Malaria kills millions of people every year, and new control measures are urgently needed. The recent demonstration that (effector) genes can be introduced into the mosquito germ line to diminish their ability to transmit the malaria parasite offers new hope toward the fight of the disease (Ito, J., Ghosh, A., Moreira, L. A., Wimmer, E. A. & Jacobs-Lorena, M. (2002) Nature, 417, 452–455). Because of the high selection pressure that an effector gene imposes on the parasite population, development of resistant strains is likely to occur. In search of additional antiparasitic effector genes, we have generated transgenic Anopheles stephensi mosquitoes that express the bee venom phospholipase A2 (PLA2) gene from the gut-specific and blood-inducible Anopheles gambiae carboxypeptidase (AgCP) promoter. Northern blot analysis indicated that the PLA2 mRNA is specifically expressed in the guts of transgenic mosquitoes with peak expression at ~4 h after blood ingestion. Western blot and immunofluorescence analyses detected PLA2 protein in the midgut epithelia of transgenic mosquitoes from 8 to 24 h after a blood meal. Importantly, transgene expression reduced Plasmodium berghei oocyst formation by 87% on average and greatly impaired transmission of the parasite to naive mice. The results indicate that PLA2 may be used as an additional effector gene to block the development of the malaria parasite in mosquitoes.

Worldwide mortality due to malaria has increased in the past decade mainly because of parasite and mosquito resistance to drugs and insecticides, respectively, and the lack of effective vaccines (1). Genetically engineered mosquito vectors for refractoriness to malaria parasites is a strategy for reducing disease transmission that should be explored. Plasmodium, the causative agent of malaria, has to complete a complex developmental program in the mosquito for transmission to occur. The first interactions between the parasite and the mosquito occur in the midgut lumen, where the parasite has to traverse two barriers, the peritrophic matrix, and the midgut epithelium (2, 3). Because the gut is a closed compartment that limits diffusion, antimalarial compounds secreted into the midgut lumen are expected to efficiently target the initial stages of parasite development.

Previous studies have demonstrated that venom phospholipases A2 (PLA2s) strongly inhibit oocyst formation when administered to mosquitoes with an infectious blood meal (4). Although the mechanism of inhibition has not been established, it is known that PLA2 does not kill ookinetes and does not interfere with their development in vitro. Furthermore, inhibition of oocyst formation did not depend on PLA2 enzymatic activity. It is possible that PLA2 inhibits ookinete invasion by modifying the properties of the midgut epithelial membranes that are invaded by the parasite.

The best candidates for driving the expression of foreign gene products to be secreted into the mosquito midgut are the promoters of bloodmeal-inducible midgut genes because of their strength, tissue specificity, and synchrony of expression with parasite ingestion by the mosquito. In this context, we have shown that the Anopheles gambiae and Aedes aegypti carboxypeptidase promoters can be used to drive strong expression of recombinant protein in the midgut of transgenic mosquitoes (5) and also that they can drive the expression of a parasite blocking peptide (6) in transgenic mosquitoes (7). Although the latter results indicate that genetic manipulation of mosquito vectors is a promising strategy for reducing malaria transmission, it is also important to consider that the use of a single effector gene is likely to lead to rapid selection of resistant parasites. Thus, we have searched for additional effector genes whose products interfere with parasite development by mechanisms different from the previously developed ones (6, 7). The results reported in this article suggest that bee venom PLA2 may be used as an alternate effector gene.

**EXPERIMENTAL PROCEDURES**

**Cloning of a Full-length Bee Venom Phospholipase A2 cDNA**—The full-length cDNA of the honeybee phospholipase A2 gene was cloned for the first time by relying on the previously published partial sequence (GenBank™ accession number X16709). Messenger RNA was isolated from 105 venom glands dissected out of newly emerged honeybees using the Micro Fast-Track mRNA isolation kit from Invitrogen. The mRNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) using the SMART cDNA synthesis kit (Clontech) in the presence of a primer specific to the previously published 3’ end of the honeybee PLA2 cDNA (5’-ggagcgtagataaataacgatctcgaagtggtactc-3’), as well as a custom-synthesized SMART primer (5’-ggagcgtagataaataacgatctcgaagtggtactc-3’), dual-labeled primers to amplify the full-length cDNA. The cDNA was cloned into the pET15b vector (Novagen) for expression in E. coli DH5α. The resulting recombinant plasmid was transformed into the BL21 (DE3) strain of E. coli. The expressed recombinant PLA2 was purified from the inclusion bodies as described (8).

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**The nucleotide sequences(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF438408.**

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1. The abbreviations used are: PLA2, phospholipase A2; AgCP, A. gambiae carboxypeptidase; UTR, untranslated region; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; EGFP, enhanced GFP; DAPI, 4’,6-diamidino-2-phenylindole.
cagagtggccattatggccgg-3'). The first strand cDNA was PCR-amplified using the same two primers and Platinum-Tag high fidelity polymerase (Invitrogen), and the PCR product was cloned with the TOPO-TA cloning kit (Invitrogen). The resulting construct BV PLA2 5-7 consisted of the full-length bee venom PLA2 open reading frame flanked by 5'– and 3'-untranslated sequences inserted into the pCR-TOPO vector (Invitrogen). The PLA2 sequence thus obtained included the entire signal peptide, which was only partially present in previously published sequences, as well as a portion of the 5'-untranslated region (UTR). Inserts from several identical clones were sequenced, and the sequence was used to prepare PCR primers for the construction of the mosquito expression constructs.

Carboxypeptidase-PLA2 Construct—A 3.9-kb fragment from the AgCP genomic subclone using AgCPKpn (5'–GGTACCTGGCCGCGCT-TGGACACT-3') and 27 (5'–GATAACGACTCTATAGGGG-3') primers and cloned into pGemT-easy. The AgCP5 plasmid was then cut with these enzymes and cloned into pGemT-easy. Both the AgCP5 and pSLfa1180fa plasmid was then cut with these enzymes and cloned into pGemT-easy. Bee venom PLA2 coding region (450 bp) was cloned into pGemT-easy (Promega) from a cDNA clone using primers PLA2K (5'–GGTACCTGGCCGCTTAACATCGGAT-3') and MLA2B (5'–GGTACCTTTATACATTCCGGAAGATC-3'). The AgCP5 in pGemT-easy was digested with KpnI, and the resulting 1.8-kb fragment was ligated into the PLA2 plasmid (KpnI-digested). AgCP5'- (555 bp) (untranslated 3' region) was obtained by PCR amplification with primers AgCP5BH (5'–GGTACCTGGCCGCTTTACATTCCGGAAGATC-3') or AgCP5HI (5'–GGTACCTGGCCGCTTTACATTCCGGAAGATC-3') and pBluescript subclone genome and cloned into pGemT-easy. The AgCP5 and AgCP in pGemT were digested with BamHI and SacI and the AgCP 3'-UTR fragment ligated to AgCP5/pGemT-easy (pGemT-easy). A NolI fragment containing AgCP/pL AvaI fragment was cloned into pSLfa1180fa plasmid (9) that contains unique NcoI and AsclI sites. The plasmid was then cut with these enzymes and cloned into piggyBac[3xP3-EGFP(AgCPPLA2)]. The transposon construct (0.5 mg/ml) and of the promoter (see “Experimental Procedures” and the legend for Fig. 2), b indicates the probe used for Southern analysis.

Blocking Malaria Transmission with PLA2

Experimental Procedures—Individual G. pseudoscutellaris mosquitoes were separated into small feeding cups, and each mosquito was allowed to feed on a single naive mouse (Swiss Webster (CFW), Charles River Laboratory). After feeding, mosquitoes were cold-anaesthetized, salivary glands were dissected and homogenized in a small volume of PBS, and sporozoites were counted with a hemacytometer. The infection status of each mouse was followed by counting the number of infected erythrocytes. Mice were killed by cervical dislocation and brains were removed and stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the liver stages before and after feeding. The brains were mounted onto glass slides, covered with a drop SlowFade Antifade (Molecular Probes), and examined by fluorescence microscopy.

Immunofluorescence Assays of Midgut Sections—Guts from blood-fed mosquitoes were dissected in 4% paraformaldehyde in PBS, fixed for 2 h at room temperature, and washed three times in PBS. After dehydration in a graded ethanol/PBS series, the guts were treated with xylene and embedded in Paraplast (Oxford Laboratory), and 7–14-μm sections were mounted onto glass slides. The slides were washed twice in xylene at room temperature followed by rehydration in a graded ethanol/PBS series. To reduce autofluorescence, slides were dehydrated and rehydrated in a graded methanol/PBS series. The slides were blocked in 10% nonfat milk and 0.1% Triton X-100 in PBS for 2 h at room temperature and incubated overnight at room temperature with an anti-venom phospholipase A2 antibody (Accurate; 1:2,000 dilution). The slides were washed seven times and incubated in the dark for 2 h with a fluorescein isothiocyanate-conjugated anti-rabbit IgG (Sigma; 1:600 dilution). The gut sheets were mounted onto glass slides, covered with a drop SlowFade Antifade (Molecular Probes), and examined by fluorescence microscopy.
RESULTS AND DISCUSSION

A. stephensi Transformation—For the expression of PLA2 in the A. stephensi midgut, we constructed the AgCP-PLA2 gene that consists of the promoter, the 5'-UTR, and the signal peptide from the AgCP gene (8) linked to the coding sequence of the bee venom PLA2 gene and the AgCP 3'-UTR (Fig. 1). This gene was inserted into the pSL1180fa shuttle plasmid, which has unique restriction sites for cloning into the 3xP3-EGFP piggy- 

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TABLE I

Effect of PLA2 expression on P. berghei oocyst formation

Transgenic and non-transgenic (control) mosquitoes were fed on the same P. berghei-infected mouse. On day 15, transgenic and non-transgenic mosquitoes (positive and negative GFP eye fluorescence, respectively) were separated and after gut dissection and the number of oocysts per gut was determined.

| Experiment | Mosquitoes | Prevalencea | No. oocysts/gutb | Inhibitionc |
|------------|------------|-------------|-----------------|-------------|
| 1          | Control    | 84% (31/37) | 28.8 ± 44.8     | 98.8%       |
|            | AF1 line  | 14% (3/21)  | 0.3 ± 0.96      |             |
| 2          | Control    | 96% (28/29) | 23.7 ± 22.1     | 76.5%       |
|            | AF1 line  | 44% (7/16)  | 5.6 ± 15.6      |             |
| 3          | Control    | 95% (19/20) | 41.2 ± 37.2     | 76.9%       |
|            | BM1 line  | 63% (20/32) | 9.5 ± 15.1      |             |
| 4          | Control    | 100% (27/27) | 184.7 ± 137.8 | 95.7%       |
|            | AF1 line  | 10% (2/21)  | 7.9 ± 25.8      |             |
| 5          | Control    | 100% (10/10) | 102.9 ± 45.0  | 84.7%       |
|            | BM1 line  | 56% (5/9)   | 15.8 ± 26.6     |             |

a Percentage of infected mosquitoes (actual numbers in parentheses).
b Average number of oocysts per midgut and standard deviation.
c 100 − [(average oocyst number per gut in transgenic mosquitoes/average oocyst number per gut in control mosquitoes) × 100]. Reduction of oocyst number/gut was significant (p < 0.001) for all experiments, as calculated by the Mann-Whitney test.

TABLE II

The vectorial capacity of transgenic mosquitoes is severely impaired

For each experiment, control non-transgenic and transgenic mosquitoes were fed on the same P. berghei-infected mouse. To measure transmission, single mosquitoes were fed on individual naive mice 25 days after the infectious blood meal. The salivary gland of each mosquito was dissected immediately after feeding on the mouse, and the number of sporozoites per salivary gland was determined (results reported in columns 2 and 3). The infection status of each mouse was established by examining a smear of tail vein blood on alternate days. Mice that had no parasites by day 25 were considered to be non-infected (results reported in column 4).

| Experiment | % infected mosquitoes (number infected/total) | Mean sporozoite numberd (range) | % infected mice (number infected/total) |
|------------|----------------------------------------------|---------------------------------|----------------------------------------|
| 1. Control | 90% (10/11)                                  | 569 (0–1,800)                   | 90% (10/11)                            |
| BF4 line   | 30% (3/10)                                   | 10 (0–50)                      | 0% (0/10)                              |
| 2. Control | 100% (16/16)                                 | 1,976 (38–9,500)               | 88% (14/16)                            |
| BF4 line   | 56% (5/9)                                    | 10 (0–38)                      | 0% (0/9)                               |
| 3. Control | 88% (14/16)                                  | 884.5 (0–3,450)                | 44% (7/16)                             |
| AM3 line   | 50% (5/10)                                   | 6.5 (0–13)                     | 0% (0/10)                              |
| 4. Control | 100% (12/12)                                 | 1,774 (50–6,800)               | 83% (10/12)                            |
| BF4 line   | 90% (4/5)                                    | 39.2 (0–120)                   | 20% (1/5)                              |

a This is a minimum estimate. Sporozoites from only an aliquot of the salivary gland homogenate were counted. The number of sporozoites was significantly lower (p < 0.01) in transgenic than in non-transgenic mosquitoes in all experiments, as calculated by the Mann-Whitney test.

and 4% for the following combinations: A. stephensi with piggyBac (14), A. aegypti with Hermes (5, 15, 16), and A. aegypti with mariner (5, 17).

Characterization of the Transgenic Lines—To confirm that the transgene was stably integrated and to determine the number of integrated transposons per genome, DNA from each mosquito line was analyzed by Southern blot hybridization. Mosquito genomic DNA was digested with Bgl II, fractionated, blotted, and hybridized with a probe from the piggyBac left arm (Fig. 1, probe a). As shown in Fig. 2, each mosquito line carried a single transgene integrated at a different position, indicating that independent integration events had occurred in each of the four families (note that AM3 and BM1 originated from different families). No signal was detected with DNA from non-transformed mosquitoes (data not shown).

Expression of the AgCP-PLA2 mRNA was investigated by Northern analysis (Fig. 3). A single band of the expected size (∼800 bases) was detected (18). The AgCP-PLA2 mRNA was present in the midgut of sugar-fed mosquitoes and was strongly induced by blood ingestion (peak at ∼4 h), consistent with the pattern of expression of the A. gambiae carboxypeptidase gene (8). Enhanced expression at 48 h was also observed, but the reasons for this are not clear. Robust mRNA expression as well as tissue and sex specificity together indicate that the 1.7-kb AgCP upstream sequence contains the necessary regulatory elements.

Immunoblot analysis showed that the anti-PLA2 antibody detected a protein that was produced in a blood-inducible manner in the midguts of transgenic mosquitoes (Fig. 4). The electrophoretic mobility of the protein (17 kDa) on acrylamide gels was identical to that of an high pressure liquid chromatography-purified bee venom PLA2 protein (data not shown). Less than 0.5 gut equivalent of protein per lane was sufficient for PLA2 detection. Importantly, the recombinant protein showed a peak of expression between 8 and 24 h after a blood meal, coinciding with the time of ookinete invasion of the midgut (3). By 48 h, the protein was not detectable anymore, which contrasts with the Northern data.

Immunofluorescence assays detected the protein in whole-mount gut sheets (Fig. 5) and in gut sections (Fig. 6). In sheets, the strongest signal appeared in epithelia prepared from guts 6 h after a blood meal, whereas in sections, a much stronger signal was detected at 24 h. Presumably, by 24 h, the majority of the PL2A2 had been secreted into the lumen and was lost during washing of the gut sheets (Fig. 5). Secreted PLA2 can be seen as a thick FITC signal between the epithelium and the blood meal in 24-h transgenic gut sections (Fig. 6), and this agrees with the Western data (Fig. 4).

Effect of PLA2 Expression on the Progression of Parasite Development in the Mosquito and on Mosquito Vectorial Capacity—To investigate the effect of recombinant PLA2 expression on P. berghei development, we fed both transgenic and non-transgenic mosquitoes on the same infected mouse and counted the number of oocysts that formed in each group of mosquitoes. As indicated in Table I, infection prevalence (column 3) and oocyst formation (column 4) were strongly reduced in transgenic mosquitoes. In five independent experiments, oocyst formation was inhibited from 77 to 99% (average inhibition 87%, column 5).

The effect of recombinant gene expression on the ability of
mosquitoes to transmit the parasite to uninfected animals (vectorial capacity) was assessed by letting single infected mosquitoes feed on individual naive mice and determining whether these mice became infected. As reported in columns 2 and 3 of Table II, in every experiment, fewer transgenic than non-transgenic mosquitoes became infected, and the number of sporozoites in salivary glands of transgenic mosquitoes was correspondingly lower. More importantly, the ability of transgenic mosquitoes to transmit the parasite to naive mice was strongly inhibited. In three out of four experiments, transmission of *P. berghei* parasites from infected transgenic mosquitoes to naive mice was completely blocked, and in a fourth experiment, the proportion of transgenic mosquitoes that transmitted the parasite was much lower than in control wild type mosquitoes (Table II, column 4). We considered the possibility that inhibition of parasite development was a consequence of the fortuitous disruption of a mosquito gene upon transposon insertion or of marker GFP expression. The following considerations strongly argue against these possibilities: 1) the same phenotype (inhibition of parasite development and transmission) was observed in different mosquito lines, in which the transposon integrated at different positions of the mosquito genome (Fig. 2 and Tables I and II); 2) the same phenotype was observed when PLA2 was provided exogenously to wild type mosquitoes (4); and 3) *P. berghei* developed equally well (oocyst and sporozoite numbers) in transgenic mosquitoes that express only GFP and hygromycin resistance gene (10) as in wild type mosquitoes. The PLA2 protein secreted in the midgut lumen of transgenic mosquitoes is most likely responsible for inhibition of ookinete midgut invasion, as observed with the exogenously administered protein (4).

The binding of phospholipases to their substrates, such as aggregated phospholipids and membrane surfaces, is dependent of their enzymatic activity (19). Indeed, it has been shown that PLA2 inhibited *Plasmodium* development even when its enzymatic activity was inhibited (4), suggesting that PLA2 acts primarily via its binding to exposed membrane lipids. Moreover, PLA2 had no effect on exflagellation and zygote formation and did not affect normal ookinete motility on glass slides, suggesting that this enzyme does not kill the zygote (4). These considerations and the lipophility of the enzyme support the hypothesis that PLA2 may be acting by interfering with the interactions between *Plasmodium* and the midgut cell surface. PLA2-expressing mosquitoes were normal in appearance and lived as long as the non-transformed counterparts, although an abnormal coloration of the blood meal was observed even 24 h after feeding (bright red instead of dark brown). Egg laying may also be affected. A detailed assessment of the fitness of transgenic as compared with wild type mosquitoes is under way.

In summary, these experiments demonstrate that expression of the bee PLA2 gene in the midgut of transgenic mosquitoes seriously compromises their ability to sustain *Plasmodium* development and to transmit the parasite to other vertebrate hosts. Since this PLA2 also interferes with development and transmission of *Plasmodium falciparum* in *A. gambiae* (4), we presume that PLA2 will be equally effective in curtailing transmission in this most important parasite-vector combination. Whereas reports of attempts to interfere with *Plasmodium* transmission by expression of defensin (20) or single chain antibodies (21) in *A. aegypti* have appeared, their effectiveness in transgenic mosquitoes remains to be demonstrated. Separate work from this laboratory indicates that expression of SM1, a midgut and salivary gland binding peptide, also effectively inhibits development and transmission of the parasite (6, 7). The availability of multiple targets to inhibit *Plasmodium* development is crucial for future implementation of the transgenic mosquito approach to reduce malaria transmission in the field. This is because the *Plasmodium* genome is known for its plasticity, and the possibility of the emergence of resistant parasite strains needs to be avoided at all cost. Before one can consider actual release assays, much work remains to be done. Issues that need to be addressed include determination of wild mosquito population structures, an evaluation of the ability of gene(s) to spread through populations, considering the likelihood that the parasites will develop resistance to the foreign effector gene product, and concerns about horizontal transfer. The experiments reported here strongly suggest that genetic modification of mosquito vectorial capacity is feasible and represent a major step toward the goal of containing the spread of malaria. However, for maximum effectiveness, we will have to rely on a multipronged approach that may include drugs, insecticides, vaccines, and mosquito vectors expressing a combination of effector genes.

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