Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito
Anopheles gambiae

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We show that, in the malaria vector Anopheles gambiae, expression of Cecropin 1 is regulated by REL2, an NF-κB-like transcription factor orthologous to Drosophila Relish. Through alternative splicing, REL2 produces a full-length (REL2-F) and a shorter (REL2-S) protein isoform lacking the inhibitory ankyrin repeats and death domain. RNA interference experiments show that, in contrast to Drosophila Relish, which responds solely to Gram-negative bacteria, the Anopheles REL2-F and REL2-S isoforms are involved in defense against the Gram-positive Staphylococcus aureus and the Gram-negative Escherichia coli bacteria, respectively. REL2-F also regulates the intensity of mosquito infection with the malaria parasite, Plasmodium berghei. The adaptor IMD shares the same activities as REL2-F. Microarray analysis identified 10 additional genes regulated by REL2, including CEC3, GAM1, and LRIM1.

From insects to mammals, NF-κB-like transcription factors play central roles in induction and regulation of innate immune responses. In Drosophila, three NF-κB-like proteins have been identified. Dorsal is largely engaged in early developmental processes, whereas Relish and Dif are involved in immune signaling leading to transcriptional activation of genes, including those encoding antimicrobial peptides (AMPs) (1). The Toll pathway, which is used, principally, to counteract infections by fungi (2) and Gram-negative bacteria, is regulated by the immune signaling pathways leading to transcriptional activation of AMPs (3), including Cecropins A and B, Drosomycin (4, 5). In contrast, the immunodeficiency (Imd) pathway primarily mediates defense against the Gram-positive bacterium Staphylococcus aureus, whereas REL2-S is involved in defense against the Gram-negative bacterium Escherichia coli. Through alternative splicing, Anopheles uses a single NF-κB gene (REL2) to mediate immune reactions, for which Drosophila employs two distinct genes, Relish and Dif. We show that the Anopheles Imd pathway leading to REL2-F activation is also involved in limiting Plasmodium parasite infections. Thus, a classical immune signaling pathway is implicated in parasite survival in the Anopheles mosquito vector. Finally, REL2-F interacts with CEC1 or KIN1 reporter (300 ng per well) and reference

Materials and Methods
cDNA Production. Total RNA was extracted from 25 larvae, pupae, or adult Anopheles gambiae and ~5,000 embryos with TRIzol reagent (Invitrogen) and treated with DNaseI, and 0.2 μg were used for reverse transcription using the Omniscript kit (Qiagen, Santa Clarita, CA).

dsRNA Construction. PCR amplicons tailed with T7 promoter sequences (see Table 2, which is published as supporting information on the PNAS web site) were used to synthesize dsRNAs with the MEGAshortscript kit (Ambion) that were DNaseI-treated, cleaned up by phenol:chloroform extraction, precipitated with isopropanol, and resuspended in nuclease-free water.

Luciferase Assays. Cells were plated in 24-well plates and, upon reaching ~70% confluence, were incubated for 30 min at room temperature with 7 μg of dsRNA in FBS-free medium. Transfections with CEC1 or KIN1 reporter (300 ng per well) and reference
Gene Silencing in Adult Anopheles gambiae. One- to two-day-old adult female mosquitoes were injected with 207 ng of dsRNA, as described in ref. 15, and, after 4 days, was subjected to infection assays.

Immune Challenge in Mosquito Cell Lines. Lipopolysaccharides from E. coli O26:B6 (Sigma Aldrich) were dissolved in water and added to cells at 10 μg/ml. Micrococcus luteus and E. coli (DH5α) bacteria and Beauveria bassiana spores (American Type Culture Collection) were heat-inactivated by boiling for 5 min in PBS and added to cells at 100 microbes per cell.

Bacterial and Malaria Infection of Adult Anopheles gambiae. Bacteria were grown to OD600 = 0.7, precipitated, washed, and resuspended in PBS. Bacterial suspensions of 69 nl (E. coli at OD600 = 0.01 and S. aureus at OD600 = 0.4) were injected into the mosquito thorax with a nanojector (Nanoject, Drummond Scientific, Broomall, PA). For malaria infections, mosquitoes were fed for 30 min at 21°C and infected with Plasmodium berghei (16, 17).

Quantitative RT-PCR. cDNA was produced as described above. Transcript abundance was measured with an Applied Biosystems 7500 Real-Time PCR system according to the manufacturer’s instructions. PCRs of 25 μl consisted of 1X SYBR green mix, 900 nM forward and reverse primers, and cDNA, corresponding to 0.02 μg of total RNA. Primer sequences are shown in Table 2.

Microarray Analysis. Slides were printed as described in ref. 18; prehybridized in 5X standard saline citrate (SSC) (1X SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), 0.1% SDS, and 1% BSA at 42°C for 45 min; washed at room temperature with deionized water; dipped in 100% isopropanol; and dried. mRNA samples were prepared by using the PolyTract isolation system (Promega) and labeled as described in ref. 19. Equal volumes of Cy-3- and Cy-5-labeled samples were combined in hybridization buffer supplemented with poly(A)-DNA and hybridized to microarray slides at 42°C overnight. Hybridized arrays were washed, air-dried, and scanned by using the ScanArray 3000 (GSI Lumonics, Billerica, MA). Two independent experiments with dye swaps were performed. Expression ratios were calculated and analyzed by using the TM4 microarray software package (www.tigr.org/software/tm4/). After spot quality filtering, expression data were normalized by using the Lowess (Locfit) algorithm, and clustered with k-mean and hierarchical clustering with the program TMEV (The Institute for Genomic Research) (19). Elements with at least 500 signal intensity values were considered.

Results

Genomic Organization and Expression of REL2. RT-PCR and RACE using RNA from adult female Anopheles gambiae were used to determine the structural organization of REL2. This gene consists of 11 exons, encompassing 10.9 kb (Fig. 1). It encodes putative protein(s) with a glutamine- and histidine-rich region, potentially implicated in protein–protein interactions, followed by RHD, IPT/TIG (DNA binding), ANK and death domains, and a nuclear localization signal.

We detected two REL2 transcripts: full-length REL2-F, and a shorter form, REL2-S, encompassing a unique exon at its 3’ end. For mRNA splicing, this exon uses a splice acceptor that is 4 bp 3’ upstream to that used in REL2-F and results in a downstream early termination codon. Thus, REL2-F encodes a protein with all of the above features, whereas REL2-S is missing the ANK and death domains. Additional alternative splicing at the 5’ end of REL2 may also produce two transcript variants for each form (Fig. 1C). REL2-F shows significant similarity to the Aedes (55%) and Drosophila (29%) Relish (see Fig. 7, which is published as supporting information on the PNAS web site).

REL2-F and -S transcripts are expressed constitutively throughout Anopheles gambiae development, albeit at different levels: REL2-F is expressed more strongly (Fig. 2A). Both transcripts are also expressed in cultured cell lines, Sua1B (Fig. 2B) and 4a3A (data not shown). Cells challenged with heat-killed E. coli (Gramnegative) or purified E. coli lipopolysaccharides (LPS) up-regulate
REL2-F significantly and REL2-S to a lower level. We cannot exclude the possibility that induction after LPS challenge was due to the common peptidoglycan contaminants of LPS preparations (20). No significant up-regulation was detected after cell challenge with the fungus Beauveria bassiana or the Gram-positive bacterium M. luteus.

Expression of CEC1 Is Regulated by REL2-F. A putative AMP target gene of Anopheles immune signaling pathways is CEC1 (previously designated as Cecropin A) (14, 21). Compared with Drosophila Cecropins, which are mostly active against Gram-negative bacteria, CEC1 has a wide spectrum of antibacterial activities and is induced by Plasmodium and both Gram types of bacteria (21). Sua1B and 4a3A cells have high levels of constitutive CEC1 expression that are further enhanced by treatment with heat-killed E. coli or M. luteus (data not shown). We examined the effect of REL proteins on CEC1 gene expression in Sua1B cells after transfection with a CEC1-promoter–luciferase-reporter construct (14), silencing of REL1 and REL2 genes by RNAi, and subsequent monitoring of luciferase activity. Targeting either the RHD or ANK domains of REL2 resulted in substantial reduction of luciferase activity, typically 5- to 10-fold. In contrast, REL1 knockdown (KD) had no significant effect (Fig. 3A).

Because Anopheles gambiae shows no transitive RNAi effects, i.e., dsRNAs are effective only against mRNAs bearing the corresponding sequences (22), targeting the RHD should silence both REL2 forms, whereas ANK-domain KD should affect only REL2-F. We observed that KDs using ANK dsRNA result in a major (>91%) reduction in CEC1-promoter expression, essentially indistinguishable from that observed with RHD dsRNA. Thus, it appears that REL2-F largely controls constitutive CEC1-promoter expression in Sua1B cells, whereas REL2-S has no major effect (Fig. 3A). Similar results were obtained for the 4a3A cells (data not shown). Proteolytic cleavage is necessary to remove the inhibitory ANK domain of Drosophila Relish, and this processing would also be expected for REL2-F activation. By extension, these results suggest that REL2-F is constitutively activated in these mosquito cells.

Other Anopheles orthologues of Drosophila Imd-pathway components, the Imd adaptor (IMD) and the caspase Dredd (CASPL1), were identified in ref. 11; orthologues of IKKβ (IKK1) and IKKγ (IKK2) are reported here (see Table 3, which is published as supporting information on the PNAS web site). To examine the degree of functional Imd-pathway conservation between Anopheles and Drosophila, we silenced these genes in Sua1B and 4a3A cells by using RNAi and observed significant down-regulation (Fig. 3 B and C). In both cell lines, IMD KD reduced CEC1-promoter expression (∼60% and 68%, respectively), although not as efficiently as REL2 KD. This is consistent with evidence from Drosophila imd mutants, in which Cecropin A expression is reduced but not abolished (23).

Surprisingly but consistently, silencing of CASPL1 or IKK1 or IKK2 had opposite effects in the two cell lines. In Sua1B cells, KD of CASPL1, but not of IKK1 or IKK2, substantially reduced CEC1 promoter expression, whereas, in 4a3A cells, KD of IKK1 or IKK2, but not of CASPL1, reduced CEC1 promoter expression. We used semiquantitative RT-PCR to estimate the level of gene silencing (Fig. 4A). Importantly, KD of IKK1 and IKK2 in Sua1B was highly effective, suggesting that these genes are not implicated in the regulation of CEC1 in Sua1B cells. KD of CASPL1 in the same cells was less effective (∼40%) and, yet, associated with substantial reduction in CEC1 activity (Fig. 3B). A plausible explanation that requires further investigation is that IKK1/IKK2 and CASPL1 are implicated in alternative branches.
of the CEC1-activation pathway that are differentially active in these two cell types.

**REL2 Regulates the Expression of AMPs and Other Immunity Genes.**

We performed a DNA microarray analysis (18) to identify genes regulated by REL2 in *Anopheles* 4a3A cells by comparing the expression profiles of cells treated with either REL2 (RHD domain) or control lacZ dsRNAs. Cells were challenged, 24 h after dsRNA addition, by a 12-h exposure to heat-killed *E. coli*, and expression profiles were assessed at 0, 2, 6, and 12 h. From 3,840 EST clones present on the microarray, nine were consistently down-regulated in REL2 KD cells, and these genes have been categorized previously as defense/immunity-related (24). They encode the AMPs CEC3 and GAM1 (previously CecB and gambicin, respectively), the serine protease CLIPB14, another two CLIP-domain serine proteases, a fibrinogen-domain lectin, a Brix-domain protein implicated in ribosome bio-

**REL2 Is Required for Antibacterial Defense of Adult *Anopheles gambiae*.** We investigated, by RNAi analysis, the possible role of REL2 and other components of the *Imd* pathway in defense against bacterial infection in adult mosquitoes. dsRNA was injected into newly emerged adult females that were infected, 4 days later, with bacteria representative of the Gram-positive (*S. aureus*) or the Gram-negative (*E. coli*) types, as described in ref. 15. Real-time quantitative RT-PCR confirmed that RHD dsRNA injection silences both REL2-S and REL2-F transcripts equally (∼50%), whereas ANK dsRNA decreases the levels of only REL2-F (Fig. 4B). Interestingly, REL2-F transcript levels were up-regulated by 1.5-fold in dsANK-injected mosquitoes.

Mosquito survival was assessed daily for 7 days after bacterial infection (Fig. 5). Simultaneous KD of REL2-F and REL2-S transcripts (RHD dsRNA) significantly compromised mosquito defense against both *S. aureus* and *E. coli*. In contrast, KD of REL2-F alone (ANK dsRNA) reduced the survival of *S. aureus*-but not of *E. coli*-infected mosquitoes. Similar results were obtained with IMD KD, suggesting that IMD acts in concert with REL2-F in defense against *S. aureus*. Survival of CAPSL1-KD mosquitoes did not detectably decrease after infection (data not shown). Preliminary results indicated that KD of REL1 had no measurable effect on mosquito survival, suggesting that REL2 is sufficient to confer resistance to bacterial infections.

**The *Anopheles* Imd Pathway Regulates Malaria Parasite Infection.** It has been reported previously that CEC1 and other AMP genes are up-regulated when *Plasmodium* crosses the *Anopheles* midgut (11, 21, 26). Furthermore, our microarray results indicated that REL2 regulates expression of the major *Plasmodium* antagonist LRIM1 (see below). We tested the effect of REL2 and IMD KD on *P. berghei* survival to the oocyst stage in the mosquito midgut. At 4 days after dsRNA treatment, mosquitoes were infected with GFP-marked parasites.

**Table 1. Effect of REL2 pathway gene silencing on parasite development**

<table>
<thead>
<tr>
<th>Gene KD</th>
<th>Midguts</th>
<th>Parasites per midgut</th>
<th>Prevalence, %</th>
<th>Mel, %</th>
<th>P (KS)</th>
<th>P (t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (5)</td>
<td>79</td>
<td>86</td>
<td>87.34</td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMD (5)</td>
<td>79</td>
<td>153</td>
<td>96.20</td>
<td>0.13</td>
<td>0.044</td>
<td>0.017</td>
</tr>
<tr>
<td>Control (4)</td>
<td>49</td>
<td>51</td>
<td>87.76</td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>REL2-RHD (4)</td>
<td>49</td>
<td>104</td>
<td>91.84</td>
<td>4.39</td>
<td>0.147</td>
<td>0.035</td>
</tr>
<tr>
<td>Control (3)</td>
<td>42</td>
<td>82</td>
<td>97.62</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>REL2-ANK (3)</td>
<td>42</td>
<td>166</td>
<td>95.24</td>
<td>4.87</td>
<td>0.093</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Numbers of oocysts and melanized parasites are reported as three experimental datasets for knocked-down genes or REL2 domains and control dsRNA-treated mosquitoes (GFP). The number of replicates is indicated in parenthesis in the first column. Each replicate used different batches of mosquitoes, which were fed on the same infected mouse. Identical numbers of midguts from control and KD mosquitoes were assessed for each replicate (see also Table 5). P values indicate whether the distributions of parasites in KD and control midguts in each dataset are similar and were determined by KS and Student’s t test. Mel, melanized parasites (ookinetes); KS, Kolmogorov–Smirnov test. Parasites per midgut are oocysts and melanized ookinetes.
mosquitoes to Gram-positive S. aureus (A) and Gram-negative E. coli (B) infections after KD of IMD, REL2-RHD, and REL2-ANK. GFP dsRNA-treated mosquitoes were used as a control. The survival assay was repeated at least 3 and up to 10 times per gene. Error bars represent the standard error. 

Fig. 5. Mosquito survival to bacterial infection. Survival of adult mosquitoes infected with GFP-tagged P. berghei. Arrows point to melanized ookinetes.

Discussion

Previously, our comparative genomic analysis of immunity-related genes in the fruit fly Drosophila melanogaster and the malaria mosquito Anopheles gambiae (11) revealed that, in general, intracellular components of immune signaling pathways are well conserved between the two insects. However, differences were noted, particularly in potential components of the Toll pathway, which may indicate important variations in immune signaling. Importantly, the NF-κB-like transcription factor Dif, which activates gene expression upon recognition of Gram-positive bacteria and fungi, has no orthologue in Anopheles. This may imply that in a putative Toll pathway, one of the other two Anopheles NF-κB-like proteins, REL1 (the Dorsal orthologue) or REL2 (the Relish orthologue), may substitute for the role of Dif. Furthermore, Toll genes have evolved through independent gene duplication and sequence divergence, and, thus, the Drosophila Toll receptor has no clear orthologue in Anopheles. In contrast, intracellular components of the Imd pathway, which is activated mainly in response to Gram-negative bacteria, are highly conserved between the two insects. Diversification of proteins implicated in immune recognition upstream of both pathways is very pronounced. This category includes peptidoglycan-recognition proteins (PGRPs) and Gram-negative binding proteins. For example, the Drosophila PGRP-LC, the main receptor of the Imd pathway (27, 28), and its Anopheles orthologue have similar architectures, but their PGRP domains have apparently evolved independently from a single domain-containing ancestral protein (11). Thus, recognition properties of PGRP-LC could differ substantially between the two species. Interestingly, the other Imd-pathway-associated receptor, PGRP-LE, has no Anopheles orthologue.

Here, we provide evidence from detailed gene-structure determination and reverse genetic analysis of function, illuminating the structural and functional evolution of immune signaling in Drosophila and Anopheles gambiae. The first major difference we detected in Anopheles is alternative splicing of the REL2 gene. This gives rise to two isoforms, REL2-F and REL2-S, which are differentially involved in defense against Gram-positive and Gram-negative bacteria, respectively. In this way, the single mosquito gene REL2 mediates alternative immune responses, which, in the fly, require two genes: REL2’s orthologue Relish and Dif. Thus, through posttranscriptional processing of REL2, Anopheles appears to compensate for the absence of Dif.

In addition to this gene substitution, we discovered a switch in the roles of the NF-κB transcriptional factors during the ~250 million years of evolution (29, 30) since the last common ancestor of
Drosophila and Anopheles. The Anopheles REL2-F is used for defense against the Gram-positive bacterium S. aureus, whereas the orthologous and structurally similar Relish in the fruit fly is required for defense against Gram-negative bacteria. Likewise, REL2-S deals with Anopheles defense against the Gram-negative bacterium E. coli, whereas Dif, which, like REL2-S, does not have an inhibitory domain, deals with fruit fly responses against Gram-positive bacteria. However, both REL2-F and Relish mediate their functions in concert with the death-domain adaptor IMD, indicating that the pathway structure has remained conserved.

REL2-F, unlike its fly counterpart Relish but similar to orthologous genes in other higher eukaryotes, encodes a death domain located at the carboxyl terminus of the deduced protein. This domain might be used for oligomerization with the respective domain of IMD or other death-domain-encoding proteins. Pertinent information is available from studies on the Relish gene of Aedes aegypti, the vector of yellow and dengue fever, which belongs to a different mosquito subfamily (Culiciniæ rather than Anopheliniæ). The Relish gene of Aedes is structurally similar to Anopheles REL2, in that it encodes a death domain. Aedes REL2 produces three alternatively spliced transcripts: two are similar to Anopheles REL2-F and REL2-S, and the third encodes a protein encompassing only the N-terminal domain (9).

In Aedes, transgenic overexpression of a truncated version of the REL2-S homologue (C8), which lacks the putative glutamine- and histidine-rich transactivator domain, results in susceptibility to Gram-negative but not to Gram-positive bacteria (31). Additional studies are required to determine whether C8 acts as a dominant-negative allele of REL2-S. In this case, our postulated switch in pathway function would probably have occurred in the common ancestor of anopheline and culicine mosquitoes.

Whether Aedes, also Anopheles, also lacks Dif is not yet resolved. However, recent studies have shown that the Aedes orthologue of REL2 is not involved in defense against bacteria but only against fungal infections (32). Likewise, our preliminary results in Anopheles indicate that REL2 is the only NF-κB factor implicated in the antibacterial defense against E. coli and S. aureus, thus, it is likely that the two mosquitoes use similar strategies to deal with bacterial infections.

Control of NF-κB activation is achieved through inhibitory IκB domains by the use of two alternative strategies. In the fly, RELish contains its own inhibitory IκB domain, deals with fruit fly responses against Gram-negative bacteria (31), whereas Dif, which, like REL2-S, does not have an inhibitory domain, deals with fruit fly responses against Gram-positive bacteria. However, both REL2-F and Relish mediate their functions in concert with the death-domain adaptor IMD, indicating that the pathway structure has remained conserved.

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Control of NF-κB activation is achieved through inhibitory IκB domains by the use of two alternative strategies. In the fly, RELish contains its own inhibitory IκB domain (ANK) located at the carboxyl terminus of the protein, whereas the inhibitory domain for Dif is provided by another protein, Cactus. Accordingly, Anopheles REL2-S, in noninduced condition, might be constitutively acti-