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Protein trafficking and host cell remodeling in malaria parasite infection

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ABSTRACT

Plasmodium endurance depends on the ability of the parasite to reorganize the cytosol of the erythrocyte, a terminally differentiated cell, and remodels its skeleton membrane immediately after invasion. In this way the parasite can organize the import/export of the molecules necessary to its survival. The comprehension of cellular trafficking mechanisms which occur during *Plasmodium* infection is a very important step and fundamental contribute to understand the biology of the malaria parasite.

We identified in the rodent malaria parasite *Plasmodium berghei* the gene family *sep*, corresponding to *etramp* in *P. falciparum*, encoding small exported proteins conserved in the genus *Plasmodium*. SEP proteins (13–16 kDa) contain a predicted signal peptide at the NH2-terminus, an internal hydrophobic region while they differ in their C-terminal region; the genes share the upstream regulative region while they differ in the 3'UTR. Despite this, we showed that SEPs have a different timing of expression and a different localization: in the erythrocytic cycle PbSEP1 and PbSEP3 start to be expressed at trophozoite and the same amount of protein is detected also in schizonts and gametocytes, while PbSEP2 is highly detected in mature trophozoites and even more in gametocytes. In mosquitoes stages PbSEP1 and PbSEP3 are expressed only in ookinetes, while PbSEP2 is very abundant in ookinetes, oocysts and in sporozoites of the salivary glands. SEPs also have a different localization in the iRBC: PbSEP1 is targeted to the membrane of the parasitophorous vacuole, while PbSEP2 and 3 are exported beyond the parasite membrane and translocated to the host cell compartment in association with vesicle-like structures. In this study we identified the specific signals necessary for the correct timing of expression and to direct SEP proteins to the vacuolar membrane and to the host cell compartments. The second part of the work was carried out in Montpellier II University and aims to identify the localization of two RBC membrane skeleton components, dematin and adducin, during *Plasmodium falciparum* infection. Our purpose is to recognize a possible mechanism of internalization of host cytoskeleton components to the parasite compartments. In fact, IFA experiments carried on iRBCs showed that dematin and adducin start to be internalized at trophozoite stage and localize at the periphery of the parasite, most probably at the parasitophoruos vacuole (PV) membrane/lumen. Dematin and adducin internalization during *Plasmodium* infection is also demonstrated by subcellular fractionation and proteinase K (PK) assay: while dematin is fully internalized, adducin is partially protected and suggesting a localization of the protein at the periphery of the parasite where it can be exposed to PK degradation.

RESUME

Pour assurer ses besoins de croissance, multiplication, et survie, *Plasmodium* modifie sa cellule hôte, l'érythrocyte, après l'invasion. Le parasite met en place ainsi un système d'échanges (import/export) avec sa cellule hôte et le milieu extérieur.

Nous avons identifié dans la base de données de *Plasmodium berghei*, le parasite de rongeurs, une famille de gènes, *sep*, correspondant à la famille *etramp* chez *Plasmodium falciparum*. Cette famille de gènes code pour des petites protéines exportées, et conservées dans tout le genre *Plasmodium*. Les protéines SEP (13–16 kDa) contiennent en N-terminal un peptide signal prédit, un domaine hydrophobe interne, et elles diffèrent au niveau des régions C-terminal et 3' UTR. Toutefois, les protéines SEP sont exprimées à différents moments du cycle de *Plasmodium*. Durant le cycle érythrocytaire, PbSEP1 et PbSEP3 sont exprimées à partir du stade trophozoïte, et la même quantité de protéine est détectée au stade schizonte et gamétocyte, pendant que PbSEP3 est hautement détectée dans les trophozoïtes mûrs et les gamétocytes. Chez le moustique, PbSEP1 et PbSEP3 sont détectées seulement chez les ookinètes, alors que PbSEP2 est très abondante dans les ookinètes, oocystes, et sporozoïtes des glandes salivaires. Les protéines SEP ont également des localisations différentes. Dans l'érythrocyte, PbSEP1 est localisée dans la membrane de la vacuole parasitophore, alors que PbSEP2 et PbSEP3 sont exportées au-delà de cette vacuole, et sont ainsi localisées dans la cellule hôte, en association avec des structures vésiculaires. Dans cette étude, nous avons identifié les signaux d'adressage des protéines SEP dans la vacuole parasitophore et dans la cellule hôte, chez *Plasmodium berghei*.

L'autre partie du travail, effectuée à l'Université de Montpellier II, a consisté à étudier la localisation de deux protéines du squelette sous- membranaire de l'érythrocyte, la dématine, et l'adducine, durant le développement intra-érythrocytaire de *Plasmodium falciparum*. Le but de cette étude étant d'identifier un mécanisme potentiel d'internalisation des composants du squelette sous-membranaire de l'érythrocyte dans le parasite. Des études d'immuno-localisation ont montré que la dématine et l'adducine sont internalisées à partir du stade trophozoïte, et sont localisées probablement à la vacuole parasitophore (membrane et/ou lumière). Cette internalisation a été confirmée par des études de fractionnement cellulaire et d'accessibilité à la protéinase K, montrant que la dématine est totalement internalisée, alors l'adducine ne l'est que partiellement, suggérant une localisation de la protéine à la périphérie du parasite.

RIASSUNTO

Il Plasmodio della malaria si riproduce nell'eritrocita, una cellula che ha perso capacità di sintesi poiché è altamente specializzata nel trasporto di ossigeno. La sopravvivenza del parassita è strettamente collegata alla riorganizzazione del citoplasma e della superficie del globulo rosso tramite la generazione di strutture membranose composte sia da molecole esportate dal parassita, che da altre importate dalla cellula ospite. Plasmodio garantisce così i nutrienti necessari al suo sviluppo e la possibilità di esportare fattori di virulenza alla superficie della cellula ospite. Tre membri della famiglia genica *sep* di *P. berghei* denominate SEP (Small Exported Proteins- 13-16 KDa), che presentano similarità con le proteine ETRAMP di *P. falciparum*, mostrano caratteristiche peculiari: condividono quasi totalmente la sequenza codificante, contenente un peptide segnale e una regione transmembrana, mentre differiscono nella porzione C-terminale. Anche la regione del promotore è pressoché identica, mentre variano gli specifici 3'UTR. Con la generazione di anticorpi specifici della regione al C-terminale, è stato possibile definire che si tratta di proteine integrali di membrana, la cui localizzazione e stadio specificità sono sorprendentemente diverse: in particolare la SEP2 e la SEP3 vengono esportate nella cellula ospite, mentre la SEP1 risiede alla membrana del vacuolo parassitoforo, struttura di interfaccia tra il parassita ed l'eritrocita. Inoltre è stato osservato che la SEP1 e 3 sono espresse in livelli confrontabili negli stadi intraeritrocitari, mentre la SEP2 è abbondantemente espressa nei gametociti, dato significativo in quanto questi sono le uniche forme ingerite dalla zanzara anofelina durante il pasto di sangue che daranno origine a parassiti in grado di svilupparsi nell'insetto vettore. Anche nell'insetto le 3 SEP hanno un profilo di espressione differente: SEP1 e 3 sono presenti negli ookineti, mentre la SEP2 è abbondantemente espressa in ookineti, oocisti e sporozoiti. L'identificazione dei motivi proteici necessari alla localizzazione delle proteine SEP2 e SEP3 nei vari distretti dell'eritrocita infetto si è basata sulla generazione di linee transgeniche di *P. berghei* in grado di esprimere porzioni diverse delle proteine SEP fuse alla proteina reporter GFP: la contemporanea presenza del peptide segnale della regione transmembrana è necessaria per traslocare le proteine di fusione SEP/GFP nell'eritrocita. La seconda parte del lavoro è stata svolta presso l'Università di Montpellier 2, e si propone di caratterizzare la localizzazione di due proteine del complesso giunzionale del citoscheletro dell'eritrocita umano, la dematina e l'adducina, in quanto recenti esperimenti svolti con il parassita murino hanno suggerito la possibilità di un meccanismo di internalizzazione di entrambe. Avvalendosi dell'impiego di anticorpi specifici e dell'utilizzo del microscopio confocale e apotome, è stato osservato che entrambe sono associate a comparti del parassita. Il processo di internalizzazione di queste due proteine è stato inoltre dimostrato attraverso esperimenti di frazionamento subcellulare e successiva digestione con proteinasi K: La dematina è interamente protetta e quindi completamente internalizzata dal parassita mentre l'adducina è in parte digerita dalla proteinasi K e quindi parzialmente esposta nel citoplasma dell'eritrocita.

Keywords: malaria, *Plasmodium falciparum*, *Plasmodium berghei*, cellular trafficking, erythrocyte remodeling, exported proteins.

Mots-clés: paludisme, *Plasmodium falciparum*, *Plasmodium berghei*, trafficking cellulaire, modification de l'érythrocyte, protéines exportées.

Parole chiave: malaria, *Plasmodium falciparum*, *Plasmodium berghei*, trafficking cellular, rimodellamento dell'eritrocita, proteine esportate.

1. INTRODUCTION

Malaria is an infective disease caused by protozoan parasites belonging to the genus *Plasmodium* (Greenwood et al. 2005). More than 100 species of *Plasmodium* can infect different vertebrate hosts such as reptiles, birds, and various mammals, but in nature only four species are infective for humans: *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*; the first two species cause the most infections worldwide.

According to the World Health Organization's World Malaria Report 2005, malaria is considered the most important parasitic infection in Humans causing 300-500 million of new cases worldwide and more than 1 million deaths every year (more than 80% occur in Africa south of the Sahara) so that the study of this disease has become a priority for the international health community.

The most dangerous manifestations of malaria disease depend on *P. falciparum* infection in almost all cases. Erythrocyte rupture induces periodic waves of fever in patients as the disease progresses. Symptoms range in severity from headaches, hypoglycaemia, anaemia and fevers to renal failure, cerebral malaria and death.

Cerebral malaria is a complication that is observed in a small subset of *P. falciparum* infections and is associated with changes in mental status and coma. The mortality ratio is between 25–50%. The histopathological feature of this encephalopathy is the sequestration of parasitized and uninfected red blood cells (RBCs) to cerebral capillaries and venules. (Maier et al. 2009).

Despite strategies for developing malaria vaccines have been targeted at specific points in the parasite life cycle during which the organism appears particularly susceptible to the host's immune system (Purcell et al. 2008), the complexity of *Plasmodium* life-cycle and the climatic and environmental factors play a negative role in the malaria challenge.

Development of new medicines for malaria is a challenging and important topic of applied research because malarial parasites rapidly adapt to man-made interventions. The best-studied examples are widely used drugs such as chloroquine, sulfadoxin-pyrimethamine, and, recently, artemisinins; all of them can lose efficacy after large-scale introduction.

1.1 - *Plasmodium* life cycle

Plasmodium is an obliged intracellular parasite which develops in a vertebrate host and is transmitted through female mosquitoes of the *genus Anopheles* (Tuteja R. 2007).

Malaria infection starts with the bite of an infected mosquito, which injects salivary gland sporozoites (*Plasmodium* infective stage) into the bloodstream of the vertebrate host.

In about 30 minutes sporozoites invade hepatocytes, where they grow and differentiate into trophozoites, which undergo asexual multiplication (schizogony).

Mature hepatic schizonts (14 days for *P. falciparum*) produce thousands of merozoites equipped with conoid, an apical structure necessary for the red blood cell (RBC) invasion. Only in the case of *P. ovale* and *P. vivax* infections, a fraction of sporozoites stay quiescent in liver cells and develop after a latency period (hypnozoites) causing relapses.

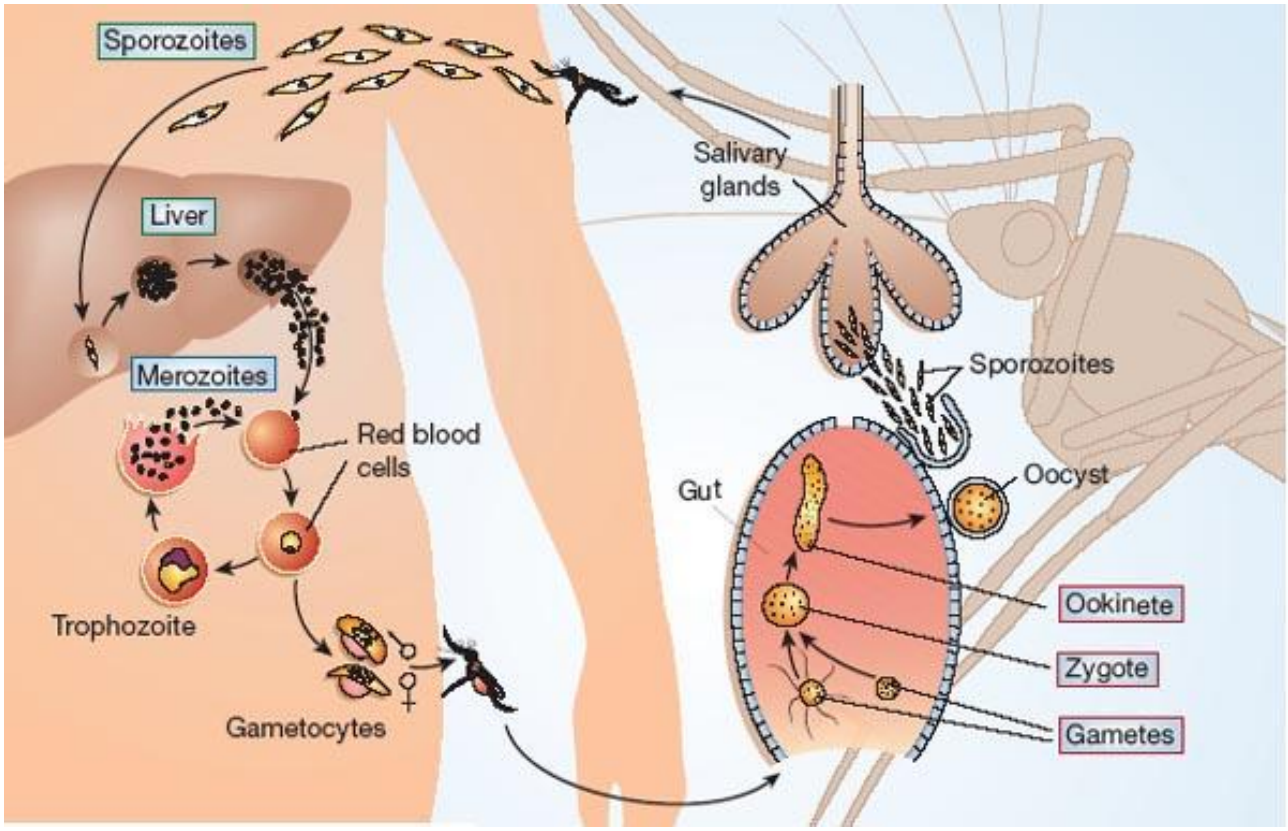
Merozoites are detected in the peripheral blood 7-15 days after the mosquito bite, depending on *Plasmodium* species.

They invade RBCs and develop into trophozoites. Mature trophozoites undergo nuclear division (schizont stage) and when merozoites are mature inside the iRBC, the membrane is dissolved releasing parasites ready to invade new RBCs. Repetitive feverish abscesses each 72 hours in *P. malariae* and 48 hours in the other three species are due to the synchronised rupture of infected RBCs (iRBC).

A fraction of blood stages undergoes sexual differentiation with the production of male/female gametocytes which stay in the peripheral blood until a new mosquito ingests them during the blood meal on an infected host.

When infected blood is sucked by a mosquito, male gametocytes undergo a rapid nuclear division, producing 8 flagellated microgametes which fertilize female macrogametes.

The resulting “ookinete” traverses the mosquito gut wall and encysts. After maturation (7-13 days) the oocyst ruptures, releasing hundreds of sporozoites which migrate to the salivary glands. (Figure 1.1 – *Plasmodium* life-cycle).



Greenwood et al. 2008

Figure 1.1. *Plasmodium* life cycle

1.2 - Malaria in the world

The diffusion of malaria depends mainly on climatic factors such as temperature, humidity, and rainfalls. Malaria is transmitted in tropical and subtropical areas, where *Anopheles* mosquitoes can survive and multiply and parasites can complete their growth cycle in the mosquitoes (Figure 1.2).

Temperature is particularly critical; for example, at temperatures below 20°C, *Plasmodium falciparum* (which causes severe malaria) cannot complete the life cycle in the mosquito, and thus cannot be transmitted (Kreier J. 1980).

According to geographic and climatic factors, even within tropical and subtropical areas, there is no transmission at high altitudes, during cooler seasons, in deserts (excluding the oases), in some areas where local *Anopheles* species, capable of transmitting malaria, are not present; there, *P. vivax* might be more prevalent because it is more tolerant of lower ambient temperatures.

In many temperate areas, such as Western Europe and the United States, economic development and public health measures contributed in eliminating malaria (de Zulueta J. 1973).

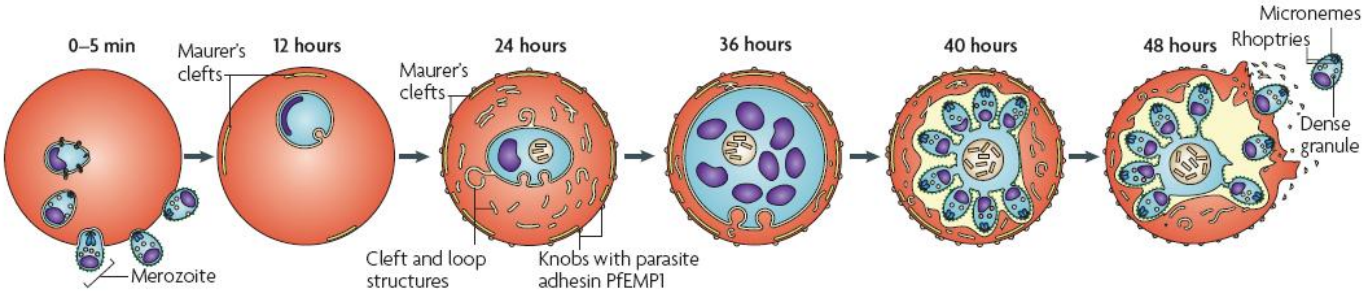
However, *Anopheles* mosquitoes that can transmit malaria are still present in most of European and American areas, and the migration of people from endemic areas represents a constant risk of malaria reintroduction due to the presence of human "tank" (WHO. 2007).

Figure 1.2. Geographic distribution of malaria in the world.

1.3 - Erythrocyte remodeling after *Plasmodium* invasion

Plasmodium is an intracellular parasite, able to enter and grow inside the RBC, a terminal differentiated cell, which lacks the ability to synthesise new proteins and to perform intracellular trafficking. As *Plasmodium* enter the host cell, it reorganizes the cytoplasm of RBC generating new membrane compartments such as the parasitophorous vacuole (PV), in which it resides surrounded by the PV membrane (PVM), which offers a semi-permeable barrier between host and parasite.

Early after invasion *Plasmodium* also forms structures and compartments, well identified by electron microscopy (Maier et al. 2009), which are extensions of the PVM called the Tubulo-Vesicular Membrane Network (TVN), which play a role in the import/export of molecules from/to the erythrocyte surface (Figure 1.3).



Maier et al. 2009.

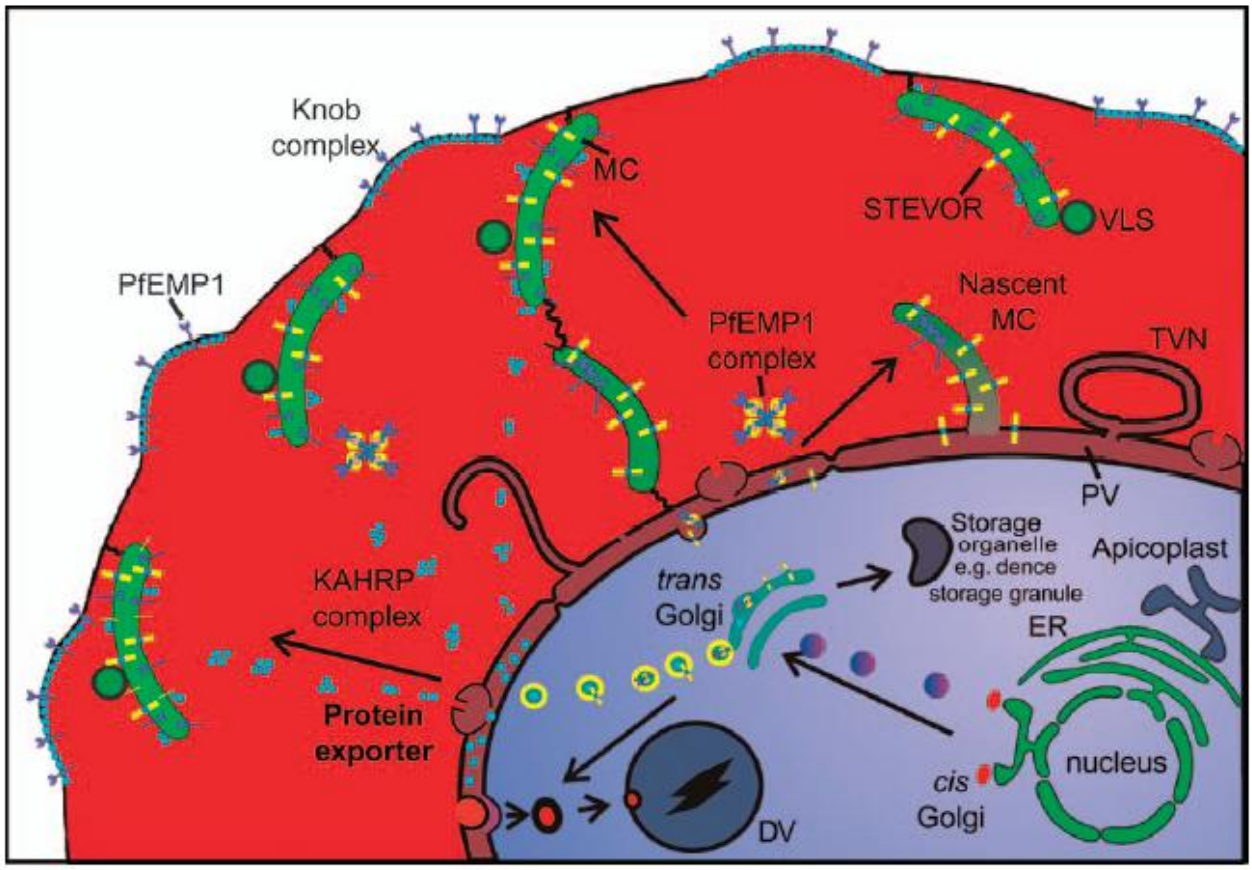
Figure 1.3. Remodeling of the human erythrocyte during *Plasmodium falciparum* infection.
After merozoites invasion of the RBC, the parasite develops as ring (0-24 hours) into the parasitophorous vacuole (PV). At trophozoite (24–36 hours) stage membrane-bound structures appear in the RBC cytoplasm and knobby deformations are formed at the RBC membrane. After approximately 48 hours (mature schizonts) the infected RBC ruptures, releasing 16–32 daughter merozoites. Degradation of hemoglobin results in the deposition of crystals of haemozoin in a digestive vacuole.
PfEMP1: *Plasmodium falciparum* erythrocyte membrane protein 1.

In the cytosol of *P. falciparum* iRBCs another set of structures, with a different protein composition, have been identified, the so called Maurer's clefts (MCs). MCs are of extended tanks surrounded by a membrane (Taraschi et al. 2001), that appear to act as secretory organelles concentrating virulence factors to be delivered to the host RBC membrane (Wickam et al. 2001).

The origin of the MCs is not known, and it is not clear if they are independent structures or subdomains of the TVN (Lanzer et al. 2006).

MCs have a central role in protein sorting and are important for the assembly of 'knobs', electron-dense structures underlying the erythrocyte membrane involved in the cytoadherence of the infected host cells to the vascular endothelium, thus contributing to severe pathogenesis of *P. falciparum* malaria (Sam-Yellowe, 2009).

The exported proteins cause morphological changes to the erythrocyte, including increased host cell rigidity and altered erythrocyte mechanical properties (Glenister et al. 2002).



Tilley et al 2008.

Figure 1.4. Schematic representation of certain trafficking mechanisms in the iRBC. Soluble proteins destined for export are directed into the ER, pass through Golgi compartments and routed to the parasitophorous vacuole (PV). Some proteins are retrieved from the plasma membrane or diverted from the ER or Golgi to intracellular organelles, such as the digestive vacuole (DV) and the apicoplast or (in the schizont stage) to regulated secretory compartments, such as the dense granules, rhoptries and micronemes. Following transport across the PV membrane, soluble proteins (such as KAHRP) may form complexes as they diffuse across the RBC cytoplasm and interact with the cytoplasmic surface of the MC before redistribution to the RBC membrane skeleton. *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) may also be trafficked in protein complexes and may become membrane-embedded by inserting into MC from the RBC cytoplasm. Integral membrane proteins destined to the MC (such as STEVOR) may be transferred to the PV membrane and then accumulate in nascent MC. Vesicle-like structures are observed in the infected RBC cytoplasm; however, their role in protein trafficking is unclear. TVN, tubulovesicular network.

1.4 - *Plasmodium* secretory pathways

How the parasite can move proteins to the different compartments and the surface of the host cell represents is an important topic for understanding the biology of *Plasmodium* parasite.

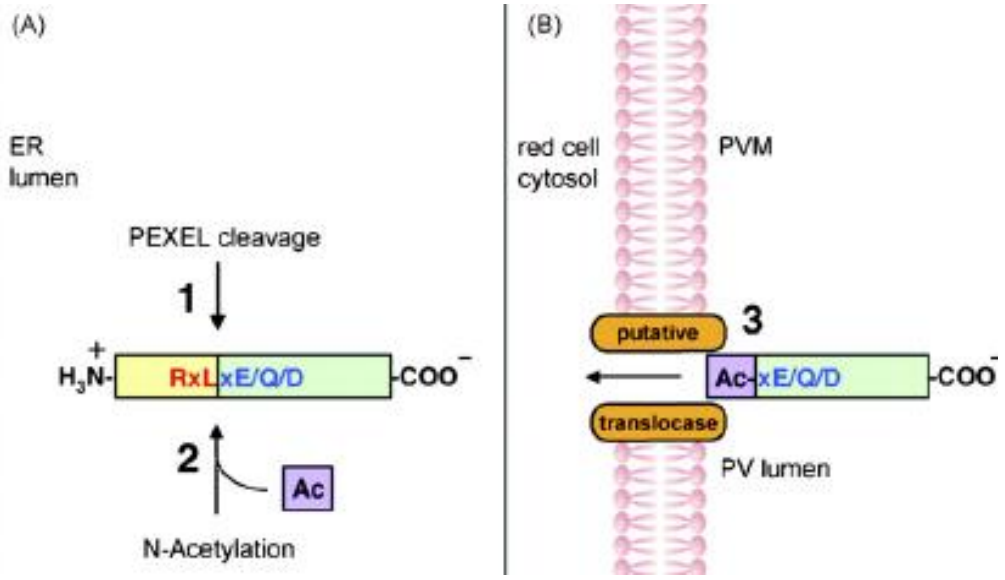
It is known that the malaria parasite exports proteins from within the parasite to the red blood cell surface moving them across both the parasite membrane and the parasitophorous vacuole membrane (Lopez-Estrano et al. 2003).

The presence of a signal sequence at the N-terminus directs parasite proteins to the endoplasmic reticulum (ER) and then to the parasitophorous vacuole (Epp and Deitsch. 2006). The conserved motif RxLxE/Q/D, termed *Plasmodium* export element (PEXEL) (Marti et al. 2004) or vacuolar translocation signal (VTS) (Hiller et al. 2004), is required for targeting the exported proteins beyond the parasitophorous vacuole membrane to the erythrocyte or hepatocyte cytosol (Singh et al. 2007). This motif, usually found approximately 20 aminoacids downstream of a signal peptide, was used to predict the malaria "exportome" which includes more than 300 proteins in *P. falciparum* (Sargeant et al. 2006).

The PEXEL motif has been identified in other *Plasmodium* species such as *P. vivax* and *knowlesi* and also in rodent malaria parasite *Plasmodium berghei, P. chabaudi* and *P. yoelii* suggesting that PEXEL-mediated export is conserved in the genus (Hiller et al. 2004; Sargeant et al. 2006; van Ooij et al. 2008). Interestingly, recent studies (Mackenzie et al. 2008; Haase et al. 2010) showed that the PEXEL motif, present in the *P. falciparum* proteins HRPII or KAHRP, promotes GFP export in *P. berghei*.

It was recently described (de Koning-Ward et al. 2009) a translocation machine (translocon) at the *P. falciparum* PVM that is able to interact with PEXEL-containing proteins and transfer them to the RBC cytosol. It consists of a *Plasmodium*-specific ClpB ATPase orthologue, EXP2 probably involved in pore formation, the novel protein PTEX150, HSP101 and potentially PTEX88 and thioredoxin 2. The translocon components are specific of the genus *Plasmodium*, they are absent in other organisms, including other Apicomplexans (de Koning-Ward et al. 2009). This is in accordance with the absence of PEXEL proteins in these organisms.

The PEXEL motif is not the sequence recognized by the translocon, it is instead a protease recognition site cleaved in the endoplasmic reticulum (ER) of the parasite before export (Chang et al 2008; Boddey et al. 2009). Cleavage of the motif occurs after the L of the PEXEL motif (Figure 1.5); this generates a new N-terminus starting with xE/Q/D, and the terminal residue bears an acetyl group at its N-terminus (Chang et al. 2008; Boddey et al. 2009). The ER aspartic protease plasmepsin V (Boddey et al. 2010; Russo et al. 2010) was recently identified as the ER protease responsible for the cleavage.



Chang et al. 2008.

Figure 1.5. N-terminal processing of PEXEL-positive proteins. A) Hypothetical exported protein is N-terminal processed in the ER lumen. B) The processed protein is recognized by translocon members as PTEX150 and HSP101 and can be exported to the RBC cytosol.

However, protein export in *Plasmodium* most probably involves more than one pathway, as several PEXEL-negative exported proteins (PNEPs) in *P. falciparum* have been described. A number of them localize at the MCs, such as the skeleton binding protein 1 (SBP1) (Blisnick et al. 2000), the membrane associated histidine-rich protein 1 (MAHRP1) (Spycher et al.2003), the ring-exported protein 1 and 2 (REX1 and 2) (Hawthorne et al. 2004; Spielmann et al. 2006).

SBP1, MAHRP1 and REX2 share a common structure containing a single transmembrane (TM) domain but lack a signal peptide. REX1 contains a single hydrophobic stretch, but this region most probably represents a recessed signal peptide. In addition, recent studies using GFP fusions indicate that a variety of sequences can support PNEP export. How the limited sequence specificity in the PNEP export regions or the mature PEXEL N-terminus mediates export is still an open question.

1.5 - Remodeling of the erythrocyte cytoskeleton

The membrane bilayer and the network of membrane-associated proteins, able to establishes vertical and horizontal interactions, regulate the characteristic shape, stability and elastic properties of red blood cell (Lux and Palek, 1995; Elgsaeter et al. 1986).

When membrane skeleton is prepared in the presence of a high concentration of monovalent salt, the protein core consists of spectrin, actin, protein 4.1, adducin and dematin (Sheetz et al. 1979).

The role of most of them has been extensively studied (Luna and Hitt. 1992; Bennett et al. 1989). Spectrin and actin work as scaffolding for the double lipid layer and confer shape and deformability to the red blood cell. Spectrin is constituted of two subunits, α and β, and works as tetramer anchored to the plasma membrane through two major protein bridges: the one connects the integral membrane protein band 3 to spectrin via ankyrin (Lux and Palek, 1995), while the other involves the junctional complex, which connects the C-terminal end of spectrin to short actin protofilaments (Siegel and Branton, 1985; Derick et al. 1992). Protein 4.1, dematin, and adducin are components of the junctional complex (Salomao et al. 2008). Protein 4.1, p55 and the transmembrane glycophorin C, form a well-characterized ternary complex, which tethers the junctional complex to the plasma membrane.

Adducin and dematin are peripheral membrane proteins which interact with actin filaments. They are substrate of many kinases and the phosphorylation status influences their interaction with membrane and cytoskeleton (Husain-Chishti, 1988; Khan et al. 2008).

Dematin works as a trimer molecule consisting in two subunits of 48 kDa and one of 52 kDa; the actin-binding domain at the C-terminus is homologous to the villin "headpiece" (Rana et al. 1993). It also includes a cAMP-kinase phosphorylation site, and plays an essential role in dematin self-assembly.

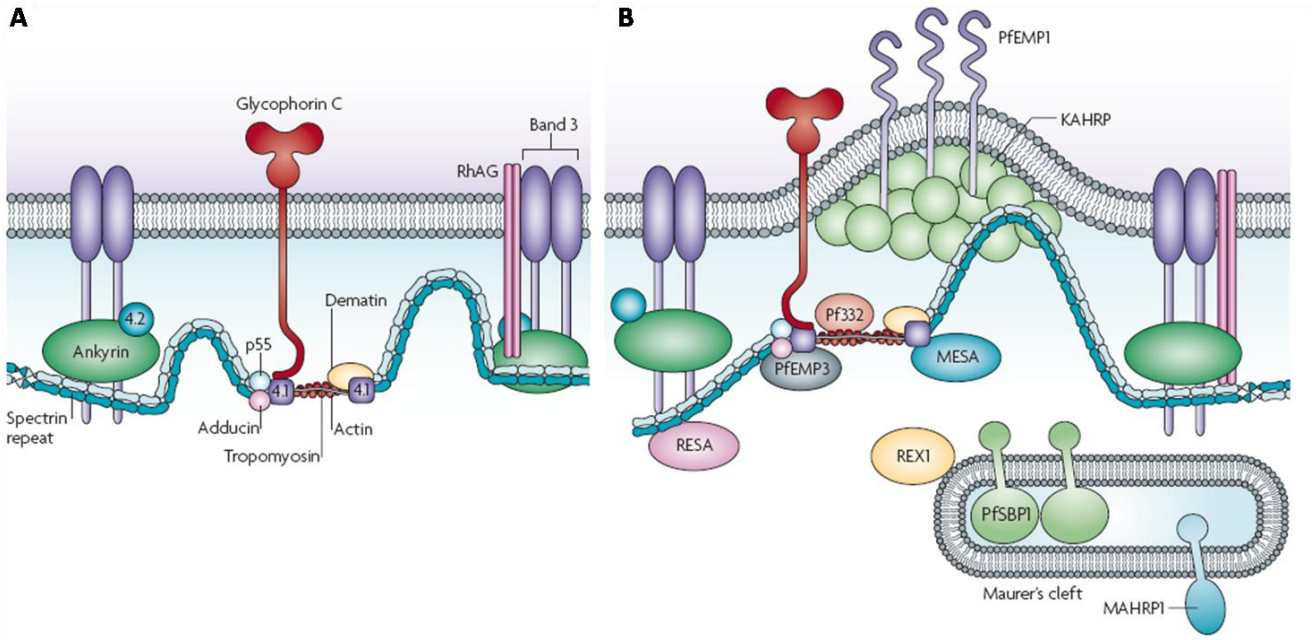
Erythrocytes from transgenetic mice containing a deletion of dematin headpiece showed reduced deformability, osmotic fragility and reduced structural integrity (Khanna et al. 2002). A similar phenotype was observed in transgenic mice knocked-out for the β-adducin gene (Gilligan et al. 1999). Conversely, double knockout mutant mice display severe defects in erythrocyte shape, membrane instability and hemolysis suggesting that dematin and β-adducin may serve redundant functions (Chen et al. 2007).

The erythrocyte membrane skeleton is a site of extensive remodeling upon *Plasmodium* infection. The interaction with exported parasite proteins modifies structural flexibility, morphology, permeability and adhesive properties of the RBC (Figure 1.6). For example the ring-infected erythrocyte surface antigen (RESA), routed to the RBC membrane skeleton

immediately after parasite invasion, interacts with spectrin and stabilizes the RBC membrane during febrile shock (Da Silva et al. 1994).

A second *Plasmodium* protein, which interacts with the RBC skeleton, is the mature parasite-infected erythrocyte surface antigen (MESA) which binds protein 4.1R (Black et al. 2008). The precise function of MESA remains unknown; however the disruption of MESA/4.1R interaction results in accumulation of unbound MESA within the RBC cytosol causing parasite death (Magowan et al. 1995).

Changes in the RBC adhesive properties are associated with the formation of the knob protrusions on the RBC membrane. Knob-associated histidine-rich protein (KAHRP) is a parasite-exported protein at knobs platforms which interacts with spectrin and actin thus playing an important role in the exposition of the virulence factor PfEMP1 (Oh et al. 2000) which is implicated in cytoadherence to vascular endothelium.



Maier et al. 2009

Figure 1.6. The membrane skeleton in uninfected and *Plasmodium falciparum*-infected red blood cells. A) Uninfected red blood cells (RBCs). Spectrin heterodimers, comprising repeat units linked by flexible hinges, can expand and unfold in response to deformation stress. Spectrin dimers are linked head-to-head to form tetramers and at their tails by junction complexes comprising actin oligomers that are stabilized by protein 4.1R and other molecules. Vertical interactions connecting the underlying skeleton to the membrane proper include the band 3–ankyrin–spectrin link and the ternary complex between protein 4.1R, p55 and glycophorin C. B) Infected RBCs. In ring-stage-infected RBCs, ring-infected erythrocyte surface antigen (RESA) associates with spectrin and stabilizes the membrane skeleton. In mature-stage parasitized RBCs, knob-associated His-rich protein (KAHRP) molecules self-associate to form conical structures that interact with spectrin. Pf332 and mature-parasite-infected erythrocyte surface antigen (MESA) bind to the junction complex while *Plasmodium falciparum* erythrocyte membrane protein 3 (PfEMP3) binds to spectrin, further compromising RBC membrane deformability. The cytoadherence-mediating protein PfEMP1 is concentrated in the region of the knobs by an interaction of its cytoplasmic domain with KAHRP. The aminoterminal domain of PfEMP1 is presented at the extracellular surface where it can interact with host cell receptors to mediate cytoadherence. Parasite-derived, membrane-bound Maurer's clefts are involved in PfEMP1 trafficking. The formation and architecture of the organelles is controlled by resident proteins such as the ring exported protein 1 (REX1), membrane-associated His-rich protein 1 (MAHRP1) and *P. falciparum* skeleton binding protein 1 (PfSBP1), and they are attached to the RBC membrane by tether-like structures (not shown).

1.6 - Host raft components are recruited by *Plasmodium* parasite

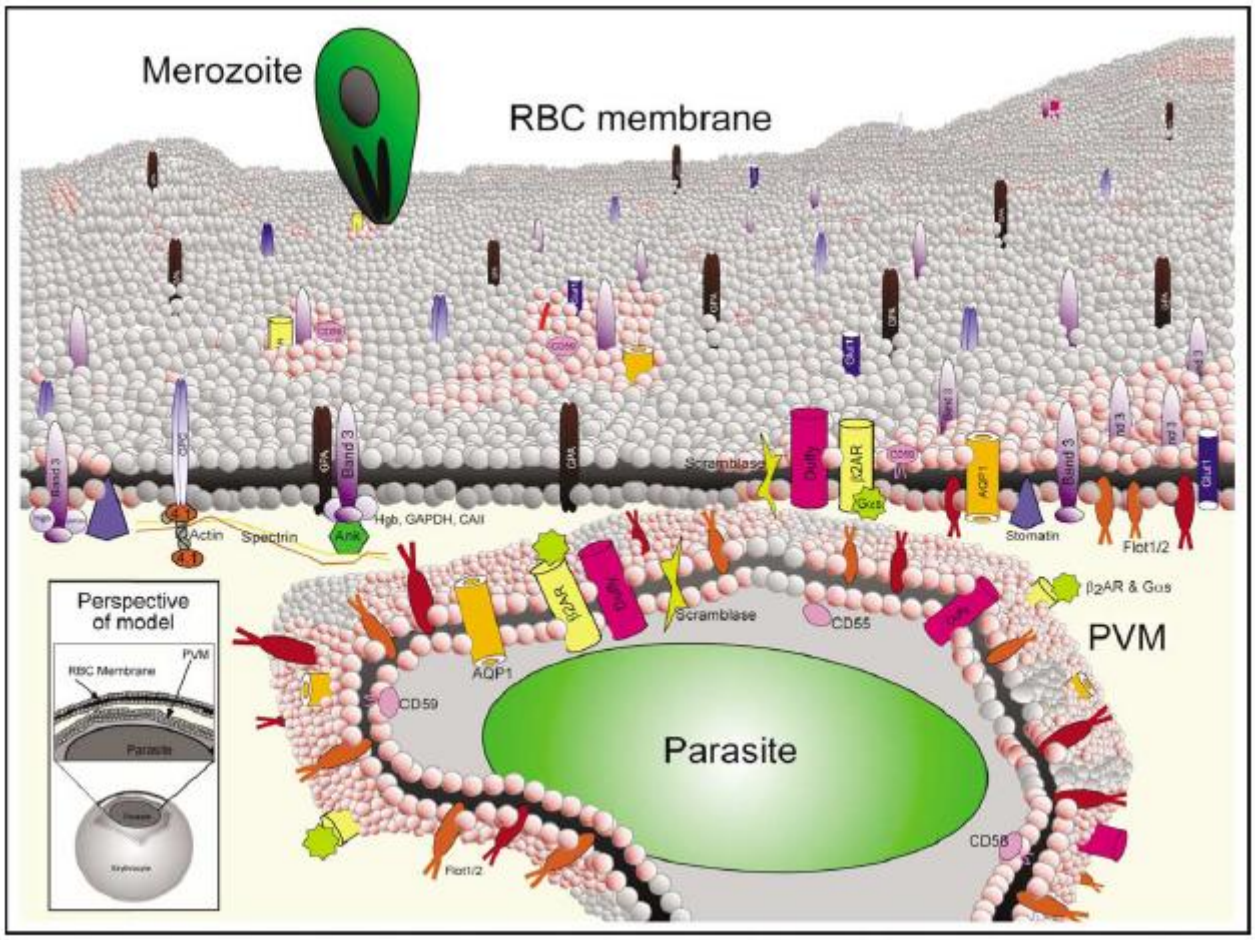
The parasite vacuolar membrane (PVM) is generated immediately after *Plasmodium* merozoite invasion as an invagination of the erythrocyte membrane bilayer. Insights into host molecular determinants that regulate vacuole formation have come from studies on the identification and characterization of cholesterol-rich membrane microdomains (lipid rafts) isolated from erythrocytes (Salzer et al. 2002; Murphy et al. 2004).

The traditional view that the plasma membrane is a uniform lipid bilayer containing randomly distributed membrane proteins has given way to a more complex model in which association of sphingolipids, cholesterol and proteins enables the formation of special microdomains called lipid rafts (Simons et al. 1997). Rafts are thought to constitute floating 'islands' of proteins and lipids held together by the cholesterol-rich microenvironment. Lipid rafts within the plasma membrane have distinct biophysical properties and restrict free diffusion of membrane proteins. They have a major role in cellular signaling and trafficking of proteins and lipids to and from cellular compartments (Lai 2003; Golub et al. 2004).

The resistance of lipid rafts to solubilization with nonionic detergents at low temperature has been well defined experimentally (van Meer, 2002). This property allows separating these detergent-resistant membranes (DRMs) from other detergent-resistant cytoskeletal components by density gradient centrifugation.

At least 19 erythrocyte proteins have been identified as DRM-associated. They include membrane proteins such as stomatin and flotillin 1 and 2, proteins involved in complement regulation (i.e., CD55, CD59) and water transport (i.e., aquaporin-1). They also comprise major integral membrane proteins such as band 3, but not others such as glycophorin A.

Immunolocalization studies showed that at least 10 of these host raft-associated proteins are selectively recruited by the parasite, most probably at the early stages of invasion, and re-localized at the PVM (Haldar et al. 2001; Haldar 2002). In contrast, all non-DRM proteins are excluded from the PVM. Residence in a DRM is necessary but not sufficient for recruitment of a host protein to the PVM. Band 3, which is abundantly represented in membrane microdomains of the RBC, is not internalized by the parasite. Thus, there must be active mechanisms which allow to sort and traffic selected erythrocyte DRM raft proteins.



Murphy et al. 2006

Figure 1.7. Model of erythrocyte DRM rafts and their enrichment in the malarial vacuolar membrane. The uninfected erythrocyte membrane contains a variety of generalized lipid domains (grey spheres) and raft microdomains (pink spheres), containing various proteins. Some proteins partition mostly into DRM raft domains (i.e., flotillins), while others are only minimally present there (i.e., band 3). During malaria infection, merozoite-stage parasites invade erythrocytes to reside in a membrane-bound parasitophorous vacuole. The PVM becomes selectively cholesterol-enriched, and ten of the known raft proteins are internalized to the PVM (flotillin-1 and -2, Gs, b2AR, AQP1, Duffy, CD55, CD58, CD59, scramblase). Major integral membrane proteins are not internalized to the PVM (i.e., glycophorins A and C, cytoskeleton-associated band 3, etc.). The lower left inset shows the perspective of the model, depicting a single infected erythrocyte with a magnified view of the plasma membrane and PVM. Since the PVM is formed by invagination of the plasma membrane, proteins that are cytoplasmically-oriented in uninfected cells remain so upon infection; protein structures exposed to the extracellular space face the vacuolar space upon infection. 4.1 indicates protein 4.1.

Parasite ligands that reside in invasion-associated apical organelles are also enriched in parasite DRM rafts that insert into the nascent vacuole, suggesting that they interact with host rafts there. This has led to a model (Figure 1.7) that erythrocyte raft-associated signaling and parasite ligands act in conjunction to modulate host rafts, catalyzing endovacuolation of the erythrocytic membrane during malarial infection.

The generation and dynamics of these functional membrane microdomains in the infected host cell has a crucial role for host/parasite interplay and the success of the infection. *P.*

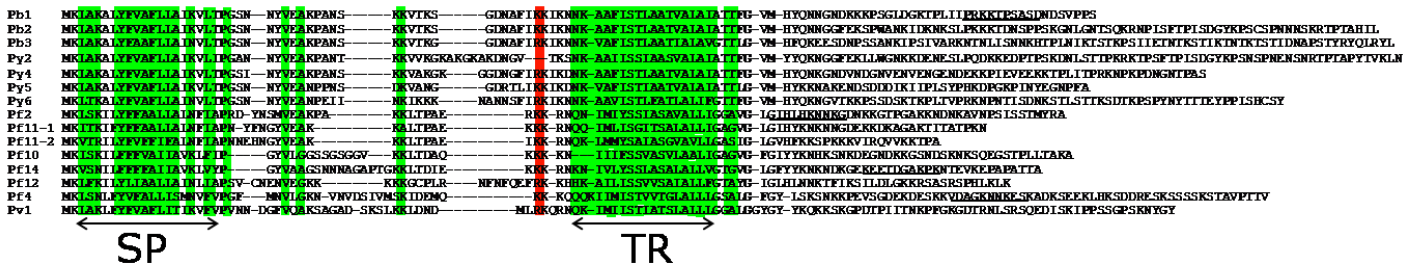
falciparum invasion is, in fact, hampered by cholesterol depleting agents such as methyl-β-cyclodextrin (MBCD) (Samuel et al. 2001) or by agents which disrupt lipid rafts without altering the content of cholesterol (Koshino and Takakuwa, 2009).

1.7 - Aim of the work

The identification of *Plasmodium* secretory pathways and proteins involved in host cell remodeling is an important step for the understanding of parasite biology. Here we contributed to tackle these issues defining:

1) Timing of expression and motifs required for the export of members of the conserved *Plasmodium* family of small proteins (13-16 KDa), SEP/ETRAMP:

The gene family encoding exported proteins SEP (Small Exported Proteins) /ETRAMP (Early TRAnscribed Membrane Proteins) is characterized by the presence of a N-terminal signal peptide, a hydrophobic domain and a highly charged C-terminal region. These features are well conserved among members of this family within *Plasmodium* genus, as shown by multiple alignment (Figure 1.8).



Birago et al. 2003

Figure 1.8. Sequence alignment of *Plasmodium* SEP/ETRAMPs

Database search identified 13 predicted genes encoding members of the ETRAMP family of *P. falciparum* (Spielmann et al. 2003). By the use of specific antibodies, ETRAMP10.1 and ETRAMP10.2 were localized at the PVM of the iRBCs, with their C-terminal domains facing the host cell cytoplasm. This orientation was postulated as a general feature of the ETRAMP family members. *Plasmodium falciparum* ETRAMP5 was detected at the PVM of liver stages where it specifically interacts with the human apolipoproteins ApoA1, ApoB, and ApoE, as demonstrated in a two hybrid system experiment in yeast (Vignali et al. 2009).

The structural organization of ETRAMPs at the PVM was analyzed by *in vivo* cross-linking procedure for *Plasmodium*-infected RBCs; ETRAMP4 and ETRAMP2 are involved in the formation of both omo-complexes and complexes with EXP1, a protein exported at the parasite-host cell interface (Spielmann et al. 2006).

Despite SEP/ETRAMPs do not contain the PEXEL motif which allows proteins to be exported to the host cell compartment, members of this family were exported to the red blood cell (Birago et al. 2003; MacKellar et al. 2010).

Aim of this work is the characterization of SEP/ETRAMP family members in *P. berghei*. In this rodent model three *sep* genes have been identified, which share the upstream regulatory sequence and the region encoding the first 85 amino acids while differing in their C-terminal region and 3'UTR. A previous study (Birago et al. 2003) characterized PbSEP1 as a protein expressed early after erythrocyte invasion and localized at the vacuolar membrane of *P. berghei* blood stages. We take advantage of the limited expansion of this protein family in *P. berghei* and the high conservation between family members to define their expression pattern throughout the parasite cycle and to identify protein motifs requited for their subcellular localization.

2) Subcellular localization of two host proteins, dematin and adducin, components of the erythrocyte spectrin-based membrane skeleton, in the course of *P. falciparum* infection:

The analysis of lipid rafts of the iRBC showed that *Plasmodium* recruits raft-associated proteins from the RBC surface and relocates them to the PVM. The inward movement of host proteins to parasite compartment is a poorly explored aspect of host-parasite interplay. Recent studies, conducted in our laboratory showed that dematin and β-adducin, components of the erythrocyte membrane skeleton, are internalized by *P. berghei* parasite. In addition, recruited dematin interacts with *Plasmodium* 14-3-3. 14-3-3s are members of a large family of small acidic proteins with molecular mass of 28-33 kDa, usually active as dimers (Aitken 2006). These highly conserved proteins are expressed ubiquitously from yeast to mammals. There are seven isoforms encoded by seven distinct genes in mammals, ten in plants, two in yeast, and one in *Plasmodium*; 14-3-3s are phosphoserine-binding proteins and are implicated in numerous cellular processes such as protein trafficking, cellular signalling, apoptosis, cell cycle control.

Here we analyze localization and topology of these two skeletal proteins in *P. falciparum* iRBCs.

2. EXPERIMENTAL PROCEDURES

2.1 - Materials

2.1.1 - Parasites

Plasmodium berghei: ANKA strain, clone 8417 HP (high producer of gametocytes);

Plasmodium falciparum: Clone 3D7

2.1.2 - Bacteria

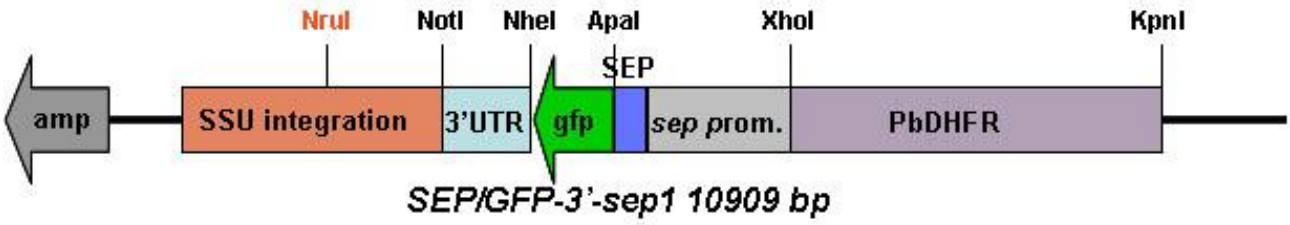
Escherichia coli K-12: SURE (Stratagene) has been used for all the cloning procedures relevant to both transfection constructs and expression ones.

2.1.3 - Plasmids

Plasmids already available in the lab have been used for the cloning procedures or as donors of reporter genes. In particular:

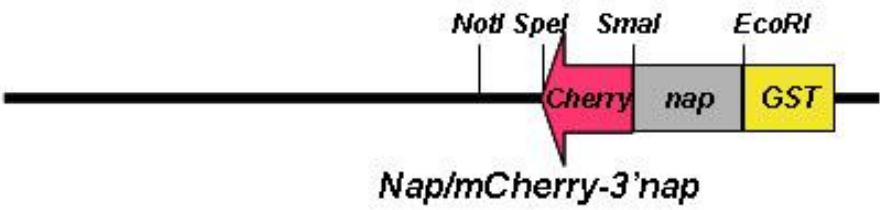
SEP/GFP-3' sep1:

The main features are *sep* promoter, SEP-GFP (green fluorescent protein) fusion coding region, *sep*1-3'UTR, the beta-lactamase gene for ampicillin selection, SSU for genomic integration (spacer of rDNA unit located in both chromosome 5 and 6 of *Plasmodium berghei*) and pyrimetamine resistance gene.



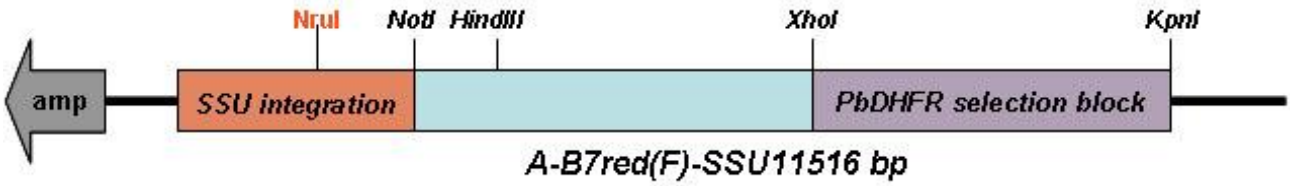
set/nap-cherry-3'nap:

This is an expression plasmid in which the resident EcoRI-SmaI region coding for gene *set/nap* (nucleosome assembly protein) has been replaced with *sep* family coding regions to engineer coding regions for fusion SEPs/mCherry proteins.



A-B7red(F)SSU:

This is a transfection vector which helped us in all the mCherry (Shaner et al. 2004) constructs: it has the beta-lactamase gene for ampicillin selection, SSU (spacer in rDNA unit) for genomic integration in chromosome 5 or 6 of *Plasmodium berghei* and pyrimetamine resistance gene.



pGEX-6P-1:

This is a commercial expression vector used in this work to clone C-termini of Sep2 and Sep3 (pGST-2 and pGST-3) in order to produce recombinant fusion proteins for further immunisations of mice.

2.2 - Methods

2.2.1 - DNA cloning techniques

Transformation by electroporation was performed with a Bio-Rad *E. coli* Pulser apparatus on *E. coli* SURE cells previously made competent for the procedure.

The Millipore Montage Gel Extraction System was used for the purification of DNA fragments from agarose gel.

Plasmid DNAs were prepared using the QIAGEN Plasmid Mini kit or Midi kit.

The sequence of the coding regions of all the plasmids used in transfection experiments have been determined by external service (MWG, Germany); for each plasmid the sequencing has been carried out on the DNAs of three independant colonies.

PCR reactions were performed according to the following parameters of amplification: 2 minutes of initial denaturation at 94°C were followed by 30 cycles (30 seconds at 94°C, 1 min. at 55°C, 1 min. at 68°C) of amplification and by a final extension of 7 minutes at 68°C. About 50 micrograms of *P. berghei* genomic DNA were used in each reaction.

2.2.2 - *Plasmodium berghei in vivo* infection

The study of the animal model is very important in learning the biology of the malaria parasite. Routine infection is achieved by intraperitoneal (IP) injection of 0,2-0,3 ml of infected blood in Swiss CD1 mice. After some days, when parasitemia is about 20-50%, mice are anaesthetized with 0,01-0,015 ml/g IP of Avertin (2,5%) (Tribromoethanol) and the infected blood is collected by cardiac puncture (with eparine 500 U/ml).

2.2.3 - Determination of parasitemia

Parasitemia is verified and measured by Giemsa staining; a drop of blood from the tail of the animal is smeared on a slide; blood is fixed in methanol for 1' and then stained with Giemsa 5% solution in Weise buffer pH 7.2 for 15'; the slide is washed with water, air-dried and observed at the microscope. To determine parasitemia many similar fields are counted (100x) to establish the percentage of iRBCs. If there are many poliinfected RBC, it is necessary to count all the parasites to calculate the possible yield of nucleic acid and/or proteins in a given sample. Moreover it may be useful to count the different developmental stages (rings, trophozoites, gametocytes).

2.2.4 - *Plasmodium berghei* freezing

350 µl of infected blood at 20-50% parasitemia are mixed to equal volume of glycerol 30% in PBS 1x, kept 15' at 4°C and then put in liquid nitrogen for storage. Each tube is enough for the infection of 2 mice.

2.2.5 - *Plasmodium berghei* cultures and synchronized infections

The asexual cycle of *P. berghei* takes about 23 hours to complete and all the different parasite stages (but not schizonts which are retained in the inner organs). are present in peripheral blood. In particular, *P. berghei* schizonts are not able to break the RBC *in vitro* and therefore purified schizonts can be used to start a synchronous infection of *P. berghei* in mice. Schizonts are the parasite forms which are used for DNA transfection too. Blood from infected mice (parasitemia 1-3%) is spun down for 10 minutes at 420g at room temperature. After the removal of the supernatant, pelleted RBC are resuspended in a little volume of complete culture medium and than added to cultures; each flask will contain 0.25-0.5 ml of packed RBC (in 50 ml culture); flasks are gassed (2% O_2, 5% CO_2, 93% N_2) for 1 minute and then kept overnight at 37°C with constant and gentle shaking for 19-22 hours. Shaking is then interrupted and a small aliquot is takes and spread on a slide for Giemsa staining to check the development of parasites: when schizonts are at least 80% of the culture, they are purified on Nycodenz [5-(N-2,3-diidrossipropilacetamido)-2,4,6-tri-iodio-N-N'-bis(2,3 diidrossipropil) isoftalamide, PM 821] gradients. Nycodenz is routinely used to separate schizonts and/or gametocytes (interface) from rings and non-infected RBC (pellet).

2.2.6 - Purification of schizonts

When the most of the schizonts are mature, Nycodenz cushions are prepared in 50ml Falcon tubes (10 ml of 55% Nycodenz in PBS) and carefully overlaid with 25-40 ml of the culture. The cultures should not contain more than 1 ml of initial blood per flask otherwise the maturation can be affected; moreover the gradients will result overloaded and the schizont purification will not be optimal. Then samples are spun down at 300g for 20' at 20-22°C with very slow acceleration/deceleration. After removing the supernatant, schizonts (the brownish band located at the boundary of the Nycodenz layer) are collected in a clean 50 ml Falcon tube. Enough PBS 1x is added to fill the tubes which are then centrifuged at 560 g for 20'. After removing supernatant pellet is resuspended in about 300 µl PBS 1x (about $2x10^8$ schizonts) for each transfection or to inject into mice tail intravenously to start an *in vivo*

synchronized infection. 3-5 hours later it is possible to verify the synchronicity of the infection by Giemsa staining: only small rings will be present.

PBS 10x: NaCl 1,37 M, KCl 0,027 M, $Na_2HPO_4*2H_2O$ 0,043 M, KH_2PO_4 0,015 M.

Incomplete medium: RPMI 1640 Medium (51800-019 GIBCO-BRL) with L-glutamine pH 6.5-6.8 in glass-distilled water.

Complete medium pH 7.2, 100 ml: incomplete medium 75,3 ml, $NaHCO_3$ 5% 4,2 ml, Foetal Calf Serum 20 ml; Neomicine (10 mg/ml) 0,5 ml.

Nycodenz stock solution: Nycodenz 276 g in 1 l H_2O.

2.2.7 - *Plasmodium berghei* transfection

As stated above, *Plasmodium* is an intracellular parasite thus the integration of external DNA depends on the crossing of four biological membranes. For transfections mature schizonts are used, and in *P. berghei* homologue recombination frequently occurs so that transfections are frequently successful.

2.2.8 - Transfection protocol

When most of the cultured schizonts are mature they can be purified. Each gradient (10 ml of 55% Nycodenz in PBS in 50ml Falcon tubes) is carefully overlaid with 25-40 ml of the culture, as described above. Schizonts for each transfection are resuspended in 300 µl PBS.
30-50 µg of the plasmid is linearized with the appropriate restriction enzyme, phenol-chloroform extracted and ethanol precipitated and eventually resuspended in 100 µl PBS are added to purified schizonts (300 µl, see above) and mixed. The blood is then put in a 0.4ml cuvette for electroporation (BIORAD GENE PULSER) and subjected to the electric pulse: 1.1 kvolts, 25µF capacitor; time constant should be 0,6-0,9 msec. Immediately 400 µl of non-infected blood from a phenylhydrazine-treated mouse (treated five days before with a single PHZ dose, 80 mg/kg body weight) are added to the sample, the sample is kept for 25 minutes at 37°C before the intraperitoneal injection into the recipient mice (400 µl per mouse). The day after parasitemia is confirmed by Giemsa staining of tailblood and selection is applied (0.07 mg/ml pyrimethamine in drinking water) for 4-5 days. The drug is prepared as a stock solution (7 mg/ml in DMSO) which is diluted 100 times in acidified water, pH 3.5-5. For

special purposes it is possible to use WR99210 which has to be administered subcoutaneously. Resistant parasites usually emerge at day 3-4 after selection removal.
When parasitemia is around 1-3 % mice are bled and the blood used both for aliquots to be stored in liquid nitrogen (first selection) and to infect new mice in order to run a second selection round.

2.2.9 - Parasite preparation

Two main procedures are currently used to prepare parasites from infected blood. The first one uses the action of the detergent saponin and leads to the isolation of parasites devoid of any contaminants of the erythrocyte but most of the parasites are damaged at the level of parasitophorous vacuole; this kind of preparation is reliable if cytoplasmic or nuclear proteins are the object of the study but not if the interest is in molecules involved in trafficking or located at the periphery of the parasite. This method is however indicated when DNA or RNA must be prepared. A second method is much milder and it is based upon osmotic lysis and gives intact parasites with their parasitophorous vacuole; a disadvantage is represented by the presence of a certain degree of contamination of membrane and proteins from RBC, therefore this method can be used for samples containing a very low number of uninfected host cells; to this purpose RBC infected with trophozoites or gametocytes can be collected at the interface of Nycodenz cushion centrifugation (see above Schizont preparation) while RBC infected by ring stages and uninfected cells are in the pellet.
The first step is common to the two procedures: infected blood is passed through a Plasmodipur filter (Europroxima) which almost completely retains white cells.

Saponin method: blood after Plasmodipur filtration is diluted ten times with ice-cold PBS (supplemented with protease inhibitor cocktail Roche N. 11836145001) and kept in ice. Saponin is added to 0.03% final concentration (stock solution 5% in water) and lysis is achieved in few minutes in ice. The sample is centrifuged for ten minutes at 4°C at 8000g, the supernatant is discarded and the pellet washed several times in PBS (with inhibitors) up to a stage in which the supernatant is absolutely colorless. A small sample is taken before the last centrifugation to count the parasites at the microscope in the Thoma-Burker chamber. Parasite pellets are stored at -80°C for many months.

Osmotic lysis: blood after Plasmodipur filtration is diluted 50 times with PBS, layered onto Nycodenz cushions and centrifuged for 20 minutes at 300 g at room temperature. Parasitised cells at the interface are collected and washed once with PBS (with inhibitors). Cells are then resuspended in 50 volumes of ice-cold lysis buffer (with protease inhibitors) and kept in ice for 20-30 minutes. The sample is then centrifuged for ten minutes at 4°C at 8000g, the supernatant is discarded and the pellet washed several times in PBS (with inhibitors) up to a stage in which the supernatant is absolutely colorless. A small sample is taken before the last centrifugation to count the parasites at the microscope in the Thoma-Burker chamber. Parasite pellets are stored at -80°C for many months.

Lysis buffer 10x:	NH_4Cl	1,5M
	$KHCO_3$	0,1M
	EDTA	0,01M

2.2.10 - Parasite lysate preparation

Parasite pellets were lysed by freeze-and-thaw method:
pellets of $5*10^8$ parasites are resuspended in 200 microlites TBS (50mM Tris-Cl pH 7.5, 150mM NaCl) containing protease inhibitor cocktail (Complete, Roche) and the sample is frozen in liquid nitrogen for thirty seconds and then incubated for 5 minutes at 37°C. This procedure is repeated five times. The sample is then centrifuged at 20000g at 4°C for 20 minutes in Eppendorf centrifuge. Supernatant is kept as the soluble fraction and the pellet is forcedly resuspended in TBS and spun again in the same conditions. This washing step is repeated and the final pellet is regarded as the insoluble fraction. It can be resuspended in SDS-PAGE loading buffer and boiled for five minutes.

2.2.11 - Recombinant proteins preparation

Strains pGST-bis and pGST-ter which harbor the expression plasmids for the production of GST-Sep2 and GST-Sep3 have been grown for three hours in the presence of 1 mM IPTG to induce the synthesys of the fusion proteins. The bacterial pellets were sonicated in ice with 10 strokes of 10 seconds each (10 seconds for each pause too) at the half of the intensity. The lysate was centrifuged at 20000 g at 4°C for ten minutes; supernatants and pellet were run on acrylamide gel to check for the presence of recombinant proteins with uninduced samples as negative controls. Both fusion proteins were present in the supernatant and this made relatively easy to purify them by using a glutathione-sepharose resin (4 Fast Flow, Amersham).

2.2.12 - Production of polyclonal antibodies

Polyclonal antibodies against GST-Sep2 and GST-Sep3 were produced in Balb/c mice using the protocol described below:

- Day 0: Collection of pre-immune serum form mice tail as negative control. IP injection of an emulsion made of 150 µl physiological solution (containing 25-50 µg of protein) + 150 µl of Complete Freund Adjuvant)
- Day 14: IP injection of 10-25 µg of protein (in 150 µl physiological solution + 150 µl of Incomplete Freund Adjuvant)
- Day 28: IP injection of 10-25 µg of protein (in 150 µl physiological solution + 150 µl of Incomplete Freund Adjuvant)
- Day 42: Bleeding of mice by intracardiac puncture.

Blood samples were kept 1 h at 37°C, than at +4°C ON.

The day after sera were prepared by spinning down the samples for 10 minutes at 10000 g at +4°C; the supernatants were transferred to new tubes, aliquoted and stored at -20°C.

2.2.13 - Indirect immunoFluorescence Assay (IFA)

The whole procedure is performed at room temperature and the centrifugations are made in Eppendorf centrifuge at 600 g.

Infected blood was washed with PBS, then resuspended in 10 volumes paraformaldehyde 4% and Glutaraldehyde 0,0075% in PBS and incubated 1 hour. Fixed blood was washed once with PBS and permeabilized for 10 minutes in 5 volumes of 0,1% Triton X-100 in PBS. After two washes in PBS the sample was resuspended in blocking solution (3% BSA in PBS) and incubated 1 hour (after the blocking step the sample can be washed once with PBS and kept in PBS at +4°C for several days). Then the sample is centrifuged and resuspended in PBS containing the appropriate amount of primary antibody and incubated for 1 hour; unbound antibody is removed by 2 or 3 washes with PBS and then the pellet is resuspended in PBS containing the appropriate secondary antibody (Rhodamine- or fluoresceine-conjugated) and DAPI 1 µg/ml and incubated for one hour. At least three washes with PBS are necessary before mounting the slide.

2.2.14 - Protein polyacrylamide gel electrophoresis and immunoblotting

Immunoblotting assay were used to detect the presence of the protein of interest on different samples of *Plasmodium* previously transferred on a membrane after separation on SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis).

Aliquots of the samples were mixed with loading buffer and heated 5 minutes at 99°C before loading on the gels. SeeBluePlus2 (Invitrogen) prestained molecular weight marker is added to visualize the migration.

Electrophoresis run is at constant voltage (200 V) for about 45 minutes (with BIO-RAD Mini-PROTEAN II apparatus) in running buffer 1X.

Running buffer 5x: Tris-HCl 15 g, Glycine 72 g, SDS 5 g, H_2O up to 1 liter

Western Blotting was performed using MINI TRANS-BLOT® Bio-Rad apparatus: transfer is performed at constant voltage (100 V) for 30 minutes if analyzing SEPs proteins (13-16 Kda) or one hour for dematine and adducin proteins (50 Kda, 100 Kda) in transfer buffer (20% methanol, Tris 0,025 M, Glycine 0,192 M) onto Protran 0.22 microns membrane (Whatman).

The whole immunoblotting procedure is performed at room temperature unless specified and all the solutions are in a ratio of one ml. per square centimetre of membrane. First the membrane is wet in PBS/Tween and then incubated in blocking solution (4% non-fat milk in PBS 1x/Tween-20 0,05%) for one hour; then it is incubated with a primary antibody (usually in PBS/Tween but in some cases 2% milk is added to reduce aspecific background signal) for one hour. After 3-4 washes with PBS/Tween the membrane is incubated with HRP-conjugated appropriate secondary antibody for one hour and after three-four washes with PBS/Tween the blot is developed using the ECL system (SuperSignalWest Pico, Thermo Scientific)according to manufacturer's instructions.

2.2.15 - Plasmid constructions with Green Fluorescent Protein (GFP)

To understand which are the genetic elements involved in different timing of expression and localization, we generated constructs, which use different regulative regions of *sep* genes, able to confer the ability to express fluorescent reporter proteins in the transfected *P. berghei* parasites, and/or different parts of *sep* coding region.

Common features of all these constructs are: 1) DHFR gene with its regulative regions which confers the resistance to the drug pyrimethamine for the selection of transfectants, 2) a region for the genomic integration (the spacer of rDNA gene), 3) GFP coding region

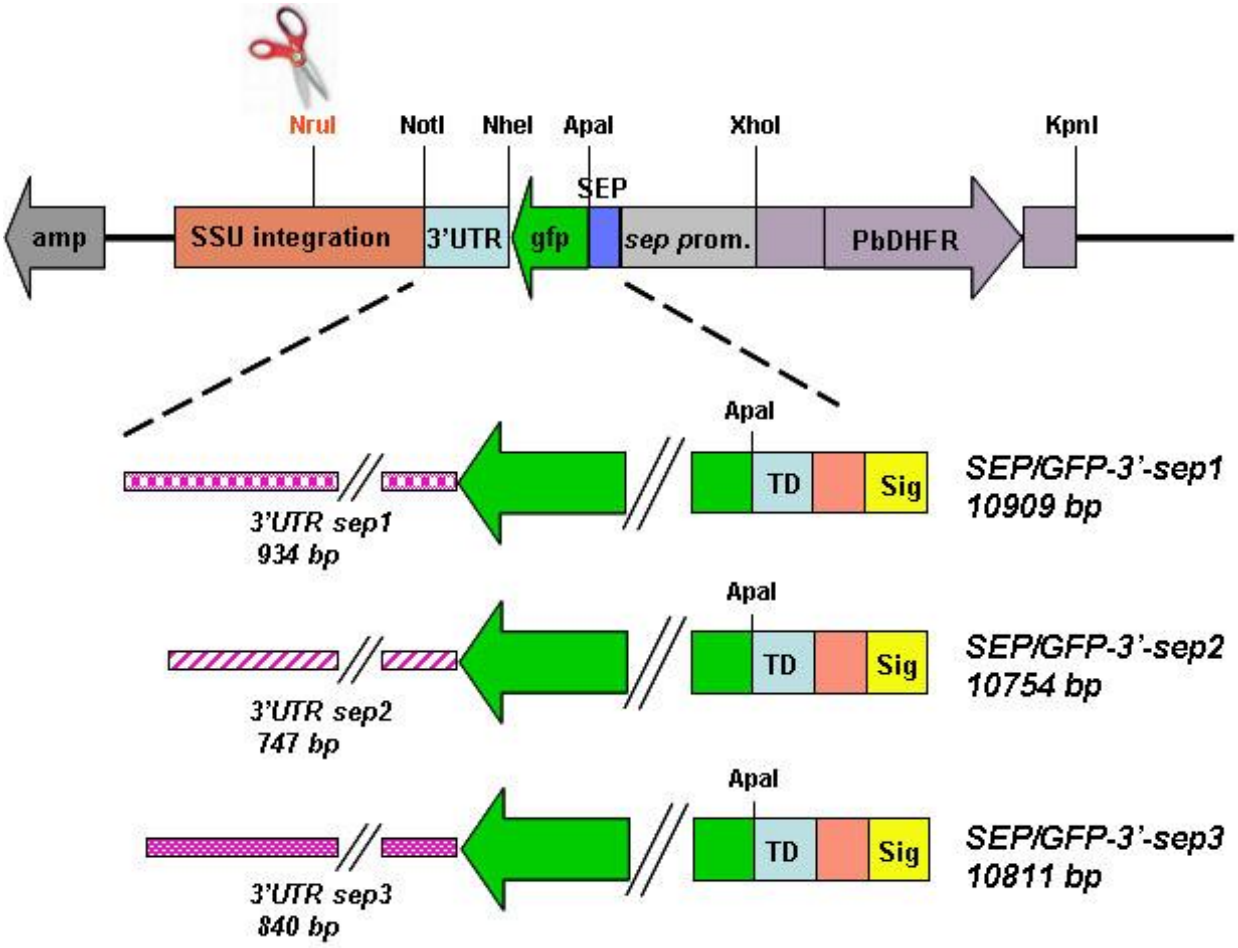
First group:

The first series of three construct share the sep1 promoter and 5'UTR region and the coding region of a fusion protein made of the first 92 residues of SEP1 upstream the GFP sequence; the difference among them is the 3'UTR which is specific of each of the three *sep* genes.
Starting from **SEP/GFP-3'-sep1** plasmid (see Materials), by cloning the PCRs of the specific 3'UTR region of each gene into NotI-NheI site we generated **SEP/GFP-3'-sep2** plasmid:

PCR primers:
150bis-3A: gaccggatcc**gctagc**ccaactgcccatatactgt
150bis-3B: gacc**gcggccgc**actagtattgtagtactattattgcga

and **SEP/GFP-3'-sep3** plasmid:

PCR primers:
150ter-3A:gaccggatcc**gctagc**gtagattctggtggatattatc
150ter-3B: gacc**gcggccgc**actagtcaatttaccataatcaaacagt

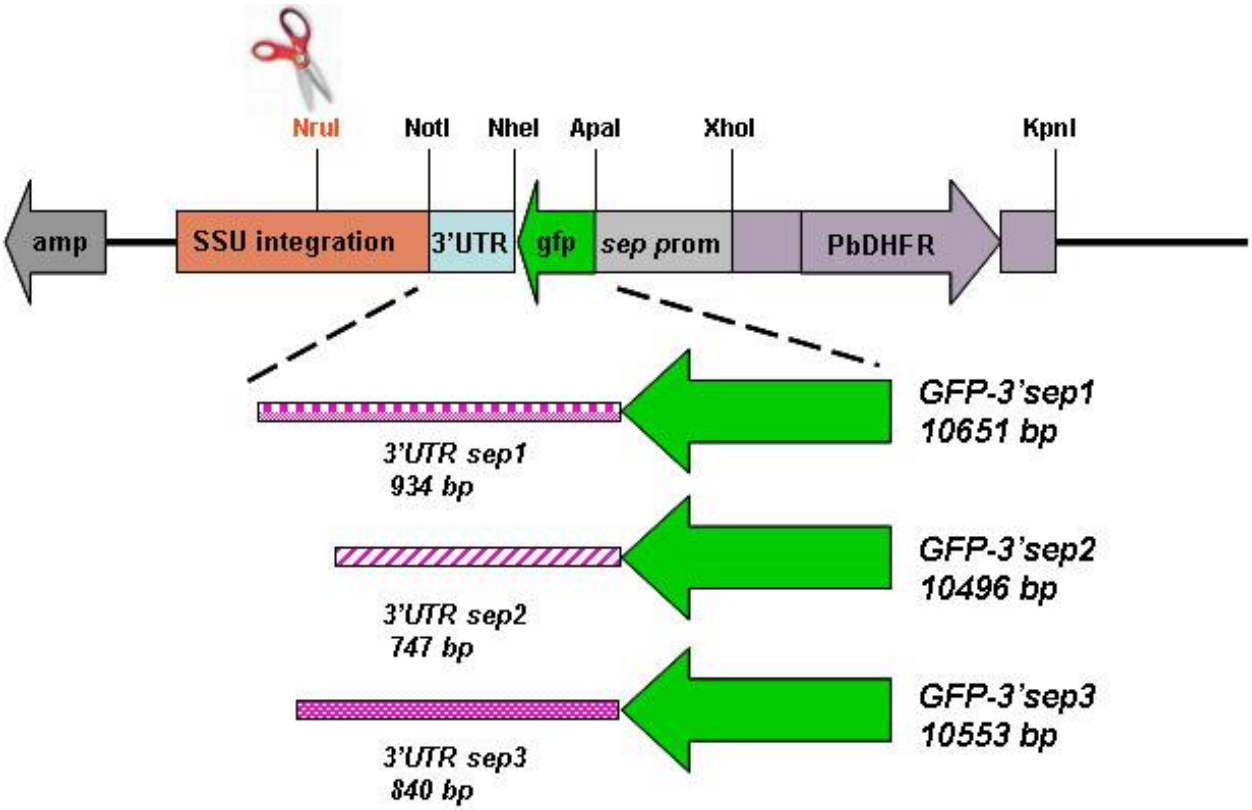


Second group:

A new series of constructs was made to study the possible role of different 3'UTRs; to this aim the XhoI-ApaI fragment containing [prom-5'UTR-coding region] of the three plasmids drawn above was replaced with the PCR product Xho-Prom-5'UTR-Apa

PCR primers:
sep-5?-for: gacc**ctcgag**ctagtaatggcaataaattgc
sep-5?-rev: gacc**gggccc**tgatgctagtttcatttttgtataagtaa

In this way we deleted the *sep* coding portion thus generating 3 new plasmids, **GFP-3'sep1**, **GFP-3'sep2** e **GFP-3'sep3,** which express GFP under *sep* promoter and 3' UTR is specific of each of 3 genes.



Third group:

A third series of constructs was made to study the role of different parts of SEP2 for the localization of the protein itself. By using XhoI-ApaI restriction site of the GFP-3'sep2 plasmid, we inserted a PCR product containing the promoter, 5'UTR and the first 84bp (28 aa) of *sep2* gene coding for the signal peptide, thus obtaining **SEPsignal/GFP-3'sep2.**

PCR primers:

Sep-5?-for: gaccctgcag**ctcgag**ctagtaatggcaataaattgc

Sep-sig-rev: gacc**gggccc**ttcaacataattattagatcctgg.

In a similar way we generated plasmid **SEP(53)/GFP-3'sep2** containing the signal peptide along with other 25 residued, with the exclusion of transmembrane region

PCR primers:
Sep-5?-for: gaccctgcag**ctcgag**ctagtaatggcaataaattgc
sep-TM-no: gacc**gggccc**tttgttatttttaatttttttaataaatgcattatcacc)

and **SEP(81)/GFP-3'sep2** containing the signal peptide along with other 53 residued, which includes transmembrane region

PCR primers:
Sep-5?-for:gaccctgcag**ctcgag**ctagtaatggcaataaattgc
sep-TM-si: gacc**gggccc**tccattattttgataatgcattacacc)

and **SEP2total/GFP** containing the whole *sep2* coding sequence

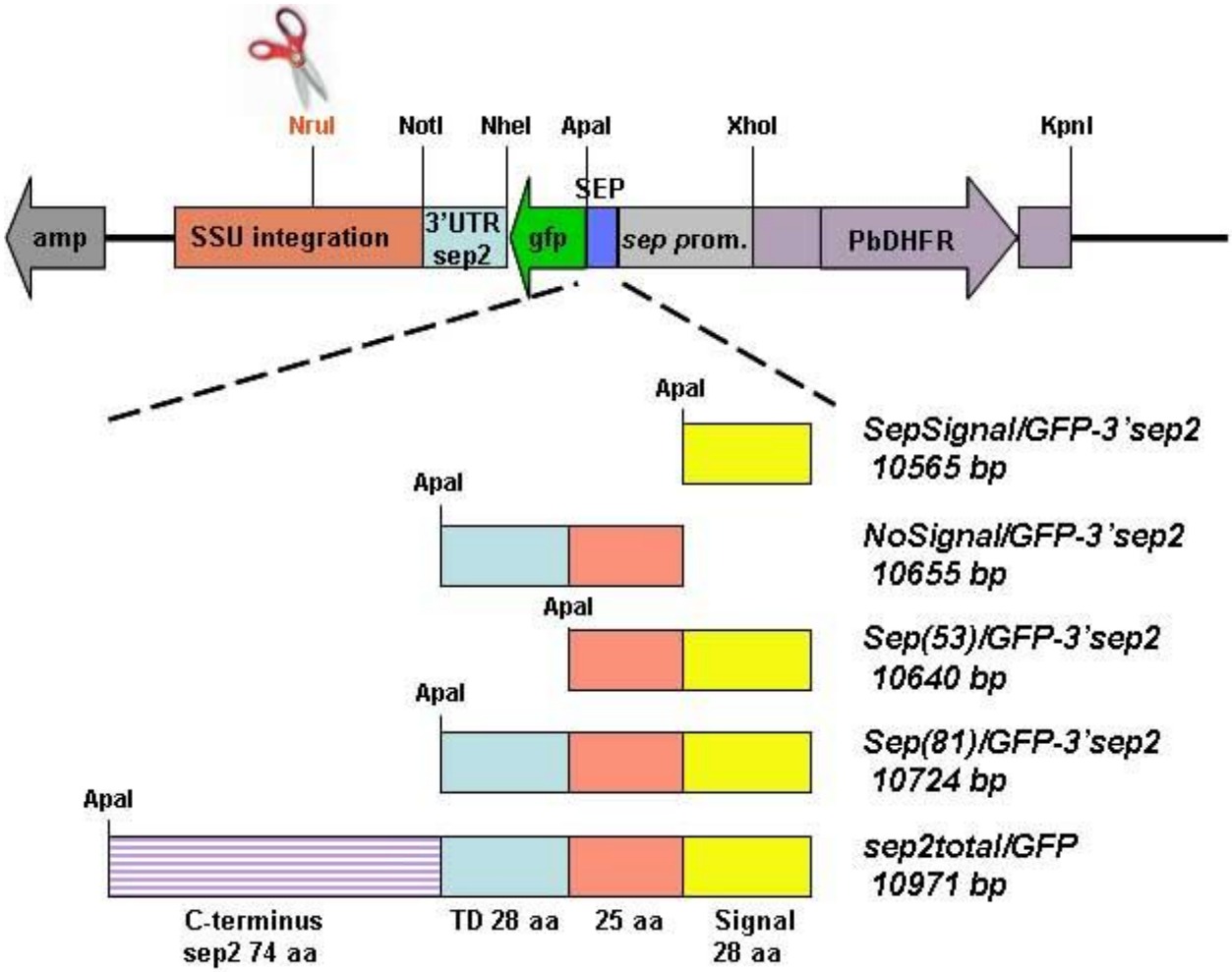
PCR primers:
Sep-5?-for:gaccctgcag**ctcgag**tagctatgctgatattatgc
Bis-cod-rev: gacc**gggccc**cagtatatgggcagttggggttcg)

A control without signal peptide was needed therefore the PCR of promoter digested XhoI-SmaI and the PCR of SEP coding region without the signal peptide, digested SmaI-ApaI, were mixed with the 9200bp XhoI-ApaI fragment from the plasmid SEP/GFP-3'sep2 and ligated. The expected plasmid is called **NoSignal/GFP-3'sep2**.

PCR primers for the promoter:
Sep-5?-for: gacc**ctcgag**ctagtaatggcaataaattgc
Sep-prom-rev: and gacc**cccggg**gaattcttttgtataagtaaaaaaatt

PCR primers for the coding region without the signal peptide:
sep-NOSIG: gacc**cccggg**atggctaaacccgcaaattca
sep-TM-si: gacc**gggccc**tccattattttgataatgcattacacc

All the plasmids of the three groups were linearized with NruI before transfection.



2.2.16 - Plasmid constructions with flag

All the constructs above described are based on the presence of a fluorescent reporter which can immediately give information on the expression timing and localisation. However this approach has an intrinsic limitation due to the somehow large size of the fluorescent reporter which might hamper the correct localisation of the fusion protein; this can be especially true in the case of SEP proteins which harbor a transmembrane domain and can be expected to be involved in some traffic mechanisms. In order to reduce the size of the transgenic proteins we designed a series of constructs in which the GFP moiety is replaced with a much simpler element, the octapeptide FLAG M2, DYKDDDDK; this peptide is easily recognised both in Western blot and IFA by the use of specific antibodies and can also be used in pull-down experiments when possible interactors are looked for.

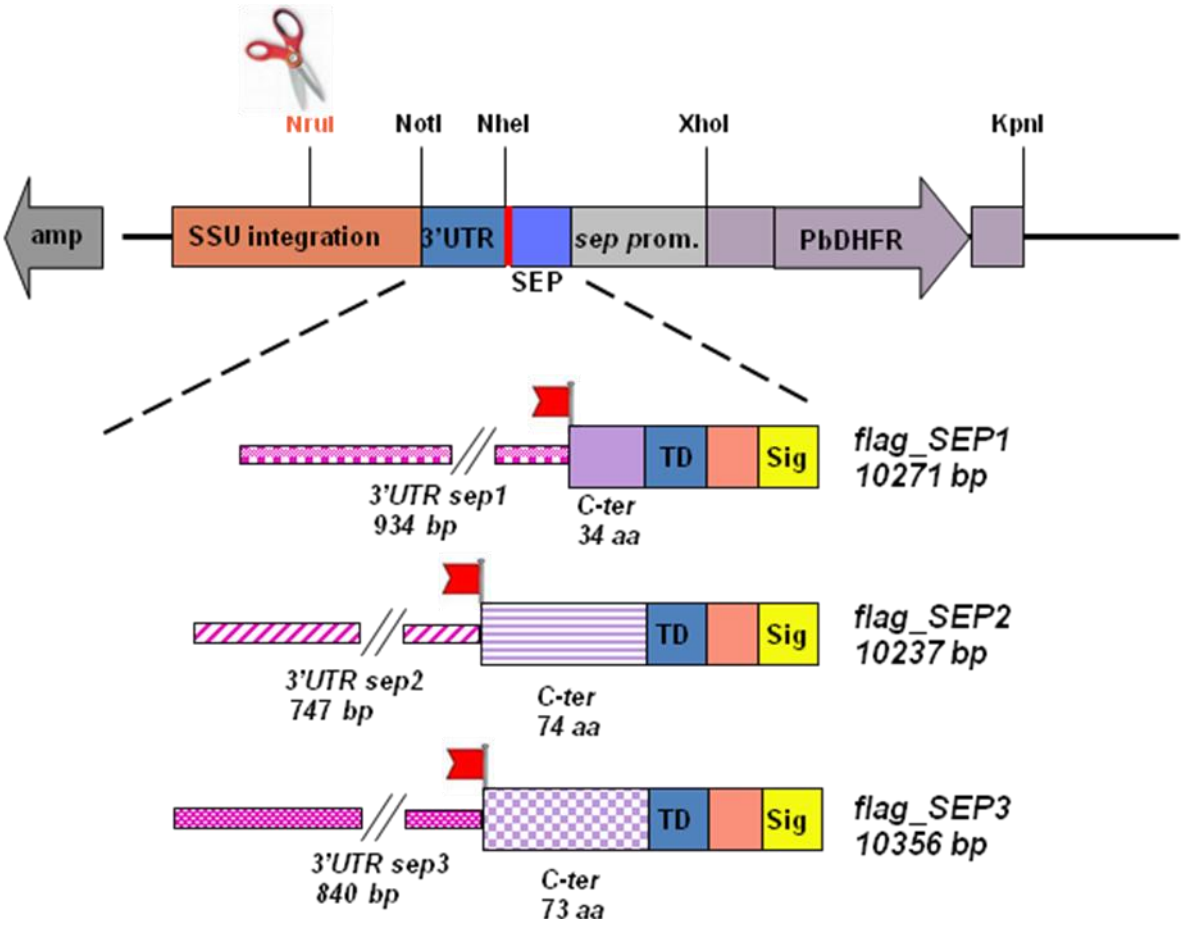
Three constructs were made with the insertion of FLAG-tag at the C-terminus of the SEP proteins.

These plasmids are directly derived from the three initial plasmids SEP/GFP-3'sep1, SEP/GFP-3'sep2, SEP/GFP-3'sep3 in which the XhoI-NheI fragment (promoter, 5'UTR, SEP/GFP fusion protein) is replaced by genomic PCRs [XhoI-promoter, 5'UTR, SEP coding sequence plus FLAG at the C-terminus-NheI]

Primers for Sep1tot-flag
1&ter-prom-sin: gacc**ctcgag**ctagtaatggcaataaattgc
1cod-flag-rev: gacc**gctagc**ttacttatcatcatcatccttataatctgatggtggtacactgtcattatc

Primers for Sep2tot-flag
Bis-prom-sin: gacc**ctcgag**gtgtatgaatttaagaatttc
bis-cod-flag-rev: gacc**gctagc**ttacttatcatcatcatccttataatcattcaattttacagtatatgg

primers for Sep3tot-flag
1&ter-prom-sin: gacc**ctcgag**ctagtaatggcaataaattgc
Ter-cod-flag-rev: gacc**gctagc**ttacttatcatcatcatccttataatccaaataacgtaattgatagcg



2.2.17 - Generation of *mCherry*_Pbsep1, *mCherry*_Pbsep2 and *mCherry*_Pbsep3 plasmids

The generation of transgenic *mCherry*_PbSEPs *Plasmodium* lines proved to be very useful in the study of SEPs expression and localisation, particularly in mosquito stages where IFA experiments may suffer high background.

The whole cloning procedure has involved five cloning steps and the use of plasmids already available in the lab (see Materials).

The first step utilises the expression plasmid set/nap-mCherry-3'nap, already available in the lab (see Materials), which contains a mCherry-fusion protein; in particular the SpeI-NotI *set/nap* 3'UTR was replaced with the 3'UTRs region of *sep* genes amplified by PCR. 3'UTR PCRs of the three genes are 931bp for sep1, 743 bp for sep2 and 832 bp for sep3 and all include a new AatII restriction site just close to SpeI site.

PCR primers for sep1:
3'-1-spe: gacc**actagt**gacgtctctagcacaaaaccagtacc
3'-1-not: gacc**gcggccgc**gtcgaacattcaatttacc

PCR primers for sep2:
3'-bis-spe: gacc**actagt**gacgtctataattatccaaaccaataagacg
3'-bis-not: gacc**gcggccgc**attgtagtactattattgcg

PCR primers for sep3:
3'-ter-spe: gacc**actagt**gacgtcgtagattctggtggatattatc
3'-ter-not: gacc**gcggccgc**caatttaccataatcaaacag

Second step: we amplified the sep1 coding region (345 bp), sep2 coding region (465 bp) and the sep3 coding region (462 bp) and cloned them in EcoRI-SmaI restriction sites of the plasmid *set/nap*-mCherry-3'nap

PCR primers for sep1:
sep1&2Hind-cod-for: gacc**gaattc**aagcttatgaaactagcaaaagcatt
sep1Sma-cod-rev: gacc**cccggg**tgatggtggtacactgtcatt

PCR primers for sep2:
sep1&2Hind-cod-for: gacc**gaattc**aagcttatgaaactagcaaaagcatt
sepbisSma-cod-rev: gacc**cccggg**attcaattttacagtatatgg

PCR primers for sep3:
SepterHind-cod-for: gacc**gaattc**aagcttatgaaattagcaaaagcatt
septerSma-cod-rev: gacc**cccggg**caaataacgtaattgatagcg

thus replacing the *nap/set* coding region; a HindIII site next to EcoRI was added in the forward primers to be used in a further cloning step. At this point the plasmids are able to drive the expression of *mCherry*_SEPs fusion proteins in bacteria under IPTG induction; six colonies for each *sep* were examined for fluorescence and the lysates were analysed in SDS-

PAGE to check the size of the fusion protein. The DNAs from candidate colonies were sent for sequencing and those with no errors chosen for further cloning steps.

Third step: EcoRI-SmaI inserts containing the region coding for *mCherry*_SEPs has been cloned in the same sites of the plasmids obtained in the first cloning step which contain the 3'UTRs of the three *sep* genes. Positive clones have been again controlled for fluorescence and DNA restriction analysis.

Fourth step: the promoter region of *sep1* (1187), *sep2* (1118 bp) and *sep3* (1253 bp)

PCR primers for sep1:
1&ter-prom-sin: gacc**ctcgag**ctagtaatggcaataaattgc
1&bisprom-Hind-rev: gacc**aagctt**ttttgtataagtaaaaaaattataat

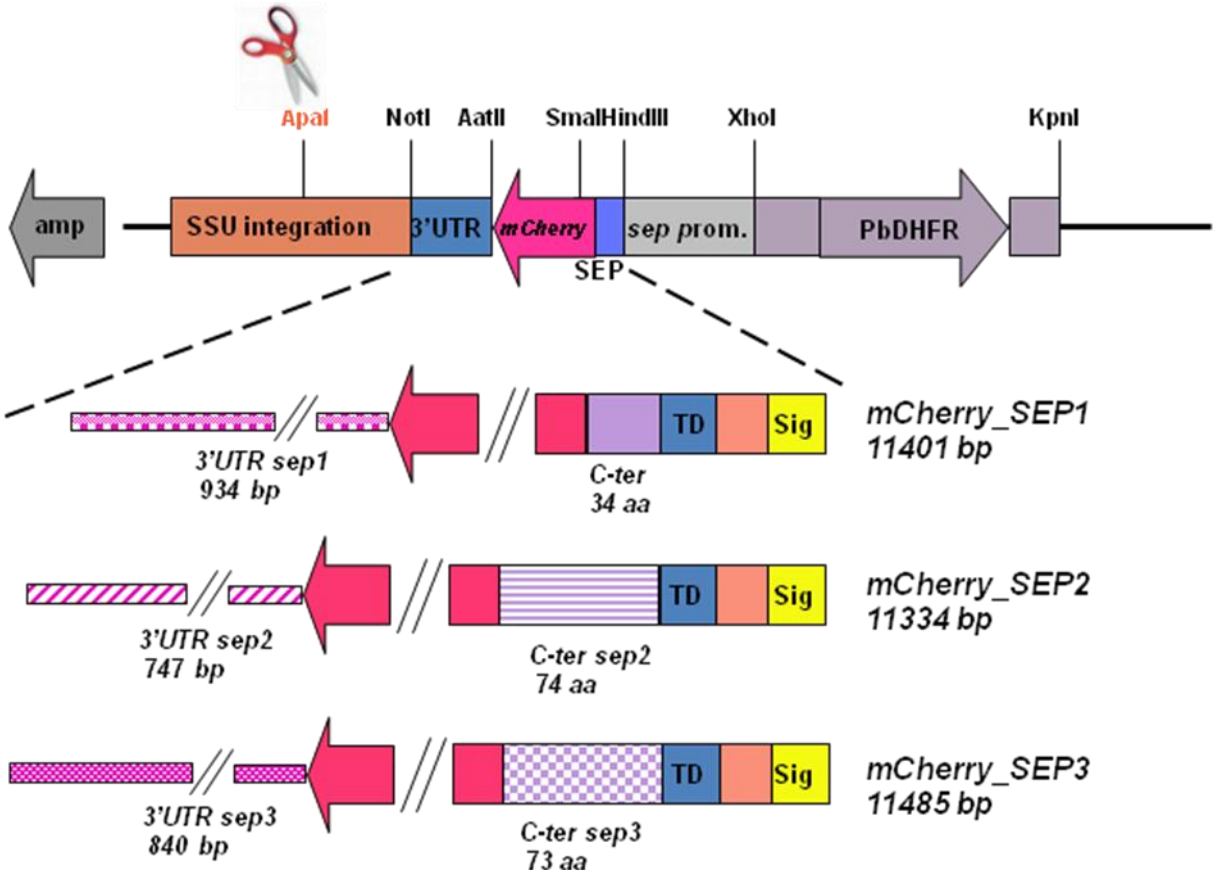
PCR primers for sep2:
Bis-prom-sin: gacc**ctcgag**gtgtatgaatttaagaatttc
1&bisprom-Hind-rev: gacc**aagctt**ttttgtataagtaaaaaaattataat

PCR primers for sep3:
1&ter-prom-sin: gacc**ctcgag**ctagtaatggcaataaattgc
Ter-prom-Hind-rev: gacc**aagctt**tttcgtataattaagtaaaaaaa

were amplified and the genomic PCRs were inserted in XhoI-HindIII site of the available transfection vector A-B7red(F)SSU plasmid (see Materials). The positive clones were chosen after DNA restriction analysis.

Fifth and last step: in the final ligation the inserts HindIII-NotI containing the coding sep and the 3'UTR (third cloning step) were cloned in the plasmids with the specific *sep* promoter (fourth step) to obtain the final plasmids *mCherry*_Sep1, *mCherry*_Sep2 and *mCherry*_Sep3 used for transfections. The positive clones have been chosen after DNA restriction analysis.

Plasmids were ApaI linearized and used to transfect *P. berghei* schizonts.



2.2.18 - RNA extraction and Northern blot analysis

RNA from *P. berghei* parasites was isolated using Rneasy kit (Qiagen) according to the manufacturer's instructions. Total RNA, size fractionated on 1% agarose under denaturing conditions, was transferred to Hybond-C extra membrane (Amersham) and prehybridised at 58°C in 7% SDS and 0,5M phosphate buffer in the presence of 1% BSA for 3 hours. The hybridisation was run in the same solution containing the radioactive the probe at 10^6 cpm/ml.

2.2.19 - Parasite mosquito stages

Ookinetes were cultured *in vitro* according to a routinely used procedure (Sinden, 1997): to set up an *in vitro* culture of ookinetes, the starting blood should have high parasitemia (around 30%) with high gametocytemia. Infected blood is mixed 1 to 10 to ookinete medium (for 1 liter: 10.4 g RPMI 1640 (+glutamine), 5,9 g HEPES (25 mM), 2 g NaHCO3, 50 mg Hypoxanthine) and incubate at 19°C for 20-24 hours. Then the culture is examined under a phase contrast microscope for the presence of ookinetes. Infections in *Anopheles stephensi* vector were performed according to Methods in Malaria research (2008. Fifth Edition edited by Kirsten Moll, Inger Ljungström, Hedvig Perlmann, Artur Scherf, Mats Wahlgren). Briefly,

both a *P. berghei* control strain and mCherry_SEPs- expressing lines were propagated in mice using standard protocols, and the presence of gametocytes assessed by Giemsa staining of tail blood. *Anopheles stephensi* mosquitoes were reared, females were selected and subdivided into different cages, considering sufficient a starting number of 60-70 mosquitoes for each experiment. Mosquitoes were infected with all different *Plasmodium* strains by direct feeding on infected mice. Ten days post-infection oocysts were detected in mosquito midguts and after 21 days sporozoites were present in collected salivary glands.

2.2.20 - *Plasmodium falciparum* cultures

P. falciparum 3D7 line was cultured in human 0^+ erythrocytes, at 5% hematocrit under 5% CO_2, 2% O_2, 93% N_2 (Trager and Jensen, 1976). Cultures were grown in medium containing: RPMI 1640 medium (Gibco) supplemented with 25 mM Hepes (Sigma-Aldrich), 50 µg/ml hypoxanthine, 0,25 mM NaHCO3, 50 µg/ml gentamicin and 10% pooled heat-inactivated 0^+ human serum. To enrich mature parasites we used Percoll gradient or magnetic purification (MIDIMACS, Variomacs, Milteny Biotec).

2.2.21 – Percoll gradient purification

To purify mature parasite we used a 60% Percoll gradient. Cultures were spun down 5' at 600 g at room temperature and supernatant was discarded. Cells were then resuspended in PBS or RPMI 1640 to 20 % haematocrit. 2 ml of RBC were layered onto 5 ml of 60% Percoll in a 15 ml centrifuge tube and spun down at 600 g for 10' without brake. After this centrifugation the schizonts usually form a distinct reddish/brown band at the top of the Percoll, while the uninfected RBCs and ring/trophozoites stages form a pellet at the bottom of the tube. Carefully schizonts were collected into a clean tube and washed once in warm complete medium; then parasites were added to flask(s) containing pre-warmed medium.

2.2.22 - Subcellular fractionation of iRBC and proteinase K digestion assay

Proteinase K digestion assay combined with different lysis procedures makes it possible to determine the subcellular compartment in which proteins of interest are located. For subcellular fractionation experiments, late stages *P. falciparum* infected RBCs (around 7% parasitemia) were enriched to 95 to 99% using 60% Percoll gradient. Parasites were treated 30' at 37° with Streptolysine-O (La technique biologique – 4 haemolytic units) or recombinant equinatoxin (1 µg/µl) which generates pores on the erythrocyte plasma membrane. Erythrocyte cytoplasm and parasites were separated by low speed centrifugation;

the cytoplasm of iRBC (the soluble portion) was successively spun down at high speed to remove ghost contamination. This lysis makes the erythrocyte cytosol accessible to proteinase K digestion but parasites with parasitophorous vacuole are still intact. Treated parasites were divided into 4 tubes (final volume of each tubes was 100 µl) to proceed to different kind of lysis in ice for 1 h:

i) the control, no proteinase K added to SLO-treated parasites; ii) SLO-treated parasites plus proteinase K 50 mg/ml, which digests soluble proteins in RBC cytosol, but not in parasitophorous vacuole iii) parasites treated with 0.05% Saponin plus proteinase K, to open the parasitophorous vacuole membrane, solubilise and digest molecules inside it, iv) sample treated with 0.1% Triton-TX plus proteinase K to solubilise and digest all proteins inside the parasite. Fifteen µl of 200 mM phenyl-methyl-sulfonyl-fluoride (PMSF) were then added to inhibit the proteinase activity.

2.2.23 - Preparations of Ghosts from Infected Erythrocytes

Infected erythrocytes were washed extensively in PBS and lysed with EquinotoxinII (1 µg for 10^8 cells) for 10 minutes at RT. The lysate was then separated by centrifugation at 15000 g, 4°C for 30' into a cytosolic fraction and a pellet containing ghosts and free parasites; ghosts were recovered from the pellet and washed several times in PBS until supernatant appear clear.

3. RESULTS and DISCUSSIONS

3.1 - Characterization of PbSEP2 and PbSEP3 in *Plasmodium berghei* and possible role in cellular trafficking in the iRBC

3.1.1 - PbSEP2 and PbSEP3 are integral membrane proteins

The three members of *Plasmodium berghei* gene family *sep* share the upstream regulatory sequence, the region coding for a predicted signal peptide and a transmembrane domain while they differ in their C-terminal region and 3'UTR. One of the encoded proteins, PbSEP1, characterized in a previous study (Birago et al. 2003), is expressed throughout the erythrocytic cycle and localizes at the parasitophorous vacuole membrane (PVM).

Our first purpose was to analyze timing of expression and subcellular localization of PbSEP2 and PbSEP3. To this aim we raised polyclonal mouse immune sera (α-PbSEP2; α-PbSEP3) against the specific C-terminal portions of both proteins. We also constructed three *Plasmodium berghei* transgenic lines (*flag*_Pbsep1, *flag*_Pbsep2; *flag*_Pbsep3), containing a second integrated copy of the three genes, fused at the C-terminus with the sequence encoding an immuno-tag (FLAG). The chimeric proteins were expressed under the control of the upstream and downstream regulatory regions of the endogenous genes. α-FLAG monoclonal antibody was used to detect the FLAG-tagged versions PbSEP2-F and PbSEP3-F.

In order to verify whether PbSEP2 and 3 are integral membrane proteins we purified wild type (wt) HP and transgenic *flag*_Pbsep2 and *flag*_Pbsep3 asynchronous parasites and prepared fractionated protein extracts. Blotted proteins were probed with the specific immune sera or the α-FLAG monoclonal antibody. As shown (Figure 3.1, panels A and B), α-PbSEP2 and α-PbSEP3 detected a protein band at the expected size of 16 KDa mainly in the insoluble fraction of the wild type parasites and a doublet corresponding to the endogenous protein and the FLAG-tagged version of PbSEP2 and 3 in the insoluble fraction of the transgenic lines *flag*_Pbsep2 and *flag*_Pbsep3. As expected, the α-FLAG revealed only the higher band of the doublet in the insoluble fraction of the *flag*_Pbsep2 and *flag*_Pbsep3 extracts while the wild type line, which doesn't contain the extra copy of the genes, was negative. Our results confirmed the specificity of the polyclonal antibodies and indicated that PbSEP2 and 3, as in the case of PbSEP1, are integral membrane proteins.

To define the timing of expression of the three SEP proteins, we performed synchronous infections in mice and obtained parasites at different developmental stages: ring, trophozoite, schizont and gametocyte. Schizont preparation contained a certain and variable amount of gametocytes, which could not be eliminated during the purification procedures. Western blot analysis using specific immune sera showed that PbSEP2 is expressed in all the asexual stages but it is very abundant in gametocytes. PbSEP1 and 3 were detected at the trophozoite, schizont and gametocyte stages at approximately the same level of expression (Figure 3.1, panels C - E).

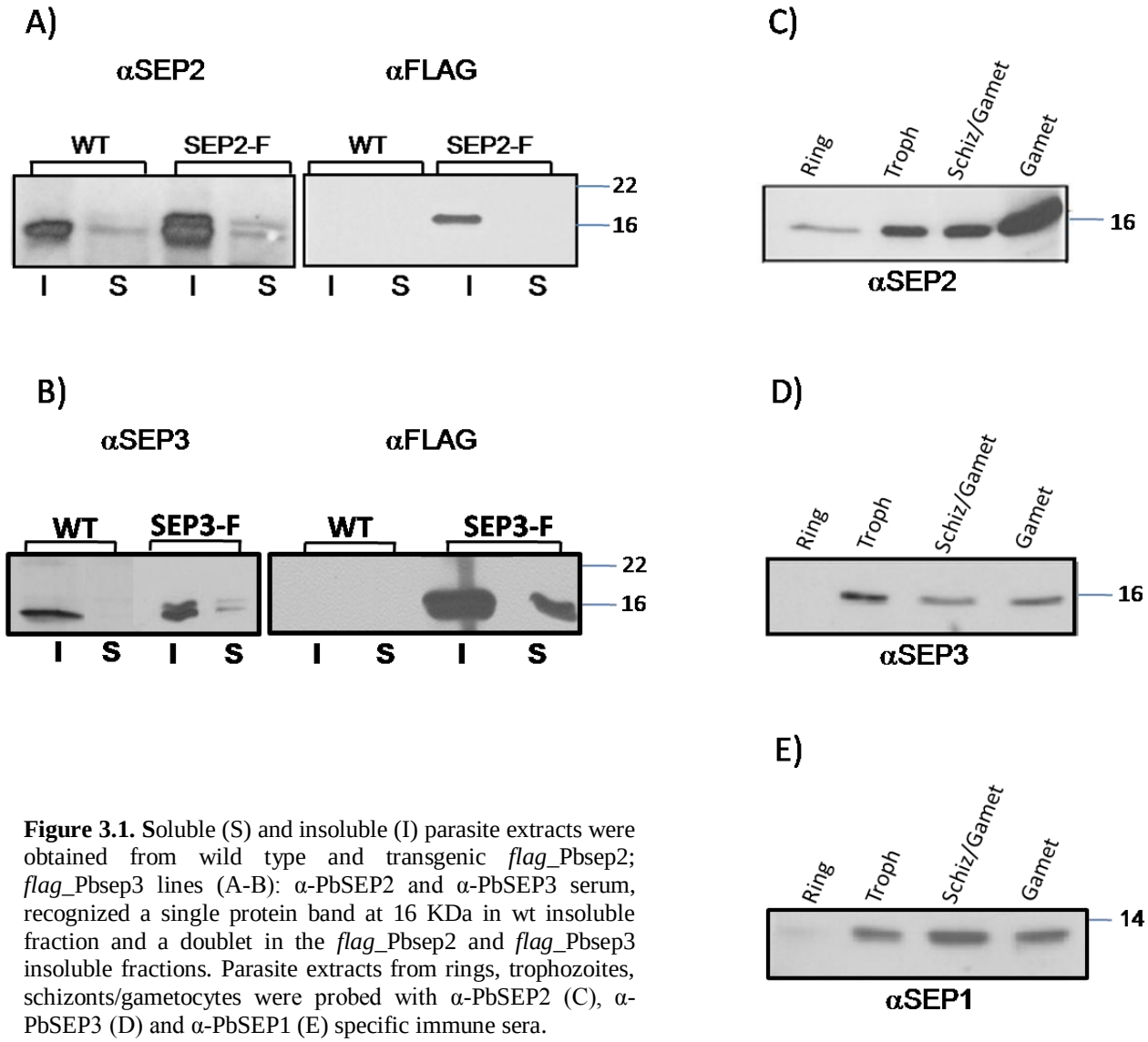


Figure 3.1. Soluble (S) and insoluble (I) parasite extracts were obtained from wild type and transgenic *flag*_Pbsep2; *flag*_Pbsep3 lines (A-B): α-PbSEP2 and α-PbSEP3 serum, recognized a single protein band at 16 KDa in wt insoluble fraction and a doublet in the *flag*_Pbsep2 and *flag*_Pbsep3 insoluble fractions. Parasite extracts from rings, trophozoites, schizonts/gametocytes were probed with α-PbSEP2 (C), α-PbSEP3 (D) and α-PbSEP1 (E) specific immune sera.

To define the subcellular localization of PbSEP2 and PbSEP3, we performed immunofluorescence assay (IFA) on fixed parasites from the wt line HP or the transgenic *flag*_Pbsep2 and *flag*_Pbsep3 lines using specific polyclonal immune sera or α-FLAG monoclonal antibody. Co-immunolocalization experiments (Figure 3.2, panel A) showed that PbSEP2 specific fluorescence decorated the periphery of parasites, well overlapping that of PbSEP1. We did not obtain information on the subcellular localization of PbSEP3 as the specific immune serum gave only a very weak background signal (not shown). α-FLAG monoclonal antibody, used on fixed *flag*_Pbsep2 and *flag*_Pbsep3 lines, decorated mainly the periphery of young and mature trophozoites (Figure 3.2, panel B) suggesting that also the PbSEP3 localizes at the PVM. In schizonts, specific signal was confined to few dots flanking the dividing nuclei.

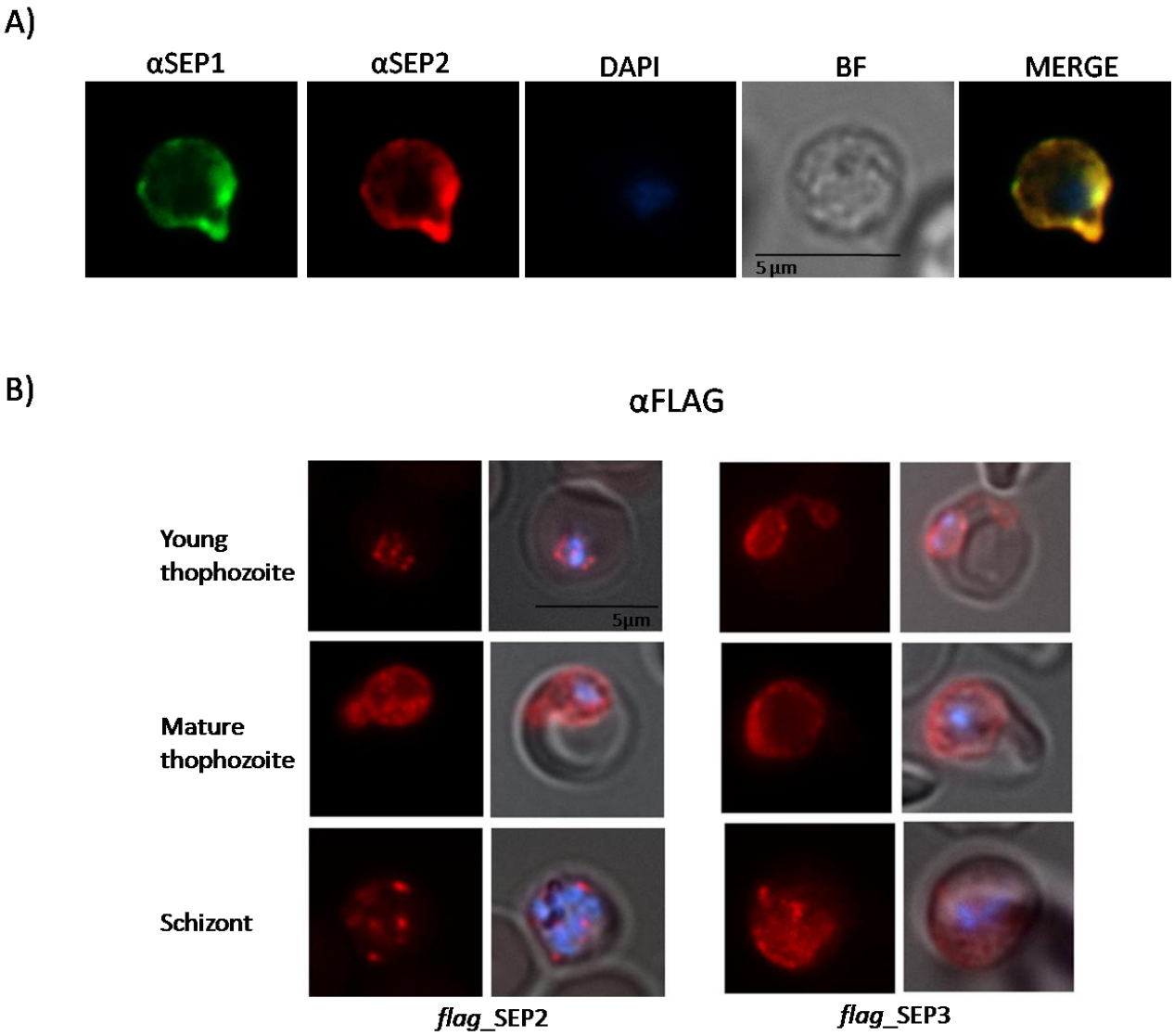


Figure 3.2. A) Immuno co-localization assay of PbSEP1 (green) and PbSEP2 (red). B) IFA assay have been performed on fixed and permeabilized transgenic *flag*_Pbsep2 and *flag*_Pbsep3 parasites using a specific α-FLAG serum. FLAG_SEP2 and FLAG_SEP3 are expressed in mature parasites with a very similar pattern.

3.1.2 - Localization and timing of expression of PbSEP2 in parasite blood stages

Western blot analysis indicated that the relative abundance of PbSEP2 differs in asexual and sexual blood stages of *P. berghei* while the other two members of the family, SEP1 and 3, are expressed at a similar level throughout the erythrocytic cycle. To track localization and timing of expression of PbSEP2, we performed IFA experiments during a synchronous infection in mice. Few drops of blood were collected from mice tail: 4 hours post invasion (hpi) at the ring stage; 8, 13 hpi (young trophozoites), 15, 17 and 19 hpi (mature trophozoites), 21, 23 hpi (replicating parasites), 27 hpi (gametocytes and rings of the next generation).

Interestingly, we observed a peak of PbSEP2 expression in mature trophozoites (17 hpi) and in gametocytes, where the protein seems to be accumulated in structures inside the parasite. In the other stages it clearly localizes at the periphery of the cell (Figure 3.3, panel A).

We asked whether the peak of expression observed at 17 hpi occurred in parasites committed to gametocytogenesis or in asexual trophozoites. To answer this question we performed synchronous infections of the wt and the mutant line HPE, unable to undergo sexual differentiation. We collected parasites at 13 hpi and 17 hpi and compared the abundance of PbSEP2 in both parasite lines; we prepared soluble (S), membrane-associate (A) and insoluble (I) fractions from the non-gametocyte producer (HPE) and from the wild type (HP). As shown, (Figure 3.3, panel B) PbSEP2 was detected in the insoluble fraction after high salt extraction (performed to solubilise associated proteins) in all samples. Consistent with immune-localization results, in mature trophozoites (17 hpi) PbSEP2 is more abundant than in young trophozoites (13 hpi). This was observed in both lines indicating that a transient peak of expression occurs in mature asexual parasites. It is conceivable that an increase in the amount of PbSEP2 might be required before schizogony to guarantee its correct distribution to the daughter merozoites (Figure 3.3, panel B).

To have an approximate evaluation of the relative abundance of PbSEP2 in gametocytes and mature trophozoites, we performed Western blot analysis on estimated numbers of gametocytes (10^6 and 10^7) and mature trophozoites (10^7) and compared the relative intensity of the specific bands. As shown, (Figure 3.3, panel C), PbSEP2 is at least ten times more abundant in the insoluble fraction of gametocytes than in trophozoites. This difference in abundance does not depends on the transcription level or stability of the specific mRNA since we didn't notice significant variations in the intensity of hybridization signal comparing different blood stages in Northern blot analysis (Figure 3.3, panel D).

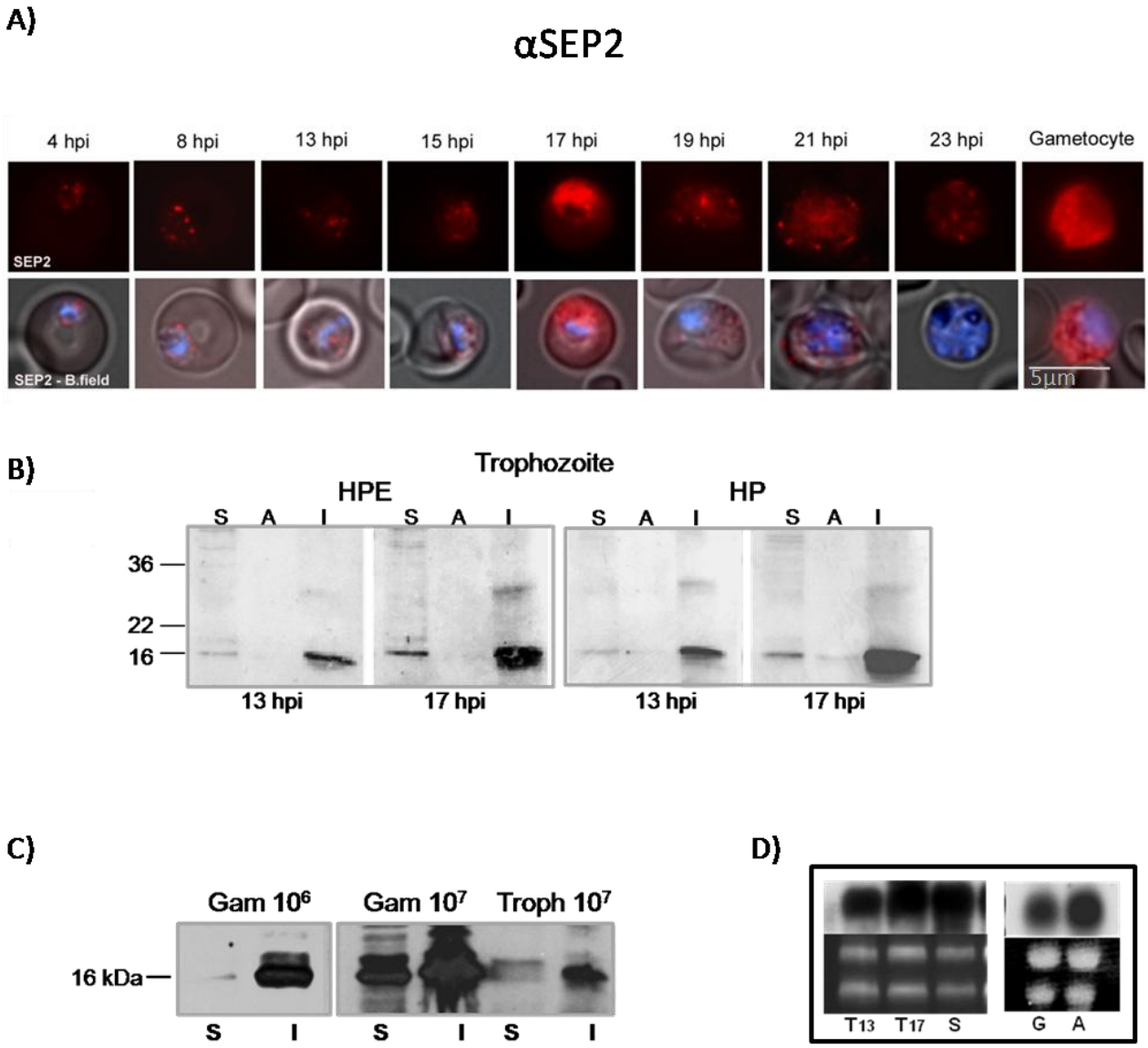


Figure 3.3. A**)** Immunofluorescence on *Plasmodium berghei* synchronous parasites using α-PbSEP2 specific immune serum. B) Western blot analysis of fractionated parasite extracts prepared from the non-gametocyte producer line (HPE) and the wt line (HP), probed with α-PbSEP2 immune serum. Soluble (S), membrane-associated (A) and insoluble (I) fractions were prepared from young (13hpi) and mature (17hpi) trophozoites. C) Soluble and insoluble fractions of trophozoites (10^7) and gametocytes (10^6 and 10^7) probed with α-PbSEP2 immune serum. D) Northern blot analysis (upper panels) of mRNA extracted from trophozoites at 13 and 17 hpi (T13; T17), schizonts (S) and gametocytes (G) and mixed asexual stages (A). Total RNA loaded from each sample is shown in lower panels.

3.1.3 - SEP proteins are exported to the erythrocyte and are detected in ghosts prepared from iRBCs

Although SEP/ETRAMPs don't contain a PEXEL motif, previous studies reported that *P. falciparum* members are translocated beyond the parasite membrane to the RBC compartments: PfSEP14 of *Plasmodium falciparum* was shown to co-localize with Maurer's Cleft marker (Birago et al. 2003) and ETRAMP10.3 was detected in erythrocyte sites in parasite blood stages (MacKellar et al. 2010). As shown above, we did not detect PbSEP2 and PbSEP3 specific fluorescence associated with erythrocyte compartments in IFA experiments on fixed parasites. One possible explanation is that the conformation of SEP proteins may changes, when exported to the RBC cytosol, thus affecting antigen recognition by the specific antibodies. Since the PfSEP14 localization at the Maurer's clefts was detected in IFA experiments on unfixed dried smears of iRBCs (Birago et al. 2003), we tried this procedure on RBCs infected with *flag*_Pbsep2 and *flag*_Pbsep3 parasites using α-FLAG antibody. As shown (figure 3.4, A upper panels), both FLAG_SEP2 and FLAG_SEP3 are associated with parasite compartments, but they are also clearly exported to the RBC in dot-like structures, which resemble Maurer's clefts in *P. falciparum*. We did not observe translocation of FLAG_SEP1 to the host compartment when we analysed *flag*_Pbsep1 parasites (data not shown).

To understand whether SEP proteins are anchored to the erythrocyte membrane we prepared ghosts from purified *flag*_Pbsep2 and *flag*_Pbsep3 iRBCs as well as from normal erythrocytes as a control. Unfixed ghost preparations were subjected to IFA using α-FLAG antibody. Figure 3.4 shows that PbSEP2 and 3 are associated with the membrane of the infected erythrocyte ghosts (lower part of panel A) while no signal was detected in ghosts from normal RBCs (panel B). This indicated that FLAG_SEP2 and FLAG_SEP3 are exported to the periphery of the red blood cell where they probably interact with the host cell membrane skeleton.

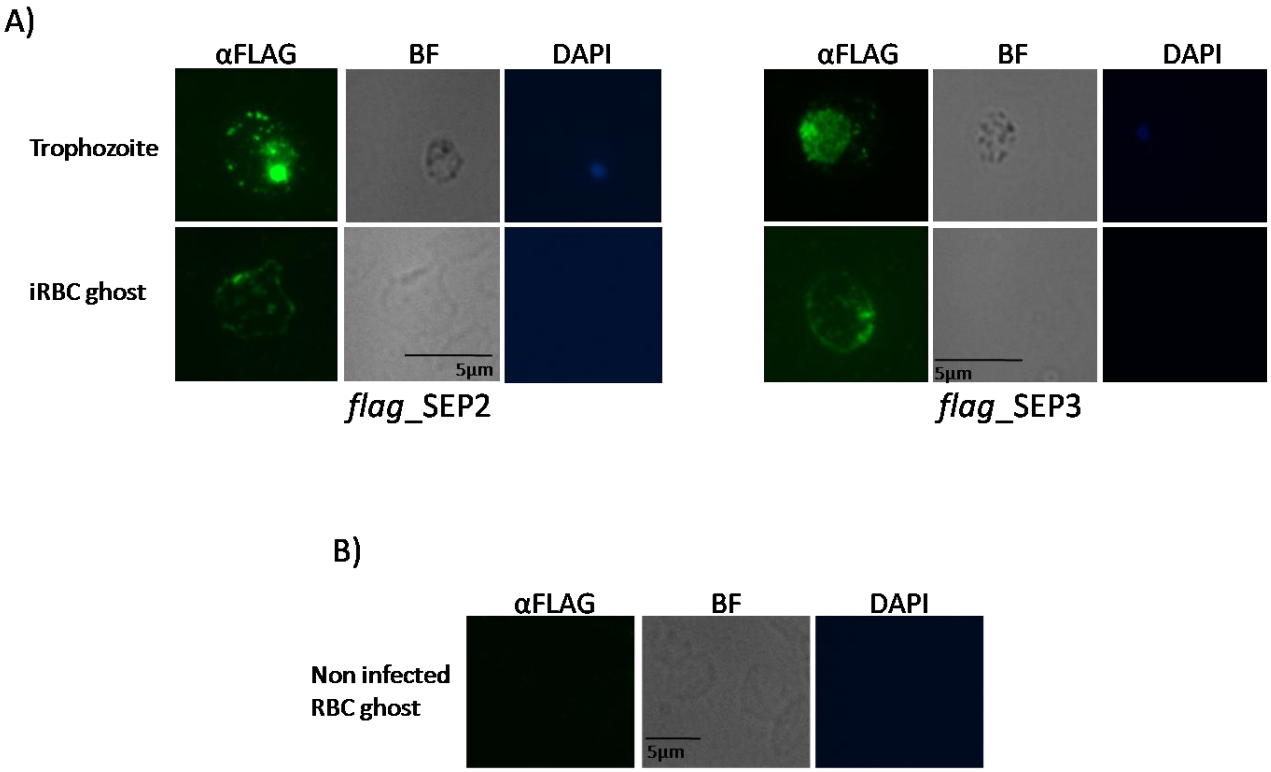


Figure 3.4. A) IFA using the α-FLAG monoclonal antibody on unfixed blood smears (upper panels) and ghosts (lower panels) of the transgenic lines *flag*_Pbsep2 and *flag*_Pbsep3. B) Ghosts from normal RBCs tested as a control with α-FLAG. Bright field (BF).

To confirm the association of SEPs with the host cell membrane/cytoskeleton, we purified iRBCs to prepare ghosts and parasite soluble/insoluble fractions. Specific immune sera, used in Western blot analysis of fractionated iRBCs, detected PbSEP2 and PbSEP3 both in ghost and parasite fractions (Figure 3.5, panel A). The reliability of fractionated extracts was tested using immune sera against not exported parasite proteins: the cytoplasmic Pb14-3-3 (Di Girolamo et al.2008) and the nuclear protein PbSET (Pace T. et al. 2006). Specific signals were detected in parasite soluble and insoluble fractions and not in ghost preparation (Figure 3.5, panel B).

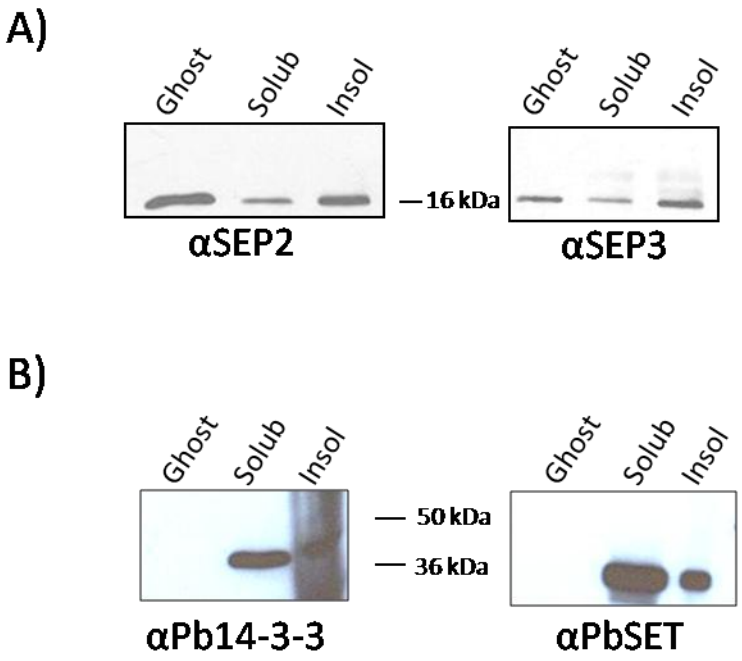


Figure 3.5. A) Western blot analysis of fractionated iRBCs: ghosts and parasite soluble and insoluble fractions probed with α-PbSEP2 and α-PbSEP3 immune sera (A) or with α-Pb14-3-3 and α-PbSET (B).

3.1.4 - Generation of SEP/GFP transgenic lines

Preliminary data obtained from IFA and Western blot analyses indicated that PbSEPs differ in timing of expression, relative abundance and subcellular localization, despite the three proteins share the first 85 aminoacids, which include localization motifs such as the N-terminal signal peptide and the internal transmembrane region. PbSEP1 localizes at the PVM, while PbSEP2 and PbSEP3 are also exported to the RBC compartment. PbSEP1 and PbSEP3 maintain the same level of expression in all blood stages while PbSEP2 has two peaks of expression in mature trophozoites and in gametocytes. In addition, the encoding genes share the upstream 5'UTR and promoter region suggesting the absence of a specific transcriptional control. At the protein level the three SEPs differ only in their C-terminal region while, at the gene level, they only differ in the 3' UTRs. To understand the role of the specific regulatory regions and to identify motifs required for the correct localization of SEPs, we designed different transfection constructs, which included the fluorescent GFP reporter (details are in Methods). Transgenic parasites generated after transfection were analysed by fluorescence microscopy to assess the expression pattern and subcellular localization of the GFP.

To evaluate a possible role of the specific 3' UTRs in the expression level of the *sep* genes, we prepared three plasmids containing the GFP coding region flanked by the common upstream region and the downstream 3'UTR specific for each *sep* gene. The three transgenic lines generated (Figure 3.6) expressed the GFP at the same level in asexual and sexual blood stages. This indicated that the specific 3'UTRs do not affect mRNA stability and/or translatability. The higher abundance of SEP2 in sexual stages might be explained by a different stability of the protein or by a reduced translocation of SEP2 to the host cell compartment.

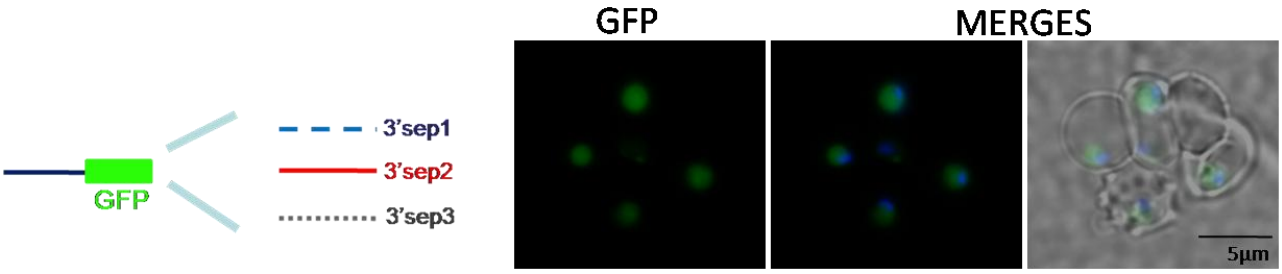


Figure 3.6. Transgenic lines expressing the GFP reporter under the control of the common promoter and the 3'UTR specific for each *sep* gene. The exemplar image shows that the GFP is expressed at the same level in all blood stages.

To define protein motifs required for translocation of SEPs beyond the PVM, we generated new GFP constructs in which the fluorescent reporter was appended to growing portions of the common *sep* coding region. The chimeric proteins were expressed under the control of the common promoter and the specific 3'UTR of *sep*2. Transgenic parasites expressing the GFP-signal peptide fusion or the GFP-signal peptide plus the next 25 aminoacids routed the chimeric proteins to the parasite periphery, most probably the PV (Figure 3.7, panels A and B). Transgenic parasites expressing the GFP fused to the internal hydrophobic region, without the signal peptide, maintain the GFP inside the parasite in part associated with membrane structures (Figure 3.7, panel C). Only when the fusion included both signal peptide and internal transmembrane domain (SEP/GFP-3'-sep2 construct) the chimera protein was efficiently exported outside the parasite in dot-like structures at the periphery of the host cell (Figure 3.7- D, top panel). SEP/GFP-3'-sep2 construct was also used to transfect a transgenic line (SET/DS-RED), available in the lab, which contains an additional copy of the nuclear protein SET fused with a red fluorescent reporter and expressed under a gametocyte-specific promoter. Red fluorescence allows the identification of gametocytes in a mixed parasite population. In this transfected line, we observed a bright GFP fluorescence associated with gametocytes, suggesting that the stage-specific abundance is also restored (Figure 3.7- D, bottom panel). Similar results were obtained with the GFP fused to the entire SEP2 protein (not shown).

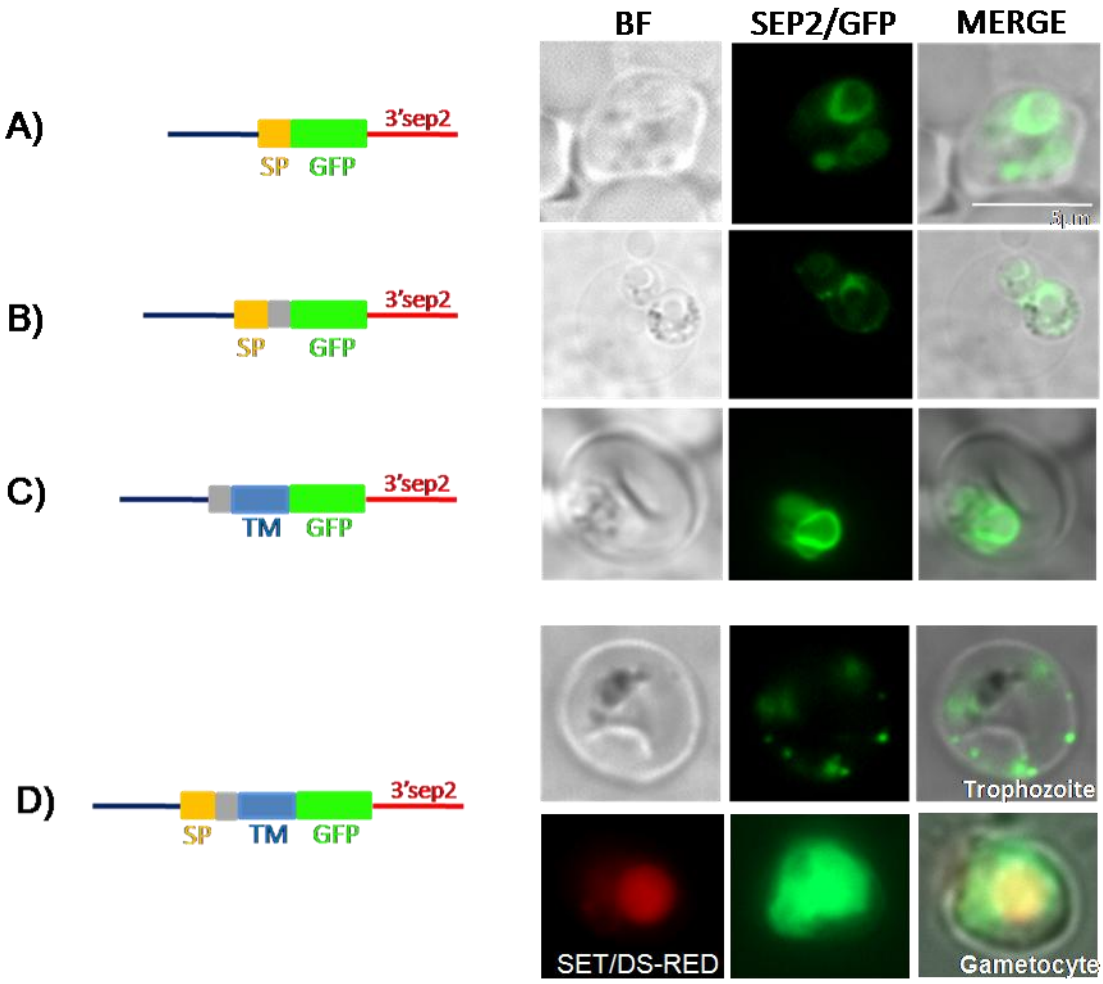


Figure 3.7. Transgenic lines containing the *gfp* appended to different portions of the common *sep*-coding region, expressed under the control of the upstream common promoter and the downstream 3'UTR specific for *sep*2. The signal peptide (A) or the signal peptide plus the next 25 aminoacids (B) drive GFP to the parasite periphery. The internal transmembrane domain drives the reporter to membranous structures inside the parasite (C). When both signal peptide and transmembrane region are present in the construct, the GFP is exported to the erythrocyte compartment (D, top panel) and abundantly present in gametocytes (D, bottom panel). Signal peptide (SP); Transmembrane domain (TM); Bright field (BF).

We also generated two additional constructs: SEP/GFP-3'-sep1 and SEP/GFP-3'-sep3, which differ from SEP/GFP-3'-sep2 only for the presence of the downstream regulatory region specific for SEP1 and 3 respectively. In the presence of Sep1-specific 3'UTR (Figure 3.8, panel A) the GFP fluorescence localizes at parasitophorous vacuole in all blood stages. In parasites expressing GFP fusion in the presence of 3'UTR of sep3 (Figure 3.8, panel B), a dot-like specific fluorescence was detected outside the parasite at the RBC periphery.

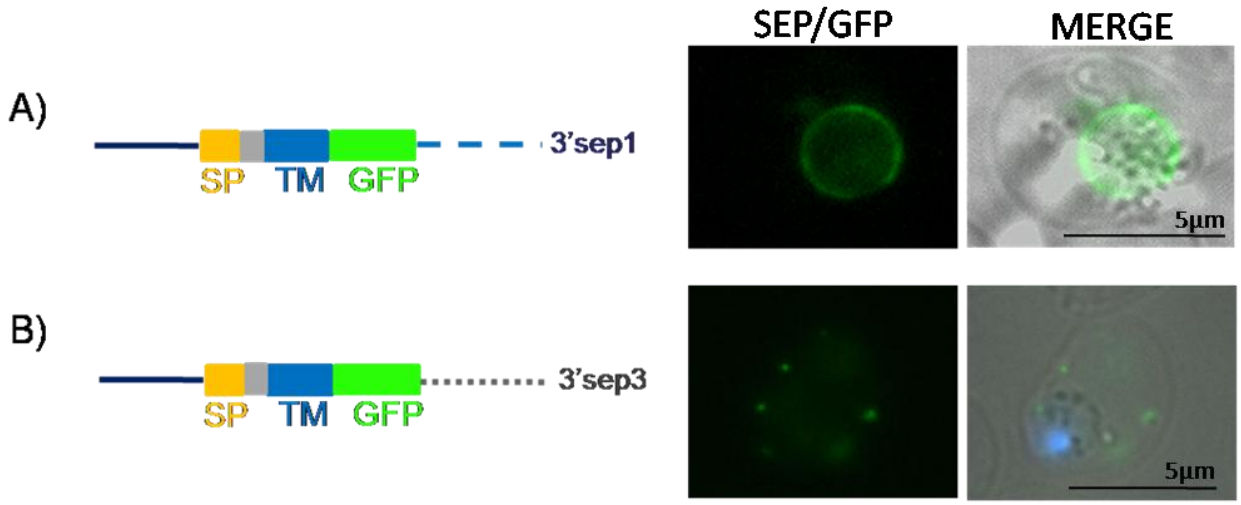


Figure 3.8. A) The coding box containing the signal peptide (SP), the transmembrane region (TM) fused with GFP was placed upstream the 3'UTR specific for *sep*1. Transgenic parasites expressing this construct drive the chimeric protein to the parasitophorous vacuole. B) When the same coding box was placed upstream the 3'UTR of *sep*3 the fusion protein is exported to the RBC.

We can conclude that the common protein region (85 amminoacids) is necessary to promote the export of SEPs and their localization at the erythrocyte periphery. This region is also sufficient to restore the higher abundance of SEP2 in gametocytes which might be due to its preferential retention inside the parasite. Still completely unknown remains the mechanism by which transgenic parasites expressing the construct SEP/GFP-3'-sep1 (which differ only for the presence of the specific 3'UTR) retain the GFP fusion at the PVM without translocating it to the RBC compartment as is the case of the two constructs which contain 3'UTRs of the other two members of the family. We can speculate that the downstream regulatory regions may direct mRNA localization to different domains of the ER, which decide the final destination of the encoded protein.

Our results indicate that the variable C-terminal regions do not contribute to determine the subcellular localization of SEPs. It is conceivable that they may participate to establish protein-protein interactions.

3.1.5 - Characterization of PbSEP1, PbSEP2 and PbSEP3 in mosquito stages

Repeated attempts to disrupt *Pbsep2* and *Pbsep3* in *P. berghei* blood stages were unsuccessful. This suggested that the genes might be essential for intraerythrocytic development. Differently, it is possible to delete *Pbsep1* gene (it also characterized a natural mutant line with a large deletion of chromosome 5 which includes *Pbsep1*) and obtain transformed parasites able to grow up in blood stages. We verified that the *Pbsep1* gene product is dispensable also in mosquito stages: in fact we infected *Anopheles stephensi* with the *Plasmodium berghei* transgenic line deleted for the left arm of chromosome 5 which included *Pbsep1* gene (Δ-Pbsep1). Ten days after the blood meal on mice, we found similar numbers of oocysts in mosquitoes midguts of Δ-Pbsep1 line and in the wild type HP used as a control. Twenty-one days after infection a similar number of sporozoites was also found in salivary glands (Figure 3.9). Sporozoites of the Δ-Pbsep1 line gene were able to infect mice and to develop again in blood stage with the same efficiency of the wild type HP.

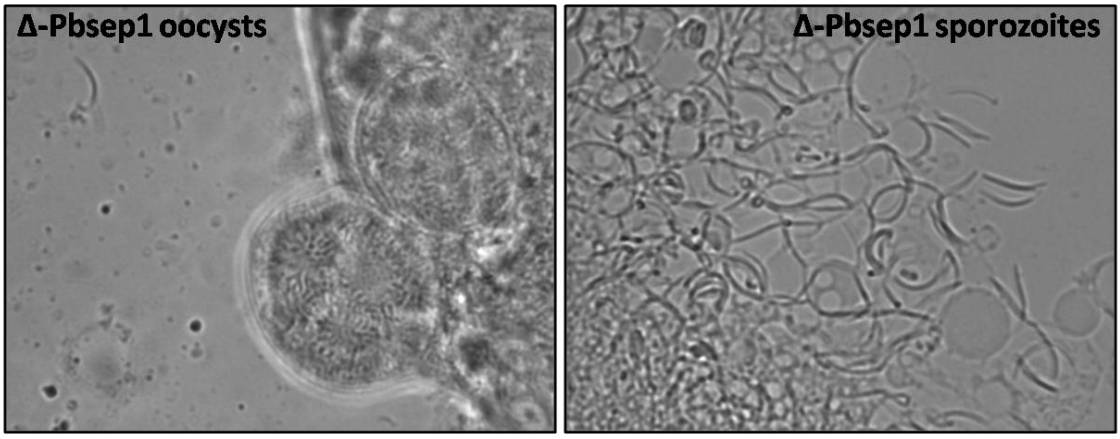


Figure 3.9. Pb*sep*1-deleted parasites were able to infect mosquitoes and develop into midgut oocysts and salivary gland sporozoites.

When we performed IFA experiments using α-PbSEP1 rabbit polyclonal serum we detected specific fluorescence in ookinetes developed *in vitro*. PbSEP1 is assembled in dot-like structures inside the parasite with no overlay with Pb70, a protein marker of the ookinete

surface (Figure 3.10); PbSEP1 is not expressed in oocysts in *Anopheles* midgut as well as in salivary gland sporozoites.

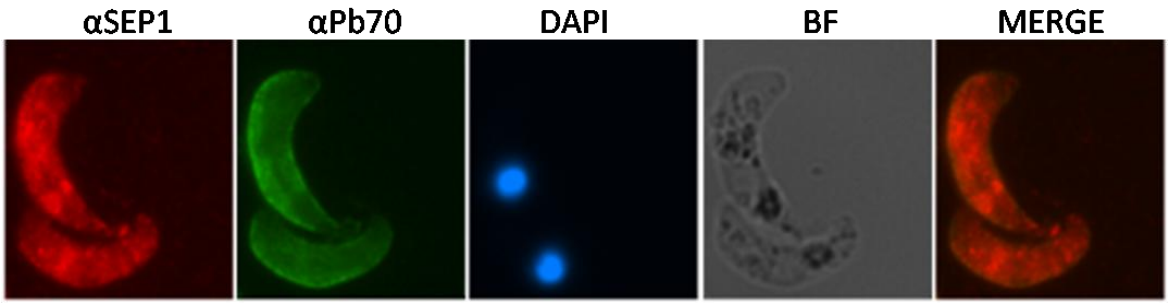


Figure 3.10. Co-immunolocalization experiments using immune sera specific for α-PbSEP1 and the ookinete surface marker Pb70. Bright field (BF).

To investigate the expression and subcellular localization of PbSEP2 and PbSEP3 in mosquito stages we generated transgenic lines expressing PbSEP2 and 3 fused with the mCherry red fluorescent reporter, named *mCherry*_Pbsep2 and *mCherry*_Pbsep3 respectively. In ookinetes produced *in vitro* (Figure 3.11, panel A), PbSEP2/mCherry was detected in few dot-like structures in parasite cytoplasm while PbSEP3/mCherry was more abundantly expressed with a punctuate pattern uniformly distributed inside the parasite. We also infected female *Anopheles stephensi* with *mCherry*_Pbsep2 and *mCherry*_Pbsep3 transgenic lines and dissected mosquitoes to analyze midgut oocysts (Figure 3.11, panel B) and salivary gland sporozoites (Figure 3.11, panel C).

In midgut oocysts PbSEP2/mCherry was expressed at high level, with a signal spread through the entire cyst. An intense red fluorescence was also detected in salivary gland sporozoites. PbSEP3/mCherry was detected as a residual fluorescence inside the cysts. The protein gave a very weak signal only in a small percentage of salivary gland sporozoites.

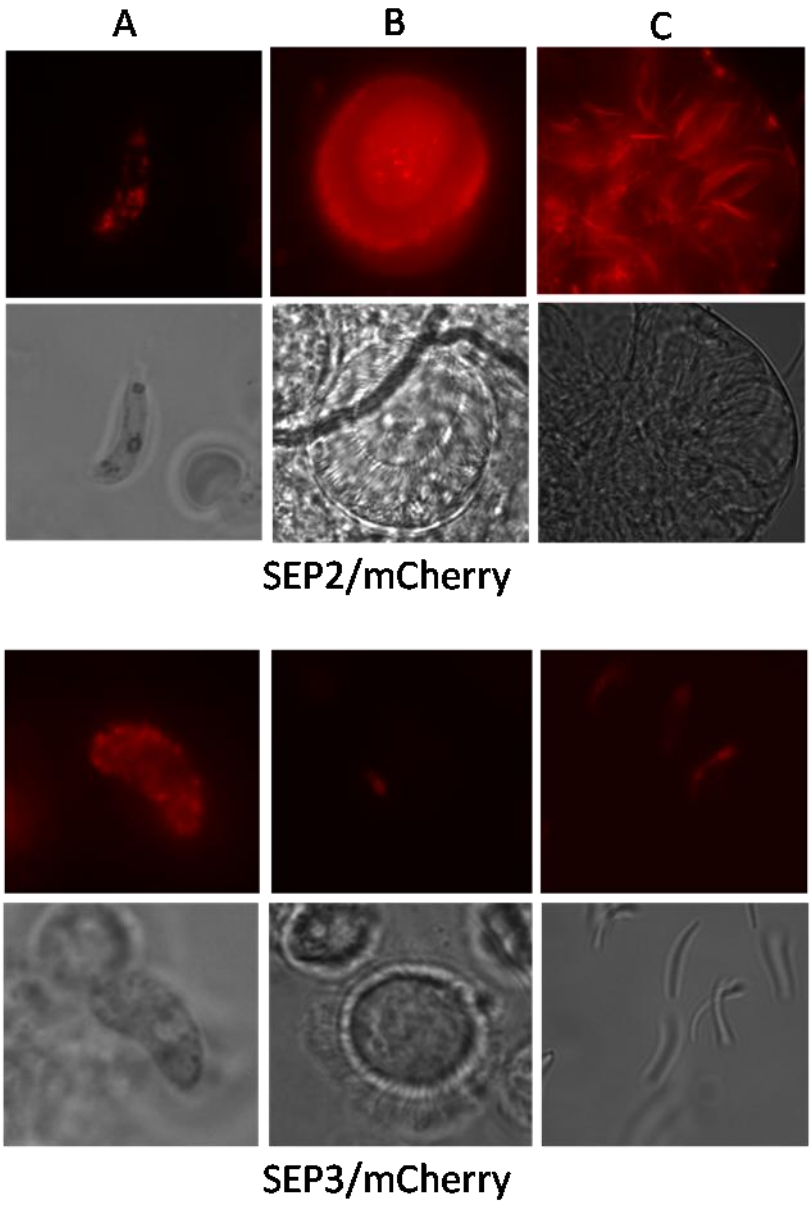


Figure 3.11. Expression of PbSEP2/mCherry and PbSEP3/mCherry proteins in mosquito stages: ookinete (A), mature oocyst (B) and salivary gland sporozoites (C).

We can conclude that in mosquito stages SEP proteins reside in punctuate structures inside the parasite and their expression is modulated. SEP1 and 3 are abundant in ookinetes and nearly absent in midgut oocysts and salivary gland sporozoites. SEP2 is expressed at low level in ookinetes and abundant in the other mosquito stages.

3.1.6 – Discussion

In this study we focused on a *Plasmodium berghei* gene family (*sep*) encoding 3 small exported proteins. *Sep* members share similarities with a larger family (*etramp*) characterized in *Plasmodium falciparum.* SEP/ETRAMP proteins (13–16 kDa) contain a predicted signal peptide at the N-terminus, an internal hydrophobic region and a variable C-terminal portion. Comparative analysis identified predicted *sep/etramp* genes in the genome of *P. yoelii*, *P. vivax*, *P chabaudi* and *P. knowlesi*, thus suggesting that this family, conserved within *Plasmodium* genus, may have an important role in parasite development. In both rodent and human *Plasmodium* species it was shown that SEP/ETRAMP proteins localize at the PVM (Birago et al. 2003, Spielmann et al. 2003, Spielmann et al. 2006). It was also reported (Birago et al. 2003, MacKellar et al. 2010) that members of this family translocate beyond the PVM to the iRBC cytosol/surface during blood stage development. The expression of SEP/ETRAMPs is not limited to parasite blood stages. In rodent parasites, a family member (UIS 3) is specifically expressed in infectious sporozoites. Notably, transgenic lines knocked out for this gene develop normally in the mosquito vector but they are not able to grow and multiply into the hepatocytes (Mueller et al. 2005). Overall these data indicate that SEP/ETRAMPs exert specific functions throughout parasite life cycle.

We decided to better define expression pattern and subcellular localization of the three family members of *P. berghei* (*Pbsep*1-3) as they share the upstream regulatory region and the coding portion (85 amino acids) including the signal peptide and the hydrophobic region but differ in their C-terminus and 3'UTRs.

This study was aimed at defining: a) timing of expression and subcellular localization of SEP proteins in blood and mosquito stages b) motifs responsible for subcellular localization of SEPs in blood stages.

Timing of expression and subcellular localization of SEP proteins in blood and mosquito stages

This part of the study was tackled by generating immune sera specific for PbSEP2 and 3 proteins, which were not characterized in previous studies. Both sera identified, in Western blot analysis, protein bands at the expected molecular weight (16 KDa) associated to the insoluble fraction of parasite extracts. This suggested that, as the case of PbSEP1, PbSEP2 and 3 are integral membrane proteins. These results were confirmed using monoclonal α-FLAG serum on transgenic lines *flag*_Pbsep2 and *flag*_Pbsep3 expressing an extra copy of the genes with a flag at the C-terminal portion. Synchronous parasite infections were also

performed and the expression of the three SEP proteins assessed at different time points: rings, young and late trophozoites, schizonts and gametocytes. PbSEP1 and 3 were uniformly expressed in asexual and in sexual stages. Notably, we observed a peak of PbSEP2 expression in late trophozoites and gametocytes. It is conceivable that new synthesis of PbSEP2 in late asexual stages might guarantee a certain level of this protein to the daughter invasive forms. In gametocytes, PbSEP2 is at least 10 times more abundant than in asexual stages and we may hypothesize that it might be required during mosquito stages.

Immunofluorescence assays, performed using either specific immune sera or anti-FLAG monoclonal antibody on fixed iRBCs showed that PbSEP2 and 3 are distributed at the periphery of the parasite. Specific fluorescence well overlaps that of PbSEP1, indicating that the three SEP proteins localize at the PVM as also described for the ETRAMPs in *P. falciparum* (Spielmann et al. 2006). In *Plasmodium* the PVM is a very peculiar compartment at the interface between host cell and parasite (Charpian and Przyborski, 2008), which works as a sorting center for exported parasite proteins. Due to the discrepancy of reports about the subcellular localization of SEP/ETRAMP, we performed IFA experiments on unfixed, air-dried blood smears of *P. berghei* iRBCs to verify whether immunogenic epitopes might be affected by fixation. Using this procedure we showed that both PbSEP2 and PbSEP3 are exported beyond the PVM, to the erythrocyte cytosol in dot-like structures which resemble the Maurer's clefts detected in *P. falciparum*. We also performed immunofluorescence assay using α-FLAG antibody on unfixed ghosts obtained from *flag*_Pbsep2 and *flag*_Pbsep3 transgenic lines iRBCs. The characteristic punctuate pattern was still detected, indicating that the structures recognized by the antibody are most probably attached to the erythrocyte membrane skeleton. Association with cytoskeleton has been described for Maurer's clefts of *P. falciparum*. The molecular basis of this interaction is only partially elucidated. It was shown (Blisnick et al; 2000) that the Maurer's cleft-resident protein PfSBP1 interacts with the erythrocyte peripheral membrane protein LANCL1; this interaction is restricted to the schizonts stage and depends on the phosphorylation of PfSBP1 protein. Since Maurer's clefts remain attached to the ghosts throughout the erythrocyte development of *P. falciparum*, it was proposed that SEP/ETRAMP family members might be responsible for this interaction (Lanzer et al; 2006). Maurer's cleft-like structures have not been described up to now in RBCs infected by the rodent parasite *P. berghei* and more experiments are required to confirm that the compartments we detect at the periphery of the host erythrocytes have a role in assembly and translocation of parasite factors to the RBC surface as described for Maurer's clefts. PbSEP1, which share with the other two members of the family most of the coding

sequence, is a dispensable gene, as indicated by the identification of natural mutants harboring a subtelomeric deletion of chromosome 5 including *Pbsep1*. In this study we showed that the lack of *Pbsep1* gene product does not affect the development of parasite mosquito and hepatic stages. Conversely, repeated attempts to knock out *Pbsep2* and *Pbsep3* were unsuccessful, indicating that both genes are necessary for parasite development.

Localization studies in the mosquito stages detected PbSEP1 only in ookinetes and PbSEP3 in ookinetes and at a very basal level in salivary gland sporozoites; PbSEP2 was instead abundantly expressed throughout the mosquito cycle. These results indicate that the level of the three SEP proteins highly differ in sexual and mosquito stages.

Motifs responsible for subcellular localization of SEPs in blood stages

We showed that PbSEP2 and 3 are efficiently translocated to the host erythrocyte cytosol and routed to the erythrocyte periphery. SEP/ETRAMP family members do not contain the PEXEL sequence, which mediates the translocation of *P. falciparum* proteins from the PVM to the erythrocyte cytosol. To identify motifs required for the correct sorting of these proteins, we generated transgenic lines expressing the GFP fused to different portions of the region encoding the first 85 amino acids shared by the three SEPs under the control of the common promoter and the 3'UTR specific for *sep2*. Signal peptide sequence drives as expected the GFP fluorescence to the periphery of the parasite, most probably the PV. However, an efficient export of the chimeric protein occurs only when both signal peptide and transmembrane region are present. The presence of the internal transmembrane domain is not sufficient to direct the GFP to the PVM. We can conclude that, also in the case of PEXEL negative proteins, translocation to the host cell cytosol is a two-step process: the protein is firstly routed to the PVM and later to the host compartment. A possible alternative mechanism of protein export is still object of investigation. Furthermore, the export of the GFP reporter was not affected when our constructs did not include the variable C-terminal portion of the coding region. Conversely, the substitution of the 3'UTR of the *sep2* with that of *sep1*, changes the subcellular localization of the encoded protein confining it to the PVM. We can speculate that the specific 3'UTRs might direct the mRNAs to distinct domains of the ER thus influencing the successive localization of the encoded protein. This challenging hypothesis will drive future experimental work.

3.2 - Host protein internalization during *Plasmodium* infection in human erythrocytes

Erythrocyte remodeling is a very important step for *Plasmodium* development and survival inside the erythrocyte host. Preliminary results obtained in our laboratory on the rodent model *P. berghei* indicated that remodeling of the host cytoskeleton, upon parasite infection, involves also the mobilization of the host dematin and adducin, components of the junctional complex of the erythrocyte membrane skeleton (Khan et al. 2008) and their recruitment by the parasite. Co-immunoprecipitation experiments showed that internalized dematin interacts with the parasite protein Pb14-3-3, a multifunctional adaptor molecule which mediates protein-protein interactions (Aducci et al, 2002). As part of this thesis, we performed experiments on the human parasite *P. falciparum* to assess whether recruitment of components of the erythrocyte skeleton is a conserved feature in *Plasmodia*. *Plasmodium falciparum* is the most dangerous specie affecting human beings as it exports virulence factors to the surface of iRBC leading to endothelial cytoadherence and obstruction of the vascular circulation. Several *P. falciparum* exported proteins, which bind and modify properties of the erythrocyte membrane skeleton, have been characterized (Haase and de Koning-Ward, 2010). This study was carried out in the laboratory directed by Prof. Catherine Braun-Breton in Montpellier. Her group studies the remodeling of the human erythrocyte after parasite infection, focusing on parasite proteins exported to the host erythrocyte and involved in the genesis of Maurer's clefts such as PfSBP1 (Saridaki et al, 2009) and RhopH complex which is also transferred to defined domains of the red blood cell cytoplasm, and possibly transiently associated with Maurer's clefts (Vincensini et al, 2008).

3.2.1 - Host dematin and adducin are internalized by *Plasmodium falciparum* during erythrocyte infection.

To establish whether *P. falciparum* infection causes mobilization of erythroid skeleton components and its successive recruitment inside the parasite, we performed double immunofluorescence assay on fixed *P. falciparum* infected erythrocytes using α-dematin immune serum in conjunction with antibodies specific for the PVM marker EXP1. Similarly to what observed in *P. berghei*, mobilization of the erythroid dematin and translocation of this host protein in parasite compartments was observed in all parasite blood stages (Figure 3.12).

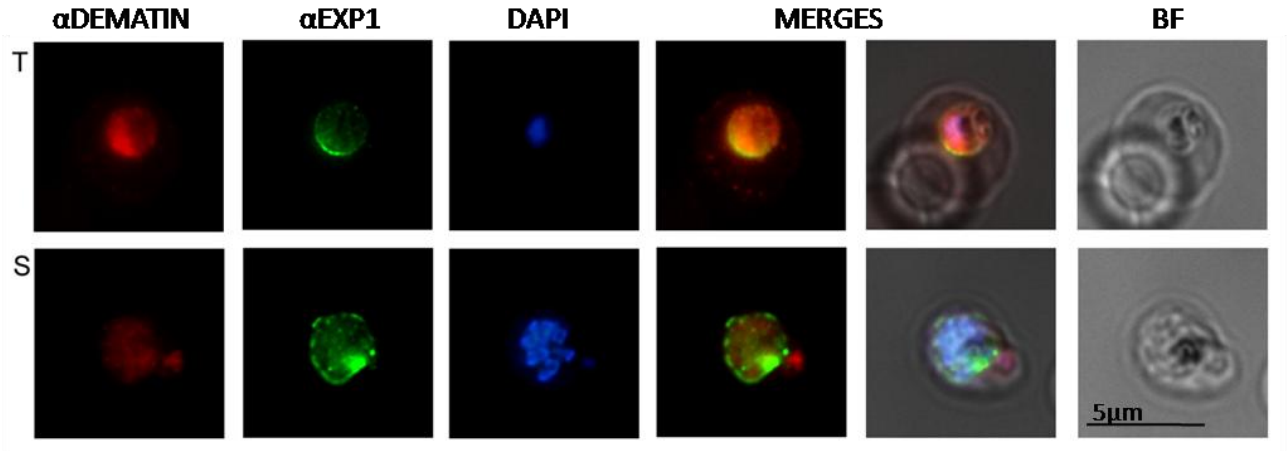


Figure 3.12. Double immunofluorescence analysis was performed on fixed erythrocytes infected with *P. falciparum* trophozoite (T) and schizont (S) using mouse α-dematin and rabbit α-EXP1. Nuclei were stained with DAPI (shown in pseudocolor blue). Bright field acquisitions (BF) are also reported.

As β-adducin is part of the junctional complex and participates with dematin to the maintenance of the actin cytoskeleton/plasma membrane interaction in the RBC (Chen et al, 2007; Khan et al, 2008), we also analyzed the intracellular localization of this protein in *P. falciparum* infected erythrocytes at different stages of parasite development using antibodies against the human β-adducin (α-β-adducin). To distinguish parasite compartments from the host cytosol, we take advantage of the immune serum specific for Pb14-3-3 which recognized also the *P. falciparum* ortholog. Results showed that also β-adducin was detected in parasite compartments with a punctuate pattern (Figure 3.13).

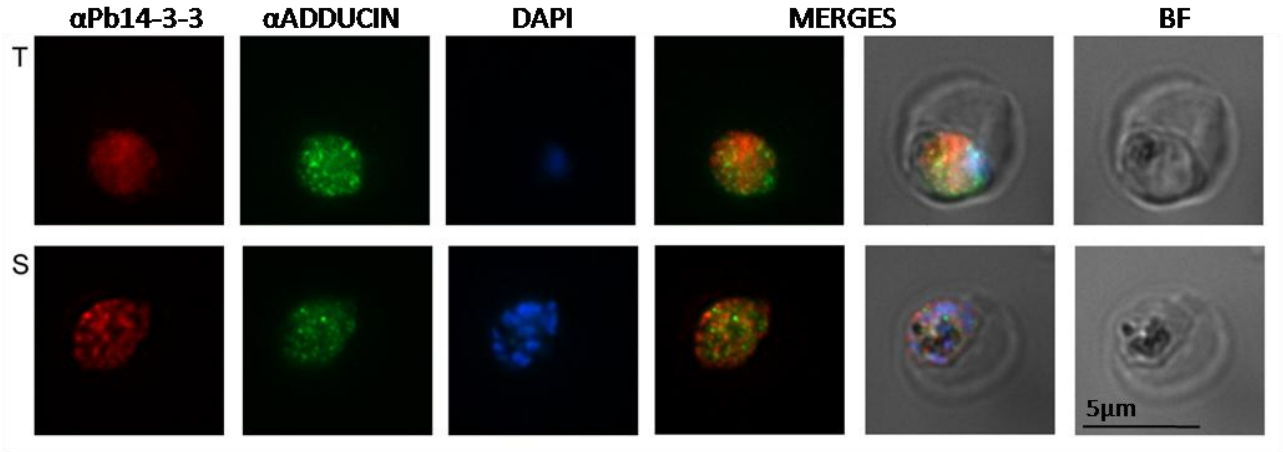


Figure 3.13. Double immunofluorescence analysis was performed on fixed erythrocytes infected with *P. falciparum* trophozoite (T) and schizont (S) using mouse α-Pb14-3-3 and rabbit α-βadducin. Nuclei were stained with DAPI (shown in pseudocolor blue). Bright field acquisitions (BF) are also reported.

To further analyze the localization and topology of internalized dematin and β-adducin in *P. falciparum* iRBC we applied a well-validated procedure (Jackson et al, 2007) to separate intact parasite surrounded by the parasitophorous vacuole (vacuolar parasites) and erythrocyte ghosts by selective permeabilization of purified iRBC membrane with Streptolysin-O. Vacuolar parasites were further fractionated in Triton-X100 soluble and insoluble fractions. Obtained samples were separated in SDS-PAGE and subjected to Western blot analysis using different antibodies. As shown (Figure 13.14), *Plasmodium* 14-3-3 was equally distributed in Triton-soluble and insoluble parasite fractions while it was not detected in ghosts prepared from iRBCs. Conversely, antibodies specific for human 14-3-3 isoforms, used as a control, recognized exclusively the ghost fraction.

As expected, β-adducin and dematin were detected in ghost and parasite preparations. In fractionated parasite extracts, they were both detected mainly in Triton-insoluble fractions suggesting that, inside the parasite, these host proteins may participate to large protein complexes.

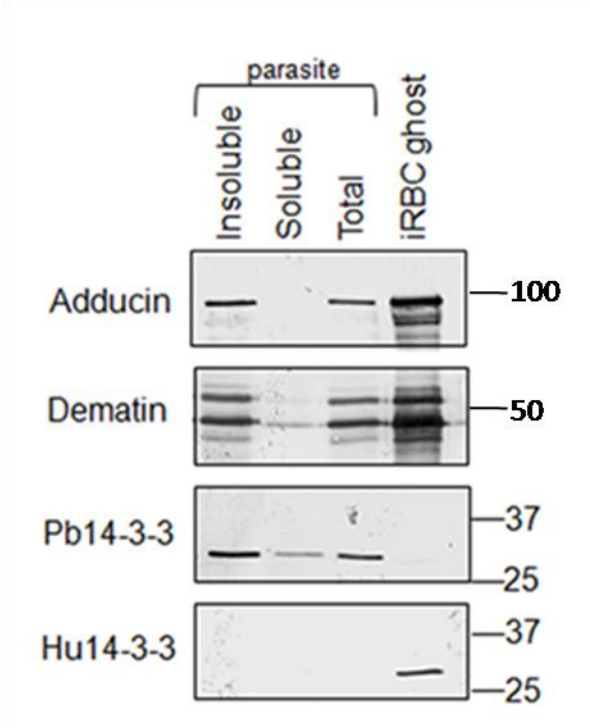


Figure 3.14. Vacuolar parasites (parasite) and iRBC membrane ghosts were prepared from SLO-treated iRBC. Unfractionated parasite (total) and soluble and insoluble parasite fractions after Triton-X100 treatment were tested in Western blot with α-dematin, α-βadducin, α-Pb14-3-3 and α-Hu14-3-3γ (hu14-3-3) serum, specific for human 14-3-3 isoforms.

To confirm the translocation of these host skeletal proteins to parasite compartments, we solubilized iRBCs and normal RBC with streptolisin-O, separated vacuolar parasites and ghosts from infected and non-infected RBC and then submitted the samples to Proteinase K

(PK) digestion. Treated samples were probed with different immune sera (Figure 3.15, panel A). In ghosts from infected and non-infected RBC dematin and β-adducin were both sensitive to PK treatment. In parasite preparation we expected protection from protease degradation only if dematin and β-adducin were inside the parasite. This held true for dematin; specific antibodies recognized, in fact, protein bands of the same size and relative intensity both in treated parasites and in control sample. In the case of β-adducin, we observed multiple bands in the PK treated sample at a lower molecular size compared to the main band observed in the control. This indicated that β-adducin was only partially protected and suggested a localization of the protein at the periphery of the parasite where it can be exposed to PK degradation. Since α-βadducin antibodies utilized in this study recognize the C-terminus of the protein, the N-terminus should face the host cell cytosol. This external localization of β-adducin was confirmed by the analysis of single stacks in immunolocalization studies conducted with an apotome-equipped fluorescence microscopy (Figure 3.15, panel B).

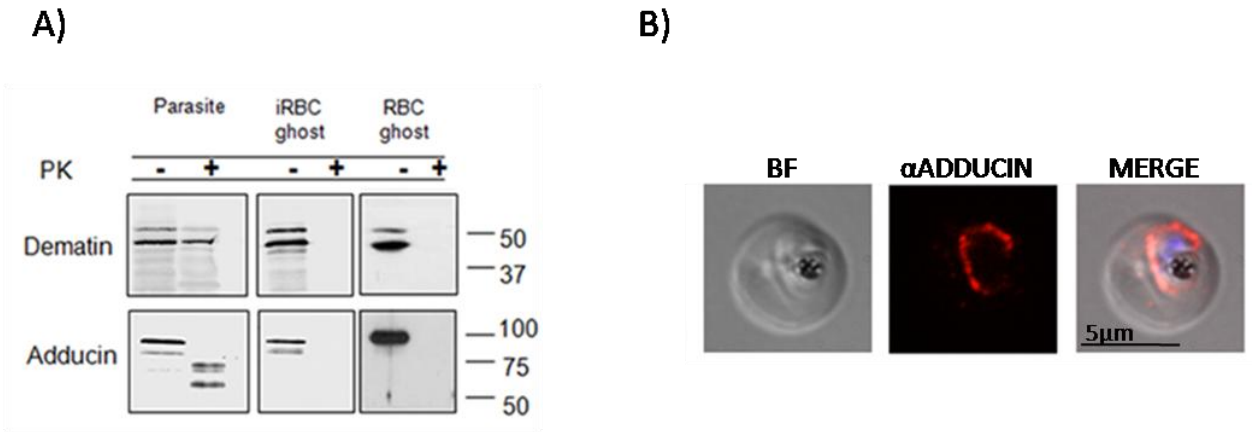


Figure 3.15. A) Ghosts from SLO-treated infected and normal RBCs and vacuolar parasites incubated with Protease K (+) and subjected to Western blot analysis using antibodies specific for dematin and β-adducin. Control samples (-) were run in parallel. B) Immunofluorescence with α-βadducin, on fixed *P. falciparum* trophozoite-infected erythrocytes. Nuclei were stained with DAPI (shown in pseudocolor blue). Bright field acquisitions (BF) are reported. Displayed micrographs were acquired with an apotome-equipped Axiovert 200 M and correspond to the single stack encompassing the center of the nucleus.

Internalization of host proteins does not include band 3, which is an abundant integral membrane protein, which connects the erythrocyte skeleton to the phospholipids bilayer via ankyrin. As shown in Figure 3.16, double immunofluorescence assay using specific antibodies showed that β-adducin is mobilized and recruited by the parasite while band 3 remains at the periphery of the erythrocyte. It would be interesting to extend the analysis to

other host skeletal proteins to establish whether recruitment upon parasite infection is limited to components of the junctional complex.

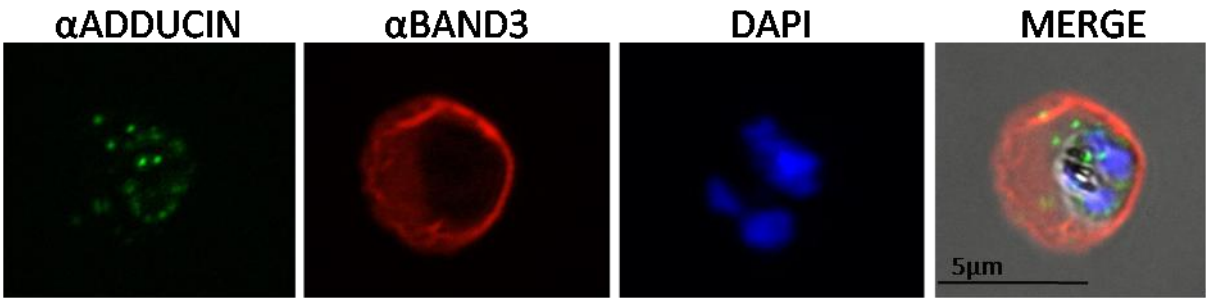


Figure 3.16. Double immunofluorescence analysis was performed on fixed erythrocytes infected with *P. falciparum* using rabbit α-βadducin and mouse α-band3. Nuclei were stained with DAPI (shown in pseudocolor blue).

We showed above that β-adducin remained at the periphery of the parasite while dematin is entirely internalized. We asked whether recruitment of dematin might occur via endocytic pathway, a well-described route through which host proteins, mainly represented by hemoglobin, are internalized during parasite growth. Endocytic vesicles release their content to a digestive food vacuole (FV), readily detected by light microscopy for the presence of large haemozoin crystals derived by the heme detoxification mechanism. In immunofluorescence FV can be identifiable using antibodies against the *P. falciparum* CRT protein, the Chloroquine Resistance Transporter, which localizes to the membrane of the parasite's internal digestive vacuole (Fidock et al. 2000). To ascertain whether dematin could be internalized through this pathway, FV formation and dematin internalization was followed in IFA experiments. Results clearly showed that dematin does not co-localize with the FV marker CRT at any stage of *P. falciparum* development (Figure 3.17). This suggested that a distinct pathway might regulate host skeletal protein entry and localization within the parasite.

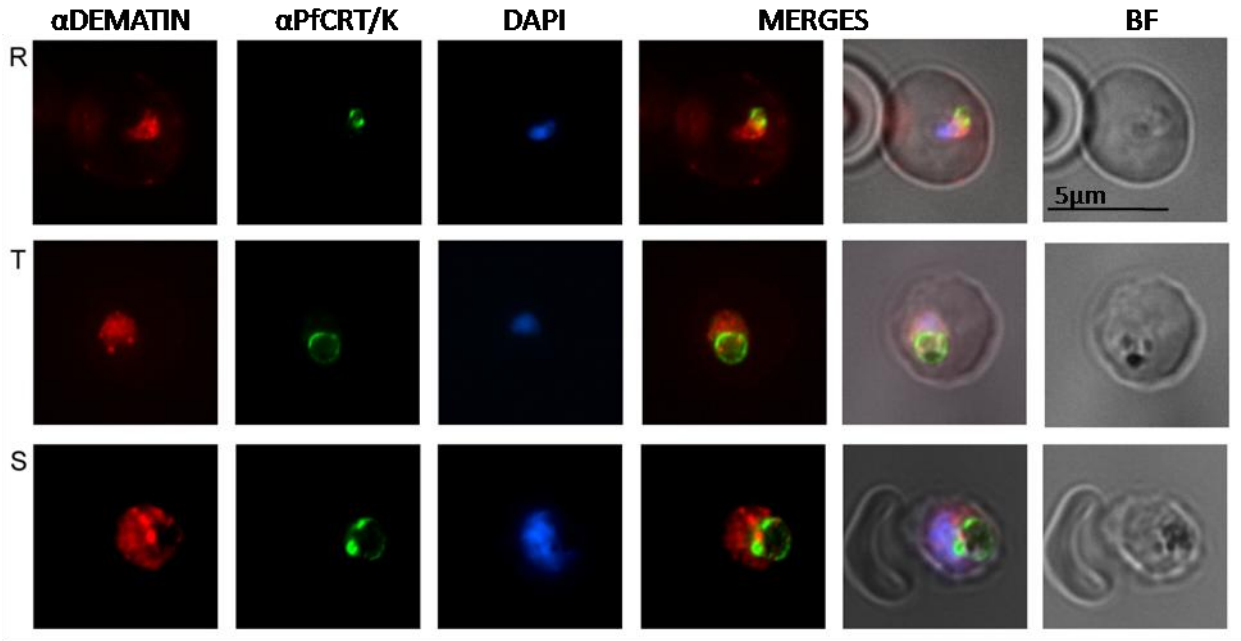


Figure 3.17. Double immunofluorescence analysis was performed on fixed erythrocytes infected with *P. falciparum* ring (R), trophozoite (T) and schizont (S) using mouse α-dematin, and rabbit α-PfPCRT/K, that marks the food vacuole. Nuclei were stained with DAPI (shown in pseudocolor blue), bright field acquisitions (BF) were also reported.

3.2.2 – Discussion

In this study we showed that the actin-binding and -bundling proteins dematin and β-adducin, components of the spectrin-based erythrocyte membrane skeleton, are both mobilized, after *Plasmodium falciparum* infection, and redirected to parasite compartments.

The RBC membrane owes its remarkable deformability and durability to the membrane skeleton. The erythrocyte skeleton is anchored to the phospholipids bilayer through two major protein bridges: the one connects the integral membrane protein band 3 to spectrin via ankyrin, while the other involves the junctional complex, which connects the tail end of spectrin to short actin protofilaments (Lux and Palek. 1995). Protein 4.1R, dematin, and adducin are components of the junctional complex (Derick et al. 1992). Protein 4.1R, p55 and the transmembrane glycophorin C, form a well-characterized ternary complex, which tethers the junctional complex to the plasma membrane (Marfatia et al. 1994). More recent studies demonstrated that the membrane receptors glucose transporter-1 GLUT1 (Khan et al. 2008) directly bind to dematin and/or adducin providing alternate linkage of spectrin-actin junctions to the erythrocyte plasma membrane (Khan et al. 2008). This suggested that the two actin-binding cytoskeletal proteins could perform a function that is similar to the vertical interactions of protein 4.1R.

We showed in immunolocalization studies on fixed *P. falciparum* infected erythrocytes that dematin and β-adducin specific fluorescence are associated with parasite compartments. In particular, β-adducin localized at the parasite periphery while dematin was mainly detected inside the parasite. In *P. falciparum* extracts both skeletal proteins were recovered in Triton-insoluble fractions suggesting that inside the parasite they are incorporated in large protein complexes. Topology of the internalized host proteins was established through PK treatment of fractionated infected and normal erythrocytes. We showed that dematin and β-adducin associated with ghosts were sensitive to PK digestion; while dematin associated with parasite compartment was protected indicating that it is fully internalized by the parasite. β-Adducin was in part affected by PK treatment as we detected multiple bands at a molecular weight lower than the main band observed in the control sample. This indicated that the recruited protein localizes at the periphery of the parasite where it can be exposed to PK degradation. Since α-βadducin antibodies utilized in this study recognize the C-terminus of the protein, the N-terminus should face the host cell cytosol.

One possible route for dematin internalization could be via membrane invaginations (cytostomes) and subsequent formation of endocytic vesicles. This is a well-characterized pathway through which erythrocyte cytosol and hemoglobin are internalized and routed to the digestive vacuole (FV). We verified this hypothesis through double immunofluorescence using antibodies specific for dematin in conjunction with antibodies against the *P. falciparum* CRT protein (Fidock et al. 2000) that localizes at the FV membrane. Our results clearly showed that the recruited dematin does not enter the endocytic pathway at any stage of parasite development.

To date host membrane proteins shown to enter the parasitophorous vacuole are localized in DRMs (Haldar et al. 2001). Most probably, their uptake occurs at the parasite entry and vacuole formation. However, the role of these recruited proteins in *Plasmodium* development has been poorly investigated. It was shown, in the case of aquaporin 3 (Bietz et al. 2009) that this host protein is not accumulated during parasite growth but its recruitment is confined to the early stages of invasion. At difference, we observed that, in early stages of infection, dematin and adducin specific fluorescence was mainly detected at the periphery of the infected erythrocytes, similarly to that observed in uninfected erythrocytes. With the progression of the infection, we observed an increase of the specific fluorescence associated with parasite compartments. This suggests that depletion of dematin and adducin from the junctional complex and their accumulation to parasite sites is a progressive process. Despite the two host proteins are routed to different subcellular compartments they were both

recovered in Triton-insoluble parasite fractions suggesting that they are incorporated in large and distinct protein complexes. In conclusion, this study demonstrated that the coordinate remodeling of the erythrocyte skeleton not only includes the export and binding of parasite proteins but also depletion of resident components of the host junctional complex and their recruitment by the parasite.

4. CONCLUSIONS and PERSPECTIVES

Malaria, caused by intracellular parasites of the genus *Plasmodium,* is a worldwide problem for human health. In the vertebrate host, *Plasmodium* grows and multiplies in the erythrocyte, a terminally differentiated cell that completely lacks intracellular organelles. The success of parasite infection depends on its ability to re-organize the host cell surface and cytosol during the early stages of development by *de novo* generation of membrane compartments and organelles, which sustain protein trafficking within the infected erythrocyte. The parasitophorous vacuole, in which *Plasmodium* resides after invasion, is the site where proteins routed to the erythrocyte cytosol are sorted (Lopez-Estrano et al. 2003). The Maurer's clefts, identified in the cytosol of the RBCs infected by the human parasite *P. falciparum*, are another set of structures characterized by extended tanks surrounded by a membrane (Taraschi et al. 2001). They act as secretory organelles that concentrate virulence proteins and deliver them to the host RBC membrane (Lanzer et al. 2006). Maurer's clefts have a central role in protein sorting and are important for the assembly of 'knobs', electron dense structures underlying the erythrocyte membrane involved in cytoadherence of the infected host cells to the vascular endothelium, thus contributing to the severe pathogenesis of *P. falciparum* (Sam-Yellowe. 2009). A number of *P. falciparum* proteins exported beyond the PVM bring about the coordinate remodeling of the spectrin-based erythrocyte membrane skeleton (Maier et al 2009). One of the best-characterized examples is the Knob-Associated Histidine-Rich Protein (KAHRP), which is a major component of knobs, conical protrusions on the cytoplasmic side of the host plasma membrane. Multimeric clusters of KAHRP associate with spectrin and actin and act as attachment point in cytoadherence. Pf332 protein, the mature-parasite-infected erythrocyte surface antigen (MESA) and the erythrocyte membrane protein 3 (PfEMP3) are exported parasite proteins, which interact with the erythrocyte skeleton and contribute to increase its rigidity. Moreover, a recent reverse genetic screen identified additional proteins, which influence erythrocyte deformability, most probably by acting directly or indirectly on erythrocyte skeleton (Maier et al 2008).
In this study we focused in:

1 - *Defining timing of expression and motifs required in for the export of three members of the conserved Plasmodium family SEP/ETRAMP, in the rodent malaria model P. berghei:*

sep/etramp family encodes small (13-16 KDa) integral membrane proteins characterized by the presence of a predicted signal peptide, a hydrophobic region and a highly charged C-terminal region. Previous work (Spielmann et al. 2003) carried out on 4 members of the *P. falciparum* family showed that the ETRAMP2, ETRAMP10.1, ETRAMP4 and ETRAMP10.2 localize at the PVM of parasite blood stages, where they form domains distinct from those formed by the PVM resident integral protein EXP-1.

Other studies identified members of the *P, falciparum* family at the PVM and at the periphery of the host erythrocyte: ETRAMP14.1 was found associated with the Maurer's clefts (Birago et al. 2003); while ETRAMP10.3 was localized in vesicular structures at the periphery of *P. falciparum* infected erythrocytes (MacKellar et al. 2010).

Two recent reports detected ETRAMP10.3 (MacKellar et al. 2010) and ETRAMP5 (Vignali et al. 2009) at the PVM of *Plasmodium* liver stages.

Plasmodium berghei is a valid model to study SEP/ETRAMP protein family, since it is represented by only three members, SEP1-3. Moreover, the encoding genes share the upstream regulatory region and most of the coding region including the predicted signal peptide and the hydrophobic region. They differ only in the C-terminal portion of the proteins and in the 3'UTR.

Immunolocalization experiments showed that the *P. berghei* SEP proteins are routed to the PVM but two of them, SEP2 and 3 are also exported to the host cell, in vesicular structures, which remind the Maurer's clefts of *P. falciparum*.

SEP/ETRAMPs do not contain a PEXEL motif (Marti et al. 2004; Hiller et al. 2004), a short consensus sequence R/KxLxE/Q/D (where x is any aminoacid) which is needed to export parasite proteins beyond the PVM to the host cell. In order to identify motifs required for the correct localization of these proteins we generated transgenic *P. berghei* lines, which contain the fluorescent reporter GFP fused with different portions of the common coding region. The chimeric proteins were expressed under the control of the conserved upstream regulatory region and the downstream gene-specific 3'UTRs. We observed that, as already described for the *P.falciparum* protein RESA (Rug et al. 2004), the signal peptide region is necessary and sufficient to drive the GFP to the parasitophorous vacuole, while the inclusion of the transmembrane domain is required for the GFP to be exported to the host compartment. A third element, which seems to have a role in subcellular localization of the encoded proteins is the specific 3'UTR. In fact we observed that the chimeric protein, which includes signal peptide and transmembrane domain, is translocated beyond the PVM when the transfection construct contains the 3'UTRs specific for *Pbsep*2 and 3, while it remains at the PVM when

the construct contains the 3'UTR specific for *sep*1. We can speculate that the downstream regulatory regions may direct the specific mRNAs to different compartments of the ER, thus deciding the final destination of the encoded protein.

PbSEP2 is extremely abundant in gametocytes suggesting a specific role of this protein in parasite sexual stages. PbSEP2 is in fact expressed at a very high level in all mosquito stages, including salivary gland sporozoites while PbSEP1 and 3 are expressed in ookinetes and at a very low level in sporozoites.

Future experiments will be planned to understand how SEPs may contribute to the organization of different secretory compartments. A structural role at the PVM of these proteins is suggested by the fact that they compartmentalize forming omo-complexes (Spielmann et al. 2006). Immune-precipitation assays aimed at identifying possible interactors of SEPs in fractionated iRBCs might provide further hints on the potential role of these proteins.

Since PbSEP2 is very abundant in salivary gland sporozoites, we planned to use the transgenic line expressing SEP2 fused with a fluorescent reporter to analyze subcellular localization in liver stages. Inhibition assays using α-SEP2 specific immune sera to assess a possible role of this protein in hepatocyte invasion will also be carried out.

2 - *Establishing subcellular localization of dematin and adducin, components of the eryhrocyte spectrin-based membrane skeleton, in the course of P. falciparum infection:*

The second part of the thesis focused on subcellular localization of the host dematin and β-adducin in *P. falciparum* iRBCs. In normal red blood cells these two proteins interact with actin at the spectrin-based membrane skeleton of the erythrocyte participating to the formation and dynamics of the junctional complex.

Recent data demonstrated that host cell molecules are of crucial importance in key steps of parasite development. Erythrocyte calpain-1 is required *in vitro* for egress of *P. falciparum* merozoites from the infected erythrocyte (Chandramohanadas et al. 2009) while *de novo* polymerization of host actin is required for entry of *Toxoplasma gondii* tachyzoites and *Plasmodium berghei* sporozoites (Gonzalez et al. 2009).

Immunolocalization studies allowed us to establish that, during *P. falciparum* infection, dematin and adducin are both mobilized and redirected to parasite compartments. Topological studies on fractionated iRBCs suggested that dematin is completely internalized while β-adducin remains at the surface of the parasite, partially exposed to the erytrocyte cytosol.

Cell phenotype associated to depletion of host dematin and β-adducin might be inferred by extensive studies on transgenic mice knocked out for these genes. A limited phenotypic effect on erythrocyte shape and membrane stability was observed in mice knocked out for either dematin "headpiece" domain (Khanna et al. 2002) or β-adducin (Muro et al. 2009) suggesting that these proteins serve redundant functions at the junctional complex. On the other hand, double knockout mutant mice display severe defects in erythrocyte shape, membrane instability and hemolysis (Chen et al. 2007). In view of that, we speculate that depletion of these junctional components, after the parasite infection, may determine a temporary instability of the spectrin-actin skeleton and membrane cohesion thus providing space for new interactions based on parasite proteins, which may stabilize and confer novel properties to the erythrocyte membrane skeleton.

Further experiments will be planned to understand whether phosphorylation at specific sites, triggered by *Plasmodium* entry, might be at the basis of the displacement of dematin and β-adducin from the erythrocyte membrane skeleton. Immunolocalization experiments will be performed to investigate whether additional components of the junctional complex are recruited by the parasite. Immunoprecipitation assays will be also attempted to identify parasite/host molecules, interacting with internalised dematin and β-adducin.

In conclusion our results suggest that the coordinated remodeling of the erythrocyte skeleton not only includes the export and binding of parasite proteins but also the depletion of resident components of the host junctional complex and their recruitment by the parasite.

Understanding the role of key erythrocyte molecules may contribute to design strategies that prevent a cascade of events leading to the host cell remodeling and/or parasite development during malaria infection.

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