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Malaria Parasite Pre-erythrocytic Infection: Preparation Meets Opportunity

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SUMMARY

For those stricken with malaria, the classic clinical symptoms are caused by the parasite's cyclic infection of red blood cells. However, this erythrocytic phase of the parasite's life cycle initiates from an asymptomatic pre-erythrocytic phase: the injection of sporozoites via the bite of a parasite-carrying Anopheline mosquito, and the ensuing infection of the liver. With the increased capabilities of studying liver stages in mice, much progress has been made elucidating the cellular and molecular basis of the parasite's progression through this bottleneck of its lifecycle. Here we review relevant findings on how sporozoites prepare for infection of the liver and factors crucial to liver stage development as well as key host/parasite interactions.

Introduction

Malaria is a major global health burden, causing 300–500 million clinical cases and 800,000 deaths annually. *Plasmodium* species, the etiologic agents of malaria, cycle between a mosquito vector and vertebrate host, and undergo several stages of replication and development. *Plasmodium* infection occurs when sporozoites are injected into the host's skin when bitten by an infected *Anopheles* mosquito. The sporozoites migrate to the liver where they infect a hepatocyte. One replicating parasite within a hepatocyte generates tens of thousands of erythrocyte-infectious merozoites to initiate blood-stage infection. This entire process takes 5–7 days for parasites infecting humans, and remarkably only 2 days for parasites infecting rodents. In this review, we discuss recent advances in understanding this most elusive of the life cycle stages of the parasite: the infection of the mammalian liver. Specifically, we review our current understanding of how *Plasmodium* infects and develops in the livers of mice and men and the interactions between the host and the pathogen during liver stage infection.

The Making of Highly Infectious Sporozoites in the Mosquito

Transmission of the infectious sporozoite to the mammalian host occurs through the bite of a female Anopheline mosquito after a 2–3 week complex developmental process within the mosquito. Our current understanding of this portion of the life cycle has been recently reviewed in detail (Aly *et al.*, 2009). Here we highlight two recently discovered essential aspects of the production of highly infectious sporozoites that adequately prepare the parasite to infect and develop within a host hepatocyte.

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An early observation relevant to the maturation of sporozoite infectivity was the finding that sporozoites are only highly infectious to mammals after entry into the mosquito salivary glands (Vanderberg, 1975). Screens using subtractive cDNA hybridization or comparative microarrays demonstrated that the increase in sporozoite infectivity coincided with changes in gene expression (Matuschewski *et al.*, 2002, Mikolajczak *et al.*, 2008). Many of the genes for which expression is up-regulated in infectious sporozoites (UIS genes) have proven to be critical for successful infection of the liver. Deletion of some UIS genes effectively attenuates sporozoites by severely impairing liver stage development, providing a novel means to pursue the design of live-attenuated parasite strains that are useful for vaccination (Vaughan *et al.*, 2010).

Recently, sporozoite infectivity was found to depend upon the protection and translational silencing of specific mRNAs, which include transcripts of some (but not all) UIS genes. This process likely involves packaging mRNAs into large complexes termed storage granules that form similarly as in other eukaryotes: through phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) by a stimulus-specific kinase. Within the sporozoite, this process is governed by the stage-specific expression of UIS1, also called the eIF2 α kinase (IK2) (Zhang *et al.*, 2010). Two recent studies have demonstrated that specific mRNAs are targeted to these granules by the Pumilio/FBF family Puf2 RNA-binding protein (also a UIS gene) (Gomes-Santos *et al.*, 2011, Muller *et al.*, 2011). These mRNA/protein complexes might be maintained by the SAPI/SLARP protein while in the sporozoite's salivary glands, and thus may help maintain the infectious state (Aly *et al.*, 2011, Silvie *et al.*, 2008). Deletion of Puf2 or UIS1/IK2 leads to the premature transformation of sporozoites within the salivary glands into a form that resembles a liver stage trophozoite both physically and transcriptionally, and consequently causes their subsequent reduction/loss of infectivity. In contrast, deletion of SAPI/SLARP brings about the degradation of specific mRNAs from both the 5' and 3' ends and an early liver stage developmental arrest, but without premature transformation. Lastly, while a kinase responsible for the formation of storage granules has been identified (UIS1/IK2), a specific phosphatase has yet to be implicated in the release of these complexes.

Together, these studies suggest that numerous proteins are critical to the parasite immediately after hepatocyte infection, but if prematurely translated before transmission they will have a detrimental effect upon its infectivity by ectopically initiating liver stage development. The parasite's strategy is to transcribe these genes in the sporozoite, but to protect and silence transcripts until some stimulus (discussed below) triggers their release and translation after hepatocyte infection. Additional work to understand the regulation of the infectious state in sporozoites may provide additional means to interfere with infection.

Sporozoite Infection of the Liver

When an infected mosquito takes a blood meal, up to hundreds of sporozoites are injected into the skin and use a form of locomotion (gliding motility) to reach the host vasculature and a form of host interaction (cell traversal) to enter it. Sporozoites passively travel to the liver sinusoids, which are unique blood vessels with a fenestrated endothelium and are lined with liver-resident macrophages called Kupffer cells. Sporozoites actively traverse the sinusoidal barrier to gain access to hepatocytes. This has elegantly been shown by the discovery of cell-traversal defective parasites lacking *spect 1,2*/Perforin-Like Protein (PLP) and *celTOS*, which were less infective *in vivo* when injected intravenously. Infectivity was restored after depletion of Kupffer cells, which produces temporary gaps in the sinusoidal cell layer (Reviewed in (Ejigiri *et al.*, 2009)). These experiments also suggest that Kupffer cells may be the gateway through which sporozoites cross the liver sinusoids. In support of this, *P. berghei* sporozoites have been visualized traversing Kupffer cells to access

hepatocytes (Frevet *et al.*, 2005). Furthermore, fewer *P. yoelii* sporozoites reached the liver of Kupffer cell-deficient transgenic op/op mice than in control mice (Baer *et al.*, 2007b). It has also been shown that Kupffer cells undergo apoptosis following exposure to sporozoites (Klotz *et al.*, 2008). Thus, an alternate theory is that traversal of and subsequent death of Kupffer cells may serve as a mechanism to resist phagocytosis, rather than as a specific gateway to the liver.

Once a sporozoite has traversed the sinusoidal barrier, it traverses several hepatocytes before invading one that serves as a host cell (Mota *et al.*, 2001). There is much debate as to what induces the switch from migration to invasion. One hypothesis suggests that it is the act of traversal that renders the sporozoite invasion-competent. Indeed, it was reported that sporozoites that have traversed cells are more infectious than non-traversing parasites, likely due to induction of exocytosis of sporozoite apical organelles necessary for invasion (Mota *et al.*, 2002). On the other hand, the above mentioned cell traversal mutants infect host cell at wild-type levels, thus contradicting the finding that traversal is activating sporozoites for infection (reviewed in (Ejigiri *et al.*, 2009)). During traversal, sporozoites are exposed to increased levels of potassium inside the traversed cells. Prolonged exposure to potassium, such as would be experienced by the sporozoite after traversal of multiple cells, causes sporozoites to become less migratory and more infective (Kumar *et al.*, 2007). Further studies have shown that wounded/traversed hepatocytes secrete hepatocyte growth factor (HGF), which conditions neighboring cells to be more susceptible to infection via the binding of its receptor, Met (Carrolo *et al.*, 2003), suggesting that it is a change in the host hepatocyte rather than the sporozoite that enhances infection. However, the *P. berghei* rodent model used for these studies does not accurately predict what occurs in *P. falciparum*, as a recent study showed that hepatocyte traversal by *P. falciparum* sporozoites does not activate Met (Kaushansky *et al.*, 2011). Furthermore, traversal-deficient parasites can invade hepatocytes normally *in vitro*, implying that traversal is not a prerequisite for sporozoite invasion (Ejigiri *et al.*, 2009). Another hypothesis as to how the sporozoite switches to an infectious mode is that an environment-specific signal primes sporozoites for invasion. In an elegant set of studies, Coppi *et al.* show that traversal is the default behavior of sporozoites and that it is only upon interaction with highly sulfated-heparan sulfate proteoglycans (hsHSPGs) present on hepatocytes that the sporozoite switches to an invasive mode. Exposure to hsHSPGs induces the proteolytic processing of the surface circumsporozoite protein (CSP) by a cysteine protease (Coppi *et al.*, 2007), which is distinct from the processing that results in the shedding of CSP during gliding motility. While in traversal mode, a cell-adhesive domain of CSP (type I thrombospondin repeat, TSR) that is necessary for cell invasion (Plassmeyer *et al.*, 2009) is masked by the N-terminus of CSP. Upon interaction with hsHSPGs, cysteine protease(s) cleave the N-terminus from CSP and expose the TSR, thus allowing the sporozoite to shift to an invasive state (Coppi *et al.*, 2011). Why then do sporozoites traverse hepatocytes? The data suggest that sporozoites traverse hepatocytes until sufficient CSP is cleaved to allow invasion of the host hepatocyte.

Only in a properly invaded hepatocyte will the parasite develop into a liver stage. The invasion process relies on the parasite's membrane-associated actin-myosin motor and requires the formation of a moving junction to form an interface between the parasite and the hepatocyte. The proteins necessary for host cell invasion are stored and released from specialized apical organelles called micronemes. One such protein, thrombospondin-related anonymous protein (TRAP) is necessary for invasion. TRAP forms a connection between the host and the parasite by using its cytoplasmic tail to indirectly bind parasite actin via aldolase, while simultaneously binding hepatocytes with its extracellular portion. The importance of TRAP has been shown using both gene deletion and mutagenesis of functional domains (Morahan *et al.*, 2009). During invasion, TRAP is secreted from micronemes at the sporozoite's anterior end and is translocated to the posterior end, thus

providing the propulsion used by the sporozoite during invasion. Additionally, some proteins stored in the rhoptries, a second type of secretory organelle situated at the anterior end of invasive sporozoites and merozoites, also have demonstrated roles in the invasion of red blood cells by merozoites (Gaur *et al.*, 2011). These proteins are also likely necessary for sporozoite invasion of hepatocytes, however this has not yet been shown experimentally.

In addition to parasite proteins, several host factors are important for invasion of hepatocytes. Parasites induce the formation of a ring-shaped F-actin structure in the host cell at the moving junction, which requires the actin nucleating complex Arp2/3 for sporozoite invasion (Gonzalez *et al.*, 2009). These data imply that successful invasion requires not only the parasite motor but also *de novo* polymerization of host actin at the entry site, which is likely involved in anchoring the tight junction so that the parasite can pull upon it to penetrate the host cell. The tetraspanin cluster of differentiation 81 (CD81) is also critical for sporozoite invasion. CD81 is present in cholesterol-rich lipid rafts on the surface of hepatocytes and HepG2 cells expressing CD81 are more permissive to invasion by *P. yoelii* (Silvie *et al.*, 2006a). How CD81 facilitates invasion is not yet known, as CD81 is not required for invasion by *P. berghei* (Silvie *et al.*, 2007) and its presence is not sufficient for invasion by *P. falciparum* (Silvie *et al.*, 2006b). However, its function may be linked to that of another hepatocyte surface protein: scavenger receptor BI (SR-BI). SR-BI is a high-density lipoprotein (HDL) receptor that, along with CD81, is present in cholesterol-rich micro domains on the hepatocyte surface and is necessary for efficient sporozoite invasion as well as intracellular development (Yalaoui *et al.*, 2008, Rodrigues *et al.*, 2008). SR-BI normally functions to facilitate the uptake of HDL cholesteryl-esters, and thus may indirectly promote sporozoite invasion by providing the cholesterol necessary for appropriate CD81 cell-surface localization.

The Parasitophorous Vacuole: A Critical Host-Parasite Interface

During invasion of hepatocytes, the parasite forms a parasitophorous vacuole (PV), within which it resides throughout liver stage development. Hence, the membrane of this vacuole constitutes the principal interface between the parasite and its host. The PV membrane (PVM) is derived from the host plasma membrane, but is significantly remodeled with proteins, and possibly lipids, of parasite origin (Bano *et al.*, 2007) but little is known about this process in hepatocyte infection. Factors necessary for the induction of the liver stage PV are relatively unknown, however the parasite proteins P36 and P52 (P36p) appear to be involved in this process, as a *P. yoelii* *p52/p36* double gene knockout is unable to form a PVM and arrests early in liver stage development (Labaied *et al.*, 2007). Single gene deletion parasites of *p52* in *P. berghei* and *P. falciparum* are similarly arrested early in liver stage development and are also unable to form a PVM (van Schaijk *et al.*, 2008, van Dijk *et al.*, 2005). Recently, a secreted protease from *P. yoelii*, ROM1, was found to be involved in liver stage PV formation, as 50% of *rom1* knockout parasites developed abnormal vacuoles and aborted growth within the first 12 hours of infection (Vera *et al.*, 2011). Several liver stage PVM-resident proteins have been identified, including UIS3, UIS4, Hep17 (EXP1), and two members of the early transcribed membrane protein (ETRAMP) family (Mackellar *et al.*, 2011, Mueller *et al.*, 2005, Doolan *et al.*, 1996). The importance of PVM resident proteins for parasite growth and survival is highlighted by the observation that deletion mutants of UIS3 or UIS4 cannot develop beyond early liver stages *in vivo*. This arrest may be due to the fact that the carboxyl-termini of UIS3 and UIS4 reside in direct contact with host hepatocyte and are likely involved in host-parasite interactions. In support of this, UIS3 was shown to bind host liver fatty acid binding protein, indicating an involvement in parasite uptake of fatty acids (Mikolajczak *et al.*, 2007). It is likely that other PVM proteins are also involved in host-pathogen interactions, such as nutrient uptake or other forms of host cell

manipulation. Further study of PVM structure and composition will provide essential insight into how the parasite manipulates and survives in the hepatocyte.

Liver Stage Trophozoite and Schizont Development

Upon successful invasion of the hepatocyte with the formation of a PVM, the sporozoite dedifferentiates from an invasive form to an active replicative form: the liver stage trophozoite. Trophozoite formation begins with the breakdown of the sporozoite's inner membrane complex (IMC), a cytoskeletal structure located beneath the plasma membrane, which harbors the invasion motor. This leads to the formation of a central spherical bulb around the nucleus with the simultaneous retraction of the sporozoite's two distal ends (Jayabalasingham *et al.*, 2010). At the same time, the parasite expels the majority of its contents by an active exocytic process, including the remnants of its invasion machinery. Only those organelles that are necessary for parasite replication within the hepatocyte are retained (e.g. apicoplast, mitochondria, endoplasmic reticulum (ER)). Dedifferentiation to a trophozoite can occur outside of the hepatocyte (Kaiser *et al.*, 2003) and recent work comparing two defined medias with differential effects on this process demonstrated that extracellular bicarbonate concentrations and an elevation in temperature that mimics host conditions are the major triggers of this process (Hegge *et al.*, 2010). These findings were extended by the use of a FRET-based sensor to demonstrate that intracellular calcium levels increase as the parasite dedifferentiates, with peak calcium levels occurring in the center of the spherical bulb (Doi *et al.*, 2011). Additionally, the translational repression of specific mRNAs packaged into storage granules (discussed above) also plays a major role in sporozoite transformation. Once dedifferentiated, the trophozoite enters schizogony, undergoing rapid growth and numerous rounds of DNA replication. It also replicates its organelles (e.g. apicoplast, mitochondria, ER) and forms a multinucleate syncytium that grows within the infected hepatocyte to a size exceeding that of an uninfected hepatocyte. The division and segregation of liver stage organelles was recently described in a visually stunning paper by Stanway and colleagues by expressing organelle-targeted fluorescent proteins to show that during nuclear division, the apicoplast and mitochondrion become two extensively branched and intertwining structures. The organelles then undergo morphological and positional changes prior to cell division, and finally segregate into individual merozoites (Stanway *et al.*, 2011). This cellular and genomic expansion happens at a remarkable speed: 2 days for rodent-infective species (*P. yoelii* and *P. berghei*). Unfortunately, almost nothing is known about how the parasite is able to orchestrate this rapid development. We know that parasites derive nutrients from the host hepatocyte both by passive diffusion through pores in the PVM, and by active processes such as those that take up glucose, via hexose transporters (Blume *et al.*, 2011, Slavic *et al.*, 2011), fatty acids, whose uptake might be mediated by UIS3 (Mikolajczak *et al.*, 2007), and cholesterol, which is diverted continuously from the host hepatocyte (Labaied *et al.*, 2011). Microarrays of *P. berghei* infected hepatoma cells and *P. yoelii*-infected hepatocytes that were isolated from mice revealed that the parasite modulates the host cell's transcriptome towards biosynthetic pathways including carbohydrate and fatty acid metabolism (Albuquerque *et al.*, 2009). Furthermore, Portugal and colleagues recently demonstrated that iron availability is also necessary for liver stage development. Blood stage parasite density, when present above a certain threshold parasitemia, can inhibit the next wave of sporozoite invasion and development through modulation of the host iron regulatory hormone hepcidin to redistribute iron away from the liver (Portugal *et al.*, 2011). While uptake of iron, glucose and fatty acids are critical for liver stage development, it remains unknown what other nutrients are also acquired from the host.

Exo-erythrocytic Merozoite Formation and Release

Formation of the individual liver stage merozoites begins with repeated invaginations of the parasite plasma membrane, eventually packaging individual daughter merozoites with a single nucleus and necessary organelles. This process requires an enormous expansion of the parasite plasma membrane, a feat that is in part reliant upon the endogenous generation of fatty acids via the type II fatty acid synthesis (FAS-II) pathway. The components of the bacterial-type FAS-II pathway are trafficked to the apicoplast, and FAS-II genes are upregulated in liver stages (Tarun *et al.*, 2008). Interestingly, deletion of FAS-II components, such as FabB/F or FabI in rodent malaria parasites, resulted in the arrest of liver stage development prior to merozoite formation (Vaughan *et al.*, 2009, Yu *et al.*, 2008). Similar results were seen in parasites deficient in components of the pyruvate dehydrogenase (PDH) complex (Pei *et al.*, 2010), which provides metabolic precursors to the FAS-II pathway. This indicates that fatty acid synthesis is essential for late liver stage development. Recently, another apicoplast-targeted protein, PALM, was implicated in efficient merozoite formation and segregation (Haussig *et al.*, 2011) because gene deletion resulted in a significant defect in late liver stage development. Also in this study, the application of antibiotics that inhibit the bacterial-like protein translation processes of the apicoplast (e.g. azithromycin) inhibited the development of this organelle, which in turn disrupted the maturation of the parasite. As mentioned earlier, the late liver stage schizont exceeds the size of the host hepatocyte, yet it remains ensconced within the confines of the hepatocyte plasma membrane. How the parasite contributes to and regulates enlargement of the host cell remains unknown.

During or just following merozoite formation, the PVM becomes damaged and is rapidly degraded, releasing merozoites into the host cell cytoplasm (Sturm *et al.*, 2006). Destruction of the PVM is dependent upon cysteine proteases, as this process can be completely blocked with the cysteine protease inhibitor E64 (Sturm *et al.*, 2006). Likely candidates for the cysteine proteases involved in these processes are the serine repeat antigen (SERA) proteases, which are present in all *Plasmodium* species and are necessary for merozoite egress from erythrocytes, and sporozoite egress from oocysts in the mosquito midgut (Blackman, 2008). In *P. berghei*, SERA-1 (Putianti *et al.*, 2010) and SERA-3 (Schmidt-Christensen *et al.*, 2008) leak into the hepatocyte cytoplasm during merozoite development, likely caused by breakdown of the PVM. Protease inhibitors may also be important for coordinating the proteases involved in PVM breakdown. A *P. berghei* cysteine protease inhibitor is released into the host cell cytoplasm during merozoite development in a similar manner to *P. berghei* SERA-1 and -3 (Rennenberg *et al.*, 2010). It is unknown if these proteases and protease inhibitors are involved in the breakdown of the PVM or if they play a role during merozoite release from the hepatocyte.

Following the breakdown of the PVM, merozoites are packaged into vesicles termed merosomes that are extruded from the infected hepatocyte into the liver sinusoid (Sturm *et al.*, 2006, Tarun *et al.*, 2006). It is estimated that anywhere from a few to several thousand merozoites are packaged per merosome (Sturm *et al.*, 2006). The parasite induces a non-apoptotic/non-necrotic cell death program in the hepatocyte causing it to detach from surrounding tissue, while also delaying the fragmentation of the host genomic DNA (*ibid*). Additionally, the parasite inhibits phosphatidylserine display on the outer leaflet of the plasma membrane of these dying hepatocytes (Sturm *et al.*, 2006), and on the surface of merosomes, whose membrane is comprised of the host hepatocyte plasma membrane (Graewe *et al.*, 2011). This allows daughter merozoites to evade detection and engulfment by immune cells present in the liver. Merosomes then travel passively in the blood stream through the heart and into the narrow vasculature of the lung (Baer *et al.*, 2007a), where they burst and release merozoites into the blood stream to initiate blood stage infection.

How does the host respond to infection, and how does the parasite counter these responses?

In order to be successful, *Plasmodium* parasites must counter innate responses from both immune cells and the host hepatocyte. Following the infectious mosquito bite, the host mounts an appreciable immune response to the large number of sporozoites that never exit the dermis (Chakravarty *et al.*, 2007). For those sporozoites that travel to the liver, Kupffer cells (resident macrophages in of the liver) are able to engulf and digest them. However, sporozoites resist clearance by these macrophages. Interestingly, *Plasmodium* utilizes the ubiquitous surface protein CSP to subvert respiratory bursts generated by Kupffer cells, which is mediated by parasite-induced production of the signaling molecule cyclic AMP (cAMP). cAMP signaling also shifts cytokine expression of Kupffer cells towards an anti-inflammatory profile. Furthermore, Kupffer cells undergo apoptosis upon co-incubation with *P. yoelii* sporozoites *in vitro* (Klotz *et al.*, 2008). While these observations have not been confirmed *in vivo*, they suggest that parasites manipulate macrophage function in several complementary ways to avoid clearance. *Plasmodium* can also modulate the host immune response by inducing expression of the anti-inflammatory factor heme oxygenase-1 (HO-1), which is involved in the catabolism of free heme, and has potent antioxidant and anti-inflammatory functions. HO-1 is up-regulated in the livers of sporozoite-infected mice and is necessary for liver stage development, as HO-1 deficient mice (*Hmox1*^{-/-}) do not develop a blood stage infection. This pro-survival effect on liver stages is due to the dampening of the innate inflammatory response of the host, as HO-1 over-expressing mice contained fewer and smaller inflammatory foci, and less pro-inflammatory cytokine production following infection compared to *Hmox1*^{-/-} mice (Epiphanio *et al.*, 2008). How *Plasmodium* induces the expression of HO-1 has yet to be determined.

One host cell response *Plasmodium* must be able to control is hepatocyte apoptosis, which would result in collateral death of the liver stage parasite due to the death of its host cell. Several groups have shown that *Plasmodium* can inhibit apoptosis (Luder *et al.*, 2009), but how the parasite achieves this is still largely unknown. One mechanism might be through HGF/Met signaling via the host hepatocyte. *P. berghei* sporozoite-traversed hepatocytes release HGF, which binds to the MET receptor on the host cell and induces pro-survival signaling via a PI3-kinase/Akt pathway. RNA silencing of MET in host cells or inhibition of the PI3-kinase signaling pathway by chemical inhibitors early during infection resulted in increased host cell death (Leiriao *et al.*, 2005). However, PI3-kinase signaling is not necessary for cell survival later during liver stage development (van de Sand *et al.*, 2005). As discussed earlier, liver stage parasites manipulate host cell death, first by preventing it, and then by inducing a unique form of cell death during merozoite release (Sturm *et al.*, 2006). How the parasite accomplishes these feats is unknown. One possibility is that the parasite utilizes protease inhibitors to inhibit host cathepsins and caspases, and induces apoptosis via its own cysteine proteases. One candidate for an inhibitor of pro-apoptotic host proteases is the cysteine protease inhibitor falstatin (termed inhibitor of cysteine proteases, ICP, in *P. berghei*) (Pandey *et al.*, 2006). Falstatin is located in the host hepatocyte cytoplasm during merozoite release when the host cell would attempt programmed cell death, and may prevent the activation of caspases or cathepsins that would normally induce apoptosis. This scenario is supported by the observation that mammalian cells transfected with *P. berghei* falstatin can resist apoptosis (Rennenberg *et al.*, 2010). However, we found that *P. yoelii* falstatin is contained within the PV in early liver stages, suggesting that other factors are involved in the inhibition of cell death prior to PVM permeabilization (Pei, Miller and Kappe, unpublished data). Thus, although it is well established that *Plasmodium* can manipulate host cell death for its own ends, the molecular mechanisms of the feat are only slowly emerging.

Conclusions

We have discussed recent advances in understanding how the malaria parasite invades and develops within hepatocytes, and the means by which it interacts with and defeats the host's responses. However, many important questions remain. For instance, what comprises the complete invasion machinery of the sporozoite? Experimental evidence exists for the involvement of CSP, TRAP, P52, P36, and ROM1 in this process, but what are the host cell ligands and what other parasite proteins are involved? Do these proteins act individually, or in complexes? Also, it is important to determine what factors regulate storage granules in the infectious sporozoite, as understanding this process may provide an understanding to how the parasite spatially and temporally regulates infectivity and differentiation. Translational repression is also used by *Plasmodium* in the transition from the vertebrate host to the mosquito. Could this mechanism be similarly employed to transition between a dormant hypnozoite, a key feature of the *Plasmodium vivax* life cycle, and an active (trophozoite/schizont) infection of the hepatocyte?

We also have an incomplete understanding of how the parasite utilizes and exploits the host hepatocyte. For instance, it remains to be shown what the range of nutrients the parasite can provide for itself is versus what it must scavenge from the host cell. Additionally, little is known about how the parasite interacts with and obtains such factors from the host cell. At least one PVM resident protein, UIS3, interacts with the host. Other than CSP, no parasite factors have been found to be secreted into the hepatocyte prior to PVM breakdown. Secretion of CSP into the hepatocyte may be dependent on a PEXEL amino acid sequence motif (Singh *et al.*, 2007), however contradictory evidence has recently been reported (Cockburn *et al.*, 2011). Thus, in absence of such secreted parasite factors, it is tempting to suggest that the majority of parasite-hepatocyte interactions happen at the PVM. However, the lack of experimental evidence makes it difficult to be certain at this time. In addition to nutrient uptake, *Plasmodium* must also manipulate its environment in order to subvert host clearance mechanisms (e.g. inhibition of host cell apoptosis). How the parasite does this, and what molecules it utilizes, is still largely unknown.

Lastly, much of our understanding of pre-erythrocytic stages is derived from experiments with rodent-infective species; however few of these findings have been corroborated in *P. falciparum* or *P. vivax* due to experimental challenges in directly observing their liver stages. Recent developments in humanized mouse models that support *P. falciparum* infection, and improved *in vitro* culture systems for hypnozoites, should enable a direct analysis of human parasite liver stages (Chattopadhyay *et al.*, 2010, Demele *et al.*, 2011, Sacci *et al.*, 2006). The continuing investigation and improvement of all these models is necessary to further unravel fundamental features of malaria parasite pre-erythrocytic stage infection.

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Figure 1.

Key events of the liver stage developmental program. (Left-to-Right) A highly infectious sporozoite travels to the liver within minutes, where it traverses through the endothelium, wounds several hepatocytes without invading them, and finally invades one hepatocyte. During invasion, the parasite forms a parasitophorous vacuole (gray) around itself, actively expels/disassembles invasion-related organelles, and dedifferentiates into a trophozoite. Infection by *P. vivax* also can produce a dormant “hypnozoite” form that can reactivate months or years later. Liver stage parasites consume the contents of the hepatocyte and extensively replicate their genome and organelles, which are segregated during schizogony into each of tens of thousands of merozoites. The parasite grows to a size approximately 10 times that of an uninfected hepatocyte. Late in schizogony, the PV membrane breaks down and merozoites are enclosed in vesicles called merozoites, which form from the hepatocyte’s plasma membrane. Merozoites are released into the liver sinusoid, enter the circulation and eventually burst in the lung microvasculature to release the merozoites. The approximate time required for each developmental stage in rodent and human malaria is noted.