therefore, represents a physical barrier that needs to be traversed before midgut invasion. Indeed, Billingsley and Rudin (1992) found that the infectivity of *Ae. aegypti* by *Plasmodium gallinaceum* was reduced when the thickness of the PM increased, indicating that the PM does act as a partial barrier to *Plasmodium* development. Interestingly, the PM may also play a role by protecting the parasite from the action of digestive enzymes as suggested by the decreased survival of *Leishmania* in the absence of a PM (Pimenta et al., 1997).

How do parasites penetrate the PM then? There is histological evidence to suggest that a chitinase secreted by *Leishmania* disrupts the PM allowing it to escape from the midgut of its sand fly host (Schlein et al., 1991). Early studies of the malaria parasite indicated that *P. gallinaceum* secretes a chitinase to penetrate the PM (Huber et al., 1991). Further support for the essential role of chitinase for parasite penetration of the PM came from the finding that allosamidin (a chitinase inhibitor) completely blocks development of the ookinete to oocyst in the mosquito midgut (Shahabuddin et al., 1993).

P. gallinaceum chitinase activity increases by treatment with midgut proteases (Shahabuddin et al., 1993), which suggests that the enzyme is secreted as an inactive pro-enzyme. In support of this model, an anti-trypsin antibody blocked parasite development but had no deleterious effect on the mosquito (Shahabuddin et al., 1996). These results suggested that trypsin may be a candidate antigen for a transmission blocking vaccine. However, recent analysis of *P. gallinaceum* chitinase activity indicated that ookinetes express more than one chitinase (Vinetz et al., 2000). One of these chitinases, PgCHT1, has been cloned and, although it is secreted as a pro-enzyme, the ookinete itself appears to be capable of processing this into the fully active form independent of mosquito midgut proteases (Vinetz et al., 2000). Moreover, it was discovered that a *P. falciparum* chitinase, PfCHT1, may not be synthesized as a pro-enzyme (Vinetz et al., 1999), thus precluding the use of trypsin as a transmission blocking antigen for this human parasite. Of course, *Plasmodium* chitinase itself is a potential transmission blocking target.

Cloning the *Plasmodium* chitinase genes will allow further investigation of the biological function of chitinases during parasite penetration of the PM. However, it still remains to be shown if the mosquito midgut chitinase (Shen and Jacobs-Lorena, 1997) or other proteases secreted by the parasite play a role in penetration of the PM by *Plasmodium*. Further studies are required to characterize other chitinase genes and determine the gene product(s) necessary for mosquito invasion.

CONCLUDING REMARKS

In summary, while significant progress has been made in recent years in our understanding of the peritrophic matrix, much remains to be learned. The almost ubiquitous occurrence of this structure in insects argues that it is important for insect fitness or survival. The PM serves as a protective barrier that some viruses and parasites such as *Plasmodium* and Leishmania "learned" how to traverse. These pathogens appear to do so by using hydrolytic enzymes that destroy PM structure locally. The molecular characterization of the PM is still in its infancy. The development

of new tools promises to bring fresh insights for our understanding of the PM. For example, the yeast two-hybrid system has been widely used to study protein-protein interactions (Van Aelst et al., 1993; Kuo et al., 1997; Lie and Macdonald, 1999). We are currently adapting this technique to identify novel proteins that interact with known PM components. This should help in understanding the role played by protein-protein interactions in the assembly of the PM. It is safe to predict that in the next few years we will learn a good deal more about the structure and function of the PM.

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