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Characterization of the Rel2-regulated transcriptome and proteome of *Anopheles stephensi* identifies new anti-*Plasmodium* factors

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ABSTRACT

Mosquitoes possess an innate immune system that is capable of limiting infection by a variety of pathogens, including the *Plasmodium* spp. parasites responsible for human malaria. The *Anopheles* immune deficiency (IMD) innate immune signaling pathway confers resistance to *Plasmodium falciparum*. While some previously identified *Anopheles* anti-*Plasmodium* effectors are regulated through signaling by Rel2, the transcription factor of the IMD pathway, many components of this defense system remain uncharacterized. To begin to better understand the regulation of immune effector proteins by the IMD pathway, we used oligonucleotide microarrays and iTRAQ to analyze differences in mRNA and protein expression, respectively, between transgenic *Anopheles stephensi* mosquitoes exhibiting blood meal-inducible overexpression of an active recombinant Rel2 and their wild-type conspecifics. Numerous genes were differentially regulated at both the mRNA and protein levels following induction of Rel2. While multiple immune genes were up-regulated, a majority of the differentially expressed genes have no known immune function in mosquitoes. Selected up-regulated genes from multiple functional categories were tested for both anti-*Plasmodium* and anti-bacterial action using RNA interference (RNAi). Based on our experimental findings, we conclude that increased expression of the IMD immune pathway-controlled transcription factor Rel2 affects the expression of numerous genes with diverse functions, suggesting a broader physiological impact of immune activation and possible functional versatility of Rel2. Our study has also identified multiple novel genes implicated in anti-*Plasmodium* defense.

1. Introduction

Mosquitoes are vectors for many important human pathogens, including viruses, filarial worms, and apicomplexan parasites. A number of *Plasmodium* spp. parasites, vectored exclusively by *Anopheles* spp. mosquitoes, cause human malaria. Because of difficulties in the distribution of anti-malarial chemotherapeutics and the rise of drug resistance in the parasite, vector control remains at the forefront of malaria control efforts. However, after decades of insecticide spraying, bed net distribution, and habitat remodeling, the disease remains established, so novel vector-control methods must be developed. Recently, methods have

Abbreviations: IMD, immune deficiency; PRR, pattern recognition receptors; PAMPs, pathogen associated molecular patterns; CP15, carboxypeptidase gene promoter-driven Rel2 line 15; VG, vitellogenin gene promoter-driven Rel2 line; GO, gene ontology; RNAi, RNA interference; iTRAQ, isobaric tags for relative and absolute quantitation; dsRNA, double-stranded RNA; qRT-PCR, quantitative Real-Time PCR; CFUs, colony forming units; PBM, post-blood meal; PSMs, peptide spectrum matches; AgMDL6, *An. gambiae* MD2-like protein 6; NLRR3, neuronal leucine-rich repeat protein 3; SCRBQ1, scavenger receptor BQ1 domain; AGBP1, bacteria response protein 1; NPC2, Neimann-Pick type C-2; A2MRAP, alpha-2-macroglobulin receptor-associated protein; LRTP, leucine-rich transmembrane protein; CRQ, Croquemort; SRPN10, serine protease inhibitor 10; R2RSP1, Rel2-responsive serine protease 1; R2RSP2, Rel2-responsive serine protease 2; SEPRP1, serine protease precursor 1; TRYPP, trypsin precursor; ACEP, angiotensin converting enzyme precursor; SEPRP2, serine protease precursor 2; GFP, green fluorescent protein; SRPN10, serpin 10; R2RSP1, Rel2-responsive serine protease 1; R2RSP2, Rel2-responsive serine protease 2; SEPRP1, serine protease precursor 1; TRYPP, trypsin precursor; ACEP, angiotensin-converting enzyme precurser; SEPRP2, serine protease precursor 2.

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been developed to generate genetically modified mosquitoes (Ito et al., 2002), and various strategies based on their release are being investigated for malaria control. Conversion of a natural mosquito population to a transgenic population that overexpresses anti-*Plasmodium* immune system activators or effector molecules could represent one such method, and multiple mosquito lines expressing such transgenes in different tissues have already been developed (Dong et al., 2011, 2012).

Mosquitoes possess an innate immune system that is capable of responding to, and controlling, infection by diverse pathogens, including bacteria, viruses, and apicomplexan parasites (Cirimotich et al., 2009; Garver et al., 2009). Two immune pathways, the Toll and immune deficiency (IMD) pathways, have been extensively studied in both *Drosophila* and mosquitoes. Both pathways recognize invading pathogens through the association of host pattern recognition receptors (PRR) with pathogen associated molecular patterns (PAMPs), leading to a signaling cascade, nuclear localization of transcription factors, and subsequent induction of the expression of numerous immune effector molecules and anti-microbial peptides. Invading pathogens are then killed by various mechanisms, such as phagocytosis and complement-like killing.

The nuclear translocation of the NF-κB transcription factor Rel2 leads to an induction of immune gene expression that constitutes the IMD pathway-mediated immune response (Meister et al., 2005). The IMD pathway has been shown to regulate the mosquito's resistance to *Plasmodium falciparum* infection (Garver et al., 2009), and numerous mosquito lines with inducible overexpression of the constitutively active short form of Rel2 have been created (Dong et al., 2011). One such line (henceforth referred to as the CP15 line) uses the carboxypeptidase gene promoter to limit Rel2 overexpression to the midgut following a blood meal, while another line (the VG1 line) overexpresses the same Rel2 transgene under the control of the vitellogenin gene promoter, leading to fat body-specific expression after a blood meal (Dong et al., 2011). Both these lines exhibit a greatly reduced susceptibility to *Plasmodium* infection following an infected blood meal and may represent viable tools for future release as part of a malaria control program. However, immune pathways and their downstream transcription factors can regulate a large variety of both immunity and non-immunity related processes (Dong et al., 2006; Xi et al., 2008). Hence, the overexpression of the Rel2 transcription factor affects the immune system, but it is also likely to regulate other physiological processes entailing genes of diverse functions.

A majority of studies on the insect immune system have relied on infection with a pathogen and observation of the insect's response to the insult (Imroze and Prasad, 2012; McKean et al., 2008; Zerofsky et al., 2005). Using transgenic mosquitoes that overexpress Rel2 in an inducible fashion provides a pathogen-independent system to study IMD pathway-regulated immune response and eliminates any confounding factors brought about by the presence of the infecting organism. Zou and colleagues used a Rel2-overexpressing *Aedes aegypti* to study the IMD pathway-regulated transcriptome (Zou et al., 2011).

We used whole-genome oligonucleotide microarrays to study recombinant Rel2-induced changes in mRNA abundance, as well as isobaric tags for relative and absolute quantitation (iTRAQ) to study changes in protein abundance after Rel2 overexpression in transgenic mosquitoes. Measuring the expression levels of both mRNA and protein allowed us to look for correlations between transcript and protein abundance following up-regulation of a transcription factor. We then used RNA interference (RNAi) assays to investigate a subset of genes, both with and without known immune function, for anti-*Plasmodium* and anti-bacterial activity, leading to the identification of multiple novel anti-*Plasmodium* effectors.

2. Materials and methods

2.1. Mosquito rearing

Anopheles stephensi Liston strain wild-type, CP, and VG transgenic Rel2-overexpressing lines (Dong et al., 2011) were maintained according to standard insectary procedures. In brief, larvae were reared at low densities in trays and fed a combination of ground fish flakes (Tetra) and cat food pellets (Purina). Upon emergence, adults were maintained on a 12 h/12 h light/dark cycle at 27 °C with 80% humidity and constant access to a 10% solution of sucrose in water. In order to stimulate egg production, adults were fed on ketamine-anesthetized mice according to IACUC-approved protocols.

2.2. RNA extraction and microarrays

One-week-old adult female mosquitoes were given a human blood meal from water-jacketed membrane feeders maintained at 37 °C. Mosquito tissues were dissected in sterile PBS as follows: midguts were collected at 6 and 12 h after blood feeding, while fat bodies were collected at 12 and 18 h after the blood meal. Total mosquito RNA from dissected tissues was extracted using RNeasy kits (Qiagen) according to the manufacturer's protocols and quantified on a NanoDrop spectrophotometer before quality assessment on an Agilent Bioanalyzer 2100. Probes were synthesized using 200 ng of RNA and the Low-Input RNA Labeling Kit (Agilent) according to the manufacturer's protocol. These probes were hybridized to a custom-designed Agilent microarray slide, which was scanned with an Axon GenePix 4200AL scanner at 2-μm resolution. After scanning, statistical analysis was performed using the TIGR, MIDAS, and TMEV software packages (Dudoit et al., 2003), following standard laboratory protocol (Dong et al., 2006), and analysis was performed using a *t*-test, with a significance level of $\alpha = 0.05$. Changes in gene expression were considered significant if the absolute value of the gene regulation was ≥ 0.75 on a $\log_2$ scale. For each treatment, three biological replicates and one psuedoreplicate were performed. The array was designed using Array Designer software (Premier Biosoft, www.premierbiosoft.com) and based on an early version of the *An. stephensi* transcriptome obtained from Dr. Jake Tu of Virginia Polytechnic Institute and State University, and putative function and gene ontology (GO) terms were assigned to transcript sequences based on homology to previously annotated *Anopheles gambiae* genes discovered by a blastn search (Altschul et al., 1990). The blast search was performed against gene set AgamP3.7, downloaded from vectorbase.org; for each gene, the most significant hit was used for annotation, with a maximum *e*-value of 0.0001 used as a cutoff. Any genes that did not have significant homology to any previously annotated *An. gambiae* genes were used for a blastn (Altschul et al., 1990) search against the non-redundant nucleotide database from NCBI to assign putative function if similar genes or conserved sequences were identified in other species. While the gene with the highest blast homology between *An. stephensi* and *An. gambiae* may not represent a true orthologue, this is our best prediction given the early state of the annotation of the *An. stephensi* genome. Seven genes were analyzed by qRT-PCR to verify the results of the microarray (Fig. S4).

2.3. Protein extraction and iTRAQ

One-week-old adult female mosquitoes from the WT, CP, and VG lines were given a human blood meal from membrane feeders at 37 °C. Prior to the blood meal and 24 h afterward, mosquitoes were dissected in sterile PBS and their midguts and fat bodies collected.