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Determination Of The Mechanism Of Integral Membrane Protein Translocation By PTEX

by

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A Research Paper
Submitted in Partial Fulfillment of the
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Abstract:

Malaria has been devastating major civilizations worldwide and is still a major concern for large population of people. It is responsible for more than 215 million cases and 445,000 deaths each year (CDC n.d.). This infectious disease is caused by *Plasmodium* parasites, which invade and reproduce within human erythrocytes, inducing the clinical symptoms of malaria. These symptoms result from the hundreds of effector proteins the parasite exports into the host erythrocytes cytoplasm. These effector proteins remodel the erythrocyte to become more suitable for the parasite survival. During the erythrocytic stage of malaria, protein export mechanisms are critical and require many signaling sequencing and transport proteins to deliver the effector proteins. The only protein translocon that has been identified thus far is PTEX (Ho, et al. 2018). PTEX is a membrane protein complex over 1.2MDa in size consisting of three core proteins HSP101 ATPase, PTEX150, and EXP2 (Ho, et al. 2018). In order to study the mechanism of translocation that PTEX utilizes, first a fluorescence tag (m-NG) was fused to the SBP-1 in plasmids that contain Dihydrofolate reductase-based destabilizing domain fused to HSP101 which will serve as a conditional knockdown system of PTEX when the small molecule Trimethoprim is removed from growth media. These plasmids were transfected into parasite lines. The mechanism by which PTEX translocate the SBP-1 membrane protein will be determined based on the location at which it is held relative to PTEX after the conditional knockdown system has been activated. Due to the time constraints, no results have been obtained at the time of this report.
Introduction:

Malaria is an infectious disease that affects and estimated 216 million people worldwide and 445,000 people die every year, which were mostly children in the African region. There are cases of malaria diagnosed in the United States each year due to travelers and immigrants returning from endemic areas (CDC n.d.). There are five different species within the Plasmodium genus that have the causative agents of malaria; namely, Plasmodium falciparum which is responsible for the most severe form of malaria thus highest mortality rate This parasite is transmitted by the females of anopheles mosquitoes (Tania F. de Koning-Ward 2016).

There are three different stages of malaria life cycle (figure 1), one mosquito stage and two stages in the human host, asymptomatic infection of hepatocytes and malaria causing red blood infection stage. The transmission cycle starts when the infected mosquito takes a blood meal from a human host injecting sporozoites into the blood stream. These sporozoites find their way to the liver and infect hepatocytes where they divide rapidly for the next three days to form schizonts. The schizonts bud off the hepatocyte into circulation and once they reach the pulmonary circulation, they rupture from the sheer stress of the blood stream releasing

![Figure 1: A schematic of the malaria life cycle (CDC n.d.).](image)
merozoites that infect red blood cells forming a vacuole during this process, in this stage they are referred to as trophozoites. Immature trophozoites mature in the red blood cells and remodel their structure which causes the symptoms of malaria. For example, fever, shivering and chills, anemia, pain in the joints, headache, and severe vomiting.

Malaria parasites have developed along with human for many years which allowed them to gain many sophisticated methods to evade immune system detection. One of these mechanisms is the modification of the red blood cell structure and membrane protein composition forcing the infected cells to stick to the endothelial cells of the vasculature wall until the cell ruptures. This allows the parasites to avoid being filtered out of the blood stream by the spleen. Also during the blood stage, gametocytes are formed; they do not have any function in the human host but when another mosquito takes a blood meal, they are taken up along with some blood and continue the sexual life cycle of the parasite in the mosquito. Once in the mosquito, the male gametocytes fertilize the female gametocytes forming ookinete that reside in the mid gut of the parasite. Ookinetes mature into oocysts and once they rupture, they migrate to the salivary glands of the mosquito ready to be injected into another host.

The focus of this research project will be on the Erythrocytic stage of the malaria. During this stage, the parasite exports proteins targeting the red blood cell membrane and cytoplasm to remodel the shape of the cell making it more suitable for the parasite’s survival. The most pronounced structural change is the presence of three different membranes, parasite plasma membrane (PPM), parasitophorous vacuole membrane (PVM), and the red blood cells plasma membrane. The presence of these membranes requires the parasite to have secretory pathway mechanisms to facilitate protein passage through the different membranes; figure (2) shows a schematic of the different membranes.

Soluble secreted proteins have been shown to have recessed amino-terminal signal sequence known as plasmodium export element (PEXEL) which is sufficient to direct co-translations transport into the ER. However, another downstream sequence is facilitates the export across the PVM (Mark E. Wickham 2001). Not all proteins contain the PEXEL; known as PEXEL-negative proteins (PNEPs) which include Plasmodium falciparum erythrocyte
membrane protein I (PfEMP1) family of proteins. Generally, these proteins possess a transmembrane domain. PEXEL motif has been shown to be cleaved co-translationally or soon after; further modification via acetylation occurs at the N-terminus of the protein. But these modification (cleavage and acetylation) are not sufficient for allowing the protein to cross the PVM (Boddey JA 2009). In order for exported proteins and perform their functions they must cross the PVM, *Plasmodium* Translocon of Exported Proteins (PTEX) has been shown to play a critical role in this process (Ho, et al. 2018). PTEX is a membrane protein complex over 1.2MDa in size consisting of three core proteins HSP101 ATPase, PTEX150, and EXP2; other proteins are involved in this complex like Thioredoxin 2 and PTEX88 that are not critical for the functioning of PTEX (Ho, et al. 2018). Conditional knockdown experiments of HSP101 (Beck, et al. 2014), PTEX150 (Elsworth B 2014) and EXP2 (Garten, et al. 2018) (Ho, et al. 2018) has shown to be lethal in infecting parasites.

A model of PTEX-mediated translocation has been proposed for soluble secreted proteins (Ho, et al. 2018). It hypothesized that HSP101 unfolds and threads proteins through an oligomeric EXP2 transmembrane channel spanning the PVM, PTEX150 forms an adaptor that has a structural interaction with EXP2 to mediate this translocation between HSP101 and EXP2. Structural investigation has shown the complex with a 6:7:7 stoichiometry. Each EXP2 monomer contributes a single transmembrane helix to form a seven-fold-right-handed-spiral-symmetric-protein-conducting channel. Six HSP101 protomers are tethered on top of PTEX150 and is
oriented in such a way that forms a single unbroken channel extending from the top of HSP101 to the bottom of EXP2 pore.

However, the mechanism of export for transmembrane proteins via PTEX has not been resolved yet. This project attempts to create a conditional knockdown system of PTEX to help determine the mechanism of translocation for transmembrane proteins; specifically Skeleton-binding protein 1 (SBP-1). SBP-1 is a parasite encoded transmembrane protein essential for the correct positioning and insertion of PfEMP1 into the red blood cell plasma membrane. PfEMP1 are high molecular weight, antigenically-diverse, parasite-encoded proteins that are transcribed from the var multi-gene family. They are presented on the surface of red blood cells to mediate the adhesion of infected red blood cells to the vascular endothelium (Kats, et al. 2015) (Cooke BM 2006). SBP-1 is localized in Maurer’s clefts (MC’s) which are parasite-induced membranous structures within the infected red blood cell cytoplasm (Blisnick T 2000) (Figure 4).

The topology of SBP-1 is critical for this investigation; the N-terminus is located within the lumen of the MC while the C-terminus is exposed to the red blood cell cytoplasm (Saridaki T 2009). The C-terminus of SBP-1 is fused a monomeric Neon-green (m-NG) fluorescence marker which will be used to determine the location of the protein before and following the knockdown of PTEX. The location of fluorescence will help determine the mechanism by which PTEX is contributing to the translocation and thus the export of SBP-1 and other transmembrane proteins.
The conditional knockdown system that will be utilized uses dihydrogenfolate reductase (DHFR)-based destabilizing domain (DDD) fused to the C-terminus of HSP101 component of PTEX. The DDD is stabilized in the presence of a small molecule trimethoprim (TMP) and allows HSP101 to form the interactions necessary for its function. When TMP is not present in the media the folding of DDD is disturbed and inhibits HSP101 from forming the required interactions (Figure 5). The degree of inhibition depends on the stage of parasitism of culture, if TMP was removed before the ring-stage in an asynchronous culture the parasites will accumulate as late ring-stage forms. However, if TMP was removed at or after the beginning of the trophozoite stage then the growth arrest does not occur until the subsequent ring stage, until which the parasite development and re-invasion occurs normally (Beck, et al. 2014).
Methods:

Two plasmids from different parental lines were used to construct the fusion protein (SBP1-mNG). The first target plasmid yEOE-attp-SBP1-mNG-2xMYC (yEOE), was constructed using pLN-SBP1-HA wt as the vector to PCR amplify the SBP1 gene using primers BI1 and BI2. The SBP1 insert was then inserted into the receiving plasmid yEOE-attp-EXP1-mNG-2xMYC using Gibson assembly replacing the EXP1 gene. The resulting plasmid, yEOE-attp-SBP1-mNG-2xMYC, contained the target fusion protein (SBP1-mNG) which expression was controlled by HSP86 promoter. The second target plasmid, pLN-SBP1-mNG-HA wt (pLN), was constructed using yEOE-attp-EXP1-mNG-2xMYC as the insert source of mNG gene using PCR amplification. Then, Gibson assembly was used to insert the mNG insert into the pLN-SBP1-HA vector.

The two plasmids were transformed into ultra-competent XL Gold E. coli cells and plated with ampicillin selection marker. The plates were screen for colonies; three colonies were isolated from the pLN transformed E. coli and five from the yEOE transformed E. coli. These colonies were then incubated for 48 hours in LB media. The plasmids were isolated from the cells using miniprep protocol.

Diagnostic digest was done using two newly constructed plasmids to ensure the inserts were positioned correctly. NcoI HF restriction enzyme was used to digest the parent plasmid pLN-SBP1-HA wt and the target plasmid pLN-SBP1-mNG-HA wt; this enzyme has one cut site in the parent plasmid and two cut sites in the target plasmid. PstI HF restriction enzyme was used to digest the other parent plasmid, yEOE-attp-EXP1-mNG-2xMYC, and the target plasmid, yEOE-attp-SBP1-mNG-2xMYC; this enzyme has two cut sites on the parent plasmid and one on the target plasmids. Both digestion reactions were allowed to run for 24 hours to ensure complete digestion. Gel electrophoresis was used to separate the different fragments of the digested fragments. The target plasmids were also sequenced to ensure the region of the fusion protein was constructed correctly. The two target plasmids were also sequenced to ensure the conservation of gene code of the SBP-1 protein, linker, and mNG fusion tag.

The two sequenced bacterial clones were expanded to 1L cultures each and the target plasmids were isolated using the midiPrep protocol. These plasmids were transfected into two different parasite lines (AC2Bpγ and WA1AcBII) using a standard protocol designed by Dr. Beck. In previous studies, the two parasite lines were engineered to have a conditional
knockdown system which requires the present of trimethoprim (TMP) in the growth media. In addition to this system, the target plasmids contain different drug selection markers which were exploited to select for the parasites that took up the target plasmids. pLN plasmids contains BSD selection marker and yEOE plasmid contains BSD and DSM-1 selection markers. The yEOE plasmid was transfected into both parasite lines and the pLN plasmid was only transfected into the WAIAcBII parasite line. Two replicates were made of each transfection; a total of 6 cultures were produced.
Results and discussion

The diagnostic digests of the target plasmids were used to ensure the correct integration of the inserts into the parental vectors. In Figure 7 shows the results after gel electrophoresis; lanes pLN1,2,3 show 2 bands. One of the bands was slightly higher larger than 5kb and the other slightly smaller than 3kb, which is in agreement with the simulated digestion results shown in figure 6 in lane pLN\textsubscript{T}. Also shown in figure 7 a single band slightly larger than 8kb which is in agreement with the simulated digestion results shown in figure 6 in lane yEOE\textsubscript{T}. Both parental plasmids in lanes yEOE\textsubscript{P} and pLN\textsubscript{P} in figure 7 did not appear, which was due to loading error. If the experiment was to be repeated the results should be similar to the simulated digestion shown in lanes yEOE\textsubscript{P} and pLN\textsubscript{P}. One of each target plasmid clone was sequenced to ensure the conservation of the gene code for SBP-1 protein, linker, and m-NG fusion tag. Sequencing results showed no modification of the DNA sequence.

The transfection replicates are currently in the recovery phase; the tissue cultures usually take between 3-6 weeks to present any reliable parasitemia results. Therefore, no experiments can be done at the time of this report. However, there are theoretical results that could be discussed as potential outcomes of the conditional knockdown of PTEX that will help understand its mechanism of action.

SBP-1 protein is a membrane protein that is targeted for the Maurer’s clefts in the cytosol of the RBC, thus it must cross three membranes in order to reach Maurer’s clefts. These membranes are parasite ER (P-ER), PPM and PVM. First, SBP-1 is synthesized into the P-ER and transported into the PPM via vesicular transport (figure 8-1). At this point, there are three possible mechanism of PTEX action. First, PTEX could be responsible for extracting the membrane protein from the PPM and threatring it through (figure 8-2). Second, there could be a
specialized protein that extracts SBP-1 protein from the PVM (figure 8-3) and either delivers it directly to PTEX (figure 8-4) or to a chaperon protein (figure 8-5) which is responsible for the delivery (figure 8-6). Once SBP-1 is delivered to PTEX, it can be inserted into the PVM using a lateral gating mechanism (figure 8-7) and gets delivered to Maurer’s clefts via vesicular transport (figure 8-8,9). The other option would be a chaperon protein that receives SBP-1 from PTEX and delivers it to another protein in Maurer’s clefts that is responsible for its integration (figure 8-10,11).

When the knockdown system is activated, the fluorescence of SBP1-mNG and the mRuby tag fused to the EXP2 subunit will be used to identify the location of the SBP-1 protein. If PTEX is directly responsible for extracting the membrane protein, then the fluoresce signal should be localized on the parasite cytosolic side (the m-NG is attached to the C-terminus of SBP-1); the mRuby tag will help differentiate between the PPM and PVM. If the fluorescence was observed within the vacuole, then the SBP-1 extraction is PTEX independent and most likely there is a protein responsible for the extraction of SBP-1 protein from the PPM.

*Figure 8: Schematic of the possible mechanisms by which PTEX functions as a translocon*
References


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