Identification of the *Aedes aegypti* Peritrophic Matrix Protein AeIMUCI as a Heme-Binding Protein†

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ABSTRACT: The gene *Aedes aegypti* intestinal mucin 1 (*AeIMUC1*) encodes a putative peritrophic matrix (PM) protein that is expressed in the midgut of mosquito larvae and adults and is upregulated in response to exposure to heavy metals. The AeIMUC1 protein has a predicted secretory signal peptide and three putative chitin-binding domains (CBDs) with an intervening mucin-like domain. Immunofluorescence and immunoelectron microscopy experiments established that AeIMUC1 is a bona fide PM protein, and binding of the recombinant protein to chitin was demonstrated in vitro. Previous experiments suggested that the *Ae. aegypti* PM can bind toxic heme molecules generated during blood digestion. However, the identity of the binding molecule(s) was unknown. Using of heme−agarose beads and spectrophotometric and microcalorimetric titrations, we show that recombinant AeIMUC1 can bind large amounts of heme in vitro, suggesting for the first time a role for a PM protein in heme detoxification during blood digestion. Binding of heme to AeIMUC1 was accompanied by an altered circular dichroism spectrum indicating a change in protein conformation, consistent with an increase in secondary structure. Heme-binding activity was mapped to the AeIMUC1 CBDs, suggesting that these domains possess dual chitin- and heme-binding activity.

The mosquito midgut is the only portion of the digestive tract that is not covered by a protective cuticle. For protection, the midgut epithelial cells produce the peritrophic matrix (PM),[1] a semipermeable extracellular layer made of chitin, protein, and proteoglycans that lines the epithelium (*1*). The PM separates the contents of the midgut lumen from the surrounding gut epithelium. Thus, the PM is thought to be a physiological analogue of the gastrointestinal mucus in mammals, and many of the functions performed by these two layers are deemed to be the same (*2*−*5*). These functions are mainly protective and include resistance to digestive enzymes, pathogens, and toxins and protection from abrasion by food particles.

Mosquitoes use blood as a source of nutrients for egg and energy production (*6*). Digestion of hemoglobin results in the release of large amounts of free heme in the gut lumen of these insects. Heme is essential as a prostetic group, participating in many biological reactions such as oxygen transport, photosynthesis, drug detoxification, and respiration. Nevertheless, even in micromolar concentrations, heme is able to promote oxidation of lipids (*7*, *8*) and proteins (*9*, *10*), DNA degradation (*11*), and to interfere in the stability of biological membranes because of its amphiphilic nature (*12*−*15*). Thus, blood-feeding organisms, such as mosquitoes, face major oxidative challenges that must be counteracted by antioxidant defenses or heme detoxification mechanisms. The malarial parasite, *Plasmodium*, faces similar challenges, because it relies mainly on hemoglobin from blood for nutrition and must inactivate the released heme by formation of a harmless crystalline aggregate called hemozoin (*16*). Páscoa et al. (*17*) reported that the adult type-1 PM from *Aedes aegypti* can bind heme, suggesting that the PM acts in antioxidant defense by preventing heme from reaching the cells of the midgut epithelium. However, a key unresolved issue concerns the identity of the PM molecule(s) that bind heme.

Despite proteins being major constituents of the insect PM (21−55.5% of total PM mass; ref *1*), little is known about their function (*18*). The presence of chitin-binding domains (CBDs) in several PM proteins of different insects suggests a structural role in the spatial organization of chitin fibrils (*18*). The first *Ae. aegypti* putative peritrophic matrix protein

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[1] Abbreviations: AeIMUC1, *Aedes aegypti* intestinal mucin 1; PM, peritrophic matrix; CBD, chitin-binding domain; HRM, heme-regulatory motif; P_2, poly(proline) II-type helix; ITC, isothermal titration calorimetry; CD, circular dichroism; GlcNAc, *N*-acetylglucosamine; BCIP, 5-Bromo-4-Chloro-3′-Indolyphosphate p-Toluidine Salt; NBT, Nitro-Blue Tetrazolium Chloride; PCR, polymerase chain reaction.

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described, the *Ae. aegypti* intestinal mucin 1 (AeIMUC1), is a mucin-like protein that is induced by metal feeding in both larvae and adults and by a blood meal in adults (*19*). This protein has a secretory signal peptide as well as three CBD-like domains and an intervening mucin-like domain, rich in proline, serine, and threonine (*19*), all of which are features characteristic of PM proteins (*18*). However, the evidence is circumstantial, and a direct association of AeIMUC1 with the PM has not been demonstrated.

Here, we demonstrate that AeIMUC1 is an integral PM protein and provide evidence that the recombinant protein can bind chitin in vitro. Importantly, we show, for the first time for a PM protein, that recombinant AeIMUC1 is also capable of binding large amounts of heme in vitro and use deletion constructs to identify the protein domains that interact with heme. We hypothesize that AeIMUC1 plays a dual role in providing structural integrity to the PM while protecting the midgut epithelium from the harmful effects of heme.

EXPERIMENTAL PROCEDURES

Mosquitoes. Ae. aegypti larvae (Rockefeller strain) were reared on dry cat food. Adults were maintained on a 14/10 h light−dark period at 27 °C and 80% relative humidity. The adults were offered 20% sucrose solution ad libitum. The 3−4 day-old females were blood-fed on anesthetized mice.

AeIMUC1 Sequence Analysis. DNA and protein sequences were analyzed and manipulated using Molecular BioComputing Suite (MCBS; ref *20*). Protein sequence alignments were performed using the Clustal W program on the EBI server (http://www.ebi.ac.uk/clustalw/).

Northern Blot Analysis. Total RNA from guts of sugar-fed adult *Ae. aegypti*, as well as from guts of adult females dissected at different times after a blood feeding, was extracted using the TRI reagent (Molecular Research Center, Inc.). Approximately 3 μg of total RNA from each sample was fractionated by electrophoresis on a denaturing 1.5% agarose−formaldehyde gel and transferred to a nylon membrane (Gene Screen, New England Nuclear). The membrane was hybridized with a full-length ^{32}P-labeled AeIMUC1 cDNA probe.

Antibody Preparation. The *AeIMUC1* coding sequence was amplified by polymerase chain reaction (PCR), cloned into the pGEX-4T-1 expression vector (Pharmacia Biotech), and expressed as a GST fusion protein in *Escherichia coli* BL21 cells. The fusion protein was isolated as an inclusion body from the expressing cells, and approximately 500 μg of each sample was size-fractionated by electrophoresis on a 10% polyacrylamide/sodium dodecyl sulfate (SDS) gel. Polyclonal anti-serum was generated in rabbits through three intradermal injections of the purified protein at 1 month intervals. For the primary injections, full-length fusion protein was excised from the gel, homogenized in 500 μL of PBS, and mixed with an equal volume of Freund's complete adjuvant. For the boosts, the protein was mixed with an equal volume of Freund's incomplete adjuvant. The final immune serum was collected 1 month after the final boost.

Western Blot Analysis. Guts were dissected from sugar-fed mosquitoes as well as from mosquitoes at different times after a blood meal, and proteins equivalent to approximately 0.5 gut were fractionated by electrophoresis on a 10−20% polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane-bound proteins were subsequently refolded by denaturing in a 6 M guanidine hydrochloride solution in basic buffer (20 mM HEPES at pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1% Nonidet P-40) followed by slow renaturation through serial dilution with basic buffer according to the protocol by Einarson (*21*). The membrane was then processed using standard protocols and incubated with the anti-AeIMUC1 polyclonal antibody (1:250 dilution) followed by detection with an alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Promega, 1:7500 dilution) in the presence of BCIP/NBT substrates.

Immunofluorescence of Midgut Sections. Adult *Ae. aegypti* posterior midgut sections were prepared and stained for immunofluorescence microscopy as described previously (*22*). The anti-AeIMUC1 serum was used at a dilution of 1:500.

Immunoelectron Microscopy. Adult *Ae. aegypti* posterior midgut sections were prepared and stained for immunoelectron microscopy as described previously (*22*). Antibodies were used at dilutions of 1:∼50−200.

Generation of the AeIMUC1 Deletion Proteins. The *AeIMUC1* deletion construct coding regions were amplified by PCR using the following primers: B1d1 forward, 5′-CGGGATCCTGCCCCGATATCTTCGACTCC-3′; B1d1 reverse, 5′-AACTCGAGCTAAATTTCAATTGGTTTCGAGTG-3′; B1d2 forward, 5′-CGGGATCCTGCCCCGATATCTTCGACTCC-3′; B1d2 reverse, 5′-CCCTCGAGCTACTTGTTGGTTGGTGCCACAG-3′; B1d3 forward, 5′-CGGGATCCTGTCCTGAATTCTTCAACCCC-3′; B1d3 reverse, 5′-AACTCGAGCTAAATTTCAATTGGTTTCGAGTG-3′; B1d4 forward, 5′-GGATCCGGCAAATGCCCCGATATCTTCG-3′; B1d4 reverse, 5′-CTCGAGTCATCCACCACTACATCCGGCCAAC-3′. In each case, the forward primer incorporates a *Bam* HI restriction site and the reverse primer incorporates a stop codon and a terminal *Xho* I restriction site. PCR fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI), digested with *Bam* HI and *Xho* I, and cloned into the pGEX-4T-1 expression vector (Pharmacia Biotech). Proteins were expressed as GST fusions in *E. coli* BL21 cells and purified with glutathione-sepharose 4B beads according to the protocol of Frangioni and Neel (*23*). GST tags were removed from the recombinant proteins by cleavage with thrombin while attached to the glutathione-sepharose columns, and the AeIMUC1 deletion proteins were recovered in the eluate.

Chitin-Binding Assays. Approximately 20 μg of recombinant protein in a total volume of 1.0 mL of Tris-buffered saline plus 0.1% Triton X-100 (TBST) was mixed with approximately 100 μL of chitin beads (New England Biolabs) or cellulose (Sigma; 20% stock solution in TBS) that had been prewashed with TBST. The protein and beads were rocked for 3 h at room temperature, and the beads were recovered from the unbound protein (flow through) by centrifugation for 1 min at 4000 rpm. The chitin beads and cellulose matrix were then washed 4 times with 100 μL of TBST followed by centrifugation, and bound proteins were eluted by boiling in 100 μL of 5% β-mercaptoethanol and 2% SDS. Proteins from each fraction were separated by electrophoresis on polyacrylamide gels and detected by silver

staining. For additional assays, the protein was preincubated with TBST supplemented with 2% SDS/5% β-mercaptoethanol, 0.3 M GlcNAc, or 5 mM heme for 30 min at room temperature. Washing was performed with the same solutions, except for the heme competition assay, where the dark color of the 0.5 mM heme solution impairs the recovery of the chitin/cellulose, and therefore, washing was performed with TBST.

Heme−Agarose Binding Assays. Heme-binding assays were performed essentially as described by Tsutsui and Mueller (*24*). Briefly, approximately 20 μg of recombinant protein in a total volume of 1.0 mL of high ionic strength buffer (0.5 M NaCl, 10 mM sodium phosphate, and 0.1% Triton X-100 at pH 7.5) was mixed with approximately 100 μL of heme−agarose beads (Sigma) or unconjugated agarose beads (Sepharose CL4B-200; Sigma), which had been prewashed with high ionic strength buffer. The protein and beads were rocked for 1 h at 4 °C, and the beads were recovered from the unbound protein by centrifugation for 2 min at 10 000 rpm. The beads were washed 6 times with high ionic strength buffer, and the bound proteins were eluted by boiling the beads in 100 μL of 5% β-mercaptoethanol and 2% SDS. Fractions (50 μL) of the input protein and the eluted protein were analyzed by electrophoresis on polyacrylamide gels and detected by silver staining.

Spectrophotometric Analysis of Heme Binding. Binding of heme to the AeIMUC1 constructs was followed by progressively adding 0.5 nmol of heme and taking wavelength spectra 1 min after each addition in a cuvette containing 1 nmol of each protein in 1 mL of 0.135 M NaCl and 60 mM sodium phosphate buffer at pH 7.4. Wavelength spectra were obtained with a GBC 920 UV/vis spectrophotometer (Victoria, Australia). Differential spectra before and after each addition were obtained with the equipment software, and titration curves were constructed using the shift of the absorption maximum.

Heme-Binding Assay by Isothermal Titration Calorimetry (ITC). Heat of heme binding to B1d1 was measured in a MCS titration microcalorimeter from MicroCal, Inc. (Northamptom, MA) at 22.5 and 30.0 °C. Titration experiments were performed by successive injections of heme (4×10 and 21×4 μL) into a 1.34 mL sample cell containing 2 μM B1d1 in 60 mM sodium phosphate buffer and 135 mM NaCl at pH 7.4. All injections were done with a 250 μL syringe, with constant stirring at 200 rpm. The hemin solution (1 mM) was prepared from a 5 mM stock in 0.1 N NaOH diluted in the sample buffer. The calorimetric enthalpy (ΔH^{cal}) for each injection was calculated after correction of the heat of heme dilution obtained in control experiments by titrating heme into buffer. Calorimetric data were analyzed using the Origin 5.0 software provided by MicroCal. Changes in heat capacity upon binding (ΔC_p) was determined by the temperature dependence of ΔH and corresponds to the slope in a linear fit of ΔH^{cal} as a function of the temperature as described by

$$\Delta C_p = dH/dT \quad (1)$$

Circular Dichroism (CD) and Secondary Structure Analysis of B1d1 in the Presence and Absence of Heme. B1d1 (7 μM) was incubated with the excess of heme (16:1), from a 10 mM stock solution made in 0.1 N NaOH, or without heme, in 60 mM sodium phosphate buffer and 135 mM NaCl at pH 7.4. The CD spectra (6 accumulations) were taken from 190 to 260 nm, at 50 nm/min in a 0.2 cm path-length cell in a Jasco J715 (Jasco, Japan) CD spectrometer at room temperature. Evaluation of the secondary-structure profile was performed by spectra deconvolution with SELCON 3 software (*25*), using database set (IBasis) number 2. The prediction of the secondary structure based on the protein sequence used the SOPMA software (*26*) available on the Expasy website (http://au.expasy.org/tools).

RESULTS

Isolation and Sequence Analysis of the AeIMUC1 B1 Clone. The *AeIMUC1* cDNA was identified by immunoscreening of an *Ae. aegypti* midgut cDNA expression library with a rabbit polyclonal antibody prepared against whole dissected PMs (*27*), providing initial support for the designation of AeIMUC1 as a PM protein. The *AeIMUC1* gene is polymorphic, and three allelic size variants (*AeIMUC1*^{R1}, AeIMUC1^{R2}, and AeIMUC1^{M1}) were previously reported from the *Ae. aegypti* MOYO-R and RED strains (*28*, *29*). The variant isolated in our screen of the Rockefeller strain was designated B1. AeIMUC1^{B1} shares the highest sequence identity with AeIMUC1^{R1}; both proteins have the same length, and they differ by only three amino acids (Figure 1).

AeIMUC1 Expression during the Course of Blood Digestion. On Northern blots, the *AeIMUC1* probe detects a single band of approximately 1 kb only in the RNA extracted from female adult midguts (data not shown), in agreement with previously published data (*28*). Using Northern blot analysis, AeIMUC1 mRNA abundance was found to be constant throughout the course of blood meal digestion (Figure 2A). This is in contrast with data of Rayms-Keller et al. (*19*), who reported an increase in gene expression following a blood meal.

To further characterize AeIMUC1 expression during the course of blood digestion, specific antibodies were prepared against a recombinant protein and used in Western blot analysis (Figure 2B). While mRNA is abundant, no AeIMUC1 protein was detected prior to blood feeding. The protein is first detected at around 1 h after a blood meal and reached its highest level of expression at around 4 h. Migration of the protein (Figure 2B) is much slower than of a protein of its predicted mass (27.7 kDa). This could be due to abnormal migration of the protein and/or to O-linked glycosylation, a common occurrence in mucin-like proteins. At 12 and 18 h after blood feeding, two bands are present. The faster-migrating band may represent a degradation product of the larger protein, perhaps on account of the increased levels of proteolytic enzymes during the course of blood digestion (*30*). AeIMUC1 breakdown could serve to decrease chitin fibril cross-linking and facilitate permeability to digestive enzymes and hydrolytic products that need to transverse the PM during digestion (*31*). Preimmune sera from the same rabbit did not detect any proteins on Western blots (data not shown).

Immunofluorescence Microscopy and Immunoelectron Microscopy Demonstrates that AeIMUC1 Is an Integral PM Protein. Indirect immunofluorescence microscopy of midguts from blood-fed mosquitoes localized the AeIMUC1 protein to the PM, a distinct layer between the midgut epithelium

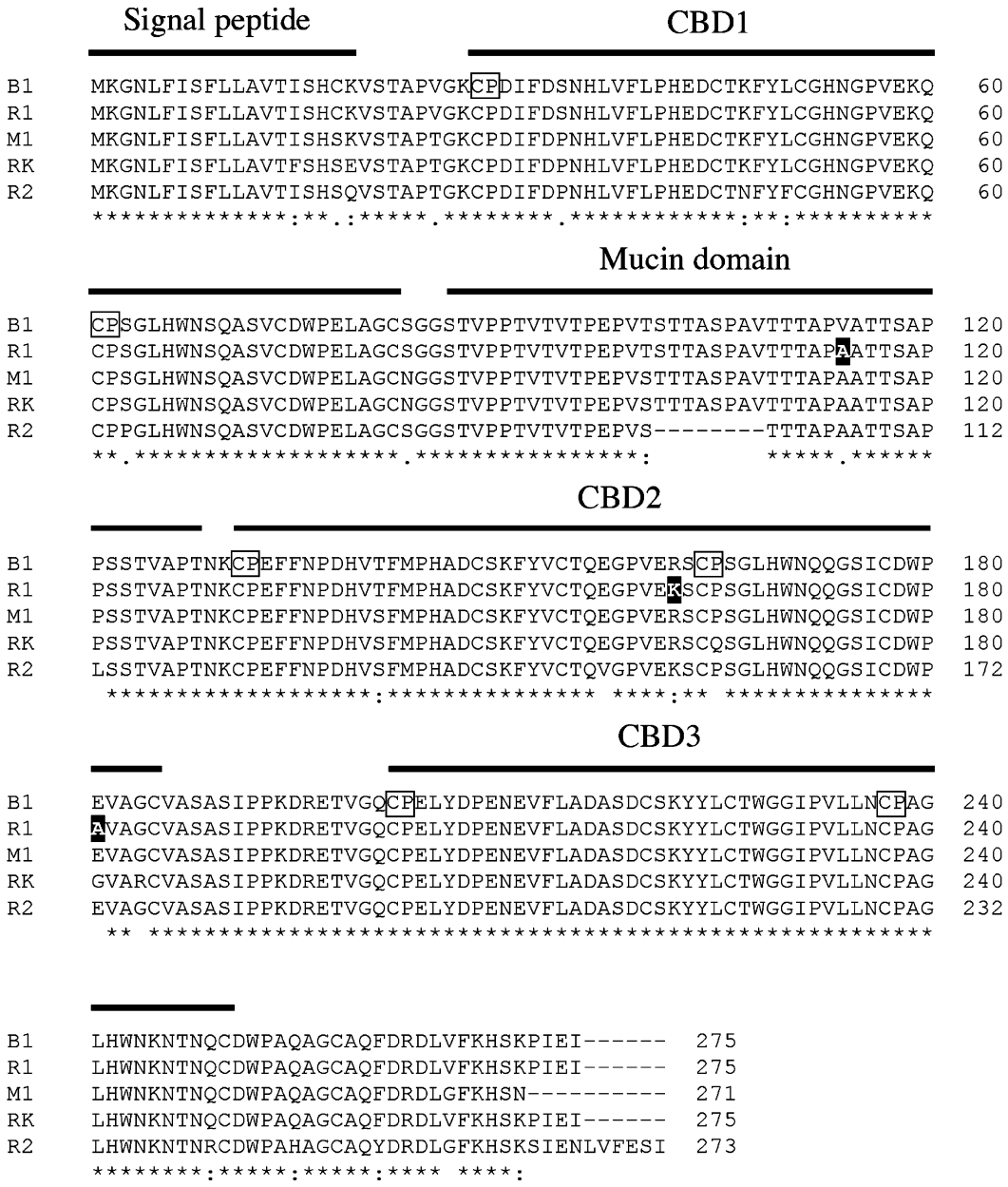

FIGURE 1: Alignment of the AeIMUC1 protein sequences. The amino acid sequences of the AeIMUC1^{B1}, AeIMUC1^{R1} (GenBank accession AF308863), AeIMUC1^{R2} (GenBank accession AF308864), AeIMUC1^{M1} (GenBank accession AF308862), and AeIMUC1^{RK} (Rayms-Keller; GenBank accession AF125984) proteins are aligned. The secretory signal peptide, mucin-like domain, and CBDs are indicated. The three amino acid differences between the B1 and R1 sequences are shown in white against a black background. Cysteine−proline residues belonging to putative HRMs are boxed. Asterisks indicate conserved amino acids; colons indicate conserved substitutions; and periods indicate semiconserved substitutions.

and the ingested blood meal (Figure 3). Localization of AeIMUC1 was further analyzed by immunoelectron microscopy. As expected from Western blot analysis, no protein was detected in the midguts of sugar-fed mosquitoes (Figure 4A). At 6 h after blood feeding, the protein was detected over the PM as well as microvilli (Figure 4B). Detection of AeIMUC1 over microvilli may represent newly synthesized protein that is being secreted into the midgut lumen and in the process of being assembled into the PM (chitin is synthesized by a microvillar membrane-bound chitin synthase; ref *32*). Formation of the PM requires a period of maturation during which its thickness gradually increases. The amount of time required for PM maturation is determined empirically, and estimates of the time this takes in *Ae. aegypti* varies from 5 to 12 h (*33*−*35*). Accordingly, both at 12 and 24 h, the protein was found predominantly over the maturing PM (parts C and D of Figure 4), confirming the designation of AeIMUC1 as a PM protein. No signal was observed with preimmune sera (data not shown).

Binding of AeIMUC1 to Chitin in Vitro. The presence of putative CBDs in AeIMUC1 suggests that the protein integrates into the PM by binding to chitin fibrils. To investigate this hypothesis, we measured the chitin-binding ability of recombinant full-length AeIMUC1 (B1d1) and deletion constructs (B1d2−4) made with selected regions of the protein (Figure 5A). The B1d2 construct corresponds to the first half of the protein (i.e., the first CBD and the mucin-like domain); B1d3 corresponds to the second half of the protein (i.e., the last two CBDs); and the B1d4 construct

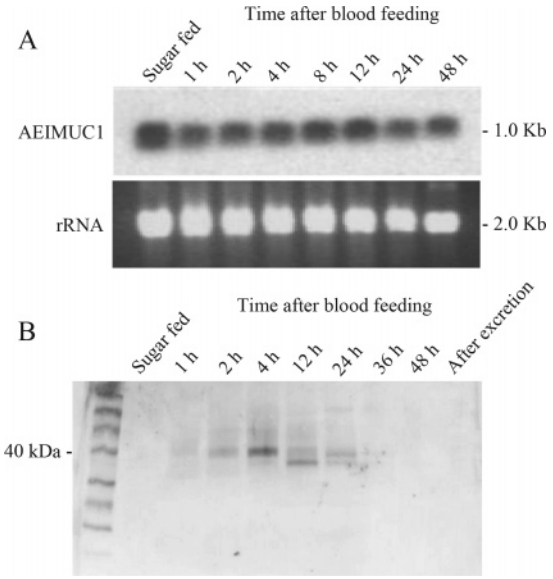

FIGURE 2: Temporal patterns of *AeIMUC1* expression after blood feeding. Adult female midguts were dissected before a blood meal (sugar fed) and at different times after a blood meal as indicated, and AeIMUC1 expression was analyzed. (A) Northern blot of total midgut RNA hybridized with a ^{32}P-labeled *AeIMUC1* cDNA probe (upper panel). The lower panel is a ethidium-bromide-stained gel showing the ribosomal RNA before transfer to the membrane to verify the amount of RNA loaded on each lane. (B) Western blot of proteins isolated from the midguts detected with an anti-AeIMUC1 antibody.

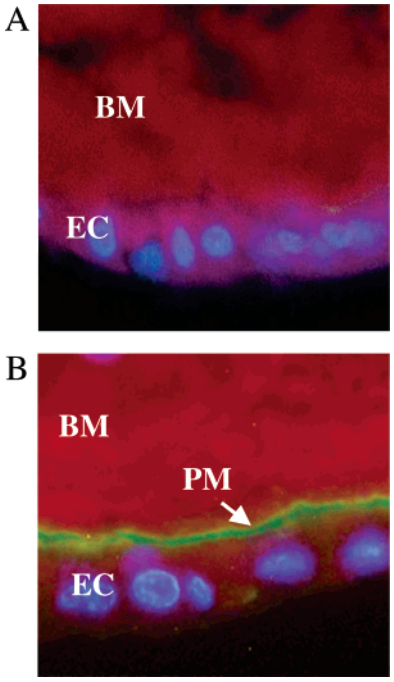

FIGURE 3: Localization of AeIMUC1 by immunofluorescence. Midgut sections from adult female mosquitoes dissected 24 h after a blood meal were incubated with preimmune serum (A) or anti-AeIMUC1 serum (B) and detected by immunofluorescence using an FITC-conjugated secondary antibody. EC, epithelial cell layer; BM, blood meal. Cell nuclei (blue) were revealed by DAPI staining.

corresponds to the first CBD alone. The four recombinant proteins specifically bind chitin beads but not cellulose, another carbohydrate polymer (Figure 5B), demonstrating that at least two and possibly all three of the AeIMUC1 CBDs are able to bind chitin in vitro. These results are consistent with those of Wang et al. (*36*), who have shown

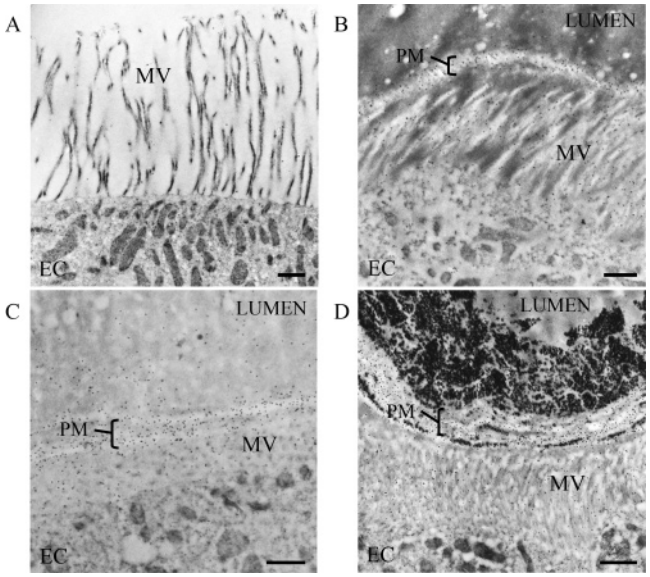

FIGURE 4: Localization of AeIMUC1 by immunoelectron microscopy. Immunoelectron microscopy of ultrathin sections from adult female midguts. (A) Sugar fed, (B) 6 h after a blood meal, (C) 12 h after a blood meal, and (D) 24 h after a blood meal. The sections were exposed to anti-AeIMUC1 immune serum followed by gold-conjugated secondary antibodies. The PM, epithelial cell cytoplasm (EC), microvilli (MV), and blood meal (BM) are indicated. Scale bars = 1 μm.

that a single CBD expressed in insect cells can bind to chitin in vitro, although this is the first time the chitin-binding activity of a bacterially expressed protein is demonstrated.

Chitin is a linear polymer of *N*-acetylglucosamine (GlcNAc). To test whether GlcNAc inhibits the binding of the full-length recombinant AeIMUC1 protein to chitin beads, the chitin-binding assay was performed in the presence of a high concentration of GlcNAc. Binding of AeIMUC1 to chitin was not inhibited in the presence of 0.3 M GlcNAc (Figure 5C). This is unlike peritrophin-44, a type-2 PM protein with multiple CBDs from the fly *Lucilia cuprina*, that can be eluted from reacetylated chitosan with an excess of GlcNAc (*37*) and similar to the invertebrate intestinal mucin (IIM), a type-1 PM protein from the lepidopteran *Trichoplusia ni*, whose binding to regenerated chitin was unaffected by GlcNAc (*38*). However, binding of AeIMUC1 to chitin was ablated by reducing (β-mercaptoethanol) and denaturing (SDS) agents (Figure 5C), suggesting that protein folding is important for this function and indicating that the recombinant protein is folded in a functional manner.

AEIMUC1−Heme Interactions. A group of unrelated proteins contain a class of heme-binding sites characterized by the presence of a cysteine−proline dipeptide, called the heme-regulatory motif (HRM; ref *39*). Six putative HRMs were identified in the AeIMUC1 sequence, two in each of the three CBDs (Figure 1). This finding, together with the observation that the synthesis of the AeIMUC1 protein is upregulated in response to a blood meal (Figures 2−4), raised the possibility that AeIMUC1 can bind heme and thus protect the midgut from the harmful effects of heme. This hypothesis was investigated by measuring the ability of AeIMUC1 to bind heme−agarose beads. The full-length AeIMUC1 protein and single CBD deletion construct were both able to bind to heme−agarose beads but not to unconjugated beads (Figure 6). To further identify the protein region(s) responsible for

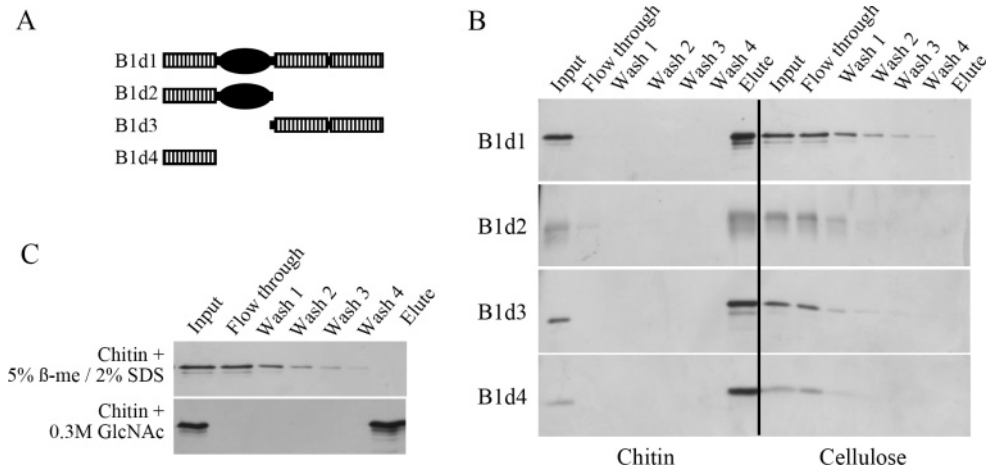

FIGURE 5: Chitin-binding assay. (A) Schematic representation of the AeIMUC1 constructs. The striped boxes are CBDs, and the ovals are the mucin-like domain. (B) Full-length recombinant AeIMUC1 protein (B1d1) or its deletion construct proteins (B1d2−4) (input) were incubated with chitin beads (left panels) or cellulose (right panels). Unbound protein (flow through) was removed, and the substrate was washed 4 times. Bound protein was eluted by boiling the substrate in the presence of 5% β-mercaptoethanol and 2% SDS. Proteins were analyzed by polyacrylamide gel electrophoresis and detected by silver staining. (C) Chitin binding of the full-length B1d1 protein was also performed in the presence of either 5% β-mercaptoethanol plus 2% SDS or 0.3 M GlcNAc, as indicated.

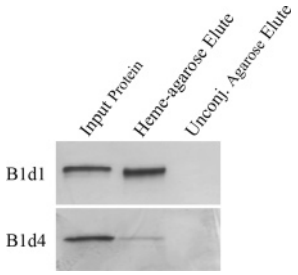

FIGURE 6: Heme−agarose binding assay for recombinant AeIMUC1 protein. Either the full-length AeIMUC1 (B1d1) or a single CBD of AeIMUC1 (B1d4) (input proteins) was incubated with heme−agarose or unconjugated agarose beads. After washing, bound protein was eluted and analyzed by polyacrylamide gel electrophoresis and detected by silver staining.

binding heme, binding of all four AeIMUC1 protein constructs to heme was assayed by light absorption spectrum analysis. Adding heme to AeIMUC1 shifts its Soret band from 390 to 368 nm (Figure 7A), resulting in spectra similar to heme bound to a HRM (*39*). All four proteins caused a similar shift in the absorption spectrum of heme (Figure 7A), indicating that the HRM-like motifs within the CBDs are responsible for heme binding.

The spectral changes induced upon association of heme with AeMUC1 were used to estimate the number of heme-binding sites. After each addition of heme, differential spectra (spectrum after addition minus spectrum before addition) were obtained (Figure 7B). After saturation of heme-binding sites, absorption maxima shifts from that of heme bound to protein (368 nm) to values similar to that of free heme (390 nm). The results indicated a heme-binding stoichiometry of 3:1 for B1d2, 9:1 for B1d3, and up to 12:1 for B1d1 (Figure 7B). Significantly, the stoichiometry of the two protein halves is together equal to that of the full-length protein.

Heme association to the full-length B1d1 protein was also investigated by ITC (Figure 8). The thermodynamic parameters of heme binding to B1d1 were studied by ITC at two different temperatures, 22.5 and 30.0 °C. The binding of heme to B1d1 was exothermic at both temperatures with a binding stoichiometry of $n = 12$ that fully agrees with the result obtained by spectrophotometric analysis. Because of

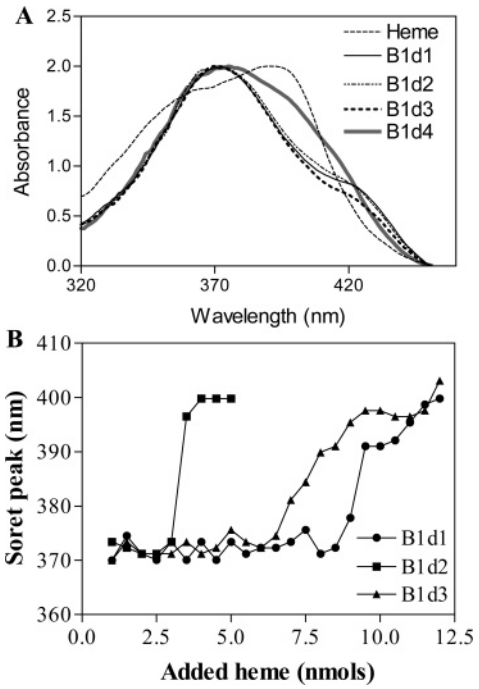

FIGURE 7: Spectrophotometric analysis of heme binding to recombinant AeIMUC1 proteins. (A) Absorption spectra of 1 nmol of heme were measured in the absence of added protein (heme) or in the presence of 1 nmol of each of the four AeIMUC1 constructs (B1d1−4; cf. Figure 5A). Normalized spectra are shown. (B) Stoichiometry of heme binding to these proteins was measured by the stepwise addition of 0.5 nmol of heme to 1 nmol of each protein. Wavelength spectra were taken after each addition; the differential spectrum was calculated; and the values of the maximum absorption (Soret peak) were graphed.

the large number of binding sites, it was difficult to determine the best fitting model for the binding isotherms. The best fit was achieved with the sequential binding sites model, although the values for the association constants and binding enthalpy did not differ for each binding site. The association constants where on the order of 10^5 M^{-1} ($K_d = 10^{-5}$ M). The heat capacity change (ΔC_p), obtained by the temperature dependence of the binding enthalpy (see the eq 1 in the Experimental Procedures), gives an indication of the type

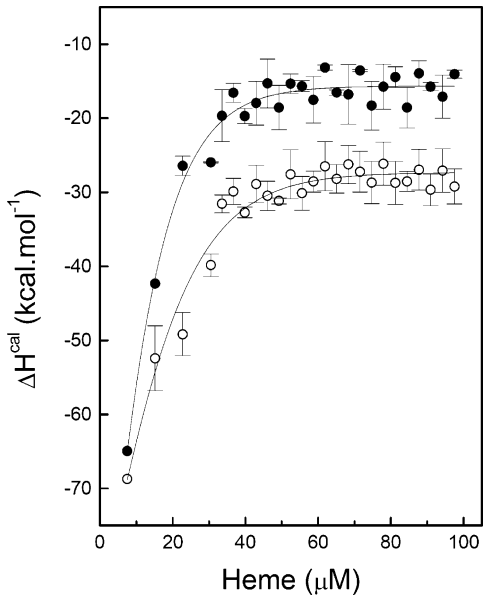

FIGURE 8: ITC of heme binding to B1d1. Heme titration was performed in a calorimetric cell containing 2 μM B1d1 in 60 mM sodium phosphate buffer and 135 mM NaCl at pH 7.4 and 22.60 ± 0.05 °C (●) and 30.01 ± 0.03 °C (○). The calorimetric enthalpy (ΔH^{cal}) for each injection is plotted against the heme concentration. The values were corrected for the heat of heme dilution, obtained in control experiments by the addition of heme to the buffer in the absence of protein. Data are means ± standard deviation (SD).

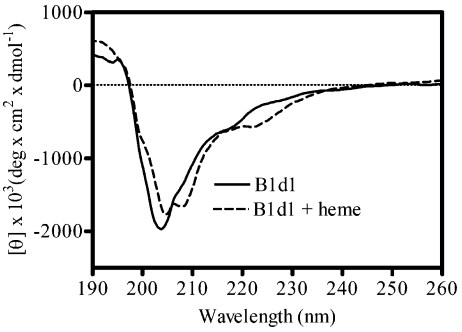

FIGURE 9: CD spectra of B1d1. CD spectra of B1d1 (7 μM) were taken in buffer alone and after the addition of 112 μM heme, a 16:1 heme/AeIMUC1 molar ratio.

of noncovalent interactions involved in the binding of heme to B1d1. Results obtained at both temperatures indicate that ΔC_p is negative, which strongly suggests that hydrophobic effects because of the burial of nonpolar surfaces provide a major energy contribution to the binding.

The CD spectra of B1d1 in the presence of heme is shown in Figure 9. In the absence of heme, B1d1 has negative ellipticity at 203 nm and a discrete small positive band at 190 nm, suggesting the presence of a significant amount of β-strand secondary structure. Nevertheless, it is not a classical all β-strand protein spectrum, because of the marked intensity of the negative band at 203 nm. This feature could be explained by the presence of unordered regions in the protein, together with poly(proline) II-type helix (P_2) secondary structure, as suggested by deconvolution of the spectrum using the SELCON 3 software (*25*). This indicates a content of 42% of unordered structure, 20% of β sheet, 14% of helical P2 structure, and 13% of β turn. Prediction of the B1d1 secondary structure using its amino acid sequence and the SOPMA software (*26*) indicated that 68% of the protein

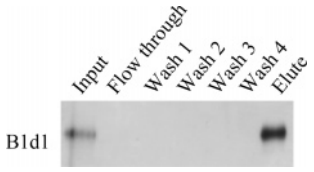

FIGURE 10: Binding of B1d1 to chitin after preincubation with heme. Full-length AeIMUC1 protein was preincubated with 5 mM heme and then incubated with chitin beads (input). Unbound protein (flow through) was removed, and the beads were washed 4 times. Bound protein was eluted by boiling the beads in the presence of 5% β-mercaptoethanol and 2% SDS and analyzed by 12% polyacrylamide gel electrophoresis followed by silver staining.

had unordered (random-coil) structure, 20% had β sheet, 2.4% had α helix, and 8.8% had β turn, a result close to the CD deconvolution. The most important difference between the two datasets is that the CD deconvolution spectra allows for the calculation of the P2 helix content, in contrast to the SOPMA software that does not distinguish P2 structures and instead assigns them as random coil. The addition of saturating amounts of heme (16:1) led to a small increase in the positive band intensity at 190 nm, together with a shift of the negative band to longer wavelengths and with the appearance of a negative shoulder at 224 nm. This suggests a decrease in unordered secondary-structure content and a concomitant increase in ordered structures upon the binding of heme, which was confirmed by deconvolution analysis.

Our results indicate that the heme-binding function is conferred by the HRMs embedded within the CBDs. Therefore, we investigated whether heme could compete with chitin-binding activity. As shown in Figure 10, preincubation of the recombinant protein with heme did not inhibit chitin binding. This result supports the concept of a dual role for AeIMUC1: structural by cross-linking chitin fibers to form the PM and protective by trapping heme released during blood digestion to defend the mosquito against heme toxicity.

DISCUSSION

AeIMUC1 has been described as a putative peritrophic matrix protein based exclusively on sequence similarity to other PM proteins (*19*). Here, we provide the first direct evidence that AeIMUC1 is an integral PM protein (Figures 3 and 4). The PM of *Ae. aegypti* is produced de novo in response to blood feeding (*35*, *40*). Prior to blood feeding, the midgut epithelial cells have characteristic whorls of rough endoplasmic reticulum. After a blood meal, these whorls unfold, correlating with the activation of protein synthesis (*41*, *42*). Thus, mRNAs encoding PM proteins (and some digestive enzymes) have been hypothesized to be stored in guts from sugar-fed mosquitoes and their translation induced by blood feeding. Here, we show that, in support of this hypothesis, AeIMUC1 mRNA is present in midguts from sugar-fed mosquitoes but the protein is not produced until after blood feeding, when it is secreted to form the PM (Figure 2). Thus, AeIMUC1 mRNA appears to be translationally regulated during the blood-feeding cycles. The mechanism of translational regulation is presently unknown.

The binding of toxic compounds by the PM for elimination by excretion has been observed in other insects, particularly those that feed on plants (reviewed in refs *43* and *44*). In the midgut of the blood-feeding hemipteran *Rhodnius prolixus*, heme is aggregated into hemozoin and ultimately

excreted with the feces (*45*). Aggregates of heme are also present in the midgut of *Ae. aegypti*, and these accumulate as blood digestion proceeds (Figure 4). The exact nature of these aggregates is unknown, but they do not appear to have the same biochemical properties as hemozoin (unpublished observations). *Ae. aegypti* aggregates are relatively small and occur mainly on the lumenal side of the PM, although they can also be observed on the side facing the midgut epithelium (Figure 4D). This is in contrast with the mosquito *Anopheles gambiae*, where heme aggregates are much larger and found exclusively on the lumenal side of the PM (*22*).

Páscoa et al. (*17*) demonstrated that the PM of *Ae. aegypti* is able to bind heme and that one PM could bind about the same amount of heme as that present in a blood meal. However, the identity of the binding molecule(s) remained unknown. With the present set of experiments, we identify for the first time a specific PM protein, AeIMUC1, which is able to bind substantial amounts of heme. Recently, another peritrophin was described in the midgut of *Ae. aegypti* (27). Similar to AeIMUC1, this protein, Ae-Aper 50, has five CBDs, each containing two Cys−Pro putative HRMs. This finding raises the possibility that AeIMUC1 may be one member of a multigene family of proteins able to bind both heme and chitin.

The PM completely surrounds the blood meal and acts as a sieve through which digestive enzymes, hydrolytic products, and other small molecules can freely traverse (*31*). However, the potentially toxic heme that is generated during digestion appears to be trapped by the PM, impeding its access to the midgut epithelial surface. At the end of the digestion cycle, the PM remnants are excreted together with the bound heme, thus providing protective and detoxification functions.

The multiple CBDs of PM proteins are thought to function in cross-linking of chitin fibers, providing structure and strength to the PM (*5*, *46*, *47*). CBDs or peritrophin-A domains (*19*) are defined by the spatial conservation of six cysteine residues that conform to the pattern CX_{13-20}-$CX_{5-6}CX_{9-19}CX_{10-14}CX_{4-14}C$ (where X is any amino acid; Figure 1). Three putative CBDs were identified in AeIMUC1, and we confirmed that the full-length protein and the three deletion constructs, each of which contained at least one CBD, were able to bind chitin in vitro. In addition, we noticed that each AeIMUC1 CBD has Cys−Pro motifs that are hallmarks of certain heme-binding proteins.

Heme binding to some proteins such as catalase, cytochromes, and globins involves a histidine−methionine pair or bishistidine (*39*, *48*). Binding of heme to other proteins, such as heme oxygenase-2, yeast heme activator protein 1 (HAP-1), and mammalian δ-aminolevulinate synthase (ALAS), appears to be mediated by a short peptide motif containing a conserved cysteine−proline dipeptide, the so-called HRM (*39*, *49*−*51*). Significantly, the first and fourth cysteines in each of the three AeMUC1 CBDs (Figure 1) resemble HRM-like Cys−Pro motifs, providing six potential heme-binding sites in the full-length protein, and the spectral shifts observed upon the binding of heme to all AeIMUC1 constructs (Figure 7A) are similar to the spectrum of heme bound to the Cys−Pro residues in HRMs of other proteins. However, spectrophotometric titration of heme binding to the AeMUC1 constructs suggests that this protein may bind up to 12 heme molecules per polypeptide chain, 3 to the first CBD (B1d2) and 9 to the remaining two CBDs (B1d3; Figure 7B). Results obtained by ITC assays confirmed the binding stoichiometry of 12:1 (heme/B1d1). Binding of heme to HRMs is probably mediated by the interaction of the sulfur of the cysteine side chain with the iron atom from the heme; the proline residue is not essential to binding but appears to increase the affinity of this interaction (*39*, *51*). Therefore, the non-Cys−Pro-binding sites of AeMUC1 could, at least in part, provide lower affinity binding sites involving cysteine residues without neighboring prolines. Although we were not able to measure individual binding constants for each one of the multiple heme-binding sites, ITC data (Figure 8) indicated that binding constants are in the micromolar range. This binding constant value agrees with the physiological role proposed here for AeIMUC1 because vertebrate blood has about 10 mM heme (bound to globin) that is released upon digestion of the blood meal.

The B1d4 spectrum displayed a small shoulder suggesting the presence of some free heme, indicating weaker binding of heme to this protein domain than to the full-length protein. This is consistent with the relatively small amount of protein eluted from the heme−agarose beads (Figure 6). The reduced binding capacity may be due to the apparent lower stability of this single-domain construct in comparison with B1d1, because the heme-binding capacity of B1d4 is quickly lost even during short-term storage of a recombinant B1d4 protein solution (data not shown).

CBDs of both plant and invertebrate proteins were hypothesized to have little, if any, secondary structure (*52*). However, we found a significant amount of ordered structure both by CD spectra and by in silico prediction from the primary structure. More importantly, the change of the CD spectrum upon the addition of excess heme indicated that ligand binding increased the content of the ordered secondary structure of the protein (Figure 9). Similar observations have been made for several other proteins (*53*), including heme binding to *Plasmodium falciparum* histidine-rich protein (HRP) II, a parasite protein that mediates heme aggregation and detoxification in red blood cells (*54*). Formation of β structures is thought to be driven mainly by hydrophobic amino acid residues. Thus, the negative ΔC_p observed by the ITC assay is consistent with an increase of the secondary structure and a predominance of hydrophobic interactions upon heme binding to B1d1. We hypothesize that the presence of multiple heme-binding sites in AeIMUC1 will promote heme interaction and aggregation. This hypothesis is supported by the heme-binding data obtained by ITC, where the best fit model of sequential binding sites (Figure 8) leads to the proposal that AeIMUC1 acts as a heme nucleation center for the formation of the aggregates that are found in the mosquito midgut (Figure 4 and ref *17*).

In summary, we provide evidence that AeIMUC1 is an integral PM protein and that it can serve dual functions of binding chitin and heme. Both functions were mapped to the three CBDs of the protein. This is the first identification of a heme-binding PM protein in any insect. The *Ae. aegypti* PM may have as many as 30−40 major proteins (*55*), and other proteins with cysteine-rich CBDs, such as the Ae-Aper 50 *Aedes* peritrophin (*27*), may also bind heme, thus accounting for the large heme-binding capacity of PM (*17*). We hypothesize that the PM acts as a net through which digestive enzymes and hydrolytic products freely traverse

but traps the heme released during hemoglobin digestion, impeding its access to the midgut epithelial surface. Because the PM completely surrounds the blood meal, the ability of PM proteins to bind heme would provide an efficient protective mechanism. Further research is required to determine how heme binding leads to aggregation and presumed inactivation of its toxic effects, not unlike HRP-II-mediated hemozoin formation in the digestive vacuole o*f P. falciparum*-infected red blood cells (*54*). The ability of AeIMUC1 to bind multiple heme molecules may be important for this process.

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