The Roles of Two Clip Domain Serine Proteases in Innate Immune Responses of the Malaria Vector Anopheles gambiae

Jennifer Volz, Mike A. Osta, Fotis C. Kafatos, and Hans-Michael Müller

The malaria vector Anopheles gambiae is capable of multiple immune responses against Plasmodium ookinetes. Accumulating evidence in several insect species suggests the involvement of serine protease cascades in the initiation and coordination of immune responses. We report molecular and reverse genetic characterization of two mosquito clip domain serine proteases, CLIPB14 and CLIPB15, which share structural similarity to proteases involved in prophenoloxidase activation in other insects. Both CLIPs are expressed in mosquito hemocytes and are transcriptionally induced by bacterial and Plasmodium challenges. Functional studies applying RNA interference revealed that both CLIPs are involved in the killing of Plasmodium ookinetes in Anopheles. Studies on parasite melanization demonstrated an additional role for CLIPB14 in the prophenoloxidase cascade. We further report that both CLIPs participate in defense toward Gram-negative bacteria. Our findings strongly suggest that clip domain serine proteases serve multiple functions and play distinctive roles in several immune pathways of A. gambiae.

Extracellular proteolytic cascades involving clip domain serine proteases (CLIPs) are implicated in diverse arthropod innate immune responses against physical or biological challenges. Specific CLIPs have been shown to process immune signals rapidly, leading to localized reactions such as hemolymph clotting in the horsehoe crab (1), activation of antimicrobial peptide synthesis in Drosophila (2), and melanization in insects and crayfish (3–9).

Melanization is a potent insect defense response, in which activation of a serine protease cascade leads to proteolytic conversion of prophenoloxidase (PPO) into active phenoloxidase (PO). In turn, PO catalyzes the formation of quinone reactive intermediates for melanin synthesis (10), which serves several protective functions. CLIPs involved in PPO activation have been well studied in some insect species. They share common structural features including a regulatory N-terminal clip domain and a C-terminal catalytic serine protease domain that contains the catalytic triad His, Asp, Ser as well as a relatively conserved activation cleavage site R(I/V)XG and two additional cysteine residues positioned after the catalytic His (5–7, 9).

In certain genetic backgrounds of Anopheles gambiae (the major vector of human malaria in sub-Saharan Africa), notably the L3-5 strain, activation of the PPO cascade leads to melanotic encapsulation of Plasmodium ookinetes as they egress from the midgut epithelium; this phenotype is a prime example of refractory mechanisms associated with a compromised parasite cycle (11–13). In susceptible strains such as G3, parasite melanization is absent or extremely rare. Previous bioinformatic analysis of the A. gambiae genome has allowed identification of 41 genes encoding CLIPs, which were classified into four subfamilies, A, B, C, and D, according to distinct sequence signatures (14). Strikingly, A. gambiae subfamily B members share significant features with known insect CLIPs involved in PPO activation. Transcriptional profiling of RNA extracted from whole mosquitoes revealed that two CLIPs, CLIPB14 and CLIPB15, are strongly induced by Plasmodium as well as microbial challenge (15), suggesting that they may be central players in mosquito innate immune responses.

Here, we report the molecular and functional characterization of A. gambiae CLIPB14 and CLIPB15. Both CLIPs are expressed exclusively in mosquito hemocytes, from which they are secreted into the hemolymph. Functional analysis by our validated dsRNA knockdown procedure (11, 16) revealed that both are involved in parasite killing in the susceptible (G3) as well as refractory (L3-5) strains. Silencing the respective genes, singly or jointly, induced a significant increase in the number of developing oocysts in G3 and of melanized ookinetes in L3-5 mosquitoes. Interestingly, neither CLIPB14 nor CLIPB15 silencing prevented ookinete melanization in L3-5 mosquitoes, indicating that these CLIPs do not function in the melanization response of that strain. To explore whether these genes are involved in melanization in a different genetic background, we used as a model the melanizing CTL4 knockout G3 mosquitoes (11). Double knockdown studies of CTL4 and CLIPB14 revealed a substantial reduction in the proportion of melanized ookinetes and a corresponding increase in the proportion of developing, non-melanized parasites. These phenomena were not observed in CTL4/CLIPB15 double knockdowns. Thus, in certain genetic backgrounds, CLIPB14 is indeed specifically involved in the melanization response to Plasmodium berghei ookinetes. In L3-5 mosquitoes, ookinete melanization may either involve different CLIPs or be promoted by a chronic state of oxidative stress as suggested previously (17). Interestingly, we also demonstrate that CLIPB14 and CLIPB15 are involved in defense against Gram-negative but not Gram-positive bacteria. Taken together, our findings suggest that individual CLIPs can serve multiple reactions, suggesting the existence of cross-talk between distinct mosquito immune responses.
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MATERIALS AND METHODS

Mosquito Rearing and P. berghei Infections—Mosquito rearing and parasite infections were performed essentially as described previously (18). A P. berghei (ANKA strain) clone expressing GFP constitutively under control of the ef-1 promoter (19) was passaged in CD1 mice. Parasitemias of infected mice were determined from Giemsa-stained blood films.

Double-stranded RNA Preparation and Parasite Survival Assays—Double-stranded RNA was prepared as described previously (16) using the pl6 vector for expression of control dsGFP and the modified pl10 vector for expressing cloned gene fragments of interest: a 590-bp sequence of CLIPB14 (nucleotide positions 569–1159) or a 546-bp sequence of CLIPB15 (nucleotide positions 542–1088) flanked by two T7 promoters. These fragments were obtained by amplification of CLIPB14 and CLIPB15 full-length clones with the following primers containing restriction sites XhoI (5’)- and HindIII (3’): CLIPB14, Fwd 5’-CCC CCT CGA GGA CTG CAA GCA GGT CAA AGG C-3’ and Rev 5’-TAT CAA GGT TCT CAC GGA ACA TCT CCC G-3’; CLIPB15, Fwd 5’-CCC CCT CGT GAC GGA GAA CGA TGA GAG GTC-3’ and Rev 5’-TAT CAA GGT TCT CAC ACA AAT ACA G-3’. In each of the RNA interference experiments (see TABLE ONE), mosquitoes of all tested genotypes were fed on the same P. berghei-infected mouse 4 days after dsRNA injection, and midguts were dissected and fixed 5–7 days later. Oocysts were counted using a UV-light fluorescence microscope, and melanized parasites were counted by light microscopy (Zeiss).

RNA Isolation and Quantitative Real-Time RT-PCR—Total RNA from whole mosquitoes or dissected tissues was isolated with TRIzol reagent (Invitrogen), and first strand cDNA synthesis was performed using SuperScript™II RNase H⁻ reverse transcriptase (Invitrogen) according to the supplier’s instructions. Specific primers were designed using the Primer Express Software (Applied Biosystems): CLIPB14, Fwd 5’-CAG TGT TTT ACC GCA GCA AAA-3’ and Rev 5’-GGT CCA GGC CAC CGA GTG ATT TTT-3’; CLIPB15, Fwd 5’-ACC GAA CTG GAA GCT TTA C-3’ and Rev 5’-TTT CCA GTG GAG CAA TTA TCC-3’. The isolated CLIPB14 and CLIPB15 clones contained full-length sequences of 1242 and 1158 bp, respectively (GenBank™ accession numbers CAB90819 (CLIPB14) and CAB90818 (CLIPB15)), which were confirmed later by whole genome analysis (14).

Probes) for 45 min and analyzed with a fluorescence imager (Fuji FLA-3000) and first strand synthesis and semiquantitative RT-PCR were performed as described previously (16) using the pl6 vector for expression of control dsGFP and the modified pl10 vector for expressing cloned gene fragments of interest: a 590-bp sequence of CLIPB14 (nucleotide positions 569–1159) or a 546-bp sequence of CLIPB15 (nucleotide positions 542–1088) flanked by two T7 promoters. These fragments were obtained by amplification of CLIPB14 and CLIPB15 full-length clones with the following primers containing restriction sites XhoI (5’)- and HindIII (3’): CLIPB14, Fwd 5’-CCC CCT CGA GGA CTG CAA GCA GGT CAA AGG C-3’ and Rev 5’-TAT CAA GGT TCT CAC GGA ACA TCT CCC G-3’; CLIPB15, Fwd 5’-CCC CCT CGT GAC GGA GAA CGA TGA GAG GTC-3’ and Rev 5’-TAT CAA GGT TCT CAC ACA AAT ACA G-3’. In each of the RNA interference experiments (see TABLE ONE), mosquitoes of all tested genotypes were fed on the same P. berghei-infected mouse 4 days after dsRNA injection, and midguts were dissected and fixed 5–7 days later. Oocysts were counted using a UV-light fluorescence microscope, and melanized parasites were counted by light microscopy (Zeiss).

For time point analysis, 20–25 midguts were dissected, and the remaining carcasses were collected from mosquitoes every 3 h between 18–30 h after blood feeding or Plasmodium infection in two independent biological experiments. For time point analysis of bacteria-challenged mosquitoes, 20–25 intact mosquitoes were collected 2, 4, 6, and 12 h after challenge in two independent biological experiments. The efficacy of the CLIP knockdown was analyzed by collection of intact dsCLIPB14, dsCLIPB15, and control dsGFP-treated mosquitoes 4 days after dsRNA injection.

For semiquantitative RT-PCR analysis total RNA from various mosquito developmental stages was purified as described previously (20), and first strand synthesis and semiquantitative RT-PCR were performed (21). The ribosomal gene 5S was amplified in a separate tube and served as internal control for normalization. After electrophoresis on agarose, gels were stained with the sensitive SYBR green dye (Molecular Probes) for 45 min and analyzed with a fluorescence imager (Fuji FLA-3000). The primer sequences and corresponding annealing temperatures in the RT-PCR experiments were as follows: 5S (60 °C), Fwd 5’-GGC GAT CAT CAT CTA CGT-3’ and Rev 5’-GTA GCT GCT GCA AACT TTC GG-3’; CLIPB14 (60 °C), Fwd 5’-TGT GGT TGG TCT TGC TTT G-3’ and Rev 5’-CTT GCT CTT TGC TGG GTA-3’; CLIPB15 (60 °C), Fwd 5’-GGA GAC TCC GGT GGA CCG CTG ATG CG-3’ and Rev 5’-CCA CAT TGT AGA AAG CAT ATC ATA C-3’.

Cloning and Sequencing—Prior to the genome release, gene-specific amplification primers were available based on the A. gambiae expressed sequence tag sequence 4A3B-aad-d-12-3’ for CLIPB14 and 4A3B-aad-b-06-3’ for CLIPB15 (22). Partial sequences of the protease domain of these CLIPs including the His-Asp-Ser catalytic triad sequence were amplified out of the ZAP Express vector from a pool of the A. gambiae cell line cDNA library 4a-2/3B using the T3 primer and the corresponding reverse expressed sequence tag primers (see above). For CLIPB15, this fragment (1037 bp) was used directly as a probe to screen the cDNA library. For CLIPB14, a new sequence-specific primer was generated (5’-GGG CGA GTG CCT ATG CCT GCC G-3’) based on the obtained sequence and was used with a T3 primer to amplify a 584-bp fragment; the latter was used to screen the cDNA library. The isolated CLIPB14 and CLIPB15 clones contained full-length sequences of 1242 and 1158 bp, respectively (GenBank™ accession numbers CAB90819 (CLIPB14) and CAB90818 (CLIPB15)), which were confirmed later by whole genome analysis (14).

Generation of Polyclonal CLIPB14 and CLIPB15 Antiserum—CLIPB14 and CLIPB15 sequence-specific primers were designed for PCR amplification of the complete protease domain using as template full-length sequences subcloned in pBK-CMV phagemid vectors. Forward and reverse primers contained Ncol and XhoI restriction sites, respectively, corresponding to cloning sites within the pETM11 protein expression vector. The CLIPB14 primers were: Fwd, 5’-CGG CGG CCT GCC TCT CCC ATG GTC ATC GGT GGC AAC GAT-3’ and Rev, 5’-GGT GCT GGA CAC GAA AAA TTC CAC G-3’. The CLIPB15 primers were: Fwd, 5’-GGC ATG GTC TAC TGT GGC GAA AGG-3’ and Rev, 5’-GGT GCT GGA GTT ACA AAT ATC GCT CTT C-3’. PCR products were digested with Ncol and XhoI and cloned into the corresponding sites of pETM11. Fusion proteins containing an N-terminal His tag were expressed in Escherichia coli strain BL21 and purified as described previously (23) followed by tobacco etch virus cleavage to remove the His tag. For each protein, three rats were immunized with 40 μg of protein each using R-700 adjuvants (RIBI Immunochem Research, Inc.) and were boosted every third week until the final bleed.

Immunoblotting—Hemolymph was collected into Schneider medium (without fetal calf serum and protease inhibitors) by proboscis clipping, and the serum after pelleting hemocytes was used for protein analysis (shown in Fig. 2F). Alternatively, hemolymph including hemocytes was collected directly into SDS loading buffer. Proteins were resolved by 12% SDS-PAGE, transferred to Hybond-P membranes (Amersham Biosciences), blocked with 5% milk, and detected using CLIPB14 and CLIPB15 antiserum at 1:1000 dilution. Anti-rat IgG conjugated to horseradish peroxidase (Promega) was used as secondary antibody (1:10,000), and blots were developed using Western blot Chemiluminescence Reagent Plus kit (PerkinElmer Life Sciences). After CLIPB14 and CLIPB15 analysis, protein loading and efficient transfer were monitored by blot staining with Coomassie. Hemolymph was collected into PBS containing a protease inhibitor mix (Roche Applied Science) and treated with N-glycosidase F (Roche Applied Science) as described previously (24) to cleave off putative asparagine-linked glycan chains of the CLIPs.

Immunostaining—Hemocytes were collected in chilled 5-μl Schneider medium (without fetal calf serum and protease inhibitors) on a glass slide by proboscis clipping. After settling for 15 min, hemocytes were fixed in PBS and 4% paraformaldehyde for 10 min. After treatment with
0.1% Triton-X in PBS for 5 min, samples were blocked with 5% bovine serum albumin for 1 h. Primary rat antibody against CLIPB14 1:500, rat antibody against CLIPB15 1:500, and rabbit antibody against PPO6 1:500 (20) were added, and samples were incubated overnight at 4 °C and treated with secondary antibodies (The Jackson Laboratory, 1:1000) for 1 h at room temperature. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (Roche Applied Science, 1:2000), and samples were mounted with Dabco/Moviol. For sectioning, mosquito abdomens were cut off, fixed for 3 h in ice-cold PBS with 4% paraformaldehyde, dehydrated, incubated for 20 min in a 1:1 paraffin/xylene emulsion at 42 °C, changed to fresh paraffin at 60 °C, incubated overnight, and embedded at 4 °C. Sagittal sections (6 μm) were taken using a microtome (Microm HM310) and stretched on poly-L-lysine-coated glass slides and allowed to dry overnight at 42 °C. After paraffin removal and rehydration of the samples, antigens were unmasked by the high temperature technique by cooking glass slides in unmasking solution (0.01 M citrate buffer, pH 6.4) for 20 min in a 600-watt microwave oven. Samples were blocked (1% bovine serum albumin, 0.01% Triton X-100, 5% goat serum in PBS) for 1 h and subjected to immunostaining.

**RESULTS**

**Cloning and Molecular Characterization of CLIPB14 and CLIPB15—** To obtain full-length sequences of CLIPB14 and CLIPB15, a cDNA library (4a-2/3B) constructed from an A. gambiae cell line was screened with CLIPB14- and CLIPB15-specific probes that were amplified from the same cDNA library, using a gene-specific reverse primer and a T3 primer. This allowed the identification of full-length CLIPB14 (1242 bp) and CLIPB15 (1158 bp) clones, corresponding to the predicted cDNA sequences (14). CLIPB14 and CLIPB15 belong to subfamily B clip domain serine proteases, which are most similar in sequence architecture to the known PPO-activating CLIPs (5–7, 9) of *Holotrichia diomphalia*, Bombyrx mori, and *Manduca sexta* (Fig. 1A). Protein sequence alignment of CLIPB14 and CLIPB15 reveals that they share ~40% sequence identity (Fig. 1B). Both proteins contain the catalytic serine protease triad as well as all conserved cysteine residues. Several predicted N-glycosylation sites suggest that CLIPB14 and CLIPB15 are glycosylated proteins.

Developmental Expression—Expression analysis in mosquito developmental stages revealed that CLIPB14 is strongly expressed in adult females; only modest expression is detected in the second and third larval as well as pupal stages. (Fig. 2A). In contrast, the CLIPB15 transcript is detected throughout development and is relatively abundant in the late larval and pupal stages. The presence of CLIPB15 transcripts in freshly laid eggs indicates additional maternal inheritance.

**CLIPB14 and CLIPB15 Are Hemocyte-specific—** To identify the immune tissues that synthesize CLIPB14 and CLIPB15, we utilized paraffin-embedded sections prepared from whole mosquitoes 30 h after blood feeding (Fig. 2B). Immunostainings indicated that both CLIPB14 and CLIPB15 are produced by a subpopulation of mosquito hemocytes that also stain positive for PPO (Fig. 2C, lower panels). Some of the circulating hemocytes in hemolymph, harvested by proboscis clipping, also expressed these CLIPs, which invariably co-localized with PPO (Fig. 2D, left upper and lower panels). No CLIPB14 or CLIPB15 expression was observed in pericardial cells, the midgut epithelial layer, fat body cells, muscle cells, or ovaries (data not shown). Thus, in adult mosquitoes CLIPB14 and CLIPB15 are produced exclusively by the hemocyte subpopulation that produces PPO. These CLIPs were almost completely absent from hemocytes isolated from CLIPB14 and CLIPB15 knockdown mosquitoes, respectively (Fig. 2D, right upper and lower panels). At the transcript level, the efficiency of the knockdown was confirmed 4 days after dsRNA injection in G3 mosquitoes and was on the order of 90% for both genes (Fig. 2E). No cross-silencing was observed between CLIPB14 and -B15, confirming that the knockdowns are gene-specific (data not shown). Reproducibility of silencing at the RNA level was confirmed by use of two different preparations of dsRNA with consistent results in both the G3 and L3-5 strains.

The predicted sequences of CLIPB14 and CLIPB15 reveal the presence of a signal peptide, suggesting that these proteins are secreted (Fig. 3A). A. gambiae CLIPs differ from their counterparts in *B. mori* and *H. diomphalia* in that they lack the Cys5-Cys6 disulfide motif that is diagnostic of many CLIPs and that is present in all other species. In addition, the two cysteine residues that are diagnostic for subfamily B CLIPs are indicated by blue vertical lines. A linker region (horizontal line) connects the two domains. A triangle indicates the putative activation cleavage site. Conserved cysteines are connected with brackets according to the disulfide bonding pattern that has been determined in the proclotting enzyme of the horseshoe crab (36). Two cysteine residues that are diagnostic for subfamily B CLIPs are indicated by blue vertical lines. A, alignment of the amino acid sequences of CLIPB14 and CLIPB15. The numbers refer to residues counted from the initiation methionine. Residues identical in both sequences are marked with asterisks. The catalytic triad residues are indicated with closed diamonds, and the conserved regions comprising each residue are boxed. Yellow highlighting marks the positions of conserved cysteine residues, and blue marks the two cysteine residues characteristic of subfamily B. Asparagine residues predicted as N-glycosylation sites are colored in red, and green lettering indicates the signal peptide.
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We confirmed this prediction by detecting both proteins in immunoblots on hemolymph extracted from adult female mosquitoes, which was separated from hemocytes. Immunoblots with the CLIPB14 antibody reveal a major band at 55 kDa and two weaker bands at 80 and 37 kDa (Fig. 2F, left lane). These bands are specific, as they are significantly reduced in hemolymph extracts from CLIPB14 knockdown mosquitoes (Fig. 2G, left panel and data not shown). The 55-kDa band that represents full-length CLIPB14 is larger than the theoretical mass of the predicted protein (41.1 kDa), suggesting that the native protein might be glycosylated. The sequence predicted four N-glycosylation sites in CLIPB14 (Fig. 1B), but hemolymph treatment with N-glycosidase F induced only a slight reduction in the size of full-length CLIPB14. This suggests that N-linked glycosylation is not the major contributor to the increased apparent size of the protein detected in immunoblots (Fig. 2H, lanes 1 and 2). The weaker 80-kDa band (Fig. 2F) may represent a stable protein complex involving CLIPB14; it is known that serine proteases associate with serpins to form SDS-resistant complexes (25, 26). The 37-kDa band may represent a cleaved form of CLIPB14.

Similarly, the CLIPB15 antibody recognizes a major band at 45 kDa corresponding to full-length CLIPB15 and minor bands of lower apparent molecular mass, most probably corresponding to cleavage forms (Fig. 2F, right lane). The slower mobility of CLIPB15 relative to the theoretical mass of the predicted protein (40.6 kDa) could well be attributed to N-linked glycosylation, as a significant reduction in apparent size occurs after treatment with N-glycosidase F (Fig. 2H, lanes 3 and 4). The abundance of CLIPB15 is significantly and reproducibly reduced in hemolymph from CLIPB15 knockdown mosquitoes (Fig. 2G, right panel).

The Role of CLIPB14 and CLIPB15 in Antibacterial Immunity—The involvement of several clip domain serine proteases in insect innate immunity (5, 7, 27) prompted us to test by QRT-PCR whether CLIPB14 and CLIPB15 expression is induced by bacterial challenge. A. gambiae female mosquitoes of the G3 strain were injected with E. coli or S. aureus, and the transcript levels of both genes were assessed in whole mosquitoes at several time points post-challenge (Fig. 3A). The CLIPB15 transcript was rapidly induced by both E. coli and S. aureus already at 2 h; induction relative to controls seemed to decline after 4 h. CLIPB14 was also induced by both bacteria with somewhat slower kinetics; induction relative to controls was highest at 12 h. PBS injection alone transiently induced expression, especially of CLIPB14, indicating that this gene may also respond to sterile injury. Regulation at the protein level was less apparent suggesting that gene induction primarily serves replenishment rather than enrichment (data not shown).

We performed G3 mosquito survival assays to assess whether CLIPB14 and CLIPB15 function in antimicrobial defense. Expression of CLIPB14 and/or CLIPB15 was silenced by injecting corresponding dsRNAs into the body cavity of newly emerged females in the susceptible G3 strain; controls received dsRNA of an unrelated gene (GFP). The CLIPB14, CLIPB15, CLIPB14/1B15, and control GFP knockdown mosquitoes were infected with E. coli or S. aureus, and survival was monitored for a period of 7 days post-challenge. The survival of CLIP knockdown mosquitoes challenged with S. aureus was comparable with that of controls (data not shown). However, depletion of either CLIP gene followed by E. coli challenge compromised significantly the survival of mosquitoes throughout the course of the experiment (Fig. 3B).

FIGURE 2. Developmental profiles and tissue localization of CLIPB14 and CLIPB15. A, semiquantitative RT-PCR was performed on RNA extracted from A. gambiae developmental stages: Eg, eggs; Em, 18-h embryos; L1–L4, first to fourth instar larvae; Pu, pupae; F, adult females. The number of amplification cycles for each primer pair is indicated. The ribosomal RNA (rS7) transcript was used for data standardization. B, sagittal paraffin section of a female mosquito 30 h after a non-infectious blood meal, stained with myotoxin and eosin, including the midgut epithelium (mg), midgut lumen (ml), fat body (fb), ovaries (ov), and cuticle (cu). C and D, immunolocalization of CLIPB14 and CLIPB15 in sagittal paraffin sections (C) and in isolated hemocytes (D). CLIPB14 and CLIPB15 are stained in green, nuclei in blue, and prophenoloxidase in red. Co-localization of PPO with CLIPB14 and CLIPB15 appears in yellow. CLIPB14 and CLIPB15 were absent from hemocytes isolated from dsCLIPB14 (dsB14) and dsCLIPB15 (dsB15) mosquitoes, respectively. D, differential interference contrast, E, efficiency of CLIPB14 and CLIPB15 knockdown (KD) in G3 mosquitoes using QRT-PCR; the levels of CLIPB14 (B14) and CLIPB15 (B15) transcripts were normalized to the internal ribosomal transcript S7, and dsGFP-treated mosquitoes served as control (C, 1.0). F–H, immunoblot analysis of CLIPB14 and CLIPB15. F, naive female mosquito hemolymph collected on ice in Schneider medium (without fetal calf serum) in the absence of protease inhibitors. G, hemolymph from dsCLIPB14 and dsCLIPB15 mosquitoes extracted directly in SDS loading buffer, demonstrating equal mobility but reduced amount in specific versus nonspecific knockdowns. RNAi; RNA interference. H, hemolymph collected in PBS containing protease inhibitors from female mosquitoes and treated with N-glycosidase F (Ng−). Compared with untreated hemolymph (Ng−), reduced sizes of CLIPB14 and CLIPB15 are observed in Ng−-treated hemolymph.
CLIPB14 and CLIPB15 Expression Is Also Induced by Parasite Infection—Transcriptional expression of these CLIPs was assessed in infected and in non-infected but blood-fed mosquito midguts and carcasses (mosquito remnants following midgut isolation) dissected at five time points in a 12-h window encompassing the period when parasites invade the midgut epithelium. The carcass is enriched in hemocytes, some of which produce these CLIPs, and some hemocytes are also attached to the midgut, the organ where parasite melanization occurs. For these infections, we used \( P.\) berghei, which is the best studied rodent malaria parasite. It infects \( A.\) gambiae easily and is available as fluorescently labeled reporter strains. Thus, this species serves as the model for biological studies pertaining to mosquito-Plasmodium interactions. The CLIP15 profiles were simpler and will be described first. In the carcass the transcript induction and dynamics were modest; indeed, CLIPB15 levels were higher in blood-fed than in infected carcasses at 21–27 h (Fig. 4B). However, the transcript level at 27 h after parasite ingestion, i.e. immediately following epithelial crossing of the midgut epithelium by the parasites, was substantially higher in infected midguts. In the case of CLIPB14, the levels in the carcass increased significantly between 27 and 30 h in both infected and non-infected blood-fed mosquitoes (Fig. 4A). More dramatic changes were evident in the midgut, where CLIPB14 transcript levels were negligible between 18 and 27 h but increased remarkably between 27 and 30 h, specifically in infected mosquitoes (7-fold by 30 h). These results indicate that CLIPB14 and CLIPB15 are immune-responsive to \( P.\) berghei and correlate with previous microarray data that showed immune inducibility of these genes in whole \( P.\) berghei-infected mosquitoes at 28 h post-infection (14). However, comparable regulation could not be observed at the protein level (data not shown; see “Discussion”).

Both CLIPs Limit Parasite Development—The strong up-regulation of CLIPB14 and CLIPB15 following oocYTE invasion of the midgut cells prompted us to investigate whether these proteins are implicated in immune defense against the late ookinete stage of Plasmodium, which was shown previously to be highly vulnerable to mosquito immune responses (11, 13). To this purpose we performed in vivo functional analysis of both genes by reverse genetics. Four days after \( ds\)RNA injection, mosquitoes were fed on mice infected with a transgenic \( P.\) berghei parasite that expresses GFP constitutively (19), and developing (fluorescent) oocysts or killed and melanized oocysts (Fig. 5, A–D) were counted 5–7 days post-infection. The experimental CLIPB14 and CLIPB15 knockdown mosquitoes showed consistent (2.8- and 2.0-fold, respectively) and highly significant (\( P_{OC}<0.001\)) increases in mean oocyst numbers relative to the GFP controls (TABLE ONE, Experiment A). In an experiment where smaller numbers of mosquitoes were analyzed (TABLE ONE, Experiment B), a comparable effect of the simultaneous double CLIPB14/B15 knockdown was observed (1.8-fold increase in oocysts, \( P_{OC} = 0.057\)). In all of these experiments no effect was observed on melanization, which is very rare in the G3 strain and remained sporadic after all knockdowns, at an average level of less than 0.1 melanized parasite per midgut or less than 0.002 of the total number of parasites. We conclude that in G3 mosquitoes, CLIPB14 and CLIPB15 significantly limit the number of ookinete that can develop into oocysts and that the “missing parasites” are killed by unknown processes involving CLIPs and are subsequently cleared through lysis rather than melanization (13).

In the melanizing-refractory strain L3–5 (Fig. 5, H–K), the observed parasite numbers per midgut also increased significantly in the CLIP knockdowns, but in this case parasites were killed and melanized rather...
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The difference between these distributions is significant. Five bins according to their number of parasites. A pronounced shift was evident toward all replicates were pooled and showed similar distributions. Midguts were grouped in susceptible mosquitoes and from 7 independent replicates in refractory mosquitoes, as accompanied by a clear decrease in melanized ookinetes (2.3-fold, as compared with 8.2% in dsCTL4/CLIPB14). Remarkably, 77.3% of the parasites developed as oocysts in dsCTL4/CLIPB15, whereas the number of developing oocysts remained negligibly low (data not shown) suggesting that the transcriptional induction may serve protein replenishment rather than enrichment during immune responses. Functional analysis using in vivo RNA interference revealed that both dsCLIPB14 and dsCLIPB15 in response to Gram-negative and -positive bacterial challenge, indicating that both of these genes may be implicated in microbial defenses but are not co-regulated. Interestingly, dsCLIPB14 was also induced by sterile injury suggesting a potential role in wound healing. However, protein levels in hemolymph extracts from mosquitoes infected with E. coli or S. aureus did not change substantially (data not shown) suggesting that the transcriptional induction may serve protein replenishment rather than enrichment during immune responses. Functional analysis using in vivo RNA interference revealed that both dsCLIPB14 and dsCLIPB15 are involved in defense against Gram-negative bacteria. The survival of either dsCLIPB14- or dsCLIPB15-treated mosquitoes was compromised following E. coli challenge. Despite the transcriptional induction of both genes in S. aureus-infected mosquitoes, survival was not affected, indicating that these CLIPs may have no major role in the clearance of Gram-positive bacteria. In Drosophila, a proteolytic amplification cascade is believed to link fungal and Gram-positive bacterial infections to the cleavage of the cytokine-like molecule Spatzle and subsequent activation of the Toll pathway, ultimately culminating in the synthesis of antimicrobial peptides. Indeed, the clip domain serine protease Persephone, which displays structural features of the A. gambiae CLIP subfamily C (14), is required for Toll activation in response to fungal infections. The involvement of CLIPB14 and 15.8% in dsCTL4 mosquitoes (Fig. 5, E–G; TABLE ONE, Experiment C). These data indicate a strong contribution of CLIPB14 but not of CLIPB15 in ookinete melanization in the G3/CTL4 knockdown genetic background.

DISCUSSION

The phenoloxidase cascade is an integral part of insect physical and innate immune defenses, including cuticle sclerotization and melanization of large pathogens. Melanization requires the limited proteolysis of inactive PPO to active PO by PPAEs, trypsin-like clip domain serine proteases similar to the Drosophila Easter protease. Much is known on the biochemistry of PPO activation by CLIPs from in vitro reconstitution studies using proteins purified from the cuticle or plasma of several large insect species, including B. mori (28), M. sexta (27, 29, 30), H. diomphalia (5, 6), and Hyalophora cecropia (31). However, the involvement and specific contribution of PPAE-like CLIPs to immune responses to microbes and parasites has not been genetically investigated, primarily because of the absence of genetic tools for rapid analysis of gene function in the insect species that serve as models for biochemical studies. Here, we report the cloning and in vivo functional characterization of CLIPB14 and CLIPB15, two A. gambiae clip domain serine proteases that were identified previously from expressed sequence tags generated from immune-competent cell lines (22). These CLIPs are synthesized by hemocytes and secreted into the hemolymph. Both CLIPB14 and CLIPB15 have a domain organization similar to that of M. sexta prophenoloxidase-activating proteinase 1 (27), H. diomphalia prophenoloxidase-activating factor 1, and Pacifastacus leniusculus PPAE (8). They are characterized by a single N-terminal clip domain and a C-terminal protease domain, in contrast to other PPAEs including M. sexta prophenoloxidase-activating proteinase 2 (29) and prophenoloxidase-activating proteinase 3 (30) and B. mori PPAE (7), which contain two N-terminal clip domains each. Several biological roles have been suggested for the clip domain but have not been proven experimentally to date (32). In a single case, the P. leniusculus clip domain exhibited antibacterial activity against Gram-positive bacteria, suggesting that such domains may impart a second immune function to the CLIPs (8). QRT-PCR revealed the distinct induction kinetics of CLIPB14 and CLIPB15 in response to Gram-negative and -positive bacterial challenge, indicating that both of these genes may be implicated in microbial defenses but are not co-regulated. Interestingly, CLIPB14 was also induced by sterile injury suggesting a potential role in wound healing. However, protein levels in hemolymph extracts from mosquitoes infected with E. coli or S. aureus did not change substantially (data not shown) suggesting that the transcriptional induction may serve protein replenishment rather than enrichment during immune responses. Functional analysis using in vivo RNA interference revealed that both CLIPB14 and CLIPB15 are involved in defense against Gram-negative bacteria. The survival of either dsCLIPB14- or dsCLIPB15-treated mosquitoes was compromised following E. coli challenge. Despite the transcriptional induction of both genes in S. aureus-infected mosquitoes, survival was not affected, indicating that these CLIPs may have no major role in the clearance of Gram-positive bacteria. In Drosophila, a proteolytic amplification cascade is believed to link fungal and Gram-positive bacterial infections to the cleavage of the cytokine-like molecule Spatzle and subsequent activation of the Toll pathway, ultimately culminating in the synthesis of antimicrobial peptides. Indeed, the clip domain serine protease Persephone, which displays structural features of the A. gambiae CLIP subfamily C (14), is required for Toll activation in response to fungal infections. The involvement of CLIPB14 and
CLIPB15 in defense against Gram-negative bacteria points to a possible link with downstream synthesis of antimicrobial peptides or other effectors, which remains to be explored in the future.

We have shown previously that ookinete killing and subsequent lysis is a potent *Anopheles* immune reaction that eliminates ~80% of invading ookinetes and requires the pattern recognition receptor TEP1 (13, 24) and the leucine-repeat protein LRIM1 (11). Here we report that this lytic immune module also involves a serine protease cascade comprising CLIPB14 and CLIPB15 and probably additional unidentified serine proteases.

Our in vivo RNA interference analysis has established that both CLIPB15 and CLIPB14 are involved in the killing and lysis of *Plasmodium* ookinetes. The knockdown of one or both genes induced a significant increase in the number of oocysts or melanized ookinetes in L3-5 mosquitoes, respectively. Interestingly, these CLIPs appear to act differently in parasite killing within the two strains. In L3-5 mosquitoes, double CLIPB14/CLIPB15 knockdowns induce a dramatic increase in melanized ookinetes, 7-fold relative to dsGFP controls and 2–3.4-fold relative to the single CLIP knockdowns (TABLE ONE, Experiment D). The much stronger phenotype of the double knockdown strongly suggests that in the L3-5 strain CLIPB14 and CLIPB15 function at least additively and possibly synergistically to promote parasite killing rather than acting at successive steps in the same pathway. In contrast, in the G3 strain the 2-fold increase in oocysts in this double knockdown as compared with dsGFP controls is modest and not higher than the increases in single CLIP knockdowns (TABLE ONE, Experiment B); this implies that in G3 mosquitoes CLIPB14 and CLIPB15 promote parasite killing through the same pathway. Taken together, the evidence suggests that the killing/lysis-promoting serine protease cascade has at least partially different functional architecture in the L3-5 versus G3 strains, presumably reflecting their different genetic backgrounds. Similarly, melanization is under different genetic control in two different melanization backgrounds, L3-5 and G3/dsCTL4. Despite sharing sequence features with insect PPAEs that are known to activate melanization, neither CLIPB14 nor CLIPB15 is essential for melanization in L3-5. In this strain, knockdowns of these CLIPs, singly or jointly, do not lead to a significant increase in non-melanized, developing oocysts (TABLE ONE, Experiment D). However, in the dsCTL4 background the mean frequency of developing oocysts increases dramatically (more than 8-fold) when CLIPB14 but not CLIPB15 is silenced (TABLE ONE, Experiment C), again emphasizing the effect of genetic background.

In this context, it is worth noting that genetic mapping studies have identified three quantitative trait loci as implicated in ookinete melanization in L3-5 mosquitoes (34) and suggested that additional QTLs

### TABLE ONE

Numbers of melanized ookinetes and developing oocysts in gene knockdown mosquitoes

| Strain | Exp. | Knockdowns | Mg | TP | MeanTP | OC | MeanOC | ML | MeanML | P_ML |
|--------|------|------------|----|----|--------|----|--------|----|---------|-------|
| G3     | A    | GFP        | 150| 9195| 61     | 9186| 61     | 9  | 0.05    | 1.0   |
|        |      | CLIPB14    | 150| 25257| 168   | <0.001| 25249| 168| <0.001  | 8     |
|        |      | CLIPB15    | 150| 17777| 119   | <0.001| 17770| 119| <0.001  | 7     |
| B      |      | GFP        | 58 | 2745 | 47     | 2738 | 47     | 5  | 0.09    | 1.0   |
|        |      | CLIPB14    | 58 | 9814 | 169   | <0.001| 9813 | 169| <0.001  | 1     |
|        |      | CLIPB15    | 58 | 6209 | 107   | <0.001| 6205 | 107| <0.001  | 4     |
|        |      | CLIPB14/15 | 58 | 4797 | 83    | 0.057 | 4793 | 83  | 0.057   | 4     |
| C      |      | GFP        | 46 | 2790 | 61     | 2788 | 61     | 2  | 0.04    | 1.0   |
|        |      | CLIPB14    | 46 | 7408 | 161   | 0.005 | 7408 | 161| 0.005   | 0     |
|        |      | CLIPB15    | 46 | 5806 | 126   | 0.022 | 5803 | 126| 0.022   | 3     |
|        |      | CTL4       | 35 | 5653 | 162   | 0.014 | 895  | 17.9| 4758    | 95    |
|        |      | CTL4/CLIPB14| 46| 9182 | 200   | <0.001| 7101 | 154| <0.001  | 2081  |
|        |      | CTL4/CLIPB15| 46| 6961 | 151   | 0.005 | 574  | 13.0| 6387    | 139   |
| L3–5  | D    | GFP        | 56 | 1429 | 6     | 3    | 0.05  | 1426| 26      |
|        |      | CLIPB14    | 56 | 2914 | 52    | <0.001| 31   | 0.6 | 0.991   | 2883  |
|        |      | CLIPB15    | 56 | 5006 | 89    | <0.001| 25   | 0.5 | 0.786   | 4981  |
|        |      | CLIPB14/15 | 56 | 9801 | 175   | <0.001| 22   | 0.4 | 0.991   | 9777  |

Our genetic background Phenotype Control dsCLIPB14 dsCLIPB15 dsCLIPB14/CLIPB15

| Genetic background | Phenotype | Control | dsCLIPB14 | dsCLIPB15 | dsCLIPB14/CLIPB15 |
|--------------------|-----------|---------|-----------|-----------|------------------|
| G3                 | Relative oocyst no. | 1.0 | 3.6     | 2.3 | 1.8 |
| L3–5               | Relative melanized oocyst no. | 1.0 | 2.0     | 3.4 | 6.7 |
| L3–5               | % Melanization | 99.8 | 98.9    | 99.5 | 99.8 |
| G3/dsCTL4          | % Melanization | 84.2 | 22.7    | 91.8 | ND |

The much stronger phenotype of the double knockdown suggests that one or both genes are involved in the killing and lysis of *Plasmodium* ookinetes. The knockdown of one or both genes induced a significant increase in the number of oocysts or melanized ookinetes in L3-5 and L3-5 mosquitoes, respectively. Interestingly, these CLIPs appear to act differently in parasite killing within the two strains. In L3-5 mosquitoes, double CLIPB14/CLIPB15 knockdowns induce a dramatic increase in melanized ookinetes, 7-fold relative to dsGFP controls and 2–3.4-fold relative to the single CLIP knockdowns (TABLE ONE, Experiment D). The much stronger phenotype of the double knockdown strongly suggests that one or both genes are involved in the killing and lysis of *Plasmodium* ookinetes. The knockdown of one or both genes induced a significant increase in the number of oocysts or melanized ookinetes in L3-5 and L3-5 mosquitoes, respectively. Interestingly, these CLIPs appear to act differently in parasite killing within the two strains. In L3-5 mosquitoes, double CLIPB14/CLIPB15 knockdowns induce a dramatic increase in melanized ookinetes, 7-fold relative to dsGFP controls and 2–3.4-fold relative to the single CLIP knockdowns (TABLE ONE, Experiment D). The much stronger phenotype of the double knockdown strongly suggests that in the L3-5 strain CLIPB14 and CLIPB15 function at least additively and possibly synergistically to promote parasite killing rather...
are likely involved differentially in responses to different malaria parasites (35). Highlighting the complexity of the melanization response in the L3-5 strain, it has been reported that L3-5 mosquitoes are in a chronic state of oxidative stress, resulting in high steady-state levels of reactive oxygen species, which favor ookinete melanization (17).

Intriguingly, no enhanced systemic activation cleavage of CLIPB14 or CLIPB15 was observed in hemolymph extracts following Plasmodium or bacterial infections (data not shown), unlike the reports (7, 25, 30) for other insect PPAEs. Our interpretation is that during infection, activation of CLIPB14 and CLIPB15 is highly localized in tissue foci (probably in the vicinity of pathogen surfaces) and does not occur in the circulating hemolymph where the chance of propagated, potentially fatal melanization would be enhanced. The fact that both CLIPB14 and CLIPB15 are involved in the killing of P. berghei (TABLES ONE and TWO) as well as E. coli suggests the existence of cross-talk between mosquito immune responses to Plasmodium and Gram-negative bacteria. Furthermore, the strong additional contribution of CLIPB14 to parasite melanization in CTL4 knockdown G3 mosquitoes suggests the existence of cross-talk between parasite killing responses and the melanization cascade. Whether CLIPB14 acts upstream of as yet unidentified PPAEs or is directly involved in PPO activation is not yet elucidated and merits future investigation using biochemical approaches. In light of this work, we are engaged in conducting a large scale in vivo functional analysis of all A. gambiae clip domain serine proteases to identify those that are involved in defense against Plasmodium as well as bacteria and to define their hierarchy in the respective cascades. Understanding the A. gambiae immune modules, in particular those underlying ookinete killing/lysis and melanization, will not only shed light on the mechanisms that govern the survival of Plasmodium in its vector but may also lead to novel transmission-blocking strategies.

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