Dissecting the genetic basis of resistance to malaria parasites in *Anopheles gambiae*

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Abstract

The ability of *Anopheles gambiae* mosquitoes to transmit *Plasmodium* parasites is highly variable between individuals. However, the genetic basis of this variability has remained unknown. We combined genome-wide mapping and reciprocal allele-specific RNA interference (rasRNAi) to identify the genomic locus that confers resistance to malaria parasites, and demonstrate that polymorphisms in a single gene encoding the antiparasitic thioester-containing protein 1 (TEP1) explain a substantial part of the variability in parasite killing. The link between *TEP1* alleles and resistance to malaria may offer new tools for controlling malaria transmission. The successful application of rasRNAi in *Anopheles* suggests that it could also be applied to other organisms where RNA interference is feasible to dissect complex phenotypes to the level of individual quantitative trait alleles.

Anopheles gambiae mosquitoes are major vectors of *Plasmodium falciparum*, a protozoan parasite causing the most severe form of human malaria in Africa. The fact that mosquito strains completely wide variety of parasite species (1). Previous genetic analyses of crosses between this strain and the susceptible 4Arr strain infected with two simian parasite species focused on resistant to malaria parasites can be selected (1,2) indicates that genetic factors in mosquitoes control the level of parasite transmission. Understanding the genetic basis of this resistance has been a long-standing question. The L3-5 resistant strain kills and melanises a the melanotic encapsulation phenotype and identified several quantitative trait loci (QTLs), whose relative contributions varied with parasite species and between F2 generation families (3,4). Recently it became clear that melanisation occurs after parasite killing, as a means to dispose of dead parasites in some strains, while in others, killed parasites are only cleared by lysis (Fig. S2A) (5–7). In this study, we aimed at mapping the genomic regions and identifying genes that control resistance (i.e., the absence of live parasites) of mosquitoes to the rodent malaria parasite *Plasmodium berghei*.

We set up reciprocal crosses of the resistant L3-5 and susceptible 4Arr strains. F1 mosquitoes were intercrossed and individual females were isolated to lay eggs, yielding 10 F2 families.

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Summary sentence: Polymorphisms in a mosquito complement-like factor confer resistance to malaria parasites.

Females were blood fed on mice infected with *PbGFPcon*, a transgenic clone of *P. berghei* expressing *GFP* constitutively (8). Fluorescent live and dead melanised parasites were counted on dissected midguts 7 to 9 days post infection (Fig. 1A and supporting online text). As expected, parental L3-5 females displayed only melanised parasites (with the exception of one that bore one live parasite), and 4Arr mosquitoes displayed only live parasites. Most of the 111 F1 mosquitoes exhibited an intermediate phenotype (mix of live and melanised parasites). Both parental and F1 phenotypes were present in the 402 F2 females. Percentages of resistant (i.e. devoid of live parasite) and melanising (bearing at least one melanised parasite) mosquitoes in each generation (Fig. 1B) did not follow the segregation pattern of simple Mendelian traits (p-values < 0.001 in both cases (9)), indicating that the killing of *P. berghei* and the mode of clearance of dead parasites are complex traits that are each likely to result from the segregation of several alleles.

To map loci controlling resistance to parasites, we genotyped 39 informative markers spanning the entire genome in 206 selected F2 individuals with extreme phenotypes (Fig. S1 and supporting online text). Linkage analysis comparing resistant and non-resistant mosquitoes identified a single region on chromosome 3L (Fig. 1C). We interpreted this region, covering approximately 19Mb, as a major locus responsible for resistance to *P. berghei*, and named it *Pbres1*, for *P. berghei* resistance locus 1. We further compared the genotypes of melanising and non-melanising mosquitoes, and detected two intervals that are likely to contain regulators affecting the mode of clearance of dead parasites, i.e., the balance between lysis and melanisation (Fig. 1C): a major QTL on chromosome 2R, which we named *Pbmel1* (5Mb), for *P. berghei* melanisation locus 1, and a minor pericentromeric QTL on chromosome 3, *Pbmel2* (17Mb), which partially overlaps with *Pbres1*. Linkage mapping using the actual counts of live or melanised parasites identified the same loci as above (Fig. S2B and supporting online text). Interestingly, the newly identified QTLs overlap with regions previously identified as controlling melanisation of *P. cynomolgi* and *P. berghei* in L3-5 mosquitoes (Fig. 1C) (3, 4,10), indicating that the major mechanisms underlying parasite elimination in L3-5 are likely partially conserved and independent of parasite species. Nevertheless, clear quantitative differences exist between the four studies, probably, at least in part, because previous studies did not consider resistance and melanisation as distinct traits.

Because of its major role in parasite transmission, we investigated the resistance QTL on chromosome 3L in more detail. *Pbres1* contains ~975 genes, among which 35 can be classified as "immune-related" (11). This category includes the gene encoding the thioester-containing protein 1 (TEP1), a complement-like molecule circulating in the hemolymph with key antiparasitic activity (12). Two features make it an attractive candidate: it binds to, and promotes the killing of midgut stages of the rodent parasite *P. berghei*, and it is highly polymorphic (5). To examine *TEP1* polymorphism in the L3-5 and 4Arr strains, we cloned and sequenced the full ORF (Figs. 2 and S3). We renamed the previously known *TEP1r* (or *TEP16*) from the L3-5 strain (5,13), *TEP1*R1*, and *TEP1s* (or *TEP1*) from the PEST strain (14), *TEP1*S1*. All *TEP1* sequences in L3-5 mosquitoes were identical to *R1*. Sequences from the 4Arr strain appeared to be chimeras of *TEP1*S* and *TEP1*R*: one was closer to *S1*, we named it *TEP1*S2*; the second allele clustered with *R1* in the phylogenetic tree, and was therefore named *TEP1*R2*. We also sequenced *TEP1* from our G3 colony and confirmed that it was closely related, although not identical, to *TEP1*S1*. We named this allele *TEP1*S3*.

To determine whether the diverse alleles of *TEP1* have a phenotypic effect, we compared the degree of resistance of mosquitoes that differed solely in the expression of *TEP1* alleles. For this, we developed an assay similar to reciprocal hemizygosity analysis in yeast (15): we used reciprocal allele-specific RNA interference instead of chromosomal deletions to silence each allele separately in F1 mosquitoes, enabling to compare the function of each allele in the same genetic background (Fig. 3A). We crossed resistant L3-5 and susceptible G3 mosquitoes as

these strains are homozygous for *TEP1*, and bear representative alleles of the *TEP1*R* and *TEP1*S* classes. We also note that G3 and 4Arr mosquitoes share the same susceptible phenotype.

We designed three pairs (a–c) of short dsRNAs (*dsR/dsS*) targeting specifically **R1* and **S3*, and tested them in the parental L3-5 and G3 strains (Fig. S4 and supporting online text). We used *dsLacZ* as a negative control, and *dsTEP1* that targets both alleles as a positive control (5). Pair a (*dsRa* and *dsSa*) was selected for further experiments: 3–4 days after dsRNA treatment, TEP1*R1 was depleted from L3-5 mosquitoes upon treatment with *dsRa* but not *dsSa*, and reciprocally, injection of *dsSa* but not *dsRa* in G3 mosquitoes reduced *TEP1*S3* levels. In the F1 progeny of reciprocal crosses between L3-5 and G3 mosquitoes, both alleles were silenced to a similar level by allele-specific RNAi, allowing to specifically study the function of each allele (Figs. 3B, S4B and supporting online text).

DsRNA-treated F1 mosquitoes were infected on mice carrying *PbGFPcon* (Figs. 3C and S4C). Control *dsLacZ*-treated F1 mosquitoes bore a mixture of live and melanised parasites. *R1-depleted mosquitoes (*dsRa*) were significantly more susceptible than *S3-depleted (*dsSa*) mosquitoes, and were completely unable to melanise. Moreover *S3-depleted mosquitoes were consistently more resistant than *dsLacZ* controls, containing fewer live parasites. Thus TEP1*R1 is more efficient than TEP1*S3 in promoting parasite killing and melanisation of dead parasites. The reversal of the F1 phenotype towards the susceptible parent phenotype upon depletion of TEP1*R1, or towards the resistant parent phenotype upon depletion of TEP1*S3, indicates that polymorphisms in *TEP1* are a major determinant of resistance to *P. berghei* in these mosquito strains.

To examine whether the two allelic variants of the 4Arr strain, **S2* and **R2*, which belong to the *TEP1*S* and *TEP1*R* classes, also differ in their efficiency in parasite killing, we further refined our association analysis of the F2 progeny of the QTL mapping crosses, and genotyped all F2 progeny for *TEP1* (Fig. 4 and supporting online text). Most **R1/R1* F2 mosquitoes (81%) were fully resistant, and those that were not carried only a few live parasites. In contrast, 90% of **S2/S2* mosquitoes were susceptible, containing high parasite loads. **R2/R2* mosquitoes had an intermediate phenotype, suggesting that although **R2* is closely related to **R1*, the few polymorphisms between these two alleles affect its efficiency in parasite killing. Further studies are required to precisely identify the essential SNP(s) and the molecular mechanisms that underlie this resistance. In addition, **R1/R2* mosquitoes were more resistant than **R1/S2* mosquitoes, indicating that the two 4Arr alleles confer different degrees of resistance, with **R2 > *S2*. Thus the complexity of the resistance inheritance in our crosses is partially explained by the segregation of the three *TEP1* alleles. Still, other genes besides *TEP1* must contribute. This is apparent from comparing phenotypes of groups from different generations with the same *TEP1* genotypes (Figs. 1B and 4A): e.g. 50–70% of **R1/R2* and **R1/S2* mosquitoes were resistant in F2, while <7% were resistant in F1. Thus, this additional locus/ loci appear to be unlinked to TEP1, and to have a limited impact in mosquitoes homozygous for the extreme alleles **R1* and **S2*, which have similar resistance as the parental strains, but are essential to support resistance in heterozygotes. Future work may focus on identifying secondary QTL(s) and potential candidate *TEP1* suppressor gene(s).

Importantly, the single locus identified here that controls resistance to *P. berghei* and includes *TEP1*, does not overlap with previously reported QTLs controlling the intensity of infection of natural populations by the human malaria parasite *P. falciparum*, and in particular, not with the major *Plasmodium* resistance island (PRI) (16–18) (Fig. 1C). Two leucine-rich repeat proteins encoded in the PRI, APL1 and LRIM1, form a complex with TEP1. These proteins maintain mature TEP1 in circulation and regulates its binding to parasites and their subsequent killing (19,20). Polymorphisms in proteins that control TEP1 function, or in TEP1 itself, might

both contribute to the efficiency of TEP1 antiparasitic activity. The differences between the QTLs identified in laboratory strains and in field mosquitoes might thus reflect the sampling of determinant polymorphism(s) in various players of the same pathway, rather than different mechanisms employed to limit development of human and rodent malaria parasite species. Consistently, silencing of *TEP1* increases *A. gambiae* susceptibility to both murine and human *Plasmodia* (5,21). Haplotypes of the "susceptible" and "resistant" alleles of *TEP1*, as well as recombinants between these forms, exist in field populations from East and West Africa (22). Understanding the genetic basis of resistance to malaria parasites, and how the determinant polymorphisms are maintained and selected in field populations, will be of tremendous importance for the control of malaria transmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References and Notes

1. Collins FH, et al. Science Oct 31;1986 234:607. [PubMed: 3532325]

2. Vernick KD, et al. Exp Parasitol Jun;1995 80:583. [PubMed: 7758539]

3. Zheng L, et al. Science Apr 18;1997 276:425. [PubMed: 9103203]

4. Zheng L, et al. BMC Genet Oct 24;2003 4:16. [PubMed: 14577840]

5. Blandin S, et al. Cell Mar 5;2004 116:661. [PubMed: 15006349]

6. Volz J, Muller HM, Zdanowicz A, Kafatos FC, Osta MA. Cell Microbiol Sep;2006 8:1392. [PubMed: 16922859]

7. Shiao SH, Whitten MM, Zachary D, Hoffmann JA, Levashina EA. PLoS Pathog Dec;2006 2:e133. [PubMed: 17196037]

8. Franke-Fayard B, et al. Mol Biochem Parasitol Sep;2004 137:23. [PubMed: 15279948]

9. Materials and methods are available as supporting material on Science online.

10. Gorman MJ, Paskewitz SM. Am J Trop Med Hyg 1997;56:446. [PubMed: 9158056]

11. Waterhouse RM, et al. Science Jun 22;2007 316:1738. [PubMed: 17588928]

12. Blandin SA, Marois E, Levashina EA. Cell Host Microbe Jun 12;2008 3:364. [PubMed: 18541213]

13. Baxter RH, et al. Proc Natl Acad Sci U S A Jul 10;2007 104:11615. [PubMed: 17606907]

14. Levashina EA, et al. Cell Mar 9;2001 104:709. [PubMed: 11257225]

15. Steinmetz LM, et al. Nature Mar 21;2002 416:326. [PubMed: 11907579]

16. Niare O, et al. Science Oct 4;2002 298:213. [PubMed: 12364806]

17. Riehle MM, et al. Science Apr 28;2006 312:577. [PubMed: 16645095]

18. Riehle MM, et al. PLoS ONE 2008;3:e3672. [PubMed: 18989366]

19. Fraiture M, et al. Cell Host Microbe Mar 19;2009 5:273. [PubMed: 19286136]

20. Povelones M, Waterhouse RM, Kafatos FC, Christophides GK. Science. Mar 5;2009

21. Dong Y, et al. PLoS Pathog Jun;2006 2:e52. [PubMed: 16789837]

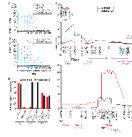22. Obbard DJ, et al. BMC Evol Biol 2008;8:274. [PubMed: 18840262]

Fig. 1.
Loci associated with resistance and clearance of dead parasites. (**A**) Numbers of melanised (x-axis) and live (y-axis) parasites per mosquito in reciprocal crosses of the resistant L3-5 and susceptible 4Arr strains. (**B**) Percentages of resistant (devoid of live parasites) and melanising (bearing at least one melanised parasite) mosquitoes in each generation. (**C**) Linkage mapping for the resistant (red) and melanising (black) traits, with estimated LOD score thresholds represented as dotted lines (3.00 and 2.88, respectively). Genetic markers, centromere positions (C), chromosome arms and the *TEP1*, *LRIM1* and *APL1* loci (in cyan) are indicated below axes. Previously identified QTLs for resistance against simian parasites (light purple) or *P. falciparum* (dark purple) are positioned below chromosomes. The PRI corresponds to the region covered by the QTLs Pfin1, Pfin4, Pfin5 and Pfmel2.
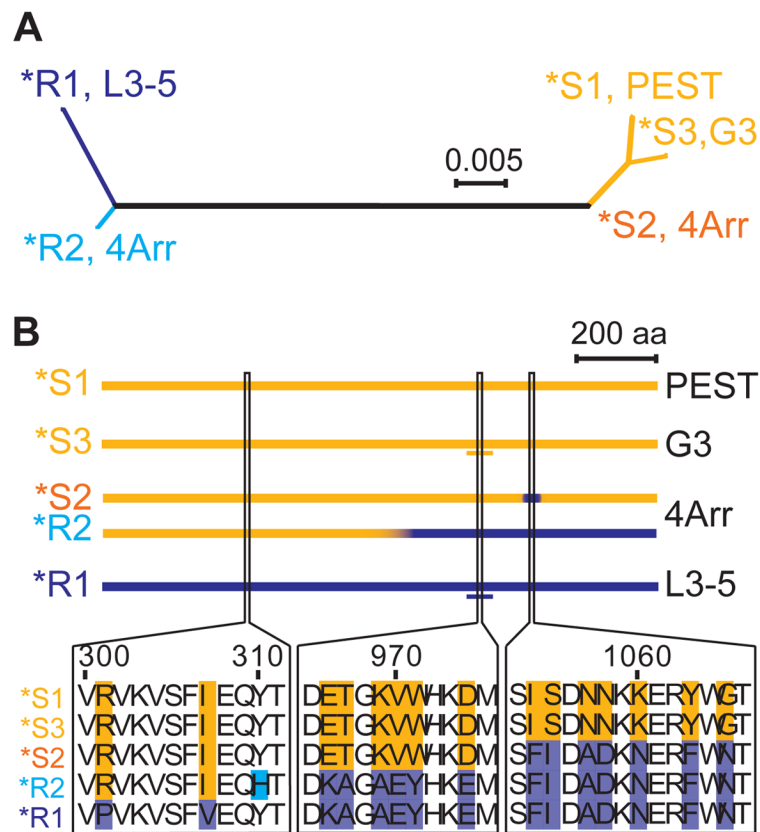
Fig. 2.
TEP1 polymorphism. (**A**) Phylogenetic tree built from the global alignment of complete amino acid sequences of TEP1 alleles from L3-5 (*R1), 4Arr (*R2 and *S2) and G3 (*S3) mosquitoes and the previously described *S1 allele from the PEST strain. Scale bar: estimated amino acid substitutions per site. (**B**) Schematic representation of TEP1 sequences. Amino-acid sequences of *S1 and *S3 are represented by orange horizontal bars, *R1 by a blue bar. The 4Arr alleles are combinations of *S1/S3 and *R1, as illustrated by short stretches of aligned sequences. The short horizontal lines below *R1 and *S3 indicate the regions targeted by *dsRa* and *dsSa*, respectively.
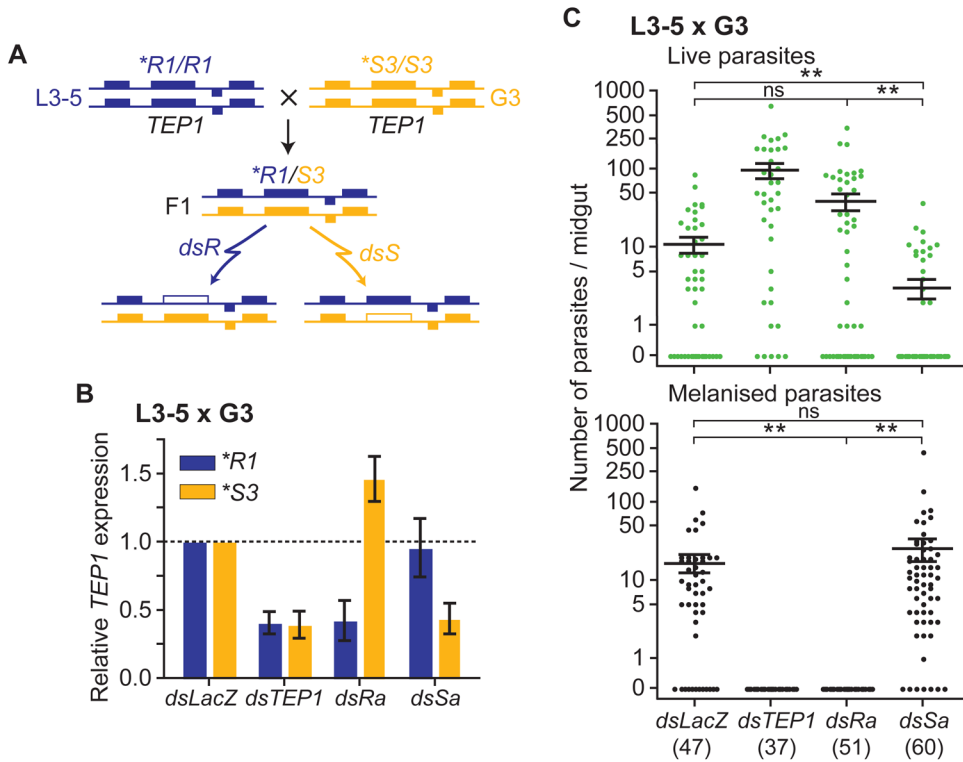
Fig. 3.
TEP1*R1 is more efficient than TEP1*S3 in parasite killing. (**A**) Reciprocal allele-specific RNAi. Each box represents a gene. With the use of short dsRNA probes specifically directed against *R1* (*dsR*) or *S3* (*dsS*), each *TEP1* allele is silenced separately in F1 mosquitoes (open box) allowing to compare the function of each allele in the same genetic background. (**B**) *TEP1* expression in the F1 progeny of crosses between L3-5 females and G3 males (L3-5 x G3) was measured by allele-specific quantitative real-time PCR 3 days after dsRNA-treatment. Expression levels of *TEP1*R1* and *S3* were normalized to their levels in the *dsLacZ* control. Mean (central bar) ± SEM (error bar) of three independent experiments. (**C**) Parasite counts in the F1 progeny of L3-5 x G3. Results of three independent experiments were pooled, sample sizes are shown in brackets. Mean (central bar) ± SEM (error bar). Significance for differences between groups are indicated (Mann-Whitney on key comparisons): **, $p < 0.001$; ns, not significant.
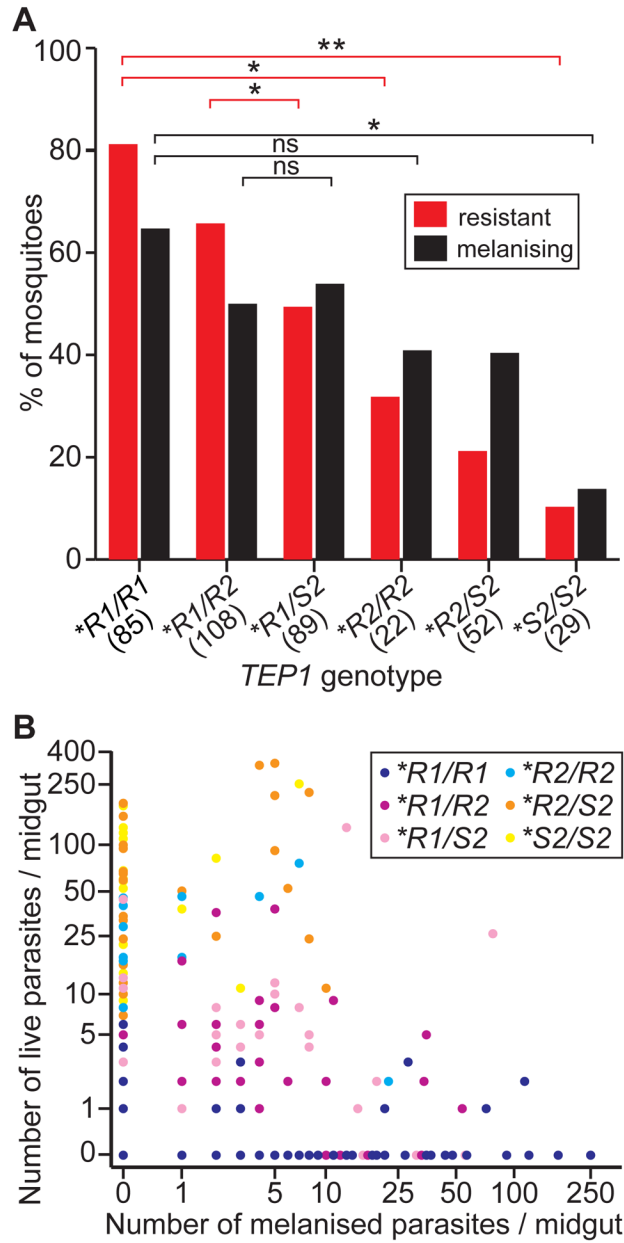
Fig. 4.
Correlation between *TEP1* genotype and phenotype upon *P. berghei* infection in the F2 generation. (**A**) Percentages of resistant and melanising mosquitoes for each genotype. Sample sizes are shown in brackets. Significance for differences between groups were calculated taking into account F2-family structure (9): **, p<0.001; *, p<0.05; ns, not significant. (**B**) Parasite counts in F2 mosquitoes as in Fig. 1A.