Understanding the Role of Plasmodium falciparum VAMP8 SNARE Homologue

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UNDERSTANDING THE ROLE OF PLASMODIUM FALCIPARUM VAMP8 SNARE HOMOLOGUE

by

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A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Molecular Biology and Microbiology in the Burnett School of Biomedical Sciences and in the Burnett Honors College at the University of Central Florida Orlando, Florida

Fall Term 2013

Thesis Chair: Dr. Debopam Chakrabarti
ABSTRACT

Malaria is one of the worlds most deadly infectious diseases and results in almost a million deaths each year, largely in children under the age of five in Sub-Saharan Africa. Outside Africa, malaria is responsible for a large number of cases in the Amazon rainforest of Brazil, Middle East, and in some areas of Asia [37]. According to the World Health Organization, there was an estimated 655,000 deaths from malaria in 2012. Malaria is caused by a eukaryotic Apicomplexan parasite, Plasmodium, which has three distinct life cycles occurring in the midgut of the female Anopheles mosquito, the liver of the human host, and human erythrocytes.

When the parasite infects the erythrocyte, some induced cell host modifications are made in order to accommodate growth. During its intra-erythrocytic life cycle, the malaria parasite traffics numerous proteins to a set of unique destinations within its own plasma membrane including the digestive vacuole, the apicoplast, rhoptries, and micronemes.

Vesicular transport is an essential process in eukaryotic cells. This coordinated process is responsible for moving thousands of proteins between compartments within the cell. Essential to the targeting and fusion of protein transport vesicles in eukaryotes are SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptors), a family of fusogenic proteins that are localized to distinct intracellular compartments [11]. Studies performed in our laboratory have identified 18 proteins putatively belonging to the PfSNARE family [2]. To date the exact role of PfSNAREs in the unique trafficking pathways of malaria is undetermined. Of particular interest to our study is PfVAMP8. In model eukaryotic organisms, VAMP8 containing vesicles deliver cargo to lysosomes and are involved in endocytosis. The food vacuole of the parasite is very similar to that of lysosomes and is essential to parasite survival. The study aims to identify
the organelle(s) to which PfVAMP8 is localized and characterize membrane-association properties of this parasite’s R-SNARE protein.

We believe that PfVAMP8 would localize to unique compartments in the parasite protein network flow. An in depth understanding of its mechanisms and localizations could be a key in developing novel anti-malarials.

This study aims to identify the organelle(s) to which PfVAMP8 are localized, determine the trafficking determinants of this protein and determine this proteins’ expression and membrane association during the intra-erythrocytic stages of *Plasmodium falciparum*. Our immunofluorescence studies with known biological markers reveals that, PfVAMP8 passes through the endoplasmic reticulum, Golgi, and localizes to the food vacuole during trophozoite and schizont stage. Further characterization of the membrane association properties of the protein in this study reveals that PfVAMP8 is a soluble integral membrane protein with amphipathic characteristics.
ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest gratitude to my committee chair Dr. Debopam Chakrabarti for granting me the opportunity to work on this project and providing me with invaluable critical thinking skills, knowledge, and guidance throughout my education as an undergraduate researcher.

I am also very grateful to my committee members, Dr. Zixi Cheng and Dr. Dmitry Kolpashchikov for their insightful comments and suggestions that facilitated the completion of my work.

A special thank you goes to Dr. Jude Przybroski and Dr. Shadab Siddiqi, collaborators of our lab, whose suggestions helped with the progress of my project.

I would also like to thank all members of the Dr. Chakrabarti lab, especially Brittany Pease, for her constant support, friendship, and assistance.

Last but not least, I would like to thank my family who entered in this journey with me and never let me give up on my dreams. Thank you for supporting me and encouraging me to always dream big and work hard to achieve my goals.
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CHAPTER 1: INTRODUCTION

1.1 Hypothesis and Objectives

The objective of this study is to elucidate the molecular mechanisms of SNARE-mediated vesicle trafficking pathways in *Plasmodium falciparum*. The central hypothesis in this study is that *Plasmodium* R-SNAREs residing on transport vesicles will function at novel trafficking routes in parasitized erythrocytes. Cloning of GFP-tagged PfVAMP constructs was performed in our laboratory in order to express the GFP-tagged proteins under endogenous levels. GFP-tagged constructs were created, sequence confirmed, and transfected into the Dd2 cell culture line, which is resistant to quinolone, and where further biological analysis can be performed. We will achieve our objective by determining the subcellular distribution and dynamics of the *P. falciparum* R-SNARE VAMP8 and analyzing co-localization of PfVAMP8 by pursuing two specific aims.

Our first aim is to characterize PfVAMP8 expression patterns. This first objective aims to assess subcellular distribution of the Pf R-SNARE by immunofluorescence assay and GFP-chimera localization. This will identify the cellular components in which PfVAMP8 is located with the use of known biological markers.

Our second aim is to analyze membrane-association properties of the Pf R-SNARE by Triton X-114 phase partitioning. The samples obtained through differential centrifugation will be further analyzed by Western Blot analysis with an anti-PfVAMP8 antibody.
1.2 Literature Review

1.2.1 Malaria and *Plasmodium falciparum*

Malaria is transmitted through the bite of an infected female Anopheles mosquito, which transmits *Plasmodium falciparum*, an apicomplexan parasite. This parasite, which has hundreds of different species, is a eukaryotic-single celled organism. Currently, only five species can infect humans [37]. These species are classified based on morphology, immunological response, drug response, and their geographical distribution into: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, and Plasmodium knowlesi* [37]. However, *Plasmodium falciparum* is the species responsible for the majority of malaria death cases due to the rupture of erythrocytes. This process results in fever, severe anemia followed by coma, and death.

The disease has a broad distribution, especially in the tropics and subtropics where the weather has an effect on the mosquito’s abundance [30] including countries of sub-Saharan Africa, India, Brazil, Afghanistan, Sri Lanka, Thailand, Indonesia, Vietnam, Cambodia, and China [37].

Worldwide, the parasite has gained resistance to the majority of the drugs that are widely available on the market [2]; as a result, malaria is causing a significant impact on the global economy. The lack of vaccines, limited number of anti-malarials and low investments on malaria research has cost Africa alone 12 billion dollars annually [37]. Malaria endemic countries are trying to reduce the spread of malaria vectors, but the rapid spread of drug-resistance and the reduced amount of tools to treat the disease are exasperating the problem. In order to create new
drugs and vaccines to eradicate malaria, it is crucial for researchers to gather more information about the parasite’s biology and how it interacts with the mosquito and its cell host.
Figure 1: Distribution of malaria in the world. From Health Center Diseases Information, Web.
1.2.2 Life Cycle of Plasmodium falciparum

The life cycle of *Plasmodium falciparum* constitutes one of the most intriguing and complex life cycles compared to other organisms since it involves sexual and asexual phases in two different hosts – a human and a female Anopheles mosquito [1, 17]. Since the erythrocytes are an inactive environment devoid from nucleus and protein machineries, malaria parasites require specialized protein expression produced on its own in order to survive inside the host’s cells and evade immune responses [37]. The life cycle of the malaria parasite can be divided into three stages: tissue schizogony, erythrocytic schizogony, and sporogony.

1.2.2.1 Tissue Schizogony

The first stage starts when the mosquito bites the human host and releases sporozoites that are present in its salivary gland. Once in the bloodstream, sporozoites are directed to the liver where they will invade hepatocyte cells and undergo asexual replication. After thousands of rounds of replications, the parasite induces the death of liver cells and release merozoite-filled vesicles (merosomes), to the host’s bloodstream [1].

1.2.2.2 Erythrocytic Schizogony

Merozoites play a key role in the erythrocytic schizogony stage of the parasite. They are released following the rupture of merosomes and they invade red blood cells through a four step process as follows: 1) recognition and reversible attachment of the merozoite to the erythrocyte
membrane 2) formation of the parasitophorous vacuole 3) invagination of the erythrocyte membrane around the merozoite and 4) resealing of the parasitophorous vacuole and erythrocyte membranes after completion of merozoite invasion [3, 8].

After insertion into the RBC, the parasite goes through three different morphological stages: ring, trophozoite, and schizont. During the ring stage, the parasite starts to occupy a large portion of the cell by engulfing the host’s cytoplasm in a process occurring 16-24 hours after red blood cell infection. The next stage, the trophozoite stage, occurs 36 hours after infection and is characterized by high metabolism and breakdown of hemoglobin, which fulfills the need of amino acids for the parasites. The metabolite resultant from the digestion of hemoglobin is a heme group that is toxic to the cell. The food vacuole is capable of transforming this heme group into hemozoin, which is stored inside the parasite’s food vacuole in the form of a crystal. The end of the trophozoite stage is followed by schizogony, where multiple rounds of DNA replication occur without cytokinesis; therefore, the schizont is characterized as a multinucleated cell. The multiple rounds of replication that occur inside the schizont release new merozoites into the bloodstream in order to infect new erythrocytes. This process coincides with a sharp rise of the body’s temperature. In *P. falciparum*, the intraerythrocytic cycle repeats every 48 hours [1].

Since the invasion of the *P. falciparum* parasites requires a complex series of specific molecular interactions, it is regarded as an important target for interventions to combat malaria [17].
1.2.2.3 Sporogony

Due to factors and stimuli not yet understood, some merozoites do not re-infect RBCs and instead enter gametocytogenesis to form micro- and macrogametocytes. These gametes present in the human blood are transmitted to another anopheles mosquito that bites an infected host. This marks the initial sexual stage of the parasite. Inside the mosquito, the male gametocyte fertilizes the female gametocyte forming an ookinite, which will be transported to the midgut of the mosquito [17]. Upon rupture of the oocyst, the released sporozoites migrate to the salivary gland of the mosquito where they remain awaiting to be transferred as sporozoites to the vertebrate host during the next blood meal.
Figure 2: Diagram of the malaria parasite *Plasmodium falciparum*’s life cycle. From Cowman et al., 2006.
1.2.3 Host Cell Modifications

Life inside red blood cells (RBCs) offers the parasite protection from the host’s immune system. However, to survive and replicate inside RBCs, the parasite has to export proteins that interact with and modify the properties of the host red blood cells. It appears that the parasite establishes a system within the red blood cell cytosol that allows the correct trafficking of parasite proteins to their final cellular destinations via vesicular trafficking. After infecting RBCs, the parasite induces host cell modifications that are vital to the growth and the survival of the parasite. As the parasite grows within the confines of a parasitophorous vacuole, some dramatic alterations start to take place in order to facilitate the acquisition of nutrients from the extracellular environment that are not provided by the host cell. These modifications contribute to the symptoms of malaria and avoid clearance by the spleen [29].

Among the various host cell modifications that take place, the formation of the parasitophorous vacuole (PV) and the parasitophorous vacuole membrane (PVM) constitutes the first event. There were some debates to decide whether the PV is derived from the RBCs cell surface or from the parasites’ merozoites. Analysis of electron microscopy suggests that the PV is derived from structures present in merozoites with the following rationale: (1) the PVM is more resistant to lysis by saponin when compared to the RBCs, (2) the rhoptries and micronemes from the apical end of the parasite contain abundant amounts of lipid bodies to form a bilayer with the area of the PVM, (3) when fluorescent lipid precursors from the parasite are incorporated into lipids, fluorescent signals can be visualized in the PVM following invasion, and (4) the PVM is devoid of RBCs major cell membrane proteins [13].
Another host cell modification occurs when the parasite creates surface protrusions, called knobs, on the surface of the RBC [32]. The main components of these knobs are histidine-rich proteins (KAHRP), which alter mechanical properties of the parasite and are the site of attachment for the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is a protein responsible for the adhesion of infected RBCs to endothelial cells [28,32] causing vascular problems in the human host and some of the symptoms of the disease.

Interestingly, as the parasite matures, the ER creates a mesh like network throughout the cytosol [17]. The ER of *P. falciparum* has been shown to be involved in many cellular processes including calcium storage, phospholipid synthesis, and trafficking of proteins [37]. Similarly to the ER, the Golgi becomes more structured as the parasite matures.

During the trophozoite stage, extensions of the parasitophorous vacuole to the RBC cytosol begin to form a network of tubular structures called the tubovesicular network (TVN). The TVN is believed to mediate molecular transport and play a key role in nutrient import [39].

Another organelle formed during host cell modifications is the Maurer’s cleft (MC), single membrane structures in the RBCs’ cytoplasm [41]. The role of the Maurer’s cleft is currently under debate but various theories have already been proposed [31]. The currently accepted model suggests that the Maurer’s cleft acts as an intermediate in protein transport from the parasite to the RBC cell surface by delivering proteins in packed vesicles [41].

*P. falciparum* has a single mitochondria during the intra-erythrocytic stages. The shape of the mitochondria varies as the life cycle of the parasite progresses. In the schizont stage, the mitochondria divide and each new merozoite receives a new organelle. The functions of the
mitochondria in *P. falciparum* include: electron transport, coordination of pyrimidine biosynthesis and oxidative phosphorylation [26].

In the ring stages, the apicoplast appears bound to the mitochondrion [25]; however, throughout the life cycle of the parasite, the apicoplast transforms its structure from an elongated shape to a branched shape [36].

The food vacuole (FV), also known as digestive vacuole, is critical for the survival of the malaria parasite. The organelle resembles a lysosome and contains many proteins involved in the acidic digestion of host cell metabolites [27]. Unlike other organelles, the FV is not present in all stages of intra-erythrocytic development and is discarded at the end of each cycle. The isolation of the parasite within the confines of the PV requires the need of permeability pathways. However, if the RBC becomes too permeable, the high concentrations of hemoglobin would encourage fluid to move inside the cell lysing the infected RBC. To prevent this event from happening, the parasite digests a large portion of hemoglobin in order to make its own proteins [12]. The remaining heme is toxic to the cell; as a result, the proteins present in the FV convert the heme to an inner crystal or malarial pigments named hemozoin [27].
Figure 3: *Plasmodium* resides in a membrane vacuole within an erythrocyte. From Osbourne, 2010.
CHAPTER 2: SNAREs

2.1 Vesicular trafficking and the SNARE hypothesis

Transport vesicles play a central role in the traffic of molecules between different compartments of the secretory pathways; thus, it is a major cellular activity responsible for molecular targeting and trafficking with minimal errors. Specificity and selectivity are key to maintain the functional organization of a cell and its understanding can elucidate molecular trafficking pathways in *P. falciparum* [9].

During the formation of a vesicle, cargo vesicles start to assemble at the surface of a donor compartment. Then, a set of coating proteins attach around the surface of the circularized vesicle. This process is followed by scission; that is, a complete detachment of the vesicle from the donor membrane. Uncoating and recycling of the coat proteins occurs and is followed by tethering with the aid of rab proteins followed by fusion of the cargo vesicles to the target membrane (Figure 4).

COPI and COPII vesicles traffic between the ER and the Golgi apparatus. COPI vesicles traffic primarily from the Golgi to the ER, and COPII, from the ER to the Golgi. Coat assembly and vesicle budding also requires GTPase, adaptor proteins (i.e., AP-2 and Beta-arrestin), and SNARE proteins [34].

Analysis of the proteins involved in vesicle fusion, led Rothman to propose a model called the SNARE hypothesis [9]. In this hypothesis, vesicle fusion is mediated by interactions between specific proteins called SNAREs that are present on both the vesicle and on the target membranes; these proteins are key components that drive membrane fusion.
Figure 4: Steps of vesicle budding and fusion. From Stolle et al., 2005.
2.2 The SNARE superfamily

Since parasites are eukaryotic cells, they need to transport synthesized proteins to their appropriate location. The classical pathway for the secretion of proteins in eukaryotic cells, called vesicular trafficking, requires a series of steps involving budding and fusion of small vesicles. Proteins are transported to the endoplasmic reticulum and then transit through the Golgi apparatus before being released to their specific destinations throughout and out of the cell [11]. Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are key elements in membrane fusion; they seem to mediate all the trafficking steps of the secretory pathway [11].

SNAREs are responsible for mediating the fusion of transport vesicles into the target membrane. It is a superfamily of small proteins with a simple structure characterized by at least one SNARE motif (a conserved stretch of 60-70 amino acids [2]), a transmembrane (TM) domain or a lipid modification at the C-terminus domain, and a N-terminus oriented towards the cytoplasm [2]. Moreover, vesicle-associated membrane proteins (VAMPs) can also have a longin or brevin domain at the N-terminus [23]. Our protein of study is characterized as a longin VAMP.

SNAREs were initially referred to as v- or t-SNAREs based on their localization. Usually, one SNARE protein, called a v-SNARE, is located exclusively on the transport vesicle, whereas two or more t-SNAREs are located on the target membrane [23]. V- and t-SNAREs are able to recognize each other assembling a stable SNARE complex that allows the vesicle to get closer to the target membrane and mediate assembly and target fusion [43]. This process necessitates specificity and interaction between the hydrophobic repeats present on a SNARE
motif since they will serve as a point of contacts between interacting SNAREs. Four SNARE motifs form a complex called a SNARE-pin [40], which is a highly stable quaternary structure.

The hydrophobic repeats are numbered from -7 to +8 and the “0” layer is highly conserved [2]. Depending on the classification of the “0” layer, SNAREs are structurally classified into R- or Q- types [40] (Figure 6).

Q-SNAREs are subdivided as Qa-, Qb-, or Qc-SNAREs [23]. It is believed that functional SNARE complexes must contain one member of each class (R, Qa,Qb,Qc) for fusion, with three SNARES located on one membrane and one SNARE on a second membrane, following a “3Q:1R” rule [2].

Bioinformatic analysis of the P. falciparum genome in our laboratory revealed 18 SNARE-like proteins [2]. These proteins, depending on the number of SNARE motifs, the sequence of the SNARE motifs, and the type of flanking domains, can be classified into five main groups: the membrin-like, Bet1-like, VAMP-like, Syntaxin 5-like, and the Plasmodium specific subfamilies [2]. The vesicle-associated membrane protein, PfVAMP 8, has not yet been characterized. It contains some unique characteristics that are uncommon to the eukaryotic homologue highlighting the need for an in depth characterization of this protein.
Figure 5: Structure features of PfSNAREs. From Ayong L. et al., 2007.
Figure 6: Sequence Alignment of PfSNAREs. From Ayong L. et al., 2007.
2.3 Protein trafficking in *Plasmodium falciparum*

Since RBCs do not have a nucleus, they are not able to produce their own proteins; as a result, they are an attractive host for the parasite to establish its own machinery. In order to survive and replicate, the parasite needs to export a number of proteins that will either interact or modify the host cell membrane [8]. Studies show that the parasite exports about 5% of its genome-encoded proteins into the host cell cytosol [12].

The parasite has to export proteins beyond its own plasma membrane (PM) through the parasitophorous vacuole (PV), the parasitophorous vacuole membrane (PVM), and the RBC cytoplasm, until the proteins get to the host cell membrane [8]. Some of these proteins interact with the erythrocyte membrane while others are exposed to the surface of the RBC. The proteins that are exported are crucial to the pathogenicity of *P. falciparum*.

All proteins that were analyzed to date, and which are transported into the cytoplasm of the parasitized red blood cells, transverse the parasitophorous vacuole (PV) by fusion of the transport vesicles with the parasite plasma membrane [8]. Some proteins will remain in this compartment, such as the serine-rich antigen (SERA) family, whereas others, such as KHRP, will be directed outwards across the PVM [8]. It was suggested that proteins that are targeted to transit throughout the host cell cytosol, might be segregated into specialized secretory compartments within the parasite [8,20,21]. It is possible that PV-resident and forward-destined proteins might be separated into different compartments [8]. Recent electron microscopy studies revealed that the presence of double membrane bound vesicles with low density contents appear to bud from the ER and fuse with the parasite membrane. Following budding from the PV, these
vesicles might fuse with the PVM and bud with the RBC providing a possible mechanism of the export of vesicle proteins from the parasite [8].

Proteins destined to the ER, parasite PM, PV, PVM, and apical organelles seem to have a hydrophobic N-terminal signal sequence, a stretch of approximately 15 hydrophobic amino acids. However, proteins that are destined beyond the PVM, to the RBC cytosol, have a longer hydrophobic stretch of amino acids that is recessed from the N-terminus [8]. Nevertheless, not all proteins have a recessed hydrophobic region. Chaperone-like proteins have also been identified within the PV and play a key role in protein unfolding as a prerequisite for membrane translocation.

It appears that proteins destined to be exported from the parasite, arrive at the parasite plasma membrane and are exported into the vacuolar lumen by a one step process or a two-step process. In the latter case, there is a need for a protein channel to translocate the protein across the membrane and a pentapeptide, RxLxE/Q/D, towards the N terminus of the protein. This pentapeptide is known as the *Plasmodium* export element (PEXEL) [12]. PEXEL is responsible for directing proteins to locations beyond the PVM [12]. Some proteins that are exported from the parasite and enter the RBCs’ cytosol induce heterogeneous structures that are tethered to the erythrocyte membrane; these structures are called the Maurer’s cleft.
Figure 7: Trafficking Pathways in *Plasmodium falciparum*. From Cooke et al., 2004.
CHAPTER 3: MATERIALS AND METHODS

3.1 Parasite culture and isolation

Plasmodium falciparum GFP tagged cell lines were grown in human A+ red blood cells at 4% hematocrit in RPMI supplemented with 0.5% Albumax at 37°C in 5% CO₂, and 95% air. Cultures had their media changed daily and were split every 48 hours to maintain parasitemia levels around 10%. Parasites were released from infected erythrocytes by treatment with 0.05% saponin for 5 minutes. Pellets were washed three times in DPBS to remove all erythrocyte components.

3.2 Use of constructs for analysis of trafficking determinants

In order to study the trafficking determinants of our targeted protein, PfVAMP8, it was necessary to generate fluorescent-tagged fusion proteins. Plasmodium transfected constructs already created in our lab express N-terminal GFP-tagged proteins. The importance of the GFP-tagged proteins being located at the N-terminus relies on the fact that the C-terminus contains transmembrane domains; as a result, placing GFP-tagged proteins at this site might interfere with the proteins site of insertion.

The constructs obtained to further progress our studies were created by inserting the cDNA encoding the respective VAMP8 into a modified pDC-rep 20 transfection vector. The expression of the protein was under control of the promoter regions of PfVAMP8 (Figure 8).
Figure 8: Construct of PfVAMP8
3.3 Immunofluorescence assays and confocal microscopy

The subcellular localization of PfVAMP8-GFP was determined in transgenic parasites by immunofluorescence analysis through the utilization of the following antibodies: anti-PfSERP (parasitophorous vacuole marker), anti-PfErd-2 (Golgi marker), and anti-PfBip (ER marker).

Infected erythrocytes were fixed at 37° C for 30 minutes in a 4% paraformaldehyde/0.0075% gluteraldehyde solution followed by permeabilization in a solution of 0.1% Triton X-100 in PBS for 10 minutes at room temperature. The cells were incubated in 125 mM Glycine for 10 minutes at room temperature. Cells were blocked for one hour at room temperature in a 3% BSA/PBS solution. Primary antibodies were incubated with the cells in the blocking solution overnight at 4° C. The following dilutions were used: mouse-αGFP (1:75 dilution in a 200 microliters volume) and rabbit-αERD2 (1:75 dilution in a 200 microliters volume); mouse-αGFP (1:75 dilution in a 200 microliters volume) and rabbit-αBip (1:75 dilution in 200 microliters volume); and mouse-αGFP (1:75 dilution in a 200 microliters volume) and rabbit-αSERP (1:75 dilution in 200 microliters). On the next day, cells were washed three times in blocking solution, and then incubated with the appropriate secondary antibody for 2 hours at room temperature as follows: α mouse-GFP (1:500 dilution in a 500 microliters volume) and α rabbit- RFP Alexa Fluor-555 (1:500 dilution in a 500 microliters volume). Cells were washed three times in PBS and adhere to glass coverslips coated in 0.1 mg/ml Poly L- Lysine for 20 minutes. Coverslips were mounted on slides covered with 10 microliters of Dapi fluoromount. Cells were imaged using a laser scanning confocal microscope and the excitation/emission spectra settings were 543/555 For Alexa Fluor-555 conjugated secondary antibodies.
3.4 Biochemical fractionation: Analysis of membrane-association properties of PfVAMP8

3.4.1 By Freeze/thaw fractionation of soluble proteins from membrane proteins

*Plasmodium falciparum* 3D7 parasites were re-suspended in a TBS buffer (10mM Tris-HCl, pH 7.4 + 150 mM NaCl), containing a 1X protease inhibitor cocktail (Pierce). The cells were subjected to five cycles of freezing and thawing in liquid nitrogen (10 seconds) and five minutes at 37°C in a water bath. This cycle was followed by 15 seconds of sonication in order to release both the cytosolic and the luminal proteins. The disrupted cells were centrifuged at 100,000 x g for 1 hour at 4°C to separate the soluble proteins from the membrane-associated fractions. The pellet sample was then normalized with TBS buffer to the volume of the supernatant and equivalent volumes analyzed by western blotting.

3.4.2 Alkaline extraction of peripheral proteins from integral membrane proteins

A membrane pellet was prepared as above and the sample was diluted with TBS and centrifuged at 100,000 x g for 1 hour at 4°C. The pellet obtained was re-suspended in 1 ml of 0.1M Na₂CO₃, pH 11 and incubated on a rotary mixer for 30 minutes at 4°C. The suspension was centrifuged at 1000,00 x g for 1 hour at 4°C in order to separate the peripheral membrane proteins in the supernatant from the integral membrane proteins in the pellet. The resulting pellet was then normalized with Na₂CO₃ and 50 microliters of each sample volume was analyzed by western blot.
3.4.3 Phase separation of hydrophobic proteins from hydrophilic membrane proteins

Integral membrane fractions were solubilized using the Membrane Protein Extraction Reagent kit (Pierce). The pellet (0.164 g) was re-suspended in 150 microliters of Mem-Per reagent A and incubated at room temperature for 10 min. Then reagent D (a mixture of 300 microliters of Reagent C + 150 microliters of Reagent B) was added to the pellet for an incubation period of 30 minutes on ice vortexing every 5 minutes. The sample was then centrifuged at 10,000 x g for 3 minutes at 4°C. The supernatant was transferred to a new tube and incubated in a 37°C water bath for 20 minutes followed by centrifugation at 10,000 x g for 2 minutes at room temperature. Phase separation had to be performed as quickly as possible because the interface between the hydrophobic and hydrophilic layers slowly disappears at room temperature. 50 microliters of each sample was analyzed by western blot.
CHAPTER 4: RESULTS

4.1 Structure of PfVAMP8

Since human SNAREs have been extensively characterized, PfSNAREs were named based on their sequence homologies with the human SNAREs. The designated names were assigned to the parasite’s proteins by adding the prefix Pf to the corresponding human SNARE homologue [2]. The sequence of P. falciparum MAL13P1.16 has a high similarity to the human gene VAMP8 (approximately 31.5%); as a result, it was designated as PfVAMP8 [2].

As discussed previously, PfVAMP8 contains a single SNARE motif, an arginine residue at the “0” layer, and a single C-terminal hydrophobic domain. Contrarily to the human VAMP8, PfVAMP8 lacks a structured N-terminus and has a profilin-like longin domain. As a result, PfVAMP8 is classified as a longin VAMP (Figure 9).
Figure 9: Structure of PfVAMP8
4.2 Co-localization studies of PfVAMP8

Immunofluorescence analysis of infected erythrocytes with *P. falciparum* was performed in order to detect PfVAMP8 during all stages of the intra-erythrocytic life cycle. We did not detect expression of PfVAMP8 at ring stage in any of the immunofluorescence assays. However, in trophozoite and schizont stage, the localization with ER and Golgi is apparent until we detect an end co-localization of our protein of interest to the food vacuole. This suggests that PfVAMP8 may be transported through the parasite as the cell cycle progresses.
Figure 10: Co-localization of PfVAMP8 with the ER
Figure 11: Co-localization of PfVAMP8 with anti-PfErd2
Figure 12: Co-localization of PfVAMP8 with anti-PfSERP
Figure 13: Hypothesis of the trafficking pathways of PfVAMP8 within the red blood cells
4.3 Biochemical fractionation

A biochemical fractionation was performed with PfVAMP8 asynchronous parasite culture to analyze membrane-association properties. Parasite’s soluble membrane proteins were separated from membrane associated proteins by several rounds of freezing and thawing in liquid nitrogen and incubation in a 37° C water bath. Following separation of membrane associated proteins from soluble membrane proteins, the proteins were treated with sodium carbonate buffer at an alkaline pH to separate peripheral membrane proteins from integral membrane proteins. To further analyze the membrane-association properties of PfVAMP8, a membrane protein extraction kit was utilized to fractionate hydrophilic proteins from hydrophobic proteins.
Figure 14: Biochemical fractionation of PfVAMP8
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

*Plasmodium falciparum* protein trafficking pathways are unique among all eukaryotes. This intracellular parasite of human red blood cells target nuclear-encoded proteins to several unique destinations within its cytoplasm that include the micronemes, the rhoptries, various dense granules, the digestive food vacuole, and the apicoplast. Although very little is known about the molecular machineries that mediate protein trafficking in *P. falciparum*, intracellular transport of proteins is expected to occur via classical secretory and endocytic pathways mediated by vesicle budding and fusion events.

Essential to the targeting and fusion of protein transport vesicles in eukaryotes are SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptors), a family of fusogenic proteins. Distinct SNAREs localize to different intracellular compartments where they selectively interact with other SNARE members.

Studies in our laboratory identified that the presence of arginine at the ‘0’ layer is a characteristic of SNAREs called VAMPs (Vesicle-Associated Membrane Proteins), whereas glutamine at the ‘0’ layer position is a characteristic of syntaxins and synaptosome-associated protein (SNAP)-like SNAREs [2]. Depending on the presence of a profilin-like fold, VAMPs can be further divided into longins (long-VAMPs) or brevins (short-VAMPs) [2]. Variations within the SNARE motif leads to the subdivision of Q-SNAREs into Qa (Syntaxin), Qb (SNAP25N/Membrin-like), Qc (SNAP25C/Bet1-like) [2].
The exact roles of PfSNAREs in the trafficking pathways of malaria are unknown. We initially hypothesize that *P. falciparum* VAMP8, an R-SNARE, residing on transport vesicles would function at multiple transport pathways. For the analysis of the immunofluorescence assay, it was expected that the protein would be detected during all stages of the intra-erythrocytic life cycle of *P. falciparum*. As the parasite develops, PfVAMP8 should be detected moving away from the nuclei, passing through endoplasmic reticulum (ER), and finally ending at the Golgi.

Immunofluorescence analysis of a PfVAMP8 GFP-tagged transgenic Dd2 cell line demonstrates and confirms that PfVAMP8 moves away from the nucleus, passes through ER, Golgi, and finally localizes to the digestive food vacuole during trophozoite and schizont stage.

In addition, the biochemical fractionation demonstrates that PfVAMP8 is a soluble integral membrane protein and we hypothesize that the smear present at the P2 lane (integral protein sample) might be due to the viscosity of the pellet sample, which couldn’t properly migrate through the SDS-PAGE gel. In future experiments, this sample will be diluted in order to obtain more clear results.

Also, the insertion of a GFP tag, which has a size of 26kD, might have disrupted the VAMP8 longin domain; therefore, affecting the results obtained. Further studies need to be performed in order to determine if GFP in fact influenced the trajectory of the protein as the parasite goes through some morphological changes. Experiments with smaller tags such as a FLAG tag, might be an option for further analysis.
Another strategy that we will be performing is to compare the IFAs from PfVAMP8 wild type cell lines and PfVAMP8 dominant negative cell lines in order to compare co-localizations properties.

The understanding of the biological processes that underlines infected erythrocytes by *Plasmodium falciparum* parasites has achieved great advances largely due to the complete sequencing of the *P. falciparum* genome, the generation of transfection systems, morphological analysis of the parasite with electron and confocal microscopy, and the identification of the parasite’s exported proteins. However, its complex molecular understanding is still not complete. Characterizing trafficking proteins, such as PfVAMP7 will be our next step in order to better understand the trafficking pathways of PfSNAREs in *Plasmodium falciparum*.
REFERENCES


