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Molecular Regulation of *Plasmodium* Sporozoites Exocytosis and Infection

Laura Cristina Cabrita dos Santos

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Prof. Ana Rodriguez, PhD
New York University School of Medicine
Prof. Maria Manuel Mota, PhD
Faculdade de Mediicina da Universidade de Lisboa

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À minha avó

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Preface

This dissertation assembles data obtained during my PhD research project, developed at the New York University School of Medicine, Department of Medical Parasitology, under the supervision of Doctor Ana Rodriguez, from August 2003 to June 2007.

This thesis is structured in 5 chapters, preceded by a summary, both in Portuguese and English, outlining the aims, results and outcomes of this project.

The first chapter provides an insight on malaria liver stage research field and the aims of this work.

The two following chapters (second and third) contain the original data regarding this project.

The fourth chapter encloses an overall discussion and conclusion of the studies performed.

Chapter five contains the description of the methods and material employed to carry out the present work.

In Appendix 1 and 2 are included the publications that derived from this project.

The data presented in this dissertation is the result of my own work. This work has not been previously submitted for any degree at this or any other University.

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SUMÁRIO

A malária é a doença parasitária mais relevante a nível mundial, uma vez que é responsável por mais de 1 milhão de mortes anualmente. O agente causador da doença é o parasita protozoário do género *Plasmodium*. Aquando da picada de um mosquito fêmea *Anopheles*, o vector de transmissão da doença, o parasita é introduzido na pele do hospedeiro sob a forma de esporozoíto. Os esporozoítos são células móveis que migram na pele até alcançarem a circulação sanguínea. Uma vez em circulação, os esporozoítos atingem o fígado do hospedeiro onde atravessam a barreira sinusoidal e invadem os hepatócitos. Aqui multiplicam-se e desenvolvem-se até atingirem o seu próximo estado de maturação, o merozoíto.

O desenvolvimento dos esporozoítos nas formas exo-eritrocíticas ocorre exclusivamente em hepatócitos, o que torna o percurso desde o local da inoculação na derme até ao fígado um passo imprescindível na conclusão do ciclo de vida do parasita. Durante este trajecto os esporozoítos necessitam de atravessar várias barreiras celulares. Estudos prévios mostram que os esporozoítos interagem com as células do hospedeiro de duas formas: podem invadir a célula formando um vacúolo parasitóforo no qual o parasita se irá replicar , ou podem atravessar a célula, rompendo a membrana plasmática no processo de migração (Mota et al. 2001). Neste último caso, os parasitas entram em contacto directo com o citoplasma dos hepatócitos. Este processo de migração conduz à exocitose regulada de organelos secretórios no parasita, o que resulta na exposição de proteínas, tal como a TRAP (thrombospondin-related anonymous protein), no pólo apical do esporozoíto (Mota et al. 2002). Devido às suas propriedades de adesão, a TRAP

é considerada um intermediário essencial na invasão do fígado, uma vez que sua exposição no pólo apical dos esporozoítos é necessária durante o processo de invasão dos hepatócitos (Mota et al. 2002).

De modo a aprofundar o conhecimento dos diferentes processos moleculares envolvidos na interacção dos esporozoítos com os hepatócitos, propusemo-nos a fazer as seguintes caracterizações:

1) Como é activada a exocitose nos esporozoítos durante a migração através dos hepatócitos?

2) Este processo de migração/activação é regulado? Como?

3) Quais as vias de sinalização no esporozoíto que intervêm na activação da exocitose?

Usando um modelo murino, observámos que a exocitose apical nos esporozoítos é induzida por nucleótidos derivados de uracilo. Estes nucleótidos, que se encontram em concentrações elevadas no citoplasma de qualquer célula, contactam e consequentemente activam os esporozoítos durante o processo de migração. Contudo , os esporozoítos parecem possuir um mecanismo regulador que previne a sua activação prematura, uma vez que a exocitose apical só ocorre quando estes atingem os hepatócitos, as células alvo para a infecção. É conhecido que a albumina, uma proteína presente em elevadas concentrações no sangue e nos tecidos, aumenta a motilidade dos esporozoítos (Vanderberg 1974). Observámos que, na presença de albumina, os esporozoítos não são activados pelos nucleótidos derivados de uracilo, mantendo-se os níveis basais de exocitose apical. Desta forma, concluímos que a albumina previne a ocorrência de exocitose induzida pelos nucleótidos derivados de uracilo. Assim, quando os esporozoítos são depositados na pele, a presença da

albumina promove um aumento na sua mobilidade ao mesmo tempo que inibe a sua activação prematura. No entanto, na presença de hepatócitos, o efeito inibitório da albumina desaparece, permitindo desta forma a activação dos esporozoítos e subsequente infecção dos hepatócitos. Concluímos que a passagem dos esporozoítos através de células que não os hepatócitos, não contribui para a activação de exocitose nos esporozoítos nem aumenta a sua infectividade.

As cadeias de glicosaminoglicanos dos proteoglicanos de sulfato de heparina (HSPG), presentes na superfície dos hepatócitos, são consideradas os receptores dos esporozoítos na ligação aos sinusóides hepáticos (Sinnis et al. 1996). A proteína CS (circumsporozoite protein), que se encontra na superfície dos esporozoítos, liga-se com elevada eficiência aos proteoglicanos de sulfato de heparina das células hepáticas antes dos parasitas atravessarem para o parenquima (Pradel et al. 2002). Os nossos resultados demonstram que o grau de sulfatação das cadeias de HSPGs está directamente relacionado com a capacidade do parasita em superar o efeito inibitório da albumina na exocitose dos esporozoítos induzida por nucleótidos derivados de uracilo. Deste modo, na migração através dos hepatócitos, os esporozoítos podem ser activados por nucleótidos derivados de uracilo presentes no citoplasma destas células, e iniciar a exocitose apical necessária para a invasão e infecção.

O parasita utiliza processos de sinalização de forma a alterar o seu comportamento dependendo do ambiente em que se encontra,. Por exemplo, Os esporozoítos têm de mudar de um estado inicial em que migram por diferentes tipos de células na pele e nos sinusóides hepáticos, para outro em que invadem hepatócitos e formam um vacúolo necessário para a infecção. De facto, observámos que uma elevação nos

níveis de cálcio (Ca^{2+}) e de AMP cíclico (cAMP) nos esporozoítos induz exocitose apical, passando os esporozoítos de um estado migratório para um estado infeccioso.

Com os resultados apresentados nesta tese esperamos contribuir para a compreensão das interacções que se estabelecem entre os esporozoítos de *Plasmodium* e o seu hospedeiro no decurso de uma infecção.

Palavras-chave: malária, esporozoítos, *Plasmodium*, exocitose regulada, infecção, hepatócitos, sinalização.

Abstract

Malaria remains one of the most prevalent and severe human infectious diseases in the world and is responsible for more than a million infant deaths per year. The causative agent of malaria is the protozoan parasite *Plasmodium*. It is transmitted by the bite of infected mosquitoes that deposit the sporozoite form of the parasite in the skin of the mammalian host. Sporozoites are motile and travel from the skin into the circulation, from where they reach the host's liver. Liver infection is the first obligatory step and is clinically silent.

Plasmodium sporozoites are able to invade all sorts of cells but they only develop inside hepatocytes. Sporozoites can enter cells by two distinct routes, either through a tight moving junction with the target cell that leads to the formation of a parasitophorous vacuole, where development proceeds, or by disrupting their plasma membrane (Mota et al. 2001). In the latter case, the parasite glides in the cytoplasm, and exits the cell again rupturing the plasma membrane. Migration through cells triggers the secretion of micronemes in the sporozoite. This process is called apical regulated exocytosis and is triggered when sporozoites are in contact with host cells (Mota et al. 2002). Thrombospondin-related anonymous protein (TRAP) is an essential mediator of hepatocyte invasion due to its adhesive properties, and it is believed that exposure of this protein in the apical end of the sporozoites is required for invasion of the host cell (Mota et al. 2002).

In an effort to broaden our knowledge of the molecular processes involved in the malaria sporozoite - host hepatocyte interactions, we addressed the following questions:

1) How does migration through cells induce exocytosis in *Plasmodium* sporozoites?

2) Is this process regulated and how?

3) What are the signaling pathways that mediate the activation of exocytosis in sporozoites?

We determined that uracil and its derived nucleotides, which are found in the cytosol of traversed cells, induce apical regulated exocytosis in *P. yoelii* and *P. falciparum* sporozoites. However, sporozoites seem to have a regulatory mechanism preventing a premature activation, since exocytosis only occurs when sporozoites reach the liver. Albumin is a protein present at high concentrations in circulation and in the tissue and it has been described to increase the motility of *Plasmodium* sporozoites (Vanderberg 1974). We determined that exocytosis is specifically inhibited by albumin, since in its presence sporozoites no longer respond to uracil and its derived nucleotides. However, the inhibitory effect is no longer active once sporozoites contact hepatocytes, allowing activation of sporozoites for infection. We conclude that sporozoite migration through cells other than hepatocytes does not activate exocytosis or increase their infectivity.

Glycosaminoglycan chains of heparan sulfate proteoglycans (HSPGs), on the surface of hepatocytes, are considered the main receptors for *Plasmodium* attachment in the liver sinusoids (Sinnis et al. 1996). Circumsporozoite (CS) protein in the surface of sporozoites binds efficiently to liver HSPGs before parasites traverse into the parenchyma (Pradel et al. 2002). We found that the level of sulfation at the HSPGs chains is directly related to its capacity to

overcome albumin inhibition of exocytosis by uracil nucleotides.

In order to change the behavior according to the surrounding environment, sporozoites use signaling processes. We have analyzed the role of the cAMP signaling pathway in sporozoite apical exocytosis and infection and showed that apical regulated exocytosis is induced by increases in cAMP in sporozoites of rodent (*P.yoelii* and *P.berghei*) and human (*P. falciparum*), which activates sporozoites for host cell invasion.

In summary, data presented in this thesis contributes to a wider understanding of the interactions established between the *Plasmodium* sporozoites and its host in the course of a malaria liver infection.

Keywords: malaria, *Plasmodium* sporozoites, regulated exocytosis, hepatocyte infection, signaling.

ABBREVIATIONS

AC	Adenylyl cyclase
ADP, ATP	Adenosine 5′ – Di/TriPhosphate
Alb	Albumin
cAMP	Cyclic adenosine monophosphate
CelTOS	Cell Traversal protein for Ookinete and Sporozoite
CHO	Chinese Hamster Ovary cell line
CS	Circumsporozoite protein
CTRP	Circumsporozoite protein and TRAP related protein
EEF	ExoErythrocytic Form
Hepa1-6	mouse hepatoma cell line
HepG2	human hepatoma cell line
HGF	Hepatocyte Growth Factor
HSPG	Heparan Sulfate Proteoglycan
IMC	Inner Membrane Complex
MDF	Mouse dermal fibroblasts
MTRAP	Merozoite specific TRAP analogue
MTs	Microtubules
PbPL	*Plasmodium berghei* Phospholipase
PKA	cAMP-dependent Protein Kinase
PV	Parasitophorous Vacuole
SPECT	Sporozoite Protein Essential for Cell Traversal
TRAP	Thrombospondin- Related Anonymous Protein
UD	Uracil nucleotides and its derivatives
UMP,UDP, UTP	Uridine 5′- mono/di/triphosphate
wt	Wild type

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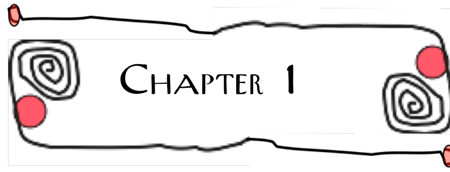


General Introduction

1.1. General overview ~

Malaria infection is caused by an intracellular protozoan parasite of the genus *Plasmodium* and is transmitted by an *Anopheles* mosquito vector.

The earliest medical writers in China, Assyria, and India described malaria-like intermittent fevers, which they attributed to evil spirits. In medieval times it was believed that vapors and mists arising from swamps and marshes caused the disease. The names malaria (mal, bad; aria, air) and paludism (palus, marsh) reflect these beliefs.

All concepts of malaria changed within 20 years after Laveran's 1880 description of the unicellular parasite *Plasmodium falciparum* in the fresh blood of an infected soldier. Golgi described asexual development in 1886, and MacCallum observed the sexual cycle of the parasite in 1897. In 1898 Ross, Grassi and colleagues showed that the parasite developed in the mosquito and was transmitted to the human within the small amount of salivary fluid secreted by that insect (Wahlgren 1999).

The full understanding of malaria parasite's cycle was achieved only in 1948 when Shortt and Garnham described the exoerythrocytic liver stage, after observing malaria parasites developing in livers of sporozoite-infected monkeys and in livers of human volunteers bitten by mosquitos infected with *Plasmodium* vivax (Shortt HE 1948).

Malaria is a widespread infectious human disease, with an estimated annual worldwide toll of 350-500 million acute episodes, resulting in more than a million deaths. Most of these are caused by *P. falciparum* infections, which are the leading cause of Africa's mortality in the under-five (20%) and represents 10% of the continent's overall disease burden.

It also accounts for 40% of public health expenditure, 30-50% of inpatient admissions, and up to 50% of outpatient visits in areas with high malaria transmission. The vast majority of malaria deaths occur south of the Sahara, where it presents major obstacles to social and economic development. For instance, malaria has been estimated to cost Africa more than US$ 12 billion every year in lost Gross Domestic Product - GDP, even though it could be controlled for a fraction of that sum (W.H.O., 2005).

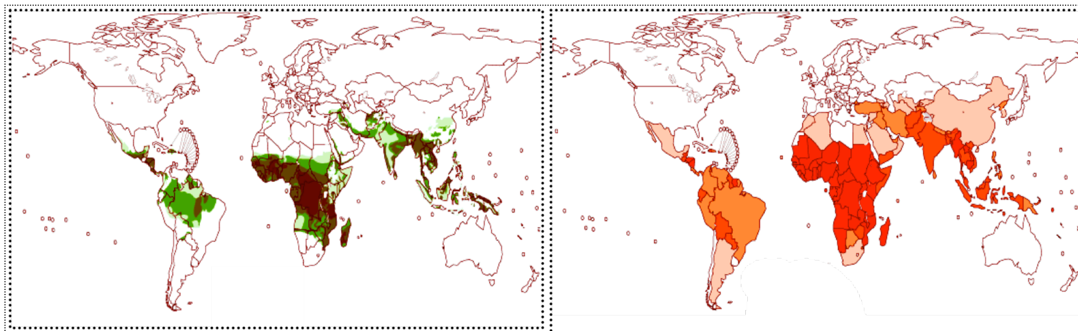


Figure 1.1| Global distribution of malaria (A) World's malaria transmission risk in 2003 and (B) the estimated incidence of clinical malaria episodes caused by any *Plasmodium* species, resulting from local transmission, country level averages in 2004. (Adapted from Roll Back Malaria partnership report, 2005).

The fight against malaria is currently being pursuit, among others, by The Roll Back Malaria, an international partnership launched by the World Health Organization (W.H.O). Its goal is to halve malaria-associated mortality by 2010 and again by 2015. Four action steps are being taken in four different areas: prevention is to be achieved by the use of protection against mosquito bites; prompt treatment by using effective anti malarial medicines; protection of pregnant women and their unborn children and, in areas of high risk, preventive medication; and pre-empting epidemics by predicting outbreaks and acting swiftly (W.H.O. 2005).

1.2. Plasmodium and its life cycle~

Malaria parasites (*Plasmodium* spp.) are part of the phylum Apicomplexa that includes other important intracellular pathogens such as *Toxoplasma*, *Cryptosporidium*, *Eimeria*, *Babesia* and *Theileria*. Different *Plasmodium* species can infect different vertebrates, including human, other primates, rodents, birds, and reptiles.

The malaria parasite life cycle is complex and involves both a vertebrate and an invertebrate host, the *Anopheles* mosquito vector. The interaction between them results in transmission, which in turn allows the infection to endure. Of the approximately 400 species of *Anopheles* throughout the world, about 60 are malaria vectors under natural conditions, 30 of which are of major importance (W.H.O. 2005).

While probing to find blood, a malaria-infected female *Anopheles* mosquito injects salivary fluids into the skin and inoculates sporozoites into the human host. Sporozoites migrate through the skin and enter into the circulation, a step that can take up as long as a few hours (Yamauchi et al. 2007), circulate for a short time in the blood stream, and then infect liver cells where they undergo asexual division followed by maturation into schizonts. Some parasites, such as *Plasmodium vivax* and *P. ovale,* have a dormant stage instead, the hypnozoite (Krotoski et al. 1982; Wahlgren 1999), that can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.

Although primary infection occurs in the liver, no pathology is associated with the hepatic stage of malaria.

After this initial replication in the liver (exo-erythrocytic schizogony), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony). The asexual cycle is synchronous and periodic (parasite species dependent and can take 48 or 72 hours) and is the stage where clinical manifestations of the disease occur and individuals get sick.

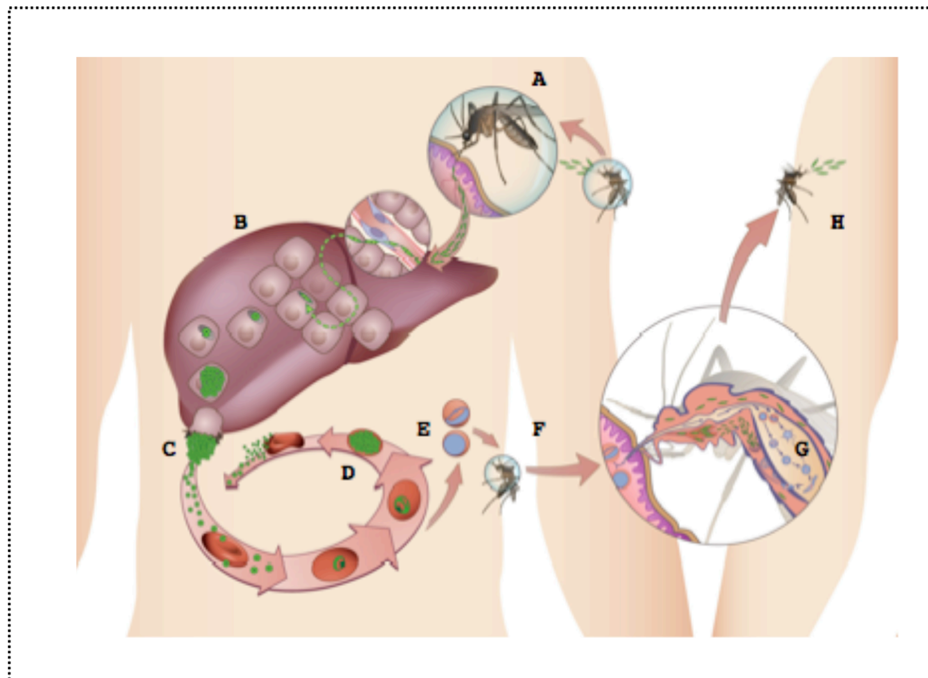


Figure 1.2|*Plasmodium* life cycle (A) An infected female *Anopheles* mosquito feeds on a host injecting *Plasmodium* sporozoites into the blood stream (B) Sporozoites arrest at the liver, glide along sinusoidal endothelia and breach through several hepatocytes before finally developing into liver schizonts (merozoites) within the hepatocyte. (C) Upon merozoite formation, merozomes are extruded into liver sinusoids and liberated into the blood stream (D) where they will cyclically infect erythrocytes. (E) Repeated infection cycles occur with some parasites developing into gametocytes. (F) When an Anopheles mosquito takes a blood feed on this host, it will collect these gametocytes (G) Fertilization takes place in the mosquito gut and an ookinete, and later oocyst, are formed. Oocysts will give rise to sporozoites, which will migrate and invade the mosquito salivary glands. (H) Infective sporozoites are ready to be inoculated in a new host upon the mosquito's next blood meal.

An exoerythrocytic schizont contains 10.000 to 30.000 merozoites, which once released invade the red blood cells in about 30 seconds. This process is dependent on the

interactions of specific receptors on the erythrocyte membrane with ligands in the surface of the merozoites. Once within the cell, the parasite begins to grow, first forming the ring-like early trophozoite, and eventually enlarging to fill the cell. The parasites are nourished by the hemoglobin within the erythrocytes and produce a characteristic pigment called hemozoin. The erythrocytic cycle is completed when the red blood cell ruptures and releases merozoites that proceed to invade other erythrocytes.

Not all merozoites divide asexually. Some differentiate into the sexual forms, the macrogametocytes (female) and microgametocytes (male) and can only complete their development within the gut of an *Anopheles* mosquito. The duration of gametocytogony is assumed to be approximately 4 to 10 days depending on the *Plasmodium* species. Upon ingestion by the mosquito, and once in the gut, the microgametes penetrate the macrogametes generating zygotes. Within 18 to 24 hours the zygotes become motile and elongated ookinetes which in turn invade the midgut wall of the mosquito where they develop into oocysts. It takes between 7-15 days for the oocysts to grow, rupture, and release sporozoites, which then make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

1.2.1. Study of host infection with mouse models.

Clinical cases of malaria in humans are caused by four different species of *Plasmodium*: *P. falciparum, P. vivax, P. ovale and P. malariae*. *P. falciparum* is, by far, the deadliest of the four, accounting for most of the mortality and morbidity associated with this disease. Several studies involving *Plasmodium* pre-erythrocytic stages have made use of *Plasmodium* species that infect rodents, specifically *P. berghei* and *P. yoelii*. These two

species have striking differences in sporozoites infectivity in inbred mouse strains. *Plasmodium yoelii* is often favored as a model for human malaria because, similarly to *P. falciparum* in humans, a low number of *P. yoelii* sporozoites is enough to establish an infection (Khusmith et al. 1991), whereas at least 100 *P. berghei* sporozoites are required to ensure blood stage infection (Burkot et al. 1988); (Khan and Vanderberg 1991). Nevertheless, *P. berghei* remains the most widely used rodent parasite since the technology enabling its transfection was developed earlier (van Dijk et al. 1996) than for *P. yoelii* (Mota et al. 2001a).

1.3 Liver Stage Biology~

Although intense research on the cell biology of *Plasmodium* liver stages has considerably advanced our understanding of basic events in the host, from sporozoite deposition into the skin, to liver stage maturation and merozoite differentiation, the lack of a large-scale culture system for infectious sporogonic stages, the difficulty in isolating sporozoites, the need to study the interaction of the parasites with complex tissues of the host and problems obtaining pure preparations of infected hepatocytes, have delayed the progress of this field.

There is still great controversy on why *Plasmodium* has elected the liver and the hepatocyte as a first cellular home inside mammalian hosts. However, it is possible that the reason is related to the hepatocyte's highly complex metabolism (hepatocytes are small storehouses of glycogen and serum protein factories, hence a great supply of nutrients), capable of fulfilling parasite replication needs (Frevert 2004). Another plausible reason is that the immunologic characteristics of the liver permit parasites to survive and pursue infection. In an effort to maintain

immunological silence to harmless material from the gut, the liver favors a tolerogenic response towards incoming antigens (Knolle and Gerken 2000; Crispe 2003). Other aspects to take into consideration are the morphology of the liver itself and the fact that hepatocytes are heterogeneous, allowing easy access to venules and arteries separated by the space of Disse (Wisse et al. 1985).

1.3.1. The *Plasmodium* Sporozoite

Malaria life cycle consists of three major invasive stages: the ookinete, the sporozoite and the merozoite. There is a large degree of conservation in their organization, including a surface designed to interact with the host cell and a cytoskeleton against which the actomyosin exerts its power. Additionally, secretory organelles modify the host cell to permit entry (the micronemes) and establish a parasitophorous vacuole (PV), in which the parasite may replicate (the rhoptries) (Sinden and Matuschewski 2005).

Between day 7 and 17 after infection, depending on the *Plasmodium* species and environmental temperature, the single-celled ookinete transforms into a mature oocyst, which contains hundreds or even thousands of sporozoites. Mature sporozoites exit from the oocyst to the body cavity and invade the salivary glands. The first morphological evidence for sporozoite bud formation is the appearance of the inner membrane complex (IMC) and the associated microtubules (MTs) under the cytoplasmatic face of the sporoblast plasma membrane. The sporozoite plasma membrane is derived from the sporoblast plasma membrane whilst the IMC is presumably made *de novo* from Golgi-derived cytoplasmatic vesicles that fuse and flatten together with sporozoite outgrowth (Sinden and Strong 1978; Beier and Vanderberg 1998).

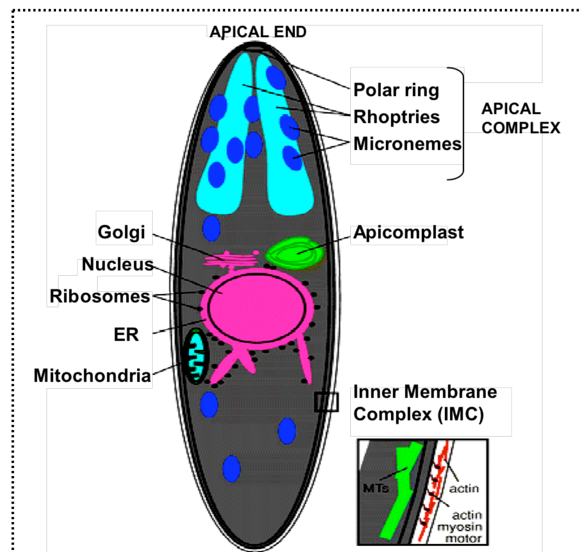


Figure 1.3|Schematic representation of a *Plasmodium* sporozoite (showing some of its organelles and subcellular structures).

The fully formed sporozoite has a crescent shape ranging from 9 to 16.5 μm in length and 0.4 to 2.7 μm in width, depending upon the species (Sinden and Strong 1978). Sporozoites contain an elongated nucleus, mitochondria, endoplasmatic reticulum and Golgi apparatus. Additionally, an actin-myosin motor, essential for parasite motility and invasion, is located in the narrow space between the plasma membrane and the outer membrane of the IMC (Kappe et al. 2004b). The anterior half side of the sporozoite contains two classes of electron-dense tubules: (i) Micronemes are small vesicles of varying electron density in *Plasmodium* sporozoites that frequently show a neck-like extension; and (ii) Rhoptries are large, usually paired, pear-shaped organelles filled with proteins and phospholipids. Both organelles discharge at the anterior tip of the parasite, and their contents (and that of dense granules, not yet identified in *Plasmodium* sporozoites) are involved in apicomplexan motility, host cell invasion, and generation of the non-phagosomal parasitophorous vacuole, where the parasite resides and replicates inside the host cell.

Two well-characterized sporozoite proteins are

circumsporozoite protein (CS) and thrombospondin-related anonymous protein (TRAP).

CS, not found in any other Aplicomplexa besides *Plasmodium*, is encoded by a single copy gene and covers the entire surface of sporozoites (Nussenzweig and Nussenzweig 1989). Following sporozoite invasion of hepatocytes, CS is also detected in the plasma membrane of early Exoerythrocytic forms (EEFs) and in the cytoplasm of infected cells (Hamilton et al. 1988; Singh et al. 2007). Due to its abundance, surface localization, immunogenicity, and key role in parasite invasion, CS constitutes the leading candidate molecule for the development of malaria pre-erythrocytic vaccines (Alonso et al. 2004; Saul et al. 2004).

TRAP is a member of a type I trans-membrane protein family (Menard 2001). In *Plasmodium,* three TRAP proteins have been identified: TRAP in sporozoites stage, TRAP homologue (MTRAP) in merozoite stage and CS- and TRAP-related protein (CTRP) in ookinetes (Baum et al. 2005). In sporozoites, TRAP is found in micronemes and on the plasma membrane, with a characteristic patchy distribution. Furthermore, the TRAP family members connect the host cell receptors with the molecular motor, driving Apicomplexa motility and cell invasion (Buscaglia et al. 2003).

1.3.2. Sporozoite gliding motility

Plasmodium sporozoites, as many of other invasive stages of the Apicomplexa phylum, present a form of locomotion that is not based on cilia or flagella. They cannot swim in liquid medium but they can glide on solid substrates, including host cell surfaces. Substrate dependent gliding motility is defined by the absence of any obvious modifications in the shape of the moving cell.

During gliding motility, CS protein is secreted at the apical end of the parasite, translocated along the

sporozoite's surface by an actin-dependent process, and shed on the substrate from the posterior end (Stewart and Vanderberg 1988). In this way gliding sporozoites leave surface proteins and membrane lipids on the substrate, resulting in characteristic spiral trails that can be visualized by CS protein staining (Fig. 1.4)

Plasmodium sporozoites can also enter and exit host cells by breaching their plasma membrane, a process that also requires gliding motility and is used for migration through host cells and tissues (Mota et al. 2001b).

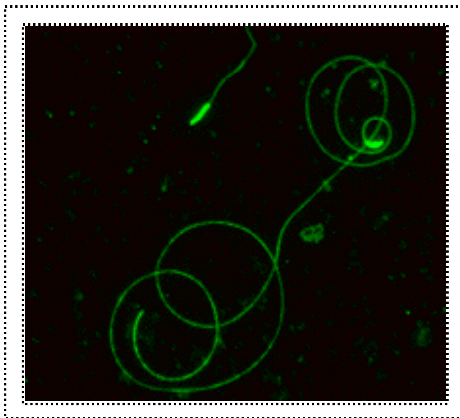


Figure 1.4| Gliding *Plasmodium* sporozoites leaving trails of CS protein. (Adapted from Mota, 2002)

1.3.3. In the skin

When a female Anopheles mosquito bites the mammalian host, it probes for a blood source under the skin. While probing, the mosquito injects saliva containing vasodilators and anti-coagulants to facilitate the blood ingestion (Griffiths and Gordon 1952; Ponnudurai et al. 1991), along with *Plasmodium* sporozoites. Although mosquitoes can harbor hundreds of sporozoites in their salivary glands, they typically inoculate only small numbers (Medica and Sinnis 2005). The majority of these sporozoites are injected into the dermis and not directly into circulation. They can remain in the skin for a long time (Yamauchi et al. 2007) and probably traverse skin cells before entering the blood circulation from where they reach the liver (Vanderberg and Frevert 2004; Amino

et al. 2006). An alternative route for the sporozoite journey to the liver is via the lymphatic system, possibly inside leukocytes (Vaughan et al. 1999; Krettli and Dantas 2000). Videomicroscopic analysis of GFP- expressing sporozoites in the skin revealed their high motile activity and subsequent active penetration through the vascular endothelium. Also, recent intravital microscopy using the *Plasmodium berghei* rodent model of malaria showed that sporozoites deposited into avascular dermal tissue use gliding motility to migrate within the skin and into dermal vessels, covering distances of many micrometers for several minutes before reaching circulation (Vanderberg and Frevert 2004). There are indications that a sporozoite surface phospholipase (PbPL) is required to breach host cell membranes during migration in the skin, as parasites deficient in PbPL are impaired in their ability to cross epithelial cell monolayers, and their infectivity is greatly decreased when they are transmitted by mosquito bite (Bhanot et al. 2005). During migration in the skin the parasite is vulnerable to antibodies against *Plasmodium* surface proteins, which may act as the first line of the host's immune response against the parasite (Vanderberg and Frevert 2004).

Amino *et al* (Amino et al. 2006) showed that a significant proportion of mosquito-injected sporozoites remain in the dermis after exhausting their gliding motility. Of those that leave the area of the bite within 1 hour of injection, approximately 70% enter blood vessels and the remaining 30% invade lymphatic vessels. The majority of the latter do not reach the blood circulation, as had been previously assumed. Instead, they are trapped in the lymph nodes, where most are phagocytosed by dendritic cells. Some of these lymphatic sporozoites were found to partially develop into small-sized EEFs before eventually being degraded (Amino et al. 2006). These studies were

performed with the rodent malaria parasite *P. berghei*. Studies on *P. yoelii* showed that the majority of injected sporozoites remain at this site for several hours and exit in a slow trickle rather than a rapid burst. Similar to what was found for *P. berghei*, about 20% of the *P. yoelii* sporozoite innoculum traffic through the draining lymph node, a process that is likely to have an effect on the immune response generated against the sporozoite stage of infection (Yamauchi et al. 2007). Taken together these studies suggest that there are significant interactions between sporozoites and their mammalian host at the injection site.

1.3.4. Getting to the liver

Most invasive stages of Apicomplexan parasites are released in close proximity to of their target and therefore do not required to move long distances. *Plasmodium* sporozoites differ from other zoites in this regard since, in the mammalian host, they must make their way from the dermis to the hepatocyte. The majority of the circulating sporozoites are arrested in the liver after a single passage, suggesting that specific receptors are present on the cells lining the sinusoids (Shin et al. 1982). The liver sinusoid lining consists mostly of a fenestrated endothelium and Kupffer cells. The reduced speed in the blood circulation, while percolating through the liver, facilitates the encounter of the parasites with the putative sinusoidal receptors. Because CS covers the entire sporozoite plasma membrane, it is very likely that it contains the postulated liver ligand(s). Numerous observations indicate that the ligand is contained in the stretch of positively charged residues of region II-plus of CS, and that the binding sites in the liver are heparan sulfate proteoglycans (HSPGs) (Sinnis and Nardin 2002; Tewari et al. 2002). In addition to region II-plus, the

positively charged region I of CS may also bind to HSPGs and contribute to sporozoite arrest in the liver (Rathore et al. 2002). In addition to the liver, HSPGs are ubiquitously distributed in extracellular matrices and on cell surfaces. Among other functions, HSPGs bind growth factors and cytokines, are involved in the lipoprotein metabolism and participate in the viral entry into cells. The multiple roles of HSPGs are associated with extensive chemical variation, imparting specificity to the various interactions (Iozzo 2001). Liver HSPGs include two members of the syndecan family (syndecam 1 and syndecan 2), which are the type I integral membrane proteins that can function as co-receptors (Couchman 2003). Interestingly, Syndecan 1 knockout mice are as susceptible to sporozoite infection as the wild type controls suggesting that syndecan 1 is not necessary for the infection to occur (Bhanot and Nussenzweig 2002). Thus, syndecan 2 is more likely to be the CS receptor. It is an unusual member of the HSPGs family, with a large proportion of heparin-like, highly sulfated structures at the distal end of glycosaminoglycans chains (Pierce et al. 1992; Lyon et al. 1994). Notably, among glycosaminoglycans, heparin is the most efficient inhibitor of CS binding to the human hepatoma cell line HepG2 (Kappe et al. 2004a).

1.3.5. In the Liver

The first step of infection is the establishment and full development of *Plasmodium* sporozoites inside hepatocytes, which, although symptomatically silent, gives rise to thousands of merozoites in each hepatocyte.

The molecular signals that allow sporozoites to determine their position in the mammalian host are not known. It was suggested by recent studies on the proteolytic cleavage of the sporozoite's major surface protein, CS, that sporozoites recognize different cell types (Coppi et al. 2005).

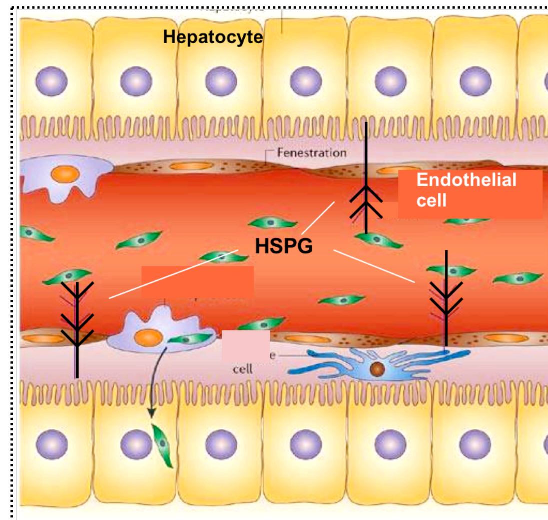


Figure 1.5| Sporozoites arrest in the liver. Once sporozoites (in green) reach the liver sinusoids they glide along the endothelium of the blood vessel and interact with the heparan sulfate proteoglycans from hepatocytes and stelate cells. They cross the sinusoidal layer by traversing either endothelial cells or Kupffer cells (as represented).

After being sequestered in the sinusoids, sporozoites must reach and invade the hepatocytes. They encounter two different cell types on the way: endothelial and Kupffer cells. Although liver endothelial cells have fenestrations, these are too small (about one tenth of the diameter of a sporozoite) to allow sporozoite passage (Wisse et al. 1985).

As sporozoites are able to migrate through all nucleated cell types examined to date, it is possible that sporozoites can traverse either endothelial or Kupffer cells. However, there is increasing evidence indicating that sporozoites cross the sinusoidal layer primarily through Kupffer cells (Frevert et al. 2006).

1.3.6. Sporozoite Migration and Apical Regulated Exocytosis

Sporozoites can enter cells by two distinct routes, either through a tight moving junction with the target cell that leads to the formation of a parasitophorous vacuole (PV) where EEF development proceeds, or by disrupting their plasma membrane (Mota et al. 2001b). In the latter case, the parasite glides in the cytoplasm and exits the cell again rupturing the plasma membrane. Using a cell-wounding assay, it has been shown, both *in vitro* and *in vivo,* that during migration through cells, *Plasmodium* spp. sporozoites breach the plasma membranes of several hepatocytes, which can rapidly be repaired (Mota et al. 2001b). Recently, sporozoite migration in the liver was confirmed by intravital microscopy (Frevert et al. 2005). Migration through cells is also observed in other parasites at similar stages of the life cycle: *Toxoplasma* and *Eimeria bovis* (a cattle pathogen) sporozoites are also able to migrate through cells by disrupting the membrane (Mota and Rodriguez 2001).

Breaching of the cell membranes by the *Plasmodium* parasite is likely to involve specific lipases, proteases and pore-forming proteins. Four distinct *P. berghei* proteins have been shown to have important roles during cell traversal: sporozoite protein essential for cell traversal (SPECT), SPECT2, cell traversal protein for ookinete and sporozoite (CelTOS) and the phospholipase PbPL (Ishino et al. 2004; Bhanot et al. 2005; Ishino et al. 2005b; Kariu et al. 2006). At least two of these proteins, SPECT2 and PbPL, seem to be involved in pore formation (Ishino et al. 2005b), whereas CelTOS has been proposed to be required for movement through the host-cell cytosol (Kariu et al. 2006)

Entering hepatocytes by breaching the cell membrane might be advantageous to *Plasmodium* parasite, since it offers an unimpeded view of the local host cytoplasmatic environment. In *Plasmodium* sporozoites, migration through cells induces apical regulated exocytosis. Exocytosis is observed as an accumulation of micronemal proteins, such as TRAP, in the apical end of the sporozoite. TRAP is a

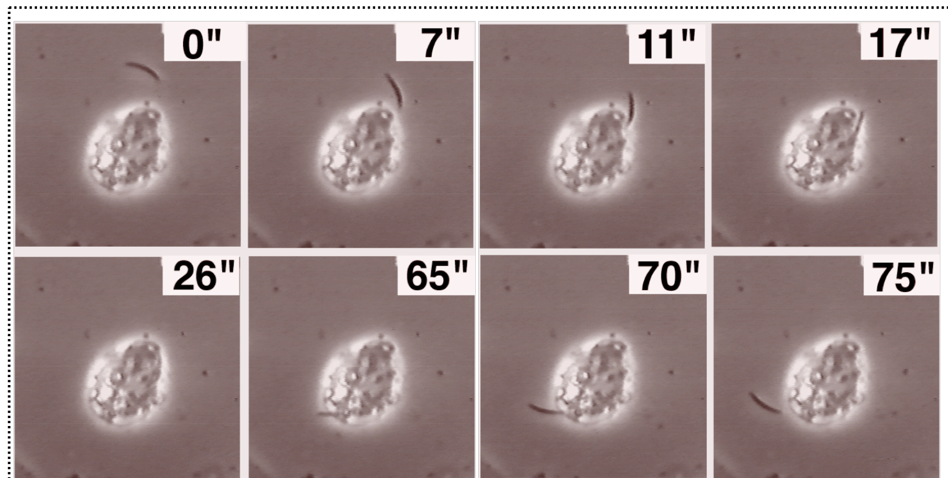


Figure 1.6| *Plasmodium* sporozoites migration through cells. Time lapse video images of a *Plasmodium berghei* sporozoite entering and exiting a HepG2 cell (hepatocyte) in about a minute (adapted from Mota *et al*, 2001).

transmembrane protein with most of its aminoacid sequence located in the lumen of the micronemes. When micronemes fuse with the plasma membrane of the sporozoite, TRAP is incorporated in the apical plasma membrane, with its main domains exposed to the extracellular medium. Since TRAP is an essential mediator of hepatocyte invasion and presents adhesive properties, it is believed that exposure of this protein in the apical end of the sporozoites is required for invasion of the host cell (Mota et al. 2002). This type of secretion occurs in response to a stimulus, and can be visualized as a "cap" structure on the apical end of the sporozoite (Gantt et al. 2000; Mota et al. 2002).

Invasion with formation of a parasitophorous vacuole is tightly associated with exocytosis of apical organelles in different Apicomplexa (Rick et al. 1998; Carruthers et al. 1999). In *Toxoplasma*, this process is accompanied by sequential discharge of micronemes, rhoptries and dense granules (Carruthers and Sibley 1997). Attachment of toxoplasma tachyzoites to host cells triggers a transient cytosolic Ca^{2+} increase that is required for invasion (Vieira and Moreno 2000).

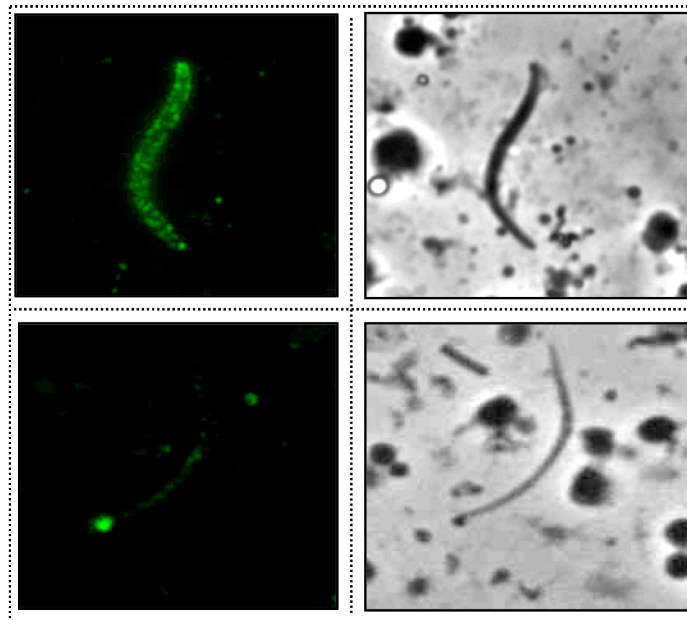


Figure 1.7|*Plasmodium yoelii* sporozoite exocytosis. Sporozoites were incubated with (lower panel) or without (upper panel) a hepatocyte lysate. Surface staining with monoclonal antibody against TRAP. Right panels show the same microscopic field in phase contrast. Exocytosis is observed as a cap at the apical end of the parasite.

In *Plasmodium* sporozoites, activation of exocytosis is induced by migration through cells but can also be activated in an artificial manner by incubating sporozoites with a Ca^{2+} ionophore (Gantt et al. 2000) or with host cell lysates (Mota et al. 2002), suggesting that factors from the host cell activate signaling cascades in sporozoites leading to exocytosis. Activation of exocytosis leads to increased infectivity of sporozoites, by enabling the release of key factors required for

hepatocyte invasion. Many of the proteins found in the micronemes contain cell adhesive domains that function in zoite-host cell interactions required for invasion (Soldati et al. 2001). Another aspect of sporozoite migration in the liver is that the wounding of the cells induces the secretion of "Hepatocyte Growth Factor" (HGF), that renders the surroundings more susceptible to parasite growth (Carrolo et al. 2003).

In summary, migration through cells is involved in at least four sporozoite activities necessary to achieve efficient liver infection: exit from the skin into circulation (Bhanot et al. 2005), entry from circulation into the liver (Ishino et al. 2004), generation of HGF in the liver to increase host cell susceptibility (Carrolo et al. 2003) and activation of sporozoites before infection (Mota et al. 2002).

1.3.7. Hepatocyte Invasion and Intrahepatic development

Intense secretion of TRAP and CS accompanies the final invasion of the hepatocyte and the parasite finds itself surrounded by a PV, in which it replicates and develops (Meis et al. 1983; Mota et al. 2002; Silvie et al. 2004). CS seems to have an active role in sporozoite attachment rather than internalization (Pradel et al. 2002), whereas TRAP contributes to sporozoite internalization and not attachment (Matuschewski et al. 2002). Other proteins have recently been implicated in the invasion of hepatocytes: AMA-1 (apical membrane antigen 1) is required for hepatocyte invasion by *P. falciparum* parasites (Silvie et al. 2004); two *P. berghei* proteins, Pb36p and Pb36, seem necessary for sporozoites to recognize hepatocytes and commit to infection (Ishino et al. 2005a) but also for sporozoite early development (van Dijk et al. 2005) . One host protein that seems to interact with sporozoites is the tetraspanin CD81. It is required for *P. yoelii*

invasion of mouse hepatocytes and for *P. falciparum* invasion of human hepatocytes.

After the final invasion each *Plasmodium* sporozoite develops and multiplies inside the hepatocyte, thereby generating thousands of merozoites. Recently, it has been observed that removal of either protein UIS3, UIS4 or Pb36p (Mueller et al. 2005b; Mueller et al. 2005a; van Dijk et al. 2005) (UIS stands for upregulated in infective sporozoites) leads to impairment of parasite development in hepatocyte.

The final important step in the life cycle of intracellular pathogens is the exit from the host cell after replication, but the molecular mechanisms involved in this process are poorly understood. It has been recently reported that *P.berghei* merozoites are not released by the rupture of the hepatocyte, but by the formation of merozoite filled vesicles (merosomes), which bud of from the infected hepatocytes into the lumen of the liver sinusoids (Sturm et al. 2006).

1.4. Aims and strategies~

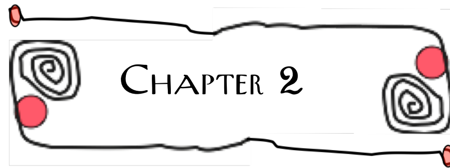
Plasmodium sporozoites are found for extended periods of quiescence in the mosquito's salivary gland lumen before being subjected to a sudden change in environment when inoculated into the warm-blooded host. In the mammalian host, the inoculated sporozoites enter a journey from the skin to the liver. During this period, sporozoites are activated to a state of readiness for hepatocyte invasion and expose molecules necessary in the process of invasion.

The main questions proposed in this thesis are concerned with the activation of exocytosis upon migration through host cells and its importance in host cell invasion. We posed three specific questions:

1) How does migration through cells induce exocytosis in *Plasmodium* sporozoites? We propose to determine which host cell molecules are responsible for the activation of exocytosis.

2) Is this process regulated and how? Since sporozoites activation only occurs in the liver, we propose that sporozoites make use of regulatory mechanisms that permit a sequential and specific preparation for infection.

3) What are the signaling pathways that mediate the activation of exocytosis in sporozoites? It was observed in a previous study that an intracellular increase of calcium in sporozoites leads to an increase in apical exocytosis (Mota et al. 2002) and a subsequent boost in infectivity. We propose to elucidate the role of signal transduction in sporozoites exocytosis and infection.



HOST MOLECULES INVOLVED IN THE REGULATION OF *PLASMODIUM* SPOROZOITES EXOCYTOSIS AND INFECTION.

2.1. Introduction~

Plasmodium sporozoites and other apicomplexan parasites, such as *Eimeria* sporozoites and *Toxoplasma* tachyzoites, have small vesicles – micronemes that contain proteins involved in host cell infection (Sibley 2004). These proteins, such as MIC-2 in *Toxoplasma* or TRAP in *Plasmodium,* become exposed on the apical surface of the parasite upon exocytosis of the micronemes, which is triggered by incubation of these parasites with host cells (Carruthers et al. 1999; Gantt et al. 2000). Exocytosis of micronemal proteins, resulting in the appearance of TRAP on the apical surface of *Plasmodium* sporozoites, is induced during the process of migration through cells and precedes infection with the formation of an internalization vacuole (Mota and Rodriguez 2004). This process, similarly to *Toxoplasma* secretion of MIC2 (Huynh et al. 2006), is thought to facilitate invasion of the host cell (Mota et al. 2002). Migration through host cells is therefore considered an early step in activation of sporozoites for infection (Mota and Rodriguez 2004). During this process sporozoites are not surrounded by a vacuolar membrane and therefore are in direct contact with the cytosol of the traversed cell. Because apical regulated exocytosis can also be induced by incubation of sporozoites with host cell lysates, it was proposed that cytosolic factors in the mammalian cell activate exocytosis in the parasite (Mota et al. 2002).

The results presented in this chapter include the identification of host cell cytosolic factors that induce exocytosis of the rodent parasite *P. yoelii* and the human parasite *P. falciparum*. We found that uracil, uridine and uracil-derived nucleotides, at concentrations that are

normally found in the cytosol of mammalian cells, induce exocytosis in sporozoites and increase their infectivity. We have also characterized the regulation of this process. As sporozoites are deposited in the host skin, where they apparently traverse host cells (Vanderberg and Frevert 2004; Amino et al. 2006), it is likely that they encounter high concentrations of uracil-derived nucleotides before reaching their target cells in the liver. However, exocytosis is only expected to take place just before hepatocyte infection, as it exposes high concentrations of adhesive molecules on the surface of the parasite, such as TRAP, which are required for internalization and formation of a parasitophorous vacuole.

In this study, we found that exocytosis is inhibited specifically by albumin, a protein found in the skin, blood and liver of the mammalian host. This finding suggests that during infections *in vivo,* sporozoites don't undergo apical regulated exocytosis in the presence of physiological concentrations of this protein. Additionally we observed that this inhibitory effect of albumin is reversed when sporozoites are in contact with hepatocytes, suggesting that after arrival in the liver, sporozoites become susceptible to stimulation by uracil-derived nucleotides that will in turn induce apical regulated exocytosis and facilitate hepatocyte infection. In fact, the reversion of albumin inhibitory effect appears to be mediated by HSPGs present in the surface of hepatocytes.

2.2. Results~

2.2.1. Uracil derivatives induce apical regulated exocytosis in *Plasmodium* sporozoites

Exocytosis in the rodent parasite *P. yoelii* is induced by migration through host cells, as well as by incubation with lysates of a hepatoma cell line (Hepa1-6), which is susceptible to sporozoite infection (Mota and Rodriguez 2000; Mota et al. 2002). Apical regulated exocytosis in *P. yoelii,* (Mota et al. 2002) and in the human parasite, *P. falciparum*, is observed as the surface exposes TRAP protein in the apical end of the sporozoites (Fig.2.1).

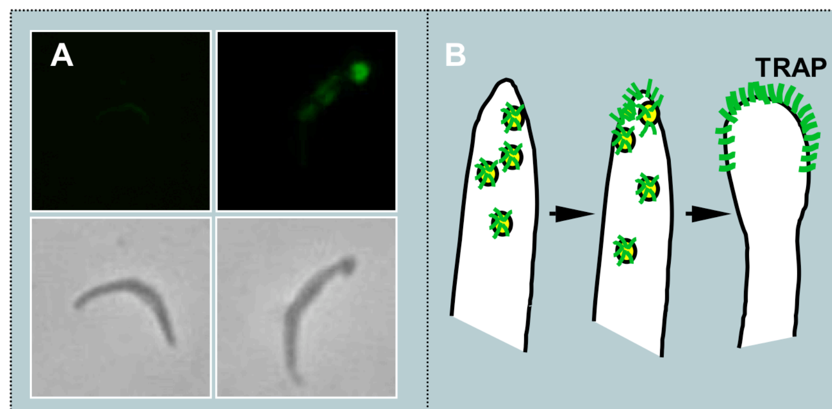


Figure 2.1| Schematic of Apical regulated exocytosis in *Plasmodium* sporozoites. (A) Upper panels show surface staining of *P. falciparum* sporozoites with anti-TRAP mAb. Lower panel shows the same microscope field in phase contrast. Apical regulated exocytosis is observed as a 'cap' in one end of the sporozoite (right panels). (B) Model of apical regulated exocytosis. After activation, *Plasmodium* sporozoites recruit TRAP-containing micronemes to their apical end, which fuse with the apical membrane of the parasite.

Regulated exocytosis in mammalian cells can be induced by a wide variety of molecules, ranging from proteins to nucleotides. In particular, uracil and adenine nucleotides (UDP, ADP, UTP and ATP) bind to specific receptors of the P2X and Y families and induce regulated exocytosis in different cell types (Lazarowski et al. 2003). Since these nucleotides are found in high concentrations in the

cytosol of cells and therefore are in direct contact with migrating sporozoites, we tested their ability to induce exocytosis in *P. yoelii* sporozoites. We found that UDP and UTP, but not ADP or ATP, induce sporozoite exocytosis (Fig.2.2.A). Furthermore, UDP induced exocytosis in *Plasmodium* sporozoites in a dose dependent manner and physiological concentrations of UDP in the cytosol (app. 100 μM) (Traut 1994) were sufficient to efficiently induce exocytosis in sporozoites (Fig.2.2.B). Additionally, this induction by UDP was observed already 5 min after incubation and reached it maximum by 10 to 20 min (Fig.2.2.C).

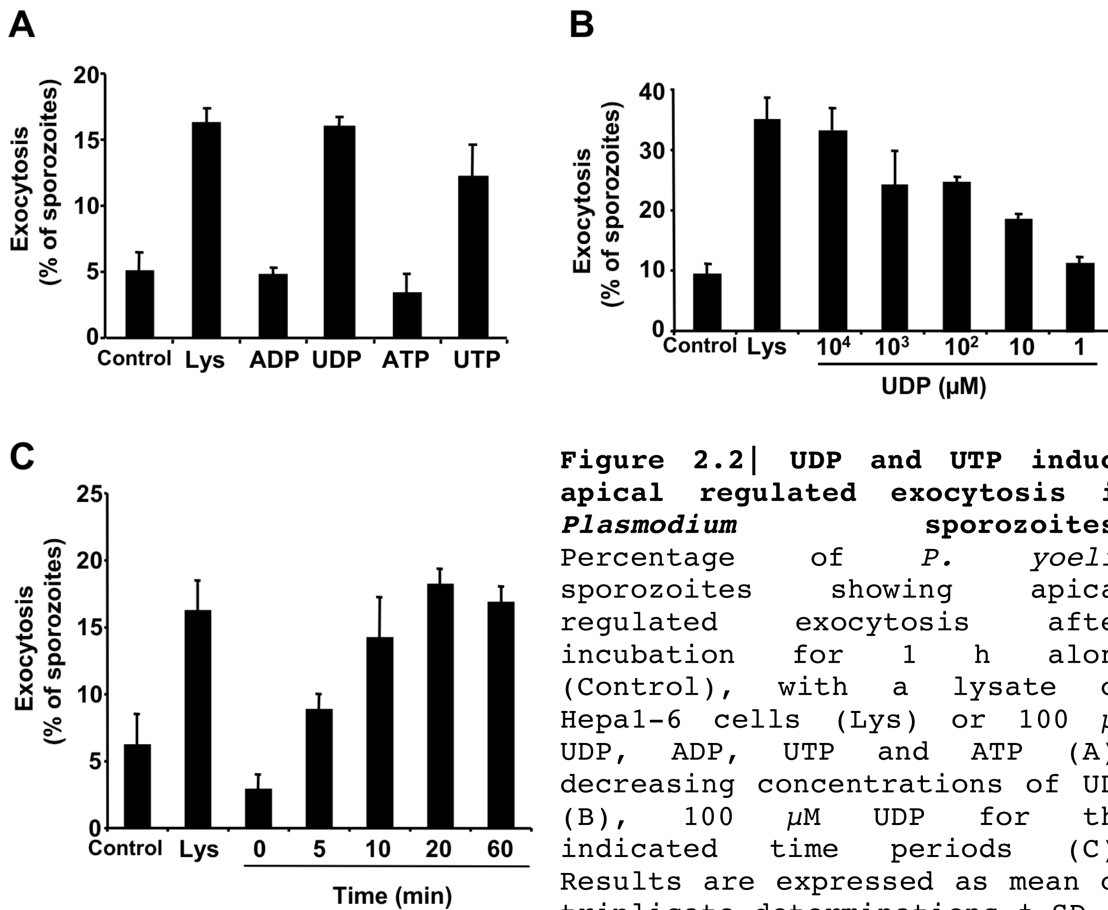


Figure 2.2| UDP and UTP induce apical regulated exocytosis in *Plasmodium* sporozoites. Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis after incubation for 1 h alone (Control), with a lysate of Hepa1-6 cells (Lys) or 100 μM UDP, ADP, UTP and ATP (A), decreasing concentrations of UDP (B), 100 μM UDP for the indicated time periods (C). Results are expressed as mean of triplicate determinations ± SD.

We also investigated whether other pyrimidines were able to induce exocytosis in sporozoites. Similar concentrations of thymine, uracil and their derivative nucleosides and nucleotides (100 μM) showed identical capability of stimulating exocytosis (Fig.2.3.A). No significant activity was detected with cytosine derivatives (Fig.2.3.B). As the physiological concentrations of thymine and its derivatives are very low (<5 μM) in mammalian tissues (Traut 1994), uracil and its derivatives are likely to be the major effectors in activating sporozoite exocytosis during migration through host cells.

We next analyzed the effect of a mixture of uracil and its derivatives (uridine, UMP, UDP and UTP) at the physiological concentrations found in the cytosol of mammalian cells (from 30 to 300 μM, described in methods) (Traut 1994), and observed that exocytosis is efficiently induced (Fig.2.4.A).

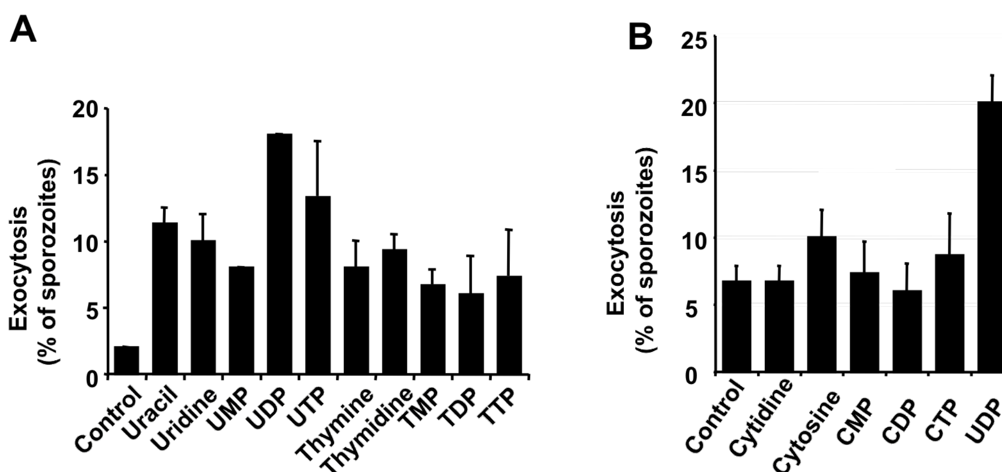


Figure 2.3| Effect on *Plasmodium* sporozoites exocytosis of Uracil, Thymine and Cytosine derivatives. Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis after incubation for 1 h alone (Control) or 100 μM of the indicated pyrimidines of the thimidine and uracil families (A) and the cytosine family (B). Results are expressed as mean of triplicate determinations ± SD.

Migration through hepatocytes induces sporozoite apical regulated exocytosis, which facilitates invasion of the host cell (Mota et al. 2002). Stimulation of exocytosis by other means, such as calcium ionophores or Hepa1-6 cells lysates, overcomes the need for migration through host cells and increases infection (Mota et al. 2002).

To examine whether this was also the case for physiological concentration of uracil and its derivatives we tested whether stimulation of exocytosis by physiological concentrations of uracil and its derivatives, would also overcome the need for migration through hepatocytes before infection, we incubated *P. yoelii* sporozoites with these molecules to induce regulated exocytosis before incubation with Hepa1-6 cells. Migration through host cells was determined as the percentage of cells wounded by sporozoite migration and that in turn became positive for a soluble impermeant tracer (dextran) (McNeil et al. 1989).

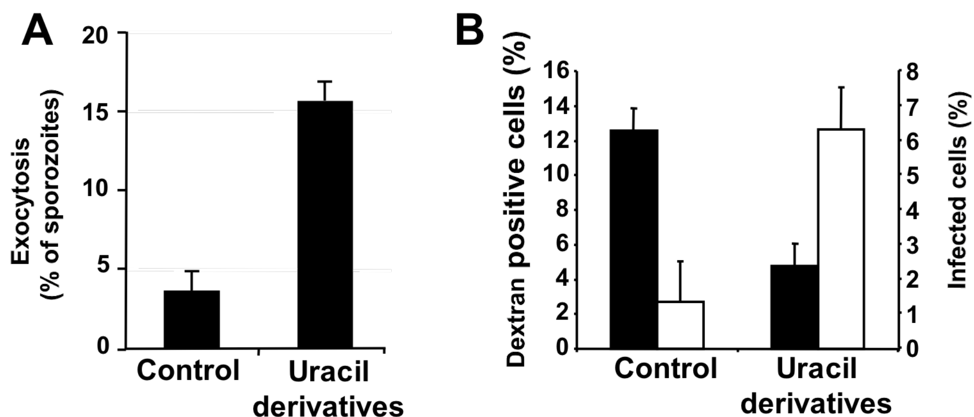


Figure 2.4| Physiological concentrations of uracil derivatives induce apical regulated exocytosis in *P. yoelii* sporozoites and activate them for infection. (A) Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis after incubation with physiological cytosolic concentrations of uracil and its derivatives, as described in methods. (B) *P. yoelii* sporozoites were incubated with uracil derivatives mix and added to monolayers of Hepa1-6 cells. Percentage of dextran-positive cells (black bars) and infected cells (white bars) are shown. Results are expressed as mean of triplicate determinations ± SD.

We found an increase in the number of infected cells, indicating that stimulation of regulated exocytosis increases infectivity, in sporozoites. In addition, reduced migration through hepatocytes was observed, suggesting that such migration is not necessary when exocytosis is previously induced by these nucleotides (Fig.2.4.B).

2.2.2. Albumin inhibits exocytosis induced by uracil nucleotides.

A malaria infection starts with the bite of an infected mosquito that deposits saliva containing *Plasmodium* sporozoites in the skin of the host. Motile sporozoites move freely in the dermis (Vanderberg and Frevert 2004), where they most likely encounter high concentrations of uracil-derived nucleotides. This would then lead to stimulation of apical regulated exocytosis long before sporozoites have reached their target cells. However, exocytosis only happens once sporozoites have reached liver cells, suggesting that host factors that sporozoites encounter during the journey from the skin to the liver regulate sporozoite exocytosis. To test this hypothesis we first analyzed the effect of mouse serum on sporozoite exocytosis. Pre-incubation of sporozoites with mouse serum completely inhibited exocytosis induced by uracil-derivatives (Fig.2.5.A). Since albumin is found at high concentrations in the serum and specifically regulates sporozoite activity, by inducing gliding motility (Vanderberg 1974), we tested the effect of this protein on sporozoites exocytosis. Interestingly, we observed that albumin completely prevents activation of exocytosis by uracil derivatives (Fig.2.5.B).

Albumin has previously been described as a carrier protein that binds lipids (Kragh-Hansen et al. 2002). Therefore, we next tested the effect of highly purified

fatty acid-free albumin on sporozoite exocytosis and observed a similar inhibitory effect (Fig.2.5.C). The inhibitory effect of albumin was found to be dose dependent (Fig.2.7.A), with physiological concentrations in the interstitial fluid of the dermis (35 mg/ml) (Reed et al. 1989) or in blood (28-37 mg/ml) (Don and Kaysen 2004) being sufficient to prevent sporozoite stimulation for exocytosis (Fig.2.6).

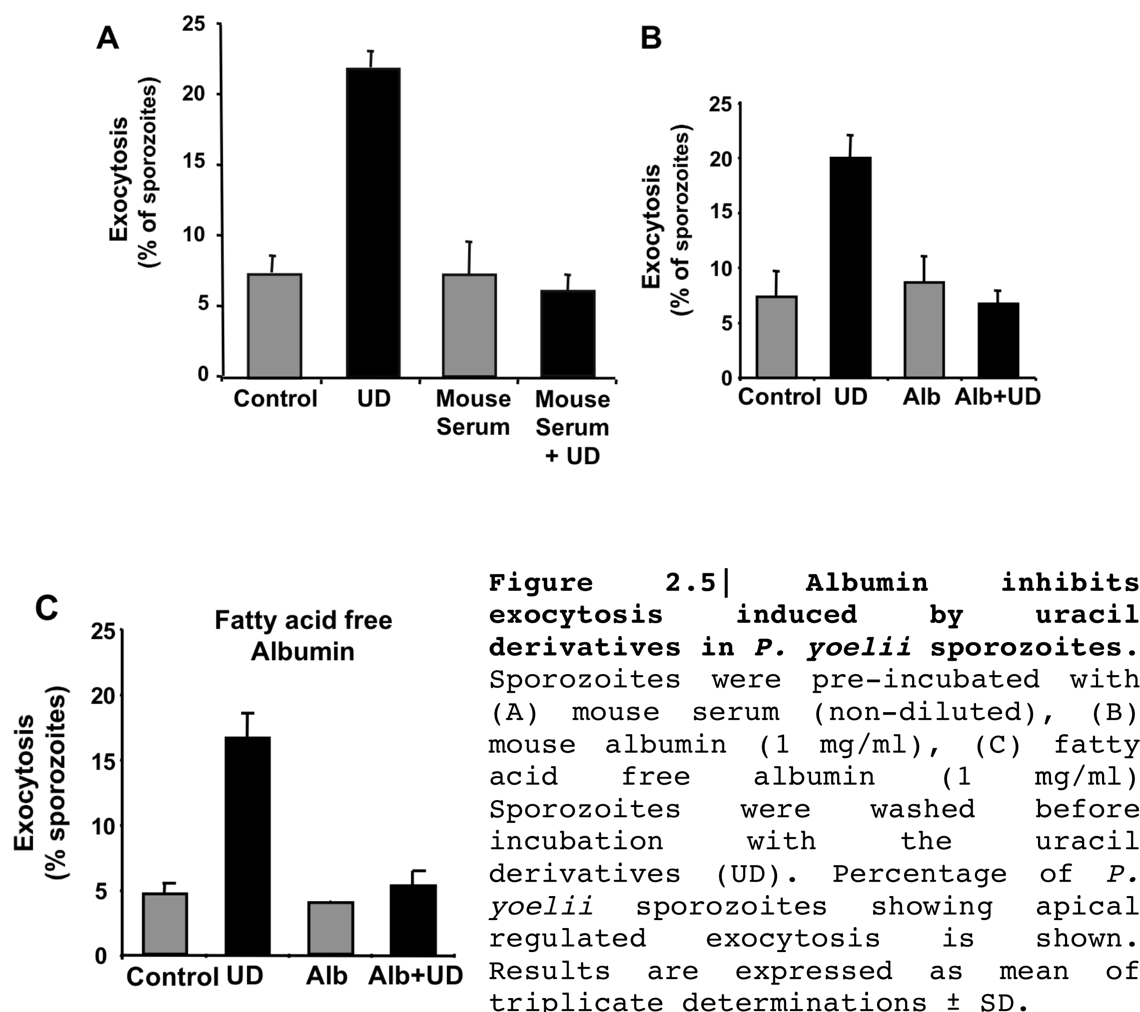


Figure 2.5| Albumin inhibits exocytosis induced by uracil derivatives in *P. yoelii* sporozoites. Sporozoites were pre-incubated with (A) mouse serum (non-diluted), (B) mouse albumin (1 mg/ml), (C) fatty acid free albumin (1 mg/ml) Sporozoites were washed before incubation with the uracil derivatives (UD). Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis is shown. Results are expressed as mean of triplicate determinations ± SD.

In contrast, other proteins, such as gelatin or the serum proteins alpha2-macroglobulin and transferrin, did not inhibit sporozoite exocytosis (Fig.2.6). To confirm that the inhibitory activity observed is specifically due to

the presence of albumin, we tested the effects of neutralizing antibodies, which show reversal of the inhibitory effect of albumin (Fig.2.7).

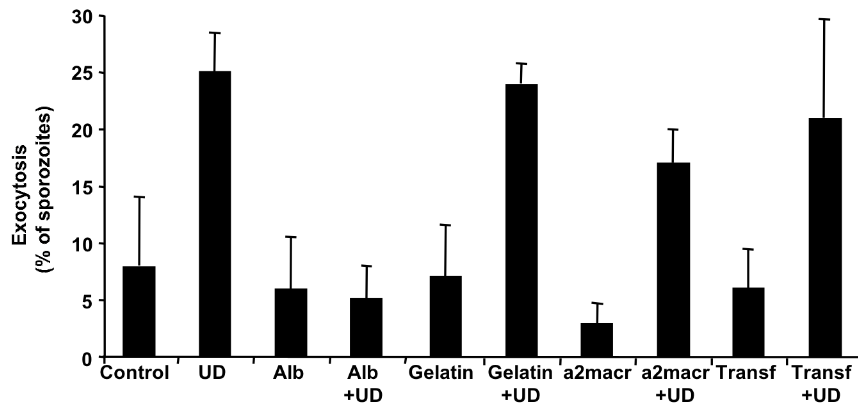


Figure 2.6| Effect of some serum proteins on exocytosis induced by UD. *P. yoelii* sporozoites were pre-incubated with gelatin at 35 mg/ml or serum physiological concentrations of albumin (35 mg/ml), a2-macroglobulin (1.64 mg/ml) and transferrin (2.5 mg/ml) Sporozoites were washed before incubation with the uracil derivatives (UD). Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis is shown. Results are expressed as mean of triplicate determinations ± SD.

2.2.3. The inhibitory effect of albumin on sporozoites exocytosis is reversed in the presence of hepatocytes.

Since albumin is at high concentrations in the interstitial fluids of the skin tissues (Reed and Burrington 1989) our results would suggest that following inoculation of sporozoites in the mammalian host, albumin inhibits the exocytosis response to a stimulus, such as uracil derivatives, preventing premature activation of sporozoites for infection. However hepatocytes contain high concentrations of albumin (Reed et al. 1989) which would interfere with the infectivity of the parasite.

To analyze the regulation of exocytosis by albumin in the presence of hepatocytes, we added albumin pre-incubated sporozoites to monolayers of mouse or human hepatoma cell lines. In the presence of these cells the inhibitory

effect of albumin was no longer detectable, resulting in efficient activation of exocytosis (Fig.2.8.A). This finding indicates that in the presence of hepatocytes, sporozoites are no longer susceptible to the inhibitory effect of albumin and can be activated by uracil derivatives.

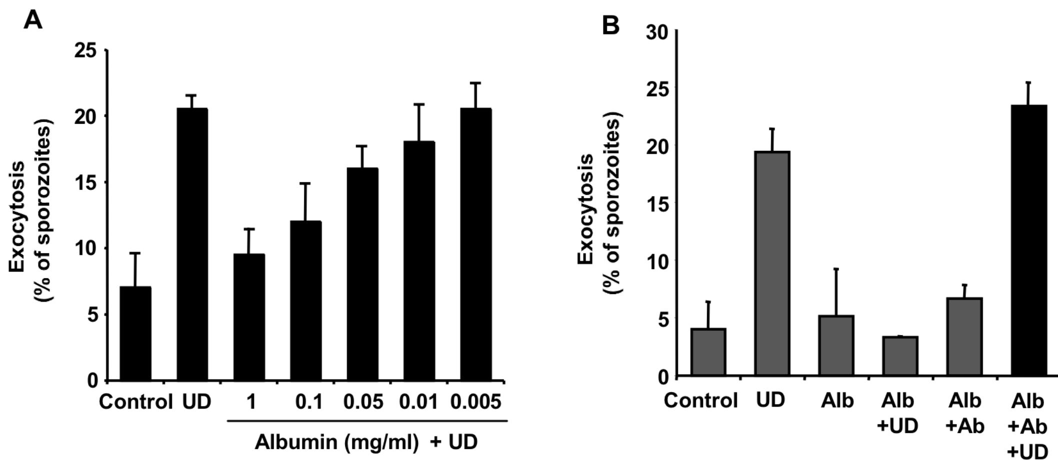


Figure 2.7| Albumin inhibitory effect on exocytosis is specific and dose dependent. Sporozoites were pre-incubated with (A) decreasing concentrations of mouse albumin or (B) mouse albumin (1 mg/ml) pre-incubated or not with anti-albumin specific antiserum. Sporozoites were washed before incubation with the uracil derivatives (UD). Percentage of P. yoelii sporozoites showing apical regulated exocytosis is shown. Results are expressed as mean of triplicate determinations ± SD.

In order to prevent internalization of sporozoites inside host cells, where exocytosis cannot be detected, we inhibited sporozoite motility with a myosin inhibitor (BDM). This way sporozoites, although in contact with the surface of hepatocytes, were no longer able to migrate through or infect these cells and instead the exocytosis stimulus was provided by addition of uracil derivatives to the medium. We then tested whether hepatocytes had to be alive and whether a hepatocyte lysate or hepatocyte membrane fraction could mediate the reversal of albumin inhibition in uracil derivatives-induced exocytosis. We found that both paraformaldehyde fixed hepatocytes and

sporozoites pre-incubated with either hepatocyte lysate or its membrane fraction, could also reverse the inhibitory effect of albumin on uracil derivatives induced exocytosis (Fig.2.8.B). These results suggest that a molecule localized in the extracellular side of the hepatocyte membrane mediates the hepatocyte effect on exocytosis. Conversely, primary cultures of skin dermal fibroblasts did not reverse the inhibitory effect of albumin, resulting in the lack of exocytosis activation (Fig.2.8.C). Together, these results indicate that different cell types have different effects on the regulation of parasite activity, and suggest that sporozoites when migrating through cells in skin dermis are not able to undergo exocytosis in response to the cytosolic uracil nucleotides present in these cells. On the other hand, contact with hepatocytes seems to counteract the inhibitory effect of albumin resulting in exocytosis activation after migration through these cells.

To further confirm this hypothesis, we analyzed the capacity of different cell types to induce sporozoite exocytosis in the presence of albumin. *P. yoelii* sporozoites were incubated with cells cultured on Transwell filters. Sporozoites migrate through cells on the filter and are collected on coverslips placed underneath the filters (Mota et al. 2002). The assay is performed in the presence of fluorescent dextran to confirm sporozoite migration. We found that migration through hepatocytes results in the activation of sporozoite exocytosis, while migration through dermal fibroblasts or other non-hepatic cell types does not (Fig.2.9). During infection of the host, this differential capacity to activate sporozoites may play a role in ensuring stimulation of exocytosis only after sporozoites have reached their target cells in the liver.

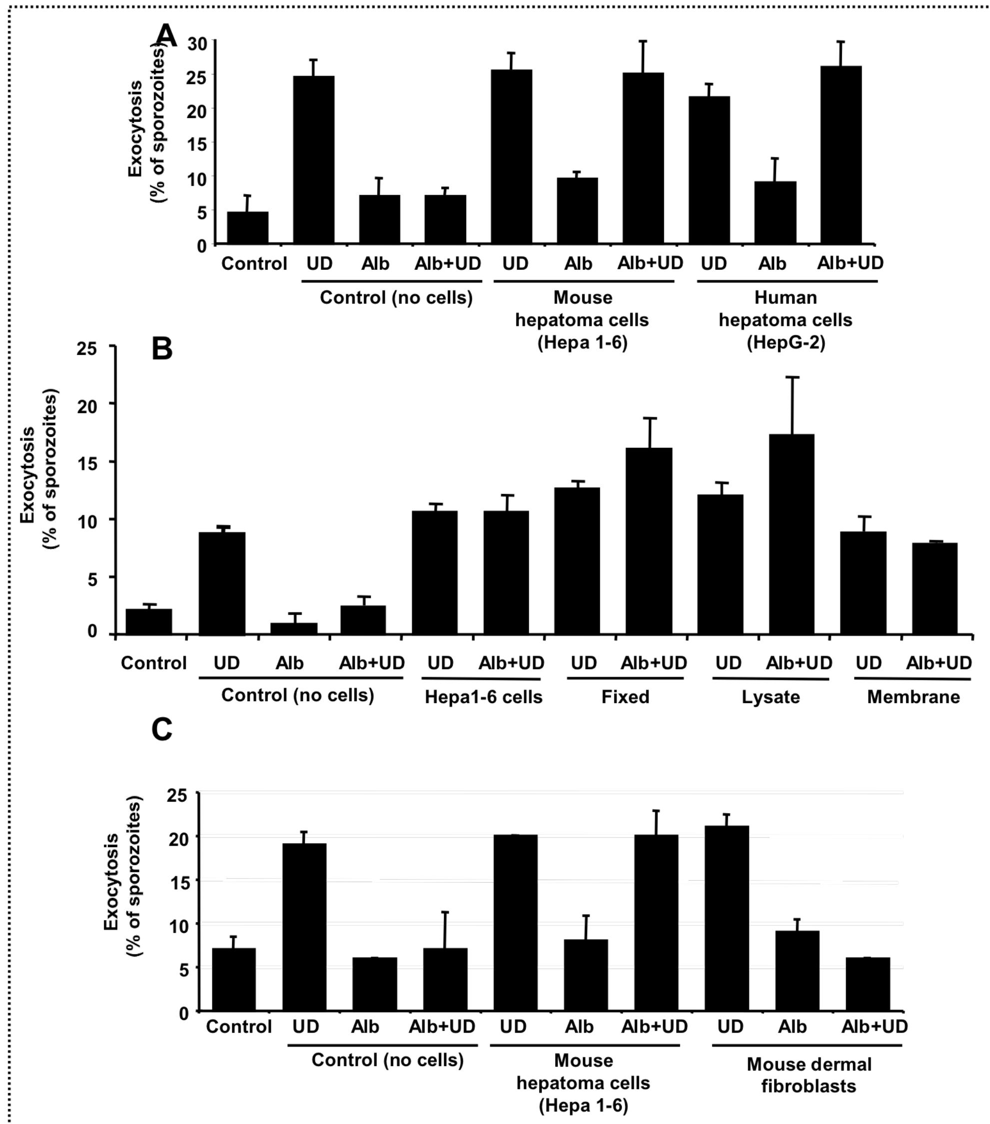


Figure 2.8| The inhibitory effect of albumin on sporozoite exocytosis is reversed in the presence of hepatocytes. (A) Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis. Sporozoites were pre-incubated or not with mouse albumin (1 mg/ml), washed and incubated with BDM to inhibit parasite motility before incubation with monolayers of mouse (Hepa1-6) and human (HepG2) hepatoma cell lines, in the presence or absence of the uracil derivatives (UD). As negative control in each condition, we used sporozoites incubated with albumin (Alb) but not stimulated with UD. (B) *P. yoelii* sporozoites were pre-incubated or not with mouse albumin, washed and incubated with intact or fixed monolayers of mouse Hepa1-6 cells, a lysate or the membrane fraction of Hepa1-6 cells. (C) Sporozoites were pre-incubated or not with mouse albumin, washed and incubated with BDM before incubation with monolayers of mouse (Hepa1-6) or mouse dermal fibroblasts (MDF). Results are expressed as mean of triplicate determinations ± SD.

To analyze whether lack of exocytosis activation by skin cells actually results in lack of sporozoite activation for infection, we compared sporozoites after migrating through dermal fibroblasts and hepatocytes. *P. yoelii* sporozoites were added to filters containing confluent dermal fibroblasts or Hepa1-6 cells. Sporozoites that traversed the filters encountered Hepa1-6 cells on coverslips placed underneath. In this way, we could distinguish between sporozoites that migrated through Hepa1-6 cells or through dermal fibroblasts before encountering the cells on the coverslip.

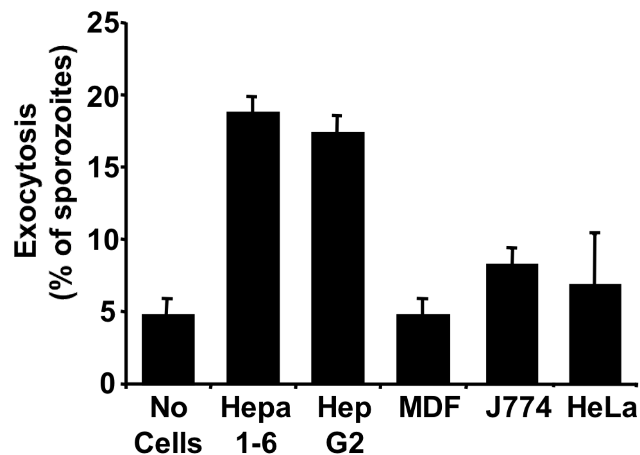


Figure 2.9| Migration through hepatocytes reverses the inhibitory effect of albumin on exocytosis. *P. yoelii* sporozoites were pre-incubated with mouse albumin, washed and added to filter insets containing the indicated cell types. Sporozoites were collected on empty coverslips placed underneath the filters in the lower chamber. Percentage of sporozoites in coverslips showing apical-regulated exocytosis is shown. Results are expressed as mean of triplicate determinations ± SD.

We found that sporozoites that traversed filters with Hepa1-6 cells migrated through fewer cells before infection in the coverslips when compared to sporozoites that migrated through dermal fibroblasts (Fig.2.10.A). Additionally, whilst sporozoites that migrated through Hepa1-6 cells appeared to be ready to infect host cells in

the coverslips underneath, with no need for further migration, the ones that migrated through dermal fibroblasts still required migration through Hepa1-6 in the coverslips to be infective. As an alternative way to analyze sporozoite infectivity after migration through different types of host cells, we incubated *P. yoelii* sporozoites with Hepa1-6 cells or mouse dermal fibroblasts for 30 min, before transferring them to new Hepa1-6 cell monolayers and analyze their infectivity.

Sporozoites pre-incubated with Hepa1-6 cells migrated through fewer cells before infection when they contacted cell monolayers a second time, as compared to sporozoites that migrated through mouse dermal fibroblasts that still needed to migrate through Hepa1-6 cells before infection (Fig.2.10.B).

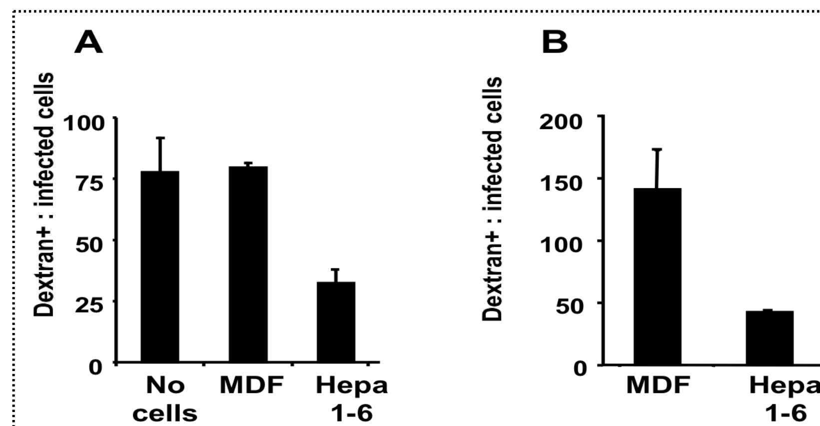


Figure 2.10| Migration through hepatocytes overturns inhibitory effect of albumin on sporozoites and activates them for infection.
(A) Hepa1-6 cells or MDF were cultivated on filters and coverslips with Hepa1-6 cells were placed underneath the filters in the lower chamber. *P. yoelii* sporozoites were added to the filter insets. As a control, sporozoites were added to filters containing no cells. The ratio of dextran-positive cells to infected cells is shown for coverslips placed under filters. (B) *P. yoelii* sporozoites were incubated with monolayers of Hepa1-6 cells or MDF, before transfer of the supernatants containing sporozoites to new Hepa1-6 monolayers. The ratio of dextran-positive cells to infected cells is shown for each condition. Results are expressed as mean of triplicate determinations ± SD.

These results suggest that while migration through hepatocytes activates sporozoites for infection, migration through dermal fibroblasts does not. Since all cells have high concentrations of uracil derivatives in their cytosol, these data are consistent with the existence of a regulatory mechanism that would allow exocytosis when sporozoites migrate through hepatocytes, but not through other cell types.

In order to determine whether this is specific for *P. yoelii* sporozoites or reflects a more general mechanism of the malaria parasite, we looked at the induction of exocytosis in *Plasmodium falciparum* sporozoites. *P. falciparum* is the human malarial parasite that causes most of the mortality associated with this disease. Its sporozoites also migrate through host cells (Mota et al. 2001b), but apical regulated exocytosis has not been studied in this species of the parasite. We observed that physiological concentrations of uracil and its derivatives also induce exocytosis in these sporozoites, which is inhibited by albumin (Fig.2.11.A).

We also found that migration through a hepatocyte cell line that is susceptible to infection by *P. falciparum* sporozoites (Sattabongkot et al. 2006) induces exocytosis, while migration through other cells does not activate sporozoites (Fig. 2.11.B).

These results suggest that *P. falciparum* sporozoites also activate exocytosis in response to uracil-derived nucleotides that they encounter in the cytosol of host cells during migration. Similarly to *P. yoelii,* exocytosis is also inhibited by albumin and seems to be reversed by the presence of hepatocytes, resulting in efficient activation of exocytosis.

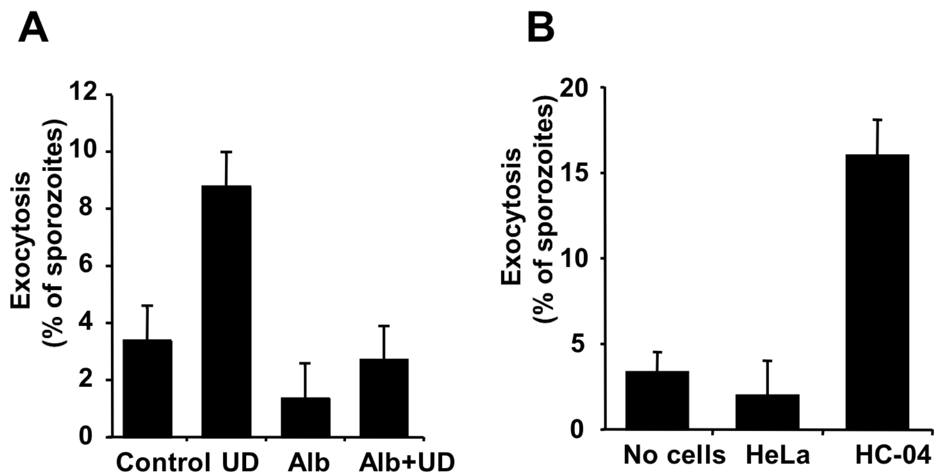


Figure 2.11| *P. falciparum* sporozoites apical regulated exocytosis is induced by uracil derivatives or migration through human hepatocytes and it is inhibited by human albumin. Percentage of *P. falciparum* sporozoites showing apical regulated exocytosis when pre-incubated with fatty-acid free human albumin followed by washing and (A) uracil derivatives (UD) or (B) addition to filter insets containing no cells, non-hepatic cells (HeLa) or the human hepatocyte cell line (HC-04). Sporozoites were collected on empty coverslips placed underneath the filters in the lower chamber. Results are expressed as mean of triplicate determinations ± SD.

2.2.4. Highly sulfated HSPGs in hepatocytes reverse inhibitory effect of albumin on sporozoite exocytosis.

As the interaction between HSPGs and CS protein determines the liver specificity for *Plasmodium* infection, we next tested whether HSPGs expressed on the surface of hepatocytes are responsible for the hepatocyte-specific reversion of the inhibitory effect of albumin. Previous work has shown that sporozoites bind to the heparan sulfate glycosaminoglycans (GAGs) of HSPGs (Frevert et al. 1993). In addition, the sulfate moieties of the GAGs are critical for sporozoite binding and a high overall density of sulfation is required (Pinzon-Ortiz et al. 2001). To determine whether highly sulfated heparan sulfate mediates the recovery from the inhibitory effect of albumin, we

treated Hepa1-6 cells with chlorate, a metabolic inhibitor of sulfation that decreases the extent of GAG sulfation (Humphries and Silbert 1988). Previous studies in hepatoma cells indicated that treatment with 10 mM and 30 mM chlorate decreased incorporation of $^{35}SO_4$-sulfate into proteoglycans by 60% and 75% respectively, with no effect on protein synthesis or cell growth (Pinzon-Ortiz et al. 2001).

We found that chlorate treatment decreases the ability to overcome the inhibitory effect of albumin, resulting in lack of exocytosis activation (Fig.2.12.A). Since chlorate is a general inhibitor of macromolecular sulfation, we also performed experiments with CHO (Chinese hamster ovary cells) cell mutants, derived from the parental cell line CHO K1. The mutant CHO pgsA lacks xylosyltransferase activity and produces less than 2% of wild-type levels of glycosaminoglycans. The mutant CHO pgsE has a mutation in N-deacetylase/N-sulfotransferase (Ndst1), which results in the formation of HS with less overall sulfation (Esko et al. 1985).

We observed that these CHO mutant cell lines, that present less HSPGs or low sulfation of these, have decreased ability to overcome the inhibitory effect of albumin, resulting also in lack of exocytosis activation (Fig.2.12.B). As expected, CHO *wt* cells that express HSPGs at lower levels than hepatocytes induce a partial reversion of the inhibitory effect of albumin. These results indicate that HSPGs are required to overcome the inhibitory effect of albumin on exocytosis and induce efficient sporozoite activation.

We next tested the effect of deficient HSPG expression and sulfation on the activation of exocytosis in sporozoites. When sporozoites migrate through cells with deficient sulfation or low HSPG expression, exocytosis

activation is no longer induced in these parasites whilst is efficiently induced after migration through hepatic cell lines (Fig.2.13.A).

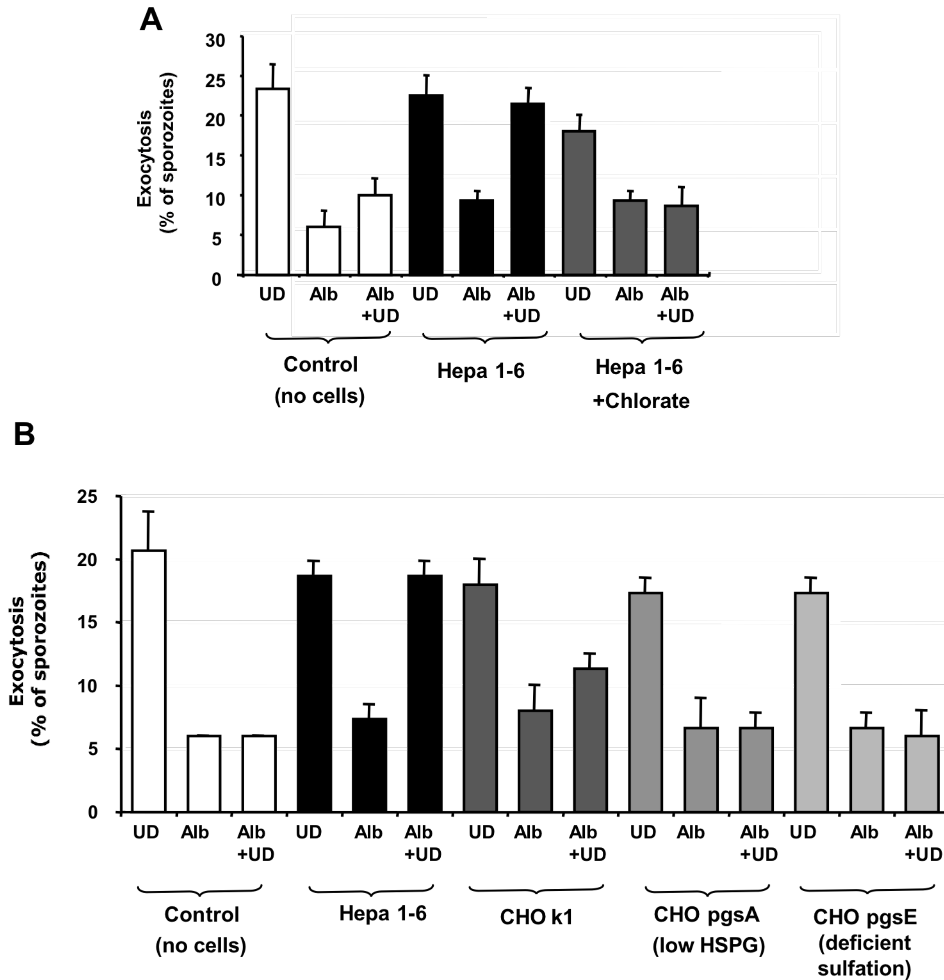


Figure 2.12| Inhibitory effect of albumin on sporozoite exocytosis is reversed in the presence of highly sulfated HSPGs present in hepatocytes. (A) Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis. Sporozoites were pre-incubated or not with mouse albumin (1 mg/ml), washed and incubated with BDM to inhibit parasite motility before incubation with monolayers of Hepa1-6 or Hepa1-6 treated with sodium chlorate **(B)** CHO k1, CHO pgsA or CHO pgsE cell lines, in the presence or absence of the uracil derivatives (UD). As negative control in each condition, we used sporozoites incubated with albumin (Alb) but not stimulated with UD. Results are expressed as mean of triplicate determinations ± SD

To determine if lack of exocytosis activation by cells with deficient HSPGs also results in lack of sporozoite activation for infection, we compared sporozoites after migrating through cells with low expression of HSPGs and hepatocytes. *P. yoelii* sporozoites were added to filter sets containing confluent CHO pgsA or Hepa1-6 cells. Sporozoites that traversed the filters encountered Hepa1-6 cells on coverslips placed underneath. We found that sporozoites that traversed filters with Hepa1-6 cells migrated through fewer cells before infection in the coverslips when compared to sporozoites that migrated through CHO pgsA cells (Fig.2.13.B).

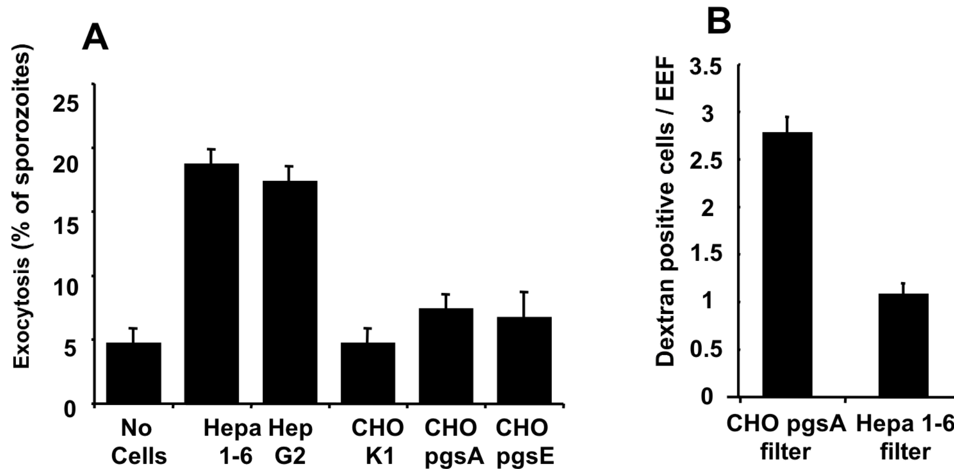


Figure 2.13| Migration through cells with highly sulfated HSPGs overcomes the inhibitory effect of albumin on exocytosis.(A) *P. yoelii* sporozoites were pre-incubated with mouse albumin, washed and added to filter insets containing the indicated cell types. Sporozoites were collected on empty coverslips placed underneath the filters in the lower chamber. Percentage of sporozoites in coverslips showing apical-regulated exocytosis is shown. (B) Hepa1-6 cells or CHO pgsA cell lines were cultivated on filters, and coverslips with Hepa1-6 cells were placed underneath the filters in the lower chamber. *P. yoelii* sporozoites were added to the filter insets. The ratio of dextran-positive cells to infected cells is shown for coverslips placed under filters. Results are expressed as mean of triplicate determinations ± SD

2.3. Discussion ~

The completion of a successful liver infection by *Plasmodium* sporozoites involves multiple steps, as these parasites need to traverse different host tissues before reaching the liver parenchyma where they finally invade a non-phagocytic cell, the hepatocyte. Sporozoites perform this journey with high rates of success, as very low numbers of sporozoites are able to initiate a malaria infection (Ungureanu et al. 1977). The capacity of sporozoites to sense their environment and react accordingly seems essential to complete this task with high efficiency. Signaling pathways are probably activated in sporozoites regulating activities such as motility, migration through cells and exocytosis. Our results suggest that *Plasmodium* sporozoites can sense and react to the extracellular environment modulating their infectivity.

We have found different molecules that regulate the behavior of *Plasmodium* sporozoites. Uracil, uridine and uracil-derived nucleotides, at concentrations that are normally found in the cytosol of mammalian cells, induce exocytosis in sporozoites and increase their infectivity. We have also characterized the regulation of this process.

Immediately after being injected into the dermis, sporozoites will encounter albumin, as this protein is found in the interstitial fluids of the dermis in high concentrations (Reed and Burrington 1989). In addition, the blood pool formed after mosquito bite (Sidjanski and Vanderberg 1997) must contain albumin normally present in serum. Albumin specifically induces *Plasmodium* sporozoites motility (Vanderberg 1974), suggesting that sporozoites are able to sense the presence of this protein. Albumin is not present in mosquitoes, where sporozoites move at a

slow speed (<2 μm/s) (Frischknecht et al. 2004), however, it is abundant in mammals, where sporozoites need to initiate active motility. At the same time, our results indicate that albumin prevents sporozoite exocytosis. These observations are consistent with the requirements of an infection *in vivo*, where sporozoites in the skin need to move actively in order to reach the circulation but also need to prevent premature activation of exocytosis before reaching the liver.

There are several observations suggesting that sporozoites migrate through cells in the dermis after mosquito inoculation. Intravital microscopy of the skin has revealed that sporozoites move through the dermis and through endothelial cells (Vanderberg and Frevert 2004; Amino et al. 2006). Additionally, mutant sporozoites with reduced ability to migrate through cells have low infectivity in the host when deposited in the dermis by mosquito bites (Bhanot et al. 2005). It has also been observed that sporozoites migrate through several hepatocytes in the liver before infecting a final one (Mota et al. 2001b; Frevert et al. 2005) and that mutant parasites with defective migration have reduced infectivity after intravenous injection (Ishino et al. 2004; Ishino et al. 2005b). As migration through cells leads to the activation of sporozoite exocytosis (Mota et al. 2002), albumin would prevent this process before sporozoites reach the liver. In fact, we found that migration through skin dermal cells does not induce exocytosis and does not activate sporozoites for infection. Sporozoites must enter in contact with high concentrations of uracil derivatives while migrating through the cytosol of these cells, but exocytosis is not induced, presumably due to the inhibitory effect of

albumin. Our results indicate that migration through cells can occur without sporozoite activation, a situation probably occurring *in vivo* during migration in the skin of the host.

We have confirmed that sporozoite stimulation and regulation of exocytosis is similar in *P. falciparum,* the human parasite with highest clinical importance. It seems likely that this is a common mechanism in different species of *Plasmodium*, as the molecules involved, uracil derived-nucleotides and albumin, are highly conserved among different host species (Baker 1989). It is noteworthy that *Plasmodium* uses these essential, highly conserved molecules to regulate its behavior towards infection. This may represent an advantage for the parasite, as it limits the possibility of encountering host variants that would be more resistant to infection.

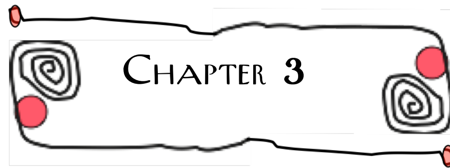
Plasmodium sporozoites may require specific surface receptors or transporters to respond to uracil derivatives. Several putative nucleoside transporters have been identified within the *P. falciparum* genome (Bahl et al. 2003), but only one (PfNT1) has been functionally characterized, showing preferential affinity for purines (El Bissati et al. 2006). Mammalian cells have pyrimidine receptors, the P2Y family, that activate signaling cascades and exocytosis in specific cell types (Brunschweiger and Muller 2006) however, no sequence homology is found for this type of receptor in the *Plasmodium* genome (Bahl et al. 2003). Our results also do not exclude the possibility of alternative signals to trigger exocytosis provided by host cells.

After reaching the liver, sporozoites need to undergo exocytosis to release or expose on their surface molecules necessary to invade hepatocytes forming a parasitophorous

vacuole. We have observed that after contact with hepatocytes, sporozoites recover their capacity to exocytose regardless of the presence of albumin. Accordingly, migration through hepatocytes induces sporozoite exocytosis, activating parasites for infection. The reversion of the inhibitory effect of albumin therefore must be necessary to establish an infection in the host, as there are high concentrations of albumin in the liver, both in the cytosol of hepatocytes and in interstitial tissues. The activation of exocytosis during migration through hepatocytes would also represent an advantage to the parasite, since molecules that are required for host cell invasion, such as TRAP, would only be exposed to the cytosol of traversed hepatocytes and not to the extracellular environment, avoiding the potential inhibitory effect of antibodies. In fact, although TRAP is required for host cell invasion, antibodies to TRAP do not inhibit the infectivity of sporozoites, even at high concentrations (Gantt et al., 2000).

Our results suggest that sporozoites are able to differentiate hepatocytes from other cell types. The reversion of albumin inhibitory effect appears to be mediated by HSPGs present in the surface of hepatocytes, as treatments that inhibit sulfation or mutant cells with low HSPGs or deficient sulfation fail to revert the inhibitory effect of albumin on sporozoite exocytosis. This mechanism would allow for the fine regulation of sporozoite activation, as it would only take place after sporozoites have reached their target cells in the liver. In this way, when sporozoites contact hepatocytes in the liver, HSPGs would interact with sporozoites making them susceptible to the stimulatory effect of nucleotides and resulting in exocytosis, which is required for infection of hepatocytes. Recently, it was shown that HSPGs in the

surface of hepatocytes induce signaling cascades in sporozoites, resulting in the cleavage of the surface protein CS and enhancing sporozoite infectivity (Coppi et al. 2007). It appears that HSPGs may be the key signaling event marking sporozoite recognition of the liver and triggering the initiation of mechanisms required for infection.



cAMP signaling in *Plasmodium* sporozoites Exocytosis and Infection.

3.1. Introduction~

Plasmodium belongs to the phylum apicomplexa, a group of parasites that share conserved mechanisms of motility and cell invasion machinery (Kappe et al. 1999). Apical exocytosis is another common feature that has been characterized in *Toxoplasma* tachyzoites (Carruthers and Sibley 1999) and sporozoites from *Eimeria* (Bumstead and Tomley 2000), *Cryptosporidium* (Chen et al. 2004) and *Plasmodium* (Gantt et al. 2000). This process has been most extensively studied in *Toxoplasma* tachyzoites, where active invasion of host cells involves the secretion of transmembrane adhesive proteins from the micronemes, which congregate on the anterior surface of the parasite and bind host receptors to mediate apical attachment (Carruthers 2006).

Sporozoites of different human and rodent *Plasmodium* species have the ability to migrate through host cells. Sporozoites enter and exit cells by breaching the plasma membrane of the traversed cell. This process results in sporozoites traversing host cells by moving through their cytosol without any surrounding membranes. Migration through host cells induces apical exocytosis in *Plasmodium* sporozoites, resulting in the exposure of high concentrations of TRAP/SSP2 in the apical end of the parasite (Mota et al. 2002). This process, similarly to *Toxoplasma* secretion of MIC2 (Huynh and Carruthers 2006), is thought to facilitate invasion of the host cell (Mota et al. 2002).

During migration through host cells sporozoites are not surrounded by any host membranes, and as a result, they are in direct contact with the cytosol of the host cell (Mota et al. 2001). Incubation of *Plasmodium* sporozoites with a lysate of host cells activates apical exocytosis in

the parasite, suggesting that host cell molecules induce the activation of exocytosis in migrating parasites (Mota et al. 2002). We have studied the role of uracil nucleotides in sporozoite exocytosis, since these molecules induce exocytosis in other cellular systems (Lazarowski et al. 2003) and are found in the cytosol of mammalian cells in high concentrations. We found that uracil and its derived nucleoside and nucleotides (UMP, UDP and UTP) at the physiological concentrations found in the cytosol of mammalian cells, activate apical regulated exocytosis and increase the infectivity of sporozoites (Chapter 2; Cabrita-Santos L. et al.). Addition of uracil derivatives *in vitro* induces apical regulated exocytosis within the first ten minutes after addition of the stimulus (Cabrita-Santos L. et al.). In certain mammalian cell types, UTP and UDP can activate signaling cascades by binding to P2Y receptors, which in turn can activate adenylyl cyclase and increase cAMP levels. Activation of P2Y receptors by nucleotides leads to exocytosis in different cells, from insulin release from pancreatic islet b cells to the release of histamine from mast cells (Abbracchio et al. 2006).

Here we have analyzed the role of the cAMP signaling pathway in sporozoite apical exocytosis and infection. We found biochemical evidences indicating that increases in cAMP levels in sporozoites mediate apical regulated exocytosis, which activates sporozoites for host cell invasion. A role for migration through cells and apical regulated exocytosis in infection was proposed before (Mota et al. 2002), but it had been questioned in view of transgenic sporozoites that were able to infect cells *in vitro* without performing the previous migration step (Ishino et al. 2004). Here we show that apical regulated exocytosis contributes significantly to host cell

invasion, but the parasite seems to have alternative mechanisms to establish successful infections in host cells.

3.2. Results~

3.2.1. Exocytosis in *P. yoelii*, *P. berghei* and *P. falciparum* sporozoites is mediated by increases in intracellular levels of cAMP.

To investigate the signaling pathways mediating *Plasmodium* sporozoite exocytosis, we used a mix of uracil and its derivatives (uridine, UMP, UDP and UTP) at the concentrations normally found in the cytosol of mammalian cells (described in Materials and Methods), which induces exocytosis in sporozoites (Chapter 2; Cabrita-Santos L. et al.). Apical regulated exocytosis has been characterized in *Plasmodium* sporozoites by the exposure of high concentrations of TRAP/SSP2 in the apical end of the parasite and also by the release of this protein into the medium (Mota et al. 2002). We confirmed that exocytosis occurs at the apical end of the sporozoite by staining the trails left behind after gliding motility. Trails are always behind the posterior end because sporozoites move with their apical end in the front (Fig. 3.1).

We first investigated whether cAMP induces or modulates sporozoite regulated exocytosis by preincubating *P. yoelii* sporozoites with a membrane permeant analogue of cAMP (8Br-cAMP). Exocytosis is quantified as the percentage of sporozoites that present a defined accumulation of extracellular TRAP/SSP2 in their apical end (Mota et al. 2002).

We found that 8Br-cAMP induces sporozoite exocytosis to a similar level than uracil derivatives. Addition of both stimuli to sporozoites did not increase the level of exocytosis (Fig. 3.2A), suggesting that both stimuli may

be using the same pathway to induce exocytosis. As an alternative way to increase cytosolic cAMP in sporozoites, we used forskolin, an activator of adenylyl cyclase (AC), the enzyme that synthesizes cAMP.

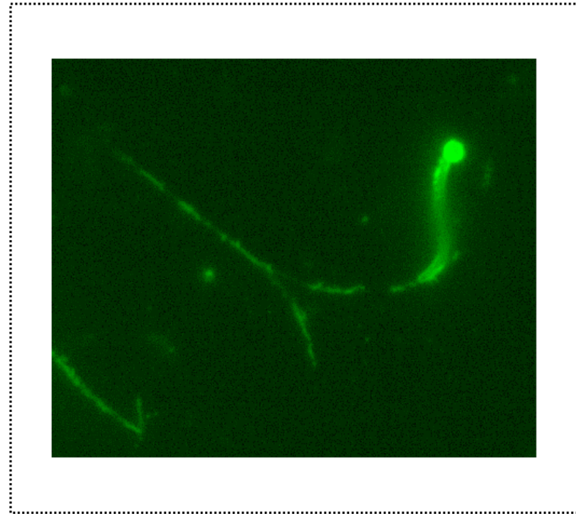


Figura 3.1| Exocytosis of TRAP occurs in the apical end of sporozoites. *P. berghei* sporozoites were incubated on coverslips coated with anti-CS antibodies for 20 min before addition of forskolin. After another 30 min, sporozoites were fixed and stained for CS protein.

This treatment also induced apical regulated exocytosis in sporozoites (Fig.3.2B). Incubation of sporozoites with MDL-12,330A, an inhibitor of AC (Guellaen et al. 1977) prevented activation of exocytosis by uracil derivatives (Fig.3.2B). We confirmed that this treatment did not increase sporozoite lysis when compared to the control (Table 3.1).

Genetically manipulated sporozoites that are deficient in their capacity to migrate through cells (*spect*-deficient), infect hepatic cell lines *in vitro*, questioning the role of migration through cells in the activation of sporozoites for infection (Ishino et al. 2004). In order to analyse the exocytosis response, these sporozoites were stimulated with uracil derivatives or treatments that modulate cAMP levels. Incubation of *P. berghei* *wt* or

spect-deficient sporozoites with uracil derivates induced apical regulated exocytosis. However, neither forskolin nor 8-Br-cAMP induced exocytosis in *spect*-deficient sporozoites and MDL-12,330A had only a partial effect in the inhibition of exocytosis (Fig. 3.3). These results suggest that, in contrast to *wt P. berghei* sporozoites, *spect*-deficient sporozoites do not use cAMP-mediated signaling pathways to activate exocytosis.

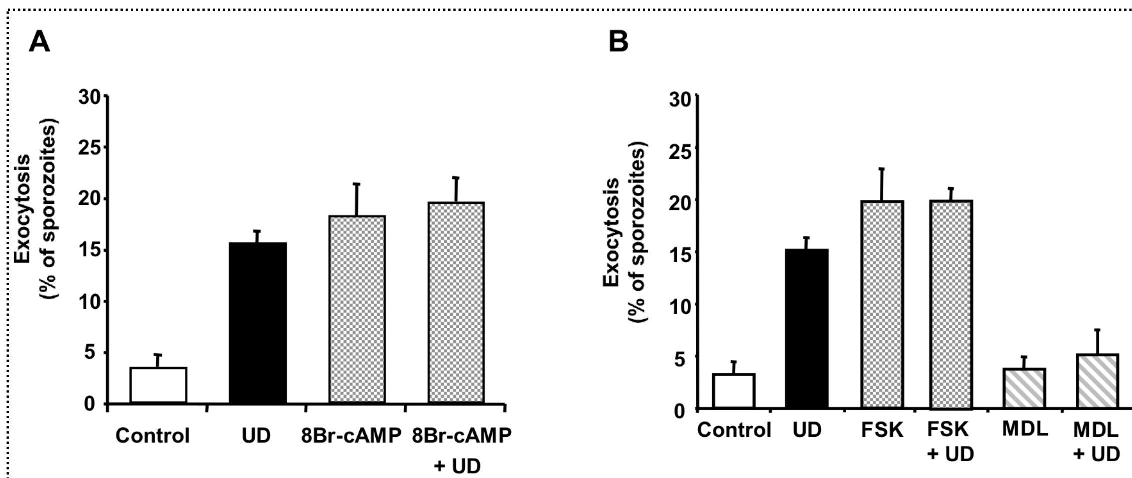


Figure 3.2| Increases in cytosolic cAMP induce exocytosis in *Plasmodium yoelii* exocytosis. *P. yoelii* sporozoites were pre-incubated for 15 min with 8Br-cAMP (A), forskolin (FSK) or MDL-12.330A (B) to activate or inhibit adenylyl cyclase respectively, followed by addition or not of uracil derivatives (UD). Sporozoites were incubated for 1 h before fixation and quantification of exocytosis. Results are expressed as mean of triplicates ± SD.

We have used the rodent malaria parasites *P. yoelii* and *P. berghei* as a model for *P. falciparum*, the human parasite responsible for the mortality associated with this disease. *P. falciparum* sporozoites also migrate through host cells (Mota et al. 2001), a process that induces apical regulated exocytosis in this species (Cabrita-Santos L. et al.). Similar to the rodent parasites, uracil and its derivatives induce exocytosis in *P. falciparum* sporozoites (Chapter 2; Cabrita-Santos L. et al.).

Here, we found that elevated cAMP levels also induce exocytosis in *P. falciparum* sporozoites and that exocytosis induced by uracil derivatives is inhibited by MDL-12,330A (Fig.3.4), suggesting that this pathway is conserved in the human and murine parasites.

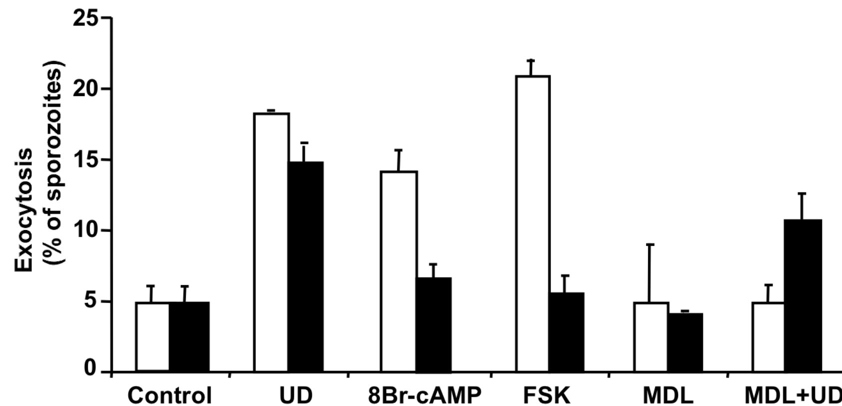


Figure 3.3| Exocytosis response in *P. berghei spect* 1- deficient sporozoites. *P. berghei wt* (white bars) or *spect 1*-deficient (black bars) sporozoites were pre-incubated for 15 min with 8Br-cAMP, forskolin (FSK) or MDL-12.330A to activate or inhibit adenylyl cyclase respectively, followed by addition or not of uracil derivatives (UD). Sporozoites were incubated for 1 h before fixation and quantification of exocytosis. Results are expressed as mean of triplicates ± SD.

To directly demonstrate that cAMP levels are increased in *P. yoelii* sporozoites in response to exocytosis-inducing stimuli, we measured cAMP concentration in sporozoites after incubation with uracil derivatives. Salivary glands dissected from uninfected mosquitoes and processed in a similar way, were used as negative control. We found that uracil derivatives significantly increase the levels of cAMP in sporozoites (Fig.3.5). No increases were found when control material from uninfected mosquitoes was stimulated with uracil derivatives (not shown).

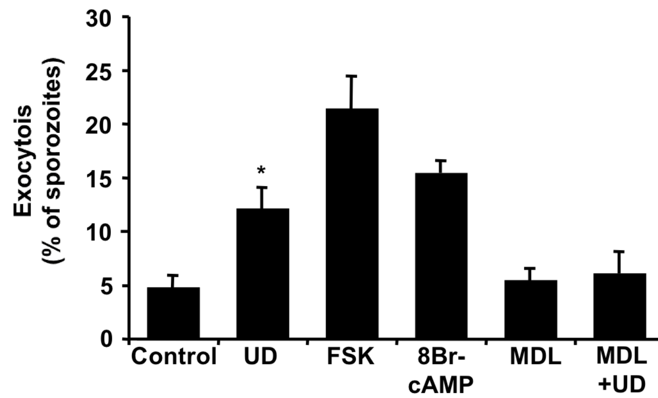


Figure 3.4| Increases in cAMP induce exocytosis in *Plasmodium falciparum* sporozoites. *P. falciparum* sporozoites were pre-incubated for 15 min with, forskolin (FSK), 8Br-cAMP or MDL-12.330A to activate or inhibit adenylyl cyclase, followed by addition or not of uracil derivatives (UD). Sporozoites were incubated for 1 h before fixation and quantification of exocytosis. Results are expressed as mean of triplicates ± SD.

Migration through host cells induces sporozoite apical regulated exocytosis, which activates sporozoites for infection. Stimulation of exocytosis by other means, such as host cells lysate (Mota et al. 2002) or uracil derivatives (Chapter2; Cabrita-Santos L. et al.), overcomes the need for extensive migration through cells and increases infection. To test whether stimulation of exocytosis by increases in intracellular cAMP in the sporozoite would also overcome the need for migration through host cells before infection, we incubated *P. yoelii* sporozoites with forskolin or 8Br-cAMP to induce regulated exocytosis before addition of sporozoites to intact Hepa1-6 cells. Migration through host cells is determined as the percentage of cells that are wounded by sporozoite migration and, as a result, become positive for a soluble impermeant tracer (dextran) (McNeil et al. 1999). We observed an increase in the number of infected cells, indicating that stimulation of regulated exocytosis by cAMP in sporozoites increases their infectivity

(Fig.3.6.A, black bars). In addition, activation of sporozoite exocytosis with increased cAMP levels reduces sporozoite migration through host cells, confirming that such extensive migration is no longer necessary when exocytosis is induced by elevations in the level of cAMP (Fig.3.6.A, white bars). These results indicate that cAMP-induced exocytosis contributes to the activation of sporozoites for infection.

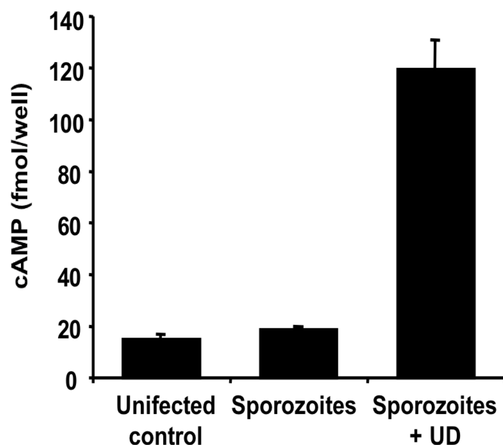


Figure 3.5| Intracellular levels of cAMP in *P. yoelii* sporozoites stimulated with UD. *P. yoelli* sporozoites were incubated or not with uracil derivatives for 45 min. Same number of uninfected salivary glands were processed in a similar way and used as a control (uninfected). Results are expressed as mean of triplicates ± SD.

Since sporozoites appear to activate the cAMP signaling cascade to stimulate apical regulated exocytosis, inhibition of cAMP production by MDL-12,330A, the inhibitor of AC, should decrease sporozoites infectivity. We actually found a significant reduction in their infectivity after treatment with this inhibitor (Fig.3.6.B). MDL-12.330A does not appear to have a toxic effect on sporozoites, since migration through cells was not affected (Fig.3.6.B).

3.2.2. PKA mediates sporozoites exocytosis and is activated downstream of cAMP .

The major downstream effector of cAMP is PKA, a serine/threonine kinase that activates other kinases and transcription factors in the cell. This protein is likely to be present in *Plasmodium* because PKA activity has been

detected in *P. falciparum* during the blood stage of the parasite (Syin et al. 2001; Beraldo et al. 2005). In addition, a gene sequence with high homology to PKA is expressed in *P. falciparum* and conserved in all species of *Plasmodium* analyzed (Li and Cox 2000; Bahl et al. 2003). However no functional assays have yet determined the PKA activity of this putative protein.

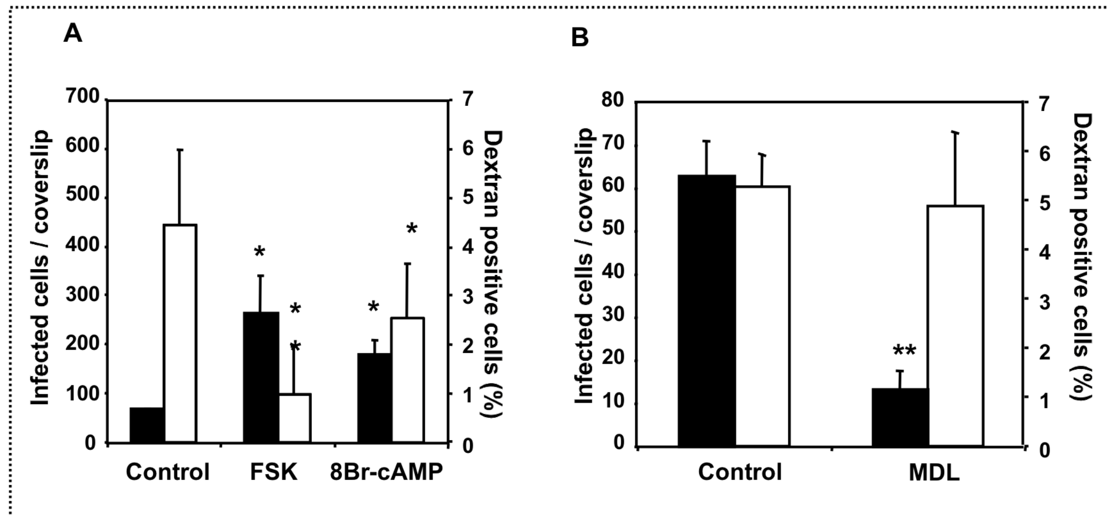


Figure 3.6| Stimulation of exocytosis mediated by cAMP increases sporozoite infection and decreases migration through host cells. *P. yoelii* sporozoites were pretreated with forskolin or 8Br-cAMP (A) or MDL-12.330A (B) before addition to monolayers of Hepa1-6 cells. Percentage of dextran-positive cells (white bars) and number of infected cells/coverslip (black bars) are shown as mean of triplicates ± SD. *, $p < 0.05$; ** $p < 0.01$ when compared to control by ANOVA.

To investigate whether sporozoite exocytosis is mediated by PKA activity, we treated sporozoites with H89, a PKA inhibitor already shown to inhibit this kinase in a different stage of the parasite (Syin et al. 2001; Beraldo et al. 2005). We found that H89 inhibits sporozoite exocytosis induced by uracil derivatives (Fig.3.7.A), suggesting that this process is mediated by the activation of PKA. The infectivity of sporozoites pretreated with H89 is reduced, probably as a consequence of the inhibition of exocytosis (Fig.3.7.B), while parasite migration through

host cells is not affected, confirming that H89 treatment is not toxic for sporozoites (Fig.3.7.C).

Activation of PKA should occur after cAMP has been generated in the signaling cascade. To analyze this step of the pathway, we pretreated sporozoites with H89 before increasing cAMP levels with the addition of 8Br-cAMP. As expected, we found that exocytosis was fully inhibited (Fig.3.7.D), suggesting that PKA is activated down-stream of cAMP. Incubation of sporozoites with genistein, an inhibitor of tyrosine kinases, did not affect regulated exocytosis (Fig.3.7.E), indicating that tyrosine kinases are not involved in the signaling cascade. In fact, no sequences with homology to tyrosine kinases have been found in the *Plasmodium* genome (Bahl et al. 2003)

To strengthen the evidence that the cAMP signaling pathway mediates the activation of exocytosis in sporozoites and at the same time reduce the possible non-characterized effects of the inhibitors on exocytosis, we made use of alternative inhibitors with unrelated chemical structures from the ones used previously. We found similar inhibitory results using 2′, 5′-Dideoxyadenosine or SQ22536, which inhibit adenylyl cyclase. The addition of a competitive inhibitor of cAMP (cAMP Rp-isomer), which inhibits PKA, also results in inhibition of apical regulated exocytosis in sporozoites (Fig.3.7.F).

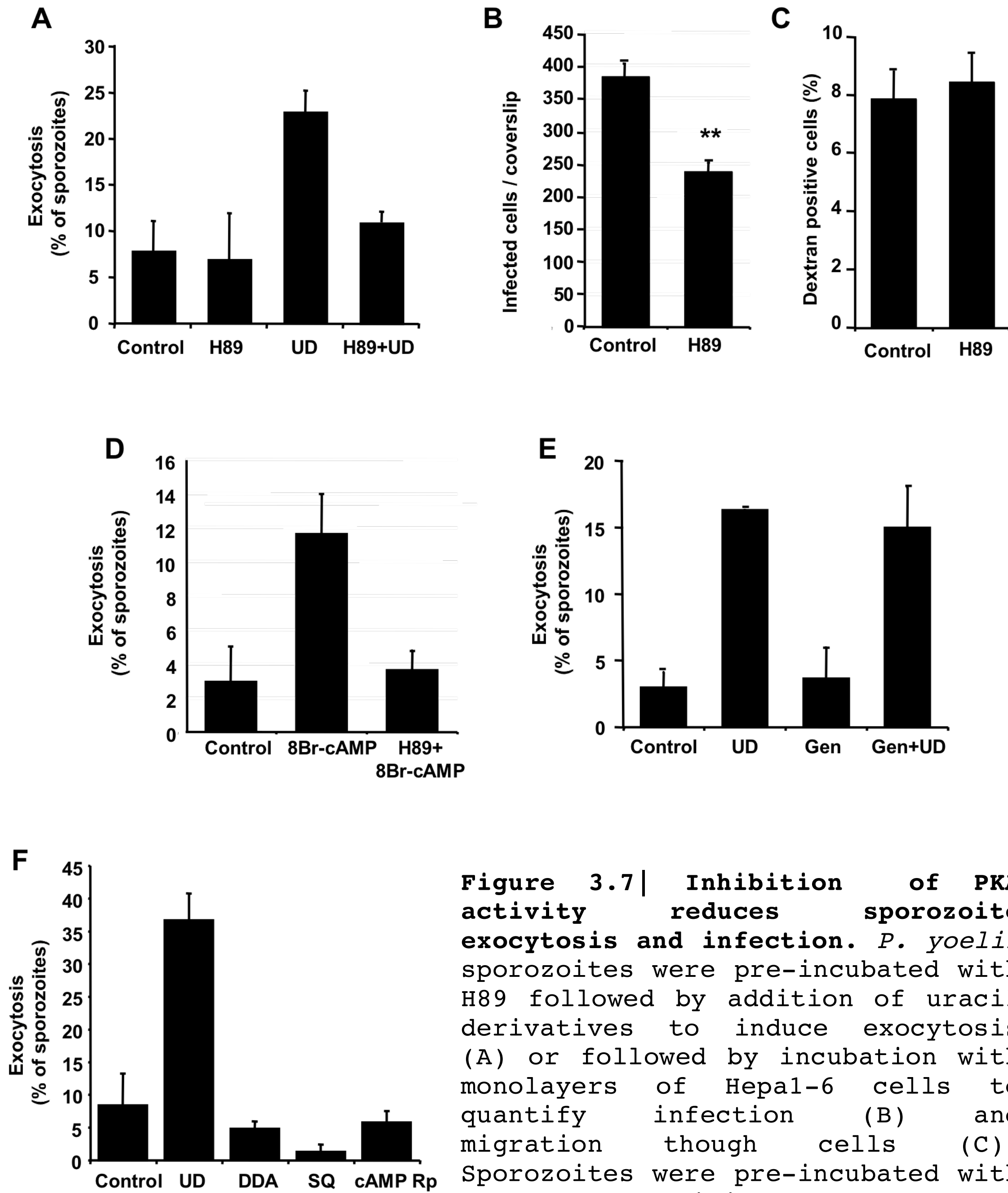


Figure 3.7| Inhibition of PKA activity reduces sporozoite exocytosis and infection. *P. yoelii* sporozoites were pre-incubated with H89 followed by addition of uracil derivatives to induce exocytosis (A) or followed by incubation with monolayers of Hepa1-6 cells to quantify infection (B) and migration though cells (C). Sporozoites were pre-incubated with H89 before addition of 8Br-cAMP to induce exocytosis (D). Sporozoites were pre-incubated with genistein (Gen) before addition of uracil derivatives (E). *P. yoelii* sporozoites were pre-incubated with 2′, 5′-Dideoxyadenosine (DDA) or SQ22536 (SQ) to inhibit adenylyl cyclase activity or with cAMP Rp-isomer to inhibit PKA, before addition of uracil derivatives to induce exocytosis(F). Results are expressed as mean of triplicates ± SD. ** $p < 0.01$ when compared to control by ANOVA.

3.3.3. Extracellular K^+ is required for sporozoites exocytosis.

Since cAMP signaling appears to mediate the activation of apical exocytosis, we searched for ACs in the malaria genome. Two different genes with high homology to ACs (ACα and ACβ) have been identified in *Plasmodium*. In particular, ACα was shown to have AC activity in *P. falciparum* (Muhia et al. 2003; Weber et al. 2004). Interestingly, ACα genes from *Plasmodium, Paramecium* and *Tetrahimena* are closely related and their sequence includes a domain with high homology to K^+ channels (Weber et al. 2004). In *Paramecium*, where the purified AC protein also has K^+ channel activity, generation of cAMP is regulated by K^+ conductance (Schultz et al. 1992). It is thought that ACα presents a transmembrane K^+-channel domain and an intracellular AC domain, which are functionally linked (Baker 2004).

Given that cAMP in *Plasmodium* sporozoites induces apical exocytosis, we first tested whether extracellular K^+ is required for this process. High concentrations of K^+ are found in the cytosol of eukaryotic cells; therefore sporozoites are likely to remain in a high K^+ during migration through cells (Alberts B 2002). The existence of K^+ channels has been predicted for *Plasmodium* parasites from electrophysiological (Allen and Kirk 2004) and genomic sequence data (Bahl et al. 2003).

To determine whether extracellular K^+ is required for sporozoite exocytosis, we stimulated exocytosis in *P.yoelii* sporozoites in either regular (containing K^+) or K^+-free medium. We found that exocytosis stimulated with uracil derivatives was inhibited in K^+-free medium (Fig.3.8.A). To confirm that sporozoites were not impaired by K^+-free medium incubation, sporozoites were transferred to regular medium after the K^+-free medium incubation. We found that exocytosis in these sporozoites was similar to

exocytosis in those that were never incubated in K^+-free medium (Fig.3.8.B). Moreover, pre-incubation of sporozoites with different K^+-channel inhibitors resulted in inhibition of exocytosis (Fig.3.8.C, D), suggesting that K^+ is required for the activation of this process.

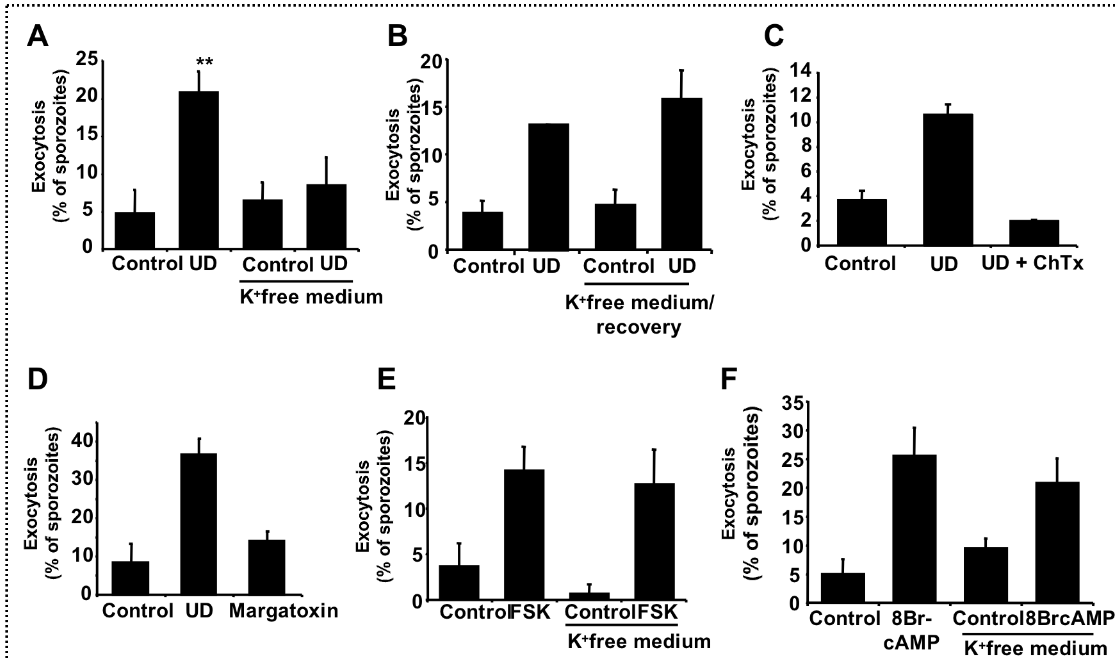


Figure 3.8| Extracellular K^+ is required for sporozoite apical regulated exocytosis. (A) *P. yoelii* sporozoites were pre-incubated for 15 min in regular medium or K^+-free medium before addition or not of uracil derivatives (UD) for 45 min. (B) Sporozoites were incubated with regular medium or K^+-free medium for 45 min, followed by incubation in regular medium in the presence or absence of UD for another 45 min. (C, D) Sporozoites were pre-incubated with the K^+-channel inhibitors charybdotoxin (C) or margatoxin (D) for 15 min before addition of UD for 45 min. (E,F) sporozoites were pre-incubated for 15 min in regular medium or K^+-free medium before addition or not of forskolin (E) or 8Br-cAMP (F). Results are expressed as mean of triplicates ± SD.

Next we analyzed the requirement for extracellular K^+ in sporozoite exocytosis induced by 8Br-cAMP or forskolin. We found that in these cases extracellular K^+ is not required (Fig.3.8.E, F), suggesting that extracellular K^+ is required upstream of cAMP in the signaling cascade. Removal of K^+ from the medium may alter the electrochemical

gradient of sporozoites affecting exocytosis induced by UD. However, since the response to forskolin and 8Br-cAMP in K^+ free medium is not affected, it suggests that the sporozoite exocytosis pathway is perfectly functional in the absence of extracellular K^+. Also, the viability and capacity of exocytosis response (Fig.3.8.B) of sporozoites after this treatment was found to be unaffected.

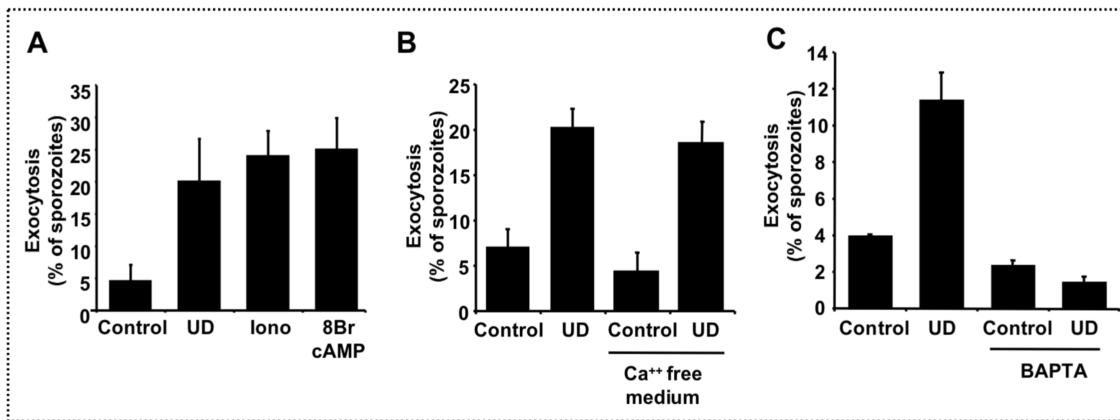


Figure 3.9| Extracellular Ca^{2+} is not required for sporozoites exocytosis. *P. yoelii* sporozoites were incubated with UD, ionomycin or 8Br-cAMP for 45 min (A). Sporozoites were pre-incubated for 15 min in regular medium or Ca^{2+}-free medium before addition or not of UD for 45 min. (B) Sporozoites were pre-incubated with the membrane permeant calcium chelator BAPTA-AM for 15 min before addition of UD for 45 min. Results are expressed as mean of triplicates ± SD.

A Ca^{2+} ionophore can induce apical regulated exocytosis in *P. yoelii* (Mota et al. 2002), suggesting that Ca^{2+} signaling may be involved in exocytosis. To test this, we first compared the magnitude of the cAMP-induced to the Ca^{2+}-induced exocytosis, and found no difference (Fig.3.9.A). To study whether Ca^{2+} is also involved in the signaling induced by UD, we induced exocytosis with UD in Ca^{2+}-free medium. Again, we found that exocytosis is not inhibited in Ca^{++}-free medium (Fig.3.9.B). Taken together these results suggest that extracellular Ca^{2+} is not

required for this process. However, when sporozoites were incubated with a membrane-permeant Ca^{2+} chelator, a strong inhibition of exocytosis was detected, suggesting that intracellular Ca^{2+} is required for exocytosis (Fig.3.9.C). A possible model for the signaling mediating exocytosis is proposed (Fig.3.10).

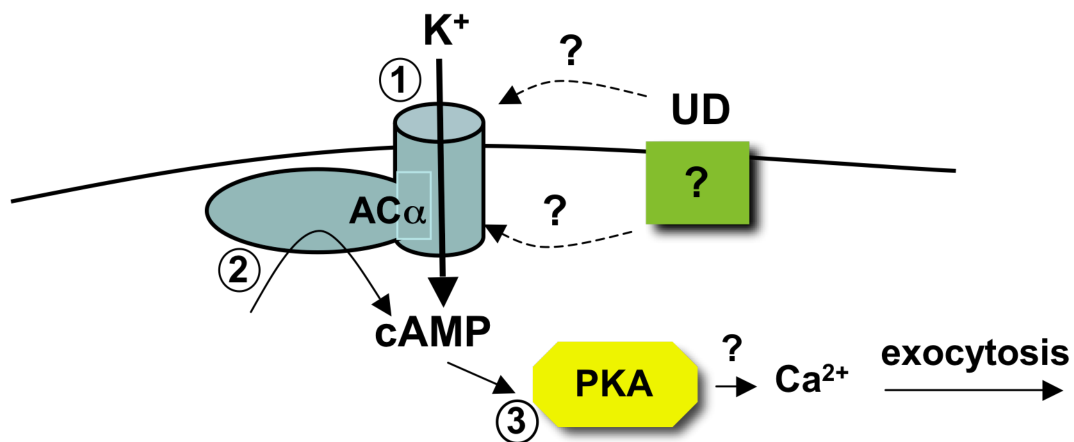


Figure 3.10| Possible model for the signaling cascade mediating exocytosis. Consistent with our results: UD activate directly or indirectly the K+ channel domain of ACα (1) and trigger the activation of AC activity (2). The increase in cAMP activates PKA (3), which leads to the activation of exocytosis.

Table 3.1| Sporozoite viability after drug treatment. *Plasmodium yoelii* sporozoites were incubated with the different conditions indicated. Dead sporozoites were quantified using propidium iodide staining. An untreated control was performed for each condition since the background do dead sporozoites may vary on each batch of dissected mosquitoes.

DRUGS	**% DEAD SPZ**
Uracil Derivatives	0.00
Control	2.13
Forskolin 100 μM	5.21
Control	3.57
8-Br-cAMP 500 μM	4.94
Control	1.40
MDL 100 μM	1.11
Control	3.09
SQ22536 50 μM	3.90
Control	4.24
Dideoxyadenosine 50 μM	0.00
Control	3.95
H-89 10 μM	3.83
Control	0.00
cAMP Rp isomer 5μM	1.72
Control	2.99
Charybdotoxin 100 nM	0.00
Control	2.50
Margatoxin 1nM	0.89
Control	1.12
K^+ free medium	4.66
Control	1.90
BAPTA 20 μM	1.71
Control	1.29
Control heated sporozoites	100.00

3.3. Discussion~

Using a rodent malaria model we have identified a role for cAMP signaling pathway in *Plasmodium* sporozoite exocytosis. The similar response observed in *P. falciparum* sporozoites suggests that the cAMP-dependent signaling pathway leading to exocytosis is conserved in the human parasite.

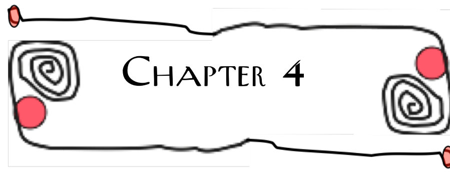
Regulated exocytosis in mammalian cells is frequently triggered by an elevation of intracellular Ca^{2+} levels and is modulated by cAMP, which acts synergistically with Ca^{2+}, but cannot induce exocytosis by itself. However, in some specific cell types exocytosis is triggered solely by elevations in cAMP concentrations (Fujita-Yoshigaki 1998). Increases in cytosolic Ca^{2+} induced with ionophores can trigger exocytosis in *Plasmodium* sporozoites (Mota et al. 2002), suggesting that Ca^{2+} stimulation is also sufficient to induce this process. The signaling pathways of Ca^{2+} and cAMP are interrelated inside eukaryotic cells (Borodinsky and Spitzer 2006). In particular, in *P. falciparum* blood-stages, a cross talk between Ca^{2+} and cAMP has been observed, where increases in cAMP induce the elevation of intracellular Ca^{2+} concentrations through the activation of PKA (Beraldo et al. 2005). Our results suggest that the cAMP and Ca^{2+} pathways are also interconnected in the sporozoite stage and that intracellular, but not extracellular Ca^{2+}, is required for exocytosis.

It has been previously observed that activation of sporozoite exocytosis increases their infectivity and reduces the need for migration through cells (Mota et al. 2002). This work confirms that activation of exocytosis by cAMP-mediated pathways increases exocytosis infectivity reducing migration through cells. Accordingly, inhibitors of this pathway inhibit sporozoites regulated exocytosis

and decrease their infectivity. Interestingly, *spect*-deficient sporozoites, which do not migrate through host cells (Ishino et al. 2004), responded to uracil derivatives but were not able to respond to either an activator of AC or to a permeant analogue of cAMP, suggesting that cAMP-induced signaling leading to exocytosis is different in these mutant sporozoites. The positive exocytosis response observed in the presence of the inhibitor of AC, indicates that these parasites are able to respond to uracil derivatives by activating cAMP-independent pathways that are not normally activated in *wt* sporozoites, where cAMP is required for exocytosis. It is still not clear how this relates to their impaired capacity to migrate through cells, but suggests that they may up-regulate the alternative mechanisms that are independent of migration through cells and exocytosis to infect hepatocytes. These results are consistent with the concept that sporozoites can use alternative pathways to invade hepatocytes, as the infection experiments with *PbACα*- sporozoites suggest (Ono et al. 2008).

Two genes with high homology to ACs have been identified in the *Plasmodium* genome: *ACα* and *ACβ* (Baker 2004). ACα activity as an AC has been demonstrated for *P. falciparum*, where the catalytic domain was expressed independently (Muhia et al. 2003). Interestingly, the *ACα* gene contains a N-terminal domain with high homology to voltage-gated K^+ channels. Other apicomplexans and also the ciliates *Paramecium* and *Tetrahymena* have an *ACα* gene homologous to the one in *Plasmodium* (Weber et al. 2004). In *Paramecium* it has been demonstrated that the purified ACα protein also has K^+ channel activity, and the generation of cAMP is regulated by K^+ conductance (Schultz et al. 1992). Although functional K^+ channel activity has not been demonstrated for ACα in *Plasmodium*, our results are consistent with a

role for K^+ conductance in sporozoite exocytosis. Uracil derivates do not induce exocytosis in K^+ free medium, but activation of AC with forskolin or addition of the permeant analogue of cAMP overcomes the requirement for extracellular K^+. Therefore, it seems likely that increased K^+ permeability may induce activation of ACα and synthesis of cAMP.



General Discussion

4.1. Discussion~

The completion of a successful liver infection by *Plasmodium* sporozoites involves multiple steps, as these parasites need to traverse different host tissues before reaching the liver parenchyma where they finally invade a non-phagocytic cell, the hepatocyte. Sporozoites perform this journey with high rates of success, as very low numbers of sporozoites are able to initiate a malaria infection (Ungureanu et al. 1977). The capacity of sporozoites to sense their environment and react accordingly seems essential to complete this task with high efficiency.

In this study we were particularly interested in the signaling pathways regulating sporozoites activities such as motility, migration through cells and exocytosis. Our results suggest that *Plasmodium* sporozoites can sense and react to the extracellular environment modulating their infectivity.

The role of exocytosis of apical organelles in invasion of host cells has been extensively studied in *Toxoplasma* tachyzoites. Our knowledge of *Plasmodium* sporozoite exocytosis and infection is less advanced, as this parasite stage can only be obtained by dissection of infected mosquitoes, and this procedure provides limited numbers of sporozoites.

Exocytosis of apical organelles is associated with apicomplexan parasite invasion of host cells. Different stages of *Plasmodium*, *Eimeria* and *Toxoplasma* present apical exocytosis triggered by incubation with host cells (Bannister and Mitchell 1989; Carruthers et al. 1999; Carruthers and Sibley 1999; Bumstead and Tomley 2000; Mota et al. 2002) or Ca^{2+} ionophores (Carruthers and Sibley

1999; Mota et al. 2002). Previous work on *Plasmodium* sporozoites showed that signaling exocytosis is induced by signals provided during migration through host cells (Mota et al. 2002). Most probably this process takes place while sporozoites migrate through hepatocytes in the liver before infection occurs. Here we have identified uracil-derived nucleotides as host molecules that can signal in the sporozoite inducing apical exocytosis. In other related parasites such as *Plasmodium* merozoites and *Toxoplasma* tachyzoites that do not migrate through host cells before infection, exocytosis is induced after contact with the host cell membrane (Carruthers and Sibley 1997; O'Donnell and Blackman 2005). Since exocytosis in *Plasmodium* sporozoites is activated during the process of migration through cells (Mota et al. 2002) this form of the parasite may have specific surface receptors or transporters to respond to uracil derivatives or other host signaling molecules that can trigger exocytosis . Several putative nucleoside transporters have been identified within the *Plasmodium falciparum* genome (Bahl et al. 2003), but only one (PfNT1) has been functionally characterized, showing preferential affinity for purines (El Bissati et al. 2006). Mammalian cells have pyrimidine receptors, the P2Y family, that activate signaling cascades and exocytosis in specific cell types (Brunschweiger and Muller 2006) however, no sequence homology is found for this type of receptor in the *Plasmodium* genome (Bahl et al. 2003).

Additionally, we found that, in sporozoites, the signaling induced by uracil derivatives leading to exocytosis is mediated by elevated levels of cAMP. In mammalian cells, regulated exocytosis is frequently triggered by an elevation of intracellular Ca^{2+} levels and is modulated by cAMP, which acts synergistically with Ca^{2+},

but cannot induce exocytosis by itself. On the other hand, in some specific cell types exocytosis is triggered solely by elevations in cAMP concentrations (Fujita-Yoshigaki 1998). In the case of sporozoites, cAMP seems to be sufficient to trigger exocytosis. However, since elevations of intracellular Ca^{2+} are also able to induce exocytosis in sporozoites (Mota and Rodriguez 2002), it is likely that both pathways are interconnected in the parasite and act synergistically to achieve efficient activation for infection.

Activation of sporozoite exocytosis increases their infectivity and reduces the need for migration through cells (Mota and Rodriguez 2002). In our studies, we confirmed that activation of exocytosis by cAMP-mediated pathways increases sporozoite infectivity reducing migration through cells. Accordingly, inhibitors of this pathway prevent sporozoite exocytosis and decrease their infectivity. These results indicate that sporozoites need activation of exocytosis for host cell invasion, and that this activation is provided by stimulation of the cAMP pathway. The physiological ligands capable of stimulating Ca^{2+} signaling in the sporozoite are not yet known, although uracil derivatives are possible candidates.

Genetically manipulated sporozoites that are deficient in their capacity to migrate through cells (SPECT), present very low infectivity of hepatocytes *in vivo*, but they are able to infect hepatic cell lines *in vitro*, questioning whether migration through cells is necessary to induce exocytosis before infection (Ishino et al. 2004; Amino et al. 2008). We have found that uracil and its derivatives induce apical regulated exocytosis in these mutant parasites. However, SPECT-deficient parasites show altered signaling responses and seem to use different signaling pathways to activate exocytosis that are not used by *wt*

sporozoites, suggesting that these parasites are activated using alternative mechanisms, which may be independent of migration through cells (Ono et al. 2008). Another factor contributing to the apparently contradictory results found using SPECT deficient sporozoites might be the fact that all SPECT mutants were performed in *P. berghei* background. Our experiments of sporozoite infectivity are performed with *P. yoelii,* a parasite that is more restricted to infection of hepatocytes, and therefore, more similar to *P. falciparum*. It is possible that the regulation of exocytosis and its role in infection is more important in *P. yoelii* infection than in *P. berghei*. Since we found that the ACα deficient sporozoites, which are also *P.berghei*, have only a 50% decrease in their infectivity (Ono et al. 2008), it is possible that alternative infection strategies, that are independent of apical exocytosis and are not regulated by migration through cells, are used by this strain of parasites.

Two genes with high homology to ACs have been identified in the *Plasmodium* genome (Baker 2004). The generation of ACβ -deficient parasites failed, as the gene seems to be essential for the asexual blood-stages of *Plasmodium* (Ono et al. 2008). *Pb*ACα- sporozoites were generated in our laboratory and it was found that ACα is required for the stimulation of apical exocytosis. *Pb*ACα- sporozoites are able to stimulate exocytosis in response to the permeant analogue of cAMP, but not to forskolin, the activator of ACs, confirming that the defect is caused by the lack of a functional AC and can be compensated by artificially increasing intracellular concentrations of cAMP (Ono et al. 2008). The results obtained with *Pb*ACα- sporozoites also suggest that ACα is sensitive to forskolin stimulation, as the increase in exocytosis induced by this drug is lost in the genetically deficient sporozoites.

Since AC activity is insensitive to forskolin in asexual blood-stages (Read and Mikkelsen 1991) and ACβ is preferentially expressed in this stage of the parasite cycle (Baker 2004), it seems likely that ACβ, rather than ACα, is required for cAMP formation during erythrocyte infection. Ono *et al* also found that the growth of *Pb*ACα- parasites in the asexual blood-stages was indistinguishable from control, consistent with the lack of activity of ACα during this stage.

Interestingly, the *ACα* gene contains a N-terminal domain with high homology to voltage-gated K^+ channels. Other apicomplexans and also the ciliates *Paramecium* and *Tetrahymena* have an *ACα* gene homologous to the one in *Plasmodium* (Weber et al. 2004). In *Paramecium* it has been demonstrated that the purified ACα protein also has K^+ channel activity, and the generation of cAMP is regulated by K^+ conductance (Schultz et al. 1992). Although functional K^+ channel activity has not been demonstrated for ACα in *Plasmodium*, our results are consistent with a role for K^+ conductance in sporozoite exocytosis. Uracil derivates do not induce exocytosis in K^+ free medium, but activation of AC with forskolin or addition of the permeant analogue of cAMP overcomes the requirement for extracellular K^+. Therefore, it seems likely that increased K^+ permeability may induce activation of ACα and synthesis of cAMP. Recently, it has been reported that exposure of *Plasmodium* sporozoites to the intracellular concentration of potassium enhances their infectivity (Kumar et al. 2007), reinforcing the role of host intracellular K^+ in sporozoite biology.

Our work represents one of the first studies describing signaling in *Plasmodium* sporozoites. As mentioned before, signaling is required for sporozoites to complete their

journey from the skin to the liver. Specific signals are required for parasites to traverse different tissues and specifically enter the liver in order to complete infection. How can a sporozoite differentiate skin cells from hepatocytes? Why do soporozoites enter the circulation rather than being lost in the skin? How can sporozoites specifically enter the liver and not other organs? How can sporozoites infect hepatocytes but not other cell types? All these questions are probably answered by the capacity of sporozoites to sense their environment by transducing signals from extracellular receptors. Well-coordinated signaling cascades that lead to specific reactions in the sporozoites are essential to achieve high levels of infectivity. For example, when mosquitoes deposit sporozoites their motility increases (Amino et al. 2006). This is likely mediated by albumin, known to increase sporozoite motility (Vanderberg 1974) and found in the skin of the mammalian host, but not in the mosquito. In this case albumin functions as the signal for the parasite, when in the mammalian host, to move rapidly to reach the liver. The same way, we found that albumin can also signal to prevent activation of exocytosis. In this case, host albumin signals to trigger parasite sensors, which consequently modify the behavior of the sporozoite towards a more efficient infection (i.e. higher motility with inhibition of exocytosis activation). Once in the liver sporozoites contact highly sulfated heparan sulfate proteoglycans (HSPGs) and as a consequence modify their behavior, resulting in the removal of albumin inhibition.

After contact with HSPGs, sporozoites are ready to be activated for exocytosis. At this point, the parasite migrates through several hepatocytes where, as mention above, uracil derivatives present in the cytosol, induce

exocytosis by activation of the cAMP cascade. The activation of this pathway leads to the fusion of micronemes with the apical end of the sporozoite and results in the extracellular exposure of adhesive molecules, therefore facilitating invasion of the hepatocyte. The regulation by albumin and HSPGs would then ensure that exocytosis only occurs after sporozoites have reached the liver and available host cells are in the surroundings. If this regulatory mechanism was not in place, activation of exocytosis in the skin would lead to premature activation and lack of efficient infection because there are no available hepatocytes. It is still not clear which signaling pathways mediate these regulatory mechanisms, but it is likely that the sporozoite signaling cascades are tightly regulated.

Recently, it was shown that signaling in *Plasmodium* sporozoites can be induced by HSPGs resulting in the cleavage of the sporozoites surface protein CS (Coppi et al. 2007). Since cleavage of CS is required for infection and is supposed to take place after the sporozoite arrival to the liver, it appears that HSPGs may constitute the recognition signal that sporozoites need to acknowledge that they have arrived to the liver. It is not clear yet the relation between apical exocytosis and cleavage of CS, however, since both events take place just before infection, are required for it and are regulated by HSPGs, it seems likely that they are related. It is possible that apical exocytosis may contribute to the activation of a protease that cleaves CS protein.

We have confirmed that sporozoite stimulation and regulation of exocytosis is similar in *P. falciparum,* the human parasite with highest clinical importance. It seems likely that this is a common mechanism in different strains of *Plasmodium*, as the molecules involved, uracil

derived-nucleotides and albumin, are highly conserved among different host species (Baker 1989). It is noteworthy that *Plasmodium* uses these essential, highly conserved molecules to regulate its behavior towards infection. This may represent an advantage for the parasite, as it limits the possibility of encountering host variants that would be more resistant to infection. Therefore, our results appear to be relevant for the human parasite *P. falciparum* and may be applied to understand the infection of humans by this parasite. We believe our results open a door to develop novel clinical interventions against malaria. The finding that exocytosis is stimulated by uracil derivatives and inhibited by albumin indicates that these molecules must have receptors in the sporozoite that could be used as drug targets for putative interventions.

4.2 Conclusions and perspectives ~

In summary we can conclude that the infection of hepatocytes by *Plasmodium* sporozoites is a tightly regulated mechanism that involves sensing of the environment by the sporozoite. Signaling cascades in the parasite are essential to achieve efficient infection and will provide a number of new targets for interventions against the disease.

When exocytosis is inhibited by the AC or the PKA inhibitors, the reduction in sporozoite infectivity is comparatively lower than the reduction in exocytosis. Similar results were obtained with the *Pb*ACα- sporozoites, where exocytosis is reduced to background levels, but infection is reduced by 50% (Ono et al. 2008). Taken

together these results suggest that sporozoites have alternative pathways to invade host hepatocytes that do not require apical regulated exocytosis. However, we cannot exclude the possibility that low levels of exocytosis, which are not sensitive enough for our assays, still occur in the *Pb*ACα- sporozoites and are sufficient to mediate infection of hepatocytes.

The analysis of host cell molecules required for sporozoite infection has provided evidence that sporozoites use more than one unique pathway to achieve hepatocyte infection (Silvie et al. 2007), suggesting that sporozoites may take advantage of this phenomenon to overcome polymorphisms in host receptors or to escape from immune mechanisms inhibiting one particular pathway of infection.

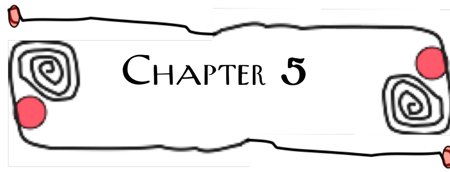
The next step in the development of this project is to identify of the parasite molecules that interact specifically with uracil derivatives and albumin to modulate infection. Theoretically, inhibition of a putative uracil receptor would impair infectivity of sporozoites. Similarly, inhibition of a putative albumin receptor in the sporozoite would also allow early activation of sporozoites inhibiting infection in the liver. In addition, the binding partner of HSPGs in the sporozoite is very well characterized, that is, the circumsporozoite (CS) protein (Rathore et al. 2002; Sinnis and Nardin 2002; Tewari et al. 2002). Currently, one of the anti-malaria vaccine design approaches is focusing in the inhibition of the interaction between CS protein and HSPGs in the liver, in an attempt to inhibit liver infection. Our findings suggest that this approach would

also lead to inhibition of sporozoite exocytosis activation having a stronger inhibitory effect.

So far, inhibition of infection of hepatocytes by sporozoites has proven a difficult task. One factor to consider is that when sporozoites migrate through host cells they are not accessible to the action of immune defense mechanisms such as antibodies or complement fixation. In addition, once sporozoites activate exocytosis, the exposure of critical molecules such as TRAP occurs only inside the cytosol of the traversed host cells. Consequently, essential parasite molecules are not exposed to antibodies that the host may use as defense against infection. The long co-evolution of *Plasmodium* parasites and humans suggest that the mechanisms of infection of this parasite are finely tuned to achieve the maximal efficiency of infection.

It is becoming apparent that *Plasmodium* has developed different redundant strategies to achieve similar milestones that are required for infection. Therefore, the parasite ensures that inhibition of one single pathway does not result in complete inhibition of infection. An example of this is the stimulation of exocytosis by both cAMP elevations and by Ca^{2+}. Even in the case of complete inhibition of a single cellular pathway required for infection, it seems likely that sporozoites would be able to up-regulate alternative pathways to achieve infection. These observations are promoting the idea that efficient intervention strategies should target more than one physiological target affecting more than one cellular process. Strategies that affect more than one stage of the parasite are also welcome because they would increase the effectiveness. Studies in this field have shown that some similar events take place in the invasion of hepatocytes by sporozoites and in the invasion of erythrocytes by

merozoites. Specific inhibitors or vaccines targeting these common events between sporozoites and merozoites are currently being evaluated. One of these mechanisms is the cleavage of surface proteins in sporozoites and merozoites that precedes infection, targeting exocytosis in *Plasmodium* could also be a common approach for the inhibition of both sporozoite and merozoite infectivity.



Materials and Methods

5.1. Materials~

5.1.1. Parasites.

Plasmodium yoelii yoelii sporozoites (cell line 17X NL), *P. berghei* ANKA *wt* and *spect-1* deficient sporozoites (Ishino et al. 2004) and the NF54 isolate (Ponnudurai et al. 1981) of *P. falciparum* were used to produce sporozoites in *A. stephensi* mosquitoes. Salivary glands were dissected from the mosquitoes. The *P. falciparum* sporozoites were extracted from the salivary glands, purified, and cryopreserved. Prior to being used in assays, the sporozoites were thawed and suspended in RPMI medium.

3-5 day-old *Anopheles stephensi* mosquitoes were fed on Swiss-Webster mice infected with either P. *yoelii*, *P. berghei* (ANKA wt or SPECT-1 mutant). On days 14 to 16 for *P. yoelii* and 18 to 20 for *P. berghei*, post-infective blood meal, mosquitoes were anesthetized on ice, rinsed in 70% ethanol, washed in RPMI 1640 medium (Gibco) and the salivary glands were removed. Tissue was mechanically disrupted and homozenized to free the parasites. The debris was pelleted by centrifugation at 80 x g for 3 minutes and sporozoites were collected, counted in a hemocytometer and maintained on ice until use.

5.1.2. Cells.

Hepa1-6 (ATCC CRL-1830), a hepatoma cell line derived from a C57L/J mouse, which is efficiently infected by rodent malaria parasites (Mota and Rodriguez 2000) was used for *in vitro* hepatocyte infections. Hepa1-6, HepG2 (ATCC, HB-8065; human hepatocelular carcinoma cell line), J774 (ATCC, TIB-67; monocyte/macrophage cell line) and HeLa cells were maintained at 37ºC with 5% CO_2 in DMEM medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1mM glutamine. HC-04 cells

were maintained as described (Sattabongkot et al. 2006). CHO cells were grown in Ham's F-12 medium supplemented with 7.5% FCS. Mouse dermal fibroblasts (MDF) were isolated from Balb/C mice as previously described (Freshney 2000) with some modifications. Briefly, 1 cm x 1 cm strips of skin from the back of a male Balb/C mouse were soaked in penicillin/streptomycin for 3 minutes before mincing into 1 mm x 1 mm pieces under sterile conditions. Skin pieces were then incubated in Liberase III (Roche Applied Sciences) in PBS for 1 hr at 37°C with agitation followed by grinding with PBS/0.2% BSA and centrifugation for 10 minutes at 100 x g. The tissue was then filtered through 70 mm mesh, centrifuged, resuspended in DMEM/FCS and transferred to a 25 cm2 culture flask.

5.1.3. HEPA1-6 CELL LYSATES.

Hepa1-6 cells (4 x 10^5 cells per ml) resuspended in culture medium were repeatedly passed through a 28G syringe until more than 95% of the cells were lysed, as determined by Trypan blue staining. For membrane extraction, the Hepa 1-6 cells lysate was centrifuged at 3,600 x g to remove debris and nuclei. The supernatant was centrifuged at 110,000 x g for 40 minutes to pellet the membrane fraction.

5.1.4. URACIL DERIVATIVES.

Exocytosis was induced by incubation of sporozoites with a mixture of physiological concentrations of uracil derivatives in the cytosol of mammalian cells (Traut 1994) consisting of 180 mM uracil, 280 mM uridine, 300 mM uracil monophosphate (UMP), 50 mM uracil diphosphate (UDP) and 30 mM uracil triphosphate (UTP) (ICN Biomedicals), prepared in RPMI 1640 and pH adjusted to 7.

5.2. Methods~

5.2.1. Chlorate treatment of cells.

Hepa1-6 cells were seeded on glass coverslips (2,5 x 10^5/ well) and grown overnight in a low sulfate medium (Ham's F-12, 1 mM glutamine and 2% FCS that had been dialyzed extensively versus 150 mM NaCl, 10 mM HEPES, [pH 7.3] with 20 mM of sodium chlorate (Sigma). An appropriate amount of medium was replaced with water to maintain normal osmolarity. Cells were washed twice with DMEM not containing chlorate next day.

5.2.2. Apical regulated exocytosis.

Plasmodium sporozoites (10^5 for *P. yoelii*, *P. berghei* or 5 x 10^4 for *P. falciparum*) were centrifuged for 5 minutes at 1,800 x *g* on glass coverslips before addition of uracil derivatives mixture or conditioned medium, with or without a monolayer of 2x10^5 Hepa1-6 cells, HepG2 cells or mouse dermal fibroblasts. In one experiment as indicated, Hepa1-6 cells were fixed with 4% paraformaldehide for 2 hours and washed before use. After 45 minutes incubation at 37 °C, sporozoites were fixed with 1% paraformaldehyde (non-permeabilization conditions) for 20 minutes before staining with anti-TRAP mAb (F3B5 for *P. yoelii* or *Pf*SSP2.1 for *P. falciparum* (Charoenvit et al. 1997) and a specific TRAP/SSP2 rabbit anti-serum for *P. berhgei*).

Sporozoite regulated exocytosis was quantified as the percentage of total sporozoites that present a TRAP/ SSP2 stained "cap" in their apical end. Results are expressed as mean of triplicate quantifications of a minimum of 50 sporozoites with standard deviation. Background level of exocytosis was measured in sporozoites after dissection from mosquitoes, before incubation *in vitro*. Background exocytosis was always lower than 8% and was subtracted from all values.

Digital pictures were acquired using an inverted Olympus 1x70 with a 63x oil-immersion objective at room temperature with a Hammatsu Photonics C4742-95 camera using Metamorph Imaging Systems software. Images were not modified other than adjustment of brightness and contrast to the whole image.

Albumin from mouse serum, essentially fatty acid-free human and mouse albumin (0.005% fatty acid content) solutions were prepared at 35 mg/ml in RPMI 1640. Gelatin from bovine skin was used at 35 mg/ml in RPMI 1640, alpha2-macroglobulin at 1.64 mg/ml and apo-transferrin at 2.5 mg/ml. All proteins were from Sigma. Sporozoites were pre-incubated with albumin or the other proteins for 15 minutes at room temperature in an eppendorf tube, spun down at 8,600 x*g* and resuspended in fresh medium before incubation with the uracil derivatives at 37ºC for 45 minutes. Rabbit anti-albumin antiserum (4-6 mg/ml) (Sigma) was pre-incubated for 1 h at 37ºC with mouse albumin at 1mg/ml before addition of the complex to sporozoites. When indicated, sporozoites were pre-incubated for 15 minutes with the myosin inhibitor butanedionemonoxime (BDM) (1 mM) to inhibit gliding motility.

5.2.3. Drug treatments.

Sporozoites (10^5) were incubated with 100 μM forskolin, 100 μM MDL-12.330A, 500 μM 8Br-cAMP, 10 μM H89, 30 μM genistein, 100 nM charybdotoxin, 50 μm SQ22536, 50 μm 2′,5′-Dideoxyadenosine, 5 μm Adenosine 3′, 5′-cyclic monophosphoriothioate 8Br-Rp-isomer, 1 nM margatoxin, 20 μM BAPTA, ionomycin 1μM (all from Calbiochem) before addition, or not, of uracil derivatives mixture for 1 hour, followed by fixation and quantification of exocytosis. For exocytosis assays, sporozoites were

pretreated with the drug for 15 minutes and concentrations were kept constant throughout the experiment. For infection and migration, treatment with drugs was performed for 15 minutes before washing and spinning sporozoites on Hepa1-6 cells grown on coverslips placed in 24-well dishes containing 1 ml of culture medium/well.

For assays in K^+-free medium: 10^5 *P. yoelii* sporozoites were incubated for 45 minutes in regular medium (RPMI 1640, that contains 5.3 mM KCl and 100 mM NaCl), K^+-free medium (modified RPMI 1640 with no KCl and 110 mM NaCl to maintain osmolarity) in the presence or absence of stimulus, before fixation and quantification of exocytosis. To assay sporozoites viability after incubation in K^+-free medium, sporozoites centrifuged at 20,800 x g and resuspended in regular medium with uracil derivatives to induce exocytosis. All experiments were performed twice showing similar results.

5.2.4. Determination of live/dead sporozoites with Propidium Iodide.

P. yoelii sporozoites were incubated with the indicated drugs for 20 minutes before addition of propidium iodide (1 mg/ml) for 10 minutes. Sporozoites were washed and observed directly with a fluorescence microscope. Propidium iodide positive sporozoites were considered dead and quantified. At least 100 sporozoites were counted in each condition.

5.2.5. Intracellular cAMP levels.

Intracellular levels of cAMP in *P. yoelii* sporozoites were determined using a cAMP Biotrack Enzymeimmunoassay system from Amersham Bioscience. For each sample 2 x 10^6 *P. yoelii* sporozoites were incubated with uracil derivatives for 45 minutes at 37ºC. All experiments were performed twice showing similar results.

5.2.6. Migration through cells and infection.

Sporozoites (10^5 sporozoites/coverslip) were added to monolayers of 2 x 10^5 Hepa1-6 cells for 1 hour in the presence of 1 mg/ml of rhodamine-dextran lysine fixable (10,000 MW; Molecular Probes). Sporozoites breach the plasma membrane of host cells during migration and as a result fluorescent dextran enters in their cytosol, allowing detection of wounded cells (McNeil et al. 1989; McNeil et al. 1999). In a different set of experiments, P. *yoelii* sporozoites (10^5 per coverslip) were added to monolayers of 2 x 10^5 Hepa1-6 cells or mouse dermal fibroblasts for 30 minutes. Sporozoites were then transferred to a new monolayer of Hepa1-6 cells and incubated for an additional 30 minutes in the presence of the tracer dextran. Cells were washed and incubated for another 24 hours before fixation and staining of infected cells with the mAb (2E6) recognizing HSP70 to detect infected cells (Tsuji et al. 1994), followed by anti-mouse IgG-FITC antibodies. Migration through host cells is quantified as percentage (or total number) of dextran-positive cells. Infection was quantified as the number of infected cells per coverslip. All experiments were performed twice showing similar results.

5.2.7. Transwell filter assays.

Cell lines or primary cultures of mouse dermal fibroblasts ($5x10^5$) were cultivated on 3 μm pore diameter Transwell filters (Costar, Corning, New York) until they form a continuous monolayer. Empty coverslips or coverslips containing Hepa1-6 cells monolayers ($2x10^5$ Hepa1-6) were placed underneath the filters. *P. yoelii* sporozoites ($2x10^5$) were added to filter insets containing Hepa1-6 cells, mouse dermal fibroblasts, other cell lines or no cells. Filters and coverslips were fixed after 2 h of incubation with sporozoites, before staining for

surface TRAP. To determine migration through host cells, FITC-dextran (1 mg/ml) was added before addition of sporozoites. Coverslips were washed after 2 h of incubation with sporozoites and further incubated for 24 h before fixation, staining and quantification of dextran positive cells and infected cells with anti-HSP70. All experiments were performed twice showing similar results.

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Appendix I

Adenylyl Cyclase α and cAMP Signaling Mediate *Plasmodium* Sporozoite Apical Regulated Exocytosis and Hepatocyte Infection

Takeshi Ono[1]◑, Laura Cabrita-Santos[1]◑, Ricardo Leitao[1], Esther Bettiol[1], Lisa A. Purcell[1], Olga Diaz-Pulido[1], Lucy B. Andrews[2], Takushi Tadakuma[3], Purnima Bhanot[4], Maria M. Mota[5], Ana Rodriguez[1]*

1 Department of Medical Parasitology, New York University School of Medicine, New York, New York, United States of America, **2** Sanaria Inc., Rockville, Maryland, United States of America, **3** Department of Parasitology and Immunology, National Defense Medical College, Tokorozawa, Saitama, Japan, **4** Department of Microbiology and Molecular Genetics, New Jersey Medical School, Newark, New Jersey, United States of America, **5** Faculdade de Medicina da Universidade de Lisboa, Instituto de Medicina Molecular, Lisboa, Portugal

Abstract

Malaria starts with the infection of the liver of the host by *Plasmodium* sporozoites, the parasite form transmitted by infected mosquitoes. Sporozoites migrate through several hepatocytes by breaching their plasma membranes before finally infecting one with the formation of an internalization vacuole. Migration through host cells induces apical regulated exocytosis in sporozoites. Here we show that apical regulated exocytosis is induced by increases in cAMP in sporozoites of rodent (*P. yoelii* and *P. berghei*) and human (*P. falciparum*) *Plasmodium* species. We have generated *P. berghei* parasites deficient in adenylyl cyclase α (*AC*α), a gene containing regions with high homology to adenylyl cyclases. *PbAC*α-deficient sporozoites do not exocytose in response to migration through host cells and present more than 50% impaired hepatocyte infectivity *in vivo*. These effects are specific to *AC*α, as re-introduction of *AC*α in deficient parasites resulted in complete recovery of exocytosis and infection. Our findings indicate that *AC*α and increases in cAMP levels are required for sporozoite apical regulated exocytosis, which is involved in sporozoite infection of hepatocytes.

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* E-mail: rodria02@popmail.med.nyu.edu

◑ These authors contributed equally to this work.

Introduction

Plasmodium, the causative agent of malaria, is transmitted by the bite of infected mosquitoes that inoculate the sporozoite form of the parasite in the host. Sporozoites rapidly migrate to the liver, where they infect hepatocytes, replicate and develop into merozoites, the blood-stage form of the parasite. *Plasmodium* belongs to the phylum apicomplexa, a group of parasites that share conserved mechanisms of motility and cell invasion machinery [1]. Apical exocytosis is another common feature that has been characterized in *Toxoplasma* tachyzoites [2] and sporozoites from *Eimeria* [3], *Cryptosporidium* [4] and *Plasmodium* [5]. This process has been most extensively studied in *Toxoplasma* tachyzoites, where active invasion of host cells involves the secretion of transmembrane adhesive proteins from the micronemes, which congregate on the anterior surface of the parasite and bind host receptors to mediate apical attachment [6]. One of these adhesive proteins, MIC2, which plays a central role in motility and invasion [7] is closely related to *Plasmodium* Thombospondin-Related Anonymous Protein, TRAP (also known as Sporozoite Surface Protein 2, SSP2) [8], which is also exposed in the apical end of the parasite upon microneme exocytosis [5,9] and is also required for *Plasmodium* sporozoite motility and invasion [10].

While in *Toxoplasma* tachyzoites microneme secretion is strongly up-regulated upon contact with the host cell, in *Plasmodium* sporozoites contact with host cells is not sufficient to activate this process and migration through cells is required to induce apical regulated exocytosis [9]. Sporozoites of different human and rodent *Plasmodium* species have the ability to migrate through host cells. Sporozoites enter and exit cells by breaching the plasma membrane of the traversed cell. This process results in sporozoites traversing host cells by moving through their cytosol without any surrounding membranes. Ultimately, sporozoites establish infection in a final hepatocyte through formation of a vacuole within which the parasite replicates and develops [9]. Migration through host cells induces apical exocytosis in *Plasmodium* sporozoites, resulting in the exposure of high concentrations of TRAP/SSP2 in the apical end of the parasite [9]. This process, similarly to *Toxoplasma* secretion of MIC2 [7], is thought to facilitate invasion of the host cell [9].

During migration through host cells sporozoites are not surrounded by any host membranes, and as a result, they are in direct contact with the cytosol of the host cell [11]. Incubation of *Plasmodium* sporozoites with a lysate of host cells activates apical exocytosis in the parasite, suggesting that host cell molecules induce the activation of exocytosis in migrating parasites [9]. We

Author Summary

Malaria is transmitted through the bite of an infected mosquito that deposits *Plasmodium* sporozoites under the skin. These sporozoites migrate from the skin into the circulation and then enter the liver to start a new infection inside hepatocytes. Sporozoites have the capacity to traverse mammalian cells. They breach their membranes and migrate through their cytosol. This process is required for infection of the liver and triggers the exposure of adhesive proteins in the apical end of sporozoites, a process that facilitates invasion of hepatocytes. We found that elevations of cAMP inside sporozoites mediate the exposure of adhesive proteins and therefore the infection process. Mutant sporozoites that do not express adenylyl cyclase, the enzyme that synthesizes cAMP, are not able to expose the adhesive proteins and their infectivity is reduced by half. Reinsertion of adenylyl cyclase gene in the mutant sporozoites recovers their capacity to expose adhesive proteins and to infect hepatocytes, confirming the specific role of this protein in infection. These results demonstrate the importance of cAMP and the exposure of adhesive proteins in sporozoites, but also show that *Plasmodium* sporozoites have other mechanisms to invade host hepatocytes that are not inhibited in the mutant parasites.

have studied the role of uracil nucleotides in sporozoite exocytosis, since these molecules induce exocytosis in other cellular systems [12] and are found in the cytosol of mammalian cells in high concentrations. We found that uracil and its derived nucleoside and nucleotides (UMP, UDP and UTP) at the physiological concentrations found in the cytosol of mammalian cells, activate apical regulated exocytosis and increase the infectivity of sporozoites [13]. Since sporozoites are in contact with the cytosol of the traversed host cells, it is likely that the high concentrations of uracil derivatives that they would encounter, probably participate in the activation of sporozoites during migration through cells. Addition of uracil derivatives *in vitro* induces apical regulated exocytosis within the first ten minutes after addition of the stimulus [13]. In certain mammalian cell types, UTP and UDP can activate signaling cascades by binding to P2Y receptors, which in turn can activate adenylyl cyclase and increase cyclic adenosine monophosphate (cAMP) levels. Activation of P2Y receptors by nucleotides leads to exocytosis in different cells from insulin release from pancreatic islet β cells to the release of histamine from mast cells [14].

Here we have analyzed the role of the cAMP signaling pathway in sporozoite apical exocytosis and infection. We found biochemical evidences indicating that increases in cAMP levels in sporozoites mediate apical regulated exocytosis, which activates sporozoites for host cell invasion. By creating a parasite line deficient in adenylyl cyclase α (ACα), we confirmed that the cAMP signaling pathway is essential to induce apical exocytosis, which is activated during migration through cells. In addition, this recombinant parasite provides a tool to determine the precise contribution of apical exocytosis to sporozoite infection. A role for migration through cells and apical regulated exocytosis in infection was proposed before [9], but it had been questioned in view of transgenic sporozoites that were able to infect cells *in vitro* without performing the previous migration step [15]. Here we show that apical regulated exocytosis contributes significantly to host cell invasion, but the parasite seems to have alternative mechanisms to establish successful infections in host cells.

Results

To investigate the signaling pathways mediating *Plasmodium* sporozoite exocytosis, we used a mix of uracil and its derivatives (uridine, UMP, UDP and UTP) at the concentrations normally found in the cytosol of mammalian cells (described in Experimental Procedures), which induce exocytosis in sporozoites [13]. Apical regulated exocytosis has been characterized in *Plasmodium* sporozoites by the exposure of high concentrations of TRAP/SSP2 in the apical end of the parasite and also by the release of this protein into the medium [9]. We confirmed that exocytosis occurs at the apical end of the sporozoite by staining the trails left behind after gliding motility. Trails are always next to the posterior end because sporozoites move with their apical end in the front (Fig. S1).

We first investigated whether cAMP induces or modulates sporozoite regulated exocytosis by preincubating *P. yoelii* sporozoites with a membrane permeant analogue of cAMP. Exocytosis is quantified as the percentage of sporozoites that present a defined accumulation of extracellular TRAP/SSP2 in their apical end [9]. We found that 8Br-cAMP induces sporozoite exocytosis to a similar level than uracil derivatives. Addition of both stimuli to sporozoites did not increase the level of exocytosis (Fig. 1A), suggesting that both stimuli may be using the same pathway to induce exocytosis. As an alternative way to increase cytosolic cAMP in sporozoites, we used forskolin, an activator of the enzyme that synthesizes cAMP, adenylyl cyclase (AC). This treatment also induced apical regulated exocytosis in sporozoites (Fig. 1B). Incubation of sporozoites with MDL-12,330A, an inhibitor of AC [16] prevented activation of exocytosis by uracil derivatives (Fig. 1B). We confirmed that these treatments did not increased sporozoite lysis compared to control (Table S1 and Fig. S2).

Genetically manipulated sporozoites that are deficient in their capacity to migrate through cells (*spect*-deficient) infect hepatic cell lines *in vitro*, questioning the role of migration through cells in the activation of sporozoites for infection [15]. To analyze the exocytosis response of these sporozoites, we stimulated them with uracil derivatives or treatments that modulate cAMP levels. Incubation of *P. berghei wt* or *spect*-deficient sporozoites with uracil derivates induced apical regulated exocytosis. However, forskolin and 8-Br-cAMP did not induce exocytosis in *spect*-deficient sporozoites and MDL-12,330A only has a partial effect in the inhibition of exocytosis (Fig. 1C). These results suggest that, in contrast to *wt P. berghei* sporozoites, *spect*-deficient sporozoites do not use cAMP-mediated signaling pathways to activate exocytosis.

We have used the rodent malaria parasites *P. yoelii* and *P. berghei* as a model for *P. falciparum*, the human parasite responsible for the mortality associated with this disease. *P. falciparum* sporozoites also migrate through host cells [11], a process that induces apical regulated exocytosis in this species of the parasite [13]. Similar to the rodent parasites, uracil and its derivatives induce exocytosis in *P. falciparum* sporozoites [13]. We found that elevated cAMP levels also induce exocytosis in *P. falciparum* sporozoites and that exocytosis induced by uracil derivatives is inhibited by MDL-12,330A (Fig. 1D), suggesting that this pathway is conserved in the human and murine parasites.

To directly demonstrate that cAMP levels are increased in *P. yoelii* sporozoites in response to exocytosis-inducing stimuli, we measured cAMP concentration in sporozoites after incubation with uracil derivatives. Salivary glands dissected from uninfected mosquitoes and processed in a similar way, were used as negative control. We found that uracil derivatives significantly increase the levels of cAMP in sporozoites (Fig. 1E). No increases were found

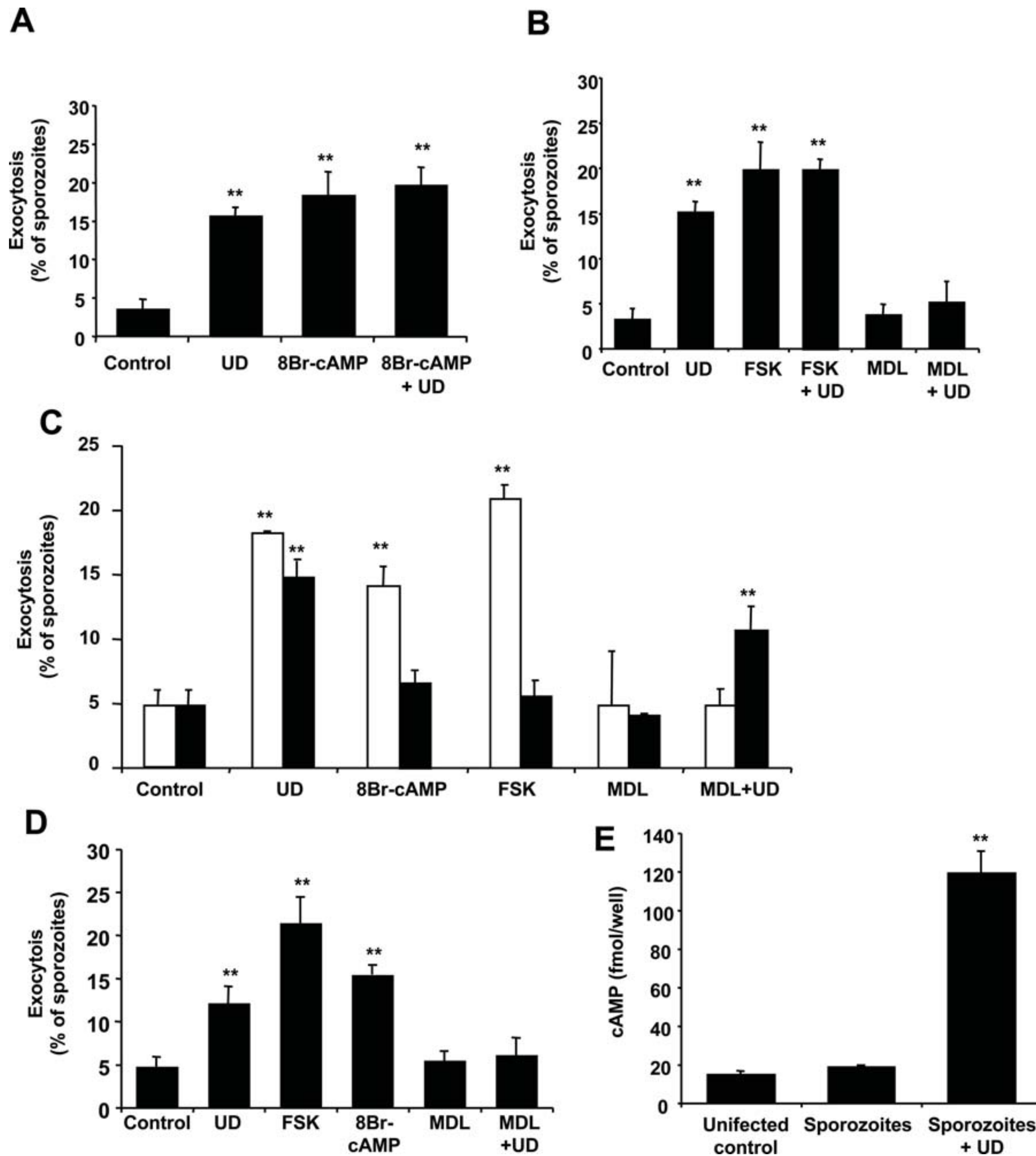

Figure 1. Increases in cytosolic cAMP induce *Plasmodium* sporozoite exocytosis. (A–B) *P. yoelii* sporozoites were pre-incubated for 15 min with 8Br-cAMP, forskolin (FSK) or MDL-12.330A to activate or inhibit adenylate cyclase respectively, followed by addition or not of uracil derivatives (UD). Sporozoites were incubated for 1 h before fixation and quantification of exocytosis. (C) *P. berghei wt* (white bars) or *spect 1*-deficient (black bars) sporozoites were pre-incubated with the different activators and inhibitors as in (A,B). (D) *P. falciparum* sporozoites were pre-incubated with the different activators and inhibitors as in (A,B). (E) Intracellular levels of cAMP in *P. yoelii* sporozoites incubated or not with uracil derivatives for 45 min. Same number of uninfected salivary glands were processed in a similar way and used as a control (uninfected). Results are expressed as mean of triplicates±SD. *, $p<0.05$; ** $p<0.01$ when compared to control by ANOVA.
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when control material from uninfected mosquitoes was stimulated with uracil derivatives (not shown).

Migration through host cells induces sporozoite apical regulated exocytosis, which activates sporozoites for infection. Stimulation of exocytosis by other means, such as host cells lysate [9] or uracil derivatives [13], overcomes the need for extensive migration through cells and increases infection. To test whether stimulation of exocytosis by increases in intracellular cAMP in the sporozoite would also overcome the need for migration through host cells before infection, we incubated *P. yoelii* sporozoites with forskolin or 8Br-cAMP to induce regulated exocytosis before addition of sporozoites to intact Hepa1-6 cells. Migration through host cells is determined as the percentage of cells that are wounded by sporozoite migration and as a result become positive for a soluble impermeant tracer (dextran) [17]. We found an increase in the number of infected cells, indicating that stimulation of regulated exocytosis by cAMP in sporozoites increases their infectivity (Fig. 2A, black bars). In addition, activation of sporozoite

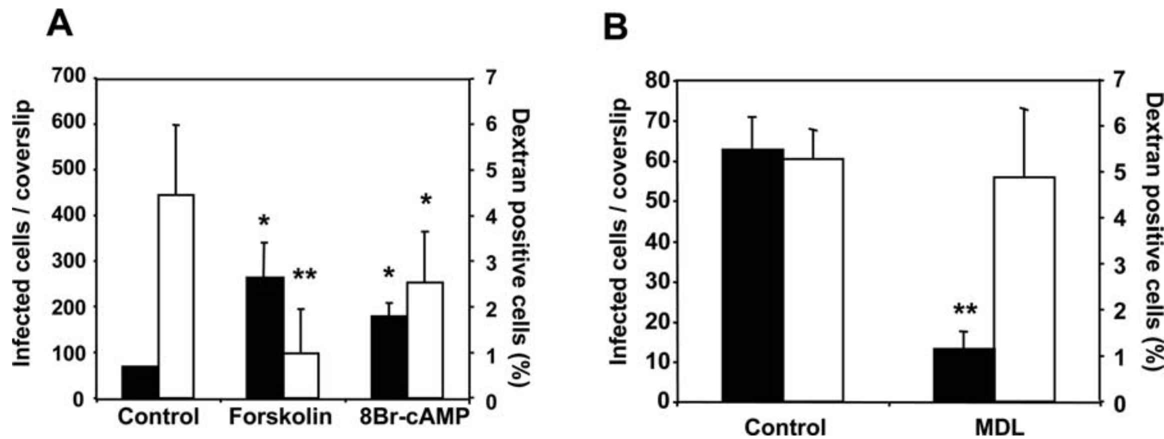

Figure 2. Stimulation of exocytosis increases sporozoite infection and decreases migration through host cells. *P. yoelii* sporozoites were pretreated with forskolin or 8Br-cAMP (A) or MDL-12.330A (B) before addition to monolayers of Hepa1-6 cells. Percentage of dextran-positive cells (white bars) and number of infected cells/coverslip (black bars) are shown as mean of triplicates±SD. *, $p<0.05$; ** $p<0.01$ when compared to control by ANOVA.

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exocytosis with increased cAMP levels reduces sporozoite migration through host cells, confirming that such extensive migration is no longer necessary when exocytosis is induced by elevations in the level of cAMP (Fig. 2A, white bars). These results indicate that cAMP-induced exocytosis contributes to the activation of sporozoites for infection.

Since sporozoites appear to activate the cAMP signaling cascade to stimulate apical regulated exocytosis, inhibition of cAMP production in sporozoites by MDL-12,330A, the inhibitor of AC, should decrease their infectivity. We actually found a significant reduction in their infectivity after treatment with this inhibitor (Fig. 2B). MDL-12.330A does not appear to have a toxic effect on sporozoites, since migration through cells was not affected (Fig. 2B).

We also observed that gliding motility of sporozoites is greatly decreased 18 to 24 min after addition of the exocytosis inducing stimulus (UD or forskolin), but not during earlier time points, while exocytosis is presumably occurring (0 to 8 min after addition of the stimulus) (Fig. S3).

The major downstream effector of cAMP is protein kinase A (PKA), a serine/threonine kinase that activates other kinases and transcription factors in the cell. This protein is likely to be present in *Plasmodium* because PKA activity has been detected in *P. falciparum* during the blood stage of the parasite [18,19] and there is a gene sequence with high homology to PKA expressed in *P. falciparum* and conserved in all species of *Plasmodium* analyzed [20,21], however no functional assays have yet determined the PKA activity of this putative protein. To investigate whether sporozoite exocytosis is mediated by PKA activity, we treated sporozoites with H89, a PKA inhibitor already shown to inhibit this kinase in a different stage of the parasite [18,19]. We found that H89 inhibits sporozoite exocytosis induced by uracil derivatives (Fig. 3A), suggesting that this process is mediated by the activation of PKA. The infectivity of sporozoites pretreated with H89 is reduced, probably as a consequence of the inhibition of exocytosis (Fig. 3B), while parasite migration through host cells is not affected, confirming that H89 treatment is not toxic for sporozoites (Fig. 3C).

Activation of PKA should occur after cAMP has been generated in the signaling cascade. To analyze this step of the pathway, we pretreated sporozoites with H89 before increasing cAMP levels with the addition of 8Br-cAMP. As expected, we found that exocytosis was completely inhibited (Fig. 3D), suggesting that PKA is activated down-stream of cAMP. Incubation of sporozoites with genistein, an inhibitor of tyrosine kinases, did not affect regulated exocytosis (Fig. 3E), indicating that tyrosine kinases are not involved in the signaling cascade. In fact, no sequences with homology to tyrosine kinases have been found in the *Plasmodium* genome [20].

To strengthen the evidence that the cAMP signaling pathway mediates the activation of exocytosis in sporozoites and reduce the probability of inhibitors affecting exocytosis due to non-characterized effects of the drugs, we used alternative inhibitors with unrelated chemical structures from the ones used before to inhibit adenylyl cyclase and PKA. We found similar inhibitory results using 2′, 5′-Dideoxyadenosine or SQ22536, which inhibit adenylyl cyclase. The addition of a competitive inhibitor of cAMP (cAMP Rp-isomer), which inhibits PKA, also results in inhibition of apical regulated exocytosis in sporozoites (Fig. 3F).

Since cAMP signaling appears to mediate the activation of apical exocytosis, we searched for ACs in the malaria genome. Two different genes with high homology to ACs (ACα and ACβ) have been identified in *Plasmodium*. In particular, ACα was shown to have AC activity in *P. falciparum* [22,23]. Interestingly, ACα genes from *Plasmodium*, *Paramecium* and *Tetrahimena* are closely related and their sequence includes a domain with high homology to K^+ channels [23]. In *Paramecium*, where the purified AC protein also has K^+ channel activity, generation of cAMP is regulated by K^+ conductance [24]. It is thought that ACα presents a transmembrane K^+-channel domain and an intracellular AC domain, which are functionally linked [25].

Since cAMP in *Plasmodium* sporozoites induces apical exocytosis, we first tested whether extracellular K^+ is required for this process. In fact, sporozoites must remain in a high K^+ environment during migration through cells, because the cytosol of eukaryotic cells has high concentrations of this ion [26]. The existence of K^+ channels has been predicted for *Plasmodium* parasites from electrophysiological [27] and genomic sequence data [20].

To determine whether extracellular K^+ is required for sporozoite exocytosis, we stimulated exocytosis in *P. yoelii* sporozoites in regular medium (containing K^+) or in K^+-free medium. We found that exocytosis stimulated with uracil derivatives was inhibited in K^+-free medium (Fig. 4A). To confirm that sporozoites were not impaired by the incubation in K^+-free medium, we transferred sporozoites to regular medium after the

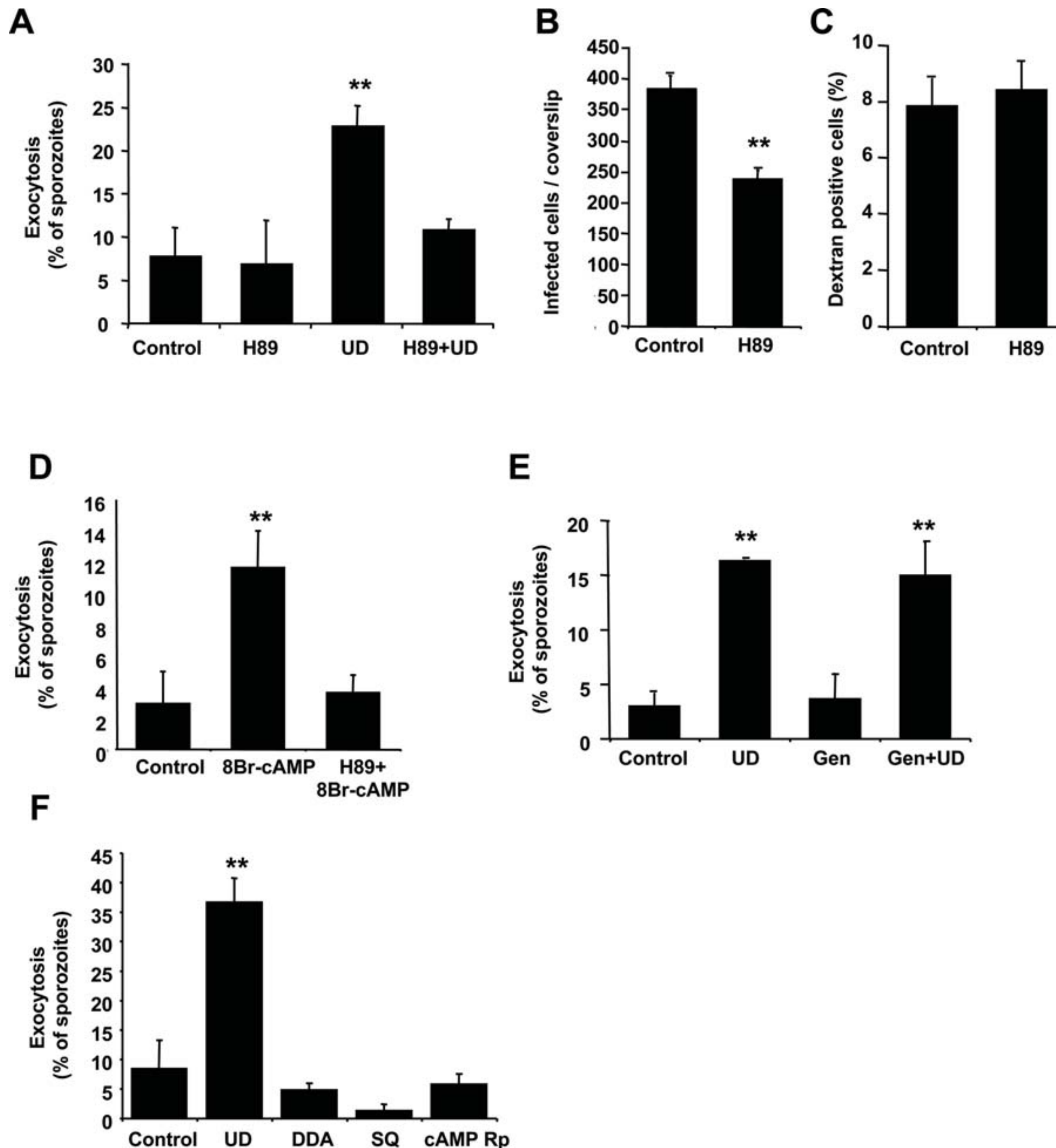

Figure 3. Treatment with an inhibitor of PKA reduces sporozoite exocytosis and infection. *P. yoelii* sporozoites were pre-incubated with H89 followed by addition of uracil derivatives to induce exocytosis (A) or followed by incubation with monolayers of Hepa1-6 cells to quantify infection (B) and migration though cells (C). (D) Sporozoites were pre-incubated with H89 before addition of 8Br-cAMP to induce exocytosis. (E) Sporozoites were pre-incubated with genistein (Gen) before addition of uracil derivatives. (F) *P. yoelii* sporozoites were pre-incubated with 2′, 5′-Dideoxyadenosine (DDA) or SQ22536 (SQ) to inhibit adenylyl cyclase activity or with cAMP Rp-isomer to inhibit PKA, before addition of uracil derivatives to induce exocytosis. Results are expressed as mean of triplicates±SD. * $p<0.05$; ** $p<0.01$ when compared to control by ANOVA.
doi:10.1371/journal.ppat.1000008.g003

K^+-free medium incubation. We found that exocytosis in these sporozoites was similar to exocytosis in sporozoites that were never incubated in K^+-free medium (Fig. 4B).

Exocytosis was inhibited when sporozoites were pre-incubated with different K^+-channel inhibitors (Fig. 4C,D), suggesting that K^+ is required for the activation of exocytosis. We also analyzed the requirement for extracellular K^+ in sporozoite exocytosis induced by 8Br-cAMP or forskolin. We found that in these cases extracellular K^+ is not required (Fig. 4E,F), suggesting that extracellular K^+ is required upstream cAMP in the signaling cascade. Removal of K^+ from the medium may alter the electrochemical gradient of sporozoites affecting UD-induced exocytosis. However, since the response to forskolin and 8Br-cAMP in K^+ free medium is not affected, it suggests that the sporozoite exocytosis pathway is perfectly functional in the absence of extracellular K^+. Also, the viability (Table S1) and capacity of exocytosis response (Fig. 4B) of sporozoites after this treatment was found to be unaffected.

A Ca^{++} ionophore can induce apical regulated exocytosis in *P. yoelii* [9], suggesting that Ca^{++} signaling may be involved in exocytosis. We first compared the magnitude of the cAMP-induced to the Ca^{++}-induced exocytosis, finding similar results

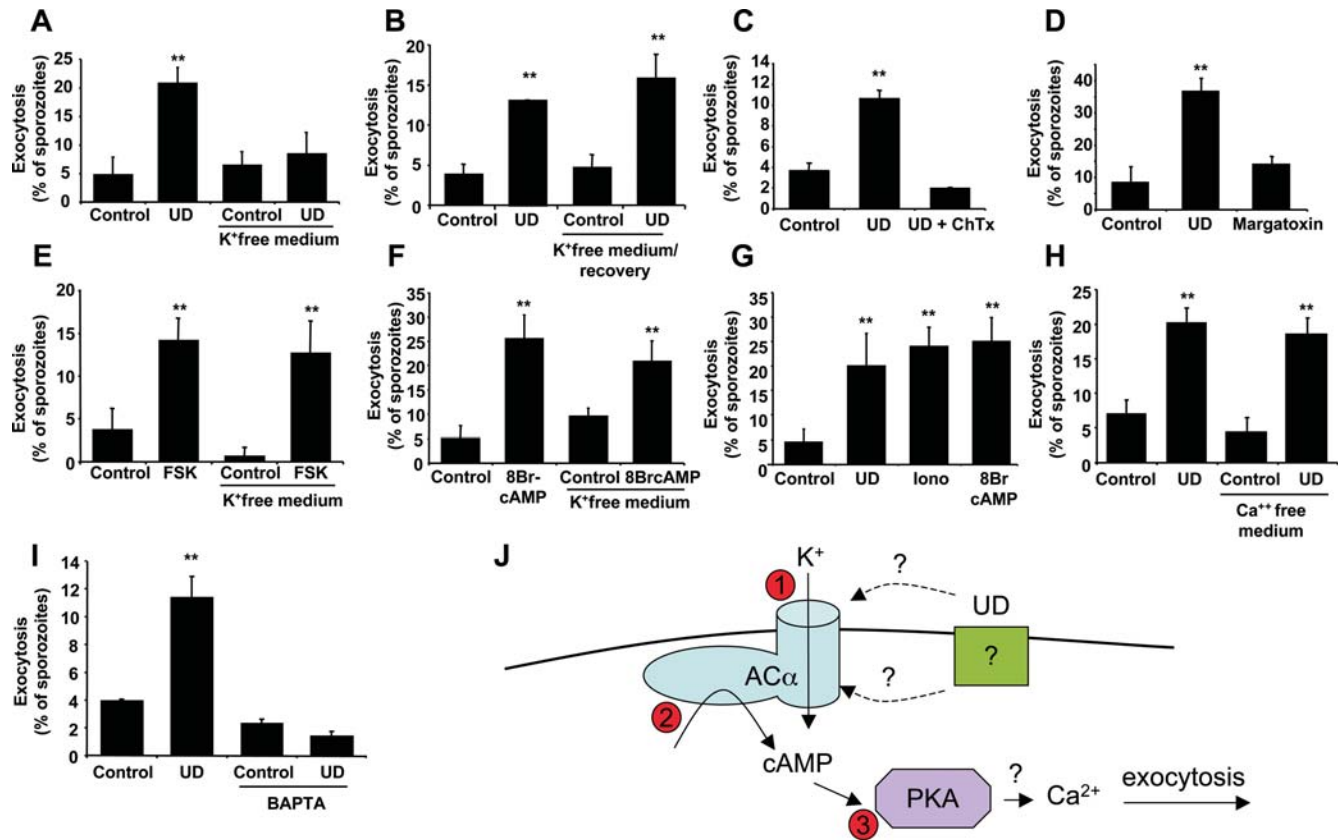

Figure 4. Extracellular K^+ is required for sporozoite apical regulated exocytosis. (A) *P. yoelii* sporozoites were pre-incubated for 15 min in regular medium or K^+-free medium before addition or not of uracil derivatives (UD) for 45 min. (B) Sporozoites were incubated with regular medium or K^+-free medium for 45 min, followed by incubation in regular medium in the presence or absence of UD for another 45 min. (C,D) Sporozoites were pre-incubated with the K^+-channel inhibitors charybdotoxin (C) or margatoxin (D) for 15 min before addition of UD for 45 min. (E,F) sporozoites were pre-incubated for 15 min in regular medium or K^+-free medium before addition or not of forskolin (E) or 8Br-cAMP (F). (G) Sporozoites were incubated with UD, ionomycin or 8Br-cAMP for 45 min. (H) Sporozoites were pre-incubated for 15 min in regular medium or Ca^{++}-free medium before addition or not of UD for 45 min. (I) Sporozoites were pre-incubated with the membrane permeant calcium chelator BAPTA-AM for 15 min before addition of UD for 45 min. Results are expressed as mean of triplicates±SD. ** $p<0.01$ when compared to control by ANOVA. (J) Possible model consistent with the results. UD activate directly or indirectly the K+ channel domain of ACα (1) and trigger the activation of AC activity (2). The increase in cAMP activates PKA (3), which leads to the activation of exocytosis.
doi:10.1371/journal.ppat.1000008.g004

(Fig. 4G). To study whether Ca^{++} is also involved in the signaling induced by UD, we induced exocytosis with UD in Ca^{++}-free medium. We found that exocytosis is not inhibited in Ca^{++}-free medium (Fig. 4H), suggesting that extracellular Ca^{++} is not required for this process. However, we found a strong inhibition of exocytosis when sporozoites were incubated with a membrane-permeant Ca^{++} chelator, suggesting that intracellular Ca^{++} is required for exocytosis (Fig. 4I). A possible model for the signaling mediating exocytosis is proposed (Fig. 4J).

Since *Plasmodium* sporozoite regulated exocytosis requires both extracellular K^+ and cAMP, we decided to test whether *ACα* is involved in the process of sporozoite exocytosis and activation for infection by producing recombinant parasites deficient for this enzyme. We identified the sequence encoding *PbACα*, the *P. berghei* orthologue of *PfACα*, in the PlasmoDB database (http://www.plasmoDB.org/). Complete *PbACα* sequences were retrieved from Sanger sequencing genomics project (http://www.sanger.ac.uk/). We found that *PbACα* is 60% identical to *PfACα* at the amino-acid level of the full-length predicted protein, and 79% in the AC catalytic domain.

Microarray analysis had detected expression of *PfACα* in sporozoites [28]. To analyze the expression of *PbACα*, we isolated mRNA from *P. berghei* sporozoites and performed reverse transcription followed by PCR. We also found expression of this gene in sporozoites (Fig. 5A). Thus, we decided to pursue a targeted gene disruption at the blood stages to study the importance of *ACα* for the *Plasmodium* pre-erythrocytic life cycle stages. We created two independent cloned lines of *P. berghei* parasites that are deficient in *ACα* (*PbACα-*) by using targeted disruption of the *ACα* gene through double crossover homologous recombination (Fig. 5B). *PbACα*-deficiency of the mutant parasites was confirmed by RT-PCR and Southern Blotting (Fig. 5C).

We examined the phenotype of *PbACα-* parasites during the *Plasmodium* life cycle. We compared the two *PbACα-* lines with *WT P. berghei* parasites also cloned independently. *PbACα-* parasites were indistinguishable from *WT* parasites in growth during red blood cell stages in mice (Fig. 6A). We next analyzed parasite growth in the mosquito by determining oocyst development and sporozoite salivary gland invasion. Similar oocyst and salivary gland sporozoite numbers were obtained for *PbACα-* and the *WT* control, indicating that *PbACα* is not involved in oocyst development and sporozoite salivary gland invasion (Table 1).

Gliding motility, the characteristic form of substrate-dependent locomotion of salivary gland sporozoites, was unaffected in *PbACα-* parasites. Stimulation of gliding motility with albumin [29] was

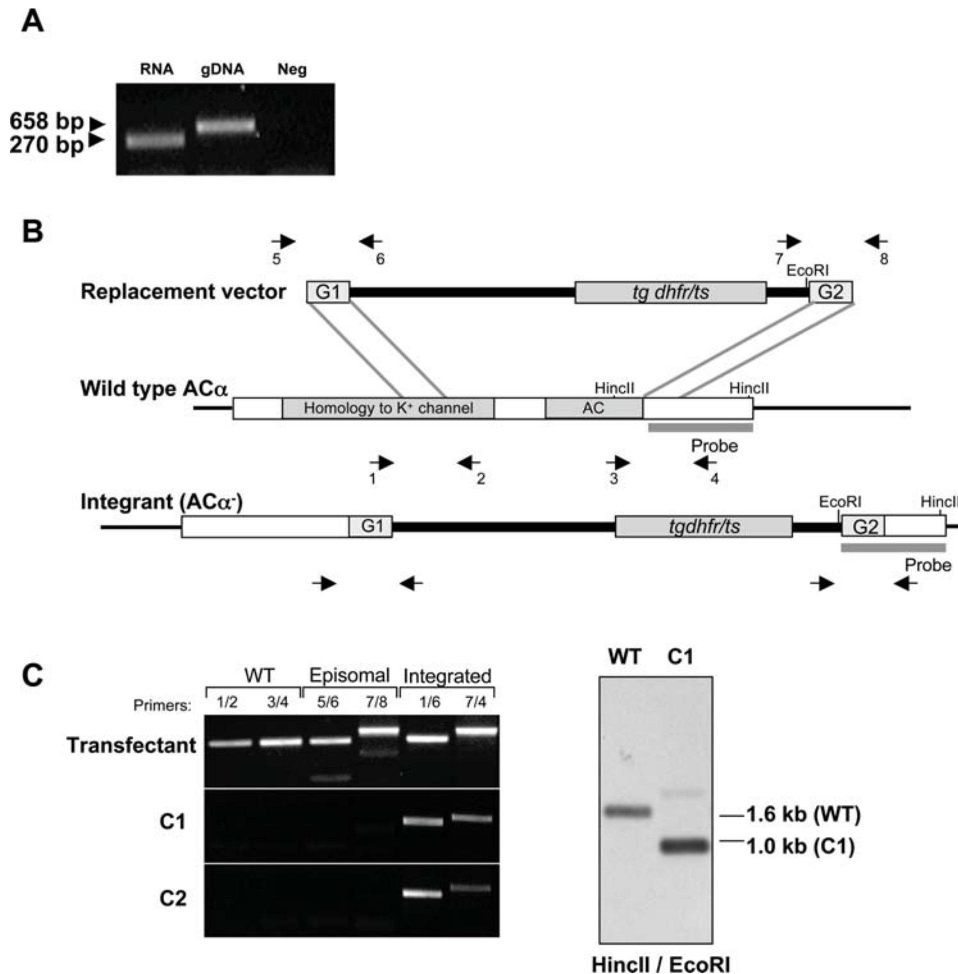

Figure 5. Generation of *PbACα*- parasite lines. (A) RNA from WT *P. berghei* sporozoites was reverse transcribed into cDNA and used as template to amplify *ACα*. Water was used as negative control (Neg) and wild type *P. berghei* genomic DNA (gDNA) as positive control. (B) Schematic representation of the *ACα* locus and the replacement vector. Correct integration of the construct results in the disrupted *ACα* gene as shown. Arrows indicate the position of the primers used for PCR in C. (C) Disruption of *ACα* was shown by PCR (*left*) and by Southern analysis (*right*). PCR on DNA of WT transfected population (before cloning) and *PbACα*- clones (C1 and C2) results in the amplification of two 0.7-kb WT fragments and a 0.8 and a 0.9-kb disrupted fragments when using the primers indicated in (B). Genomic Southern blot hybridization of WT and the *PbACα*- C1. The probe used for hybridization is represented in B. Integration of the targeting plasmid causes reduction in size of a 1.6-kb fragment in WT parasites to a 1.0-kb fragment in the *PbACα*- parasites. Similar results were found for *PbACα*- C2.
doi:10.1371/journal.ppat.1000008.g005

also similar in *WT* and *PbACα*- sporozoites (Fig. 6B). We also tested whether deletion of the *ACα* gene affect sporozoites ability to migrate through cells. We found that the cell-traversal activity of *PbACα*- sporozoites was slightly lower, but not significantly different from *WT* sporozoites (Fig. 6C).

We then tested whether apical regulated exocytosis was affected in *PbACα*-sporozoites. Activation of exocytosis by the mix of uracil derivatives or by forskolin, was greatly reduced in the two different clones of *PbACα*- sporozoites analyzed (Fig. 7A). Addition of a membrane permeant analogue of cAMP (8-Br-cAMP), which induces exocytosis in *WT* parasites, also stimulated exocytosis in *PbACα*- sporozoites (Fig. 7B). This result indicates that all sporozoite components required for exocytosis downstream of cAMP are functional in *PbACα*- sporozoites; however, the lack of *ACα* inhibits proper response upon activation with uracil derivatives or activators of AC activity. Migration through host cells induces apical regulated exocytosis in *Plasmodium* sporozoites [9]. To confirm that *ACα* is also required for exocytosis stimulated by migration through hepatocytes, we measured the response of *WT* and *PbACα*- sporozoites after migration through Hepa1-6 cells. We found that regulated exocytosis was not activated in sporozoites deficient in *ACα* (Fig. 7C).

To examine the role of apical regulated exocytosis and *ACα* in sporozoite infection, we first analyzed the infectivity of *PbACα*- sporozoites *in vitro* using Hepa1-6 cells. We found that *PbACα*- sporozoites are approximately 50% less infective than *WT* sporozoites (Fig. 7D). As the infectivity of *Plasmodium* sporozoites can be noticeably different depending on each particular mosquito infection, we repeated the experiment using sporozoites from three different batches of infected mosquitoes. Similar results were found, confirming that *PbACα*- sporozoites have reduced infectivity in hepatocytes (not shown).

We also tested the infectivity of *PbACα*- parasites *in vivo* in C57/Bl6 mice, which are highly susceptible to infection by *P. berghei* sporozoites [30]. To quantify the infectivity of *PbACα*-, we used real time PCR to measure parasite load in the liver by determining the levels of the parasite-specific 18 S rRNA [31]. Remarkably, 50% decrease of parasite rRNA was detected by this method (Fig. 7E). We repeated the experiment using sporozoites from three different batches of infected mosquitoes finding similar results (not

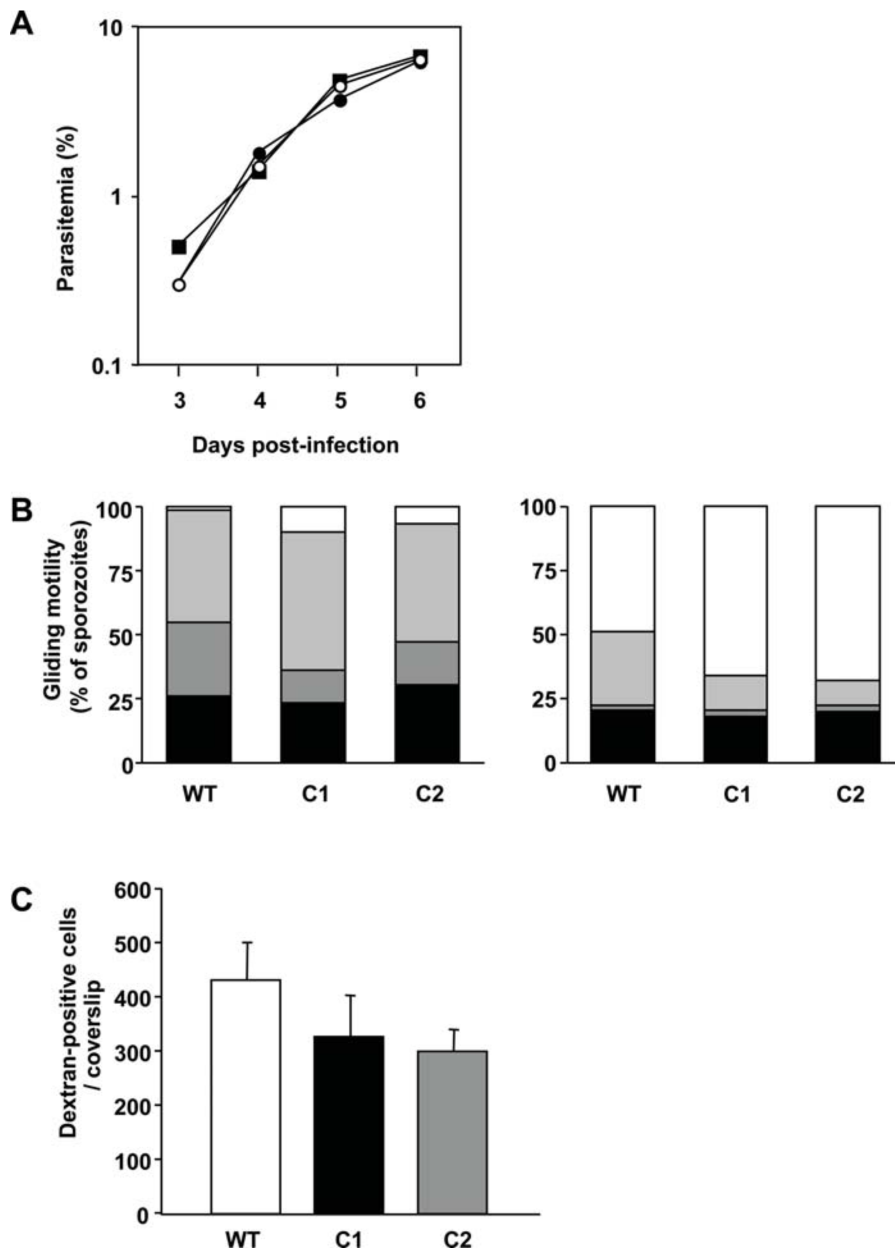

Figure 6. *PbACα-* has normal blood-stage growth rates and sporozoite motility. (A) Growth curves of *P. berghei WT* (black squares), *PbACα-* C1 (black circles) and C2 (white circles) in mice. (B) Gliding motility of sporozoites from *WT*, *PbACα-* C1 and C2 in the presence (right panel) or absence (left panel) of mouse albumin. Percentage of sporozoites that do not glide or do less than a complete circle (black bars), gliding sporozoites exhibiting 1 (dark gray bars), 2 to 10 (light gray bars), or >10 (white bars) circles per trail. (C) Migration through Hepa1-6 cells was measured as the number of dextran positive cells per coverslip. The difference between C1 or C2 and *WT* is not significantly different ($p>0.05$).
doi:10.1371/journal.ppat.1000008.g006

shown). These results suggest that *Plasmodium* sporozoites use apical regulated exocytosis to infect host cells and that *ACα* is an important protein involved in *Plasmodium* liver infection.

To confirm that the phenotype observed in the *PbACα-* sporozoites is caused specifically by depletion of the *PbACα* gene, we complemented one of the *PbACα-* parasite lines with *ACα*. The correct replacement event was confirmed by PCR and Southern blot hybridization (Fig. 8A). No differences were found between the complemented parasite line and *WT* or *PbACα-* parasites during blood stage infection in mice or in mosquito oocyst development and salivary gland sporozoite numbers (not shown). We found that apical regulated exocytosis response to uracil derivatives was recovered in the complemented sporozoites (Fig. 8B). The infectivity of sporozoites was restored by complementation of the *PbACα* gene (Fig. 8C), confirming the role of PbACα in sporozoite exocytosis and infection.

Discussion

The role of exocytosis of apical organelles in invasion of host cells has been extensively studied in *Toxoplasma* tachyzoites. Our knowledge of *Plasmodium* sporozoite exocytosis and infection is less advanced, as this parasite stage can only be obtained by dissection of infected mosquitoes, and this procedure provides limited numbers of sporozoites. Sporozoite purification methods have been recently developed (S. L. Hoffman, personal communication)

Table 1.

	Midgut		Salivary glands
	Number of oocysts per infected mosquito (day 11)	**Percentage of infected midguts (day 11)**	**Number of salivary gland sporozoites per mosquito (day 18)**
WT	36	76	3,157
C1	37	80	3,653
C2	33	80	3,333

doi:10.1371/journal.ppat.1000008.t001

allowing us to use highly purified *P. falciparum* sporozoites in our studies. Gene deletion technology has opened the possibility of dissecting the role of complex pathways into their individual protein components. Using a rodent malaria model we have first identified that the cAMP signaling pathway is involved in *Plasmodium* sporozoite exocytosis. The similar response observed in *P. falciparum* sporozoites suggests that the cAMP-dependent signaling pathway leading to exocytosis is conserved in the human parasite. Based on these results, we have generated a transgenic parasite that is deficient in an essential protein in the cAMP signaling pathway. This approach allowed us to evaluate the role of apical regulated exocytosis in hepatocyte infection by sporozoites *in vitro* and *in vivo* using a mouse model.

Regulated exocytosis in mammalian cells is frequently triggered by an elevation of intracellular Ca^{2+} levels and is modulated by cAMP, which acts synergistically with Ca^{2+}, but cannot induce exocytosis by itself. However, in some specific cell types exocytosis is triggered solely by elevations in cAMP concentrations [32]. Increases in cytosolic Ca^{2+} induced with ionophores can induce exocytosis in *Plasmodium* sporozoites [9], suggesting that Ca^{2+} stimulation is also sufficient to induce this process. The signaling pathways of Ca^{2+} and cAMP are interrelated inside eukaryotic cells [33]. In particular, in *P. falciparum* blood-stages, a cross-talk between Ca^{2+} and cAMP has been observed, where increases in cAMP induce the elevation of intracellular Ca^{2+} concentrations through the activation of PKA [18]. Our results suggest that the cAMP and Ca^{2+} pathways are also interconnected in the sporozoite stage and that intracellular, but not extracellular Ca^{2+}, is required for exocytosis.

When exocytosis is inhibited by the AC or the PKA inhibitors, the reduction in sporozoite infectivity is comparatively lower than the reduction in exocytosis. Similar results were obtained with the *PbACα*- sporozoites, where exocytosis is reduced to background levels, but infection is reduced by 50%. Taken together these results suggest that sporozoites have alternative pathways to invade host hepatocytes that do not require apical regulated exocytosis. However, we cannot exclude the possibility that low levels of exocytosis that cannot be detected in our assays still occur in the *PbACα*- sporozoites and are sufficient to mediate infection of hepatocytes.

The analysis of host cell molecules required for sporozoite infection has provided evidence that sporozoites use more than one unique pathway to achieve hepatocyte infection [34], suggesting that sporozoites may take advantage of this phenomenon to overcome polymorphisms in host receptors or to escape from immune mechanisms inhibiting one particular pathway of infection.

We had previously observed that activation of sporozoite exocytosis increases their infectivity and reduces the need for migration through cells [9]. Here we confirmed that activation of exocytosis by cAMP-mediated pathways increases exocytosis infectivity reducing migration through cells. Accordingly, inhibitors of this pathway inhibit sporozoite exocytosis and decrease their infectivity. Interestingly, *spect*-deficient sporozoites, which do not migrate through host cells [15], responded to uracil derivatives but were not able to respond to either an activator of AC or to a permeant analogue of cAMP, suggesting that cAMP-induced signaling leading to exocytosis is different in these mutant sporozoites. The positive exocytosis response observed in the presence of the inhibitor of AC, suggests that these parasites are able to respond to uracil derivatives by activating cAMP-independent pathways that are not normally activated in *wt* sporozoites, where cAMP is required for exocytosis. It is still not clear how this relates to their impaired capacity to migrate through cells, but suggests that they may up-regulate the alternative mechanisms that are independent of migration through cells and exocytosis to infect hepatocytes. These results are consistent with the concept that sporozoites can use alternative pathways to invade hepatocytes, as the infection experiments with *PbACα*- sporozoites suggest.

Apical regulated exocytosis in the transgenic parasites deficient in *ACα* is dramatically decreased in response to uracil derivatives or migration through host cells, indicating that *ACα* is necessary to induce high levels of exocytosis and confirming the essential role of the cAMP signaling pathway in this process. Complementation of the genetically deficient parasites with the *ACα* gene confirms that the defect in exocytosis and infection observed in *PbACα*- sporozoites is caused by deletion of the *ACα* gene and not by other modifications resulting from the genetic manipulations of these parasites.

Two genes with high homology to ACs have been identified in the *Plasmodium* genome: *ACα* and *ACβ* [25]. ACα activity as an AC has been demonstrated for *P. falciparum*, where the catalytic domain was expressed independently [22]. A second putative AC gene, called *ACβ*, has been identified in the *Plasmodium* database. We tried to generate *ACβ*-deficient parasites; however the *ACβ* gene seems to be essential for the asexual blood-stages of *Plasmodium*.

ACα- sporozoites are able to stimulate exocytosis in response to the permeant analogue of cAMP, but not to forskolin, the activator of ACs, confirming that the defect is caused by the lack of a functional AC and can be compensated by artificially increasing intracellular concentrations of cAMP. The results obtained with *PbACα*- sporozoites also suggest that ACα is sensitive to forskolin stimulation, as the increase in exocytosis induced by this drug is lost in the genetically deficient sporozoites. Since AC activity is insensitive to forskolin in asexual blood-stages [35] and AC*β* is preferentially expressed in this stage of the parasite cycle [25], it seems likely that AC*β*, rather than ACα, is required for cAMP formation during erythrocyte infection. We also found that the growth of *PbACα*- parasites in the asexual blood-stages was indistinguishable from control, consistent with the lack of activity of ACα during this stage.

Interestingly, the *ACα* gene contains a N-terminal domain with high homology to voltage-gated K^+ channels. Other apicomplexans and also the ciliates *Paramecium* and *Tetrahymena* have an *ACα* gene homologous to the one in *Plasmodium* [23]. In *Paramecium* it has been demonstrated that the purified ACα protein also has K^+ channel activity, and the generation of cAMP is regulated by K^+ conductance [24]. Although functional K^+ channel activity has not been demonstrated for ACα in *Plasmodium*, our results are consistent with a role for K^+ conductance in sporozoite exocytosis. Uracil derivates do not induce exocytosis in K^+ free medium, but activation of AC with forskolin or addition of the permeant analogue of cAMP overcomes the requirement for extracellular K^+. Therefore, it seems likely that increased K^+ permeability may induce activation of ACα and synthesis of cAMP.

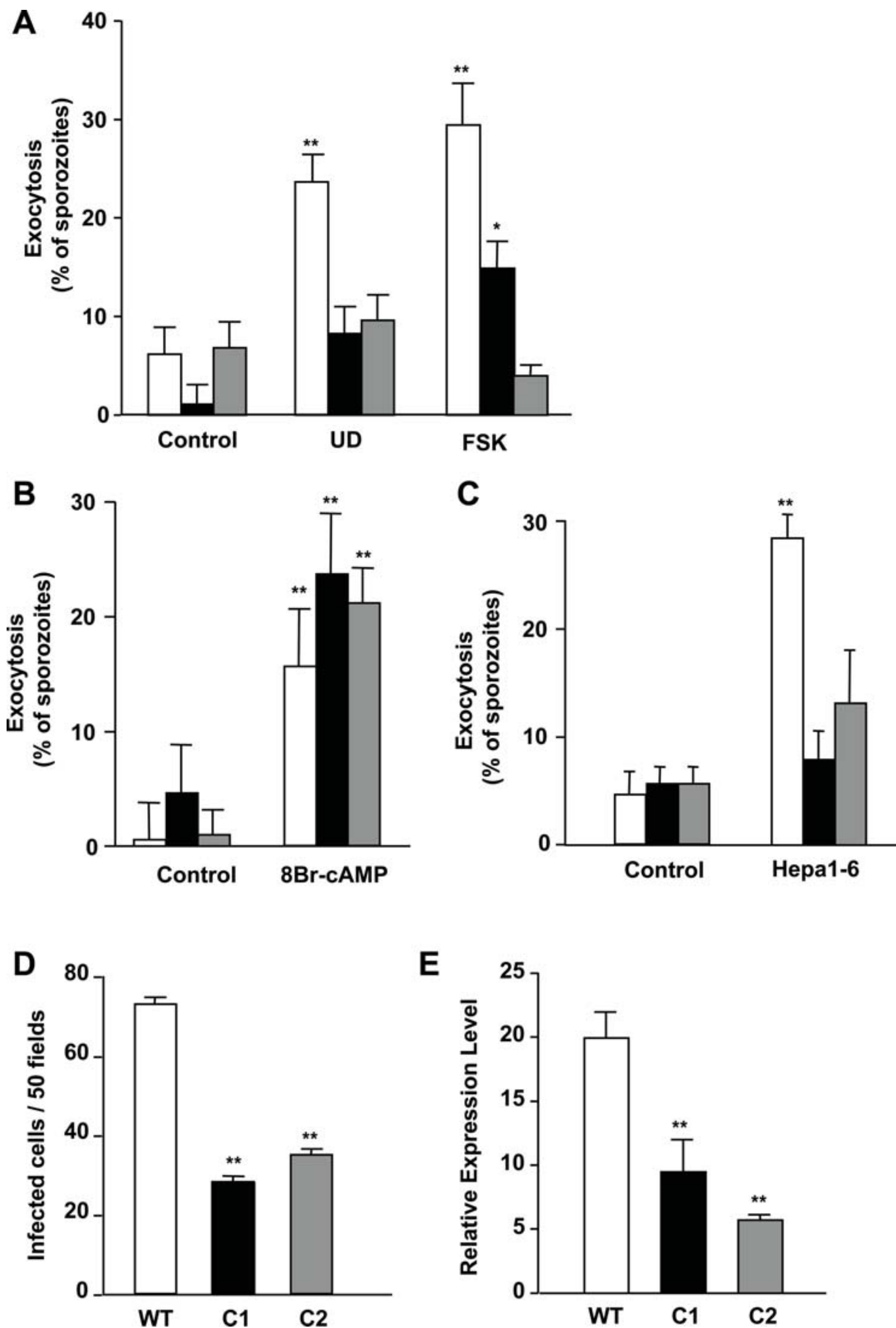

Figure 7. *PbAC*α- sporozoites have defective exocytosis and infection. Exocytosis and infectivity of *P. berghei WT* (white bars), *PbAC*α- C1 (black bars) and C2 (gray bars) sporozoites was analyzed. (A, B) Sporozoites were incubated or not with uracil derivatives (UD) or forskolin (FSK) (A) or 8Br-cAMP (B) for 1 h before fixation and quantification of exocytosis. (C) Sporozoites were added to filter insets containing confluent Hepa1-6 cells and collected on empty coverslips placed underneath the filters in the lower chamber. Percentage of sporozoites in coverslips showing apical-regulated exocytosis is shown. (D) Infection of Hepa1-6 cell by sporozoites *in vitro* was determined by counting the number of infected cells after 24 h incubation. (E) Infection of mice was determined by real-time PCR amplification of 18S rRNA in the liver 40 h after inoculation of sporozoites.
doi:10.1371/journal.ppat.1000008.g007

Materials and Methods

Host cells and parasites

Hepa 1-6 (ATCC CRL-1830), a hepatoma cell line derived from a C57L/J mouse, which is efficiently infected by rodent malaria parasites [36] was used for *in vitro* hepatocyte infections. *Plasmodium yoelii yoelii* sporozoites (cell line 17× NL), *P. berghei* ANKA *wt* and *spect-1* deficient sporozoites [15] and the NF54 isolate [37] of *P. falciparum* were used to produce sporozoites in *A. stephensi* mosquitoes. Salivary glands were dissected from the

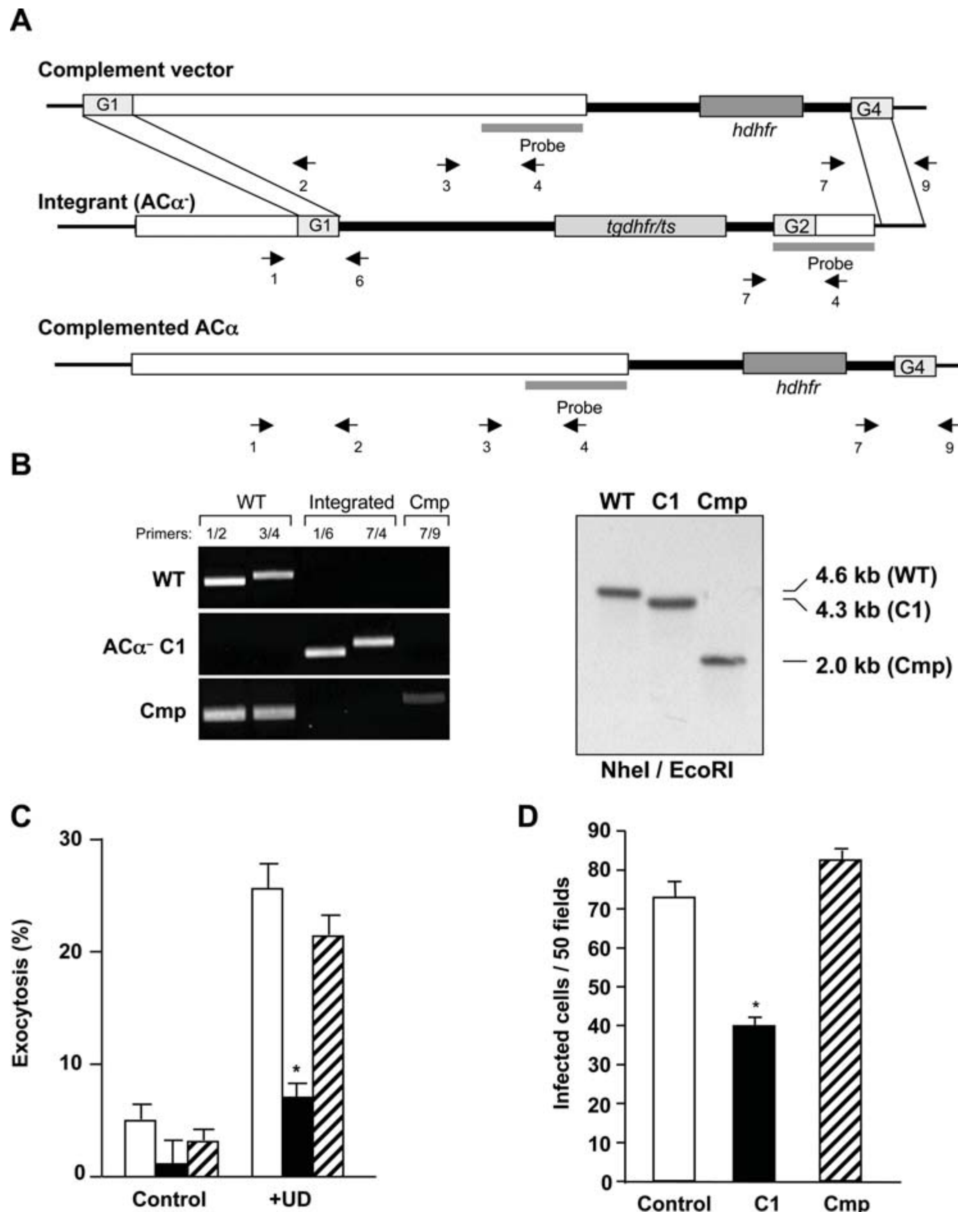

Figure 8. *PbACα*- complemented sporozoites recover the *WT* phenotype. (A) Schematic representation of the complement replacement vector, the *ACα*- disrupted locus and the complemented *ACα* locus. Correct integration of the construct results in the reconstitution of the disrupted *ACα* gene as shown. Arrows indicate the position of the primers used for PCR in B. (B) Complementation of *ACα* was shown by PCR (*left*) and by Southern analysis (*right*). PCR on DNA of *WT*, *PbACα*- C1 and complemented *ACα* (Cmp) results in the amplification of a fragment of 1 kb when using the primers indicated in (A). Genomic Southern blot hybridization of *WT*, *PbACα*- C1 and complemented *ACα*. The probe used for hybridization is represented in A. Integration of the complementation plasmid causes reduction in size of a 4.3-kb fragment in *PbACα*- C1 parasites to a 2.0-kb fragment in the $AC\alpha^{-}$ complemented parasites. (C) Exocytosis of WT (white bars), *PbACα*- C1 (black bars) and complemented *ACα* (stripped bars) sporozoites in response to uracil derivatives (UD). (D) Infection of Hepa1-6 cells *in vitro* by WT, *ACα*- C1 (black bars) and complemented *ACα* (stripped bars) sporozoites was determined by counting infected cells 24 h after addition of sporozoites. * significant difference ($p<0.01$, ANOVA) compared to *WT* and complemented *ACα*.
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mosquitoes. The *P. falciparum* sporozoites were extracted from the salivary glands, purified, and cryopreserved. Prior to being used in assays, the sporozoites were thawed and suspended in RPMI medium.

Uracil derivatives

Exocytosis was induced by incubation of sporozoites with a mixture of the physiological concentrations of uracil derivatives (ICN Biomedicals) consisting of 180 μM uracil, 280 μM uridine, 300 μM uracil monophosphate (UMP), 50 μM uracil diphosphate (UDP) and 30 μM uracil triphosphate (UTP) was prepared in RPMI 1640 and pH adjusted to 7.

Regulated exocytosis

Sporozoites (10^5 *P. yoelii*, *P. berghei* or 5×10^4 *P. falciparum*) were centrifuged for 5 min at 1800 × *g* on glass coverslips before addition

of uracil derivatives or conditioned medium. After incubation at 37°C for 1 h, sporozoites were fixed with 1% paraformaldehyde for 10 min (non-permeabilizing conditions) before staining for surface TRAP/SSP2 with the monoclonal antibody (F3B5) for *P. yoelii*, *Pf*SSP2.1 for *P. falciparum* [38] and a specific TRAP/SSP2 rabbit anti-serum for *P. berghei*. Sporozoite regulated exocytosis was quantified as the percentage of total sporozoites that present a TRAP/SSP2 stained 'cap' in their apical end. Results are expressed as the average of triplicate determinations counting at least 50 sporozoites for each condition. Background level exocytosis was measured by staining sporozoites after dissection from mosquitoes, before incubation *in vitro*. Background exocytosis was always lower than 8% and was subtracted from all values. All experiments were performed twice showing similar results.

Western blot

4×10^5 *P. yoelii* sporozoites were incubated alone or with the different exocytosis stimuli for 1 h at 37°C before spinning at 20,000 *g* for 10 min. The supernatants were collected and separated in a 7.5% gel in reducing conditions. After semi-dry transfer to a PDVF membrane, proteins were stained with anti-*P. yoelii* MTIP antiserum followed by anti-rabbit conjugated to horseradish peroxidase. Bound antibodies were detected by chemiluminescence using ECL (GE Healthcare Bio-Sciences).

Drug treatments

Sporozoites (10^5) were incubated with 100 μM forskolin, 100 μM MDL-12.330A, 500 μM 8Br-cAMP, 10 μM H89, 30 μM genistein, 100 nM charybdotoxin, 50 μM SQ22536, 50 μM 2′, 5′-Dideoxyadenosine, 5 μM Adenosine 3′, 5′-cyclic monophosphorothioate 8Br-Rp-isomer, 1 nM margatoxin, 20 μM BAPTA, ionomycin 1 μM (all from Calbiochem) before addition or not of uracil derivatives for 1 h, followed by fixation and quantification of exocytosis. For exocytosis assays sporozoites were pretreated with the drug for 15 min and concentrations were kept constant throughout the experiment. For infection and migration, treatment with drugs was performed for 15 min before washing and spinning sporozoites on Hepa1-6 cells grown on coverslips placed in 24-well dishes containing 1 ml of culture medium/well. For assays in K^+-free medium: 10^5 *P. yoelii* sporozoites were incubated for 45 min in regular medium (RPMI 1640, that contains 5.3 mM KCl and 100 mM NaCl), K^+-free medium (modified RPMI 1640 with no KCl and 110 mM NaCl to maintain osmolarity) in the presence or absence of stimulus, before fixation and quantification of exocytosis. To assay sporozoites viability after incubation in K^+-free medium, sporozoites centrifuged at 20,800 g and resuspended in regular medium with uracil derivatives to induce exocytosis. All experiments were performed twice showing similar results.

Intracellular cAMP levels

Intracellular levels of cAMP in *P. yoelii* sporozoites were determined using a cAMP Biotrack Enzymeimmunoassay system from Amersham Bioscience. For each sample 2×10^6 *P. yoelii* sporozoites were incubated with uracil derivatives for 45 min at 37°C. The experiment was performed twice showing similar results.

Migration through cells and infection

Sporozoites (10^5 sporozoites/coverslip) were added to monolayers of 2×10^5 Hepa1-6 cells for 1 h in the presence of 1 mg/ml of rhodamine-dextran lysine fixable, 10,000 MW. Sporozoites breach the plasma membrane of host cells during migration and as a result fluorescent dextran enters in their cytosol, allowing detection of wounded cells [17]. Cells were washed and incubated for another 24 hours before fixation and staining of infected cells with the mAb (2E6) recognizing HSP70 to detect infected cells [39], followed by anti-mouse IgG-FITC antibodies. Migration through host cells is quantified as percentage (or total number) of dextran-positive cells. Infection was quantified as the number of infected cells per coverslip or per 50 microscopic fields. For transwell filter assays Hepa1-6 cells (5×10^5) were cultivated on 3 μm pore diameter Transwell filters (Costar, Corning, New York) until they form a continuous monolayer. Empty coverslips were placed underneath the filters. *P. berghei* sporozoites (2×10^5) were added to filter insets containing Hepa1-6 cells. Coverslips were fixed after 2 h of incubation with sporozoites, before staining for surface TRAP/SSP2. All experiments were performed twice showing similar results.

Determination of live/dead sporozoites with propidium iodide

P. yoelii sporozoites were incubated with the indicated drugs for 20 min before addition of propidium iodide (1 μg/ml) for 10 min. Sporozoites were washed and observed directly with a fluorescence microscope. Propidium iodide positive sporozoites were considered dead and quantified. At least 100 sporozoites were counted in each condition.

Motility of live sporozoites

Live *P. yoelii* sporozoites were observed directly under the microscope in a heated stage at 37°C before or after addition of different stimuli. As control, the same volume of medium with the same solvent used for the stimuli was added. At least one hundred sporozoites were counted in each condition and they were classified as immobile, twisting or gliding, depending on their type of motility observed.

Generation of the *PbACα*- parasite lines

To disrupt the *ACα* locus an *ACα* replacement vector was constructed in vector b3D.D_T.^H.^D_b (pL0001, MRA-770) containing the pyrimethamine-resistant *Toxoplasma gondii* (*tg*) *dhfr/ts* gene. To complement *ACα* into the genome of *PbACα*- parasites, a vector was constructed with the human (*h*) *dhfr* selectable marker and two fragments of 4.3kb (5′) and 0.5 kb (3′) of the *ACα* gene of *P. berghei*. The linearized vector can integrate in *ACα*. Further details are described in Fig. 5. *P. berghei-ANKA* (clone 15cy1) was used to generate *PbACα*-parasites. Transfection, selection, and cloning of *PbACα*- parasites was performed as described [40]. Two clones (C1 and C2) were selected for further analysis. *PbACα*- C1 parasites were transfected with the complement vector to create *ACα*- complement. Selection of transformed parasites was performed by treating infected animals with WR99210 (20 mg/kg bodyweight) as has been described [41]. One parasite clone (Cmp) in which the *ACα* gene was integrated into the *ACα* locus was selected for further analysis. Correct integration of constructs into the genome of transformed parasites was analyzed by RT-PCR and Southern analysis of restricted DNA. PCR on DNA of *WT* and $AC\alpha^-$ parasites was performed by using primers specific for the *WT* 5′ (flG1F 5′-AGCGCATTAGTTTATGATTTTTG-3′ and flG1R 5′-TTGTGAATTAGGGATCTTCATGTC-3′; amplifying a fragment of 0.7 kb) and *WT* 3′ (flG2F 5′-ATGCGCAAACCCGTTAAAT-3′ and flG2R 5′-TTTGATTCATTCCACTTTCCA-3′; amplifying fragment of 0.7 kb) and disrupted 5′ (flG1F and Pb103 5′-TAATTATATGTTATTTTATTTCCAC-3′; amplifying a fragment of 0.8 kb) and disrupted 3′ (flG2R and Pb106a 5′-TGCATGCACATGCATGTAAA-

TAGC-3′; amplifying fragment of 0.9 kb) locus. PCR on DNA of complement was performed by using primers specific for INT3′ (Pb106a and flG4R 5′-GCAGAGAGAGCGTTAAAAAC-TATTG-3′, amplifying a fragment of 1.0 kb). RT-PCR was performed on RNA isolated from *WT* sporozoites. Primers 02-F (5′-AGGGTGACATTGAAGGGATG-3′) and 02-R (5′-ATTCCTCGGGATATTCCACC-3′) were used to amplify cDNA or genomic DNA derived from the *PbAC*α gene, amplifying a fragment of 270 bp and 658 bp, respectively.

Genomic Southern hybridization

Genomic DNA of *P. berghei* (2 μg) was digested with HincII / EcoRI or NheI / EcoRI, separated on 0.9% agarose gel and then transferred onto a nylon membrane. DNA probe was labeled with digoxigenin using the DIG PCR labeling kit (Roche Diagnostics) using genomic DNA as template with the following primer pair, 5′-TCCTTCGTGGAATTTACACTTG-3′ and 5′-CCAGAC-GAGGAACTAATGCAG-3′. Signals were detected using the DIG/CPSD system (Roche Diagnostics).

Phenotype analysis of the *PbACα*- parasite during blood stage and mosquito stage development

Parasitemia in mice was determined by examination of a Giemsa-stained blood smear. Oocyst formation and sporozoite development were quantified in infected *Anopheles stephensi* mosquitoes as described [42]. The number of salivary gland sporozoites per mosquito was determined by dissecting salivary glands from 10 infected mosquitoes in each condition [43]. Blood stage infections were studied in mice (male Swiss Webster or C57/Bl6 mice, 20–25 g) infected with 200 μl of blood at 0.5% parasitemia. Experiment was performed twice showing similar results.

Gliding motility of sporozoites

Gliding motility of sporozoites was analyzed by counting the average number of circles performed by single sporozoites [44]. Sporozoites (2×10^4) were centrifuged for 10 min at 1,800 × *g* onto glass coverslips previously coated with anti-CS 3D11 antibody, followed by incubation for 2 h at 37°C and staining with biotin-labeled 3D11 antibody followed by incubation with avidin-FITC for sporozoite and trail visualization. Quantification was performed by counting the number of circles performed by 100 sporozoites in three independent coverslips. When indicated 3% mouse albumin was present in the assay.

Transwell filter assays

Hepa1-6 cells were cultivated on 3 μm pore diameter Transwell filters (Costar, Corning, New York) until they form a continuous monolayer. Empty coverslips were placed underneath the filters. Sporozoites (2×10^5) were added to filter insets containing Hepa1-6 cells or no cells. Coverslips were fixed after 2 h of incubation with sporozoites, before staining for surface TRAP to determine exocytosis. Experiment was performed twice showing similar results.

Sporozoite infectivity *in vivo*

Groups of three C57/Bl6 mice were given i.v. injections of 20,000 sporozoites. 40 h later, livers were harvested, total RNA was isolated, and malaria infection was quantified using reverse transcription followed by real-time PCR [31] using primers that recognize *P. berghei*–specific sequences within the 18S rRNA 5′-AAGCATTAAATAAAGCGAATACATCCTTAC and 5′-GGA-GATTGGTTTTGACGTTTATGT. Experiment was performed three times showing similar results.

Accession numbers/ID numbers for genes and proteins

P. falciparum ACα: UniProtKB/TrEMBL accession number: Q8I7A1. PlasmoDB identifier: PF14_0043

P. berghei ACα: PlasmoDB identifier: PB001333.02.0. Complete *PbACα* sequences (contig 1047, 5680) were retrieved from Sanger sequencing genomics project. *P. falciparum* PKA: PlasmoDB identifier PFI1685w.

Supporting Information

Figure S1 Exocytosis of TRAP occurs in the apical end of sporozoites. *P. berghei* sporozoites were incubated on coverslips coated with anti-CS antibodies for 20 min before addition of forskolin. After another 30 min, sporozoites were fixed and stained for CS protein.
Found at: doi:10.1371/journal.ppat.1000008.s001 (5.64 MB TIF)

Figure S2 Control for sporozoite lysis. *P. yoelii* sporozoites (4×10^5) were incubated for 1 h with UD, forskolin (FSK) or 8Br-cAMP. Culture media (upper panel) and pellet containing sporozoites (lower panel) were analyzed by Western blot against myosin A tail domain interacting protein (MTIP), which is localized to the inner membrane complex. A unique band at 25 kDa was found.
Found at: doi:10.1371/journal.ppat.1000008.s002 (1.20 MB TIF)

Figure S3 Motility of sporozoites before and after exocytosis. Live *P. yoelii* sporozoites were observed directly under the microscope before or after addition of forskolin (A) or UD (B). Sporozoite motility was classified as immobile, twisting or gliding. There is a clear shift in sporozoite motility profile from gliding to immobile at later times after addition of the stimuli. As expected, a certain decrease in motility is observed over time even in control sporozoites, however, the decrease induced by the exocytosis stimuli is significantly more pronounced. No significant changes were observed in twisting motility.
Found at: doi:10.1371/journal.ppat.1000008.s003 (1.23 MB TIF)

Table S1 Determination of sporozoite viability after drug treatments. *P. yoelii* sporozoites were incubated in the different conditions indicated. Dead sporozoites were quantified using propidium iodide staining. An untreated control was performed for each condition because the background level of dead sporozoites may vary on each batch of dissected mosquitoes.
Found at: doi:10.1371/journal.ppat.1000008.s004 (1.05 MB TIF)

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Author Contributions

Conceived and designed the experiments: TO LC EB PB MM AR. Performed the experiments: TO LC RL EB LP OD. Analyzed the data: TO LC EB AR. Contributed reagents/materials/analysis tools: LA. Wrote the paper: TO LC AR. Provided advice: TT.

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Appendix II

Manuscript submitted for publication

Cellular Microbiology

Plasmodium sporozoites exocytosis and infection are regulated by uracil-derivatives and albumin

Running title: *Plasmodium* sporozoites exocytosis and infection

Laura Cabrita-Santos, Esther Bettiol, Olga Diaz-Pulido, Alida Coppi, Dabeiba Bernal-Rubio, Salim Merali, Maria M. Mota* and Ana Rodriguez#

New York University School of Medicine, Department of Medical Parasitology, 341 E. 25th St. New York, NY 10010, USA.*Faculdade de Medicina da Universidade de Lisboa

Instituto de Medicina Molecular. Av. Prof. Egas Moniz 1649-028 Lisboa, Portugal. #Corresponding author.

Characters: 19,707

Contact: Ana Rodriguez. rodria02@med.nyu.edu. Tel: 212 263 6757, Fax: 212 263 8116

Key words: Plasmodium, malaria, exocytosis, sporozoite, uracil, albumin, hepatocyte.

Abstract:

Malaria is transmitted through the bite of a mosquito that deposits *Plasmodium* sporozoites in the skin, from where they migrate into circulation and later into the liver. Sporozoites traverse hepatocytes before infection, a process that activates them for infection by inducing regulated exocytosis at the apical end of the parasite. Here we show that uracil and its derived nucleotides, which are found in the cytosol of traversed cells, induce apical regulated exocytosis in *P. yoelii* and *P. falciparum* sporozoites. Exocytosis is specifically inhibited by albumin, which is present in host tissues, but this inhibitory effect is no longer active once sporozoites contact hepatocytes, allowing activation of sporozoites for infection. In this way, sporozoite migration through cells other than hepatocytes does not activate exocytosis or increase their infectivity. We have identified two host molecules that regulate sporozoite exocytosis and infectivity. Our results indicate that sporozoites regulate exocytosis in response to specific molecules in their environment and may use this capacity to distinguish between different tissues to successfully establish infection in the liver.

Introduction:

The causative agent of malaria is the protozoan parasite *Plasmodium*. It is transmitted by the bite of infected mosquitoes that deposit the sporozoite form of the parasite in the skin of the mammalian host. Sporozoites are motile and travel from the skin into the circulation, from where they reach the host's liver (Mota and Rodriguez, 2004). We have previously observed that *Plasmodium* sporozoites traverse several cells in the liver before infecting a final hepatocyte. Sporozoites migrate through host cells by disrupting their plasma membranes and traversing their cytosol. *In vitro*, sporozoites can migrate through different types of cells, in what appears to be a non-specific type of cell invasion (Mota *et al*., 2001). This is in contrast to infection, in which sporozoites are more selective for hepatocytes and enter these cells forming a parasitophorous vacuole where they replicate (Mota and Rodriguez, 2004).

Plasmodium sporozoites and other apicomplexan parasites such as *Eimeria* sporozoites and *Toxoplasma* tachyzoites have small vesicles called micronemes that contain proteins involved in host cell infection (Sibley, 2004). These proteins, such as MIC-2 in *Toxoplasma* or thrombospondin-related anonymous protein (TRAP) in *Plasmodium,* become exposed on the apical surface of the parasite upon exocytosis of the micronemes, which is triggered by incubation of these parasites with host cells (Gantt *et al*., 2000; Carruthers *et al*., 1999). Exocytosis of micronemal proteins resulting in the appearance of TRAP on the apical surface of sporozoites is induced during the process of migration through cells and precedes infection with formation of an internalization vacuole. This process, similarly to *Toxoplasma* secretion of MIC2 (Huynh and Carruthers, 2006), is thought to facilitate invasion of the host cell (Mota *et al*., 2002).

Migration though host cells is therefore considered an early step that activates sporozoites for infection (Mota and Rodriguez, 2004).

During the process of migration through cells sporozoites are not surrounded by a vacuolar membrane and therefore are in direct contact with the cytosol of the traversed cell. Because apical regulated exocytosis can also be induced by incubation of sporozoites with host cell lysates, it was proposed that cytosolic factors in the mammalian cell activate exocytosis in the parasite (Mota *et al*., 2002). In this work we have identified host cell cytosolic factors that induce exocytosis of the rodent parasite *P.*

yoelii and the human parasite *P. falciparum*. We found that uracil, uridine and uracil-derived nucleotides at concentrations that are normally found in the cytosol of mammalian cells induce exocytosis in sporozoites and increase their infectivity. We have also characterized the regulation of this process. As sporozoites are deposited in the skin of the host where they traverse host cells (Amino *et al*., 2006; Vanderberg and Frevert, 2004), it is likely that they encounter high concentrations of uracil-derived nucleotides before reaching their target cells in the liver. However, exocytosis is only expected to take place just before hepatocyte infection, as it exposes high concentrations of adhesive molecules on the surface of the parasite, such as TRAP, which are required for internalization and formation of a parasitophorous vacuole. We found that exocytosis is inhibited specifically by albumin, a protein found in the skin, blood and liver of the mammalian host, suggesting that during infections *in vivo* sporozoites would not undergo apical regulated exocytosis in the presence of physiological concentrations of this protein. The inhibitory effect of albumin is reversed when sporozoites are in contact with hepatocytes, suggesting that after arrival in the liver, sporozoites become susceptible to stimulation by uracil-derived nucleotides that will induce apical regulated exocytosis and facilitate hepatocyte infection.

Results:

Apical regulated exocytosis in the rodent parasite, *P. yoelii* (Mota *et al*., 2002) and in the human parasite, *P. falciparum* is observed as the surface exposure of TRAP protein in the apical end of the sporozoites (Fig. 1A and B). Exocytosis in *P. yoelii* is induced by migration through host cells, but also by incubation with lysates of a hepatoma cell line (Hepa1-6), which is susceptible to sporozoite infection (Mota *et al*., 2002; Mota and Rodriguez, 2000). Regulated exocytosis in mammalian cells can be induced by a wide variety of molecules, ranging from proteins to nucleotides. In particular, the uracil and adenine nucleotides (UDP, ADP, UTP and ATP) bind to specific receptors of the P2X and Y families and induce regulated exocytosis in different cell types (Lazarowski *et al*., 2003). Since these nucleotides are found in high concentrations in the cytosol of cells and therefore migrating sporozoites are in direct contact with them during migration, we tested their ability to induce exocytosis in *P. yoelii* sporozoites. We found that UDP and UTP induce sporozoite exocytosis, but not ADP or ATP (Fig. 1C). We also found that UDP induces exocytosis in *Plasmodium* sporozoites in a dose dependent

manner and that the physiological concentration of UDP found in the cytosol of cells (app. 100 μM) (Traut, 1994) is sufficient to induce efficient exocytosis in sporozoites (Fig. 1D). UDP induces exocytosis in sporozoites already 5 min after incubation and reaches maximum stimulation by 10 to 20 min (Fig. 1E).

We also determined whether other pyrimidines could induce exocytosis in sporozoites. Using the same concentration of 100 μM, we found that uracil and thymine and their derivative nucleosides and nucleotides also induce exocytosis in sporozoites (Fig. 1F). No significant activity was found with cytosine derivatives (not shown). We next tested a mix of uracil and its derivatives (uridine, UMP, UDP and UTP) at the concentrations normally found in the cytosol of mammalian cells (from 30 to 300 μM, described in methods) (Traut, 1994), and found that it efficiently induced exocytosis in sporozoites (Fig. 2A). As the physiological concentrations of thymine and its derivatives are very low (<5 μM) in mammalian tissues (Traut, 1994), uracil and its derivatives are likely to be the major effectors in this pathway to activate sporozoite exocytosis during migration through host cells.

Migration through hepatocytes induces sporozoite apical regulated exocytosis, which facilitates invasion of the host cell (Mota *et al.*, 2002). Stimulation of exocytosis by other means, such as calcium ionophores or Hepa1-6 cells lysates, overcomes the need for migration through host cells and increases infection (Mota *et al.*, 2002). To test whether stimulation of exocytosis by physiological concentrations of uracil and its derivatives, would also overcome the need for migration through hepatocytes before infection, we incubated *P. yoelii* sporozoites with these molecules to induce regulated exocytosis before incubation with Hepa1-6 cells. Migration through host cells was determined as the percentage of cells that were wounded by sporozoite migration and as a result became positive for a soluble impermeant tracer (dextran) (McNeil *et al.*, 1999). We found an increase in the number of infected cells, indicating that stimulation of regulated exocytosis in sporozoites increases their infectivity. In addition, activation of sporozoite exocytosis by uracil and its derivatives reduced sporozoite migration through hepatocytes, suggesting that such migration is not necessary when exocytosis is previously induced (Fig. 2B).

A malaria infection starts with the bite of an infected mosquito that deposits saliva containing *Plasmodium* sporozoites in the skin of the host. Motile sporozoites move freely in the dermis (Vanderberg and Frevert, 2004), where they probably encounter high concentrations of uracil-derived nucleotides. This would lead to the stimulation of apical regulated exocytosis long before sporozoites have reached their target cells in the liver. To study whether sporozoite exocytosis might be regulated by host factors that sporozoites encounter during the journey from the skin to the liver of host, we first tested the effect of mouse serum on sporozoite exocytosis. We found that pre-incubation of sporozoites with mouse serum completely inhibits exocytosis induced by uracil-derivatives (Fig. 3A). Since albumin is found in high concentrations in the serum and specifically regulates sporozoite activity inducing gliding motility (Vanderberg, 1974), we tested the effect of albumin on sporozoites exocytosis. We found that albumin completely prevents activation by uracil derivatives (Fig. 3B). Because albumin is a carrier protein normally found binding lipids (Kragh-Hansen *et al.*, 2002), we next tested the effect of highly purified fatty acid-free albumin, which presented a similar inhibitory effect (not shown). We also found that other proteins such as gelatin, or the serum proteins a2-macroglobulin and transferrin did not inhibit sporozoite exocytosis (Fig. 3C). The inhibitory effect of albumin was found to be dose dependent (Fig. 3D), with physiological concentrations found in the interstitial fluid of the dermis (35 mg/ml) (Reed *et al.*, 1989) or in blood (28-37 mg/ml) (Don and Kaysen, 2004) completely inhibiting sporozoite stimulation for exocytosis (Fig. 3C).

To confirm that the inhibitory activity observed is specifically due to the presence of albumin, we pre-incubated albumin with specific antibodies to neutralize its effect. We found that anti-albumin antibodies specifically reverse the inhibitory effect of albumin (Fig. 3E). As albumin is found in high concentrations in the interstitial fluids of the skin tissues (Reed *et al.*, 1989) our results suggest that after sporozoites are inoculated in the mammalian host, albumin would inhibit the exocytosis response to a stimulus such as uracil derivatives, preventing premature activation of sporozoites for infection.

This inhibitory mechanism, however, would interfere with the infectivity of the parasite, since hepatocytes contain high concentrations of albumin. To analyze the regulation of exocytosis by albumin in the presence of hepatocytes, we first added sporozoites pre-incubated with albumin or not to monolayers of mouse or human hepatoma cell lines.

We found that in the presence of these cells the inhibitory effect of albumin on exocytosis was no longer detectable, resulting in efficient activation of exocytosis (Fig. 4A). This result indicates that in the presence of hepatocytes, sporozoites are no longer susceptible to the inhibitory effect of albumin and can be activated by uracil derivatives. In these experiments, we inhibited sporozoite motility with a myosin inhibitor (BDM) to inhibit internalization of sporozoites inside host cells where exocytosis cannot be detected. Therefore, sporozoites were in contact with the surface of hepatocytes, but were not able to migrate through or infect these cells and the exocytosis stimulus was provided externally by addition of uracil derivatives in the medium. We then tested whether hepatocytes had to be alive and whether a hepatocyte lysate or the membrane fraction of hepatocytes could also mediate the reversal of albumin inhibition in uracil derivatives-induced exocytosis. We found that paraformaldehide fixed hepatocytes could also reverse the inhibitory effect of albumin (Fig. 4B). Incubation of sporozoites with a hepatocyte lysate or only its membrane fraction also prevented the inhibitory effect of albumin on uracil derivatives induced exocytosis (Fig. 4B), suggesting that the hepatocyte effect on exocytosis is mediated by a molecule localized in the extracellular side of the hepatocyte membrane.

Conversely, primary cultures of skin dermal fibroblasts did not reverse the inhibitory effect of albumin on exocytosis, resulting in the lack of exocytosis activation (Fig. 4C). These results indicate that different cell types have different effects on the regulation of parasite activity, and suggest that when sporozoites migrate through cells in skin dermis, they would not be able to undergo exocytosis in response to the cytosolic uracil nucleotides present in these cells. Conversely, contact with hepatocytes seems to counteract the inhibitory effect of albumin resulting in exocytosis activation after migration through these cells.

To test this hypothesis, we analyzed the capacity to induce sporozoite exocytosis of different cell types in the presence of albumin. *P. yoelii* sporozoites were incubated with cells cultured on Transwell filters. Sporozoites migrate through cells on the filter and are collected on coverslips placed underneath the filters (Mota *et al.*, 2002). The assay is performed in the presence of fluorescent dextran to confirm sporozoite migration through cells on the filter. We found that migration through hepatocytes results in the activation of sporozoite exocytosis, while migration through dermal fibroblasts or other

non-hepatic cell types does not (Fig.4D). During infection in the host, this differential capacity to activate sporozoites may play a role to achieve timely stimulation of exocytosis only after sporozoites have reached their target cells in the liver.

To analyze whether lack of exocytosis activation by skin cells actually results in lack of sporozoite activation for infection, we compared sporozoites after migrating through dermal fibroblasts or through hepatocytes. *P. yoelii* sporozoites were added to filters containing confluent dermal fibroblasts or Hepa1-6 cells. Sporozoites that traversed the filters encountered Hepa1-6 cells on coverslips placed underneath. In this way, we can distinguish between sporozoites that migrated through Hepa1-6 cells or through dermal fibroblasts before encountering the cells on the coverslip. We found that sporozoites that traversed filters with Hepa1-6 cells migrated through fewer cells before infection in the coverslips when compared with sporozoites that migrated through dermal fibroblasts (Fig. 4E, left panel). Sporozoites that migrated through Hepa1-6 cells appear ready to infect host cells in the coverslips underneath without need for further migration, whereas sporozoites that migrated through dermal fibroblasts still required migration through Hepa1-6 in the coverslips to be infective. As an alternative way to analyze sporozoite infectivity after the migrating through different types of host cells, we incubated *P. yoelii* sporozoites with Hepa1-6 cells or mouse dermal fibroblasts for 30 min, before transferring them to new Hepa1-6 cell monolayers to analyze their infectivity. Sporozoites that were pre-incubated with Hepa1-6 cells migrated through fewer cells before infection when they contact cell monolayers the second time, as compared to sporozoites that migrated through mouse dermal fibroblasts that still need to migrate through Hepa1-6 cells before infection (Fig. 4E, right panel). These results suggest that while migration through hepatocytes activates sporozoites for infection, migration through dermal fibroblasts does not. Since all cells have high concentrations of uracil derivatives in their cytosol, these results are consistent with the existence of a regulatory mechanism that would allow exocytosis only when sporozoites migrate through hepatocytes, but not through other cell types.

P. falciparum is the human malarial parasite that causes most of the mortality associated with this disease. *P. falciparum* sporozoites also migrate through host cells (Mota *et al.*, 2001), but apical regulated exocytosis has not been studied in this species of the parasite. We observed that physiological concentrations of uracil and its derivatives also

induce exocytosis in these sporozoites, which is inhibited by albumin (Fig. 5A). We also found that migration through a hepatocyte cell line that is susceptible to infection by *P. falciparum* sporozoites (Sattabongkot *et al.*, 2006) induces exocytosis, while migration through other cells did not activate sporozoites (Fig. 5B). These results suggest that *P. falciparum* sporozoites also activate exocytosis in response to uracil-derived nucleotides that they encounter in the cytosol of host cells during migration. Similarly to *P. yoelii,* exocytosis is also inhibited by albumin and seems to be reversed by the presence of hepatocytes, resulting in efficient activation of exocytosis.

Discussion:

The completion of a successful liver infection by *Plasmodium* sporozoites involves multiple steps, as these parasites need to traverse different host tissues before reaching the liver parenchyma where they finally invade a non-phagocytic cell, the hepatocyte. Sporozoites perform this journey with high rates of success, as very low numbers of sporozoites are able to initiate a malaria infection (Ungureanu *et al.*, 1977). The capacity of sporozoites to sense their environment and react accordingly seems essential to complete this task with high efficiency. Signaling pathways are probably activated in sporozoites regulating activities such as motility, migration through cells and exocytosis. Our results suggest that *Plasmodium* sporozoites can sense and react to the extracellular environment modulating their infectivity.

We have found two different molecules that regulate the behavior of *Plasmodium* sporozoites. Immediately after being injected into the dermis, sporozoites will encounter albumin, as this protein is found in the interstitial fluids of the dermis in high concentrations (Reed *et al.*, 1989). In addition, the blood pool formed after mosquito bite (Sidjanski and Vanderberg, 1997) must contain albumin normally found in serum. Albumin specifically induces *Plasmodium* sporozoites motility (Vanderberg, 1974), suggesting that sporozoites are able to sense the presence of this protein. Albumin is not present in mosquitoes, where sporozoites move at a slow speed (<2 μm/s) (Frischknecht *et al.*, 2004), however, it is abundant in mammals, where sporozoites need to initiate active motility. At the same time, our results indicate that albumin prevents sporozoite exocytosis. These observations are consistent with the requirements of an infection *in vivo*, where sporozoites in the skin need to move actively in order to reach the circulation and but also need to prevent premature activation of exocytosis before

reaching the liver.

There are several observations suggesting that sporozoites migrate through cells in the dermis after mosquito inoculation. Intravital microscopy of the skin has revealed that sporozoites move through the dermis and through endothelial cells (Amino *et al.*, 2006; Vanderberg and Frevert, 2004). Also, mutant sporozoites with reduced ability to migrate through cells have low infectivity in the host when deposited in the dermis by mosquito bites (Bhanot *et al.*, 2005). It has also been observed that sporozoites migrate through several hepatocytes in the liver before infecting a final one (Frevert *et al.*, 2005; Mota *et al.*, 2001) and that mutant parasites with defective migration have reduced infectivity after intravenous injection (Ishino *et al.*, 2005; Ishino *et al.*, 2004). As migration through cells leads to the activation of sporozoite exocytosis (Mota *et al.*, 2002), albumin would prevent this process before sporozoites reach the liver. In fact, we found that migration through skin dermal cells does not induce exocytosis and does not activate sporozoites for infection. Sporozoites must enter in contact with high concentrations of uracil derivatives while migrating through the cytosol of these cells, but exocytosis is not induced, presumably due to the inhibitory effect of albumin. Our results indicate that migration through cells can occur without sporozoite activation, a situation that probably occurs *in vivo* during migration in the skin of the host.

After reaching the liver, sporozoites need to undergo exocytosis to release or expose on their surface molecules necessary to invade hepatocytes forming a parasitophorous vacuole. Probably several parasite and host cell molecules are involved in this interaction. We have used TRAP as a marker for apical regulated exocytosis, as it is one of the best-characterized parasite proteins that is found in the micronemes (Bhanot *et al.*, 2003) and is involved in host cell invasion (Jethwaney *et al.*, 2005; Sultan *et al.*, 1997). We have observed that after contact with hepatocytes, sporozoites recover their capacity to exocytose regardless of the presence of albumin. Accordingly, migration through hepatocytes induces sporozoite exocytosis, activating parasites for infection. This reversion of the inhibitory effect of albumin must be necessary to establish an infection in the host, as there are high concentrations of albumin in the liver, both in the cytosol of hepatocytes and in interstitial tissues. The activation of exocytosis during migration through hepatocytes would also represent an advantage to the parasite, since molecules that are required for host cell invasion, such as TRAP, would only be

exposed to the cytosol of traversed hepatocytes and not to the extracellular environment, avoiding the potential inhibitory effect of antibodies. In fact, although TRAP is required for host cell invasion, antibodies to TRAP do not inhibit the infectivity of sporozoites, even at high concentrations (Gantt et al., 2000).

Our results suggest that sporozoites are able to differentiate hepatocytes from other cell types. This mechanism allows the parasite to respond to exocytosis stimuli only after being in contact with hepatocytes. Sporozoites probably recognize hepatocyte surface molecules, as they become responsive to uracil derivatives after incubation with hepatocytes when sporozoite motility was inhibited to avoid host cell invasion. Once they start migrating through host hepatocytes, uracil derivatives in their cytosol would induce apical exocytosis, activating sporozoites for infection. This mechanism probably allows sporozoites to sense that they have reached an intracellular cytosolic environment, as the concentration of uracil derivatives is very low in extracellular fluids (Traut, 1994). *Plasmodium* sporozoites may require specific surface receptors or transporters to respond to uracil derivatives. Several putative nucleoside transporters have been identified within the *P. falciparum* genome (Bahl *et al.*, 2003), but only one (PfNT1) has been functionally characterized, showing preferential affinity for purines (El Bissati *et al.*, 2006). Mammalian cells have pyrimidine receptors, the P2Y family, that activate signaling cascades and exocytosis in specific cell types (Brunschweiger and Muller, 2006) however, no sequence homology is found for this type of receptor in the *Plasmodium* genome (Bahl *et al.*, 2003). Our results also don't exclude the possibility of alternative signals to trigger exocytosis provided by host cells.

Genetically manipulated sporozoites that are deficient in their capacity to migrate through cells (SPECT), present very low infectivity of hepatocytes *in vivo*, but they are able to infect hepatic cell lines *in vitro*, questioning whether migration through cells is necessary to induce exocytosis before infection (Ishino *et al.*, 2004). We have found that uracil and its derivatives induce apical regulated exocytosis in these mutant parasites. However, SPECT-deficient parasites present altered signaling responses and seem to use different signaling pathways to activate exocytosis that are not used by *wt* sporozoites, suggesting that these parasites are activated using alternative mechanisms, which may be independent of migration through cells (Ono *et al.*).

We have confirmed that sporozoite stimulation and regulation of exocytosis is similar in *P. falciparum,* the human parasite with highest clinical importance. It seems likely that this is a common mechanism in different species of *Plasmodium*, as the molecules involved, uracil derived-nucleotides and albumin, are highly conserved among different host species (Baker, 1989). It is noteworthy that *Plasmodium* uses these essential, highly conserved molecules to regulate its behavior towards infection. This may represent an advantage for the parasite, as it limits the possibility of encountering host variants that would be more resistant to infection.

Experimental Procedures:

Cells and parasites. Cell lines were maintained at 37ºC with 5% CO_2 in DMEM medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1mM glutamine. HC-04 cells were maintained as described (Sattabongkot *et al*., 2006). *P. yoelii yoelii* (parasite line 17 XNL) and *P. falciparum* (parasite line NF54, clone 3D7) sporozoites were obtained from dissection of infected female *Anopheles stephensi* mosquito salivary glands. Mouse dermal fibroblasts were obtained from a Balb/c mouse.

Hepa1-6 cell lysates and membrane fraction. Hepa1-6 cells (4 x 10^5 cells per ml) resuspended in culture medium were repeatedly passed through a 28G syringe until more than 95% of the cells were lysed, as determined by Trypan blue staining. For membrane extraction, a Hepa1-6 cells lysate was centrifuged at 3,600 *g* to remove debris and nuclei. The supernatant was centrifuged at 110,000 *g* for 40 min to pellet the membrane fraction.

Uracil derivatives. A mixture of the physiological concentrations of uracil derivatives in the cytosol of mammalian cells (Traut, 1994) consisting of 180 μM uracil, 280 μM uridine, 300 μM uracil monophosphate, 50 μM uracil diphosphate and 30 μM uracil triphosphate (ICN Biomedicals) was prepared in RPMI 1640 and pH adjusted to 7.

Apical regulated exocytosis. *Plasmodium* sporozoites (10^5) were centrifuged for 5 min at 1,800 x *g* on glass coverslips with or without a monolayer of 2x10^5 Hepa1-6 cells, HepG2 cells or mouse dermal fibroblasts. In one experiment as indicated, Hepa1-6 cells

were fixed with 4% paraformaldehide for 2 h and washed before use. After 45 min incubation at 37 °C sporozoites were fixed with 1% paraformaldehyde for 20 min before staining with anti-TRAP mAb (F3B5 for *P. yoelii* or *Pf*SSP2.1 for *P. falciparum* (Charoenvit *et al.*, 1997)), followed by FITC-labeled anti-mouse secondary antibodies. Sporozoite regulated exocytosis was quantified as the percentage of total sporozoites that present a TRAP stained "cap" in their apical end. Results are expressed as mean of triplicate quantifications of a minimum of 50 sporozoites with standard deviation. Background level of exocytosis was measured in sporozoites after dissection from mosquitoes, before incubation *in vitro*. Background exocytosis was always lower than 8% and was subtracted from all values. Digital pictures were acquired using an inverted Olympus 1x70 with a 63x oil-immersion objective at room temperature with a Hammatsu Photonics C4742-95 camera using Metamorph Imaging Systems software. Images were not modified other than adjustment of brightness and contrast to the whole image. Albumin from mouse serum, essentially fatty acid-free human and mouse albumin (0.005% fatty acid content) solutions were prepared at 35 mg/ml in RPMI 1640. Gelatin from bovine skin was used at 35 mg/ml in RPMI 1640, alpha2-macroglobulin at 1.64 mg/ml and apo-transferrin at 2.5 mg/ml. All proteins were from Sigma. Sporozoites were pre-incubated with albumin or the other proteins for 15 min at room temperature in an eppendorf tube, spun down at 8,600 x*g* and resuspended in fresh medium before incubation with the uracil derivatives at 37ºC for 45 min. Rabbit anti-albumin antiserum (4-6 mg/ml) (Sigma) was pre-incubated for 1 h at 37ºC with mouse albumin at 1mg/ml before addition of the complex to sporozoites. When indicated, sporozoites were pre-incubated for 15 min with the myosin inhibitor butanedionemonoxime (BDM) (1 mM) to inhibit gliding motility.

Migration through cells and infection *in vitro*. *P. yoelii* sporozoites (10^5 per coverslip) were added to monolayers of $2x10^5$ cells for 1 h in the presence of 1 mg/ml of FITC-conjugated, lysine-fixable dextran (Mr 10,000; Molecular Probes). Cells were washed and incubated for another 24 h before fixation and staining with anti-HSP70 mAb (2E6) to detect infected cells (Tsuji *et al.*, 1994). Migration through host cells is quantified as percentage of dextran positive cells. In a different set of experiments, *P. yoelii* (10^5 sporozoites per coverslip) were added to monolayers of $2x10^5$ Hepa1-6 cells or mouse dermal fibroblasts for 30 min. Sporozoites were then transferred to a new monolayer of Hepa1-6 cells and incubated for an additional 30 min in the presence of the tracer dextran.

Transwell filter assays. Cell lines or primary cultures of mouse dermal fibroblasts ($5x10^5$) were cultivated on 3 μm pore diameter Transwell filters (Costar, Corning, New York) until they form a continuous monolayer. Empty coverslips or coverslips containing Hepa1-6 cells monolayers ($2x10^5$ Hepa1-6) were placed underneath the filters. *P. yoelii* sporozoites ($2x10^5$) were added to filter insets containing Hepa1-6 cells, mouse dermal fibroblasts or no cells. Filters and coverslips were fixed after 2 h of incubation with sporozoites, before staining for surface TRAP. To determine migration through host cells, FITC-dextran (1 mg/ml) was added before addition of sporozoites. Coverslips were washed after 2 h of incubation with sporozoites and further incubated for 24 h before fixation, staining and quantification of dextran positive cells and infected cells with anti-HSP70.

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Abbreviations:

Thrombospondin-related anonymous protein (TRAP)

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Figure 1

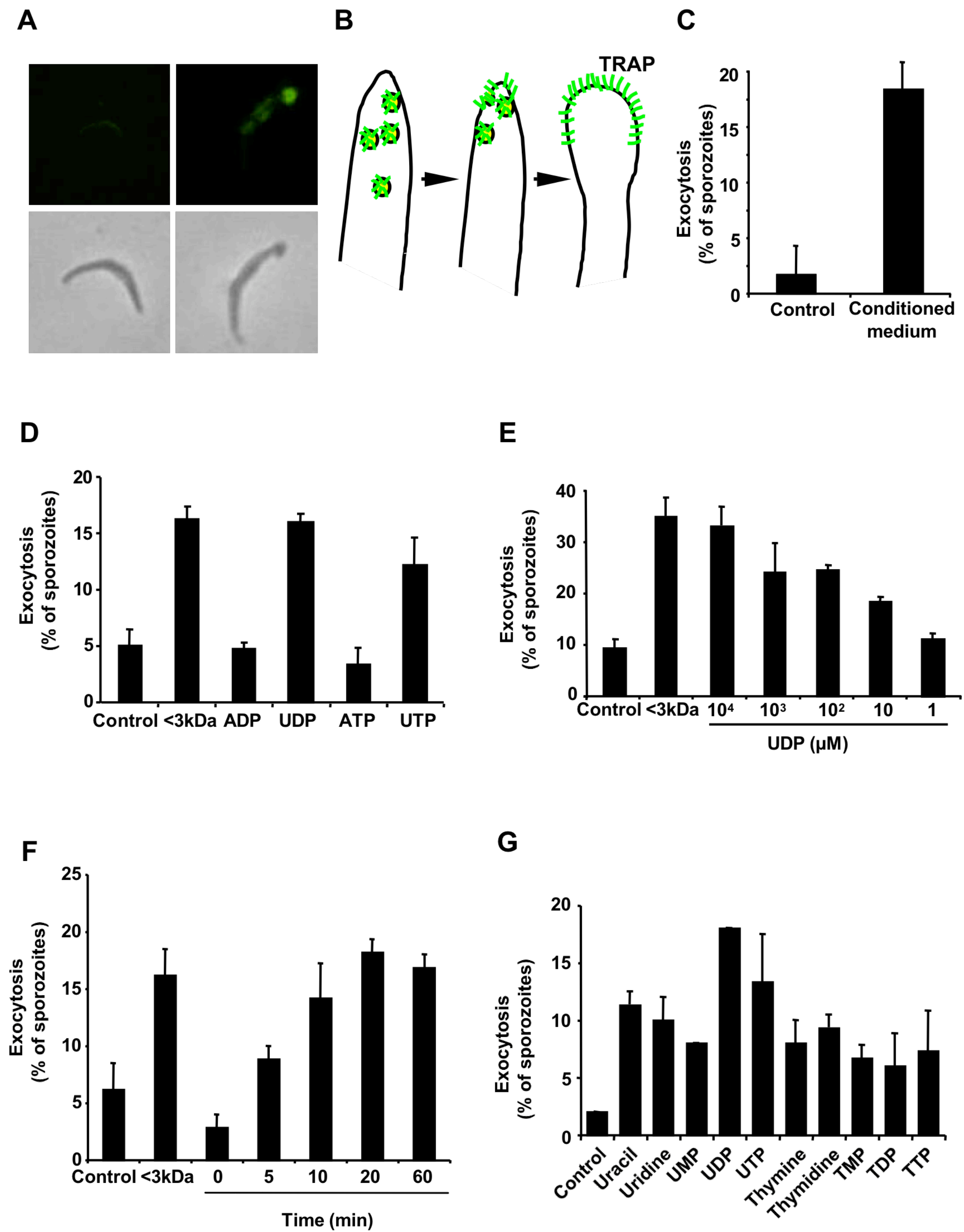
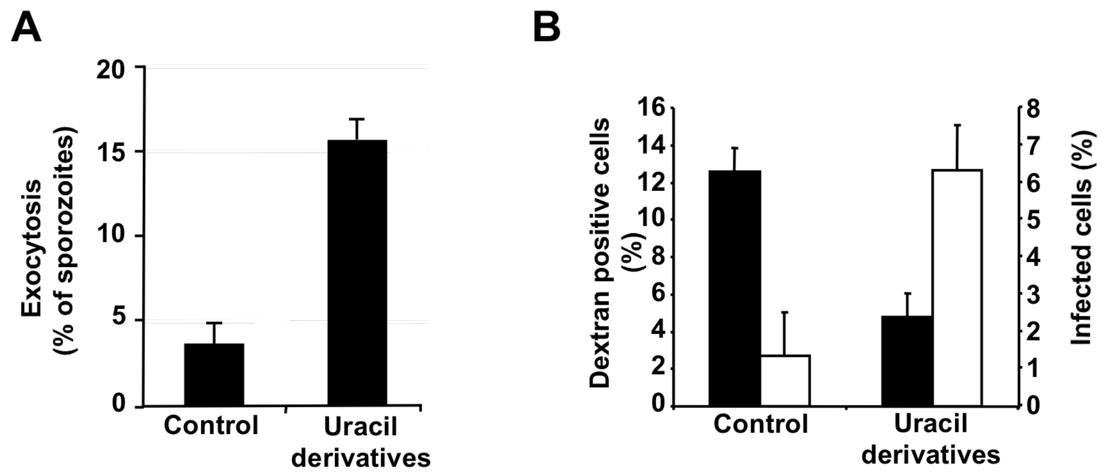


Figure 2



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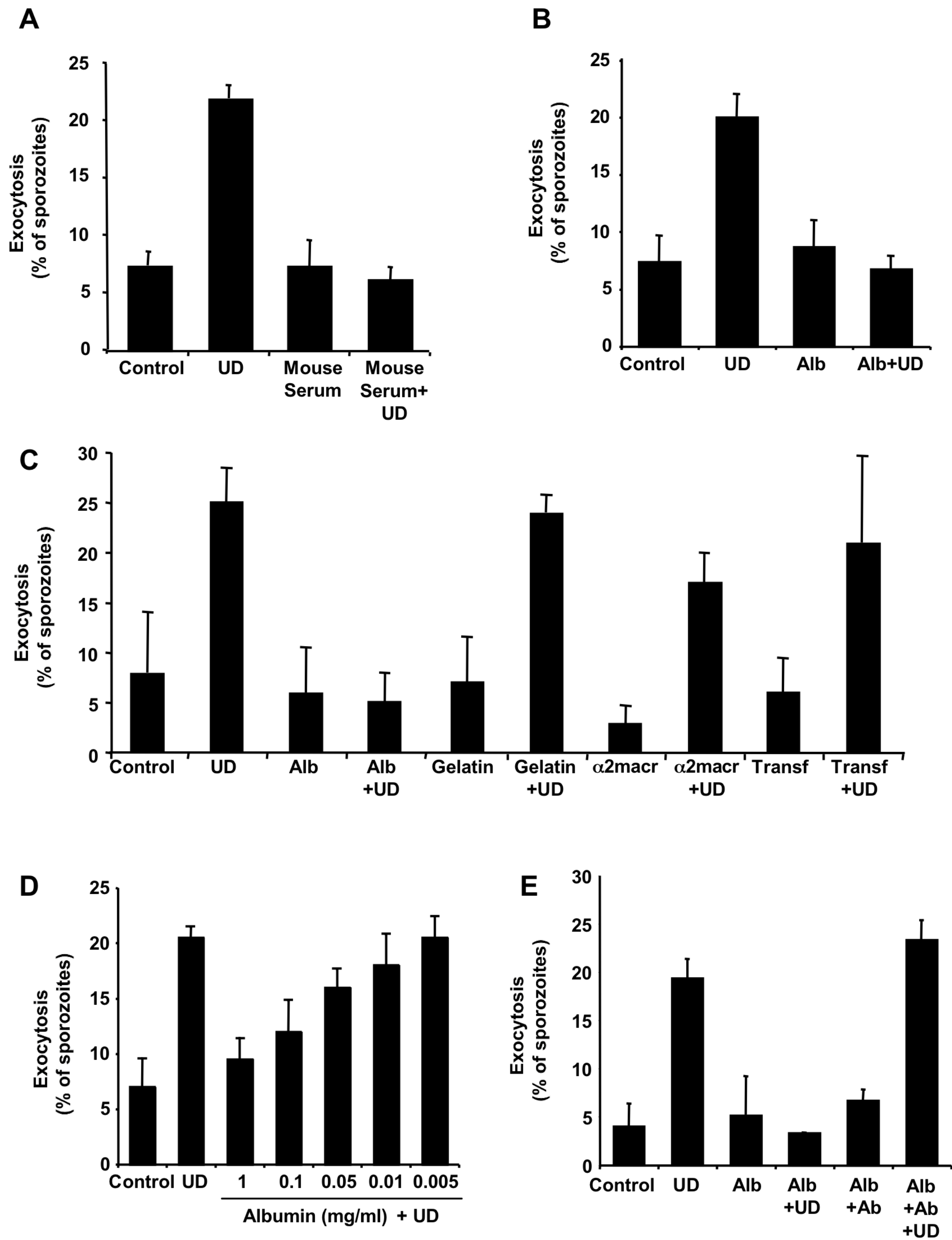

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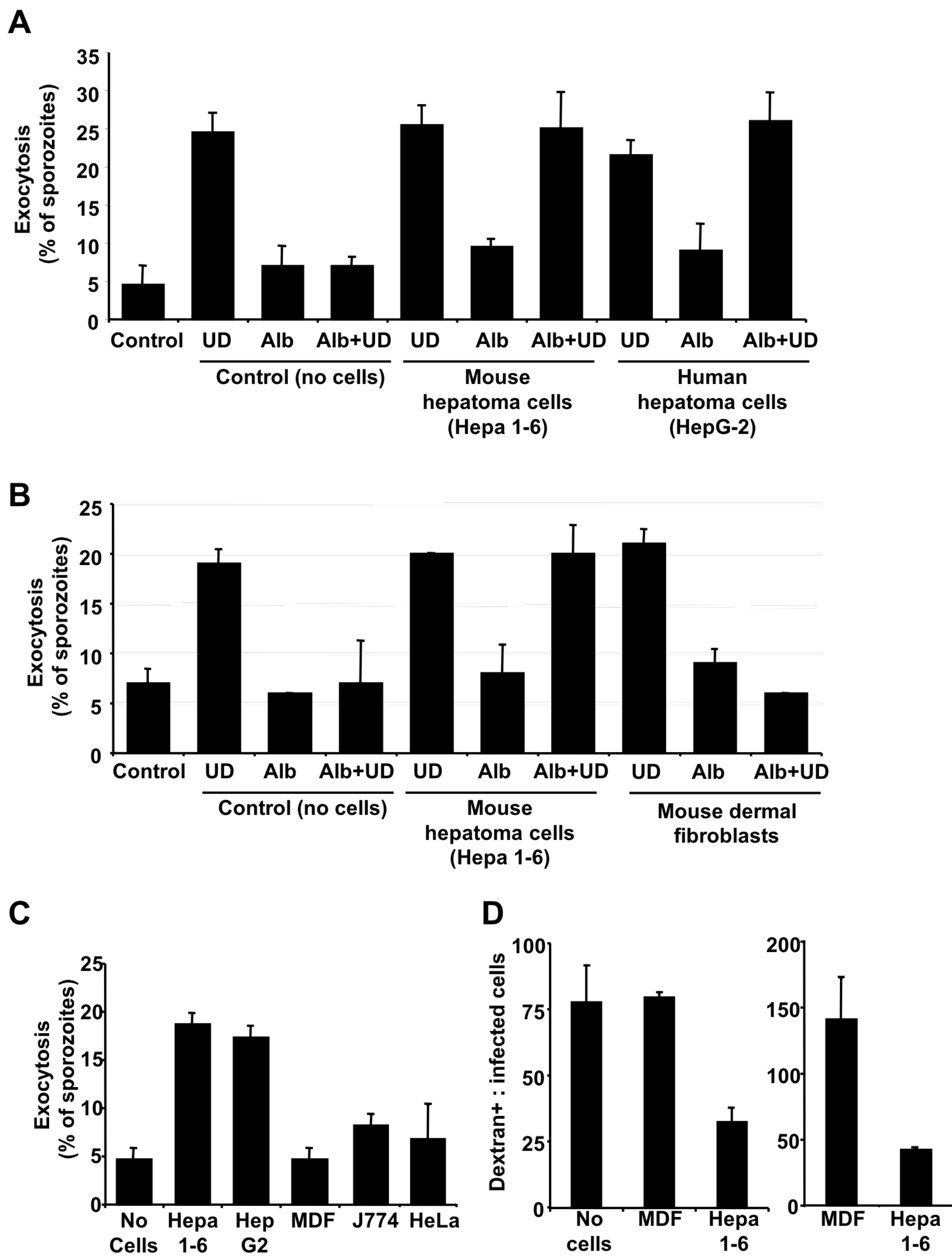

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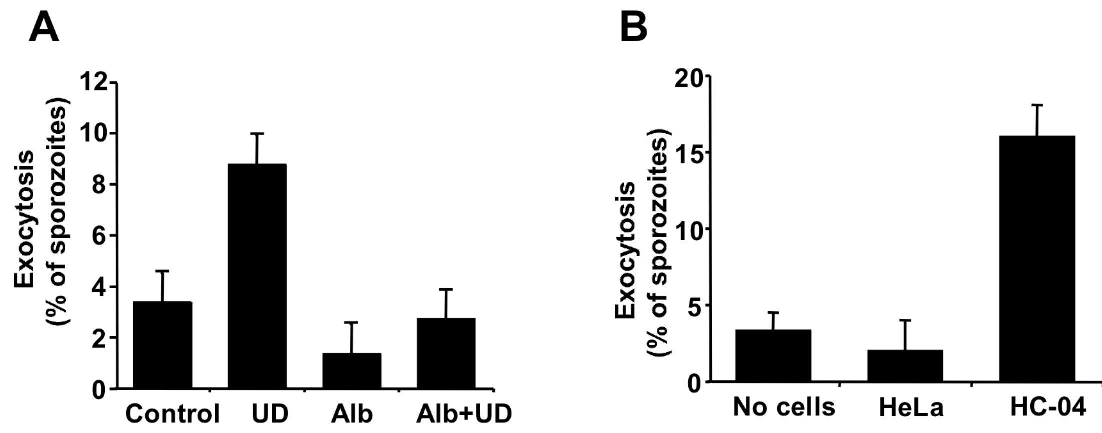

Figure legends:

Fig. 1. Uracil derivatives induce apical regulated exocytosis in *Plasmodium* sporozoites. (A) Upper panels show surface staining of *P. falciparum* sporozoites with anti-TRAP mAb. Lower panel shows the same microscope field in phase contrast. Apical regulated exocytosis is observed as a 'cap' in one end of the sporozoite (right panels). (B) Model of apical regulated exocytosis. After activation, *Plasmodium* sporozoites recruit TRAP-containing micronemes to their apical end, which fuse with the apical membrane of the parasite. (C-F) Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis after incubation for 1 h alone (Control), with a lysate of Hepa1-6 cells (Lys) or 100 μM UDP, ADP, UTP and ATP (C), increasing concentrations of UDP (D), 100 μM UDP for the indicated time periods (E), 100 μM of the indicated pyrimidines (F). Results are expressed as mean of triplicate determinations ± SD.

Fig. 2. Physiological concentrations of uracil derivatives induce apical regulated exocytosis in *P. yoelii* sporozoites and activate them for infection. (A) Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis after incubation with physiological cytosolic concentrations of uracil and its derivatives, as described in methods. (B) *P. yoelii* sporozoites were incubated with uracil derivatives mix and added to monolayers of Hepa1-6 cells. Percentage of dextran-positive cells (black bars) and infected cells (white bars) are shown. Results are expressed as mean of triplicate determinations ± SD.

Fig. 3. Albumin inhibits exocytosis induced by uracil derivatives in *P. yoelii* sporozoites. Sporozoites were pre-incubated with (A) mouse serum (non-diluted), (B) mouse albumin (1 mg/ml), (C) gelatin (35 mg/ml) or serum physiological concentrations of albumin (35 mg/ml), a2-macroglobulin (1.64 mg/ml) and transferrin (2.5 mg/ml), (D) increasing concentrations of mouse albumin, (E) mouse albumin (1 mg/ml) pre-incubated or not with anti-albumin specific antiserum. Sporozoites were washed before incubation with the uracil derivatives (UD). Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis is shown. Results are expressed as mean of triplicate determinations ± SD.

Fig. 4. The inhibitory effect of albumin on sporozoite exocytosis is reversed in the presence of hepatocytes. (A) Percentage of *P. yoelii* sporozoites showing apical regulated exocytosis. Sporozoites were pre-incubated or not with mouse albumin (1 mg/ml), washed and incubated with BDM to inhibit parasite motility before incubation with monolayers of mouse (Hepa1-6) and human (HepG2) hepatoma cell lines, in the presence or absence of the uracil derivatives (UD). As negative control in each condition, we used sporozoites incubated with albumin (Alb) but not stimulated with UD. (B) *P. yoelii* sporozoites were pre-incubated or not with mouse albumin, washed and incubated with intact or fixed monolayers of mouse Hepa1-6 cells, a

lysate or the membrane fraction of Hepa1-6 cells. (C) Sporozoites were pre-incubated or not with mouse albumin, washed and incubated with BDM before incubation with monolayers of mouse (Hepa1-6) or mouse dermal fibroblasts (MDF). (D) *P. yoelii* sporozoites were pre-incubated with mouse albumin, washed and added to filter insets containing the indicated cell types. Sporozoites were collected on empty coverslips placed underneath the filters in the lower chamber. Percentage of sporozoites in coverslips showing apical-regulated exocytosis is shown. (D) Left panel: Hepa1-6 cells or MDF were cultivated on filters and coverslips with Hepa1-6 cells were placed underneath the filters in the lower chamber. *P. yoelii* sporozoites were added to the filter insets. As a control, sporozoites were added to filters containing no cells. The ratio of dextran-positive cells to infected cells is shown for coverslips placed under filters. Right panel: *P. yoelii* sporozoites were incubated with monolayers of Hepa1-6 cells or MDF, before transfer of the supernatants containing sporozoites to new Hepa1-6 monolayers. The ratio of dextran-positive cells to infected cells is shown for each condition. Results are expressed as mean of triplicate determinations ± SD.

Fig. 5. *P. falciparum* sporozoites apical regulated exocytosis is induced by uracil derivatives or migration through human hepatocytes and it is inhibited by human albumin. Percentage of *P. falciparum* sporozoites showing apical regulated exocytosis when pre-incubated with fatty-acid free human albumin followed by washing and (A) uracil derivatives (UD) or (B) addition to filter insets containing no cells, non-hepatic cells (HeLa) or the human hepatocyte cell line (HC-04). Sporozoites were collected on empty coverslips placed underneath the filters in the lower chamber. Percentage of sporozoites in coverslips showing apical-regulated exocytosis is shown. Results are expressed as mean of triplicate determinations ± SD.