**RESEARCH ARTICLE**

**Hemoglobin S and C affect protein export in *Plasmodium falciparum*-infected erythrocytes**

Nicole Kilian1,§, Sirikamol Srismith1,§, Martin Dittmer1, Djeneba Ouermi2, Cyrille Bisseye2, Jacques Simpore2, Marek Cyrklaff1, Cecilia P. Sanchez1 and Michael Lanzer1,*

**ABSTRACT**

Malaria is a potentially deadly disease. However, not every infected person develops severe symptoms. Some people are protected by naturally occurring mechanisms that frequently involve inheritable modifications in their hemoglobin. The best studied protective hemoglobins are the sickle cell hemoglobin (HbS) and hemoglobin C (HbC) which both result from a single amino acid substitution in β-globin: glutamic acid at position 6 is replaced by valine or lysine, respectively. How these hemoglobinopathies protect from severe malaria is only partly understood. Models currently proposed in the literature include reduced disease-mediating cytoadherence of parasitized hemoglobinopathic erythrocytes, impaired intraerythrocytic development of the parasite, dampened inflammatory responses, or a combination thereof. Using a conditional protein export system and tightly synchronized *Plasmodium falciparum* cultures, we now show that export of parasite-encoded proteins across the parasitophorous vacuolar membrane is delayed, slower, and reduced in amount in hemoglobinopathic erythrocytes as compared to parasitized wild type red blood cells. Impaired protein export affects proteins targeted to the host cell cytoplasm, Maurer’s clefts, and the host cell plasma membrane. Impaired protein export into the host cell compartment provides a mechanistic explanation for the reduced cytoadherence phenotype associated with parasitized hemoglobinopathic erythrocytes.

**KEY WORDS:** Hemoglobinopathy, Protein export, Malaria, *P. falciparum*

**INTRODUCTION**

Malaria has plagued mankind since prehistoric times and will continue to do so in the foreseeable future as an effective vaccine is not yet available and resistance to currently used antimalarial chemotherapeutics is spreading. According to the latest estimates, malaria causes 225 million disease episodes and approximately 0.66 million deaths in the year 2012 (World Health Organization, 2013). The high death toll from malaria, particularly among young children, has placed a strong selective force on the human population, which, in turn, led to the emergence of polymorphisms in the human genome that protect carriers from severe malaria-related disease and death (Kwiatkowski, 2005; Taylor et al., 2013; Williams, 2006). The best established example of such a survival benefit is the sickle cell trait, an inheritable hemoglobin modification. Normal hemoglobin (HbA) consists of two α- and two β-globin chains. In sickle cell hemoglobin, the β-globin chain is altered at position 6 by a glutamate to valine substitution (Weatherall and Provan, 2000). Heterozygous carriers of the sickle cell hemoglobin (HbS) have a 10-fold lower risk of dying from malaria (Aidoo et al., 2002; Taylor et al., 2012; Williams, 2006; Williams et al., 2005). As a consequence, the sickle cell trait is highly prevalent in malaria endemic areas, particularly in Sub-Saharan Africa, with up to 25% of the native population carrying the allele despite the lethal consequences for homozygotes who frequently die of sickle cell disease at a young age (Nagel and Fleming, 1992; Piel et al., 2010; Simpore et al., 2002; Taylor et al., 2013). In addition to the sickle cell trait, there are several other hemoglobinopathies that confer a survival advantage in malaria infections. These include hemoglobin C (HbC), which, like HbS, contains an alteration at position 6 in the β-globin chain but instead of valine, glutamate is replaced by lysine, as well as α-thalassemia, a quantitative hemoglobinopathy where carriers produce reduced amounts of the α-globin chain, leading to an excess of unpaired β-globin in their red blood cells (Taylor et al., 2013; Williams, 2006).

Severe malaria is attributed to the intraerythrocytic life cycle of the protozoan parasite *Plasmodium falciparum* and the pathological cytoadhesive behavior of parasitized erythrocytes, which sequester in the deep vascular bed of inner organs (Beeson et al., 2002; Mackintosh et al., 2004; Miller et al., 2013; Silamut et al., 1999). By cytoadhering to the endothelial lining of venular capillaries, the parasite avoids splenic clearance mechanisms, but causes pathological sequelae in the affected blood vessel, such as diminished tissue perfusion, tissue hypoxia, and systemic microvascular inflammation (Mackintosh et al., 2004; Miller et al., 2013).

Hemoglobinopathies are thought to impact on the parasite’s virulence through mechanisms that might include impaired intraerythrocytic development, reduced cytoadherence, and/or modulation of the host’s immune responses, although the underpinning molecular processes are still under investigation and the relative contribution of the different models to protection remains to be established. For instance, several but not all studies have noted a reduced multiplication rate of *P. falciparum* in hemoglobinopathic erythrocytes (Friedman, 1978; Glushakova et al., 2014; Pasvol et al., 1978). LaMonte et al. (LaMonte et al., 2012) showed that certain microRNAs, miR-451 and miR-233,
are enriched in sickle cell erythrocytes and can translocate into the parasite, where they inhibit mRNA translation and consequently affect the parasite growth (LaMonte et al., 2012). Studies conducted in a sickle cell murine model system have implicated accelerated turn-over of cytotoxic free heme in protection (Ferreira et al., 2011). Because of their variant hemoglobin, sickle cells release more heme into the plasma than do normal erythrocytes, which, in turn, stimulates the synthesis of hemoxygenase 1 by hematopoietic cells. Hemoxygenase 1 catalyzes the breakdown of heme, resulting in the production of the gasotransmitter carbon monoxide, which is thought to modulate the malaria-induced disease-mediating inflammatory reactions in the brain and other vital organs (Ferreira et al., 2011).

Recent evidence has pointed towards a role of hemoglobinopathies in interfering with cytoadhesion. Parasitized HbS, HbC and -thalassemic erythrocytes display substantially reduced cytoadherence to human venular endothelial cells compared to infected wild type erythrocytes (Cholera et al., 2008; Fairhurst et al., 2005; Fairhurst et al., 2012; Krause et al., 2003; Krause et al., 2012; Taylor et al., 2012). The reduced capability to cytoadhere correlates with a number of other phenotype alterations. For effective cytoadhesion, the major adhesion molecule PFE-MPI needs to be placed in parasite-induced knob-like protrusions on the erythrocyte plasma membrane (Baruch et al., 1995; Goldberg and Cowman, 2010; Maier et al., 2009). Infected hemoglobinopathic erythrocytes, however, possess fewer and abnormally enlarged knobs (Cholera et al., 2008; Fairhurst et al., 2005; Fairhurst et al., 2003; Krause et al., 2012). Moreover, the amount of PFE-MPI molecules exposed on the cell surface is reduced, and the PFE-MPI molecules that are presented are aberrantly displayed (Cholera et al., 2008; Fairhurst et al., 2005; Fairhurst et al., 2003).

We have recently described further phenotypic anomalies in infected HbS and HbC erythrocytes, namely those that affect structural elements of the protein trafficking and sorting machinery that the parasite establishes within the host cell cytoplasm to direct proteins, such as PFE-MPI, to the erythrocyte plasma membrane (Cyrklaff et al., 2011). For instance, Maurer’s clefts, which serve as intermediary compartments for exported proteins and which usually form stacks of unilamellar membranes (Lanzer et al., 2006), have an amorphous appearance and their movements are aberrant in parasitized HbS and HbC erythrocytes (Cyrklaff et al., 2011; Kilian et al., 2013). Moreover, the parasite-induced actin filaments that normally connect the Maurer’s clefts with knobs and which guide cargo vesicles from the Maurer’s clefts to the erythrocyte surface are unusually short and unattached (Cyrklaff et al., 2011).

The finding that structural elements of the protein export system are aberrant in HbS and HbC erythrocytes begs the question of whether protein trafficking of parasite-encoded proteins to the host cell compartment is dysfunctional in hemoglobinopathic erythrocytes. If parasite proteins are not delivered to their destination at the right time and in the right amount, this might underpin the aberrant organization of the knobs, Maurer’s clefts, the actin cytoskeleton, and eventually the impaired cytoadhesive behavior of parasitized hemoglobinopathic erythrocytes. To verify this model, we have studied the kinetics of protein trafficking from the parasite to different host cell compartments, including the cytoplasm, the Maurer’s clefts and the plasma membrane. Our data show that protein export is delayed, slower, and with reduced amounts of exported protein in parasitized HbS and HbC erythrocytes.

**MATERIALS AND METHODS**

**Ethical clearance**

The study was approved by the ethical review boards of Heidelberg University and the Biomolecular Research Center (CERBA/Labiogene) at the University of Ouagadougou in Burkina Faso. Written informed consent was given by all blood donors.

**Red blood cells**

The different hemoglobin genotypes were determined by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) and cellulose acetate electrophoresis as previously described (Modiano et al., 2001). The hemoglobinopathic erythrocytes were donated in Burkina Faso and immediately shipped at 4°C. After the arrival, the cells were washed 3 times with cold AB-transfusion medium (RPMI 1640 medium supplemented with 2 mM l-glutamine, 25 mM Hepes, 100 μM hypoxanthine, 20 μg ml⁻¹ gentamicin) and stored at 4°C. The cells used for infection experiments were not older than 3 weeks in total.

**Parasite strains**

The Plasmodium falciparum strains 3D7 were used to monitor the conditional export of PISBP1 CAD and SOL CAD. The GFP-tagged proteins PISBP1 CAD and SOL CAD have recently been described (Saridaki et al., 2008). To examine the presentation of PfEMP1 on the erythrocytic surface, the P. falciparum strain FCR3 CSA, preselected for high capacity binding to CSA, was used (Scherf et al., 1998). The var2csa knockout strain FCR3 var2CSA, which exhibits a null CSA-binding phenotype, has been described (Viebig et al., 2005).

**Culture of Plasmodium falciparum**

Hemoglobinopathic erythrocytes were infected with the MACS-purified (magnetic activated cell sorting, Miltenyi Biotech GmbH) late stages of P. falciparum (Sanchez et al., 2003). The eluted infected erythrocytes were washed twice in AB-transfusion medium and used to inoculate a culture with a hematocrit of 5.0% of the appropriate red blood cells. The infected erythrocytes were maintained at 37°C under controlled atmospheric conditions (5% O₂, 3% CO₂, 92% humidity). The parasites containing PISBP1 CAD and SOL CAD-plasmids were selected for using 5 nM WR99210. The parasitemia in these cultures was kept below 5% throughout. Cultures were synchronized using 5% d-sorbitol and/or gelatin flotation (Goodyer et al., 1994; Lambros and Vanderberg, 1979). The P. falciparum strain FCR3 CSA was selected for the adhesive phenotype every three weeks by adhesion to plastic flasks coated with 10 mg ml⁻¹ CSA as previously described (Viebig et al., 2005).

**Induction of protein export and imaging**

Trophozoite-stage parasites (18–22 h post invasion) were used to monitor the export of PISBP1 CAD and SOL CAD. For the induction of the protein export, the anti-aggregation ligand AP21998, also known as D/D-Solubilizer (CloneTech), was used at a concentration of 1 μM as previously described (Saridaki et al., 2008). Cells were fixed in 4% paraformaldehyde and 0.0075% glutaraldehyde for 15 min in order to preserve the export phenotype of the early time points. These parasites were stored in phosphate buffered saline (PBS, pH 7.3) at 4°C prior to imaging. The infected erythrocytes were transferred into a chamber containing either physiological Ringer’s solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂, 11.0 mM d-glucose, 1.0 mM NaH₂PO₄ and 25.0 mM Hepes) supplemented with 5 mM WR99210 and 1 μM anti-aggregation ligand (live cells) or PBS (fixed cells). The protein export was examined using an LSM510 confocal laser scanning microscope (Carl Zeiss). The GFP in PISBP1 CAD and SOL CAD was excited at a wavelength of 488 nm with an argon laser (laser power 40%, transmission 3%), Plan-Apochromat 100x/1.4 oil DIC. The emission was captured with a 505 to 550 band pass filter. Images were analyzed using the Carl Zeiss AIM Image Examiner Version 4.2. Rates of protein export into the erythrocytic cytoplasm were determined using Fiji 1.45b 64-bit (http://Fiji.sc/Fiji). The deconvolution of the acquired images was performed with the software Huygens Essential 3.4 (Scientific Volume Imaging, SVI).
**Adhesion assay**

The adhesion of *Plasmodium falciparum* FCR3CSA-infected erythrocytes to CSA was determined throughout the entire parasitic life cycle using static adhesion assay as previously described (Buffet et al., 1999; Viebig et al., 2005). Plastic petri dishes were prepared by pre-coating several areas of 20 mm² overnight with 1 mg ml⁻¹ purified CSA or 1% BSA as a control. Samples were taken at specified time points and 5×10⁶ infected erythrocytes were applied onto these spots for 1 h. After multiple washing steps, the adherent cells were fixed with 2% glutaraldehyde for at least 2 h. After staining for 10 min with 2% Giemsa, three randomly selected areas from each spot were imaged using Zeiss Axiovert 200M (objective 10×0.25 air DIC).

**Flow cytometry**

The presentation of total antigens was monitored using flow cytometry. Erythrocytes were taken from the culture at specific time points and fixed with 0.05% glutaraldehyde for 15 min. Afterwards, the cells were washed with 2% fetal calf serum in PBS. The cells were then labeled for 30 min with 3 μl of either pooled immune serum from 2 adult individuals living in a hyperendemic region in Burkina Faso or one uninfected adult as a control in a final volume of 50 μl PBS/fetal calf serum. After multiple washing steps, the erythrocytes were resuspended in 50 μl PBS/fetal calf serum containing 1:100 fluorescein (FITC)-conjugated affinity-pure F(ab')2 fragment donkey anti-human IgG (H+L), (Jackson ImmunoResearch Laboratories) and 1:100 propidium iodide for 30 min. Uninfected erythrocytes were similarly treated in parallel as a control. After further washing steps, the fluorescence signal was measured. Briefly, the fluorescence displayed on the surface of 400 infected erythrocytes was determined using a FACScalibur (Becton Dickinson) and the CellQuest Pro Software 6.04 BD (Franklin Lakes) was used to further process and analyze the data.

**Scanning electron microscopy**

Infected erythrocytes were deposited on a coverslip and fixed with 2.5% glutaraldehyde and 1% osmium tetroxide for 1 h. The cells were then dehydrated in aceton series (30–100%) and dried using critical point dryer. Alternatively, the dehydration was done by using ethanol series (30–100%) followed by soaking in 100% hexamethyldisalzan overnight. Dehydrated samples were mounted on studs and sputter-coated with 5–10 nm Au coat. The samples were viewed using a SEM Leo1530 at 10,000× magnification.

**Statistical analyses**

The beginning of export and the export rate of PfSBBr1/CAD and SOL/CAD were determined from the fluorescence exported to the erythrocytic cytoplasms by the parasite. The raw data were subsequently fitted using the sigmoid function:

\[ y(x) = \frac{k - \rho_0 e^{-x}}{k + \rho_0 (e^{-x} - 1)} \]

where \( y \) is the export in percent and \( x \) is the time in minutes. The parameters \( k \) (plateau value), \( \rho_0 \) (intersection with y-axis) and \( r \) (a variable that decides the steepness of the ascent of the curve) were determined by fitting. With these three parameters, a high-resolution (2000 individual points) curve was calculated and numerically differentiated twice using the proper commands in the statistical program “R” (R Development Core Team, 2011). The \( x \)-value at which second derivative has its maximum was taken as onset of the protein export (Luu-The et al., 2005). To determine the export rate, the linear part of each sigmoid curve was extracted and fitted with a linear equation:

\[ y = mx + t \]

The slope (m) of this linear fit is the export rate in min⁻¹. Error bars of the individual bars are the standard error of mean of the estimated slope as returned by “R”. Principal component analysis (Fig. 6) was performed using the appropriate commands in “R”. As input variables, we used the values for “export onset”, “export rate” and “amount of protein exported” compiled in Fig. 5. In addition, we calculated the Pearson correlation coefficient between each property according to:

\[ Cor(x,y) = \frac{n \sum_{i=1}^{n} (x_i - \bar{x})(y_i - \bar{y})}{sd(x)sd(y)} \]

\( n \): number of individual data points, \( x \): mean of \( x \), \( y \): mean of \( y \), \( sd() \): standard deviation of data points. Statistical significance was subsequently determined using the Student’s t-test. \( p\)-values < 0.05 were considered significant.

**RESULTS**

**Different kinetics of antigen presentation and cytoadherence in parasitized HbAS and HbCC erythrocytes**

We initially reproduced the previously described differential adhesion behavior of parasitized HbAA, HbAS, HbAC, and HbCC erythrocytes (Cholera et al., 2008; Fairhurst et al., 2003), using the *P. falciparum* strain FCR3CSA (preselected for high capacity binding to bovine chondroitin sulfate A) and chondroitin sulfate A (CSA) as a widely used surrogate receptor for cytoadherence. In static binding assays performed concurrently, parasitized HbAS and HbAC erythrocytes showed a significant reduction (44% and 28%, respectively) in adherence compared to parasitized HbAA erythrocytes (Fig. 1A, \( p < 0.001 \)). Parasitized HbCC erythrocytes showed little to no cytoadherence. This was comparable to the null CSA-binding phenotype displayed by FCR3Av2CSA (Viebig et al., 2005), where the var2CSA gene that exclusively confers binding to CSA was disrupted (Fig. 1A). Reduced cytoadherence correlated with altered knob morphology and density; parasitized HbAS, HbAC, and HbCC erythrocytes had fewer and abnormally enlarged knobs, compared to infected wild type erythrocytes, as determined by scanning electron microscopy (Fig. 1B). This was consistent with previous reports (Cholera et al., 2008; Cylklafl et al., 2011; Fairhurst et al., 2003). Next, we investigated the time course of adherence to CSA by determining the amount of adherent cells throughout the intraerythrocytic life cycle of *P. falciparum*, with samples taken in time intervals of 2 to 4 h, using tightly synchronized cells. In parasitized HbAA erythrocyte, the adherence phenotype appeared 16 h post invasion and then increased until 24 h post invasion before reaching a plateau value (Fig. 2A), consistent with previous reports (Gardner et al., 1996; Kriek et al., 2003). Time courses of parasitized HbAS erythrocytes, which were performed in parallel with infected wild type red blood cells, revealed a significantly slower temporal increase in the number of adherent cells and a lower final plateau level (Fig. 2A; \( p < 0.01 \)). Fitting linear functions to the rising sections of the data points provided a quantititative readout, in terms of the slopes, for the differential adherence kinetics displayed by parasitized HbAA and HbAS erythrocytes. In parasitized HbAA erythrocytes, adherence rose with a slope of 61±4 cells h⁻¹, as compared to 20±2 cells h⁻¹ for parasitized HbAS erythrocytes (\( p < 0.01 \) in Fig. 2A). Parasitized HbCC erythrocytes did not show any significant binding to CSA during the intraerythrocytic life cycle (Fig. 2A).

We determined, in paired and parallel assays, the temporal appearance of total antigens on the surface of HbAA, HbAS and HbCC erythrocytes infected with FCR3CSA during the intraerythrocytic life cycle, using pooled sera from residents of a malaria holoendemic region in Burkina Faso. The FACS analysis revealed clear differences in the time courses of antigen presentation between the different parasitized red blood cells. In
HbAA infected erythrocytes, the first surface antigens were detected 16 h post infection. The amount of presented antigens then rose with a slope of 30 arbitrary fluorescence units h$^{-1}$ before it reached a plateau level 30 h post invasion (Fig. 2B). In parasitized HbAS erythrocytes, antigen presentation rose with a significantly flatter slope of 10 arbitrary fluorescence units h$^{-1}$ (p<0.01). Moreover, the final plateau value was only half that of infected wild type erythrocytes (Fig. 2B). Even more pronounced were the differences in parasitized HbCC erythrocytes. Antigens were not detected on the cell surface before 36 h post invasion and the total amount of antigen presented was even lower than that seen in parasitized HbAS erythrocytes (Fig. 2B). These data indicate major deviations in the timing and the amount of cytoadherence and antigen presentation in parasitized HbAS and HbCC erythrocytes, relative to the wild type controls.

**Aberrant protein trafficking across the parasitophorous vacuolar membrane**

We have recently described a conditional protein export system in *P. falciparum* based on the conditional aggregation domain...
that allows protein trafficking to destinations within the host erythrocyte to be controlled (Saridaki et al., 2008). Proteins of interest fused to the CAD domain self-aggregate in the parasite’s ER in a reversible manner and only continue their trafficking path upon the addition of a small membrane-permeable ligand. We used this conditional export system to further analyze potential differences in protein trafficking associated with erythrocytes containing HbS and HbC. Two proteins were studied: (i) an artificial, soluble, PEXEL-containing protein (consisting of the first 80 amino acids of a STEVOR protein), termed SOL(CAD), destined for export into the cytoplasm of the host cell (Saridaki et al., 2008); and (ii) a membrane bound non-PEXEL PISBP1 fusion protein, termed PISBP1(CAD), targeted to Maurer’s clefts (Saridaki et al., 2008). Both proteins were tagged with the green fluorescence protein to follow their trafficking. The corresponding genes were episomally expressed in the P. falciparum strain 3D7, which was continuously cultured in vitro using the following erythrocyte variants: HbAA, HbAC, HbAS, HbCC, and HbSC.

In the absence of the anti-aggregation ligand, both SOL(CAD) and PISBP1(CAD) were retained in the parasite’s ER, consistent with previous reports (Saridaki et al., 2008). To study the kinetics of protein export, the anti-aggregation ligand was added to highly synchronized parasite cultures at the early trophozoite stage (18–22 h post invasion) and the time courses of protein export to the erythrocyte cytosol and the Maurer’s clefts, respectively, were monitored over the next 960 min. In the case of SOL(CAD), fluorescence spread from its initial focus in the parasite’s ER to the parasitophorous vacuolar lumen within 90 min following the addition of the anti-aggregation ligand (Fig. 3). No major differences in intra-parasitic protein trafficking were apparent between wild type and hemoglobinopathic erythrocytes at this stage. After 120 min, a homogenous fluorescence signal was detected in the erythrocyte cytosol but only in parasitized HbAA erythrocytes and not in erythrocytes containing hemoglobin variants. In parasitized HbAC, HbAS, HbCC, and HbSC erythrocytes, SOL(CAD) remained in the parasitophorous vacuole, and it was not until the 600 min time point that a clear fluorescence signal was detected in the host cell cytoplasm.

Similar results were obtained for PISBP1(CAD). Again, the protein was trafficked from the parasite’s ER to the parasite’s periphery within 60 min upon addition of the anti-aggregation ligand, with no apparent differences in the time courses between wild type and variant erythrocytes. Between 60 to 90 min upon the addition of the anti-aggregation ligand PISBP1(CAD) had reached the Maurer’s clefts, but only in parasitized wild type erythrocytes. In parasitized erythrocytes containing the hemoglobin variants S and C, export beyond the parasitophorous vacuolar membrane was substantially delayed (Fig. 4).

To better assess the kinetics of protein export in the different parasitized erythrocytes, we quantified the data by determining the fraction of fluorescence that was present in the erythrocyte compartment (in reference to the total cellular fluorescence) in at least 40 cells, obtained from three independent biological replicates, for each time point and parasitized erythrocyte variant. Logistic functions were then fitted to the data points, which yielded the plateau value of protein export plotted in Fig. 5A,B for SOL(CAD) and PISBP1(CAD), respectively. The onset of export across the PVM was subsequently determined, using the second derivative maximum method (Luu-The et al., 2005), and the export rate was calculated by fitting a linear function to the linear part of each sigmoidal curve.
Fig. 3. Kinetics of export of SOL in various *P. falciparum*-infected erythrocytes. *P. falciparum*-infected erythrocytes were taken from culture at the time points indicated after the addition of the anti-aggregation ligand and immediately analyzed by live cell confocal fluorescence microscopy. Tightly synchronized cultures at the trophozoite-stage (18–22 h post invasion) were used. Images shown are representative examples for the export phenotypes that were used for later quantification.

| Time (min) | HbAA | HbAC | HbAS | HbCC | HbSC |
|------------|------|------|------|------|------|
| 0          |      |      |      |      |      |
| 10         |      |      |      |      |      |
| 30         |      |      |      |      |      |
| 90         |      |      |      |      |      |
| 120        |      |      |      |      |      |
| 240        |      |      |      |      |      |
| 480        |      |      |      |      |      |
| 600        |      |      |      |      |      |
| 960        |      |      |      |      |      |

Fig. 4. Kinetics of export of PfSBP1 in various *P. falciparum*-infected erythrocytes. *P. falciparum*-infected erythrocytes were taken from culture at the time points indicated after the addition of the anti-aggregation ligand and immediately analyzed by live cell confocal fluorescence microscopy. Tightly synchronized cultures at the trophozoite-stage (18–22 h post invasion) were used. Images shown are representative examples for the export phenotypes that were used for later quantification.

| Time (min) | HbAA | HbAC | HbAS | HbCC | HbSC |
|------------|------|------|------|------|------|
| 0          |      |      |      |      |      |
| 30         |      |      |      |      |      |
| 60         |      |      |      |      |      |
| 90         |      |      |      |      |      |
| 120        |      |      |      |      |      |
| 240        |      |      |      |      |      |
| 480        |      |      |      |      |      |
| 600        |      |      |      |      |      |
| 960        |      |      |      |      |      |
the case of PfSBP1CAD, the export rate was independent of the two other variants (≈0.17 and >0.5, and 0.33 and >0.5, respectively). The principal component analysis further revealed similarities in the protein export behaviors of the different parasitized erythrocytes.

For SOL CAD, two clusters can be observed. The first cluster consists of hemoglobinopathic erythrocytes carrying HbS, i.e., HbAS and HbSC erythrocytes, and is characterized by delayed onset and reduced amount of protein export (Fig. 6A). The second group is formed by erythrocytes carrying HbAC and HbCC and has lower export rates and slightly increased (though not statistically significant) amounts of protein export than the first group (Fig. 6A). Apparently, HbS has a strong dominating effect over HbA and HbC, in that it severely impacts on onset and amount of protein export into the host cell compartment. HbAA wild type can be clearly distinguished from all the hemoglobinopathic erythrocytes (Fig. 6A).

The principal component analysis of PfSBP1CAD revealed a similar pattern; again, the HbAA wild type is clearly separated from all variant hemoglobins (Fig. 6B). However, a slightly different clustering pattern is observed amongst the hemoglobinopathies. Here, the heterozygous HbA erythrocytes are grouped together, indicating that the effects of the remaining HbA are the deciding factor for the export kinetics of the membrane-bound PfSBP1CAD. The absence of HbA, such as in HbCC and HbSC erythrocytes, creates a different cluster, which...
shows a strong impairment in PfSBP1\textsuperscript{CAD} export kinetics (Fig. 6B).

**DISCUSSION**

Here we explored the hypothesis that hemoglobin S and C affect trafficking of exported parasite-encoded proteins within the host cell compartment. The study was inspired by our recent finding that structural constituents of the protein trafficking and sorting machinery are malformed in parasitized HbCC and HbSC erythrocytes (Cyrklaff et al., 2012; Cyrklaff et al., 2011). We found that the kinetics of protein export was anomalous when parasites were grown in hemoglobinopathic erythrocytes, as exemplified for surface antigens, the Maurer’s clefts-associated membrane protein PfSBP1, and an artificial soluble protein targeted into the host cell cytoplasm.

Aberrant protein export is consistent with most models posited to explain the protective effect of hemoglobinopathic erythrocytes on severe malaria. *P. falciparum* exports a few hundred proteins into the host erythrocyte compartment (Goldberg and Cowman, 2010; Heiber et al., 2013; Maier et al., 2009; van Ooij et al., 2008). If these proteins are delivered at the wrong moment in time or in insufficient quantity then this is likely to impact on physiological and pathophysiological functions of the parasite. As a consequence, intraerythrocytic development of the parasite might be impaired, particularly if parasite-encoded transporters and channels are affected that create permeation pathways for nutrient uptake and ion homeostasis across the host erythrocyte cytoplasm (Nguitragool et al., 2011). Indeed several studies have reported lower intraerythrocytic multiplication rates of *P. falciparum* in HbS and HbC containing erythrocytes, although other studies failed to confirm this result (Fairhurst et al., 2003; Friedman et al., 1979; LaMonte et al., 2012; Olson and Nagel, 1986; Pasvol, 1980; Pasvol et al., 1978) and a meta-analysis revealed that malaria patients harboring hemoglobinopathies can have parasitemias as high as those seen in patients with normal hemoglobin (Taylor et al., 2013; Taylor et al., 2012). Our own data suggest that the parasite develops normally in the different red blood cell variants under continuous in vitro culture, although there is a statistically insignificant trend of a slightly lower replication rate when parasites are grown in hemoglobinopathic erythrocytes (Kilian et al., 2013). Similarly, delayed and insufficient export of the knob-associated histidine-rich protein, the major constituent of the knobs, and PIEMP1 into the host compartment may, at least in part, explain why parasitized hemoglobinopathic red blood cells possess fewer and abnormally enlarged knobs, why they present less PIEMP1 on the surface, and why their ability to cytoadhere is diminished (Cholera et al., 2008; Fairhurst et al., 2005; Fairhurst et al., 2012) (Fig. 2A).

Cryotomographic images have recently shown that structural features of the protein trafficking and sorting system established by the parasite in the host cell cytoplasm are malformed in parasitized HbCC and HbSC erythrocytes (Cyrklaff et al., 2011). For instance, the Maurer’s clefts, serving as intermediary compartments for proteins en route to the plasma membrane, have an amorphous appearance (Cyrklaff et al., 2011), which is...
quite distinct from the typical stacked unilamellar membrane profiles (Lanzer et al., 2006). In addition, host actin reorganization progresses only marginally in parasitized HbCC and HbSC erythrocytes. Usually the parasite mines the actin of the erythrocyte membrane skeleton to generate a network of long filaments that connect the Mauer’s clefts with the knobs and which seem to direct cargo vesicles towards the erythrocyte plasma membrane (Cyrklaff et al., 2012; Cyrklaff et al., 2011). The parasite-induced actin filaments are much shorter in parasitized HbCC and HbSC erythrocytes and they do not link the Mauer’s clefts with the host cell plasma membrane (Cyrklaff et al., 2012; Cyrklaff et al., 2011). Although tomographic images are not yet available for parasitized HbAS and HbAC erythrocytes, it is conceivable that Mauer’s clefts morphology and host actin reorganization are also affected in these hemoglobinopathic cells.

The degree of functional impairment seems to vary among the haemoglobinopathies, as suggested by principal components analyses of the three export parameters: onset of export, rate of export, and amount of protein exported, which were recorded for each of the different erythrocytes. In the case of SOL CAD, parasitized HbAS and HbSC erythrocytes and parasitized HbAC and HbCC erythrocytes form a similarity cluster each. In the case of the Mauer’s clefts associated protein PSBP1 CAD, parasitized HbAS and HbAC erythrocytes cluster and parasitized HbCC and HbSC erythrocytes cluster. These differences in groupings might be explained by the different classes of exported protein that SOL CAD and PSBP1 CAD represent. SOL CAD is a soluble PEXEL containing protein (Marti et al., 2004) and PSBP1 CAD is a membrane-associated PEXEL-negative exported protein (Heiber et al., 2013; Saridaki et al., 2009). Although both PEXEL and PEXEL-negative exported proteins share many functional and structural features along their trafficking pathway, including unfolding and translocation across the parasitophorous vacuolar membrane via a common translocon (Beck et al., 2014; Elsworth et al., 2014; Heiber et al., 2013), there may be slight differences in protein handling and processing that are affected by the various hemoglobinopathies to different degrees. Irrespective, the principal component analysis of SOL CAD and PSBP1 CAD revealed some common principles: Firstly, a late onset of export is generally highly correlated with a low amount of protein export. Secondly, the export rate is correlated with the onset of export or the amount of protein export only for SOL CAD, but not for PSBP1 CAD. This finding might again point towards some differences in the protein export pathways between a PEXEL and a PEXEL-negative protein. Thirdly, HbCC seems to be the most effective hemoglobin variant when it comes to affecting protein export kinetics of both SOL CAD and PSBP1 CAD. The highly impaired protein export displayed by HbCC erythrocytes correlates well with the almost null adhesion phenotype of these cells (compare Fig. 6A,B with Fig. 1A). HbSC erythrocytes displayed an even more impaired protein export phenotype than did HbCC erythrocytes but only for PSBP1 CAD not for SOL CAD.

While malfunctioning protein trafficking from the Mauer’s clefts to the erythrocyte plasma membrane provides a plausible explanation for diminished total antigen presentation and reduced cytoadhesion, the results obtained using the conditional protein trafficking system suggest a more nuanced model. Unexpectedly, trafficking of fluorescently labeled PSBP1 CAD and SOL CAD was delayed at the parasitophorous vacuolar membrane. This membrane, which separates the parasite from the host cell, is a highly selective barrier that lets only those parasite-encoded proteins pass via a translocon that are allotted for export into the host cell compartment (Beck et al., 2014; Elsworth et al., 2014). Why proteins are held up in the parasitophorous vacuolar lumen is unclear, but might involve a translocation process that is malfunctioning in hemoglobinopathic erythrocytes.

HbS and HbC containing erythrocytes are characterized by a redox imbalance due to the instability of their hemoglobin (Chaves et al., 2008; Dargouth et al., 2011). Unlike normal hemoglobin, HbS and HbC are prone to oxidation, resulting in increased amounts of irreversible hemichromes, free heme, and free iron, which themselves act as oxidants (Bauminger et al., 1979; Chaves et al., 2008; Hebbel, 1991). For instance, ferryl hemoglobin can oxidize actin, which was shown to reduce actin polymerization rates and affect actin dynamics (Abraham et al., 2002; Cyrklaff et al., 2011; Farah et al., 2011; Jarolim et al., 1990). On the basis of these considerations, we have recently proposed that the redox imbalance triggered by the instability of HbS and HbC interferes with host actin reorganization and subsequently with the organization of Mauer’s clefts in hemoglobinopathic erythrocytes (Cyrklaff et al., 2012; Cyrklaff et al., 2011). Whether components of the translocon or factors assisting translocation are affected by the increased oxidative state present in hemoglobinopathic erythrocytes remains to be seen. Interestingly, several membrane protein supercomplexes recruit actin to stabilize their assembly (Abrahi et al., 2010). This includes the ribosome-translocon complex of the endoplasmic reticulum which, after binding of the palmitoylated chaperone calnexin, binds to the actin cytoskeleton (Lakkaraju et al., 2012). It is tempting to speculate that the impaired protein translocation across the parasitophorous vacuolar membrane relates not only to a malfunctioning translocon but also to the inability of the translocon to recruit actin in hemoglobinopathic erythrocytes.

Acknowledgements

We thank Stefan Prior and Marina Müller for technical assistance. We are grateful to Ross Douglas for proofreading the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

M.L. and C.P.S. conceived the study and analyzed data. M.L. wrote the manuscript. N.K., S.S., M.D., and C.P.S. performed experiments and analyzed data. D.O., C.B., and J.S. performed hemoglobin genotyping, identified appropriate blood donors, and collected blood from donors.

Funding

This work was funded by the Deutsche Forschungsgemeinschaft under the Sonderforschungsbereich 1129; and the European Community’s Seventh Framework Programme [Cluster of Excellence “EvMalaria”; FP7/2007–2013; grant 242095]. M.D. was supported by fellowships from the Konanz-Stiftung and EvMalaria and SS by a fellowship from the Graduate School of Molecular and Cellular Biology at Heidelberg University (HBiGS). M.L. is a member of the German Excellence Cluster “CellNetworks”.

References

Abdi, H. and Williams, L. J. (2010). Principal component analysis. Wiley Interdiscip. Rev. Comput. Stat. 2, 433-459.

Abraham, A., Bencsath, F. A., Shartava, A., Kakhniashvili, D. G. and Goodman, S. R. (2002). Preparation of irreversibly sickled cell beta-actin from normal red blood cell beta-actin. Biochemistry 41, 292-296.

Abrami, L., Bischofberger, M., Kunz, B., Groux, R. and van der Goot, F. G. (2010). Endocytosis of the anthrax toxin is mediated by clathrin, actin and unconventional adaptors. PLoS Pathog. 6, e1000792.

Aldoo, M., Terlouw, D. J., Kolczak, M. S., McElroy, P. D., ter Kuile, F. O., Kariuki, S., Nahlen, B. L., Lal, A. A. and Udhayakumar, V. (2002). Protective effects of the sickle cell gene against malaria morbidity and mortality. Lancet 359, 1311-1312.

PLoS Pathog. 8, e1003243.
haemoglobin C may protect against malaria. Variants: fresh insights into malaria pathogenesis and protection.

In Plasmodium falciparum-infected erythrocytes containing hemoglobin 101 cells: implications for the malaria protective effect of the homozygous state.

P. R., Chisholm, S. A., Counihan, N. A., Shaw, P. J., Pino, P. et al.

PTEX is an essential nexus for protein export in malaria parasites.

Olivier, M. F., Bartolucci, P., Benkerrou, M., Pissard, S. et al.

(2011).

Remodeling and protection from malaria by hemoglobinopathies.

In Plasmodium falciparum-infected erythrocytes.

F. and Lanzer, M.

Impaired cytoadherence of Plasmodium falciparum-infected erythrocytes containing sickle hemoglobin.

Hemoglobins S and C interfere with actin remodeling in Plasmodium falciparum-infected erythrocytes. Science 343, 1283-1286.

Cyrklafl, M., Sanchez, C. P., Kilian, N., Bisseye, C., Simpore, J., Frischknecht, F. and Lanzer, M. (2011). Hemoglobin S and C interfere with actin remodeling in Plasmodium falciparum-infected erythrocytes. Science 343, 1283-1286.

Kilian, N., Dittmer, M., Cyrklafl, M., Ouermi, D., Bisseye, C., Simpore, J., Frischknecht, F., Sanchez, C. P. and Lanzer, M. (2013). Haemoglobin S and C affect the motion of Maurer’s clefts in Plasmodium falciparum-infected erythrocytes. Cell. Microbiol. 15, 1111-1126.

Krause, M. A., Diakite, S. A., Ofer, S. and Rachmilewitz, E. A. (1979). Quantitative studies of ferritin-like iron in erythrocytes of thalassemia, sickle-cell anemia, and hemoglobin Hammarsmith with Mössbauer spectroscopy.

Proc. Natl. Acad. Sci. USA 76, 9394-9373.

Kriek, N., Tilley, L., Horrocks, P., Elford, B. C., Ferguson, D. J., Lingelbach, K. and Newbold, C. I. (2003). Characterization of the pathway for transport of the cytoadherence-mediating protein, PFE01P, to the host cell surface in malaria parasite-infected erythrocytes. Mol. Microbiol. 50, 1215-1227.

Kwiatkowski, D. P. (2005). Pne60 has affected the human genome and what new human genetics can teach us about malaria. Am. J. Hum. Genet. 77, 171-192.

Laloue, A. K., Abrami, L., Le Minven, T., Blaskovic, S., Kunz, B., Khira, A., Dal Peraro, M. and van der Goot, F. (2012). Palmitoylated cavinex is a key component of the rhomboide-sinister complex. EMBO J. 31, 1823-1835.

Lambros, C. and Vanderberg, J. P. (1979). Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 65, 418-420.

LaMonte, G., Philip, N., Reardon, J., Lascina, J. R., Majoros, W., Chapman, L., Thornburg, C. D., Telen, M. J., Ohler, U., Nicchita, C. V. et al. (2012). Translocation of sickle cell erythrocyte microRNAs into Plasmodium falciparum inhibits parasite translation and contributes to malaria resistance. Cell Host Microbe 12, 187-199.

Lanzer, M., Wickert, H., Krohne, G., Vincensini, L. and Braun Breton, C. (2006). Maurer’s clefts: a novel multifunctional organelle in the cytoplasmin of Plasmodium falciparum infected erythrocytes. Int. J. Parasitol. 36, 23-39.

Luu-The, V., Paquet, N., Calvo, E. and Cumps, J. (2005). Improved real-time RT-PCR method for high-throughput measurements using second derivative calculation and double correction. Biotechniques 38, 287-293.

Mackintosh, C. L., Best, L. and Schumacher, H. (2008). Pathophysiology of sickle cell disease is mirrored by the red blood cell metabolism. Blood 111, e57-e66.

Lewsoth, B., Matthews, K., Nie, C. Q., Kalamon, M., Charnaud, S. C., Sanders, P. F., Ghosh, M., Cunningham, N., Shap, J. P., Pyy, P. et al. (2014). Pathophysiology of sickle cell disease is mirrored by the red blood cell metabolism. Blood 111, e57-e66.

Fairhurst, R. M., Beale, S., Collins, K. F. and Wellems, T. E. (1999). Identification of the P. falciparum gene PfSBP1 to the Plasmodium falciparum Maurer’s clefts. EMBO J. 18, 137-152.

T. R., Smith, A., Le, M. V., Le, T. T., Pino, P., Benkerrou, M., Pissard, S. et al. (2011). Pathophysiology of sickle cell disease is mirrored by the red blood cell metabolism. Blood 111, e57-e66.

Fairhurst, R. M., Fujioka, H., Smith, A., Le, M. V., Le, T. T., Pino, P., Benkerrou, M., Pissard, S. et al. (2011). Pathophysiology of sickle cell disease is mirrored by the red blood cell metabolism. Blood 111, e57-e66.

Fairhurst, R. M., Fujioka, H., Hayton, K., Smith, A., Le, M. V., Le, T. T., Pino, P., Benkerrou, M., Pissard, S. et al. (2011). Pathophysiology of sickle cell disease is mirrored by the red blood cell metabolism. Blood 111, e57-e66.

Fairhurst, R. M., Fujioka, H., Hayton, K., Collins, K. F. and Wellems, T. E. (1999). Identification of the P. falciparum gene PfSBP1 to the Plasmodium falciparum Maurer’s clefts. EMBO J. 18, 137-152.
Taylor, S. M., Parobek, C. M. and Fairhurst, R. M. (2012). Haemoglobinopathies and the clinical epidemiology of malaria: a systematic review and meta-analysis. *Lancet Infect. Dis.* 12, 457-468.

Taylor, S. M., Cerami, C. and Fairhurst, R. M. (2013). Hemoglobinopathies: slicing the Gordian knot of Plasmodium falciparum malaria pathogenesis. *PLoS Pathog.* 9, e1003327.

van Ooij, C., Tamez, P., Bhattacharjee, S., Hiller, N. L., Harrison, T., Lilios, K., Kooij, T., Ramesar, J., Balu, B., Adams, J. et al. (2008). The malaria secretome: from algorithms to essential function in blood stage infection. *PLoS Pathog.* 4, e1000084.

Viebig, N. K., Gamain, B., Scheidig, C., Lépolard, C., Przyborski, J., Lanzer, M., Gysin, J. and Scherf, A. (2005). A single member of the Plasmodium falciparum var multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A. *EMBO Rep.* 6, 775-781.

Weatherall, D. J. and Provan, A. B. (2000). Red cells I: inherited anaemias. *Lancet* 355, 1169-1175.

Williams, T. N. (2006). Human red blood cell polymorphisms and malaria. *Curr. Opin. Microbiol.* 9, 388-394.

Williams, T. N., Mwangi, T. W., Wambua, S., Alexander, N. D., Kortok, M., Snow, R. W. and Marsh, K. (2005). Sickle cell trait and the risk of Plasmodium falciparum malaria and other childhood diseases. *J. Infect. Dis.* 192, 178-186.

World Health Organization (2013). *World Malaria Report 2013.* Geneva: WHO Press.