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5
The Kinomics of Malaria

Mathieu Brochet, Andrew B. Tobin, Oliver Billker, and Christian Doerig

5.1
Introduction

5.1.1
Malaria Parasites: Highly Divergent Eukaryotes

Malaria is caused by unicellular eukaryotic parasites of the genus *Plasmodium*. Humans can be infected through the bite of an infected *Anopheles* mosquito carrying any one of five *Plasmodium* species (*Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*), *P. falciparum* being the most virulent (see Figure 5.1 for a description of the life cycle of these parasites). Recently, the global burden from malaria has significantly decreased through programs such as Roll Back Malaria, the distribution of insecticide-treated bednets and artemisinin-based combination therapies (ACTs) [1, 2]. Despite this progress, it is still estimated that more than 1.2 million deaths a year are the result of malaria, with over 300 million infected people worldwide [3]. Resistance to artemisinin derivatives, the latest-generation frontline antimalaria therapeutics [4, 5], is clearly emerging. Drugs with novel modes of action are thus urgently needed if malaria is to be effectively controlled or even eradicated [1, 2].

Malaria parasites descended from photosynthetic cyanobacteria that were subjected to endosymbiosis by a biciliate eukaryotic phagotroph to generate the Plantae (Archaeplastida) supergroup, ancestor to algae and the land plants. This primary endosymbiotic event was subsequently followed by a second endosymbiosis where algae were engulfed by a second phagotroph to give rise to the Chromalveolata superphylum that contains the Apicomplexa phylum which includes the *Plasmodium* species [6, 7]. It appears that Apicomplexa diverged very early (>1 billion years ago) from the main eukaryotic lineage, possibly adopting a parasitic lifestyle more than 500 million years ago [6].

Kinomics: Approaches and Applications, First Edition. Edited by Heinz-Bernhard Kraatz and Sanela Martic.

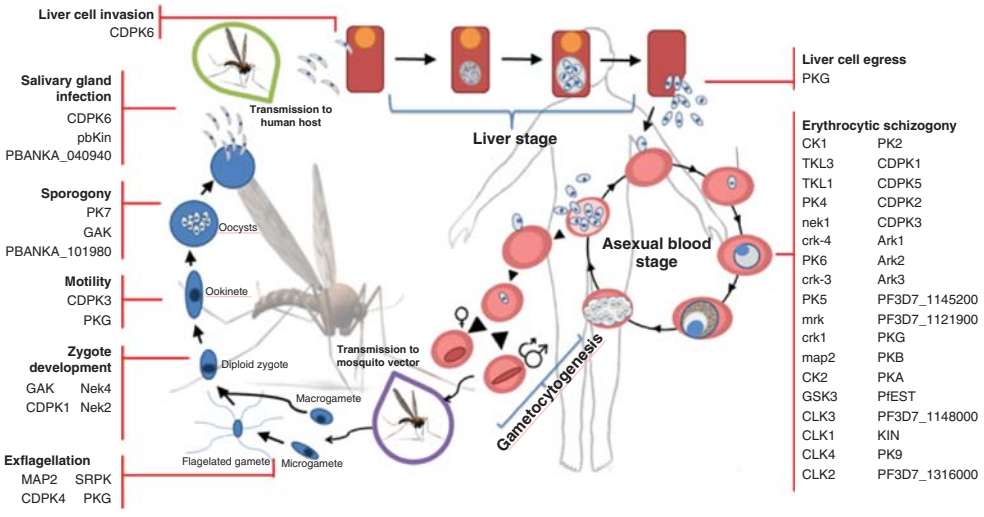

Figure 5.1 The life cycle of malaria parasites. *Plasmodium* sporozoites injected into the human host through the bite of an infected *Anopheles* mosquito travel via the bloodstream to the liver, where they invade a hepatocyte. Intense schizogony (repeated nuclear division in the absence of cytokinesis, with eventual ontogeny of individual progeny cells) ensues, producing several thousand merozoites that are released to the bloodstream and invade erythrocytes. Malaria pathogenesis is caused by the asexual multiplication of parasites in erythrocytes through schizogony. Some merozoites, after invasion of the host red blood cell, arrest their cell cycle and differentiate into male or female gametocytes. These sexual cells mediate transmission to the mosquito vector. Once ingested by the insect, they develop into gametes (for the male gametocyte, this involves a process called exflagellation, whereby eight flagellated gametes are formed from each male gametocyte). Fertilization occurs in the mosquito's midgut; the zygote develops into a motile ookinete, where meiosis occurs. The ookinete crosses the midgut epithelium and establishes an oocyst, which is the site of a new round of asexual proliferation resulting in the generation of sporozoites. These accumulate in the insect's salivary glands, where they are primed to infect a new human host during a subsequent blood meal. Protein kinases that have been demonstrated to control specific life cycle transitions, or to be involved in processes at various stages of the cycle, are indicated at the sides.

5.1.2
Posttranslational Modifications of Proteins: An Essential Multiplier of Proteome Complexity

The relatively limited number of genes in a mammalian genome (20–25 000) is transcribed into an estimated 100 000 different coding transcripts, a result of alternative promoters and alternative splicing. The proteome translated from these transcripts is made of >1 000 000 proteins, a jump in complexity that is a direct consequence of the numerous posttranslational modifications (PTMs) of proteins [8]. PTMs are covalent alterations of specific residues on a polypeptide, many of which are reversible. The vast majority of PTMs (addition and removal of chemical

groups) are mediated by specific enzymes, and such enzymes represent a nonnegligible fraction of the proteome; for example, genes encoding protein kinases make up to about 2% of the mammalian and yeast genome [9, 10]. Reversible phosphorylation regulates many aspects of protein function and properties, such as proper folding, localization, binding potential, enzymatic activity, or stability. It is essential for cell survival, and dysregulation can lead to disease such as neoplasia; indeed, many anticancer drugs target kinases. In view of the phylogenetic distance between malaria parasite and their hosts, *Plasmodium* mediators of protein phosphorylation certainly represent attractive targets for selective inhibition [11–13].

Here, we focus on the *Plasmodium* kinome and on the biology of protein phosphorylation in *Plasmodium*, and we discuss the potential and initial progress in antimalarial drug discovery based on the inhibition of the protein kinases of both the parasite and its host erythrocyte.

5.2 The *Plasmodium* Kinome: Salient Features

The *Plasmodium* kinome and its potential as a druggable target have been the subject of recent reviews [13, 14]. Soon after the sequences of the 14 chromosomes that make up the *P. falciparum* genome were made available [15], two independent studies were published reporting the result of genome mining based on kinase hidden Markov model (HMM) profiles [16, 17]. There were some discrepancies between the two studies, resulting largely from the threshold value for inclusion of borderline sequence, with one study proposing 85 [17] and the other 99 [16] kinase-compliant sequences (i.e., 1.57% or 1.83% of the ~5400 genes, close to the value found in other eukaryotes). A third study subsequently proposed a 91-sequence kinome [18]. All three reports concurred on the salient points of the plasmodial kinome, which revealed a picture of profound divergence between *Plasmodium* and mammalian protein kinases (PKs). Such divergences occur at three levels.

First, at the Level of the Set of Groups and Families. Comparative analysis of the distribution of the PK sequences into the groups and families that have been established for the mammalian kinome [19] indicates that several plasmodial eukaryotic protein kinases (ePKs) cluster within the established AGC, CK1, CMGC, CamK, and tyrosine kinase-like (TKL) groups (Figure 5.2). However, there are also significant divergences. On one hand, entire groups and families that have numerous members in mammalian kinomes are absent in *Plasmodium*, and on the other hand, the parasite's kinome comprises families that are not present in mammals. Missing kinase groups and families include:

- The tyrosine kinase group, which is prominent in metazoan kinomes and has been expanded in metazoans in response to the need for intercellular communication (although there are a few examples of unicellular eukaryotes possessing members of this group).


TKL
NEK
PEK
FIKK
CMGC
CAMK
AGC
STE
CK1
PF3D7_0923000
PF3D7_1331000
PF3D7_1428500
PF3D7_0628200 (PfPK1)
PF3D7_0107600 (PfeIK1)
PF3D7_1444500 (PfeIK2)
PF3D7_1247500
Hs GCN2
PF3D7_0902100 (FIKK-9.2)
PF3D7_0902300 (FIKK-9.4)
PF3D7_0902600 (FIKK-9.7)
PF3D7_0902400 (FIKK-9.5)
PF3D7_0902500 (FIKK-9.6)
PF3D7_0902200 (FIKK-9.3)
PF3D7_0902000 (FIKK-9.1)
PF3D7_1016400 (FIKK-10.1)
PF3D7_1149300 (FIKK-11)
PF3D7_0424500 (FIKK-4.1)
PF3D7_0424700 (FIKK-4.2)
PF3D7_1039000 (FIKK-10.2)
PF3D7_1317700
PF3D7_0301200 (FIKK-12)
PF3D7_1200600 (FIKK-3)
PF3D7_0506900 (FIKK-7)
PF3D7_0726200 (FIKK-1)
PF3D7_0857100 (FIKK-8)
PF3D7_0102600 (FIKK-6)
PBANKA_122500 (PbFIKK)
PF3D7_0923800
PF3D7_1428300
PF3D7_1539500
PF3D7_1443200 (PfCLK-1)
Hs CLK1
PF3D7_1114700 (PfCLK-4)
PF3D7_0312400 (PfCLK-2)
PF3D7_1109600 (PfCLK-3)
PF3D7_1318500 (PfSRPK1)
PF3D7_0821100 (PfSRPK2)
PF3D7_0302100 (PfCK2)
PF3D7_1431500 (Pfmap-1)
Pf3D7_1113900 (Pfmap-2)
Hs ERK1
PF3D7_0615500 (Pfcrk-5)
PF3D7_1337100 (PfPK9)
PF3D7_1356800 (PfPK5)
Hs CDK2
PF3D7_0417800 (Pfcrk-1)
PF3D7_1014400 (Pfmrk)
PF3D7_0415300 (Pfcrk-3)
PF3D7_0317200 (Pfcrk-4)
PF3D7_1338900
PF3D7_1121900
PF3D7_1474700
PF3D7_0213400 (PfPK7)
Hs CaMK1a
At CDPK1
PF3D7_0217500 (PfCDPK1)
PF3D7_0717500 (PfCDPK4)
PF3D7_0610600 (PfCDPK2)
PF3D7_0310100 (PfCDPK3)
PF3D7_1337800 (PfCDPK5)
PF3D7_1315100 (PfPK9)
PF3D7_1454300 (PfKIN)
PF3D7_1238900 (PfPK2)
PF3D7_1122800 (PfCDPK6)
PF3D7_1423600
PF3D7_1450000
PF3D7_0715300
PF3D7_0314400
PF3D7_0214600
PF3D7_0704500
PF3D7_1104900
PF3D7_1441300
PF3D7_1148000
PF3D7_1246900 (PfPKG)
PF3D7_1458200 (PfPK8)
Hs PKACa
PF3D7_1456600 (PfPKG)
PF3D7_0929200 (PfPKA)
PF3D7_0929400 (PfeIK-2)
PF3D7_1358900 (PfeIK-1)
PF3D7_0803500 (PfeIK-3)
PF3D7_0931100 (PfPK6)
Hs PKA
PF3D7_0936000
PF3D7_0821400
PF3D7_0826100
PF3D7_0826400
PF3D7_1139600
PF3D7_0306900 (PfTKL-3)
PF3D7_1349300 (PfTKL-2)
Hs RAF1
PF3D7_1108800
PF3D7_0211700 (PfTKL-1)
PF3D7_0623800 (PfTKL-4)
PF3D7_1121300 (PfTKL-2)
Hs IRAK1
PF3D7_1228300 (Pfnek-1)
Hs NEK1
PF3D7_1201600 (Pfnek-3)
PF3D7_0928600 (Pfnek-2)
PF3D7_0719200 (Pfnek-4)
Hs CK1
PF3D7_1139600

- The STE group is essentially absent, although there is one borderline sequence; of particular interest is the total absence of MAP kinase kinases (MAPKKs/MEKs), which constitute a prominent multimember family within the metazoan STE group.

Among the atypical kinases (aPKs, which comprise enzymes that diverge from the ePKs at the primary structure level but nevertheless possess protein kinase activity), the RIO, PHDK, and PIKK families are present in malaria parasite, but the Alpha family is absent.

Conversely, several plasmodial protein kinases do not have orthologs in mammalian cells; these include:

- The FIKKs, a family that is strictly restricted to Apicomplexa, with one single member in most species of this phylum but has expanded to 20 members in *P. falciparum* and thus represents an astonishing 20–25% of this parasite's kinome [16, 17, 20]. Most members display a signal peptide and a so-called PEXEL motif that directs them to the host erythrocyte, a localization that has been confirmed in a number of instances [21–23]; some members have been implicated in virulence through their participation in the modification of the properties of the infected erythrocyte [24].
- The calcium-dependent protein kinases (CDPKs), a family that is present in plants and alveolates but absent from metazoans and characterized by the presence of a calmodulin-like calcium-binding regulatory domain attached to the kinase catalytic domain (reviewed in [23, 25]).

Second, at the Level of the Classification of Individual Kinases into ePK Groups or Families. Several individual kinases in the OPK group that play specific roles in metazoan cell biology, such as the Wee1, Myt1, or mammalian target of rapamycin (mTOR), do not have clear orthologs in the plasmodial kinome; it is likely that some of the functions these enzymes play in fundamental eukaryotic biology processes are assigned, in the parasite, to kinases that cannot be recognized on a phylogenetic basis. Conversely, many individual kinases that are found in the *Plasmodium* kinome do not cluster with any of the metazoan ePK groups or families (including the OPK group); these represent approximately 25% of the plasmodial ePKs (FIKKs excluded) and are called "orphan" kinases.

Third, at the Level of Sequence of Individual Kinases. These can actually be clearly assigned to an ePK group/family but possess unique characteristics that distinguish them from their metazoan counterparts. Such features include long

Figure 5.2 The kinome of *Plasmodium falciparum*. Phylogenetic tree of the *P. falciparum* kinome. Circular tree of all 91 eukaryotic protein kinases (ePK) in *P. falciparum* as defined by Talevich *et al.* [14]. Representative genes from human (Hs), *Arabidopsis thaliana* (At), and *Plasmodium berghei* (Pb) are indicated with labels colored gold, green, and purple, respectively. Branch and arc colors indicate kinase classification by ePK major group, with minor modifications in group assignment according to the gene tree. A gray circle on a branch indicates bootstrap support greater than 50; larger circles indicate greater bootstrap values. Taken from Ref. [14], with permission.

and often disorganized C-terminal or N-terminal extensions to the catalytic domain, as well as insertions within the catalytic domain that can be several hundred residues long and contain homopolymeric stretches (such as dozens of adjacent Asp residues in a Pfcrk-4, a CDK-related sequence); such insertions are found in loops between secondary structure elements and presumably do not interfere with the kinase fold. Another intriguing divergence between metazoan and plasmodial kinases consists of the apparent swapping of specific subdomains within the catalytic domain between different kinase families. For example, the N-terminal lobe of the kinase called PfPK7 clearly clusters with fungal cAMP-dependent kinases, whereas its C-terminal lobe clusters with the mammalian MAPKK6 subfamily [26]. Similarly, the NIMA-related kinase Pfnek-1 clearly belongs to the NIMA/Nek family of the OPK group; however, the phosphorylation motif in its activation loops reads SMAHS, very similar to the SMANS motifs found in MAPKKs but clearly distinct from the FXXT motif shared by all other NIMA/Nek enzymes; it thus appears that Pfnek-1 is a NIMA/Nek enzyme with a MAPKK activation site [27]. To our knowledge, there has been no systematic search for such "composite" kinases in the plasmodial kinome, but it is likely that these two examples are just the tip of the iceberg.

Many plasmodial kinases thus either do not cluster within established ePK groups or families or, for those that do, branch off near the base of their cluster [14, 17]. In the latter case, although assignment to the groups or family concerned is clear, the lack of orthology to specific metazoan enzymes within the cluster makes it very difficult or impossible to predict precise cellular functions for the kinases of malaria parasites. Elucidating the function of these enzymes requires *de novo* experimental data, mostly based on reverse genetics.

5.3 Reverse Genetics of the *Plasmodium* Kinome

Methods to manipulate the *Plasmodium* genome have been developed only relatively recently [28–30]. The most commonly used methods rely on the transfection of asexual erythrocytic stages of the life cycle, in which the parasite is haploid and replicates continuously, facilitating genetic manipulation and selection of recombinants. However, experimental genetics in *Plasmodium* is complicated by the difficulty of delivering DNA to the nucleus of the intraerythrocytic parasite, by the relative inefficiency of homologous recombination, and by the extremely high AT content of most *Plasmodium* genomes. The use of zinc finger and CRISPR-Cas9 endonucleases for genome editing in *P. falciparum* has begun to have a great impact by increasing recombination efficiency dramatically [31–33] and has enabled the first stable genome modification in *P. vivax* [34]. The most tractable malaria parasite for genetic modification is still *Plasmodium berghei*, a species infecting rodents and for which a genome-scale resource of barcoded knockout and targeting vectors is now available to enable reverse genetic screening (*http://plasmogem.sanger.ac.uk/*) [35, 36].

Since *P. berghei* has the additional advantage of being easily transmitted through the entire life cycle in the laboratory, most of our knowledge on protein kinase functions during the sexual and mosquito stages comes from this species.

Systematic gene knockout approaches of protein kinases can serve an important validation function for drug and vaccine development by identifying gene targets that may be essential for parasite growth. Kinome-wide reverse genetic analyses have been conducted in both *P. berghei* [37] and *P. falciparum* [38], leading to the establishment of a list of enzymes that are likely essential for asexual proliferation or for specific transmission stages. However, failure to disrupt a gene does not on its own provide sufficient evidence for its essentiality, and further experiments are required for such statements. With these caveats in mind, it appears that approximately half of the kinome comprises enzymes that are required for asexual proliferation in the erythrocyte. Kinases required for asexual intraerythrocytic proliferation include almost all of the CMGC, AGC, and Aurora-related kinases, while most NimA-related kinases and CamK can be knocked out but show developmental phenotypes in the sexual stages, suggesting that distinct stages of the life cycle have different requirements for signaling functions. Intermediate phenotypes resulting in a decreased rate of asexual growth in erythrocytes were also observed. Disruptions of the "orphan" kinase PK7 [39], the cyclin-dependent kinase Pfcrk-5 [40], and the calcium-dependent kinase CDPK7 [41] were, for instance, associated with a lower number of daughter merozoites generated per schizont in *P. falciparum*. A genetic screen in *P. berghei* has revealed additional mutants whose competitive fitness is compromised *in vivo* [42]. While studies in both species show significant overlap, there are also some notable differences in the targetability of orthologous genes, which may be considered surprising given the high overall conservation of the two kinomes. It remains to be seen which of these are true species differences resulting from different physiological needs and which reflect differences between *in vitro* and *in vivo* growth conditions in *P. falciparum* and *P. berghei*, respectively.

Kinase genes that are not essential for asexual erythrocytic growth can be knocked out, and stable clonal KO parasite lines obtained, whose phenotype can then be examined during sexual development and in the mosquito vector. Half of the enzymes that are not essential in asexual blood stages are required for transmission through the mosquito vector or for the liver-stage infection. Mosquito infection relies on the developmentally arrested gametocytes circulating in the blood. Upon mosquito ingestion, gametocytes resume their development in response to various mosquito factors. Following initial activation, a cascade of protein kinases including CDPK4 [43], MAP2 [44, 45], and SRPK [37] regulates cell cycle events that, in male gametocytes, eventually result in the release of eight flagellated gametes. After fertilization, two NimA-related kinases are required for the replication to the tetraploid level that precedes meiosis [46, 47]. Further differentiation of zygotes into motile ookinetes is controlled by CDPK1 [48]. In-depth translational and transcriptional profiling of CDPK1-arrested parasites revealed a role of this kinase for the activation of translationally arrested mRNA species. Another CDPK, CDPK3, is further required for efficient gliding motility

ookinetes rely on to colonize the midgut epithelium [49, 50]. Successful ookinetes differentiate into oocysts. Growth and division of each oocyst to produce thousands of sporozoites require the "orphan" kinase PK7 (which, interestingly, is also involved in the control of proliferation in asexual blood stages; see the preceding text) [39], a cyclin G-associated kinase (GAK) [37], and a CDPK-like kinase [51]. Control of sporozoite egress from the oocyst to reach the mosquito salivary glands further requires two CamK kinases and CDPK6 [37]. Finally, following a mosquito bite, invasion of the liver cells by sporozoites also requires CDPK6 [52].

Genes essential for asexual proliferation cannot be constitutively deleted, posing a major challenge to further investigate their functions. Widespread approaches such as inducible knockdown based on siRNA are not implementable in malaria parasites, which lack the necessary enzymatic machinery [53]. However, new conditional genetic technologies have been developed recently to study the role of genes essential for asexual proliferation. One such approach, which uses a short degron sequence fused to the protein of interest to enable protein degradation to be controlled through a small molecule [54], was used to show that CDPK5 is clearly required for egress of *P. falciparum* merozoites from the red blood cell at the end of schizogony [55]. However, another member of the same kinase family, CDPK1, illustrates some of the challenges the field still faces to identify essential gene functions. CDPK1 is abundantly expressed in asexual blood stages and has been implicated through *in vitro* phosphorylation studies to be a kinase of the molecular motor that drives RBC invasion [56]. It is thought to be the primary target of a compound that blocks parasite development [57] and has been proposed to function in schizont growth, based on the dominant interfering effect of overexpressing a peptide that links the regulatory and kinase domains of the protein [58]. All this evidence is indirect and protein knockdown using the same inducible degron system as for CDPK5 failed to reveal an obvious phenotype [58]. The *P. berghei* ortholog of CDPK1 is dispensable for blood-stage growth [48], and more decisive genetic evidence is needed to define its function in *P. falciparum*.

A powerful approach that is now available to reveal functions for CDPK1 and other blood stage-expressed kinase genes relies on site-specific recombinases. A Cre recombinase of bacteriophage P1, which has been broken down into two subunits that can be dimerized by rapamycin, provides a powerful generic system to tightly regulate essential gene expression in *P. falciparum* blood stages [59]. Another recombinase, FLP of *Saccharomyces cerevisiae*, which recognizes *FRT* sites, has been adapted to delete essential genes in sporozoite and liver stages of *P. berghei* [60]. This approach relies on expression of FLP via developmentally regulated promoters active in parasite stages that inhabit the mosquito midgut or salivary glands. Regulated expression of FLP leads to the excision of the DNA sequence flanked by FRT sites. This approach has been successfully used to conditionally disrupt in sporozoites the cyclic GMP-dependent protein kinase, PKG. Transgenic parasites in which the *pkg* gene is disrupted are not transmitted to their mammalian host after a mosquito bite due to a block in late liver stages [61].

Exceptionally elegant, if they can be implemented, are chemical genetic methods developed by Shokat and Velleca [62] that rely on a mutation of the so-called gatekeeper residue, an amino acid that controls access of small molecules to the adenosine triphosphate (ATP)-binding pocket of the kinase. The original approach used in yeast and mammals replaces this amino acid, which tends to be a large side-chain residue in wild-type kinases, with a small side-chain residue that maintains activity of the enzyme but also sensitizes the kinase to "bump" inhibitors that do not affect the wild-type kinase. This direct approach has been attempted with very limited success, presumably because several *Plasmodium* kinases have short side-chain residues, such as a threonine or a serine, at their gatekeeper position, and are therefore sensitive to bump inhibitor. Interestingly, the *Plasmodium*-related apicomplexan parasite *Toxoplasma* possesses the only known kinase with the smallest possible gatekeeper residue, a glycine; the *Plasmodium* ortholog, CDPK4, has a serine residue at the gatekeeper position, which has been exploited in drug discovery (see the following text). Nevertheless, this principle was successfully exploited in a "reverse" fashion in the case of PKG. This essential enzyme has a small threonine gatekeeper residue. Parasite lines were generated in *P. falciparum* and *P. berghei*, where this threonine was replaced with a large glutamine, making the kinase resistant to Compound **1** and Compound **2**. These two related molecules selectively inhibit plasmodial PKG but not metazoan PKG homologs, which have a large gatekeeper. Comparative examination of the effect of Compound **1** or **2** in control and resistant lines allowed to determine that PKG plays essential roles not only during erythrocytic schizogony [63] but also in the early events of gametogenesis [64] and ookinete motility [65]. This combination of the specificity of reverse genetics with the speed and versatility of chemical intervention opened the possibility to dissect with a high temporal resolution the role of PKG in these stages. For example, deep molecular phenotyping of specific PKG transient inhibition revealed this enzyme as a stage-transcending regulator of critical Ca^{2+} signals [65].

5.4 Lessons from Phosphoproteomics

The rapid expansion of mass spectrometry-based proteomic techniques has provided a method of producing a snapshot of the global phosphorylation status of organisms such as yeast [66] and bacteria [67, 68], as well as cultured eukaryotic cells [69], tissues such as the liver [70], and even whole animals [71] and plants [72]. This has also been applied to apicomplexan parasites, including *P. falciparum* [38, 73–77] and *Toxoplasma gondii* [76], which has revealed many thousands of phosphorylation sites on proteins involved in nearly every aspect of parasite biology. In this regard, protein phosphorylation in malaria can certainly be viewed as having a wide ranging regulatory role on fundamental biological processes such as cell division, protein synthesis, and cellular metabolism (as is clearly the case in higher eukaryotes), as well as specialized, parasite-specific functions such as egress, invasion, and cytoadhesion. We are, however, only at the very start of

understanding the role played by phosphorylation in maintaining the complex life cycle of malaria parasites.

5.4.1 Phosphorylation Cascades

It is clear from many decades of research in mammalian systems that protein kinases are organized within phosphorylation signaling cascades where kinases regulate the activity of other downstream kinases [78, 79]. Such phosphosignaling networks allow for the integration and cross talk between signaling pathways and offer a mechanism whereby signaling outputs can be tailored to produce a specific physiological response [69]. Whereas mammalian phosphosignaling networks are being progressively well understood and are undoubtedly contributing to our ability to effectively target key phosphosignaling pathways in diseases such as cancer [80], the understanding of phosphosignaling in malaria is still at a rudimentary level.

We are however making progress, and from the examination of the phosphoproteomes published to date [38, 73, 75, 76], it is now clear that many of the malaria protein kinases are themselves phosphoproteins (Figure 5.3). Some of the most interesting examples of this phenomenon is the phosphorylation of the second messenger-regulated protein kinases PfPKA and PfPKG [38]. These kinases are phosphorylated within the activation loop in a manner consistent with phosphorylation regulating protein kinase activity [38]. Thus, it is very likely that PfPKA and PfPKG are not only regulated by changes in cyclic nucleotide levels, but also by phosphorylation mediated by as yet unidentified upstream kinases.

There are other examples of malaria kinases being phosphorylated. This includes the CDPK PfCDPK1 which is phosphorylated within the glycine-rich loop of domain I of the kinase domain [38, 73, 75, 76]. In contrast, other kinases are phosphorylated outside the kinase domain, such as PfARKs and PfCDPK6, or within large insertions within the kinase domain as exemplified by PfSRPK1 and PfSRPK2 [38, 73, 75, 76]. In addition, protein kinase regulatory subunits are phosphorylated as seen for regulatory subunit of PfPKA [38]. These findings illustrate that *Plasmodium* protein kinases, like the mammalian counterparts, are subject to regulation by upstream kinases and as such are very likely to sit within phosphosignaling cascades. The challenge is to define the nature and role of these cascades and thereby identify the key phosphosignaling hubs that will provide novel targets for pharmacological intervention.

5.4.2 Evidence for Tyrosine Phosphorylation *Plasmodium*

Only in rare cases such as choanoflagellates, a group of aquatic flagellate unicellular eukaryotes considered to be the closest relatives to metazoans, have tyrosine kinase families been identified in protozoan organisms [81, 82]. Thus, yeast (*S. cerevisiae*) [9] and protists such as *Trypanosoma brucei* (the causative agent

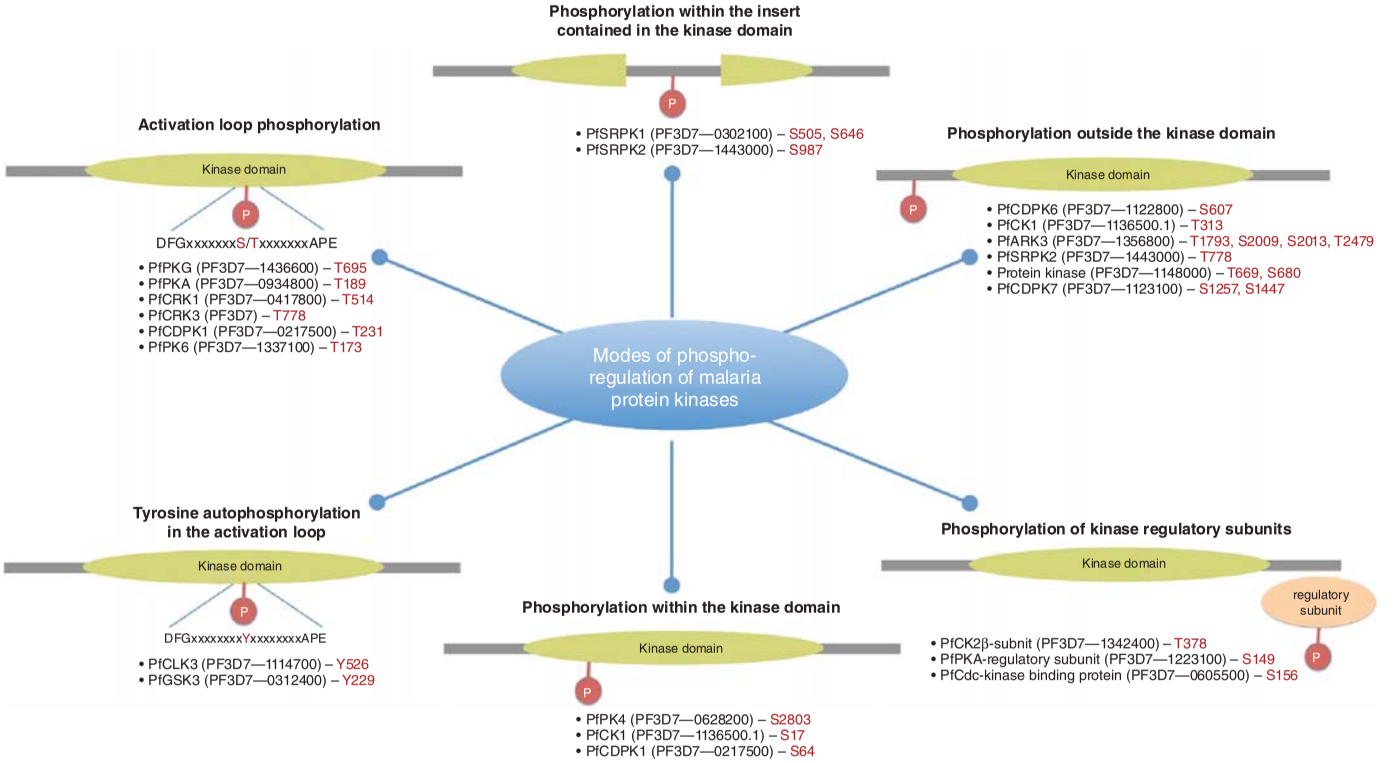

Figure 5.3 Phosphosites detected by mass spectrometry on *Plasmodium* kinases. Data compiled from the published global phosphoproteomic studies indicate that many of the malaria protein kinases are themselves phosphoproteins. This suggests that parasite protein kinases (like the mammalian counterparts) are under phosphorylation-dependent regulation. These phosphorylation events might be the result of autophosphorylation or of the action of upstream protein kinases in a phosphorylation cascade. Indicated are the modes of phosphorylation identified together with specific examples. Also shown is an illustration of the kinase domain, a classic activation loop starting with a DFG and ending in APE (please note that this is illustrative and that for some of the *Plasmodium* kinases listed, the DFG/APE motifs are atypical).

of sleeping sickness) [83, 84] and *Giardia lamblia* (a human gut parasite) [85] as well as parasites of the Apicomplexa phylum including *T. gondii* [86] and *P. falciparum* [14, 17] do not possess any members of the tyrosine kinase group. This is mirrored by a lack of key phosphotyrosine signaling elements such as proteins containing Src homology 2 domains (SH2 domains) [14] and phosphotyrosine phosphatases [17]. These findings are consistent with the notion that tyrosine phosphosignaling pathways evolved in metazoan organisms to meet the demands of intercellular communication [9]. Interestingly, global phosphoproteomic analyses have reported tyrosine phosphorylation in yeast [87], *T. brucei* [83], *G. lamblia* [85, 88], and *T. gondii* [76]. Likewise, despite the lack of a tyrosine kinase family in *P. falciparum,* tyrosine phosphorylation has been reported to occur with a frequency (1–4%, depending on the study [38, 73, 75, 76]) that is similar to that observed in metazoa [89, 90]. However, on manual inspection, only a fraction of the relevant spectra proved to be unambiguous tyrosine-phosphorylated peptides (reviewed in [74]). In light of this, it is likely that the true extent of the tyrosine phosphorylation in *P. falciparum* is very low. Using radiolabeled phosphoamino acid analysis indeed suggested that tyrosine phosphorylation accounted for ~0.5% of the total phosphoproteome [38].

The most solid evidence for tyrosine phosphorylated malaria proteins has emerged from examination of autophosphorylation of protein kinases. In particular, PfCLK3, which is closely related to the human kinase PRP4 [17, 18], has been designated as a dual-specificity tyrosine phosphorylated-regulated kinase (DYRK) [18]. Kinases within the DYRK subfamily are characterized by the fact that they autophosphorylate on tyrosine at translational intermediate – after which the mature DYRK is a serine/threonine kinase [91, 92]. Consistent with its designation as a DYRK, PfCLK3 has been shown to autophosphorylate on tyrosine in a manner that is essential for full kinase activity [38]. Similarly, *P. falciparum* PfGSK3 is autophosphorylated at a tyrosine in the activation loop during translation [38] in a manner similar to that seen in the mammalian ortholog glycogen synthase kinase 3 [93].

Even though they are low, the reported levels of tyrosine phosphorylation in *Plasmodium* phosphoproteome are not accounted for by solely DYRK autophosphorylation, suggesting the presence of genuine dual-specificity kinases able to phosphorylate substrate proteins on tyrosine. *P. falciparum* does contain four members of the TKL group [94, 95], from which the true tyrosine kinases found in metazoan are thought to have originated. Although these might have the potential for tyrosine phosphorylation, the tyrosine kinase activity of these enzymes has not been investigated. Prominent among the dual-specificity kinases in mammalian systems is protein kinase CK2. In mammals, there are two genes encoding the catalytic α-subunit (α and α′) and a single regulatory β-subunit gene – the products of which form a tetrameric complex consisting of two α-subunits and two β-subunits [96]. The *P. falciparum* kinome includes a single CK2 α-subunit and the two regulatory β-subunits (β_1 and β_2) [97]. Like the mammalian counterpart, PfCK2 has dual serine/threonine and tyrosine kinase activity *in vitro*: a minichromosome maintenance (MCM) complex

subunit, PfMCM2, was used as a substrate and shown to be phosphorylated by PfCK2 on tyrosine 16 (Y^{16}) [98]. PfMCM2 was chosen since there was *in vivo* phosphoproteomic data that suggest Y^{16} on PfMCM2 is phosphorylated in the schizonts [38]. However, following the generation of phosphospecific antibodies and in-depth examination of the phosphorylation of Y^{16} *in vivo*, it was concluded that despite the fact that PfCK2 could phosphorylate Y^{16} on PfMCM2 *in vitro*, *in vivo*, this phosphorylation event likely did not occur and that, in fact, PfCK2 was more likely to phosphorylate the nearby S^{13} [98]. Thus, although PfCK2 has the potential to phosphorylate on tyrosine, there is currently no evidence that this actually occurs in living parasites.

In conclusion, there is evidence for tyrosine phosphorylation in malaria parasites, but the extent of tyrosine phosphorylation appears to be lower than that seen in mammalian systems. In the absence of a tyrosine kinase family, it is still unclear which protein kinases are involved in tyrosine phosphorylation in *Plasmodium*. The fact that PfCK2 has some tyrosine kinase activity *in vitro* might suggest that at least this protein kinase has the potential to mediate a proportion of the *in vivo* tyrosine kinase activity, although the cellular substrates for this activity have yet to be established. It is also possible that some of the "orphan" kinases that have no orthologs in other systems, such as the FIKKs, may display tyrosine kinase activity.

5.5 Host Cell Kinomics in Malaria Infection

It is well established that many intracellular pathogens rely on, and modulate, signaling pathways of their host cells to facilitate their replication and survival [99], and this has been documented in liver stages of malaria infection: an siRNA screen focused on the human kinome showed that downregulating some kinases in the host hepatocyte impaired liver-stage parasite proliferation [100]. Because mature red blood cells are generally viewed as largely passive players in the infection process, few studies have been devoted to the role of host erythrocyte signaling molecules during infection. Nevertheless, erythrocyte heterotrimeric G proteins, which constitute a well-characterized class of signal transduction proteins in mammalian cells but have no homologs in *P. falciparum*, have been shown to play a role in the invasion by the parasite [101]; the downstream signaling and effectors of heterotrimeric G proteins in *Plasmodium*-infected erythrocytes remain to be characterized.

As discussed earlier, the parasite does not possess MEK homologs [17]. Surprisingly, however, it was found that structurally distinct, highly selective inhibitors of mammalian MEK1 had a parasitocidal effect in *P. falciparum* cultures, with IC_{50} values similar to those these molecules display on various mammalian cellular systems [102]. Consistently, host erythrocyte MEK1 was hyperphosphorylated in infected (vs. noninfected) erythrocytes; both on the activation loop dual serines and on the regulatory residue Ser-297 that is the known

target for the p21-activated kinase (PAK). In other systems, phosphorylation of PAK1 on Ser-144, which lies on a kinase autoinhibitory domain, is known to significantly contribute to its activation; PAK is indeed phosphorylated on its activating residue, Ser-144, in *Plasmodium*-infected erythrocytes, and treatment of parasite cultures with a PAK allosteric inhibitor abolishes MEK1 Ser-297 phosphorylation and impairs parasite proliferation [102]. These observations suggest that hijacking of the host erythrocyte PAK–MEK pathway is crucial for parasite survival. Together with the data showing reliance of the parasite on host erythrocyte heterotrimeric G proteins [101], these results suggest that malaria parasites modulate, and rely on, the activity of signaling pathways present in mature erythrocytes; proteomic studies [103–105] have identified a large number of signaling molecules that are indeed maintained in these cells, despite their anucleated status.

5.6 Targeting Protein Kinases in Antimalarial Drug Discovery

The holy grail of *Plasmodium* biology is to develop a definitive cure for malaria. This in turn requires the design of suitable assays for individual steps in gene control that can be used to test the activity of small-molecule inhibitors. That novel drugs targeting *Plasmodium*-specific processes can be developed is supported by the large degree of phylogenetic diversity between malaria parasites and their human hosts that ensues from this ancient lineage split. This is not based solely on the fact that many processes and enzymes characterized in the parasite (including many protein kinases) have no counterpart in human cells: even in the case where orthologs do exist in both the parasite and its host, sufficient divergence exists to lend hope that selective inhibition of the plasmodial (vs. human) enzyme is achievable, as developed in the last section of this chapter. Specific inhibitors of key players in *Plasmodium* protein phosphorylation will represent not only urgently needed lead compounds for antimalarials with novel modes of action, but also very useful tools for further dissecting the fascinating biology of this parasite.

5.6.1 Targeting the Parasite Kinome for Curative and Transmission-Blocking Intervention

As discussed previously, although many of parasite kinases fall broadly into the familiar protein kinase groups, the considerable differences from the mammalian kinome mean that many of the parasite kinases have no clear mammalian orthologs [14, 18]. Even where orthologs can be identified, significant differences are evident that indicate different regulatory processes, substrate specificities, and modes of subcellular localization [14, 18]. Based on the fact that protein kinases have been successfully targeted in the treatment of human disease, particularly cancer [80, 106], it has been suggested that drug development programs targeting malaria protein kinases might be a strategy with significant therapeutic benefits

[11, 12, 107]. A number of target-based biochemical screens have been implemented over recent years (reviewed in [13, 107, 108]), in some instances yielding hits that show some promise in terms of selectivity with respect to human homologs, when they exist; as a recent example, the group of C. Kunick reported the identification of a series of compounds selectively targeting the *P. falciparum* (*versus* human) glycogen synthase kinase 3, PfGSK3 [109].

The small gatekeeper residue of some apicomplexan protein kinases has been exploited successfully to generate inhibitors that are apparently selective over host protein kinases. As mentioned earlier, two anticoccidial compounds were discovered to target the relatively small Thr gatekeeper of apicomplexan PKG [110] giving them great selectivity over the mammalian PKG orthologs, and both compounds have since become important tools to dissect the essential function of this kinase at different life cycle stages of *Plasmodium*. In another example, bumped ATP analogs were designed to interact with the small Ser gatekeeper of CDPK4 [111], the first essential function of which is early during the mosquito phase of the life cycle. Consequently, a CDPK4 inhibitor does not kill the RBC stages of *Plasmodium*, but was the first compound to selectively block parasite transmission to the mosquito when administered to the host [111, 112].

Phenotypic screens of large libraries (>2M compounds) on cultured *P. falciparum*, coupled with counterscreens on human cell lines, have yielded a large number (>30 000) of compounds with demonstrated selective parasitocidal activity [113–115]. In one of these studies, chemoinformatic examination of the hits indicated that large proportion of the antiplasmodial compounds from cellular screens are ATP analogs, which may act through targeting parasite kinases. These provide a rich resource of starting points for drug development, and work is ongoing in several laboratories to identify targets, notably through secondary biochemical screens using recombinant *P. falciparum* kinases.

5.6.2 Targeting Host Kinases?

Recent findings that infection of erythrocytes with *P. falciparum* requires activation of host cell phosphosignaling pathways open new perspectives in antimalarial drug development. Human signaling protein kinases are currently targeted with success in several diseases, notably cancer [106]. In particular, MEK is a validated target for cancer therapy, and MEK inhibitors have been the subjects of Phase 1 and 2 clinical trials. Molecules initially developed for cancer, and which passed preclinical development and early clinical phase trials, could be repositioned as potential antimalarials. This would have great benefits in terms of speed and economics of new antimalarials development. Importantly, targeting a human enzyme would deprive the parasite of the most straightforward mechanism for emergence of drug resistance, namely, the selection of genotypes expressing a mutated, resistant target. That both *P. falciparum* and *P. berghei* are susceptible to MEK inhibitors [102] indicates that reliance on host RBC signaling pathways is widespread across the genus *Plasmodium* and suggests that other species infecting

humans (e.g., *P. vivax*, which, like *P. berghei*, infects preferentially reticulocytes) are likely to share this feature.

5.7 Concluding Remarks

Overall, investigations on the kinome of malaria parasites have revealed on the one hand features that are conserved across the vast phylogenetically distances that exist between Apicomplexa and mammalian cells and on the other hand some unique peculiarities. This not only is fascinating in terms of fundamental and evolutionary biology; it also offers potential for selective pharmacological intervention. Furthermore, the intricate relationship between the parasite and its host erythrocyte includes a dependence of the former on signaling pathway components encoded by the host's genome. This represents a captivating aspect in the context of biological adaptation of parasites to their hosts, as well as another distinct set of opportunities for novel strategies for drug discovery strategies aimed at one of the most devastating infectious diseases the world is facing.

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