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Trafficking and assembly of the cytoadherence complex in *Plasmodium falciparum*-infected human erythrocytes

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After invading human erythrocytes, the malarial parasite *Plasmodium falciparum*, initiates a remarkable process of secreting proteins into the surrounding erythrocyte cytoplasm and plasma membrane. One of these exported proteins, the knob-associated histidine-rich protein (KAHRP), is essential for microvascular sequestration, a strategy whereby infected red cells adhere via knob structures to capillary walls and thus avoid being eliminated by the spleen. This cytoadherence is an important factor in many of the deaths caused by malaria. Green fluorescent protein fusions and fluorescence recovery after photobleaching were used to follow the pathway of KAHRP deployment from the parasite endomembrane system into an intermediate depot between parasite and host, then onwards to the erythrocyte cytoplasm and eventually into knobs. Sequence elements essential to individual steps in the pathway are defined and we show that parasite-derived structures, known as Maurer's clefts, are an elaboration of the canonical secretory pathway that is transposed outside the parasite into the host cell, the first example of its kind in eukaryotic biology.

Keywords: fluorescence recovery after photobleaching/green fluorescent protein/KAHRP/malaria/protein trafficking

Introduction

Plasmodium falciparum causes the most severe form of malaria in humans. Each year, several hundred million people become infected with this deadly pathogen and 2–3 million will die. Once in the blood, continuous multiplication of the parasite inside erythrocytes is responsible for almost all the clinical symptoms of malaria and the associated morbidity and mortality. Dramatic structural and morphological changes occur in erythrocytes after invasion by the parasite. These changes play a pivotal role in the development of the severe complications that accompany falciparum malaria.

As the parasite develops inside the erythrocyte, several parasite-encoded proteins involved in its pathogenicity become associated with the erythrocyte cytoskeleton or are inserted into the host cell membrane. The *P.falciparum* erythrocyte membrane protein-1 (PfEMP1) (Su *et al*., 1995), the knob-associated histidine-rich protein (KAHRP) (Pologe *et al*., 1987; Triglia *et al*., 1987) and *P.falciparum* erythrocyte membrane protein-3 (PfEMP3) (Pasloske *et al*., 1993) are three such proteins. PfEMP1 is an antigenically-variant protein family responsible for both immune evasion and adherence of *P.falciparum*-infected erythrocytes to host receptors such as CD36 (Baruch *et al*., 1997). KAHRP is essential for the formation of knob structures that deform the erythrocyte surface (Crabb *et al*., 1997). The function of PfEMP3 is unknown although truncation of the protein has been shown to affect the trafficking of PfEMP1 to the parasite-infected erythrocyte membrane by accumulation in structures underlying the membrane (Waterkeyn *et al*., 2000).

The knob consists of an electron-dense, cup-shaped structure that underlies a protrusion of the erythrocyte membrane (Kilejian, 1979; Nagao *et al*., 2000), the main component of which is KAHRP. Several other parasite proteins are associated with the knob structure at the erythrocyte surface including the transmembrane protein PfEMP1, which has a large extracellular adhesive domain and a cytoplasmic tail, known as the acidic terminal sequence (ATS) (Su *et al*., 1995). KAHRP has been shown to interact with the ATS of PfEMP1 and there is evidence suggesting that in addition to undergoing self-association (Waller *et al*., 1999) it also interacts with spectrin and F-actin (Kilejian *et al*., 1991). Thus self-associated KAHRP scaffolds are thought to anchor onto the erythrocyte membrane skeleton and to provide a raised platform that facilitates the presentation of PfEMP1 at the erythrocyte surface. As a consequence, KAHRP-expressing parasitized erythrocytes cytoadhere to endothelial cell receptors under physiologically relevant flow conditions more efficiently than erythrocytes infected with KAHRP-knockout parasites (Crabb *et al*., 1997).

Merozoite forms of *P.falciparum* invade human erythrocytes and the parasite develops in this intracellular location within a parasitophorous vacuole. The parasite directs the trafficking of newly synthesized proteins to a series of different organelles within the boundaries of its own plasma membrane. In addition, it exports proteins beyond the confines of its own plasma membrane into the surrounding erythrocyte. Proteins exported beyond the confines of the parasite presumably contain 'signals' that allow them to traverse both the parasite plasma membrane and parasitophorous vacuole membrane. However, the nature of these sequence signals is unknown.

The export of parasite proteins to the erythrocyte cytoplasm is all the more remarkable given that human

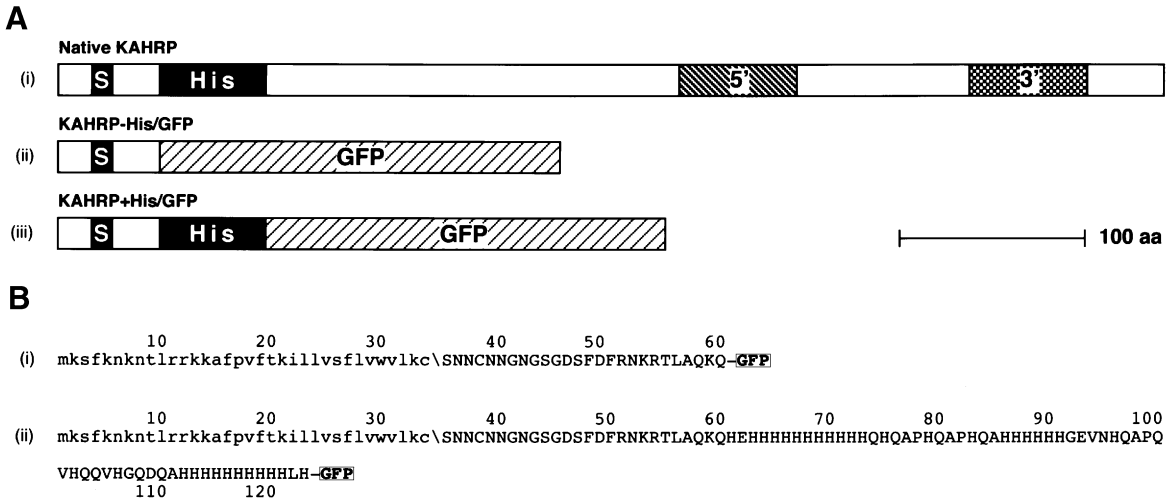

Fig. 1. Structure of the KAHRP–GFP chimeric proteins expressed in transgenic *P.falciparum*. (**A**) The domain structure of the endogenous KAHRP protein is shown in (i), where (S) depicts the hydrophobic core of the putative signal sequence, (His) is the histidine-rich region, and (5′) and (3′) are the 5′ repeat and the 3′ repeat regions. The KAHRP(–His)–GFP chimeric protein expressed from the transfection plasmid pHH2-KAHRP(–His)–GFP is shown in (ii). This chimeric protein contains the putative hydrophobic signal (S) within the first 60 amino acids of the KAHRP protein fused to GFP. The KAHRP(+His)–GFP chimeric protein expressed from transfection plasmid pHH2-KAHRP(+His)–GFP is shown in (iii). This chimeric protein contains the putative signal sequence (S) and the histidine-rich region (His) fused to GFP. (**B**) The sequences of the KAHRP–GFP fusions expressed in the 3D7–His (i) and 3D7+His (ii) transgenic parasites. N-terminal lower-case sequence represents the putative signal peptide. The cleavage point predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/) is indicated by (\). Upper-case sequence represents the mature peptide fused with GFP.

erythrocytes are devoid of the endocytic and biosynthetic–secretory machinery present in other cells. Thus, it has been suggested that the parasite must transpose the secretory machinery required for protein traffic outside the confines of its own plasma membrane and into the host cytoplasm: a novel paradigm in eukaryotic biology. Indeed, it has been shown that populations of the plasmodial homologues of the COPII vesicle coat protein, Sar1p, and NSF, a protein involved in controlling the fusion of protein trafficking vesicles, are located within the erythrocyte cytoplasm (Albano *et al*., 1999; Hayashi *et al*., 2001). However, the detailed molecular mechanisms of transport and the nature of the vesicular structures involved in the trafficking of proteins within the extracellular sector of the parasite's export pathway remain largely uncharacterized.

Inhibitors of vesicle-mediated protein trafficking have been employed in an effort to dissect the protein trafficking pathway in parasitized erythrocytes. Brefeldin A, a fungal metabolite that causes the Golgi complex to disassemble and redistribute to the endoplasmic reticulum (ER) in mammalian cells, has been shown to inhibit the export of a number of proteins that are trafficked to the erythrocyte cytoplasm, including the glycophorin binding protein, GBP, and the Maurer's cleft-associated antigen, Pf332 (Benting *et al*., 1994; Hinterberg *et al*., 1994). Thus, export of these proteins from the parasite is assumed to involve a classical secretory pathway. However, Wiser *et al*. (1997) have argued that proteins destined for the erythrocyte cytosol are diverted into a specialized secretory organelle before export. Moreover, there is evidence to suggest that many proteins, including KAHRP, are trafficked via a brefeldin A-insensitive pathway (Hinterberg *et al*., 1994).

A major advance in our understanding of the molecular dynamics of cellular systems has derived from recent elegant studies in which the gene encoding the green fluorescent protein (GFP) is appended to a gene encoding a protein of interest and transfected into a living cell (Lippincott-Schwartz *et al*., 2000; Klonis *et al*., 2001). Here, we investigate the function, trafficking and assembly of KAHRP–GFP chimeras in erythrocytes infected with transfected parasites. We identify separate N-terminal sequence elements in KAHRP responsible for the trafficking of KAHRP into the parasitophorous vacuole and for further trafficking across the parasitophorous vacuole membrane and assembly into the knob structure. We use fluorescence recovery after photobleaching (FRAP) (Axelrod, 1977) to measure the physical state of the KAHRP chimeras in different locations. We also show that KAHRP, PfEMP3 and PfEMP1 are transiently located in structures in the erythrocyte cytosol, referred to as Maurer's clefts. Our data suggest that these structures play a major role in trafficking and sorting of proteins assembled in the knob complex and co-localization studies indicate that Maurer's clefts represent an elaboration of the parasite's early canonical secretory pathway that has been exported into the host cell.

Results

Expression of KAHRP–GFP fusion proteins in P.falciparum-infected erythrocytes

To identify the sequence elements in the KAHRP protein responsible for targeting to the erythrocyte cytosol, we generated C-terminal fusions with the reporter GFP (Figure 1A) (Waller *et al*., 2000). A portion of the *KAHRP* gene that encodes the first 60 amino acids of the protein was joined upstream of the *GFP* coding sequence in the transfection vector pHH2 [pHH2-KAHRP(–His)–GFP] (Reed *et al*., 2000; Waller *et al*., 2000) (Figure 1). This region of KAHRP includes a putative hydrophobic

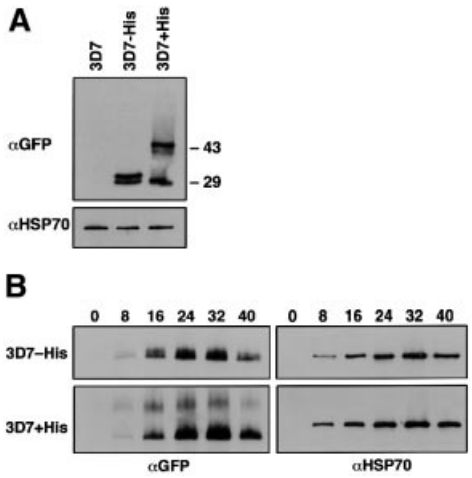

Fig. 2. Expression of GFP fusion proteins in transgenic *P.falciparum* transfected with pHH2-KAHRP(–His)–GFP and pHH2-KAHRP(+His)–GFP. (**A**) Western blot analysis of GFP fusion protein expression in Percoll-purified preparations of erythrocytes infected with the untransfected parent line 3D7 and the 3D7 lines stably transfected with pHH2-KAHRP(–His)–GFP (3D7–His) and pHH2-KAHRP(+His)–GFP (3D7+His). (**B**) Time-course of KAHRP–GFP fusion expression in the KAHRP(–His) and KAHRP(+His) transgenic parasite transfectants over 40 h. Parasites were synchronized by two consecutive sorbitol treatments 4 h apart (time zero corresponds to the second sorbitol lysis), aliquots taken 8-hourly and saponin lysed. Saponin lyses the erythrocyte plasma and parasitophorous vacuole membranes, hence the depletion of undegraded GFP products. As a control, the levels of expression of the housekeeping protein HSP70 were also examined.

signal sequence of 11 amino acids flanked by lysine residues (Figure 1). A second transfection construct was made in pHH2 that contains a region of the gene encoding the first 123 amino acids of KAHRP [pHH2-KAHRP (+His)–GFP] including the histidine-rich region (Figure 1). The resultant GFP fusion proteins would be expressed under the control of the *HSP86* gene promoter from stably maintained episomes within the transformed 3D7 *P.falciparum* blood stage parasites. 3D7 parasites demonstrate cytoadherence under both static and physiologically relevant flow conditions (Crabb *et al*., 1997).

To confirm expression of the KAHRP–GFP fusion proteins in the 3D7 *P.falciparum* transfectants containing the KAHRP(–His)–GFP (3D7–His) and KAHRP(+His)–GFP (3D7+His) plasmids, we performed western blots on parasite-infected erythrocytes (Figure 2A). When probed with antibodies recognizing GFP, no reactivity was observed in the untransfected parental line 3D7 whereas 3D7–His expressed a doublet of ~31 and 29 kDa. This is consistent with the 33.9 kDa size expected for the protein encoded by the KAHRP(–His)–GFP plasmid; however, a predicted cleavage site after the short hydrophobic stretch of amino acids would result in a mature protein of 29.8 kDa. The 3D7+His parasites also expressed two GFP protein bands; however, these were of 44 and 29 kDa. The expected size of this protein was 41.6 kDa for the full-length KAHRP–GFP protein and 37.6 kDa after cleavage at the predicted site. The 44 kDa KAHRP–GFP protein band was larger than expected for the cleaved product but this altered electrophoretic mobility may be due to the histidine-rich nature of the KAHRP domain. It is likely that the 29 kDa GFP protein bands identified in both 3D7–His and 3D7+His represent GFP degradation products described previously for other *P.falciparum* protein–GFP fusion proteins (Waller *et al*., 2000). It was important to confirm that the 3D7–His and 3D7+His parasites expressed the KAHRP(–His)–GFP and KAHRP(+His)–GFP fusion proteins at the same time throughout the development of the asexual life cycle to ensure that any differences in the subcellular localization of the fusions are not attributable to differences in timing of expression. The parasite transfectants were synchronized and cells sampled every 8 h followed by western blot analysis with anti-GFP antibodies (Figure 2B). Expression of the KAHRP–GFP proteins in 3D7–His and 3D7+His parasites was first detected at 8 h and the level of expression increased throughout the trophozoite stage. Importantly, the KAHRP–GFP proteins in both 3D7–His and 3D7+His were expressed at the same time throughout the asexual life cycle at approximately equal levels compared with the HSP70 control (Figure 2). The timing of expression of endogenous KAHRP was examined in the 3D7 line used to derive 3D7–His and 3D7+His (data not shown) and was identical to that published previously (Waterkeyn *et al*., 2001).

The N-terminal domain is sufficient for trafficking of KAHRP to the parasitophorous vacuole of the P.falciparum-infected erythrocyte

Expression of the KAHRP–GFP fusion proteins in both 3D7–His and 3D7+His resulted in green fluorescence in well-defined regions within the parasitized erythrocyte (Figure 3). This enabled direct visualization of the subcellular localization of GFP and permitted tracking of its movement during the asexual propagation of parasites in human erythrocytes. Figure 3 shows the expression of the KAHRP–GFP fusion proteins from young parasites (ring stages) through to cell division (schizogony) in both transgenic parasite lines.

Transgenic parasites expressing the KAHRP(–His)–GFP fusion protein showed a 'necklace of beads' pattern around the parasite in young rings (Figure 3A). As the parasite developed to young trophozoites, this was filled in to obtain a more uniform pattern surrounding the cell, consistent with a location in the parasitophorous vacuole (Figure 3B and C). Fluorescence was also consistently observed in the food vacuole of the trophozoite and schizont stages (Figure 3D, arrow). In the schizont stage a 'bunch of grapes' pattern of fluorescence was obtained (Figure 3E). This indicates that the fusion protein surrounds the individual merozoites, which again is consistent with trafficking of the protein to the parasitophorous vacuole (Figure 3E). These results show that the first 60 amino acids of the KAHRP protein are sufficient for entry into the secretory system and secretion from the parasite into the parasitophorous vacuolar space but insufficient to allow trafficking of the protein into the parasite-infected erythrocyte cytosol.

The histidine-rich domain is required for trafficking of KAHRP to the erythrocyte cytosol

The KAHRP(+His)–GFP protein contained the N-terminal 123 amino acids of KAHRP [Figure 1B(ii)]. Interestingly, young ring stage parasites expressing this protein showed a similar 'necklace of beads' pattern to that seen for the

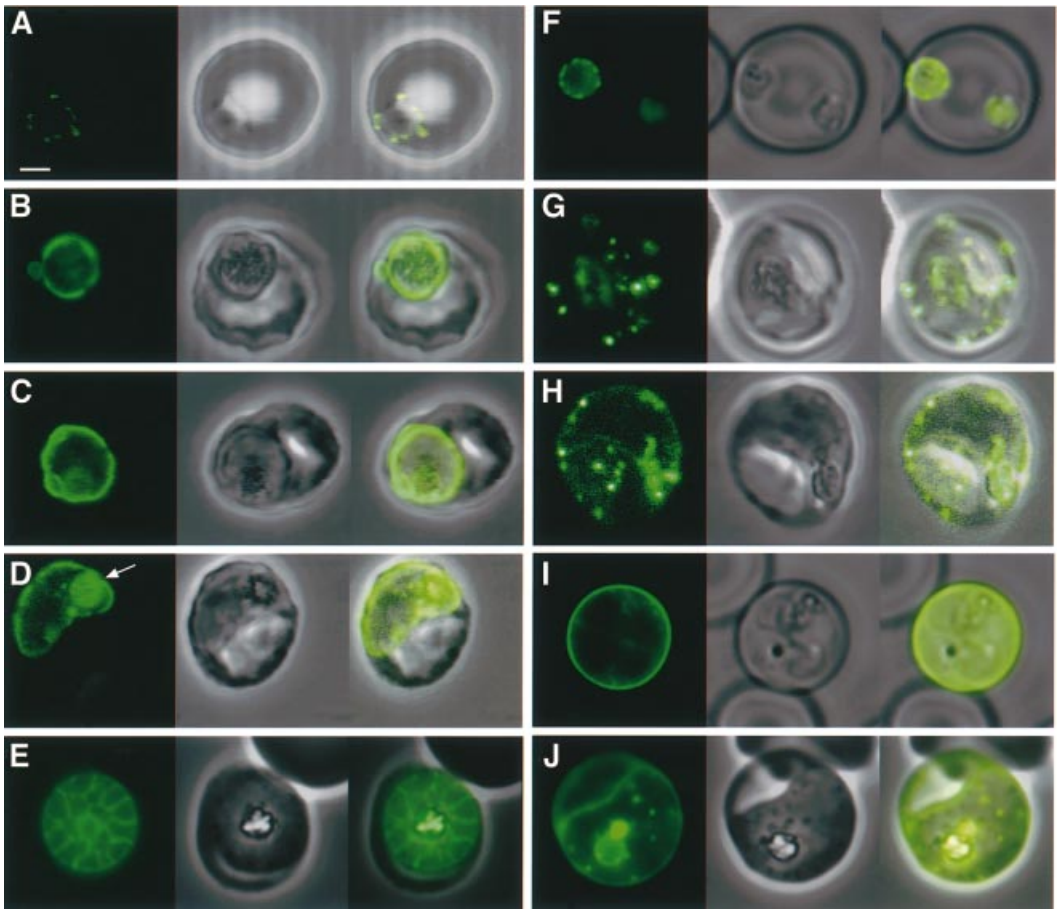

Fig. 3. Expression of KAHRP(–His)–GFP and KAHRP(+His)–GFP chimeric proteins at different stages of the intra-erythrocytic life cycle of *P.falciparum.* (A–E) Expression of KAHRP(–His)–GFP in 3D7–His and (F–J) expression of KAHRP(+His)–GFP in 3D7+His. The first image in each set represents the fluorescence signal from the GFP chimeric protein, the second is the bright field image, with an overlay of these images in the third panel. The KAHRP(–His)–GFP-expressing parasites, 3D7–His, traffic GFP to the parasitophorous vacuole, showing a 'necklace of beads' pattern in ring stages (**A**). In trophozoite stages, distortions and evaginations of the parasitophorous vacuole can be observed (**B** and **C**). In late trophozoites, GFP can be seen in association with the parasitophorous vacuole and the food vacuole (arrow) (**D**). Schizonts show a 'bunch of grapes' pattern (**E**). KAHRP(+His)–GFP-expressing parasites, 3D7+His, traffic GFP to the parasitophorous vacuole early in development (i.e. early ring stages, **F**). In later stages, the chimeric protein is located in the host cell cytoplasm (G–J). The trafficking appeared to involve transit through the parasitophorous vacuole to foci in the erythrocyte cytosol (**G** and **H**). The foci then appeared to dissipate, and the GFP chimera became concentrated at the cytoplasmic side of the erythrocyte plasma membrane (**I** and **J**).

KAHRP(–His)–GFP construct (Figure 3F). However, the protein is very rapidly trafficked into the erythrocyte cytosol resulting in a weak generalized fluorescence signal within the erythrocyte cytosol and more concentrated foci of fluorescence associated with punctate structures within the erythrocyte cytosol (Figure 3G). As the parasite matures, these structures are arrayed around the cytoplasmic face of the erythrocyte membrane (Figure 3H). This can be visualized by rotating a 3-dimensional reconstruction of a Z-section series taken through a young trophozoite stage parasite-infected erythrocyte (see supplementary data). These fluorescent structures are apparently located at the periphery of the infected cell, suggesting they may be very close to, or in contact with, the erythrocyte membrane. As the young trophozoites develop, the fluorescent chimera redistributes from these vesicle-like structures and appears to become associated with the erythrocyte membrane (Figure 3I and J) to yield a similar pattern to that seen with endogenous full-length KAHRP in immunofluorescence experiments (Culvenor *et al*., 1987; Pologe *et al*., 1987). To exclude the possibility that the trafficking of the KAHRP(+His)–GFP fusion is attributable to association with endogenous KAHRP, we have gelatin-selected 3D7+His parasites to enrich for knobless parasites that do not express KAHRP (Waterkeyn *et al*., 2001). These KAHRP-negative parasites traffic the KAHRP(+His)–GFP fusion into the erythrocyte via the same mechanism as unselected 3D7+His parasites (data not shown). Therefore, information within the 63 amino acid histidine-rich region is sufficient to direct the trafficking of KAHRP into the erythrocyte cytosol, as well as permitting association with vesicle-like structures in the erythrocyte cytoplasm, and eventually association with the erythrocyte membrane.

To confirm the subcellular localization of the KAHRP(–His)–GFP and KAHRP(+His)–GFP proteins in the two transgenic parasite lines we performed immunoelectron microscopy with anti-GFP antibodies (Figure 4). The KAHRP(+His)–GFP protein was localized to the periphery of the infected erythrocyte and frequently associated with the knob structure, showing that the histidine-rich domain is sufficient for assembly of the

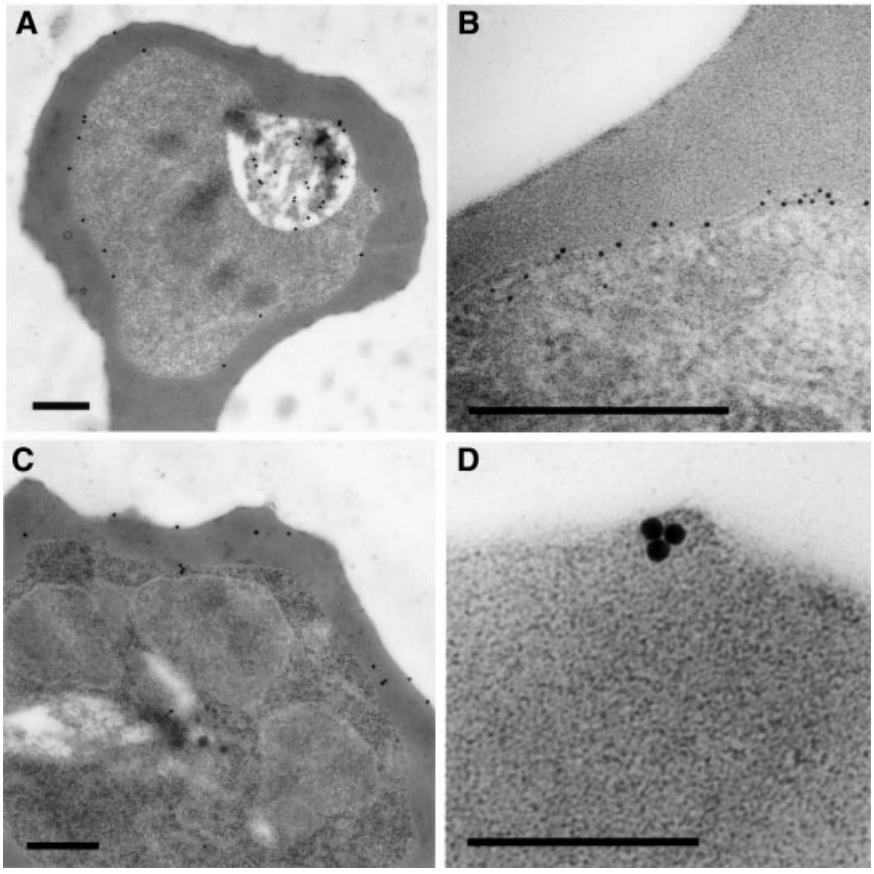

Fig. 4. Detection of GFP by immunogold labelling with anti-GFP of ultra-thin sections of 3D7–His (**A** and **B**) and 3D7+His (**C** and **D**) parasites. In KAHRP(–His)–GFP expressing parasites GFP localizes to the parasitophorous vacuole (A and B) and can also be seen in the food vacuole (A). In KAHRP(+His)–GFP-expressing parasites, GFP localizes to the erythrocyte cytoplasm (C and D) and can be seen associated with the knob structure (D). Scale bars are 400 nm in (A–C) and 250 nm in (D).

KAHRP protein into knobs (Figure 4C and D). This fusion protein was also present within the cytosol of the infected erythrocyte. The KAHRP(–His)–GFP protein showed localization around the periphery of the parasite, but few immunogold particles were observed in the cytosol of the infected erythrocyte (Figure 4A and B). The protein was also localized to the food vacuole of the parasite (Figure 4A). These results are consistent with fluorescence microscopy of live 3D7+His and 3D7–His transgenic parasites (Figure 3).

The 'necklace of beads' pattern observed for the GFP chimeras in young ring stage parasites (Figures 3A and F, and 5A) is intriguing. These structures do not represent artefacts of the particular constructs used in this work, as similar images have been observed previously when *P.falciparum*-infected erythrocytes were transfected with another parasitophorous vacuole-targeted GFP chimera (Waller *et al*., 2000). The foci of fluorescence may represent individual subcompartments within or near the parasitophorous vacuole. In more mature stages of 3D7–His, the GFP chimera was often present in short protrusions of the parasitophorous vacuole (Figure 5I–K and M–O). These may be evaginations of the parasitophorous vacuole into blind appendices that form part of the tubulovesicular network (Elford and Ferguson, 1993; Elmendorf and Haldar, 1994). However, it is also possible that at least some of these evaginations are closed compartments. Thus, there may be barriers to diffusion of the chimera between one region of the parasitophorous vacuole and another.

To study this further, we selectively photobleached the GFP molecules in sections of the parasitophorous vacuole to determine the mobility of the KAHRP(–His)–GFP chimera in 3D7–His parasite-infected erythrocytes (Lippincott-Schwartz *et al*., 2000). As shown in Figure 5A–D, exposure of one of the parasitophorous vacuole-associated 'beads' to a 5 s laser pulse resulted in extensive localized bleaching of the 'bead' and in significant quenching of other regions of the parasitophorous vacuole, including other 'beads'. A quantitative analysis of the images indicates a progressive increase in bleaching of the 'beads' closest to the exposed region with no recovery of the fluorescence in the most extensively bleached regions (data not shown). The 'beads' might represent vesicular compartments involved in the transport of the fluorescing protein to the parasitophorous vacuole, or subcompartments with defined geometry within the parasitophorous vacuole. Either way, there appears to be at least a partial connection between the different parasitophorous vacuole-associated compartments.

In most of the more mature parasites, the parasitophorous vacuole had a smoother appearance. The mobility of the KAHRP(–His)–GFP chimeric protein population in the parasitophorous vacuole of these parasites was also

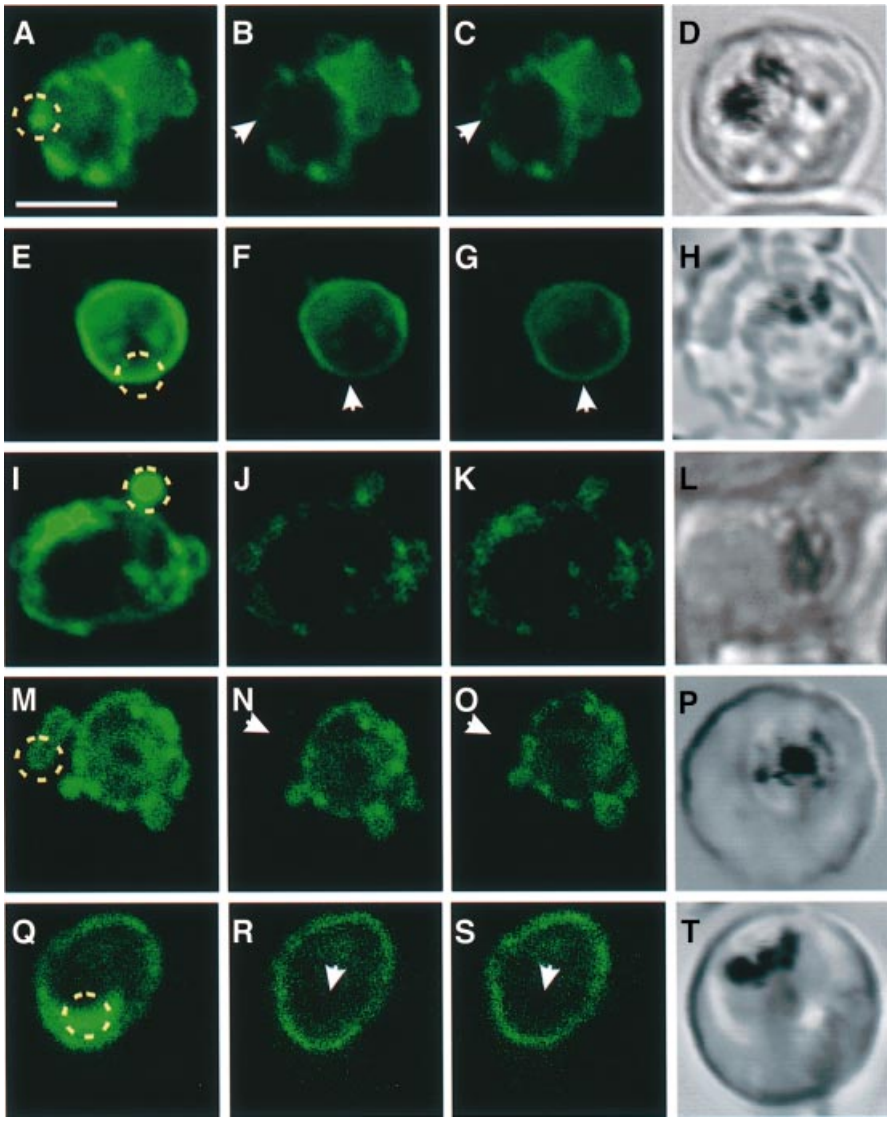

Fig. 5. Confocal fluorescence microscopy and selective FRAP of *P.falciparum*-infected erythrocytes expressing KAHRP(–His)–GFP. In each row, the left-hand panels represent pre-bleach images collected at low laser power. The second panels represent images collected 1 s after exposing a defined area (circle, first panels) to a high intensity laser pulse (100% of the laser power). The third panels represent images collected 0.5–1 min after the bleach pulse and the right-hand panels show differential interference contrast images of the cells. (**A–D**) A young trophozoite stage-infected erythrocyte showing a 'necklace of beads' fluorescence pattern. A region of the parasitophorous vacuole was subjected to a 5 s bleach pulse (A and B) and re-imaged after 1 min (C). The bleach site is indicated with an arrow. (**E–H**) A trophozoite stage parasite showing a more homogeneous parasitophorous vacuole fluorescence pattern. A region of the parasitophorous vacuole was subjected to a 3 s bleach pulse (E and F) and re-imaged after 30 s (G). Partial recovery of fluorescence into the bleached area was observed (arrow). (**I–L**) A trophozoite stage-infected erythrocyte showing a parasitophorous vacuole evagination was subjected to a 10 s bleach (I–L) and re-imaged after 1 min (K). Pixel values in (J) and (K) have been multiplied by four for the image to be visible. (**M–P**) A trophozoite stage-infected erythrocyte showing a parasitophorous vacuole evagination was subjected to a 3 s bleach pulse (M and N) and re-imaged after 1 min (O). No recovery of fluorescence into the bleached area was observed (arrow). (**Q–T**) A late trophozoite stage-infected erythrocyte showing a fluorescent compartment within the parasite was subjected to a 3 s bleach (Q and R) and re-imaged after 1 min (S). The parasitophorous vacuole did not undergo bleaching and there was no recovery of fluorescence into the internal compartment (arrow). The bar in (A) represents 5 μm.

examined. Figure 5E shows an example of a parasite in which the parasitophorous vacuole exhibited a smooth appearance without any associated blebs or beads. In this case, a 3 s bleach of a region at the edge of the parasitophorous vacuole led to extensive bleaching localized to the region of illumination, but also resulted in less, but significant, reduction of fluorescence throughout this entire compartment (Figure 5F). In addition, there was recovery of fluorescence in the bleached region after a period of 30 s (Figure 5G). These observations can be explained if the GFP chimera exhibited a recovery time of seconds as this would be sufficient to cause both the general reduction in fluorescence throughout the parasitophorous vacuole and the extensive localized bleaching. Such a recovery time would require the GFP to be in the form of large molecular aggregates or associated with other structures within the parasitophorous vacuole, or perhaps to be associated in some way with the parasite plasma membrane or the parasitophorous vacuole membrane.

In more mature stages of the intra-erythrocytic development of the parasite, protrusions and blebs from the

parasitophorous vacuole were commonly observed (Figure 5I–L and M–P). Many of the protrusions appear to remain fully connected to the parasitophorous vacuole. For example, as shown in Figure 5I–L, bleaching of the contents of a parasitophorous vacuole protrusion by exposure to a 10 s laser pulse resulted in rapid bleaching of the entire parasitophorous vacuole. This suggests rapid diffusion of proteins between the parasitophorous vacuole and the protruding arm (Figure 5K).

In some cells, the protrusions appeared to have blebbed from the parasitophorous vacuole. Figure 5M–P shows an example in which bleaching of a protruding arm of the parasitophorous vacuole containing the GFP chimera completely eliminated fluorescence from the protrusion but caused only very limited bleaching of the fluorescence of the rest of the parasitophorous vacuole (Figure 5N, arrow). It is clear that there is no rapid diffusion of GFP molecules between the protruding arm and the parasitophorous vacuole proper since there is no recovery of fluorescence in this region during a 3 min period after the bleach pulse (Figure 5O). These data indicate that while there is free diffusion within the parasitophorous vacuole, it is possible for constrictions to occur between these evaginations of the parasitophorous vacuole and the vacuole proper. These evaginations remain associated with the parasitophorous vacuole, and we have seen no evidence for fusion of these blebs with the erythrocyte membrane, indicating that they do not play a role in the trafficking of proteins to the erythrocyte membrane. While it is possible that the protrusions in Figure 5I–L and M–P represent different structures, it is likely that the difference in the connectivity of these protrusions are different stages in their development.

To investigate further the nature of the peripheral compartment that was frequently observed within the parasite cytosol, we have also selectively photobleached fluorescent molecules within this compartment (Figure 5Q–T). Exposure to a 3 s bleach pulse eliminated the fluorescence in this region without perturbing the fluorescence within the parasitophorous vacuole. Moreover, no recovery of fluorescence was observed over a 3 min period (Figure 5S). This again indicates that this region of localized fluorescence within the parasite cytosol is not linked by a fluid connection to the parasitophorous vacuole. It is possible that this region represents a compartment within the secretory pathway where the GFP chimera is located before export to the parasitophorous vacuole.

Fluorescence from the KAHRP(+His)–GFP chimeric protein expressed in young ring stage 3D7+His parasites is largely restricted to the parasite (Figure 6A–D). However, even in ring stage parasites, there appears to be a population of fluorescent molecules that are present in the erythrocyte cytosol. Bleaching of a region of the erythrocyte cytosol resulted in an even decrease in fluorescence of this compartment (Figure 6A–C), indicating rapid diffusion of the KAHRP(+His)–GFP in the erythrocyte cytosol in this early stage of the intra-erythrocytic cycle. The physical state of the parasite-associated GFP in this same cell was examined by subjecting a region of the parasite cytosol to a 3 s bleach pulse (Figure 6E–H). This resulted in complete bleaching of the parasite-associated fluorescence, indicating rapid diffusion of the chimera within the parasite. This is consistent with the presence of the protein within a connected network of endoplasmic reticulum.

In young trophozoite stage parasites, the fluorescence appeared to be associated with both the parasite and the parasitophorous vacuole, along with a very diffuse fluorescence pattern in the erythrocyte cytosol (Figure 6I–L). Selective bleaching of a region within the parasite was achieved with no recovery of fluorescence from the parasitophorous vacuole into the compartment within the parasite cytosol (Figure 6K). The remnant fluorescence within the parasitophorous vacuole again showed evidence of a non-homogeneous or 'necklace of beads' pattern (Figure 6I–J). These data are consistent with the suggestion that both the KAHRP(–His)–GFP and KAHRP(+His)–GFP chimeric proteins transit through a secretory network within the parasite and thence to the parasitophorous vacuole, before export of the KAHRP-(+His)–GFP chimera to the erythrocyte cytosol.

As discussed above, in more mature-stage parasites, foci of KAHRP(+His)–GFP fluorescence were transiently observed in the erythrocyte cytosol (Figure 6). This pattern appeared to be short-lived, consistent with the suggestion that it is an interim organization that occurs before redistribution of protein to the erythrocyte membrane. Selective bleaching of two of the foci with a 3 s exposure to a laser pulse is shown in Figure 6M–P. Interestingly, the fluorescence associated with these compartments was consistently observed to partly recover with time. This indicates an equilibrium between a freely diffusing soluble population of KAHRP(+His)–GFP molecules and a population that is immobilized (and concentrated) by association with a vesicular compartment within the erythrocyte cytosol. These data suggest that the KAHRP(+His)–GFP chimera becomes transiently associated with the external surface of these membrane-bound compartments.

In more mature stage parasites, a rim-like fluorescence pattern develops (Figure 6Q–T) consistent with association of KAHRP(+His)–GFP molecules with the knob structure as seen by immunoelectron microscopy analysis. Exposure of a region of the rim-located fluorophores to a 3 s bleach pulse, resulted in loss of fluorescence from the bleached region. Interestingly, it was again noted that the fluorescence of the bleached region recovered slowly, reaching about 50% of control levels in a 3 min period (Figure 6R–S). The partial immobilization of the KAHRP-(+His)–GFP is consistent with the suggestion that the His-rich region of KAHRP is sufficient to allow an interaction with an erythrocyte membrane-associated component. The partial recovery of fluorescence suggests a dynamic interaction of the KAHRP(+His)–GFP fragment with the erythrocyte membrane. An explanation for the observed recovery is that the KAHRP(+His)–GFP may be associated with an integral membrane protein (such as PfEMP1) that is diffusing laterally within the membrane. However, analysis of the profile of the bleached region indicated that recovery occurred evenly throughout the bleached region (data not shown). This suggests that the recovery of fluorescence probably occurs due to dissociation of bound bleached GFP fusion protein from the cytoskeletal network on a time-scale of minutes and association of

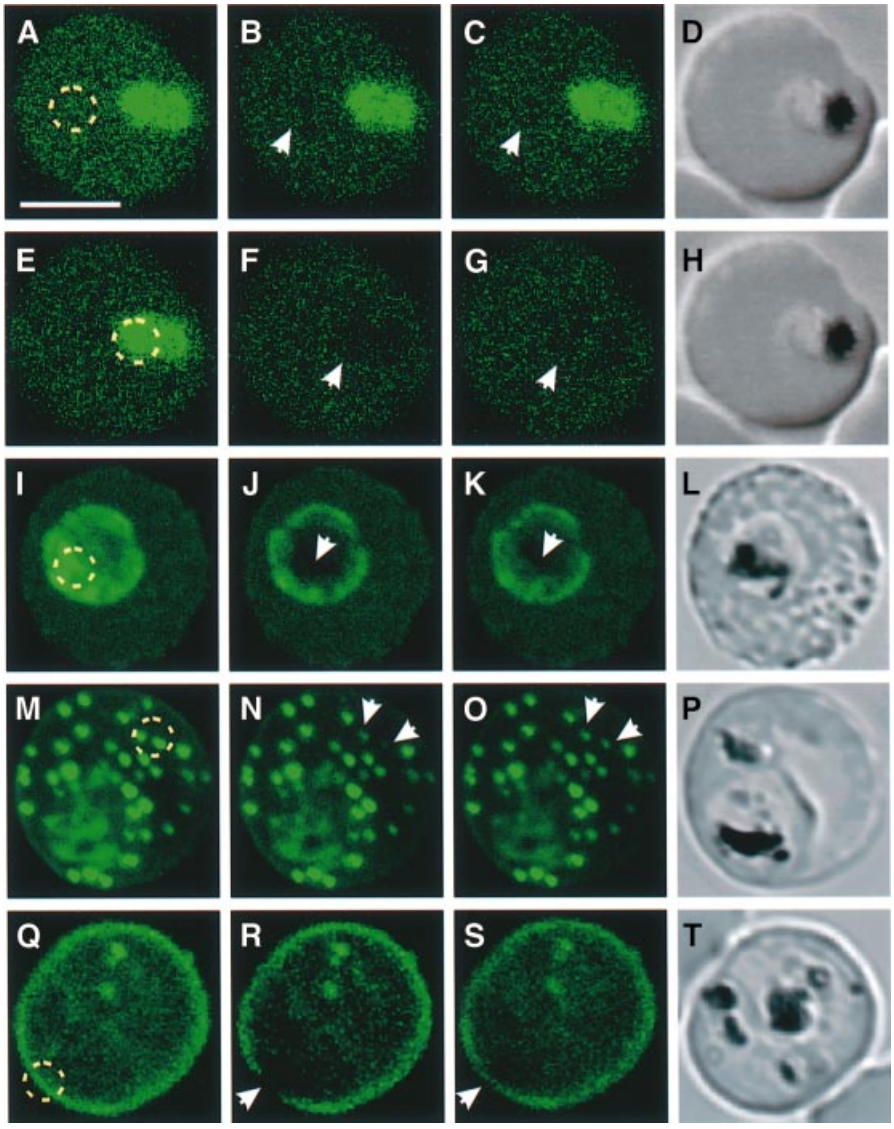

Fig. 6. Confocal fluorescence microscopy and selective photobleaching of different intra-erythrocytic stages of *P.falciparum*-infected erythrocytes expressing the KAHRP(+His)–GFP. (**A**–**D**) A ring stage-infected erythrocyte showing bright fluorescence within the parasite and weak diffuse fluorescence within the erythrocyte cytosol. A region within the erythrocyte cytosol was subjected to a 3 s bleach pulse (A and B) and re-imaged after 1 min (C). A diffuse pattern was observed after bleaching with no recovery within the time-frame examined (arrow). (**E**–**H**) The same ring-stage parasite was subjected to a 3 s laser pulse targeting the parasite (E and F) and re-imaged after 1 min (G). The parasite-associated fluorescence was completely ablated (arrow). (**I**–**L**) A trophozoite stage-infected erythrocyte showing a fluorescent compartment within the parasite was subjected to a 3 s bleach (I and J) and re-imaged after 2 min (K). No recovery of fluorescence into the bleached area was observed (arrow). (**M**–**P**) A trophozoite stage-infected erythrocyte showing punctate fluorescence within the erythrocyte cytosol. Two foci of fluorescence were subjected to a 3 s bleach pulse (M and N) and re-imaged after 2 min (O). Some recovery of the fluorescence associated with the vesicular-like compartments was observed (arrows). (**Q**–**T**) A trophozoite stage-infected erythrocyte showing rim-like fluorescence associated with the erythrocyte membrane. A region of membrane-associated fluorescence was subjected to a 3 s bleach (Q and R) and re-imaged after 1 min (S). Partial recovery of fluorescence into the bleached area was observed (arrow). The bar in (A) represents 5 μm.

unbleached KAHRP(+His)–GFP from a cytosolic pool on to this region.

Trafficking of KAHRP is brefeldin A sensitive

In higher eukaryotes, brefeldin A blocks secretion and disrupts Golgi morphology resulting in redistribution of Golgi markers back to the ER (Lippincott-Schwartz *et al*., 1989). Brefeldin A has been shown to cause a reorganization of a parasite homologue of the Golgi marker, ERD2 (Elmendorf and Haldar, 1993), into a compartment that appears to be the ER as judged by immunofluorescence microscopy using antibodies to the ER-resident protein, GRP90/BIP (Elmendorf and Haldar, 1993). To determine the effect of brefeldin A on the trafficking of the KAHRP(–His)–GFP and KAHRP(+His)–GFP fusion proteins transfected parasites were treated with brefeldin A for 16 h and followed visually after treatment (Figure 7A). The trafficking of both fusion proteins was sensitive to brefeldin A [Figure 7A(a and b)] and unaffected by the carrier solvent, ethanol, which was added to control samples [Figure 7A(c and d)]. An intense focus of fluorescence was observed near the periphery of the parasite with a more diffuse pattern surrounding it. Removal of brefeldin A resulted in normal growth and

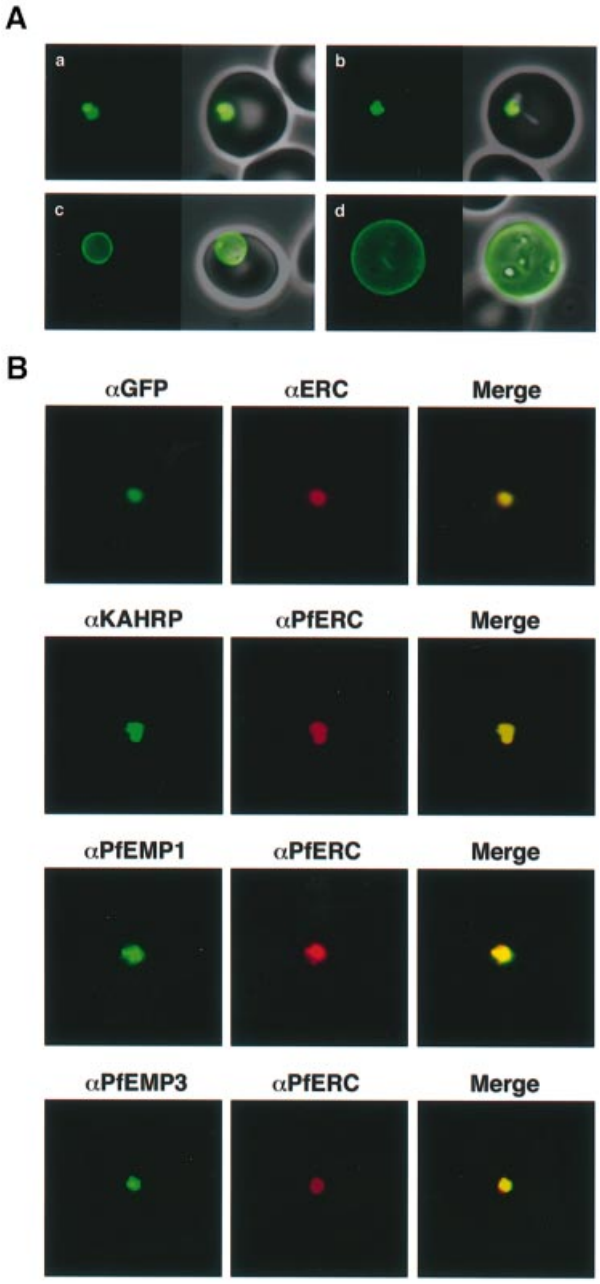

Fig. 7. Brefeldin A inhibition of protein trafficking in *P.falciparum*-infected erythrocytes. (**A**) Brefeldin A inhibition of KAHRP(–His)–GFP trafficking (a) and KAHRP(+His)–GFP (b) visualized in live parasites. Control cultures were incubated in the presence of equivalent amounts of ethanol to ensure that it had no effect on growth and cell morphology (c and d). (**B**) Brefeldin A inhibition of trafficking of KAHRP(+His)–GFP and endogenous KAHRP, PfEMP1 and PfEMP3 and co-localization of these antigens with the ER marker, PfERC (La Greca *et al*., 1997). First row: co-localization of GFP (green) with the ER marker PfERC (red) in brefeldin A-treated parasites. Second row: co-localization of KAHRP (green) with PfERC (red). Third row: co-localization of PfEMP1 (green) with PfERC (red). Fourth row: co-localization of PfEMP3 (green) with PfERC (red). The third window in each panel represents the merging of the red and green channels. Yellow areas represent regions of co-localization.

development being established and trafficking of KAHRP(–His)–GFP to the parasitophorous vacuole and KAHRP(+His)–GFP to the parasite-infected erythrocyte membrane. Thus the effect of the drug appears to be reversible. These data suggest that both KAHRP(–His)–GFP and KAHRP(+His)–GFP are trafficked through the ER compartment.

We have also examined whether the KAHRP–GFP chimeras and a series of endogenous proteins that are exported to the erythrocyte co-localize with the ER-resident protein, PfERC (La Greca *et al*., 1997; Albano *et al*., 1999), in brefeldin A-treated parasitized cells. Immunofluorescence microscopic analysis of KAHRP-(–His)–GFP and KAHRP(+His)–GFP (Figure 7B) showed an identical pattern of fluorescence to that observed using anti-PfERC antiserum providing further evidence that both proteins are retained within the ER after brefeldin A treatment. The trafficking of endogenous KAHRP, PfEMP1 and PfEMP3 were also shown to be brefeldin A sensitive. These proteins were retained in what appears to be the same compartment as that seen for the KAHRP–GFP fusion proteins (Figure 7B). In ethanol-only controls, KAHRP, PfEMP1 and PfEMP3 trafficking is unaffected and these molecules, which localize to the erythrocyte plasma membrane, reach their destination (data not shown). These rather surprising results are consistent with the suggestion that KAHRP, PfEMP1 and PfEMP3 are each trafficked via the 'classical' vesicle-mediated secretory system rather than via an alternative pathway(s) as has been suggested in previous studies (Hinterberg *et al*., 1994; Mattei *et al*., 1999). They also indicate that the contracted compartment observed within the cytoplasm of brefeldin A-treated parasites represents an early stage in the elaboration of the ER, rather than a secondary ER compartment as has been proposed previously (Wiser *et al*., 1997).

KAHRP is associated with Maurer's clefts before assembly into knob structures

It is likely that the KAHRP(+His)–GFP fusion protein is trafficked out of the parasite to the erythrocyte cytosol via the same route as that used by the endogenous KAHRP protein. Interestingly, in early stage parasites, the KAHRP(+His)–GFP protein is associated with vesicle-like structures within the erythrocyte cytoplasm, suggesting that these structures are involved in the pathway for trafficking from the parasitophorous vacuole to the erythrocyte membrane. To characterize these structures further, we examined immunofluorescence patterns obtained using antibodies to endogenous KAHRP and a parasite antigen known as PfSBP1, which has previously been localized to Maurer's clefts (Blisnick *et al*., 2000). In ring stage-infected erythrocytes, KAHRP is associated with large vesicle-like structures that also contain PfSBP1 (Figure 8). These data indicate that KAHRP is transiently associated with Maurer's clefts before being relocated to the erythrocyte membrane. PfEMP1 is also associated with the same structures in ring stage parasite-infected erythrocytes (Figure 8) suggesting that this protein also accumulates in Maurer's clefts before transit to and insertion into the erythrocyte membrane.

Sar1p is involved in the non-clathrin-based COPII protein coat, which mediates vesicle transport between the ER and the Golgi apparatus (Barlowe *et al*., 1994). It has been shown previously that some of the plasmodial homologue of Sar1p is associated with Maurer's cleft-like

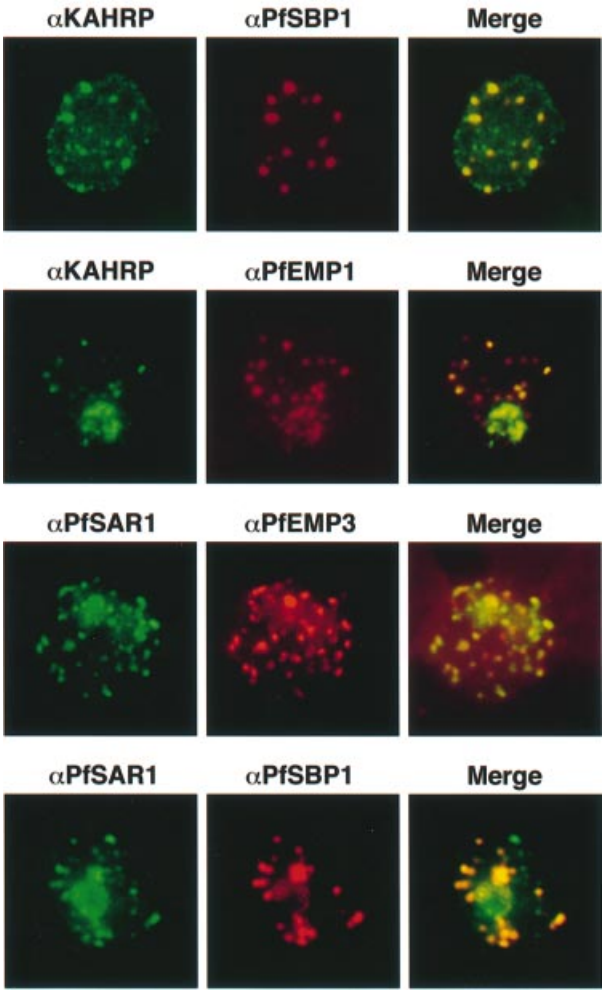

Fig. 8. Transient co-localization of KAHRP, PfSBP1, PfEMP1, PfEMP3 and PfSAR1p to Maurer's clefts in ring stage *P.falciparum*-infected erythrocytes. First row: co-localization of KAHRP (green) with PfSBP1 (red). Second row: co-localization of KAHRP (green) with PfEMP1 (red). Third row: co-localization of PfSAR1p (green) with PfEMP3 (red). Fourth row: co-localization of PfSAR1p (green) with PfSBP1 (red). The third window in each row represents the merging of the red and green channels. Yellow areas represent regions of co-localization.

structures (Albano *et al*., 1999). Immunofluorescence experiments with anti-PfSAR1p antibodies showed that this protein co-localizes with PfSBP1 (Figure 8). This supports the suggestion that Maurer's clefts play a role in vesicle-mediated transport of proteins to the erythrocyte membrane (Figure 9). Additionally, PfEMP3, which has structural similarity to Uso1p, a tethering protein identified in *Saccharomyces cerevisiae* (Waterkeyn *et al*., 2000), also appears localized to Maurer's clefts in ring stage parasite-infected erythrocytes (Figure 8) before it moves to the inside of the parasite-infected erythrocyte. In trophozoite stage parasites, PfEMP1, PfEMP3 and KAHRP show no obvious fluorescent signal associated with Maurer's clefts, suppporting the suggestion that this association is transient (data not shown). These results suggest that KAHRP, PfEMP1 and PfEMP3 are transiently associated with Maurer's clefts *en route* to their final subcellular locations.

Discussion

Sequence elements required for trafficking of KAHRP to P.falciparum-infected erythrocytes and assembly into knobs

Functional analysis of putative targeting signals in *P.falciparum* has recently become possible with the advent of transfection protocols to introduce exogenous genes into this parasite. For example, we have analysed trafficking of GFP fusions to the apicoplast (relict plastid of *P.falciparum*) and shown that transport to this organelle also commences via the secretory system. Moreover, we showed that this initial trafficking event is mediated by an N-terminal pre-sequence consisting of a signal and transit peptide (Waller *et al*., 2000). In this work we have used GFP fusions with regions of the KAHRP protein to analyse the trafficking pathway of this exported protein. The N-terminal region of the KAHRP protein contains a recessed putative signal sequence with 21 amino acids at the N-terminus followed by an 11 amino acid hydrophobic core. This region also contains a predicted cleavage site at amino acid 37, which presumably results in removal of the N-terminal region during translocation of the protein into the ER. A number of exported parasite proteins contain non-canonical, 'internal' hydrophobic signals beginning 20–50 amino acids from the N-terminus (Lingelbach, 1993). These recessed hydrophobic signals are not recognized by the translocation machinery of higher eukaryotes as KAHRP is not translocated across the ER membrane in cell-free systems using mammalian microsomes (Lingelbach, 1993). Nevertheless, our data indicate that the first 60 amino acids of the KAHRP protein allow entry into the parasite's secretory system and trafficking to the parasitophorous vacuole. Although the first 60 amino acids are sufficient to allow trafficking of KAHRP to the secretory system and across the parasite plasma membrane, they are not sufficient for trafficking across the parasitophorous vacuole membrane into the erythrocyte cytoplasm. The presence of sequence elements within the additional 63 amino acids of the histidine-rich region is required for this process. It is unlikely that the trafficking of KAHRP(+His)–GFP fusion across the parasitophorous vacuole into the erythrocyte cytosol is attributable to the association of the histidine-rich region with the ATS of PfEMP1 since previous work has demonstrated that PfEMP1, but not KAHRP, is associated with lipid vesicles that traffic into the erythrocyte cytoplasm (Gormley *et al*., 1992; Trelka *et al*., 2000). Additionally, we have shown that KAHRP(+His)–GFP is freely diffusable in the erythrocyte cytoplasm before interaction with Maurer's clefts. It seems unlikely that the histidine repeat sequence itself is the signal required to mediate transit across the parasitophorous vacuolar membrane as this sequence is found only in KAHRP and not in other exported parasite proteins. Candidate sequence elements are the short stretches of non-histidine-containing regions within this domain.

The ER and the parasitophorous vacuole are intermediate compartments in the export of KAHRP

Previous studies have indicated that trafficking of KAHRP is brefeldin A insensitive, leading to the suggestion that the

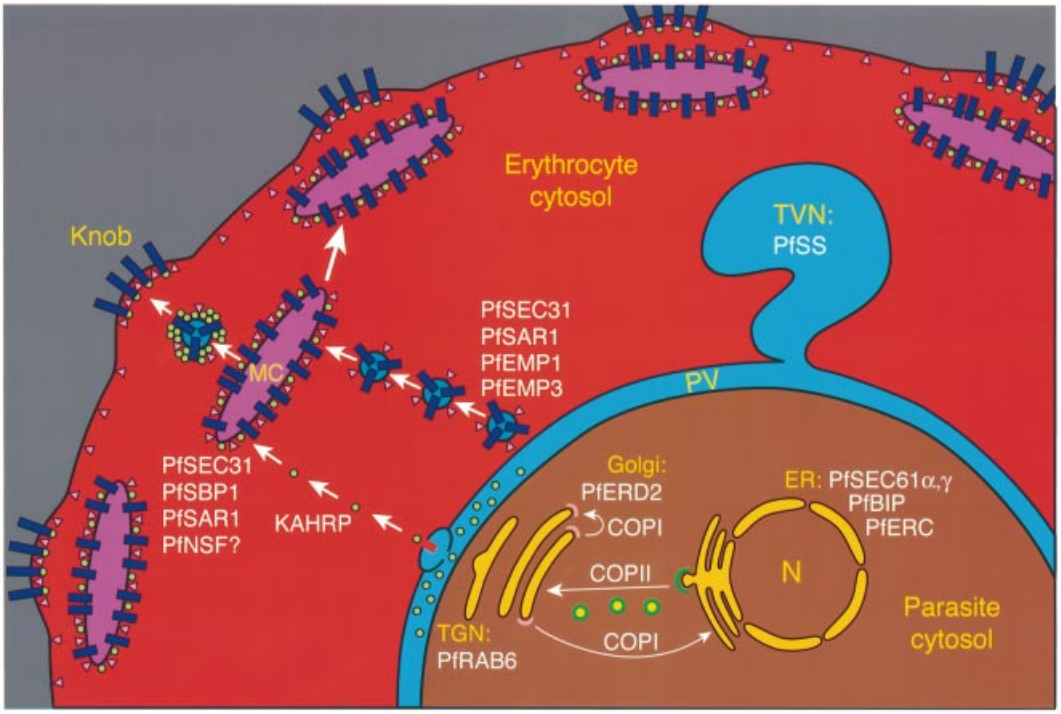

Fig. 9. A proposed model of the trafficking of KAHRP to the knobs of *P.falciparum*-infected erythrocytes. Proteins destined for export are routed through the ER to the parasitophorous vacuole (PV), which presumably involves cleavage of the signal peptide upon translocation into the ER and trafficking through the classical secretory pathway. Recognition of a 'translocation motif' in the histidine-rich region of KAHRP (green circles) would result in translocation of this protein across the parasitophorous vacuolar membrane, via a putative ATP-dependent transporter. Freely diffusable KAHRP located in the parasite-infected erythrocyte cytoplasm would be recruited on to Maurer's clefts (which contain PfSBP) most likely by interaction of the histidine-rich region with the cytoplasmic tail of PfEMP1 (blue rectangles), which may be exposed on the cytoplasmic surface of Maurer's clefts (MC) in ring stages. Maurer's clefts, which co-localize with homologues of components of the canonical secretory pathway such as Sar1p and Sec31p (Adisa *et al*., 2001), and possibly proteins involved in SNARE-mediated membrane fusion such as NSF (Hayashi *et al*., 2001), are important structures involved in protein traffic and knob complex formation, and their interaction with the erythrocyte plasma membrane appears to involve PfEMP3 (pink triangles), which has structural similarity to Uso1p, a yeast tethering protein involved in ER to Golgi traffic. Our evidence suggests that Maurer's clefts are involved directly in the deposition and assembly of parasite proteins and come into close proximity with the cytosolic face of the parasite-infected erythrocyte membrane. *P.falciparum* homologues of proteins associated with the ER (PfSEC61 α and γ, GRP90/BIP, PfERC), Golgi [sphingomyelin synthase (SS), PfERD2], the trans-Golgi network (TGN) (PfRAB6) and those transposed beyond the parasite into the host cell (PfSAR1p, PfSEC31 and PfNSF) that have been localized in infected erythrocytes are marked. TVN refers to tubovesicular network.

KAHRP protein transits the parasite and parasitophorous vacuole membranes via an alternate pathway, i.e. via a non-classical mechanism (Hinterberg *et al*., 1994; Mattei *et al*., 1999). In this study, we found that KAHRP export is sensitive to brefeldin A. Previous inhibition studies involve the addition of brefeldin A 14–20 h post-erythrocyte invasion (Hinterberg *et al*., 1994; Mattei *et al*., 1999), resulting in no inhibition of KAHRP trafficking. It has been concluded from these studies that malaria utilizes a brefeldin A-insensitive 'alternative' secretory pathway. We have examined the effect of brefeldin A addition at different points in the *Plasmodium* cell cycle, and here there is clearly no contradiction with the work of Hinterberg *et al*. (1994); addition of brefeldin A after secreted proteins have exited the ER and Golgi results in no inhibition of trafficking of these proteins. Complete inhibition of the export of endogenous KAHRP required very early addition, immediately following reinvasion, of brefeldin A to tightly synchronized parasites. We demonstrate inhibition not only of KAHRP, but also of PfEMP1 and PfEMP3 trafficking. We also show that brefeldin A treatment trapped both KAHRP–GFP fusion proteins within a compartment labelled by the ER marker PfERC. This adds further support to the suggestion that trafficking of KAHRP indeed proceeds via a classical vesicle-mediated pathway.

KAHRP(–His)–GFP and KAHRP(+His)–GFP show a very similar subcellular location in early ring stage parasites, suggesting that these proteins initially follow the same route. It is likely that the first step in the pathway for KAHRP trafficking would be co-translational translocation into the lumen of the ER, followed by cleavage of the signal sequence at the predicted amino acid and trafficking to the parasitophorous vacuole. KAHRP-(–His)–GFP, which lacks the additional signal, becomes trapped in this compartment. Outwards transport from the parasitophorous vacuole presumably requires recognition of the appropriate sequence element(s) within the histidine-rich region of KAHRP. It is likely that trafficking across the parasitophorous vacuole involves monomer transfer through a proteinaceous pore because the KAHRP(+His)–GFP in the erythrocyte cytosol of early ring stage parasite is freely mobile, which is consistent with a soluble species. This suggestion is also consistent with a previous analysis of trafficking in *P.falciparum*-infected erythrocytes permeabilized using streptolysin O lysis (Ansorge *et al*., 1996). This elegant permeabilization

study demonstrated that translocation across the parasite plasma membrane and parasitophorous vacuole membrane is a two-step process, with the second step involving an ATP-dependent putative translocator located within the parasitophorous vacuole membrane (Ansorge *et al*., 1996).

An interconnected tubovesicular network (TVN) that extends from the parasitophorous vacuole membrane has been described and reported to be involved in the uptake of exogenous nutrients and drugs (Lauer *et al*., 1997). Visualization of the parasitophorous vacuole using the KAHRP(–His)–GFP fusion protein showed some membrane-limited extensions that are likely to correspond to the TVN. Some of these are clearly connected to the parasitophorous vacuole. However, others appear to be closed and may have blebbed from the main structure but remain in contact with it. These evaginations were often evident in trophozoite stage parasites, but were not extensive and were not observed to make connections with the erythrocyte membrane. Our observations support the previous conclusion (Waller *et al*., 2000) that these structures probably do not play a direct role in delivery of proteins to the erythrocyte membrane.

Our immunoelectron and immunofluorescence microscopic analyses suggest that subpopulations of each of the KAHRP–GFP chimeras are located in the food vacuole of the parasite. We have previously shown that GFP fused to a hydrophobic signal sequence was trafficked to the parasitophorous vacuole, but that a subpopulation was associated with the parasite digestive vacuole (Waller *et al*., 2000). It is likely that the GFP fusion proteins are taken up during the parasite's feeding process, which involves the use of a cytostome (mouth) to ingest small packets of haemoglobin from the host cytoplasm. These endocytic compartments are surrounded by a double membrane originating from the parasite plasma membrane and the parasitophorous vacuolar membrane and thus are likely to contain proteins from the parasitophorous vacuole as well as the erythrocyte cytoplasm. These vesicles are transported to and fuse with the food vacuole, which would deliver the fusion proteins to this compartment. It is possible that the population of cleaved fusion products observed by western analysis (Figure 2A) arises from GFP chimeras that have been trafficked to the food vacuole.

KAHRP associates transiently with Maurer's clefts

Previous immunofluorescence microscopy studies of KAHRP in the *P.falciparum*-infected erythrocyte have shown that it is not associated with lipid-containing structures in the cytoplasm and is present as aggregates of <0.2 μm (Gormley *et al*., 1992). This is consistent with our studies of KAHRP(+His)–GFP diffusion in early ring stage parasites. However, in more mature stage parasites, KAHRP(+His)–GFP is transiently associated with Maurer's clefts. A number of proteins have been shown previously to be associated with Maurer's clefts including Pf332 (Hinterberg *et al*., 1994) and PfSBP1 (Blisnick *et al*., 2000) and it has been suggested that these structures may play a role in trafficking. FRAP analysis revealed that the fluorescence signal for Maurer's cleft-associated KAHRP-(+His)–GFP recovers quickly following photobleaching. This suggests that the fusion protein is present in the erythrocyte cytosol in an equilibrium between a freely diffusable population and a population associated with Maurer's clefts.

The transient recruitment of KAHRP(+His)–GFP on to Maurer's clefts may occur by the binding of KAHRP to the cytoplasmic ATS of PfEMP1 (Waller *et al*., 1999), which may be exposed on the exterior of Maurer's clefts. This is consistent with the transient co-localization of KAHRP and PfEMP1 in these structures in immature stage-parasitized erythrocytes. Indeed, the co-localization of KAHRP and PfEMP1 in Maurer's clefts suggests that assembly of the knob structure may take place in Maurer's clefts before insertion under the erythrocyte membrane. The histidine-rich domain appears to be sufficient for assembly of the KAHRP–GFP chimera into knob structures. This is consistent with previous data showing high-affinity binding of this domain to the cytoplasmic domain of PfEMP1 (Waller *et al*., 1999). It has been shown that the 5′ repeat region of KAHRP can also bind to the ATS region of PfEMP1 (Waller *et al*., 1999) and it seems likely that the histidine-rich region and the 5′ repeats act co-operatively to enhance the interaction of KAHRP with PfEMP1. Photobleaching of KAHRP(+His)–GFP that is associated with the erythrocyte membrane was followed by a gradual recovery of the fluorescence signal. This may indicate exchange of free and bound forms of the fusion protein, which suggests that the KAHRP(+His)–GFP chimera is bound only rather weakly to the erythrocyte membrane. It is likely that although the histidine-rich region of KAHRP is sufficient for binding to Maurer's clefts, the 5′ repeat region greatly strengthens the interaction of KAHRP with PfEMP1.

As Maurer's clefts develop they appear to be positioned close to the periphery of the parasite-infected erythrocyte membrane in 3-dimensional reconstructions (see supplementary data). Immunoelectron microscopic analysis of the cleft-associated protein Pf332 (Hinterberg *et al*., 1994) and of truncation mutants of PfEMP3 (Waterkeyn *et al*., 2000) has been used previously to suggest that these structures may attach to the erythrocyte membrane. Indeed, previous work has shown that a Maurer's cleft protein co-localizes with the skeletal protein, actin (Etzion and Perkins, 1989), and that ankyrin may also be associated with Maurer's clefts (Atkinson *et al*., 1988), although it is difficult to rule out the possibility that the apparent connection between the Maurer's clefts and the erythrocyte membrane is an artefact due to procedures used during the sample preparation. However, it is clear from the 3-dimensional reconstruction of live 3D7+His parasites expressing the KAHRP(+His)–GFP fusion protein in Maurer's clefts that these structures are closely apposed to the membrane of the host cell and may have a direct association with the underlying membrane skeleton. It is interesting that PfEMP3 has structural similarity to Uso1p, a yeast cytoskeleton-related tethering protein involved in ER to Golgi vesicular traffic, and targeted disruption of both genes result in trafficking defects (Waterkeyn *et al*., 2000). The demonstration that PfSAR1p, a small GTPase that is involved in vesicle-mediated trafficking (Barlowe *et al*., 1994; Albano *et al*., 1999), is also associated with Maurer's clefts suggests that these structures are an elaboration of the canonical secretory and sorting machinery that has been transposed beyond the parasite into the host cell. A schematic

summarizing a proposed pathway for the trafficking of KAHRP from the parasite followed by recruitment on to Maurer's clefts and insertion on the parasite-infected erythrocyte membrane is shown in Figure 9.

In conclusion, transfection of *P.falciparum*-infected erythrocytes with a gene construct encoding GFP fused to fragments of the KAHRP protein has allowed us to monitor the trafficking of this protein within parasitized erythrocytes. Using photobleaching techniques, we have monitored the physical state of the GFP chimeras and tested for connectivity between different compartments. We have identified sequence elements in KAHRP that are sufficient for exit from the parasite, for transfer across the parasitophorous vacuole membrane and for assembly into knob structures. We have shown that the pathway involves passage via a classical, vesicle-mediated pathway through the ER, with the parasitophorous vacuole as an intermediate compartment in the export process. Importantly, we have shown that proteins destined for the erythrocyte membrane or knob complex co-localize transiently in Maurer's clefts with PfSAR1p, a protein typically involved in the process of vesicle budding in ER to Golgi traffic within eukaryotic cells, but which in *P.falciparum* has assumed a role in protein transport beyond the parasite cell in that of its host. These results suggest that Maurer's clefts are an important site of assembly and sorting of proteins destined for the knob structure.

Materials and methods

Plasmid constructs, parasite culture and transfection

To generate transgenic *P.falciparum*-infected erythrocytes expressing KAHRP–GFP chimeric proteins we made transfection constructs in the plasmid vector pHH2 as described previously (Reed *et al*., 2000; Waller *et al*., 2000). The 5′ regions of the *kahrp* gene were amplified by PCR of cDNA and subcloned as *Bgl*II and *Avr*II fragments into the plasmid vector pHH2. Plasmid pHH2-KAHRP(–His)–GFP was constructed by amplifying the region of *kahrp* encoding its N-terminus and the region proximal to the histidine-rich region using the oligonucleotides ATG*Bgl*II 5′-GGGG**AGATCT**ATGAAAAGTTTTAAGAAC-3′ and –His*Avr*II 5′-CCCC**CCTAGG**TTGCTTTTGTGCTAAAGTTCTC-3′. Plasmid pHH2-KAHRP(+His)–GFP was constructed by amplifying the region of *kahrp* encoding its N-terminus and the histidine-rich using the oligonucleotides ATG*Bgl*II 5′-GGGG**AGATCT**ATGAAAAGTTTTAAGAAC-3′ and (+His)*Avr*II 5′-CCC**CCTAGG**GTGTAAGTGATGATGGTGG-3′. The *Avr*II and *Bgl*II restriction sites introduced are shown in bold. The 3D7 cloned *P.falciparum* parasites were transfected by electroporation and drug selected using 0.25 mM WR99210 as described previously (Fidock and Wellems, 1997).

Parasite extraction and western blotting

To examine GFP expression in transfected lines, parasites were synchronized by two consecutive sorbitol treatments 4 h apart, and 32 h later trophozoites were purified by Percoll, washed twice in phosphate-buffered saline (PBS) and resuspended in Laemmli sample buffer. For time course of expression experiments, parasites were synchronized by two consecutive sorbitol treatments 4 h apart (time zero corresponds to the second sorbitol lysis), and aliquots were taken 8 hourly and lysed in 1.5 vol of 0.15% saponin for 10 min on ice, centrifuged and the pellet washed in PBS and subsequently resuspended in Laemmli sample buffer. Proteins were transferred to polyvinylidene fluoride and visualized by ECL using mouse anti-GFP antiserum (1:1000) or rabbit anti-HSP70 antiserum (1:4000).

Brefeldin A treatment

Ring stage parasites were synchronized using two consecutive sorbitol treatments 4 h apart, cultured for 40 h and allowed to reinvade. Brefeldin A (Sigma) was added upon re-invasion, to a final concentration of 5 μg/ml from a 10 mg/ml solution in ethanol. Control cultures were incubated in the presence of equivalent amounts of ethanol to ensure that it had no effect on growth and cell morphology. Brefeldin A was removed after 16 h and the parasites were cultured for 24 h to ensure viability after treatment. Brefeldin A-treated *P.falciparum*-infected erythrocytes were smeared on to glass slides and fixed with methanol.

Microscopy

Green fluorescence of GFP-expressing transfectant lines was observed and captured in live cells using a Leica TCS 4D confocal microscope or a Carl Zeiss Axioskop with a PCO SensiCam and Axiovision 2 software. FRAP measurements described previously (Lippincott-Schwartz *et al*., 1989, 2000) were made with a Leica TCS-NT laser scanning confocal microscope equipped with a 60 mW krypton/argon laser using the emission at 488 nm. The confocal scan head was mounted on a Leica TCS microscope fitted with a ×100 planapo objective (NA 1.4). Samples were imaged at 20°C within 20 min of mounting the sample in culture medium under a coverslip on to a glass slide. Individual transfected parasitized erythrocytes were subjected to a 1–10 s exposure to the laser at 100% of full power focused as a diffraction limited spot. Photobleaching studies were performed using the time-lapse feature of the Leica TCS NT software and data were analysed using ImageJ v1.22 (http://rsb.info.nih.gov/ij/index.html). Immunoelectron microscopy was performed with anti-GFP antibodies as described in Waller *et al*. (2000). For indirect immunofluorescence assays of brefeldin A-treated parasites, slides were incubated sequentially with one of the following antibodies raised in mice: anti-GFP (1:800), PfEMP1 (1:100) or PfEMP3 (1:25), or rabbit anti-KAHRP (1:200). These slides were washed and incubated with appropriate anti-PfERC antibodies raised in rabbit (1:100) or mouse (1:75). The slides were then incubated with an appropriate mixture of anti-mouse or anti-rabbit antibodies conjugated to fluorescein isothiocyanate (FITC) or rhodamine (1:1000). For indirect immunofluorescence assays untreated parasites were synchronized and ring stages smeared on to glass slides and fixed with methanol. Slides were incubated sequentially with one of the following antibodies raised in mice: anti-PfSBP1 (1:200), PfEMP1 (1:100) or PfEMP3 (1:25). These slides were washed and incubated with one of the following antibodies raised in rabbits: anti-KAHRP (1:200) or PfSAR1p (1:50). The slides were then incubated with a mixture of anti-rabbit antibodies conjugated to FITC and anti-mouse antibodies conjugated to rhodamine (1:1000).

Supplementary data

Supplementary data for this paper can be found at *The EMBO Journal* Online.

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