Evidence for a role for a *Plasmodium falciparum* homologue of Sec31p in the export of proteins to the surface of malaria parasite-infected erythrocytes

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Accepted 10 June 2001
Journal of Cell Science 114, 3377-3386 (2001) © The Company of Biologists Ltd

SUMMARY

The malaria parasite, *Plasmodium falciparum*, spends part of its life cycle inside the enucleated erythrocytes of its human host. The parasite modifies the cytoplasm and plasma membrane of its host cell by exporting proteins beyond the confines of its own plasma membrane. We have previously provided evidence that a plasmodial homologue of the COPII protein, Sar1p, is involved in the trafficking of proteins across the erythrocyte cytoplasm. We have now characterised an additional plasmodial COPII protein homologue, namely Sec31p. Recombinant proteins corresponding to the WD-40 and the intervening domains of the PfSec31p sequence were used to raise antibodies. The affinity-purified antisera recognised a protein with an apparent relative molecular mass of 1.6×10^5 on western blots of malaria parasite-infected erythrocytes but not on blots of uninfected erythrocytes. PfSec31p was shown to be largely insoluble in nonionic detergent, suggesting cytoskeletal attachment. Confocal immunofluorescence microscopy of malaria parasite-infected erythrocytes was used to show that PfSec31p is partly located within the parasite and partly exported to structures outside the parasite in the erythrocyte cytoplasm. We have also shown that PfSec31p and PfSar1p occupy overlapping locations. Furthermore, the location of PfSec31p overlaps that of the cytoadherence-mediating protein PfEMP1. These data support the suggestion that the malaria parasite establishes a vesicle-mediated trafficking pathway outside the boundaries of its own plasma membrane – a novel paradigm in eukaryotic biology.

Key words: Malaria, *Plasmodium*, COPII, Sec31p, Membrane trafficking, Secretion

INTRODUCTION

Protein trafficking in eukaryotic systems involves a sequence of tightly controlled vesicle-mediated processes that direct proteins through a series of membrane compartments. Export from a donor compartment is initiated by the recruitment of a series of coat proteins (COPs) that cause the budding of a cargo-containing small vesicle. The vesicle then uncoats and fuses with its target membrane (Barlowe et al., 1994; Stephens et al., 2000). The COPs that are responsible for trafficking between the endoplasmic reticulum (ER) and the Golgi are the COPI and COPII complexes (Barlowe, 1998; Wieland and Harter, 1999; Scales et al., 2000; Glick, 2000). COPII initiates the formation of buds at ER exit sites, whereas COPI proteins are thought to be involved in transit from the ER-Golgi intermediate compartment (ERGIC), retrograde transport from the Golgi to the ER and trafficking between different compartments within the Golgi (Scales et al., 2000).

The intraerythrocytic malaria parasite faces a special protein-trafficking problem. The mature human erythrocyte lacks the machinery for protein synthesis or protein trafficking, but the parasite achieves the export of a series of proteins that bring about major alterations of properties of its host cell. For example, in the mature stages of the intra-erythrocytic cycle, the membrane of the erythrocyte becomes distorted with knobby protrusions. These 'knobs' are involved in cytoadherence of infected erythrocytes to the vascular endothelium. Two major components of these knobs are the knob-associated histidine-rich protein (KAHRP), which forms the major structural element of the knob, and an integral membrane protein, the *Plasmodium falciparum* erythrocyte membrane protein 1, PfEMP1, which is inserted into the erythrocyte membrane and acts as the ligand for binding to endothelial cell receptors (Baruch, 1999). An intriguing question is: how are these proteins trafficked across the two membranes that separate the parasite and erythrocyte cytoplasm and transported to locations underneath or within the erythrocyte membrane?

Trafficking of proteins within the parasite itself appears to involve elements of a classical vesicle-mediated secretory pathway. Homologues of a number of trafficking components have been found in *Plasmodium*, including the ER-located proteins, Pfgrp/BiP (Kumar et al., 1991) and PfERC (La Greca et al., 1997), the KDEL-binding protein ERD2 (Elmendorf and Haldar, 1993) and trafficking-associated GTPases, such as Rab6 (Alves de Castro et al., 1996). Moreover, the parasitophorous vacuolar membrane (PVM) protein, Exp1, has a signal sequence that appears to be cleaved both in vitro and in vivo (Kara et al., 1990; Gunther et al., 1991) and brefeldin A (BFA), a drug that specifically blocks vesicle-mediated

trafficking, has been shown to block the secretion of a number of proteins (Elmendorf et al., 1992; Hinterberg et al., 1994; Albano et al., 1999a; Albano et al., 1999b). However, the molecular machinery and the mechanisms of transport that are responsible for trafficking of proteins within the external sector of the export pathway remain largely uncharacterised.

Recently, we have identified a *P. falciparum* homologue of yeast Sar1p (Albano et al., 1999b). Sar1p is a member of the COPII protein translocation machinery (Nakano and Muramatsu, 1989; Barlowe, 1998). Interestingly, a subpopulation of PfSar1p molecules was found to be located within the erythrocyte cytoplasm. This led us to suggest that the malaria parasite exports at least some components of the machinery for vesicle-mediated trafficking into the erythrocyte cytoplasm. To test this suggestion further, we have now characterised the *P. falciparum* homologue of Sec31p, another member of the COPII complex.

MATERIALS AND METHODS

Analysis of the sequence of *P. falciparum SEC31*

A putative *P. falciparum* Sec31p homologue was identified by searching the *P. falciparum* database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). The sequence of the *P. falciparum* WEB-1 orthologue, WD-40 (accession no. PFB0640c), was compared with that of *S. cerevisiae* Sec31p (accession no. NP_010086) using the BLAST 2 sequences algorithm (http://www.ncbi.nlm.nih.gov/gorf/bl2.html). The amino acid sequence of PfSec31p was aligned with that of the Sec31p sequence from *Saccharomyces cerevisiae* using the Multalin program (http://protein.toulouse.inra.fr/multalin.html). Further analysis was performed using the Genedoc program (http://www.psc.edu/biomed/genedoc/). The Simple Modular Architecture Research Tool (SMART; http://coot.embl-heidelberg.de/; Schultz et al., 1998) was used identify WD-40 domains within the N-terminal region of PfSec31p.

Parasite culture and metabolic labelling

Parasites (D10, 3D7, K1 and FAC8 strains) were continuously cultured as described previously (Raynes et al., 1996) using erythrocytes and serum obtained from the Red Cross Transfusion Service (Melbourne, Australia). Cultures were synchronised as described by Raynes et al. (Raynes et al., 1996) and erythrocytes infected with mature stage parasites were collected by floatation on a Percoll-sorbitol cushion (Desneves et al., 1996). BFA treatment was performed as described previously (Albano et al., 1999b). Briefly, BFA (final concentration, 5 μg ml^{-1}) was added, from a concentrated stock in methanol, to erythrocytes infected with tightly synchronised parasites (2-10 hours ring stages, 8% parasitemia). In preliminary experiments, we found that it was necessary to incubate the parasitised erythrocyte for 15-18 hours to ensure significant levels of export of proteins in controls cells, so an 18 hour period was chosen for all incubations. Smears were prepared for immunofluorescence microscopy and the BFA was removed and the parasites cultured for a further 24 hours to ensure viability. Solubility studies of PfSec31p were as described previously (Albano et al., 1999b). Briefly, mature Percoll-purified parasitised erythrocytes (~10^6 cells) were either sonicated in the presence of phosphate-buffered saline (PBS) or 50 mM $NaHCO_3$ (pH 10), or agitated in 2% Triton X-100 in PBS and kept at 4°C for 30 minutes. The soluble and particulate fractions were separated by centrifugation (100,000*g*, 30 minutes, 4°C). For metabolic labelling of parasite proteins, erythrocytes infected with highly synchronised FAC8 strain parasites (12% parasitemia) were washed with excess methionine- and cysteine-free RPMI 1640 (Gibco) and cultured in the presence of 100 μCi ml^{-1} L-[^{35}S]-methionine (73%), L-[^{35}S]-cysteine (22%) and unradiolabelled methionine/cysteine (5%) (Geneworks, Australia). Aliquots were removed at 16-hour intervals and subjected to an immunoprecipitation protocol as described below.

Genomic DNA preparation, PCR and Southern blot analysis

Genomic DNA from an asynchronous *P. falciparum* culture (FAC8 strain) was extracted using the QIAmp blood kit (Qiagen, Melbourne, Australia) and used as a template for PCR amplification of a fragment within the 5′ region of the *PfSEC31* gene, encompassing bp 67-1095 (encoding amino acids 23-365) (referred to as *PfSEC31(WD)*) and of a fragment within the 'intervening' region of the *PfSEC31* gene, encompassing bp 1808-2781 (encoding amino acids 604-927) (referred to as *PfSEC31(int)*). The oligonucleotides incorporated 5′ *Bam*HI and 3′ *Eco*RI restriction endonuclease sites. The PCR products were sequenced in both directions using fluorescent dideoxynucleotide termination. For Southern blot analysis, α-[^{32}P]-dATP (Amersham Pharmacia) was incorporated into *PfSEC31(WD)* using the Gigaprime DNA Labelling kit (Bresatec, Adelaide, Australia). The [^{32}P]-labelled *PfSEC31(WD)* gene probe was hybridised to Southern blots of genomic DNA (2 μg) of *P. falciparum* (FAC8 strain) restricted with *Eco*RI, *Hin*dIII, *Kpn*I or *Nde*I (Promega).

Cloning, expression and purification of recombinant PfSec31p fragments

The *PfSEC31(WD)* and *PfSEC31(int)* gene fragments were cloned into the pGEX-5×-3 vector (Amersham Pharmacia) and transformed into *Escherichia coli* BL21. The constructs were verified by DNA sequencing. The gene products, expressed as glutathione *S*-transferase (GST) fusion proteins, were purified on glutathione (GSH)-Sepharose columns (Amersham Pharmacia). The N-terminal sequence of the recombinant proteins (referred to as PfSec31(WD) and PfSec31(int)) were confirmed by Edman sequencing.

Immunochemical techniques

Purified recombinant PfSec31(WD) and PfSec31(int) was used to generate antisera in rabbits (four inoculations of 90-150 μg protein for each rabbit) or in mice (three inoculations of 25 μg protein for each mouse). The rabbit antisera were affinity purified by absorbing the reactive antibodies onto recombinant protein immobilised on polyvinylidene difluoride (PVDF) membrane. The membrane was washed extensively with Tris-buffered saline (pH 7.4), bound antibodies were eluted with 0.1 M glycine pH 2.6 and the eluate was immediately neutralised with 1.5 M Tris (pH 10). For western analyses, aliquots of a synchronous culture of malaria-parasite-infected erythrocytes (FAC8 strain, ~10% parasitemia) were harvested by floatation on a Percoll-sorbitol cushion as described by Desneves et al. (1996). Alternatively, haemoglobin-free parasites were prepared by mixing washed samples of parasite cultures with 0.05 volumes of 1% saponin and 0.01 volumes of gentamycin (50 mg ml^{-1}) as described previously (Raynes et al., 1996). Aliquots (~10^6 parasites) were subjected to SDS-PAGE (7.5% acrylamide) and western blotting was performed using enhanced chemiluminescence (ECL) detection as described previously (La Greca et al., 1997). For immunoprecipitation studies, cultures of metabolically labelled *P.-falciparum*-infected erythrocytes (FAC8 strain) were harvested and solubilised in 2% SDS. The SDS was then sequestered with the addition of Triton X-100 to achieve a final concentration of 0.2% SDS and 0.7% Triton X-100, in 50 mM Tris, 150 mM NaCl, pH 7.4, containing protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 0.5 μg ml^{-1} leupeptin, 0.7 μg ml^{-1} pepstatin). Immunoprecipitation of PfSec31p, using rabbit polyclonal antiserum, was achieved from the SDS/Triton X-100-solubilised parasite samples as previously described (Foley et al., 1991). Samples were subjected to SDS-PAGE (7.5% acrylamide) and radiolabelled proteins were visualised in dried gels by phosphorimage analysis (Molecular Dynamics, USA).

Confocal fluorescence microscopy

Confocal microscopy was performed at the Confocal Microscopy Facility, Monash University, Australia, using a Leica TCS-NT laser scanning confocal microscope equipped with a krypton/argon laser with main emissions at 488 nm and 568 nm. The confocal scan head was mounted on a Leica TCS microscope. Smears of cultured infected erythrocytes (K1 or FAC8 strain) were probed with affinity-purified anti-PfSec31(WD) or anti-PfSec31(int) antiserum and antisera recognising QF116/exp1 (Kara et al., 1990), PfERC (La Greca et al., 1997), PfSar1p (Albano et al., 1999b), PfEMP1 (Reeder et al., 1999) or PfEMP3 (Waterkeyn et al., 2000). As controls, smears were probed with pre-immune antisera, an anti-GST antibody or anti-PfSec31p antisera that had been pre-adsorbed with recombinant PfSec31p. For dual labelled samples, the images were collected simultaneously. Binding of rabbit antibodies was visualised using goat anti-rabbit immunoglobin G (IgG) conjugated with fluorescein (Sigma) or Alexa Fluor 568 (Molecular Probes). Binding of murine antibodies was visualised using goat anti-mouse IgG conjugated with rhodamine (Sigma) or Alexa Fluor 568 (Molecular Probes). Rabbit anti-PfEMP3 was kindly donated by J. Waterkeyn (Walter and Eliza Hall Institute of Medical Research, Australia) and a murine monoclonal antibody recognising QF116/exp1 was kindly donated by A. Saul (Queensland Institute of Medical Research, Australia). A mouse polyclonal antiserum recognising PfERC was generated against recombinant PfERC, which was prepared as described by Albano et al. (Albano et al., 1999b), and was kindly donated by A. Cowman (Walter and Eliza Hall Institute of Medical Research, Australia). Semi-quantitative image analysis of the 8-bit confocal images was performed using ImageJ v. 1.22 (http://rsb.info.nih.gov/ij/index.html). Images used for analysis were collected with pixel values below saturation in regions of interest and were background corrected. Image sizes were 512×512 pixels. The area occupied by the parasite was defined by examination of transmission images and/or images of PfERC staining that were collected simultaneously. Average pixel intensities obtained for the region of the parasite and the entire parasitised erythrocyte were multiplied by the respective pixel areas to obtain total pixel intensity values.

RESULTS

Identification of a *P. falciparum* homologue of *SEC31*

A search of the *P. falciparum* sequence database was performed with the amino acid sequence of *S. cerevisiae* Sec31p (accession no. NP_010086) using the BLAST algorithm. The search revealed a *P. falciparum* protein with significant similarity to yeast Sec31p (28% amino acid identity, 56% similarity over a 277 amino acid sequence within the N-terminal region of the proteins; 31% identity, 48% similarity over a 300 amino acid region in the central region and 21% identity, 40% similarity over the entire C-terminal 790 amino acids). The *P. falciparum* gene sequence is present as two open reading frames with a combined length of 4317 bp on chromosome 2 (Gardner et al., 1998; accession no. PFB0640c) and is referred to as the WEB-1 orthologue. The gene sequence codes for a protein of 1438 amino acids with a predicted relative molecular mass (M_r) of 163,374 (Fig. 1B). The SMART algorithm (Schultz et al., 1998) was used to analyse the PfWEB-1 orthologue protein sequence, revealing that the N-terminal region (which is the region of highest homology with yeast Sec31p) contains four WD-40 motifs (Fig. 1A). N-terminal WD-40 domains have previously been noted in Sec31p sequences from other organisms (Salama et al., 1997; Shugrue et al., 1999; Tang et al., 2000). The *P. falciparum* WEB-1 orthologue also shows significant similarity (27% identity, 54% similarity in the WD-40 domain; 29% identity, 46% similarity in a 330 amino acid region in the central region and 21% identity, 37% similarity over a 1368 amino acid segment spanning most of the protein sequence) to a human Sec31p homologue (Tang et al., 2000). An alignment of the WD-40 domain of the *P. falciparum* WEB-1 orthologue and the equivalent domain of human Sec31A is shown in Fig. 1A. The PfWEB-1 orthologue sequence can be partitioned into four major domains, these being a WD-40 domain at the N-terminus, an intervening region, a proline-rich region and a short C-terminal region (Fig. 1B). An equivalent domain organisation has been reported for other Sec31 proteins (Salama et al., 1997; Shugrue et al., 1999; Tang et al., 2000). Furthermore, a hydropathy plot of PfSec31p generated using the Kyte and Doolittle algorithm is similar to that of *S. cerevisiae* Sec31p further suggesting a level of structural similarity (data not shown). Taken together, these data indicate that the *P. falciparum* WEB-1 orthologue is a candidate for the plasmodial homologue of Sec31p. We have therefore tentatively renamed this protein, PfSec31p.

PfSEC31 exists as a single copy gene in *P. falciparum*

A fragment within the 5′ region of the *PfSEC31* gene, encompassing bp 67-1095 (referred to as *PfSEC31(WD)*) and a fragment within the central region of the *PfSEC31* gene, encompassing bp 1808-2781 (referred to as *PfSEC31(int)*) were amplified from *P. falciparum* (FAC8 strain) genomic DNA by PCR and completely sequenced. The sequences of these regions of *PfSEC31* from the FAC8 strain exactly matched the published 3D7 sequence (Gardner et al., 1998). The *PfSEC31(WD)* PCR product was labelled by incorporation of α-[^{32}P]-dATP and hybdridised to a Southern blot of a sample of *P. falciparum* (FAC8 strain) genomic DNA that had been digested with *Eco*RI, *Hin*dIII, *Kpn*I and *Nde*I. As expected, the probe hybridised to a single fragment in the *Eco*RI, *Hin*dIII and *Kpn*I digestions, whereas *Nde*I cut within the gene sequence, yielding fragments of ~1.6 kb and 3.7 kb, respectively (data not shown). The observed banding pattern indicates a single copy of the *PfSEC31* gene in *P. falciparum*. *S. cerevisiae* also encodes only a single isoform of Sec31p, but two isoforms are expressed in humans (Tang et al., 2000).

Identification of PfSec31p in *P.-falciparum*-infected erythrocytes

The *PfSEC31(WD)* and *PfSEC31(int)* fragments were cloned into the pGEX-5×-3 vector and expressed as GST fusion proteins in *E. coli*. The recombinant proteins were purified using GSH-Sepharose and used to raise antibodies in rabbits and mice. The anti-PfSec31(WD) rabbit antiserum specifically recognised the ~66 kDa recombinant GST-PfSec31(WD) fusion protein in the *E. coli* lysate (Fig. 2A). The rabbit antiserum was affinity purified by binding to immobilised PfSec31(WD) and used to probe western blots of samples of erythrocytes infected with the FAC8 strain of *P. falciparum* (Fig. 2B). The purified antiserum recognised a major protein band with the expected M_r of 1.6×10^5, as well as a slower-migrating doublet with an apparent M_r of ~2.4×10^5 (Fig. 2Bc,d). In some samples, smaller species, especially a band with an apparent M_r of ~0.9×10^5, were also

A

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P.fal: 124 GIIVGGLTNGDIVLLNAKNLFETNRNYDNFILSKTNIHDNGINCLEYNRHKNNLIATGGN 183
           G+++ G  NG+I+L +   +   ++     ++++ + H   +  L+ N  + NL+A+G N
Human:  87  GVLIAGGENGNIILYDPSKIIAGDKEV---VIAQNDKHTGPVRALDVNIFQTNLVASGAN 143

P.fal: 184 DGQLFITDIENLYSPTSYDPYLDKNNLQKITCLNWNKKVSHILATSSNNGNTVIWDLKIK 243
           + +++I D+ N  +P +  P       + I+C+ WN++V HILA++S +G   +WDL+
Human: 144 ESEIYIWDLNNFATPMT--PGAKTQPPEDISCIAWNRQVQHILASASPSGRATVWDLRKN 201

P.fal: 244 KSAVSFRDPHSRTKTSSLSWLSNQPTQVLISYDDDKNPCLQLWDLRNSNYPIKEIIGHSK 303
           +  +   D  +R   S L+W  +  TQ++++ +DD+ P +Q+WDLR ++ P++ +  H++
Human: 202 EPIIKVSDHSNRMHCSGLAWHPDVATQMVLASEDDRLPVIQMWDLRFASSPLRVLENHAR 261

P.fal: 304 GINNICFSPIDTNLLLSSGKDVTKCWYLDNNNFDIFNEINNSANNIYS-KWSPYIPDLFA 362
           GI  I +S  D  LLLS GKD  K    + N  ++  E+  +    +  +W P  P + +
Human: 262 GILAIAWSMADPELLLSCGKD-AKILCSNPNTGEVLYELPTNTQWCFDIQWCPRNPAVLS 320

P.fal: 363 SSTNMDTIQINS 374
           +++    I + S
Human: 321 AASFDGRISVYS 332
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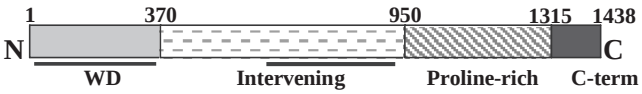
Fig. 1. Analysis of the sequence of Sec31p from *P. falciparum*. (A) Alignment of the amino acid sequences of the WD-40 domains of *P. falciparum* Sec31p and human Sec31A. Identical amino acids are repeated in the central line. Similar amino acids are denoted with a +. The residues that form the four WD-40 motifs of *P. falciparum* Sec31p and five of the six human WD-40 motifs are underlined. (B) Domain structure of PfSec31p. Approximate boundaries between the WD40 domain, the intervening domain, the proline-rich domain and the C-terminal domain are indicated. Regions produced as recombinant proteins for the generation of antibodies are indicated by solid lines.

B



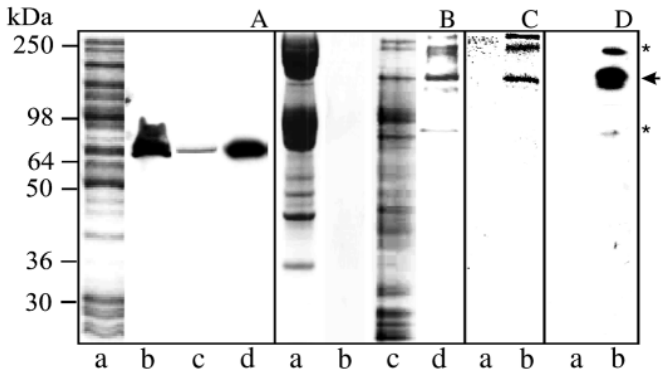


Fig. 2. Western blot and immunoprecipitation analysis of PfSec31p. (A) Lysates of *E. coli* expressing PfSec31(WD) (lanes a,b) and recombinant PfSec31(WD) isolated by affinity chromatography on GSH-Sepharose (lanes c,d) were subjected to SDS-PAGE (10% acrylamide) and visualised by Coomassie blue staining (lanes a,c) or transferred to nitrocellulose filters and incubated with rabbit antiserum against PfSec31(WD) followed by horse radish peroxidase (HRP)-conjugated anti-rabbit IgG and visualised using ECL (lanes b,d). (B) Saponin-lysed uninfected red blood cells (lanes a,b) or harvested saponin-lysed parasitised erythrocytes (lanes c,d) were subjected to SDS-PAGE (10% acrylamide) and stained with Coomassie blue (lane a,c) or transferred to nitrocellulose and probed with rabbit antiserum against PfSec31(WD) (lanes b,d). (C) [^{35}S]-methionine/cysteine-labelled infected erythrocytes were solubilised in detergent and the sample was subjected to an immunoprecipitation protocol using either pre-bleed rabbit serum (lane a) or rabbit anti-PfSec31(WD) antiserum (lane b). (D) Harvested saponin-lysed parasitised erythrocytes (lanes a,b) were subjected to SDS-PAGE (10% acrylamide) and transferred to nitrocellulose and probed with either pre-bleed rabbit serum (lane a) or rabbit antiserum against PfSec31(int) (lane b). An ~160 kDa band that might correspond to a full-length monomeric form of PfSec31p in the parasite samples is marked with an arrow. Higher and lower molecular mass species are indicated with asterisks.

observed; these might represent proteolytic fragments because they varied in intensity between different preparations and similar proteolytic fragments of PfSec31p have been observed in other organisms (Shugrue et al., 1999). Bands of similar apparent M_r were observed in parasites of the 3D7, D10 and K1 strains (data not shown). In uninfected erythrocytes, no immunoreactive bands were observed (Fig. 2Ba,b). This confirms that the 160 kDa protein is of parasite origin. In an effort to examine the specificity of the antiserum further, *P.-falciparum*-infected erythrocytes (FAC8 strain) were cultured in the presence of [^{35}S]-methionine and [^{35}S]-cysteine to radiolabel parasite proteins. The anti-PfSec31(WD) antiserum immunoprecipitated a 160 kDa protein and higher molecular species from SDS/Triton X-100-solubilised samples of this metabolically labelled preparation (Fig. 2Cb). By contrast, no proteins were precipitated using the pre-bleed antiserum (Fig. 2Ca). To characterise the nature of the reactive species further, the antiserum raised against PfSec31(int) was also used in western blot analysis. This antiserum recognised proteins of equivalent apparent M_r (Fig. 2Db), which indicates that the reactive species of higher and lower apparent M_r are likely to be Sec31p aggregates and Sec31p fragments, respectively, rather than cross-reactive proteins

P. falciparum Sar1p has been shown to be largely insoluble in nonionic detergents and aqueous buffers (Albano et al., 1999b). In this work, the solubility characteristics of PfSec31p were examined. PfSec31p was largely insoluble in 2% Triton X-100 (Fig. 3Ac,d) and also largely insoluble in PBS (Fig. 3Aa,b). However, PfSec31p was successfully solubilised using a high-pH buffer (Fig. 3Ae,f). In these samples, an increased level of aggregation of PfSec31p was observed, which reflected an increased period of storage of the samples prior to analysis. Similar SDS-resistant aggregates have been observed in studies of Sec31p from other species (Salama et al., 1997). The timing of expression of PfSec31p was examined by immunoprecipitation of the metabolically labelled PfSec31p from a synchronised culture of FAC8-infected erythrocytes. These data indicated that PfSec31p is expressed in all intraerythrocytic stages and that the level increases with increasing maturity (Fig. 3Ba-c). A further increase in the expression of PfSec31p was observed in the ring stage of the second cycle (Fig. 3Bd) owing to the increased level of parasitemia.

PfSec31p is localised to a novel trafficking pathway in malaria-parasite-infected erythrocytes

Confocal immunofluorescence microscopy was used to assess the subcellular location of PfSec31p in erythrocytes infected

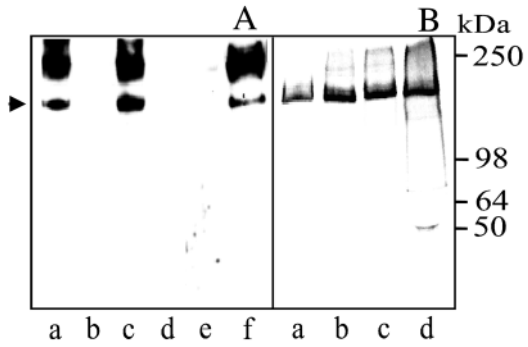


Fig. 3. Solubility studies and time-course of expression of PfSec31p. (A) Mature-stage-parasitised erythrocytes (FAC8 strain, ~10^6 cells) were either sonicated in PBS (lanes a,b), or agitated in 2% Triton X-100 (lanes c,d) for 20 minutes at 4°C or sonicated in 50 mM $NaHCO_3$, pH 10 (lanes e,f) and the samples kept for 30 minutes at 4°C. The soluble and particulate fractions were separated by centrifugation and the supernatant (lanes b,d,f) and pellet (lanes a,c,e) fractions were subjected to SDS-PAGE (7.5% acrylamide) and transferred to a nitrocellulose membrane. The filters were incubated with antiserum against recombinant PfSec31(WD) followed by HRP-conjugated anti-rabbit IgG and visualised using ECL. (B) Aliquots of a synchronous culture of [^{35}S]-methionine/cysteine-labelled parasitised erythrocytes (FAC8 strain, 10% parasitemia) were harvested at the ring stage (lane a), the trophozoite stage (lane b), the schizont stage (lane c) or the ring stage of the second cycle (lane d). The samples were solubilised, immunoprecipitated with anti-PfSec31(WD) antiserum, subjected to SDS-PAGE (7.5% acrylamide) and visualised by phosphorimage analysis. A ~160 kDa band that might correspond to a full-length monomeric form of PfSec31p is marked with an arrow.

with K1 strain parasites. High concentrations of the rabbit pre-bleed serum gave a very weak generalised labelling of malaria-parasite-infected erythrocytes, although no labelling was observed at the dilutions used for the immune sera (data not shown). Nonetheless, the anti-PfSec31(WD) antiserum was affinity purified on immobilised recombinant PfSec31(WD) prior to use in immunofluorescence studies. All intraerythrocytic stages of the malaria parasite were labelled with the affinity-purified anti-PfSec31(WD) antiserum (Fig. 4). In ring-stage parasites, a slice through the centre of a parasitised erythrocyte revealed labelling of a compartment(s) within the parasite cytoplasm. In addition, punctate structures were observed within the erythrocyte cytosol (Fig. 4a). A confocal slice taken near the surface of the infected erythrocyte provided evidence that the punctate structures are often closely connected to the erythrocyte membrane (Fig. 4b). In more mature stage-infected erythrocytes, the localisation of PfSec31p in compartments within the erythrocyte cytosol was more marked. An uneven or polarised labelling pattern was still observed within the parasite cytoplasm, but PfSec31p appeared to be largely associated with structures in the erythrocyte cytosol that appear vesicular in nature (Fig. 4c,d). This labelling pattern suggests that PfSec31p is exported into the host cell cytoplasm.

The affinity-purified anti-PfSec31(int) antiserum gave a similar labelling pattern. Peripheral foci of fluorescence staining were observed in the erythrocyte cytoplasm, with an increase in the number of punctate structures with increasing maturity of the parasite (Fig. 4i-l). The fact that equivalent patterns were observed with two antibodies recognising different regions of PfSec31p adds further support to the suggestion that PfSec31p is indeed exported to the erythrocyte cytosol. Smears probed with an anti-GST antibody or with anti-PfSec31(WD) antisera that had been pre-adsorbed with recombinant PfSec31(WD) showed only background fluorescence (data not shown). Similar labelling patterns were observed using anti-PfSec31(WD) antisera to probe two other strains of the parasite, 3D7 and FAC8 (data not shown).

Export of PfSec31p to the erythrocyte cytoplasm is BFA sensitive

We have previously shown that the export of PfSar1p to the erythrocyte cytosol is sensitive to treatment with the fungal inhibitor BFA. In this work, we have examined the BFA sensitivity of PfSec31p export. Synchronous ring stage parasites (~2-10 hour rings) were incubated in the presence of 5 μg ml^{-1} of BFA for 18 hours. In control parasites, which reach the trophozoite stage after the 18 hour incubation period, PfSec31p is exported to structures within the erythrocyte cytoplasm (Fig. 4t). In BFA-treated cells, we found that PfSec31p was retained within compartments within the parasite cytosol (Fig. 4r, blue arrow). This compartment has a similar morphology to the one observed using PfSar1p as a marker (data not shown). It is important to realise that the development of the parasites was somewhat retarded by the BFA treatment. The retardation of growth was indicated by the fact that they accumulated very little haemozoin, presumably owing to an inability to traffic proteases to the food vacuole. However, if the BFA was removed, these parasites were viable and were able to continue through the cycle (not shown). This inhibition of export of PfSec31p was only observed if BFA was added to the infected erythrocytes in the early ring stage. Once the PfSec31p-containing structures have been formed within the erythrocyte cytosol, incubation with BFA did not cause collapse of the vesicles back to the parasitophorous vacuole (PV; data not shown).

PfSec31p location only partially overlaps that of the ER-located protein PfERC

The location of PfSec31p was compared with that of the ER-located protein PfERC (La Greca et al., 1997; Albano et al., 1999). A mouse polyclonal anti-PfERC antiserum identified a reticular structure that is confined to the parasite cytoplasm (Fig. 5c,g). Within the parasite cytosol, the location of PfERC partly (but not completely) overlapped that of PfSec31p (Fig. 5d,h). Optical slices collected at 0.6 μm intervals from the bottom to the top of young trophozoite stage parasitised erythrocytes (Fig. 5i-p) provided further support for the suggestion that a substantial proportion of the anti-PfSec31p reactivity lies outside the parasite in the erythrocyte cytosol. In order to perform a semiquantitative analysis of the amount of PfSec31p that is exported to the erythrocyte cytosol, the total pixel intensities in the region of the cell occupied by the parasite were estimated relative to the total pixel intensities in the entire parasitised erythrocyte. Analysis of a series of 15 different young trophozoite-stage-infected erythrocytes (similar to those shown in Fig. 5) indicated that the parasite-associated fluorescence was 35 ± 13% (range: 18-55%). This suggests that more than half of the PfSec31p that is produced by the parasite might be exported to the erythrocyte cytosol.

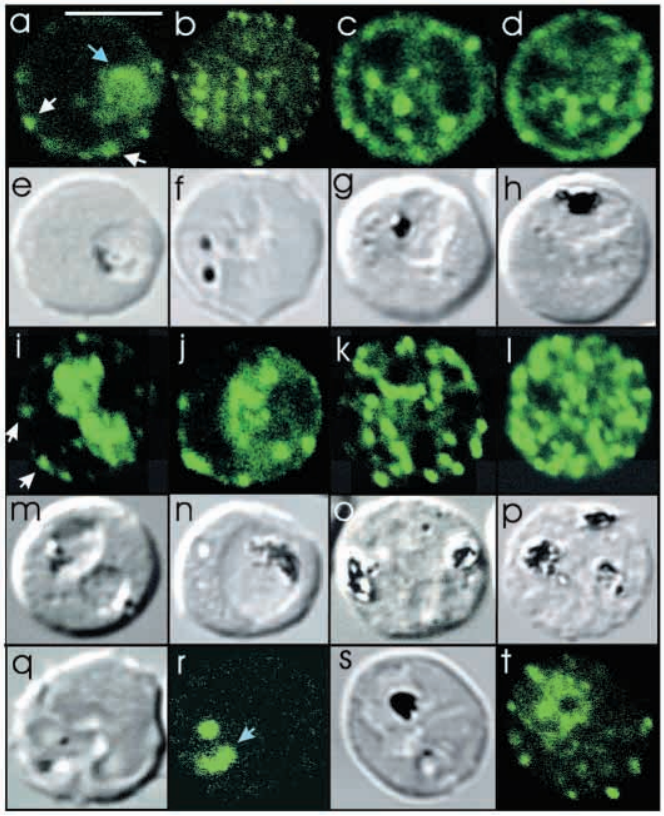


Fig. 4. Intracellular location of PfSec31p and effect of BFA treatment on export of PfSec31p. Erythrocytes infected with ring stage (a,b,i) or mature (c,d,j-l) malaria parasites (K1 strain) were labelled with affinity purified rabbit anti-PfSec31(WD) antiserum (a-d) or affinity-purified rabbit anti-PfSec31(int) antiserum (i-l) followed by a fluorescein-conjugated anti-rabbit IgG. Transmission images of a-d are shown in e-h. Transmission images of i-l are shown in m-p. Optical slices were collected by confocal microscopy through the centre of the parasite (a,i,j) or near the surface of the parasitised erythrocyte (b-d,k,l). In (a) and (i), some PfSec31p-containing structures in the erythrocyte cytosol are marked with white arrowheads. A PfSec31p-containing structure in the parasite cytosol in (a) is marked with a blue arrowhead. Erythrocytes infected with synchronised ring stage parasites (FAC8 strain, 2-10 hour rings) were incubated for 18 hours in the presence of 5 μg ml^{-1} BFA (q,r) or an equivalent volume of methanol (s,t). Smears were prepared for immunofluorescence microscopy and visualised using affinity-purified rabbit anti-PfSec31(WD) antiserum. Bar in (a), 5 μm.

PfSec31p is associated with another COPII protein in the erythrocyte cytosol and might be involved in the transport of PfEMP1

Further definition of the intraerythrocytic location of PfSec31p was sought by comparing its fluorescence-labelling pattern with the profile for that of a number of other parasite antigens. The exported parasite protein Exp1 has been shown to be located in the PV and, occasionally, in vesicular structures in the erythrocyte cytosol (Kara et al., 1990; Albano et al., 1999b). As shown in Fig. 6c,d, the PfSec31p-containing compartment(s) within the parasite cytoplasm (green fluorescence, blue arrow) appears to lie within the area defined by Exp1 (red fluorescence). The population of PfSec31p that is located in the erythrocyte cytosol (white arrows) is clearly outside the PV (Fig. 6b,d).

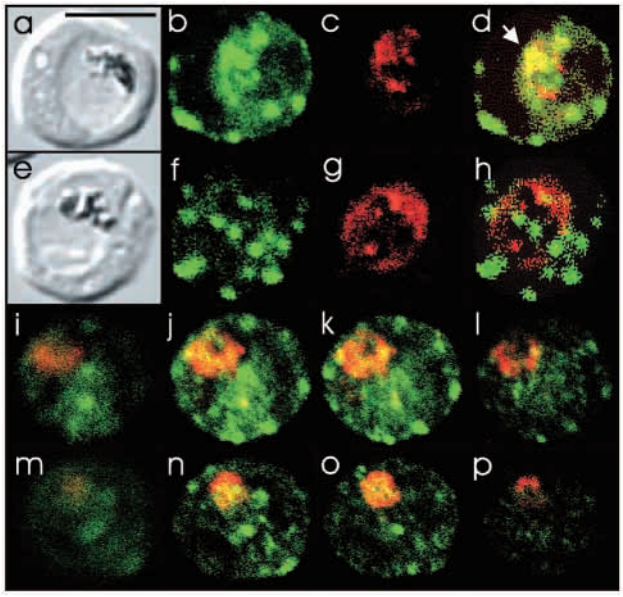


Fig. 5. Comparison of the location of PfSec31p with the ER-located protein PfERC in *P.-falciparum*-infected erythrocytes and semiquantitative analysis of the amount of PfSec31p in the erythrocyte cytosol. (a-h) Asynchronous infected erythrocytes (K1 strain) were probed with affinity-purified rabbit anti-PfSec31(WD) antiserum followed by a fluorescein-conjugated anti-rabbit IgG (green fluorescence) and a murine antiserum recognising the ER-located protein PfERC followed by Alexa-Fluor-568-conjugated anti-mouse IgG (red fluorescence). A region of partial overlap of PfSec31p and PfERC in the parasite cytosol in (d) is marked with an arrowhead. (i-p) Asynchronous infected erythrocytes (K1 strain) were probed with rabbit anti-PfSec31(WD) antiserum (green fluorescence; i-l) or a murine anti-PfERC (red fluorescence; m-p). Four optical slices (0.6 μm) were collected by confocal microscopy from the bottom to the top of the parasitised erythrocytes.

A polyclonal mouse antiserum against PfSec31(WD) gave an equivalent labelling pattern to that observed with the rabbit antiserum (Fig. 6f,j). A rabbit polyclonal antiserum that recognises the COPII protein, PfSar1p, gave a very similar labelling pattern (Fig. 6g,k), as judged by the almost complete overlap of the fluorescence patterns obtained using probes for these two proteins (Fig. 6h,l). This supports the suggestion that PfSec31p is present, in the erythrocyte cytosol, as a COPII complex that coats vesicular structures.

PfEMP3 is a large (>300 kDa) parasite protein that is deposited underneath the erythrocyte membrane (Pasloske et al., 1994). Recent transfection studies have shown that the accumulation of a mutated form of PfEMP3 blocks the insertion of PfEMP1 into the erythrocyte membrane, although complete disruption of the PfEMP3 gene did not prevent protein export. These data have been taken to suggest that PfEMP1 and PfEMP3 might share a common trafficking pathway (Waterkeyn et al., 2000). We found that there was little overlap of the location of PfEMP3 with that of PfSec31p. Occasional structures were observed that contained both proteins (Fig. 6p), but the proteins largely occupied separate compartment. This might indicate that PfEMP3 is only transiently associated with the COPII trafficking machinery. Data indicating transient overlap of PfEMP3 and PfEMP1 have been reported previously (Trelka et al., 2000; Waterkeyn et al., 2000).

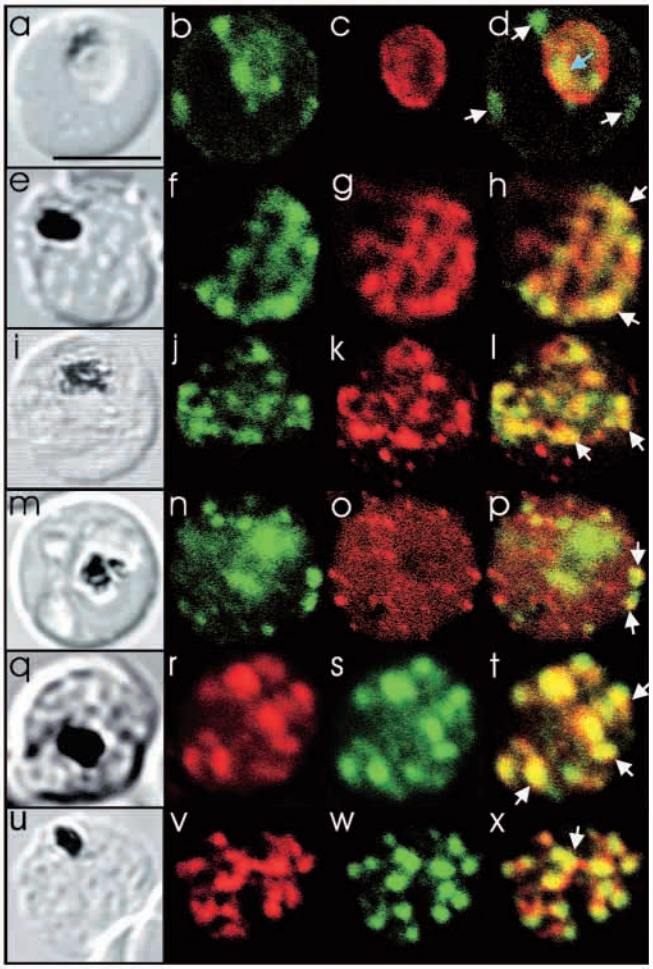


Fig. 6. Comparison of the location of PfSec31p with that of other parasite antigens in *P.-falciparum*-infected erythrocytes. (a-d) Asynchronous infected erythrocytes (K1 strain) were probed with affinity-purified rabbit anti-PfSec31(WD) antiserum followed by a fluorescein-conjugated anti-rabbit IgG (b; green fluorescence) and a murine monoclonal antibody recognising the PV-located protein Exp1 followed by rhodamine-conjugated anti-mouse IgG (c; red fluorescence). An optical slice was collected through the centre of the parasite by confocal microscopy. Some PfSec31p-containing structures in the erythrocyte cytosol are marked with white arrowheads and a PfSec31p-containing structure in the parasite cytosol is marked with a blue arrowhead (d). (a) A transmission image of (b-d). (e-l) Asynchronous infected erythrocytes (K1 strain) were probed with mouse anti-PfSec31(WD) antiserum followed by a fluorescein-conjugated anti-mouse IgG (green fluorescence) and a rabbit anti-PfSar1p antiserum followed by Alexa-Fluor-568-conjugated anti-rabbit IgG (red fluorescence). Optical slices were collected near the surface of the parasitised erythrocytes. Some PfSec31p- and PfSar1p-containing structures are marked with arrowheads. (m-p) Asynchronous infected erythrocytes were probed with affinity-purified rabbit anti-PfSec31(WD) antiserum followed by fluorescein-conjugated anti-rabbit IgG (green fluorescence) and a murine monoclonal antibody recognising PfEMP3, followed by Alexa-Fluor-568-conjugated anti-mouse IgG (red florescence). An optical slice was collected near the surface of the parasitised erythrocyte. Two structures in which there is partial overlap of PfSec31p and PfEMP3 in the erythrocyte cytosol are marked with arrowheads. (q-x) Asynchronous infected erythrocytes were probed with a mouse anti-PfSec31(WD) antiserum followed by Alexa-Fluor-568-conjugated anti-mouse IgG (red fluorescence) and an affinity-purified rabbit anti-PfEMP1 antibody followed by fluorescein-conjugated anti-rabbit IgG (green florescence). Optical slices were collected near the surface of the parasitised erythrocytes. Some PfSec31p- and PfEMP1-containing structures in the erythrocyte cytosol are marked with arrowheads. Bar in (a), 5 μm.

PfEMP1 is a protein with a single transmembrane domain that is inserted into the erythrocyte membrane and acts as a ligand for binding to receptors on the vascular endothelium (Baruch, 1999). The transport of this integral membrane protein to the erythrocyte membrane presumably involves the budding of a vesicular compartment from the PV, followed by the trafficking of this vesicular compartment across the erythrocyte cytosol and, eventually, fusion of at least a subpopulation of vesicles with the erythrocyte membrane. We have examined the relative locations of PfSec31p and PfEMP1. As can be seen in Fig. 6t,x, there is almost complete overlap of the locations of these proteins. This suggests that COPII proteins might be involved in the export of PfEMP1. For example, these vesicular structures might represent a ‘storage’ depot for PfEMP-1 that is en route to the erythrocyte membrane.

DISCUSSION

Maturation of the intraerythrocytic malaria parasite is accompanied by major structural and functional alterations of the host membrane. The altered membrane properties are due to the deposition of parasite proteins underneath and insertion of proteins into the erythrocyte membrane bilayer. Of particular importance is the transport and display of the parasite-encoded cytoadherence-mediating protein PfEMP1 (a member of the family of antigenically diverse integral membrane proteins). A major gap in our understanding of the ways in which the malaria parasite interacts with the erythrocyte relates to the mechanism by which proteins such as PfEMP1 are trafficked to the erythrocyte membrane. How does the parasite transport proteins across the cytosol of the erythrocyte, a cell that is itself devoid of trafficking machinery?

We have previously reported that a homologue of Sar1p is located, in part, within the erythrocyte cytosol (Albano et al., 1999a; Albano et al., 1999b). Sar1p is a small GTP-binding protein that controls the formation and dissociation of the COPII family of coat proteins. The identification of PfSar1p in the erythrocyte cytosol provided the first evidence that the malaria parasite exports components of a vesicle-mediated trafficking machinery to the cytosol of its host cell.

In addition to Sar1p, the COPII complex consists of the Sec23p complex, comprising Sec23p and Sec24p, and the Sec13p complex, comprising Sec13p and Sec31p. Each of these components has been shown to be essential for protein trafficking in yeast (Salama et al., 1997; Shugrue et al., 1999; Tang et al., 2000). Recently, the complete sequence of chromosome 2 of *P. falciparum* has been published (Gardner et al., 1998). One of the proteins identified was a homologue of the WEB-1 protein from *Schizosaccharomyces pombe.* The function of *S. pombe* WEB-1 has not been established, but recent studies suggest that a WEB-1 orthologue from humans performs an equivalent function to *S. cerevisiae* Sec31p (Tang

et al., 2000). Proteins of the Sec31/WEB-1 family display only limited sequence similarity (20-30% identity) but they have a similar domain structure, characterised by the presence of a WD-40 domain in the N-terminal region (Tang et al., 2000; Shugrue et al., 1999; Salama et al., 1997). The WD-40 repeat is a sequence motif of ~31 amino acids that encodes a structural element (Smith et al., 1999). The structure of one member of the WD-repeat family has been solved and the data indicate that each of the WD-40 domains folds into a small anti-parallel β sheet and that the sheets are organised around a central axis to form a β propeller (Wall et al., 1995). Analysis of the *P. falciparum* WEB-1 orthologue indicates that it conforms to the domain pattern expected for the Sec31 family and possesses four WD-40 motifs in the N-terminal region. We therefore propose that the *P. falciparum* WEB-1 orthologue be renamed PfSec31p.

In this work, we have expressed two different fragments of PfSec31p as fusion proteins with GST. Some regions of the PfSec31p sequences are rich in asparagine (the intervening domain) or proline (the Pro-rich domain) as has been reported for a number of other parasite proteins (Enea et al., 1984; Zhang et al., 1999). Moreover, various trafficking proteins might contain WD-40 domains (Smith et al., 1999). Thus, it is possible that antibodies raised against particular regions of PfSec31p might cross-react with other parasite proteins. Therefore, fragments from two independent regions – the WD-40 domain and the intervening domain – were chosen for the generation of antibodies. The fusion proteins were successfully expressed in *E. coli* and used to immunise mice and rabbits. The antisera recognised a protein in preparations of parasitised erythrocytes with an apparent M_r of approximately 1.6×10^5. The antisera also precipitated a protein of this size from an SDS/Triton-X-100 solubilised parasite preparation. The observed M_r is very similar to the size predicted from the deduced amino acid sequence and also similar to the M_r observed for yeast and human Sec31 proteins (Salama et al., 1997; Tang et al., 2000). The level of expression of PfSec31p increased during the intraerythrocytic development of the parasite, which is consistent with the pattern observed for the expression of PfSar1p (Albano et al., 1999b) and with the reported increased levels of protein trafficking to the erythrocyte cytosol in more mature stage parasite (Gormley et al., 1992).

PfSar1p was shown to be largely insoluble in the nonionic detergent, Triton X-100 (Nishikawa and Nakano, 1991). In this work, we have shown that PfSec31p is also insoluble in the nonionic detergent, Triton X-100. This detergent insolubility might result from a tight association of the COPII complex with cytoskeletal elements (Barnes et al., 1992). PfSar1p is also insoluble in aqueous buffers suggesting a tight association with lipid. We found that PfSec31p is associated with the membrane fraction when incubated with buffers of physiological pH, but was displaced from the membrane by treatment with a high pH buffer. This suggests that PfSec31p behaves as a peripheral membrane protein as might be predicted from its putative role in the COPII complex.

It has recently been reported that the human Sec31p homologue is localised to ER exit sites (Tang et al., 2000). In this work, confocal immunofluorescence microscopy studies indicated that part of the population of PfSec31p is associated with a compartment(s) within the parasite cytosol, which is consistent with a role for this protein in ER to Golgi trafficking, even though the locations of PfSec31p and the ER-located protein PfERC do not completely overlap. Moreover, a semiquantitative analysis revealed that at least 50% of the PfSec31p is associated with compartments within the erythrocyte cytoplasm. Double labelling studies with the PVM-located protein Exp1 indicated that the structures in the erythrocyte cytosol were discrete structures rather than blebs from the PV.

The export of PfSec31p to the erythrocyte cytosol is sensitive to treatment with the fungal metabolite BFA. BFA is an inhibitor of the activation of ARF, a GTP-binding protein involved in COPI-mediated vesicle formation (Lippincott-Schwartz et al., 1989). This suggests that the mechanism for export of COPII proteins to the erythrocyte cytosol itself involves a vesicle-mediated pathway. The inhibition of PfSec31p might be direct but, given that PfSec31p lacks an export signal sequence, it is perhaps more likely that BFA acts by inhibiting the export of components that are required for the transport of the COPII proteins across the PVM. We found that BFA treatment did not cause a redistribution of PfSec31p molecules that had already been exported to erythrocyte cytosol, as has previously been shown for PfSar1p and various parasite antigens (Benting et al., 1994; Albano et al., 1999b). This might indicate that COPI proteins do not play a role in transport of proteins across the erythrocyte cytosol.

The location of PfSec31p was found to overlap substantially with that of PfSar1p. This is consistent with a role for COPII proteins in vesicle trafficking both within the parasite cytoplasm and in the erythrocyte cytosol. We propose that, following synthesis within the parasite cytoplasm, some COPII complexes become associated with specialised budding structures at the plasma membrane of the parasite. Budding of large vesicles, containing these structures, into the PV and their subsequent fusion with the PVM would release the COPII proteins into the erythrocyte cytosol. Olliaro and Castelli (Olliaro and Castelli, 1997) have provided electron micrographic evidence for the presence of vesicles in the PV. Once released into the erythrocyte cytosol, the COPII proteins could play a role in the budding of vesicles from the PVM. Thus, we anticipate that there would be no exchange of the exported population of PfSec31p with the intraparasitic population.

Although it is difficult to obtain an accurate determination of the sizes of cellular structures by immunofluorescence microscopy, it is apparent that the PfSec31p-containing structures in the erythrocyte cytosol are much larger than the small, coated vesicles involved in ER to Golgi transport in other eukaryotic systems (Kuehn et al., 1998; Pagano et al., 1999). It is possible that PfSec31p is associated with large membrane-bound structures, such as ‘Maurer’s clefts’ (Aikawa, 1971) that have been observed in the host cytosol of parasitised erythrocytes by electron microscopy. Indeed, preliminary immunofluorescence microscopy studies (F. Albano and L.T., unpublished) indicate that the COPII protein, PfSar1p, co-localises with Pf332, a protein that has been shown by immunoelectron microscopy studies to be associated with Maurer’s clefts (Hinterberg et al., 1994). It remains possible, however, that smaller vesicles are involved in the delivery of PfEMP1 from these structures to the erythrocyte membrane (Trelka et al., 2000).

While this manuscript was in review, a report appeared providing evidence that a plasmodial homologue of the *N*-ethylmaleimide-sensitive factor (PfNSF) is exported to the erythrocyte cytosol (Hayashi et al., 2001). NSF is an ATPase that brings about dissociation of soluble NSF attachment receptor (SNARE) complexes following fusion of protein trafficking vesicles with their target membranes (May et al., 2001). Immunofluorescence studies suggest that PfNSF is located in structures that are morphologically similar to the vesicle-like compartments reported in this study (Hayashi et al., 2001). At the level of the electron microscope, PfNSF was associated with structures that appear as electron-lucent 30-70 nm vesicles and larger membrane-bound structures. These structures might be the same compartments that are occupied by PfSar1p and PfSec31p. Thus, the parasite appears to export several different components of the trafficking machinery to the erythrocyte cytosol.

The observation that the locations of PfSec31p and PfEMP1 overlap supports the suggestion that COPII proteins are involved in the export of this integral membrane protein. PfEMP1 is presumably inserted into the ER membrane following synthesis on ER-associated ribosomes, despite the fact that it has no obvious N-terminal signal sequence (Smith et al., 1995). It is possible that the transmembrane-spanning region of PfEMP1 functions as a 'start transfer' signal. The long transmembrane domain of PfEMP1 might indicate that the protein is inserted into the ER with its C terminus facing the parasite cytoplasm (for a review of export signal sequences, see Martoglio and Dobberstein, 1998). Once the protein is embedded in the ER, a series of budding and fusion events that bring about its passage across the parasite plasma membrane and the PVM would allow the N terminus of PfEMP1 eventually to face the exterior of the erythrocyte (Albano et al., 1999a). We propose that COPII proteins are involved in the transport of PfEMP1 to the erythrocyte membrane by promoting the budding of proteins from membranous structures within the erythrocyte cytosol.

In conclusion, the data presented here support a novel paradigm in eukaryotic cell biology: the export of a trafficking pathway. This novel pathway might represent a target for the development of antimalarial strategies.

This work was supported by the National Health and Medical Research Council, Australia. Expert technical assistance was provided by M.-A. Siomos. We thank I. Harper (Confocal Microscopy Facility, Monash University, Australia) for technical advice.

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