EFFECT OF PERITROPHIC MATRIX C- TYPE LECTIN (AdPMCTL) ON BLOOD-MEAL SIZE IN *ANOPHELES DIRUS*

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Abstract. The peritrophic matrix (PM) is penetrated by *Plasmodium* ookinete to permit transition to oocyst in the mosquito midgut, the manner by which the ookinete interacts with glycoproteins on the PM remains poorly understood. We partially characterized peritrophic matrix C-type lectin (PMCTL) from *An. gambiae* (CTL10) and *An. dirus* (AdPMCTL). AdPMCTL protein was produced specifically in blood-fed mosquitoes. The 320 amino acid AdPMCTL exhibits 72% identity with a putative secreted *An. gambiae* ortholog (AGAP009316, CTL10). *AdPMCTL* was cloned and its expression profile determined in sugar- and blood-fed midguts. RNAi was used to determine the effect of AdPMCTL on blood meal size and on mosquito survival. *AdPMCTL* mRNA was present in midguts of sugar-fed mosquitoes and exhibited up-regulation following a blood meal, and *AdPMCTL* silencing significantly influenced the blood-meal size of engorged mosquitoes, suggesting a role for AdPMCTL as a stabilizing linker molecule, which limits PM distension after blood feeding.

Keywords: *Anopheles dirus*, *Anopheles gambiae*, C-type lectin, gene silencing, peritrophic matrix

INTRODUCTION

The incidence of malaria continues unabated in Southeast Asia (WHO SEARO, 2010). *Anopheles dirus* is one of the most efficient vectors of malaria (Baimai, 1998; Manguin *et al*, 2008). Members of *An. dirus* complex are widespread throughout Southeast Asia, especially in Indochina (Manguin *et al*, 2008). In order for transmission to be successful, the *Plasmodium* parasite must complete its complex life cycle in the *Anopheles* mosquito. *Plasmodium* gametes present in a blood meal mate and develop into motile ookinetes (~16-24 hours post-blood feeding, depending on the *Plasmodium* species) in the mosquito midgut. In order to be established in the mosquito, *Plasmodium* ookinetes must interact with and penetrate the peritrophic matrix (PM) before contacting midgut epithelium (Devenport and Jacobs-Lorena,

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2005). As PM maturation coincides with ookinete development and subsequent penetration of the PM by *Plasmodium* ookinetes (Han *et al*, 2000), PM is assumed to be involved in modulating parasite infection of the mosquito (Shahabuddin *et al*, 1993; Abraham and Jacobs-Lorena, 2004).

The PM is an extracellular matrix composed of chitin fibrils, chitin-binding proteins (peritrophins) and other secreted glycoproteins, which line the area between ingested blood bolus and midgut epithelium cells in the gut of adult hematophagous arthropods (Lehane, 1997). Midgut epithelial cells secrete Type I PM, which is triggered by the mechanical distension resulting from ingestion of a bloodmeal (Shao *et al*, 2001). The PM completely surrounds the food bolus, thereby separating it from the epithelium and acts as a molecular sieve mediating the diffusion of molecules passing into and from the bloodmeal during digestion (Villalon *et al*, 2003). Although ookinetes secrete a chitinase to allow penetration through the PM barrier (Huber *et al*, 1991), it remains unclear as to the role the PM plays in triggering chitinase secretion or the manner in which it acts as a potential barricade to the ookinete. The abundance of glycans on PM-associated glycoproteins helps create and thicken the molecular sieve by occupying the interstitial spaces between chitin fibrils (Rostand and Esko, 1997; Hegedus *et al*, 2009). The structural organization of adult *An. gambiae* PM and the complete *An. gambiae* PM proteome have been described, and it has been proposed that the secreted glycoproteins (non-chitin-binding proteins) may be involved in both protein-protein and protein-glycan interactions required to form the 3-dimensional PM structure (Dinglasan *et al*, 2009; Hegedus *et al*, 2009).

C-type lectins (CTLs) are members of a large family of extracellular Ca^{2+}-dependent binding proteins containing conserved C-type carbohydrate recognition domains (CRDs) (Weis *et al*, 1998; Vasta *et al*, 2004). Interactions between glycans and CTLs occur on cell surface of the extracellular matrix or on soluble secreted glycoproteins and may mediate biological processes, such as cell adhesion, cellular interaction, glycoprotein turnover, and pathogen recognition, leading to mosquito innate immune responses (Vasta *et al*, 2004). CTLs circulating in the mosquito hemolymph is not only essential in protecting oocysts from the mosquito's immune response (Osta *et al*, 2004) but are also involved in defending the mosquito against bacterial infection of the hemocoel (Schnitger *et al*, 2009). However, the PM-associated CTLs secreted by the midgut epithelium remain uncharacterized. Moreover, the functions of other members of the CTL class of lectins have not been clearly elucidated.

In the present study, secreted *An. dirus* peritrophic matrix C-type lectin (AdPMCTL), an ortholog of *An. gambiae* CTL10 (AGAP009316), was examined to ascertain its association with the PM.

MATERIALS AND METHODS

Mosquito

The laboratory colony of *An. dirus* (Peyton and Harrison) used in this study was maintained under previously described methods and conditions at the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand (Sattabongkot *et al*, 2003).

Quantitative real-time PCR (qRT-PCR) analysis of *AdPMCTL* gene expression

The mRNA expression profile of *AdPMCTL* in the midgut of *An. dirus* in response to blood feeding was measured

using qRT-PCR relative to mRNA level of *An. dirus* ribosomal protein gene *S7* (GenBank accession no. AY369135). Twenty midguts were dissected and isolated at 30 minutes, and at 2, 5, 8, 12, 24, 48, and 72 hours post-blood feeding (PBF). Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, and contaminating genomic DNA was removed by DNase I treatment (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed using SuperScript™ III RT as described by the manufacturer (Invitrogen, Carlsbad, CA). qRT-PCR was performed using Platinum® SYBR® GREEN qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Relative quantification of *AdPMCTL* mRNA was calculated by using the comparative C_T method as described in the ABI Prism 7700 Sequence Detection System User Bulletin #2. For each sample, qRT-PCR was conducted in triplicate for both *AdS7* and *AdPMCTL* determinations. The amplification program was as follows: 95ºC for 2 minutes; 40 cycles of 95ºC for 15 seconds, and 60ºC for 30 seconds. At the end of the last cycle, a melting curve was generated by starting fluorescence acquisition at 65ºC and taking measurements every 0.2ºC until 90ºC was reached. Primers used for amplification were AdS7-F4, AdS7-R170, AdPMCTL-F212, and AdPMCTL-R369 (Table 1).

Identification of *An. dirus PMCTL* (*AdPMCTL*)

Due to the absence of readily available sequence information for *An. dirus*, we used the *An. gambiae* amino acid sequences for CTL10 to BLAST the GenBank genome database in order to clone the *AdPMCTL* sequence. The CTL conserved region was analyzed using *in silico* homology search and alignment strategies on the amino acid sequences of *An. gambiae* (GenBank accession no. AGAP009316), *Aedes aegypti* (GenBank accession no. AAEL009338), and *Culex quinquefasciatus* (GenBank accession no. CPIJ007062). Degenerate primers were designed based on the conserved region across the mosquito CTLs. Primers corresponding to a conserved region of the CTL domain, AdPMCTL-F21, AdPMCTL-F373, AdPMCTL-R705, and AdPMCTL-R889 were used to amplify a partial *An. dirus* CTL gene. PCR amplicons were separated by 1% agarose gel-electrophoresis and purified DNA fragments were subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) for sequence analysis.

Isolation of full-length cDNA of *AdPMCTL* by RACE-PCR

Based on the fragment sequences obtained by PCR as described above, sequence-specific primers were designed for AdPMCTL-F41, AdPMCTL-F412, AdPMCTL-R289, and AdPMCTL-R540 (Table 1) to perform RACE (rapid amplification of cDNA end). RACE-PCR of *An. dirus* CTL was conducted for both 5′ and 3′ directions using a BD SMART RACE cDNA Amplification Kit (BD Clontech, Mountain View, CA) according to the manufacturer's protocols. A pair of primers (AdPMCTL-F1 and AdPMCTL-R930) was designed to amplify a single fragment containing the complete open reading frame (ORF) of *AdPMCTL*. Sequence analysis of the full-length cDNA was performed to obtain the signal peptide sequence and complete CTL domain. The nucleotide sequences of *AdPMCTL* were deposited in GenBank, National Center for Biotechnology Information (NCBI), accession number JN797605.

Table 1
Primer sequences used in the study.

Primer	Sequence
AdS7-F4	5′-GTG TTC GGT TCC AAG GTG ATC AAA G-3′
AdS7-R170	5′- GCC TTC TTG TTGTTG AAC TCG ACC T-3′
AdPMCTL-F212	5′- GCG AAC AGA ACA GGA CCA ACA AGG-3′
AdPMCTL-R369	5′- GTA GTA ATC AAA CCG CGT GTC GTT CA-3′
AdPMCTL-F21	5′-TGY CAR CGR TCG AAT CGT A-3′
AdPMCTL-F373	5′- TGT CCS GAG ATC GRA ACC T-3′
AdPMCTL-R705	5′- SGT CTC RTT YAA YCC CAC RTA-3′
AdPMCTL-R889	5′- GWW GTG TGC AGY TCA CAG A-3′
AdPMCTL-F41	5′-CAT CGT CCA ACT TCA CCT GTG TAC CTT CG-3′
AdPMCTL-F412	5′-ACG CGG TTT GAT TAC TAC ACG CTT TAT GC-3′
AdPMCTL-R289	5′-GAT TCT TCT CCG ACT CGT TCA GAC CAA CGT-3′
AdPMCTL-R540	5′-GGA AGG AGC TAT TCT GTT GTT GGG ACG CTT GAC AAA GCA -3′
AdPMCTL-F1	5′-ATG CGT GTG TCG GTT GTA CTA GCA GCA CT-3′
AdPMCTL-R930	5′-TTA TCA GGC TGT GTG CCT CCG G-3′
AdPMCTL-F	5′-ACG CGG TTT GAT TAC TAC ACG CTT-3′
AdPMCTL-R	5′-AGC TAT TCT GTT GTT GGG ACG CTT-3′
dsAdPMCTL-F-T7	5′-**TAA TAC GAC TCA CTA TAG GG**A CGC GGT TTG ATT ACT ACA CGC TT-3′
dsAdPMCTL-R-T7	5′-**TAA TAC GAC TCA CTA TAG GG**A GCT ATT CTG TTG TTG GGA CGC TT-3′
dsLacZF	5′-AGC GCC CAA TAC GCA AAC CGC C-3′
dsT7F- LacZ	5′-**TAA TAC GAC TCA CTA TAG GGA GC**G CCC AAT ACG CAA ACC GCC-3′
dsLacZR	5′-CAT TCG CCA TTC AGG CTG CGC A-3′
dsT7R-LacZ	5′-**TAA TAC GAC TCA CTA TAG GG**C ATT CGC CAT TCA GGC TGC GCA-3′

Silencing of *AdPMCTL*

We produced double-stranded RNA (dsRNA) for both *AdPMCTL* and β-galactosidase (*LacZ*) as a control dsRNA (Table 1) using the MEGAscript RNAi kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. To knock down *AdPMCTL* expression, 400 ng of *AdPMCTL*-dsRNA in sterile phosphate-buffered saline (PBS) was injected intrathoracically into cold-anesthetized 2 day-old individual female *An. dirus*, following previously described methods (Blandin *et al*, 2002). As a control, female *An. dirus* of the same age were injected with equivalent concentrations of *LacZ*-dsRNA. In order to determine the silencing efficiency of *AdPMCTL*-dsRNA, expression levels of *AdPMCTL* in midguts of mosquitoes were examined post-blood feeding. Both *AdPMCTL*- and *LacZ*-dsRNA-injected mosquitoes were maintained for 48, 72, and 96 hours before being fed a normal blood meal through a water-jacketed membrane feeder. Total RNA and protein were isolated from pools of ten mosquitoes per time point and analyzed by qRT-PCR and western blotting, respectively.

Determination of effect of AdPMCTL on *An. dirus* longevity

Three groups of one hundred 2 day-old *An. dirus* females were separately cold anesthetized and intrathoracically injected with 400 ng of *AdPMCTL*-dsRNA

per mosquito. *LacZ*-dsRNA-injected mosquitoes were used as injection dsRNA control, and one group of mosquitoes was not injected. Mosquitoes were maintained for 96 hours to ensure that knockdown had occurred before being fed a normal blood meal as described above. Subsequently, mosquitoes were provided a diet of 10% sucrose and survival was monitored daily for 30 days.

Determination of effect of AdPMCTL on blood meal size (on PM structure)

At day 4 post-dsRNA injection, *An. dirus* in *LacZ*-dsRNA control and *AdPMCTL* test groups were provided with normal blood for 30 minutes as described above and kept at 25-27ºC until dissection at 12, 24, 36, and 48 hours post-blood feeding (PBF) to measure blood-meal size. At each time point, 15 mosquitoes from each group were randomly selected, midguts dissected and using the hemiglobincyanide (HiCN) method (Briegel *et al*, 1979), blood-meal size was estimated indirectly by measuring hemoglobin concentration ingested by the mosquito. Midgut containing blood from a single mosquito was added to 1 ml Drabkin's' reagent and absorbance at 540 nm measured. Midguts taken from unfed females were also analyzed and the average optical density was subtracted from that of the blood-fed dissected midguts.

Statistical analysis

The cumulative survival of dsRNA-injected mosquitoes was analyzed by Kaplan-Meier survival analysis. Statistical significance for comparing survival distribution across all time points was calculated by log-rank test. Survival is considered significantly different at $p < 0.05$. The effect of silencing of *AdPMCTL* on blood-meal size, based on hemoglobin content of the dissected midgut, was determined by Mann-Whitney *U* test, to compare differences in blood-meal sizes between *AdPMCTL*- and *LacZ*-knockdown groups. Blood-meal size is considered significantly different at $p < 0.05$.

RESULTS

Identification and sequence analysis of AdPMCTL full-length cDNA

There are 23 putative CTL molecules in *An. gambiae,* as reported by VectorBase, release 6, 2010 (www.vectorbase.org). All 23 contain the InterPro classification IPR001304, which refers to proteins containing carbohydrate-binding activity, or possess a CTL domain. CTL10 (930 base pairs) is found on chromosome 3R (31, 241, 289-31, 242, 351) and encodes a 310 amino acid polypeptide. Amino acid sequence analysis by SMART indicated that CTL10 contains a single predicted CTL domain at its C-terminus at position 151 to 292; the E-value of similarity with the IPR001304 CTL domain is $5.3x10^{-10}$.

The full length *AdPMCTL* cDNA is composed of 960 nucleotides and an ORF encoding a putative protein of 320 amino acids (Fig 1). Similar to its homolog in *An. gambiae* CTL10, AdPMCTL contains a signal peptide at the N-terminus, positions 24-25 from the putative initiating methionine and a C-terminal C-type lectin domain at amino acid positions 149 to 286 with E-value equal to $1.57x10^{-8}$. The calculated molecular mass of AdPMCTL, excluding the signal peptide, is 33,605 Da. *AdPMCTL* contains no predicted membrane or GPI anchor sequence. A comparison of AdPMCTL with CTL10 revealed a high degree of CTL-domain identity (~80% sequence similarity) between these two orthologs (Fig 2), and to a lesser degree with CTLs from *Aedes aegypti*, *Culex quinquefasciatus*, *Drosophila melanogaster*,

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ATGCGTGTGTCGGTTGTACTAGCAGCACTTGCTTGCACGATATTCCCCCCAGAGCTAGTG
 M  R  V  S  V  V  L  A  A  L  A  C  T  I  F  P  P  E  L  V

AGCGCACTTGTGAAGCGAACGCCCGCAAGATCCATCGATTCCATCCGTTGCGGCACTGTC
 S  A  L  V  K  R  T  P  A  R  S  I  D  S  I  R  C  G  T  V

AGCGTTCGAATCACCGTGGTTGGACTGACGAATGCCAAAACGGTTCGGGAGTGTGCTGAC
 S  V  R  I  T  V  V  G  L  T  N  A  K  T  V  R  E  C  A  D

TTTGCGCGGGACAAGCAGGCCCTGGCGTTCAACTATGCGCCGGTGGGACGCAACAACACC
 F  A  R  D  K  Q  A  L  A  F  N  Y  A  P  V  G  R  N  N  T

AATTGGTACGATGTGGTGAAGGAGCGCGAACAGAACAGGACCAACAAGGCGCCATGGAAA
 N  W  Y  D  V  V  K  E  R  E  Q  N  R  T  N  K  A  P  W  K

CCGCAACCGCCTGCTGTGAGTAACGCGTTCGGGTTTGAGGATTTCTACAACTGCCACGTG
 P  Q  P  P  A  V  S  N  A  F  G  F  E  D  F  Y  N  C  H  V

CTCGACTGTCCGGAGTATCGTAACCTTTCGACGATGGTGAACGACACGCGGTTTGATTAC
 L  D  C  P  E  Y  R  N  L  S  T  M  V  N  D  T  R  F  D  Y

TACACGCTTTATGCAAGAAATTTGCCATCGTCCAACTTCACCTGTGTACCTTCGATTGGG
 Y  T  L  Y  A  R  N  L  P  S  S  N  F  T  C  V  P  S  I  G

ATGTTTCTTTTCGAGGACACAAAGCTAAACTATTCGAATGCCTACAATGCTTGTGTCGCT
 M  F  L  F  E  D  T  K  L  N  Y  S  N  A  Y  N  A  C  V  A

GCTGGTGGTAGCCTCGCACACATTGCCAGTGATGCGCGTACGTTTCATCTGTCCAAGTAC
 A  G  G  S  L  A  H  I  A  S  D  A  R  T  F  H  L  S  K  Y

ATCGCGGAGCTACCGCCCGCGAACAACTCAGCATCAAACGCTACCACAGTTGAGCCCCTG
 I  A  E  L  P  P  A  N  N  S  A  S  N  A  T  T  V  E  P  L

TACTACGTTGGTCTGAACGAGTCGGAGAAGAATCGTTTCTTCACATCCGCGAACGAACGA
 Y  Y  V  G  L  N  E  S  E  K  N  R  F  F  T  S  A  N  E  R

TTGGATTGCTTTACATTCCGGGCGTGGGCACCAAAGCATCCGGACCGTAATCGACATCCT
 L  D  C  F  T  F  R  A  W  A  P  K  H  P  D  R  N  R  H  P

CCCAGCTGTACCGCTCTGACTGACGAAGGGTCCTGGAAGGTTTTCGACTGTAACCGTACC
 P  S  C  T  A  L  T  D  E  G  S  W  K  V  F  D  C  N  R  T

TTACCGTACATCTGTGAGCTGCACACATCTGGTCCGGCCTTGTACGAACCGAAACTGAAA
 L  P  Y  I  C  E  L  H  T  S  G  P  A  L  Y  E  P  K  L  K

CGGAGATGCTTTGTCAAGCGTCCCAACAACAGAATAGCTCCTTCCCGGAGGCACACAGCC
 R  R  C  F  V  K  R  P  N  N  R  I  A  P  S  R  R  H  T  A

TGATAA
 * *
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Fig 1–Nucleotide sequence of cDNA and predicted encoded protein sequence of *AdPMCTL*. Amino acid residues in the signal peptide are underlined; the C-type lectin domain is thick underlined; and putative N-glycosylation sites (Asn-Xaa-Thr/Ser), analyzed by NetNGlyc 1.0 server, are enclosed in rectangles.

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CTL10      MRLTAVLAALVLSHHPPEGATDRHCEANARKIVHRFYPLRHCQRSNR-TVIGLVNVQSVR
AdPMCTL    MRVSVVLAALACTIFPPELVSALVKRTPARSIDS----IRCGTVSVRITVVGLTNAKTVR
           **::.*****. : .*** .:    .: **.*      :*    * * **:**.*.::**

CTL10      ECADFARDKQGLAFNFAPVKRNSTNWYDVVREREQNQSTVSSWKPASPRVA-SFGFEDFY
AdPMCTL    ECADFARDKQALAFNYAPVGRNNTNWYDVVKEREQNRTNKAPWKPQPPAVSNAFGFEDFY
           **********.****:*** **.*******:*****::. :.*** .* *: :*******

CTL10      NCHVLDCPEYRNLSTIVNDTRFDYYTLYARLLPAGNATCIPSIGMFLFEDTKLNYSNAYN
AdPMCTL    NCHVLDCPEYRNLSTMVNDTRFDYYTLYARNLPSSNFTCVPSIGMFLFEDTKLNYSNAYN
           ***************:************** **:.* **:********************

CTL10      ACVSAGGSLAHIASDARTFQLSKYIASLPETNYTTNNSTDTSSAVEPLYYVGLNETVKNR
AdPMCTL    ACVAAGGSLAHIASDARTFHLSKYIAELPP----ANNSASNATTVEPLYYVGLNESEKNR
           ***:***************:******.**     :***:..:::***********: ***

CTL10      FFTSADERLDCFRFHAWAPGHPARNRTSPSCAALTDEGSWKVFNCNRTLPYICELHTSGP
AdPMCTL    FFTSANERLDCFTFRAWAPKHPDRNRHPPSCTALTDEGSWKVFDCNRTLPYICELHTSGP
           *****:****** *:**** ** *** .***:***********:****************

CTL10      ALYAPKLKRKCF----------------
AdPMCTL    ALYEPKLKRRCFVKRPNNRIAPSRRHTA
           *** *****:**
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Fig 2–Alignment between CTL10 and AdPMCTL protein sequences. The signal peptide sequences are thick underlined. The 80% similarity of the single CTL domain, analyzed by Clustal W, for *An. gambiae* and *An. dirus*, is underlined. Asterisks indicate conserved amino acids.

and the body louse, *Pediculus humanus corporis*, at 53, 38, and 30%, respectively (data not shown). The predicted potential *N*-linked glycosylation sites using NetNGlyc 1.0 server showed that AdPMCTL contains 8 predicted N-linked glycosylation sites (Asn^{78}, Asn^{92}, Asn^{128}, Asn^{134}, Asn^{152}, Asn^{170}, Asn^{213}, and Asn^{278}), but no putative *O*-linked glycosylation site was found using NetOGlyc 3.1 server analysis.

Transcript expression profile of *AdPMCTL* in *An. dirus* mosquitoes post-blood feeding

The abundance of *AdPMCTL* transcripts in mosquito midgut was monitored following blood feeding (Fig 3A, 3B). Semi-quantitative and qRT-PCR analyses demonstrated that *AdPMCTL* mRNA levels were up-regulated from the first 30 minutes of blood uptake through to 8 hours PBF followed by a decrease in expression until 72 hours PBF. *AdPMCTL* transcript was about 50-fold higher at 5-8 hours PBF compared with sugar-fed mosquitoes and then declined at 12 hours PBF and remained low till 72 hours. *S7* mRNA levels remained constant throughout the entire experimental period.

Effect of AdPMCTL on longevity of *An. dirus* mosquitoes

AdPMCTL has been hypothesized as a PM-associated protein and the PM is directly involved in blood bolus containment and digestion, we posed the notion that an absence of this protein may have a marked effect on nutrient uptake, and consequently, mosquito survival. We measured daily survival of *AdPMCTL*dsRNA-injected mosquitoes, *LacZ*dsRNA-injected control mosquitoes, and non-injected mosquitoes, for 30 days. Cumulative survival rate up to day 30 for *AdPMCTL*dsRNA-injected *An. dirus*, *LacZ*dsRNA-

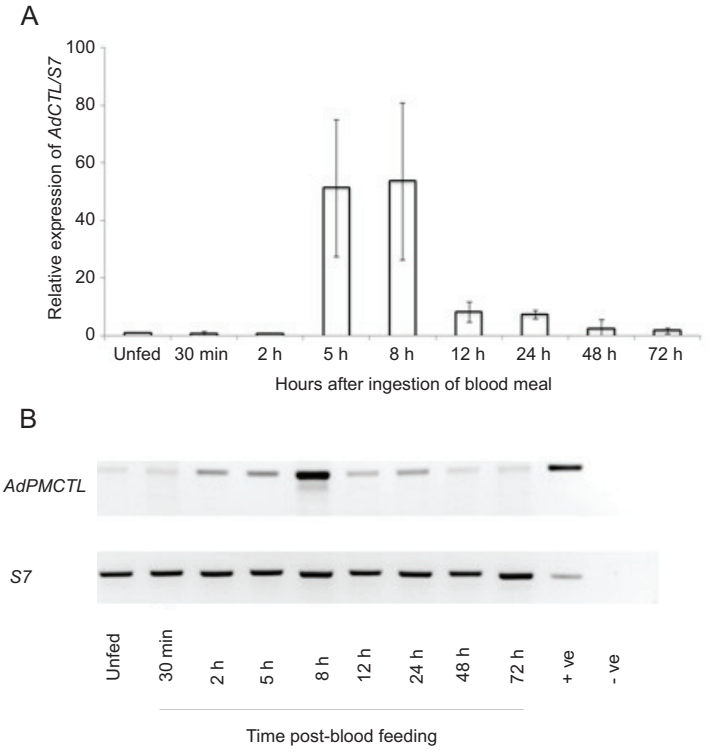

Fig 3–Expression profile of *AdPMCTL* in *An. dirus.* (A) Quantitative real-time PCR analysis of the expression profile of *AdPMCTL* in blood-fed compared with sugar-fed (unfed) mosquitoes at 30 minutes, and 2, 5, 8, 12, 24, 48, and 72 hours post-blood feeding (PBF). Relative mRNA expression of *AdPMCTL* is normalized with the *S7* expression level. Means are calculated from 3 independent experiments. Error bars represent mean ± SEM. (B) Semi-quantitative RTPCR expression analysis of *AdPMCTL* (upper panel) and *S7* (lower panel) in sugar-fed (unfed) and blood-fed adult female mosquito midguts at 30 minutes, and 2, 5, 8, 12, 24, 48, and 72 hours PBF. Positive controls were amplicons generated from a plasmid clone of *AdPMCTL* and *S7*. Negative controls are labeled as -ve.

injected control group, and non-injected *dsRNA,* was 90, 96, and 93%, respectively (Fig 4). Although survival was affected for all 3 groups, log-rank testing shows a statistically significant difference ($p < 0.05$) in survival between *LacZ*dsRNA-injected and *AdPMCTL*dsRNA-injected mosquito groups.

Effect of RNAi gene silencing of *AdPMCTL* on blood-meal size/volume

We sought to determine if RNAi silencing of *AdPMCTL* would affect blood-meal size, by measuring the amount of blood ingested by individual females injected with ds*AdPMCTL* in comparison with the amount of blood ingested by ds*LacZ* controls at 12 hours intervals. There is a statistically significant difference in blood-meal size of ds*AdPMCTL*-injected mosquitoes compared with controls ($p < 0.0001$) (Table 2). At 12 hours PBF and thereafter, when the PM gradually increases in thickness and matures, *AdPMCTL*ds RNA-injected mosquitoes had smaller blood-meals than *LacZ*dsRNA-injected control group.

DISCUSSION

Proteomic data from the adult midgut PM of *An. gambiae* identified CTL10 (AGAP009316) as a putative PM-associated protein that is secreted into the lumen by the midgut epithelial cells (Dinglasan *et al*, 2009). We identified, cloned and expressed a C-type lectin protein and putative ortholog of the PM associated CTL10 from *An. dirus* (AdPMCTL), a malaria vector present throughout Southeast Asia (Baimai, 1998; Manguin *et al*, 2008). AdPMCTL sequence contains 6 conserved cysteine residues involved in the formation of

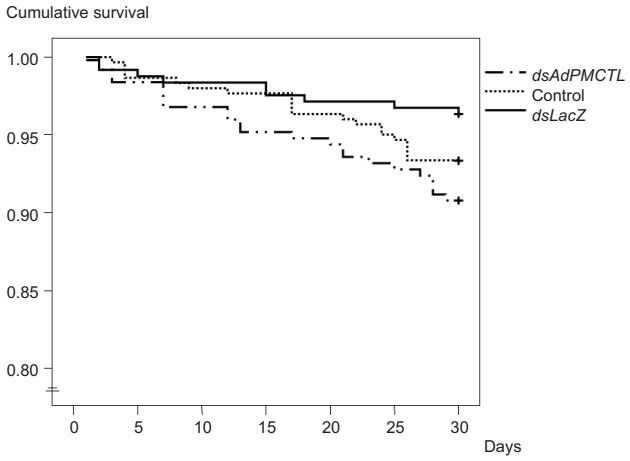

Fig 4–RNA silencing of *An. dirus AdPMCTL* on *An. dirus* daily survival. Kaplan-Meier plot represents the survival of *Lac*ZdsRNA- and *AdPMCTL*dsRNA-treated mosquitoes over 30 days compared with non-treated mosquitoes. Statistical significance of the survival rate among the 3 groups was calculated by log-rank test. Statistical analysis of percent survival of *Lac*ZdsRNA and *AdPMCTL*dsRNA-injected mosquito groups indicated that disruption of *AdPMCTL* expression significantly influences *An. dirus* longevity ($p = 0.0125$). Differences in survival curves are considered significant when $p < 0.05$.

CRD intermolecular-disulfide bridges and 3 additional cysteine residues at the N-terminus acting as the glycan-binding motif typical of CTL domain protein family (Weis *et al*, 1998). Aromatic amino acid residues, found within the CRD region, are conserved across all CTLs, and are probably critical for hydrophobic interactions with glycans (Shen and Jacobs-Lorena, 1998; Wang and Granados, 2001). Taken together with the expression profile data, it is likely that *AdPMCTL* mRNAs are stored in sugar-fed midguts and their translation is induced by blood feeding, as expected for a PM-associated glycoprotein. In general, adult hematophagous insects form a type I PM in response to abdominal distension resulting from the uptake of a blood meal (Dana *et al*, 2005; Devenport and Jacobs-Lorena, 2005). Transcription and translation profiles of AdPMCTL appear to coincide with PM AeIMUC1 protein, *Ae. aegypti* intestinal mucin 1, which is an integral mucin-like PM protein induced by metal feeding in both larvae and adults, and by blood meal in adults (Devenport *et al*, 2006).

As with AeIMUC1 (Devenport *et al*, 2006), AdPMCTL is localized at the interface between the blood meal and the midgut epithelium (data not show), presumably at the PM at 28 hours PBF. The exact roles of AdPMCTL and AeIMUC1 remain unknown. However, the small size of AdPMCTL and its predicted CTL domain suggest that it may be involved in binding to glycan moieties on peritrophins, which are bound to chitin fibrils and/or *N*-acetylglucosamine present in other PM-associate glycoproteins. In doing so, it can modulate the structure and porosity of the PM (Wang and Granados, 2001; Dinglasan *et al*, 2009; Hegedus *et al*, 2009). However, we suggest that AdPMCTL may influence PM structure as a molecular linker, rather than as a recognition molecule, with the glycan-binding domain and the rich N-glycosylated region connecting PM chitin fibrils into a 3-dimensional network, thereby promoting expansion of the PM layer. The absence of AdPMCTL from the PM probably influences the 3-dimensional structure of the PM, indirectly affecting thickness, porosity and elasticity (Dinglasan *et al*, 2009). Moreover, it was previously shown

Table 2
Effect of AdPMCTL-silencing on midgut blood-meal size.

Time PBF (hours)	Mean blood-meal size (µl) ± SEM		*p*-value[a]
	*LacZ*dsRNA	*AdPMCTL*dsRNA	
12	3.43 ± 0.10	2.55 ± 0.07	$3.07E^{-06}$
24	2.62 ± 0.09	1.46 ± 0.14	$6.70E^{-06}$
36	2.77 ± 0.09	1.62 ± 0.14	$1.00E^{-05}$
48	2.42 ± 0.15	1.43 ± 0.08	$1.46E^{-05}$

[a]The blood-meal size for the *AdPMCTL* gene and *LacZ* knockdown was analyzed using the Mann-Whitney *U* test. Level of significance, $p < 0.0001$. Means are calculated from three independent biological experiments. PBF, post-blood feeding

that the PM limits the rate of digestion (Villalon *et al*, 2003) and that the disruption of the PM results in an increased rate of blood digestion. Additionally, we hypothesize that, without AdPMCTL, PM porosity remains unregulated, allowing rapid passage of hydrolytic enzymes from the midgut epithelium into the blood meal, resulting in rapid digestion and ingestion of a reduced blood volume. In the same light, digestion by-products, which can be harmful to mosquito midgut epithelia, can pass through the PM, which in turn, would result in a feedback mechanism that prevents the mosquito from imbibing more blood. However, the reduction in blood meal size appears not to have a commensurate negative effect on mosquito survival. The survival rate for laboratory mosquito colony in a standard transmission blocking experiment is normally reduced by 10%. This suggested that the reduction in lifespan observed for *AdPMCTL*-silenced *An. dirus* is normal. We routinely have observed this reduction in our laboratory colony and, importantly, a reduction in lifespan for *AdPMCTL*-silenced *An. dirus* was comparable to the *LacZ*-silenced control, and/or non-injected *An. dirus*.

Although the exact function of AdPMCTL in *An. dirus* remains unclear, our findings nonetheless have elucidated the location of AdPMCTL on the PM as well as confirming its regulated expression after blood ingestion. We observed that PM integrity, determined by blood-meal size, decreased when *AdPMCTL* expression was silenced. Additional structure-function studies of AdPMCTL in the formation and elastic framework of the PM should provide critical insights into mosquito biology.

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