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Proteases as Regulators of Pathogenesis: Examples from the Apicomplexa

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Abstract

The diverse functional roles that proteases play in basic biological processes make them essential for virtually all organisms. Not surprisingly, proteolysis is also a critical process required for many aspects of pathogenesis. In particular, obligate intracellular parasites must precisely coordinate proteolytic events during their highly regulated life cycle inside multiple host cell environments. Advances in chemical, proteomic and genetic tools that can be applied to parasite biology have led to an increased understanding of the complex events centrally regulated by proteases. In this review, we outline recent advances in our knowledge of specific proteolytic enzymes in two medically relevant apicomplexan parasites: *Plasmodium falciparum* and *Toxoplasma gondii*. Efforts over the last decade have begun to provide a map of key proteotolyic events that are essential for both parasite survival and propagation inside host cells. These advances in our molecular understanding of proteolytic events involved in parasite pathogenesis provide a foundation for the validation of new networks and enzyme targets that could be exploited for therapeutic purposes.

Keywords

apicomplexans; proteases; pathogenesis; *Plasmodium*; *Toxoplasma*; malaria; invasion; egress

1. Introduction

The primary function of all proteases is to catalyze the simple process of peptide bond hydrolysis. While this process is the same for all proteases, each family utilizes a different general mechanism to accomplish this goal. Although proteases were originally thought to act as downstream mediators of protein turn-over, we now know that distinct proteolytic processing events can also regulate or initiate key biological processes such as cell death [1], cell cycle progression [2] and cell migration [3]. Given that proteases regulate such basic biological processes, it is not surprising that apicomplexan parasites, with their complex life cycles, also depend on proteolytically regulated processes. Decades of research in parasite biology have begun to define the roles of proteases in all stages of the parasite life cycle. In this review, we will provide a general overview of the current knowledge of proteases found in the apicomplexan parasites *Plasmodium spp*. and *Toxoplasma spp*, with special focus on

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three important cellular events: host-cell invasion, general catabolism and host cell rupture (also known as egress). While the roles of proteases in various aspects of parasite biology have been reviewed in the past [4–7] we will focus on recent discoveries that have contributed to our understanding of critical proteolytic events in these two important human pathogens.

The apicomplexan parasites: *Plasmodium* and *Toxoplasma*

The Apicomplexa comprise a phylum of highly diverse eukaryotic protozoa that exists as parasites in animals. Apicomplexans are morphologically characterized by the presence of an eponymous apical complex of organelles consisting of micronemes, rhoptries and dense granules, as well as other less-well characterized secretory organelles such as exonemes. These organelles contain proteins that are secreted around the time of egress and invasion to facilitate parasite motility, host cell adhesion and subsequent remodeling of host cells (see [8] for detailed review). These parasites also have common, yet parasite-specific features such as a chloroplast-like organelle termed the apicoplast that has a role in fatty acid and isoprenoid synthesis [9]. *Plasmodium spp*., the causative agent of malaria, affects 500 million worldwide and therefore attracts a considerable amount of medical and research attention [10]. Classically at least five species of *Plasmodium* infect humans: *Plasmodium vivax, P. ovale, P. malariae, P. knowlesi* and *P. falciparum*. Each species has a highly regulated life cycle inside the intermediate human host as well as inside the definitive insect vector. In addition all *Plasmodium spp*. within the intermediate host primarily infect red blood cells. *Toxoplasma gondii* is related to *Plasmodium spp*. however a notable difference is that *T. gondii* infects virtually any nucleated host cell. Up to one third of the entire population of the world is infected by *T. gondii* [11] and although primary infection by *T. gondii* is typically subclinical, it can cause serious illness in immuno-compromised patients and lead to fetal death if acquired during pregnancy [12].

Both *Plasmodium* and *Toxoplasma* progress through a complicated series of sexual and asexual life cycles where non-replicative extracellular forms of the parasites known as zoites first invade host cells, grow and replicate within these cells, and finally rupture to establish further infections (Figure 1). [13, 14] This variety of molecular events requires the expression of many specialized proteins to coordinate these diverse biochemical activities. Recent efforts have revealed proteases as crucial players in all stages of the parasite life cycle. The roles of various proteases in *Plasmodium* and *Toxoplasma* will be reviewed here, but the importance of proteases is not limited to the apicomplexans, and their roles in the progression of other parasitic diseases have been previously reviewed [15].

2. Role of proteases in host-cell invasion

As obligate intracellular parasites, it is vital for the extra-cellular forms of *Plasmodium* and *Toxoplasma* to rapidly invade host cells. This process of invasion occurs within a very short time frame because the extracellular forms cannot survive for long periods of time outside host cells. Invasion proceeds following an initial contact by the polar zoite, which reorients such that its apical end contacts the host cell membrane [16, 17]. A tight junction that results from the interaction of multiple receptor proteins forms between the apposed parasite and host membranes, and this junction moves from the apical to the posterior end of the zoite. The active trafficking of the receptor proteins on the parasite surface drags the host cell membrane over the parasite, creating an invagination within the host cell [5, 6]. As invasion proceeds, the zoite must shed the surface proteins initially required for attachment and penetration. Invasion is complete when the parasite is fully engulfed within the parasitophorous vacuole, which seals to create a protective environment where it can subsequently grow and replicate (see Figure 2, Step 1).

Many apical surface proteins have been confirmed to be required for successful attachment [7, 18, 19]. In *Plasmodium*, proteins such as merozoite surface proteins (MSPs), apical membrane antigen 1 (AMA1), rhoptry neck proteins (RONs) and serine repeat antigen (SERA) proteins are thought to play important roles in parasite invasion. In addition, many of these proteins are proteolytically processed either during trafficking to, or arrival at, the parasite surface. However, until recently the identities of the majority of proteases that regulate the maturation and subsequent shedding of the surface proteins during invasion were largely unknown.

2.1 Subtilases

The subtilisin-like serine proteases are a class of enzymes present in both *P. falciparum* and *T. gondii*. The *P. falciparum* genome project identified three subtilases but only two, PfSUB1 and PfSUB2, are known to have functional roles in the blood-stages of the parasite life cycle [20]. PfSUB1 has roles in both egress (see section 4.2) and invasion. This protease has a high degree of substrate specificity [21] and its expression peaks in schizont stage [22]. It was initially postulated that PfSUB1might be involved in the shedding of micronemal proteins from the surface of the parasite during invasion. However selective inhibitors of PfSUB1 do not interfere with shedding of surface proteins MSP-1 and AMA-1 [6], but can efficiently block invasion [23]. The effects of PfSUB1 inhibitors were suggested to be the result of a block in processing of surface proteins required for productive invasion. PfSUB1 was subsequently shown to effectively process MSP1/6/7 and that the maturation of these surface proteins was required for successful invasion [24]. A detailed bioinformatic and proteomic analysis has identified additional proteins processed by PfSUB1 that are putatively involved in egress and invasion [25].

While PfSUB1 is involved in maturation of MSP1 before merozoite rupture, PfSUB2 functions as a sheddase during merozoite invasion. PfSUB2 localizes to the micronemes, and is secreted and translocated to the posterior end of parasite surface during invasion [26]. During this translocation, PfSUB2 proteolytically cleaves the ectodomain of MSP1 and AMA1. This processing may disengage adhesion complexes to release the parasite into the parasitophorous vacuole. Evidence also suggests that PfSUB2 is responsible for the proteolytic processing of *Plasmodium* thrombospondin related apical merozoite protein (PTRAMP), another adhesive protein on the merozoite surface [27]. Similar to MSP1 and AMA1, PTRAMP is shed from the merozoite during erythrocyte invasion.

Given the relatedness of *T. gondii* and *P. falciparum*, it is not surprising that subtilisin-like proteases also have important functions in host cell invasion by *Toxoplasma* [28, 29]. Using a gene disruption strategy to knock out the *sub1* gene in tachyzoites, Lagal and co-workers [30] showed that loss of GPI-anchored protease TgSUB1 results in defective surface processing of micronemal proteins MIC2, MIC4 and M2AP. Since secretion of mature forms of these proteins is important for attachment to the host cell, tachyzoites lacking TgSUB1 have a reduced ability to invade. Furthermore, deletion of the *sub1* gene results in parasites with a defect in gliding motility, a key process required for invasion of the host. The highly related *T. gondii* subtilase, TgSUB2, also has been linked to invasion. TgSUB2 is a transmembrane protein that localizes to the rhoptry organelle in tachyzoites [31]. Attempts to disrupt the *Tgsub2* gene suggest that it is essential in the *T.gondii* life cycle. Interestingly, the peptide sequence where autocleavage occurs within the TgSUB2 protein matches that of cleavage sites of other rhoptry protein (e.g. ROP1). This, combined with its known rhoptry localization, is suggestive of a role for TgSUB2 in proteolytic maturation of rhoptry proteins.

2.2 Rhomboid proteases

The Rhomboid family of serine proteases was first discovered in *Drosophila* [32], and it soon became apparent that these intra-membrane proteases are conserved in both prokaryotic and eukaryotic organisms [33, 34]. The rhomboid proteases have a mechanism of proteolysis that is unique from the soluble serine proteases. Specifically, the protease active site is buried in the plane of the membrane, and proteolysis occurs in, or adjacent to, the transmembrane domain of the substrate [35].

In *Toxoplasma*, proteomic analysis of invasion-specific cleavage events first hinted at a role for rhomboid proteases in surface protein shedding [36]. Six rhomboid-like genes were identified and five rhomboid proteases, TgROM1–5, were subsequently cloned and characterized [37, 38]. These rhomboid proteases have non-overlapping substrate specificities and localize to different regions of the cell. Of these, TgROM2, TgROM4 and TgROM5 function as sheddases of micronemal adhesin proteins during the process of invasion. Recent work has uncovered a crucial role for TgROM4 in invasion as a result of processing of surface adhesins MIC2, MIC3 and AMA1 [39]. This cleavage of AMA1 from the *T. gondii* surface during invasion, in turn, triggers a switch of the parasite from an invasive to replicative state [40]. TgROM5, which like TgROM4, is localized to the cell surface of the tachyzoite, cleaves the adhesion protein MIC2 upon its trafficking to the posterior end of the parasite membrane during invasion. The rhomboid proteases in *T.gondii* thus collectively serve as sheddases that remove adhesion proteins to allow successful host cell invasion. However, it is perhaps worthwhile to note that rhomboid proteases may have functions beyond those required for invasion. For example, TgROM1 has been shown to contribute to intracellular growth of the parasite [41].

Not long after the characterization of TgROMs, rhomboid proteases were identified in *P. falciparum* and shown to also function in shedding of adhesion proteins during invasion. PfROM1 and PfROM4 are able to cleave a variety of substrates that possess a transmembrane domain, and they are likely to be involved in all invasive stages of *P. falciparum* [42–44]. PfROM1 has similar substrate preference as the *Drosophila* Spitz-cleaving rhomboid proteases, while PfROM4 is distinct in that it does not cleave Spitz-type substrate motifs and instead cleaves erythrocyte binding-like (EBL) adhesins [42]. Collectively, these results establish a role for rhomboid proteases in apicomplexan invasion. Further studies will be required to characterize the specific targets of each rhomboid protease in the life cycle of the parasites.

2.3 The cysteine proteases

Although cysteine protease inhibitors have convincingly been shown to block parasite invasion, only a few candidate proteases have been identified. In *P. falciparum*, the identification of selective inhibitors of falcipain-1, through screening of epoxide inhibitor libraries, provided a tool that implicated this protease in erythrocyte invasion [45]. Unlike the other falcipains that are involved in hemoglobin degradation, falcipain-1 localizes to distinct, granule like structures in newly formed merozoites. However, subsequent gene disruption of falcipain-1 suggests that its role in invasion may be somewhat redundant [46, 47]. The fact that broad-spectrum cysteine protease inhibitors block invasion suggests that yet undiscovered cysteine proteases may function together to ensure successful invasion.

In *T. gondii*, a cathepsin B homologue TgCPB (also known as toxopain-1 or TgCP1), may play a role in tachyzoite invasion by processing rhoptry proteins [48]. Immunoelectron microscopy using cathepsin B antibodies provides some evidence that TgCPB might be localized to the rhoptries. The use of small molecule inhibitors of cathepsin B have also been shown to disrupt rhoptry protein maturation resulting in abnormal rhoptry development

and defects in tachyzoite invasion [49]. However, the specificity of the compounds used in these studies has not been confirmed suggesting that effects could be mediated by other targets. In particular, *T. gondii* expresses a highly homologous cathepsin L, TgCPL, which has recently been shown to localize to a specialized secretory organelle termed the vacuolar compartment (VAC) where it is believed to play a role in maturation of secretory proteins targeted to the micronemes [50]. In particular, *T. gondii* deficient for TgCPL showed slower maturation of TgM2AP and TgMIC3 proproteins. As TgM2AP and TgMIC3 are required for efficient host cell invasion, it is not surprising that the TgCPL-deficient strain has reduced ability to invade host cells. Although only a limited number of cysteine proteases have been confirmed to have a role in invasion, recent tools such as the complete genome sequence of *P. falciparum* and *T. gondii*, in combination with the use of chemical inhibitors, will hopefully aid in the discovery and characterization of additional important cysteine proteases.

2.4 The metalloprotease Toxolysin 4

The metalloprotease toxolysin 4 contains a signature motif present in insulin-degrading enzymes [51]. This protease localizes to the micronemes, and its secretion coincides with discharge of micronemal contents. Thus, it is possible that toxolysin 4 can function in invasion. However, further studies will be required to assign specific roles to this protease.

3. Proteases in general catabolism

Once invasion has occurred, parasites actively metabolize host cell proteins to generate products required for growth and replication. In *Plasmodium*, this process has been extensively studied due to its potential as a target pathway for malaria drug-therapy (See Figure 2, Step 2) [52].

3.1 Hemoglobin Degradation in *Plasmodium*

During the intraerythrocytic stage of the *Plasmodium* life cycle, the parasite imports host cell hemoglobin and then progressively degrades it to generate key nutrients as the parasite grows. Degradation of hemoglobin provides free amino acids, frees up space for further growth, and also has a possible role in regulating the osmotic status of the cell [53]. This process occurs mainly in the trophozoite stage, during which time hemoglobin is endocytosed into a parasite organelle known as the food vacuole. Once inside this acidic compartment, a cascade of aspartyl and cysteine proteases degrade the hemoglobin to the final amino acid products [54].

The extended family of plasmepsin aspartyl proteases in *P. falciparum* has been the focus of intensive research mainly due to the parasite-specific nature of these enzymes [55]. Therefore, plasmepsins were initially thought to be ideal targets for development of anti-parasitic drugs. The plasmepsins are a family of aspartyl proteases made up of 4 main members: plasmepsins I, II, IV and histo-aspartic protease [56, 57]. Plasmepsin I and II are thought to initiate hemoglobin degradation, while histo-aspartic protease and plasmepsin IV function in the degradation of peptides generated by the action of upstream members of the family [58–61]. However, the fact that genetic disruption of individual plasmepsins did not result in any difference in *P. falciparum* morphology suggests that functional redundancy between the family members may make these proteases non-optimal as therapeutic targets [62]. Nevertheless, plasmepsins are important proteases in hemoglobin degradation, especially since a triple or quadruple plasmepsins gene knock-out confers a growth defect in *P. falciparum* [63].

Other proteases such as the metalloprotease falcilysin [64–66], and the cysteine proteases falcipains 2, 2’ and 3 [67–70] and dipeptidyl aminopeptidase 1 (DPAP1), are also involved

in degradation of hemoglobin after it has been cleaved into peptide fragments [71]. Falcipains are thought to function downstream of plasmepsin I and II, and evidence suggests that these proteases are involved in activation of the food vacuole plasmepsins [72]. Gene disruptions as well as studies using small molecule inhibitors of falcipain-2, have confirmed a role for this protease in hemoglobin degradation [73–75]. Furthermore, falcipains may be able to replace the functions of plasmepsins [62] making them promising targets for antimalarials [76].

The cysteine dipeptidyl aminopeptidase 1 (DPAP1) is an exopeptidase in the food vacuole [71]. DPAP1 is a homologue of mammalian cathepsin C that also removes dipeptides from protein substrates. DPAP1 functions in an acidic environment (the food vacuole) and has similar substrate specificity as cathepsin C [77]. Studies using specific inhibitors of DPAP1 have shown that inhibition of this protease leads to an accumulation of parasites at the trophozoite stage and a subsequent decrease in parasitemia [78]. These inhibitors were also tested in a mouse model of malaria and found to reduce parasitemia. Furthermore, by linking DPAP1 inhibitors to a synthetic analog of another effective antimalarial drug, it was possible to enhance killing of parasites in culture [79]. These observations, combined with the essential nature of DPAP1 expression in blood stage parasites [71], suggest that it plays a critical role in parasite survival and is therefore a potentially valuable drug target.

The final stages of hemoglobin degradation are carried out by aminopeptidases that generate single amino acids from cleaved peptides [80]. An additional family of neutral amino-peptidases PfA-M1 and PfM17LAP [81–83] has recently been suggested to degrade peptide substrates that are exported to the cytosol from the food vacuole to generate free amino acids [84–86]. These proteases may also represent promising new targets for the development of anti-malarial drugs, which may be facilitated by the recent structural characterization of members of this family [87].

3.2 Proteases involved in general peptide processing

Proteases that perform essential roles such as protein homeostasis, protein transport and host cell remodeling are also potentially valuable as targets for theraputic intervention. As these processes are required throughout the parasite life cycle, drugs that block the action of these proteases have the potential to be effective at multiple points during an infection.

The proteasome is a large multi-subunit complex that catalyzes the proteolysis of proteins that have been targeted for degradation by ubiquitination. This protease complex is conserved across the eukaryota and is also found in some prokaryotes [88]. The proteasome is involved in regulatory functions such as protein turnover and degradation of misfolded proteins. In mammalian cells, it also regulates cell cycle progression and inflammation pathways [89]. Furthermore, a small molecule proteasome inhibitor has been approved by the FDA for the treatment of multiple myeloma, suggesting that targeting the proteasome could be an effective way to treat other diseases [90].

Initial studies using the natural product lactacystin, which inhibits *P. falciparum* proteasome at nanomolar concentrations, confirmed that a block in proteasome activity leads to parasite death [91]. However, these initial studies also pointed out the overall toxicity that resulted from inhibition of the host proteasome. More recent studies with several different classes of proteasome inhibitors suggest that the proteasome may be a viable target in *Plasmodium spp*. [92–94]. Furthermore, at least two proteasome inhibitors – epoxomicin and thiostrepton – also reduce growth of the sexually differentiated *Plasmodium* gametocyte, suggesting that inhibition of the proteasome could be an effective way to block parasite transmission [95, 96]. Unfortunately, relatively little is known about the biochemical or structural properties of the proteasome from *Toxoplasma* or *Plasmodium* and this has hindered the development

of parasite-specific inhibitors. Selective inhibitors will almost certainly be required to effectively validate the proteasome as a drug target for malaria and other parasitic diseases.

An additional protease that has been suggested to function in *T. gondii* in general catabolism is cathepsin C (TgCPC). Three isoforms of this protease have been identified, and TgCP1 is implicated in growth and replication of the parasite [97]. Cathepsin C is an exopeptidase that cleaves dipeptides from the N-terminus of proteins. Treatment with a cathepsin C inhibitor led a reduction in parasitemia and reduced protein degradation in the parasitophorous vacuole (PV). However, additional studies to identify the substrates of cathepsin C will be required to define its role in general peptide degradation in *T. gondii*.

The recent placement of plasmepsin V in the *Plasmodium* protein export pathway also opens up new strategies for antimalarial chemotherapy. Unlike plasmepsin I–IV, plasmepsin V is not localized to the food vacuole, but is instead restricted to the endoplasmic reticulum (ER) membrane [98]. Recent work demonstrated the role of this aspartyl protease in cleaving the Plasmodium Export Element (PEXEL) sequence motif, required for the export of PEXEL containing parasite proteins into the erythrocyte cytosol [99, 100]. This export of proteins allows the parasite to remodel the host cell for functions such as adhesion to endothelial cells. Such remodeling could be a mechanism to evade host defenses and therefore inhibition of plasmepsin V could be a strategy for clearing parasites [101, 102].

4. Proteases have central roles in parasite egress

As obligate intracellular parasites, *Plasmodium* and *Toxoplasma* spend the majority of their lives within the confines of their host-cell environment. Once replication is complete and several mitotic cycles have taken place, specialized zoite cells are released from the host cell through a lytic process known as egress (See Figure 2, Step 3). Our current understanding of egress is that it is a rapid and tightly regulated process. However, the exact mechanisms that regulate egress remain unclear. In *Plasmodium*, there are two popular models; the first model proposes that egress occurs by initial degradation of the parasitophorous vacuole membrane (PVM), and then the erythrocyte membrane. The second model proposes the reverse, where the erythrocyte membrane is degraded prior to the release of the merozoites from the PVM [103]. Both models have experimental support, and thus it is difficult to provide a definite mechanism. However, recent advances with video microscopy supports a model whereby the PVM ruptures, followed by the rapid and explosive rupturing of the erythrocyte membrane that is thought to facilitate the dissemination of the daughter zoites from the parent host-cell [104, 105].

Despite our lack of understanding of the precise mechanistic processes underlying egress, proteases appear to play pivotal roles. For example, treatment of *P. falciparum* infected cells with serine and cysteine protease inhibitors produces a block in egress leading to accumulation of late stage schizonts [106]. However, it is important to note that these experiments used broad-spectrum inhibitors that block proteases implicated in both egress and invasion. Subsequent experiments have identified inhibitors that can specifically block egress, and these have served as useful chemical tools in further exploration of the proteases involved in egress [23, 107]. The following section reviews the role of several proteases that function in *Plasmodium* egress. Although studies on host-cell rupture in *Toxoplasma* have uncovered roles for potassium and calcium signaling [108] and the pore-forming perforin proteins [109], virtually nothing is known about proteases that may regulate egress in this organism.

4.1 Serine-repeat antigen (SERA) family

The serine-repeat antigen (SERA) family is group of highly expressed *Plasmodium* proteins that are characterized by a central papain-like protease domain. Within the family, only SERA 6–8 have the canonical 'active site' cysteine residue found in classical papain family proteases, while SERA 1–5 and 9 have a serine in place of this residue [6]. Intriguingly, this family of proteins is not found in related apicomplexan parasites such as *Toxoplasma*. SERA5 is the most abundant of the SERA family [110] and is found in the parasitophorus vacuole where it is processed around the time of egress. Antibodies against the truncated form of SERA5 and a cyclic peptide directed against the predicted enzyme domain of the protein interferes with its function and cause a reduction in parasite growth, suggesting that SERA5 is vital for *Plasmodium* egress and/or invasion [110–112]. SERA5 is one of the two SERA genes that cannot be disrupted, further highlighting its importance in the parasite life cycle [113]. As SERA5 possesses a papain-like fold with serine as the active site residue [114], it could potentially function in egress and invasion by proteolytically processing substrates. The recombinant catalytic domain of SERA5 indeed possesses some weak chymotrypsin-like activity and this activity is sensitive to protease inhibitors [115]. However, it is still presently unclear if endogenous SERA5 has proteolytic activity. Crystallographic evidence suggests that the active site in SERA5 is not conducive to substrate binding [114]. As such, although SERA5 may be an important player in egress, any catalytic function remains a mystery. The only SERA protein with a direct link to parasite egress is SERA8. In *Plasmodium berghei*, disruption of the SERA8 orthologue, ECP1, resulted in a complete block of sporozoite egress from oocytes [116]. Therefore, the SERA proteins may mediate egress in both the sexual and asexual stages of *Plasmodium*.

4.2 PfSUB1

During maturation, SERA5 is proteolytically cleaved from the full-length form of approximately 126 kDa, through two intermediates before accumulating as a 50 kDa form which is released into culture supernantant during rupture [117]. Investigation of the proteases responsible for the maturation of SERA5 linked the serine protease PfSUB1 and the cysteine protease DPAP3 to this maturation process [23, 107]. In addition to the role of PfSUB1 in priming merozoites during invasion, it has a role in parasite egress. PfSUB1 localizes to a merozoite organelle called the exoneme, and is released into the parasitophorous vacuolar space before egress. Further studies demonstrated that PfSUB1 is responsible for cleaving SERA 4, 5 and 6 during egress. Inhibition of PfSUB1 using a specific inhibitor (MRT 12113) led to significant reduction in egress and invasion. The role of PfSUB1 in processing SERA5 was also confirmed by a chemical screen that identified a chloroisocoumarin (JCP104) that serves as both an inhibitor and probe of PfSUB1 activity [107]. JCP104 specifically blocks schizont rupture further demonstrating that PfSUB1 is a regulator of *P. falciparum* egress.

4.3 DPAP3

In addition to identifying a small molecule inhibitor of PfSUB1, the small molecule screen by Arastu-Kapur and co-workers identified a cysteine protease inhibitor that specifically blocked host cell rupture [107]. The identified dipeptide vinyl sulfone hit was converted to an active site probe and used to identify the dipeptidyl aminopetidases (DPAPs) as targets of this compound. Further development efforts identified inhibitors that could specifically target the food vacuole specific protease DPAP1 as well as the related family member DPAP3, a protease with unknown functions. These compounds showed that inhibition of DPAP1 resulted in toxicity to the parasite without affecting host cell rupture, while inhibition of DPAP3 specifically blocked parasite release from the host cell. Inhibition of DPAP3 also led to reduction in levels of the mature form of PfSUB1 without causing accumulation of the precursor protein, suggesting that DPAP3 may be required for

maturation of PfSUB1. DPAP3 may therefore regulate host cell egress by controlling maturation of secretory proteins required for this process [107].

4.4 Host cell calpain

In addition to parasite derived proteases, a host protease has also recently been implicated in the regulation of parasite egress in both *P. falciparum* and *T. gondii*. Specifically, depletion of host cell calpain leads to a complete block in parasite egress [118]. In this study, human calpain-1 activity in erythrocytes was reduced by both chemical inhibition and immunodepletion. The resulting calpain-1 deficient erythrocytes were infected with *P. falciparum* and a defect in schizont rupture was observed. To confirm the role of calpain in this process, purified calpain was added back to the calpain-depleted host cells and egress was restored. The role of calpain in release of *T. gondii* tachyzoites was also investigated using siRNA knockdown in U2OS human host cells. Knockdown of both calpain 1 and calpain 2 was achieved by targeting of the regulatory subunit of both calpains. Similar to *Plasmodium*, calpain knockdown in host-cells blocked *T. gondii* egress. Therefore, it is likely that host-cell calpain facilitates parasite egress through some type of host cell remodeling, the details of which are yet to be determined.

5. Proteases as targets for anti-malarials

Proteases are attractive targets for drug development because the presence of a well-defined active site facilitates interactions with small molecules, and thus many classes of small molecule proteases inhibitors have been developed [119]. Our understanding of the biology of specific proteases in the intra-erythrocytic stages during malaria infection has advanced greatly in the past decade and this information will likely facilitate the design of specific protease inhibitors as anti-parasitic agents (Table 1). Furthermore, the rapid emergence of drug resistance in *Plasmodium spp*. suggests an urgent need for new anti-malarial compounds. Current approved drugs focus mainly on targeting processes in the apicoplast or food vacuole, which are responsible for parasite growth and metabolism [52]. Proteases involved in hemoglobin metabolism are the most commonly targeted proteases in anti-malarial drugs currently under development, yet it may not be the most optimal pathway due to highly redundant mechanisms and the ability of the parasite to actively export compounds out of the food vacuole. While much effort has been directed towards developing lead compounds to target falcipains and plasmepsins, it is important to note that redundancies exist between these two families of proteases [62, 63] and hence effective therapy would most likely require targeting of both. Recent work has highlighted the potential of DPAP1 as a target for drug development efforts [78]. The inability to disrupt the DPAP1 gene highlights its vital role in parasite catabolism. Furthermore a hybrid of a DPAP1 inhibitor and an artemisinin analogue was able to effectively reduce parasite growth at low nano-molar concentrations with low cell toxicity [79]. Such a hybrid drug is less likely to induce resistance since each fragment of the compound uses distinct mechanisms for parasite killing.

Other proteases involved in *Plasmodium* egress and invasion are gaining attention as potential targets for therapy [103]. Arguably, egress and invasion are attractive processes for inhibitor development because *Plasmodium spp*. are obligate intracellular parasites that are particularly vulnerable outside their host cells [7]. Our lack of understanding of specific players in these processes is probably the reason for slow development of compounds that target egress and invasion. While there have been a recent series of published inhibitors that target DPAP3 and PfSUB1 [23, 107], these compounds are generally not optimal drugs and significant additional efforts will be required to convert these lead compounds into more suitable therapeutic agents.

6. Tools to facilitate dissection of apicomplexan parasite proteases

The post-genomic era offers exciting prospects to begin to further define apicomplexan biology. Genome sequences [120–122] and transcriptome profiling [123, 124] of *P. falciparum* and *T. gondii* enable genetic validation of protease activities and increase the rate of discovery of novel proteases with important functions. While *T. gondii* is a relatively genetically tractable organism [125], *P. falciparum* remains challenging to study using classical genetic methods. Low transfection efficiency [126, 127], coupled with the lack of RNAi machinery [128, 129] make standard genetic tools untenable. Fortunately, recent advances in chemical genetics [107, 130] and systems biology are helping to advance the study of basic parasite biology.

One particularly exciting area of development is the use of small molecules to conditionally regulate protein stability. This technique makes use of ligands that can stabilize a protein domain known as the destabilizing domain (DD), which is otherwise degraded in the absence of a stabilizing small molecule [131]. Recent success in *P. falciparum* has allowed the conditional knockdown of falcipain-2 [132], parasite calpain [133], calcium dependent-protein kinase 5 [134] and a proteasome cap regulatory subunit Rpn6 [135]. In *P. falciparum*, the DD system has therefore proven to be more generally applicable than the previously reported tetracycline-inducible transactivator system [136]. Moreover, the DD system allows the rapid temporal control of protein levels and thus enables the study of protein function at precise stages of the parasite life cycle.

The use of activity-based probes (ABPs) as a forward chemical genetic tool [137] also represents an exciting method to study parasite protease biology [78, 107, 130]. Using inhibitor scaffolds tagged with a reporter, the activity of proteases can be monitored in intact parasites. This technique can also be applied effectively to identify selective substrates that can then be used for high throughput screens of protease targets [138]. In addition, various groups have harnessed mass spectrometry (MS) as a tool to assess global proteomic changes in *P. falciparum* [25, 139]. These approaches allow for a more global mapping of proteolytic events, and could lead to the identification of essential pathways that are controlled by proteases. The regulators of these pathways can then be prioritized as targets for small molecule therapies.

Conclusion

In this review, we have described the roles of various proteases throughout the life cycles of *Plasmodium* and *Toxoplasma*. Although we have highlighted many proteases that have been the focus of significant study over the past decade, we also chose to draw attention to proteases where further investigation is required to better understand their function in the parasite. With the help of novel genetic and chemical tools, coupled with systems-based analysis, the functions of poorly understood and novel proteases can hopefully be defined in the near future. As proteases represent very attractive targets in drug development, further studies will facilitate discovery of novel drugs against malaria and toxoplasmosis that will hopefully overcome emerging resistance to current drug therapies.

Highlights

- Discussion of Proteases as important mediators of pathogenesis in parasites
- Outline of proteases in *Plasmodium falciparum* and *Toxoplasma gondii*, and their roles in invasion, growth, replication and egress from host cells.
- Outline of advances in chemical genetics and systems biology that have advanced our understanding of the roles of proteases in parasite biology.

Discussion of how new information on proteases in parasite pathogens could lead to new anti-parasitic drug targets.

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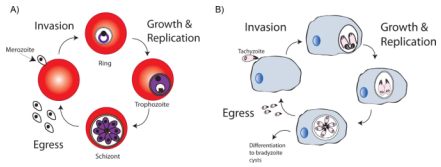

Figure 1.

Asexual life cycle of *Plasmodium* (A) and *Toxoplasma* B). During invasion, polar zoite cells randomly establish contact to host cells, followed by reorientation of apical end to host cell membrane. A tight junction forms between the parasite and host membrane, and this junction moves from the apical to the posterior end of the parasite. This results in formation of an invagination which eventually forms the parasitophorous vacuole. This is accompanied by shedding of surface proteins. Once invasion is complete, parasites rapidly grow in size in the intracellular environment, and replicate their genome to prepare for cellular division. When cell division is complete, parasites exit host cells in a process known as egress to invade other cells. In *Toxoplasma*, chronic infection can occur via transformation of the active tachyzoites to the inactive form of parasites known as bradyzoites which form tissue cysts in hosts.

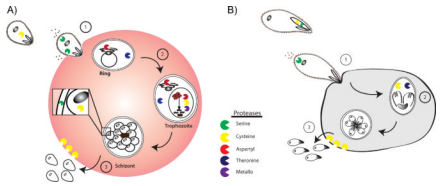

Figure 2.

Schematic depicting the role of proteases in different stages of the parasite life cycle in A) *Plasmodium falciparum* and B) *Toxoplasma gondii*. Different classes of proteases are represented in the corresponding color. Proteases are involved in proteolytic maturation of apical organelle proteins prior to invasion (not shown in figure). During invasion (1), the proteins from the apical organelles can facilitate adhesion to the host cell, and these proteins are subsequently shed in a protease-dependent manner. Proteases are also involved in general metabolism (2) to allow growth and replication. After several rounds of cell division, host cells rupture to release parasite zoite cells (3) which then re-invade other host cells and repeat the life cycle.

Table 1

List of partially characterized proteases organized by mechanism of proteolysis in *Toxoplasma gondii* and *Plasmodium falciparum*. Cited references are representative papers from studies of each protease listed.

Protease Class	Protease	Apicomplexan species	Implicated function in asexual stage	Protease function and localization	Literature
Aspartyl	Plasmepsins I–IV	*Plasmodium falciparum*	Growth and Replication	Hemoglobin degradation in food vacuole	[55], [60]
	Plasmepsin V	*Plasmodium falciparum*	Growth and Replication	PEXEL processing for export into the erythrocytic cytosol; localized to the ER	[99], [100]
Cysteine	TgCPB/Toxopain-1	*Toxoplasma gondii*	Invasion	Rhoptry protein processing; localized to the rhoptries	[48]
	TgCPCs	*Toxoplasma gondii*	Growth and Replication	Cleavage of dipeptides from N-terminal in cytosol, exact functions unknown	[97]
	TgCPL	*Toxoplasma gondii*	Invasion	Maturation of micronemal proteins; localized in the vacuolar compartment	[50]
	PfDPAP1	*Plasmodium falciparum*	Growth and Replication	Involved in later stages of hemoglobin degradation in food vacuole	[77], [78]
	PfDPAP3	*Plasmodium falciparum*	Egress	Facilitates PfSUB1 maturation in apical end of merozoites (unpublished)l	[107]
	Falcipain-1	*Plasmodium falciparum*	Invasion	Exact functions unknown; localized to apical end of merozoites	[45], [46]
	Falcipain-2,2',3	*Plasmodium falciparum*	Growth and Replication	Hemoglobin degradation in food vacuole	[69], [70], [74]
Metalloprotease	Falcilysin	*Plasmodium falciparum*	Growth and Replication	Involved in later stages of hemoglobin degradation in food vacuole	[64], [66]
	PfM1AAP and PfM17LAP	*Plasmodium falciparum*	Growth and Replication	Involved in terminal stages of hemoglobin degradation in food vacuole	[80], [82]
	Toxolysin 4	*Toxoplasma gondii*	Invasion/Egress	Exact function unknown; localized to micronemes	[51]
Serine	PfSUB1	*Plasmodium falciparum*	Invasion and egress	Processing of merozoite surface and parasitophorous vacuole (PV) constituents prior to egress; localized to the exonemes	[23], [24], [107]
	PfSUB2	*Plasmodium falciparum*	Invasion	Surface protein sheddase;localized to the micronemes	[26]
	TgSUB1	*Toxoplasma gondii*	Invasion	Processes micronemal proteins involved in host cell attachment; localized to the micronemes	[30]
	TgSUB2	*Toxoplasma gondii*	Invasion	Maturation of rhoptry proteins; localized to the rhoptries	[31]
	PfSERA5	*Plasmodium falciparum*	Egress	Potential role in egress; localized to the PV	[110], [114]
	TgROM2,4,5	*Toxoplasma gondii*	Invasion	Surface protein sheddase;localized to tachyzoite plasma membrane	[38]
	PfROM1 and 4	*Plasmodium falciparum*	Invasion	Surface protein sheddase;localized to merozoite plasma membrane	[42]
Threonine	PfHslV	*Plasmodium falciparum*	Growth and Replication	Possible function as peptidase, but targets unknown; localized to mitochondria	[141], [142]

Protease Class	Protease	Apicomplexan species	Implicated function in asexual stage	Protease function and localization	Literature
	Proteasome	*Plasmodium and Toxoplasma*	Growth and Replication	General protein degradation; Regulates protein turnover; localization unknown	[94], [140]