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Uncovering Common Principles in Protein Export of Malaria Parasites

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SUMMARY

For proliferation, the malaria parasite *Plasmodium falciparum* needs to modify the infected host cell extensively. To achieve this, the parasite exports proteins containing a *Plasmodium* export element (PEXEL) into the host cell. Phosphatidylinositol-3-phosphate binding and cleavage of the PEXEL are thought to mediate protein export. We show that these requirements can be bypassed, exposing a second level of export control in the N terminus generated after PEXEL cleavage that is sufficient to distinguish exported from nonexported proteins. Furthermore, this region also corresponds to the export domain of a second group of exported proteins lacking PEXELs (PNEPs), indicating shared export properties among different exported parasite proteins. Concordantly, export of both PNEPs and PEXEL proteins depends on unfolding, revealing translocation as a common step in export. However, translocation of transmembrane proteins occurs at the parasite plasma membrane, one step before translocation of soluble proteins, indicating unexpectedly complex translocation events at the parasite periphery.

INTRODUCTION

Malaria remains a major burden in developing countries (World Health Organization, 2011). In infected people, an asymptomatic initial replication of *Plasmodium* parasites in liver cells is followed by continuous asexual multiplication within red blood cells (RBCs) that leads to the clinical symptoms of malaria (Miller et al., 2002). The highly differentiated RBC requires extensive modifications by the parasite to support its proliferation. For this remodeling, many parasite proteins are exported into the host cell, where they reside in the cytosol, in the RBC membrane, or in parasite-induced vesicular cisternae in the host cell, termed Maurer's clefts, which have been implicated in protein trafficking to the host-cell surface (Maier et al., 2009; Tilley et al., 2008). Maurer's clefts are generated by an unknown mechanism and are detectable soon after the invasion of the parasite into the RBC. Once these are established, no new clefts are formed during further parasite development within the host cell (Grüring et al., 2011). So-called ''tethers'' attach the Maurer's clefts to other structures in the host cell such as the RBC membrane (Pachlatko et al., 2010), but there is no lipid continuum between individual Maurer's clefts and other parasite or host-cell membranes (Hanssen et al., 2008).

A five-residue export motif with the consensus RxLxE/Q/D called PEXEL (*Plasmodium* export element) or VTS (vacuolar transport signal) mediates the export of both soluble and transmembrane (TM) parasite proteins into the host RBC (Hiller et al., 2004; Marti et al., 2004). Most PEXEL proteins possess a recessed N-terminal signal peptide, followed by the PEXEL motif 20–30 amino acids further downstream. The PEXEL was reported to bind phosphatidylinositol-3-phosphate (PI3P) in the parasite's endoplasmic reticulum (ER) (Bhattacharjee et al., 2012). Also in the ER, the PEXEL is cleaved after the leucine residue by the protease plasmepsin V, leading to an N terminus that starts with xE/Q/D (henceforth termed ''mature N terminus'') (Boddey et al., 2009; Boddey et al., 2010; Chang et al., 2008; Osborne et al., 2010; Russo et al., 2010). Thus, the mature N terminus contains only the last of the conserved PEXEL residues (PEXEL position 5). Both PI3P binding and plasmepsin V cleavage are believed to be decisive for export, but they occur within the parasite, and it remains unclear how further export of the mature protein is mediated. PEXEL position 5 was shown to have a role in export in the mature N terminus (Boddey et al., 2009), but the possibility that this is due to its contribution to PI3P binding before PEXEL cleavage cannot be ruled out (Bhattacharjee et al., 2012). It is also unclear how this single residue alone would provide specificity, as the region after the PEXEL is believed to hold little export-relevant sequence

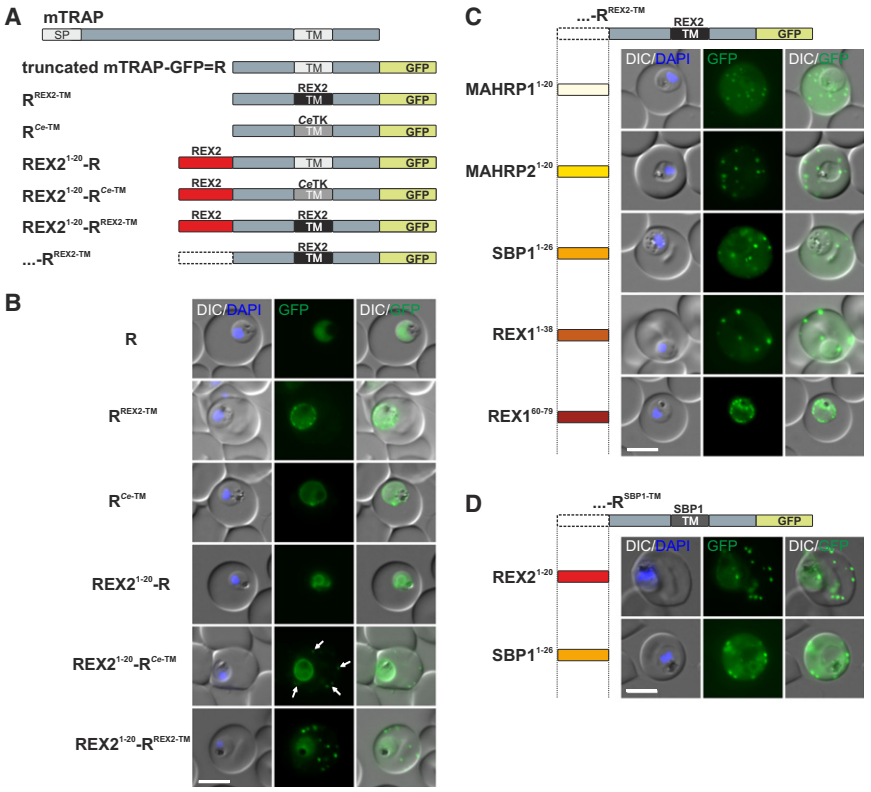

Figure 1. N Termini of PNEPs Are Sufficient for Export of $R^{REX2\text{-}TM}$

(A) Schematic of mTRAP fusion constructs.

(B) Representative images of live *P. falciparum* parasites expressing the constructs shown in (A). DIC, differential interference contrast; nuclei were stained with DAPI. Arrows indicate limited staining reminiscent of Maurer's clefts.

(C and D) Images of live *P. falciparum* parasites expressing $R^{REX2\text{-}TM}$ (C) and $R^{SBP1\text{-}TM}$ (D) fused with the PNEP N termini indicated. Panels are as in (B). Size bars represent 5 μm. The mTRAP backbone, different TMs, and the SP (signal peptide) are shown in different shades of gray; N-terminally appended regions of PNEPs are shown in shades from yellow to dark red. *Ce*-TM, *C. elegans* TK TM. See also Figure S1.

information other than being required as a spacer for spatial separation of the PEXEL from a folded domain, such as green fluorescent protein (GFP) (Bhattacharjee et al., 2006; Knuepfer et al., 2005; Przyborski et al., 2005).

In the RBC, the parasite develops in a compartment formed by a parasitophorous vacuole membrane (PVM). Exported parasite proteins therefore have to get past both the parasite plasma membrane (PPM) and the PVM to reach the host cell. Soluble PEXEL proteins were reported to cross the PVM through a translocon (de Koning-Ward et al., 2009). This is in agreement with the observed need for protein unfolding of these proteins at this step (Gehde et al., 2009). However, although a PVM translocon is a supposable gate for soluble proteins, it is unclear how TM proteins fit into this model.

A second group of exported proteins does not contain a PEXEL (Spielmann and Gilberger, 2010). These PNEPs (PEXEL-negative exported proteins) include REX1 and REX2, MAHRP1 and MAHRP2, and SBP1. All of these proteins localize to the Maurer's clefts (Blisnick et al., 2000; Hawthorne et al., 2004; Spielmann et al., 2006; Spycher et al., 2003) or, in the case of MAHRP2, to the Maurer's-cleft-associated tethers (Pachlatko et al., 2010). In contrast to PEXEL proteins, PNEPs do not contain a signal peptide but contain a single hydrophobic region found up to 214 amino acids downstream of the N terminus that is a TM in instances such as REX2 (Haase et al., 2009; Spielmann et al., 2006). Trafficking studies with different PNEPs implicated various regions in export, giving a heterogeneous picture of PNEP export (Dixon et al., 2008: Haase et al., 2009; Pachlatko et al., 2010; Saridaki et al., 2009; Spycher et al., 2006).

Here we provide evidence for similarities both in export domains and trafficking pathways of PNEPs and PEXEL proteins. Importantly, we show that TM proteins require unfolding for export, indicative of translocation events. Our data provide a mechanistic solution to the question of how TM proteins are exported. Furthermore, it links PNEP and PEXEL export and suggests a general framework for the export of different groups of proteins in malaria parasites that is characterized by vesicular trafficking to the parasite periphery followed by translocation into the host cell.

RESULTS

A Reporter for Identification of Sequences Sufficient for Mediating Export

We have previously used an N-terminally truncated version of the nonexported micronemal protein mTRAP (Baum et al., 2006) fused to GFP to show that the N terminus and the TM of the PNEP REX2 together are sufficient to mediate export of a protein in *P. falciparum* (Haase et al., 2009). To validate the mTRAP reporter for additional export studies, we generated a series of control constructs (Figure 1A). First, we confirmed that the unmodified truncated mTRAP fused to GFP (henceforth termed "R" for "reporter") does not contain any export-relevant sequences. Indeed, this protein was not exported but was found evenly distributed in the parasite cytosol (Figure 1B). When the mTRAP TM in R was replaced with that of the PNEP REX2 or a previously used heterologous TM of a *Caenorhabditis elegans* tyrosine kinase (TK) (a TM that does not promote export of REX2 and does not change topology; Haase et al., 2009), the R then entered the secretory pathway and was found in the parasite periphery (PPM, PV [parasitophorus vacuole], or PVM) but was not exported (Figure 1B). Thus, although the endogenous mTRAP TM does not promote ER entry, other TMs do.

Second, we tested the influence of the first 20 amino acids of REX2 fused N-terminally to R containing each one of the three different TMs used above. Neither the construct with the

endogenous mTRAP TM (REX2$^{1–20}$-R) nor the one with the *C. elegans* TK TM (REX2$^{1–20}$-R$^{Ce\text{-}TM}$) was exported, although the latter showed some leakiness of the phenotype, concordant with previous observations when this TM was used in REX2 (Haase et al., 2009) (Figure 1B). As expected, the construct with the REX2 TM (REX2$^{1–20}$-R$^{REX2\text{-}TM}$) was exported (Figure 1B; Haase et al., 2009). Of note, cells with a smooth pattern and cells with a necklace-of-beads pattern were both observed in all cell lines displaying fluorescence in the parasite periphery.

Interestingly, REX2$^{1–20}$-R was mainly found in the nuclear periphery, indicating an ER localization. Thus, the REX2 N terminus, although not resembling a signal peptide, has some propensity to guide the otherwise cytosolic R to the ER.

In conclusion, truncated mTRAP, if guided into the ER by a suitable TM, follows the previously shown default route to the PV or PVM (Waller et al., 2000) and is not exported. It therefore represents a neutral system for testing the capacity of sequences to promote export. Moreover, these results show that neither the REX2 N terminus nor its TM alone is sufficient to export the mTRAP reporter, demonstrating the need for both domains in PNEP export.

PNEP N Termini Promote Export

Based on the findings with REX2 (Figures 1A and 1B; Haase et al., 2009), we tested whether PNEP N termini universally mediate export. We therefore appended the N-terminal region of well-established PNEPs to R$^{REX2\text{-}TM}$ (Figure 1C). All of these N termini (REX1 amino acids [aa] 1–38, SBP1 1–26, MAHRP1 1–20, and MAHRP2 1–20) promoted export of the R$^{REX2\text{-}TM}$ reporter and displayed Maurer's clefts staining, as well as a uniform staining in the host-cell cytosol (Figure 1C). Localization of Maurer's clefts was confirmed by immunofluorescence assays (Figure S1 available online). In contrast, a control construct containing the region immediately downstream of the REX1 hydrophobic stretch, a sequence previously found to be essential for export of this protein (Dixon et al., 2008), was not sufficient to mediate export. It led to accumulation of the reporter in the parasite periphery (Figure 1C).

All PNEP N termini tested were sufficient for promoting export of our reporter, suggesting common principles in the export of PNEPs. Export was also maintained if a different PNEP TM (SBP1) was used in the reporter (tested for the REX2 and SBP1 N termini, Figure 1D). Interestingly, only the constructs containing the REX2 N terminus showed exclusive Maurer's clefts staining, whereas all other exported constructs also showed a soluble pool in the host cell, and this was independent of the type of PNEP TM (REX2 or SBP1) in the reporter (Figures 1C and 1D).

The Mature N Terminus of PEXEL Proteins Is Sufficient for Promoting Export of R$^{REX2\text{-}TM}$

We previously hypothesized that in PEXEL proteins, after being processed in the ER, the mature N terminus might be functionally equivalent to the N termini of PNEPs (Spielmann and Gilberger, 2010). To test this, we appended the first 20 amino acids of the mature N terminus of either of two soluble PEXEL proteins, GBP (aa 87–106) or PfEMP3 (aa 63–83), N-terminally to the R$^{REX2\text{-}TM}$ reporter (Figure 2A). In a third construct, we used the mature N terminus (aa 44–63) of the TM PEXEL protein STEVOR (PFF1550w) in similar manner. Although these N termini contain only the last of the conserved PEXEL residues (PEXEL position 5) and the nonconserved position 4, they promoted export of the reporter into the host cell (Figure 2A). GFP fluorescence was detected in the erythrocyte cytosol and the Maurer's clefts. Similarly to the results with the PNEP N termini (Figure 1B), export depended on the presence of a PNEP TM, in that a construct containing the *C. elegans* TK TM was not exported (Figure 2B). These data show that the full PEXEL motif is not necessary for export if the reporter contains a PNEP TM. The mature PEXEL N termini therefore appear to fulfill a comparable role in export to the N termini of PNEPs.

In contrast, the region downstream of a PEXEL-like export signal from oomycetes previously shown to promote export in *P. falciparum* (Bhattacharjee et al., 2006) failed to direct the reporter into the host cell and resulted in a localization in the parasite periphery (Figure 2A). Thus, this region seems to differ functionally from that of PEXEL proteins, which might also be expected, considering data indicating that the oomycete signal is not cleaved in *P. falciparum* (Bhattacharjee et al., 2012).

N Termini of Nonexported Secretory Proteins Mimicking Mature PEXEL N Termini Fail to Promote Export

The well-established cleavage of the PEXEL motif in the parasite's ER (Boddey et al., 2009; Chang et al., 2008) raises the question of how the parasite distinguishes mature PEXEL proteins from nonexported secretory proteins that reveal xE/Q/D after signal-peptide cleavage (i.e., nonexported protein mimicking a mature N terminus of a PEXEL protein). Our data above highlight export-relevant information in the mature N termini. We hypothesized that this contributes to the distinguishing of exported from nonexported proteins. To test this, we used the 20 amino acids after the predicted signal-peptide cleavage site of two nonexported proteins: a soluble PV protein (SERA7) and an integral PVM protein (ETRAMP5). These N termini without signal peptide reveal xE or xQ, respectively, mimicking a processed PEXEL motif. N-terminal fusion of these sequences to R$^{REX2\text{-}TM}$ did not result in any export but in a localization at the parasite periphery (Figure 2C). Thus, E or Q in the second position (corresponding to PEXEL position 5) is not sufficient to mediate export, and downstream residues also play an important role. Moreover, the mature N terminus can be sufficient for discriminating exported from nonexported proteins.

In SERA7 and ETRAMP5, the residue before the E or Q (PEXEL position 4) was not typical for PEXEL proteins. Although this position is not conserved in the PEXEL motif, it usually consists of uncharged residues (Hiller et al., 2004; Marti et al., 2004). In two new constructs, we therefore replaced it with an alanine (Q23A in SERA7; D25A in ETRAMP5), a residue frequently found in this position of the PEXEL, resulting in the N termini AE and AQ, respectively. In the case of the SERA7 N terminus, this did not change the localization of R$^{REX2\text{-}TM}$, but in the case of ETRAMP5, this resulted in export to the Maurer's clefts and the host-cell cytosol (Figure 2C). These results indicate that the extreme N terminus resembling a cleaved PEXEL motif has some role in export, but, as demonstrated by SERA7, the downstream region can be equally important.

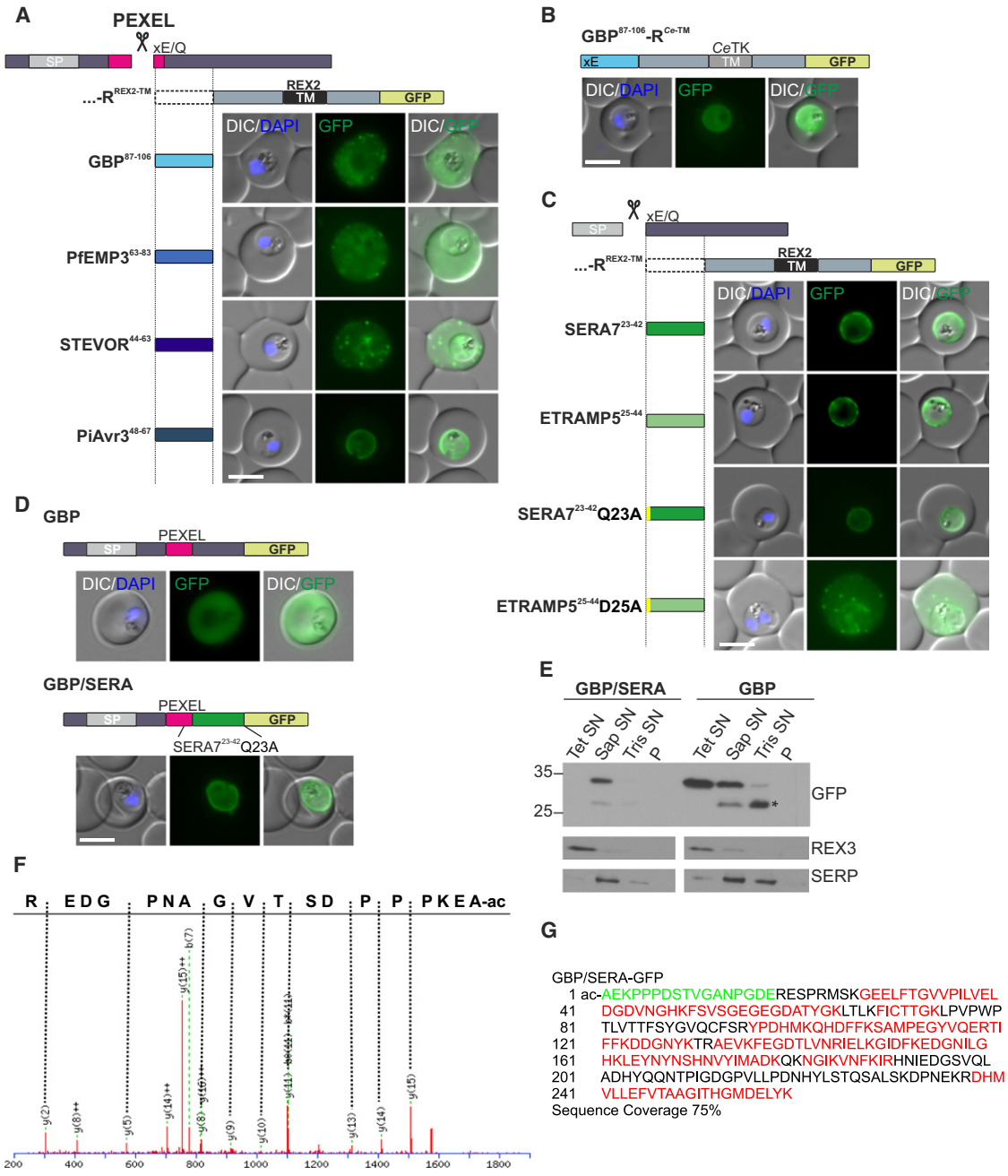

Figure 2. Mature PEXEL N Termini Promote Export of R$^{REX2-TM}$

(A–C) Images of live *P. falciparum* parasites expressing R$^{REX2-TM}$ fused with the mature N termini of PEXEL proteins (A), GBP$^{87–106}$-R^{Ce-TM} (B), or R$^{REX2-TM}$ fused with the mature N termini of nonexported secretory proteins (C). The position of the appended region in the original protein is shown above each panel.

(D) Images of live *P. falciparum* parasites expressing truncated GBP fused to GFP (GBP, top) or GBP-GFP with the mature N terminus of SERA7Q23A after the PEXEL (GBP/SERA, bottom).

(E) Western blot analysis using anti-GFP shows bands with the appropriate size for PEXEL cleavage and confirms that GBP/SERA is in the PV and GBP exported to the host cell. SN, supernatants of: Tet, tetanolysin (content of host-cell cytosol); Sap, saponin (PV content); Tris, hypotonic lysis (soluble content in the parasite); and P, pellet (final pellet). REX3, soluble parasite protein in the host cell; SERP, soluble PV marker. Asterisk, degradation product.

(F) MS-MS fragmentation spectrum of one species of the most N-terminal detected peptide of GBP/SERA after trypsin digestion. The x axis shows the mass (m/z); the y axis shows the intensity of the y and b ions.

(G) Peptides (red; N-terminal peptide in green) from GBP/SERA-GFP detected by MS. ac, acetylation.

Size bars represent 5 μm. Image panels are as in Figure 1B. PEXEL and signal-peptide cleavage sites are indicated by scissors; the PEXEL is in magenta, point mutations are in yellow, mature PEXEL N termini are in different shades of blue, and mature N termini of nonexported proteins are in green. See also Figure S2.

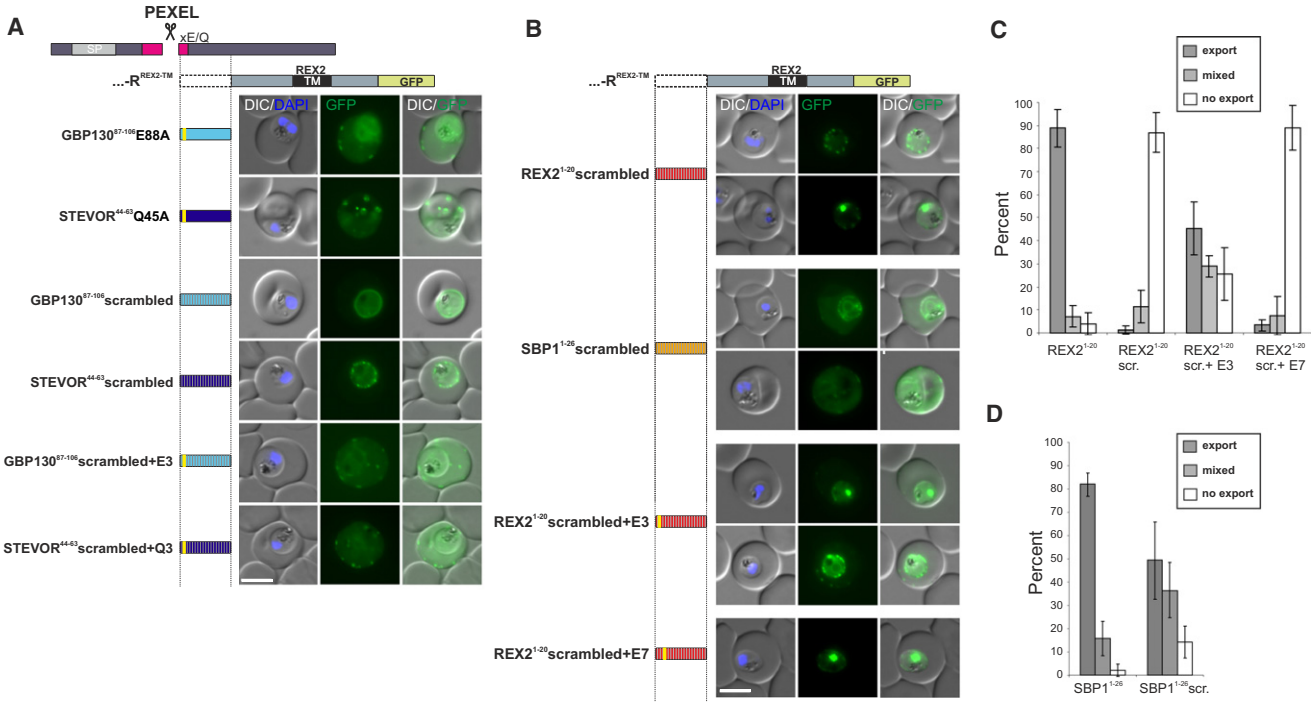

Figure 3. Sequences in N Termini Involved in Export

(A and B) Images of live *P. falciparum* parasites expressing $R^{REX2\text{-}TM}$ containing the mature PEXEL N termini (A) or PNEP N termini (B) that contain point mutations (yellow) or were scrambled (indicated by striated bars); colors of N termini are as in Figures 1 and 2. Size bars represent 5 μm.

(C and D) Comparison of export efficiency of $REX2^{1–20}$ and its modifications fused to $R^{REX2\text{-}TM}$ (C) or $SBP1^{1–26}$ and $SBP1^{1–26}$scrambled fused to $R^{REX2\text{-}TM}$ (D) by counting (blinded) the number of cells showing export only (export), export together with parasite periphery and/or internal fluorescence (mixed), or parasite periphery and/or internal fluorescence only (no export). Graphs represent countings of at least 80 cells on three different occasions; error bars represent SD. See also Figure S3.

Mature-N-Terminus-Based Discrimination for Export Still Occurs after Regular PEXEL Cleavage

Thus far, the data showing that the mature N terminus can discriminate between exported and nonexported proteins were based on our reporter. To confirm that the region downstream of the PEXEL influences export in a PEXEL protein, we inserted the mature N terminus of SERA7 containing the Q23A mutation into an established truncated version of the PEXEL protein GBP (Boddey et al., 2009). This restored a PEXEL identical to the one in the control construct containing unmodified truncated GBP; the two constructs (termed GBP/SERA and GBP, respectively) differed only in the 20 amino acids after the PEXEL. In contrast to the GBP control, the GBP/SERA hybrid was not exported but accumulated in the parasite periphery (Figure 2D). This was not due to failure of PEXEL cleavage in the GBP/SERA hybrid, because (1) the protein showed a similar migration to the GBP control (Figure 2E), and (2) cleavage of the PEXEL and N-terminal acetylation was confirmed by mass spectrometry (Figures 2F and 2G and Figure S2). Therefore, even after correct processing of a bona fide PEXEL and presentation of an N terminus starting with the typical AE, this does not overrule an export-refractory mature N terminus, and is thus not sufficient for export. This shows that the region downstream of the PEXEL is crucially important for export and validates the data obtained with our reporter approach.

Dissecting the Export Regions in Mature N Termini

To dissect the parts in the mature N terminus involved in export, we first mutated the second position (the last remaining conserved PEXEL residue) in the mature N termini of GBP and STEVOR to alanine and appended this region to $R^{REX2\text{-}TM}$. Unexpectedly, this had no detectable effect on export in both constructs (Figure 3A), suggesting that the conserved PEXEL residue remaining in the mature N terminus is not essential for export, and that the region downstream of this amino acid is important. The role of the downstream sequence was confirmed via random scrambling of the mature N termini of GBP and STEVOR (see Figure S3 for sequences), which abolished export. Finally, we changed the head group of the scrambled mature N termini to AE (GBP) or AQ (STEVOR) to artificially generate a typical mature PEXEL N terminus on the scrambled background (Figure 3A). In both GBP and STEVOR, this restored export (Figure 3A). Taken together, these data indicate that both the remaining PEXEL residues and the downstream region can influence export.

Next, we tested whether PNEP N termini behaved similarly. Scrambling of the REX2 N terminus appended to the $R^{REX2\text{-}TM}$ reporter abolished export (Figures 3B and 3C), whereas scrambling of the N terminus of SBP1 showed a more moderate reduction in export compared to the unscrambled SBP1 N terminus (Figures 3B and 3D). The quantification of export in these cell lines was done through counting (blinded) of the number of

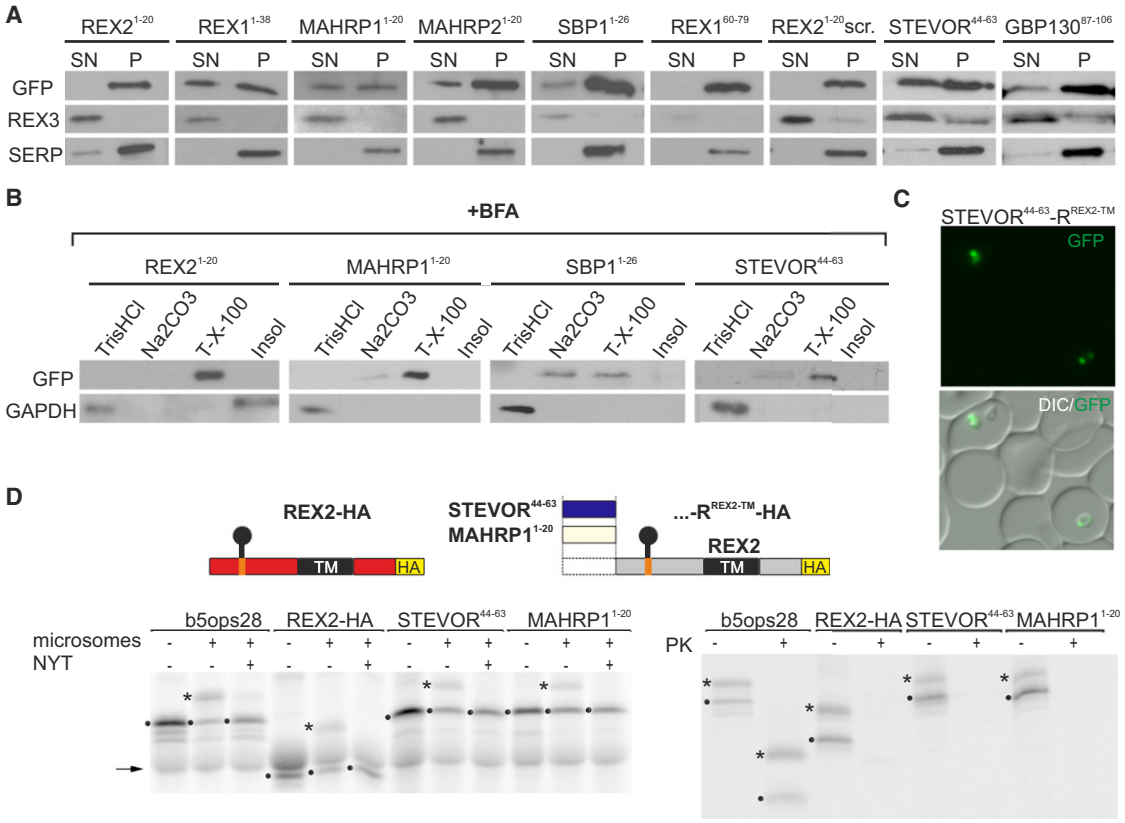

Figure 4. Solubility Shift during Export

(A) Immunoblots of cell lines expressing constructs with the indicated N termini fused to $R^{REX2\text{-}TM}$ after tetanolysin lysis and separation of the host-cell cytosol (SN) and pellet (P, containing parasites and Maurer's clefts). Two parasite internal chimeras ($REX1^{60–79}$ and $REX2^{1–20}$scrambled fused to $R^{REX2\text{-}TM}$) were analyzed as controls. Release of the host-cell cytosol was controlled with REX3, and integrity of the PVM was controlled using the PV protein SERP. See also Figure S4.

(B) Immunoblots of $R^{REX2\text{-}TM}$ constructs with the N termini indicated after BFA treatment and subjected to hypotonic (TrisHCl), carbonate (Na_2CO_3), and detergent (T-X-100) extraction. Insol, final pellet. Release of soluble protein was controlled using GAPDH.

(C) Example image of BFA treatment showing retention of $STEVOR^{44–63}$-$R^{REX2\text{-}TM}$ in the parasite (see Figure 2A for untreated parasites).

(D) In vitro translocation assays into microsomes with the constructs indicated at the top (glycosylation site indicated by a black ladle) and the control b5ops28 construct. NYT, tripeptide glycosylation inhibitor. Left panel: Autoradiography of total samples run on SDS-PAGE. The arrow on the left represents globin. Right panel: PK protection assay using anti-HA (except for b5ops28, wherein anti-opsin was used) to IP the protein either before (−PK) or after (+PK) digestion. Tail-anchored b5ops28 has the opsin tag at the C terminus that is translocated into the ER lumen. After PK digestion, a protected fragment, IPed by the opsin antibody, is generated. Asterisks and black dots indicate the glycosylated or nonglycosylated protein or protected fragments, respectively.

infected RBCs showing (1) full export, (2) no export, or (3) a mix of the two. Interestingly, many cells expressing the construct with the scrambled REX2 N terminus showed a single intense spot of fluorescence in the parasite in addition to the staining in the parasite periphery (Figure 3B) that may represent an intermediate export compartment or mistrafficking. We previously showed that E7 in the N terminus of REX2 is important for export and detected N-terminally processed forms of this protein that bring this residue into position 2 or 3 (Haase et al., 2009), creating an N terminus resembling mature PEXEL N termini (Spielmann and Gilberger, 2010). We therefore generated two constructs wherein we added an E back to the scrambled sequence in position 3 and 7, respectively. Addition of E3 in the scrambled sequence caused a mixed phenotype, whereas addition of E7 did not result in export (Figure 3B). Quantification of the export efficiency showed that adding back E3 caused a phenotype that was intermediate between $R^{REX2\text{-}TM}$ carrying the scrambled and the wild-type REX2 N terminus (Figure 3C). For both of these cell lines, the additional focus of fluorescence was also observed (Figure 3B).

These data show that the export information in the N termini resides in part in the remainder of the PEXEL (or similar residues in the respective positions in the N terminus of PNEPs) but also in the sequence immediately downstream of this.

A Solubility Shift during Export of TM Proteins

Many of the mTRAP fusion constructs showed a uniform staining in the host-cell cytosol in addition to the Maurer's clefts staining (Figures 1, 2, and 3). This could indicate a soluble pool, which would be surprising because these proteins contain a TM and backbone identical to those of the construct with the REX2 N terminus, which exclusively localizes to the Maurer's clefts. Tetanolysin lysis for release of the infected host-cell cytosol showed that there was indeed an exported soluble pool of full-length protein in these cell lines (Figure 4A). Thus, these proteins are present in two pools, one soluble in the host

cell and one associated with the Maurer's clefts. As expected, the protein pool at the Maurer's clefts behaved again like integral TM proteins (Figure S4). REX2$^{1–20}$-R$^{REX2-TM}$ did not show a soluble pool (Figure 4A), indicating that the N terminus alone affects the solubility state and localization of the R$^{REX2-TM}$ reporter in the host cell.

The soluble pool might either derive from proteins incorrectly entering the ER in a soluble state or indicate a solubility change during export. We therefore tested the solubility of these proteins in the ER, using brefeldin A (BFA) to retain the proteins in the ER (Figures 4B and 4C). These experiments showed that R$^{REX2-TM}$ constructs with the N termini of REX2, MAHRP1, or the mature N terminus of the PEXEL protein STEVOR were all found in the membrane fraction when retained in the ER (Figure 4B). This indicates that, although these proteins enter the secretory pathway as integral membrane proteins, a population of these proteins leaves the membrane to become soluble in the host cell. In the case of SBP1$^{1–26}$-R$^{REX2-TM}$, a carbonate-soluble fraction was detected in addition to the Triton X-100 fraction upon BFA treatment.

To confirm that these constructs can enter the secretory pathway as bona fide TM proteins, we used an in vitro microsome insertion assay. We generated constructs carrying an N-glycosylation consensus sequence in the N-terminal region and a hemagglutinin (HA) epitope at the C terminus (Figure 4D). Translocation of the N-terminal region is expected to result in reduced mobility in SDS-PAGE, due to glycosylation by the luminal oligosaccharyl transferase complex. Indeed, as shown in Figure 4D, the three tested constructs, like our control construct b5ops28 (Brambillasca et al., 2006), showed the expected upward mobility shift (asterisks). This was due to glycosylation, given that the shift was inhibited when a competing tripeptide (NYT) was added. To further confirm the topology of the inserted constructs, we probed for the accessibility of the C-terminal HA epitope to externally added protease K (PK). After insertion, the products were either immunoprecipitated (IPed), or the vesicles were first exposed to PK and then IPed. The control construct b5ops28 carries an opsin epitope in the translocated C-terminal region. Therefore, after insertion and PK digestion, two protected fragments, generated from the glycosylated (asterisk) and nonglycosylated but inserted protein (dot) were IPed with an opsin antibody (Figure 4D, right panel—see Brambillasca et al., 2006 for the relevant controls), demonstrating that the microsomal vesicles are impermeable to PK. Instead, although the parasite constructs were all IPed with HA antibodies, the HA epitope was fully accessible to PK, indicating that the C terminus of these constructs is exposed on the outside of the vesicles.

Taken together, these experiments indicate that the soluble pool in the host cell derives from protein that was properly inserted into the ER membrane.

Unfolding Is Required for the Export of TM Proteins

The above data indicate that a pool of our reporter changes from an integral membrane to a soluble state during export, which would be consistent with a translocation step. To test this for our constructs, we used an established system originally developed for the study of translocation into mitochondria (Eilers and Schatz, 1986). This system was recently adopted for *P. falciparum* for showing that soluble PEXEL proteins need to be unfolded to reach the host cell (Gehde et al., 2009). It is based on a murine DHFR domain (mDHFR). The folding of this domain can be stabilized with antifolate ligands such as WR99210 (Gehde et al., 2009). If a protein fused to mDHFR is transported through a translocon that requires unfolding for cargo to pass, trafficking of this protein will be blocked at this step due to ligand-induced stabilization of the mDHFR moiety. We modified the system by expressing an internal export control without the mDHFR domain (REX2mCherry) alongside the mDHFR-GFP chimera (Figure 5A). Analysis of parasites expressing REX2mDHFR-GFP, as well as MAHRP1$^{1–20}$, SBP1$^{1–26}$, and STEVOR$^{44–63}$ fused to R$^{REX2-TM}$-mDHFR-GFP, showed that these proteins were properly targeted to the Maurer's clefts (Figure 5B). Addition of WR99210 (2 nM) blocked export of all the mDHFR fusions, but not REX2mCherry, in the same individual parasites (Figure 5B), demonstrating that the block was mDHFR dependent. The export-blocked proteins were retained at the parasite periphery with some staining in the parasite cytosol, whereas REX2mCherry was detected at the Maurer's clefts (Figure 5B). Thus, REX2 and the R$^{REX2-TM}$ fusions need to be unfolded to reach the host cell. This indicates that not only PEXEL proteins but also PNEPs, including TM proteins, undergo a translocation step during export.

Unfolding Is Required for Crossing the PPM

To identify the step in export at which export of mDHFR fusions is blocked, we first investigated the solubility of the blocked protein at the parasite periphery. Treatment of infected RBCs with saponin, which lyses both red cell membrane and PVM but leaves the PPM intact, released minimal but detectable amounts of the blocked mDHFR fusion proteins, but not the exported REX2mCherry control or unblocked REX2mDHFR (Figure 6A). In addition, subsequent hypotonic lysis of the parasites revealed a soluble degradation product in the parasite, mostly in the mTRAP fusions. However, the majority of the full-length protein was found in the final pellet. These findings suggest that the main population of the blocked protein was in a membrane-associated form with a small amount of the protein soluble in the PV. The soluble degradation product after hypotonic lysis of the parasite may be derived from a soluble parasite-internal pool seen in cells expressing the mTRAP-mDHFR fusions in the presence of WR99210 (Figure 5B).

We next carried out PK protection assays on parasites with WR99210 export-blocked protein. In parasites released from the infected RBC with streptolysin O (which leaves the PVM intact), PK treatment did not result in proteolysis of the mDHFR fusion protein (Figure 6B). However, when the PVM was subsequently permeabilized with saponin (leaving the PPM intact), the protease generated a protected fragment (arrows Figure 6B, tested for REX2mDHFR-GFP as well as SBP1$^{1–26}$ and STEVOR$^{44–63}$ fused to R$^{REX2-TM}$mDHFR-GFP). This showed that the blocked protein was present in the PPM with its N terminus facing the lumen of the PV, consistent also with the presumed orientation of the protein in the ER, as deduced from the microsome assay. The small amount of protected fragment in the PK-treated streptolysin O sample was probably due to limited breach of the PVM, as was evident from some loss of the soluble PV marker SERP (Figure 6B). These results

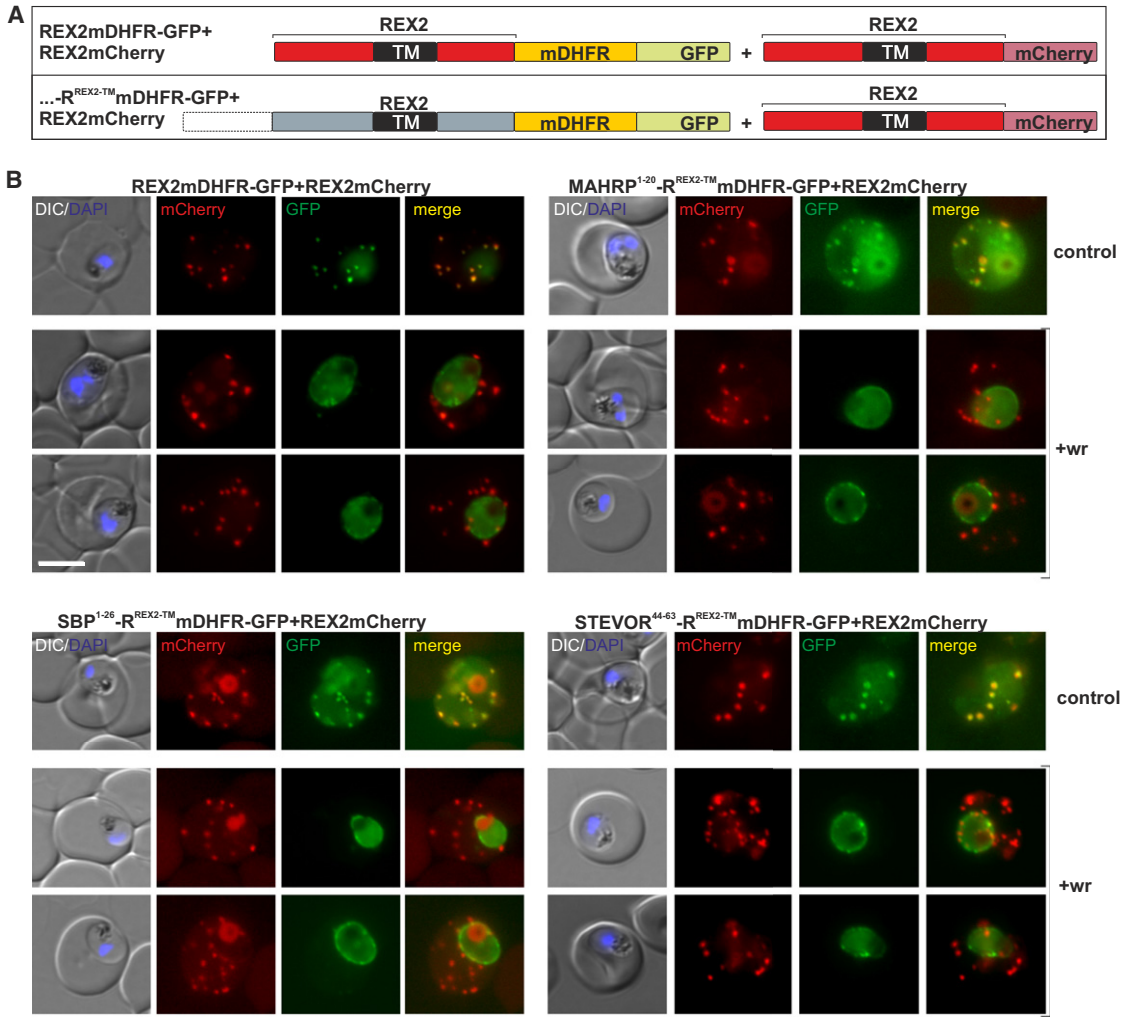

Figure 5. Blocking Unfolding Arrests Export of PNEPs

(A) Schematic of the constructs of the cell lines shown in (B).

(B) Parasites expressing REX2mCherry (red) together with the mDHFR-GFP-tagged constructs (green) indicated above each panel. Two images are shown per cell line in the WR99210 (+wr)-treated samples to demonstrate that in different cells the parasite peripheral staining of the blocked protein displayed either a smooth or a more focal pattern. Merge, overlay of the red and green signals. The size bar represents 5 μm.

suggest that the C-terminal region of these TM proteins must undergo a translocation event in an unfolded conformation at the PPM. The small amount of PV-soluble protein seen in Figure 6A therefore probably represents protein that escaped the block at the PPM. In contrast, a previously published soluble PEXEL protein (GBP) fused to mDHFR (Gehde et al., 2009) was found fully soluble in the PV upon blocking export, in that it could be released by saponin treatment (Figure 6C).

DISCUSSION

The PEXEL motif, its cleavage by plasmepsin V, and its binding to PI3P are considered to be the deciding steps in export of a protein in malaria parasites (Bhattacharjee et al., 2012; Boddey et al., 2010; Hiller et al., 2004; Marti et al., 2004; Russo et al., 2010). We show here that all of these requirements associated with the PEXEL motif can be bypassed if the protein contains a PNEP TM (but not a different TM), revealing that thereafter the mature N terminus controls export with sufficient precision to distinguish exported from nonexported proteins. This may increase the overall fidelity in sorting and suggests multiple steps in the control of protein export. Importantly, this also provides a link between PEXEL and PNEP export. N termini of PNEPs, as well as mature PEXEL proteins, were exchangeable with regards to promoting export. Hence, PNEPs can be considered to be mature PEXEL proteins that bypass the PEXEL-requiring step through the presence of an internal TM. This suggests similar principles in export or, at least in part, similar export pathways for PNEPs and PEXEL proteins (see the model in Figure 7). This is also supported by the fact that (1) both types of N termini had similar sequence requirements for export and

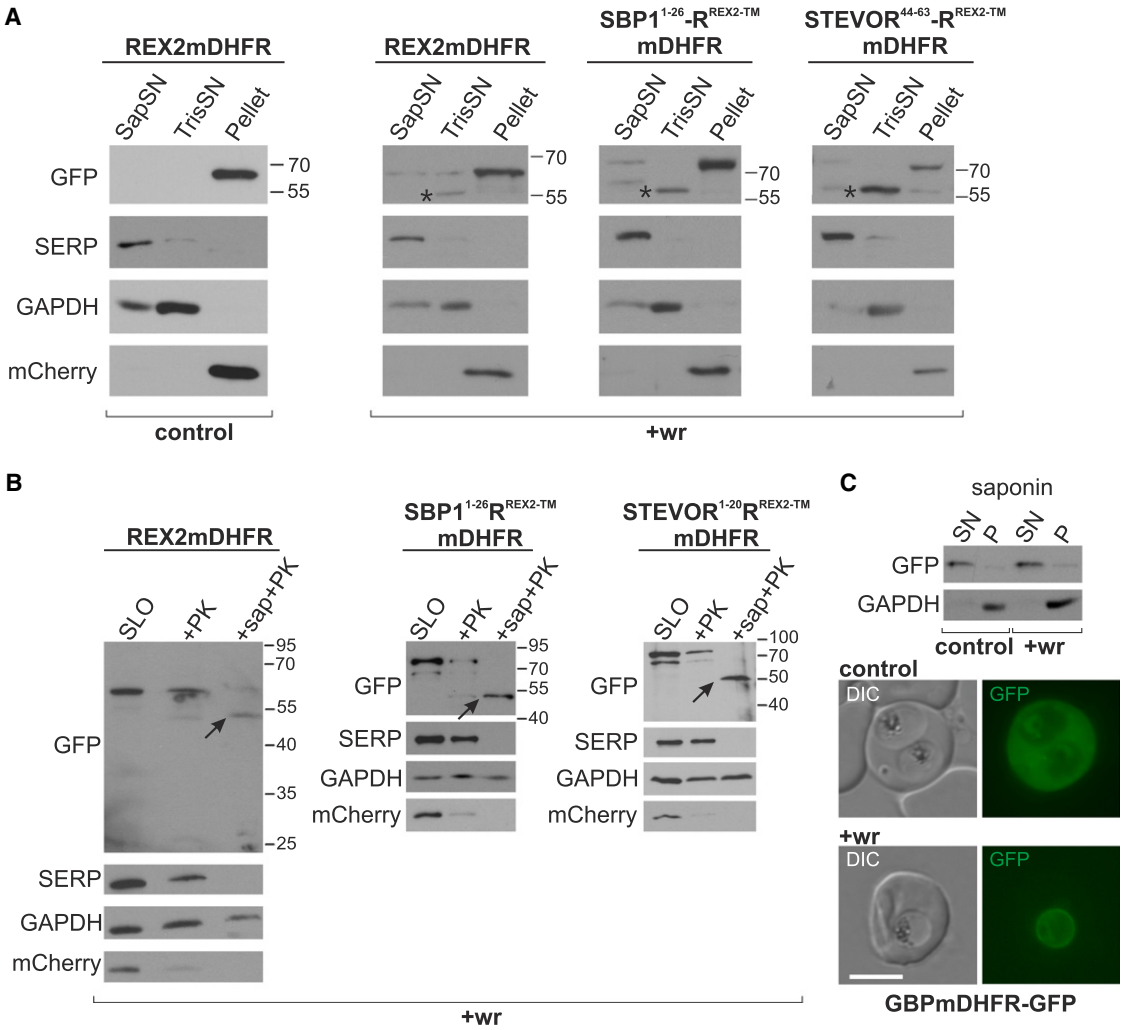

Figure 6. Site of Arrest in Export Due to Blocked Unfolding

(A) The constructs indicated are mostly membrane associated (pellet) after saponin (Sap SN) and hypotonic lysis (TrisSN) with a degradation band (asterisk) in the TrisSN (parasite cytosol) and a minimal saponin-soluble pool. Controls are as follows: SERP, for release of PV material; parasite GAPDH, for release of parasite cytosol in TrisSN; and mCherry, for detection of the internal control (REX2mCherry) at the Maurer's clefts.

(B) PK protection assay with streptolysin O (SLO)-treated cells expressing the constructs indicated. SLO creates access to the PVM; saponin (sap) creates access to the PPM. The protected fragment is shown by an arrow. Controls are as in (A) and show that mCherry was PK-accessible upon SLO treatment. Reduction in the full-length protein and appearance of a weakly detectable protected fragment in the SLO+PK fraction correlated with some loss of SERP in this sample, indicating partial lysis of the PVM by SLO. Integrity of the PPM is shown by the presence of parasite GAPDH.

(C) GBPmDHFR (green) blocked (+wr) and unblocked (control) in western blots (top) and live cells (bottom). Release of blocked GBPmDHFR in Percoll-purified infected RBCs by saponin indicates its presence as a soluble protein. The size bar represents 5 μm.

(2) an N terminus refractory to export in our reporter system prevented the export of a soluble PEXEL protein, thus translating these findings back into a PEXEL background.

Exported proteins are trafficked via the ER and the Golgi apparatus to the parasite periphery, where they have to get beyond the PPM and the PVM to reach the host cell (Maier et al., 2009). It was previously shown that unfolding is needed for the export of soluble PEXEL proteins, because a folded domain led to accumulation of the export-blocked protein in the parasite periphery (Gehde et al., 2009). This is concordant with the recently discovered translocon for PEXEL proteins at the PVM (de Koning-Ward et al., 2009).

How TM proteins are exported has thus far remained enigmatic. It was proposed that they enter newly forming Maurer's clefts by lateral diffusion in the PVM and are then carried into the host cell with the nascent cleft (Spycher et al., 2006; Tilley et al., 2008). However, our recent data using time-lapse imaging indicate that export is independent of Maurer's cleft formation (Grüring et al., 2011). Here we show that PNEPs, and thus TM proteins, need to be unfolded to reach the host cell, indicative of a translocation step at the parasite periphery. Although not specifically tested for PEXEL TM proteins, this was also the case for our reporter with a mature PEXEL N terminus. Hence, translocation appears to be

a common principle for different types of proteins for reaching the host cell.

A Translocation Step at the PPM

Although the requirement for unfolding for reaching the host cell is a shared feature of the thus-far-analyzed proteins, we found the blocked mDHFR-fused TM proteins at the PPM, not at the PVM where the PTEX translocon is situated (de Koning-Ward et al., 2009). This is not entirely unexpected: integral membrane proteins transported from the ER will inevitably end up in the PPM (see the model in Figure 7B). The orientation of the proteins in the microsome assays and the PK assays indicates that the C terminus containing the mDHFR domain faces the parasite cytosol, preventing passage through the membrane in the blocked state. Thus, a first unfolding step is required for TM proteins to clear the PPM to reach the PV and a PVM translocon. This is in contrast to soluble proteins that can be directly exocytosed into the PV to become available as substrate for PVM translocation. Thus, all of the proteins analyzed thus far follow two steps in export: vesicular trafficking to the parasite periphery, resulting in release into either the PV (soluble proteins) or the PPM (TM proteins), followed by translocation for reaching the host cell (Figure 7).

The need for unfolding at the PPM adds another dimension to protein export out of the parasite. If different translocons are involved, this may explain why coimmunoprecipitation experiments for pulling down PTEX components that use the blocked constructs have been unsuccessful thus far (F.K., C.G., and T.S., unpublished data). It is possible that the situation is similar to that of mitochondria and chloroplasts, wherein different components in the outer and inner membrane deliver both soluble as well as integral TM protein through or into these membranes (Schleiff and Becker, 2011). However, in contrast to import into these organelles, the activity at the *Plasmodium* PPM has to translocate proteins already present integral to the membrane, in this respect having a greater resemblance with the ERAD pathway (Smith et al., 2011) or import into the *Euglena* chloroplast (Sulli and Schwartzbach, 1996). The PPM and PVM are closely adjoined, which is also the case with membranes in mitochondria and chloroplasts. Translocation may be advantageous in this situation, whereas vesicle trafficking may be more beneficial in other situations, such as sorting between multiple spatially separated compartments.

Translocation through the PVM would require insertion of TM proteins at the Maurer's clefts membrane (Figure 7B). The host-cell-soluble population we found for most exported mTRAP fusions may be a result of partial failure at this step. REX2[1–20] directed the reporter to the Maurer's clefts efficiently, possibly because the short N terminus of REX2 contains properties needed for both export and cleft recruitment. In contrast, these activities may be separate in other PNEPs that have longer N termini or are not needed in soluble proteins such as GBP. The residual recruitment to the clefts might be due to the TM. Clearly, post-PVM trafficking, including insertion into the Maurer's clefts, needs further investigation. In principle the possibility that TM proteins continue to traffic via vesicles from the PVM can also not be excluded. However, in this case the soluble pool observed in the host cell would result from accidental recognition of the protein by a PVM translocase.

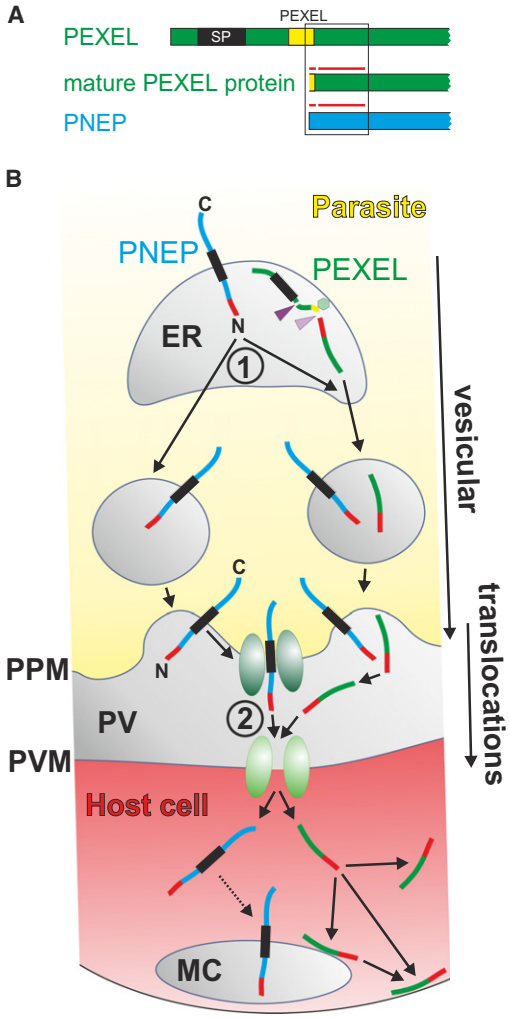

Figure 7. Model for Export

(A) Exchangeable export regions between mature PEXEL proteins and PNEPs. Red lines correspond to sequences involved in export.

(B) Model for protein export in malaria parasites. After signal-peptide cleavage, PI3P (hexagon) binding and plasmepsin V cleavage initiate export of PEXEL proteins (cleavage steps are indicated by triangles), possibly by sorting into export-competent regions of the ER for entry into a vesicular pathway. After trafficking through the parasite's secretory system (Golgi apparatus not shown), the mature PEXEL protein is released into the PV to become a substrate for the PVM translocon PTEX (light-green ellipses). PNEPs either get sorted into the same vesicular pathway (1) or are trafficked independently to the PPM where a first translocon (dark-green ellipses) releases them into the PV or directly hands the protein over to a PVM translocon (PTEX or other). Possible points of convergence of export pathways are indicated by 1 and 2. In the case of scenario 1, the shared properties in the N terminus could guide export from the ER onward; in the first and second scenario it could (also) be involved in translocation steps at the parasite periphery. Once in the host cell, soluble PEXEL proteins reach their target destination (soluble in host cell, RBC membrane or cytoskeleton, or Maurer's clefts) directly or via the Maurer's clefts (MC). PNEPs are inserted into the Maurer's clefts by a presumed additional membrane translocation (arrow with dashed line). Hydrophobic regions are shown as black bars. C, C terminus; N, N terminus.

Although trafficking relying on multiple translocation steps (PPM, PVM, and Maurer's clefts) may appear cumbersome at first glance, establishing such a system could be simpler

than installing vesicle trafficking from scratch in a host cell that has lost the intrinsic capacity for such processes. The many parasite chaperones found in the host cell and the PV might contribute by keeping these proteins in a translocation-competent form (Külzer et al., 2010; Nyalwidhe and Lingelbach, 2006). Whether there are also proteins that rely on a purely vesicular pathway remains to be determined.

Sequences in Export of PNEPs and PEXEL Proteins

Our reporter uncoupled export from the full PEXEL and made it possible to analyze the role of the mature N terminus in isolation from the initial functions attributed to the PEXEL. Unexpectedly, mutation of the last conserved PEXEL residue (position 5) remaining in the mature N terminus did not affect export, clearly demonstrating that the region downstream of the PEXEL was sufficient to mediate export. However, we found that this residue rescued export in a scrambled background, demonstrating that it can also have a crucial role. Thus, the head group and the downstream sequences both influence export. The N terminus of REX2 behaved similarly, in agreement with PNEP N termini corresponding to this region. These rather loose requirements in the mature N terminus explain the lack of an obvious common motif in PNEP N termini, which is also reflected in the limited information content in the region immediately after the PEXEL (Bhattacharjee et al., 2006). Nevertheless, we show here that this is sufficient for specificity in export. The region after the PEXEL motif has previously been found to be required as a spacer in GFP fusion constructs, but was not thought to hold specific export information (Bhattacharjee et al., 2006; Knuepfer et al., 2005; Przyborski et al., 2005). In contrast, work in *P. falciparum* that used the oomycete signal RxLR showed a role for negative charges further downstream of this motif. However, oomycete signals were recently shown not to be cleaved (Bhattacharjee et al., 2012), and this region would not be presented N-terminally. Concordantly, such a region did not promote export in our system.

Similarities in PNEP Export

All tested PNEP N termini were sufficient for promoting export depending on the presence of a PNEP TM in the protein. These findings indicate unifying principles in the export of PNEPs that thus far have been elusive (Spielmann and Gilberger, 2010). Our data disagree with a previous hypothesis that the difference in isoelectric point between N and C terminus of the protein is important for export (Saridaki et al., 2009; Spycher et al., 2006). This is based on the finding that scrambling the N-terminal sequences, which does not affect overall charge, was sufficient to abolish, or in SBP1, reduce export. It is possible that the entire (or a large part) of the N-terminal region of PNEPs needs to have export compatible properties, which would explain the heterogeneous picture of PNEP export so far. Alternatively, previous findings may have been limited due to exposing regions at the N terminus not normally found in this position, or because regions were only tested for being necessary rather than sufficient for export.

In conclusion, PNEPs and PEXEL proteins appear to share a core export domain in an export pathway that depends on the (mature) N-terminal region. This raises the question of why the PEXEL is required at all. Our finding that a PNEP TM can substitute for it could indicate a general need for membrane association in the initial steps of export. This could be provided by the PEXEL through the proposed binding to PI3P (Bhattacharjee et al., 2012) or by the signal peptide acting as a signal anchor (retention of the signal sequence was found in a PEXEL mutant by Boddey et al., 2009). Correctly timed removal of the upstream leader through Plasmepsin V, for instance to expose the mature N terminus and release the protein, could therefore be an essential function of the PEXEL. This may explain why a signal peptidase cleaved protein engineered to generate a mature PEXEL protein failed to get exported (Boddey et al., 2010), as signal peptidase may have prematurely released this protein from the membrane.

As only PNEP TMs appear to be compatible with export, specific properties of the TM may be important in substituting for the function of the PEXEL leader in the initial steps of export. For instance, the TM may facilitate delivery of the protein to export-competent regions or vesicles of the ER. Alternatively, the properties of the TM may be important for allowing for translocation at the parasite periphery.

Strikingly, unfolding appears to be a common requirement in the export for all types of proteins tested thus far, including both soluble and TM as well as PNEP and PEXEL proteins. The corresponding translocation steps therefore represent interesting drug targets and now need to be resolved further.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Primers and templates for cloning are listed in Tables S1 and S2. Details are provided in the Supplemental Experimental Procedures.

Parasite Culture and Transfection

3D7 parasites were cultured in RPMI containing 5% albumax according to standard procedures (Trager and Jensen, 1976). Transfection and selection with 4 nM WR99210 (Jacobus Pharmaceuticals) or 2 μg/ml Blasticidin S (Life Technologies) was done as described (Spielmann et al., 2006).

Live Cell Imaging

GFP-expressing parasites were viewed directly as described (Grüring and Spielmann, 2012) using a Zeiss Axio Scope M1 microscope equipped with a 100X/1,4 numerical aperture oil immersion lens. Pictures were collected with a Hamamatsu Orca C4742-95 camera and Zeiss AxioVision software. Images were processed in Corel PHOTO-PAINT X4. Parasite nuclei were stained with 1 mg/ml DAPI (Roche) for 10 min at 37°C.

Western Blotting

Western blots were done with nitrocellulose membranes (Schleicher & Schüll) using 10 mM CAPS (pH 11.2) transfer buffer and a tank blot device (Bio-Rad) as described (Spielmann et al., 2006). Antibody dilutions (in 5% milk/PBS) were: mouse monoclonal anti-GFP (Roche), 1/1,000; rat monoclonal anti-mCherry (ChromoTek), 1/5,000; rabbit anti-SERP, 1/2,000; mouse anti-REX3, 1/2,000; and mouse anti-GAPDH, 1/2,000. Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse (Roche), goat anti-rat (Dianova), both used at 1/3,000, and donkey anti-rabbit (Dianova) used at 1/2,500.

Selective Permeabilization

Percoll-purified infected RBCs were selectively lysed using 1 U/ml tetanolysin and separated into pellet and supernatant by centrifugation, and the pellet was lysed with 0.015% saponin in PBS, followed by centrifugation. The pellet was extracted with 5 mM TrisHCl (pH 8.0) and separated into pellet and supernatant, and the final pellet was extracted with 0.5X PBS containing 4% SDS and 0.5% Triton X-114. Equivalent amounts of supernatants and pellets

were used for western blot analysis. Details are provided in Supplemental Experimental Procedures.

PK Protection Assay

PK protection assays were done as described (Spielmann et al., 2006), using streptolysin O-treated infected RBCs incubated with either nothing, 1 mg/ml PK, or 0.015% saponin containing 1 mg/ml PK. The reaction was stopped and proteins were precipitated using trichloroacetic acid and analyzed via western blotting. Details are provided in Supplemental Experimental Procedures.

BFA Treatment and Solubility Assays

Newly invaded ring stages from 20 ml of culture (synchronized in the previous cycle using 5% sorbitol) were grown with 5 mg/ml brefeldin A (Sigma-Aldrich) for 16 hr. Residual nonring stages were removed with 5% sorbitol. Parasites were released with 0.03% saponin/PBS, washed in PBS, hypotonically lysed in 100 μl of 5 mM TrisHCl (pH 8.0) with complete protease inhibitor (Roche) and 1 μg/μl DNase (Sigma-Aldrich), and frozen at −20°C. Thawed pellets were centrifuged at 16,000 × *g* for 5 min, and the supernatant was saved as soluble fraction. The pellet was sequentially extracted with 100 μl each of freshly prepared 0.1 M Na_2CO_3 on ice for 30 min (peripheral fraction), ice-cold 1% Triton X-100 (integral membrane fraction), and 0.5X PBS containing 4% SDS and 0.5% Triton X-114 at room temperature (insoluble fraction). All supernatants were recentrifuged for removal of residual material. All centrifugations were at 16,000 × *g* for 5 min. Equivalent amounts were subjected to western blot analysis.

Immunoprecipitation and Mass Spectrometry Analysis

GBP/SERA was purified from infected RBC saponin supernatants using GFP-Trap-A beads (ChromoTek) and analyzed by mass spectrometry (MS) essentially as described previously (Haase et al., 2009). Details are provided in Supplemental Experimental Procedures.

In Vitro Microsome Translocation Assay

Constructs were under the control of the SP6 promoter. In vitro transcription, translation using the rabbit reticulocyte lysate system (Promega), and translocation assays into rat-liver microsomes were carried out as previously described (Brambillasca et al., 2006). Details are provided in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2012.09.010.

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