Generation of an erythrocyte vesicle transport system by Plasmodium falciparum malaria parasites

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The asexual maturation of Plasmodium falciparum is accompanied by the transport of parasite-encoded proteins to the erythrocyte plasma membrane. Activation of G proteins by treatment with aluminum fluoride produced an accumulation within the erythrocyte cytosol of vesicles coated with Plasmodium homologues of COPII and N-ethylmaleimide–sensitive factor, proteins involved in intracellular transport between the Golgi apparatus and the endoplasmic reticulum. These vesicles contain malarial proteins that appear on the erythrocyte plasma membrane, as well as actin and myosin. It is proposed that the parasite adapted a process well established for intracellular transport to mediate the extracellular movement of its proteins through the erythrocyte cytosol to the surface membrane. (Blood. 2003;102:3420-3426)

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Materials and methods

Parasites
IRBCs (Pf strain FCR-3-C5) were cultured and synchronized with sorbitol.14,16,17 In certain experiments, the IRBCs (1%-5% parasitemia) were enriched to between 80% and 90% by incubation in culture medium containing gelatin.

Antibodies
The production and characterization of the affinity-purified anti-PfSec31p,11 -PfSar1p,8 -PfNSF,13 and -PfEMP14 rabbit polyclonal antibodies was reported to be exported to structures in the RBC cytoplasm, suggested to be the Maurer clefts.11,12 A Pf homologue of N-ethylmaleimide–sensitive factor (NSF), which plays a central role in vesicular trafficking in eukaryotic cells, was localized to the parasite and associated with structures in the RBC cytosol.13 The major advance in uncovering a parasite-generated vesicle transport pathway was made by incubating IRBCs with aluminium tetrafluoride (AlF), an activator of G proteins, which revealed small (70- to 100-nm diameter), possibly coated vesicles in the RBC cytosol bearing the parasite proteins PfEMP1 and PfEMP3. These vesicles were organized in chains, which we suggested was evidence of cytoskeletal association.14 Taken together, these results led us to propose that malaria parasites export proteins into the host cell cytosol to spawn a vesicle-mediated protein trafficking pathway.14,15 We provide evidence that Pf adapted a well-established pathway for intracellular vesicle transport to mediate the extracellular movement of parasite proteins through the RBC cytosol to the surface membrane.

Introduction

During the development of the asexual stage of the human malaria parasite Plasmodium falciparum (Pf), the host cell erythrocyte is radically changed and acquires the ability to adhere to vascular endothelium. These alterations are believed to be largely because of the export of parasite proteins, which associate with the red blood cell (RBC) membrane. Protein targeting beyond the parasite plasma membrane (PPM) requires unusual pathways, given the parasite’s intracellular location within a parasitophorous vacuolar membrane (PVM) and the lack of organelles, proteins, and biosynthetic machinery in the RBC. Pf erythrocyte membrane protein 1 (PfEMP1) is a membrane-spanning protein that incorporates into the RBC membrane.1 The adhesive changes in parasite-infected red blood cells (IRBCs) are due to the expression of antigenically variant PfEMP1, which appears to be concentrated on the exterior surface of RBCs in membrane protrusions termed knobs.1,2 The transport of this protein to the RBC membrane is of considerable interest, given its prominent role in cytoadherence and the pathology of cerebral malaria.3,5

The transport of transmembrane proteins like PfEMP1 from the parasite to the RBC cytosol and surface membrane likely involves a vesicle-mediated process. An important question in proposing a vesicle-mediated trafficking pathway of parasite proteins from the PVM to the erythrocyte compartment is how vesicles form when mature RBCs do not contain proteins necessary to drive vesicle budding. For a transport vesicle to form from the PVM, a coating system would be required on its exterior surface. This coating system could be derived from the PVM. One role for the PVM is to coat in higher eukaryotes.9,10 A protein homologue of Sec31p was reported to be exported to structures in the RBC cytoplasm, suggested to be the Maurer clefts.11,12 A Sec31p homologue of N-ethylmaleimide–sensitive factor (NSF), which plays a central role in vesicular trafficking in eukaryotic cells, was localized to the parasite and associated with structures in the RBC cytosol.13 The major advance in uncovering a parasite-generated vesicle transport pathway was made by incubating IRBCs with aluminium tetrafluoride (AlF), an activator of G proteins, which revealed small (70- to 100-nm diameter), possibly coated vesicles in the RBC cytosol bearing the parasite proteins PfEMP1 and PfEMP3. These vesicles were organized in chains, which we suggested was evidence of cytoskeletal association.14 Taken together, these results led us to propose that malaria parasites export proteins into the host cell cytosol to spawn a vesicle-mediated protein trafficking pathway.14,15 We provide evidence that Pf adapted a well-established pathway for intracellular vesicle transport to mediate the extracellular movement of parasite proteins through the RBC cytosol to the surface membrane.

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Supported by National Institutes of Health grant AI41761 (T.F.T.).

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BLOOD, 1 NOVEMBER 2003 • VOLUME 102, NUMBER 9
Protein Transport in Malaria-Infected RBCs

Immunofluorescence

IRBCs were fixed onto glass slides overnight at 4°C by using periodate-lysine-paraformaldehyde. Primary antibody dilutions were P/NSF (1:200), P/Sar1p (1:25), P/Sec31p (1:500), and myosin (1:100, 1:500). Binding was visualized by using a fluorescein isothiocyanate (FITC)–conjugated goat antirabbit IgG or FITC-conjugated goat antimouse IgG as appropriate. Immunofluorescence analysis (IFA) was performed on a Biorad Radiance 2000 laser scanning imaging system interfaced to an Olympus IX70 inverted epifluorescence microscope.

Electron microscopy

Morphology. IRBCs were fixed with 2% glutaraldehyde containing 1% tannic acid in 0.1 M sodium cacodylate buffer, followed by 2% osmium tetroxide and 1% uranyl acetate. The fixed IRBCs were centrifuged in warm agarose, dehydrated in graded steps of acetone, infiltrated, and embedded in Spur's; the blocks were thin-sectioned (~70 nm) with a Diatome diamond knife on a Reichert Jung Ultra Cut E ultramicrotome; the thin sections were poststained with 2.5% uranyl acetate and 2.5% bismuth and viewed with an Hitachi H-7000 electron microscope.

Immunoelectron microscopy. IRBCs were fixed with 2% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, centrifuged in warm agarose, partially dehydrated in ethanol, and infiltrated and embedded in LR. White. Immunolabeling was performed on thin sections by incubation with antihuman β-actin (1:500), P/EMP1 (1:30), P/Sar1p (1:30), P/NSF (1:30), P/Sec31p (1:100), or P/Maser clef antibodies (1:30), and labeling was visualized with the appropriate UltraSmall gold-conjugated secondary antibody. The gold particle size was enhanced by using an Aurion R-gent SE-EM (silver enhancement reagent for electron microscopy) initiator, activator, and enhancer. To ensure specificity of antibody labeling, sections were incubated with the secondary antibodies only, which revealed a low level of nonspecific reactivity with the parasite nuclei. Sections were poststained for 5 minutes with 2.5% uranyl acetate and 2.5% sodium bismuth.

Results

Incubation of IRBCs with AIF produces vesicle chains in the RBC cytosol

Long, slender membranes with a translucent lumen termed Maurer clefts, circular unit membranes (0.2- to 1.0-μm diameter) with electron-dense contents and large, multiple membrane whorls are typically observed in the erythrocyte cytosol of IRBCs. The latter 2 structures were suggested to be PVM extensions. Typical Maurer clefts in IRBCs are shown in Figure 1A.D. Immunoelectron microscopy using monoclonal antibody (MAb) SPIA6G5, which is specific for a 130-kDa Maurer clef protein, confirmed that these structures were clefts (Figure 1D). None of these membranes resembled classical coated vesicles.

Examination of serial sections from more than 500 IRBCs by electron microscopy revealed in a few of those thousands of sections 1 or 2 of the 70- to 100-nm vesicles in the RBC cytosol, which appeared to be coated (data not shown). AIF is a valuable reagent for unraveling G protein–mediated vesicle transport between cellular compartments. The incubation of mature IRBCs with AIF caused the appearance of 70- to 100-nm vesicles with an electron dense periphery arranged in clusters (4-6 vesicles in a 70-nm section) or chains of up to 10 vesicles (Figure 1A-C) in the RBC cytosol, in agreement with previous results. Vesicles in clusters or chains were observed in a single thin section with a frequency of approximately 1 in every 25 IRBCs. These structures were not observed in serial sections from more than 500 AIF-treated uninfected RBCs. AIF had no effect on cell viability, as described previously. Preimmune rabbit serum was used as a control for nonspecific binding. A mouse monoclonal antibody SPIA6G5, specific for a 130-kDa Maurer clef protein, was provided by Tobih Sam Yelllowe (Cleveland, OH). Mouse monoclonal immunoglobulin G (IgG) against human β-actin (ab6276) and a beta terminal peptide of nonmucle myosin heavy chain (ab684) were from Abcam Limited (Cambridge, United Kingdom). Aurion GP-UltraSmall gold (0.8-nm diameter) antimouse, antirabbit, and antirat IgG and IgM antibodies were obtained from Aurion (Fort Washington, PA).

Preparation of RBC membranes and parasites from IRBCs treated with AIF

IRBCs (500 μL) were lysed with an equal volume of 0.15% saponin containing protease inhibitors (10 μg/mL leupeptin, pepstatin A, chymostatin, antipain, 0.5 mM PMSF (phenylmethylsulfonyl fluorid) fluoride, and 2.5 mM dithiobispropylfluorophosphate). Parasites surrounded by the PVM were isolated by centrifugation at 3000 rpm, and RBC membranes were isolated from the parasite-free lysate by centrifugation at 14 100 rpm. Hemozoin was removed by ultrasonically lysing the parasites followed by centrifugation at 10 000 rpm. The protein concentration of the RBC membranes and hemozoin-free parasites was determined by using the bicinchoninic acid (BCA) protein kit (Pierce, Rockford, IL).

Electrophoresis and immunoblotting

Uninfected RBCs were suspended in sample buffer (38 mM Tris (tris(hydroxymethyl)aminomethane)–HCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.1 mM DTT (dithiothreitol), 4% SDS (sodium dodecyl sulfate), 0.3 M sucrose, 0.01% bromphenol blue, 0.6% L-methionine), boiled for 5 minutes, and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). A volume of 100 μL IRBCs (~10% parasitemia) was resuspended in 500 μL Triton X-100 in phosphate-buffered saline (PBS: 1.9 mM NaH2PO4, 6.1 mM Na2HPO4, 140 mM NaCl, pH 7.4) with protease inhibitors (10 μg/mL leupeptin, pepstatin A, chymostatin, antipain, 0.5 mM PMSF, and 2.5 mM dithiobispropylfluorophosphate). The proteins were mixed and separated into Triton X-100 soluble and insoluble fractions by centrifugation for 20 minutes at 18 000g. The pellet was resuspended in 50 μL of 2% SDS in PBS and centrifuged at 18 000g for 20 minutes. The Triton X-100 and SDS soluble fractions were analyzed by SDS–PAGE and Western blotting was performed by using enhanced chemiluminescence (ECL) detection. Equal numbers (~1.5 × 105) of uninfected and infected erythrocytes (at ~10% parasitemia) were solubilized, and equal aliquots were added to each lane in the gel. The primary antibody dilutions were P/NSF (1:500), P/Sar1p (1:500), and P/Sec31p (1:1000). The secondary antibody was a donkey antirabbit antibody (Amersham, Arlington Heights, IL) used at a 1:3000 dilution.

To analyze the distribution of myosin in IRBCs, electrophoresis was performed on a 5% to 15% acrylamide linear gradient SDS gel containing 4 M urea, with a 5% stacking gel containing 2 M urea. The sample buffer contained 100 mM Tris–HCl, 10% β-mercaptoethanol, 4% SDS, 10% sucrose, 0.01% bromphenol blue, and 8 M urea. Equal amounts of protein were loaded for RBC membranes (82.5 μg) and PVM/parasite samples (39 μg) from untreated and AIF-treated IRBCs. Proteins were transferred onto Immobilon membranes and incubated with the antmyosin antibody (1:1000) followed by a horseradish peroxidase–labeled goat antimmunoglobulin (1:10 000). Contamination of the parasite fraction by RBC membranes was assayed using an antihuman glycophorin A and B mouse monoclonal antibody (ab6396; Abcam). Parasite fractions were determined to be glycophorin (RBC membrane) free.

Electron microscopy

Immunoelectron microscopy. IRBCs were fixed with 2% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, centrifuged in warm agarose, partially dehydrated in ethanol, and infiltrated and embedded in Spur's; the blocks were thin-sectioned (~70 nm) with a Diatome diamond knife on a Reichert Jung Ultra Cut E ultramicrotome; the thin sections were poststained with 2.5% uranyl acetate and 2.5% bismuth and viewed with an Hitachi H-7000 electron microscope.
parasites progressed normally through the erythrocytic cycle when AIF was removed from the culture medium. The vesicle complexes often abutted the RBC membrane (Figure 1C). The vesicles in the chains were 1 section thick (~70 nm). Inclusion of ruthenium red in the incubation medium ruled out the possibility that these were coated pits or tubuloendocytic vesicles of RBC origin, because these structures were not labeled (data not shown).

**PfSar1p** and **PfSec31p** are associated with transport vesicles and Maurer clefts in the erythrocyte cytosol

**Pf** homologues of Sar1p (Figure 2A) and Sec31p (data not shown) were detected by Western blotting in IRBCs solubilized with SDS, but not Triton X-100, in agreement with previous studies.8,11 These proteins were absent in Western blots of uninfected RBCs, although there was some ECL reactivity from higher and lower molecular weight species because of the peroxidase-like activity of hemoglobin monomers and tetramers as noted previously.8 IFA showed these proteins were localized to the parasite compartment and structures present in the RBC cytosol (**Pf**Sar1p, Figure 2A; **Pf**Sec31p, not shown). The distribution of **Pf**Sar1p and **Pf**Sec31p in the RBC cytosol of IRBCs incubated with AIF was similar to untreated IRBCs (data not shown). **Pf**Sar1p was localized to the parasite compartment, with 70- to 100-nm coated vesicles in the RBC cytosol (Figure 2B), similar to those observed in Figure 1A-C, and with Maurer clefts (Figure 2C). A similar labeling pattern was obtained for IRBCs probed with an anti-**Pf**Sec31p antibody (data not shown). Chains and clusters of coated vesicles bearing **Pf**Sar1p or **Pf**Sec31p were observed only in IRBCs treated with AIF. Thus, 2 components of a putative **Pf**COPII complex were localized to the 70- to 100-nm transport vesicles and Maurer clefts in the RBC cytosol.

**Parasite protein **Pf**EMP1** is transported by coated vesicles to the Maurer clefts and the erythrocyte plasma membrane

We suggested that the parasite proteins **Pf**EMP1 and **Pf**EMP3 were exported to the RBCs by a vesicle-mediated process.14 In IRBCs incubated with AIF, **Pf**EMP1 was detected in the parasite and associated with coated vesicles (Figure 3A) and Maurer clefts (Figure 3B) in the RBC cytosol. The vesicles transporting **Pf**EMP1 were similar in appearance to vesicles bearing **Pf**Sar1p and **Pf**Sec31p.

**Pf** homologue of NSF is associated with transport vesicles and Maurer clefts in the erythrocyte cytosol

A **Pf** homologue of NSF, which is involved in vesicle docking and fusion processes,26 was exported to the erythrocyte cytosol of IRBCs.13 In agreement with previous results, **Pf**NSF is absent in uninfected RBCs, insoluble in Triton X-100, and SDS soluble (Figure 4A), suggesting it is associated with cytoskeleton and/or membranes. IFA confirmed that **Pf**NSF was exported into the RBC cytosol (data not shown). Immunoelectron microscopy showed that **Pf**NSF was associated with vesicle chains in the RBC cytosol (Figure 4B), coated vesicles abutting the RBC plasma membrane (Figure 4C), Maurer clefts (Figure 4D-E), a large, double membrane structure in the RBC cytosol (Figure 4D), and at transitional regions between the Maurer clefts and the erythrocyte plasma membrane (Figure 4E). **Pf**NSF-coated vesicles were not observed in untreated IRBCs.

**Figure 1.** Incubation of IRBCs with AIF reveals vesicle chains. (A-B) Ultrastructural characterization of glutaraldehyde-fixed trophozoite stage IRBCs revealed vesicles with an electron-dense limiting membrane arranged in chains in the RBC cytosol (black arrowhead) or (C) closely apposed to the erythrocyte plasma membrane. A typical Maurer cleft is observed (A; white arrowhead). (D) Immunoelectron microscopy confirmed the long, slender unit membranes in the RBC cytosol are Maurer clefts. MC indicates Maurer cleft; P, parasite; PVM, parasitophorous vacuolar membrane; PPM, parasite plasma membrane, RBCM, red blood cell membrane; and V, vesicles. Scale bar = 70 nm.

**Figure 2.** **Pf** Sar1p is associated with coated vesicles and Maurer clefts. (A) Western blot analysis of uninfected and IRBCs incubated with AIF demonstrated that **Pf**Sar1p was an approximate 23-kDa Triton X-100-insoluble, SDS-soluble protein that was absent in uninfected RBCs. IFA showed **Pf**Sar1p localized to the parasite compartment and structures in the RBC cytosol. Immunoelectron microscopy revealed **Pf**Sar1p associated with (B) coated vesicles (V), black arrowhead, and (C) Maurer clefts (MC), white arrowhead, within the RBC cytosol. Scale bar = 70 nm.

**Figure 3.** **Pf** EMP1 is associated with coated vesicles and Maurer clefts. (A) Western blot analysis of uninfected and IRBCs incubated with AIF demonstrated that **Pf**EMP1 was an approximate 23-kDa Triton X-100-insoluble, SDS-soluble protein that was absent in uninfected RBCs. IFA showed **Pf**EMP1 localized to the parasite compartment and structures in the RBC cytosol. Immunoelectron microscopy and probed with an anti-**Pf**EMP1 antibody. **Pf**EMP1 associated with (A) the parasite compartment and coated vesicles in the RBC cytosol and (B) Maurer clefts. Scale bar = 70 nm.
Actin is associated with transport vesicles in the erythrocyte cytosol

The organization of coated transport vesicles in chains in AIF-treated IRBCs suggested they might be transported along a cytoskeletal network. Immunoelectron microscopy showed that actin was associated with the RBC membrane, clusters of coated vesicles in the RBC cytosol (Figure 5A-B), and vesicle chains extending to the RBC plasma membrane (Figure 5C). Other larger vesicles with electron dense contents, which were morphologically distinct from the coated 70- to 100-nm vesicles, appeared to interact with the RBC membrane through an association with actin (Figure 5D-E). Actin was localized in the vicinity of a budding, coated vesicle (Figure 5F) and other PVM protrusions (Figure 5G).

Intraerythrocytic transport vesicles do not contain erythrocyte, PVM, or Maurer cleft proteins

To gain further insight into the origin and composition of the intraerythrocytic transport vesicles in IRBCs, we used immunoelectron microscopy to investigate whether proteins from the erythrocyte membrane (glycophorin), PVM (exp-1), or the Maurer clefts (the 130-kDa protein recognized by MAb SP1A6G5) were associated with the vesicles. None of these proteins were localized to the vesicles (data not shown).

Maurer clefts in the RBC cytosol are tethered to the RBC plasma membrane via actin

We showed here that PfEMP1, PfSec31p, PfSar1p, and PfNSF associate with the Maurer clefts. Wickham et al.\(^{12}\) observed that PfEMP1, PfEMP3, PfHRP1, and PfSar1p colocalized with the Maurer clefts and suggested the clefts were an elaboration of a canonical secretory pathway that is transposed outside the parasite into the host cell. Maurer clefts were proposed to be associated with the host cell cytoskeleton,\(^{27,28}\) and this interaction may regulate the release of proteins from the clefts.\(^{29}\) The first direct evidence that Maurer clefts are connected to the RBCs is shown in Figure 5H-I. Actin was associated with the body of a cleft, and each end of the cleft appeared to extend out to actin-enriched regions of the RBC membrane (Figure 5H). In IRBCs treated with streptolysin O to remove RBC cytosol, the clefts maintained their parallel orientation to the RBC membrane. The cleft appeared to be connected to the parasite compartment and RBC membrane by actin-rich areas (Figure 5I). We suggest that the Maurer clefts are tethered to the host cell membrane by association with actin and possibly other RBC cytoskeletal proteins and play a direct role in the trafficking of parasite proteins to the host cell membrane.

Erythrocyte myosin is stabilized at the PVM in AIF-treated IRBCs

Myosins are a conserved class of actin-based motor proteins found in virtually all eukaryotes that play a role in many diverse cellular tasks, including endocytosis and exocytosis and vesicle transport. RBCs contain about 6000 copies per cell of a nonmuscle myosin II of approximately 200 kDa; parasites contain an unusual approximately 105 kDa (Myosin XIV), which is present only at the end of the erythrocytic cycle and is part of an actin-myosin motor required for parasite invasion.\(^{31}\) By immunofluorescence, myosin was associated
with the RBC membrane and was diffusively distributed in the RBC cytosol of uninfected RBCs (data not shown) in agreement with previous results.30 In IRBCs, myosin was also peripherally associated with the parasite, presumably with the PVM (Figure 6A-B). AIF treatment perturbed the myosin distribution, causing a decrease in the erythrocyte compartment and an increased accumulation at the outside edge of the parasite (Figure 6C-D). Immunoelectron microscopy of AIF-treated IRBCs showed myosin was associated with the PVM and the Maurer clefts (Figure 6E-F) and on rare occasion with coated vesicles budding from the PVM (Figure 6G).

Discussion

Treatment of IRBCs with AIF provided the key evidence for an intraerythrocytic vesicle-mediated secretory pathway and suggested that it was G-protein mediated. G proteins are divided into large, trimeric GTP-binding proteins and small, monomeric GTPases belonging to the Ras superfamily. The Ras/rab gene family are regulators of vesicle budding, motility, and fusion.32 Control of vectorial transport is mediated by these small GTPases that cycle between a membrane-associated (GTP-bound) and a soluble (guanosine diphosphate [GDP]–bound) form, which requires hydrolysis of GTP. Six Rab proteins were identified to date in the Pf genome but is uncharacterized. The Ras/rab gene family are large, trimeric GTP-binding proteins and small, monomeric GTPases belonging to the Ras superfamily. The Ras/rab gene family are regulators of vesicle budding, motility, and fusion.32 Control of vectorial transport is mediated by these small GTPases that cycle between a membrane-associated (GTP-bound) and a soluble (guanosine diphosphate [GDP]–bound) form, which requires hydrolysis of GTP. Six Rab proteins were identified to date in the Pf gene family are regulators of vesicle budding, motility, and fusion.32 Control of vectorial transport is mediated by these small GTPases that cycle between a membrane-associated (GTP-bound) and a soluble (guanosine diphosphate [GDP]–bound) form, which requires hydrolysis of GTP.

Vesicle formation and transport in the erythrocyte cytosol

Many of the steps of the proposed erythrocytic vesicle-mediated protein transport pathway in IRBCs were visualized in a single thin section by electron microscopy (Figure 7A). Vesicles that were morphologically similar to those observed in Figures 1 to 6 budded from the PVM and appeared to be transported on a network to a Maurer cleft that was tethered to the RBC membrane by actin. The synthesis of the individual results obtained in Figures 1 to 6 and 7A into a model for a parasite-generated, vesicle-mediated intraerythrocytic protein transport pathway in IRBC is presented in Figure 7B.
genome (www.plasmodb.org); the genome is devoid of heterotrimetric G proteins. Therefore, the latter are not the target of AlF action. Although human RBCs contain both classes of G proteins, the lack of effect of AlF on uninfected RBCs and the association of P/COPII homologues with the vesicles in the RBC cystol of IRBCs incubated with AlF suggests the involvement of exported parasite G proteins in the vesicle-mediated transport process.

We do not know what steps in the vesicle transport process were affected by AlF. Fluoride complexes bind next to the bound GDP on G proteins and mimic the γ-phosphate of GTP, promoting the switch to a GDP-like conformation. 23,24 Small G proteins bind fluoride complexes in the presence of their corresponding GTPase activating proteins (GAPs). 23,24 AlF inhibited GTP hydrolysis on RasGTP and RabGTP because of the formation of a quaternary G protein GDP · AlF · GAP complex, thereby inhibiting the uncoating of coated vesicles and preventing their fusion with the target membrane. AlF may interact with small P/ G proteins (eg, P/Rabs) and an as of yet undescribed P/Rab-GAP to inhibit the GTP hydrolysis of P/RabGTP, causing the coated vesicles bearing parasite proteins to back up into vesicle chains at the Maurer clefts or RBC membrane because of their inability to fuse. There is evidence for this interpretation, as uncoating vesicles appeared to be in a state of arrested fusion at the erythrocyte membrane of IRBCs treated with AlF. 14,15 It is also possible that a P/Sec23p–AlF–PfSar1p complex formed, preventing GDP hydrolysis and disassembly of the coat, thereby inhibiting fusion with target membranes.

The transport of vesicles between intracellular organelles and the cell surface is accomplished either by energy-dependent (long-range) movement on microtubules or local movement by an actin-myosin motor system. The distances between the PVM and the Maurer clefts and erythrocyte plasma membrane are small and could be traversed in seconds by random diffusion of the vesicles; we do not favor this model because transport is directional (from the PVM outward), specific parasite proteins are transported to specific destinations in the RBC cystol, and the vesicles are organized into small clusters or extended chains, suggesting they are associated with a platform. Vesicular transport along microtubules in IRBCs seems unlikely because RBCs do not contain microtubules. On the basis of the localization of actin and myosin to the PVM, chains of transport vesicles containing parasite proteins, the Maurer clefts, and erythrocyte plasma membrane, we propose that vesicle formation and transport may involve an actin-myosin–mediated process. Most of the RBC actin under normal conditions is present in stable 14-subunit oligomers, with only a small fraction in the cytosol that is below the critical concentration for assembly. Asexual parasites contain proteases that degrade the RBC cytoskeletal proteins α and β-spectrin, 4.1, actin, and ankyrin. 34,35 It is possible that the action of these proteases disrupts this balance, raising the free concentration of cytosolic actin or produces free actin filaments extending from the RBCs that could interact with other proteins. It cannot be ruled out that the actin involved in vesicle transport could be supplied by the parasite, because the antibody we used was raised against the conserved N-terminal of β-actin and cross-reacts with P/ actin.

There is some evidence for myosin II as a vesicle transporter. In mammalian cells, myosin II is Golgi associated during vesicle budding. 36–38 Myosin II binding to isolated Golgi was accompanied by the recruitment of actin. Myosin II binding to newly budded Golgi vesicles was stabilized by GTP-γS or AlF, suggesting it binds transiently to budding vesicle membranes under the regulation of G proteins. AlF stabilized myosin binding to the PVM in Figure 6. It is tempting to speculate that RBC myosin might move along actin filaments to either pull a vesicle bud or to move away vesicles from the PVM and across the RBC cystol.

We were surprised that P/NSF was membrane bound, associating with Maurer clefts, transport vesicles, and the RBC membrane. The soluble proteins α-SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein) and NSF exist predominantly in a membrane-bound form in adrenal chromaffin cells, being found predominantly at the membrane of chromaffin secretory vesicles. The observation of P/NSF-coated vesicles in the RBC cystol suggests this interaction could be important for the acquisition of competence for docking. We suggest that P/NSF may play a role in the targeting and fusion of the P/COPII-coated vesicles bearing parasite proteins (eg, P/EMP1 and P/EMP3) to the Maurer clefts or the RBC membrane.

Our results suggest that intraerythrocytic parasites export proteins into the RBC cystol to establish a vesicle-mediated system for transporting parasite proteins to the erythrocyte membrane. These proteins include P homologues of the COPII proteins Sar1p and Sec31p, well known for their involvement in intracellular transport between the Golgi and endoplasmic reticulum. This is the first report of COPII proteins being secreted to a distant location (eg, the cytosol of a host cell) to establish a vesicle-mediated protein transport system. Treatment of IRBCs with AlF resulted in the appearance of 70- to 100-nm coated vesicles arranged in extended chains or clusters in the erythrocyte cytosol of IRBCs. The localization of P/Sar1p, P/Sec31p, P/NSF, P/EMP1, P/EMP3, actin, and erythrocyte myosin to the chains/clusters of morphologically similar vesicles strongly suggests that these proteins are associated with the same vesicles. Because of the small size of the vesicles and the moderate reactivity of some of the antibodies, it was necessary to use UltraSmall gold-conjugated antibodies and silver enhancement to better visualize the antibody deposition. This use results in gold particles of varying size, making it impossible to colocalize multiple proteins to the same vesicles by immunoelectron microscopy. Further biochemical characterization of the transport vesicle proteins will require a proteomic analysis of the purified transport vesicles.

Acknowledgments

We thank Akin Adisa and Frank Albano for providing the P/Sec31p and P/Sar1p antibodies and Tobili Sam-Yellowe for the anti-Maurer cleft antibody. We thank Louis Casta for figure preparation and helpful discussions.

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Generation of an erythrocyte vesicle transport system by *Plasmodium falciparum* malaria parasites

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