Brefeldin A inhibits transport of the glycophorin-binding protein from *Plasmodium falciparum* into the host erythrocyte

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Plasmodium falciparum, a protozoan parasite of the human erythrocyte, causes the most severe form of malaria. During its intraerythrocytic development, the parasite synthesizes proteins which are exported into the host cell. The compartments involved in the secretory pathway of *P. falciparum* are still poorly characterized. A Golgi apparatus has not been identified, owing to the lack of specific protein markers and Golgi-specific post-translational modifications in the parasite. The fungal metabolite brefeldin A (BFA) is known to inhibit protein secretion in higher eukaryotes by disrupting the integrity of the Golgi apparatus. We have used the parasite-encoded glycophorin-binding protein (GBP), a soluble protein found in the host cell cytoplasm, as a marker to investigate the effects of BFA on protein secretion in the intracellular parasite. In the presence of BFA, GBP was not transported into the erythrocyte, but remained inside the parasite cell. The effect caused by BFA was reversible, and the protein could be chased into the host cell cytoplasm within 30 min. Transport of GBP from the BFA-sensitive site into the host cell did not require protein synthesis. Similar observations were made when infected erythrocytes were incubated at 15 °C. Incubation at 20 °C resulted in a reduction rather than a complete block of protein export. The relevance of our findings to the identification of compartments involved in protein secretion from the parasite cell is discussed.

INTRODUCTION

Plasmodium falciparum is a unicellular parasite which resides in human erythrocytes. Here it develops from a ring stage to a trophozoite stage and finally undergoes multiple divisions. In the erythrocyte the parasite lies within a parasitophorous vacuole, the membrane of which separates the parasite plasma membrane from the cytosol of the host cell. In the course of its development some parasite proteins are selectively transported from the parasite to the vacuole, the vacuolar membrane and to specific locations within the host cell [for reviews see Howard (1988), Barnwell (1990) and Lingelbach (1993)]. Recent studies towards an understanding of the pathways involved in the transport of proteins from the parasite into the host cell have revealed that many exported parasite proteins contain N-terminal signal sequences which mediate translocation of the respective polypeptides across the endoplasmic reticulum (ER) in heterologous cell-free systems (Feder and Blobel, 1983; Ragge et al., 1990; Günther et al., 1991), suggesting that also in the parasite the entry site into the secretory pathway is at the ER. *P. falciparum*, like other members of the apicomplexa, is a specialized eukaryotic cell. In addition to ubiquitous organelles such as a nucleus, mitochondria and a rough ER, it contains a variety of organelles such as micronemes and rhoptries which bear no obvious morphological resemblance to organelles found in higher eukaryotes (Bannister and Dluzewski, 1990), but it lacks a morphologically distinct Golgi compartment. Thus it is not clear whether proteins are directly transported from the ER to the plasma membrane or whether they pass through a compartment which is functionally equivalent to the Golgi apparatus. Protein modifications that would be specific markers for passage through the Golgi compartment are unknown in the parasite, particularly as *N*-glycosylation of secretory proteins does not occur (Dieckmann-Schuppert et al., 1992).

The antibiotic drug brefeldin A (BFA) and low temperature affect protein secretion in higher eukaryotes by blocking protein transport to the plasma membrane (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Griffiths et al., 1985; Doms et al., 1989; Lippincott-Schwartz et al., 1989). As one approach to the eventual characterization of compartments involved in the secretion of proteins in *P. falciparum*, we have investigated the effects of BFA and low temperature on the transport of proteins from the intracellular parasite into the host erythrocyte. We have used the glycophorin-binding protein (GBP) as a marker. GBP, also called the 96 kDa antigen, is a soluble protein which is synthesized by trophozoite-stage parasites and transported into the host cell cytoplasm (Kochan et al., 1986; Bianco et al., 1987; Bonnefoy et al., 1988; Perkins, 1988).

MATERIALS AND METHODS

Parasite culture and labelling of cells

Parasites, *P. falciparum* isolate FCBR (Columbia), were grown in human erythrocytes under standard conditions (Trager and Jensen, 1976). Trophozoite-infected erythrocytes (IRBCs) were enriched to a parasitaemia of 60–90% IRBC (Pasvol et al., 1978). For metabolic labelling of newly synthesized proteins, enriched IRBCs (10^8 infected cells) were washed twice in methionine-free RPMI 1640 (Gibco) and cultured for 1 h in serum-free methionine-free RPMI, in the presence of 50 μCi/ml L-[^{35}S]methionine (Amersham). For treatment with BFA, cells were preincubated with 5 μg/ml BFA for 15 min before the addition of L-[^{35}S]methionine. For pulse–chase experiments, after metabolic labelling in the presence of BFA, the medium was replaced with complete RPMI containing 50 μg/ml cycloheximide. Portions of cells were removed at several time points and immediately lysed with saponin as described below.

Lysis and fractionation of IRBCs

After metabolic labelling, cells were washed with RPMI, collected at room temperature by centrifugation for 5 min at 800 *g* and the

Abbreviations used: BFA, brefeldin A; ER, endoplasmic reticulum; GBP, glycophorin-binding protein; IRBC, trophozoite-infected erythrocyte.

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supernatant was removed. The volume of the pellet was determined and erythrocytes were lysed for 5 min on ice by adding 1.5 vol. of 0.15% ice-cold saponin (Serva) in RPMI. Subsequently, an equal volume of ice-cold RPMI was added and cells were sedimented at 1300 *g* in an Eppendorf centrifuge for 5 min at 4 °C. The supernatant was collected, the pellet was washed with 50 μl of RPMI and centrifuged as above. The supernatant from the second centrifugation was combined with the supernatant from the first centrifugation. The pellet was resuspended in PBS and subjected to three cycles of freezing and thawing.

Immunoprecipitation of radiolabelled proteins

A rabbit antiserum raised against recombinant GBP (Nolte et al., 1991) was kindly provided by E. Hundt, Behringwerke AG, Marburg, Germany. IRBCs were lysed with saponin and fractionated into pellet and supernatant. Samples corresponding to 1×10^7 IRBCs were adjusted with PBS to a final volume of 25 μl and immunoprecipitation with the antiserum was carried out by a procedure described elsewhere (Ragge et al., 1990).

Immunofluorescence microscopy

Erythrocytes infected with ring-stage parasites (5×10^7 parasites/ml, 3–8 h after invasion) were treated for 24 h under culture conditions, either in the presence of BFA (stock solution 1 mg/ml in ethanol) at a final concentration of 5 μg/ml or at low temperature. Control cells were incubated in the presence of the solvent. Cells were cooled to 4 °C, washed three times in PBS and fixed in 0.1% formaldehyde for 10 min on ice and 30 min at room temperature. The fixative was removed by three washes with PBS and blood films were air-dried. Cells were incubated with 2.5% (w/v) milk powder in PBS for 15 min and for 30 min at 37 °C with an IgG fraction of the rabbit antiserum diluted in 2.5% (w/v) milk powder in PBS. Slides were washed with PBS and incubated for 30 min at 37 °C in a mixture of fluorescein isothiocyanate-conjugated goat anti-(rabbit IgG) (Sigma) diluted 1:200 in PBS and propidium iodite (50 μg/ml) to stain nuclei. After several washes in PBS, slides were mounted in 50% (v/v) glycerol in PBS, containing 0.1% *p*-phenylenediamine as antifading agent. Fluorescence was analysed using a laser confocal microscope (Leitz).

Miscellaneous procedures

Proteins were solubilized in SDS sample buffer and separated on denaturing SDS/polyacrylamide gels (10% polyacrylamide) following standard procedures. Before autoradiography, gels were fixed in 45% methanol/10% acetic acid and treated with Entensify (DuPont). Acid-insoluble incorporation of L-[^{35}S]methionine was determined in comparable samples of the supernatant and the pellet fractions obtained after saponin lysis, following standard procedures (Clemens, 1987).

RESULTS

After lysis of IRBCs with saponin, GBP is recovered in the host cell cytoplasm

Treatment of *P. falciparum*-infected erythrocytes with saponin results in a preferential lysis of the erythrocyte membrane, whereas the plasma membrane of the parasite is less susceptible to the detergent. Thus proteins contained in the host cell cytoplasm can be separated from the parasite by centrifugation (Zuckerman et al., 1967; Siddiqui et al., 1979). As differentiated erythrocytes do not synthesize proteins, parasite-encoded proteins can be readily identified by metabolic labelling. Parasitized erythrocytes were labelled with L-[^{35}S]methionine, lysed with saponin and fractionated by low-speed centrifugation into a supernatant and a pellet (Figure 1). A quantitative comparison of supernatant and pellet showed that most metabolically labelled proteins were recovered in the pellet fraction (Figure 1, lanes 1 and 2), indicating that a large proportion of parasites had remained intact in the presence of saponin. One abundant parasite protein larger than 97.4 kDa was recovered exclusively in the supernatant fraction. Using antibodies raised against GBP (Nolte et al., 1991), this protein was immunoprecipitated from the supernatant (Figure 1, lane 3), and it was not precipitated with a preimmune rabbit serum (Figure 1, lane 4). The size of GBP is in agreement with previously reported sizes which range between 96 and 120 kDa (Perkins, 1988; Bonnefoy et al., 1988). The recovery of GBP in the supernatant after saponin lysis is consistent with reports that GBP is synthesized by the parasite and transported to and located in the host cell cytoplasm (Bianco et al., 1987; Bonnefoy et al., 1988; Perkins, 1988).

Segregation of GBP into the cytoplasm of IRBCs is inhibited by BFA

Infected erythrocytes were treated with BFA and newly synthesized proteins were metabolically labelled with L-[^{35}S]methionine. Cells were subsequently lysed with saponin. Proteins released from erythrocytes by saponin lysis were separated by centrifugation from proteins contained in the parasite, and GBP was immunoprecipitated from the respective fractions (Figure 2). As also observed in higher eukaryotic cells (Misumi et al., 1986), treatment with BFA did not inhibit synthesis of parasite proteins. In the absence of BFA, GBP was recovered in the supernatant, and the protein migrated as a single band (Figure 2, lane 2). In contrast, when IRBCs had been treated with BFA, GBP was exclusively recovered in the low-speed pellet and was not detectable in the supernatant fraction

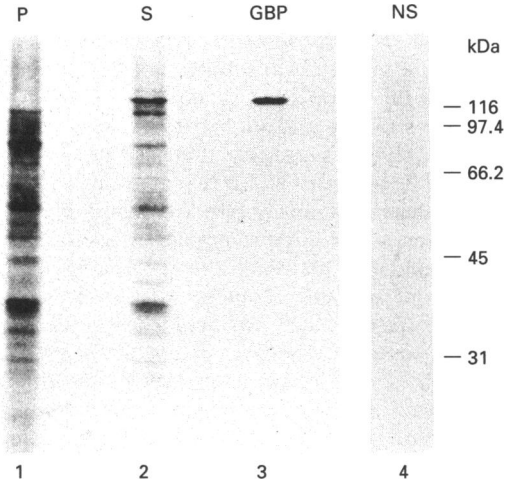

Figure 1 Distribution of GBP after saponin lysis

Infected erythrocytes were labelled metabolically with L-[^{35}S]methionine, lysed with saponin and centrifuged at 1300 *g*. The distribution of radiolabelled proteins in the pellet and the supernatant was analysed by SDS/PAGE and autoradiography. Lanes: 1, proteins contained in the pellet fraction (P); 2, proteins contained in the supernatant (S); 3, GBP precipitated with a specific rabbit antiserum from the supernatant fraction; 4, immunoprecipitation from a total lysate of parasites with a preimmune rabbit serum (NS). The sizes of the respective molecular-mass markers in kDa are indicated on the right.

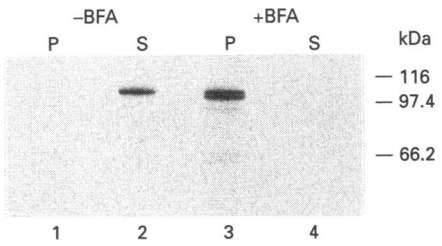

Figure 2 Effect of BFA on the segregation of GBP

Infected erythrocytes were incubated in the presence of L-[^{35}S]methionine and in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of BFA. Cells were lysed with saponin, centrifuged and GBP was immunoprecipitated from the respective fractions. P, pellet; S, supernatant. The sizes of the molecular-mass markers in kDa are indicated on the right.

(Figure 2, lanes 3 and 4). It is noteworthy that the antibody precipitated a doublet band from the pellet fraction. The larger band of the doublet corresponded in size to the product detected in the supernatant fraction from untreated cells. The following experiment was designed to exclude the formal possibility that segregation of GBP into the pellet fraction was due to its insolubility in the presence of BFA. After metabolic labelling for 1 h, IRBCs were incubated with BFA for 30 min in the absence of L-[^{35}S]methionine. Subsequently, cells were treated with saponin and immunoprecipitation was carried out on the respective fractions. Under these conditions, GBP was exclusively recovered in the supernatant (Figure 3), demonstrating that BFA acts while the protein is being transported.

Effect of BFA on the distribution of GBP is reversible

Infected erythrocytes were labelled metabolically in the presence of BFA. Subsequently the medium containing L-[^{35}S]methionine and BFA was replaced by medium without L-[^{35}S]methionine and BFA, but containing cycloheximide to inhibit further protein synthesis. At several time points, 5×10^7 infected cells were withdrawn and lysed with saponin. Portions of the supernatant fractions and of the low-speed pellets, each corresponding to 1×10^6 IRBCs, were analysed for acid-insoluble incorporation of L-[^{35}S]methionine. From each fraction, a portion, corresponding to 1×10^7 IRBCs, was analysed by immunoprecipitation for the presence of GBP (Figure 4). Immediately after BFA treatment, GBP was almost exclusively recovered in the low-speed pellet (Figure 4, lanes 1 and 2). Over a period of up to 30 min, the proportion of GBP in the supernatant increased, and after 30 min no significant increase was noticeable (Figure 4, lanes 3–8). As noticed above, two protein bands of minor size difference were precipitated from the pellet fractions, and GBP recovered from the supernatant migrated as a single band corresponding in size to the larger protein that was found in the pellet. The appearance of GBP in the supernatant is independent of ongoing protein synthesis, as cycloheximide led to a complete block of protein synthesis in the parasite (results not shown).

In order to exclude the possibility that recovery of GBP from the supernatant was the result of progressive lysis of parasite cells during the time course, the distribution of L-[^{35}S]methionine-labelled proteins in the pellet fraction and in the supernatant was monitored. The relative distribution of radiolabelled proteins did not change throughout the chase period showing that the parasite had remained intact inside the host erythrocyte. Thus export of GBP after removal of BFA reflects a selective process.

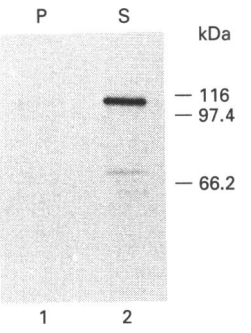

Figure 3 Radiolabelled GBP is recovered in the supernatant of saponin-lysed infected erythrocytes when BFA is added after transport into the host cell cytoplasm

Cells were labelled metabolically with L-[^{35}S]methionine for 1 h and subsequently incubated with BFA, but without L-[^{35}S]methionine, for 30 min. Cells were lysed with saponin, centrifuged and GBP was immunoprecipitated from the pellet fraction (P, lane 1) and from the supernatant (S, lane 2). The sizes of the molecular-mass markers are indicated on the right.

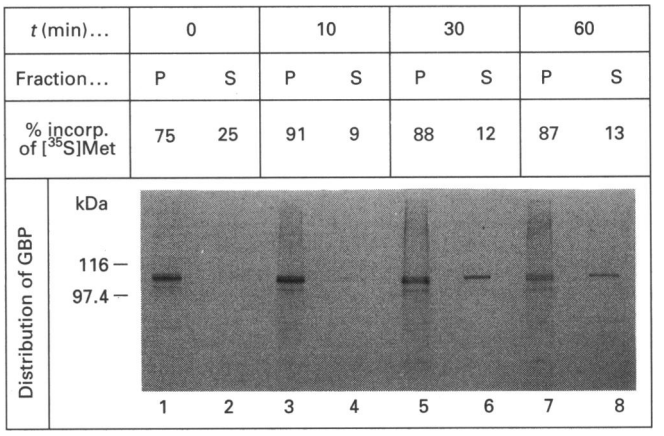

Figure 4 Effect of BFA is reversible

IRBCs were incubated in the presence of L-[^{35}S]methionine and BFA for 1 h. Subsequently, cells were washed and incubated without BFA in the presence of cycloheximide. Cells were harvested at several time points (*t*), lysed with saponin and the relative distribution of acid-insoluble L-[^{35}S]methionine was determined in the pellet fraction (P) and in the supernatant (S) of cells harvested and lysed at each time point. In parallel, GBP was immunoprecipitated from each fraction, separated by SDS/PAGE and visualized by autoradiography.

Segregation of GBP into the cytoplasm of IRBCs is temperature dependent

In mammalian cells, transport from the ER to the Golgi can be blocked by incubation of the cells at 15 °C, and in some mammalian cells transport from the *trans*-Golgi to the plasma membrane is inhibited at 20 °C (Griffiths et al., 1985). Therefore we investigated the effects of these temperatures on the appearance of GBP in the cytoplasm of the IRBCs (Figure 5). When IRBCs were labelled at 15 °C for 1 h, GBP was exclusively recovered in the low-speed pellet, migrating as a doublet band (Figure 5a, lanes 1 and 2). At 20 °C GBP was found in the low-speed pellet and in the supernatant (Figure 5a, lanes 3 and 4), and at 37 °C GBP was most abundant in the supernatant fraction (Figure 5a, lanes 5 and 6). We attribute the distribution of protein at 20 °C to a general slowing down of protein transport at low temperature rather than to a tight block at the exit site of the Golgi. This view

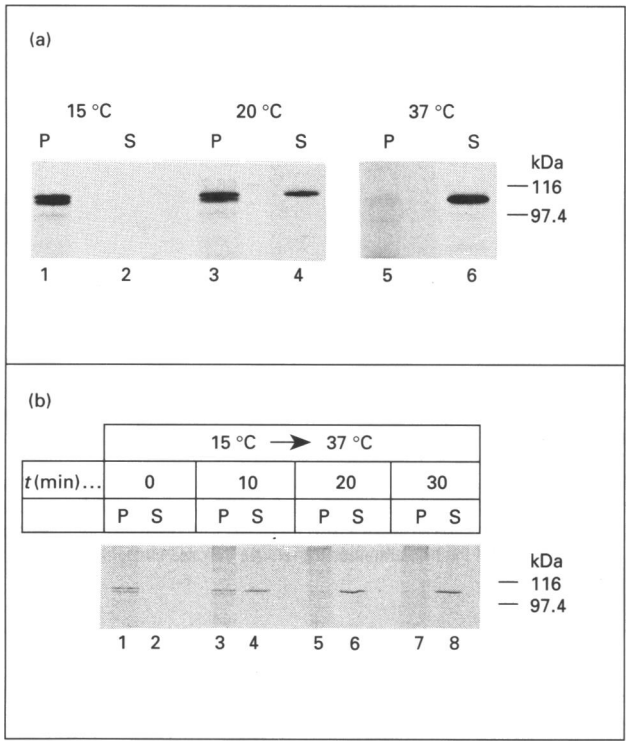

Figure 5 Effects of temperature on the distribution of GBP

(**a**) IRBCs were labelled metabolically for 1 h at 15 °C, 20 °C and 37 °C, subsequently lysed with saponin and separated into a fraction containing parasites (P) and a soluble fraction (S). GBP was immunoprecipitated from the respective fractions, separated by SDS/PAGE and visualized by autoradiography. (**b**) IRBCs were labelled at 15 °C for 1 h. The temperature was shifted to 37 °C, and the medium containing L-[^{35}S]methionine was replaced by medium containing no methionine but cycloheximide. Cells were sampled after different time points (*t*), lysed with saponin and the fractions were assayed for the presence of GBP.

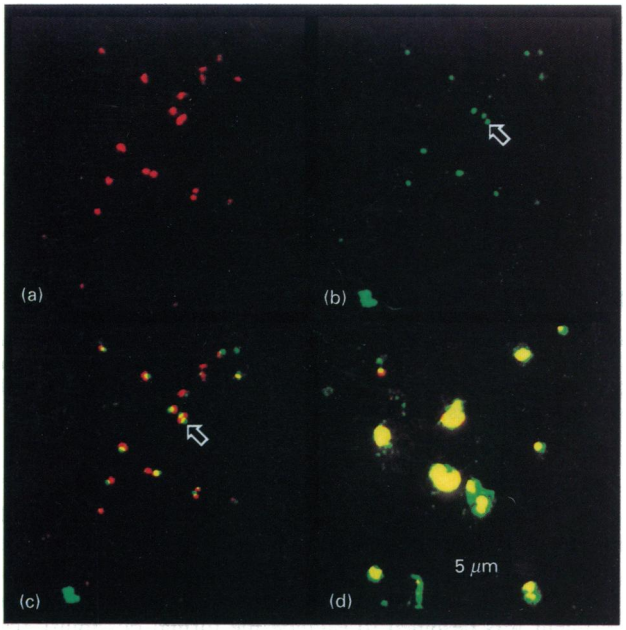

Figure 6 Intracellular distribution of GBP in the presence of BFA

Ring-infected erythrocytes were cultured in the presence (**a–c**) or absence (**d**) of BFA, fixed and incubated with antibodies to GBP, followed by a mixture of fluorescein isothiocyanate-conjugated secondary antibody and propidium iodite. The same field was analysed for staining of the nuclei (**a**) or the presence of GBP (**b**). (**c**) Superimposition of (**a**) and (**b**). (**d**) Superimposition of nuclear and protein staining in control cells. The arrows highlight the location of GBP.

was supported by the observation that, on removal of L-[^{35}S]methionine, all of the labelled protein could be chased into the supernatant fraction at 20 °C within 3 h (results not shown). In contrast, at 15 °C the protein remained associated with the parasite fraction.

The tight block induced by lowering the temperature to 15 °C was reversible. When cells were shifted from 15 °C to 37 °C and further protein synthesis was inhibited with cycloheximide, almost all radiolabelled GBP could be recovered in the supernatant fraction within 20 min of the chase (Figure 5b). Thus protein export from the parasite cell was restored somewhat faster after a temperature-induced block than after a block induced by BFA.

In the presence of BFA, GBP is retained in one discrete location in proximity of the parasite nucleus

The synthesis of GBP is developmentally regulated and begins approx. 16 h after invasion (Perkins, 1988). In order to determine the intracellular location at which transport of GBP is arrested, IRBCs were treated with BFA 6–8 h after invasion, and, after 24 h, the distribution of GBP was determined by immunofluorescence. At the onset of BFA treatment, no GBP was detectable (results not shown). After 24 h, immunofluorescence analysis showed labelling of one discrete region within each parasite cell (Figure 6b). Superimposition of fluorescence patterns

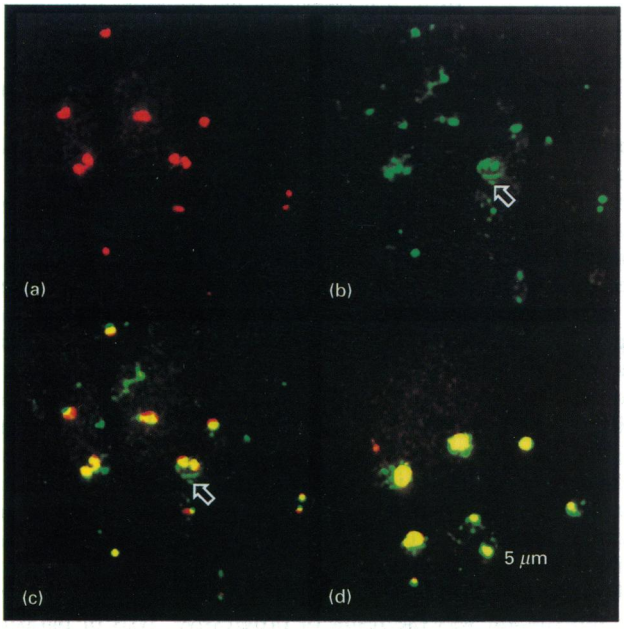

Figure 7 Intracellular distribution of GBP after incubation at 20 °C

Ring-infected erythrocytes were cultured at 20 °C and subsequently treated as described in Figure 6. (**a**) Staining of nuclei; (**b**) staining of GBP in cells grown at 20 °C; (**c**) superimposition of (**a**) and (**b**); (**d**) superimposition of nuclear and protein staining in cells cultured at 37 °C. The arrows highlight the localization of GBP in the host cell cytoplasm.

obtained by double labelling with antibodies directed against GBP and propidium iodite for the staining of nuclei (Figure 6a) revealed that the site of GBP accumulation is in direct proximity to the nucleus (arrow, Figure 6c). In control cells, the fluorescence pattern was found to be more disperse (Figure 6d), including staining in the cytoplasm of the erythrocyte. In the presence of BFA, ring-stage parasites remain viable for a prolonged period but their development to trophozoite stages is retarded (Crary and Haldar, 1992; J. Benting and K. Lingelbach, unpublished work). The retardation in growth explains the low expression of GBP in the presence of BFA compared with control cells. When IRBCs were grown at 15 °C, the intracellular distribution of GBP was similar to that observed after treatment with BFA (not shown). At 20 °C immunofluorescence showed GBP in a discrete location in the vicinity of the parasite nucleus and also in the cytoplasm of the erythrocyte (Figures 7b–7c), a finding that is in agreement with the results obtained by fractionation of IRBCs (Figure 5a).

DISCUSSION

One approach to the understanding of the mechanisms involved in the secretory pathway of eukaryotic cells has been the use of inhibitors that affect protein secretion. The drug BFA disrupts the Golgi and proteins are redistributed to the ER, possibly by a retrograde transport mechanism (Lippincott-Schwartz et al., 1990). Proteins of the *trans*-Golgi network are not returned to the ER, but nevertheless the morphology of this compartment is modified (Chege and Pfeffer, 1990; Lippincott-Schwartz et al., 1991; Wood et al., 1991; Reaves and Banting, 1992). At a molecular level, the drug seems to interfere with ADP-ribosylation factors (Donaldson et al., 1992; Helms and Rothman, 1992; Randazzo et al., 1993; Tsai et al., 1993) resulting in disassembly of non-clathrin coat proteins (Donaldson et al., 1990; Orci et al., 1991).

It has been established morphologically that *P. falciparum* contains a rough ER, and it has been shown experimentally that exported parasite proteins contain signal sequences which translocate the respective proteins across the ER membrane, at least in heterologous systems. It is unknown, however, whether secretion of these proteins proceeds via a Golgi compartment because a morphologically distinct Golgi has not been identified and Golgi-specific protein modifications are unknown in the parasite. When IRBCs were incubated with a fluorescent truncated ceramide, the ceramide was modified to sphingomyelin, suggesting that the parasite expresses sphingomyelin synthase (Haldar et al., 1991; Elmendorf and Haldar, 1993a), an enzyme found in the Golgi of higher eukaryotes (Futerman et al., 1990; Jeckel et al., 1990). Therefore inhibitors of protein secretion are currently being employed to characterize a putative Golgi compartment in the parasite and to assess its role in protein transport into the host cell. When *Plasmodium* parasites were mechanically released from the erythrocyte and incubated in the presence of BFA, secretion of the majority of exported proteins was inhibited by the drug. Some proteins, however, appeared to be secreted despite the presence of BFA (Elmendorf et al., 1992). These studies were carried out on isolated parasites which had been separated from the host cell. They were based on analyses of protein patterns on polyacrylamide gels and did not follow the transport of individual proteins known to be secreted into the cytoplasm of the intact IRBCs. In the present study, the effect of BFA on the export of a specific marker protein from the parasite into the cytoplasm of the intact erythrocyte was analysed. We chose GBP because it is synthesized by the parasite in large quantities and is exported as a soluble protein into the erythrocyte cytoplasm. Thus it could be readily detected in the supernatant of saponin-lysed IRBCs. BFA reversibly arrests export of newly synthesized GBP into the IRBCs. This effect must occur shortly after protein synthesis because GBP can be chased into the host cell cytoplasm in the presence of the translation inhibitor cycloheximide. In our experiments we noticed co-purification of a more rapidly migrating band whenever GBP was precipitated from the parasite pellet. It is unlikely that this band represents a parasite protein that cross-reacts with the antibody, because it was not precipitated when GBP was not retained in the parasite. Thus it could represent a precursor form of GBP which accumulates in the presence of the drug or it is the result of protein degradation within the parasite. In that case, the cleaved form of GBP would not be transported into the host cell cytoplasm. Alternatively, GBP might be associated with a different unrelated protein inside the parasite cell.

Export of GBP was inhibited at 15 °C, but interestingly, in contrast with some mammalian cells, incubation at 20 °C did not arrest protein secretion from the parasite. The immunolocalization studies, although carried out on different developmental stages of the parasite, correlate with the data obtained by metabolic protein labelling and subsequent fractionation. The discrete fluorescence observed inside the parasite in the presence of BFA and at low temperature suggests that the protein is contained within an intracellular compartment. However, the small size of the parasite and the limited resolution of the fluorescence microscopy do not allow an unequivocal identification of this compartment. The interpretation of our morphological observations is supported by a recent report that BFA affects the intracellular distribution of PfERD2, the parasite homologue of the XDEL receptor which is a marker protein of the *cis*-Golgi in mammalian cells (Elmendorf and Haldar, 1993b).

Although our results suggest that GBP is routed via the parasite ER, it is noteworthy that GBP belongs to a group of exported parasite proteins which, unlike other secreted parasite proteins and most secretory proteins of higher eukaryotes, contain no typical N-terminal signal sequence (Lingelbach, 1993). The open reading frame of the GBP gene encodes a single hydrophobic region which is preceded by 50 amino acids (Kochan et al., 1986). Unlike ovalbumin, a secretory protein with an internal signal sequence (Lingappa et al., 1979; Braell and Lodish, 1982; Meek et al., 1982), GBP is not translocated across the ER membrane in a heterologous system containing canine pancreatic microsomes (K. Lingelbach, unpublished work). Therefore an alternative mechanism for the translocation of GBP across the ER membrane might be operating in the parasite as discussed recently (Lingelbach, 1993). The unambiguous effects of BFA and low temperature on the secretion of GBP qualify this protein as a marker to continue the characterization of the secretory pathway in the parasite at the biochemical and ultrastructural level.

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