Intracellular structures of normal and aberrant Plasmodium falciparum malaria parasites imaged by soft x-ray microscopy

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ABSTRACT Soft x-ray microscopy is a novel approach for investigation of intracellular organisms and subcellular structures with high spatial resolution. We used x-ray microscopy to investigate structural development of Plasmodium falciparum malaria parasites in normal and genetically abnormal erythrocytes and in infected erythrocytes treated with cysteine protease inhibitors. Investigations in normal red blood cells enabled us to recognize anomalies in parasite structures resulting from growth under unfavorable conditions. X-ray microscopy facilitated detection of newly elaborated structures in the cytosol of fixed, unstained, intact erythrocytes, redistribution of mass (carbon) in infected erythrocytes, and aberrant parasite morphology. In cysteine protease inhibitor-treated, infected erythrocytes, high concentrations of material were detected in abnormal digestive vacuoles and aggregated at the parasite plasma membrane. We have demonstrated that an abnormal host erythrocyte skeleton affects structural development of parasites and that this aberrant development can be detected in the following generation when parasites from protein 4.1-deficient red blood cells infect normal erythrocytes. This work extends our current understanding of the relationship between the host erythrocyte membrane and the intraerythrocytic malaria parasite by demonstrating for the first time that constituents of the erythrocyte membrane play a role in normal parasite structural development.

Soft x-ray microscopy offers high resolution transmission images of thick biological samples maintained in aqueous solution (1–3). We applied soft x-ray microscopy to investigate development of the intraerythrocytic stages of the protozoan parasite Plasmodium falciparum in fixed, intact cells. P. falciparum is responsible for millions of deaths annually (4). Better understanding of the process of intracellular parasite maturation and the interactions between parasites and host erythrocytes can contribute to efforts to devise novel approaches to control this deadly disease.

Associations between intracellular organisms and host cells are complex and particularly difficult to examine. Enormous advances in the understanding of intraerythrocytic P. falciparum development have been achieved over the past 20 years through biochemical, molecular biological, and ultrastructural studies. Details of the substructures, organelles, and membranes of intraerythrocytic parasites have been obtained using thin section transmission electron microscopy (5–7).

With improved resolution and unique contrast from photoelectric absorption in x-ray microscopy, images show unusual features or structures not detected by other forms of microscopy. Image contrast is generated by differences in photoelectric absorption by the atoms in different areas (i.e., subcellular structures) throughout the sample. Absorption due to carbon dominates. Spatial resolution with x-ray microscopy is ∼50 nm with a 1 μm depth of focus.

We have generated the first images of the entire intraerythrocytic cycle of parasite development in normal human erythrocytes and have used these to evaluate normal structural development of parasites. We then investigated development in two different unfavorable environments, protease inhibitor-treated erythrocytes and genetically abnormal elliptocytes. Hemoglobin is degraded by malarial proteinases in the parasite digestive vacuole (8). Cysteine protease inhibitors cause the digestive vacuole to expand and fill with undegraded globin, ultimately leading to parasite death (9, 10). By bright field and electron microscopy, digestive vacuoles appear enlarged and filled with material that does not detectably differ from the contents of the erythrocyte cytosol (8). Hereditary elliptocytosis is a relatively rare red blood cell disorder that can be caused by a deficiency of protein 4.1, an 80-kDa skeletal protein that binds to spectrin and actin to provide mechanical stability to the erythrocyte membrane (11). Cysteine proteases that are totally deficient in protein 4.1 do not support normal development of P. falciparum (12–14). However, earlier investigations by bright field microscopy of parasite development in these abnormal erythrocytes did not detect morphological aberrations (12, 13, 15).

In the present study, we show previously undetected aberrations in the structure of parasites that developed in the presence of cysteine protease inhibitors. Additionally, we provide the first direct evidence for an effect of a mutation in an erythrocyte skeletal protein on morphology of intraerythrocytic stages of P. falciparum, an effect that we have shown may persist into the next generation in subsequent infection of normal red blood cells.

MATERIALS AND METHODS

Parasites. P. falciparum-infected erythrocytes were cultured in vitro as described (16). Parasites in this study either expressed or did not express histidine-rich protein 1 (HRP1) (17), and the associated P. falciparum erythrocyte membrane protein 3 (PfEMP3) (18), the mature parasite-infected erythrocyte surface antigen (MESA or PfEMP2) (19, 20) and a cytoadherence phenotype. HRP1 is localized to knob structures on trophozoite infected erythrocyte membranes (21), and binds to erythroid spectrin and actin (22). MESA binds to erythrocyte skeletal protein 4.1 (13, 23). PfEMP1 is a family of variant surface proteins that mediates cytoadherence of infected erythrocytes to venular endothelium (24–27).

Malayan Camp parasites (28) that express HRP1 and are cytoadherent were used in the time course study and the protease inhibitor experiments. FC27/D10 parasites (29) that express MESA, PfEMP3, and HRP1 and are cytoadherent

Abbreviations: HRP, histidine-rich protein; PfEMP, P. falciparum erythrocyte membrane protein; MESA, mature parasite-infected erythrocyte surface antigen.
were used to sequentially infect protein 4.1-deficient and normal erythrocytes. Clone C10, derived by limiting dilution at Lawrence Berkeley National Laboratory from D6 (30), does not express MESA, PIEMP3, or HRP1 and is not cytoadherent. Phenotypes of parasites were confirmed by indirect immunofluorescence and in cytoadherence assays as described (13, 27). We have previously shown that expression of these phenotypes is maintained during infection of protein 4.1-deficient erythrocytes (13).

To ensure a homogeneous population with respect to developmental stage, ring stage parasites were synchronized with sorbitol lysis 48 hr and 42 hr before the initiation of experiments (31) and, depending on phenotypic characteristics, trophozoite infected erythrocytes were purified either by flotation in 0.5% gelatin or on a Percoll density gradient 24 hr before the initiation of experiments (32, 33).

For time course experiments, aliquots of infected erythrocytes were removed from the cultures every 6 hr and washed three times with filtered phosphate buffered saline (PBS). In experiments with cysteine protease inhibitors, 100 μM leupeptin (Sigma), or 0.2 μM benzoyloxycarbonyl (Z)-Phe-Arg-CH₂F (ZFR) (Prototek, Dublin, CA) were added to culture media 12 hr after the initiation of experiments when parasites were early trophozoites. Erythrocytes were fixed in 1% or 2% glutaraldehyde. Ring stage parasites appeared discoidal and stippled. The darker area in the center of each parasite indicates a region of lower mass, compared with the erythrocyte cytosol, which extends from the parasite into the erythrocyte cytosol. This newly elaborated structure has not been seen before in fixed, unlabeled intact cells (exposure, 60 sec).

Normal and Protein 4.1-Deficient Erythrocytes. After informed consent, protein 4.1-deficient erythrocytes were obtained from a donor with complete deficiency of protein 4.1 (12) and normal erythrocytes were obtained from healthy volunteers and processed as described (13). To ensure that only the appropriate (protein 4.1-deficient or normal) erythrocytes were available for invasion by parasites, Percoll density gradients (33) were used to purify trophozoite-infected erythrocytes to >95% before inoculation of flasks containing uninfect ed erythrocytes (13).

The X-Ray Microscope XM-1. The x-ray microscope was built by the Center for X-Ray Optics at the Lawrence Berkeley National Laboratory. It uses x-rays from the Advanced Light Source and zone-plate optics for highest spatial resolution (1). This new instrument is optimized for biological work, as it incorporates state-of-the-art visible light microscopy for sample location, adjustment, and analysis prior to x-ray imaging of the sample. Analogous to bright field visible light microscopy, the sample is illuminated with a condenser Fresnel zone plate lens, and an enlarged image of the transmitted radiation is formed by an objective Fresnel zone plate lens. As atomic absorption cross sections are well known from other measurements (34), the images obtained with x-ray microscopy contain quantitative information on the total amount of mass per unit square (area density).

Soft x-rays of 2.4 nm wavelength were used to give a maximum of contrast. The spatial resolution for the experiments was ~50 nm (see Fig. 3E). Contrast is due to absorption of x-rays by the sample, suspended in 5–10 μm thick aqueous solution between two silicon nitride films, each 125 nm thick. Images were recorded directly on an x-ray sensitive charge-coupled device using ~1500 magnification and stored digitally with other parameters of the microscope. Field of view was about 10 microns. Exposure times of 20–60 sec were used. A 1 sec pre-exposure was always used to ensure that the sample was well centered. We observed subtle shrinkage of erythrocytes after one second exposures to x-ray radiation despite glutaraldehyde fixation. These short exposures seemed sufficient to stabilize the sample and eliminate further shrinkage.

RESULTS

Maturation of Intraerythrocytic Parasites in Normal Erythrocytes. Images of normal infected erythrocytes throughout the 48-hr cycle of development show an increase in size and structural complexity of parasites as they mature from the ring stage through the trophozoite stage to the multinucleated schizont. Infected erythrocytes collected from synchronous in vitro culture at the initiation of the experiment and at 6 hr reveal a discoid shape and a stippled, textured appearance for the ring stage parasite (Fig. 1A–C). The dark central area seen in many of the parasites is a region of higher density, probably an accumulation of material during early transport of hemoglobin into the digestive vacuole.

Samples collected from hours 12–36 show maturation of trophozoites as the parasite metabolizes hemoglobin and increases in volume (Fig. 2A–C). Trophozoites appear ellipsoidal and have regular, smooth boundaries. At 36 hr more structurally complex, multinucleated schizonts (segmenters), the asexually multiplying intraerythrocytic form, are seen (Fig. 2D). Developing individual merozoites can be seen, as well as centrally located conglomerated hemozoin, forming what is called the residual body.

Maturation of intraerythrocytic parasites was associated with progressive alterations and apparent disintegration of infected erythrocyte membranes (Fig. 2B and D). These alterations did not occur in uninfected erythrocyte membranes from the same in vitro cultures.

Elaboration of New Structures in the Erythrocyte Cytosol. Images of ring stage parasites reveal a region of reduced mass

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Fig. 1. Soft x-ray micrographs of intraerythrocytic ring stage P. falciparum malaria parasites imaged in normal erythrocytes. (A and B) Multiply infected erythrocytes from synchronized in vitro cultures were collected at the initiation of the experiment, washed three times in filtered PBS, and fixed in 1% or 2% glutaraldehyde. Ring stage parasites appeared discoidal and stippled. The darker area in the center of each parasite indicates more mass in the digestive vacuole (30 sec exposures). (C) An image of a singly infected erythrocyte removed from culture and fixed 6 hr after A and B shows a region of lower mass, compared with the erythrocyte cytosol, which extends from the parasite into the erythrocyte cytosol. This newly elaborated structure has not been seen before in fixed, unlabeled intact cells (exposure, 60 sec).
compared with the erythrocyte cytosol, having an apparently tubular structure, which seems contiguous with the parasite and extends into the erythrocyte cytosol (Fig. 1C; ref. 1). It has no visible internal structure compared with the associated parasite and seems to displace the erythrocyte cytosol. Because no such regions are detected in uninfected erythrocytes, this tubular structure is assumed to be induced by parasitization.

Images of trophozoites show a very dense area associated with and apparently surrounding the parasite that extends into the erythrocyte cytosol. This finding represents the first observation of a region of dense material peripheral to the parasite (Fig. 2A and B). We believe that this is the mature morphology of the tubular structure identified in erythrocytes infected with ring stage parasites.

Estimates of Parasite Volume. Assuming a spheroidal shape for parasites and an elliptical disk for erythrocytes, we estimated, through measurements from images, the volume occupied by the parasite. Volume of ring stage parasites is ≈5% of the host erythrocyte volumes at early stages of infection. In multiply infected erythrocytes, parasites of nearly equal size occupy up to 13% of the cell volume (Fig. 1A and B). Volume of digestive vacuoles at the ring stage is ≈11% of the parasite.

In early trophozoite images collected at 12 hr (Fig. 2A), parasites have grown to occupy ≈10% of host cell volume. As trophozoites mature, volume occupied by parasites increases, reaching ≈20% of host erythrocyte volume at 30 hr and ≈30% at 36 hr (Fig. 2B).

Effects of Protease Inhibitors. Dense regions that were visible surrounding trophozoites in normal infected erythro-

Fig. 2. Soft x-ray micrographs of intraerythrocytic trophozoite stage P. falciparum malaria parasites imaged in normal erythrocytes: untreated and treated with cysteine protease inhibitors. (A) An untreated trophozoite 12 hr after initiation of experiment. Note increase in size and structural complexity, and ellipsoidal form. Mass in the erythrocyte cytosol has been redistributed from brighter area (less mass) to dense region (indicated by arrow) surrounding the parasite (exposure, 30 sec). (B) Trophozoite at 30 hr has obvious digestive vacuole containing hemozoin (malarial pigment). Redistribution of hemoglobin at this time point is indicated by the brighter erythrocyte cytosol (less mass) and the dark, highly absorptive material in digestive vacuole. Arrows indicate dense regions surrounding the parasite (exposure, 60 sec). (C) Multiply infected erythrocyte at 36 hr (exposure, 30 sec). Individual merozoites (M) can be distinguished and the residue of the digestive vacuole [the residual body (RB)] is centrally located. Note advanced state of disintegration of erythrocyte membrane (exposure, 30 sec). (E and F) Leupeptin and (G) ZFR-treated trophozoites exhibit enlarged digestive vacuoles with extremely dense contents. Dense spheres (S) that appear to be in the parasite cytoplasm can be seen. The region surrounding the trophozoite is sheet-like and very dense, while the erythrocyte cytosol is depleted of hemoglobin (exposure, 30 sec). (H and I) ZFR-treated trophozoites exhibit unusual structures and disordered distribution of mass compared with untreated trophozoites. Cleft-like forms and apparently partitioned areas within parasites can be seen (exposure, 30 sec).
cytes became more pronounced in images of protease inhibitor-treated cells. In addition, small, dark, sharply contrasting spherical structures appear to be within the parasite cytoplasm (Fig. 2 E and G). Two previously undetected anomalies can be recognized in protease inhibitor-treated parasites. The majority of trophozoites, after being cultured for 12 hr or more in the presence of leupeptin or ZFR, provide unique and intriguing images of aberrant digestive vacuoles that occupy \(\approx25\%\) of estimated parasite volume (Fig. 2 E–G). These images show that disruption of hemoglobin utilization in the parasite results in a profound increase of mass in the vacuole, perhaps due to greatly increased concentration of undigested hemoglobin.

The second anomaly is detected in the structure of some ZFR-treated, but not leupeptin-treated, parasites. Trophozoites lack obvious digestive vacuoles and show more partitioning and have unusual cleft-like structures with redistributed mass compared with untreated parasites (Fig. 2 H and I). These structural consequences of treatment with ZFR are unique in our experience of analyzing parasite morphology in infected erythrocytes. Frequency of their occurrence can be determined using a larger sample size.

**Effects of Abnormal Erythrocyte Membranes on Parasite Morphology.** During the ring and early trophozoite stages no significant differences in parasite morphology were detectable between infected normal or protein 4.1-deficient erythrocytes. Occasionally we noted protuberances in the membranes of protein 4.1-deficient erythrocytes apposed to ring stage parasites (Fig. 3 A).

There was, however, striking variability and derangement in the structure of mature stages of intraerythrocytic parasites in protein 4.1-deficient compared with normal erythrocytes (Fig. 3 B–F). These anomalies do not resemble aberrations observed in ZFR-treated trophozoites. Instead of cleft-like structures, parasites in protein 4.1-deficient erythrocytes display complicated swirls and uneven, indistinguishable boundaries. Schizogony occurs in these abnormal erythrocytes, although organization of developing merozoites and the residual body within the protein 4.1-deficient erythrocytes appears more random compared with normal erythrocytes (Fig. 3 F).

**Anomalies in Parasites Grown in Abnormal Erythrocytes Retained in Subsequent Generations.** We used parasites that had completed one developmental cycle in protein 4.1-deficient erythrocytes to infect normal erythrocytes. Interestingly, structural derangements similar to those seen in protein 4.1-deficient erythrocytes were detected in some trophozoites during the first cycle of growth in normal erythrocytes (Fig. 4). This is the first evidence that maturation in an abnormal host cell affects structural development of the subsequent generation of parasites in a normal erythrocyte. These studies could not be extended into subsequent generations, however, because of diminisingly small numbers of rescued parasites. Indeed, this is the first report of reinvasion of normal erythrocytes after infection of erythrocytes totally deficient in protein 4.1 (12).

**DISCUSSION**

The structure seen in fixed, unlabeled, intact ring and trophozoite stage-infected erythrocytes is similar to pilus-like extensions of the parasitophorous vacuolar membrane that have been described using fluorescent lipid probes or parasites released from erythrocytes by osmotic shock (35, 36). Most
The parasite cytoplasm. Spheres probably represent small vacuoles involved in transport of material from the parasite plasma membrane to the digestive vacuole (40). Digestive vacuole abnormalities and the region of increased density surrounding parasides provide evidence of disruption by cysteine protease inhibitors of the vital pathway by which hemoglobin is transported and metabolized in *P. falciparum*. Additional abnormalities detected in some ZFR-treated trophozoites suggest either that ZFR inhibits cysteine proteases elsewhere in the parasite that function in parasite differentiation and development, or that the effects of inhibition of hemoglobin metabolism extend beyond the digestive vacuole to affect parasite development.

**Abnormal Erythrocyte Membranes Affect Parasite Structure.** Our ultrastructural studies demonstrate for the first time that an abnormal erythrocyte membrane has a direct effect on the structure of intraerythrocytic *P. falciparum* parasites. Previous studies (14, 41) suggested that red blood cell membrane constituents may be required for normal growth of parasites. In contrast to light microscopy (12, 13, 15), it is apparent with higher resolution x-ray microscopy that great disorganization in parasite structure, with internal partitioning and aberrant forms, results from maturation in protein 4.1-deficient erythrocytes.

It is not clear why there should be residual structural effects in trophozoites that develop in normal erythrocytes subsequent to infection of protein 4.1-deficient erythrocytes or why this is confined to only a subset of parasitized cells. It may be that some host skeletal proteins are actually carried over into newly invaded cells. This result suggests that a more complicated interaction exists between *P. falciparum* and the host erythrocyte membrane than previously demonstrated. It appears that, not only are host erythrocyte skeletal proteins important for structural development of parasites in an individual cell, but that host erythrocyte membranes may play an as yet undefined role in development of the subsequent generation of parasites.

**CONCLUSIONS**

These studies demonstrate that x-ray is a valuable approach that complements other microscopy technologies in investigations of intracellular organisms and subcellular structures. We have gained new insights into intraerythrocytic development of *P. falciparum* malaria parasites, including the detection of (i) newly elaborated structures in the cytosol of fixed, unstained, intact erythrocytes; (ii) redistribution of mass (carbon) in infected erythrocytes; and (iii) aberrant parasite morphology in adverse environments. Investigations in normal erythrocytes enabled us to detect anomalies in both parasites treated with potential chemotherapeutic agents and those that matured in erythrocytes with a membrane skeletal protein mutation. We have demonstrated that an abnormal host erythrocyte skeleton affects structural development of parasites and that this aberrant development can be detected in the following generation in normal erythrocytes. This finding demonstrates conclusively for the first time that constituents of the erythrocyte membrane play a role in normal parasite structural development and extends our understanding of the relationship between the host erythrocyte membrane and the intraerythrocytic parasite.

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