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OPINION ARTICLE

REVISED Diversion at the ER: How *Plasmodium falciparum* exports proteins into host erythrocytes [version 2; peer review: 3 approved]

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Abstract

Malaria is caused by parasites which live in host erythrocytes and remodel these cells to provide optimally for the parasites' needs by exporting effector proteins into the host cells. Eight years ago the discovery of a host cell targeting sequence present in both soluble and transmembrane *P. falciparum* exported proteins generated a starting point for investigating the mechanism of parasite protein transport into infected erythrocytes. Since then many confusing facts about this targeting signal have emerged. In this paper, I try to make sense of them.

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Any reports and responses or comments on the article can be found at the end of the article.

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REVISED Amendments from Version 1

In response to Tobias Spielmann's referee report, I have changed the text regarding the analysis of the N-terminal hydrophobic region of plasmepsin V by Russo *et al.*, 2010.

See referee reports

The problem

P. falciparum infects erythrocytes and causes malaria in humans. The parasite resides intracellularly in a parasitophorous vacuole (PV), and exports proteins into the erythrocyte that are important for parasite survival (Figure 1)^{1,2}. The identification of a host cell targeting signal in exported *P. falciparum* proteins was an important first step towards understanding the export mechanism, but left cell biologists puzzled: Marti *et al.* (2004) and Hiller and colleagues (2004) identified a short sequence, RxLxE/Q, which is present in many proteins in the *P. falciparum* genome known to be exported from the parasite into the erythrocyte^{3,4}. This *P. falciparum* protein export element (PEXEL) was found by both groups to be necessary and sufficient for protein export into the host cell^{3,4}.

What remains unclear to date is the mechanism by which an export signal present in both soluble and transmembrane proteins can mediate

transport of both types of protein into the erythrocyte. This issue was debated hotly, but our ideas at the time were limited, because they were solely based on the classical secretory pathway in mammalian cells⁵⁻⁷.

The fact(or)s

Since then, a lot more data related to the PEXEL sequence have been generated, but rather than clarify they seem to confuse the issue further. Meanwhile it has been shown that:

- The secretory signal peptides of the exported soluble proteins, which are located 20–40 amino acids upstream of the host cell targeting signals, are not cleaved upon endoplasmic reticulum (ER) targeting^{8,9}.
- The host cell targeting signal is cleaved and the new N-terminus is N-acetylated at the ER membrane⁹⁻¹¹.
- The protease responsible for the cleavage has been identified⁹⁻¹².
- Cleavage by this protease is a prerequisite for transport into the erythrocyte^{9,10}; if you generate the cleaved N-terminus by modifying the gene and combine it with a cleavable signal peptide, the resulting protein is secreted into the PV and remains there⁹.
- The uncleaved targeting signal binds PI3P at the ER membrane with the same specificity required for protease cleavage and host cell targeting; the cleaved signal no longer binds PI3P¹³.
- A putative 'translocator' complex resides in the PV membrane (PVM); it consists of 5 proteins that coprecipitate some of the proteins bearing a host cell targeting signal, but a function of the complex has not been demonstrated in any way, nor has it been investigated whether the association of the complex and the PEXEL proteins is mediated by the PEXEL signal^{14,15}.

The hypothesis

The authors of the respective papers assume that proteolytic cleavage, N-acetylation, and PI3P binding take place in the ER lumen (Figure 2A)^{9,10,13}. This cannot be right: N-acetylation is a cytosolic modification, based on the biochemical and sequence data characterizing the protease plasmepsin V, its active site is almost certainly on the cytoplasmic face of the membrane, and the only possible location for PI3P at the ER membrane is in the cytosolic leaflet. In detail:

All known enzyme complexes mediating N-acetylation including NatD, which is likely responsible for the N-acetylation of proteolytically cleaved PEXEL proteins, are located in the cytosol¹⁶. The presence of a secretory signal sequence indeed strongly reduces the likelihood of proteins being N-acetylated, confirming that N-acetylation does not take place inside compartments of the secretory pathway¹⁷.

In the initial characterization of plasmepsin V, Klembs & Goldberg (2005)¹² found a hydrophobic region at the N-terminus, which they described as a putative signal sequence. There is no discernible signal peptidase cleavage site C-terminal of this hydrophobic region and indeed Klembs and Goldberg did not observe processing of the N-terminus of plasmepsin V in pulse-chase experiment. Russo and colleagues showed later (2010)¹⁰ that fusion of this region of plasmepsin V to a fluorescent reporter protein resulted in ring-shaped staining around the parasite cytoplasm indicative of location in the PV or at the parasite plasma membrane, and suggesting entry of

Figure 1. The problem. *P. falciparum* (blue) resides in red blood cells (pale orange) inside a parasitophorous vacuole (white). In order to survive, *P. falciparum* needs to export numerous proteins into the red blood cell, which remodel the host cell to suit the purposes of the parasite. The mechanism by which these proteins are exported is still unclear (Figure modified from Römisch, 2005)⁶.

Figure 2. A) The hypothesis. Most proteins targeted for export into the host cell have a signal sequence (yellow) or transmembrane domain, which leads to their SRP-mediated targeting to the protein translocation channel (Sec61) in the ER membrane of the parasite (1). Many but not all of the *P. falciparum* exported proteins have an N-terminal extension (red zigzag) whose function is unknown. In addition, host cell targeted proteins contain, in a distance of 20–40 amino acids from the signal peptide, a PEXEL sequence (RxLxE), which is also required for binding to phosphoinositol-3-phosphate (PI3P; orange balls) and for cleavage by the ER-membrane associated protease plasmepsin V (PM5). The current hypothesis in the field is that, after signal-peptide mediated translocation into the ER lumen, the PEXEL sequence binds to PI3P in the luminal face of the ER membrane and is cleaved by PM5 (2). The cleaved protein is released from PM5 (3) and continues through the *P. falciparum* secretory pathway by vesicular transport (4). Note that this model cannot explain the NatD-mediated N-acetylation of the PM5-cleaved N-terminus, because NatD resides in the cytoplasm. **B) The alternative.** After SRP-mediated targeting of the protein destined for export to the parasite ER (1) and insertion into the Sec61 channel, the N-terminal extension of the signal peptide (red zigzag) delays signal cleavage, perhaps by preventing reorientation of the signal peptide in the Sec61 channel. This delay in completing translocation allows the RxLxE sequence to bind to PI3P (orange balls) on the cytoplasmic face of the ER membrane, which creates a recognition signal for PM5 and results in proteolytic cleavage (2). Cleavage releases the protein from the translocation machinery and allows N-acetylation by NatD (3). The mature protein is handed over to a PI3P-associated putative transmembrane receptor (R; 4), which may itself be a PEXEL protein.

the protein into the ER and transport to the cell surface. Since the N-terminal hydrophobic domain of plasmepsin V is not cleaved¹², it is likely a transmembrane signal anchor. Both TMHMM and TopPred predict it to insert into the ER membrane with the N-terminus in the lumen such that the subsequent soluble region containing the active site would be in the cytoplasm. In the same paper Russo and colleagues demonstrated that the C-terminal hydrophobic region of plasmepsin V was required for ER retention of the protein¹⁰. Both Russo and colleagues and Klemba and Goldberg describe this region as a transmembrane domain, but Klemba and Goldberg show that 50% of plasmepsin V can be extracted from the membrane by carbonate, pH 11.0¹². A standard feature of a transmembrane protein, however, is that it is resistant to carbonate extraction at pH 11.5¹⁸. Altogether these data suggest that plasmepsin V is tethered to the ER membrane by hydrophobic regions at both termini, and that its active site is in the cytoplasm.

Localization of PI3P has been investigated in yeast and mammalian cells where it is found on early and late endosomes and transiently at the plasma membrane¹⁹. PI3P is generated by PI3-kinases on the cytoplasmic leaflet of intracellular membranes and regulates membrane trafficking events²⁰. The localization of the kinase determines the localization of the PI3P patch²⁰. There are no PI3 kinases inside

the secretory pathway. The only known example of PI3P occurring at the ER is during formation of autophagosomal membrane precursors, the so-called omegasomes²¹. Even in this case, PI3P is generated by the Vps34 PI3-kinase in the cytoplasmic leaflet of the ER membrane²².

The alternative

An alternative explanation for most of the available data is that their secretory signal peptides target PEXEL proteins to the *P. falciparum* ER, but are inefficiently cleaved - perhaps due to their long N-terminal extensions (Figure 2B, (1)). This is similar to the biogenesis of some autotransporters in pathogenic *E. coli*, where delayed signal peptide cleavage due to N-terminal signal peptide extension allows the passenger domain to remain unfolded in the periplasm while the porin domain assembles in the *E. coli* outer membrane²³. Stuck in the protein translocation channel in the *P. falciparum* ER membrane, the PEXEL protein in transit is oriented such that the host cell targeting signal can bind PI3P at the cytoplasmic face of the ER membrane (Figure 2B, (2)). Binding creates the cleavage site for the protease plasmepsin V (Figure 2B, (2)). This possibility has also been mentioned, but not been pursued experimentally, in Bhattacharjee *et al.*¹³. After cleavage and N-acetylation (Figure 2B, (3)) the new N-terminus is not released, but the protein remains membrane-tethered or associated with a transmembrane protein (Figure 2B, (4)) until it reaches

Figure 3. The way out. Cleaved N-acetylated PEXEL proteins (red triangles) associated with their receptor (black bar) could be transported to the cell surface in a complex (1) in one of two ways. Either the receptor/soluble protein complex remains associated with the PI3P patch (green) in the ER membrane. The patch and associated proteins are transported by vesicle budding (2A) and fusion through the parasite secretory pathway to the cell surface, where a further budding event (3A) liberates vesicles containing the PI3P patch and the PEXEL proteins. These vesicles then fuse with the PVM (4A). Alternatively, similar to the formation of omegasomes during autophagy, the PI3P patch may trigger the budding of processed PEXEL protein-containing vesicles into the ER (2B). PEXEL proteins would then be transported through the secretory pathway in double membrane vesicles (DMV), and released into the PV by fusion of the outer membrane with the parasite plasma membrane (3B). The released vesicle may then fuse with the PVM (4B) (Figure modified from Römisch, 2005)⁶.

the cell surface from where it is transferred to the erythrocyte, perhaps by vesicular transport (Figure 3).

Unusual biogenesis of a host cell targeted *P. falciparum* membrane protein has already been shown: the protein PfEMP-1 remains peripherally membrane-associated throughout the *P. falciparum* secretory pathway and only becomes transmembrane in the erythrocyte²⁴. But conventional transmembrane proteins, i.e. proteins that become membrane-integrated in the parasite ER membrane, with PEXEL signals also exist²⁵. The biogenesis of a soluble PEXEL protein has not been studied in similar detail to date.

The key: PI3P in the ER

Recruitment of PEXEL proteins to specific locations within the ER membrane shows interesting parallels to autophagosomal

membrane formation at the ER membrane^{21,22}. As mentioned above, the only known example of PI3P occurring in ER membranes is during the induction of autophagosome formation²². Here the Atg14L protein recruits the Vps34 PI3-kinase to the cytosolic face of ER membranes; during amino acid starvation this leads to the formation of membrane patches or bulges, which seem to be attached to the ER and contain PI3P^{21,22}. These patches recruit proteins with PI3P-binding domains, promoting formation of so-called omegasomes, which are invaginations into the ER that ultimately lead to pinching off of a crescent-shaped membrane structure^{21,22}. The mechanism responsible for omegasome formation is not understood^{21,22,26}. In order to be recruited to the right place, the proteins binding to PI3P at the cytosolic face of the ER membrane have to contain, in addition to their PI3P-binding domains, a not yet characterized ER-targeting signal²¹.

So maybe during erythrocyte invasion, which is controlled by IP3 signalling and calcium release from the ER²⁷, a PI3-kinase is recruited to or activated at the *P. falciparum* ER. This PI3-kinase generates PI3P in patches localized in the cytoplasmic leaflet of the ER membrane in proximity to the protein translocation channel (Sec61 channel) or the SRP receptor (Figure 2B). The signal peptide of soluble PEXEL proteins promotes their targeting to the ER membrane where the signal peptide inserts into the Sec61 channel (Figure 2B). The as yet unexplained long N-terminal extensions of many of the PEXEL protein signal peptides may lead to their inefficient cleavage by signal peptidase in the ER lumen, similar to delayed signal sequence cleavage in bacterial autotransporters with long N-terminal extensions²³. Delayed cleavage may be caused by the extension preventing reorientation to an N-cytoplasmic/C-lumenal topology in the protein translocation channel as shown in Figure 2B²⁸. Alternatively, the N-terminal signal peptide extension may interact with a cytoplasmic domain of the Sec61 channel, which in turn may interfere with signal peptidase access to the cleavage site in the ER lumen (topology shown in Figure 2A). At least some of the N-terminal hydrophobic regions of PEXEL proteins also simply do not contain signal peptidase cleavage sites⁶. Delayed signal cleavage will lead to prolonged residence of the nascent PEXEL protein in the Sec61 channel, which in turn would allow interaction of the still cytosolic PEXEL signal with PI3P in the cytosolic leaflet of the ER membrane (Figure 2B). This interaction keeps the protein from entering the ER and thus the conventional secretory pathway. It may also generate a protein conformation recognized by the cytosolically located active site of plasmepsin V and result in cleavage of the PEXEL signal, which liberates the protein from the Sec61 channel, and aborts translocation into the ER (Figure 2B). Plasmepsin V might itself be a PI3P-binding protein, or interact with the Sec61 channel. After cleavage the cytosolic NatD complex would acetylate the new N-terminus (Figure 2B). Importantly, the interaction of the PEXEL protein with plasmepsin V leads to a handing over of the cleaved protein to a receptor protein (or complex) in the PI3P-patch (Figure 2B).

Transmembrane PEXEL proteins are similarly targeted to the Sec61 channel in the ER membrane, but released laterally into the lipid bilayer which allows their cytosolically exposed PEXEL sequence to bind to PI3P and be recruited into the patch (Figure 2B). In this case the topology of the PEXEL/PI3P interaction may prevent cleavage by plasmepsin V. That transmembrane proteins can be

recruited to PI3P in the ER membrane has also been shown during autophagosomal membrane formation at the ER²¹. One or more of the transmembrane PEXEL proteins may form the receptor in the PI3P patch for soluble plasmepsin V-cleaved PEXEL proteins (Figure 2B).

The way out

One option is that the PI3P patches and their associated proteins are simply packaged into a specific subset of ER-to-Golgi transport vesicles, and are then transported through the secretory pathway by a series of vesicle budding and fusion events (Figure 3, pathway A). After transport vesicle fusion at the plasma membrane, the plasma membrane then could either bud vesicles outwardly that subsequently fuse with the PVM (Figure 3), or there might be a transient fusion of parasite plasma membrane and PVM to transmit the proteins perhaps by interaction of the PI3P patch with a receptor in the PVM (Figure 3). Release of soluble proteins from the PI3P patch might be triggered by different (ion etc.) conditions in the erythrocyte cytosol. Membrane proteins would be transported by vesicular transport from the PVM to Maurer's clefts where their release from the PI3P patches could be triggered by a PI3P-phosphatase.

Alternatively, similar to what has been observed during autophagosomal membrane formation at the ER, the recruitment of specific proteins to the PI3P patch may lead to an invagination of the ER membrane, resulting in vesicles inside the ER containing the PEXEL proteins (Figure 3, pathway B). These proteins could then be transported through the secretory pathway as double membrane vesicles (DMVs) whose outer layer would ultimately fuse with the parasite plasma membrane (Figure 3, 3B). DMVs have been detected in electron micrographs of *P. falciparum*, and fusion with the parasite plasma membrane and vesicle release into the PV have been reported⁵. The released vesicles might subsequently be able to fuse with the PVM (Figure 3, 4B).

Either of the transport pathways depicted in Figure 3 would satisfy the Brefeldin A sensitivity of (at least some) protein transport into the erythrocyte^{29,30}. Either would explain how soluble and transmembrane proteins can be targeted into the host cell using the same signal^{3,4}. Both scenarios could also explain how proteins without a PEXEL signal or proteins without a signal sequence or transmembrane domain could end up in the erythrocyte: these proteins could be packaged into the PEXEL-protein containing vesicles by interaction with these on the cytoplasmic face of the ER membrane^{6,31}. Both hypotheses could also explain how the PEXEL signal leads to targeting to the erythrocyte even though the signal itself is cleaved already at the parasite ER¹³.

If the key decision - entry into the conventional secretory pathway or entry into a distinct export pathway that ultimately leads to arrival in the erythrocyte - is already made during PEXEL protein biogenesis at the parasite ER, this would explain why a protein that has been engineered to contain a conventional signal peptide and an N-terminus equivalent to the cleaved PEXEL signal ends up in the PV, not in the erythrocyte⁹. The construct with the conventional signal peptide is fully translocated into the secretory pathway, and hence separated from the PI3P patch-associated PEXEL proteins (Figure 2B). It will therefore, like conventional secretory proteins,

follow the classical secretory pathway and be secreted into the PV, from which there seems to be no direct access into the erythrocyte.

That this is true is also confirmed by a carefully done set of experiments by Gehde and colleagues (2008)³². The authors aimed to investigate whether protein folding has an effect on PEXEL protein access to the erythrocyte. They generated fusion proteins that contained the signal peptide and PEXEL region from either the transmembrane protein STEVOR or the soluble protein GBP130 fused to dihydrofolate reductase (DHFR), followed by green fluorescent protein (GFP) and expressed these fusion proteins in *P. falciparum*. They found that in the absence of folate analogues (which promote DHFR folding) these constructs were targeted to the erythrocyte. In the presence of folate analogues, the constructs were found in the PV. Strikingly, only newly synthesized proteins could be transported into the erythrocyte, i.e. it was impossible to chase pre-existing fusion proteins from the PV into the erythrocyte after washout of the folate analogue. The authors' interpretation of the data was that proteins must be unfolded in order to be transported across the PVM into the erythrocyte, that the PVM therefore contained a protein-conducting channel with similar requirements for transport as the Sec61 channel in the ER membrane, and that the time window after synthesis during which proteins were transport-competent was limited.

The scenario depicted in Figure 2B suggests a different interpretation of the data. Immediately after targeting of a PEXEL protein to the Sec61 channel in the ER membrane, there is a competition between full translocation of the fusion protein into the ER lumen and binding of the PEXEL region to PI3P on the cytoplasmic leaflet of the ER membrane, which ultimately leads to an abortion of translocation. At this stage the signal peptide and PEXEL regions of the fusion protein are already synthesized, but the ribosome is still associated with the nascent chain and protein synthesis is still going on (between step 1 and step 2 in Figure 2B, not shown). When the DHFR part of the protein emerges from the ribosome, it is initially located in the ER lumen. In the absence of the folate analogue, the DHFR chain remains sufficiently flexible for the PEXEL signal to interact with PI3P, plasmepsin V cleavage occurs, and translocation is aborted; as a result the fusion protein remains in the cytosol associated with the PI3P patch as in Figure 2B. In the presence of a folate analogue, DHFR will fold tightly in the ER lumen during its synthesis and this will interfere with or override the interaction of PEXEL with PI3P in the cytoplasm. As a result the fusion protein will be fully translocated and end up inside the secretory pathway. That folding accelerates translocation into the ER has been shown³³. If protein folding in the ER lumen and PI3P-binding at the cytoplasmic leaflet of the ER membrane interfere with each other this might also explain why some of the PEXEL proteins contain large intrinsically unstructured regions³⁴.

On the whole the model depicted in Figure 2B and Figure 3 makes sense of the vast majority of the available data on trafficking of proteins from *P. falciparum* into the host cell and suggests that the decision of where to go is made early during biogenesis of exported proteins at the parasite ER membrane. My hypothesis has a number of easily testable elements that might give the research in this field the appropriate direction for a full understanding of the mechanism of protein export from *P. falciparum* into the erythrocyte.

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Tobias Spielman

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This is a very thought provoking review on protein export in the malaria parasite *Plasmodium falciparum*. The proposed model is centered on the fact that in other systems, N-terminal acetylation of proteins is found in the cytosol, not the ER lumen.

If this is adopted for *P. falciparum*, it literally turns the current model for protein export inside out: instead of the previously assumed binding of the PEXEL export motif to PI3P and subsequent processing of the PEXEL within the ER, cytosolic acetylation of the processed N-terminus would place these events to the outside of the ER. As these events are considered to be the key requirements for export, this is of great importance to understand protein export in malaria parasites.

The review then draws on previously published models for vesicular trafficking in *P. falciparum* and autophagy-related processes to propose pathways for the de-PEXEled protein on the outer ER leaflet to reach the host cell. For instance this would make possible a previously considered elegant model (REF 7) of serial events of vesicular budding and fusion leading to delivery of the protein into the host cell without direct crossing of any membranes.

Overall this is a well written review with a clear hypothesis that adds an important new angle for the field to consider. Although the proposed model fits a lot of the data on protein export, there are also some discrepancies that become obvious when delving into the details. There are a number of points I would like to raise for discussion:

- The explanation why the mDHFR fusion constructs (REF 32) do not challenge the hypothesis (page 5, third and fourth paragraph) is difficult to understand. Is it assumed that the mDHFR domain is in the ER lumen while the PEXEL binds to the outside of the ER and that this binding would be able to rescue the unfolded domain but not the folded version out of the ER? In this case it is not entirely clear how this fits into the model for translocation of proteins into the ER. A graphical explanation of the topology of this situation with the mDHFR fusion protein and how it can ensue would be helpful. This is important, as this part of the review is central to discounting the prominently proposed protein translocation at the PVM (REF 14). As an aside on this topic:

while it is true that functional evidence for the PVM translocon is lacking, it may be worth noting that there is some earlier evidence for ATP-dependent translocation of an exported protein across the PVM (Ansorge et al., 2006; Biochem J).

- Boddey et al. (REF 8) carried out a detailed analysis of 2 proteins with mutated PEXELs. For one of these proteins they found a signal peptidase-cleaved species that was retained in/at the ER. Despite an R->A mutation in the PEXEL (position 1) this protein was N-acetylated. Thus, in this case acetylation seemed independent of PEXEL mediated PI3P binding and PMV cleavage. As the signal peptide is usually cleaved during translocation, this could also indicate N-acetylation after ER entry. For the second protein, the PEXEL cleaved E->A mutation (position 5) was analysed. This protein was found in the PV despite being N-acetylated. This would even more strongly argue that protein entering the ER can still be acetylated. Finally, N-acetylation also occurred with the signal peptidase-cleaved mutated PfEMP3 trafficked to the PV (REF 9), a construct mentioned in this review as an example not entering the proposed pathway for export. These findings seem to be at odds with the proposed model.

- The data on the exact localisation of plasmepsin V, while not exactly excluding the possibility suggested in this review (page 2), could as well indicate a localisation within the ER. If I interpret the images from the episomally expressed constructs from REF 10 correctly, then the signal peptide mediates (and is sufficient for) PV targeting and thus also for ER entry. This would indicate a failure of ER retention rather than lack of ER targeting in the mutants lacking the transmembrane domain. The conclusion here that this data 'demonstrates that it is not a signal sequence' may therefore not be correct. It is also not clear why PMV would possess an N-terminal hydrophobic region at all if it is targeted by its C-terminal hydrophobic region (*P. falciparum* contains components for the insertion of tailanchored proteins which do not require a signal peptide).

- The explanation for PEXEL negative protein (PNEP) export is a bit weak (page 5, first paragraph). Binding of PNEPs to PEXEL proteins as a means for export could occur in most models for export and is no particular feature of the alternative proposed here. In fact, for this model, I would expect PNEPs not to require a hydrophobic region at all.

- As a general consideration. If N-acetylation in *Plasmodium* parasites is expected to strictly follow the current cell biological concepts from other systems, then this should equally be considered for the vesicle trafficking needed as a result of this (i.e. to make possible the export of a protein on the outer face of the ER). In particular this concerns how proteins get across the PVM. There is no tangible evidence for the presence of the components known to mediate vesicular trafficking (e.g. coats, SNAREs, rabs etc) beyond the parasite plasma membrane (absence of evidence of course is not necessarily evidence for absence, but in contrast many classical components in anterograde and retrograde vesicular trafficking seem to be present within the parasite, see e.g. Deponte et al., MBP 2012). How the vesicle trafficking necessary to suit the model is achieved may therefore be a similarly (or even more) enigmatic cell biological problem than how proteins could be acetylated within the ER.

- Although various types of vesicles have been documented in the host cell (on the morphological level, directed trafficking has not been demonstrated), I am unaware of any report convincingly showing vesicles in the PV. The close proximity of the PPM and PVM seem to make vesicle trafficking not the most suitable way for this. Is there any precedent for vesicular trafficking in

such a situation?

- Although an opinion article, alternatives to cytoplasmic acetylation could perhaps be discussed. Possibly there still is an unknown N-terminal acetylation activity in the ER of Plasmodium parasites (for instance there is one GNAT domain containing protein, PF14_0350, with 2 hydrophobic segments). Or, at least as a formal possibility, can it be entirely excluded that the observed N-acetylation of processed PEXEL proteins is non-physiological and occurs during affinity purification of these proteins from parasite extracts? This would require posttranslational N-terminal acetylation activities which, at least in yeast, seem to exist (Helsens et al., 2011 J Proteome Res).

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 20 Nov 2012

Karin Romisch, Department of Microbiology, Faculty of Medicine, Saarland University, Germany

Dear Dr. Spielmann, thank you very much for your careful review of my opinion article. I will answer your questions in the order you raised them:

You understood correctly what I was saying about the methotrexate-mediated DHFR folding in the ER lumen competing with PEXEL binding to PI3P and plasmepsin V on the cytoplasmic face of the ER membrane. I had considered adding a drawing to the paper, but in the end I decided against it, because I felt it would distract the readers from the central issues I discuss. The paper you mention ([Ansorge et al., Biochem J 1996](#)) describes elegant work from the Lingelbach lab in which they showed ATP-dependent release of GBP from streptolysin O-permeabilized infected erythrocytes. During the permeabilization, the vacuolar membrane stays intact and GBP remains protease-protected. Upon addition of ATP and cytosol GBP is released from the PV and becomes protease-accessible. This could either be ATP-dependent translocation of GBP across the PVM, or it could be ATP-induced fusion of GBP-containing vesicles with the PVM; the data is compatible with either model.

The N-acetylation of signal-peptide cleaved proteins is indeed puzzling, and I do not have a physiological explanation for it. As you point out, one possibility is that this is a post-lysis artefact and therefore not relevant for the topology of the targeting of proteins exported from *P. falciparum* to the host cell. Topology predictions for the transmembrane GNAT-domain protein you mention are contradictory using several programmes, so it is unclear which side of the membrane the active site of the protein would be. N-acetylation within the secretory pathway, however, would also require import of Acetyl-CoA across a membrane (ER, Golgi) and a transporter. Is there such a protein? – It also remains unclear whether N-acetylation is actually part of the export signal for erythrocyte targeted proteins. Given that there is no conservation of the residue which is modified in the cleaved PEXEL (the x before the E/Q), it seems that this modification may not be important which is why I prefer not to expand this part of my review.

Thank you very much for pointing out my failure to understand the data characterizing the function of the hydrophobic N-terminal domain of plasmepsin V in [Russo et al., Nature 2010](#). To me the images of the YFP staining and the SP-YFP staining looked fairly similar and I took the text in the paper “the transmembrane domain, but no other portions of plasmepsin V were sufficient to target a reporter to the ER” to mean YFP and SP-YFP were both cytosolic and remained inside the parasite. But I agree if you blow up the images, the hydrophobic region at the N-terminus fused to YFP results in ring-shaped staining around the cytoplasm which could be the PV, or the parasite plasma membrane. I will modify this part of the text in the next version of my review. This does not, however, alter my prediction that the active site of plasmepsin V will be in the cytoplasm. The N-terminal hydrophobic domain does not have a signal peptide cleavage site, so it is likely a transmembrane domain, and both TMHMM and TopPred predict it to insert with the N-terminus in the ER lumen such that the subsequent soluble region containing the active site would be in the cytoplasm. But this is all *in silico*, and since it is a really important issue, I sincerely hope that somebody will characterize the actual topology of the enzyme in the ER membrane biochemically.

Regarding vesicles in the PV and vesicle fusion in the absence of SNAREs etc.: If these vesicles were present in the PV only transiently and given the very narrow lumen of the PV it would be exceedingly difficult to capture images of such vesicles by electron microscopy unless their fusion were inhibited by some means. Perhaps they could be accumulated in SLO-permeabilized infected erythrocytes as in [Ansorge et al., 1996](#). There are examples of vesicle fusion in the absence of the conventional eukaryotic fusion machinery: gram-negative bacteria can shed outer membrane derived vesicles which are subsequently either taken up by or fuse with eukaryotic cells. In many, but not all, cases these vesicles contain pathogenicity factors. It is conceivable that transport vesicles containing host cell-targeted *P. falciparum* proteins fuse with the PVM (which is derived from the erythrocyte plasma membrane) in a manner similar to these bacterial vesicles. The ATP-dependent vesicle fusion observed by [Ansorge et al, 1996](#), may actually be mediated by the ‘translocation complex’ isolated by Cowman’s lab which contains a protein with homology to a bacterial pore-forming protein.

One final argument against the lumen of the ER being involved in the biogenesis of proteins exported into the erythrocyte is the apparent absence of essential elements of the Unfolded Protein Response (UPR) in *P. falciparum*. The UPR is switched on in all eukaryotes under conditions that require an expansion of protein secretory capacity (e.g. insulin secretion in the pancreas, or maturation of plasma cells for antibody production) and is essential under those circumstances. One would expect *P. falciparum* to have to expand its secretory capacity when upon invasion of an erythrocyte it suddenly has to secrete hundreds of proteins into the host cell. In yeast, the UPR is mediated by a single transmembrane protein in the ER, Ire1, which upon accumulation of misfolded proteins oligomerizes and splices the mRNA for a transcription factor, Hac1, which then activates transcription of ER chaperones etc. This branch of the UPR is highly conserved in eukaryotes. Mammals have two further UPR sensors in the ER, PERK and ATF6. The last time I looked (ca 2006) in the *P. falciparum* genome there were no ORFs with significant similarity to the central UPR transcription factor Hac1 or its mammalian orthologue XBP-1, or ATF6, and only proteins with weak similarity to Ire1 and PERK. So either *P. falciparum* has evolved a novel way to adapt to increase of the load on the secretory pathway, or it does not experience much variation in

the flux through the ER, because it does not use the conventional secretory pathway for secretion of host cell targeted proteins.

Competing Interests: No competing interests were disclosed.

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Norma W. Andrews

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This is a very interesting and compelling article. In a simple and extremely clear style Karin Romisch does a beautiful job placing protein export by intracellular malaria parasites into a solid cell biological context.

She corrects important topological misconceptions (unfortunately common in the parasitology/infectious disease fields), acknowledges other authors with similar ideas, and overall makes a crystal clear and important contribution to our understanding of one of the most fascinating problems in protein transport to emerge in recent years.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Malcolm McConville

Department of Biochemistry and Molecular Biology, The University of Melbourne, Melbourne, Australia

The author proposes a new model for how proteins in intraerythrocytic stages of the malarial parasite could be transported from the parasite endoplasmic reticulum to the host cell cytoplasm.

The new model is compatible with much of the existing Plasmodium protein export literature and incorporates additional mechanistic insights from other protein trafficking process. In particular, a compelling case is made for key processing and transport steps in Plasmodium protein export occurring on the cytoplasmic leaflet of the ER and transport vesicles based on the known topology of phosphatidylinositol-3-phosphate and protein N-acetylation reactions, both of which have been shown to be involved in plasmodium protein export. Another major feature of this model is that exported soluble and membrane proteins are transported from the parasite plasma membrane to the parasitophorous vacuole via membrane vesicles. The internalization of proteins into transport vesicles draws on precedents observed during autophagy and is plausible, but raises important questions about the role of a parasite-derived multi-protein complex in the PV membrane that is thought to translocate parasite proteins across this membrane.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Comments on this article

Version 1

Author Response 16 Nov 2012

Karin Romisch, Department of Microbiology, Faculty of Medicine, Saarland University, Germany

Dear Jude,

I am aware that your chimera made of the STEVOR signal peptide & PEXEL region (STEVOR1-80) fused to GFP followed by a KDEL ER-retrieval sequence ends up in the parasite ER. I am not convinced, however, that this result tells you that the export route of PEXEL proteins into the erythrocyte is inside the conventional secretory pathway: The fluorescent images in Fig 2 of your paper ([Przyborski et al. 2005](#)) look as if there might be 2 populations of the STEVOR(1-80)-GFP, one that is targeted into the ER and ends up in the parasitophorous vacuole, and a second one that ends up in the erythrocyte. That the erythrocyte targeted population increases as the STEVOR part of the chimera lengthens could be due to the fact that this interferes increasingly with import of the chimera into the ER lumen, which will allow GFP to fold on the cytoplasmic face of the ER membrane, and the very compact folding of GFP will contribute to keeping the construct in the cytosol. Peptide fusions to the C-terminus of GFP can interfere with GFP folding (e.g. [Nicholls SB & Hardy JA 2012](#)). So an alternative interpretation of the STEVOR(1-80)-GFP-KDEL localization is that here the KDEL at the C-terminus prevents GFP from fully folding and that this increases the translocation efficiency of the chimera into the ER. Once inside the parasite secretory pathway the protein then encounters the KDEL receptor and is retained there.

Competing Interests: No competing interests were disclosed.

Reader Comment 02 Nov 2012

Jude Przyborski, Parasitology, Philipps University Marburg, Germany

An interesting article that expands on our previous hypothesis ([ref 7](#)). We have since rejected our own original model for several reasons. One major reason was evidence that parasite proteins do actually enter the ER lumen. We tagged an exported protein with a C-terminal -S/KDEL ER retrieval motif. This caused a solely ER localisation of the reporter molecule. This can only be explained if the C-terminus of the protein is within the secretory system and able to contact ERD2. Thus, exported proteins must enter the ER.

References as follows:

Przyborski JM, Miller SK, Pfahler JM, Henrich PP, Rohrbach P, Crabb BS, Lanzer M. Trafficking of STEVOR to the Maurer's clefts in Plasmodium falciparum-infected erythrocytes. EMBO J. 2005 Jul 6;24(13):2306-1

Külzer S, Gehde N, Przyborski JM. Return to sender: use of Plasmodium ER retrieval sequences to study protein transport in the infected erythrocyte and predict putative ER protein families. Parasitol Res. 2009 Jun;104(6):1535-41.

Competing Interests: No competing interests were disclosed.

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