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Cryo transmission X-ray imaging of the malaria parasite, *P. falciparum*

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ABSTRACT

Cryo transmission X-ray microscopy in the "water window" of photon energies has recently been introduced as a method that exploits the natural contrast of biological samples. We have used cryo tomographic X-ray imaging of the intra-erythrocytic malaria parasite, *Plasmodium falciparum,* to undertake a survey of the cellular features of this important human pathogen. We examined whole hydrated cells at different stages of growth and defined some of the structures with different X-ray density, including the parasite nucleus, cytoplasm, digestive vacuole and the hemoglobin degradation product, hemozoin. As the parasite develops from an early cup-shaped morphology to a more rounded shape, puncta of hemozoin are formed; these coalesce in the mature trophozoite into a central compartment. In some trophozoite stage parasites we observed invaginations of the parasite surface and, using a selective permeabilization process, showed that these remain connected to the RBC cytoplasm. Some of these invaginations have large openings consistent with phagocytic structures and we observed independent endocytic vesicles in the parasite cytoplasm which appear to play a role in hemoglobin uptake. In schizont stage parasites staggered mitosis was observed and X-ray-dense lipid-rich structures were evident at their apical ends of the developing daughter cells. Treatment of parasites with the antimalarial drug artemisinin appears to affect parasite development and their ability to produce the hemoglobin breakdown product, hemozoin.

1. Introduction

Of the different species of malaria parasite that infect humans *Plasmodium falciparum* is the most deadly; it is responsible for close to one million deaths per year (WHO, 2009). During its intra-erythrocytic development, the parasite grows and divides, feeding on the hemoglobin, and remodeling the host red blood cell (RBC) to promote its own survival. The blood stages are responsible for the clinical symptoms of the disease which range from uncomplicated fevers to life-threatening cerebral and placental malaria (Miller et al., 2002). The complications are due to the cytoadherence of infected RBCs to receptors on brain venule epithelial cells or (in pregnant women) to placental syncytiotrophoblasts. The adhesion process prevents phagocytic clearance in the spleen which contributes to virulence, while an inappropriate host immune response to the sequestered parasites can induce coma and death (Rogerson et al., 2004).

The intra-erythrocytic parasite resides within a parasitophorous vacuole (PV) that is formed during the invasion step (Bannister et al., 2000). It develops through the ring, trophozoite and schizont stages, eventually bursting to release 16–20 daughter merozoites (Bannister et al., 2000; Garcia et al., 2008). The ring stage (0 to ~20 h) is thought to be a metabolically sluggish phase during which the parasite exports a range of proteins to the RBC cytoplasm. As it grows the parasite expands its core complement of organelles needed for metabolism (nucleus, endomembrane system, mitochondria, and apicoplast), and develops novel organelles including a modified lysosome (referred to as the digestive vacuole) and a series of apical organelles (rhoptries, dense granules and micronemes) (Bannister and Mitchell, 2009; Bannister et al., 2000; Kats et al., 2006).

As the intra-erythrocytic parasite develops it needs to obtain a source of amino acid building blocks and to create sufficient space for expansion and division (Goldberg, 2005; Lew et al., 2003). To achieve this, the parasite digests its host cell from the inside, consuming ~75% of the host hemoglobin (Loria et al., 1999). Uptake of host cytoplasm is most active in the trophozoite stage

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and is thought to involve endocytic structures that bud from the surface of the parasite and deliver hemoglobin to the digestive vacuole (Slomianny, 1990; Yayon et al., 1984). Three recent electron microscopy[1] (EM)-based studies have re-examined hemoglobin uptake in infected RBC leading to somewhat conflicting views of the endocytic process (Abu Bakar et al., 2010; Elliott et al., 2008; Lazarus et al., 2008).

Artemisinin, an endoperoxide-based drug, and its derivatives, are now recommended treatments for malaria in many countries (Golenser et al., 2006; Meshnick, 2002; Tilley et al., 2006). The mechanism of action of artemisinin is still debated with some studies suggesting that the primary target lies within the ER (Eckstein-Ludwig et al., 2003; Krishna et al., 2004) or the mitochondrion (Wang et al., 2010). By contrast other studies have suggested that the digestive vacuole is an important site of artemisinin action (del Pilar Crespo et al., 2008; Maeno et al., 1993; Pandey et al., 1999).

The advent of molecular transfection technology, coupled with fluorescence microscopy analyses of fluorescent protein reporters, has greatly improved our understanding of the ways in which the malaria parasite alters its host cell (Tilley et al., 2007). However the resolution of conventional fluorescence microscopy is limited and there are concerns that the labeling of cellular components may alter their morphology and function (Marks and Nolan, 2006). EM offers superb resolution however there are concerns that the harsh fixation and sample manipulation that is required can introduce artifacts (Frey et al., 2006; McIntosh et al., 2005; Perktold et al., 2007). Thus it is important to compare data from visible light and electron microscopy with alternative techniques that provide the highest possible resolution while minimizing sample manipulation.

X-ray cryo-tomography has recently been introduced as a high resolution technique that can be performed on whole hydrated cells with a lower potential for artifacts. The differential absorption of soft X-rays by organic matter and water provides natural contrast and avoids the need for exogenous stains or chromophores. Due to the short wavelengths of X-rays this technique can achieve higher resolution than conventional optical microscopy (Larabell and Le Gros, 2004; Le Gros et al., 2005; Parkinson et al., 2008; Uchida et al., 2009). In this work we have used X-ray cryo-tomography to study the development of *P. falciparum* in infected RBCs.

2. Materials and methods

2.1. Parasites

Plasmodium falciparum parasites were cultured in vitro in RPMI medium supplemented with 4% human serum plus 0.25% AlbuMAX (GIBCO, Invitrogen) as described previously (Jackson et al., 2007). 3D7_MAHRP1-GFP transfectants were generated as described previously (Spycher et al., 2006). RBCs and pooled sera were obtained from the Red Cross Transfusion Service (Melbourne, Australia) or from volunteers (ALS, Berkeley, USA). Trophozoite stage-infected RBCs were harvested by flotation on a Percoll/sorbitol gradient (Aley et al., 1986) or using a magnetized column (Trang et al., 2004).

2.2. Immunolabeling

Freshly harvested infected RBCs were lightly fixed with 2% paraformaldehyde in RPMI medium (10 min), permeabilized with Equinatoxin II (EqtII; (Anderluh et al., 1996; del Pilar Crespo et al., 2008)), and re-fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Reactive groups were blocked with 2% BSA in PBS and cells were incubated with the primary antibody for 2 h at room temperature (rabbit anti-GFP (Humphries et al., 2005); 1:20 in 3% bovine serum albumin (BSA) in PBS). After washing the cells were incubated with Protein A gold (6 nm, Aurion, NL) and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3. The gold labeling was silver enhanced using R-Gent SE-EM (Aurion, NL).

2.3. Resealing RBCs and artemisinin treatment

RBCs were lysed in 2.5 volumes of ice-cold 5 mM phosphate, pH 7.5, 1 mM Mg-ATP (Sigma) in the presence of albumin labeled with ultrasmall gold particles (Aurion BSA gold tracer, previously concentrated and exchanged into the same buffer using a Centricon Centrifugal Filter Device) and resealed as described previously (Frankland et al., 2006). After 10 min on ice NaCl was added from a concentrated stock to a final concentration of 0.15 M and samples were incubated at 37 °C for 45 min to reseal the cells. Cells were washed and the amount of retained hemoglobin was estimated spectrophotometrically at 415 nm. The resealed cells were mixed with schizonts and the parasites were allowed to invade and develop under culture conditions. The infected RBCs were prepared for X-ray imaging, in some cases after permeabilization with EqtII, and gold enhancement of the gold particles. For drug treatment mid-ring stage parasites (~14 h invasion, D10 strain) were incubated in the presence or absence of 60 nM artemisinin for 16 h then purified using a magnetic column. The cells were washed and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3.

2.4. X-ray tomography

Aliquots of harvested infected RBCs (3D7 wild type) or EqII-permeabilized, immuno-gold-labeled infected RBCs (MAHRP1-GFP transfectants) or resealed infected RBCs were used without fixation or fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The infected RBCs were transferred into ~8 μm glass capillaries. Fiducial particles (100 nm) were added to the outside of the capillary (*note:* some enter the bottom of the tube). The samples were rapidly frozen in liquid nitrogen and mounted in a cryogenic gas stream (liquid nitrogen cooled helium gas) (Le Gros et al., 2005). Projection images were collected using a transmission soft X-ray microscope (XM-2; beamline 2.1.2 at the Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, CA). The microscope is equipped with a Fresnel zone plate condenser and objective (with 60 nm and 50 nm outer zone widths, respectively. Data were collected using X-rays with an energy of 517 eV (2.4 nm), and 16-bit images were recorded using a Peltier-cooled, back-illuminated, 2048 × 2048 soft X-ray CCD camera (Andor Technology PLC, Belfast). Projection images were collected using exposure times of 0.25 s. A full tomographic data set consisted of 90 images collected at 2° increments over 180° of rotation. In addition to the data images, 10 background images were collected (with the sample moved out of the field of view). Each data image was divided by the average of the background images, and the negative logarithm of the quotient calculated to give images whose gray values correlate directly with the X-ray absorption coefficients. The IMOD package (Kremer et al., 1996; Mastronarde, 1997) was used to align the individual images. Tomographic reconstructions were calculated using iterative reconstruction methods (Erdogan and Fessler, 1999; Stayman and Fessler, 2000). Segmentation models were generated with 3dmod (http://bio3d.colorado.edu/), Blender (www.blender.org) and Amira (Visage Imaging, USA).

[1] *Abbreviations used:* EM, electron microscopy; EqtII, Equinatoxin II; GFP, green fluorescent protein; MAHRP1, Membrane-Associated Histidine-Rich Protein-1; PfEMP1, P. falciparum erythrocyte membrane protein-1; PVM, parasitophorous vacuole membrane; RBC, red blood cell.