

ORIGINAL PAPER

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Characterization of the parasitophorous vacuole membrane from *Plasmodium chabaudi* and implications about its role in the export of parasite proteins

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Abstract Little is known about how the malaria parasite transports and targets proteins into the host erythrocyte. Parasite proteins exported into the host cell not only have to cross the parasite plasma membrane but also must traverse the parasitophorous vacuolar membrane (PVM) that surrounds the parasite. The PVM of *Plasmodium chabaudi*-infected erythrocytes was analyzed by immunofluorescence using an antibody against a known PVM protein, a fluorescent lipid probe, and electron microscopy. These analyses reveal qualitatively different membranous projections from the PVM. Some PVM projections are uniformly labeled with the antibody and with lipid probes and probably correspond to the Maurer's clefts. In contrast to this uniform labeling of the PVM and projections, a 93-kDa *P. chabaudi* erythrocyte membrane-associated protein is occasionally detected in vesicle-like structures adjacent to the parasite. These vesicle-like structures are found only coincident with protein synthesis and are located at discrete sites on the PVM. These observations suggest that the 93-kDa protein does not move along the membranous projections of the PVM toward the erythrocyte membrane. It is proposed that the 93-kDa protein is secreted directly into the erythrocyte cytoplasm at discrete PVM domains and then binds to the cytoplasmic face of the erythrocyte membrane.

Supplementary material: Additional documentary material has been deposited in electronic form and can be obtained from <http://link.springer.de/link/service/journals/00436/index.htm>

Abbreviations PVM Parasitophorous vacuole membrane · sERA Secondary endoplasmic reticulum of Apicomplexa

Introduction

Members of the phylum Apicomplexa are intracellular parasites that are characterized by specialized invasive stages (Sam-Yellowe 1996). During the invasion process the parasite is surrounded by a parasitophorous vacuolar membrane (PVM), which is retained throughout the intracellular stage in many species. The exact contribution of the host and the parasite to the composition of the PVM is unknown and controversial (Ward et al. 1993; Dluzewski et al. 1995; Susstoby et al. 1996). Regardless of its origin, the PVM presents complex problems for intracellular parasites. For example, the PVM represents an additional barrier in terms of the acquisition of nutrients from the host cell. In this regard, permeability channels on the PVM have been described in *Plasmodium* (Desai et al. 1993) and *Toxoplasma* (Schwab et al. 1994).

Apicomplexans modify the host cell during their intracellular stage, and such host modifications are probably best characterized during the intraerythrocytic stage of the malaria parasite. For example, parasite-induced membranes (e.g., Maurer's clefts) are found within the cytoplasm of the infected erythrocyte (Atkinson and Aikawa 1990). In addition, ultrastructural changes in the host erythrocyte membrane include the appearance of electron-dense knobs and/or the formation of caveola-vesicle complexes on the surface of erythrocytes infected by several *Plasmodium* species (Aikawa 1977). These alterations in the erythrocyte are

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mediated by parasite proteins that are exported and targeted to locations within the infected erythrocyte. For example, knobs on *P. falciparum*-infected erythrocytes are correlated with the expression of an approximately 90-kDa *P. falciparum* protein (Kilejian 1979; Leech et al. 1984) and proteins associated with the caveola-vesicle complexes of *P. vivax* have been described (Udagama et al. 1988; Matsumoto et al. 1988).

Little is known about how the parasite can precisely target proteins to the various compartments within the erythrocyte cytoplasm and to the host membrane. Recently we have described an endoplasmic reticulum (ER)-like organelle, called sERA, located at the parasite periphery. This organelle is involved in the early steps of extraparasite transport (Wiser et al. 1997). A 93-kDa *P. chabaudi* protein, called Pc(em)93, was used as a marker for the characterization of this alternate secretory pathway. Pc(em)93 is synthesized during the early ring stage immediately after merozoite invasion (Wiser et al. 1988; Giraldo et al. 1998), and it is rapidly transported to the erythrocyte membrane (Wiser and Lanners 1992). In this study we examined the fate of Pc(em)93 during the early ring stage to learn more about extraparasite transport processes in *Plasmodium*. Proteins, lipids, and the ultrastructure of the PVM were also characterized during the same period. The results of these studies suggest that Pc(em)93 is secreted directly into the erythrocyte cytoplasm at discrete domains of the PVM.

Materials and methods

Parasites

Plasmodium chabaudi (line 54X) were obtained from Dr. R. Walter (Bernhard-Nocht-Institut, Hamburg, Germany) and maintained by serial passage in CD-1 outbred mice (Charles Rivers Laboratories, Kingston, R.I.). Mice were housed at the Tulane University Medical Center vivarium in accordance with applicable regulations. Parasitemia was monitored by Giemsa-stained thin blood smears obtained from the tail. Following axillary incision, infected blood was collected into heparinized Pasteur pipettes, washed four times in Hanks' balanced salt solution containing 5 mM HEPES (pH 7.4) and 0.25% glucose, and processed for immunofluorescence. Unless noted otherwise, the infected erythrocytes were collected between midnight and 1 a.m., corresponding to the period of maximal schizont rupture and merozoite invasion.

Immunofluorescence

Infected erythrocytes were fixed with 0.025–0.05% glutaraldehyde for 10 min, treated with glycine, and washed in Hanks' balanced salt solution containing 1% bovine serum albumin as previously described (Wiser et al. 1993). The fixed samples were incubated with primary antibodies in the presence of 0.1% Triton X-100 for 1 h at room temperature, after which they were washed three times and incubated with fluorescein-conjugated anti-mouse or anti-rabbit IgG (Sigma, St. Louis, Mo.). Following three more washes the samples were examined for epifluorescence under ultraviolet illumination. Parasites were sometimes counterstained with ethidium bromide at $10 \mu\text{g ml}^{-1}$. The primary antibodies were either mAb-13.5 raised against Pc(em)93 (Wiser et al. 1988) or a monospecific polyclonal rabbit antiserum raised against a 24-kDa *P. chabaudi* protein (Ag-3008) localized to the PVM

(Favaloro et al. 1993). Ag-3008 is homologous with exp-1 of *P. falciparum*.

Labeling with C₅-DMB-ceramide

Infected erythrocytes were incubated for 30 min at room temperature in RPMI 1640 containing 20 μM C₅-DMB-ceramide (catalog number D-3521; Molecular Probes, Eugene, Ore.) and then fixed with 0.025% glutaraldehyde. Before examination for epifluorescence the samples were back-extracted three times on ice with RPMI 1640 containing defatted bovine serum albumin at 7 mg ml⁻¹ (Boehringer-Mannheim, Indianapolis, Ind.) as previously described (Elmendorf and Haldar 1994). Samples for scanning confocal microscopy were mounted under a coverslip in 0.1 M TRIS (pH 8.5) containing 25% glycerol, 10% polyvinyl alcohol, and 2.5% 1,4-diazabicyclo-[2.2.2]octane and examined with a Leica H20-004 confocal microscope.

Electron microscopy

P. chabaudi-infected whole blood was collected without anti-coagulants and immediately fixed in 2% glutaraldehyde containing 0.5% tannic acid in 0.1 M Na-cacodylate-HCl buffer (pH 7.2) for 3 h on ice. Following four washes with 0.1 M cacodylate buffer (pH 7.2) the samples were postfixed in 1.5% (w/v) OsO₄ for 90 min on ice. The samples were washed with buffer followed by water, treated with 0.5% (w/v) uranyl acetate, dehydrated, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEM-1200EX II electron microscope.

Results

Previous studies indicate that Pc(em)93 is transcribed (Giraldo et al. 1998) and translated (Wiser et al. 1988) exclusively during the ring stage with a maximal rate of synthesis at approximately 2 h after merozoite invasion. Therefore, infected erythrocytes were collected during

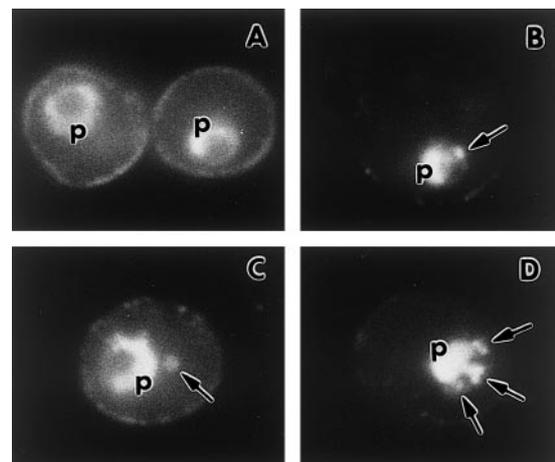


Fig. 1A–D Localization of Pc(em)93 during the early ring stage. Infected erythrocytes were collected during a period corresponding to maximal merozoite release and reinvasion and examined by immunofluorescence as previously described (Wiser et al. 1993). Parasites (p) were detected by counterstaining with ethidium bromide and the erythrocyte membrane is stained green (mAb-13.5). **B–D** Occasional green structures (arrows) are detected juxtaposed to the parasite. A supplemental color version of this figure is available

the period corresponding to the peak of merozoite release and were analyzed by immunofluorescence microscopy using mAb-13.5. The most commonly observed pattern is a fluorescence associated with the erythrocyte membrane (Fig. 1A). Occasionally, mAb-13.5 labels vesicle-like or short tubular structures that are juxtaposed to the parasite (Fig. 1B–D). These structures were never observed to extend to or fuse with the erythrocyte membrane. The rare occurrence (<1% of infected erythrocytes) of these structures suggests that they are transient, which is consistent with the rapid export of Pc(em)93 from the parasite (Wiser and Laners 1992). Occasionally, multiple projections, located close together, are labeled with mAb-13.5 (Fig. 1D).

Ag-3008 is a 24-kDa *Plasmodium chabaudi* protein localized to the PVM and membranous clefts within the cytoplasm of the host erythrocyte (Favaloro et al. 1993). Antibodies against Ag-3008 were used to determine the immunofluorescence pattern of the PVM. In contrast to the pattern obtained with mAb-13.5, anti-Ag-3008 uniformly stains the PVM and membranous extensions emanating from the PVM (Fig. 2A–C). Approximately 20% of the infected erythrocytes exhibit these PVM extensions. The most common PVM extension is a single thin extension projecting into the cytoplasm of the erythrocyte membrane (Fig. 2A). Multiple PVM extensions from a single parasite (Fig. 2B) or a single PVM extension with branches (Fig. 2C) are occasionally observed. These extensions are always continuous with the PVM and are never observed as free structures within the erythrocyte cytoplasm.

P. chabaudi-infected erythrocytes were incubated with C₅-DMB-ceramide and examined under UV illumination for further characterization of the PVM and its extensions. All membranes, especially the PVM, incorporated the fluorescent label (Fig. 2D–F). Two types of membranous projections from the PVM were detected. One type appears similar in size and morphology to the PVM extensions detected with anti-Ag-3008 (Fig. 2D). The other type of structure labeled with C₅-DMB-ceramide is substantially larger in width (Fig. 2E). Scanning confocal microscopic images of these larger structures (Fig. 2F) are similar to the previously published structures referred to as the tubovesicular membrane network (Behari and Haldar 1994; Elmendorf and Haldar 1994). The larger PVM extensions are not as common as the thinner PVM projections and are relatively rare. No vesicle-like structure labeled with C₅-DMB-ceramide was observed in the erythrocyte cytoplasm.

The PVM and its extensions were also examined by electron microscopy. Membranous extensions projecting from the PVM were observed during all stages, including the ring stage. At least two morphologically distinct PVM projections are observed. One type of projection is long and thin (Fig. 3A–C). The continuity of these extensions with the PVM is apparent (Fig. 3A). PVM projections are detectable soon after merozoite invasion (i.e., appliqué form) as evidenced by the parasite's close

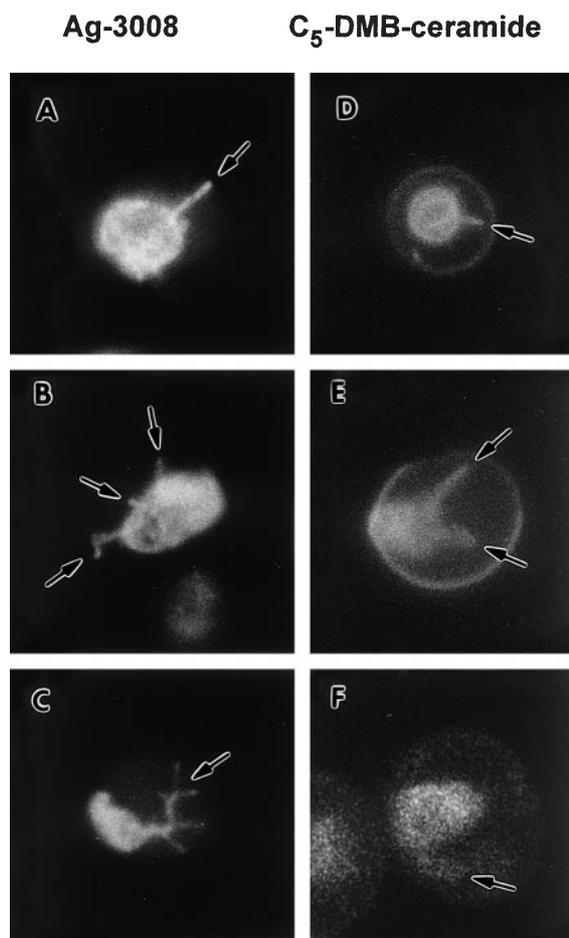
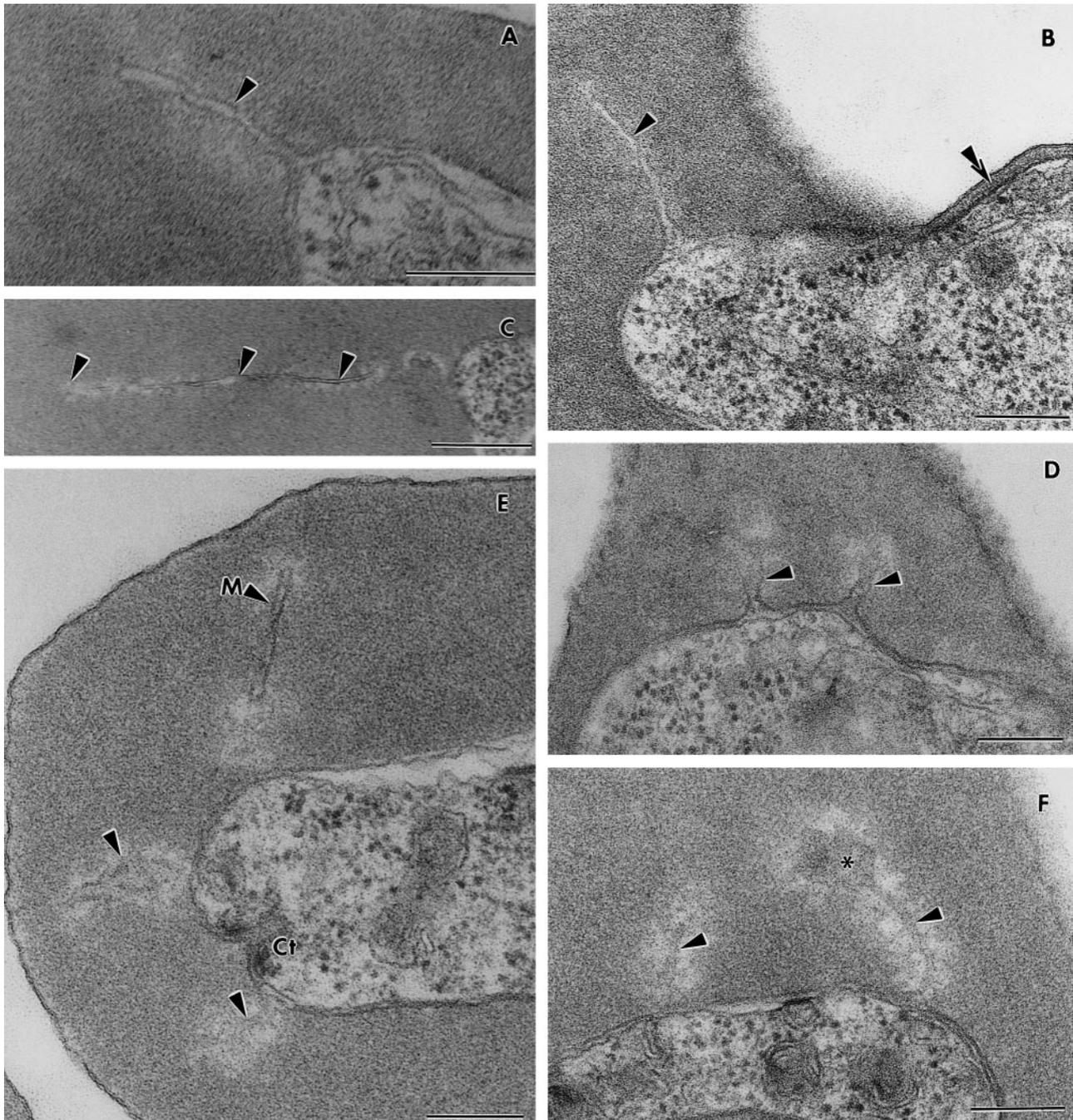


Fig. 2A–F Labeling of the PVM by immunofluorescence and lipid probes. Infected erythrocytes were examined by immunofluorescence using a monospecific rabbit serum raised against Ag-3008 (A–C). Ag-3008 is associated exclusively with the PVM and membranous projections (*arrows*) from the PVM. Infected erythrocytes were also labeled with C₅-DMB-ceramide and examined by conventional (D, E) or confocal (F) fluorescence microscopy. All membranes, including the PVM and projections (*arrows*), are labeled with C₅-DMB-ceramide

apposition to the erythrocyte membrane (Fig. 3B). These PVM extensions are likely equivalent to the projections labeled with anti-Ag-3008 (Fig. 2) as also supported by previous ultrastructure studies indicating that Ag-3008 is localized to Maurer's clefts (Favaloro et al. 1993). Some projections are quite long and are observed to extend 3.9 μ m into the erythrocyte cytoplasm (Fig. 3C). Although the membranous projections extended toward the erythrocyte membrane, fusion with the erythrocytic membrane was not observed, nor did we observe vesicles pinching off of these projections and fusing with the erythrocyte membrane.

A second type of PVM projection exhibits membranes that are initially continuous with the PVM but then become diffuse at the more distal ends (Fig. 3D) or terminate in a vesicle-like enlargement (Fig. 3F). In some cases, well-defined membranes are not detected and the PVM at the base of the projection appears diffuse (Fig. 3F). The latter projection appears to be disinte-



grating and the diffuseness at the base may be the PVM resealing. Occasionally, both types of projections are present in the same section (Fig. 3E), indicating that these diffuse projections are not due to poor preservation. Furthermore, the infected erythrocytes were fixed immediately after exsanguination and the parasites appear quite healthy, indicating that the projections are not the result of dying parasites. Regardless of the nature of the extensions, these observations indicate that there are qualitatively different projections from the PVM.

Discussion

Examination of infected erythrocytes by immunofluorescence microscopy during a period coincident with the synthesis of Pc(em)93 resulted in the occasional detection of Pc(em)93 associated with the vesicle-like structures adjacent to the parasite. The rarity of detection of these vesicle-like structures suggests that they are transient, which is consistent with the rapid transport of Pc(em)93 to the host membrane (Wiser and Lanners 1992). These vesicle-like structures are possibly related to the aggregates of RESA that are associated with the



Fig. 3A–F Electron microscopy demonstrating membranous projections extending from the PVM. *Plasmodium chabaudi*-infected erythrocytes were collected during a period of maximal schizont rupture and merozoite invasion and were immediately fixed and processed for electron microscopy as described in Materials and methods. Tubular membranous extensions emanating from the PVM are indicated by *arrowheads*. **A** Young trophozoite exhibiting tubular extension of PVM. **B** Extension of the PVM from a young trophozoite as an appliqué form. The erythrocyte membrane is separated from the PVM by 46 nm (*double arrowhead*). **C** PVM extension projecting 3.9 μm into the erythrocytic cytoplasm. Although the membrane of this extension is not evident over its entire length, the clearing of the erythrocyte cytoplasm around this extension indicates its path. **D–F** PVM extensions showing a disintegration of their membranes and a prominent clearing of the surrounding erythrocyte cytoplasm. Sometimes these projections can be seen ending in a vesicle enlargement (**F**, *asterisk*). **E** Both types of PVM extensions in the same section. The slender type is seen in the form of a traditional Maurer's cleft (*M*). (*Ct* Cytostome). Bars 200 nm

parasitophorous vacuole in newly invaded erythrocytes (Culvenor et al. 1991). The immunofluorescence pattern of Pc(em)93 as discrete structures adjacent to the parasite is distinct from the uniform staining pattern obtained with PVM markers such as Ag-3008 and C₅-DMB-ceramide. These different patterns suggest that Pc(em) 93 does not travel along the PVM and the membranous extensions as it moves to the erythrocyte membrane. Furthermore, we never observed any evidence of vesicular traffic between the PVM and the erythrocyte membrane in these studies. Other investigators have also concluded that there is no transfer of membrane material between the host erythrocyte membrane and the PVM using fluorescent lipid probes (Haldar and Uyetake 1992; Pouvelle et al. 1994). In addition, PVM extensions, called the tubovesicular membrane network, have been demonstrated not to be involved in the transport of parasite proteins but have been proposed to function in the acquisition of nutrients (Lauer et al. 1997).

Infected erythrocytes were also analyzed by pre- and postembedding immunogold electron microscopy using mAb-13.5. In both cases, label was associated only with the erythrocyte membrane, and no labeling of discrete structures adjacent to the parasite was observed (data not shown). As previously discussed (Wiser et al. 1997), labeling with mAb-13.5 requires the use of low glutaraldehyde concentrations during fixation, thus resulting in poor structural preservation. A more serious problem, however, may be the infrequency and transient nature of these parasite-associated structures detected by mAb-13.5 immunofluorescence. This infrequency in detecting the parasite-associated structures is compounded by the ultrathin sections used in electron microscopy. However, membranous extensions of the PVM that appear to be disintegrating, and thereby possibly releasing material into the host cytoplasm, are observed at the ultrastructural level (Fig. 3D–F). Despite our inability to demonstrate definitively that Pc(em)93 is localized at these disintegrating PVM projections, we hypothesize that these PVM-associated structures are the same as the

vesicle-like structures detected by immunofluorescence with mAb-13.5 (Fig. 1B–D). Definitive proof, though, must await methodological refinements or the use of another antibody against an exported protein.

On the basis of the observations reported herein and the mounting evidence against vesicular transport between the PVM and erythrocyte membrane, we feel that Pc(em)93 is released into the host erythrocyte cytoplasm at the level of the PVM. The suggestion that Pc(em)93 is not transported to the host membrane via a vesicle-mediated process raises questions about how proteins are targeted to their final destinations within the infected erythrocyte. With regard to Pc(em)93, we have previously speculated the involvement of a soluble intermediate (Wiser and Lanners 1992) and that targeting to the erythrocyte membrane is due to the ability of Pc(em)93 to bind to the cytoplasmic face of the erythrocyte membrane (Wiser et al. 1990). Several *Plasmodium falciparum* proteins, such as the knob-associated protein (Kilejian et al. 1991), RESA (Foley et al. 1991), and MESA (Lustigman et al. 1990), also specifically bind to proteins located on the cytoplasmic face of the erythrocyte membrane. Therefore, secretion of proteins into the host erythrocyte and their subsequent binding to the erythrocyte membrane may be a general mechanism by which malaria parasites modify their host cell membrane. Accessory proteins, such as molecular chaperones, could also participate in the targeting of parasite proteins to the erythrocyte membrane and other intraerythrocytic locations. Interestingly, antibodies raised against a *P. berghei* cochaperone cross-react with heat-shock protein 70 (Uparanukraw et al. 1993), and the same antibody recognizes a protein(s) in the cytoplasm of infected erythrocytes (Wiser et al. 1996). Exported chaperones could participate in the targeting of parasite proteins to their final destinations and the formation of supramolecular structures such as knobs. An analogous situation has been described for transport of proteins to the outer membrane of bacteria. For example, the pili found on the surface of some bacteria are assembled on the outer membrane from several different proteins (Hultgren et al. 1993). Chaperones found in the periplasmic space sequester the pili subunits, preventing their premature assembly, and control their ordered insertion into the outer membrane.

Maurer's clefts and other PVM extensions have been implicated in the movement of the electron-dense material of knobs (Aikawa et al. 1986), the knob protein (Taylor et al. 1987), PfEMP-1 (Crabb et al. 1997), and Pf332 (Hinterberg et al. 1994) to the erythrocyte membrane. This discrepancy with regard to our proposed model for Pc(em)93 transport might be due to different exported proteins utilizing different transport pathways to their final destinations. An alternate explanation is that exported proteins are secreted from the parasite as proposed for Pc(em)93 and then bind to the cytoplasmic face of these intraerythrocytic membranes as an intermediate step in their transport to the erythrocyte membrane. These two explanations are not mutually

exclusive, and multiple mechanisms for targeting of export proteins to their final destinations might coexist.

We have recently described a novel ER-like organelle, called sERA, which appears to be the first step in targeting of proteins for export from the parasite (Wiser et al. 1997). The sERA is located at the periphery of the parasite and is detectable in all erythrocytic stages. Presumably, exported proteins are transferred from the sERA into the parasitophorous vacuole, as has been demonstrated for the glycoprotein-binding protein of *P. falciparum* (Ansorge et al. 1996). The release of Pc(em)93 into the host cytoplasm might occur at the disintegrating PVM projections discussed above. Such structures would be transient and the PVM would need to reseal quickly to preserve its integrity as well as its membrane potential (Desai et al. 1993). The parasite-associated structures labeled with mAb-13.5 appear to be restricted to distinct domains of the PVM. Distinct domains of the PVM have previously been suggested by dual-labeling experiments (Behari and Haldar 1994). Positioning of a PVM domain for the presumptive release of Pc(em)93 may be dictated by the location of the sERA at the parasite periphery, thus suggesting that the export of parasite proteins involves their vectorial transport through the sERA and then the parasitophorous vacuole and their subsequent release into the host cytoplasm at the PVM.

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