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Review

Kinase signalling in *Plasmodium* sexual stages and interventions to stop malaria transmission

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ABSTRACT

The symptoms of malaria, one of the infectious diseases with the highest mortality and morbidity worldwide, are caused by asexual parasites replicating inside red blood cells. Disease transmission, however, is effected by non-replicating cells which have differentiated into male or female gametocytes. These are the forms infectious to mosquito vectors and the insects are the only hosts where parasite sexual reproduction can take place. Malaria is thus a complex infection in which pharmacological treatment of symptoms may still allow transmission for long periods, while pharmacological blockage of infectivity may not cure symptoms. The process of parasite sexual differentiation and development is still being revealed but it is clear that kinase-mediated signalling mechanisms play a significant role. This review attempts to summarise our limited current knowledge on the signalling mechanisms involved in the transition from asexual replication to sexual differentiation and reproduction, with a brief mention to the effects of current treatments on the sexual stages and to some of the difficulties inherent in developing pharmacological interventions to curtail disease transmission.

Contents

1. Introduction

Parasitic protists of the genus *Plasmodium* sustain a vigorous cycle of asexual reproduction in red blood cells (RBCs) which causes the symptoms of malaria. Blood stage cell division is however more the exception than the rule in the Haemosporida group of apicomplexan parasites, to which *Plasmodium* belongs [1]. Most genera in the group multiply cell numbers in solid tissues, frequently the liver, and typically cause mild or asymptomatic infections only. They use vertebrate RBCs simply as gametocyte carriers to reach the haematophagous insect vectors required for transmission to new hosts. Parasites in genera *Leucocytozoon*, *Haemoproteus* and *Hepatocystis* emerge into the bloodstream as sexually committed meronts that invade RBCs only to develop as male or female gametocytes, without additional cell divisions [2]. Considering the genus *Plasmodium* as a whole and pooling together information gained from different species, an important difference with other Haemosporida emerges and it underpins the pathology of malaria. *Plasmodium* parasites undergo tissue schizogony in the liver like their phylogenetic relatives mentioned above, but then sustain additional rounds of cell division in RBCs,

Abbreviations: CDC20, cell division cycle gene 20 product; CDH1, CDC20 homologue 1; CDPK, Ca-activated protein kinase; CITH, homolog of worm CAR-I and fly Trailer Hitch gene products; DOZI, development of zygote inhibited gene 1 product; GAK, cyclin G-dependent kinase; G-protein, guanosine nucleotide-binding proteins; MAP2, mitogen-activated protein kinase 2; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; NIMA, never-in-mitosis gene A product (a protein kinase); NEK, NIMA-related kinase; PDE, phosphodiesterase; PI-PLC, phosphoinositide-specific phospholipase C; PKA, cAMP-regulated protein kinase; PKG, cGMP-regulated protein kinase; RBC, red blood cell; SRPK, serine/arginine-rich protein kinase.

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committing just a small fraction of schizonts to sexual development during each cell cycle [3,4]. It has already been suggested that, as in other Haemosporida, sexual differentiation could indeed be the default developmental pathway for *Plasmodium* blood stages and that active signalling may be required to divert them away from gametocytogenesis and towards asexual reproduction, rather than the other way around. This suggestion was based on the failure to recover protein kinase mutants deficient in commitment to gametocytogenesis, whereas mutations affecting later steps in sexual differentiation are readily obtained [5]. It appears that commitment to sexual differentiation in *Plasmodium* occurs stochastically *in vitro*, generating a female-biased population of gametocytes [6–8], although their numbers and sex ratios can be modulated by host factors *in vivo* (Section 2). Nothing is known about sex-determining factors, but both the male and female differentiation pathways seem to be available to every asexual haploid cell (see Section 4).

The end result of sexual reproduction in the mosquito is sporogony. A single infected mosquito may carry as many as a few hundred oocysts, each able to generate thousands of mature sporozoites which distribute themselves throughout the insect body, including the salivary glands from where they are transmitted to the next vertebrate host. In nature however, transitions through the sexual life stages are far from 100% efficient and vertebrate and insect immune defences collaborate to inflict heavy losses on parasite numbers [9]. This causes population bottle-necks in the mosquito whose size determines which vectors are permissive for each *Plasmodium* species. The relatively low numbers of parasites present at some of the transitions in the life cycle are theoretically ideal points for therapeutic intervention with drugs or vaccines [10].

2. Commitment to sexual differentiation and gametocytogenesis

Gametocytogenesis is induced when a small population of merozoites (0.2–1%) do not enter the asexual replication cycle upon invasion of new RBCs but arrest cell division and develop into male or female gametocytes [6,11–14]. Intriguingly, their DNA content is close to diploid levels, although it is unclear whether that represents a fully duplicated genome or amplification of selected chromosomal regions. Gametocyte development in vertebrate blood is a step-wise process with a well-documented cytology [7,8] but one that has only recently begun to be analysed at the molecular level (see [15] and [16] for recent reviews). Early studies showed that commitment to sexual differentiation occurs prior to merozoite release from a schizont, as it was observed that all progeny from a single schizont form either sexual or asexual parasites [11,12]. Therefore, the molecular switch is probably set in the preceding trophozoite stage before the first nuclear division, resulting in a population of committed merozoites destined to differentiate into gametocytes upon subsequent RBC invasion. This scenario is supported by the detection of activated sex-specific promoters in sub-populations of schizonts, at which point sexual commitment has already taken place and differentiation has begun [17]. In *Plasmodium falciparum* sex determination occurs at the same time or soon after commitment to gametocyte development, since all merozoites from a sexually-committed schizont turn into either male or female gametocytes but not both [6,13]. *P. falciparum* gametocytes develop over a period of 10–12 days through five morphologically distinct stages (Stages I–V) that culminate in the formation of crescent-shaped, or falciform, stage V gametocytes [18].

Traditionally, sexual commitment has been proposed to be a stress response that allows the parasite to escape from an unfavourable environment [19] or a response to different external cues [20–23]. Many authors have considered different external triggers or factors that may participate in initiating gametocytogenesis. For example, there is evidence that gametocyte appearance in culture correlates with high asexual parasite density [11,24], is affected by haematological conditions [25,26], immune responses [27] and presence of competing parasite clones in the same host [28]. It was also observed early on that diffusible factors in parasite-conditioned medium increased gametocyte production and it is now thought that at least some of those factors include recently identified RBC microvesicles or exosome-like vesicles, proposed to mediate cell-cell communication between infected RBCs [20–23,29]. Purified vesicles promote sexual differentiation and mass spectrometry analysis of RBC microvesicles revealed they are composed of both RBC and parasite proteins, the latter being the best candidates for parasite gene products involved in commitment to sexual differentiation [21,22].

Although it is clear that asexual parasites transition into the gametocyte development pathway at rates influenced by environmental factors, recent evidence suggests that sexual differentiation is a constitutive pathway which is enhanced by external stimuli. A study by Eksi et al. [3] showed there is a constant commitment of parasites to gametocytogenesis in every asexual cycle in culture, indicating that a basal level of gametocyte induction occurs without specific stress stimuli. Likewise, *in vivo* data from *P. falciparum* infections show that gametocytogenesis can occur prior to symptom onset and the development of high asexual parasitemia [4]. Continuous gametocyte induction during asexual growth would facilitate transmission by providing an ongoing source of infectious forms as soon as parasites emerge from the liver [3], similarly to what happens with the other Haemosporida genera mentioned in Section 1. It also implicates the existence of a long transmission window, which reinforces the need to include the sexual parasite stages in malaria control efforts. Together, existing data strongly imply that the parasite strategy is one of ongoing sexual differentiation in every asexual cycle, open to modulation in response to environmental factors [3,19,21,22].

The specific genes and molecular mechanisms involved in determining whether a particular parasite will undergo sexual differentiation have been poorly understood until now, but the availability of parasite lines naturally variant in gametocyte production and reverse genetics technology has begun to address this important question. It is widely observed that different parasite lines produce gametocytes with different yields, strongly suggesting that parasite genetics affect gametocyte production [19,30,31]. Indeed, it was using a gametocyte-defective parasite line and genetic complementation that *P. falciparum* gametocyte development 1 gene (*Pfgdv1*) was shown to be critical for gametocytogenesis [3]. Detection of *Pfgdv1* expression in asexual parasites before the appearance of gametocytes is consistent with an early role for the protein, but there may be still earlier unrecognised steps in the process [3]. *Pf*GDV1 is distributed in sexually committed schizonts with a nuclear-associated punctate pattern, reminiscent of that of proteins known to be involved in regulating gene expression, suggesting a possible function in gametocyte induction pathways [32,33].

Similarly to *Pfgdv*1, *Pf*NEK-4, a NIMA-related kinase, is expressed in the sub-population of schizonts committed to gametocytogenesis. Following cell sorting, *Pf*NEK-4-positive parasites display high conversion rates to sexual forms, but the rate is not 100% and gene deletion studies showed that *Pfnek-4* was not required for gametocyte production [34]. Since not all asexual parasites expressing *Pf*NEK-4 appear committed to sexual development, its expression seems to mark an early reversible step in the sexual differentiation pathway. Although this particular protein kinase is not absolutely required, or it can be bypassed in the process leading to