Plasmodium falciparum apical membrane antigen 1 (PfAMA-1) is translocated within micronemes along subpellicular microtubules during merozoite development

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Summary

During the assembly of Plasmodium falciparum merozoites within the schizont stage, the parasite synthesizes and positions three sets of secretory vesicles (rhoptries, micronemes and dense granules) that are active during red cell invasion. There are up to 40 micronemes per merozoite, shaped like long-necked bottles, about 160 nm long and 65 nm at their widest diameter. On their external surfaces, they bear bristle-like filaments, each 3-4 nm thick and 25 nm long. Micronemes are translocated from a single Golgi-like cisterna near the nucleus along a band of two or three subpellicular microtubules to the merozoite apex, where they dock with the rhoptry tips. Dense granules are also formed around the periphery of the Golgi cisternae but their distribution is unrelated to microtubules. Three polyclonal antibodies raised against the recombinant PfAMA-1 ectodomain sequence recognizing both the 83 kDa and processed 66 kDa molecules label the peripheries of translocating and mature micronemes but do not label rhoptries significantly at any stage of merozoite development within schizonts. This result confirms that PfAMA-1 is a micronemal protein, and indicates that within the microneme it is located near or inserted into this organelle’s boundary membrane.

Key words: AMA-1, Electron microscopy, f-MAST, Merozoite, Microneme, Microtubule, Plasmodium falciparum, Trafficking

Introduction

Within the human bloodstream, the malaria organism Plasmodium falciparum is an intracellular parasite, cyclically invading, feeding and multiplying in red blood cells (RBCs). In its multiplicative phase (the schizont), the parasite generates in the region of 16 invasive forms (merozoites) that bud off from its perimeter and are eventually released from their surrounding membranes to invade fresh RBCs (Bannister et al., 2000). During this phase the nucleus undergoes four or more mitotic divisions, whereas, in the cytoplasm, there ensues the coordinated synthesis, targeting and assembly of invasion-specific organelles. These include three types of secretory vesicle: rhoptries, micronemes and dense granules, which have different shapes, sizes, contents and roles in invasion (for reviews, see Dubremetz et al., 1998; Preiser et al., 2000; Blackman and Bannister, 2001).

The largest of these vesicles are the rhoptries, paired club-shaped vesicles that discharge their secretions at the merozoite apex through narrow ducts. Micronemes are smaller, more elongated and more numerous, and are clustered around the rhoptry ducts and also secrete onto the merozoite apex. Dense granules are similar to micronemes in size but differ in shape, position and secretory access: they are rounded, are mostly situated non-apically and discharge their contents through the sides of the parasite after it has entered a RBC. Rhoptries and micronemes are positioned at the merozoite’s apical end, marked by a low truncated conical projection (the apical prominence). During invasion, rhoptries and micronemes discharge their contents through this region.

Although molecular studies of merozoite development are now progressing vigorously in many laboratories, the morphological events of this process are known only in broad outline [e.g. in P. falciparum (Langreth et al., 1978; Taraschi et al., 1998), Plasmodium vinckei (Vickerman and Cox, 1967) and Plasmodium elongatum (Aikawa et al., 1967)]. To address this situation, we have begun a detailed structural analysis of P. falciparum merozoite development, paying special attention to the role of the cytoskeleton. Peripheral microtubules are present in all invasive stages of Plasmodium [as in related apicomplexan genera (Morrissette and Sibley, 2002)] although, in P. falciparum merozoites, they are represented minimally by a band of just two or three (Bannister and Mitchell, 1995; Hopkins et al., 1999; Bannister et al., 2000; Bannister et al., 2001). Merozoite microtubules are destabilized by treating schizonts with colchicine and dinitroanilines, and this might be the root cause of the inhibitory effects of these compounds on merozoite...
invasion into RBCs (Bejon et al., 1997; Fowler et al., 1998; Fowler et al., 2001). However, we have been unable to find clear evidence from fluorescence imaging (FI) or electron microscopy (EM) for any dependence of merozoite organelle assembly on microtubule integrity (Bejon et al., 1997; Fowler et al., 1998).

Recently, we analysed the formation of rhoptries in *P. falciparum*, showing that they are created by fusion of vesicles derived from a simple Golgi-like structure and that they become anchored at the merozoite apex early in merozoite bud formation without apparent involvement of microtubules in this process (Bannister et al., 2000). In the present study, we focus on the development of micronemes. Micronemal proteins localized to these organelles by immuno-electron microscopy (IEM) have attracted special attention because of their potential importance in the capture of host RBCs, including the *ebi* family (Adams et al., 2001), EBA-175 in *P. falciparum* (Sim et al., 1992) and the Duffy receptor proteins DBP-135 and DBP-138 in *Plasmodium knowlesi* (Adams et al., 1990; Adams et al., 1992). Another invasion-related protein with a micronemal location is *P. falciparum* apical membrane antigen 1 (PfAMA-1) (Peterson et al., 1989; Waters et al., 1990; Narum and Thomas, 1994; Marshall et al., 1996; Triglia et al., 2000; Urquiza et al., 2000; Howell et al., 2001; Howell et al., 2003; Nair et al., 2001; Nair et al., 2002; Dutta et al., 2002; Healer et al., 2002; Kocken et al., 2002), which is one of the most promising candidates for malaria vaccine development (Collins et al., 1994; Narum et al., 2000). It is synthesised as an 83 kDa molecule and processed within the schizont by the loss of its N-terminal pro-domain to 66 kDa, and then relocates on to the merozoite surface; PfAMA-1 is further cleaved in the free merozoite prior to or at invasion (Howell et al., 2001). Within merozoites, PfAMA-1 has been localized by IEM to the apical ends of rhoptries, with indications of a micronemal involvement (Crewther et al., 1990). However, there have been doubts about its rhoptry assignment because, unlike typical rhoptry proteins, PfAMA-1 is synthesized very late in merozoite development (Crewther et al., 1990) and its *Toxoplasma gondii* homologue (TgAMA-1) is now known to be micronemal (Donahue et al., 2000; Hehl et al., 2000). Immediately before submission of this work, Healer et al. (Healer et al., 2002) published an IEM study showing that a monoclonal antibody to the full length (unprocessed) 83 kDa, but not the processed 66 kDa, form of PfAMA-1 (therefore presumably recognizing the pro-domain) labels micronemes rather than rhoptries. They concluded that 83 kDa to 66 kDa processing occurs in micronemes and that the resultant 66 kDa molecule is stored in rhoptries before release.

In the present investigation, we have used a combination of FI and EM, including serial sectioning, freeze fracture and IEM, to study the structure and development of micronemes. Particular attention has been paid to their trafficking and possible PfAMA-1 content, using a newly generated set of antibodies raised against the ectodomain of this molecule. We show that, uniquely among the three types of secretory organelle, micronemes move apically from the Golgi complex along the merozoite's subpellicular microtubules. We also confirm that PfAMA-1 is located primarily in micronemes but fail to find evidence of a rhoptry location for processed PfAMA-1 within pre-release merozoites. A brief report of these results was presented at the Gordon Research Conference on Host-Parasite Interactions in June 2002.

### Materials and Methods

#### Preparation of parasites

Cultures of the ITO4, Paolo Alto and C10 lines of *P. falciparum* were synchronized by sorbitol lysis (Lambros and Vanderberg, 1979). Schizonts were concentrated on a Percoll cushion (Dluzewski et al., 1984), washed in culture medium then allowed to grow for a further 30 minutes to 1 hour at 37°C before being fixed for electron microscopy.

#### Reagents

To detect PfAMA-1, polyclonal antibodies were raised in rabbits immunized with purified recombinant proteins engineered in *Pichia pastoris* using an optimal (non-AT-rich) codon usage (Withers-Martinez et al., 1999) to express PfAMA-1 ectodomain sequences (Kocken et al., 2002). These reagents included three sera (Pab 1, Pab 2 and Pab 3) from three rabbits, each immunized with the whole ectodomain including the N-terminal pro-domain. For controls, sera taken from an adjuvant-only treated rabbit (Pab 4) and from a rabbit immunized with contaminants from the *P. pastoris* recombinant cultures were used in parallel. For comparison, antibodies against EBA 175 were also used for IEM (obtained from the Malaria Research and Reference Reagent Resource Center, Division of Microbiology and Infectious Diseases, NIAID, NIH, courtesy of J. Adams).

For the detection of primary antibody, Protein A conjugated to 10 nm gold was obtained from the Department of Cell Biology, School of Medicine, Utrecht University. Rat anti-rabbit IgG conjugated to 10 nm gold (British Biocell, Cardiff, UK) was also used in some preparations.

#### EM for morphological analysis

**Preparation of samples for EM**

For detailed ultrastructural analysis, schizont-infected RBCs (both C10 and ITO4 lines) were fixed either at room temperature or on ice for 3 hours in 2.5% v/v glutaraldehyde and 0.75% w/v tannic acid, buffered at pH 7.0 with 0.1 M sodium cacodylate. In some samples, tannic acid was omitted from the cacodylate fixative (buffered then at pH 7.2). After washing three times by centrifugation in cacodylate buffer, pelleted cells were post-fixed for 1 hour in 1% (w/v) osmium tetroxide in cacodylate buffer, rinsed in distilled water, block stained with 0.8% or 1% (w/v) aqueous uranyl acetate for 1 hour, then dehydrated in an acetone series and embedded in TAAB epoxy resin (EMSCOPE, London, UK). Sections were cut with a Reichert Ultracut 3E ultramicrotome and stained with saturated uranyl acetate in 50% ethanol (10-12 minutes) followed by 0.4% (w/v) lead citrate (6-8 minutes). Sections were viewed in either a Hitachi H7000E or a Hitachi H7600 transmission electron microscope.

For freeze-fracture, cells were fixed in glutaraldehyde or tannic-glutaraldehyde as described above, then fractured, replicated and shadowed in a Polaron freeze fracture station, as previously described (Bannister et al., 2000).

For scanning EM, parasitized red cells were fixed, washed, pelleted by centrifugation and dehydrated as for routine transmission electron microscopy (but omitting uranyl block staining), then critical-point dried. Cell pellets were broken up with forceps and sprinkled on to stubs covered with double-sided adhesive tape and sputter-coated with gold before viewing in a Hitachi S3300 scanning electron microscope.

#### Three-dimensional reconstruction

For serial reconstruction, sections were collected on Formvar- or Pioloform-coated slotted grids, then stained with uranyl acetate and lead citrate as above. Individual schizonts needed up to 94 sections each for complete reconstruction, whereas individual merozoite buds required 15-24 sections, depending on their exact shape and orientation. Most of the specimens were of the ITO4 line, although
C10 parasites were also extensively used in this study. Much of our three-dimensional data comes from 35 developing merozoites that were serially sectioned at either 85 nm or 58 nm thickness, representing two complete late schizonts. The evidence from these was corroborated and amplified by imaging of many schizonts and mature merozoites from at least ten other separate cultures.

Reconstructions were made by tracing consecutive serial sections on to a series of acetate sheets, then superimposing them to make graphic projections, as detailed in a previous publication (Bannister et al., 2000) using the positions of neighbouring cells to ascertain registration between pictures. Measurements were taken from electron micrographs photographed at 100,000× and printed at 250,000×. For stereoscopic viewing, sections were tilted at +6°, +12° or +18° in the electron microscope and imaged at magnifications of 100,000× to 150,000×.

IEM labelling
The method followed was as described previously (Bannister and Kent, 1993), briefly as follows. Samples enriched in late schizonts were fixed in 0.1% double distilled glutaraldehyde made up in RPMI culture medium and then washed and processed by the progressive low temperature dehydration technique and embedded in Medium LR White Resin (EMSCOPE, London, UK), using ultraviolet light polymerization at room temperature. Sections mounted on nickel grids were immunostained with primary antibodies against PFAMA-1 (Pab 1, Pab 2 and Pab 3) and for controls against Pichia contaminants (Pab 4), with normal Rat IgG (dilutions for each were 1:50 and 1:100) or in the absence of primary antibody. Labelling was detected with Protein-A conjugated to 10 nm gold (a kind gift from Pauline Bennett, King’s College, London, UK). Finally they were contrasted for 5 minutes in 2% aqueous uranyl acetate before viewing.

Western blots
Percoll-purified late-stage IT04 parasites were solubilized in sample buffer for SDS-PAGE (Invitrogen) and proteins were separated on 10% polyacrylamide gels using Invitrogen NuPAGE™ Bis-Tris MOPS buffer system and blotted to nitrocellulose. As controls, uninfected RBC membranes (prepared by osmotic lysis) were used in parallel with parasitized red blood cells for each antibody. Pab 4 against Pichia expression contaminants was also used for control purposes. The blots were probed with primary antibodies diluted 1:2000 in PBS, 2% skimmed milk, 0.05% Tween 20 and visualized with a 1:3000 dilution (same buffer as primary antibody) goat anti-rabbit-IgG conjugated to alkaline phosphatase (Sigma, UK) followed by BCIP/NBT alkaline phosphatase substrate (Sigma, UK).

Results
General organization of the budding merozoite
In the developmental period studied in this investigation [immediately before the merozoites separate from central cytoplasm (residual body) of the parasite], micronemes and dense granules are being formed and positioned within the budding merozoites, whereas rhoptries have already been generated and located apically. At this stage, there are about 16 merozoite buds arranged around the parasite’s circumference, each merozoite now an ellipsoid 1.2-1.8 µm long by 0.7 µm wide, with an apical prominence pointing away from the schizont’s residual body, to which the merozoite is still attached by a narrow stalk (Figs 1, 2). Cell shapes and dimensions vary considerably, presumably because merozoites press against each other and against the residual body in the confined space of the parasitophorous vacuole (Fig. 1A).

At this stage, the merozoite’s nucleus occupies the basal third of the merozoite and often has the approximate shape of a shallow cup, with its convex surface nesting in the curve of the merozoite’s base and its concave, or sometimes flattened, face directed diagonally apex-wards or transversely across the merozoite (Fig. 1C-E, Fig. 2). A single discoidal cisterna provisionally identified as a minimal Golgi apparatus (here termed the Golgi cisterna) is situated close to the nucleus, near the spindle pole body remnant and immediately adjacent to the zone of coated vesicle budding from the nuclear envelope, similar to the situation in earlier stages of development (Bannister et al., 2000). The coated vesicles, each about 40 nm in diameter, are clustered between the nuclear budding zone and the basal surface of the Golgi cisterna (Fig. 1E). Around the perimeter of the Golgi cisterna, larger (60-120 nm) coated vesicles with dense contents occur, some apparently in the process of budding off this structure (Fig. 1E), and micronemes and dense granules are visible, identifiable by their shapes and sizes (see also below). The cytoplasm around the nucleus contains irregularly shaped profiles of rough endoplasmic reticulum, some of them visibly continuous with the nuclear envelope, which also bears groups of ribosomes on its outer perimeter. The endoplasmic reticulum persists until after merozoites have separated from the residual body, but vanishes before release from the infected RBCs.

At the merozoite’s apical end, the three characteristic densely staining polar rings are by now fully formed beneath the membrane of the apical prominence, and the pear-shaped rhoptries are in place (Fig. 1C-E). A band of two or three microtubules (which we have called the falciparum merozoite assemblage of subpellicular microtubules or f-MAST) extends down one side of the merozoite as far as the nucleus, and immediately beneath this band is the apicoplast, with the somewhat longer mitochondrion lying alongside (Fig. 2), as described previously (Hopkins et al., 1999). At the basal end of the merozoite, where a narrow cytoplasmic connection to the residual body persists, a ring of dense material undercoats the plasma membrane, indicating the constriction zone of cytokinesis.

The structure of the merozoite surface is as described for the mature merozoite by Langreth et al. (Langreth et al., 1978) with a filamentous coat attached to the plasma membrane, and two further membranes (components of the inner membrane complex, in reality a flattened membranous cisterna) underlying the circumference except at the apical prominence. A single cytosomal ring is also present about one-third of the distance from the apex to the base of the merozoite, unrelated to the f-MAST.

Microneme structure
At the late budding stage of merozoite development, micronemes are long-necked vesicles shaped like long-necked bottles (Fig. 1D,E, Fig. 3A-C,E). Their mean dimensions from a sample of 100 (22 merozoite buds from two serially sectioned schizonts, IT04 line, measured at print magnifications of 250,000×) are: length=164 nm ± 35 nm (s.d.), widest diameter=63 nm ± 10.7 nm. The bulbous base is filled with densely staining, finely granular material, whereas the narrow neck region stains only minimally (Fig. 3A,B). They number up to 40 per merozoite. On their external surfaces, all micronemes
bear numerous thin bristle-like filaments, each about 25 nm long and 3-4 nm thick (Fig. 3B,C). Where they cluster apically, the projecting filaments of adjacent micronemes often appear to attach to each other, or to similarly sized filaments projecting from the surfaces of the rhoptries (Fig. 3B,C). Rhoptries are also attached by much longer (300 nm) filaments of similar thickness (3-4 nm) to the surrounding polar rings and membranes of the apical prominence (Fig. 3D). Projecting surface filaments appear to be entirely absent from dense granules.

Freeze-fracture preparations show the typical micronemal shape and demonstrate the presence of small numbers of intramembranous particles (of about 10 nm in diameter) on their externally directed fracture face (Fig. 3E).

Translocation of micronemes: the f-MAST as a microtubule track way
The position of micronemes changes during development. They appear initially around the perimeter of the Golgi cisterna (Fig. 1E, Fig. 4A) and then cluster in a band immediately beneath the band of microtubules that constitutes the f-MAST (Fig. 2, Fig. 4A-G). Finally they mass apically, mainly positioned with their narrow ends converging radially onto and, in some instances, in contact with the rhoptry tips (Fig. 1C,D), although they are not actually confluent with them at any stage of schizont development. A few micronemes are present in more basal regions or other parts of the merozoite, unrelated to the f-MAST or the merozoite apex (Fig. 2); they might represent misrouted individuals.

As mentioned above, the f-MAST consists of two or occasionally three subpellicular microtubules. They form a single band running down one side of the merozoite in a low-pitched helix from their attachment at their third polar ring to about two-thirds the length of the merozoite, overlapping the Golgi cisterna and cluster of associated vesicles (Fig. 2, Fig. 4A-G). Before they reach the apical end of the merozoite, most micronemes are clustered beneath the f-MAST, often arranged in ranks with their narrow necks pointing transversely across
Microneme trafficking in *Plasmodium*

The present study provides structural evidence that microtubules (Fig. 4D,E). Stereopairs imaged from tilted sections show that the projecting filaments of micronemes contact the overlying microtubules (Fig. 4F,G). In later stages, most micronemes have reached the apical end of the merozoite and have detached from microtubules to dock with the apices of the rhoptries (Fig. 1E). No dense granules were found in close relation to the f-MAST at any stage of development and, indeed, they were often clustered on the opposite side of the merozoite.

**Immunolocalization of PfAMA-1 to micronemes**

IEM labelling of late schizonts with the three of the anti-PfAMA-1 sera (Pab 1, Pab 2 and Pab 3) was restricted to micronemes, both in their final apical position and in their earlier migration from the region of the Golgi cisterna. Typical labelling patterns of Pab 1 and Pab 3 are illustrated in Fig. 5A-D; Pab 2 labelling was very similar but is not shown. We found no instance of significant labelling of any part of the rhoptries within schizonts, whereas the immunolabelling of micronemes was often intense (Fig. 4A,B). Strikingly, labelling showed a strong tendency to follow the perimeters of micronemes, including their narrow necks, rather than the interior of these organelles (Fig. 5C,D), a finding seen when either Protein-A/gold (Fig. 5A,B) or IgG/gold (not shown) was used to detect the primary antibody. It might also be significant that a small number of micronemes in each merozoite remained unlabeled (e.g. Fig. 5A). Attempts using the IEM double labelling technique to determine whether there is a separate population of micronemes containing EBA-175 and not PfAMA-1 were inconclusive because of the relatively low level of EBA-175 labelling, as also reported recently by Healer et al. (Healer et al., 2002). Attempts to IEM label kinesin and dynein using commercial antibodies (Sigma, UK) were also unsuccessful in this study. Controls carried out with adjuvant-only rabbit IgG or rabbit antibodies against contaminant protein from the *P. pastoris* expression system (Pab 4), with Rat IgG or in the absence of primary antibody did not give significant immunolabelling of any structure within schizonts.

In western blots, all three antibodies used in this study detected both the full length (83 kDa) and processed (66 kDa) forms of PfAMA-1 (Fig. 6), whereas the *Pichia* contaminants controls were negative for all of them.

**Discussion**

Microtubules are implicated in micronemal trafficking

The present study provides structural evidence that...
Fig. 4. Sections through developing merozoites illustrating the route taken by micronemes from the Golgi body to the apex in relation to the subpellicular microtubules of the f-MAST. (A) Part of a merozoite transversely sectioned through the region of the vesicle budding zone at the periphery of the Golgi cisterna, an edge of which is visible in oblique section (go); three subpellicular microtubules are visible on the left (white arrows). Scale bar, 200 nm. (B) The microtubules of the f-MAST in transverse section (short arrows), close to micronemes (asterisk). The three membranes of the merozoite pellicle are also visible (long arrows); to the left of the merozoite surface is the membrane of the parasitophorous vacuole. (C) Structures similar to those in (B) with a more clearly defined microneme (asterisk). Scale bar for B and C, 100 nm. (D,E) Stereoscopic pair showing the apical region of a developing merozoite with migrating micronemes (black arrows) attached on the left to the subpellicular microtubules (white arrows) associated with a pair of subpellicular microtubules (white arrows) attached on the left to the polar rings (pr). Angle of tilt, 12°. Scale bar, 100 nm. (F,G) Another stereoscopic pair showing micronemes migrating in relation to a subpellicular microtubule (mt) attached to the apical polar rings (pr). The electron micrographs have been enhanced to show filamentous connections between the micronemes and microtubule (indicated by small arrows). Part of a rhoptry (rh) is also visible. Angle of tilt, 12°. Scale bar, 100 nm.

Fig. 5. Immunogold labelling of maturing schizonts with antibody against PfAMA-1 whole ectodomain. (A) An obliquely sectioned merozoite apex with a cluster of micronemes strongly labelled by Pab 1, surrounding the tips of two unlabelled rhoptries (rh). Scale bar, 100 nm. (B) Two micronemes (black arrows) labelled by Pab 3 and a longitudinally sectioned rhoptry devoid of labelling, including its narrow apical end (white arrow). Scale bar, 100 nm. (C,D) PfAMA-1 labelling by Pab 3 around the peripheries of micronemes. The microneme in (D) was situated near the Golgi cisterna and represents an early stage in this organelle’s migration to the apex. Scale bars, 50 nm.
sporozoites and *T. gondii* tachyzoites, in which microneme contents are secreted progressively during gliding locomotion (Ménard, 2000; Carruthers et al., 1999; Soldati et al., 2001), presumably requiring micronemal replacement from a pool of these organelles maintained within the parasite.

In contrast with the vectorial transport of micronemes, the positioning of dense granules and rhoptries in *P. falciparum* merozoites is not dependent on microtubules. Whether they are entirely diffusional processes is not clear. Rhoptries are formed by vesicular fusion relatively early in merozoite development, when the Golgi cisterna is very close to the merozoite apex (Bannister et al., 2000), and dense granules appear to be exported without any clear pathway into the general cytoplasm. However, the indications of regional clustering suggest some non-random element in their movements. A diagram illustrating the proposed trafficking pathways from the Golgi cisterna for rhoptries, micronemes and dense granules is shown in Fig. 7.

PfAMA-1 is a peripherally located micronemal protein IEM with affinity-purified antibodies directed against the

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**Fig. 6.** Western blots of the polyclonal antibodies (Pab 1, Pab 2 and Pab 3, and control Pab 4) used to study PfAMA-1 expression in extracts of late-stage ITO4 schizont-parasitized red blood cells (p) and uninfected red blood cell (r) samples for each. Lanes: (A) p and r incubated with (control) antibody against *Pichia* expression contaminants; (B-D) incubated with anti-PfAMA-1 Pab 1 (B), Pab 2 (C) and Pab 3 (D). All anti-PfAMA-1 antibodies stain both the unprocessed (PfAMA-183) and processed (PfAMA-166) forms of PfAMA-1. Lower molecular weight bands represent further cleavage products, variably detected by the three anti-PfAMA-1 antibodies.

**Fig. 7.** The trafficking routes of the three major classes of secretory organelles in the *Plasmodium falciparum* merozoite, numbered 1-3 to reflect their times of origin from the Golgi cisterna. Routes 1 (rhoptry-directed vesicles) and 3 (dense granules) are independent of microtubules, whereas route 2 represents the microtubule-related targeting of micronemes to the merozoite apex. Other details of merozoite structure, including the pellicular system and merozoite coat are not depicted.
whole ectodomain of PfAMA-1 indicates clearly that this molecule is micronemal and, at least within the schizont, not a rhoptry molecule, supporting the recent findings of a micronemal location by Healer et al. (Healer et al., 2002) based on FI and IEM with a monoclonal antibody recognizing only the unprocessed (83 kDa) form of PfAMA-1. Healer et al. proposed that, because their antibody did not detect the apical rhoptry labelling reported by Crewhter et al. (Crewhrter et al., 1990), the rhoptry tips must contain only the 66 kDa molecule. Our antibodies detect both the 83 kDa and 66 kDa forms of PfAMA-1 in micronemes but we still failed to find evidence of rhoptry labelling at any time within the schizont. The possibility that PfAMA-1 refuxes into the apices of rhoptries when the micronemes secrete their content (Healer et al., 2002) cannot be ruled out but, if this happens, it must occur in free or invading merozoites because we did not find evidence for confluence between these two organelles in pre-release merozoites within schizonts. Until a more detailed analysis of the secretory routes of rhoptry and micronemal contents in the mature merozoite is available, no definitive conclusion can be drawn about the functional relationships of the two organelles.

Another significant result from the present study is the peripheral immunolabelling of micronemes, which was seen at all levels of their translocation from the Golgi cisterna to the merozoite apex but only clearly detected when there were sufficient gold particles per microneme to see a clear pattern. Because the same result appeared when anti-rabbit IgG/gold instead of Protein-A/gold was used to detect the first antibody, it is unlikely to be a Protein-A/gold related artefact, and we have not found any evidence of similar patterns with antibodies against other organelle proteins such as RAP-1 (L. H. Bannister, unpublished). The peripheral labelling suggests that PfAMA-1 is located close to, or inserted into the microneme membrane. Such a location is supported by evidence from Triton-X114 extraction experiments (Peterson et al., 1989; Crewhter et al., 1990) in which both the unprocessed 83 kDa and processed 66 kDa forms of PfAMA-1 partitioned into the detergent fraction, as expected of membrane proteins. When micronemes secrete their contents, their membranes must fuse either directly with the merozoite plasma membrane or indirectly via the rhoptry ducts, so that a micronemal transmembrane protein could relocate onto the merozoite surface simply by diffusion along membranes.

Finally, this study emphasizes the need for a detailed knowledge of parasite ultrastructure for the correct identification of antigen locations by immunocytochemistry. It is obvious that, in the merozoite apices, the clusters of micronemes are so close to the rhoptry ducts that IEM is essential to distinguish the two types of organelle and that light microscopic methods are insufficient for this purpose. In the post-genomic era, EM will clearly be an important tool for the analysis of protein expression and function.

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