Protein sorting in *Plasmodium falciparum*-infected red blood cells permeabilized with the pore-forming protein streptolysin O

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Plasmodium falciparum is an intracellular parasite of human red blood cells (RBCs). Like many other intracellular parasites, *P. falciparum* resides and develops within a parasitophorous vacuole which is bound by a membrane that separates the host cell cytoplasm from the parasite surface. Some parasite proteins are secreted into the vacuolar space and others are secreted, by an as yet poorly defined pathway, into the RBC cytosol. The transport of proteins from the parasite has been followed mainly using morphological methods. In search of an experimental system that would allow (i) dissection of the individual steps involved in transport from the parasite surface into the RBC cytosol, and (ii) an assessment of the molecular requirements for this process at the erythrocytic side of the vacuolar membrane, we have

permeabilized infected RBCs with the pore-forming protein streptolysin O using conditions which left the vacuole intact. The distribution of two parasite proteins which served as markers for the vacuolar space and the RBC cytosol respectively was analysed morphologically and biochemically. In permeabilized RBCs the two marker proteins were sorted to the same compartments as in intact RBCs. The protein which was destined for the RBC cytosol traversed the vacuolar space before it was translocated across the vacuolar membrane. Protein transport could be arrested in the vacuole by removing the RBC cytosol. Translocation across the vacuolar membrane required ATP and a protein source at the erythrocytic face of the membrane, but it was independent of the intracellular ionic milieu of the RBC.

INTRODUCTION

Many intracellular pathogens develop inside a parasitophorous vacuole. The vacuolar membrane separates the host cell cytoplasm and the parasite plasma membrane, and it is therefore the interface at which the host–parasite interaction takes place. The mechanisms involved in the biogenesis and maintenance of the vacuolar membrane of apicomplexan parasites, a group of unicellular eukaryotic organisms that invade other eukaryotic cells, are largely unknown. Most of the current knowledge is based on studies with *Toxoplasma gondii* and *Plasmodium* spp. and can be summarized as follows [1–3]. Immediately prior to invasion, the contents of specialized secretory organelles, called rhoptries, are discharged from the apical end of the parasite when it comes into contact with the host cell membrane. The rhoptries of *Plasmodium* spp. contain lipids [4,5], and several rhoptry-specific proteins have been identified in *Plasmodium falciparum*, *T. gondii* and *Eimeria nieschulzi* (for a summary see [3]). The events following the discharge of rhoptries are rapid, and they have been studied mainly during the invasion of red blood cells (RBCs) by *Plasmodium* parasites. Upon release of the rhoptry contents, active penetration by the parasite occurs within 10–20 s, after which the parasite is found inside the RBC, surrounded by the vacuolar membrane [1,6]. The molecular mechanisms leading to the formation of this membrane have not been resolved in detail, but it is clear that they differ from those involved in the formation of endocytic compartments in several respects, not only because mature RBCs do not endocytose [7,8]. Labelled lipids incorporated into the RBC plasma membrane prior to invasion were internalized with the parasite and became constituents of the vacuolar membrane [9–11], whereas RBC membrane proteins were excluded [12,13]. Some investigators have observed the insertion of parasite lipids and proteins into the RBC membrane prior to vacuole formation [14–16], and

therefore it appears that initially the vacuolar membrane is generated as a hybrid of the RBC membrane and parasite-derived membranes. Presumably, the parasite components of the vacuolar membrane are derived from the rhoptry contents, and in *T. gondii* one rhoptry protein has recently been identified which becomes an integral component of this membrane [17]. The vacuolar space is an extracytosolic compartment for both the host cell and the parasite.

During parasite growth the vacuolar membrane extends to eventually form a tubovesicular network within the RBC cytoplasm [18–20]. Since mature RBCs do not synthesize membranes *de novo*, and as fluorescent, non-exchangeable, lipophilic markers have been found to be transported from the parasite into the tubovesicular network [9], it is most likely that the vacuolar membrane and the network are continuously built up by the parasite. Proteins which are secreted from the parasite are found in specific locations: in the vacuolar space [e.g. the serine-rich protein (SERP)], in the vacuolar membrane [e.g. exported protein 2 (exp-2)], in the RBC cytoplasm [e.g. the glycophorin-binding protein (GBP)], or associated with the cytoplasmic face of the RBC membrane (for reviews see [19,21,22]). The mechanisms that specify the destination of secreted parasite proteins (either the vacuolar space or the RBC cytoplasm) are currently unknown. Two recent models [19,23] have suggested that proteins destined for the RBC cytoplasm are targeted to specific sites where the parasite plasma membrane is either in close contact or fused with the vacuolar membrane, and translocated directly into the RBC. These models are predominantly based on morphological analyses using fluorescence microscopy. Biochemical approaches to dissect infected RBCs (iRBCs) and to study protein transport across the vacuolar membrane have so far been of limited use because standard procedures tend to partially disrupt this membrane and the tubovesicular network [20,24], and result in the release of the vacuolar contents.

Abbreviations used: BFA, brefeldin A; exp-2, exported protein 2; GBP, glycophorin-binding protein; iRBC, infected red blood cell; RBC, red blood cell; SERP, serine-rich protein; SLO, streptolysin O.

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Therefore we sought an alternative experimental system to permeabilize iRBCs in order to gain access to the erythrocytic face of the intact vacuolar membrane. We have used streptolysin O (SLO), a bacterial protein that forms large pores in membranes [25], to permeabilize the RBC membrane. We demonstrate that two marker proteins, SERP and GBP, were sorted to the same destinations in permeabilized iRBCs as in intact iRBCs. SERP was sorted to its vacuolar destination [26,27], and GBP was transported beyond the vacuolar membrane as described for intact iRBCs [28–31]. GBP accumulated in the vacuole in a first step, and was then transported across the vacuolar membrane in a second step that required a protein component and ATP at the RBC face of the membrane.

MATERIALS AND METHODS

Materials

Trypsin, haemoglobin, brefeldin A (BFA), apyrase and proteinase inhibitors were obtained from Sigma. BFA was dissolved in ethanol at 1 mg/ml and stored at 20 °C. ATP was purchased from Boehringer-Mannheim, saponin was from Serva, and L-[³⁵S]methionine (1 Ci/μmol) was from Amersham. The proteinase inhibitor cocktail contained 0.2 mg/ml each of antipain, aprotinin, chymostatin, elastatinal, pepstatin, soybean trypsin inhibitor, NaEDTA and leupeptin, and was used at 1:200 dilution. Native or recombinant SLO was prepared as described elsewhere ([32]; U. Weller and S. Bhakdi, unpublished work; details available on application to S. B.). Polyclonal rabbit antibodies directed against GBP, SERP and parasite aldolase have been described previously [27,33,34], as has the mouse monoclonal antibody McAb7.7 which recognizes exp-2 [35]. Protein A–Sepharose beads were from Pharmacia. Fluorescently labelled second antibodies were obtained from Sigma. Alkaline phosphatase-conjugated anti-rabbit antibodies were from Promega, and enzymic detection was carried out using the Promega ProtoBlot system.

Parasite cultures

P. falciparum (isolate FCBR) was maintained in human RBCs of blood group A⁺. The culture medium consisted of RPMI containing 10 % heat-inactivated (56 °C, 30 min) human serum from blood group A⁺ donors. RBCs were cultured at a haematocrit of 2–8 % (v/v) in 90 % N₂/5 % CO₂/5 % O₂. Parasites were synchronized weekly by treating RBCs, containing predominantly ring-stage parasites, in 2–3 vol. of 0.3 M L-alanine, 10 mM Hepes/KOH, pH 7.4, for 5 min at 37 °C [36]. Subsequently, cells were mixed with 10 vol. of RPMI and centrifuged at 400 g for 5 min. Cells recovered in the pellet were cultured under standard conditions.

Trophozoite-infected RBCs were enriched from cultures containing 5–10 % iRBCs to 80–95 % iRBCs [37], and the distribution of developmental stages of the parasite was quantified as described in detail elsewhere [38]. Experiments were continued only when parasite preparations contained more than 97 % trophozoites (24–30 h post-invasion) and less than 3 % polynucleated schizonts.

Lysis and fractionation of iRBCs

Lysis of iRBCs with 0.09 % saponin was carried out as described [39]. For permeabilization of iRBCs with SLO, SLO was titrated; 2 × 10⁸ iRBCs were resuspended in 200 μl of RPMI and incubated with various amounts of SLO at room temperature for 6 min. The solution was centrifuged for 5 min at 1300 g. The amount of haemoglobin released was determined spectrophotometrically at

412 nm. In parallel, 2 × 10⁸ iRBCs were lysed completely in 200 μl of water. The amount of SLO that resulted in the release of 50 % of the haemoglobin was defined as 1 haemolytic unit. For the subsequent experiments, 2 × 10⁸ iRBCs were incubated in 200 μl of RPMI containing 3–4 haemolytic units of SLO, which resulted in haemoglobin release of more than 98 %. Following permeabilization with SLO, cells were centrifuged as above and washed with 200 μl of RPMI. The cytosolic fraction obtained after lysis and the washing solution were combined. Proteins contained in the combined fractions were analysed by immunoprecipitation and immunoblotting [39]. In assays in which cytosolic fractions were used to study protein transport in permeabilized cells, the cytosol was not mixed with the washing solution. The pellet (permeabilized iRBCs) was analysed by immunoblotting and immunoprecipitation for the presence of specific marker proteins either directly or after metabolic labelling of the permeabilized iRBCs. Immunoprecipitations were routinely carried out from the RBC cytosol and from the cellular fractions of 2 × 10⁷ iRBCs; for the detection of aldolase and GBP by immunoblotting, 1 × 10⁶ and 5 × 10⁶ cells respectively were analysed.

Metabolic labelling of parasite proteins and analysis of protein transport in permeabilized iRBCs

For labelling of intact iRBCs, 2 × 10⁸ iRBCs were washed twice with methionine-free RPMI and incubated in 1 ml of RPMI containing 50–100 μCi of L-[³⁵S]methionine, without unlabelled methionine, at 37 °C for 1 h. When the effects of BFA were analysed, 2 × 10⁸ iRBCs were incubated in 1 ml of methionine-free RPMI containing 5 μg/ml BFA for 10 min; 50–100 μCi of L-[³⁵S]methionine/ml was then added. Following the labelling period, iRBCs were washed twice with RPMI at 4 °C. For labelling of permeabilized iRBCs, 2 × 10⁸ iRBCs were permeabilized with SLO, washed in methionine-free RPMI and incubated for 90 min at 37 °C in 200 μl of methionine-free RPMI supplemented with L-[³⁵S]methionine. For BFA treatment of permeabilized iRBCs, the cellular fraction was preincubated with 5 μg/ml BFA prior to the addition of L-[³⁵S]methionine. To study the requirement for RBC cytosol, the method was modified as follows. (i) For labelling in the presence of erythrocytic cytosol from iRBCs, the cytosolic fraction obtained after SLO permeabilization in methionine-free RPMI was kept on ice while permeabilized iRBCs were washed as above. Subsequently, the cytosolic fraction was supplemented with L-[³⁵S]methionine and added back to the permeabilized iRBCs. (ii) For labelling in the presence of cytosol from non-infected RBCs, 2 × 10⁸ non-infected RBCs were resuspended in a total volume of 200 μl of methionine-free RPMI, subjected to three cycles of freezing and thawing and centrifuged for 30 min at 15800 g. The supernatant was supplemented with L-[³⁵S]methionine and added to permeabilized iRBCs. (iii) For pulse-chase experiments, permeabilized iRBCs were first labelled in 200 μl of methionine-free RPMI supplemented with L-[³⁵S]methionine for 45 min. Cells were washed in RPMI, resuspended in cytosolic fractions from 2 × 10⁸ iRBCs or non-infected RBCs as described above, except without labelled methionine, and incubated. Alternatively, the cytosolic fractions were substituted with protein fractions, as described in the Results section.

Immunofluorescence

Parasite cultures were grown to parasitaemias of 10 % and parasites were harvested at the trophozoite stage, normally 24–30 h post-invasion. To investigate the effects of BFA, iRBCs were cultured in 5 μg/ml BFA for 4 h before harvesting. Cells

were collected by centrifugation at 1300 *g* and washed three times in RPMI at 4 °C. Saponin-induced lysis was carried out as described in [39], and permeabilization by SLO was performed as described for enriched iRBCs. Before fixation, both intact cells and lysed cells were washed three times in PBS. Fixation was in 0.5% formaldehyde in PBS on ice for 30 min. To remove excess fixative, cells were washed three times in PBS at 4 °C, spread on to slides and air-dried. The slides were incubated for 10 min at 37 °C in PBS/3% milk powder and subsequently reacted with the antibodies diluted in PBS/3% milk powder (1:100 for the rabbit anti-GBP antiserum; 1:400 for mouse ascites containing McAb7.7) at 37 °C for 30 min. Slides were washed three times in PBS and incubated with the second antibodies diluted 1:200 in PBS containing 50 µg/ml propidium iodide to stain parasite nuclei. The slides were washed three times in PBS and mounted with 0.1% *p*-phenylenediamine in PBS/50% glycerol. Fluorescence microscopy was performed using a Leitz fluorescence microscope.

Miscellaneous procedures

Immunoprecipitations of GBP and SERP were carried out as described previously [39]. Proteins were separated by SDS/PAGE and characterized either by immunoblotting or by autoradiography of metabolically labelled products.

RESULTS

GBP can be recovered in two separate subcellular fractions after SLO permeabilization

GBP has been localized to the RBC cytoplasm both by immunofluorescence and by immunoelectron microscopy [29–31]. When iRBCs were lysed with saponin, a procedure that allows the separation of intact parasites and RBC cytosol [40], GBP was detected in the fraction containing the RBC cytosol [39,41]. Cytoplasmic proteins of the parasite, such as parasite aldolase, segregated into the fraction containing intact parasites. Since lysis of iRBCs with saponin also results in the lysis of the vacuolar membrane [40] we sought an alternative method that would allow selective permeabilization of the RBC membrane. In initial experiments we compared the separation of the parasite cytoplasmic marker aldolase and GBP in iRBCs either lysed with saponin or permeabilized with SLO (Figure 1). Aldolase was predominantly recovered with the parasite fraction after saponin lysis and centrifugation, whereas GBP was found in the supernatant (Figure 1, lanes 1, 2, 5 and 6). As expected, after permeabilization with SLO, aldolase remained in the parasite fraction (Figure 1, lanes 3 and 4). In contrast to lysis with saponin, permeabilization with SLO resulted in a substantial amount of GBP segregating with the permeabilized iRBCs (Figure 1, lanes 7 and 8). To exclude the possibility that the incomplete release of GBP was due to only partial lysis of the cells, the haemoglobin content in the permeabilized iRBCs and in the fractions containing the released proteins were compared in parallel. Consistently, more than 95% of the total haemoglobin was released after permeabilization with SLO.

To investigate the integrity of the vacuolar membrane after SLO permeabilization and extraction of the RBC cytosol, the distribution of the vacuolar marker SERP [26,27] after saponin lysis and SLO permeabilization of iRBCs was compared. Infected RBCs were metabolically labelled, either lysed with saponin or permeabilized with SLO, and centrifuged. In iRBCs lysed with saponin, SERP was immunoprecipitated predominantly from the fraction containing the iRBC cytosol (Figure 2, lanes 1 and 2), supporting previous observations that saponin disrupts the

Figure 1 Comparison of saponin lysis and SLO permeabilization of iRBCs

iRBCs were either treated with saponin (Sap) or permeabilized with SLO using conditions that resulted in complete release of haemoglobin. The cellular fractions (P) and the fractions containing released proteins (S) were analysed by immunoblotting with an antiserum specific for parasite aldolase (ALD; lanes 1–4) or with an antiserum specific for GBP (lanes 5–8). The sizes of molecular mass markers are indicated on the left.

Figure 2 Permeabilization with SLO preserves the integrity of the vacuolar membrane

Proteins of iRBCs were metabolically labelled either in the absence (lanes 1–4) or in the presence (lanes 5–8) of BFA. iRBCs were either lysed with saponin (Sap) or permeabilized with SLO. SERP was immunoprecipitated from the cellular fractions (P) or from the fractions containing released proteins (S). Immunoprecipitated proteins were separated by SDS/PAGE and visualized by autoradiography.

vacuolar membrane. In iRBCs permeabilized with SLO, SERP was exclusively recovered in the fraction containing intact parasites (Figure 2, lanes 3 and 4), demonstrating that SLO selectively permeabilizes the RBC membrane but not the vacuolar membrane or the parasite plasma membrane. When iRBCs were labelled in the presence of BFA, i.e. under conditions that inhibit protein secretion from the parasite cell, SERP was exclusively recovered with the parasite fraction, independent of the lysis procedure (Figure 2, lanes 5–8).

The data obtained from the fractionation of iRBCs and the distribution of specific proteins indicated that GBP was present not only in the RBC cytosol but also in the parasitophorous vacuole. We therefore investigated the localization of GBP by immunofluorescence microscopy. Untreated iRBCs (Figure 3A) and iRBCs which, to give differential release of soluble proteins, were treated either with saponin (Figures 3B and 3C) or with SLO (Figures 3D, 3E and 3F) were fixed with formaldehyde to preserve soluble GBP. Fixed iRBCs were subsequently air-dried on microscope slides to allow access of antibodies to internal antigens. GBP was detected with a polyclonal antibody directed against it, followed by the addition of fluorescein-conjugated

Figure 3 Immunolocalization of GBP and exp-2 in permeabilized iRBCs

iRBCs (A), iRBCs lysed with saponin (B) and iRBCs treated with BFA for 4 h prior to lysis with saponin (C) were fixed with formaldehyde, spread on to microscope slides and air-dried. They were subsequently incubated with an antibody against GBP, followed by incubation with a fluorescein isothiocyanate-conjugated second antibody and with propidium iodide to stain parasite nuclei (N). In the experiment shown in (D)–(F), iRBCs were permeabilized with SLO, the RBC cytosol was depleted and cells were spread immediately on to microscope slides and air-dried. Cells were double-labelled with an antibody against GBP (fluorescein staining) and with an antibody against exp-2 (rhodamine staining), a marker protein of the vacuolar membrane. (D), (E) and (F) show an identical microscopical field: (D) detection of fluorescein; (E) detection of rhodamine; (F) simultaneous detection of both fluorescein and rhodamine staining. For reference, a separate microscopical field showing the distribution of exp-2 in non-permeabilized iRBCs is shown in (E').

goat anti-rabbit antibody. Parasite nuclei (N) were stained with propidium iodide. Fluorescein staining of untreated iRBCs revealed a punctate pattern, with GBP most prominent in the RBC cytosol (Figure 3A). In some cells a distinct lining of the parasite periphery could be observed. Thus the immunostaining of GBP with this antibody was very similar to the staining observed by other investigators using different antibodies to this protein. When iRBCs were lysed with saponin prior to fixation, only the nuclear staining but no immunostaining was detectable (Figure 3B). This was in accordance with previous findings that had shown total loss of GBP from cells fractionated with saponin [39,41]. When iRBCs were treated with BFA prior to saponin lysis and fixation, immunostaining was confined to a discrete location in close proximity to the parasite nucleus (Figure 3C). This result confirmed previous observations that BFA arrests the transport of GBP in a distinct intraparasite compartment [39], and it also demonstrated that saponin lysis *per se* does not abolish antibody binding in the immunofluorescence assay.

In the next experiment, iRBCs were permeabilized with SLO, fixed with formaldehyde and incubated in a mixture containing the rabbit antibody against GBP and mouse monoclonal antibody McAb7.7, which specifically recognizes exp-2, a parasite protein that is associated with the vacuolar membrane ([35]; Figure 3E'). The rabbit antibody was detected with the fluorescein-conjugated second antibody; McAb 7.7 was detected

using a rhodamine-conjugated goat anti-mouse antibody. Figures 3(D), 3(E) and 3(F) show the same microscope field of iRBCs and visualization of the different fluorescent dyes. The fluorescein staining of SLO-permeabilized cells was markedly different from the pattern observed in control iRBCs (compare Figures 3A and 3D). In the permeabilized cells the punctate fluorescence in the RBC cytosol became less prominent, and the staining of the parasite periphery was more pronounced. Visualization of rhodamine revealed a circular staining of the parasite periphery, which is in agreement with the previously reported distribution of exp-2 [35] and which was demonstrated independently by labelling non-permeabilized iRBCs with McAb7.7 (Figure 3E'). Simultaneous visualization of both dyes revealed the co-localization of the two proteins at the periphery of the parasite (Figure 3F). Thus GBP is present in the RBC cytosol and in the vacuole, but the latter location becomes apparent only after removal of the cytosol under conditions that leave the vacuolar membrane intact; i.e. after permeabilization with SLO.

In the absence of RBC cytosol, GBP accumulates in the parasitophorous vacuole

The biosynthesis and distribution of GBP were investigated in permeabilized iRBCs. iRBCs were permeabilized with SLO, washed, and RBC cytosol was replaced by RPMI containing

Figure 4 GBP accumulates in the vacuole in the absence of RBC cytosol

(A) iRBCs were permeabilized with SLO under conditions that led to the complete release of haemoglobin. Permeabilized cells were resuspended in RPMI and proteins were metabolically labelled by the addition of L-[³⁵S]methionine, either in the absence (lanes 1–4) or in the presence (lanes 5–8) of BFA. After 60 min an aliquot of the iRBCs was withdrawn from each sample and lysed with saponin (Sap; lanes 3, 4, 7 and 8). Proteins from the cellular fractions (P) and from the fractions containing released proteins (S) were immunoprecipitated with the anti-GBP antiserum and analysed by SDS/PAGE and autoradiography. (B) iRBCs were permeabilized with SLO and proteins were metabolically labelled. Permeabilized cells were then left for 60 min at 37 °C in RPMI alone (lane 1), in RPMI containing increasing amounts of trypsin (Tryp) in RPMI (lanes 2–5) or in 1 mg/ml trypsin in 0.09% saponin (lane 6). After inactivation of trypsin, permeabilized iRBCs were lysed by freezing and thawing, and GBP was immunoprecipitated.

L-[³⁵S]methionine. After a labelling period of 60 min, the distribution of GBP was analysed in the cellular fraction and in the incubation medium. Most GBP was immunoprecipitated from the cellular fraction, and hardly any GBP segregated into the incubation medium (Figure 4A, lanes 1 and 2). When the cellular fraction was subsequently lysed with saponin, the protein was recovered in the soluble fraction and only a small proportion remained associated with the parasite cells (Figure 4A, lanes 3 and 4). An internal control analogous to that described for Figure 2 was carried out; when SLO-permeabilized iRBCs were labelled in the presence of BFA, GBP was exclusively found in the cellular fraction, this was also the case after a second lysis with saponin (Figure 4A, lanes 5–8). Thus GBP can be trapped in the vacuole.

If the vacuole is bound by a sealed membrane, externally added proteinases should not have access to the vacuolar contents. To test this, iRBCs permeabilized with SLO were labelled and subsequently treated with trypsin, either in the absence or in the presence of saponin. In the absence of saponin, GBP was protected from digestion as demonstrated by comparison with cells incubated in RPMI alone (Figure 4B, lanes 1–5), but it was susceptible to the proteinase in the presence of saponin (Figure 4B, lane 6). This experiment confirmed that GBP recovered with the cellular fraction was indeed contained within a sealed compartment.

Transport of GBP across the vacuolar membrane occurs in permeabilized iRBCs

Experiments were carried out to investigate the effects of RBC cytosol on the distribution of GBP and SERP. iRBCs were

permeabilized with SLO and, after the release of haemoglobin, the permeabilized cells were washed with RPMI. A total of 2×10^8 iRBCs were then incubated and metabolically labelled for 45 min under various conditions: L-[³⁵S]methionine in (i) 200 μ l of RPMI, (ii) 200 μ l of RPMI containing the erythrocytic cytosol of 2×10^8 non-infected RBCs obtained by freeze–thawing of the cells, or (iii) 200 μ l of RPMI containing the cytosol of 2×10^8 iRBCs prepared by SLO permeabilization. The final concentrations of the cytosolic fractions corresponded to approximately 10% of the physiological concentrations. The distribution of GBP and SERP in the cellular and soluble fractions was analysed (Figure 5A). In the presence of RPMI alone, both proteins were predominantly recovered in the cellular fraction (Figure 5A, lanes 1 and 2, and 7 and 8), whereas in the presence of cytosol, either from iRBCs or from non-infected RBCs, a large proportion of the GBP was immunoprecipitated from the soluble fraction (Figure 5A, lanes 3–6). It was consistently found that translocation of GBP into the soluble fraction was more efficient in the presence of cytosol from non-infected RBCs compared with that from iRBCs. To exclude the possibility that components of the cytosolic preparations contained lytic activities resulting in the disintegration of the vacuolar membrane and release of the vacuolar contents, the distribution of SERP was assessed in parallel. In contrast to GBP, SERP was always quantitatively recovered from the parasite fraction (Figure 5A, lanes 9–12).

To investigate whether GBP could be ‘chased’ from its vacuolar location across the vacuolar membrane, iRBCs were permeabilized with SLO and the cytosol was replaced with RPMI containing L-[³⁵S]methionine. GBP was allowed to accumulate in the vacuole for 45 min. Cells were then washed and incubated for a second time in RPMI alone, in RPMI containing cytosol from non-infected RBCs or in RPMI containing RBC cytosol from iRBCs. L-[³⁵S]Methionine was omitted from the second incubation to avoid synthesis of radiolabelled protein during this period. In the presence of RPMI, GBP remained in the parasite fraction. In contrast, it was translocated in the presence of cytosol obtained from either iRBCs or non-infected RBCs (Figure 5B, lanes 1–6). Again, translocation was slightly more efficient with cytosol from non-infected RBCs and therefore, for subsequent experiments, cytosolic preparations from non-infected RBCs were used.

To assess whether a large pore is formed during the chase, allowing an unselective import of proteins into the vacuole, permeabilized iRBCs were pulse-labelled in the presence of RPMI, and subsequently incubated with RBC cytosol containing either trypsin alone or trypsin plus saponin. The susceptibility of SERP to the proteinase was analysed (Figure 5C). In the absence of the detergent, SERP was protected from digestion with trypsin. Thus externally added proteins do not gain access to the vacuolar space during ongoing translocation.

In a kinetic analysis, metabolically labelled GBP was first accumulated in the vacuolar space by incubating SLO-permeabilized iRBCs in the presence of RPMI. After 45 min the medium was replaced with RBC cytosol without L-[³⁵S]methionine, and the distribution of GBP in the soluble fraction and in the parasite fraction was determined at several time points. The protein became detectable in the soluble fraction within 10 min after addition of the cytosol, and reached a plateau concentration in this fraction after 30 min (Figure 6).

The above experiments demonstrated a requirement for one or several components contained in the RBC cytosol for the translocation of GBP. The experiments summarized in Table 1 were carried out (i) to establish the ATP requirement and (ii) to investigate the role of proteins. To deplete the cytosol of

Figure 5 Translocation across the vacuolar membrane can be restored by the addition of RBC cytosol

(A) iRBCs permeabilized with SLO were resuspended in RPMI, or in cytosol from non-infected RBCs (RBCC) or from iRBCs (iRBCC), each containing L-[³⁵S]methionine. The cellular fraction (P) and the fraction containing released proteins (S) were analysed by immunoprecipitation for the distribution of either GBP (lanes 1–6) or SERP (lanes 7–12). (B) iRBCs permeabilized with SLO were resuspended in RPMI and labelled with L-[³⁵S]methionine. Subsequently, the labelling medium was replaced with RPMI, RBCC or iRBCC and incubation was continued. The distribution of GBP in the cellular fraction and in the fraction containing released proteins was determined by immunoprecipitation. (C) iRBCs permeabilized with SLO were metabolically labelled as described for (B). Following the pulse, the labelling medium was replaced with RPMI or RBCC containing 1 mg/ml trypsin (Tryp) or RBCC containing 1 mg/ml trypsin in 0.09% saponin (Sap). After 60 min the trypsin was inactivated, and the cellular fraction was washed and lysed by freezing and thawing. The relative amounts of SERP in the different samples were determined by immunoprecipitation and subsequent electrophoretic analysis.

Figure 6 Kinetic analysis

iRBCs permeabilized with SLO were pulse-labelled in RPMI as described for Figure 5(B). Following the labelling reaction, the labelling mixture was replaced with RPMI (lanes 1 and 2) or with RBC cytosol (RBCC; lanes 3–12). A total of 2×10^7 permeabilized iRBCs and corresponding amounts of the soluble fractions were analysed at various time points for the distribution of GBP.

molecules of < 14 kDa and to hydrolyse ATP, the cytosol was respectively dialysed and treated with apyrase. Translocation was not observed with these preparations. When ATP was added back to dialysed cytosol, at a final concentration of 5 mM, GBP was translocated. Thus ATP is one essential component, but when added to RPMI it was not sufficient to restore translocation. The relevance of a protein component was supported by the observation that boiling of the cytosol resulted in the loss of translocation activity. When haemoglobin was added to RPMI, in combination with ATP, translocation was restored to the same extent as with complete cytosol.

Table 1 Assessment of the ability of various experimental conditions to restore translocation of GBP across the vacuolar membrane

iRBCs were permeabilized with SLO and metabolically labelled in the presence of RPMI. Following the labelling period, permeabilized cells were washed to remove L-[³⁵S]methionine and the incubation was continued under various conditions. The distribution of GBP was determined as described for Figure 5(B). Translocation of GBP was either as efficient as with RBC cytosol (RBCC) used as a positive control (designated 'Yes'), or the efficiency was as low as with RPMI alone (designated 'No').

Incubation mixture	Translocation
RBCC dialysed	No
RBCC/apyrase (5 units/ml)	No
RBCC dialysed/5 mM ATP	Yes
RPMI/5 mM ATP	No
RBCC heated to 90 °C	No
Haemoglobin (1–10 mg/ml)	No
Haemoglobin (1–10 mg/ml)/5 mM ATP	Yes

DISCUSSION

The vacuolar membrane that is formed by *P. falciparum* is a physical and biochemical barrier between the parasite and the RBC cytosol. Nevertheless, specific secretory proteins of the parasite are inserted into this membrane or are even transported beyond it. The characterization of the vacuolar membrane and the identification of transport pathways of lipids and proteins from the parasite into the RBC have predominantly been based on morphological approaches, because conventional methods

resulted in partial or total disruption of the vacuolar membrane. Pore-forming proteins have found widespread application as tools to study protein trafficking in eukaryotic cells because, under appropriate experimental conditions, the plasma membrane can be selectively permeabilized while the membranes of internal compartments remain intact [25,42]. We have adopted this strategy and have used SLO to study protein transport from the intracellular parasite into the cytoplasm of permeabilized iRBCs. Permeabilization of iRBCs with SLO results in the release of the RBC cytosol, as shown by complete segregation of haemoglobin into the medium, and leaves internal compartments, namely the vacuole and the parasite cell, intact. The use of SLO on iRBCs revealed three novel observations: (i) GBP, which was previously thought to be located exclusively in the RBC cytosol, was also recovered from and localized to the vacuolar space, (ii) GBP traversed the vacuolar space, where its transport could be arrested by substituting RBC cytosol with RPMI, and (iii) translocation of GBP across the vacuolar membrane required, at the erythrocytic face, protein and ATP. We anticipate that the use of pore-forming proteins to permeabilize host cells will become a generally applicable approach to study host-parasite interactions of other intracellular pathogens at the interface of the vacuolar membrane.

The vacuolar localization of GBP both by immunoblotting (Figure 1) and by immunofluorescence (Figure 3) of permeabilized iRBCs was unexpected in view of previous morphological data that showed a predominant localization of GBP in the RBC cytoplasm [29–31]. Possibly the vacuolar localization was overlooked due to the intense staining in the RBC cytoplasm. Also, in our experiments the vacuolar staining became evident only when cells were analysed after permeabilization (compare Figures 3A and 3D).

Does the detection of GBP in the vacuolar space simply reflect an alternative localization of the protein, or is the vacuole a transit compartment? When permeabilized iRBCs were labelled in the presence of medium only, GBP was transported to the vacuolar space, as shown by its release from this compartment with saponin. When the medium was then replaced with RBC cytosol in the absence of L-[³⁵S]methionine, i.e. under conditions in which no labelled protein was synthesized, labelled GBP was transported out of the vacuole. Thus transport of GBP occurred via the vacuolar space and could be arrested by substituting the host cell cytosol with medium. These data contradict alternative models which, based on morphological observations, have proposed a direct transfer of proteins from the parasite cytoplasm into the RBC cytoplasm [19,23]. Although the presence of GBP in the vacuolar space may be transient, the steady-state level of the protein in this compartment is high when compared with the half-life of proteins in transit compartments of the secretory pathway, such as the endoplasmic reticulum or the Golgi stacks. The biological significance of this observation remains to be elucidated.

Transport of GBP involved a specific process, as SERP was not exported. The transport process was unidirectional, since (i) when radiolabelled GBP was added externally to permeabilized iRBCs it was not internalized into the vacuole (results not shown), and (ii) trypsin had no access to the vacuole, as demonstrated by the protection of the vacuolar contents in the presence of RBC cytosol. In pulse-chase experiments we never noticed, in a time period of 90 min, a concentration equilibrium of GBP inside and outside the vacuole. This observation precludes the possibility of non-specific diffusion and is consistent with the high steady-state concentration of GBP inside the vacuole as demonstrated morphologically and by immunoblotting of fractionated permeabilized iRBCs. Translocation of GBP across the

vacuolar membrane also occurred when purified haemoglobin in combination with ATP was added to the medium. This finding demonstrates (i) that the contribution of specific mediator proteins at the erythrocytic face of the membrane is unlikely, and (ii) since RPMI represents an extracellular ionic milieu, that translocation is not affected by significant changes in the intra-erythrocytic ionic composition. Mature RBCs do not synthesize or translocate proteins, and during infection the permeability of the RBC membrane for ions changes considerably, thus resulting in an ionic composition of the iRBC cytosol which differs from that in non-infected RBCs. It is therefore conceivable that translocation of proteins across the vacuolar membrane does not require a highly specific and complex support at the erythrocytic face of the membrane. Other examples exist where proteins are translocated across membranes in the absence of soluble proteins at the exit side of the membrane. Translocation of some secretory proteins occurs across microsomal membranes lacking luminal proteins and across proteoliposomes reconstituted purely from phospholipids and endoplasmic membrane proteins [43–45]. On the other hand, when proteins are translocated post-translationally, either across reconstituted proteoliposomes or across mitochondrial membranes, there appears to be a requirement for soluble chaperones at the exit side which are believed to prevent back-translocation of the polypeptide chain [46,47]. Since translocation of parasite proteins across the vacuolar membrane is a post-translational event, our experiments indicate that release of the polypeptide chain from the erythrocytic face of the membrane may involve a mechanism that differs from that reported for other systems. Although it is unlikely, we cannot exclude the presence of a chaperone-like activity in the haemoglobin preparations which were used to replace the host cell cytosol.

The observation that soluble proteins are first secreted into the vacuolar space before they pass through the vacuolar membrane in a second step raises a number of mechanistical questions. Most probably, the protein is released into this vacuole in a folded conformation which renders translocation rather difficult. From studies on the post-translational translocation of precursor proteins across mitochondrial membranes it has become evident that unfolding of most proteins is necessary before insertion into the membrane can occur [48]. In addition, translocation occurs at specific protein sites [49,50]. If translocation of GBP across the vacuolar membrane follows a general principle, one would expect the necessity for a targeting sequence, soluble mediator molecules and a membrane-bound translocation apparatus. Soluble parasite proteins have been characterized in the vacuole of *P. falciparum*-infected RBCs, and integral membrane proteins in the vacuolar membrane have been identified in host cells infected with *Toxoplasma* [17] and *Plasmodium* [51,52], some of which may act as channels, at least for small molecules [53]. Thus the requirements at the insertion site of the polypeptide, i.e. at the vacuolar face of the membrane, may be far more complex than those at the erythrocytic face, and they may involve specific mediator molecules which are secreted from the parasite into the vacuolar space.

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