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The malaria digestive vacuole

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TABLE OF CONTENTS

1. ABSTRACT

During the development of malaria parasites within human erythrocytes, the fusion of digestive vesicles gives rise to a large digestive vacuole (DV). This organelle, which is maintained at low pH, processes 60–80 percent of the erythrocyte hemoglobin to provide a pool of amino acids that is crucial for parasite growth and development. During proteolysis, heme is released from hemoglobin as a toxic byproduct and is detoxified by biocrystallization to hemozoin. Proteases that contribute to hemoglobin breakdown, as well as other DV-associated proteins, arrive at this site via several different transport pathways. Antimalarial quinoline drugs, such as chloroquine, act by binding to heme and thus prevent its sequestration into hemozoin. Other drugs, such as artemisinin, may cause oxidative damage of DV macromolecules and membranes. The membrane of the DV contains ion pumps and transporters that maintain its low pH but are also pivotal in the development of parasite resistance to several antimalarial drugs. Methods for the isolation of the DV organelle have been developed to study the biogenesis and function of this important organelle.

2. INTRODUCTION

Malaria is a major cause of mortality and morbidity in tropical and subtropical regions of Latin America, Africa, the Indian subcontinent, and Southeast Asia, where approximately 40% of the world's population is at risk of infection. The disease is caused by unicellular, eukaryotic parasites of the genus *Plasmodium*. Of the five malaria parasites known to infect humans (*falciparum, vivax, malariae, ovale* and *knowlesi*), *P. falciparum* is the most virulent and responsible for an estimated 225 million clinical cases and 781,000 deaths annually (1, 2). Since resistance to a range of antimalarial drugs is spreading throughout parasite populations worldwide and a protective vaccine remains unavailable, there is an urgent need to develop novel therapeutic strategies.

The life cycle of *Plasmodium* can be divided into three phases: the pre-erythrocytic hepatic stage and the intraerythrocytic asexual cycle in humans or other vertebrate hosts, and the sexual cycle in female *Anopheles* mosquitoes. Clinical manifestations of malaria are primarily due to the infection of erythrocytes by

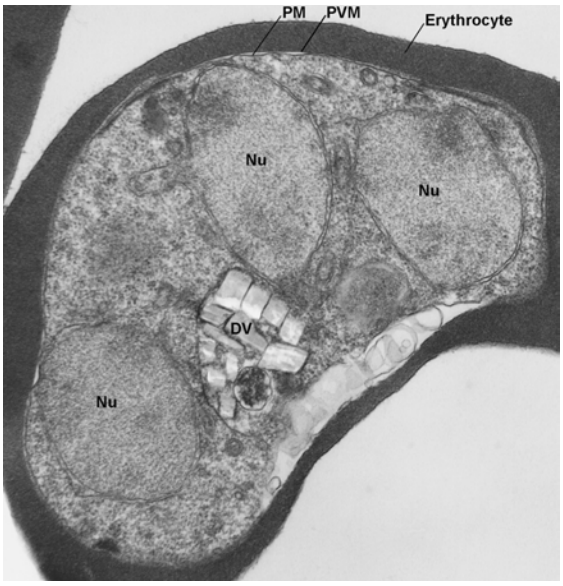

Figure 1. Electron microscopy image of a *P. falciparum*-infected erythrocyte (HB3) at the early schizont stage containing a DV with ordered hemozoin crystals. Dense hemoglobin is observed in the host erythrocyte. The samples were fixed with 2.5% glutaraldehyde, ultrathin sections of 60 nm thickness were prepared and electron micrographs were taken using a Zeiss EM10 transmission electron microscope as described (261, 262). Nu: nucleus, DV: digestive vacuole, PM: parasite plasma membrane, PVM: parasitophorous vacuole membrane. Image courtesy of Hannes Wickert.

intracellular parasites (3). The asexual intraerythrocytic cycle begins with the erythrocyte invasion by a merozoite, which is then enclosed within a newly formed parasitophorous vacuole (PV), separate from the erythrocyte cytoplasm. Three intraerythrocytic stages can be distinguished morphologically: (a) the ring stage, which lasts 22–24 h from the time of merozoite invasion in *P. falciparum* and during which metabolic activity is low, (b) the trophozoite stage, which is 10–12 h in duration and characterized by an acceleration of metabolic processes as well as the consumption of most of the hemoglobin-rich erythrocyte cytoplasm (4), and (c) the schizont stage where parasites undergo 4–5 rounds of binary division in 8–10 h, generating up to 32 merozoites. Schizonts eventually rupture their host cells, releasing the merozoites that invade new erythrocytes and repeat the cycle.

To support its growth and replication, the parasite takes up large amounts of erythrocyte cytosol and digests its primary constituent, hemoglobin, in a specialized acidic organelle called the digestive vacuole (DV) or food vacuole (5, 6). Large pigment-containing vacuoles were first described in electron microscopy studies of thin sections of *P. knowlesi* in 1956 (7) and a role for this structure in digestion of hemoglobin was first suggested by Rudzinska and Trager in 1957 (8). As in lysosomes of mammalian cells and yeast vacuoles, an acidic pH is maintained and several different proteinases are present in the DV. However, the absence of the typical lysosomal acid phosphatase and glycosidases, which release phosphate groups and smaller sugars from macromolecules, respectively, indicates that the DV of *Plasmodium* is a specialized organelle that evolved to efficiently degrade hemoglobin. The parasite apparently does not need to degrade macromolecules other than hemoglobin during its intraerythrocytic cycle (9, 10). Proteolytic degradation of hemoglobin, however, results in the release of toxic heme which is neutralized by the parasite primarily by the formation of pigments composed of hemozoin (5, 6, 11). Hemoglobin hydrolysis as well as heme detoxification are thus essential for the parasite's survival in the host erythrocyte (4, 12).

Due to the presence of two plant-type vacuolar proton pumps in the DV membrane, the vacuole has also been compared to the tonoplast, the acidic intracellular vacuole of plant cells (13), in which these H^+-pumps act in concert to maintain a low pH. Moreover, the product of the gene PF13_0353, a putative cytochrome b_5 reductase localized in the DV membrane, was found to be structurally similar to a plant nitrate reductase (14, 15). The two plant-type H^+-pumps are also involved in the acidification of the lumen of acidocalcisomes, small electron-dense granules of some protozoan parasites that store Ca^{2+} as a phosphate precipitate (16, 17). However, DVs are morphologically and functionally very distinct from acidocalcisomes, which are involved in calcium homeostasis and polyphosphate metabolism (17, 18).

DVs are also observed in unicellular organisms other than malaria parasites. Electron microscopy studies demonstrated the presence of a DV in *Babesia equi*, an intraerythrocytic parasite related to *Plasmodium* (19). The anaerobic protozoan parasite *Entamoeba histolytica,* the causative agent of amebiasis, ingests erythrocytes and degrades them with their contained hemoglobin in several acidic DVs using cysteine proteinases (20). The soil-living amoeba *Dictyostelium discoideum* (21) as well as the apicomplexan parasite *Theileria* (22), but not *Toxoplasma*, also contain a large acidic DV, although they do not degrade hemoglobin.

3. STRUCTURE AND FORMATION OF THE DV

The DV of *Plasmodium* contains hydrolytic enzymes of various mechanistic classes that digest hemoglobin (23, see Sect. 4). Microscopically, hemoglobin degradation is indicated by the alteration of the density of DVs (24). The process of hemoglobin digestion leaves insoluble waste products in the DV due to the release of heme (ferriprotoporphyrin IX), which is neutralized by the formation of coordinated hematin dimers (β-hematin) that biocrystallize to the chemically inert hemozoin, also known as the malaria pigment (11, 25-27). Hemozoin crystals can be observed by light and electron microscopy as they are very dense structures (7). These are lined up along a single axis in intact parasites (Figure 1). By contrast, disordered crystals are observed in isolated DVs, possibly due to isolation or fixation artifacts or the loss of the transmembrane pH gradient between the erythrocyte

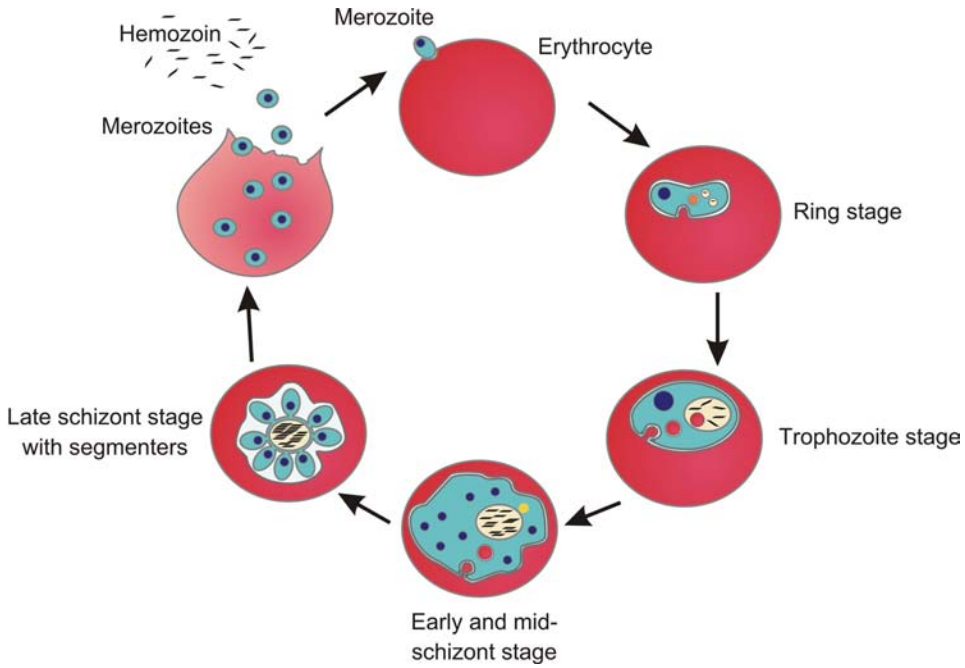

Figure 2. Biogenesis and morphology of the digestive vacuole through the asexual intraerythrocytic cycle of *P. falciparum*. Merozoites released after the rupture of the host erythrocyte invade new cells and transform to the ring stage. In early ring-stage parasites, erythrocyte cytoplasm containing hemoglobin (red) is taken up from the surface into small DVs by micropinocytosis. Hemoglobin is digested in these compartments while toxic heme is complexed as hemozoin. As the parasite develops, a single large DV (beige) is formed at the late ring stage and increases in size in trophozoites. Host erythrocyte cytoplasm is then ingested through the cytostome, a cytoskeletal ring, and transported to the DV within small double membrane-vesicles. In the mid-schizont stage, a lipid body (yellow) forms in the periphery of the DV. Maximum hemoglobin digestion is observed during the trophozoite stage; during schizogony, the DV decreases in size. Hemozoin is released into the blood circulation when the erythrocyte ruptures and can induce inflammatory responses in the host. Turquoise: parasite cytoplasm, blue: nucleus. Adapted from (30).

cytoplasm and the DV during isolation (9). It was proposed that this gradient could establish an electromotive force across the vacuole in intact parasites that causes the iron-containing crystals to align within the generated magnetic field (9).

The DV is not inherited by daughter merozoites but forms *de novo* after each round of infection (28). The biogenesis, morphology, size and number of DVs vary between developmental stages of the parasite (Figure 2). Multiple small dense vacuoles with a diameter of 150 – 300 nm are characteristic of the early and mid-stage rings (29). They are formed by micropinocytosis of host cell stroma from the parasite surface that results in double membrane-vesicles that function as individual DVs (30). The inner membrane, which develops from the parasitophorous vacuole membrane (PVM), rapidly disappears, allowing more efficient nutrient diffusion into the parasite cytoplasm. Hemoglobin is digested and hemozoin crystals are generated at this early stage (30, 31). In late ring-stage parasites, the cytostome, a cytoskeletal ring for the uptake of erythrocyte cytoplasm, appears (32, 33) and the small pigment-containing vesicles coalesce to form a single large DV (30). A recent study, using thin-section transmission electron microscopy (TEM) and 3-D reconstruction, postulated that early ring-stage parasites take on the shape of a cup that closes and internalizes a large amount of erythrocyte cytoplasm in a single event called the 'Big Gulp'. The authors suggested that the DV is derived from the so-formed vacuole rather than several smaller compartments (34). However, this hypothesis remains controversial (35, 36).

Through the cytostome, erythrocyte cytosol is internalized together with the membrane of the PV and the parasite's plasma membrane (32, 37, 38). This engulfment could increase the surface of exchange between erythrocyte and parasite (39). The outer membrane of the cytostomal system is contiguous with the parasite's surface and its inner membrane originates from the PVM (28). Portions of hemoglobin-rich host cytoplasm are pinched off from its distal end and transported to the DV within small double membrane-vesicles. The outer membrane of these vesicles is probably incorporated into the DV membrane and the inner membrane is subsequently degraded by phospholipases and proteinases inside the vacuole (30, 31, 38, 40). A knockout of the gene encoding the DV aspartic proteinase plasmepsin (*Plasmodium* pepsin) IV (PfPM4) caused abundant accumulation of electron-dense vesicles in the DV in 30% of the *P. falciparum*-infected cells as compared to wild-type Dd2 parasites, suggesting that PfPM4 might be involved in the elimination of vesicle

membranes within the DV (41). The lining membrane of the DV has been described as a mosaic of different molecular compositions with a patchy distribution of DV markers, such as the 19 kDa fragment of merozoite surface protein 1 ($PfMSP1_{19}$) and the chloroquine resistance transporter (PfCRT, 30, 42, 43), probably due to fusion with membranes from the cytostomal system (30) and Golgi-derived vesicles containing proteinases (23).

Hemoglobin degradation occurs predominantly during the trophozoite stage, at which time the DV is enclosed by a single membrane and has a diameter of up to 2.2 μm (30), occupying a significant part of the total volume of the parasite (Figure 1). Late trophozoites show dilated DVs with widely spaced hemozoin, internal membranous structures and an inward folding of the vacuolar membrane (30). During schizogony, the vacuole decreases in size (0.8 – 1.2 μm), loses the folds of its outer membrane as well as the internal membranes (44). Adjacent to the DV, a lipid body of a few hundred nanometers in size forms, whose constituents are probably retrieved from the DV interior, the DV wall, as well as the inner membrane of transport vesicles that fuse with the DV membrane (30). These neutral lipids (44) as well as histidine-rich protein II (HRPII) (45) were suggested to promote β-hematin formation.

In schizonts, large hemozoin crystals are formed, which appear closely packed (30). When the mature schizont ruptures the erythrocyte, merozoites are liberated and leave behind the malarial pigment within a membrane-bound residual body that is released into the blood stream and avidly engulfed by macrophages (30). Released native hemozoin bound to malarial DNA was shown to induce inflammatory responses via binding to Toll-like receptor 9 (TLR9) on the endosomes, resulting in cytokinemia and fever during disease (46). Furthermore, chemically synthesized hemozoin, which is structurally identical to the native crystal, was reported to be sufficient to induce the production of the pro-inflammatory cytokine interleukin-1β (IL-1β) by phagocytes via the activation of the NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome, which indicates that this metabolic product is critical in malaria pathology (47).

The structure and formation of the DV varies between different *Plasmodium* species. Whereas only one DV was observed in *P. falciparum*, *P. knowlesi* contains several smaller acidic compartments (7). In contrast to other human as well as murine *Plasmodium* species, the individual DVs fuse together very rapidly in *P. falciparum* (31). The differences between the cytostomal system of murine parasites such as *P. chabaudi*, which comprises a long tube connected with a branching tubular network, and that of *P. falciparum*, showing vesicles budding off from the cytostome (31, 48), are consistent with the phylogenetic relationships between the species (40), i.e., the feeding mechanism of *P. falciparum* is more similar to avian than either rodent or other human *Plasmodium* species (33). A cytostome with long tubular structures was also described in *Babesia equi* (19) and some species of *Theileria* (22, 49).

4. HEMOGLOBIN DIGESTION

The primary function of the DV in *Plasmodium* is the degradation of hemoglobin (9), which comprises 95% of the cytosolic erythrocyte protein, corresponding to a concentration of 310 – 350 mg/ml (50). During intraerythrocytic development, the parasite endocytoses large quantities of host cell cytosol and digests 60 to 80% of its hemoglobin (4, 51-53). The most widely suggested purpose of hemoglobin catabolism is the supply of amino acids for the synthesis of new proteins since intraerythrocytic stages of *Plasmodium* only have a limited capacity of *de novo* amino acid synthesis (6). This is supported by the observation that radiolabeled amino acids released during hemoglobin degradation are incorporated into plasmodial proteins (54, 55). However, parasite development also depends on amino acids that are rare (Met, Cys, Gln, Glu) or absent (Ile) in hemoglobin, which must be obtained elsewhere (56). Ile has been shown to be of particular significance as *P. falciparum* can be cultured in medium with Ile as the sole exogenous source of amino acids (4).

According to a study by Krugliak *et al.* (52), only 16% of the amino acids generated from hemoglobin breakdown are used for protein assembly. This observation suggests that hemoglobin uptake and catabolism could have additional functions, such as the prevention of erythrocyte lysis (57, 58). Since the parasite increases its volume 25-fold in one intraerythrocytic cycle (59), it has been suggested that degradation of host cell cytoplasm might be necessary to make room for parasite growth (57). Furthermore, the diffusion of some hemoglobin-derived amino acids into the host cell (58) could be important for the regulation of intracellular osmolarity of the infected erythrocyte during the maturation and replication of the parasite (52, 60, 61). The use on hemoglobin as a nutrient source is likely to vary with culture conditions (62), given that the entire asexual cycle of *P. falciparum*, while limited and dependent on constituents of the erythrocyte membrane and stroma, can occur extracellularly (63-66). Interestingly, even axenically grown parasites have a functional DV containing hemozoin crystals (63, 64, 66).

Hemoglobin hydrolysis in the *Plasmodium* DV is thought to be a semi-ordered process mediated by the action of a series of proteases (9). Plasmepsins (aspartic proteases) and falcipains (cysteine proteases) are involved in the initial steps of the pathway (Figure 3). Out of the ten plasmepsins encoded in the genome of *P. falciparum* (67), four are localized and function in the DV: PfPM1, PfPM2, PfHAP (histoaspartic proteinase) and PfPM4 (68). The plasmepsins PfPM1 and PfPM2 can cleave non-denatured hemoglobin at Phe^{33} and Leu^{34} in the hinge region of the α-globin chain, probably causing the globin subunits to unwind and allowing further proteolysis (9, 69). PfHAP and PfPM4 have a significantly lower capacity to digest hemoglobin as compared to PfPM1 or PfPM2 but are capable of actively cleaving globin *in vitro* (68). The four DV plasmepsins, as well as the falcipains PfFP-2, PfFP-2' and PfFP-3, cleave these large globin fragments into polypeptides (6, 70). PfFP-2 (71) and PfFP-3 (72) are also

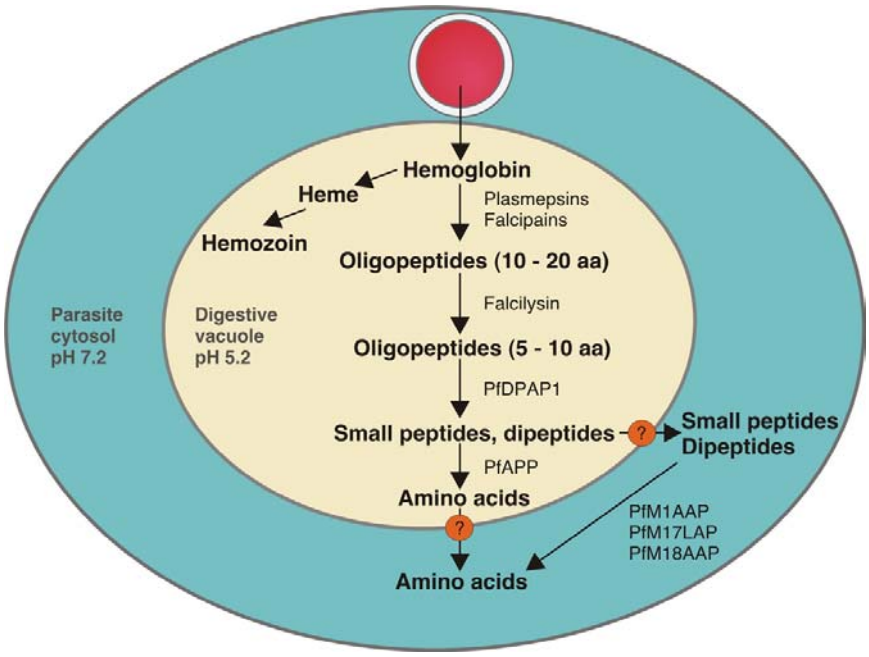

Figure 3. Generation of free amino acids from hemoglobin. Hemoglobin degradation involves initial cleavage by plasmepsins and falcipains into large fragments that are further digested into smaller peptides by falcilysin. The dipeptidyl aminopeptidase 1 (PfDPAP1) may release dipeptides from the N termini of these oligopeptides. Some of the generated small peptides might be transported out of the DV into the parasite's cytoplasm by an unknown transporter. The cleavage into amino acids might occur in the DV by the acidic aminopeptidase P (PfAPP) as well as in the cytoplasm by the aminopeptidases PfM1AAP, PfM17LAP and/or PfM18AAP.

able to digest native hemoglobin and might thus participate in its initial cleavage.

Whereas *P. falciparum* and the chimpanzee parasite *P. reichenowi* contain four DV plasmepsins, whose genes are located adjacent to one another on chromosome 14, all other examined *Plasmodium* species only have an ortholog of *PfPM4*. *PfPM1, PfPM2* and *PfHAP* are thus paralogs of *PfPM4* that may have arisen through gene duplication (73). The close phylogenetic relationship between *P. falciparum* and *P. reichenowi* strengthens with this finding (74, 75). In *P. falciparum*, the four DV plasmepsins and the falcipains are expressed at different times throughout the life cycle, which indicates that these enzymes have distinct functions at different developmental stages of the parasite. The highest transcript levels of *PfPM1* and *PfPM4* were detected in ring-stage parasites, while *PfPM2, PfHAP, PfFP-2* and *PfFP-2'* mRNAs were most abundant in trophozoites and PfFP-3 was synthesized at the late trophozoite and early schizont stage (76-78).

While the metalloprotease falcilysin, which is expressed in trophozoites (78), is unable to digest native hemoglobin or denatured globin, it can degrade polypeptide fragments of up to 20 amino acids into short peptides that are 5–10 amino acids in length (79). The DV dipeptidyl aminopeptidase 1 (PfDPAP1), a plasmodial ortholog of mammalian cathepsin C (80, 81) whose expression level is highest at the trophozoite stage (78, 82), might be involved in the cleavage of dipeptides from the N terminus of hemoglobin-derived oligopeptides (83).

How and where amino acids are released from these hemoglobin-derived dipeptides remains to be fully elucidated. Since aminopeptidase activity was detected in the parasite cytoplasm but not in the DV and was found to be optimal at neutral pH, it was suggested that short peptides are actively transported out of this acidic compartment and subsequently cleaved into amino acids by neutral aminopeptidases in the parasite's neutral cytoplasm (83-88), as seen for lysosomes (89). However, recent studies have reported the presence of aminopeptidase P (PfAPP) and M1 alanyl aminopeptidase (PfM1AAP) in the DV (90-92). Whereas the optimum pH of PfM1AAP is 7.4 (88), PfAPP is active at pH 5.0 to 7.5 and might function in the acidic environment of the DV (91). Therefore, peptides generated by the concerted action of various endo- and exopeptidases are probably cleaved into amino acids and small peptides within the DV as well as in the cytoplasm of the parasite. Small peptides that are transported into the parasite's cytoplasm likely undergo terminal degradation by aminopeptidases stationed here, such as PfM1AAP, M17 leucyl aminopeptidase (PfM17LAP) and M18 aspartyl aminopeptidase (PfM18AAP; 93, Figure 3). These enzymes function optimally at neutral pH (84, 87, 94) and are maximally expressed in early trophozoites, whereas peak expression of PfAPP occurs at the late ring stage (95, www.plasmodb.org).

It was suggested that P-glycoprotein homolog 1 (Pgh-1), a member of the ATP-binding cassette (ABC) transporter superfamily in the DV membrane, might be involved in the export of small peptides because *pfmdr1*

expressed in a heterologous yeast system was able to complement *Ste6*, which encodes an ABC-transporter that translocates a 12-amino acid peptide across the vacuolar membrane in *S. cerevisiae* (96). However, Pgh-1 was shown to be an inwardly-directed transporter, which imports solutes into the DV (97). Thus, peptide export from the DV is most likely mediated by another transporter.

The inhibition of plasmepsins and falcipains by a combination of the aspartic protease inhibitor pepstatin and the cysteine protease inhibitor E-64 leads to a complete block of hemoglobin proteolysis and parasite development, indicating that hemoglobin digestion is essential to the survival of the parasites and that these hemoglobinases are potential targets for antimalarial chemotherapy (62, 69, 98). However, knockout studies have shown that no single DV plasmepsin is essential to *P. falciparum* intracellular growth (41), which suggests that these enzymes are redundant in their functions. Furthermore, falcipains are able to compensate for the loss of all four DV plasmepsins (4, 99), revealing a functional overlap between these two protease classes. The observed redundancy within and between protease families might be a strategy by which the parasite ensures a sufficient level of proteolytic activity (70). Hemoglobin digestion can also be blocked either by inhibiting the cysteine proteinases with leupeptin and E-64 (100), or upon disruption of the gene encoding the cysteine proteinase PfFP-2 (101), both resulting in undigested hemoglobin-rich erythrocyte cytoplasm accumulated in the DV (100, 101). In PfFP-2 knockout parasites, accumulation of undigested hemoglobin only occurs at the trophozoite but not at the schizont stage, when higher expression of PfFP-3 might cause phenotypic rescue (101). As *PfFP-3* disruption has not been achieved, in contrast to the other falcipain genes, PfFP-3 might play an important role in parasite survival (102).

Inhibitors of the neutral aminopeptidases PfM1AAP and PfM17LAP have been shown to be lethal for *P. falciparum* in culture as well as for *P. chabaudi in vivo*, thus providing proof of concept for considering these enzymes as targets for future antimalarial drugs (93, 103). Both aminopeptidases are encoded by single-copy genes and gene knockout experiments (90, 93) as well as a study using enzyme-specific inhibitors (92) have shown that these are of critical importance for parasite survival and are not functionally redundant. However, it is not clear whether the site of action of the aminopeptidase inhibitors is in the DV, the cytoplasm, or both. While strong synergism was observed between inhibitors of aspartic and cysteine proteases using *in vitro* malaria killing assays, little synergism was observed between aminopeptidase inhibitors and aspartic or cysteine protease inhibitors (104).

During the proteolytic pathway, large quantities of heme are released that can disrupt membranes, inhibit enzymatic processes and initiate oxidative damage (5, 53, 105). The detoxification of this toxic byproduct can be accomplished by peroxidative degradation (53), decomposition by glutathione (106) or conversion into the biologically inert crystalline polymer hemozoin (11, 25-27). Thus, the DV has other functions in addition to hemoglobin digestion, such as detoxification of heme and oxygen radicals (39), storage of non-degradable biomolecules, already evident in hemozoin sequestration, and regulation of intracellular osmolarity of the infected erythrocyte (52, 60, 61).

5. ION HOMEOSTASIS

Ions play an important role in many biochemical reactions and the ability of cells to maintain intracellular levels of essential ions and ionic gradients is critical for a variety of cell functions. In the DV, both pH and calcium (Ca^{2+}) homeostasis have been investigated.

5.1. pH homeostasis

Many of the enzymes involved in hemoglobin degradation, such as cysteine, aspartic and metalloproteinases, have reported pH optima in the range of 4.5 to 5.5 (9, 69, 107). The DV needs to maintain a similar pH to allow for efficient hemoglobin proteolysis and the biocrystallization of heme to hemozoin (108, 109).

The mechanisms by which the DV pH (pH_{DV}) is maintained remain unclear. It was suggested that inwardly-directed proton pumps (mainly an H^+-ATPase and partially an H^+-translocating pyrophosphatase) are involved (110-112) that also work against the leak of protons out of the DV (113). Quinoline drugs, such as chloroquine (CQ), quinine (QN) and mefloquine (MQ), may increase pH_{DV} via weak base and non-weak base effects at their biologically active concentrations, effectively preventing the function of parasite acid proteases (9, 114-117). Knowledge of how new drug compounds interfere with pH regulation in the parasite, which is crucial for parasite survival, could give insights in their potential efficacy in malaria treatment.

It has long been debated if, and to what extent, pH_{DV} plays a role in determining levels of CQ accumulation and hence the CQ sensitivity of the parasite (117, 118). Since CQ and many other 4-aminoquinolines are weak bases, their uptake is influenced by passive diffusion along the pH gradient between the extracellular and intracellular compartments (38, 119). It was postulated that an increased pH_{DV} in CQ-resistant (CQR) parasites and thus a reduction in the transvacuolar pH gradient would result in a decreased uptake of CQ into the DV (114, 117, 120).

The first measurements of steady-state pH_{DV} were performed by spectrofluorometry using the pH-sensitive fluorescein-dextran and gave a value of approximately 5.2 (120). This was followed by Ginsburg *et al.* using the membrane-permeable fluorochrome acridine orange (AO), where a pH_{DV} value of approximately 4.2 was determined in a spectrofluorometric setting (121). More than ten years later, values of 5.2 − 5.6 were reported using AO and epifluorescence microscopy (122, 123). These measurements were later determined unreliable since AO was shown to be very photosensitive and causes lipid peroxidation leading to DV membrane lysis (124, 125). Using the ratiometric pH-sensitive dye

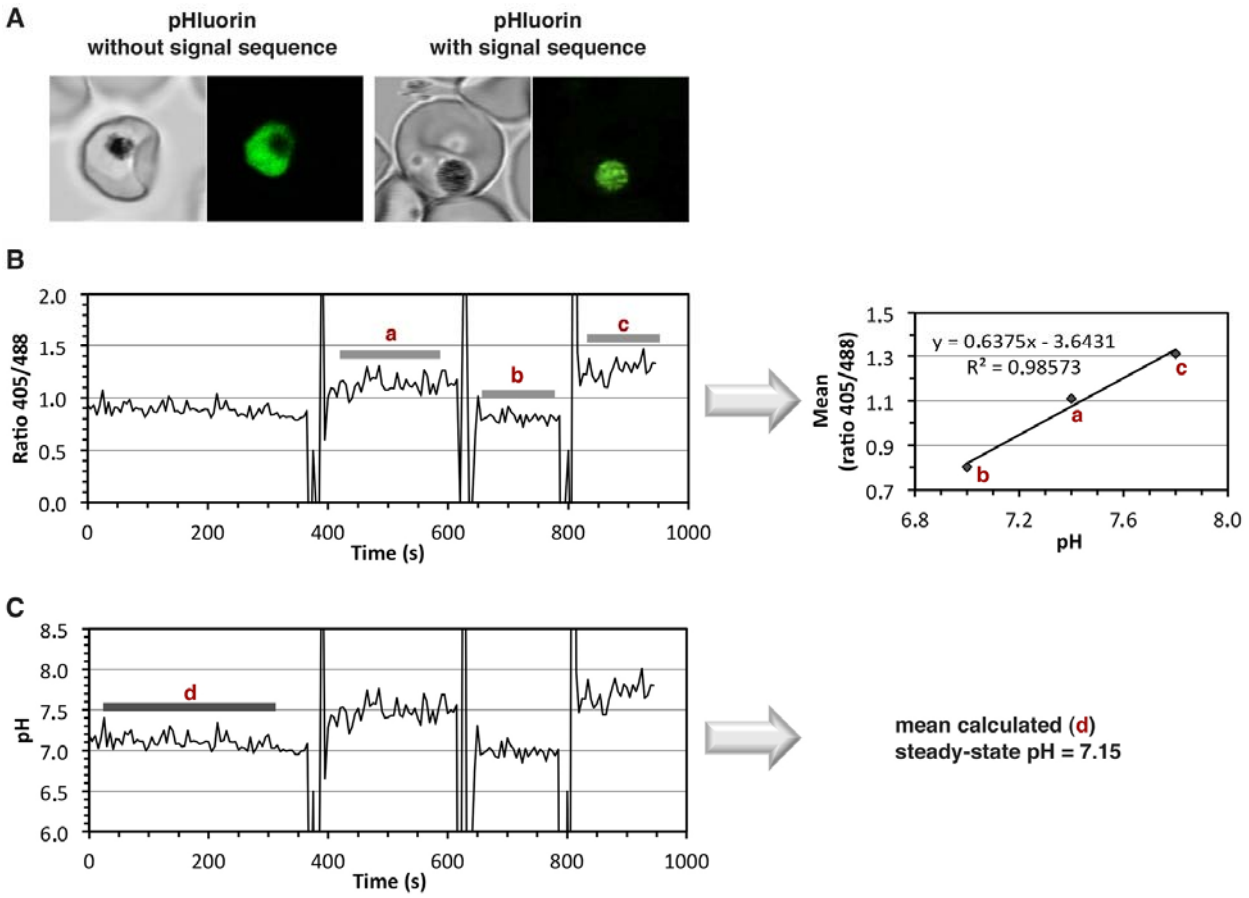

Figure 4. pH quantification within the cellular compartments in *P. falciparum*-infected erythrocytes. (A) Localizing the expression of the pH-sensitive green fluorescent protein pHluorin in *P. falciparum*. pHluorin alone is expressed in the cytosol of the parasite. By contrast, with the addition of a signal sequence, consisting of the first 70 amino acids of plasmepsin IV (PF14_0075), pHluorin was specifically targeted to the digestive vacuole. (B) Calibration of the fluorescence of pHluorin *in situ*. Live parasites expressing pHluorin were dually excited with 405 and 488 nm laser lines. A time course was initiated consisting of approximately 350 seconds of steady-state fluorescence measurements, followed by a three-point pH calibration using standardized buffers (a = pH 7.4, b = pH 7.0, and c = pH 7.8). Regions of interest, i.e. the cytosol (shown here) or the digestive vacuole, were selected and a calibration curve was plotted using the average stabilized fluorescent ratio 405/488 of each buffer used. (C) Using the equation of the calibration curve, the fluorescent ratio 405/488 of pHluorin was converted to pH values. The mean calculated pH (d) is the average pH value of the initial steady-state measurement. Using this method, a precise pH of 7.15 was determined for the parasite cytosol and pH 5.18 for the digestive vacuole.

DM-Nerf and confocal fluorescence microscopy, the pH_{DV} values of 5.2−5.6 were confirmed (126). However, these results could not be reproduced by other groups in subsequent studies using the same fluorochrome. In 2006, Hayward *et al.* determined pH_{DV} using spectrofluorometry and reported pH values that ranged from 3.7 to 6.5 depending on the fluorochrome used for measurement and what concentration was applied (118). Furthermore, the group reported comparable pH_{DV} values for CQ-sensitive (CQS) and CQR parasites (118), challenging previous studies (122, 123, 126, 127). This result was confirmed by two subsequent studies (128, 129), showing comparable pH_{DV} values for CQS and CQR parasites. Whereas Hayward *et al.* (118) and Klonis *et al.* (128) analyzed parasite populations of different strains using spectrofluometry and flow cytometry, respectively, Kuhn *et al.* (129) studied single live parasite-infected erythrocytes by confocal fluorescence microscopy and calibrated the cytoplasmic and digestive vacuolar fluorescence ratios for each cell by selecting defined regions of interest (Figure 4). This method allows for site-specific pH measurements in the intact parasite. Using *P. falciparum* expressing pHluorin, a ratiometric pH-sensitive GFP (130), pH values of 7.15 ± 0.07 in the cytoplasm and 5.18 ± 0.05 in the DV of the parasite were determined, respectively (129). These pH determinations were independent of intracellular pHluorin concentrations and the non-invasive nature of the technique was shown to preserve the physiological conditions of the parasite (125, 129). This method was validated by confirming the measured pH values of the cytoplasm using the pH-sensitive fluorochromes SNARF-1 AM and SNARF-5F AM (129).

Table 1. Structure and function of the transport proteins in *P. falciparum* that have been localized to the digestive vacuole membrane

Transporter	Structure	Function
Pgh-1 (PfMDR1)	162-kDa protein Two homologous domains, each with six transmembrane domains followed by a hydrophilic nucleotide-binding fold facing the cytoplasm (160, 263, 264) Member of ABC transporter superfamily	Implicated in resistance to several antimalarial drugs (139) Import of solutes, including some antimalarial drugs, into the DV (97, 166)
PfCRT	45-kDa protein with ten predicted transmembrane domains (234) Member of drug/metabolite transporter superfamily (177, 178)	Export of drugs and metabolites out of the DV (179-181) Polymorphisms associated with chloroquine resistance (138, 173)
V-type H^+-ATPase	Multisubunit enzyme complex Cytosolic V_1 domain with ATP catalytic site: subunits A–H Transmembrane V_0 domain: subunits a, c, c" and d (112)	Major role in acidification of DV lumen, maintenance of transvacuolar pH gradient (111, 190, 191) ATP hydrolysis (111)
V-type H^+-PPase (PfVP1)	76.4-kDa protein (111) 15-16 membrane-spanning domains (193)	Minor role in acidification of DV lumen (111) Pyrophosphate hydrolysis (13, 16, 111)

The similar pH_{DV} values of different sensitive and resistant parasite strains indicate that mechanisms other than altered drug uptake and/or pH-dependent changes in heme solubility, turn-over or biocrystallization rates are responsible for the CQR phenotype as well as the reduced drug accumulation observed in CQR parasites (131-133). A more favored explanation is that alterations in carrier-mediated drug efflux reduce the CQ concentration in the DV of CQR parasites (21, 133-136). It was suggested that mutations in the DV membrane proteins Pgh-1 and PfCRT (42, 137, 138, see Sect. 6) cause CQ resistance (138, 139).

5. 2. Calcium homeostasis

Calcium is a well-known second messenger and Ca^{2+} ions have been shown to be important for parasite development (140). In *P. falciparum*, Ca^{2+} signaling is involved in invasion (141-143), maturation (144), synchronization (140), exflagellation and gamete formation (145, 146). For the intraerythrocytic parasite, Ca^{2+} is not readily available since the Ca^{2+} concentration in the host erythrocyte cytoplasm is low. For this reason, the parasite must build up a regulatory Ca^{2+} pool to maintain its Ca^{2+} homeostasis (147, 148), a function normally associated with ATP-dependent endoplasmic reticulum (ER) pumps in eukaryotes (149). The DV was initially suggested to act as a dynamic Ca^{2+} store from fluorescence studies using the non-ratiometric green fluorescent Ca^{2+} indicator Fluo-4 (18) but confocal fluorescence studies using the ratiometric Ca^{2+} indicator Fura-Red showed that this organelle is unlikely to serve as a Ca^{2+} pool (150). X-ray spectral analysis and elemental mapping confirmed no increase in Ca^{2+} levels within this organelle (150). The intense Fluo-4 fluorescence observed from the DV was thought to be due to the pH dependence of the dye and its active transport into the DV through Pgh-1 rather than an increased Ca^{2+} content (97, 150). It is more likely that the parasitophorous vacuole (148), the acidocalcisomes (151) the mitochondria (147, 152) and/or the ER (153, 154) carry out the function of a Ca^{2+} store and regulator.

6. TRANSPORTERS IN THE DV MEMBRANE

The maintenance of ion homeostasis in the parasite, as well as nutrient uptake, is mediated by integral membrane proteins that are able to transport ions and other molecules across a biological membrane. Since these processes are important for survival, growth and replication of the parasite, transport proteins of *P. falciparum* are promising drug targets although only a few of them have been characterized to date (155). Using a genomic database approach, the 'permeome' of the malaria parasite was characterized and more than 100 transport proteins encoded by *P. falciparum* were examined for a range of organic and inorganic substrates (156). Table 1 shows a list of proteins that are known to be localized in the DV membrane and function as transporters in *P. falciparum*.

P-glycoprotein homolog 1 (Pgh-1), also known as multi-drug resistance protein 1 (PfMDR1), is a member of the ABC (ATP-binding cassette) transporter superfamily and an ortholog of mammalian P-glycoproteins that mediate multi-drug resistance in cancer cells (157). It is encoded by the *mdr* homolog *pfmdr1* (158, 159), predominantly localizes to the DV membrane (160, 161), with its ATP-binding domain facing the cytoplasm (160, 162), and is expressed during the intraerythrocytic cycle of *Plasmodium*, with a peak of *pfmdr1* mRNA abundance at the schizont stage (163). This transporter is known to play a role in resistance to several antimalarial drugs (139). A strong association has been observed between *pfmdr1* amplification, which leads to increased Pgh-1 expression, and MQ treatment failure (164). *In vitro* cross-resistance to QN, lumefantrine (LF), halofantrine (HF) and artemisinin (ART) was also reported (165-167). A higher *pfmdr1* copy number was also associated with an increased susceptibility to CQ (159, 164, 168, 169). Furthermore, since the point mutation N86Y was frequently found in parasites showing high-level CQ resistance (170-173), it is believed that *pfmdr1* mutations can enhance the degree of CQ resistance albeit are insufficient to confer complete resistance to this drug (139, 170, 172, 174). It is not fully understood how Pgh-1 mediates these effects although it might directly transport drugs across the DV membrane or indirectly affect their partitioning by altering the transport of other substrates (175). Pgh-1 might transport some drugs, including MQ, HF and ART, into the DV where they may be less harmful for the parasite (97). By contrast, increased CQ or QN import and accumulation in the DV could lead to a decreased CQ resistance level since these antimalarials are known to act in this acidic compartment (97, 166). One study showed that Chinese hamster ovary (CHO) cells expressing wild-type Pgh-1 exhibit higher intracellular CQ accumulation and are therefore more sensitive to the drug compared to cells expressing a mutant Pgh-1 bearing the amino acid changes S1034C and N1042D, which have been associated with the CQ resistance phenotype in *P. falciparum* (176). Moreover, the transport of chemically and structurally unrelated drugs into the lumen of the *Plasmodium* DV via Pgh-1 can be inhibited by the P-glycoprotein inhibitors XR-9576 (tariquidar) and ONT-093 (97).

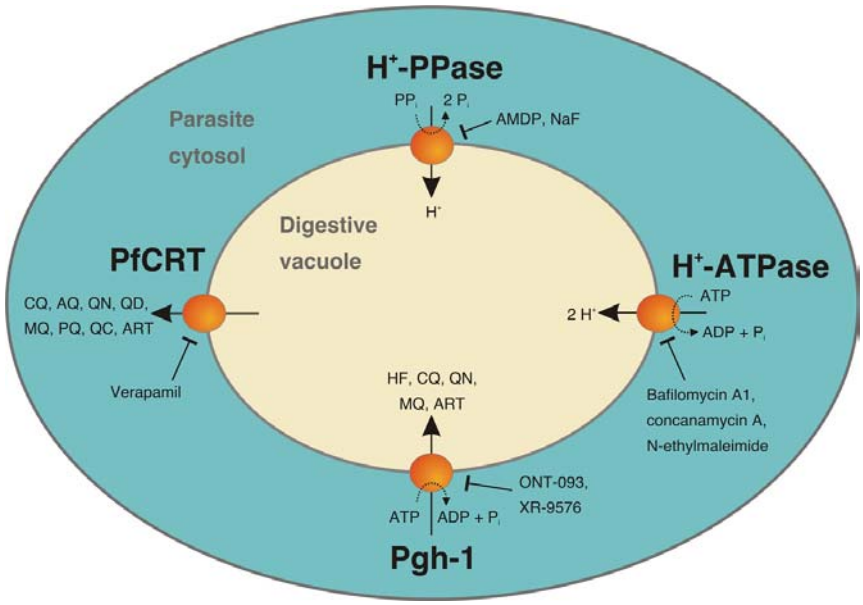

Figure 5. Function of transport proteins on the *P. falciparum* digestive vacuolar membrane. The *P. falciparum* chloroquine resistance transporter (PfCRT) transports drugs out of the DV, whereas the P-glycoprotein homolog 1 (Pgh-1) transports a range of compounds into the DV. The inwardly-acting proton pumps V-type H^+-ATPase and the V-type H^+-PPase contribute to the acidification of the DV lumen. Substrates and inhibitors of the transport proteins are indicated. AMDP: aminomethylenediphosphonate, AQ: amodiaquine, ART: artemisinin, CQ: chloroquine, HF: halofantrine, MQ: mefloquine, NaF: sodium fluoride, ONT-093: 4-[2-[4-[(E)-3-ethoxyprop-1-enyl]phenyl]-4-[4-(propan-2-ylamino)phenyl]-1H-imidazol-5-yl]-N-propan-2-ylaniline, PQ: primaquine, QC: quinacrine, QD: quinidine, QN: quinine, XR-9576: tariquidar.

The chloroquine resistance transporter (PfCRT) is a well-studied marker for the DV membrane whose expression peaks at the ring and trophozoite stage (42, 76, 138). It is a member of the drug/metabolite transporter (DMT) superfamily (177, 178) and can extrude CQ from the DV, thereby preventing the drug from accumulating to toxic levels (179-181). Polymorphisms within the *pfcrt* gene are linked to CQ treatment failure as well as *in vitro* CQ resistance (138, 173). In particular, the mutation K76T is conserved in CQ-resistant parasites and used as a molecular marker of CQ resistance (170, 182). In some CQR parasite strains carrying a mutation at position 76 in PfCRT, CQ transport out of the DV has been shown to be inhibited by verapamil (183). Different *pfcrt* alleles were also shown to influence the *in vitro* susceptibility of parasites to other antimalarial agents including QN, quinidine (QD), amodiaquine (AQ), HF, MQ and ART (42, 184-186). Furthermore, QN, QD, AQ, MF, primaquine (PQ), quinacrine (QC) and small peptides containing aromatic amino acids were capable of competing with CQ for transport via mutated PfCRT (135, 180). Thus, mutant PfCRT proteins might transport a wide range of clinically important antimalarial drugs as well as peptides out of the DV (Figure 5).

Two discrete H^+ pumps are present in the DV membrane: the V-type (vacuolar) H^+-ATPase and the V-type H^+-translocating pyrophosphatase (H^+-PPase) (111, 112). The H^+-ATPase is a heteromultimeric enzyme complex whose structure is well conserved among animals, plants, fungi and some bacteria. It is composed of a cytosolic V_1 domain, which contains the ATP catalytic site, and a transmembrane V_0 domain. In *P. falciparum,* the V_1 domain is composed of three A subunits, three B subunits, two G subunits and the subunits C, D, E, F and H, while the V_0 domain consists of the subunits a, c, c" and d (112). The genes encoding these subunits are transcribed throughout all stages of the parasite's life cycle and do not differ in the sequence of their coding regions between CQS and CQR *P. falciparum*-strains (187-189), suggesting that the H^+-ATPase is not involved in the CQ resistance phenotype (187). However, this H^+ pump has a major role in the acidification of DV and the maintenance of the transvacuolar pH gradient (111, 190, 191). Its function is dependent on ATP (111), and is sensitive to concanamycin A (111), bafilomycin A1 (127) and N-ethylmaleimide (127). Interestingly, the amino acid sequences deduced from the cloned H^+-ATPase genes of the parasite were more similar to those of plant origin than those of animal origin (112).

V-type H^+-PPases are highly conserved monomeric membrane proteins with an apparent molecular mass of 56–79 kDa and a 15- or 16-transmembrane topology. They are found in plant vacuoles, plasma membranes of phototrophic bacteria and various membranes of some protozoa. They translocate protons across a membrane by using potential energy derived from hydrolysis of the phosphoanhydride bond of pyrophosphate (111, 192, 193). The properties of *Plasmodium* H^+-PPases are similar to those of plants as well as of other protozoa including *Trypanosoma cruzi* (194), *T. brucei* (195), and

Leishmania donovani (196). As in plants, two different H^+-PPases are encoded in the genome of *P. falciparum*: PfVP1, an ortholog of the canonical plant type I H^+-PPase, and PfVP2, an ortholog of the plant type II H^+-PPase (197). While both proteins are expressed in intraerythrocytic parasites, the mRNA level of *PfVP1* is higher than that of *PfVP2* and is greatest in early trophozoite- and schizont-stage parasites (197). PfVP1 is predominantly localized in the DV membrane and to a lesser extent in the plasma membrane (13, 112, 197). The enzyme requires Mg^{2+} and modest concentrations of K^+ (50 mM) for its activity (13, 16, 197) and can be inhibited by sodium fluoride (NaF, 111) or aminomethylenediphosphonate (AMDP, 197).

The co-localization of vacuolar H^+-ATPases and H^+-PPases in the DV membrane in *P. falciparum* resembles the situation in the tonoplast of plant cells (13) and in the acidocalcisomes of a number of protozoan parasites (16) where the two H^+ pumps acidify the same compartment (112). As a byproduct of several major biosynthetic pathways, pyrophosphate is an abundant energy source and since intraerythrocytic malaria parasites rely mainly on glycolysis for ATP production (195), it is thought that its use is a mechanism to conserve ATP for vital functions in the cell (193). However, it was also postulated that the H^+-ATPases play the major role in the acidification of the DV lumen since H^+-PPases may not be capable of doing this alone (111). Due to their pivotal involvement in energy conservation and membrane transport in malaria parasites, and particularly because of the apparent absence of H^+-PPases in animals, these enzymes are considered important new antimalarial drug targets (193).

The existence of H^+-coupled transporters in the DV membrane such as a Na^+/H^+ antiporter has been suggested (18) but remains to be shown experimentally. Furthermore, it was proposed that a Ca^{2+}/H^+ antiporter and a P-type Ca^{2+}-ATPase, which is sensitive to thapsigargin and cyclopiazonic acid, function as transport proteins in the DV membrane (18), but have later been shown to be located to mitochondria (152) and a perivacuolar cytoplasmic region (198). Additionally, *P. falciparum* contains the gene PFA0375c that codes for a lipid/sterol-H^+: symporter with strong homology to Niemann-Pick type-C proteins, which mediate the efflux of lipids and cholesterol from lysosomes (www.plasmoDB.org, 199, 200). This transporter may be involved in the H^+-coupled extrusion of lipids and sterols from the DV (156). Another protein - PFE1185w - could be an H^+-driven effluxer of Fe^{2+} from the DV into the parasite's cytoplasm (156, www.plasmoDB.org) since it shows close homology to endosomal Fe^{2+} NRAMP2 transporters that are involved in the transferrin cycle (201). The function and localization of these proteins remain to be established.

7. PROTEIN TRAFFICKING TO THE DV

The trafficking pathway of parasite-derived proteinases that are responsible for hemoglobin proteolysis in the DV of *P. falciparum* takes advantage of the parasite's feeding mechanism (23). The premature enzymes contain putative N-terminal signal peptides for translocation into the ER and translation by ER-bound ribosomes, as well as a prodomain that bears additional targeting motif(s) for subsequent transport to the DV (28). These motifs are probably responsible for the targeting of these proteins to the DV (28) since green fluorescent proteins (GFPs) attached to a signal peptide can target the DV, while those without this targeting signal localize to the cytoplasm of the parasite (80, 129, 202, 203).

The proenzymes, as well as several other DV-targeted *Plasmodium* proteins, enter the secretory pathway of the parasite via the ER (6, 204) but it is unclear whether they transit a post-ER compartment such as the Golgi apparatus since they are not glycosylated (205). For the proteins that target the DV, differences in solubility and prodomain structure have been reported (28) and several trafficking routes have been hypothesized (Figure 6): for example, proteins can traffic directly from the ER to the DV membrane (Figure 6-I, e.g. PfCRT). Other proteins are transported from the ER to the cytostomal system, where they accumulate before being trafficked to the DV via double-membrane vesicles along with their substrate hemoglobin. The transport from the ER to the cytostomal system occurs either directly (Figure 6-II, e.g. PfPM2), via the parasite's plasma membrane (Figure 6-III, e.g. PfPM1, PfFP-2 and PfFP-3), via the PV (Figure 6-IV, e.g. PfDPAP1) or via the host erythrocyte's cytosol (Figure 6-V, e.g. PfHRP2). Additional proteins or substrates for nutrition could also be engulfed by the cytostome, together with those carrying a targeting sequence.

The premature DV plasmepsins are integral membrane proteins and are inserted into the ER via a transmembrane domain in their proregions. Pro-PfPM1 is initially transported to the parasite's plasma membrane before it is incorporated into the cytostomal system, possibly by lateral diffusion in the plasma membrane (206, Figure 6-III), whereas pro-PfPM2 reaches this organelle without prior trafficking to the parasite surface (Figure 6-II). Vesicles containing pro-PfPM2 are thought to fuse with the outer membrane of the cytostomal vacuole. The proenzyme is thus probably located in the space between the two vacuole membranes during transport to the DV (23). The N-terminal 70 amino acids (39 cytoplasmic, 20 transmembrane, and 11 lumenal residues) of the PfPM4 proenzyme were shown to be sufficient for targeting of a fluorescent protein version to the DV (129). Once in the DV, the proplasmepsins are anchored in the vacuolar membrane and subsequently cleaved between the residues Gly^{124} and Ser^{125} by falcipains at a low pH, resulting in their maturation and activation (23, 70, 205, 207, Figure 6). However, while this may be the prime mechanism of activation of the plasmepsins, they are capable of autoactivation when falcipain activity is inhibited. This suggests that alternate means of plasmespsin activation exists and adds a further level of redundancy between these DV proteases (70).

The cysteine proteases PfFP-2 and PfFP-3 are transported to the DV via the parasite's plasma membrane and the cytostomal system and contain large membrane-spanning prodomains with a bipartite trafficking signal

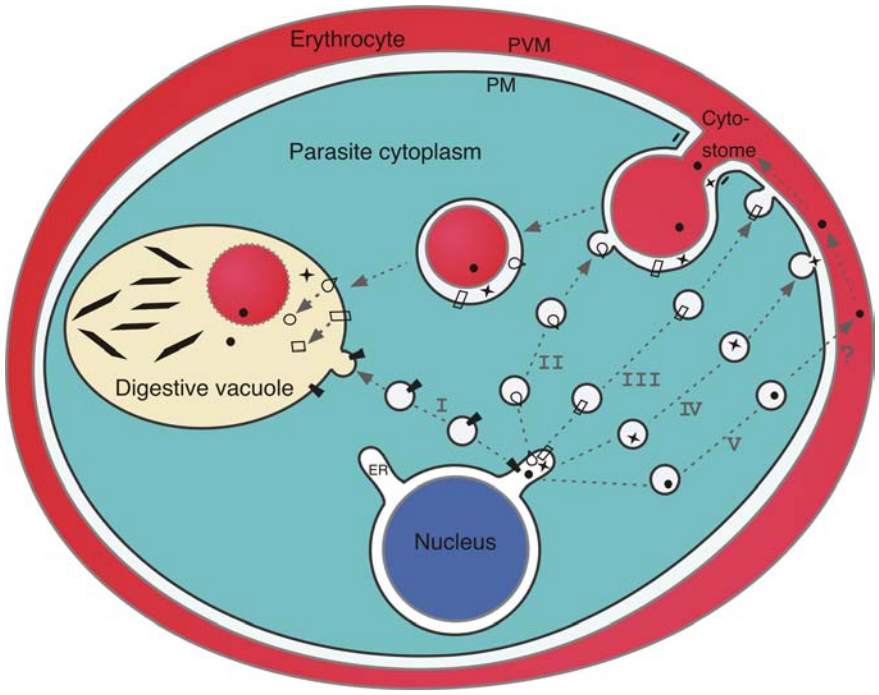

Figure 6. Protein trafficking pathways from the ER to the DV in *P. falciparum*. Most DV-targeted proteins enter the secretory pathway via the ER but it is not clear whether they traverse a post-ER compartment such as the Golgi apparatus. Several trafficking routes from the ER to the DV have been reported: direct vesicle-mediated transport (I, e.g. PfCRT) or trafficking via the hemoglobin ingestion pathway (II – V). The transport from the ER to the cytostomal system occurs either directly (II, e.g. PfPM2), via the parasite's plasma membrane (III, e.g. PfPM1), via the PV (IV, e.g. PfDPAP1) or via the host erythrocyte's cytosol (V, e.g. PfHRP2). PfPM1 and PfPM2 are cleaved and activated in the acidic environment of the DV. PM: parasite plasma membrane, PVM: parasitophorous vacuole membrane.

consisting of a cytoplasmic and luminal portion (80, 81). It was suggested that these falcipains are processed in an intracellular compartment before reaching the DV (77, 81). Dipeptidyl aminopeptidase 1 (PfDPAP1) was shown to accumulate in the PV before being ingested through the cytostome as a soluble protein and transported to the DV (Figure 6-IV). Its signal sequence does not contain a transmembrane domain and the internal prodomain is localized between an exclusion domain and the two catalytic domains (83).

DV-trafficking via the parasite's endocytic pathway has also been demonstrated for plasmodial proteins that are not involved in hemoglobin digestion. The merozoite surface proteins 1 and 8 (PfMSP1 and PfMSP8) are both initially transported to the parasite's plasma membrane, where they are cleaved. However, whereas PfMSP1 is produced in schizonts and its proteolytic cleavage comes about upon release of free merozoites and at the time of their invasion of erythrocytes, PfMSP8 is synthesized and processed at the ring stage (30, 208). The remaining non-cleaved fragment of PfMSP1 ($PfMSP1_{19}$) is endocytosed together with erythrocyte cytosol into small DVs typical of early and mid-ring-stage parasites by micropinocytosis, while the PfMSP8 fragment travels to the single DV of late rings/early trophozoites (30).

Histidine-rich protein 2 (PfHRP2), which is suggested to promote the formation of hemozoin, was reported to be secreted into the host erythrocyte cytosol before it is ingested and transported to the DV via the hemoglobin ingestion pathway (45, Figure 6-V). The integral membrane protein PfCRT (see Sect. 6) was suggested to pass through the ER and the Golgi complex and then directly traffic to the DV membrane (Figure 6-I). It was postulated that phosphorylation of Thr^{416} in PfCRT is critical for this process (209).

The molecular mechanisms and the proteins involved in trafficking of plasmodial proteins from the ER to the cytostomal system, in membrane translocation events and in the formation of the structures of the endocytic pathway remain unclear (28). Recently, a new DV trafficking route that excludes the secretory pathway has been shown for the FVYE domain-containing protein (PfFCP), a phosphatidylinositol 3-phosphate (PI3P)-binding protein without a transmembrane domain, which is directly transported from the cytoplasm to the DV via a C-terminal 44-amino acid peptide (210). However, the precise trafficking pathway of this protein is unknown. It is clear that DV-targeted proteins take a number of different routes to reach their destination and some of these pathways are still unclear.

8. MECHANISMS OF DRUG ACTION AND RESISTANCE IN THE DV

The DV plays a central role in parasite metabolism and is an important site of action of several potent antimalarial drugs that target the specialized functions of hemoglobin digestion and/or heme detoxification. Originally, CQ offered an effective low-cost treatment of falciparum malaria. However, with the spread of CQR parasites, the search for new agents capable of reversing or circumventing CQ resistance has become a major focus of malaria research (211-213). Therefore, a better understanding of the mechanism of action of CQ as well as the pathways and physiological parameters associated with CQ resistance is essential.

CQ is a lipophilic weak base that permeates membranes as an uncharged species. It readily accumulates in the DV of the parasite by passive diffusion along the pH gradient between the pH neutral cytosol and the acidic DV. In the DV lumen, CQ is protonated and becomes trapped since the vacuolar membrane is impermeable to the diprotic base (120). The drug is suggested to bind to heme, which prevents its detoxification and leads to the accumulation of toxic byproducts of hemoglobin digestion (25, 214). The resulting heme-CQ complexes are thought to disrupt membranes and result in killing of the parasite (215, 216). As mentioned in Section 5, CQ might also cause alkalinization of the DV lumen and thus inhibit or slow down hemoglobin digestion by acid hydrolases (114-117, 120, 217).

CQ resistance is associated with a decreased accumulation of the drug in the DV and, with this, its restricted access to heme (131, 136, 218). The Ca^{2+} channel blocker verapamil increases CQ accumulation in CQR parasites, attenuating resistance levels (136, 219). Mutations in PfCRT are thought to affect CQ accumulation in the DV and thus the level of CQ-sensitivity of the parasite (138, 220). The substitution of the positively charged K76 residue with a neutral residue (such as Thr, Asn or Ile) in the first transmembrane domain of PfCRT might allow the protonated drug to traverse the transporter protein and travel to the parasite's cytoplasm, thereby reducing its concentration in the DV (42, 138, 185, 221). It was proposed that this CQ efflux is associated with the H^+ leak from the DV and that it is sensitive to verapamil since CQ causes a verapamil-sensitive increase in the alkalinization rate of the DV in CQR strains after blocking the H^+-ATPase with concanamycin A (113). Additionally, mutations in *pfmdr1* (see Sect. 6) and other yet unidentified genes in addition to those in *pfcrt* may also be involved in the development of resistance to CQ (221).

Other quinoline drugs, such as QN, MQ and AQ, are also thought to affect DV function but it is not clear if their action involves interfering with hemozoin formation (222, 223). Furthermore, since these agents are weaker bases than CQ, they are not likely to accumulate to the same levels in the DV (224, 225). As previously mentioned, MQ resistance and treatment failure have been linked to *pfmdr1* amplifications, whereas the substitutions K76T in PfCRT and N86Y in Pgh-1 were found to be associated with reduced susceptibility to both CQ and AQ (226-228). A high-throughput chemical screening of 2816 compounds of the NIH Chemical Genomics Center Pharmaceutical collection that were registered or approved for human or animal use revealed that 96% of differential responses of current *P. falciparum* populations to many of the analyzed compounds were associated with mutations in *pfmdr1*, *pfcrt* or *pfdhfr* (*Plasmodium falciparum* dihydrofolate reductase). These data indicate that the differences in parasite drug sensitivity are dominated by a limited number of genes (229).

ART and its derivatives, e.g. artemether, artesunate and dihydroartemisin, are the most important class of antimalarials used today. ART-based combination therapies (ACTs) are currently used as first-line treatment of uncomplicated *P. falciparum* malaria in most malaria-endemic areas. They comprise ART derivatives in combination with one or more longer acting antimalarial drug(s) of a distinct chemical class, for example LF, MQ or AQ (230, 231). These combinations are thought to decrease the selection pressure for the development of drug resistance (231). However, strains of *P. falciparum* with a decreased sensitivity to ART have recently emerged in Bangladesh, Thailand and Cambodia (232, 233), which has raised concerns about their spread and the loss of ACT efficacy. ART susceptibility was shown to be influenced by genetic changes in *pfmdr1* and *pfcrt* (231) although studies on the exact mechanisms of decreased drug sensitivity remain to be carried out. Since point mutations in these genes and amplification of *pfmdr1* are known to affect parasite responses to different antimalarials by altering drug accumulation in the DV, ART accumulation in the DV might relate to its mode of action (234).

The mechanism of action of ART and its derivatives is poorly understood (235). It was postulated that the cleavage of the endoperoxide bridge of ART by reduced heme-iron produced during hemoglobin digestion in the DV causes the generation of toxic drug metabolites that damage parasite macromolecules (236, 237). Using live-cell microscopy, it was shown that fluorescent ART trioxane derivatives are rapidly accumulated in DV-associated neutral lipid bodies of trophozoites and schizonts, where they may be activated by neutral lipid-associated heme. Oxidative damage of parasite membranes induced by ART derivatives was observed using an oxidation-sensitive BODIPY probe and by chromatographic analysis of lipid extracts from ART-exposed parasites (238). Consistent with this, ART activity can be enhanced by oxygen and oxidizing agents and decreased by reducing agents (239). However, the activation of ART derivatives by heme may not be the only manner in which these compounds operate since the drug is also effective against ring stages, which do not contain high concentrations of heme (237).

Other studies have shown that ART and its derivatives cause heme alkylation in cultured *P. falciparum* and in mouse malaria models, suggesting that the resulting

Table 2. Characteristics of DV isolation procedures

Isolation procedure	Purification	Yield	Marker proteins in DV preparation
Developed by Choi *et al.* (110) Feeding of parasites with ferric oxide Homogenization of parasites containing ferric oxide granules Centrifugation on discontinuous tri-layer sucrose gradient Collection of DVs containing ferric oxide granules from bottom of gradient	2.6-fold[1] (110)	n.d.	Present: acid hydrolase and vacuolar ATPase activities (DV, 110) Absent: AChE (erythrocyte membrane, 110)
Developed by Goldberg *et al.* (9) Parasite lysis with D-Sorbitol Differential centrifugation Treatment with saponin and streptomycin sulfate to prevent DV adherence to other cellular components Higher-speed centrifugation Resuspension of DVs in sucrose/sodium phosphate/streptomycin sulfate solution, pH 7.1 10 triturations of DVs through a 27-G needle Percoll density gradient separation	35-fold[2] (9)	2-3%[4] (9)	Present: acid hemoglobinase (including aspartic protease) activity (DV, 9, 107) Absent: AChE (erythrocyte membrane), cytochrome c oxidase (mitochondrium), acid phosphatase, β-glucuronidase, β-galactosidase (lysosome, 9)
Developed by Saliba *et al.* (259) Exposure to saponin for release of trophozoites from erythrocytes Differential centrifugation Resuspension of trophozoites in ice-cold water, pH 4.5 4 triturations of DVs through a 27-G needle DNase I used to avoid adherence of DVs to DNA Percoll density gradient separation	10-fold[3] (260)	20%[5] (259)	Present: V-type ATPase, V-type H^+-PPase, Pgh-1, PfCRT, PfPM1, PfPM2, PfHAP, PfPM4, PfM1AAP (DV), GAPDH, PfM17LAP (parasite cytosol), HSP60 (mitochondrium), RhopH2, RhopH3 (rhoptry), Nucleoside transporter 1 (parasite plasma membrane), Exported protein 2 (PVM), ERC (ER, 260) Absent: AChE (erythrocyte membrane), pLDH (parasite cytoplasm) and oubain-sensitive ATPase (parasite plasma membrane) activities (259, 265), PfHRPI (membrane of parasitized erythrocytes, 73)

[1] Determined by assessing V-type ATPase activity in DV membranes compared to total parasite membranes. [2] Determined by comparing aspartic protease activity in isolated DVs relative to lysed parasites. [3] Determined by comparing the amount of the DV marker PfPM1 in the DV-enriched fraction with whole-parasite extracts using immunoblot analysis. [4] Counted by hemocytometer. [5] Values determined using a spectrophotometric method that correlates with counts obtained using a hemocytometer.

heme-adducts are toxic to the parasite (240, 241). It was also proposed that ART activation occurs via cleavage of peroxide bonds by intracellular iron-sulfur redox centers, which are commonly found in enzymes of *Plasmodium,* and that alkylation of these enzymes may then cause parasite death (242). By electron microscopy, it was shown that ART derivatives cause early disruption of the membranes of the DV, the nucleus and other organelles, which is followed by the loss of organellar structures (243). Furthermore, ARTs inhibited parasite endocytosis of macromolecules from the host cell (244). Studies using radiolabelled ART identified several drug-interacting parasite proteins, suggesting that their alkylation and inactivation might result in parasite killing (245, 246). Proteins that have been proposed to be the ART target include: cysteine proteases (247), proteins of the electron transport chain (248), and PfATP6, a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) ortholog (249).

Finally, recently identified inhibitors of the cysteine proteinases PfFP-2 and PfFP-3 directly target hemoglobin digestion and prevent the *in vitro* growth of *P. falciparum* at low nanomolar concentrations (250). Moreover, these small-molecule inhibitors cured *P. falciparum* infections in a human-engrafted mouse model (251, 252). HIV-1 aspartic protease inhibitors have been reported to block *P. falciparum* development *in vitro* and to reduce parasitemia in *P. chabaudi*-infected mice (253, 254). These findings are important for the development of antimalarial treatment strategies in regions that are endemic for both HIV and malaria. It was suggested that these antiretroviral drugs target the DV plasmepsins PfPM2 and PfPM4 (253, 254). However, since a disruption of the four DV plasmepsins is not lethal to *P. falciparum* (41, 255), it is unlikely that the antimalarial activity of these compounds is due to inhibition of these aspartic proteinases (256). Recently, it was shown that some HIV protease inhibitors enhance the antimalarial activity of CQ and MQ against *P. falciparum in vitro* (256-258), suggesting that the administration of both drug groups in combination therapies might potentiate their efficacy in patients (258).

9. DV ISOLATION AND PROTEOMIC ANALYSIS

Since the DV is hardly accessible to standard cellular experimental techniques, purification of intact and functionally active vacuoles is necessary to dissect the process of hemoglobin degradation, the regulation of ion homeostasis and the mechanisms of drug action and resistance (9, 259). A number of methods for the isolation of this organelle have been employed (Table 2). Most studies take advantage of the density of the iron-rich hemozoin crystals that allow the separation of DVs from other parasite fractions by differential centrifugation and Percoll density gradient separation (9, 259). The most commonly used procedures are those developed by Goldberg *et al.* (9) and Saliba *et al.* (259).

The Goldberg *et al.* method yielded 35-fold purified DVs capable of degrading hemoglobin at a pH optimum of 5.0–5.5 (9). While this preparation facilitated the study of the DV plasmepsins, it was not the method of choice for the studies of the energy-dependent accumulation of CQ in the organelle by Saliba *et al.* (259). The relevant differences in the protocol employed by these authors included: (a) ice-cold water at pH 4.5 instead of the osmotic laxative sorbitol was used for parasite lysis, (b) the

vacuoles were triturated through a 27-G needle only 4 instead of 10 times, and (c) DNase I was used to prevent the adherence of DVs to DNA, whereas Goldberg *et al.* (9) added a small amount of saponin and streptomycin sulfate to avoid vacuolar aggregation to other organelles. This apparently achieved a higher yield of isolated DVs (20% instead of 2-3%) and ATP-dependent CQ accumulation in functionally intact vacuoles from different parasite strains could be studied (259). However, according to an assessment by Lamarque *et al.* (260), this procedure only yields an approximately 10-fold enriched DV fraction. The purity and integrity of the isolated vacuoles was assessed by light and electron microscopy, immunofluorescence microscopy, immunoblotting, enzyme assays as well as mass spectrometry (9, 73, 259, 260). Table 2 details the methods employed for DV isolation and highlights some of the protein markers of the DV as well as of other compartments used to analyze the quality of the preparations.

Many of the DV proteins reported to date are implicated in the process of hemoglobin digestion (6). Knowledge of all DV-associated proteins (soluble and membrane-bound) would help to dissect the mechanisms involved in biogenesis, functions and interactions of the vacuole. Therefore, the proteome of DV-enriched fractions prepared with the method by Saliba *et al.* (259) was recently analyzed using tandem mass spectrometry by Lamarque *et al.* (260). Of the 116 proteins identified by these authors, many were known DV markers such as the DV plasmepsins, subunits of the vacuolar ATP synthase, V-type H^+-PPase, Pgh-1 and PfCRT. Furthermore, several proteins of unknown functions that are most likely localized in the DV membrane were detected. Of note, many non-DV proteins, such as PfM17LAP, Stevor, HSP60 and other mitochondrial, cytoplasmic, ER and rhoptry proteins were also detected, which indicates that the DV preparations were contaminated with proteins of other parasitic compartments (260).

10. CONCLUSIONS AND PERSPECTIVES

The DV of the malaria parasite carries out a variety of critical and specialized functions such as hemoglobin degradation in an acidic environment, peptide and/or amino acid transport, heme biocrystallization, detoxification of oxygen radicals, as well as ion homeostasis and thus offers many opportunities for novel antimalarial drug targeting (5, 39). Debates remain concerning the purpose of hemoglobin degradation, the site of amino acid generation from hemoglobin-derived peptides and the mechanisms of antimalarial drug action and resistance. A better understanding of these processes in the malaria parasite will be important for the future development of new therapeutic strategies to reduce the devastating impact of one of the most critical public health problems worldwide.

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Abbreviations: ACT: artemisinin-based combination therapy, AO: acridine orange, AQ: amodiaquine, ART: artemisinin, ATP: adenosine triphosphate, CQ: chloroquine, CQR: chloroquine-resistant, CQS: chloroquine-sensitive, DV: digestive vacuole, ER: endoplasmic reticulum, HF: halofantrine, LF: lumefantrine, MF: mefloquine, NOD: nucleotide-binding oligomerization domain, PPase: pyrophosphatase, PQ: primaquine, PV: parasitophorous vacuole, PVM: parasitophorous vacuole membrane, QC: quinacrine, QD: quinidine, QN: quinine

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