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Plasmodium falciparum: Inhibition of protein export from the parasite into the host erythrocyte coincides with retardation of parasite development

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Introduction

Plasmodium falciparum invades human red blood cells (RBC) and thus selects a host cell which is devoid of intracellular organelles, genetic information and cellular machineries capable of synthesizing proteins or lipids. Despite the limited biosynthetic activities of the RBC many morphological and physiological alterations of the host cell become apparent after infection by the parasite. These include the formation of knob-like protrusions at the RBC plasma membrane (RBCM), an increased permeability of the RBCM for certain molecules and the ability of infected RBC (IRBC) to adhere to specific receptors present on endothelial cell (cytoadherence). In the RBC cytoplasm (RBCC) new structures of various shapes and sizes, also called network of tubulovesicular membranes (TVM) are formed (8, 16). The parasite itself resides within a parasitophorous vacuole (PV), the membrane of which (PVM) separates the parasite plasma membrane from the RBCC. A biologically conceivable hypothesis would predict that many of the parasite induced alterations are essential for parasite survival. Since the erythrocyte can contribute very little, it is generally believed that the observed alterations are induced by proteins and lipids which are synthesized by the parasite in order to transform the host cell into an environment which supports parasite development. Indeed, many proteins have been characterized which are encoded by *P. falciparum* and which are exported into the IRBC (1, 20). Here they are delivered to specific locations, such as the RBCM or the TVM, or they remain soluble in the RBCC. Since some parasite proteins are restricted to the PV (7, 18), either retention of these proteins within the PV or transport of other proteins across the PVM is a selective process (22).

We have been interested primarily in two questions: (i) what are the transport pathways of proteins which are synthesized in the parasite cytosol and destined for specific locations within the IRBC? (ii) Is the presence of parasite proteins in specific locations within the host cell essential for parasite survival? In the course of our studies we have employed several marker proteins to establish a simple procedure which allowed separation of the RBCC from intact parasites and fractionation of released parasites into their soluble contents and parasite membranes. We have used this assay to devise an experimental approach for the inhibition of protein export from the parasite cell. In the following report we summarize the results of these experiments and provide preliminary data describing the effects on parasite development when protein export is inhibited.

Materials and methods

Parasites

P. falciparum (isolate FCBR) was maintained in continuous culture in an atmosphere of 5% O_2/5% CO_2/90% N_2, using human erythrocytes of blood group A^+ as host cells and RPMI medium completed with 10% human A^+ serum (complete medium). Parasites were synchronized weekly following the sorbitol method (21).

Antisera

A mouse monoclonal antibody to human band 3 protein was purchased from Sigma. Monoclonal antibody McAb 7.7 was first described by HALL et al. (14), and it was a kind gift of J. McBride, Edinburgh. It recognizes the exported protein-2 (exp-2) of *P. falciparum* (17). Rabbit polyclonal antisera specific for parasite aldolase and the parasite glycophorin binding protein (GBP), respectively, have been described previously (19, 25) and were kindly provided by E. Hundt, Behringwerke AG, Marburg, FRG. Alkaline phosphatase conjugated second antibodies to mouse IgG and rabbit IgG, respectively, were purchased from Promega.

SDS-Polyacrylamide Gel-electrophoresis (SDS-PAGE) and Western Blotting

Proteins were separated on Laemmli-type SDS-polyacrylamide gels, containing either 7.5% or 12% polyacrylamide, and transferred onto nitrocellulose filters using standard conditions. The filters were processed, incubated with first antibodies (1 : 1000 dilution of the monoclonal antibodies; 1 : 500 dilution of the antiserum against aldolase; 1 : 250 dilution of the antiserum against GBP) and immunostained with alkaline phosphatase-conjugated second antibodies using the Promega ProtoBlot system.

Subfractionation of IRBC

IRBC infected with trophozoite and schizont stage parasites were enriched by the plasmagel procedure (26) to parasitaemias ranging between 60 - 90% IRBC. Routinely, 10^9 IRBC were processed as follows. The pellet containing enriched IRBC was washed twice with RPMI and finally sedimented for 5 min at 395 × g. Cells were lysed with saponin (Serva) following a procedure described elsewhere (3) and centrifuged at 1,300 × g for 5 min at 4° C. The supernatant was removed and stored frozen (-80° C). The pellet was subjected to three cycles of freezing and thawing. Approximately 20 volumes of ice cold high salt buffer (50 mM Hepes pH 7.5/ 0.5 M KCl/5 mM DTT//50 mM lysine/3 mM $MgCl_2$) were added and samples were centrifuged for 30 min at 15,800 × g (12). The supernatant was collected, proteins were precipitated with 10% trichloroacetic acid and solubilized in SDS sample buffer. The pellet was incubated with 50 µl of 0.1 M Na_2CO_3, pH 11.5 for 15 min on ice. This solution was centrifuged through a 150 µl cushion containing 0.1 M Na_2CO_3/0.25 M sucrose, in a Beckman Airfuge at 100,000 × g for 10 min. Proteins of the supernatant were precipitated and solubilized as described above. The pellet was solubilized with SDS sample buffer without prior treatment.

Assay for effect of BFA on parasite development in vitro

IRBC were synchronized and diluted to a starting parasitaemia of approximately 2% ring stage parasites. Brefeldin A (BFA, Sigma) was added to complete medium for 24 h, at a final concentration of 5 µg/ml. IRBC were washed in complete medium and cultured in the absence of BFA. For the following 48 h blood films were prepared at several time points, stained with Giemsa and examined microscopically. To avoid experimental bias, slides were prepared in triplicates and coded. On each slide, parasitaemias were determined by counting the percentage of IRBC in 1,000 erythrocytes. In additional experiments parasites were first grown to trophozoite stages, characterized as mononuclear parasites containing visible hemozoin crystals, and then treated and processed as described above.

Results

Selection of marker proteins

In order to monitor the fractionation of IRBC, marker proteins were selected on the basis of the following criteria: (i) unequivocal localization of the individual proteins within the IRBC, (ii) expression of all proteins during the same developmental stages of the parasite, (iii) high abundance of each protein and (iv) the availability of specific antibodies. The proteins summarized in table 1 fulfill all of these criteria.

Marker proteins can be recovered in different subcellular fractions

IRBC were fractionated as described in materials and methods, and proteins contained in different fractions obtained from the same preparation were separated by SDS-PAGE, transferred onto nitrocellulose filters and assayed for the presence of marker proteins using specific antibodies (fig. 1). The size differences of the various marker proteins required electrophoresis through different concentrations of acrylamide; 7.5% acrylamide for band 3 and GBP, and 12% acrylamide for aldolase and exp-2. In initial experiments the sensitivity of the immunoblotting assay had to be determined for each combination of antibody and antigen. Stand-

Table 1:

Marker proteins used to monitor fractionation of IRBC.
Abbreviations: Exp-2, exported protein-2; GBP, glycophorin binding protein; RBCM, red blood cell membrane; PVM, parasitophorous vacuole membrane; TVM, tubulovesicular membranes; RBCC, red blood cell cytoplasm.

Marker Proteins	Molecular Mass	Location	Reference
Band 3	approx. 100 kDa	RBCM	11
Exp-2	35 kDa	PVM, TVM	17
GBP	96-130 kDa	RBCC	4, 5, 27
Aldolase	47 kDA	Parasite cytosol	19

ardized numbers of total IRBC (5×10^6 IRBC for band 3 and GBP; 1×10^6 IRBC for aldolase; 2×10^6 IRBC for exp-2) and equivalent amounts of the subcellular fractions were used to analyse the separation of the individual marker proteins. As expected, only band 3 was present in a total lysate of noninfected RBC (fig. 1, lane 1). GBP, aldolase and exp-2 were present in lysates of IRBC only (fig. 1, cf. lanes 1-2). Using appropriate experimental conditions, saponin preferentially lyses the RBCM and leaves the parasite plasma membrane intact (31). Consequently, GBP was released into the supernatant (fig. 1, lane 3) and aldolase, the marker of the parasite cytosol sedimented into the pellet (fig. 1, cf. lanes 3-4). Likewise, exp-2 which is associated with parasite derived membranes segregated with the intact parasite cells (fig. 1, lane 4). Band 3, the marker of the RBCM, was recovered predominantly with the soluble fraction (fig. 1, lane 3). The fraction containing intact parasites was subjected to freezing and thawing and consecutive extractions with high salt buffer and at high pH. The cytosol of the parasite was released as indicated by the segregation of aldolase into the fraction containing soluble proteins (fig. 1, lane 5). Exp-2, as described previously (17), remained associated with the membrane fraction and was recovered in the pellet after the final centrifugation (fig. 1, lane 7).

We also studied the segregation of the contents of the PV, using the serine rich protein (SERP) as a marker (7,18). After saponin lysis, SERP was always found in almost equal amounts in the fraction containing the RBCC and the fraction of released parasites (data not shown), suggesting that the PVM was partially disrupted.

BFA and low temperature (15° C) inhibit transport of GBP into the RBCC

The subfractionation procedure was the experimental basis of a recent study in which we investigated the effects of the fungal metabolite BFA and of low temperature (15° C) on the transport of GBP (3). BFA blocks secretion of proteins in most eukaryotic cells studied so far by interfering with the anterograde protein transport within the Golgi apparatus. In the presence of BFA, secretory proteins are redistributed to the endoplasmic reticulum (ER) (23). Incubation at 15° C inhibits transport of proteins from the ER to the Golgi apparatus (24). In trophozoite infected erythrocytes we found that, in the presence of BFA as well as at 15° C, newly synthesized GBP accumulated inside the parasite cell. This effect was reversible, if standard culture conditions were restored within 1 - 2 h (3).

Effect of BFA on the development of asexual stage parasites in vitro

The unequivocal effects on the transport of GBP and similar studies carried out by others (6) indicated that also transport of other proteins, which are synthesized by the parasite and destined for export into the IRBC, is inhibited by BFA. Protein synthesis was not affected by BFA and protein secretion was rapidly restored after removal of the drug (3). We currently believe that in the parasite, as in other eukaryotic cells, BFA predominantly affects the secretory pathway. This concept has prompted us to start a preliminary investigation into the longterm effects of BFA on parasite development, which is summarized in fig. 2. Ring stage parasites were cultured in the presence and in the absence of BFA for 24 h. Parasite development after removal of the drug was monitored microscopically. As observed previously (6), the presence of BFA blocked development of ring stage parasites to trophozoites (fig. 2, A & B). After removal of BFA, ring stage parasites developed to trophozoites. Subsequent development from trophozoites to schizonts and reinvasion were not affected, even when ring stage parasites had been treated with BFA for up to 48 hrs. In contrast, when trophozoites were cultured in the presence of BFA for more than 4 hrs, an increasing number of morphologically abnormal trophozoites were observed, and the rate of reinvasion decreased accordingly. We are currently

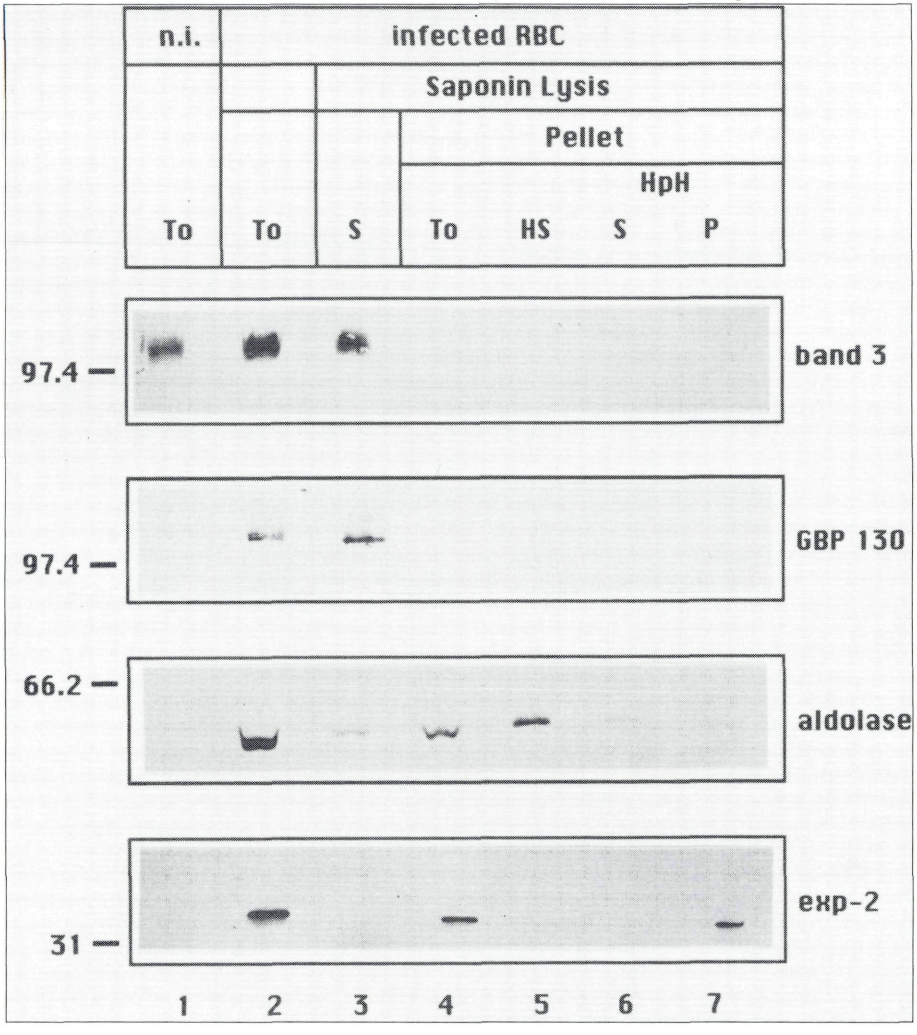

Figure 1:

Distribution of marker proteins in subcellular fractions. IRBC were lysed with saponin and centrifuged at 1,300 × g. The pellet was subjected to three cycles of freezing and thawing and was subsequently extracted consecutively with high salt buffer and at high pH. Subcellular fractions were separated by SDS-PAGE and subjected to Western-Blot analysis.
Lanes: 1, total lysate (To) of noninfected (n. i.) RBC; 2, total lysate of IRBC; 3, supernatant (S) after saponin lysis of IRBC; 4, pellet (P) after saponin lysis; 5, soluble fraction after extraction of lysed parasites with high salt buffer (HS); 6, soluble fraction after extraction at high pH (HpH); 7, membrane fraction after extraction at high pH.

pursuing an extensive and detailed study to assess the kinetics of the effects of BFA on parasite development, and to assay in parallel for general parameters of cell viability.

Discussion

In contrast to noninfected RBC, IRBC are compartmentalized as a result of the biosynthetic activities of the parasite. In the course of parasite development, IRBC gain new biochemical and physiological properties. Since attempts to stably transform *P. falciparum* have been unsuccessful it has not been possible to establish the contribution of individual exported parasite proteins to the biochemical alterations of the host cell nor to assess their significance for parasite survival. Therefore we have begun several studies to evaluate the significance of proteins exported from the parasite into the IRBC for parasite survival and development. The first requirement was the subfractionation of IRBC to monitor transport of soluble proteins. Secondly, we established the effects of known transport inhibitors on protein secretion in the parasite and, thirdly, we analysed the effects of transport inhibition on parasite development in vitro.

In the past, several efficient methods, usually including a gradient centrifugation, have been described for the separation of IRBC into various components (15). Most of these studies were carried out with *Plasmodium* parasites of rodents and they were essentially aimed at the isolation of the host cell membrane, devoid of contaminating membranes of the parasite (29, 30). We sought to establish a fast and convenient method to separate the parasite cytosol and the RBCC in order to analyse proteins contained in the two different fractions. To assess efficient separation, we use two soluble markers, aldolase and GBP, respectively. The procedure does not require centrifugation through a gradient and therefore the soluble components remain concentrated for subsequent analyses. This method is suitable if separation of the vacuolar contents is not necessary because, as already shown ultrastructurally for other *Plasmodium* species (29), the PVM is partially disrupted by saponin treatment. We have used this protocol successfully to study the appearance of parasite encoded soluble proteins in the RBCC (3) and for the identification of a novel parasite encoded membrane protein (17).

The segregation of band 3 in our experiments differs from the observation of BEAUMELLE et al. (2) who found that, employing lower concentrations of saponin (0.045%), the RBCM marker acetylcholine esterase was quantitatively associated with the parasite fraction. Although in some of our experiments, small proportions of band 3 were occasionally detected

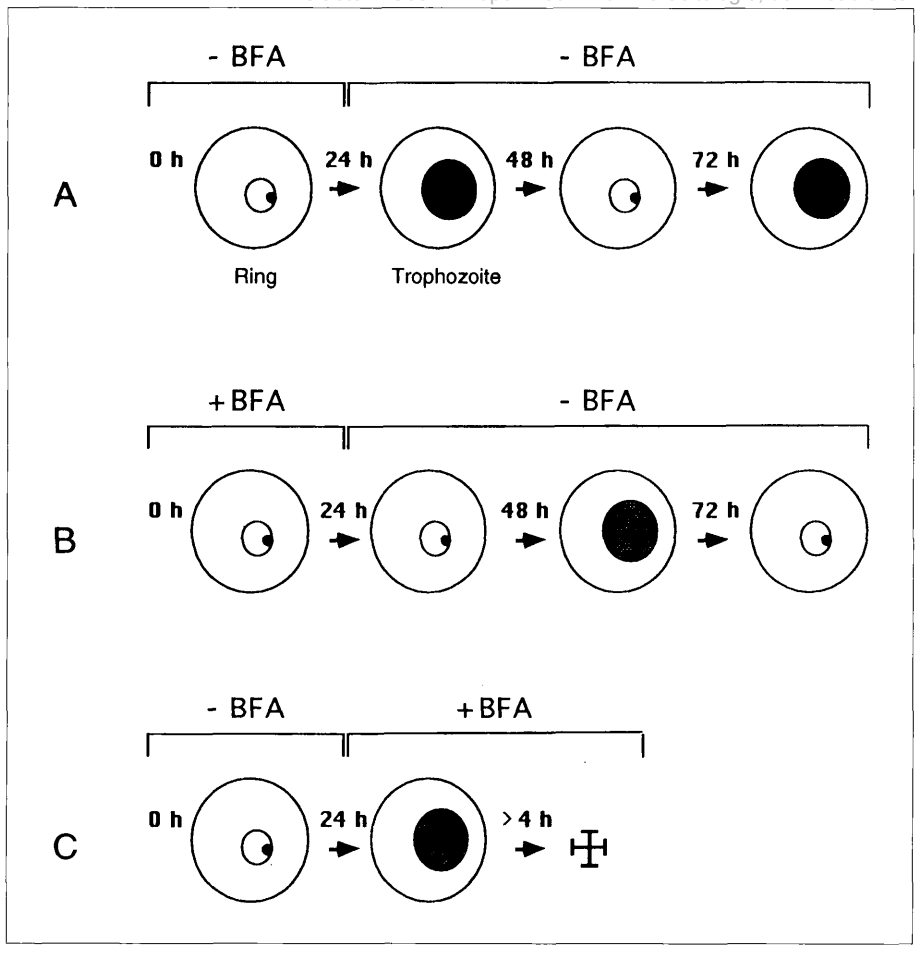

Figure 2:

Effects of BFA on different developmental stages of the parasite.

A: Parasite development in the absence of BFA.

B: Parasite development after 24 hrs treatment of ring stage parasites with BFA.

C: Parasite development after treatment of trophozoites with BFA for more than 4 hrs.

in the parasite pellet, we found that the majority of band 3 always segregated into the supernatant. We attribute the segregation of band 3 in our experiments (final concentration of saponin: 0.09%) to the formation of small vesicles derived from the plasma membrane of IRBC which did not sediment at 1,300 × g. In fact, band 3 could be sedimented in a subsequent ultracentrifugation step, and it was not solubilized by high pH, suggesting that it was membrane bound (data not shown).

Low temperature and BFA are equally effective in inhibiting protein transport from the parasite into the RBCC. It has been established morphologically by many investigators that the parasite contains an ER, and biochemical studies have shown that exported parasite proteins contain N-terminal signal sequences which direct the respective proteins into the lumen of the ER (12, 28). The subsequent steps in the parasite's secretory pathway are less clear. A morphological equivalent of a Golgi apparatus has not been demonstrated unequivocally in erythrocytic stages of the parasite. Recently the parasite homologue of the ERD 2 protein and sphingomyelin synthase activity, both markers of the Golgi apparatus in other eukaryotes, have been detected in IRBC (9, 10, 13). The existence of these proteins suggests a Golgi compartment in the parasite. We are currently investigating at which step in the parasite's secretory pathway transport of GBP is blocked at 15° C and by BFA, respectively.

Following merozoite invasion of RBC the intraerythrocytic development of *P. falciparum* can be divided into three morphologically distinct stages; the ring (0 - 24 h p. i.), the trophozoite (24 - 36 h p. i.) and the schizont (36 - 48 h p. i.). As compared to ring stage parasites, the trophozoite is metabolically more active and therefore presumably more sensitive to the inhibition of cellular functions. The effect of BFA on ring stage parasites indicates that the drug has no longterm and irreversibly toxic effect on this developmental stage. In contrast, the viability of trophozoites is affected by BFA within a comparatively short time of treatment. The most prominent effect of BFA in eukaryotic cells is an interference with protein secretion. We cannot rule out the formal possibility that BFA affects, in addition, other essential functions in trophozoites but not in ring stages of *P. falciparum*. However, it is a more likely explanation that the viability of trophozoites, unlike that of ring stage parasites, depends on a continuous transport of parasite proteins from the parasite cytosol into the host cell.

The ability to efficiently inhibit protein secretion from the parasite may eventually help to assign specific physiological alterations of the IRBC, e. g. cytoadherence or the increased permeability of the RBCM for a wide range of molecules, to a biological mechanism which includes transport of functionally essential parasite proteins to specific locations within the IRBC.

Summary *Plasmodium falciparum* invades human erythrocytes which do not contain intracellular organelles or the biochemical machineries necessary for the synthesis of proteins or lipids. Following infection, a number of morphological and physiological alterations of the erythrocyte, presumably induced by the parasite, become apparent. These alterations coincide with the transport of proteins from the parasite to specific locations within the host cell. We are interested in the mechanisms underlying the transport of these proteins to their final destinations and in their significance for parasite survival. Using specific marker proteins as indicators, we describe a simple method for the release of intact parasites from infected erythrocytes, and the separation of these parasites into two fractions, one containing soluble proteins and the other one containing membrane proteins. The effects of brefeldin A and low temperature (15° C) on protein export from the parasite cell and on parasite development are reported.

Key words Brefeldin A, *Plasmodium falciparum,* protein transport, secretion, subfractionation.

Zusammenfassung

Plasmodium falciparum: Die Hemmung der Sekretion von Parasitenproteinen in den Erythrozyten korreliert mit einer Hemmung der Entwicklung des Parasiten

Plasmodium falciparum infiziert humane Erythrozyten, die selbst über keine intrazellulären Organellen oder Syntheseapparate zur Bildung von Proteinen oder Lipiden verfügen. Nach der Infektion entstehen eine Reihe morphologischer und physiologischer Veränderungen des Erythrozyten, die vermutlich durch die Parasitenzelle verursacht werden. In diesem Zusammenhang ist auffallend, daß der Parasit viele Proteine synthetisiert, die an unterschiedlichen, spezifischen Orten der Wirtszelle lokalisiert sind. Wir untersuchen die Mechanismen, nach denen diese Proteine zu ihren Bestimmungsorten transportiert werden, sowie ihre Relevanz für das Überleben des Parasiten in seiner Wirtszelle. Im vorliegenden Bericht beschreiben wir eine einfache Methode, aus infizierten Erythrozyten intakte Parasiten freizusetzten und diese in zwei Fraktionen, eine bestehend aus löslichen Proteinen und die andere bestehend aus Membranproteinen, aufzutrennen. Der Einfluß von Brefeldin A und von niedriger Temperatur (15° C) auf den Transport von Proteinen aus der Parasitenzelle sowie auf die Entwicklung des Parasiten in vitro wird beschrieben.

Schlüsselwörter Brefeldin A, *Plasmodium falciparum,* Proteintransport, Sekretion, Subfraktionierung.

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