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Protein export in malaria parasites: many membranes to cross

Matthias Marti[1] and **Tobias Spielmann**[2]

[1]Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, USA

[2]Parasitology Section, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

Abstract

The continuous multiplication of *Plasmodium* parasites in red blood cells leads to a rapid increase in parasite numbers and is responsible for the disease symptoms of malaria. Survival and virulence of the parasite are linked to parasite-induced changes of the host red blood cells. These alterations require export of a large number of parasite proteins that are trafficked across multiple membranes to reach the host cell. Two classes of exported proteins are known, those with a conserved *Plasmodium* export element (PEXEL/HT) or those without this motif (PNEPs). Recent work has revealed new aspects of the determinants required for export of these 2 protein classes, shedding new light on the mode of trafficking during the different transport steps en route to the host cell.

Introduction

Many intracellular pathogens are taken up by host cells through endocytic or phagocytic mechanisms and subsequently establish themselves in modified host-derived vacuoles. In contrast, Apicomplexan parasites, including malaria parasites, actively penetrate their host cell to end up in a special non-fusogenic vacuole. This entry mechanism may have evolved precisely to avoid the endosomal/lysosomal pathway of the host cell. In addition, some highly specialized host cells are incapable of endocytosis or phagocytosis. For example, malaria parasites invade and replicate within red blood cells (RBCs) that provide dissemination of the parasite through the blood circulation but lack many basic features of eukaryotic cells. Malaria parasites are well adapted to this environment. They induce extensive host cell remodeling to install organelles, reinforce the host cell cytoskeleton, change the RBC surface to induce cell adhesive properties, and change the RBC permeability to nutrients. These profound alterations of the host cell require the export of a large set of parasite proteins.

As malaria parasites reside in a permanent parasitophorous vacuole (PV), exported proteins must enter the parasite secretory pathway and subsequently cross the parasite plasma membrane and the PV membrane (PVM) in order to reach the host cell. Furthermore, parasite-derived organelles in the host cell termed Maurer's clefts in *Plasmodium falciparum* are thought to be required for export of proteins to the host cell surface. One of these exported proteins is the major virulence factor PfEMP1 that localizes to particular sub-regions on the infected RBC surface termed knobs [1]. Most exported proteins contain a

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Corresponding author: Spielmann, Tobias (spielmann@bni-hamburg.de).

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simple motif termed *Plasmodium* export element (PEXEL) or host targeting signal (HT) with the consensus sequence RxLxE/Q/D [2,3] (Figure 1a). This motif, if located in the appropriate position after a signal peptide, mediates protein export. However, an increasing number of PEXEL negative exported proteins (PNEPs) [4] (Figure 1a) suggest alternative signal requirements. Here we will discuss recent advances in our understanding of the signals and the individual steps of protein export in *P. falciparum*, the causative agent of the severest form of human malaria.

Right here, right now: Protein targeting to the host cell is determined at the parasite endoplasmic reticulum (ER)

The PEXEL/HT motif is reported to be a binding site for phosphatidylinositol 3-phosphate (PI3P) [5] and a proteolytic cleavage site for an aspartic protease [6,7], Plasmepsin V (PM5), a finding based on the initial surprising discovery that the PEXEL is processed [8]. Both, PI3P binding and processing, occur in the parasite endoplasmic reticulum and are proposed to be essential for export, suggesting that the decision for export is made early in the secretory pathway [6–9]. How PI3P binding and PEXEL/HT cleavage trigger export and their relative contributions to export are topics of current debate [5,6,10]. For example, it has been proposed that PI3P binding segregates a protein into specific export competent ER sub-compartments or vesicles, where PM5 is responsible for the correctly timed release from the membrane [5]. It is currently unclear how PI3P, which normally is only found on the cytoplasmic leaflet of membranes, is transferred into the ER, and what would generate and maintain its asymmetric distribution to function in sorting PEXEL/HT proteins to ER export sites. Parallels based on function and evolutionary history have also been drawn between PI3P binding in host cell targeting of proteins in *Plasmodium* and *Oomycete* parasites [5,11,12], but it should be noted that the *Oomycete* data is at present a matter of dispute [13].

Despite the demonstrated requirement of the native PEXEL/HT motif for PI3P binding and PM5 cleavage, recent data indicate additional levels in the control of PEXEL/HT protein export. Cleavage of the PEXEL/HT occurs between position 3 and 4 of the motif. The nascent N-terminus is then acetylated, leading to a protein starting with Ac-XE/Q/D [8]. Two studies have now shown that this mature N-terminus, when exposed by other means than PM5 cleavage of a PEXEL motif, can drive protein export [14,15] (Figure 1b). Specifically, one study demonstrated that the mature PEXEL N-terminus is sufficient to mediate export of a reporter in combination with the transmembrane (TM) domain of a PNEP [14] (Figure 1b). The second study used a self-cleaving protease domain between the N-terminal signal peptide and the processed PEXEL motif, leading to the exposure of the mature PEXEL N-terminus [15]. This cleaved protein was exported, suggesting that the full PEXEL motif is not necessary as long as the mature N-terminus is presented correctly after ER entry. Together these studies argue that PI3P binding is not strictly necessary for export and that the mature N-terminus is sufficient to drive export, either upon cleavage by a protease or by direct exposure in combination with a TM domain. Notably export was abolished in a construct where a correct mature PEXEL N-terminus was created by signal peptidase cleavage [6]. The reason for this discrepancy is currently unclear. The existence of PI3P- independent export was also inferred from constructs where a surprising level of export was achieved with mutants in supposedly essential PEXEL positions that also abrogated PI3P binding [10].

Several recent studies have demonstrated that the newly exposed amino acid positions P4 and P5 in the mature PEXEL N-terminus are important for efficient protein export [14–16], confirming earlier results that P5 holds important trafficking information independent of PM5 cleavage [17]. Moreover additional redundant export signals downstream of the

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PEXEL that can compensate for P5 [14,15] and some regions from non-exported proteins can prevent export despite presence of a bona fide PEXEL [14,16] (Figure 1c). It has been proposed that the mature PEXEL N-terminus may also function through binding to PI3P [11]. However, as most export mediating residues appear to be negatively charged and can function on a scrambled or alanine background, this seems unlikely [14–16,18].

PNEPs represent a second class of exported proteins that consist of a growing number of proteins with structural similarities but no shared primary sequence features. The PNEP N-terminus lacks a signal sequence and is interchangeable with mature PEXEL N-termini in its ability to promote protein export [14]. PNEPs do not appear to be N-terminally processed, with the exception of REX2 [19]. PNEPs harbor an internal TM domain that can functionally complement the N-terminal signal sequence when combined with a mature PEXEL N-terminus [14]. PfEMP1 is also not cleaved at the PEXEL-like sequence KxLxD by PM5 and may therefore be considered a PNEP [16]. Other large antigens with similar structural features, such as Pf332 and members of the SURFIN family [20–21], appear to contain similar trafficking determinants to PNEPs [22] and therefore may all belong to this group of exported proteins. This may indicate different systems for the initial phases of export of PNEPs and PEXEL proteins that depend on a TM or N-terminal processing, respectively, to render the protein export-competent.

It is noteworthy that signal peptide cleavage seems to be inefficient in some PEXEL proteins, indicating that membrane association itself could play an important role for export [17]. Alternative membrane association through the TM may therefore have led to loss of the signal peptide and the full PEXEL motif in proteins where the TM was able to replace this function. This may have been the case for PfEMP1, as the addition of its TM seemed to reduce the strict requirement for the PEXEL residues in a reporter construct [10]. In contrast, all PEXEL residues seem to be essential in the TM protein STEVOR, which would explain why it retained the full PEXEL [23].

Insane in the membrane: Protein unfolding and translocation at the parasite host cell interface

It is not clear yet whether the different classes of exported proteins are trafficked separately or within the same cargo environment through the parasite secretory pathway. However, they all must pass the parasite host cell interface in order to reach the host cell (Figure 2a,b). After fusion of cargo vesicles with the parasite plasma membrane (PPM), soluble proteins are released into the surrounding parasitophorous vacuole, where they appear to be pumped into the host cell through a PVM-localized translocon. This hypothesis is based on a classical study analyzing transfer of an exported protein across the PVM [24], and on more recent observations demonstrating that exported proteins fused to mDHFR are blocked in the parasitophorous vacuole if the mDHFR domain is arrested in its folded state [25]. This is a well-established indicator for unfolding-dependent membrane translocation [26]. For TM proteins this concept is mechanistically more problematic because, after fusion of secretory vesicles with the PPM, they would remain embedded in this membrane. Hence, continued vesicular trafficking was considered a strong possibility [27,28]. However, fusion of exported TM proteins to mDHFR resulted in a folding-induced block of the protein at the PPM [14], suggesting presence of a second translocation activity in this membrane. Such a hypothetical PPM-localized translocon would extract TM proteins out of the PPM, after which they would become competent for translocation through the PVM-localized translocon. In support of this hypothesis, multiple chaperones that could provide the energy for this process have been identified in the PV [29]. Extraction of TM proteins out of the membrane is already known from the ER-associated degradation pathway [30].

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While the identity of the PPM translocon remains obscure, a multimeric protein complex termed PTEX fulfills several criteria qualifying it as a putative PVM translocon [31–33]. Providing formal proof of function for PTEX is however challenging. A recent study used deconvolution microscopy and 3D SIM (three-dimenional structures illumination microscopy) for a detailed co-localization analysis of PTEX components and some of its substrates [34]. The data suggested that PTEX is present at the parasite periphery in clusters that at least partially co-localize with PEXEL proteins or a PEXEL-mDHFR fusion construct arrested in a folded state. In contrast, little co-localization was observed with PTEX and PfEMP1, suggesting that PfEMP1 may take an alternative pathway. This is also supported by a recent study indicating that in early parasite stages PfEMP1 accumulates in specific PV subdomains that do not co-localize with the PTEX component HSP101 [35]. However, as PfEMP1 is a TM protein, it may require translocation at the PPM [14], which might account for the distinct localization pattern. More generally, co-localization with substrates is not sufficient to validate PTEX as a bona fide translocon. It could even be argued that exported proteins may reside only very transiently at translocation sites and accumulate to detectable levels only in regions of the PVM containing inactive PTEX or lacking it altogether. Hence, the finding that in early stage parasites PfEMP1 remains in the parasite periphery [35] could also mean that this protein is stored in inactive/non-export competent areas before moving to the translocon for correctly timed release into the host cell.

Taken together these studies provide a first glimpse at a potentially complex translocation system at the PPM and PVM (Figure 2b) that may mediate precise delivery of proteins between these compartments, possibly on a par with those found in mitochondria and chloroplasts [36], which possess similarly closely adjoined membranes.

A stairway to the surface: trafficking to and from the Maurer's clefts

Presence of parasite surface proteins in Maurer's clefts suggested early on that these organelles may act as an intermediate station for proteins en route to the host cell membrane, and this hypothesis still holds [1]. While soluble proteins can reach the Maurer's clefts by simple diffusion after crossing the PVM, the situation is again more complicated for TM proteins. Although different types of vesicular transport were initially postulated [28,37], there is increasing evidence suggesting that TM proteins are also delivered to the Maurer's clefts in a soluble state [14,38,39]. This interpretation was based on the detection of soluble TM proteins in the host cell and on a combination of time-lapse imaging and photoconvertible reporters to track pools of exported TM proteins in the host cell cytosol. Maintaining TM proteins in a soluble state requires energy, possibly provided in the form of chaperoned complexes. Candidates for such complexes are the recently identified J dots, mobile foci in the host cell cytosol that contain the exported parasite chaperones HSP40 and HSP70 [40,41]. However, vesicular transport as an alternative route for a subset of TM proteins cannot be excluded at this point.

Another key question is how surface antigens like PfEMP1 are transported from the Maurer's clefts to the infected RBC surface. Several candidate structures were recently identified, but direct evidence for their involvement in surface transport is currently lacking. Ultrastructural studies identified tether structures that connect Maurer's clefts to both the cell surface and the PVM [42,43] and often contained electron dense vesicles [44] (Figure 2c). In addition, an extensive network of actin filaments and associated vesicles (some containing PfEMP1) connects the Maurer's clefts directly with the knob structures on the infected RBC surface [45]. These filaments may therefore provide directed vesicular transport to the surface, and their rearrangement appears to be important to anchor Maurer's clefts [46], although the data about the latter is conflicting [35]. Interestingly, hemoglobin

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mutations that protect from severe malaria [47] and cause altered PfEMP1 surface display [48–50] affect rearrangements of these actin filaments and Maurer's cleft morphology [45], which may be the underlying cause for the observed effect on PfEMP1 surface exposure and clinical protection (Figure 2c).

PfEMP1 reaches the RBC surface around 24 hours post-invasion [51], inducing adherence properties of the infected RBC and coinciding with significant increases in its rigidity [52,53]. Around the same time, Maurer's clefts change from a mobile to a fixed state within a short time frame of ~30 minutes [39]. Interestingly, this phase of the life cycle also coincides with a mild echinocytosis of the host cell [39], which may reflect the proposed rearrangements of the actin cytoskeleton [45] (Figure 2c). Hence, Maurer's clefts arrest may represent a prerequisite for vesicular trafficking to the infected RBC surface.

Concluding remarks

The identification of a simple export motif in *Plasmodium* proteins has provided a rational basis for systematic investigation of the *P. falciparum* export pathway. Meanwhile other classes of exported proteins have been identified. Based on our current knowledge, it appears that these are all trafficked through the parasite vesicular pathway, unfolded at the PPM, translocated through the PVM and released into the host cell cytoplasm (Figure 2). Thereafter the pathway of surface antigens is less clear, but current evidence suggests that they remain soluble until reaching the Maurer's clefts and are then trafficked in vesicles to the surface. The recent results also support the existence of multiple checkpoints and multiple partially redundant signals to ensure selective export of proteins destined to the host cell. Whether the signals all act consecutively or to what extent there are parallel pathways remains to be determined. Future studies will provide answers to the many remaining questions: Is PM5 cleavage co-translational and in place of signal sequence processing, and how does it facilitate export? Are exported proteins segregated into separate vesicles at the ER level, similarly to GPI-anchored proteins in yeast [54]? The full PEXEL is recognized in the ER, but where does the mature PEXEL N-terminus act? What is the identity of the translocation machines in the parasite periphery? How are TM proteins unfolded at the PPM, and how is this state maintained in the host cell cytosol? Are there other exported proteins, such as multi TM proteins, that use only vesicular trafficking based pathways?

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Highlights

- Malaria parasites export a large number of proteins into their host cell
- Multiple determinants in a protein can control export
- Different groups of exported proteins follow similar export pathways
- Soluble and transmembrane proteins reach the host cell through translocation
- Maurer's clefts are important for antigens targeted to the host cell surface

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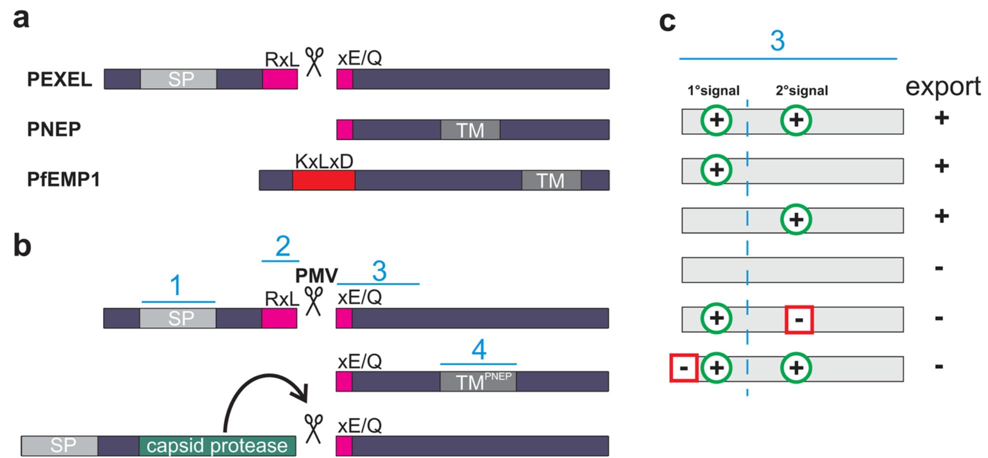

Figure 1.

Export motifs in context. **(a)** Types of exported proteins with PfEMP1 representing a potential subtype of PNEPs. **(b)** Strategies to uncouple export from plasmepsin V (PM5) cleavage. Top, PEXEL proteins; middle, construct with a PNEP TM containing the mature PEXEL N-terminus only; bottom, insertion of a self-cleaving protease domain similarly exposes a mature PEXEL N-terminus. Blue lines labeled 1–4 represent signals influencing export. **(c)** Export determinants in the mature N-terminus. The consensus from the strategies used in **(b)** to test export without the full PEXEL [14,15] suggests multiple determinants influencing export. Green circles with a plus indicate export signals, squares with a minus export inhibitory signals. The column labeled 'export' indicates whether the combination of signals leads to export of a reporter (+) or not (–). The primary (1°) signal consists of the PEXEL residues remaining in the exposed mature N-terminus.

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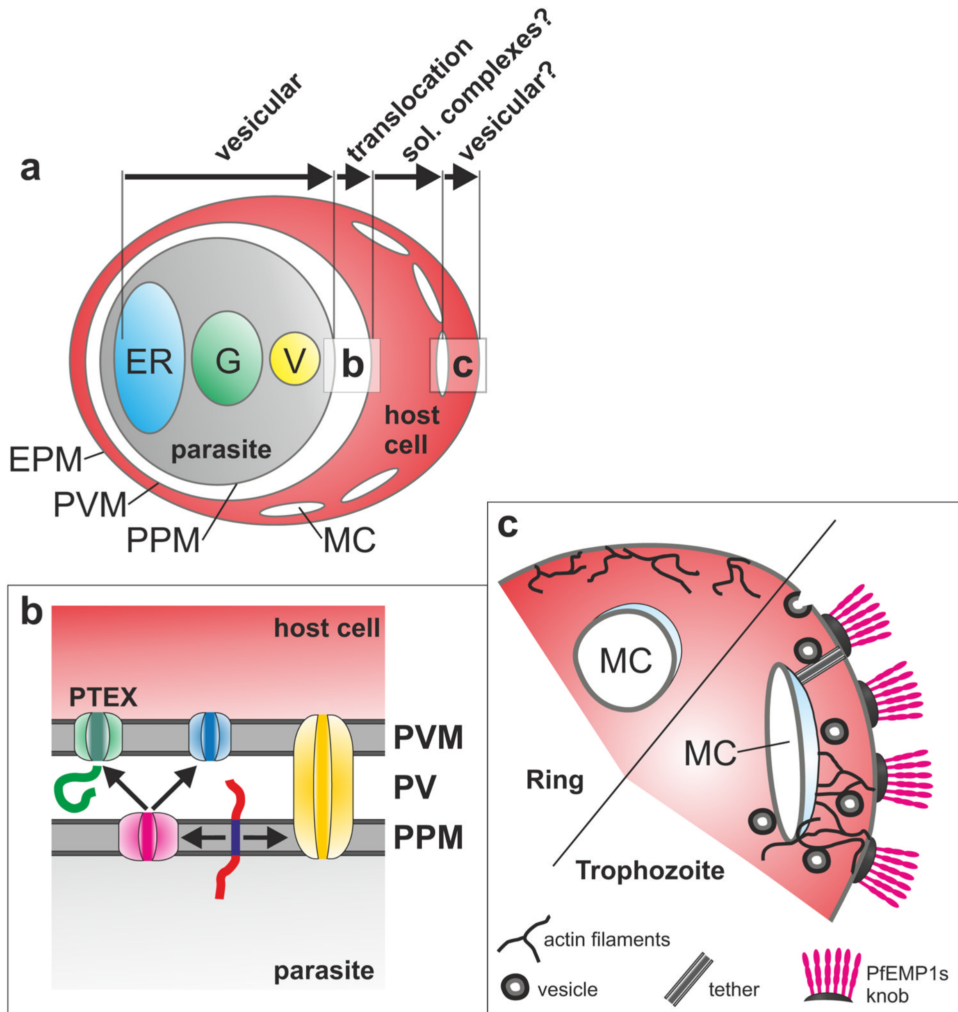

Figure 2.

General transport pathway into the host cell. **(a)** Overview of an infected RBC. Modes of transport for particular steps are indicated with labeled arrows. EPM, erythrocyte plasma membrane; ER, endoplasmic reticulum; G, Golgi, MC, Maurer's clefts; V, secretory vesicle. Squares indicate particular steps shown in **(a)** and **(c). (b)** Possible arrangements for translocons. A soluble (green) and a TM protein (red, with dark blue TM) are shown. The soluble protein can reach the host cell through PTEX (green). TM proteins either pass through a translocon spanning both the PPM and PVM (yellow), or get extracted into the PV (pink) to translocate through PTEX or a different PNEP-specific translocon (blue). **(c)** Development of Maurer's clefts. Shown are the different states of Maurer's clefts in rings (or in RBCs with mutated hemoglobin) and trophozoites (or wild type RBCs); the latter are more permissive for vesicular transport of virulence factors to the host cell surface.