Anopheles gambiae TEP1 forms a complex with the coiled-coil domain of LRIM1/APL1C following a conformational change in the thioester domain

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Abstract

The complement-like protein thioester-containing protein 1 (TEP1) is a key factor in the immune response of the malaria vector *Anopheles gambiae* to pathogens. Multiple allelic variants of TEP1 have been identified in laboratory strains and in the field, and are correlated with distinct immunophenotypes. TEP1 is tightly regulated by conformational changes induced by cleavage in a protease-sensitive region. Cleaved TEP1 forms exhibit significant variation in stability from hours to days at room temperature. In particular, the refractory allele TEP1*R1 is significantly more stable than the susceptible allele TEP1*S1. This raises the question of whether the stability of cleaved TEP1 is linked to allelic variation and varying immunophenotypes. We have analyzed the stability of the cleaved form of additional TEP1 alleles and constructs. We show that stability is correlated with allelic variation within two specific loops in direct proximity to the thioester bond. The variable loops are part of an interface between the TED and MG8 domains of TEP1 that protect the thioester from hydrolysis. Engineering specific disulfide bonds to prevent separation of the TED-MG8 interface stabilizes the cleaved form of TEP1 for months at room temperature. Cleaved TEP1 forms a soluble complex with a heterodimer of two leucine-rich repeat proteins, LRIM1 and APL1C, and precipitates in the absence of this complex. The molecular structure and oligomeric state of the TEP1/LRIM1/APL1C complex is unclear. The C-terminal coiled-coil domain of the LRIM1/APL1C complex is sufficient to stabilize the cleaved form of TEP1 in solution but cleaved forms of disulfide-stabilized TEP1 do not interact with LRIM1/APL1C. This implies that formation of the TEP1$_{cut}$/LRIM1/APL1C complex is related to the conformational change that induces the precipitation of cleaved TEP1.

Introduction

The mosquito *Anopheles gambiae* is the principal malaria vector in Sub-Saharan Africa. The immune response of *A. gambiae* is a significant factor influencing the vectoral capacity of mosquitoes to malaria parasites (genus *Plasmodium*). *Plasmodium* ookinetes invade and traverse the midgut epithelium, whereupon they face a robust complement-like immune response [1]. The complement-like factor thioester-containing protein 1 (TEP1) binds to the surface of *Plasmodium* ookinetes. TEP1-labeled ookinetes are targeted for killing by lysis and in some strains subsequent melanization.

TEP1 is a 160 kDa secreted glycoprotein comprised of eight fibronectin-fold domains called macroglobulin (MG) domains. In between MG7 and MG8 are two nested insertions of an eight stranded α-barrel domain (CUB) and the α-helical thioester domain (TED). The thioester domain contains a four amino acid motif–CGEQ–that forms a β-cysteinyl-γ-glutamyl thioester bond. The reactive thioester bond, a feature of the TEP protein family including α_2-macroglobulins and complement factors, is protected from hydrolysis in the full-length protein by an interface formed between the TED and the MG8 domain [2]. A triangular arrangement of the TED, CUB and MG8 domains forms a 'superdomain' that is conserved between TEP1 and mammalian complement factor C3 [3, 4].

Activation of thioester-containing proteins requires cleavage in a protease-sensitive region located on an extended loop within the MG6 domain [2]. The first six MG domains form a supermolecular ring spanned by the protease-sensitive region, which connects to the MG7 domain and the TED-CUB-MG8 superdomain. Cleavage within the protease-sensitive region results in dissociation of the TED-MG8 interface, exposing the thioester bond for covalent reaction with substrates on pathogen surfaces, thereby labeling the pathogen with TEP1.

TEP1 is secreted into the mosquito hemolymph as a full-length protein; both full-length and cleaved TEP1 is detected in the hemolymph [5]. The cleaved form of TEP1 (TEP1$_{cut}$) requires two leucine-rich repeat proteins, LRIM1 and APL1C, for stability in the hemolymph [6, 7]. In the absence of LRIM1 or APL1C, TEP1$_{cut}$ precipitates over time both *in vitro* and *in vivo* [6, 8]. LRIM1 and APL1C consist of an N-terminal leucine-rich repeat (LRR) domain capped by a cysteine-rich motif. The two LRR proteins form a heterodimer via a C-terminal coiled-coil domain that contains a helix-loop-helix motif [9]. The LRIM1/APL1C heterodimer binds to TEP1$_{cut}$ and stabilizes it in solution. TEP1$_{cut}$ that is in complex with LRIM1/APL1C retains an intact thioester bond that allows for covalent attachment to a pathogen surface [8]. In the absence of LRIM1, the thioester bond hydrolyzes over time, resulting in non-functional precipitation of TEP1 [6, 8].

A. gambiae forms a species complex, comprising multiple morphologically identical forms across Sub-Saharan Africa [10]. *TEP1* is among the most polymorphic genes within *A. gambiae* genomes, with multiple distinct alleles that are correlated with susceptibility to *Plasmodium* infection [11]. TEP1*R1 is an allele associated with *A. gambiae* L3-5 laboratory strain, predominately found in the West African "M" molecular form of *A. gambiae*, now named *A. coluzzii* [12]. TEP1*R1 homozygous mosquitoes are refractory to the rodent malaria parasite *P. berghei* and have decreased susceptibility to the human malaria parasite *P. falciparum* [5, 13, 14]. *TEP1*S1* is an allele associated with *A. gambiae* G3 strain, found predominantly in the "S" molecular form of *A. gambiae*, which are susceptible to both *P. berghei* and *P. falciparum* infection. Other susceptible alleles named *TEP1*R2* and *TEP1*S2* are found in the laboratory 4arr strain and field isolates [11].

TEP1 refractory and susceptible alleles are >90% identical with most variation being associated with variable loops within the TED, including two loops in direct proximity to the thioester bond. We previously reported that TEP1*S1$_{cut}$ has a half-life of thioester hydrolysis $t_{1/2} =$

8.5 h, while TEP1*R1$_{cut}$ has a half-life of thioester hydrolysis $t_{1/2}$ = 6.5 days [8]. Replacing the TED of TEP1*R1 with that of TEP1*S1 reduced the half-life of thioester hydrolysis to almost the same as full-length TEP1*S $_{cut}$, $t_{1/2}$ = 12 h. This suggests that TEP1 allelic variation within the thioester domain, specifically two hypervariable loops known as the pre-α4 and catalytic loop, respectively, contribute significantly to the stability of TEP1$_{cut}$. Both alleles bind LRIM1/APL1C on a timescale consistent with their half-life for thioester hydrolysis.

Here we compared additional alleles and by mutation analysis identify specific residues within the loops of the TED that impact TEP1 stability. We further demonstrate that the coiled-coil domain of LRIM1/APL1C is sufficient to stabilize TEP1$_{cut}$ in solution. Finally, we show that engineered disulfides within the TED-CUB-MG8 superdomain can stabilize TEP1$_{cut}$ similar to LRIM1/APL1C. These results suggest that the formation of the TEP1$_{cut}$/LRIM1/APL1C complex requires a conformational change involving dissociation of the TED-MG8 interface that is directly related to the conformational change leading to thioester hydrolysis and precipitation of TEP1$_{cut}$ in the absence of LRIM1/APL1C.

Results

Rate of thioester hydrolysis for TEP1 alleles

Polymorphisms between TEP1*R1 and TEP1*S1 are concentrated within and adjacent to the TED, and specifically in three hypervariable loops within the TEP. Two of these loops, the catalytic loop and the pre-α4 loop, are in close proximity to the thioester bond and form part of the TED-MG8 interface. The third loop, the β-hairpin loop, lies on the opposite side of the TED, surface-accessible and near the TED-MG8 interface. We previously showed that a chimeric protein replacing the TED of TEP1*R1 with TEP1*S1 had the same stability as TEP1*S1 [8], but this does not determine which of the three loops are responsible for this variable stability.

Blandin et al. (2009) reported two additional alleles, *TEP1*S2* and *TEP1*R2*, that are virtually identical to *TEP1*S1* and *TEP1*R1*, respectively, within the TED (Fig 1). The most notable difference between TEP1*S1 and TEP1*S2 is that the TEP1*S2 β-hairpin motif is identical to TEP1*R1 and TEP1*R2. Hence we used TEP1*S2 to test whether the R1/R2 β-hairpin motif has a stabilizing effect on TEP1$_{cut}$.

We produced TEP1*S2, performed limited proteolysis and measured the half-life of thioester hydrolysis in three independent experiments (Table 1). We observed that TEP1*S2$_{cut}$ had a half-life of thioester hydrolysis $t_{1/2}$ = 4.2 ± 0.2 h, half that of TEP1*S1$_{cut}$ $t_{1/2}$ = 8.5 ± 0.2 h. Hence, the β-hairpin motif is not responsible for the stabilization of TEP1*R1 relative to TEP1*S1, consistent with its location opposite the thioester bond and the TED-MG8 interface [8, 15].

TEP1*R2 is a chimeric form whose N-terminal fragment is similar to S1/S2, while the C-terminal fragment is almost identical to TEP1*R1 except for a single residue in the TED pre-α4 loop, TEP1*R1 Asn 919, which is glycine in TEP1*R2, TEP1*S1 and TEP1*S2. To test whether the R1 mutation G919N has a stabilizing effect on TEP1$_{cut}$, we produced TEP1*R2, performed limited proteolysis and measured the half-life of thioester hydrolysis. We observed that TEP1*R2$_{cut}$ had a half-life of thioester hydrolysis $t_{1/2}$ = 9.6 ± 0.4 h, comparable to that of TEP1*S1$_{cut}$, and 16 times less than that of TEP1*R1$_{cut}$ $t_{1/2}$ = 156 ± 12 h. This suggests that the differences between TEP1*R1 and TEP1*R2, specifically the point mutation N919G, have a significant effect on TEP1$_{cut}$ stability.

To confirm the role of Asn 919, we made the point mutation N919G in TEP1*R1 and measured the stability of TEP1*R1-N919G$_{cut}$. This single mutation reduced the half-life of thioester hydrolysis three times, TEP1*R1-N919G$_{cut}$ $t_{1/2}$ = 55 ± 10 h. The differences between
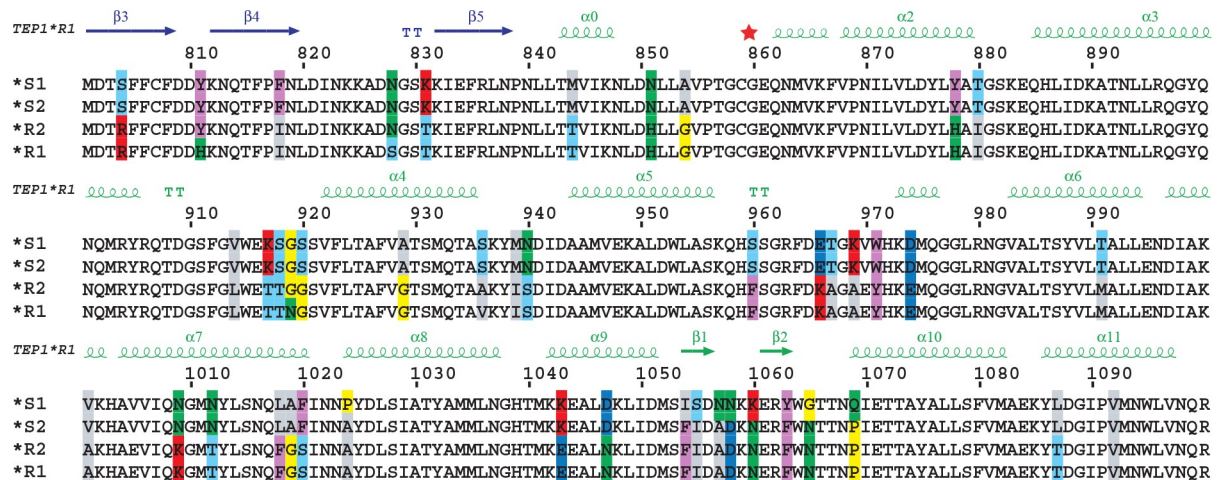
Fig 1. Sequence variation between TEP1 alleles within the TED. Multiple sequence alignment of TEP1 forms TEP1*S1, TEP1*S2, TEP1*R2 and TEP1*R1 residues 800–1100, including the hypervariable TED pre-α4 loop (917–920), catalytic loop (966–971) and β-hairpin (1054–1069). Adapted from Blandin et al. (2009) [11].

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TEP1*R1-N919G$_{cut}$ and TEP1*R2 that contribute to further destabilization of TEP1*R2$_{cut}$ lie outside of the TED and MG8 domains. The closest TEP1*R2 mutations relative to TEP1*R1 (i.e. conserved with TEP1*S1, TEP1*S2) are H811Y and S828N in the CUB domain.

The catalytic loop is the third hypervariable loop in the TEP1 TED, and is in direct proximity to the thioester bond. That catalytic loop is hypervariable between TEP1*S1/TEP1*S2 and TEP1*R1/TEP1*R2, hence we expected it to directly impact the stability of TEP1$_{cut}$. We therefore made the mutation TEP1*R1-S1$_{cat}$, substituting residues 966–971 from KAGAEY in TEP1*R1 to ETGKVW. The cleaved form of TEP1*R1-S1$_{cat}$ has a half-life of thioester hydrolysis $t_{1/2}$ = 9.1 ± 1.9 h, comparable to that of full-length TEP1*S1. Hence the catalytic loop alone is sufficient to explain the destabilization of TEP1*S1$_{cut}$ relative to TEP1*R1. Put another way, the unusual stability of TEP1*R1 compared to TEP1*S1, TEP1*S2 and TEP1*R2 is the result of specific mutations in both the pre-α4 and the catalytic loop with some contribution of TEP1*R1-specific mutations outside the TED-MG8 domains.

Stabilization of TEP1$_{cut}$ by engineered disulfide bonds

Following cleavage within the protease-sensitive region, TEP1$_{cut}$ slowly converts from a state that contains an intact thioester to one in which the thioester is hydrolyzed and which

Table 1. Rate of thioester hydrolysis of TEP1$_{cut}$.

TEP1 form	$t_{1/2}$ (h)	Ref.
*S2	4.2 ± 0.2	this work
*S1	8.5 ± 0.2	[8]
*R2	9.6 ± 0.4	this work
*R1	156 ± 12	[8]
*R1 S1$_{cat}$	9 ± 2	this work
*R1 N919G	55 ± 10	this work

Half-life for thioester hydrolysis determined by rate of precipitation for cleaved forms of TEP1. Each half-life is determined from three independent experiments.

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precipitates from solution. Cleavage of the protease-sensitive region occurs in a separate location from the TED-MG8 interface, the MG6 domain. Yet the TED-MG8 interface must separate for the thioester bond to bind to a substrate. Association of LRIM1/APL1C with TEP1 forestalls hydrolysis of the thioester, but how LRR complex binding stabilizes TEP1$_{cut}$ is unknown. We tested whether LRIM1/APL1C binding is separable from thioester hydrolysis and concomitant precipitation of TEP1$_{cut}$ by stabilizing the TED-MG8 interface with engineered disulfide bonds.

Guided by the crystal structure of TEP1*S1, we identified residues at the TED-MG8 and TED-CUB interfaces where mutation to cysteine would generate a disulfide bond with minimal perturbation of the structure [8]. We generated three disulfide mutants of TEP1*S1, varying the location of the disulfide between the TED and CUB/MG8 domains (Fig 2A). First, we joined TED residue K973 within the catalytic loop to residue Q1228 in the MG8 domain (K973C/Q1228C). Second, we joined TED residue V1102 to residue M801 in the CUB domain (M801C/V1102C). Third, we joined TED residue K969 within the catalytic loop to a residue in the MG8 domain Y1275 (K969C/Y1275C).

We confirmed formation of the engineered disulfide for TEP1*S1-M801C/V1102C using Ellman's reagent to titrate the number of cysteines forming disulfide bonds. TEP1 has three native disulfide bonds–one in the MG8 domain and two in the C-terminal anchor motif–so the disulfide mutant should have four. Hence we expect TEP1*S1 has six cysteines and TEP1*S1-M801C/V1102C has eight cysteines forming disulfide bonds. The measured number of cysteines for TEP1*S1 and TEP1*S1-M801C/V1102C were 5.71±0.42 and 8.65±0.30, respectively.

We next performed limited proteolysis and measured the half-life of thioester hydrolysis in three independent experiments (Fig 2B). The engineered disulfide K973C/Q1228C had the effect of increasing the half-life for thioester hydrolysis to $t_{1/2} = 4.0$ days (Fig 2B). The engineered disulfide M801C/V1102C increased the half-life for thioester hydrolysis to $t_{1/2} = 44$ days. The engineered disulfide K969C/Y1275C resulted in a half-life for thioester hydrolysis of $t_{1/2} > 100$ days. This confirms that precipitation of TEP1$_{cut}$ occurs after separation of the TED-MG8 domain, and preventing this conformational change can effectively stabilize TEP1$_{cut}$ indefinitely.

Stabilization of TEP1$_{cut}$ by LRIM1/APL1C coiled-coil domain

The LRIM1/APL1C heterodimer has three discrete, pseudosymmetric structural elements (Fig 3A). Each protein has an LRR domain capped by an LRRCT helix, followed by a short coiled-coil domain, an intermolecular disulfide, then a helix for LRIM1 and a flexible region for APL1C. Both proteins end with a C-terminal coiled-coil domain that contains a helix-loop-helix (HLH) motif. Multiple groups have shown that full-length LRIM1/APL1C stabilizes TEP1$_{cut}$ *in vitro* and *in vivo* [6–9, 16]. However, the LRIM1 and APL1C LRR domains were previously shown to be insufficient to stabilize TEP1*R1$_{cut}$ after treatment with methylamine (MeNH$_2$) to chemically inactivate the thioester bond, or to stabilize endogenous TEP1$_{cut}$ *in vivo* [9]. Povelones et al. (2011) expressed LRIM1/APL1C with internal deletions of the coiled-coil domain [16]. These constructs were unable to bind cleaved TEP1. In this study however, TEP1$_{cut}$ was produced by co-expression of separate N- and C-terminal fragments. As the structure of the TEP1 MG6 domain is formed by the intertwining of both N- and C-terminal fragments; co-expressing both fragments separately may result in misfolding of TEP1.

We therefore sought to test two hypotheses, (i) that LRIM1/APL1C binding is associated with conformational change of the TED-MG8-CUB superdomain by exploiting our disulfide-stabilized mutants, and (ii) that the coiled-coil domain of LRIM1/APL1C is sufficient to
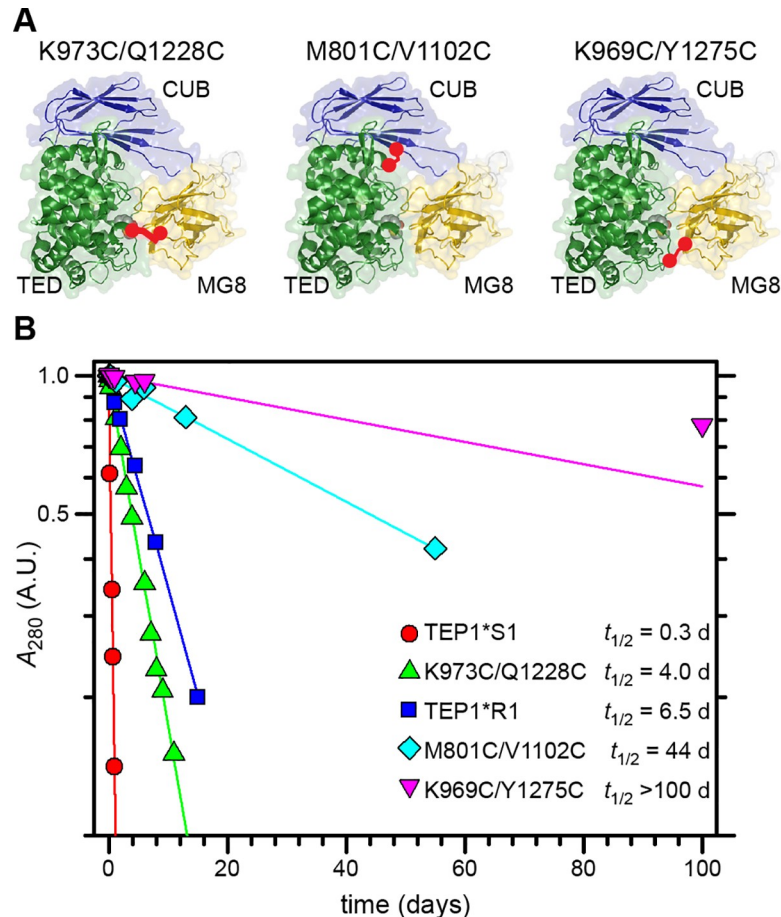
Fig 2. Engineered disulfides slow the rate of thioester hydrolysis. (A) Model of TED (green), CUB (blue) and MG8 (yellow) domains for TEP1*S1, with engineered disulfide bonds (red) K973C/Q1228C, M801C/V1102C and K969C/ Y1275C. (B) Rate of thioester hydroylsis for cleaved forms of TEP1*S1, TEP1*R1, and three engineered disulfide mutants of *TEP1*S1*.

stabilize TEP1$_{cut}$. First, we examined the ability of engineered disulfide mutants of TEP1*S1 to bind the LRIM1-FLAG/APL1C-Δ26-130-6×His construct previously found to stabilize TEP1*S1 [8, 9]. Then, to show that the C-terminal coiled-coil domain is the site of TEP1 binding we produced two C-terminal coiled-coil constructs of LRIM1/APL1C. LRIM1/ APL1C-CC1 comprises all C-terminal residues from the intermolecular cysteines LRIM1 C352 and APL1C C551. LRIM1/APL1C-CC2 comprises the C-terminal coiled-coil domain including the helix-loop-helix motif.

Despite the dramatically increased stability of the disulfide-engineered TEP1*S1 mutants, none were stabilized by or co-immunoprecipitated with LRIM1-FLAG/APL1C-Δ26-130-6×His (Fig 3B). Wild-type TEP1*S1$_{cut}$ does not IP with αFLAG immediately following cleavage. Neither full-length or cleaved TEP1*S1-K969C/Y1275C co-immunoprecipitated with LRIM1-FLAG/APL1C-Δ26-130-6×His seven days post-cleavage.

We then tested whether the coiled-coil domain of LRIM1/APL1C was sufficient to prevent the precipitation of TEP1$_{cut}$. When incubated with a mixture of TEP1*S1 and TEP1*S1$_{cut}$, both LRIM1/APL1C-CC1 and LRIM1/APL1C-CC2 were able to stabilize TEP1*S1$_{cut}$ in solution (Fig 3C). These results are consistent with those of Povelones et al. (2011), who showed that the interchain disulfide was dispensable for capture of TEP1$_{cut}$ by LRIM1/APL1C [16].
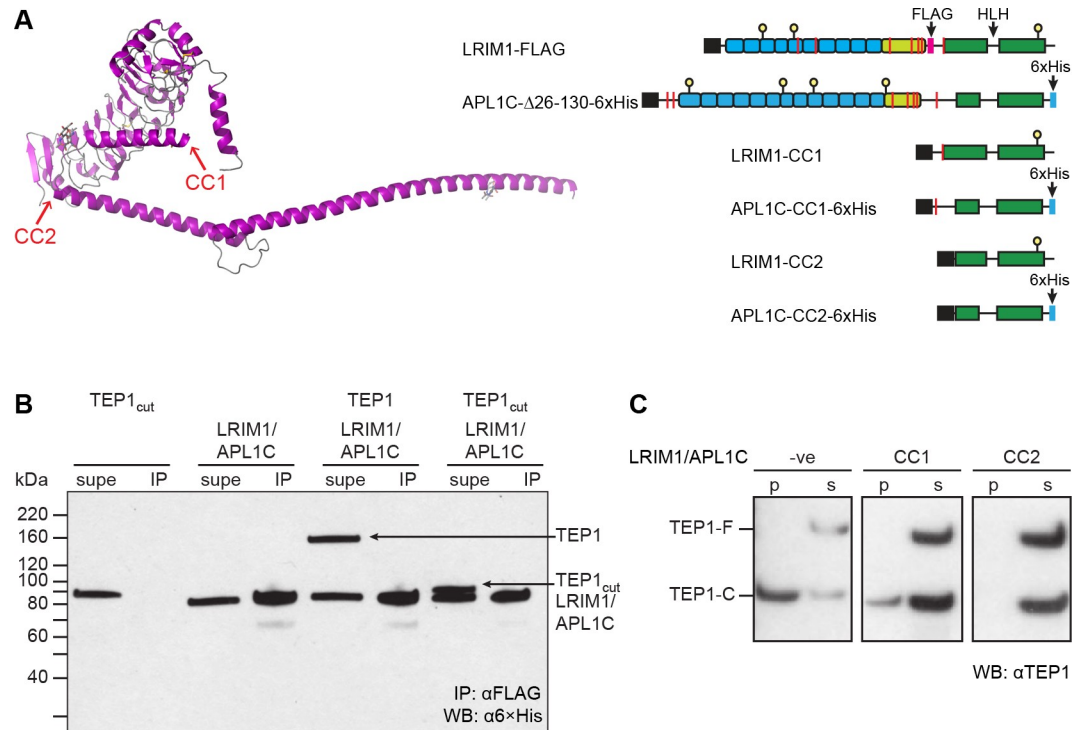
Fig 3. Disulfide-stabilized TEP1$_{cut}$ does not bind LRIM1/APL1C. (A) Model of the domain structure of LRIM1/APL1C (LRIM1 only, magenta) indicating the location of C-terminal truncations CC1 and CC2. A schematic diagram of LRIM1-FLAG/APL1C-Δ26-130-6×His construct and coiled-coil domain constructs CC1 and CC2, coiled-coil shown in green, cysteines as red bars, N-linked glycosylation site as yellow ball, 6×His tag in blue. (B) α6×His Western blot of supernatant (supe) and αFLAG immunoprecipitate (IP) of TEP1*S1-K969C/Y1275C seven days post-cleavage. The first sample is wt TEP1*S1 immediately following cleavage. Both uncleaved and cleaved TEP1 remain in the supernatatant and do not co-immunoprecipitate with LRIM1/APL1C. (C) αTEP1 Western blot of precipitate (p) and soluble (s) fractions for TEP1*S1 full-length (TEP1-F) and cleaved form (TEP1-C) after 24 h incubation with LRIM1/APL1C-CC1 and LRIM1/APL1C-CC2.

This demonstrates that the C-terminal coiled-coil domain of LRIM1/APL1C is necessary and sufficient to stabilize TEP1$_{cut}$.

Discussion

TEP1 is a key antiparasitic factor in *A. gambiae*, and allelic variation in TEP1 significantly influences susceptibility of *A. gambiae* to *Plasmodium*. Most of the variation between alleles is within the thioester domain, specifically three loops of which two–the pre-α4 loop and the catalytic loop–are proximal to the thioester bond and form part of the TED-MG8 domain interface. Variation in the pre-α4 and the catalytic loop can potentially affect the reactivity and substrate specificity of the thioester as well as the overall stability of the protein in solution. However, these inter-related properties complicate understanding the molecular mechanism of TEP1.

We previously reported a significant difference in stability for the cleaved alleles of TEP1*S1 and TEP1*R1 [8]. Here we report that genetic polymorphism in the TED domain of *TEP1* shapes its function by regulating protein stability. Interestingly, TEP1*R1, the only allele that confers mosquito resistance to *Plasmodium* infection, has an exceptionally long lifetime. Other forms display more subtle variation, ranging from 9.2 h for *R2 to 4.2 h for *S2.

Whether the increased stability of *R1 directly causes its antiparasitic activity remains to be demonstrated.

The increased stability of TEP1*R1 requires *both* changes in the catalytic loop sequence 966–971 from ETGKVW (S) to KAGAEY (R) and in the pre-α4 loop sequence 917–920 from KSGS (S) to TTNG (R), especially the mutation G919N. This is drawn from the fact that reversion of either 966–971 of 919 from R to S significantly decreases stability of cleaved TEP1. These changes are synergistic, because TEP1*R2 which is identical with TEP1*R1 in both these loops but for G919N is only marginally more stable than TEP1*S1. Yet interactions outside the TED-MG8 domain contribute to stability, because the mutant TEP1*R1-N919G is identical to TEP1*R2 in both the TED and MG8 domains yet is still more stable ($t_{1/2}$ = 2.3+0.4 days vs. 9.6 +0.4 h).

An open question is whether the conformational change in TEP1$_{cut}$ that permits LRIM1/APL1C binding is separable from that which induces thioester hydrolysis and precipitation. We sought to address this by engineering specific disulfide bonds between domains. We were able to stabilize TEP1*S1$_{cut}$ for months with engineered disulfides from the TED to either the MG8 or CUB, but in neither case did the cleaved protein interact with LRIM1/APL1C. Hence, either a single conformation or related conformations involving separation of the TED from CUB and MG8 are responsible for both LRIM1/APL1C binding and TEP1 aggregation and precipitation.

We illustrate the location of the three engineered disulfides to the TED-MG8 interface and the thioester bond in Fig 4. The first disulfide replaces K793 directly adjacent to the catalytic histidine of the TED catalytic loop and Q1228 at the top of the MG8 loop in the center of the interface near M1231, which directly abuts the thioester. Interestingly, this was the least stabilizing disulfide, less stable than TEP1*R1, suggesting that securing the catalytic loop to the top of the TED-MG8 interface does not prevent the interface from separating beneath it, possibly due to perturbation of the domains MG2 and MG6 that lie below. In comparison, the disulfide replacing K969 in the catalytic loop andY1275, one of the aromatic residues that ring the thioester bond, is indefinitely stabilizing.

Surprisingly, the third disulfide engineered between TED V1102 and CUB M801 is a highly stabilizing mutant even though it does not involve the TED-MG8 interface. In the conversion of complement C3 to C3b and C5 to C5b, the CUB domain undergoes a large motion along with the TED, hence locking the relative position of these domains may equally serve to prevent separation of the TED-MG8 interface.

In vertebrate complement factors the anaphylatoxin domain serves as a molecular wedge between the MG3 and MG8 domain, and we had speculated that LRIM1/APL1C might form an analogous complex following cleavage of TEP1. The fact that TED-MG8 engineered disulfides prevent LRIM1/APL1C binding run counter to this hypothesis. It suggests that the LRIM1/APL1C coiled-coil domain has a cryptic binding site somewhere on the TED, CUB and/or MG8 that is only exposed by dissociation of this superdomain. Povelones et al. (2011) found that both N- and C-terminal fragments were required for TEP1 to interact with LRIM1/APL1C [16]. Considering the C-terminal coiled coil domain is 17 nm end-to-end, longer than TEP1 itself, it is entirely possible that additional interactions could involve the N-terminal fragment of TEP1.

The LRIM1/APL1C heterodimer has long been known to stabilize TEP1$_{cut}$, and it has been shown that the LRR domains alone or as heterodimers with deletions in the coiled-coil domain are insufficient to stabilize TEP1$_{cut}$ [9, 16]. Here, we specifically purified heterodimers of only the coiled-coil domains of LRIM1/APL1C and show they are sufficient to stabilize TEP1*S1$_{cut}$, confirming that the coiled-coil domain is both necessary and sufficient to stabilize TEP1$_{cut}$.
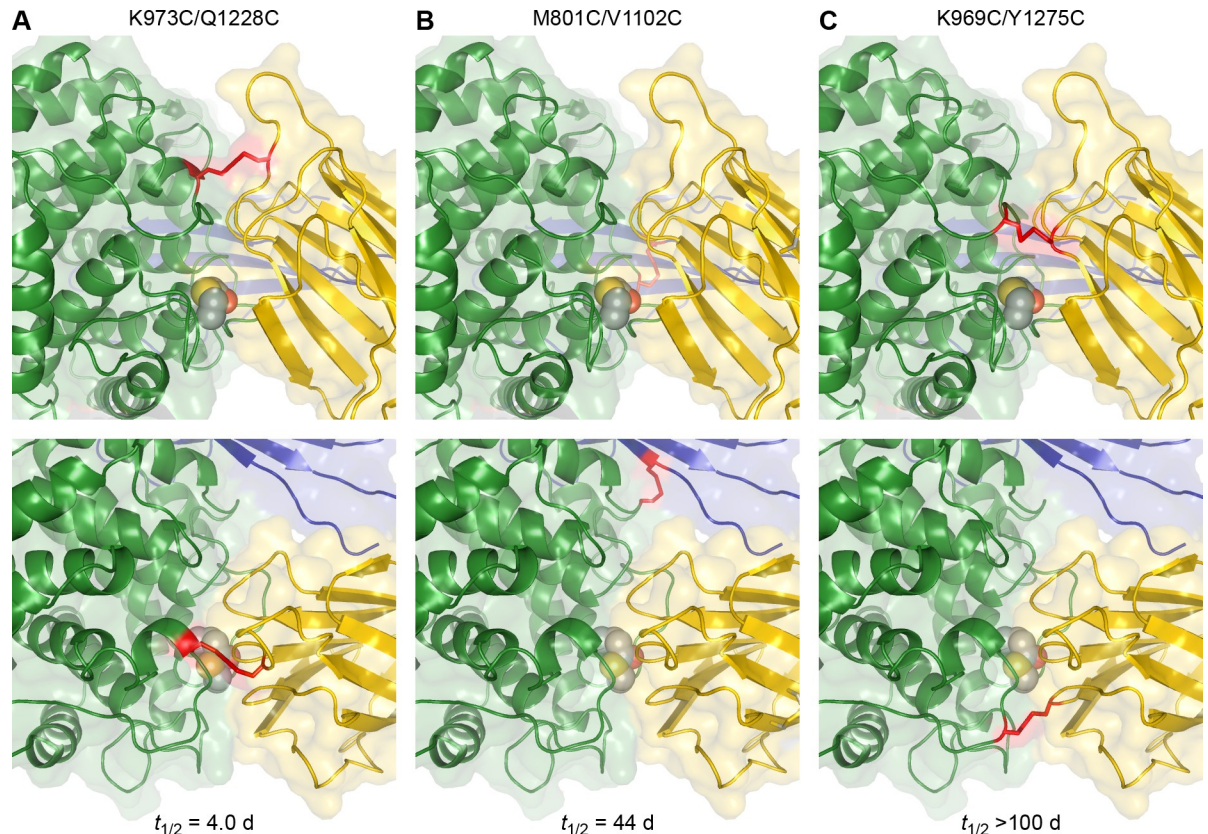
Fig 4. Illustration of engineered disulfides stabilizing the TED-MG8 interface. Front (upper) and top (lower) views of the TED-MG8 domain interface of TEP1*S1 with modeled engineered disulfides K973C/Q1228C, M801C/V1102C and K969C/Y1275C.

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This is significant because the coiled-coil domain is shared among the related genes APL1A[1] and APL1B, which also form a complex with LRIM1 [17].

As presently stands, the inability to isolate a stable complex of TEP1$_{cut}$ and LRIM1/APL1C in solution prevents structural studies that require a homogeneous and monodisperse sample. Additional engineering to solubilize TEP1$_{cut}$ or prevent its aggregation, the identification of orthologs or paralogs for the TEP1$_{cut}$/LRIM1/APL1C complex, or approaches for the analysis of heterogeneous specimens are necessary to define the molecular basis of LRIM1/APL1C regulation of TEP1 activation.

Materials and methods

Molecular cloning

DNA for *TEP1*R1* and *TEP1*S1* was obtained from cDNA clones or by total gene synthesis as previously described [8, 15]. DNA for *TEP1*R2* and *TEP1*S2* were generated by total gene synthesis from sequences in the 4arr laboratory strain [11]. All *TEP1* sequences were subcloned into pFastbac1 with a C-terminal 6×His tag. Additional mutations within the thioester domain were introduced by QuickChange site-directed mutagenesis (Stratagene). *LRIM1* and *APL1C* were subcloned into the pFastbac-Dual vector with C-terminal 6×His tag on APL1C. Construction of LRIM1-FLAG/APL1C-D26-130-6×His has been previously described [8, 9]. Truncations of the LRR and coiled-coil domains were introduced by site-directed mutagenesis.

Protein expression and purification

All TEP1 and LRIM1/APL1C constructs were expressed using the baculovirus expression system using T.ni cells in ESF-921 media (Expression Systems LLC). TEP1 was expressed for ~60 h at 27˚C, LRIM1/APL1C for ~48 h at 27˚C. Conditioned medium was concentrated and diafiltrated with 250 mM NaCl, 20 mM Tris-HCl pH 7.8 using Centramate tangential flow filtration system (Pall Biosciences). The diafiltrated medium was loaded onto 5 ml Talon resin (Clontech), washed with 10 CV of 250 mM NaCl, 20 mM Tris-HCl pH 7.8 and eluted by a 20 ml gradient to 250 mM imidazole.

TEP1 eluate from Talon resin was immediately desalted on a HiTrap 26/10 column (GE Healthcare) equilibrated with 100 mM NaCl, 20 mM Tris-HCl pH 8.0, loaded onto a MonoQ column (GE Healthcare) and eluted with a linear gradient from 100–600 mM NaCl. Further purification was achieved by gel filtration (Superdex 200) equilibrated with 100 mM NaCl 20 mM Na-Hepes pH 7.5 and cation exchange on a MonoS column. LRIM1/APL1C eluate from Talon resin was desalted into 50 mM NaCl, 20 mM Tris-HCl pH 8.5 prior to MonoQ chromatography followed by final purification on gel filtration (Superdex 200) equilibrated with 100 mM NaCl 20 mM Na-Hepes pH 7.5.

Limited proteolysis of TEP1

Purified TEP1 was cleaved using bovine pancreatic trypsin (Sigma) at a 1:20 molar ratio to TEP1 in 0.2 M NaCl and 20 mM HEPES pH 7.5. Samples were incubated for 5 min at 37˚C, then placed on ice and diluted 2-fold with 20 mM HEPES pH 7.5, 0.2 mM Leupeptin hemisulfate (Sigma), and 0.2 mM soybean trypsin-chymotrypsin inhibitor. Samples were immediately repurified on a Mono S 5/50 cation exchange column (GE Healthcare).

Thioester hydrolysis precipitation assay

TEP1 stability studies were performed as previously described [8]. Following limited proteolysis and re-purification, TEP1 samples were concentrated to an OD_{280} of 0.5–1.0 and stored at 20˚C. Samples and matching blank (filtrate from concentration) were centrifuged at $17,000{\times}g$, 20˚C for 10 min and A_{280}-A_{330} recorded. Separate time points are all derived from the same protein batch. Half-lives were calculated from samples with a decay to <25% initial value and results derived from three independent experiments.

Disulfide thiol assay

The method for titration of cysteines forming disulfide bonds was adapted from a standard procedure [18]. All reagents were prepared fresh to minimize oxidation. TEP1 (3–4 nmol, 400 μl at 1–1.6 mg/ml) was denatured with 2 ml denaturing buffer (6 M guanidinium chloride, 0.1 M Na_2HPO_4, pH 8.0), further exchanged into denaturing buffer on a PD-10 desalting column (GE Healtchare) and concentrated to ~2 ml. Free thiols were carboxymethylated with 25 mM iodoacetic acid (30 min incubation in dark, room temperature). Unreacted iodoacetic acid was removed by buffer exchange followed by reduction with 50 mM DTT (1–2 h, room temperature). Unreacted DTT was removed by buffer exchange, concentrated to 50–70 μM (<100 μl). The protein concentration was determined by A_{280} measurement on NanoDrop (Thermo Fisher) and sample used immediately. DTNB (Ellman's reagent), 10 μl at 4 mg/ml, was added to 200 μl of 2.5–3.5 μM TEP1 with a standard curve of 6.25–100 μM cysteine to verify linear response. However, the calculation of thiol concentration was made directly using the molar extinction coefficient of DTNB at 412 nm, $E_{412}TNB^{2-} = 1.37{\times}10^4$ $cm^{-1}M^{-1}$, divided

by the protein concentration to yield the number of cysteines present in disulfide bonds from three separate measurements.

Co-immunoprecipitation and western blotting

Rabbit polyclonal antibodies for TEP1*R1 have been previously described [9]. 10 µg of protein mixtures was diluted to 1 ml in IP buffer (50 mM Tris-HCl pH 7.8, 100 mM NaCl, 2 mM EDTA, 0.1 µg/ml BSA, 0.1% Tween-20). IP was performed with αFLAG-M2 agarose (Sigma). Beads were washed twice each with 50 mM Tris-HCl pH 7.8 ± 0.5 M NaCl and eluted by incubation with 2X Laemmli buffer. SDS/PAGE was run with 4–20% minigels, transferred to nitrocellulose and Western blotting performed with monoclonal α6×His/HRP (Clontech) or αTEP1.

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