The kinomes of apicomplexan parasites

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Abstract

Protein phosphorylation plays a fundamental role in the biology and invasion strategies of apicomplexan parasites. Many apicomplexan protein kinases are substantially different from their mammalian orthologues, and thus constitute a landscape of potential drug targets. Here, we integrate genomic, biochemical, genetic and evolutionary information to provide an integrated and up-to-date analysis of twelve apicomplexan kinomes. All kinome sequences are available through the Kinomer database.

1. Introduction

The reversible modification of protein by phosphorylation, as carried out by protein kinases (PKs) and phosphatases is a major mechanism that regulates virtually every aspect of cellular life [1]. Not surprisingly, when protein phosphorylation goes awry serious diseases such as neurodegenerative disorders and cancers occur as a result [2]. The protein kinase superfamily is broadly classified into eight 'conventional' PK (ePK) groups (AGC, CAMK, CK1, CMGC, RGC, STE, TK, TKL) and four 'atypical' PK (aPK) groups (Alpha, PDHK, PIKK, RIO). The ePKs possess the characteristic PK catalytic domain, as described by Hanks and Hunter [3]. Some ePKs, whilst exhibiting some degree of sequence similarity to the main ePK groups, cannot be easily classified into any such group, thus constituting a loose cluster on its own called the 'Other protein kinases' (OPK) group. The aPKs are a small set of PKs that do not share sequence similarity with ePKs, but nevertheless possess kinase catalytic activity. Given the central importance of protein phosphorylation to the cell, the characterization of the PK complement ('kinome') of an organism is bound to offer important biochemical insights and, in the case of pathogenic organisms, project a landscape of potential drug targets [4, 5]. Many PKs of parasitic organisms show a profound structural and functional divergence from their orthologues in the infected hosts (when they exist), suggesting that parasite-specific inhibition might be achieved. A striking example is given by the elegantly documented selective inhibition allowed by small gatekeeper residues displayed by some kinases from apicomplexan parasites [6-9].

The kinome of the human malaria parasite *Plasmodium falciparum* [10, 11] comprises approximately 90 PKs, many of which are not related to established families of higher eukaryotes, and lacks both tyrosine kinases and canonical MAP kinase cascades. The genus *Plasmodium* belongs to the phylum Apicomplexa, a large and diverse group of unicellular protists within the Alveolata taxonomic group. All Apicomplexa are obligate intracellular parasites. The biodiversity and wide environmental distribution of apicomplexans is astounding, with an estimated 1.2-10 million species, of which only ~0.1% have been named to date [12]. They are responsible for many debilitating diseases both in developing countries and in industrialised nations. *P. falciparum* causes 300-600 million clinical cases per year [13], resulting in 1.5-3 million deaths [14, 15]. Mortality caused by malaria has decreased in recent years, notably thanks to the introduction of artemisinin combination therapies (ACT). However, this may be a short-lived respite, as reduced sensitivity to arteminin-derived compounds has already been documented [16]. *P. vivax* also infects humans, but is less virulent than *P. falciparum* [17], and *P. knowlesi* was primarily known to cause malaria in macaques [18], but has recently been identified as capable of infecting humans [19]. The rodent malaria parasites *P. berghei*, *P. chabaudi* and *P. yoelii* are model organisms for the study of pathogenesis and parasite biology [20, 21]. Both *Theileria* species are tick-borne haemoparasites of cattle, *T. parva* causing East Coast fever [22, 23], and *T. annulata* causing tropical theileriosis [24]. Besides their economic importance, *Theileria* species are of great biological interest in the context of mammalian cell transformation, because they are the only intracellular eukaryotic pathogens known to reversibly transform their host cells through the hijacking of a number of its signalling pathways [25-27]. *Toxoplasma gondii* causes toxoplasmosis, a serious disease in immunocompromised patients that is prevalent worldwide, with an estimated 1/3 of the World's population infected with the parasite [28]. Recently, a link has been found between *T. gondii* infection and schizophrenia and paranoia [29]. The two *Cryptosporidium* species cause acute gastroenteritis and diarrhoea worldwide and differ in their host range: although both species exhibit very similar gene complements, *C. hominis* infects only humans [30], whereas *C. parvum* also infects other mammals [31]. Finally, *Babesia bovis* causes haemolytic anemia in cattle, one of the most important arthropod-transmitted diseases of cattle and for which no vaccine is available. Although babebiosis has been eradicated in the United States, it is still present in many tropical regions, posing a serious constraint to economic development [32].

Here, we exploit recent efforts in parasite genomics to compare the kinomes of twelve apicomplexan species. These include five species, whose kinomes have been described in detail: *P. falciparum* [10, 11], *P. yoelii* [33], *P. berghei* [34], *T. gondii* [35, 36] and *C. parvum* [37]. Furthermore, we report the initial characterization of seven apicomplexan kinomes, including *P. chabaudi*, *P. knowlesi*, and *P. vivax*, *C. hominis*, *T. annulata* and *T. parva*, as well as *B. bovis*. The published kinomes are reassessed and combined with the new ones by using our own validated computational tool for kinome characterisation (Kinomer). All kinomes are described with reference both to metazoan and alveolate kinomes. We outline important orthologous groups of kinases shared with other alveolates and metazoans, and a large number of Apicomplexa-specific orthologous kinases, which are likely to be important for the parasites' life cycle and invasion strategies, and thus constitute potentially attractive drug targets.

2. A comprehensive census of apicomplexan kinomes

A number of kinomes have been published to date, but their comparison is not straightforward for the following reasons: (i) they have been analysed using a variety of sequence analysis tools and pipelines with various degrees of sensitivity and classification accuracy; (ii) some kinomes only include the ePK complement (and not the aPKs); (iii) the extent of assignment of orthology relationships to higher eukaryotic kinomes vary widely. To overcome these inconsistencies, we have analysed the kinomes of twelve apicomplexan species using a validated computational tool, called Kinomer, that is specific for eukaryotic protein kinases (see Supplementary Materials and Methods). We have previously reported Kinomer to have a mis-classification rate of zero on the kinase group classification level, with the additional advantage that it is more sensitive for protein kinase database searches than general sequence analysis tools. Here, we used Kinomer to mine the latest apicomplexan genome database releases (as of February 2012) to provide a comprehensive and unified view of existing and novel apicomplexan kinomes.

2.1. Comparison with the published kinomes of *P. falciparum, P. yoelii, P. berghei, C. parvum and T. gondii*

Several independent groups have previously published their accounts of the kinomes of *P. falciparum* [10, 11], *P. yoelii* [33], *P. berghei* [34] and *T. gondii* [35]. We have performed our own analyses with the multi-level kinase HMM library and the most up-to-date genome versions, and our comparisons with the published studies above stand as follows.

The survey of the *P. falciparum* kinome by Anamika et al. [11] identified 99 PKs, including 80 ePKs and 19 FIKK enzymes. Their classification of ePKs into kinase groups essentially agrees with that of Ward et al. [10] and the refined classification presented here, except for their inclusion of PF14_0516 into the AGC group. Our study suggests that this enzyme is a CAMK related to the catalytic subunit of AMPK. Anamika et al. included 7 putative ePKs that were not retained in the study by Ward and co-workers. These sequences are very degenerate, lack several of the canonical amino acid residues of the 12 sub-domains of the kinase catalytic domain [3] and therefore, are not included in our analysis either. Ward et al. proposed a *P. falciparum* kinome comprising 65 ePKs, 3 aPKs and 20 novel FIKK enzymes. Of these, we found that PFI1290w had been fused with PFI1285w in subsequent database releases, and that the FIKK kinase PFI0125c had been removed too. Here, we have identified 3 new *P. falciparum* kinases: PF11_0079 (TKL group), plus PFD0965w and PFE0485w (both atypical kinases of the PIKK family). This brings the updated kinome of *P. falciparum* to 89 PKs, with 65 ePKs, 19 FIKKs and 5 aPKs.

With regard to the *P. yoelii* kinome, we have identified 63 PKs versus 60 from the Anamika and Srinivasan study [33]. From their study we have excluded 4 PKs: three possess truncated kinase catalytic domains (PY00266, PY04849, PY02490) and one is not a recognizable PK (PY00132). We have identified 9 additional PKs that had not been reported previously (PY05890 (CMGC), PY02456 (NEK), PY02993 (OPK), PY04175 (OPK), PY07525 (OPK), PY00334 (PIKK), PY01572 (RIO) and PY06287 (RIO)). Thus, we propose that the *P. yoelii* kinome contains 63 PKs, with 60 ePKs and 3 aPKs.

Tewari et al. recently published a description of the *P. berghei* kinome together with a kinome-wide reverse genetics analysis, revealing the role for a number of PKs in infection of the mosquito vector [34]. Our own analysis of the *P. berghei* kinome essentially agrees with that of Tewari et al.

Peixoto et al. recently analysed the *T. gondii* kinome [35]. The authors report 159 PKs, including 39 members of a novel family call ROPK (from their association with the rhoptries, apicomplexan-specific organelles involved in the process of host cell invasion). Our classification in general agrees with this analysis on the group-level classification of kinases, but not always on the homology relationships of individual PKs. This is partly because a comparative analysis including many different genomes is richer in the number of homologous sequences and is thus bound to improve the clarity of phylogenetic relationships. Our account of 135 PKs in the *T. gondii* kinome excludes a number of PKs reported by Peixoto et al. as they harbour truncated kinase catalytic domains.

Finally, inspection of the *C. parvum* kinome suggested that our classification broadly agrees with the published set of identified PKs [37], although not always on the PK group level classification, or the inclusion of fragmentary PK catalytic domains. The recent analysis by Talevich et al. [38] sought to investigate multiple apicomplexan kinomes with the purpose of analysing specific PK families both from an evolutionary and a structural point of view. Our inspection of their dataset suggests that

although a majority of true PKs were identified, their study did not include some of the most distant sequences.

2.2. Overview of apicomplexan kinomes

Apicomplexan kinomes are smaller than that of *S. cerevisiae*, with kinome sizes ranging from a surprisingly small set of 35 sequences (*B. bovis*) to 135 (*T. gondii*), with 0.8-1.7% of apicomplexan genes encoding PKs (Table 1). Apicomplexans harbour kinases of all the main ePK groups except receptor guanylate cyclase (RGC) and tyrosine kinases, and also lack members of the PDHK (except for *T. gondii*) and Alpha aPK families. The contribution of each PK group to the entire kinome is conserved between apicomplexans and higher eukaryotes (Fig. 1). Over the next sections, we describe twelve apicomplexan kinomes on a kinase group basis, and discuss the 65 orthologous groups of PKs that are shared with other alveolates and/or metazoans. Of these orthologous groups, 11 (17%) are shared with both alveolates and metazoans, 6 orthologous groups (9%) are shared with other alveolates only, and 48 groups (74%) are Apicomplexa-specific. We take the *P. falciparum* orthologues as the starting point of discussion, taking advantage of the thorough characterization of many of this parasite's PKs in recent years.

2.3. The AGC group

The AGC group (including cyclic-nucleotide and calcium/phospholipid-dependent kinases, ribosomal S6-phosphorylating kinases, G protein-coupled kinases and all close relatives of these sets) contains kinases found in all eukaryotes examined to date. The AGC group of apicomplexans comprises 6 orthologous groups, three of which are shared with alveolates and metazoans (exemplified by PfPKA, PfPKB/Akt and PfPKG), and three that are restricted to Apicomplexa (Fig. 2, Table S1). All six AGC kinases of *P. falciparum* are essential during the erythrocyte stage.

PfPKA is a universally conserved cAMP-dependent kinase (Haste et al *in press*, and see pages XX in this issue), whose expression in *P. falciparum* is developmentally regulated, with higher levels in the pathogenic asexual stages than in the sexual forms. Pharmacological data with the H89 PKA inhibitor, and the inability to produce *P. berghei* clones lacking a functional PbPKA gene [34], strongly suggest that PKA is essential for *Plasmodium* schizogony [39] (although it cannot be excluded that the inhibitor has other targets in addition to PKA). cAMP-dependent activity is implicated in the phosphorylation of the cytoplasmic tail of apical membrane antigen 1 (AMA1), a membrane protein essential for erythrocyte invasion [40], and in the regulation of an anion channel in *P. falciparum*-infected erythrocytes [41].

PfPKB/Akt is expressed mainly in schizonts and merozoites and is likely to be important for parasite development, as PKB inhibitors arrest *P. falciparum* growth, and the *P. berghei* orthologue appears to be essential to asexual blood stage parasites [34, 42]. Interestingly, PfPKB/Akt has homologues in all *Plasmodium* species and in *T. gondii*, but has been lost from piroplasmids and gregarines. The likely essential function of PfPKB/Akt in haemosporidians and coccidians must have been taken over by another PK in these taxons. PfPKB/Akt is activated by autophosphorylation, and unlike the PKBs of higher eukaryotes, all PKBs of the Apicomplexa lack lipid-binding pleckstrin-homology (PH) domains; interestingly, one of the calcium-dependent kinases, CDPK7, contains a PH domain (see below). The N-terminal region of PfPKB/Akt harbours an unconventional calmodulin-binding domain, whose activation upon calmodulin binding ultimately regulates PfPKB/Akt activity in a calcium-dependent manner [43]. The PfPKB homologues of the non-apicomplexan alveolates *Phytophthora ramorum* and *P. sojae*, however, do contain N-terminal Phox-like domains that characteristically bind phosphoinositides. This domain is not present in the homologue of the diatom *Thalassiosira pseudonana*, thus illustrating the divergent organization of signal transduction mechanisms in apicomplexans.

The cGMP-dependent protein kinase PfPKG regulates cGMP signalling in the malaria parasite, and has orthologues in all apicomplexans, alveolates and metazoans. PKG has been shown to be essential in *Eimeria* (a pathogen of poultry) and *Toxoplasma* [6], and the tri-substituted pyrrole pyrimidine Compound 1that targets *Eimeria* PKG has a much lower effect (by 3 orders of magnitude) on chicken PKG. This was shown to be due to the presence of a gatekeeper residue that is smaller in the apicomplexan (a Thr) than in metazoan (a Gln) PKG. Compound 1 was subsequently shown to inhibit *P. falciparum* proliferation *in vitro* [44]. The effect of Compound 1 on blood-stage schizogony has recently revealed that cGMP signaling and PfPKG play a central role in both asexual and sexual development of *P. falciparum* [7, 45]. The role of PKG in regulating the malaria parasite's life cycle progression has recently been reviewed by Hopp et al. (Hopp et al. *in press*, and see pages XX in this

issue). PfPKG is characterized by the presence of three cyclic nucleotide-binding domains and a fourth degenerate site which does not bind cGMP, but is nevertheless required for full activation of the enzyme. Combinatorial mutational analysis has revealed different degrees of importance of the cyclic nucleotide binding domains in PfPKG activation [46]. PKG homologues have not been characterised in *Leishmania* or *Trypanosoma*.

2.4. The CAMK group

The CAMK group (including calcium/calmodulin-regulated kinases) is the second largest kinase group in apicomplexans, in agreement with the growing experimental evidence of a central role for calcium signalling in the biology of these parasites. The CAMKs of apicomplexans consist of 18 orthologous groups, only one of which is shared with both metazoans and other alveolates, while another group is shared with other alveolates only, and a further 16 are specific to apicomplexans (Fig. 2, Table S1). This makes the CAMK the group with the lowest assignment of putative orthology both to enzymes of higher eukaryotes and to those of non-apicomplexan alveolates. Moreover, nine of the sixteen CAMKs of *P. falciparum* are essential, suggesting that Apicomplexa-specific CAMKs can be expected to carry out important functions in unique aspects of apicomplexan biology. As detailed below, this has been documented in several instances.

PfKIN, the malarial orthologue of the catalytic subunit of AMP-dependent PK (SNF1 in yeast) represents the only orthologous group that is shared with alveolates and metazoans. Yeast SNF1 plays a role in transcriptional activation and repression to respond to several forms of stress such as nutrient limitation, salt stress and heat shock (reviewed in [47, 48]). PfKIN is predominantly expressed in gametocytes [49], and accordingly reverse genetics in *P. berghei* indicates that the gene product is not required for the erythrocytic asexual cycle.

A family of seven calcium-dependent PKs (PfCDPKs) has previously been described in the malaria parasite (reviewed in [50]). CDPKs have been implicated in transmitting calcium-mediated responses to biotic and abiotic stresses and developmental signals in plants, ciliates, and trypanosomatids [51], and are represented in all apicomplexans surveyed, with the majority of orthologues harbouring the characteristic four EF-hand calcium-binding domains. Most importantly, CDPKs are absent from mammalian hosts. Structural studies on their mechanism of intramolecular activation showed that the N-terminal lobe of the calmodulin-like domain functions as the calcium sensor that triggers activation [52, 53]. As shown with the *T. gondii* CDPK1 orthologue (TgCDPK1), activation of the enzyme upon calcium binding occurs through a large conformational change. The binding of calcium reorganizes the C-terminal CDPK activation domain (CAP) causing its relocation to a site distant from the substrate binding site [54]. Moreover, TgCDPK1 has been shown to be an essential regulator of calcium-dependent exocytosis by controlling the calcium-dependent secretion of micronemes, and blocking TgCDPK1 inhibits parasite motility, host-cell invasion and egress [9]. PfCDPK1 is expressed in the asexual blood stages of *P. falciparum*, and characterized substrates include MTIP and GAP45, two components of the motor complex required for host-cell invasion. PfCDPK1 is inhibited by the kinase inhibitor K252a [55]. Since CDPK1 is conserved among apicomplexans, but is absent from mammalian hosts, it is an attractive potential target for antimalarial drug development. The subcellular localization of some CDPK isoforms is dynamic, as shown by the shift of a plasma membrane CDPK to the nucleus following stress signals (reviewed in [56]). PfCDPK1 has been shown to be myristylated and to localize at the parasitophorous vacuole [57]. An essential function in gamete formation and mosquito transmission has been ascribed to the *P. berghei* homologue of PfCDPK4 (PbCDPK4) [58]. Targeted disruption of PbCDPK3 decreases the calcium-dependent ookinete gliding mobility and ability to penetrate the layer covering the mosquito midgut epithelium, thereby decreasing the fertilization rate of the parasite [59, 60], whereas parasite lacking PbCDPK6 are impaired in their ability to invade hepatocytes [61]. The wide phylogenetic distribution of CDPKs suggests that regulation by calcium signalling is an ancient mode of regulation. Since CDPKs are an ancient family that was secondarily lost in metazoans, they represent potentially attractive targets for chemotherapeutic and transmission-blocking intervention.

The *P. falciparum* CDPK7 (PF11_0242), in addition to a calcium-binding EF-hand domain, is notable for harbouring a pleckstrin-homology domain and has orthologues in all apicomplexans (except cryptosporidians), and also in alveolates. In mammals PH domain-containing kinases are regulated by lipids, but as mentioned above none of the apicomplexans PKB/Akt orthologues contain a lipid-binding domain. This provides another striking illustration of the divergence in the organization of signalling pathways between apicomplexans and opisthokonts, as well as the difficulty in predicting function on the basis of kinase catalytic domain phylogeny alone.

2.5. The CK1 group

The CK1 group (casein kinase 1 and close relatives) enzymes are implicated in diverse cellular functions, including cell cycle progression, chromosome segregation, membrane trafficking, apoptosis and cellular differentiation [62-64]. All apicomplexans share an orthologous group of CK1 enzymes, which form a compact cluster unrelated to CK1s of other alveolates or metazoans (Fig. 2, Table S1), thus stressing the phylogenetic distance between these taxons and suggesting a potential for selective inhibition. The *P. falciparum* kinase PfCK1 is an essential enzyme, and each apicomplexan species was found to possess one CK1 enzyme. Two of the *T. gondii* CK1 enzymes (TGME49_040640 (TgCK1alpha) and TGME49_089320 (TgCK1beta)) were cloned and the recombinant molecules assayed for activity. The plasma-membrane isoforms (TgCK1beta) showed no kinase activity, whereas the cytosolic isoform (TgCK1alpha) was found to be catalytically active and inhibited *in vitro* by purvalanol B and also by the cell-permeable and related compound aminopurvalanol A with an IC_{50} of 42±7 nm for the recombinant enzyme (versus an IC_{50}=4000 nm for rat CK1delta). This suggested the existence of potentially exploitable structural differences between metazoan and parasitic CK1 enzymes [65]. Since aminopurvalanol A inhibits parasite growth, CK1 inhibitors are potential leads for anti-parasitic agents. Interestingly, the cell-impermeable purvalanol B displays activity against *P. falciparum* [66], and affinity chromatography on the immobilised inhibitor identified PfCK1 as a putative target [67]. The inhibitors tri-substituted pyrrole and imidazopyrine exhibit *in vivo* efficacy against PKG in *Eimeria tenella* in chickens and *T. gondii* in mice, but also inhibit apicomplexan CDPK1 and CK1 [68]. When tested in *Trypanosoma brucei* and *Leishmania major* (which lack PKG homologues), these PKG inhibitors were found to be selective against the trypanosomatid CK1 enzymes [69]. The *P. berghei* CK1 orthologue is likely to be essential, as suggested by the inability to obtain knock-out parasites [34].

A second, smaller, cluster includes cryptosporidian and *Toxoplasma* CK1s that are related to CK1s of *Paramecium* and *Tetrahymena*, thus highlighting the evolutionary relationship of apicomplexans and alveolates.

2.6. The CMGC group and other cell cycle control kinases

The CMGC group (including cyclin-dependent kinases, mitogen-activated PKs, glycogen synthase kinases and CDK-like kinases) is the largest PK group in apicomplexans, as well as in trypanosomatids [51]. The CMGC group harbours 22 orthologous groups of kinases, 7 of which are found in alveolates and metazoans, 4 are found in apicomplexans and non-apicomplexan alveolates, and 11 are Apicomplexa-specific (and of which 7 are found in haemosporidians only) (Fig. 2, Table S1). Therefore, ~70% of CMGC orthologous groups are specific to alveolates and not found in metazoans.

P. falciparum has two GSK-3-related enzymes (both of which are essential), one of which is found in haemosporidians only (MAL13P1.84), while PfGSK3 (PFC0525c) is shared with other alveolates and metazoans. In humans, GSK3 is a multifunctional kinase affecting a diverse range of cellular functions, including the regulation of metabolism, gene expression, and even cell fate determination and the spatial organization of the embryo [70, 71]. The clarification of GSK3's role in insulin regulation in the mid-90's has led to the development of an arsenal of very potent and specific inhibitors with therapeutic potential for the treatment of diabetes [72].

P. falciparum possesses two MAP kinases, and only Pfmap-1 (PF14_0294) has orthologues in other alveolates and metazoans, whereas Pfmap-2 (PF11_0147) is an essential enzyme shared with other alveolates only. Despite the existence of MAP kinases, apicomplexans do not seem to have complete MAP kinase cascades [73], as canonical MAPKKs appear to be missing from their kinomes.

PfCK2 (PF11_0096) is another highly conserved kinase in the CMGC group that is shared with other alveolates and metazoans, and has been implicated in critical cellular processes such as proliferation, apoptosis, differentiation and transformation [74]. The observations that PfCK2 is essential for completion of the parasite's asexual erythrocytic proliferation, and that the plasmodial and human CK2 enzymes display differential susceptibility to some inhibitors, makes PfCK2 a potential drug target [75].

The regulation of cell cycle progression in parasitic protists differs in important respects from that of higher eukaryotes (reviewed in [76]). The main kinases driving the cell cycle in higher eukaryotes are the CDK, Aurora, NEK and Polo kinases (reviewed in [77]). Seven enzymes related to cyclin-dependent kinases (CDK) are known to exist in *P. falciparum*, two of which are universally shared with other alveolates and metazoans (Pfcrk-1 and PfPK5), while Pfmrk is shared with other alveolates, and four are found in apicomplexans only (Pfcrk-3, Pfcrk-4, Pfcrk-5 and PfPK6). PfPK5 is the only apicomplexan CDK whose three-dimensional structure has been solved [78]; although its fold

is very similar to that of human CDK2, subtle differences in the mode of binding of some inhibitors (some of which significantly differ in their IC_{50} values against CDK2 and PfPK5) lend hope that the development of selective inhibitors may be achievable.

Cyclin-dependent kinase-like kinases (CLKs) are major regulators of mRNA splicing in higher eukaryotes. They function by phosphorylating Serine/Arginine-rich (SR) proteins functioning in the RNA processing pathway [79]. *P. falciparum* has 4 CLKs (PfCLK1-4, Fig. 2 and Table S1), all of which are essential enzymes. PfCLK1/Lammer (PF14_0431) and PfCLK3 (PF11_0156) have homologues both in other alveolates and metazoans. PfCLK2 (PF14_0408) and PfCLK4 (PFC0105w) are shared with other alveolates only. Given that only a handful transcription factors have been identified in *P. falciparum*, it is believed that post-transcriptional control may be crucial for the regulation of gene expression. Besides antisense RNA and translational repression, mRNA splicing has been documented in *P. falciparum* [80]. Recently, Agarwal et al. showed that PfCLK1/Lammer and PfCLK2 primarily localize to the nucleus and are transcribed throughout the asexual blood stages and in gametocytes [81]. Moreover, and as shown by reverse genetics, these two enzymes are necessary for completion of the parasite's asexual replication cycle. Dixit et al. also showed that PfCLK4 (which they refer to as PfSRPK1) inhibits mRNA splicing by phosphorylating PfSR1, a putative splicing factor known to interact with RNA [82].

Aurora kinases constitute a family of Ser/Thr protein kinases with essential roles in eukaryotic cell division, notably in the G2 to cytokinesis transition, and dysregulation of Aurora kinase function has been implicated in tumorigenesis. In metazoans, A-type Auroras regulate spindle assembly while B-type Auroras promote chromosome segregation and cytokinesis. Malaria parasites possess three kinases related to this family, one of which (PfARK1) has been characterised in detail and shown to be transiently associated with spindle pole bodies [83]. Unsuccessful attempts at disrupting all aurora kinase genes in *P. falciparum* indicate that they are essential enzymes. See [83] for a phylogenetic analysis of *P. falciparum* Aurora kinases.

Members of the NEK family are not associated to any of the established PK groups and are therefore classified with the 'Other' PK group. NEKs regulate cell cycle progression and have been implicated in the regulation of mitosis and meiosis. Humans possess 11 NEKs [84], whereas the family has expanded in trypanosomatids with 20-22 members [51]. Interestingly, the NEKs of Apicomplexa show no clear relationship to higher eukaryotic NEKs. Four NEK orthologous groups exist in apicomplexans: Pfnek-1 (PFL1370w) is an essential enzyme that phosphorylates one of the two parasite MAPKs (Pfmap-2) and may play a role in its regulation [85]. Pfnek-1 homologues are found in all apicomplexans and other alveolates (but not metazoans). Pfnek-4 (MAL7P1.100) appears to have been lost from piroplasmids and gregarines, although the *P. berghei* orthologue is essential for the completion of meiosis [86]. Pfnek-2 (PFE1290w) and Pfnek-3 (PFL0080c) are restricted to haemosporidians. Pfnek-2 is essential for transmission to the mosquito, and studies with the *P. berghei* orthologues identified a defect in meiosis in parasites lacking the enzyme, as was the case for Pbnek-4 [86, 87]. Pfnek-3 has been reported to activate Pfmap-2 *in vitro*, like Pfnek-1 [88]. A succinct phylogenetic tree of *Plasmodium* NEKs in relation to other members of the family has been published [86], and a more thorough analysis is in preparation (Luc Reininger, personal communication).

Polo kinases are required at several key points during mitosis in higher eukaryotes, starting from control of the G2/M transition through phosphorylation of Cdc25C and mitotic cyclins [89]. Yeast has one Polo kinase (Cdc5) that controls the targeting and activation of the small guanine nucleotide triphosphate-binding protein RhoA, thereby providing the link between the cell cycle machinery and the cytoskeletal proteins controlling cytokinesis [90]. Through sequence analysis we failed to identify Polo kinases in apicomplexans, and no parasitic kinases were found to harbour the characteristic Polo domain (which mediates multiple protein-protein interactions). This suggests that the Polo kinase functions are absent from apicomplexans (in striking contrast with trypanosomatids, where Polo kinases have been characterized [91]), or that they are executed in a different way. In yeast, Cdc5 phosphorylates and activates the Anaphase Promoting Complex (APC). The *P. falciparum* kinase found to be closest sequence-wise to higher eukaryotic Polo kinases is PfPKA, and yeast/mammalian PKA is also known to phosphorylate APC with the opposite effect of suppressing its activity [92]. Therefore, Polo kinases and PKA regulate mitotic progression by controlling APC activity in opposing ways. Since apicomplexans, but not trypanosomatids, appear to lack true Polo kinases, it appears that the metaphase-anaphase transition and exit from mitosis are regulated differently in these two phyla of parasitic protists.

2.7. The STE group

The STE group includes many kinases functioning in MAP kinase cascades. MAP kinases are activated by phosphorylation of their activation loops by MAP kinase kinases (MAPKK), which belong to the STE group and are entirely lacking in apicomplexans as previously suggested [10, 93]. In fact, STE kinases are sparsely represented in apicomplexans (and entirely absent from *Theileria*), and do not appear to have homologues in other alveolates or metazoan species. This suggests that these are important lineage-specific kinases (Fig. 2, Table S1). In striking contrast, STE is the second largest group in trypanosomatids, with 25 members in *T. brucei* and 31 and 34 members in *T. cruzi* and *L. major*, respectively [51]. A *Leishmania* MAPKK has been shown to regulate flagellum length [94].

2.8. The TKL group

The TKL (tyrosine kinase-like kinases) are similar to tyrosine kinases sequence-wise, but catalytically are serine/threonine kinases. Six orthologous groups of TKLs were found to be conserved in plasmodial species (Fig. 2, Table S1). PfTKL3 (PF13_0258), whose activity requires an accessory SAM domain mediating oligomerisation, is essential for asexual parasite proliferation and has recently been validated as a drug target through reverse genetics [95]. It is noteworthy that two other kinases related to TKLs, PfTKL1 (PFB050w) and PF11_0079, are both among the few proteins in the *P. falciparum* proteome that contain SAM domains, like PfTKL3 [95].

2.9. Apicomplexa-specific groups: FIKK and Rhoptry kinases

The FIKK were first identified in *P. falciparum* as a set of 20 ePKs forming a tight cluster not clearly related to any known ePK group (Ward et al., 2004). The FIKK were named after an amino acid motif (Phe-Ile-Lys-Lys) in subdomain II of their kinase catalytic domain, and all *P. falciparum* FIKK kinases have a variable extension N-terminal to their catalytic domain. This N-terminal region has no recognizable domains except for a PEXEL export signal [96]. Our analysis agrees with previous results: FIKKs are restricted to the Apicomplexa phylum (one gene per species), and are absent from *B. bovis* and *Theileria* species. The FIKK are expanded in *P. falciparum* (19 enzymes) with one likely founding member (MAL8P1.203, Fig. 2, Table S1), whose orthologue in *P. berghei* is likely essential for asexual erythrocytic proliferation [34]. The FIKK paralogues are all transcribed at some stage of the cell cycle of the malaria parasite and have been suspected to be involved in antigenic variation. Recent work showed that at least some FIKKs are exported into the cytoplasm of the red blood cell to distinct locations and that at least some of these enzymes are catalytically active (or at least associate with other PKs) [97, 98].

Rhoptry kinases (ROPK) are a recently described group of PKs of the rhoptries of *T. gondii*, with as many as 34 members [35]. ROPKs are serine/threonine PKs, and their high sequence divergence, and the fact that they seem to be restricted to *T. gondii* [35] indicates that they play specific roles in *Toxoplasma* infection and may have non-redundant substrates. A recent study implicates a secreted ROPK in the regulation of transcription of the host cell [35]. Many ROPKs are predicted to be catalytically inactive; structurally they share the typical fold of serine/threonine PKs, but they also contain a number of insertions unique to ROPKs, suggesting that they may present unique mechanisms of activation [99].

2.10. Atypical PKs

Atypical PKs (aPK) are a small set of kinases that do not share sequence similarity with ePKs, but which have been shown experimentally to possess PK activity. The four *bona fide* aPK families include: the Alpha (exemplified by myosin heavy chain kinase of Dictyostelium), PDHK (pyruvate dehydrogenase kinase), PIKK (phosphatidyl inositol 3' kinase-related kinases), and RIO ('right open reading frame', as it was one of two adjacent genes that were found to be transcribed divergently from the same intergenic region) [100]. Apicomplexans possess aPKs of the PIKK and RIO families, although none of the apicomplexan aPKs appear to have orthologues in other alveolates or metazoans. Two orthologous groups of RIO kinases exist, of which only one is found across all apicomplexan species surveyed here. RIO kinases are involved in RNA processing in *S. cerevisiae* [101]; mammalian RIOK3 has recently been shown to negatively regulate NF-kappaB signalling, a pathway of central importance in innate immunity responses [102]. PIKKs sense DNA damage, nutrient-dependent signalling and nonsense-mediated RNA decay [103]. A set of 4 lineage-specific PDHKs is found in *T. gondii*, but not in other apicomplexans, and are likely to be part of specialized tubular-shaped

mitochondrial structures called the Ovoid Mitochondrial Cytoplasmic Complex [104]. PDHKs are also present in trypanosomatids [51], suggesting that they might have been lost secondarily from most apicomplexan lineages.

2.11. Evolutionary structure-function studies

Talevich et al. recently presented a systematic approach to identify specific sequence and structural differences of apicomplexan PKs that are not found in their metazoan orthologues [38]. The authors analysed PKs from fifteen apicomplexan genomes and included the available crystallographic structures of protein kinases. Their work revealed contrastingly conserved features that were not apparent previously. For instance, when analysing the calcium-dependent PKs, the authors found that PfCDPK5 orthologues have distinguishing features not found in other CDPKs, including an arginine residue in the αC helix, and a serine or threonine residue in the activation loop. By integrating structural information, Talevich et al. concluded that the conserved arginine residue might function as a switch that interacts with the activation loop phospho-threonine to stabilize the αC helix in an active conformation, a mechanism that would be shared with human p38γ. With the multitude of apicomplexan genomes that are becoming available, and the rapid accumulation of new apicomplexan protein structures thanks in part to the Structural Genomics Consortium (SGC) [105], the integrative analysis of sequence and structural data, together with the experimental validation of the hypotheses, is likely to provide not only an entirely new perspective not only on PK selectivity issues, but also on the likely modes of activation and regulation of apicomplexan PKs. Such studies will be very valuable in informing the design of specific PK inhibitors.

2.12. Apicomplexan phosphoproteomes

Three separate mass spectrometry-based phosphoproteomic studies have recently brought a turning point to our understanding of phosphorylation networks in the malaria parasite (reviewed in Lasonder et al. (*in press* and pages XX in this issue). Solyakov et al. [106] carried out global kinomic and phosphoproteomic analyses of the erythrocytic stage of *P. falciparum*. The authors first showed by reverse genetics that 36 out of 65 PKs are essential for the parasite's survival. The gene *loci* were targeted with knockout targeting vectors that placed an epitope tag in-frame with the PK open reading frame. Therefore, the lack of integration could be attributed to the enzyme being essential. This study provided solid evidence that an abundant number of essential PKs exist in the malaria parasite and which therefore, constitute potential drug targets. The phosphoproteomes of the blood stage of *P. falciparum* were determined by Solyakov et al. [106], Lasonder et al. (*in press*), and by Treeck et al. [107] who also extended their analysis to the phosphoproteome of *T. gondii*. The technical differences among the three studies are analysed by Lasonder et al. (*in press* and pages XX in this issue) and will not be discussed here. These phosphoproteomic studies conclusively acknowledge that protein phosphorylation is widespread and plays diverse roles in the parasitic processes, including invasion and cyto-adhesion, and also metabolism, cell communication, the cell cycle, DNA replication, transcription and translation. The main findings from these studies are: (1) in *P. falciparum*, 36 out of 65 PKs are essential, and many of the orthologous PKs are also essential in the erythrocytic stage of *P. berghei* [34], which suggests that there is little functional redundancy in the parasites' kinomes; (2) in *P. falciparum* at least 23 PKs are phosphoproteins, many of which are phosphorylated in their activation loops. If these are not due to autophosphorylation events, this would suggest the existence of protein kinase cascades in the parasites; (3) although apicomplexans lack TKs, a small number of tyrosine residues (~2%) are phosphorylated in both *P. falciparum* and *T. gondii*. Important tyrosine autophosphorylated proteins include PfGSK3 and PfCLK3, both essential enzymes. Human GSK3 autophosphorylates its own activation loop, as a chaperone-dependent transitional intermediate with tyrosine kinase activity. This activity is lost upon autophosphorylation (the enzyme becomes an intermolecular serine/threonine kinase) [108]. It will be interesting to determine whether this is an ancient mode of regulation present in apicomplexans too; (4) when compared with the phosphoproteomes of higher metazoans, *P. falciparum* contains ~25% unusually phosphorylated motifs not found in humans. This could represent an evolutionary adaptation that reflects the unusual amino acid composition of the parasite's proteome and accompanying structural changes in the PKs' substrate binding regions. This could clearly be exploited to design highly specific PK inhibitors; (5) in *T. gondiii* many proteins that are exported into the host cell cytoplasm are phosphorylated, but some of these proteins only become phosphorylated once they enter the host cell. This implies that parasitic PKs exported along with these proteins regulate the secretome post-translationally, a mechanism that might be mimicked by FIKK kinases in *P. falciparum*; (6) When the phosphoproteomes of *T. gondii*

and *P. falciparum* are compared, identical phosphorylated residues are observed in homologous regions of orthologous proteins.

These phosphoproteomic studies open the way for network-based systems biology studies that will lead to a deeper understanding of the role of protein phosphorylation in essential parasitic processes, and how different signalling pathways are interconnected by reversible protein phosphorylation. This will help us understand the effect of PK inhibitors from a network-based perspective, which is a more powerful conceptual approach to parasitic intervention.

3. Conclusions

We have presented an exhaustive comparative analysis of twelve apicomplexan kinomes and show that these are among the smallest described for eukaryotes. In striking contrast, trypanosomatid kinomes are much larger, ranging from 176 PKs (*T. brucei*) to 199 PKs (*L. major*), with most PKs being orthologous across the three species [51]. Trypanosomatids lack kinases of the RGC, TK, and TKL groups, although they possess dual-specificity kinases. Few receptor serine/threonine kinases have been predicted in trypanosomatids, and protein domains accessory to the kinase catalytic domain appear to be rare too. Similarly, apicomplexans also lack RGCs and TKs and, consequently, PKs with SH2 domains. Whereas signalling via accessory domains is a major mode of regulation in human kinases and to some extent in trypanosomatids too, our analysis suggests that this is comparatively rather limited in apicomplexans. The sensing of the extracellular milieu by receptor kinases is not likely to be a major feature of signal transduction in apicomplexans, although a small number of kinases were found to contain predicted transmembrane domains.

Sixty-five distinct orthologous groups are described across apicomplexan kinomes, of which ~25% are shared with other alveolates and/or metazoans. About ~75% of apicomplexan PKs are specific to this group of parasites. Only 20 PKs are conserved across all apicomplexan lineages inspected here (16 of which are essential in the malaria parasite). This set of 20 PKs probably represents an indispensable core of PKs for all apicomplexans. The minimal kinome of *B. bovis* (35 PKs) contains extensions to this core set of apicomplexan PKs (a further 7 and 5 PKs that are shared with *P. falciparum* or *Theileria* species, and 3 lineage-specific PKs).

The small genomes of apicomplexans (containing between ~3700-8000 genes), together with their streamlined kinomes, suggests that apicomplexans, like other parasites, have lost a number of genes relative to the nearest free-living eukaryote. Parasites can tolerate massive gene losses as long as the host can supply nutrients and a sheltered environment. In fact, it has been estimated that only ~1000 genes are shared across twelve distinct apicomplexan genomes [109]. This represents less than one-third of the protein-coding genes of any apicomplexan, making the rest of the genes lineage-specific and likely reflecting adaptations to the range of apicomplexan hosts. Since these genes lack orthologues outside of the Apicomplexa, they must either have been created *de novo* or have evolved beyond recognition. The genomes of the Apicomplexa are highly dynamic [110] and besides large gene gains and losses, they also include large genome rearrangements. Gene losses and genomic rearrangements complicate the detection of syntenic relationships across apicomplexan genomes, a useful measure in less dynamic genomes of the extent of divergence that has taken place since the species last shared a common ancestor. Studies of syntenic relationships in apicomplexan genomes showed that whereas significant synteny exists between the genomes of apicomplexans in the same genus (e.g. all haemosporidians), no synteny could be detected across the entire phylum due to the extensive genome rearrangements. Limited synteny is conserved only between *Plasmodium spp*. and *Theileria* and *Babesia*, which clusters haemosporidians and piroplasmids together, followed by *Toxoplasma* and *Cryptosporidium* [109, 110]. However, synteny is only informative as to the overall conservation of genome structure, but not about orthologue conservation. In fact, examination of the orthologous groups of PKs conserved across apicomplexans (Fig. 2) would yield a less clear grouping of species and geni. This pattern reflects both the need for conservation of biochemical function over genomic rearrangements in evolution, and the existence of lineage-specific expansions. For instance, from the comparative very large kinome of *T. gondii*, only ~1/4 of its PKs are shared with *P. falciparum*, making this coccidian a less than ideal organism for the study of the malaria parasite. At the other end of the spectrum, the minimal kinome of *B. bovis* (35 PKs) still reflects many lineage-specific expansions: only 20 of its PKs are shared with *P. falciparum* (of which 16 are essential in the malaria parasite).

The apicomplexan kinomes discussed here are available through the Kinomer database [111] at http://www.compbio.dundee.ac.uk/kinomer/.

SUPPLEMENTARY MATERIALS AND METHODS

Identification and classification of apicomplexan PKs

The identification and group-level classification of PKs was done by scanning the various predicted peptide datasets with a multi-level profile hidden Markov model (HMM) library of the PK superfamily [100], under HMMER3 [112], and as detailed in [111]. Since HMMER3 uses a scoring system that is different from that of HMMER2, we re-calculated our HMM library scores to correct for HMMER3's increased sensitivity. This was done by working out the E-value score for the worst-scoring PK of each specific PK group from the kinomes of *H. sapiens, M. musculus, D. melanogaster, C. elegans, S. cerevisiae* and *D. discoideum*. These new E-value cutoff scores were uniformly applied to the apicomplexan peptide datasets to classify PKs into groups. Our HMM library has previously been applied in the kinome characterization of *Trichomonas vaginalis* [113], *Brugia malayi* [114], *E. cuniculi* [115], the fungi *Rhizopus oryzae* [116] and *Fusarium ssp*. [117], and the algae *Ectocarpus siliculosus* [118] and *Chlamydomonas reinhardtii* [119].

The apicomplexan peptide datasets were downloaded from the following public databases: PlasmoDB [120] (release 8.2) for the annotated peptides of all Plasmodium species investigated here, CryptoDB (release 4.6) [120, 121] for the annotated peptides of *C. parvum* and *C. hominis*, the Wellcome Trust Sanger Institute's FTP site (ftp.sanger.ac.uk) for *T. annulata*, the EMBL database for *B. bovis* (accession number AAXT00000000), and ToxoDB release 7.2 [36] for the annotated peptides of *T. gondii* type II strain (ME49). The *T. parva* peptide dataset had previously been downloaded from the now discontinued TIGR website (www.tigr.org/tdb/e2k1/tpa1/). Furthermore, a number of additional alveolate and metazoan kinomes were included to investigate homology. For some of these, the predicted peptides were downloaded from their respective databases and analysed as above, or their kinomes were readily available. These include the diatom *Phaeodactylum tricornutum* (JGI v. 2.0, 10,402 peptides), the water molds *Phytophthora ramorum* (JGI v. 1.1, 15,743 peptides) and *Phytophthora sojae* (JGI v. 1.1, 19,027 peptides), the model ciliate *Paramecium tetraurelia* (ParameciumDB, v. 1.43, 39,541 peptides), the ciliate *Tetrahymena thermophila* (KinBase: http://www.kinase.com/kinbase/) and the diatom *Thalassiosira pseudonana* (Kinomer v. 1.0 [111]). The fungal and metazoan kinomes were downloaded from the Kinomer v. 1.0 database [111] and include those of *S. cerevisiae, D. melanogaster, C. elegans* and *H. sapiens.* The identification of non-kinase accessory protein domains, transmembrane helices and signal peptides was performed with a local installation of InterProScan [122] run with default parameters.

Phylogenetic analysis

To identify homology relationships among apicomplexan PKs and those of alveolates and metazoans, all kinase catalytic domain sequences were compared pairwise, followed by complete clustering on z-scores calculated from randomized sequences with the Amps suite of programs [123]. This allowed the selection of homologous alveolate and metazoan sequences that were subsequently included in multiple sequence alignments of each PK group. The alignments were inspected and curated for large insertions/deletions and mis-aligned regions under Jalview [124]. Phylogenetic trees were reconstructed using three alternative strategies. Firstly, Neighbor Joining trees were reconstructed as implemented in BioNJ [125] and support for the different partitions was computed based on a bootstrap analysis of 100 alignment replicas. Secondly, Maximum Likelihood trees were reconstructed as implemented in PhyML v3.0 [126], using four alternative evolutionary models (LG, WAG, VT and JTT). The model best fitting the data according to the AIC criterion [127] was chosen for subsequent analyses. In all cases a discrete gamma-distribution model with four rate categories plus invariant positions was used, the gamma parameter and the fraction of invariant positions were estimated from the data. Support values were computed using approximate likelihood ratio test statistics (aLRT) using the option "minimum of -SH and Chi2 statistics" as implemented in PhyML [128]. Finally, Bayesian analyses were ran using PhyloBayes v. 3.2 [129] and the CAT model, two MCMC chains were ran until convergence (using maxdiff < 0.1 and effective size > 100 thresholds). Consensus trees were computed, after removing the first 20% of trees, using the majority rule. Support values for the different partitions correspond to posterior probabilities. A strict consensus of all three methods was built that shows only partitions recovered by all three approaches (ML, NJ and Bayesian).

TABLES

Table 1. The kinomes of the 12 apicomplexan genomes classified into the various ePK and aPK groups. The Rhoptry kinases of *T. gondii* are included as part of the OPK group of this parasite.

Kinase group	***P. berghei***	***P. chabaudi***	***P. falciparum***	***P. knowlesi***	***P. vivax***	***P. yoelii***	***C. hominis***	***C. parvum***	***T. gondii***	***B. bovis***	***T. annulata***	***T. parva***	**Total**
AGC	6	6	6	6	6	7	5	6	15	4	4	4	**75**
CAMK	17	18	19	18	18	18	12	15	29	7	9	10	**190**
CK1	1	1	1	1	1	1	2	2	3	1	1	1	**16**
CMGC	21	19	22	21	23	22	16	18	20	15	16	16	**229**
STE	1	1	2	2	2	1	4	6	2	1	1	1	**24**
TKL	4	7	6	7	5	4	5	4	6	0	0	0	**48**
NEK	4	3	4	4	4	3	2	3	4	1	1	1	**34**
FIKK	1	1	19	1	1	1	1	1	1	0	0	0	**27**
OPK	4	2	5	4	3	3	0	0	41	0	0	0	**62**
PDHK	0	0	0	0	0	0	0	0	4	0	0	0	**4**
PIKK	1	2	3	2	3	1	4	4	8	4	3	3	**38**
RIO	2	1	2	2	2	2	1	2	2	2	2	2	**22**
Total	**62**	**61**	**89**	**68**	**68**	**63**	**52**	**61**	**135**	**35**	**37**	**38**	**769**

Table 2. Conservation of apicomplexan PKs in non-apicomplexan alveolates and metazoans.

Group	Orthologous groups	+apicomplexans +alveolates +metazoans	+apicomplexans +alveolates -metazoans	apicomplexans only
AGC	6	3	0	3
CAMK	18	1	1	16
CK1	1	0	0	1
CMGC	22	7	4	11
STE	1	0	0	1
TKL	6	0	0	6
NEK	4	0	1	3
FIKK	2	0	0	2
PIKK	3	0	0	3
RIO	2	0	0	2
Total	65	11	6	48

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Fig. 1. Bar chart of the apicomplexan kinomes split by PK group and in comparison with the kinomes of the model organisms *H. sapiens, D. melanogaster, C. elegans* and *S. cerevisiae*. The PK groups of all species are displayed as a percentage of the organisms' entire kinome. The relative percentages of each PK group are approximately constant across the apicomplexans and the opisthokonts. The OPK

group of *T. gondii* appears disproportionately larger as it contains all the Rhoptry kinases, which are specific to Toxoplasma.

Fig. 2. Conservation of apicomplexan orthologous kinase groups in alveolates and metazoans. Empty circles indicate 'absent', whereas fully coloured circles indicate 'present'. The 36 essential PKs of *P. falciparum* are highlighted in red.

SUPPLEMENTARY MATERIAL

Table S1. Orthology relationships of the 12 apicomplexan kinomes.

LIST OF ABBREVIATIONS

PK (protein kinase); ePK (conventional PK); aPK (atypical PK); OPK ('Other' PK); HMM (profile hidden Markov model).

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The genomes analysed in this study are publicly available, and no unpublished data has been used.

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