Cell Host & Microbe
Review

Antimalarial Responses in *Anopheles gambiae*: From a Complement-like Protein to a Complement-like Pathway

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DOI 10.1016/j.chom.2008.05.007

Malaria transmission between humans depends on the ability of *Anopheles* mosquitoes to support *Plasmodium* development. New perspectives in vector control are emerging from understanding the mosquito immune system, which plays critical roles in parasite recognition and killing. A number of factors controlling this process have been recently identified, and key among them is TEP1, a homolog of human complement factor C3 whose binding to the parasite surface targets it for subsequent killing. Here, we review our current knowledge of mosquito factors that respond to *Plasmodium* infection and elaborate on the activity and mode of action of the TEP1 complement-like pathway.

Introduction

Mankind is plagued with a plethora of vector-borne diseases, among which malaria is the most widespread and devastating. The causing agents of malaria are protozoan parasites in the genus *Plasmodium*, transmitted to humans during the blood meal of female *Anopheles* mosquitoes. The combination of *Anopheles gambiae* with *Plasmodium falciparum* often causes severe cerebral malaria in Africa and is most threatening to human life. The interaction between *Anopheles* mosquitoes, and *Plasmodium* is a crucial step in the parasite's transmission cycle between humans. Current estimates suggest that 40 out of about 400 *Anopheles* species are efficient malaria vectors worldwide (Service, 1993). Moreover, transmission by a given vector species is usually restricted to only one or a few specific parasite species (reviewed in Billingsley and Sinden, 1997), suggesting that parasite infections in mosquitoes are the exception rather than the rule. Yet, a number of biological features make some *Anopheles* species very efficient vectors for the transmission of human malaria. These include a genetically determined preference for blood meals on a human host to ensure egg development, a high reproductive rate, and a long life span, combined with the ability to support parasite development. Collectively, these features constitute the vectorial capacity of a mosquito population.

Historically, approaches to control malaria have been both medical and entomological. Currently, efficient antimalarial treatments are only accessible to a fraction of malaria patients, while vaccine development has met so far with limited success in spite of an enduring great potential. On the entomological side, reduction of host mosquito populations via insecticides or water management, as well as prevention of exposure to mosquito bites using bednets and repellents, have greatly helped limiting the impact of the disease. Today, new perspectives in vector control are emerging from molecular studies on mosquito immunity against parasites.

Like in other vector/parasite systems, *Plasmodium* must undergo a series of developmental transformations inside the mosquito vector to become infectious to vertebrate hosts (Baton and Ranford-Cartwright, 2005). Within the mosquito midgut, gametocytes are rapidly activated to produce gametes. Fertilization generates diploid zygotes that initiate meiosis within 1–2 hr, without cellular division. Sixteen to thirty hours after infection, zygotes become motile ookinetes that cross the midgut epithelium and round up on the basal side of the midgut, forming protected capsules called oocysts. Over the next 10 days, parasites undergo multiple rounds of mitosis to produce thousands of sporozoites that are released in the mosquito body cavity upon rupture of the oocysts about 2 weeks after infection. A fraction of the sporozoites successfully invade the salivary glands, whereas others are lost. The parasite cycle in the mosquito is completed when the salivary gland sporozoites are injected with the saliva into the next vertebrate host during a subsequent blood meal.

During its differentiation within mosquitoes, the parasite passes through bottlenecks with massive losses in numbers during three major transition stages (Alavi et al., 2003; Blandin and Levashina, 2004a; and reviewed in Sinden, 1999): between gametocytes and ookinetes, between ookinetes and mature oocysts, and between midgut and salivary gland sporozoites (Hillyer et al., 2007). At least the two latter bottlenecks could be the consequence of mosquito immune responses to the parasite and probably explain the frequent complete failure of the parasite to accomplish its cycle. The obligate passage through a mosquito is, therefore, a vulnerable step of the infective cycle and a potential target of novel malaria control strategies. In two *A. gambiae* strains isolated in the laboratory, parasite development is always aborted at the ookinete stage. Refractoriness is manifested either by lysis of the parasites soon after they have crossed the midgut (Vernick et al., 1995) or by melanization (Collins et al., 1986). Melanization is a common defense reaction in arthropods, which results in the deposition of a black insoluble pigment, melanin, on the surface of pathogens (Cerenius et al., 2008). In the melanizing refractory strain, invading ookinetes are killed and then melanized on the basal side of the midgut (Blandin et al., 2004). In contrast, the susceptible strains, while eliminating a majority of the invading parasites, still remain infective. Thus, there are genetic factors that determine the ability of

mosquitoes to transmit the parasites, and a major goal in the field is to identify them with the purpose to use this knowledge to increase the efficiency of parasite killing.

Careful examination of the early events of mosquito midgut invasion by *Plasmodium* parasites using electron microscopy revealed that ookinetes swiftly pass through the apical region of midgut cells, exit the cells below apical junctions within minutes, and reach the basal side 16–24 hr after the infective blood meal, where they rest in hemolymph-filled cavities known as the basal labyrinth (Whitten et al., 2006). Here the ookinetes come in contact with and get exposed to soluble immune proteins produced by the mosquito blood cells—the hemocytes. The basal labyrinth has indeed been identified as the major site of ookinete killing, with only 20% of those reaching this site estimated to survive in susceptible mosquitoes (Blandin et al., 2004 ; Shiao et al., 2006). What are these ookinete-killing factors?

The mosquito antiparasitic responses have been mostly studied using an infection model of *A. gambiae* with *P. berghei*, a rodent parasite that is readily amenable to genetic manipulation (Janse et al., 2006). Two recent technological developments—the sequencing of the *A. gambiae* genome (Holt et al., 2002) and targeted gene silencing in adult female mosquitoes via injection of double-stranded RNA (Blandin et al., 2002)—have revolutionized the field of the mosquito vector biology. Thus, genome-wide transcriptional profiling using microarrays and reverse-genetics screens for genes that affect parasite development in the mosquitoes became possible (Dimopoulos et al., 2002; Kumar et al., 2003; Vlachou et al., 2005; Dong et al., 2006; Rosinski-Chupin et al., 2007). A number of laboratories initiated such screens, working their way through lists of genes compiled from homologs of immune proteins, or from microarray results comparing either susceptible versus refractory mosquitoes, or infected versus naive blood-fed females. In most cases, these screens used the *A. gambiae*/*P. berghei* model system. In some cases, promising gene candidates showing an immune phenotype in this infection model were tested for their relevance in the *A. gambiae*/*P. falciparum* interaction (Cohuet et al., 2006; Michel et al., 2006; Mendes et al., 2008). In addition to reverse-genetics approaches, forward-genetics analysis of refractoriness to *Plasmodium* in laboratory strains (Zheng et al., 1997, 2003) and natural populations (Niare et al., 2002; Menge et al., 2006; Riehle et al., 2006, 2007) identified regions in the mosquito genome, or quantitative trait loci, that underlie resistance of mosquitoes to malaria parasites. One major locus on the left arm of the second chromosome explains at least 89% of the refractoriness to *P. falciparum* and spreads over 15 megabases, encompassing 976 genes. The initial attempts to identify the gene(s) whose polymorphism confers the refractory phenotype were based on a candidate gene approach and led to the identification of APL1, a leucine-rich repeat protein whose knockdown results in a dramatic increase in the number of surviving *P. berghei* parasites (Riehle et al., 2006). This gene's role in the resistance of mosquitoes to *P. falciparum* parasites and whether it is polymorphic in natural populations and indeed responsible for the manifestation of refractoriness remain to be elucidated. With the day-to-day increase in the number of disparate genes reported to affect *Plasmodium* development within the mosquito, the overall picture of the antiparasitic responses is becoming more complex. Genes affecting parasite development belong to diverse protein families such as complement-like molecules (thioester-containing protein 1, TEP1), leucine rich-repeat proteins (LRIM1 and APL1), lectins (CTL4 and CTLMA2), serine proteases of the CLIP family, serpins (SPN2 and 6), and others. In this review, we summarize our current understanding of how these factors, and especially those with a clear-cut phenotype, integrate in the mosquito response upon parasite infection, and discuss to what extent the vertebrate complement pathway can represent a paradigm to investigate the function of the essential mosquito antiparasitic complement-like factor TEP1.

The Quest for Mosquito Factors Involved in *Plasmodium* Killing

Genes affecting parasite development can be tentatively divided into five categories based on their knockdown phenotype. In each category, we discuss several examples (the knockdown phenotypes of these genes and the associated references are summarized in Table 1), to illustrate how genes with different putative functions may integrate in the mosquito antiparasitic responses. Additional antiparasitic factors with more moderate antiparasitic phenotypes have been identified in RNAi screens (Dong et al., 2006; Garver et al., 2008), but as their mode of action remains unclear, we will not discuss them here.

The first category comprises genes with a drastic knockdown phenotype, which leads to a dramatic increase in the parasite loads in susceptible mosquitoes and converts refractory mosquitoes into susceptible. It is expected that at least some proteins described below may act in the same pathway. The category includes TEP1, the first molecule found to control parasite numbers in mosquitoes (Blandin et al., 2004). TEP1 belongs to a large family of proteins sharing significant structural similarities. The hallmark of the family is the conserved thioester (TE) motif, which underlies the unique property of these proteins to bind covalently to the target substrates. The best-characterized member of the family is the vertebrate complement factor C3 (discussed below). Leucine-rich repeat proteins LRIM1 and APL1 were found to be equally important in limiting parasite infections (Osta et al., 2004; Riehle et al., 2006). Another example in this category is WASP, a positive regulator of actin dynamics whose expression is upregulated during *Plasmodium* infection (Vlachou et al., 2005). *WASP* knockdown markedly increases *P. falciparum* and *P. berghei* survival in the mosquito at the early stages of infection, suggesting WASP impedes ookinete invasion or traversal of the midgut cells (Mendes et al., 2008). In this respect the function of WASP differs from that of CDC42 (see below), which is involved at later postkilling stages of the antiparasitic response.

A second category comprises genes whose knockdown results in a converse phenotype, i.e., makes susceptible mosquitoes refractory. The most striking example is the gene encoding Cactus, an inhibitor of NF-κB transcription factors whose silencing in susceptible mosquitoes completely aborts *P. berghei* development (Frolet et al., 2006). Similarly, the knockdown of the C-type lectin 4, *CTL4*, leads to a dramatic decrease in parasite survival, although 20%–30% of the ookinetes are not killed and further develop in oocysts (Osta et al., 2004). Silencing of the mannose binding lectin CTLMA2 also causes a decrease, albeit more moderate, in parasite survival. Interestingly, the

Table 1. Knockdown Phenotypes of a Subset of *A. gambiae* Genes Affecting the Development of *Plasmodium* Parasites

Gene	*P. berghei* (refractory mosquitoes)	*P. berghei* (susceptible mosquitoes)	*P. falciparum* (susceptible mosquitoes)	Survival upon bacterial challenge[a]	Phagocytosis (Moita et al., 2005)[a]	References
TEP1	No melanization, parasites develop	More oocysts	More oocysts	No effect (both)	Decreased phagocytosis (both)	(Blandin et al., 2004; Dong et al., 2006)
LRIM1	ND	More oocysts	No effect	ND	Decreased phagocytosis (*E.coli*)	(Osta et al., 2004; Cohuet et al., 2006)
APL1	ND	More oocysts	ND	ND	ND	(Riehle et al., 2006)
CTL4	ND	Fewer oocysts, melanization	No effect	ND	ND	(Osta et al., 2004; Cohuet et al., 2006)
CTLMA2	ND	Fewer oocysts, melanization	No effect	ND	ND	(Osta et al., 2004; Cohuet et al., 2006)
SRPN2	ND	Fewer oocysts, melanization	No effect	ND	ND	(Michel et al., 2005, 2006)
SRPN6	More melanization	Delay in lysis but no effect on oocyst numbers	ND	ND	ND	(Abraham et al., 2005; Pinto et al., 2008)
CLIPA8	No melanization but parasites are killed	No effect	ND	ND	ND	(Volz et al., 2006)
CLIPA2, CLIPA5, CLIPA7,	More melanization (not for CLIPA7)	Fewer oocysts, melanization	ND	ND	ND	(Volz et al., 2006)
CLIPB14, CLIPB15	More melanization	More oocysts	ND	Decreased survival (*E.coli*), No effect (*S.aureus*)	ND	(Volz et al., 2005)
SPCLIP1	ND	More oocysts	More oocysts	Decreased survival (both)	ND	(Dong et al., 2006)
Cactus	ND	No oocysts, melanization	ND	ND	Increased phagocytosis (*E.coli*), decreased phagocytosis (*S.aureus*)	(Frolet et al., 2006)
Rel1, Rel2	ND	More oocysts (*Rel1/Rel2* double KD)	ND	Rel2: Decreased survival (both) Rel1: No effect (both)	ND	(Meister et al., 2005; Frolet et al., 2006)
ApoII/I	Less melanization but parasites are killed	Fewer oocysts	Fewer oocysts	ND	ND	(Vlachou et al., 2005; Mendes et al., 2008)
ApoII	More melanization	No effect	No effect	ND	ND	(Mendes et al., 2008)
APOD	ND	More oocysts	More oocysts	Decreased survival (both)	ND	(Dong et al., 2006)
IRSP5	ND	More oocysts	No effect	Decreased survival (S.aureus)	ND	(Dong et al., 2006)
IRID-4	ND	No effect	More oocysts	No effect (both)	ND	(Garver et al., 2008)
IRID-6	ND	More oocysts	More oocysts	Decreased survival (S.aureus)	ND	(Garver et al., 2008)

Table 1. *Continued*

Gene	*P. berghei* (refractory mosquitoes)	*P. berghei* (susceptible mosquitoes)	*P. falciparum* (susceptible mosquitoes)	Survival upon bacterial challenge[a]	Phagocytosis (Moita et al., 2005)[a]	References
NOS, Peroxidase	ND	ND	ND	ND	ND	(Kumar and Barillas-Mury, 2005; Kumar et al., 2004)
Catalase	ND	Fewer oocysts, higher ookinete lysis	ND	ND	ND	(Molina-Cruz et al., 2008)
WASP	ND	More oocysts	More oocysts	ND	ND	(Vlachou et al., 2005; Mendes et al., 2008)
Frizzled2, Cdc42	No melanization but parasites are killed	No effect	ND	ND	ND	(Shiao et al., 2006)

ND, not determined.
[a] Bacterial species (*E. coli* or *S. aureus* or both) for which a phenotype is observed are indicated in brackets.

efficient parasite killing caused by the knockdown of these genes is invariantly accompanied by melanization of some ookinetes in the susceptible strain that normally does not melanize dead parasites. Both phenotypes (parasite killing and melanization) require the function of TEP1 in the *Cactus*-deficient background, and of LRIM1 in the *CTL4*-silenced mosquitoes. Indeed, concomitant silencing of *Cactus* and *TEP1* and/or *LRIM1*, and of *CTL4* and *LRIM1*, reverts the *Cactus* and *CTL4* knockdown phenotypes, respectively (Frolet et al., 2006; Osta et al., 2004). Taken together, these observations suggest that Cactus and CTL4 act upstream of TEP1/LRIM1 and may control one arm of the mosquito antimalarial defenses. Another gene in this category encodes the serine protease inhibitor (or serpin) SRPN2 (Michel et al., 2005). In insects, proteolytic cascades mediate such immune responses as wound healing, blood clotting, and melanization, all of which are tightly regulated by serpins to avoid potentially detrimental overreactions. In agreement with this, the knockdown of *SRPN2* provokes spontaneous melanization of the mosquito blood cells and reduces the life span of adults. Moreover, depletion of SRPN2, like that of Cactus and of the two lectins, increases parasite killing and stimulates melanization, probably as a consequence of the overactivation of the melanization cascade (Michel et al., 2005).

A third category of immune genes comprises factors whose knockdown moderately but consistently affects parasite loads. We speculate that this category, among others, comprises genes that modulate the expression level or protein activity of factors of the first category. Of course, if multiple parasite recognition pathways existed and converged on a single killing mechanism such as the one mediated by TEP1, the knockdown of any of these ''tributary'' pathways would probably also show a partial phenotype on parasite survival. This could be the case for the CLIP domain serine proteases CLIPB14, CLIPB15, and SPCLIP1 (Volz et al., 2005; Dong et al., 2006), or for the infection-responsive immunoglobulin domain genes IRID4 and IRID6 (Garver et al., 2008), whose knockdowns moderately increase parasite survival in susceptible mosquitoes. Other factors, such as the immune-responsive secreted peptide IRSP1, might directly contribute to *Plasmodium* killing by their intrinsic antimicrobial properties (Dong et al., 2006), but their mild knockdown phenotype suggests that *Plasmodium* has evolved ways to overcome their effects. Finally, another class of mosquito factors showing a mild RNAi phenotype might be required to support parasite development. For example, the lipid transporter Apolipophorin II/I (the equivalent of vertebrate LDL lipoproteins) was found to be necessary for high oocyst counts in the midgut (Vlachou et al., 2005; Mendes et al., 2008), suggesting that the developing parasite hijacks some of the host's lipophorin for its growth. Alternatively, lipophorin may somehow act as a negative regulator of immunity.

The fourth category comprises factors involved in the stress response such as the production of reactive oxygen species (ROS), notably by nitric oxide synthase and peroxidase (Kumar et al., 2004; Kumar and Barillas-Mury, 2005). Interestingly, the ROS-detoxifying catalase enzyme is induced in the midgut following a noninfective blood meal or after supplying H_2O_2 to mosquito females, but is repressed during *P. berghei* infection. Despite this lack of catalase after an infective bloodmeal, systemic H_2O_2 levels are kept in check, probably via the activity of additional ROS-detoxifying enzymes specifically induced in the fat body, the insect equivalent of the vertebrate liver, following infection (Molina-Cruz et al., 2008). The knockdown of the gene-encoding catalase results in lower parasite survival probably by promoting ookinete lysis, suggesting that ROS stimulate parasite killing. Although several theories have been proposed to explain the ROS mode of action against parasites (Han et al., 2000; Han and Barillas-Mury, 2002; Kumar et al., 2003, 2004; Kumar and Barillas-Mury, 2005), it is unclear whether the oxidative burst has a direct toxic effect toward *Plasmodium*, or whether it potentiates other killing mechanisms that culminate in parasite lysis.

Finally, the fifth category of immune genes is not involved in parasite killing, but instead affects melanization of doomed or killed ookinetes. This group comprises factors involved in diverse biological processes. For instance, Apolipophorin III (ApoIII) has recently been found to inhibit ookinete melanization in refractory mosquitoes (Mendes et al., 2008). In insect orders other than Diptera, ApoIII homologs participate in lipid transport, notably to developing oocytes, together with Apolipophorin II/I (Weers and Ryan, 2006). However, ApoIII (unlike ApoII/I) is

dispensable for egg development in mosquitoes (Mendes et al., 2008); therefore, its function might have drifted to other processes such as melanization. FZ2 and CDC42 (see below), which have putative roles in development and cytoskeleton dynamics, respectively, act downstream of TEP1 to isolate killed parasites from healthy mosquito tissues: they control the formation of an actin zone and promote melanotic encapsulation in the case of refractory mosquitoes (Shiao et al., 2006). Another member of this category is Serpin 6 (SRPN6), a serine protease inhibitor whose expression is strongly upregulated by parasite infection, both in invaded midgut and salivary gland epithelial cells (Abraham et al., 2005; Pinto et al., 2008). *SRPN6* knockdown shifts the balance from lysis toward melanization during clearance of killed ookinetes. Consistent with this, several CLIP serine proteases, such as CLIPA2, A5, A7, and A8, have been shown to affect the efficiency of melanization (Volz et al., 2006). Of note, silencing of *SRPN6* before the release of sporozoites from the oocysts leads to a 2-fold increase in the number of salivary gland sporozoites, suggesting that SRPN6 could also act at later stages of parasite development, for instance by inhibiting a parasite-derived serine protease required for invasion (Pinto et al., 2008). The intricate network of interactions between the CLIP proteases and serpins that is relevant for *Plasmodium* development calls for further investigation.

Conserved Complement-like Function of TEP1 in Antimicrobial Responses

TEP1 stands apart among the known molecules that are required for efficient *Plasmodium* killing, as it is the only one that binds directly to the surface of invading ookinetes and mediates killing of parasites. TEP1 belongs to the family of TE-containing proteins and is homologous to the vertebrate complement factors C3/C4/C5 and to members of the α2-macroglobulin family (A2Ms). In vertebrates, these proteins play important roles in immune responses as components of the complement system, in the case of factors C3/C4/C5, or as universal protease inhibitors, in the case of A2Ms (Dodds and Law, 1998). The vertebrate complement system (schematically depicted in Figure 1A) comprises about 35 serum and cell-surface molecules that react with one another in a cascade to opsonize pathogens and to induce a series of inflammatory responses that help to fight infections. There are three distinct pathways through which complement can be activated on pathogen surfaces, all starting with the recognition and binding of circulating factors to target surfaces: (1) the classical pathway initiated by the recruitment of the collectin C1q to antibody:antigen complexes; (2) the lectin pathway triggered by the direct binding of the mannan-binding lectin, another collectin, to mannose and other sugar moieties present on the surface of many microbes; and (3) the alternative pathway initiated by the spontaneous hydrolysis of complement factor C3. All three pathways trigger proteolytic cascades that converge at the activation of the central complement factor C3 and lead to the generation of a series of molecules that fulfill the major functions of the complement system: (1) the small anaphylatoxins C3a, C4a, and C5a, clipped off from the α chains of factor C3, C4, and C5, respectively, promote inflammation and recruitment of phagocytes at the site of complement activation; (2) opsonization of microbial surface by C3b, the larger proteolytic fragment of C3, initiates the assembly of the membrane attack complex composed of factors C5–C9, which forms a pore in pathogen membranes, and causes their lysis; (3) iC3b, resulting from the cleavage of C3b by factor I and bound to target surfaces through the TE, is recognized by complement receptors and facilitates the uptake of pathogens by phagocytes; and (4) further degradation products of iC3b that contain the C3d fragment attached to target surfaces, are recognized by complement receptors and activate B lymphocytes. A2Ms, on the other hand, function as pan-protease inhibitors (reviewed in Armstrong, 2006). In contrast to most protease inhibitors that are highly specific for target enzymes displaying similar specificities and catalytic mechanisms, A2Ms inhibit a wide range of proteases by a unique steric mechanism that shields the active site of the targeted protease inside a cage formed by A2M dimers or tetramers upon proteolytic activation. Once a protease has reacted with an A2M, the complex is rapidly cleared from the circulation via receptor-mediated endocytosis.

In *A. gambiae*, TEP1 is constitutively secreted by hemocytes (the mosquito blood cells), and is present in the hemolymph (equivalent of vertebrate blood) as a full-length form of 165 kDa, and a processed fragment of about 80 kDa (Levashina et al., 2001). Septic injury transiently enhances TEP1 cleavage and induces transcription of the *TEP1* gene, which results in replenishment of the full-length molecule. Similar to complement factors, the cleaved C-terminal part of TEP1 binds to the surface of Gram-negative and Gram-positive bacteria in a TE-dependent manner. However, in the absence of a functional TE, only the full-length form is detected on bacteria, suggesting that this form might bind microbial surfaces in a TE-independent manner. Binding of the C-terminal TE-containing part of TEP1 to bacteria promotes their phagocytosis, both by mosquito cells in culture, and by hemocytes in vivo. Indeed, the uptake of *Escherichia coli* by mosquito cells is decreased 2-fold after incubation with conditioned medium (CM) specifically depleted of TEP1, or with CM treated with methylamine, which inactivates all TE bonds, including that of TEP1 (Levashina et al., 2001). Likewise, the knockdown of *TEP1* in adult mosquitoes dramatically impairs phagocytosis of *E. coli* and *Staphylococcus aureus* (Moita et al., 2005). Taken together, these data suggest that TEP1 is constitutively cleaved by a mosquito protease in the absence of any experimental stimuli, and that septic injury further enhances TEP1 cleavage. Moreover, cleaved TEP1 binds to the surface of bacteria and promotes their phagocytosis in a TE-dependent manner. In this respect, TEP1 plays a very similar role to C3 in promoting uptake of opsonized bacteria by phagocytes.

Interestingly, parasite killing in mosquitoes is also mediated by the binding of TEP1 to parasite surfaces (Blandin et al., 2004). TEP1 is detected on the surface of invading ookinetes by immunofluorescence analysis of *P. berghei*-infected midgut samples using the antibodies directed against the C-terminal part of TEP1. However, it is unclear at present whether a proteolytic activation is required for TEP1 binding to parasites. Further investigations are also needed to demonstrate the covalent attachment of the molecule to pathogen surfaces, and to examine whether the N-terminal part is released upon TEP1 binding or remains associated with the C-terminal fragment. The knockdown of *TEP1* in susceptible mosquitoes increases by 3- to 5-fold the burden of developing oocysts, while it converts refractory mosquitoes into susceptible. Importantly, the kinetics and efficiency of TEP1

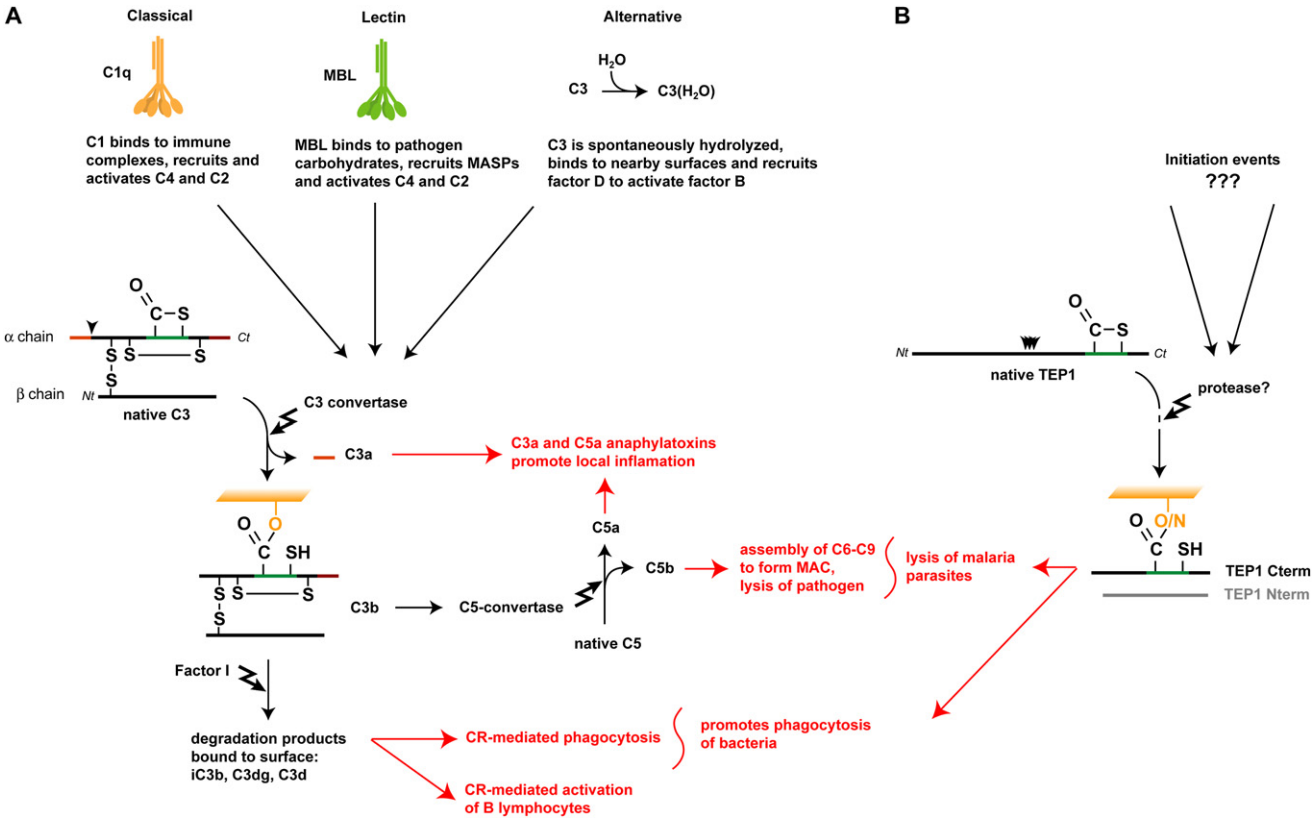

Figure 1. Parallel between the Complement System in Mammals and a Putative Complement-like System in Mosquitoes

(A) Schematic representation of the complement system in vertebrates. Complement activation proceeds through three distinct pathways: the classical, lectin, and alternative pathways, all converging at the activation of the central complement factor C3 by proteolytic cleavage, and leading to the same set of effector mechanisms (in red). Refer to main text for further details. Note that the native circulating form of C3 is intracellularly processed in two chains (α and β) linked by a unique disulfide bridge before it is secreted in the blood stream. The C3 convertase clips off the small anaphylatoxin (C3a, red fragment) from C3 (position indicated by an arrowhead), which triggers a major rearrangement of the molecule and allows the covalent attachment of the large fragment C3b to its target surface (orange) via the reaction of the thioester bond with a hydroxyl group.

(B) A model for TEP1 activity in mosquitoes. In contrast to C3, TEP1 is secreted as a single chain in the mosquito hemolymph and is devoid of the anaphylatoxin and CUB domains (red and brown fragments in native C3, respectively). The molecular events leading to the proteolytic activation and binding of TEP1 to target surfaces (orange) remain unknown, as well as the protease(s) that can cleave TEP1. It is likely that this is not achieved by a specific convertase as with complement factors, as TEP1 harbors a protease-sensitive region that contains several potential cleavage sites (arrowheads on native TEP1) for a wide range of proteases. Biochemical analyses will be required to characterize the reaction of TEP1 thioester with target molecules. Similarly to C3, TEP1 harbors a catalytic histidine about 100 amino acids downstream of the thioester, which is absent in A2Ms, suggesting it could preferentially react with hydroxyl rather than amine groups. TEP1 opsonization of bacterial surfaces is achieved through the binding of the C-term fragment of TEP1 and requires a functional TE. Whether this is also the case of malaria parasites awaits further investigation. It is also presently unclear whether proteolytic cleavage of TEP1 is sufficient to remove the N-terminal moiety (shaded in gray) or whether the two forms remain associated on the pathogen surfaces. TEP1 binding promotes phagocytosis of bacteria and lysis of parasite, which is reminiscent of the complement system. Effector molecules mediating these functions are still to be identified.

binding to and subsequent killing of parasites are higher in refractory than in susceptible mosquitoes. The proportion of dead parasites labeled with TEP1 determined by immunostaining assays is higher in refractory mosquitoes at early time points after infection, and by 48 hr postinfection virtually all parasites are TEP1-marked and killed. At the same time point in susceptible mosquitoes, about 20% of ookinetes transform into oocysts and develop further. Interestingly, the refractory and susceptible strains differ in the allelic forms of TEP1. The majority of the substitutions between the two forms are located in the vicinity of the TE site (see below, and Blandin et al., 2004; Baxter et al., 2007), suggesting that *TEP1* polymorphism accounts for the more efficient binding of TEP1 to and killing of parasites in refractory mosquitoes.

Another major difference between susceptible and refractory mosquitoes pertains to the clearance of dead parasites. In susceptible mosquitoes, parasites are undergoing lysis so that 3 days after the infectious blood meal only the live parasites are detectable in the midgut. In refractory mosquitoes, some parasites are lysed but those that are melanized remain in the midgut throughout the mosquito's life (Blandin et al., 2004). In both strains, a filamentous actin ring surrounds dead or dying ookinetes (Shiao et al., 2006). The formation of this zone is more prominent in refractory than in susceptible mosquitoes, and is invariantly accompanied by melanin deposition in refractory mosquitoes, suggesting that both processes are linked. Silencing of *TEP1* strongly reduces parasite killing and actin polymerization. Often a small proportion of parasites that have been lysed independently of TEP1 can still be observed. Interestingly, even in the refractory mosquitoes, these parasites are devoid of a surrounding actin zone and are not melanized. In keeping with these

results, TEP1 was reported to be necessary for the melanization of inoculated Sephadex beads in mosquitoes, an assay used to study melanization (Warr et al., 2006). Thus, in addition to being required for parasite killing, TEP1 activity is also necessary for the actin zone formation and ookinete melanization. Two additional genes controlling the formation of the actin zone around parasites have been identified: the gene encoding the transmembrane receptor *Frizzled 2* (*FZ2*) and the gene cell division cycle protein 42 (*CDC42*) (Shiao et al., 2006). The knockdown of both genes inhibits actin polymerization and melanization, but does not affect the number of developing parasites. This indicates that actin polymerization and melanization are two linked processes that are operating on already dead parasites and therefore do not constitute a mechanism of parasite killing. Both processes might represent a form of wound healing response, which isolates moribund parasites from surrounding tissues. It is important to note that actin polymerization might also play a role at the early stages of parasite invasion. As discussed above, knockdown of the positive regulator of actin polymerization WASP increases parasite loads, a phenotype that was correlated with the formation of an actin ring around invading parasites (Vlachou et al., 2005; Mendes et al., 2008). Further cell biology studies are required to establish whether WASP is also involved in the actin zone formation after parasite death.

Thus similar to complement factors, TEP1 is an opsonin (Figure 1B): it attaches to the surface of bacteria and parasites, and this binding activates two distinct types of immune responses—phagocytosis of bacteria and parasite killing via lysis, followed by actin polymerization and melanization in the refractory strain. Two essential questions remain to be answered. (1) How is the specificity of TEP1 binding to the surface of invading bacteria or parasites achieved? And (2) how does TEP1 binding to microbial surfaces molecularly translate into parasite lysis? Could these processes be homologous to the complement cascade described in vertebrates? First answers to these questions can be drawn from the comparison of the TEP1 crystal structure with that of complement factor C3.

The C3-like Structure of TEP1

Several features are specific to the family of TE-containing proteins. These large proteins (about 1500 amino acids) (1) are secreted as an inactive form; (2) undergo proteolytic activation to become functional, which leads to major conformational changes (note that complement factors are activated by specific proteolytic complexes termed convertases, whereas a wide range of proteases can cleave A2Ms); (3) bind covalently to nearby targets (pathogens for complement factors, proteases for A2Ms) through the highly conserved hyperreactive TE motif; and (4) undergo further proteolysis, which leads to the clearance of labeled entities via receptor-mediated phagocytosis or endocytosis.

TEP1 is a typical member of the TE-containing protein family: it displays a hydrophobic N-terminal signal peptide characteristic of secreted proteins and the canonical TE motif. At the level of the primary sequence, TEP1 is equidistant from the complement factors and A2Ms, and forms a distinct clade with other insect and *Caenorhabditis elegans* TEPs (Blandin and Levashina, 2004b). However, the recent analysis of the crystal structure of TEP1 identified clear structural similarities to complement factor C3 (Baxter et al., 2007). A comparison of both structures is shown in Figure 2. The overall structure of TEP1 resembles that of complement factor C3 (Janssen et al., 2005), with six macroglobulin domains (MGs) forming a β ring attached to the core of the protein, termed α ring, that contains the thioester domain (TED) and three β sheet domains (one CUB and 2 MGs). Two domains are absent from TEP1 compared to C3: an anaphylatoxin (ANA) domain that stabilizes the interaction between a MG and the TED in the core, thus protecting the TE from inadvertent hydrolysis, and a C-terminal C345C domain hanging from the core. In the absence of the ANA in TEP1, the watertight chamber protecting the TE is maintained through the rotation of several MG domains compared to C3, and this inactive conformation is believed to be stabilized by the threading through the β ring cavity of a protease-sensitive region, located 250 aa upstream of the TE. The precise position of this region could not be mapped on the electron density, as it appears to be disordered. In contrast to C3, which is activated by the C3 convertase at a precise site in the β ring at the basis of ANA, the protease-sensitive region of TEP1 contains several potential cleavage sites and is likely to loop out of the β ring cavity, suggesting that it could be activated by a range of proteases rather than a specific convertase (Baxter et al., 2007). The precise model for the activation of TEP1 awaits further insights from the structural analysis of cleaved TEP1.

Additional structural differences could be evidenced between TEP1 and C3, although their consequences on the function of the proteins remain unclear (Levashina et al., 2001; Baxter et al., 2007). TEP1 (like A2Ms) is secreted as a single chain, whereas C3 is posttranslationally processed into several chains. Moreover, the number and localization of the disulfide bridges, as well as the glycosylation sites, are not conserved between TEP1 and C3.

The structure of TEP1 provides a formidable tool to map the substitutions between the refractory (TEP1r) and susceptible (TEP1s) isoforms of TEP1 that are found in refractory L3-5 and susceptible G3 mosquitoes, respectively (see above). The β ring and protease-sensitive region are virtually identical, suggesting that the proteolytic activation is similar in both molecules. Most substitutions occur in the TED (50% of all substitutions) and in the nearby MG (16%), both domains forming the watertight pocket around the TE. The substitutions between TEP1r and TEP1s could explain, at least partially, the differences observed in the kinetics and efficiency of binding of the two molecules to the parasite, and in the subsequent parasite killing and elimination (Baxter et al., 2007): (1) Differences located at the interface between the TED and MG could affect the rate of dissociation between the two domains upon activation, and therefore the speed at which the TE is exposed; (2) those in the vicinity of the TE could modify the reactivity of the molecule; (3) those on the concave face of the TED that interacts directly with target surfaces could modify the reactivity and selectivity of TEP1 for available sites; and (4) the variations of surface exposed residues could affect the binding of downstream effectors to the TED, after its attachment to target surfaces. It is possible that the different forms of TEP1 are more efficient in binding different types of microbial surfaces, and that they were selected in different mosquito populations through exposure to divergent pathogenic environments.

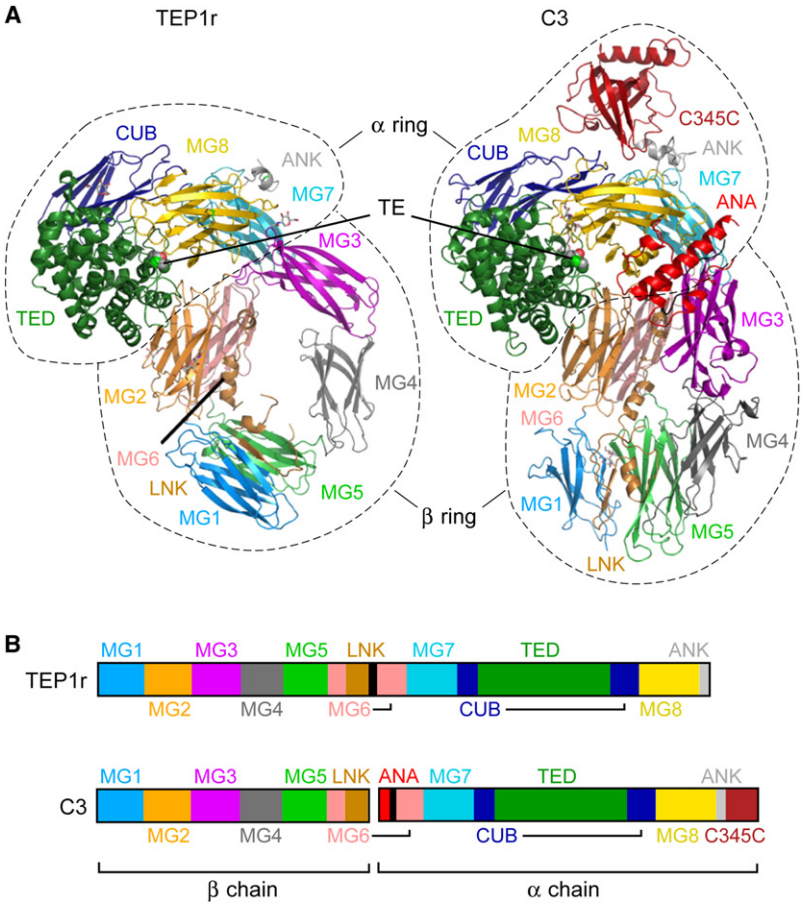

Figure 2. Similarity between TEP1r and C3 Structures

(A) Comparison of *A. gambiae* TEP1r (PDB ID 2PN5) and human C3 (PDB ID 2A73) crystal structures. In both proteins, the β chain (see B.) forms a β ring composed of 6 β sheet domains named macroglobulin domains (MG1-6) and of a linker (LNK) located at the end of the β chain. The β ring is attached to the α-ring that is formed by 5 (or 7 in the case of C3) domains of the α chain: the α-helical thioester domain (TED), 3 β sheet domains (CUB and MG7, 8) and a short anchor region (ANK). The reactive thioester bond (TE) is located in the TED and is protected from precocious inactivation in a hydrophobic pocket formed by the TED and MG8 domains. Note that two domains in the α-ring are absent in TEP1r: the anaphylatoxin (ANA) and the C-terminal C345C domain.

(B) Domain sequence and arrangement in TEP1r and C3. Complement factor C3 is posttranslationally processed in two chains (α and β) that remain linked by disulfide bridges. The color scheme matches that of (A). TEP1r and C3 Structures modified from Baxter et al. (2007).

Even though TEP1 is equidistant from C3 and A2Ms at the sequence level, the uncovered structural and functional similarities between TEP1 and complement factor C3 indicate that a complement-like cascade may exist in mosquitoes. However, the differences in the structural details suggest that these homologous proteins interact with analogous rather than with homologous factors. This is indirectly supported by the failure to identify in the mosquito genome other factors of the complement system (such as those involved in recognition or effector mechanisms) by searches based on sequence similarity. In some cases, however, potential homologs of complement factors (e.g., factor B, a protease that associates to C3b to form a convertase) are numerous in the mosquito genome and form the basis of new RNAi screens for TEP1-activating proteases.

The Concept of Basal Immunity

Just as complement factors represent a constitutive fraction of normal vertebrate serum, major antiparasitic genes, exemplified by TEP1, are already expressed prior to parasite infection in mosquito hemolymph (Levashina et al., 2001). The analysis of promoter regions of *TEP1* pinpointed the existence of sites potentially recognized by NF-κB transcription factors (Frolet et al., 2006). In *Drosophila*, Dif and Dorsal—paralogues of the NF-κB transcription factor Rel1 in *Anopheles*—are retained in the cytoplasm through interaction with IκB/Cactus, while Relish, the *Drosophila* ortholog of *Anopheles* Rel2, harbors a self-inhibitory C-terminal ankyrin domain. Dif and Relish are the transcription factors of the Toll and Imd pathways, respectively, that regulate the systemic expression of antimicrobial peptides and other genes after bacterial or fungal challenges.

In *A. gambiae*, both NF-κB factors Rel1 and Rel2 are required for the constitutive expression of *TEP1* prior to parasite invasion (Frolet et al., 2006). During this phase, hemocytes constitutively secrete TEP1 and other molecules in the hemolymph. These molecules accumulate in the basal labyrinth that is separated from the hemocoele by a basal lamina. During invasion, when ookinetes cross the midgut epithelium, expression of *TEP1* is induced via an as-yet-unidentified factor. This induction is probably necessary for the replenishment of TEP1 and other antiparasitic factors that were depleted upon reaction with the invading parasites. The expression of *TEP1* seems to be tightly regulated, as in the postinvasion phase its expression reverts to initial levels. The basal expression of the major antiparasitic factor (and of other genes) prior to infection is crucial for the mosquito resistance to *Plasmodium*. Indeed, simultaneous silencing of both NF-κB factors leads to a drop in the basal transcription levels of these genes in naive mosquitoes and diminishes resistance of *A. gambiae* to the rodent malaria parasite *P. berghei* (Frolet et al., 2006; Meister et al., 2005). Conversely, depletion of Cactus, the negative regulator of Rel1, enhances the expression of *TEP1* and other immune factors, and results in the complete abortion of parasite development (Frolet et al., 2006).

Thus, it appears that in contrast to the inducible antibacterial defenses, an efficient antiparasitic response relies on factors that are poised in the hemolymph prior to parasite infection, a status that we refer to as the basal immunity of the mosquito and that is reminiscent of the complement system. Regulation of the antiparasitic genes during the preinvasion phase is dependent on the NF-κB factors Rel1 and Rel2; however, it is still unclear whether this regulation is constitutive, or if it requires priming through previous encounters with microbes during larval or adult life. In keeping with the latter proposition, *Aedes aegypti* mosquitoes challenged with bacteria prior to parasite infection have a reduced

parasite burden compared to controls (Lowenberger et al., 1999). It will be insightful to compare the mosquito responses to parasites and to bacteria, two types of microorganisms that differ dramatically in their mode of development within the mosquito. However, the paucity of the data on the role of antimalarial genes in antibacterial responses reflected in Table 1 makes this a difficult task at this stage. Yet, an intriguing positive correlation was reported between the genes regulating phagocytosis and antimalarial responses (Blandin and Levashina, 2007).

Conclusions

The past few years have seen a spectacular increase in the number of mosquito factors controlling parasite loads in laboratory *P. berghei* infections. These molecules exert distinct roles regarding parasite survival. A current challenge is to order these factors into defined molecular pathways, some of which will act independently from each other, whereas others are likely to interact or feed into each other. The emerging picture is that of a mosquito equivalent of the vertebrate complement pathway (Figure 1B), with TEP1 paralleling complement factor C3 in promoting phagocytosis of bacteria and lysis (in some cases followed by melanotic encapsulation) of *Plasmodium* parasites. The proteolytic activation of TEP1 resulting in specific opsonization of foreign surfaces remains a poorly characterized event. The TEP1-activating protease(s) paralleling C3 convertase remain to be identified. Analysis of the TEP1 crystal structure suggests that the protease-sensitive domain is susceptible to a wide range of proteases; therefore, it is likely that TEP1 could be activated by a variety of proteases, of both microbial and mosquito origin. In vertebrates, a series of complement inhibitory factors protect self-membranes from destruction by inadvertent activation of the factor C3. Self-factors that prevent TEP1 binding to mosquito tissues probably also exist but remain to be found. Downstream of opsonization, the stimulation of phagocytosis and of lytic destruction are two functions that TEP1 shares with C3. We can therefore expect the future discovery of phagocytosis-promoting TEP1 receptors on mosquito hemocytes, and of pore-forming protein complexes analogous to the complement membrane attack complex. In contrast to the vertebrate complement system, dissection of the molecular mechanisms acting upstream and downstream of TEP1 has so far been hindered by the limited amount of mosquito material available for biochemical analyses. New genetic manipulation tools partially inspired from work in *Drosophila melanogaster*, including targeted gene disruption and site-specific transgene integration (Windbichler et al., 2007; Gong and Golic, 2003; Venken et al., 2006; Bischof et al., 2007), are under development in *A. gambiae* and should soon facilitate further identification of TEP1 pathway components.

We also note that, even though TEP1 is responsible for most parasite killing, a non-negligible number of parasites are eliminated in a TEP1-independent manner. Indeed, dead parasites that are not opsonized by TEP1 can be detected by immunofluorescence assays, both in TEP1-silenced and control mosquitoes. This indicates that additional TEP1-independent parasite killing mechanisms, such as the early parasite killing mediated by WASP, must be at work. A fraction of these parasites may also be eliminated by their own failure to progress to the next developmental stage.

Comparing the RNAi phenotypes of identified immune factors in *Plasmodium berghei* and *P. falciparum* infections revealed intriguing differences between parasite species in the face of the vector immune system. For instance, although the depletion of LRIM1 strongly increases the number of *P. berghei* oocysts, it does not affect *P. falciparum* development (Cohuet et al., 2006), nor do the *CTL4, CTLMA2*, and *SRPN2* knockdowns lead to melanization of *P. falciparum* ookinetes (Cohuet et al., 2006; Michel et al., 2006). In contrast, other factors such as TEP1, the apolipophorin II/I, the actin cytoskeleton regulator WASP, the lipoprotein homolog APOD or the member of the immunoglobulin superfamily IRID6, do affect the development of both species (Dong et al., 2006; Garver et al., 2008; Mendes et al., 2008). The contrasting results obtained for mosquito immune factors with various *Plasmodium* species is prompting researchers to be cautious when trying to extrapolate findings, but may at the same time serve as a tool to more precisely dissect mosquito defense pathways. Still, as several mosquito factors, including TEP1, affect the development of diverse parasite species, it is likely that the main mechanisms of the antiparasitic responses are conserved, whereas the species-specific differences pertain to the fine-tuning of these responses.

ACKNOWLEDGMENTS

The authors acknowledge the continuous support and interest of Professor Jules A. Hoffmann and members of the Strasbourg laboratory, especially Malou Fraiture and Annika Rademacher, for constructive discussions. We thank the three anonymous reviewers for their constructive comments. This work was supported by grants from CNRS, INSERM, Schlumberger Foundation For Education and Research (FSER), European Molecular Biology Organization (EMBO), and by the Sixth European Commission Programme "Networks of Excellence" BioMalPar. E.M. received support from the Fondation Recherche Médicale (FRM) and S.A.B. from EMBO. E.A.L. is an International Scholar of the Howard Hughes Medical Institute.

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