A secreted *Plasmodium falciparum* kinase reveals a signature motif for classification of tyrosine kinase-like kinases

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Thorough bioinformatic and phylogenetic analyses of *Plasmodium falciparum* tyrosine kinase-like kinase (TKL) sequences revealed a clear evolutionary relationship of PF3D7_1121300 (thereafter called PfTKL2) to the IL-1 receptor-associated kinase (IRAK)/receptor-like kinase (RLK)/Pelle protein family. We identified a novel conserved motif that is unique to this family, as well as an insertion whose length allows distribution of its members into two distinct subfamilies, in a way that matches exactly the dichotomy between ‘Tube/Tube-like kinases’ (TTLKs) and ‘Pelle-like kinases’ (PLKs) distinguished previously on the basis of features in accessory domains. The PfTKL2 protein is expressed ubiquitously in asexual blood stages and in gametocytes, and the recombinant enzyme displays kinase activity in vitro. The protein is exported to the host erythrocyte; furthermore, in accordance with data from a previous study of the extracellular proteome of *Plasmodium*-infected erythrocytes, we show that PfTKL2 is secreted into the culture medium. Considering the functions of other members of the RLK/Pelle family in immunity, and its secretion to the extracellular medium, we speculate that PfTKL2 functions may include an immunomodulatory role promoting parasite survival in the human host.

INTRODUCTION

*Plasmodium falciparum* is the causative agent of the most severe form of malaria, a disease responsible for huge morbidity and mortality in the tropics. The pathology of malaria has been linked to excessive production of pro-inflammatory cytokines and chemokines by the innate immune system in response to parasite products (reviewed by Mens et al., 2010; Schofield & Grau, 2005). Whilst adequate and regulated production of inflammatory factors is necessary for the protection of the host against invading pathogens, excessive stimulation of inflammatory pathways can be detrimental to host survival and, by extension, to the parasite. Pathogenic organisms have been reported to subvert these pathways in a way that modulates their virulence and prolongs their survival time (Cirl et al., 2008).

Protein kinases play a central role in the regulation of the immune response (Manning et al., 2002). A conserved family of protein kinases belonging to the tyrosine kinase-like kinase (TKL) group (Hanks, 2003), well characterized in plants, insects and mammals, has been linked to the signalling pathways implicated in defence against pathogens and production of pro-inflammatory factors. They are represented in plants by receptor-like kinases (RLKs), in *Drosophila melanogaster* by the so-called ‘Pelle kinase’ and in mammals by ‘IL-1 receptor-associated kinases’ (IRAKs). The RLKs constitute the largest gene family in plants; for example, 1100 enzymes in the 1446-enzyme kinome of rice (*Oryza sativa*) are RLKs (Lehti-Shiu et al., 2009). In contrast to plants, mammals have only four members of this family, named IRAK1 to IRAK4 (Shiu & Bleecker, 2001), while the Pelle kinase is the sole member of this family in *Dr. melanogaster* (Towb et al., 2009). The expansion of RLKs in terrestrial plants is thought to have occurred via serial tandem gene duplication to increase the ability to recognize and perceive environmental challenges, including pathogens, salinity and UV light (Lehti-Shiu et al., 2009).
et al., 2009). Some members of plant RLKs are transmembrane receptors, with an extracellular region containing various domains and an intracellular kinase domain, while others are intracellular cytoplasmic kinases referred to as ‘receptor-like cytoplasmic kinases’ (RLCKs) (Lehti-Shiu et al., 2009). In mammals and insects, all members of this family are intracellular and act downstream of Toll/Toll-like receptors (TLRs), IL-1 receptors and IL-18 receptors (Towb et al., 2009; Wang et al., 2009). These proteins are essential mediators of the innate immune and inflammatory responses.

In addition to defence responses, a role in various developmental processes has been attributed to the RLK/Pelle family. Thus, in Drosophila, the Pelle enzyme, in addition to its role in innate immunity, also plays a vital role in embryonic development, including dorso-ventral patterning (Shelton & Wasserman, 1993; Towb et al., 2009). Several plant RLKs are also required for both immunity and developmental processes, whilst others are dedicated to either immunity or development (reviewed by Afzal et al., 2008). Members of this family are absent from unicellular organisms such as yeast (Shiu & Bleeker, 2003) and Dictyostelium (Goldberg et al., 2006). We previously reported that the P. falciparum genome contains four sequences that cluster with TKLs from higher organisms, and provided a detailed characterization of one of these, PfTKL3 (PlasmoDB identifier PF13_0258/PF3D7_1349300) (Abdi et al., 2010). Here, we present the characterization of PfTKL2 (PlasmoDB identifier PF11_0220/PF3D7_1121200), a protein we identified as belonging to the RLK/Pelle family – quite an unusual observation for a unicellular organism. We provide a detailed bioinformatic analysis of the protein and identify a signature motif for the RLK/Pelle family, and demonstrate that the kinase domain is indeed endowed with phosphorylation activity. We also show that the enzyme is exported to the host erythrocyte and is detectable in culture supernatants.

**METHODS**

**Bioinformatics**

**Sequence alignment.** Sequence alignment was performed using T-Coffee software (http://www.ebi.ac.uk/Tools/t-coffee/), with manual optimization when necessary. Shading of residues was performed using GeneDoc (K. B. Nicholas and H. B. J. Nicholas, unpublished, 1987) and bootstrap neighbour-joining phylogenetic trees were generated with version 4 of the MEGA program (Tamura et al., 2007).

**Structural modelling.** A 3D structure model was generated by aligning the PfTKL2 kinase domain sequence with that of human IRAK4. The aligned sequence was submitted to the SWISS-MODEL program (Arnold et al., 2006) using as template the IRAK4 X-ray crystal structure (PDB identifier 2NRU) (Kuglstatter et al., 2007; Wang et al., 2006). As an independent approach, the PfTKL2 kinase domain (without the insertions described in Fig. S1, available in Microbiology Online) was submitted to the Phyre2 program (http://www.sbg.bio.ic.ac.uk/phyre2). The retrieved PfTKL2 model structures were manipulated using PyMOL software (DeLano Scientific, www.pymol.org).

**HMM profile of a unique RLK/Pelle motif.** The kinase domain protein sequences of PfTKL2 and 47 homologues from mammals, plants and insects were aligned using T-Coffee. We used an HMMER software package (Eddy, 1998) to generate a hidden Markov model profile for a region corresponding to amino acids 146–178 of PfTKL2, that comprises part of the N-terminal extension and the glycine triad in subdomain I of the kinase domain (the glycine triad was included to restrict the search to protein kinases). This profile was then used to search several proteomic databases.

**Parasite culture.** Asexual parasites of the P. falciparum 3D7 clone were grown as described previously (Dorin et al., 1999) and gametocytes were prepared according to the protocol of Carter et al. (1993).

**Plasmid construction**

**PfTKL2 kinase domain WT.** The region encoding the PfTKL2 kinase domain (nucleotides 592–1843 of the predicted coding sequence) was amplified by PCR from a P. falciparum asexual cDNA library (strain IT04; Berendt et al., 1989) using primers 504 and 505 containing BamHI and SalI sites, respectively (Table S1). The amplified fragment was cloned into the pET28a vector between the BamHI and SalI restriction sites.

**PfTKL2 kinase-dead mutant pET28a.** Mutations of the specific residues (K247→M and D409→N) were introduced following the method of Ho et al. (1989) by two rounds of PCR with specific primers (K→M: primers 668 and 667; D→N: primers 654 and 655) containing the mutations (Table S1).

**Expression and purification of recombinant proteins.** The expression vectors were transformed into Rosetta2DE3pLysS cells (Stratagene). pET28-PfTKL2KD-transformed cells were grown in LB medium containing 10 μg kanamycin ml⁻¹ and 34 μg chloramphenicol ml⁻¹, and expression was induced with 1 mM IPTG at 20 °C overnight. Purification of the His₆-tagged proteins was performed on Ni-NTA agarose beads (Qiagen).

**Generation of custom antibody.** A PfTKL2 peptide (599-CINMYLEDMERGEQ-612) located outside the kinase domain was selected in collaboration with Biogenes (http://www.biogenes.de) to maximize immunogenicity, whilst minimizing cross-reactivity with other P. falciparum proteins, and used to immunize rabbits. The antibodies were immunopurified against the peptide.

**Western blotting**

**Cell extracts.** Parasite pellets were obtained by saponin lysis from synchronized cultures of asexual parasites and gametocytes. Red blood cell (RBC) ghosts were prepared by hypotonic lysis as described in Blisnick et al. (2000). Briefly, infected RBCs were suspended in 40 volumes of incomplete RPMI diluted 5-fold with distilled water and supplemented with a protease inhibitors cocktail (Roche), and then incubated for 30 min on ice. For uninfected RBCs, 10-fold diluted incomplete RPMI was used instead. The lysate was centrifuged at 10 000 g at 4 °C, for 45 min; the ghost fraction was collected from the top of the parasite pellet. To separate ghosts from parasite pellets, the ghost layer was collected into a new tube, and both ghosts and parasite pellets were washed three times with lysis buffer and frozen at −80 °C until use. The resulting samples must be seen as enriched preparations rather than pure fractions.

To obtain protein extract, the RBC ghosts or the parasite pellets were resuspended in lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 0.1 mM EDTA, 0.5 % Triton X-100 and Roche protease inhibitor cocktail) for 30 min on ice, briefly sonicated and centrifuged at 16 000 for 10 min at 4 °C. Then 10 μg (when the experiment required normalization for protein quantity) or equal volumes of
RBC ghost or parasite pellet (when the experiment required normalization for cell number) from supernatants were boiled in Laemmli sample buffer, run on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane (transfer buffer: 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol and 0.025% SDS). Membranes were blocked in 5% skimmed milk (Bio-Rad) in PBS/0.25% Tween 20 for 1 h at room temperature. Washes were performed in PBS/0.25% Tween 20. Rabbit immune sera raised against the peptide (see above) or pre-immune sera from the same animal (1:1000), the affinity purified rabbit sera (1:1000) and anti-rabbit IgG-HRP (Sigma, 1:10 000) were used for Western blot analysis. Detection was performed by chemiluminescence using the ECL system (Perkin Elmer). Rabbit anti-PGKR (1:15 000) and anti-P. falciparum thioredoxin peroxidase 1 (1:4000) antibodies (a gift from Professor Sylke Müller), mouse human anti-MEK1 (1:500), and rabbit anti-spectrin (1:100; a gift from Professor Leann Tilley), and mouse Alexa Fluor 488 and rabbit Alexa Fluor 647 (1:1000; Molecular probes). Images were acquired with a DeltaVision Elite confocal microscope (Applied Precision). Deconvolution, maximum projections and 3D volume reconstitution were performed with Softworx 5.0.0. The Fiji and Photoshop CS5 software were utilized for further image handling.

**Kinase assays.** Kinase assays were performed in a 30 μl reaction mixture containing the recombinant (either WT or mutant) enzyme, 5 μg of substrate [myelin basic protein (MBP), histone H1, α-casein or β-casein], 20 mM MgCl₂, 2 mM MnCl₂, 10 μM ATP (containing 0.075 μBq [1-32P]ATP), 10 mM NaF and 10 mM β-glycerophosphate in 20 mM Tris, pH 7.5). The mixture was incubated for 30 min at 30 °C, and the reaction was stopped by adding Laemmli buffer and heating at 100 °C for 3 min. The samples were resolved by SDS-PAGE gel, and the gel was stained with Coomassie blue, dried and exposed to an X-ray film (Kodak) for 24 h for autoradiography.

**RESULTS**

PfTKL2 is a member of the RLK/Pelle family

In the initial *P. falciparum* kinome study, PfTKL2 clearly clustered within the TKL group, but was not assigned homology to any specific human TKL (Ward et al., 2004). To find its closest homologues in other systems, BLASTP analyses were carried out using as a query the PfTKL2 kinase domain (without the insertions described below). Among mammalian proteins, the top-scoring sequence (Table 1) is an IRAK (31 % identity, E value 6e–27), whilst in arthropods (*Dr. melanogaster* and *Viridiplantae*, the top scorers were the Pelle kinase (31 % identity, E value 3 e–25) and a plant RLK (33 % identity, E value 2 e–38), respectively (data not shown). Reciprocal BLASTP analysis using the kinase domains of human IRAK1, *Drosophila* Pelle and a plant RLK to query the *P. falciparum* predicted proteome confirmed this finding (Table 1; data not shown for Pelle).

The PfTKL2 kinase domain was aligned with representatives of all human TKL families, and the alignment was used to generate a phylogenetic tree (Fig. 1a), which revealed clear clustering of PfTKL2 within the IRAK family.

### Table 1. BLASTP analysis

<table>
<thead>
<tr>
<th>Query</th>
<th>Hits (accession no./identifier)</th>
<th>Maximum identity (%)</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PfTKL2 kinase domain (without the insertion highlighted green in Fig. S1) was used as a query to search the <em>Homo sapiens</em> protein database hosted at the National Center for Biotechnology Information (<a href="http://blast.ncbi.nlm.nih.gov/Blastcgi">http://blast.ncbi.nlm.nih.gov/Blastcgi</a>)</td>
<td>NP_001020413.1 (IRAK1, isoform 2)</td>
<td>33</td>
<td>6e–27</td>
</tr>
<tr>
<td></td>
<td>NP_001138728.1 (IRAK4, isoform b)</td>
<td>29</td>
<td>2e–27</td>
</tr>
<tr>
<td></td>
<td>NP_001135995.1 (IRAK3, isoform b)</td>
<td>28</td>
<td>2e–21</td>
</tr>
<tr>
<td>2. Human IRAK1 kinase domain was used as query in PlasmoDB (plasmodb.org)</td>
<td>PF3D7_1121300 (PfTKL2)</td>
<td>36</td>
<td>1.5e–21</td>
</tr>
<tr>
<td></td>
<td>PF3D7_1315100 (PIPK9)</td>
<td>28</td>
<td>7.4e–16</td>
</tr>
<tr>
<td></td>
<td>PF3D7_0525900 (PINEK2)</td>
<td>25</td>
<td>2.9e–13</td>
</tr>
<tr>
<td>3. <em>Arabidopsis</em> RLK (Q9C8I7/Q9C8I7_ARATH) kinase domain was used as a query in PlasmoDB [Note: PfTKL2 (PF3D7_1121300) has an E value that is 10³- and 10⁶-fold lower than the second-best hit (PF3D7_1315100)]</td>
<td>PF3D7_1121300 (PfTKL2)</td>
<td>40</td>
<td>1.6e–37</td>
</tr>
<tr>
<td></td>
<td>PF3D7_1315100 (PIPK9)</td>
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<td>2.6e–21</td>
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<td></td>
<td>PF3D7_1454300 (PIKIN)</td>
<td>28</td>
<td>1.2e–18</td>
</tr>
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(bootstrap value 69%). A further phylogenetic analysis of the kinase domains of PfTKL2 and RLK/Pelle family members from a variety of organisms (Fig. 1b) clearly showed clustering of the PfTKL2 sequence with RLKs, with a strong bootstrap value of 82%, while human and insect IRAK/Pelle appear together in a distinct cluster. The behaviour of the other mammalian TKL families used as an outgroup confirmed our initial observation (Fig. 1a) that PfTKL2 is more related to RLK/Pelle family than to any other family in the TKL group.
The 5028 bp pftkl2 gene encodes a predicted protein of 1675 residues. The central kinase domain is flanked by 224- and 1097-residue N-terminal and C-terminal extensions, respectively (Fig. 2). No annotated functional motif is detectable within these extensions, but a pentapeptide repeat region of unknown function is present within the C-terminal extension. BLASTP searches of apicomplexan genomes hosted in EuPathDB (http://eupathdb.org) using the PfTKL2 kinase domain as a query detected potential orthologues in all Plasmodium species whose genome has been sequenced (for Plasmodium yoelii, only a partial sequence is available), as well as in Toxoplasma gondii, Neospora caninum and Eimeria tenella (Table 2), but not in Babesia, Cryptosporidium or Theileria spp. Alignment between the predicted proteins of Plasmodium spp., To. gondii and Ne. caninum revealed little conservation outside the kinase domain (not shown). No clear orthologues were detected in other protozoa.

The proteins of insects (Pelle) and mammals (IRAK) share a similar domain organization consisting of an N-terminal death domain and a central kinase domain. Plant RLKs possess a similar structure, but the N-terminal extension contains a diverse array of domains, the most common being leucine-rich repeats (LRRs). The apicomplexan PfTKL2 orthologues possess an extraordinarily long C-terminal extension not found in the homologues from other organisms and have no detectable transmembrane domain (Fig. 2). Subdomain I does not contain the full glycine triad (GXGXXG) involved in ATP binding that is found in many (but not all) eukaryotic protein kinases. Instead, the corresponding motif reads AKGGNG in PfTKL2, with the first G in the triad being substituted with an alanine residue (Fig. S1); likewise, 11 % of human protein kinases lack the first G of the triad (Kostich et al., 2002) and this clearly does not affect enzymic activity. Other P. falciparum protein kinases with an incomplete glycine triad have been shown to possess kinase activity (Ward et al., 2004).

**Identification of unique motifs conserved throughout the RLK/Pelle family**

To detect sequence features that might be used as a signature for members of this family, an alignment of these proteins from apicomplexans and other organisms was generated (Fig. S1). This revealed (i) a novel motif that is conserved throughout the family, (ii) the conservation of a tyrosine residue at the gatekeeper position, and (iii) apicomplexan-specific divergent features (Fig. 3a).

**Novel conserved motif.** This was identified immediately upstream of the glycine triad in subdomain I. In this region, three positions are highly conserved; a leucine (L) or any branched-chain amino acid residue, an invariant threonine (T) and a phenylalanine (F) or a tryptophan
Fig. 3. Protein sequence alignment of representative members of the RLK/Pelle family. (a) The novel conserved motif of unknown function (UC, unknown, conserved) was identified N-terminal to the glycine triad (GXGXXG) in subdomain I (see complete alignment in Fig. S1). The three residues shaded in black are the most conserved. The tyrosine gatekeeper residue (GK) is shown in pink. The residues corresponding to those necessary for activation of IRAK4 are indicated in green with a black background. The threonine residue conserved only in apicomplexan sequences is shown in red. The apicomplexan sequences possess a divergent APE motif (highlighted with a yellow background). In *Plasmodium*, the conserved glutamate is substituted by tyrosine, and in *Toxoplasma* and *Neospora* by lysine (shown in red with a yellow background). Some plant RLKs have an aspartate as the first residue of the motif, like apicomplexans. The regions corresponding to PfTKL2 amino acid residues 206–220, 286–292 and 427–455 are shown. (b) Hidden Markov model logo of the conserved novel motif with unknown function (UC). The height of each letter is proportional to the frequency of the amino acid at that position. An asterisk labels the three most highly conserved residues. In total, 48 RLK/Pelle sequences from various organisms were used to generate the logo (http://www.sanger.ac.uk/cgi-bin/software/analysis/logomat-m.cgi).
Tyrosine gatekeeper. The so-called ‘gatekeeper residue’ controls the binding of nucleotides and small molecule inhibitors to protein kinases. An atypical tyrosine gatekeeper was identified as unique to IRAKs among human protein kinases (Wang et al., 2006) and is conserved in all members of RLK/Pelle family examined, including PfTKL2 (Fig. 3, shown in pink).

Apicomplexan-specific features. Sequence alignment and overall domain organization concur to reveal divergence between apicomplexan PfTKL2 orthologues and RLK/Pelle from higher eukaryotes. The APE motif in subdomain VIII in which the glutamate residue is highly conserved in eukaryotic protein kinases is substituted by DPY in Plasmodium and DPK in To. gondii and Ne. caninum (Fig. 3, highlighted in red with a yellow background). IRAK4 kinase activity is regulated by autophosphorylation at three sites in the activation domain (T342, T345 and S346) (Cheng et al., 2007). These residues are conserved in most plant and insect but not in apicomplexan sequences, except for Ne. caninum where the serine residues corresponding to T342 is conserved (Fig. 3, highlighted in green). Instead, all apicomplexan sequences contain a threonine (T444 for PfTKL2) that is not found in RLK/Pelle (Fig. 3, highlighted in red). Moreover, PfTKL2 contains two insertions in the catalytic domain (Fig. S1) unique to Plasmodium spp. (insertion I, residues 305–380; insertion II, residues 482–513).

Similarity of the PfTKL2 model structure to the IRAK4 crystal structure

The 3D crystal structure of IRAK4 has been solved (Kuglstatter et al., 2007; Wang et al., 2006). Sequence identity >30% allows accurate modelling of folding (Baker & Sali, 2001; Martí-Renom et al., 2000). Therefore, using the IRAK4 structure as a template (Fig. 4a), a PfTKL2 model structure was generated (Fig. 4b) with the SWISS-MODEL program (Arnold et al., 2006). Alignment of the modelled PfTKL2 structure with that of IRAK4 revealed significant overlap, especially in the N-terminal lobe (Fig. 4c). Interestingly, this is evident particularly in the novel conserved motif described above (coloured in blue and red in Fig. 4a and b, respectively). Very similar results were obtained with a distinct modelling server, Phyre2 (Fig. S4).

The tyrosine in the DPY motif of PfTKL2 is at the same position as the glutamate of the APE motif and may play a similar role (see Discussion), while threonine T444 is close to T345 and S346 of IRAK4; since phosphorylation of the latter residues is known to regulate enzymic activity, the apicomplexan-specific T444 may functionally substitute for these residues. Based on the model structure, insertions I and II are located between secondary structural elements in the C-lobe, and are predicted not to affect the overall folding of the protein.

Position of the novel conserved motif classifies RLK/Pelle proteins into two distinct groups

The IRAK4 structure contains a short loop named the ‘Schellman loop’ consisting of the solvent-exposed motif 284-PISVGG-289 (Kuglstatter et al., 2007), which is absent from the PfTKL2 model structure (Fig. 5a, top panel, motif in pink). Compared to PfTKL2, IRAK4 contains an extra six residues between the novel conserved motif and the glycine triad in subdomain I of the kinase domain (Fig. 5a, bottom panel), in line with the lack of the Schellman loop in PfTKL2. Detailed examination of a large panel of RLK/Pelle proteins revealed that the proteins fall into two groups: (i) those that contain 10–18 residues in this region, which includes IRAK4, and (ii) those (including PfTKL2) containing four to six residues (Fig. 5b). A recent study on mammalian and insect members of this family classified the proteins into ‘Tube/Tube-like kinases’ (TTLKs) and ‘Pelle-like kinases’ (PLKs)’ on the basis of dichotomic similarities in their death and kinase domains (Towb et al., 2009). Interestingly, the two classifications perfectly match, as all Pelle-like proteins possess four to six residues, while all TTLKs possess 10–18 residues, in the region between the novel motif and subdomain I.

PfTKL2 is a constitutively expressed and exported kinase

Elucidating the spatio-temporal expression profile of PfTKL2 might shed some light on the function of the protein, notably with respect to a possible receptor role. A rabbit polyclonal antiserum was raised against a peptide derived from the PfTKL2 sequence, whose specificity was tested by Western blot on a mixed blood-stage parasite extract. As shown in Fig. 6(a), a band with an apparent molecular mass of 250 kDa (predicted size 200 kDa) was detected with the immune serum (lane 2), while no signal was seen with the pre-immune serum (lane 1). The immunopurified antibody yielded a clean signal (lane 3) and was used in subsequent Western blot analyses.

Western blot analysis of protein extracts from rings, trophozoites, schizonts and gametocytes shows that PfTKL2 is expressed in all these stages (Fig. 6b), in line with microarray data available in PlasmoDB. The protein seems to be more abundant in rings than in schizonts. To determine if PfTKL2 is exported from the parasite into the host erythrocyte, synchronized schizont-infected RBCs were hypotonically lysed to obtain RBC ghost- and parasite-enriched fractions. Proteins extracted from both
fractions were analysed by Western blot, using a ghost extract from uninfected RBCs as a negative control. The result clearly demonstrates that PfTKL2 is more abundant in the infected RBC ghost than in the parasite pellet (Fig. 6c), suggesting the protein is exported to the RBC. Antibodies against human MEK1 and *P. falciparum* thioredoxin peroxidase 1 were used as control for RBC and parasite cytosolic proteins, respectively, and behaved as expected in the Western blot analysis.

Unfortunately, our antibody did not work in immunofluorescence assays (data not shown). Immunofluorescence assays were therefore performed using another anti-PfTKL2 antibody, which has previously been shown to also yield a single band at the expected size in Western blot analysis (Singh et al., 2009). A co-localization experiment using a parasitophorous vacuole marker (Exp1) clearly shows that a fraction of the PfTKL2 protein is exported into the RBC cytoplasm (Figs 7a and S5a; Movie S1). Further, the dual staining of PfTKL2 with RBC spectrin shows that PfTKL2 is present in the RBC cytosol (Figs 7b and S5b; Movie S2). Visualization through Movies S1 and S2 clearly shows association of distinct dots of PfTKL2 within the infected erythrocyte cytosol and at (or very near) its surface, in line with Western blot data (Fig. 6c) indicating that a large fraction of the PfTKL2 pool is detectable in the ghost fraction.

A recent report on the unbiased proteomics-based detection of proteins in parasite culture supernatants suggested that at least a fraction of PfTKL2 is secreted out of the infected erythrocyte (Singh et al., 2009). In line with the proteomics data, Western blot and immunofluorescence assays allowed us to detect PfTKL2 (i) in the culture medium (unlike *P. falciparum* glutathione reductase, a cytosolic enzyme used as a control) (Fig. 6d) and (ii) in the RBC cytoplasm (Fig. 7). Thus, our results are in agreement with those of Singh et al. (2009) and concur to indicate that at least a fraction of PfTKL2 protein is secreted outside of the infected erythrocyte.

**In vitro activity of recombinant PfTKL2**

The PfTKL2 kinase domain (residues 198–578) was expressed in *Escherichia coli* with an N-terminal His6-tag. The predicted molecular mass of the recombinant

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**Fig. 4. PfTKL2 model 3D structure versus IRAK4 crystal structure.** (a) Crystal structure of IRAK4 (PDB identifier 2NRU). (b) model structure of PfTKL2. (c) Overlay of (a) and (b). The region inclusive of the novel motif (UC) in Fig. 3, labelled ‘N-extension’ in the IRAK4 structure (Wang et al., 2006), the tyrosine gatekeeper (GK), the phosphorylated residues (T345, S346) in the IRAK4 structure and PfTKL2 T444, the insertion I in PfTKL2, the glutamate of the APE motif of IRAK4, and the aspartate and tyrosine in the corresponding PfTKL2 DPY motif are all highlighted with colours different from the main body of the structures. (d) Comparison of the positions of the coloured residues in the APE of IRAK4 and DPY of PfTKL2 and the R of subdomain XI: blue, PfTKL2; red, IRAK4.
Fig. 5. Classification of IRAK/RLK/Pelle proteins into TTLK and Pelle. (a, top panel) The structure of IRAK4 and PTKL2 (model) starting from the start of the novel conserved motif (UC) to the glycine triad (GXGXXG), inclusive. Starting from the N-terminus, the green represents the TNNF residues, blue the DER (IRAK4) or SEY (PTKL2); this is followed by the Schellman loop (pink) which consists of the residues PISVGG; the six additional residues that IRAK4 has compared to PTKL2 in this region account for the absence of this loop in PTKL2 model structure (see the alignment in the bottom panel). According to Kuglstatter et al. (2007), IRAK4’s TNNF forms a motif named the ST motif and DER forms an ASX motif. The novel motif covers only the TNNF and extends to the N-terminus. (a, bottom panel) Alignment of the amino acid sequence forming the above structure. Note IRAK4 contains six more residues (pink) in the region between the UC and the GXGXXG. (b) The region between the end of the novel conserved motif and the start of the glycine triad classifies IRAK/RLK/Pelle proteins into two groups: TTLKs and PLKs. TTLKs contain 10 or more residues in this region, whilst Pelle-like proteins contain four to six residues. The red arrows point to the boundaries of this region. Apicomplex, apicomplexans.
His6-PfTKL2 protein is 49 kDa, but the expressed protein runs at 43 kDa on SDS-PAGE (Fig. 8a). The activity of the recombinant protein was tested against the non-physiological substrates MBP, histone H1 and α/β-caseins. The enzyme showed activity against MBP and caseins (Fig. 8b), and exhibited autophosphorylation activity, clearly demonstrating that PfTKL2 is a genuine kinase. Two ‘kinase dead’ mutants (K247A and D409A) were produced to rule out that the observed activity is due to co-purified bacterial contaminants rather than to recombinant PfTKL2. The K is involved in the ATP orientation and also in stabilizing the active enzyme conformation by forming a salt bridge with the glutamate (E) in subdomain III. The aspartate is directly involved in phosphotransfer (Kostich et al., 2002). The D→A mutant (Fig. 8b) is a genuine activity-dead mutant, while the K→M mutant (Fig. 8c) still displays weak autophosphorylation and weak activity against MBP. In IRAK4, interaction between the Glu in subdomain III and the ‘Y’ gatekeeper residue (instead of the K in subdomain II) stabilizes the active conformation. The presence of the ‘Y’ gatekeeper may underlie the maintenance of a weak activity of the K→M mutant, which, in PfTKL3, completely abolishes activity (Abdi et al., 2010) (see Discussion).

As shown in Fig. 1, PfTKL2 is closer to plant RLKs; some recombinant RLKs, such as Pto, prefer manganese to magnesium as the metal ion cofactor (Xing et al., 2007). Assaying PfTKL2 activity in the presence of various divalent metal cations clearly shows that manganese stimulates the activity of PfTKL2 five times more efficiently than cobalt, and >30-fold more efficiently than magnesium (Fig. 9).

**DISCUSSION**

**Phylogeny, domain organization, amino acid sequence comparison and structure-based homology modelling**

We demonstrated by phylogenetic analysis that PfTKL2 is a member of the RLK/Pelle family, and detected potential orthologues in the apicomplexans To. gondii, Ne. caninum and E. tenella. The pathways in which RLK/Pelle enzymes operate are conserved in evolutionarily divergent systems (Lehti-Shiu et al., 2009; O’Neill & Greene, 1998; Towb et al., 2009). Furthermore, the overall domain organization of the proteins is largely conserved, although the apicomplexan homologues display some degree of divergence (Fig. 2).

**Tyrosine gatekeeper.** The gatekeeper is a pivotal residue that controls access to an internal hydrophobic pocket at
the back of the ATP-binding site that nucleotides do not exploit. The most abundant gatekeeper residue in kinases is methionine, followed by leucine, threonine and phenylalanine (Kuglstatter et al., 2007). A tyrosine gatekeeper is rare in human protein kinases, being restricted to the IRAK family. In contrast, tyrosine gatekeepers are quite common in plant protein kinases, a direct consequence of the expansion of the RLK family. The alignment file for the published *P. falciparum* kinome (Ward et al., 2004) shows the presence of three more sequences, in addition to PfTKL2, that contain a tyrosine gatekeeper: PlasmoDB identifiers PF3D7_1121900, PF3D7_0213400 (PfPK7) and PF3D7_0615500 (Pfcrk-5). PF3D7_1121900 and PfPK7 are orphan kinases and Pfcrk-5 is an atypical, apicomplexan-specific cyclin-dependent protein kinase (Dorin-Semblat et al., 2013). In IRAK4, the tyrosine gatekeeper forms a direct hydrogen bond interaction with the conserved glutamate in subdomain III (Fig. 3), allowing the protein to assume the active conformation (Kuglstatter et al., 2007; Wang et al., 2009). In other protein kinases, this stabilizing interaction involves a lysine in subdomain II and the same glutamate in subdomain III. This indicates the transition between active and inactive conformation of these proteins is regulated differentially compared to other protein kinases. As suggested earlier, this may explain the weak activity observed with the K→M ‘kinase dead’ PfTKL2 mutant. Crystal structures of PfPK7 (Merckx et al., 2008) and IRAK4 (reviewed by Wang et al., 2009) bound to inhibitors show, in both proteins, that the aromatic side chain of the tyrosine gatekeeper interacts with that of inhibitory ATP analogues. Moreover, a nitrile group of an inhibitor was also shown (in the case of PfPK7) to interact with the hydroxyl group of the tyrosine. This suggests that this tyrosine residue might be exploited in the design of antimalarial drugs that target tyrosine gatekeeper-containing *P. falciparum* kinases. Structure-based inhibitor design using the crystal structure of PfPK7 has indeed started (Klein et al., 2009). Highly potent inhibitors targeting IRAK4 have been developed (Buckley et al., 2008a, b, c) and their potential for the treatment of inflammatory diseases is being evaluated (reviewed by Cohen, 2009; Wang et al., 2009). The activity of these inhibitors against recombinant PfTKL2 deserves investigation.

**Unique conserved N-terminal extension.** We identified in the N-terminal extension a novel motif conserved in the RLK/Pelle family (Fig. 3). As stated earlier, this region has been associated with high expression levels and activity of recombinant IRAK4 (Kuglstatter et al., 2007; Wang et al., 2006), suggesting functional relevance of this motif. Conservation of the motif across evolutionarily very divergent systems is a further indication of its importance.

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**Fig. 7.** Intracellular localization of PfTKL2 by immunofluorescence assay of WT 3D7 parasites. (a) Co-localization of PfTKL2 and the parasitophorous vacuole protein Exp1. (b) Co-localization of PfTKL2 and the RBC cytoskeleton protein spectrin. DAPI was used to stain the parasite DNA. Bar, 2 μm.
Fig. 8. Expression and kinase activity of PfTKL2 recombinant protein. (a) In vitro expression of PfTKL2 kinase dead: left, Coomassie stain of the gel; right, Western blot using an anti-His antibody. Lane 1, uninduced lysate; lane 2, induced lysate. Lane 3, purified recombinant PfTKL2. (b) In vitro kinase activity assay: lane 1, WT+H1+MBP; lane 2, WT+β/γ-caseins; lane 3, D→N mutant+H1+MBP; lane 4, D→N mutant+α/β-caseins. Left panel, Coomassie; right panel, autoradiograph. (c) Kinase assay. Lane 1, WT+MBP; lane 2, K→M mutant+MBP. The phosphorylated band between 34 and 25 kDa is likely a PfTKL2 degradation product as it is not detectable by Western blot.

Divergent APE motif. The otherwise highly conserved glutamate of the APE motif in subdomain VIII is not present in apicomplexan TKL2 orthologues. In Plasmodium, it is replaced by a tyrosine, and by a lysine in To. gondii and Ne. caninum. About 93% of human eukaryotic protein kinases contain a glutamic acid in the APE motif (Kostich et al., 2002) and crystallographic data indicate this residue plays a structural role, forming a salt bridge with the conserved arginine of subdomain XI and thereby stabilizing the C-terminal lobe of the kinase domain (reviewed by Coulombe & Meloche, 2007). Based on the role of the glutamate, we can speculate that the hydroxyl group of the tyrosine in Plasmodium may interact with the guanidinium group of the arginine in subdomain XI – a possibility that is supported by the model structure of PfTKL2 (Fig. 4d). Enzymes such as human ERK3 and ERK4 possess an arginine, while human casein kinase 1δ has an asparagine (reviewed by Coulombe & Meloche, 2007), implying that the APE motif can accommodate a non-glutamate residue without compromising the activity of the enzyme. Furthermore the aspartate present within the motif in apicomplexan sequences (DPY and DPK; Fig. 4d) might provide an alternative interaction site with the arginine of subdomain XI. Clearly, experimentally solving the PfTKL2 structure will be of great interest in the context of our understanding of atypical eukaryotic protein kinase structure–function relationships.

Classification and evolution of RLK/Pelle proteins

We identified a region that categorizes RLK/Pelle in the same way as proposed by Towb et al. (2009) on the basis of the death and kinase domains (Fig. 5). Metazoan proteins of this family possess an accessory death domain, plant homologues have a diverse array of domains, and apicomplexans have large extensions that lack recognizable, annotated domains (Fig. 2). In contrast to such variations, the central kinase domain is shared across systems and displays features unique to this family (Fig. 3). It has been hypothesized that metazoan TTLKs (RD, i.e. an R residue immediately precedes the invariant D residue that is essential for catalytic activity) and PLKs (non-RD) arose by gene duplication pre-dating the split between the vertebrate and invertebrate lineage (Towb et al., 2009). It is also thought that land plants expanded RLK members through gene duplication, and acquired diverse accessory domains in response to increasing diversity of biotic and abiotic challenges (Lehti-Shiu et al., 2009). This seems to be supported by the fact that Chlamydomonas reinhardtii (a
primitive plant) has only two RLKs (one RD and one non-RD), both of which are devoid of receptor kinase configuration (Lehti-Shiu et al., 2009). In contrast, O. sativa japonica possesses 1100 RLKs out of 1446 total protein kinases, of which 786 have a receptor kinase configuration. The number of non-RLK protein kinases in C. reinhardtii is almost equal to that in O. sativa, indicating the difference in their kinome size is a direct consequence of RLK expansion (Lehti-Shiu et al., 2009). This is similar to the expansion of tyrosine kinases in metazoans as an adaptation to multicellular life.

**Activity regulation of PfTKL2**

The mode of regulation of the activity of PfTKL2 is not known. The recombinant PfTKL2 kinase domain displays autophosphorylation activity (Fig. 8). Although several studies highlight the importance of IRAK4 kinase activity (Kim et al., 2007; Koziczak-Holbro et al., 2007a, b; Li et al., 2002; Lye et al., 2004), there is ongoing debate on whether physical protein interaction rather than (or in addition to) kinase activity is important for the signalling function of IRAKs (reviewed by Wang et al., 2009). Similarly, for Pto (a plant RLK involved in innate immunity), autophosphorylation on a residue within the activation domain is critical for binding to its substrate, AvrPto (Xing et al., 2007; Ntoukakis et al., 2009). Autophosphorylation may thus lead to conformational change essential for both interaction with substrates and trans-activity of both IRAK4 and Pto, and this is likely to be applicable to PfTKL2 as well.

**What is the possible function of an RLK/Pelle protein in apicomplexans?**

Based on the function of RLK/Pelle proteins in other systems, we hypothesize that PfTKL2 may have both immunomodulatory and developmental functions. PfTKL2 is exported to the RBC (Figs 6c and 7), and a proportion of it appears to be exported and secreted into the medium (Fig. 6d) (Singh et al., 2009). We speculate that PfTKL2 may be involved in modulating the host immune response through molecular mimicry. Malaria parasites are known to produce molecules involved in immune regulation; for example, *P. falciparum* macrophage migration inhibitory factor (PfMIF; PlasmoDB identifier PF3D7_1229400) is also exported to the surface of the RBC and constitutively secreted to the medium (Shao et al., 2008), and the *Plasmodium berghei* orthologue was shown to bind to mammalian MIF receptor (Dobson et al., 2009). Overexpression of PfMIF was shown to decrease severity of the disease in *P. yoelii*-infected mice (Thorat et al., 2010). A more recent study suggested that PfMIF interferes with the development of immunological memory (Sun et al., 2012).

*Schistosoma mansoni* has also been shown to produce a soluble factor that acts as a decoy receptor to evade immune response (Smith et al., 2005), and bacteria and viruses also employ molecular mimicry for evasion from immune response (Cirl et al., 2008; Mantovani et al., 2006). Virulent pathogenic *E. coli* was demonstrated to directly inhibit host TLR signalling by secreting a structural homologue of the signalling domain of human TLRs (Cirl et al., 2008). The secreted bacterial protein was able to enter nearby immune cells, thereby enabling long-range modulation of immune cell function. Similarly, HIV-1 is known to secrete a protein known as Nef that is able to enter nearby B-cells, suppressing CD40-dependent immunoglobulin class switching (Qiao et al., 2006). It is possible that the secreted fraction of PfTKL2 may be able to access bystander immune cells and modify their response to infection.

As mentioned in the Introduction, *Drosophila* Pelle and several plant RLKs, in addition to their role in innate immunity, are also involved in various developmental processes, such as dorso-ventral axis embryonic patterning in *Drosophila* (Shelton & Wasserman, 1993; Towb et al., 2009) and zygote elongation role before the start of cell division in *Arabidopsis thaliana* (Bayer et al., 2009). A possible developmental function of PfTKL2 deserves further investigation, especially in the transition between the apolar zygote and the polar ookinete. Functional studies carried out on the *P. berghei* orthologue showed no phenotype (Tewari et al., 2010), but at the time of that study only partial sequence at the extreme C-terminus was available and it is this region that was deleted, leaving the kinase domain intact. We are in the process of implementing a reverse genetics approach in *P. falciparum* to address these important questions.

In conclusion, we established PfTKL2 to be an exported and secreted protein kinase belonging to a family of protein kinases involved in immune response referred to as RLK/Pelle. We hypothesize it might be involved (i) in immunomodulatory functions to favour parasite survival in the host and (ii) in developmental processes. A role in immune modulation and cell development will be a fascinating focus for future studies on PfTKL2, and may provide a novel basis for chemotherapeutic intervention.

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