REL1, a Homologue of *Drosophila* Dorsal, Regulates Toll Antifungal Immune Pathway in the Female Mosquito *Aedes aegypti*

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Sang Woon Shin, Vladimir Kokoza, Guowu Bian, Hyang-Mi Cheon, Yu Jung Kim, and Alexander S. Raikhel‡

From the Department of Entomology and the Institute for Integrative Genome Biology, University of California, Riverside, California 92521

Signaling by *Drosophila* Toll pathway activates two Rel/NF-κB transcription factors, Dorsal (Dl) and Dorsal-related immune factor (Dif). Dl plays a central role in the establishment of dorsoventral polarity during early embryogenesis, whereas Dif mediates the Toll receptor-dependent antifungal immune response in adult *Drosophila*. The absence of a Dif ortholog in mosquito genomes suggests that Dl may play its functional role in the mosquito Toll-mediated innate immune responses. We have cloned and molecularly characterized the gene homologous to *Drosophila* Dl and to *Anopheles gambiae* REL1 (Gambif1) from the yellow fever mosquito *Aedes aegypti*, named AaREL1. AaREL1 alternative transcripts encode two isoforms, AaREL1-A and AaREL1-B. Both transcripts are enriched during embryogenesis and are inducible by septic injury in larval and female mosquitoes. AaREL1 and AaREL2 (*Aedes* Relish) selectively bind to different κB motifs from insect immune gene promoters. Ectopic expression of AaREL1-A in both *Drosophila* mbn-2 cells and transgenic flies specifically activates Drosomycin and results in increased resistance against the fungus *Beauveria bassiana*. AaREL1-B acted cooperatively with AaREL1-A to enhance the immune gene activation in Aag-2 cells. The RNA interference knock-outs revealed that AaREL1 affected the expression of *Aedes* homologue of *Drosophila* Serpin-27A and mediated specific antifungal immune response against *B. bassiana*. These results indicate that the homologue of Dl, but not that of Dif, is a key regulator of the Toll antifungal immune pathway in *A. aegypti* female mosquitoes.

Mosquitoes transmit many diseases, including malaria, which is a particularly threatening disease that is responsible for over two million deaths each year (1, 2). Diseases caused by mosquito-borne viruses, most importantly Dengue fever, are reaching disastrous levels in Central and South America and Southeast Asia (3). Since arriving on the eastern coast of the United States in 1999, the West Nile encephalitis virus has swept westward, killing almost 100 people (4). Lymphatic filariasis, a nematode-based disease transmitted by mosquitoes, affects millions of people in tropical regions of the world (5).

The major reasons for this tragic situation are the unavailability of effective vaccines for malaria and other mosquito-borne diseases and the lack of insecticides and drug resistance to vectors and pathogens, respectively (6, 7).

Immunity plays a key role in the interaction between a pathogen and its vector host. Many genes and their products involved in mosquito innate immunity have been identified and characterized either independently or on the basis of their relationships to known immune genes of the model organism, *Drosophila melanogaster*. The genomic DNA sequences of *Anopheles gambiae*, a major disease vector involved in the transmission of *Plasmodium falciparum* malaria in Africa, have been revealed, and they encompass more than 278 million base pairs, which suggests the presence of ~14,000 protein-encoding genes (8). Of these, 242 *A. gambiae* genes from 18 gene families have been implicated in innate immunity by comparative genomic analysis to the *Drosophila* immune systems (9). The mosquito immune genes involved in recognition, signal modulation, and effector systems diverge widely from those in *Drosophila*. In addition, these families of immune factors have undergone significant expansion during the evolution of mosquitoes, which possibly reflects different selection pressures to a variety of pathogens encountered in the distinct lifestyles of these insects. However, the components of two principal immune transduction pathways, the Toll and IMD, have principally been conserved between these two insects. This points to the evolutionary requirement of preserving the integrity of key factors in intracellular immune signal transduction and gene activation pathways (9).

In *Drosophila*, three Rel/NF-κB molecules, Dorsal-related immunity factor (Dif), 1 Dorsal (Dl), and Relish, affect the expression of numerous immune genes, including those encoding antimicrobial peptides (AMPs) (for review, see Refs. 10 and 11). These Rel/NF-κB molecules are involved in two distinct innate immune pathways, the Toll pathway, which is mediated by Dl and Dif and responds primarily to fungal and Gram-positive bacterial infections, and the IMD pathway, which is regulated by Relish and is predominantly directed against Gram-negative bacteria. In the Toll-signaling pathway, Dif stimulates the production of the antifungal factor Drosomycin (12). In loss-of-function mutants affecting the Toll signaling pathway, the inducibility of *Drosomycin* in immune-challenged adults is severely compromised, whereas those of *Defensin*, *Cecropin*, and *Attacin* are reduced to a lesser extent. In contrast, the expres-
sion of Diptericin, Drosocin, and Methchnikouin are completely unaffected (13, 14). In Drosophila relish mutants, the induction of immune defense is severely reduced, and insects become extremely sensitive to bacterial and fungal infection (15). Immune effector genes under the regulation of Relish include Cecropin, Diptericin, Attacin, Defensin, and Methchnikouin (15, 16). Recent microarray studies have shown that in addition to AMP genes many other immune genes are regulated cooperatively or independently by the Toll and IMD pathways (17). These genes include recognition molecules like peptidoglycan recognition proteins and Gram-negative bacteria-binding protein, which are components involved in both the IMD and Toll pathways; the protease cascade proteins; and putative components of the phenoloxidase or blood clotting pathways.

Dl, the first insect member of NF-κB family, was identified in a screen for genes required for Drosophila embryonic development. Dl is a key regulator in determining dorsoventral polarity (18). A gradient of nuclear Dl spatially restricts the expression of zygotic genes along the dorsoventral axis and functions both as a transcriptional activator and a repressor (19). The remarkable structural and functional similarities between the mechanisms of activation of Dl during Drosophila morphogenesis and NF-κB during the mammalian acute phase response have implicated Dl and Dif in the host defense of Drosophila (20, 21). However, two independent studies clearly show that Dif, the possible deterrent of the Dl gene during Drosophila evolution, is the essential regulator of the Drosophila immune Toll pathway. The rescue experiments of Drosophila DI and Dif double mutants have shown that the ectopic expression of Dif without Dl is sufficient to mediate the induction of Drosomycin (22). In addition, genetic epistasis studies with Dif mutant flies have demonstrated that Dif mediates the Toll-dependent control of the inducibility of the Drosomycin gene (23).

The Drosophila Toll pathway regulates many aspects of the immune response (for review, see Ref. 24). In addition to activating antifungal defenses, this pathway is also required for resistance against Gram-positive bacteria, for regulation of early humoral immune response, and for activation of late humoral immune response. The Toll pathway governs many aspects of the immune response, including the expression of Toll family genes, the expression of other genes involved in the immune response, and the regulation of other developmental processes. The Toll pathway is also involved in the regulation of other immune processes, such as the regulation of the innate immune response and the regulation of the adaptive immune response.

**Isolation of cDNA and Genomic DNA Clones and RACE**—

**Northern Hybridization and RT-PCR**—Adult 2- or 3-day-old A. aegypti females were injected with a stationary phase culture of Enterobacter cloacae and/or Micrococcus luteus. For the stage-specific study, adult males, females, and fourth instar larvae were collected with or without bacterial challenge. The vitellogenic mosquitoes were collected prior to blood feeding. The unfertilized eggs were obtained by inseminating virgin female mosquitoes with fertilized males. The vitellogenic mosquitoes were collected 1 and 2 days after blood feeding. The unfertilized eggs were collected 1 day after egg laying. Total RNA was prepared by the TRIzol technique (Invitrogen). Samples of 2 μg of total RNA were separated on a formaldehyde gel, blotted, and hybridized with a corresponding DNA probe. RT-PCR was performed by using the Titan one-step RT-PCR kit (Roche Diagnostics) with samples of 0.2 μg of total RNA as templates. Each reaction contained 2 μg of total RNA and 1× Taq DNA polymerase (Promega). The reverse transcription reaction was performed at 42 °C for 30 min followed by 2 min at 94 °C for denaturation and 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min.

**Electrophoretic Gel Mobility Shift Assay (EMSA)**—Each protein was synthesized by a coupled in vitro transcription-translation (TnT) system (Promega). The corresponding cDNA clones were subcloned into pcDNA3.Zeo (+) (Invitrogen). The in vitro transcription-translation reactions programmed by the circulam plasmid DNA utilized the T7 promoter. To confirm the synthesis of proteins with expected size, the control TnT reactions of each protein were performed in the presence of [35S]methionine, and the resulting reactions were analyzed by SDS-PAGE and autoradiography. The annealed deoxyoligonucleotide (5′-CTGGATATTCTTGCAGTCAAGAA-3′) specific to the Dl coding region was used for making transformation vectors for each NF-κB protein.

**Transfection Assay in Drosophila mnb-2 Cell Line**—Coding region sequences of A. aegypti, REL1 (Relish C8 cDNA, which encodes truncated Rel-type protein without N-terminal transactivation domain, Ref. 29), and REL2 (Rel-type cDNA with N-terminal transactivation domain of Aedes REL2, Ref. 29), AaREL1-A (D4 cDNA), and AaREL1-B (A RT-PCR product with open reading frame of 844 amino acids), were PCR-amplified and inserted into pMVT5-His (Invitrogen). They were used for making transformation vectors for each NF-κB protein. Drosophila mnb-2 cells were seeded at 2 × 106 cells/ml in 35-mm plates of Schneider’s medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen) and 1× Gluta Max-1 (Invitrogen). Twenty-four hours later, the cells were co-transfected with 2 μg of expression plasmid. The cells were incubated for 4 h in serum-free Schneider’s medium, which was then removed and replaced with complete medium. Overexpression was induced by 48 h of incubation with the addition of 500 μM CuSO4. The immune response was activated by the addition of heat-inactivated E. coli. The Escherichia coli (E. coli) cells were then used to activate the cell line.

**Transfection Assay in Aag-2 Cell Line**—Cell line Aag-2 derived from A. aegypti was maintained in Schneider’s medium supplemented with 10% fetal bovine serum (Hyclone). Aedes REL2+A, AaREL1-A, and AaREL1-B were inserted into the pAex5.IVS/HisA (Invitrogen) vector. Cells were incubated at 26 °C for 36 h prior to transfection. After transfection, the cells were incubated at 26 °C until they reached at least 70% confluence (<24 h). Following transfection, the bacterial
challenge, heat-inactivated E. coli was added to the well and incubated for an additional 4 h, then cells were harvested for RNA extraction.

**Generation of Transgenic Flies—**AaREL1-A cDNA (D4) was subcloned downstream of the upstream activating sequence in piggyBac 3xP3-EGFP-based vector. Transformants were established by microinjection of wild-type Canton-S strain homozygous for white mutation. Two independent *Drosophila* lines were examined, and both produced similar results in overexpression of AaREL1-A transgene driven by Hsp70-GALA control element-using driver line FGalA-Hsp70.PB2, which was kindly provided by the Indiana University Drosophila stock center (Bloomingdon, IN).

**Synthesis and Microinjection of dsRNA—**The synthesis of dsRNA was accomplished by simultaneous transcription of both strands of template DNA with a HiScribe RNAi Transcription Kit (New England Biolabs). The plasmid LITMUS 28iMal containing a nonfunctional portion of the E. coli malE gene that encodes a maltose-binding protein was used to generate control dsRNA. After RNA synthesis, the samples were treated with phenolchloroform extraction and ethanol precipitation. The dsRNA was then suspended in diethyl pyrocarbonate-treated distilled water with a final concentration of 5 μg/μl. The formation of dsRNA was confirmed by running 0.2 μl of these reactions in a 1.0% agarose gel in TBE. A Picospritzer II (General Valve, Fairfield, NJ) was used to add the 3′-truncated region of the D6 cDNA. Both short and long form cDNA clones were 3,433 and 4,110 bp in size, which encoded the same protein. The additional 3′-UTR of 263 bp of D6 cDNA was obtained by 5′-RACE PCR. Two major PCR products, short and long form, obtained by 3′-RACE PCR, added the 3′-truncated region of the D6 cDNA. Both short and long RT-PCRs products were subcloned and additional transcript sequences of 1,290 and 1,967 bp were obtained. The complete short and long form transcripts were 3,433 and 4,110 bp in size, which encoded the same protein. The additional 3′-UTR of 4,110-bp transcript was identical to the 3′-region of D4 cDNA clone, indicating that long form transcript might be the precursor mRNA of the D4-type transcript. The 844-residue protein encoded by these transcripts lacked N-terminal 39 residues that were encoded by the AaREL1-A transcript, which indicated the presence of an alternative transcription start site. We were able to isolate 2,894-bp (844 residues) and 2,968-bp (883 residues) cDNA fragments by RT-PCR that yielded polypeptides with or without N-terminal 39 residues. Northern blot analysis clearly showed that all *A. aegypti* REL1 transcripts used both start sites, which resulted in doublet bands for each alternate transcript (Fig. 2A).

The CTD of the second *A. aegypti* REL1 form (named AaREL1-B) was highly conserved with the rear portion of *Drosophila* DI-B CTD, which possessed trans-activating properties (Fig. 1A). A crucial region for the transactivation properties of *Drosophila* DI-B, located between amino acids 576 and 683, contains two putative acidic activation modules (30). These two modules and a bipartite NLS were highly conserved in the CTds of *Drosophila* and AaREL1-B. A Q-rich region, present in the CTD of *Drosophila* DI-B, was absent in AaREL1-B. The Rel domains of DI-B and AaREL1-B lacked an NLS at their ends. Instead, both DI-B and AaREL1-B had a bipartite NLS in their CTds. This was a common variant for NLS in which a small cluster of basic residues positioned 10–12 residues at the N- terminal of a monopartite-like NLS sequence. The additional binding energy contributed by the upstream cluster of basic residues relaxes the requirements for the downstream monopartite-like sequences (31). As shown in Fig. 1A, the monopartite NLS sequence of AaREL1-A consisted of all basic residues, KRKKKR, whereas the monopartite-like sequence of AaREL1-B was SRKSKK and included an upstream cluster of basic residues, KNKK.

Two overlapping PCR products spanning 13,355 bp in length were isolated and sequenced. This genomic region did not contain a 5′-UTR, N-terminal region (M1-C150), and short 3′-UTR as shown in the D4-type transcript. The sequence result, however, gave us sufficient information about how the RNA was processed in generating the different types of AaREL1 transcripts (Fig. 1C). These exon-intron structures and the alternative splicing of the AaREL1 gene were very similar to the *Drosophila* DI gene (30), showing that both insect genes originated from a common ancestor gene.

The **Inducible AaREL1 Isoform Transcripts Are Expressed in Adult *A. aegypti* Females—**Northern blot analyses were performed to examine the expression of AaREL1 transcripts. Utilization of the total RNA revealed three transcript bands of ~2.5, ~3.5, and ~4.3 kb in size that were constitutively expressed during the normal unchallenged state of the naive larvae in adult males and females (Fig. 2A). The probe from the AaREL1-B-specific CTD region (F400-L568 of AaREL1-B) did not hybridize to the ~2.5-kb band, suggesting that it represented the AaREL1-A transcript (Fig. 2A). According to the sizes of the transcripts, the ~3.5- and ~4.3-kb bands corresponded to the short and long AaREL1-B transcripts, respectively. The expression level of all transcripts in larvae and adult female mosquitoes were more elevated 5 h after septic injury with a mixture of stationary-phase culture of *E. cloacae* and *Micrococcus luteus*. AaREL1 transcripts remained unchanged upon bacterial challenge in adult males.

Without the immune challenge, the expression level of AaREL1 transcripts was very low in the fat body and mainly occurred in the ovary of naive previtellogenic female mosquitoes. The AaREL1 gene expression was elevated after blood feeding as well as after bacterial challenge, and it increased further during egg development. All REL1 transcripts showed the highest level in unfertilized eggs that were dissected from the ovaries of female mosquitoes 3 days post-blood feeding and in 1-day post-oviposition eggs (Fig. 2A). These AaREL1 expression data in the ovary and during vitellogenesis, and in eggs of *A. aegypti*, indicated REL1 participation in the Toll-mediated
FIG. 1. Molecular characterization of two Aedes REL1 isoforms, AaREL1-A, and AaREL1-B. A, amino acid sequence comparison of AaREL1-A and AaREL1-B to Drosophila Dl, Dl-B, and Anopheles REL1 (AgREL1). Alignments were done by ClustalW and were manually adjusted. CTDs with a Gln (Q)-rich or Gln/His (Q/H)-rich region that was shown to be important for transactivation in Drosophila Dl and Dif are
early embryogenesis similar to that of *Drosophila* DI. In addition, *AaREL1* expression was greatly induced after septic injury in the fat body of previtellogenic female mosquitoes, which implicated *AaREL1* involvement in innate immunity at this stage of the mosquito life cycle (Fig. 2A).

Comparative Analysis of *A. aegypti* REL1 Isoforms and REL2 (Relish) Binding to *xb* Motifs—We expressed *AaREL1-A* and *AaREL1-B* by using an *in vitro* coupled transcription-translation assay. We used D4 cDNA for *AaREL1-A* and a 2,894-bp RT-PCR product containing the alternative open reading frame of 844 residues for *AaREL1-B*. *In vitro* translation of D4 cDNA resulted in two REL1-A protein bands, indicating that an alternative start site could be used as the translation initiation codon (data not shown). Only a single major band was observed after *in vitro* translation of the REL1-B RT-PCR product. The sizes of translated proteins matched well with those expected from deduced proteins (data not shown). For *in vitro* translation of *AaREL2*, we used our previous construct (29). Seven *xb* motifs, including those from *Drosophila Cecropin A1* and *A. aegypti Defensin* promoters (29), were designed based on the comparison of promoter sequences of various insect immune genes (Fig. 2B).

In EMSA, *in vitro* expressed DI-A specifically bound to four *xb* motifs among seven tested motifs (Fig. 2B). The highest affinity of *AaREL1-A* binding was to the *xb* motif (I) from *Drosophila CG16978* promoter. This *xb* motif, 5′-GGGAAAT-TCC-3′, from the promoter of *Drosophila* unknown immune gene *CG16978*, matched well to *xb* motif consensus, GGG-(W/nCCM, of *Drosophila zen* ventral repression element (32). This type of *xb* motif was present in the promoter regions of *Drosophila* immune genes whose expression was activated only by the Toll pathway (17). Similar *xb* motifs were found from *Drosophila GNBP-like* (CG13422) and Nee gene promoters (Fig. 2B). The other six sites were representatives of various *xb* motifs found in upstream promoter regions of *Drosophila* and mosquito immune genes (Fig. 2B). The addition of 25-fold excess of the unlabeled specific oligonucleotide (I) effectively completed binding to the labeled probe, whereas the addition of a nonspecific competitor, AP2, affected binding very weakly (Fig. 2C). The competition of *AaREL1-A* binding to motif I by three *xb* motifs (V, VI, and VII) was similar to that of the nonspecific competitor, indicating that binding of *AaREL1-A* to these motifs was nonspecific (Fig. 2D). The binding specificities of *AaREL1-A* and *AaREL2* to various *xb* motifs from insect immune gene promoters were clearly different (Fig. 2B). To test whether *AaREL1-A* and *AaREL2* could bind to *xb* motifs as heterodimers, equal amounts of both these Rel proteins, co-translated *in vitro* (data not shown), were subjected to EMSA. Formation of additional complexes was not detected, indicating that *AaREL1-A* and *AaREL2* bound to tested *xb* motifs only as homodimers (Fig. 2A). Binding activity of *in vitro* translated *AaREL1-B* was not detected with any *xb* motif (data not shown). In addition, its co-translation with either *AaREL1-A* or *AaREL2* did not affect *xb* binding of these two factors.

*Relish* Binding to *Drosomycin* Expression in *Drosophila* mbn-2 Cell Line and Transgenic Flies—*Drosophila* transgenic blood cells (mbn-2 line) can be induced to express *Diptericin* and *Cecropin* genes by the addition of lipopolysaccharide to the culture medium (33, 34). Moreover, expression of the *Drosomycin* gene is highly activated by immune challenge in this cell line (35). We examined the expression of *Drosophila* AMP genes in the presence of exogenous mosquito NF-*xb* proteins. The overexpression of mosquito *AaREL1-A* in mbn-2 cells activated strong expression of the *Drosophila* *Drosomycin* gene in the absence of bacterial challenge (Fig. 3A). The expression pattern of *Diptericin*, *Cecropin*, and *Attacin* genes remained constant during overexpression of any mosquito Rel protein (Fig. 3A, data not shown).

Next, the mutant flies, which overexpressed *AaREL1-A* by the heat shock (hs)-GAL4/UAAS system, were constructed to show *in vivo* activity of *AaREL1-A*. Because of the leaky promoter, *AaREL1-A* had a background expression which could activate the *Drosomycin* gene independent of septic injury with fungal infection (Fig. 3B). The overexpression of *AaREL1-A* by heat shock resulted in an increased expression level of the *Drosomycin* gene but not other *Drosophila* AMP genes such as *Diptericin*, *Cecropin*, and *Attacin* (data not shown). The level of *Drosomycin* expression is significantly higher in heat-shocked flies overexpressing *AaREL1-A* than that fully induced by immune challenge in the presence or absence of bacterial challenge (Fig. 3B). These transgenic flies exhibited increased resistance to the infection with *B. bassiana* spores (Fig. 3C).

*REL1-A* and *REL1-B* Synergistically Activate the Immune Genes in Aag-2 Mosquito Cells—*Aag-2* cell line originated from *A. aegypti* embryonic tissues is responsive to the immune challenges (36). Immune factors such as cecropin, defensin, lysozyme, and transferrin were inducibly expressed in this cell line. *Drosophila Serpin-27A*, which regulates the melanization cascade through the specific inhibition of the prophenoloxidase-activating enzyme, is an acute immune-responsive gene mainly regulated by the Toll pathway (37). We have isolated the mosquito homologues of *Drosophila Serpin-27A* from *A. aegypti.* In addition to *Aedes Cecropin A* and *Defensin A*, the expression of mosquito *Serpin-27A* was induced by heat-killed bacteria in *Aag-2* cells (Fig. 4A).

Transfection of *AaREL1-A* alone in *Aag-2* cells could weakly activate mosquito immune genes. However, when both *AaREL1-A* and *AaREL1-B* were co-transfected together, the same immune genes were strongly activated (Fig. 4A). This experiment showed that *AaREL1-B* acted cooperatively with *AaREL1-A* in enhancing the immune gene activation. This response was similar to that observed for *Drosophila DI-B*, which increased *in vitro* activation of *xb*-like promoters when it was co-expressed with *DI-A* (30). Because *AaREL1-B* did not bind to any *xb* motifs directly, it might act as a cofactor to *AaREL1-A*.

*Aedes REL1 Regulates Antifungal Immune Response in Vivo—*We tested the expression profiles of several immune genes in *A. aegypti* mosquitoes after infection with Gram-negative bacteria (*E. cloacae*), Gram-positive bacteria (*M. luteus*), and fungal spores (*B. bassiana*). The expression levels of all tested immune genes were highly elevated after any type of infection. However, the specific response of *A. aegypti REL1* and *Serpin-27A* was elicited only by the fungal challenge (Fig. 4B).
FIG. 2. Molecular characterization of *Aedes* REL1. A, expression of *Aedes* REL1 transcripts during infection and embryo development. The DNA sequence of RHDs coding regions, which were identical in both D4 and D6 cDNAs, was used as a probe. L, fourth instar larvae; M, adult male; F, adult female; C, naïve; FB, fat body; OV, ovary; 5 or 24, 5 or 24 h after septic injury with the suspension mixture of *E. cloacae* and *M. luteus*. Right panel, a DNA fragment encoding AaREL1-B-specific CTD was used as a probe. *Aedes* actin gene was used to reprobe the same blot as an RNA loading control. B, mobility shift assay for the binding of AaREL1-A and AaREL2 to various insect NF-κB motifs. Each NF-κB motif used in this experiment was listed in this figure. The putative NF-κB binding sites from various insect AMP and other immune genes were collected. Motifs similar in their sequences and patterns were grouped, and the representative NF-κB motif from each group was used for EMSA assay. D4 cDNA (AaREL1-A) and AaREL2 C8 cDNA (29) were used for in vitro transcription-coupled translation. The binding affinity of seven representative NF-κB binding motifs to AaREL1-A and AaREL2 were compared. Equal quantities of D4 and C8 cDNAs were transcribed in one reaction to test any interaction between two NF-κB proteins. The unbound primers were shown as the labeling and loading control of each NF-κB motif (Input). C, the binding of AaREL1-A to κB motif (I) from the *Drosophila CG16978* gene promoter is almost abolished by the addition of 25-fold (25×) of unlabeled specific competitor but not by the nonspecific probe AP2. D, relative binding affinity of each κB motif (I–VII) to AaREL1-A was shown by competition assay. The binding of AaREL1-A to κB motif (I) from the *Drosophila CG16978* gene promoter was competed with 25-fold unlabeled κB motifs (I–VII).
The flies (only hs-GAL4 mycinspores. Wild-type (B. bassiana) after the septic injuries with Cells expressing each NF-κ expression of AaREL1-A rp49 shutoff; (Drosomycin to be partially affected by the IMD pathway after bacterial challenge. Moreover, the tissue-specific expression in female mosquitoes, the enriched ovary expression in the naïve state, and the elevated fat body expression after septic injury suggest AaREL1 as an immune factor of female mosquitoes and a morphogen during egg development.

Although many complications have been reported concerning specificity of response and gene regulation by IMD or Toll pathways (24), the fungal-specific late induction of Drosomycin expression and antifungal activity against some fungi including B. bassiana are still two representative characteristics of the Drosophila Toll immune pathway. We overexpressed mosquito AaREL1-A in both in vivo and in vitro Drosophila systems and examined the expression of Drosomycin in their native chromosomal environment. Our results demonstrated that AaREL1-A could fully up-regulate Drosomycin expression prior to bacterial challenge. In addition, the ectopic expression of AaREL1-A increased the resistance against B. bassiana in transgenic flies. In Drosophila S2 cells, the Drosomycin expression was more affected by Dif, than by Dl (39). Moreover, Dif, not Dl, was sufficient to mediate the induction of Drosomycin and antifungal immunity against B. bassiana in mutant adult flies (22, 23). The characteristics of AaREL1 in both a Drosophila cell line and transgenic flies clearly showed that AaREL1-A, a mosquito Dl homologue, could function as Drosophila Dif in antifungal immune response. However, there were differences between AaREL1 and Drosophila Dif and/or Dl. The expression of Dif or Dl prior to lipopolysaccharide incubation caused a modest up-regulation of Drosomycin expression in S2 cells, and, after lipopolysaccharide treatment, further up-regulated the expression (39). With immune challenge, the overexpression of Dif or Dl under a heat shock promoter rescued the lack of Drosomycin inducibility in larval fat body cells of TW119 mutant flies with a deficiency uncovering both Dif and Dl genes (40). In contrast, the overexpression of AaREL1-A fully up-regulated the Drosomycin expression without the immune challenge, and no further up-regulation was found after the challenge. These results suggest that AaREL1 might not interact with some components of the Drosophila Toll pathway. In Drosophila loss-of-function mutant of cactus, a Drosophila I-kB inhibitor specific to the Toll pathway, the Drosomycin genes are constitutively transcribed (14, 41), and the overexpression of Drosophila Dl could fully activate the Drosomycin expression without immune challenge (40).

Because of the absence of Drosomycin homologue in mosquitoes, we surveyed other mosquito immune genes possibly regulated by Toll pathway. In general, the Drosophila immune genes dependent upon Toll pathway show a specific activation profile by fungal challenge. Drosomycin expression was shown to be partially affected by the IMD pathway after bacterial infection but to be regulated predominantly by the Toll path-

**FIG. 3.** Ectopic expression of AaREL1-A in Drosophila mbn-2 cells and transgenic flies affects on Drosophila antifungal immunity. A. AaREL1-A activates Drosomycin (Drs) gene expression in Drosophila mbn-2 cells. Drosomycin expression was induced upon heat shock promoter rescued the lack of Drosomycin inducibility in larval fat body cells of TW119 mutant flies with a deficiency uncovering both Dif and Dl genes (40). In contrast, the overexpression of AaREL1-A fully up-regulated the Drosomycin expression without the immune challenge, and no further up-regulation was found after the challenge. These results suggest that AaREL1 might not interact with some components of the Drosophila Toll pathway. In Drosophila loss-of-function mutant of cactus, a Drosophila I-kB inhibitor specific to the Toll pathway, the Drosomycin genes are constitutively transcribed (14, 41), and the overexpression of Drosophila Dl could fully activate the Drosomycin expression without immune challenge (40).

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**DISCUSSION**

We have investigated the contribution of REL1 in the antifungal immune defense in adult females of A. aegypti mosquitoes. This investigation has been prompted by the lack of a homologue of Drosophila Dif in mosquitoes (9). Dif is the immune factor responsible for the antifungal immune defense in adult Drosophila (12, 13). In this report, we have cloned and characterized a homologue to Drosophila DI from the yellow fever mosquito A. aegypti, which we named AaREL1. Although A. gambiae REL1 (Gambf1) has previously been cloned, the conclusive in vivo data on its role in adult A. gambiae had been lacking (28). Our study has convincingly demonstrated that mosquito AaREL1 serves as a functional Dif analog in adult A. aegypti females.

The AaREL1 expression profile is different from Drosophila DI. In Drosophila, DI transcripts were previously shown to be markedly enhanced in adult males upon bacterial challenge (20). In a later study by Gross et al. (30), Drosophila DI-B transcript was increased upon bacterial challenge in larvae and adult males. AaREL1 transcripts, however, were elevated in larvae and adult females after bacterial challenge. Moreover, the tissue-specific expression in female mosquitoes, the enriched ovary expression in the naïve state, and the elevated fat body expression after septic injury suggest AaREL1 as an immune factor of female mosquitoes and a morphogen during egg development.

Next, dsRNA complementary to the RHD of AaREL1 was synthesized in vitro and injected into the thorax of newly emerged female mosquitoes. When AaREL1 dsRNA was introduced into the mosquitoes, the mRNA level of both AaREL1 transcripts greatly decreased (Fig. 4C). The mRNA quantity of A. aegypti Serpin-27A in AaREL1 dsRNA-treated mosquitoes also significantly declined, suggesting that AaREL1 was involved in the regulation of A. aegypti Serpin-27A gene expression. The mRNA level of AaREL2 was not affected by the treatment of AaREL1 dsRNA.

Genetic analyses have shown that Drosophila Toll pathway mutants are sensitive to fungal infection (13). To address the role of Aedes Dorsal homologue in mosquito immune response, the susceptibility of either AaREL1 or AaREL2 dsRNA-treated mosquitoes was compared after bacterial or fungal challenge. After 2–4-day recovery following injection with either dsRNA, the treated mosquitoes were challenged by either the spores of entomopathogenic fungus, B. bassiana, or the mixture of E. cloacae and M. luteus. As illustrated in Fig. 4D, the AaREL1 dsRNA-treated mosquitoes were significantly more sensitive to the fungal infection than AaREL2 or MalE dsRNA-treated mosquitoes. In contrast, only AaREL2 dsRNA-treated mosquitoes were susceptible to the bacterial challenge (Fig. 4E). We did not observe any increased susceptibility to Gram-positive bacteria in either AaREL1 or AaREL2 dsRNA-treated mosquitoes (data not shown).

**FIG. 3.** Ectopic expression of AaREL1-A in Drosophila mbn-2 cells and transgenic flies affects on Drosophila antifungal immunity. A. AaREL1-A activates Drosomycin (Drs) gene expression in Drosophila mbn-2 cells. Drosomycin expression was induced upon heat shock promoter rescued the lack of Drosomycin inducibility in larval fat body cells of TW119 mutant flies with a deficiency uncovering both Dif and Dl genes (40). In contrast, the overexpression of AaREL1-A fully up-regulated the Drosomycin expression without the immune challenge, and no further up-regulation was found after the challenge. These results suggest that AaREL1 might not interact with some components of the Drosophila Toll pathway. In Drosophila loss-of-function mutant of cactus, a Drosophila I-kB inhibitor specific to the Toll pathway, the Drosomycin genes are constitutively transcribed (14, 41), and the overexpression of Drosophila Dl could fully activate the Drosomycin expression without immune challenge (40).
way during fungal infections (17, 23). In addition, the microarray analysis shows that the Toll pathway controls most of the late genes induced by fungal infection (17). To find the mosquito immune genes regulated by Toll pathway, A. aegypti females were challenged by different types of microorganisms (E. cloacae, M. luteus, and B. bassiana spore). The results showed that the expression of Aedes Serpin-27A was elicited specifically by the fungal challenge, suggesting regulation by the Toll pathway similar to Drosophila Serpin-27A (37).

The increased fungal susceptibility of AaREL1 dsRNA-treated mosquitoes more clearly indicated that AaREL1 is an essential factor of the Toll antifungal immune response. Our dsRNA knock-down results have demonstrated that mosquito antifungal immune response by AaREL1 can be distinguished from the immune response against Gram-negative bacteria mediated by AaREL2, previously reported as a key regulator of mosquito IMD pathway (38). Transgenic mosquitoes with a stable dominant negative immune-deficient phenotype for AaREL2 showed a marked susceptibility to Gram-negative bacteria infection, which severely compromised induction in the expression levels of both Defensin A and Cecropin A. This indicated that the IMD pathway is generally conserved between Drosophila and mosquitoes (38). Our RNAi experiments demonstrated the AaREL1 regulated antifungal immune response, distinctly separated from the IMD pathway immune response regulated by REL2.

In vitro comparative binding studies, transfection assays in both Drosophila mbn-2 cells and mosquito A. aegypti Aag-2 cells, and the ectopic overexpression in transgenic flies provide conclusive evidence to implicate AaREL1 as a Dif analog of the Toll-mediated antifungal immune pathway in adult female mosquitoes. In addition, in vivo studies by the RNAi knock-outs in mosquitoes strongly suggest that AaREL1 serves as a key regulator of mosquito Toll immune pathway but not as a redundant factor of the immune response.

REFERENCES
1. Beier, J. C. (1998) Annu. Rev. Entomol. 43, 519–543
2. Bremen, J. (2001) Am. J. Trop. Med. Hyg. 64, (suppl.) 1–11
3. Beaty, B. (2000) Proc. Natl. Acad. Sci. U. S. A., 97, 10295–10297
4. Enserink, M. (2002) Science 297, 1988–1989
5. Wattam, A. R., and Christensen, B. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6502–6505
6. Curtis, C. P., and Lines, J. D. (2000) Parasitol. Today 16, 119–121
7. Lyette, G. J., and Kafatos, F. C. (2002) Nature 417, 387–388
8. Holt, R. A. Subramanian, G. M., Halpern, A., Sutton, G. G., Carlab, R., Nusskern, D. R., Wincker, P., Clark, A. G., Ribeira, J. M., Wides, R., Salzberg, S. L., Loftus, B., Yandell, M., Majors, W. H., Rusch, D. B., Lai, Z., Kraft, C. L., Abril, J. F., Anthourard, V., Arensburger, P., Atkinson, P. W., Baden, H., de Berardinis, V., Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R., Boscus, D., Barnstead, M., Cai, X., Center, A., Chaturverdi, R., Christophides, G. K., Chrystal, M. A., Clamp, M., Cravek, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, C. A., Flannigan, M., Grundschopfer-Freimoser, A., Frideli, L., Gu, Z., Guan, P., Guigo, R., Hillmenner, M. E., Hudan, S. L., Hogan, J. R., Hong, Y. S., Hoover, J., Jaillen, O., Ke, Z., Kodira, C., Kokoza, E., Koots, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J. J., Lobo, N. P., Lopez, J. R., Malek, J. A., McIntosh, T. C., Meister, S., Miller, J., Moharry, C., Mongin, E., Murphy, S. D., O’Brochta, D. A., Pfannkoch, C., Qi, R., Regier, M. A., Remington, K., Shao, H., Sharakhova, M. V., Sitter, C. D., Shetty, J., Smith, T. J., Strong, R., Sun, J., Thomasova, D., Ton, L. G., Topalis, P., Tu, Z., Unger, M. F., Walenz, B., Wang, A., Wang, J.,
Aedes REL1 in Mosquito Immunity

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Wang, M., Wang, X., Woodford, K. J., Wortman, J. R., Wu, M., Yao, A., Zdobnov, E. M., Zhang, H., Zhao, Q., Zhao, S., Zhu, S. C., Zhimulev, I., Coluzi, M., della Torre, A., Roth, C. W., Louis, C., Kafatos, F. C., Collins, F. H., and Hoffmann, S. L. (2002) *Science* **298**, 129–149

9. Christophides, G. K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blass, C., Brey, P. T., Collins, F. H., Danielli, A., Dimopoulos, G., Hetru, C., Hoa, N. T., Hoffmann, J. A., Kanisz, S. M., Letunic, I., Levashina, E. A., Loukeris, T. G., Lycett, G., Meister, S., Michel, K., Moita, L. F., Muller, H. M., Osta, M. A., Paskewitz, S. M., Reichhart, J. M., Rahetsky, A., Trefler, L., Vlachou, D., Volz, J., von Mering, C., Xu, J., Zheng, L., Bork, P., and Kafatos, F. C. (2002) *Science* **298**, 159–165

10. Hultmark, D. (2003) *Curr. Opin. Immunol.* **15**, 12–19

11. Hoffmann, J. A. (2003) *Nature* **426**, 33–38

12. Lemaitre, B., Reichhart, J. M., and Hoffmann, J. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14614–14619

13. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996) *Cell* **86**, 973–983

14. Levashina, E. A., Ohresser, D., Lemaître, B., and Imler, J. L. (1998) *J. Mol. Biol.* **278**, 515–527

15. Hedengren, M., Asling, B., Dushay, M. S., Ando, I., Ekengren, S., Wihlborg, M., and Hultmark, D. (1999) *Mol. Cell* **4**, 827–837

16. Leulier, F., Rodriguez, A., Khush, R. S., Chen, P., Abrams, J. M., and Lemaitre, B. (2000) *EMBO Rep.* **1**, 353–358

17. De Gregorio, E., Spellman, P. T., Tzou, P., Rubin, G. M., and Lemaître, B. (2002) *EMBO J.* **21**, 2568–2579

18. Drier, E. A., and Steward, R. (1997) *Semin. Cancer Biol.* **8**, 83–92

19. Pan, D., and Courrey, A. J. (1999) *EMBO J.* **18**, 1387–1402

20. Reichhart, J. M., George, S., Meister, M., Lemaître, B., Kappler, C., and Hoffmann, J. A. (1993) *C. R. Acad. Sci. (Paris)* **316**, 1218–1224

21. Ip, Y. T., Roth, M., Engstrom, Y., Kadaliyil, L., Cui, H., Gonzalez-Crespo, S., Tatei, K., and Levine, M. (1999) *Cell* **75**, 753–763

22. Meng, X., Khanuja, B. S., and Ip, Y. T. (1999) *Genes Dev.* **13**, 792–797

23. Rutschmann, S., Jung, A. C., Hetru, C., Reichhart, J. M., Hoffmann, J. A., and Ferrandon, D. (2000) *Immunity* **12**, 569–580

24. Brennan, C. A., and Anderson, K. V. (2004) *Annu. Rev. Immunol.* **22**, 457–483

25. Qiu, P., Pan, P. C., and Govind, S. (1999) *Proceedings of the National Academy of Sciences of the United States of America* **96**, 12999–13004

26. Canton, Y., and Nappi, A. J. (2003) *Immunogenetics* **55**, 157–164

27. Osta, M. A., Christophides, G. K., Vlachou, D., and Kafatos, F. C. (2004) *J. Exp. Biol.* **207**, 2551–2563

28. Brillas-Mury, C., Charlesworth, A., Gross, I., Richamn, A., Hoffmann, J. A., and Kafatos, F. C. (1999) *EMBO J.* **18**, 1491–1497

29. Shim, S. W., Kokoza, V., Ahmed, A., and Raikhel, A. S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 9978–9983

30. Gross, I., George, P., Oertel-Buchheit, P., Schnarr, M., and Reinhard, J. M. (2000) *Gene (Amst.)* **249**, 233–242

31. Hodel, M. R., Corbett, A. H., and Hodel, A. E. (2001) *J. Biol. Chem.* **276**, 1317–1325

32. Markstein, M., Markstein, P., Markstein, V., and Levine, M. S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 763–768

33. Samakovlis, C., Aeling, B., Boman, H. G., Gateff, E., and Hultmark, D. (1992) *Biochem. Biophys. Res. Commun.* **188**, 1169–1175

34. Kopper, C., Meister, M., Lagueux, M., Gateff, E., Hoffmann, J. A., and Reichhart, J. M. (1993) *EMBO J.* **12**, 1561–1568

35. Lindmark, H., Johansson, K. C., Steven, S., Hultmark, D., Engstrom, Y., and Soderhall, K. (2001) *J. Immunol.* **167**, 6920–6923

36. Fallon, A. M., and Sun, D. (2001) *Insect. Biochem. Mol. Biol.* **31**, 263–278

37. De Gregorio, E., Han, S. J., Lee, W. J., Baek, M. J., Osaki, T., Kawahata, S., Lee, B. I., Iwanaga, S., Lemaître, B., and Brey, P. T. (2002) *Dev. Cell* **3**, 581–592

38. Shin, S. W., Kokoza, V., Lobkov, I., and Raikhel, A. S. (2003) *Proceedings of the National Academy of Sciences of the United States of America* **100**, 2616–2621

39. Han, Z. S., and Ip, Y. T. (1999) *J. Biol. Chem.* **274**, 21355–21361

40. Manfruelli, P., Reichhart, J. M., Steward, R., Hoffmann, J. A., and Lemaître, B. (1999) *EMBO J.* **18**, 3380–3391

41. Nicolas, E., Reichhart, J. M., Hoffmann, J. A., and Lemaître, B. (1998) *J. Biol. Chem.* **273**, 10463–10469