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Leucine-rich repeat protein complex activates mosquito complement in defense against *Plasmodium* parasites

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Abstract

Leucine-rich repeat containing proteins are central to host defense in plants and animals. We show that in the mosquito *Anopheles gambiae*, two such proteins that antagonize malaria parasite infections, LRIM1 and APL1C, circulate in the hemolymph as a high molecular weight complex held together by disulfide bridges. The complex interacts with the complement C3-like protein, TEPI, promoting its cleavage or stabilization, and its subsequent localization on the surface of midgut-invading *Plasmodium berghei* parasites, targeting them for destruction. LRIM1 and APL1C are members of a protein family with orthologs in other disease vector mosquitoes and appear to be important effectors in innate mosquito defenses against human pathogens.

Anopheline mosquitoes are the vectors of malaria that is caused by protozoan *Plasmodium* parasites and claims the lives of 1-3 million people annually (1). Parasites enter female mosquitoes during blood feeding and develop into ookinetes that on traversing the midgut epithelium and encountering the hemolymph are attacked by the mosquito immune system (2). A few survive to transform into oocysts, which generate sporozoites capable of re-infecting humans.

The *Anopheles gambiae* leucine-rich repeat (LRR)-containing protein LRIM1 is a potent *Plasmodium berghei* antagonist that is also involved in phagocytosis of bacteria and melanization of parasites and Sephadex beads (3-5). Since the discovery of LRIM1, two other LRR proteins, APL1 (6) and LRRD7 (7), have been shown to limit *Plasmodium* infection. LRIM1 and APL1 (also called LRIM2) also mediate *Plasmodium* lysis and melanization in *Anopheles quadriannulatus* species A, contributing to the natural refractory phenotype of these mosquitoes to parasites (8). The *APL1* locus encompasses three distinct yet highly similar genes, which originate from recent duplications. Of these, *APL1C* is the sole *P. berghei* antagonist; *APL1A* and *B* do not influence infection intensities (9).

We used single and double gene knockdowns (KDs) to compare the quantitative effects of *LRIM1* and *APL1* on *P. berghei* infections in susceptible *A. gambiae* and obtained an approximately 50-fold increase in parasite infection intensities and no significant difference between double and single KDs (Fig. 1A). Quantitative real-time RT-PCR confirmed equal and efficient silencing of *LRIM1* and *APL1* (using primers amplifying all three *APL1* genes) and no detectable cross-silencing (fig. S1). The known role of *LRIM1* in melanization (3, 4) prompted us to examine whether *APL1* is also required in this immune reaction. We silenced *LRIM1* and *APL1* in *A. gambiae* L3-5 mosquitoes which melanize virtually all invading *P. berghei* ookinetes (10). Indeed, both KDs produced identical phenotypes: no melanization and an approximately 80-fold increase in live oocyst numbers (Fig. 1B-C). Thus, in both *Plasmodium* susceptible and refractory mosquitoes, the effects of

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LRIM1 and *APL1* are qualitatively and quantitatively indistinguishable, suggesting that the two genes function in a single genetic pathway, which is disrupted by silencing either gene alone.

Sequencing multiple *LRIM1* and *APL1C* cDNA clones from adult female mosquitoes verified their strong similarities in gene architecture. Each gene encompasses a short first exon separated from a long second exon by a short intron. Their deduced amino acid sequences also show striking structural similarities: signal peptides indicating secretory proteins; N-terminal regions with 10 and 12 predicted LRR repeats for *LRIM1* and *APL1C*, respectively; and C-terminal regions with two closely spaced coiled-coil domains (Fig. 2A). The LRR domains are flanked at their C-termini by a cysteine-rich region. In addition, both proteins have consensus sites for putative N-linked glycosylation. The N-terminal region of the mature *APL1C* encompasses multiple repeats of the consensus amino acid sequence PANGGL. These repeats are not found in *LRIM1* or any other *A. gambiae* protein. Preliminary data indicate that laboratory *A. gambiae* colonies have *APL1C* alleles encoding variable numbers of these repeats.

We generated antibodies against *LRIM1* and *APL1C* peptides (orange lines in Fig. 2A), and found that they specifically recognize single protein bands of the predicted sizes in mosquito hemolymph: 55kDa and 80kDa, respectively (Fig. 2B). PNGase-F treatment increased the band mobility indicating that both proteins are N-glycosylated. Since *LRIM1* and *APL1C* contain several cysteine residues, we used western blot analysis of hemolymph to examine if they exist in disulfide-bonded complexes. Importantly, under non-reducing conditions both antibodies detected major protein bands of similar molecular weights of approximately 260kDa, which resolved into the expected monomers under reducing conditions (Fig. 2C). These data indicated that *LRIM1* and *APL1C* are either in the same complex or in different complexes with fortuitously identical molecular weights.

We used *APL1C* antibody to immunoprecipitate the 260kDa *APL1C*-containing hemolymph complex, which was then analyzed by western blot (Fig. 2D). *LRIM1* was found to be part of this complex when assayed under non-reducing conditions and migrated at the monomer's size of 55kDa under reducing conditions. These data suggest that *LRIM1* and *APL1C* are partners in a disulfide-bonded complex.

Next, we tested what effect removing either component would have on the formation of the complex. Neither the complex nor the monomers were detectable in hemolymph following depletion of either protein alone (Fig. 2E), indicating that formation of the complex is required for secretion of these proteins. Taken together these data demonstrate that the *LRIM1/APL1C* complex is the only hemolymph form of the two proteins.

We used affinity purification to investigate whether the *LRIM1/APL1C* complex interacts with other secreted proteins. Since such interactions would be expected to be dynamic and transient, we used hemocyte-like *A. gambiae* Sua4.0 cells that possess immune properties, such as phagocytosis of bacteria and induction of immune genes (11, 12), to circumvent the problem of limited amounts of hemolymph extracts. Cells were co-transfected with transgenic *LRIM1* and *APL1C* carrying C-terminal 10xHis-tags or with control *GFP*, expressed via constitutive promoters, and then allowed to condition serum-free medium for 3 days. Capture using the His-tag revealed that, as in hemolymph, *LRIM1*^{HIS} and *APL1C*^{HIS} in conditioned medium are detected in the 260kDa complex (Fig. 3A). Additional complexes containing *LRIM1*^{HIS} or *APL1C*^{HIS} as well as *LRIM1*^{HIS} and *APL1C*^{HIS} monomers were also detected. This secretion pattern is not due to tagging or overexpression but an intrinsic property of cultured cells (fig. S2).

Silver staining of captured proteins revealed 3 additional, prominent protein bands (Fig. 3A). Two of these had molecular weights that matched the cleaved form of another important *P. berghei* antagonist, the complement C3-like molecule TEP1 (13). TEP1 is secreted into the hemolymph as a 165kDa precursor (TEP1-F) and then processed by an unidentified mechanism to generate an 80kDa C-terminal fragment (TEP1-C) that bears a reactive thioester motif (14, 15). Through the thioester, TEP1 opsonizes bacteria promoting their phagocytosis (5, 14). It also localizes to the surface of ookinetes during midgut invasion targeting them for lysis or melanization (13). The indistinguishable loss-of-function phenotypes of TEP1 and LRIM1 (16) on *P. berghei* infections led us to investigate whether either of these two bands corresponds to TEP1. Indeed, there was a selective enrichment of TEP1-C in the material co-captured with LRIM1^{HIS}/APL1C^{HIS} compared to the control (Fig. 3A). This indicates that the complex interacts with TEP1 and may be involved in TEP1 processing.

To address this, we probed the hemolymph of *LRIM1* and *APL1* KD mosquitoes with TEP1 antibody. A decrease was detected in the abundance of TEP1-C in KDs compared to *GFP* dsRNA-treated controls (Fig. 3B). Thus, the LRIM1/APL1C complex appears to be involved in TEP1 processing in the mosquito hemolymph. However, the lack of a parallel increase in TEP1-F may alternatively indicate that the complex is required for the stabilization of TEP1-C in the mosquito hemolymph. This might serve to prevent the reactive TEP1-C from non-specific interaction with self-surfaces and/or to direct TEP1-C to foreign surfaces during infection.

Binding of TEP1 to invading parasites is proposed to mediate their killing via a complement-like pathway (2). To investigate whether TEP1 binding is affected by the loss of the LRIM1/APL1C complex, we immunolocalized TEP1 on GFP-expressing *P. berghei* ookinetes 30 hours post infection of mosquitoes lacking LRIM1, APL1 or TEP1. As reported previously (16), three distinct classes of parasites were observed in the midguts of control mosquitoes: live (GFP positive), dead (TEP1 positive), and dying (GFP and TEP1 positive). In contrast, TEP1-positive parasites were never detected in *LRIM1* or *APL1* KD mosquitoes (Fig. 3C), despite the presence of TEP1-F in the hemolymph. Lack of TEP1 parasite staining was as complete as in TEP1 KD mosquitoes, which entirely lack TEP1 in the hemolymph. These data demonstrate that the LRIM1/APL1C complex is necessary for TEP1-mediated parasite killing during midgut invasion and indicate that TEP1 binds parasites only after it is processed.

The *APL1* locus has been implicated in mosquito resistance to the human malaria parasite, *Plasmodium falciparum* (6), and TEP1 has been shown to act against *P. falciparum* in laboratory infections (7). Mosquito defense against *Plasmodium* is likely to be influenced by vector/parasite co-evolution and adaptation, thus the observation that LRIM1 did not affect *P. falciparum* in experimental field infections (17) may suggest that parasites have evolved to evade this pathway. Proteins such as the Fibrinogen-related FBN9 (18) or other LRR proteins may provide alternative mechanisms for TEP1-mediated parasite killing.

Bioinformatic searches for proteins related to LRIM1 and APL1C using their shared structural features (signal peptide, LRRs, cysteine pattern and coiled-coils; see supporting online material) detected over 20 *LRIM*-like genes in each of the available mosquito genomes, *A. gambiae*, *Aedes aegypti*, and *Culex quinquefasciatus*, but not in any other species (Table S1). Several of these genes were previously implicated in *A. gambiae* immune responses (3, 6, 7, 9, 19). Phylogenetic analysis in conjunction with pairwise comparisons, examination of orthologous genomic neighborhoods and protein domain analysis revealed four distinct LRIM sub-families (figs. S3 and S4). Thus, LRIM1 and APL1C are members of a family of putative recognition receptors, which appears to be

unique and greatly expanded in mosquitoes. Nevertheless, structural integrity of both LRRs and coiled-coils rests with only a few key amino acids, allowing considerable sequence variation that may hinder identification of functional equivalents in other organisms.

The versatile LRR motif mediates recognition of diverse pathogen-associated molecules in host innate defense in plants and animals (20). For example, the repertoire of variable lymphocyte receptor (VLR) antibodies in jawless vertebrates is generated via combinatorial assembly of LRR modules instead of immunoglobulin segments as in jawed vertebrates (21). Similarly to LRIM1/APL1C, the VLR antibodies are secreted as disulfide-linked multimers (22).

LRIMs form a family of mosquito LRR receptors with putative roles in defense against human and animal pathogens. LRIM1 and APL1C exist as a complex that mediates immunity against malaria parasites through activation of mosquito complement. The multimeric nature of the complex indicates the potential to bind multiple targets similarly to mammalian multi-subunit receptors that robustly activate complement, i.e. immunoglobulin M, lectin and C1q. Bound LRIM1/APL1C complex may then undergo conformational changes inducing the recruitment of additional cascade components, such as a TEP1-activating protease. In-depth study of these interactions will provide insights into complement activation in mosquitoes and novel tools towards blocking disease transmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Silencing of *LRIM1* and *APL1* together has the same effect on midgut infections of GFP-expressing *P. berghei* as silencing either gene alone. Experiments were performed 7 days post infection with injection of *dsGFP* serving as the control. Horizontal lines indicate the median. **(A)** Oocyst numbers in susceptible mosquitoes. Whether injected together or separately *dsLRIM1* and *dsAPL1* treated mosquitoes show a significant ($P < 0.001$) increase of oocysts numbers compared with the control but no difference between them. **(B)** Live oocysts (green dots) and melanized ookinetes (grey squares) in refractory mosquitoes. Both the increase in live and the decrease in melanized parasites in *dsLRIM1* and *dsAPL1* injected mosquitoes are significant ($P < 0.001$) compared with the control. **(C)** Phase contrast (top row) and fluorescent (bottom row) photomicrographs of parasites in the midguts of refractory mosquitoes following injection of dsRNA. Inset in the control shows a melanized ookinete at higher magnification.

Fig. 2.

LRIM1 and APL1C circulate in the hemolymph as a disulfide-bonded multimeric complex. **(A)** Schematic representation and predicted molecular weights of the LRIM1 and APL1C proteins. Features indicated: asterisk, predicted N-linked glycosylation site; red line, cysteine residue; black bracket, conserved cysteine-rich region. Colored boxes denote: black, signal peptide; white, PANGGL repeat; blue, LRR repeats; green, coiled-coil domain. Orange horizontal lines show the location of peptides used to generate antibodies. **(B)** LRIM1 and APL1C western blots of hemolymph before (-) and after (+) treatment with PNGase-F. Arrows indicate the position of the endogenous proteins and open arrowheads indicate deglycosylated forms. **(C)** LRIM1 and APL1C western blots of hemolymph under non-reducing (NR) and reducing (R) conditions **(D)** LRIM1 co-immunoprecipitates with the APL1C complex. Hemolymph (H) was used for immunoprecipitation with an antibody against APL1C (+) or mock treated (-). Left and middle panels show western blot analysis of samples run under NR conditions and probed with APL1C and LRIM1 antibodies, respectively. Right panel shows western blot under R conditions, probed with LRIM1 antibody. Immunoprecipitated complex detected with APL1C antibody (white arrowhead), LRIM1 antibody (white arrowhead) and LRIM1 monomer (arrow) are indicated. **(E)** Silencing by injection of either *dsLRIM1* or *dsAPL1* results in specific loss of the LRIM1/APL1C complex from the hemolymph compared to control (*dsGFP*). Probing with SRPN3 served as a loading control. The LRIM1 and APL1C blots were cropped at the position of the complex since no other bands were detected.

Fig. 3.

The LRIM1/APL1C complex interacts with TEP1-C. (A) *A. gambiae* cultured cells were co-transfected with transgenes for *LRIM1*^{HIS} and *APL1C*^{HIS} (+) or *GFP* (–) as a control. His-tagged proteins were affinity purified from conditioned medium (CM). Starting CM and captured (Bound) samples were analyzed by western blot with anti-His (left panel) or TEP1 antibodies (right panel), or silver stained (middle panel). Indicated are: black arrows, LRIM1^{HIS} and APL1C^{HIS} monomers; black arrowhead, LRIM1^{HIS}/APL1C^{HIS} complex (black); white arrowheads alternative LRIM1^{HIS} and APL1C^{HIS} complexes; red arrow, putative TEP1-C bands; black double, TEP1-F and TEP1-C. (B) TEP1 western blot of hemolymph extracted from *dsGFP*, *dsLRIM1*, *dsAPL1* and *dsTEP1* injected mosquitoes. Probing with SRPN3 served as a loading control. (C) Confocal analysis of TEP1 immunolocalization (red) in midgut epithelia from susceptible mosquitoes 30 hours post-infection with GFP-expressing *P. berghei* (green). A mixture of live (GFP⁺, open triangles), dead (TEP1⁺, white arrows) and dying (GFP⁺ and TEP1⁺, box and inset) parasites are detected in control injected (*dsGFP*) mosquitoes. In contrast, no dead or dying parasites were observed in mosquitoes injected with *dsLRIM1*, *dsAPL1*, or *dsTEP1* in three independent experiments. Scale bars in *dsGFP* panel and inset are 10 μ m.