Blocking of *Plasmodium* transmission by cooperative action of Cecropin A and Defensin A in transgenic *Aedes aegypti* mosquitoes

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To overcome burden of mosquito-borne diseases, multiple control strategies are needed. Population replacement with genetically modified mosquitoes carrying antipathogen effector genes is one of the possible approaches for controlling disease transmission. However, transgenic mosquitoes with antipathogen phenotypes based on overexpression of a single type effector molecule are not efficient in interrupting pathogen transmission. Here, we show that co-overexpression of two antimicrobial peptides (AMP), Cecropin A, and Defensin A, in transgenic *Aedes aegypti* mosquitoes results in the cooperative antibacterial and anti*Plasmodium* action of these AMPs. The transgenic hybrid mosquitoes that overexpressed both Cecropin A and Defensin A under the control of the *vitellogenin* promoter exhibited an elevated resistance to *Pseudomonas aeruginosa* infection, indicating that these AMPs acted cooperatively against this pathogenic bacterium. In these mosquitoes infected with *P. gallinaceum*, the number of oocysts was dramatically reduced in midguts, and no sporozoites were found in their salivary glands when the mosquitoes were fed twice to reactivate transgenic AMP production. Infection experiments using the transgenic hybrid mosquitoes, followed by sequential feeding on naive chicken, and then naive wild-type mosquitoes showed that the *Plasmodium* transmission was completely blocked. This study suggests an approach in generating transgenic mosquitoes with anti*Plasmodium* refractory phenotype, which is coexpression of two or more effector molecules with cooperative action on the parasite.

vector-borne disease | malaria | immunity | antimicrobial peptide | vitellogenin

Mosquito-borne diseases are among the major concerns of public health. Malaria is a particularly threatening disease that is responsible for over one million deaths per year. Dengue fever affects hundreds of millions of people. Other viral and filarial diseases transmitted by mosquitoes are prevalent in many areas of the world (1, 2). Therefore, there is an urgent need to explore every avenue for developing unique control strategies against mosquito-borne diseases.

Population replacement with genetically modified mosquitoes carrying an antipathogen effector gene has been suggested as one of strategies for disrupting mosquito-borne disease transmission (3, 4). Development of genetic transformation has been accomplished for *Aedes aegypti*, the vector of Dengue fever, *Anopheles stephensi*, the vector of Asian malaria, and recently for the vector of most deadly form of malaria in Africa, *Anopheles gambiae* (5–8). Genetic modification of mosquitoes by means of transposable elements provides an outstanding tool for experimental testing of concepts of vector biology, including pathogen-vector interactions. Utilization of transgenesis to generate mosquitoes with altered immunity has been instrumental in deciphering major immune pathways and their role in antipathogen defense (7, 9–11). Transgenic mosquitoes with antipathogen refractory phenotypes have been developed by utilizing blood meal-activated, tissue-specific promoters, and effector molecules or

RNAi-constructs (11–15). However, complete interruption of pathogen transmission in these transgenic models has not been achieved. In the case of malaria parasite, only a few sporozoites present in mosquito salivary glands were sufficient to infect a vertebrate host (15). These transgenic mosquitoes with antipathogen refractory phenotypes have been based on expression of a single type effector molecule in a single organ or tissue. Overexpression of two or more effector molecules with additive antipathogen action in the same transgenic mosquito strain could dramatically increase effectiveness of antipathogen-vector phenotypes. To provide a proof for this hypothesis, we have tested whether co-overexpression of two effector molecules in the transgenic *Ae. aegypti* could block transmission of *Plasmodium gallinaceum*.

Studies in *Drosophila melanogaster* have provided the basis of our knowledge about the insect innate immune response (16). Antimicrobial peptides (AMPs) are an important part of the humoral immunity. In *D. melanogaster*, there are seven distinct AMP families, which differ widely in their specificities against microorganisms (16). Mosquito AMPs significantly differ from *Drosophila* and are mainly represented by defensins and cecropins (17). Mosquito defensins are primarily active against Gram-positive bacteria, although a Gram-negative bacterium, *Enterobacter cloacae*, is susceptible to *Aedes* Defensin A (DefA) (18). Mosquito cecropins have a broad spectrum of antimicrobial activity (19, 20). In vivo, AMPs, and other components of the immune system could work cooperatively against invading pathogens. To test this hypothesis, we have chosen DefA and Cecropin A (CecA) for ectopic overexpression in transgenic mosquitoes, because they are representatives of two major classes of mosquito AMPs (17). Both these AMPs were expressed highly after immune challenge or ectopic expression of REL2, the mosquito orthologue of *Drosophila* Relish, in *Ae. aegypti* mosquitoes (11).

Here, we report the generation of two transgenic *Vg-CecA* strains of *Ae. aegypti* with blood meal-activated overexpression of *Aedes* CecA. Obtaining these new strains has permitted us to test whether the two major mosquito AMPs, CecA and DefA, could have cooperative antimicrobial and anti*Plasmodium* action. The hybrid *Vg-CecA/Vg-DefA* mosquitoes (CxD), which expressed both CecA and Def A, exhibited an elevated resistance to *Pseudomonas aeruginosa* infection, indicating that these AMPs acted cooperatively against this highly pathogenic bacterium. Significantly, we show that coproduction of these AMPs in the hybrid mosquito completely blocked the proliferation cycle of *Plasmodium* parasites and interrupted their transmission to a

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vertebrate host. Thus, this work has provided proof of principal of the transgenic approach for effective interruption of the parasite transmission cycle by means of coexpression of antiparasite effector molecules with additive action.

Results

We first generated transgenic *Ae. aegypti* strains overexpressing *CecA*. The *Vg-CecA* construct, obtained by linking the 1.8 kb *Vg* gene 5' regulatory region, *CecA* cDNA, and SV40 polyadenylation signal, was inserted into the pBac[3xP3-EGFP afm] vector (Fig. S14). Two independent transgenic strains (C2 and C32) were selected for and established as homozygous for the *Vg-CecA* transgene. Southern blot and PCR analyses confirmed stable integration of the transgene into the mosquito genome for C2 and C32 strains. These analyses indicate that in each strain the transposition occurred as a single copy insertion in different genomic sites (Fig. S1 and Table S1).

In C2 and C32 strains, the *Vg-CecA* transgene was under the control of the *Vg* gene regulatory region, which is activated by a blood meal in a strict sex-, stage-, and tissue-specific manner (21). The *Vg-CecA* transcript was detected only in females of both C2 and C32 strains after blood feeding and expressed in the fat body with a peak at 24 h post blood meal (PBM) similar to that of the endogenous *Vg* gene (Fig. S2). Immunoblot analysis showed that there was a high level of *CecA* peptide 24 h PBM but not at pre-tellogenesis fat bodies, thus confirming stage-specific production of *CecA* peptide in transgenic mosquitoes (Fig. S2F). Both C2 and C32 *Vg-CecA* mosquitoes exhibited higher level of resistance than the wild-type (wt) mosquitoes to Gram-negative bacterium, *E. cloacae*, and two Gram-positive bacteria, *Staphylococcus*

aureus and *Staphylococcus epidermidis*, which demonstrated functionality of overexpressed *CecA* peptide (Fig. S3).

To examine whether *CecA* and *DefA* could act cooperatively on pathogens in vivo, we produced hybrid transgenic mosquitoes by crossing the *Vg-CecA* C32 with the *Vg-DefA* X1 transgenic strain that produces high levels of *DefA* in response to a blood meal (7, 22). To generate *Vg-CecA/Vg-DefA* (CxD) hybrid mosquitoes, the two homozygous strains, *Vg-CecA* (C32) and *Vg-DefA* (X1), were maintained as homozygous for four generations before crossing. The hybrid strain was established by crossing C32 females (*Vg-CecA*) to X1 males (*Vg-DefA*), and F1 hybrid females were used for experiments. Northern blot analysis indicated that both *Vg-CecA* and *Vg-DefA* transgenes were expressed in the CxD mosquitoes (Fig. 1A). To confirm that *CecA* and *DefA* peptides were correctly processed in the hybrid mosquitoes, we used mass spectrometry to analyze the components present in their hemolymph 30 h PBM. The mass spectrum from 3,000–4,500 m/z was used to compare the peptide composition of the hemolymph of CxD transgenic and wt mosquitoes (Fig. 1B). Three additional peaks were observed in the hemolymph of CxD mosquitoes that were absent in samples from wt mosquitoes. The mass value of the large 4 kDa peak was only slightly smaller than that calculated for the 40-mer *DefA* peptide, possibly due to an existing disulfide bond modification in the in vivo peptide (Fig. 1B). The occurrence of two 34-mer and 35-mer peptides is likely related to unprocessed and processed forms of *CecA*. In *Hyalophora cecropia*, a modification of C-terminus of recombinant cecropin was necessary to restore broad-spectrum of antimicrobial activity, which was shown in a naturally occurring peptide (23). The naturally occurring *CecA* in the mosquito

Fig. 1. Characterization of the *Aedes aegypti* hybrid transgenic strain expressing both *CecA* and *DefA*. (A) Northern blot analysis of the *Vg-CecA* C32, *Vg-DefA* X1, and *Vg-CecA/Vg-DefA* CxD hybrid mosquitoes. The transgenic and control wild-type (wt) mosquitoes were analyzed 24 h PBM. Total RNA was hybridized with *CecA*, *DefA*, or actin cDNA probes wt parental strain. (B) MALDI-TOF mass spectrometry analysis of the hemolymph of the hybrid CxD (Upper Panel) and parental wt (Lower Panel) mosquitoes. Peptide sequences below MALDI-TOF image represented processed mature 34-mer and 35-mer *CecA* and 40-mer *DefA* peptides. (C) Survival rate of transgenic and control wt mosquitoes after infection with *P. aeruginosa*. Means and standard deviations of results reflect data from two independent experiments. *P* value between the CxD hybrid with other groups was <0.001. (D) Bacterial proliferation test for *P. aeruginosa* in adult transgenic and wt mosquitoes. Three mosquitoes per sample were collected and used for colony forming unit determination. Analysis of wt, the transgenic C32, X1, and CxD hybrid female mosquitoes was performed. Means and standard deviations of results reflect data from two independent experiments. Asterisk (*) represents *P* < 0.05 (*t* test), while CxD was compared to other groups.

Fig. 3. RNA interference depletion of CecA to test the functional effect of CecA overexpression on *P. gallinaceum* infection. Wt and transgenic *Vg-CecA* female mosquitoes (C32 strain) were injected with CecA or control luciferase dsRNA. One week later mosquitoes from each treated group were blood fed on the same infected chick. Mosquito midguts were dissected 7 days postinfection and scored for oocyst numbers: *Vg-CecA/iLuc*, *Vg-CecA* transgenic mosquitoes treated with luciferase dsRNA (control); *Vg-CecA/CecA*, *Vg-CecA* transgenic mosquitoes treated with CecA dsRNA; *wt/iLuc*, wt mosquitoes treated with luciferase dsRNA (control for infection). Data represents the result of 3 independent experiments for each transgenic and control strains.

of the mosquitoes were then blood fed on a naive chick at 7 d PIBM. On the 14th day PIBM, the number of sporozoites per salivary gland was determined. Both *Vg-CecA* and *Vg-DefA* transgenic strains had significantly reduced numbers of sporozoites in their salivary glands compared with the wt mosquitoes (Table 2). Number of sporozoites in the *Vg-CecA* C32 strain was significantly less than in the *Vg-DefA* X1 strain suggesting that CecA exhibited a higher anti-*Plasmodium* activity than DefA (Table 2). Importantly, no sporozoites were detected in salivary glands of the CxD mosquitoes after reactivation of both transgenes by feeding on a naive chick at 7 d PIBM (Table 2).

To determine whether transgenic mosquitoes could still transmit *Plasmodium*, we blood fed each mosquito on a naive chick at 14 d PIBM prior to examination for sporozoites. After 9 d, the parasitemia level in individual chicks was determined, then each chick was used for blood feeding of naive wt mosquitoes, and finally the number of oocysts in each mosquito midgut was determined 7–8 d later (30–31 d PIBM) (Table 2). Although we observed a significant decline in the number of oocysts in the midgut and sporozoites in the salivary gland of the *Vg-CecA* and *Vg-DefA* strains compared with the control wt mosquitoes, both of these transgenic strains were still able to transmit the

Plasmodium. However, when we used the CxD mosquitoes as a vector, we could detect neither any parasitemia in tested chicks nor oocysts in the midguts of naive wt mosquitoes fed on these chicks (Table 2). Therefore, we observed that the systemic co-overexpression of DefA and CecA hindered the development of *Plasmodium*, causing a significant reduction in the number of oocysts and sporozoites in the transgenic mosquitoes. Most importantly, in the CxD mosquitoes, the second simultaneous reactivation of these two AMPs by a blood meal completely blocked *Plasmodium* transmission.

Discussion

We used the yellow fever mosquito, *Ae. aegypti*, as a vector model because it is amenable to both transgenesis and reverse-genetic analysis. Its eggs can be stored for many months, making it possible to maintain a long-term large genetic stock. Moreover, *Ae. aegypti* pair wise crossing makes developing hybrid strains possible. The 5' regulatory region of the *Ae. aegypti* *Vg* gene sufficient for a high level blood meal-activated, sex- and tissue-specific expression (21) was used in this study for expression of effector molecules. The *Vg* gene is highly activated exclusively in the fat body, the tissue central for production of AMPs. The *Vg* gene expression is cyclic as it is tightly linked to separate blood meals making it possible to manipulate expression of effector molecules by repeated blood feedings (21).

The site of expression of antipathogen effector molecules is an important consideration in enhancing antipathogen phenotype in transgenic mosquitoes. Overexpression of effector molecules via the fat body-specific promoter results in secretion of these products into the hemolymph affecting two stages of *Plasmodium*: oocysts and sporozoites. It has been shown that after the release from mature oocysts and prior to invasion of the mosquito salivary glands, large number of sporozoites undergoes massive destruction by humoral factors in the hemolymph (27). Simultaneous utilization of transgenes with promoters driving effector molecules in different tissues (i.e., the midgut and the fat body), affecting different stages of the parasite development could be even more effective for transmission blocking.

In this work, we have shown that co-overexpression of two effector molecules with antipathogen action in the same transgenic mosquito results in a total refractory phenotype. Two AMPs, CecA and DefA, completely blocked *P. gallinaceum* transmission when they are produced by the *Ae. aegypti* fat body and secreted into the hemolymph in a blood meal-activated manner. These experiments have clearly demonstrated cooperative action of these AMPs against *Plasmodium*. Interestingly, in the in vivo

Table 2. Blocking of *Plasmodium* transmission in transgenic mosquitoes co-overexpressed DefA and CecA

		Wild-type	Vg-CecA	Vg-DefA	Vg-CecA/DefA
Exp 3	*Sporozoite # per a SG	12210 (8410–17430)	350 (0–560)	660 (40–1240)	0 (0–0)
	*Parasitemia (%) /chicks #	15% (5–20%) /9	1% (0–4%) /9	3% (0–9%) /8	0% (0–0%) /11
	*Oocyst # per a midgut	42 (11–79)	1 (0–6)	2 (0–11)	0 (0–0)
Exp 4	*Sporozoite # per a SG	8950 (5840–15740)	315 (0–610)	573 (30–970)	0 (0–0)
	*Parasitemia (%) /chicks #	12% (0–18%) /10	0.5% (0–2%) /9	2.1% (0–4%) /9	0% (0–0%) /10
	*Oocyst # per a midgut	29 (0–62)	1 (0–4)	1.5 (0–7)	0 (0–0)
Exp 5	*Sporozoite # per a SG	12950 (7820–17210)	380 (0–770)	1542 (10–11200)	0 (0–0)
	*Parasitemia (%) /chicks #	14% (0–22%) /9	2% (0–3%) /9	3% (0–5%) /9	0% (0–0%) /10
	*Oocyst # per a midgut	34 (0–88)	1 (0–5)	2 (0–12)	0 (0–0)

**Plasmodium*-infected mosquitoes from independent cohorts of EXP3–5 (Table 1) were fed on naive chickens on day 14 postinfection. This was done to reactivate the production of CecA and DefA in transgenic strains. The mean sporozoite number per a salivary gland. The range of observed values is indicated in parentheses.

*Mosquitoes from the same batches were then fed on naive chickens. Parasitemia of these chickens was established by examining red blood cells for the presence of *Plasmodium* infection. Parasitemia is expressed as the mean percentage of infected to total red blood cells examined. The range of observed value in an individual chick is indicated in parentheses.

*Wt naive mosquitoes were blood fed on an individual chicken tested for parasitemia levels in each column of†. The oocyst number in there midguts was 7 days after blood feeding. The mean oocyst number per midgut and the range of observed values (in parentheses) are indicated.

environment of the transgenic *Ae. aegypti*, CecA alone had notably higher negative effect on *Plasmodium* development than DefA. Further tests of the mosquito-*Plasmodium* system (particularly, *An. gambiae*-*P. falciparum*) should reveal whether anti-*Plasmodium* action by CecA can be augmented by another effector even stronger than by DefA.

Although the mechanism of AMP action on parasitic protozoa is not well understood, they appear to possess potent antiprotozoan properties. In earlier studies, exogenous AMPs, purified from other insect species, have been tested for activity against *Plasmodium*. Two defensins, one from the dragon fly *Aeschna cyanea* and the other from the flesh fly *Phormia terranova*, have been reported as having a profound toxic effect on the development of *P. gallinaceum* oocysts in the midgut of *Ae. aegypti* as well as on isolated sporozoites (28). The midgut-specific activation of CecA in the transgenic *An. gambiae* resulted in 60% reduction of *Plasmodium* oocysts (13). In addition, the adverse effects of cecropins have been observed on other pathogens such as *Leishmania* (29).

In *Ae. aegypti*, transgenic overexpression of REL2 by the fat body leads to increased resistance to bacterial infection as well as partial refractoriness toward *P. gallinaceum* (11). The Imd/REL2 is also important in *An. gambiae* for resisting *P. berghei* (30). RNA interference experiments to deplete Caspar, the negative regulator of Imd pathway, have shown that the pathway is involved in regulating resistance of *A. gambiae* and other anopheline species to the human malaria parasite *P. falciparum* (31). Nevertheless, these studies have not provided a clear link between AMPs and the anti-*Plasmodium* defense. Indeed, defensins and cecropins do not appear to be a part of natural mosquito defense against *Plasmodium* species. Transcriptomic analyses have shown that the infection of *An. gambiae* by either *P. falciparum* or *P. berghei* does not elicit the activation of defensins and cecropins (32, 33). In agreement, RNA interference depletion of defensins and cecropins did not affect in vivo *Plasmodium* development in mosquitoes (32, 33). It is, however, different from the transgenic activation of these AMPs linked to blood feeding. Appearance of the high level of AMPs, CecA and DefA, at the time of parasite acquisition by a mosquito clearly has a detrimental effect on *Plasmodium* development.

In conclusion, we report the genetic engineering of transgenic *Ae. aegypti* mosquitoes with a blood meal dependent systemic activation of two major mosquito AMPs, CecA and DefA. Co-overexpression and cooperation of CecA and DefA in these mosquitoes limit the proliferation of the highly pathogenic bacteria *P. aeruginosa*. These AMPs appear to work cooperatively against *Plasmodium* developmental stages, completely blocking the parasite transmission. Our present study suggests a unique approach in generating transgenic mosquitoes with anti-*Plasmodium* refractory phenotype, which is coexpression of two or more effector molecules with cooperative action on the parasite. Based on our study, it appears that a combinatorial overexpression of several antiparasitic effector molecules in genetically modified mosquitoes will provide a potent tool for preventing transmission of *Plasmodium* in human malaria vectors.

Methods

Generating Transgenic Mosquitoes. The *Ae. aegypti* wt Rockefeller/UGAL strain used in these experiments was reared at 27 °C and 80% humidity. Three- or four-day old previtellogenic females were fed on anaesthetized rats. Newly laid eggs were collected and prepared for microinjection as described previously (8, 10). The 293 nucleotide-long *Ae. aegypti* CecA cDNA was isolated by means of RT-PCR and used for construction of transformation vector. A DNA fragment containing the 1.8 kb 5' regulatory region of the *Vg* gene linked to the CecA cDNA was subcloned into the pBac[3xP3-EGFP *afm*] in the *Ascl* unique cloning sites. The resulting pBac[3xP3-EGFP *afm*, *Vg*-CecA] donor plasmid was purified using the QIAfilter kit (Qiagen). The pBac[3xP3-EGFP *afm*, *Vg*-Cec] donor plasmid and the phsp-pBac helper plasmid were

mixed in a buffer containing 5 mM KCl and 0.1 mM NaH₂PO₄ (pH 6.8) at final concentrations of 0.35 µg/mL and 0.25 µg/mL, respectively. Transformation of *Ae. aegypti* and selection of transformed strains have been performed by a well-established methodology [(5, 22), and *SI Text*].

Southern, Northern, and immunoblot (Western) blot analyses were performed using standard methodologies (*SI Text*).

RT-PCR, Quantitative Real-Time PCR Analysis, and Inverse PCR. To remove any genomic contamination, 3 µg total RNA was mixed with amplification-grade Dnase I (Gibco BRL/Invitrogen). Then, 2 µg RNA treated with Dnase I was directly used in a cDNA synthesis reaction using the Omniscript Reverse Transcriptase kit (Qiagen). Levels of cDNA in the different samples were quantified using real-time PCR. The real-time PCR master mix, iQ Supermix (BioRad) was utilized. All reactions for real-time PCR were run in triplicate using 2 µL cDNA per reaction. The PCR reactions were performed using an iCycler real-time PCR machine (BioRad). For sequences of primers and probes used in the RT-PCR reactions, see *SI Text*.

Inverse PCR analysis was done with genomic DNA isolated from transgenic female mosquitoes. Endonucleases Taq I and Msp I were used for digestion of genomic DNA to identify 5' and 3' junctions, respectively. For inverse PCR reactions the following primers were used: forward, 5'-TCTTGACCTTGCCA-CAGAGG-3', reverse, 5'-TGACACTTACCGATTGACA-3' (for TaqI digestion); and forward, 5'-GTCAGTCCAGAAACAACCTTTGGC-3', reverse, 5'-CCTCGATA-TACAGACCGATAAAACACATG-3' (for Msp I digestion).

Survival Test and Bacterial Proliferation Study. Transgenic and control female mosquitoes, 24 h PBM, were injected with different strains of Gram-negative and Gram-positive bacteria. In survival test experiments, groups of 20 females were inoculated by pricking the mosquitoes with a Hamilton 33S needle that had been dipped into medium containing freshly growing bacteria in their exponential phase after 6 h of culturing. The survival rate was calculated 72 h after bacterial injection, because mortality no longer occurred beyond this time.

For the bacterial proliferation study of *P. aeruginosa*, the overnight-grown bacterial medium was diluted 10-fold with distilled water to a final concentration 3×10^8 CFU/mL, before being used for infection. Infected mosquitoes, collected 5 h after bacterial challenge, were ground up in distilled water, and the resulted supernatant was plated onto LB agar plates. Bacterial colonies were grown for 14–16 h at 37 °C, after which colony counts were scored.

Mass Spectrometry. For hemolymph collection *Ae. aegypti* female mosquitoes were decapitated, and their last two abdominal segments were cut off. Mosquitoes prepared in this way were then centrifuged at 4,000 rpm for 5 min, and the collected supernatant was used for analysis. Hemolymph samples were pulled from 35–40 females of each of the wt and hybrid CxD mosquitoes and subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis.

Plasmodium Infection. *P. gallinaceum* was maintained by transmission between mosquitoes of the wt strain and White Leghorn chicks. All the experimental animals were housed in cages in the psychology building vivarium, and all procedures were preapproved by the Institutional Animal Care and Use Committee (see *SI Text* for detail).

RNA Interference Gene Depletion. For CecA gene silencing experiments we generated cDNA by RT-PCR and then produced the double stranded RNA (dsRNA) using T7 RNA polymerase. RNA interference gene depletion experiments were essentially performed as previously described [(34) and *SI Text*].

Statistics. Individual survival rate curves after bacterial injections were built by the approach of Kaplan–Meier; meanwhile, curves of different groups were compared by both the log-rank and the Gehan–Breslow–Wilcoxon tests.

P. gallinaceum infection data from three independent experiments were combined and the Kolmogorov–Smirnov goodness of fit test was done essentially as described (34). Statistically significant difference between samples was evaluated using the Mann–Whitney *U* test (Graphpad 5.0).

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