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New insights into parasite rhomboid proteases

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ABSTRACT

The rhomboid-like proteins constitute a large family of intramembrane serine proteases that are present in all branches of life. First studied in *Drosophila*, these enzymes catalyse the release of the active forms of proteins from the membrane and hence trigger signalling events. In protozoan parasites, a limited number of rhomboid-like proteases have been investigated and some of them are associated to pathogenesis. In Apicomplexans, rhomboid-like protease activity is involved in shedding adhesins from the surface of the zoites during motility and host cell entry. Recently, a *Toxoplasma gondii* rhomboid was also implicated in an intracellular signalling mechanism leading to parasite proliferation. In *Entamoeba histolytica*, the capacity to adhere to host cells and to phagocytose cells is potentiated by a rhomboid-like protease. Survey of a small number of protozoan parasite genomes has uncovered species-specific rhomboid-like protease genes, many of which are predicted to encode inactive enzymes. Functional investigation of the rhomboid-like proteases in other protozoan parasites will likely uncover novel and unexpected implications for this family of proteases.

1. Introduction

Regulated intramembrane proteolysis (RIP) is a precise and irreversible mechanism by which integral membrane proteins that are inactive in their membrane-tethered form are activated by proteolytic cleavage within their transmembrane domain (TMD) to release their functional cytoplasmic or luminal/extracellular domains (reviewed in [1]). RIP is catalysed by four distinct protease families, collectively named intramembrane cleaving proteases (i-CLiPs), comprising two families of aspartyl proteases – presenilin and signal peptide peptidases; one family of serine proteases – rhomboids; and one family of zinc metalloproteases – site-2 proteases. All i-CLiPs are multi-spanning membrane proteins that cleave their substrates at residues buried inside or in proximity to the TMD. In light of the current findings, rhomboids are thought to be the only i-CLiPs that do not require previous cleavage of the substrates prior to their recognition.

The conservation of rhomboid-like genes in almost all organisms suggests that these proteases are implicated in important biological processes. Investigation of their role in many species is still at an early stage and little is known about the mechanisms behind substrate recognition, substrate specificity and regulation of proteolytic activity. This review will discuss the most recent findings on the biological roles of rhomboids and, in particular, their importance for protozoan parasites.

2. The rhomboid family of proteases

2.1. Common features and classification

Drosophila melanogaster Rhomboid-1 was the first rhomboid protease described [2,3]; since then, rhomboid-like genes have been found throughout the six kingdoms of life [4]. The overall sequence identity between rhomboids from different organisms

Abbreviations: MIC, microneme protein; ROM, rhomboid protease; PV, parasitophorous vacuole; RON, rhoptry neck protein; ROP, rhoptry bulb protein; MJ, moving junction; TRAP, thrombospondin-related anonymous protein; TgMIC1, micronemal protein 1; TgMIC2, micronemal protein 2; TgMIC3, micronemal protein 3; TgMIC4, micronemal protein 4; TgMIC6, micronemal protein 6; TgMIC8, micronemal protein 8; TgAMA1, apical membrane antigen 1; TgM2AP, MIC2-associated protein; PVM, parasitophorous vacuole membrane; RIP, regulated intramembrane proteolysis; TMD, transmembrane domain; i-CLiP, intramembrane cleaving protease; PARL, presenilin associated rhomboid-like; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; MPP1, micronemal protein protease 1; DCI, 3,4-Dichloroisocoumarin (DCI); TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

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is rather low [4,5] except for the presence of 6–7 TMDs and of the family signature motifs. These conserved sequences include the catalytic dyad composed of a histidine residue followed by a GxxxG motif in one of the TMDs and a serine residue within a GxSx motif in another TMD; a trpyptophan–arginine motif (WR) in the first extracellular loop, and a HxxxxHxxxN motif. These sequence motifs are not only conserved in different rhomboids but also have been assigned structural and functional significance [6]. Nevertheless, because similar sequence motifs can be found in unrelated transmembrane proteins, the identification of genes that encode putative new rhomboid proteases remains difficult. New phylogenetic analysis indicates that the rhomboid-like proteases belong to a Rhomboid-like superfamily or clan (pfam clan CL0207), including the derlins, involved in endoplasmic reticulum-associated degradation of proteins [7,8].

The most recent classification of the rhomboid family in eukaryotes [5] proposed an organisation into three groups: Rhomboid-like proteases (active rhomboids); iRhoms (inactive rhomboids) harbouring three specific features [9] – a longer N-terminal cytoplasmic domain, a large and conserved loop 1 domain (corresponding to the iRhom homology domain IRHD), and an invariant proline residue N-terminus to the predicted catalytic serine (GPxx replacing the GxSx rhomboid catalytic motif usually present in the transmembrane domain 4); and other inactive rhomboid-like proteins that cannot be assigned to either of the previous groups. The rhomboid-like proteases can be further classified according to their intracellular localization in the secretory pathway or mitochondria. In the secretory pathway, the secretase rhomboids are further divided into a secretase type A group that includes the *bona fide* rhomboids with all the signature motifs characteristic of the family and 6 TMDs fused to an extra TMD at the C-terminus (6 + 1 TMD topology) and the secretase type B group containing the 6 TMD topology rhomboids and, with few exceptions, only a conserved arginine residue in the WR motif [5]. The mitochondrial rhomboids – called PARL-like rhomboids after the presenilin-associated rhomboid like protein found in mammals – have a 1 + 6 TMD topology, with an extra TMD fused N-terminus to the 6 core TMDs, and a mitochondrion-targeting motif. Typically, there is only one PARL-like rhomboid in each species [4,5].

2.2. Cleavage site and substrate recognition

Rhomboid substrates are usually type I or type III transmembrane proteins with conserved cleavage sites at or in proximity to the TMD. Star is the only type II transmembrane protein reported to date to be cleaved by a rhomboid [10]. At least three studies suggest that the cleavage site can be located outside the TMD; the *D. melanogaster* protein Gurken [11] and the human EGF ligand [12], when cleaved *in vitro*; an artificial fusion construct of the second TMD domain of the *Escherichia coli* LacY protein [11,13] and a series of engineered TatA variants [11] that are cleaved at sites at the junction of the TMD with the extracellular domain. It remains unclear whether rhomboid-mediated cleavage can occur outside the TMD *in vivo* because the few rhomboid crystal structures that have been solved so far point towards an intramembrane cleavage mechanism (reviewed in [14]). One possibility is that when cleavage occurs outside the TMD, the substrate sequence is flexible enough to gain access to the rhomboid active site [11].

Distantly related rhomboid proteases can cleave the same synthetic model substrates at identical positions [11], and rhomboids of one species can cleave substrates from another species, indicating either that rhomboids have little substrate specificity or that there are shared structural features in the proteases, in the substrates, or both [15]. Initial studies with the *D. melanogaster* Rhomboid-1 indicated that substrate recognition relied mainly on helix-breaking residues because fusion of the transmembrane residues of the Rhomboid-1 substrate Spitz converted several tested proteins into a rhomboid substrate [16]. Nevertheless the distance between the substrates rhomboid cleavage site and the helix-breaking motif could be moved [11,13,17]. Despite being conserved, these cleavage site determinants are not present in all rhomboids [15–17] and it has been suggested that the presence of helix-destabilising motifs in the substrate is less important for cleavage to occur if the cleavage site is outside the membrane instead of within the TMD [11]. More recently, a cleavage motif was identified that is recognised by rhomboid proteases from evolutionarily distant species, but again this motif is not universal and it is insufficient for cleavage by all rhomboids [11]. These findings suggest that the proteases recognise a common conformation rather than a specific amino acid sequence. Erez and Bibi suggested that tightly folded substrates, or substrates in complexes with other proteins might be inaccessible to the protease, thus specificity would be determined by the intrinsic conformational properties of the substrate [18]. In support of this idea, these researchers showed that GlpG can cleave type III proteins as long as they are presented to the rhomboid in a truncated, possibly unfolded form [18].

At least in some cases, regions outside the cleavage site were also shown to play a role in substrate recognition: the bacterial GlpG rhomboid requires helix-destabilising residues both in the hydrophobic TMD region and in the cleavage site region of its substrates [17]; *D. melanogaster* Rhomboid-1 can use a secondary recognition motif in Spitz when the primary one is disabled [11]; and cleavage of the mammalian protein thrombomodulin by the vertebrate RHBDL2-like rhomboids is directed not by sequences in the TMD but in the cytoplasmic domain [19].

Substrate recognition by the PARL-like rhomboids does not require helix-destabilising residues in the cleavage region but proteolytic processing only occurs when a second sequence composed of a stretch of strongly negatively charged Asp/Glu residues is present C-terminal to the rhomboid cleavage site [20].

The rhomboid proteases characterized to date appear to require no co-factors [13,21,22]; their activity is regulated, most likely, by regulation of their gene expression and by the compartmentalization of the substrates in a subcellular localization distinct from that of the proteases (reviewed in [23]). In addition, experiments have shown that the membrane environment regulates the activities of several rhomboid proteases [22]. Since these experiments were performed *in vitro*, it remains to be seen if this phenomenon has any role in a physiological context. The human rhomboid RHBDL2 is expressed as an inactive proenzyme and its activity is regulated by proteolysis [24], but it is unclear if other members of the family are regulated in this way. The cytosolic regions of the proteases may also play a role in regulating rhomboid activity. These domains are often large and conserved between rhomboid family members but are not essential for catalysis, suggesting a function in substrate recognition/specificity or regulation of activity (reviewed in [23]). Consistent with this hypothesis, the removal of the N-terminal cytosolic domain of RHBDL2 affects its activity *in vitro* [19] and the N-terminal domain of the *Pseudomonas aeruginosa* GlpG is also important for its function *in vitro* [25]. In the case of GlpG, Sherratt et al. suggested that the N-terminus of the protease interacts with the catalytic core domain, enhancing its activity by altering the conformation/stability of the active site and/or aiding anchorage of the protease to the membrane [25]. The iRhom family of pseudoproteases, which evolved from rhomboid proteases that lost their catalytic activity but retained their localization and the ability to bind their substrates, may provide additional regulatory functions. These pseudoproteases were recently shown to regulate the function of their active counterparts by promoting endoplasmic reticulum-associated degradation of the substrates [9]. Phylogeny studies revealed that the iRhoms are highly conserved in all animal species and are hence under selective pressure [23].

3. Biological functions

3.1. Role in signalling

Rhomboid-mediated RIP is most often associated with intercellular signal transduction pathways. The archetypal rhomboid, *D. melanogaster* Rhomboid-1 regulates signalling of the epidermal growth factor receptor (EGFR) by mediating cleavage of its ligand Spitz [2]. Full-length Spitz is trapped in the endoplasmic reticulum until Star chaperones it to the Golgi apparatus, where it is activated by Rhomboid-1-mediated cleavage. Rhomboid-1 also cleaves Star to regulate the levels of secreted Spitz [10]. EGFR signalling in the flour beetle *Tribolium castaneum* is also mediated by rhomboid cleavage of a Spitz homologue, which traffics in a Star-dependent manner, but in this case Star is not susceptible to rhomboid cleavage [26]. In the nematode *Caenorhabditis elegans*, an EGFR-like pathway also involves a rhomboid protease. *C. elegans* cells express an EGF-like signalling factor called LIN-3, which has similarities to Spitz. LIN-3 is sensed by the EGFR-like receptor LIN-23 on neighbouring cells thus activating the RAS/mitogen-activated protein kinase (MAPK) pathway [27]. In this case, cleavage by rhomboid increases the range of the LIN-3 signal [28]. Less is known about the signalling function of rhomboid proteases in mammals. The human rhomboid, RHBDL2, when overexpressed in human cells, can cleave the receptor tyrosine kinases ephrinB2 and ephrinB3 [29], thrombomodulin [19] and EGF [12] in an *in vitro* assay, but whether this cleavage occurs in physiological conditions remains unknown.

In the bacterium *Providencia stuartii*, cleavage of a small inhibitory N-terminal peptide from the twin-arginine translocase TatA by the rhomboid protease AarA initiates quorum sensing by activating TatA's function as a channel for export of the quorum-sensing signal [30,31]. Interestingly, most bacteria do not encode a TatA protein with an N-terminal extension and therefore do not have to be activated by cleavage, suggesting that the role of AarA is a rare adaptation rather than a general mechanism (reviewed in [32]). In *E. coli*, the GlpG rhomboid protease has been widely studied in terms of its protein structure and substrate specificity, however, its natural substrates remain to be discovered (reviewed in [32]).

3.2. Role in mitochondria

The archetype of the mitochondrial rhomboids is the yeast Rbd1p (or Pcp1p) rhomboid protease. Rbd1p mediates secondary proteolytic processing of cytochrome C peroxidase (Ccp1p), which is involved in oxidative-stress signalling, and of the dynamin-like GTPase Mgm1p, which is implicated in mitochondrial membrane fusion (reviewed in [23,33]). In *D. melanogaster*, the mitochondrial rhomboid Rhomboid-7 (also known as Rho7) has been shown to cleave the membrane-tethered protease Omi/HhtrA2 and the kinase PINK1 [34]. The mammalian Rhomboid-7 homologue – PARL – also mediates cleavage of Omi and PINK1 [35–37]. These proteins have been previously associated with the mitochondrial defects seen in Parkinson's disease and most recently, a mutation in the auto-catalytic processing site of PARL was shown to be more prevalent in Parkinson's patients than in control individuals [37].

Investigations of *Parl* knockout mice have revealed that cells lacking PARL are more susceptible to apoptosis [38] due to a defect in remodelling of the cristae, a process that is controlled by the dynamin-related protein OPA1. Although cristae remodelling depends on the catalytic activity of PARL, most data suggest that OPA1 is not the substrate and OPA1 cleavage is predominantly mediated by a PARL-independent mechanism [39].

The plant *Arabidopsis thaliana* encodes at least thirteen rhomboid-like genes, one of which, AtRBL2, is a putative PARL-like rhomboid [4]. AtRBL2 can cleave the *D. melanogaster* substrate Spitz *in vitro* [40] and the plastid translocon component Tic40, which is located in the mitochondria appears to be one of the physiological substrates [41].

4. Rhomboid proteases in protozoan parasites

4.1. Apicomplexans

Initial insights into rhomboid function in apicomplexan parasites came from studies on the micronemal protein protease 1 (MPP1). Microneme proteins (MICs) are secreted onto the parasite surface during host cell invasion and the MPP1 activity is responsible for the proteolytic shedding of the MICs (reviewed in [42,43]). The exact function of the MPP1 activity remains unknown but several hypotheses have been suggested: preventing the accumulation of the parasite's adhesin proteins at the cell surface to avoid them becoming the target of neutralizing antibodies in the host [43]; contributing to the proper reorientation of the parasite preceding penetration of the host cell by generating a gradient of adhesins at the parasite surface; disengagement of the parasite from its tight attachment to the host cell receptors at the end of the invasion process. In *Toxoplasma gondii*, MPP1 was shown to cleave TgMIC2, TgMIC6, TgMIC12 and TgAMA1, which are implicated in distinct steps of the invasion process [44–47]. The MPP1 cleavage site was first mapped by mass spectrometry to the TMD of TgMIC6 [46] and is conserved in other transmembrane micronemal proteins [42]. The MPP1 activity is essential at least for TgMIC2 since overexpression of a cleavage site mutant of TgMIC2 blocked invasion [44]. The first experimental evidence that a rhomboid protease was responsible for the MPP1 activity came for the analysis of TgMICs in cell based cleavage assays [50,52,53]. Subsequently, *Plasmodium falciparum* PfEBA-175 [48], PfAMA1 [45], PfRh1, PfRh4 [49] and PfTRAP (P. Sinnis, personal communication) were reported to be cleaved by a rhomboid-like activity *in vivo*, whereas PfCTRP, PfMTRAP and PfMAEBL were shown to be rhomboid substrates *in vitro* [50]. Importantly, this group of substrates includes critical *Plasmodium* adhesins implicated in each of the invasive stages of the parasite and in all of the alternative pathways of invasion (reviewed in [51]). The broad range of putative MPP1 substrates also extends to the other Apicomplexans [42], suggesting that the MPP1 activity is ubiquitous throughout the phylum and across life stages.

Phylogenetic analyses have revealed that rhomboid-like proteases comprise an abundant and diverse family in apicomplexan parasites. With the exception of a PARL-like rhomboid (*ROM6*), all the other genes are unique to the phylum of Apicomplexa suggesting that they are involved in processes related to parasitism [42] (Table 1). ROM4/ROM5 are the only rhomboid-like proteases conserved across the phylum; ROM2 is restricted to some coccidians, *ROM7* is only present in the haemosporidia, whereas ROM9 and ROM10 are exclusive to *Plasmodium* species (Fig. 1). Interestingly, in phylogenetic analyses *Plasmodium* ROM9 groups with the PARL-like rhomboids and it contains a putative mitochondrion-targeting motif, suggesting that the malaria parasite might have two PARL-like rhomboids.

Studies of several of the *T. gondii* and *Plasmodium* rhomboid-like proteases have found that the different proteases localize to distinct subcellular organelles in the parasites. TgROM1 is a micronemal protein, which is catalytically active *in vitro* against Spitz, but its physiological substrate(s) are unknown [52,53]. In *P. falciparum*, ROM1 has been localized to either a new secretory organelle called mononeme [54] or to the micronemes [48]. PfROM1 is an active protease but, again, its physiological substrates remain to be identified [50]. Deletion of the *ROM1* gene in *T. gondii* [55] or *Plasmodium berghei* [56] revealed no significant defect whereas in *Plasmodium yoelii*, the absence of ROM1 leads to malformation of the parasitophorous vacuole in some parasites

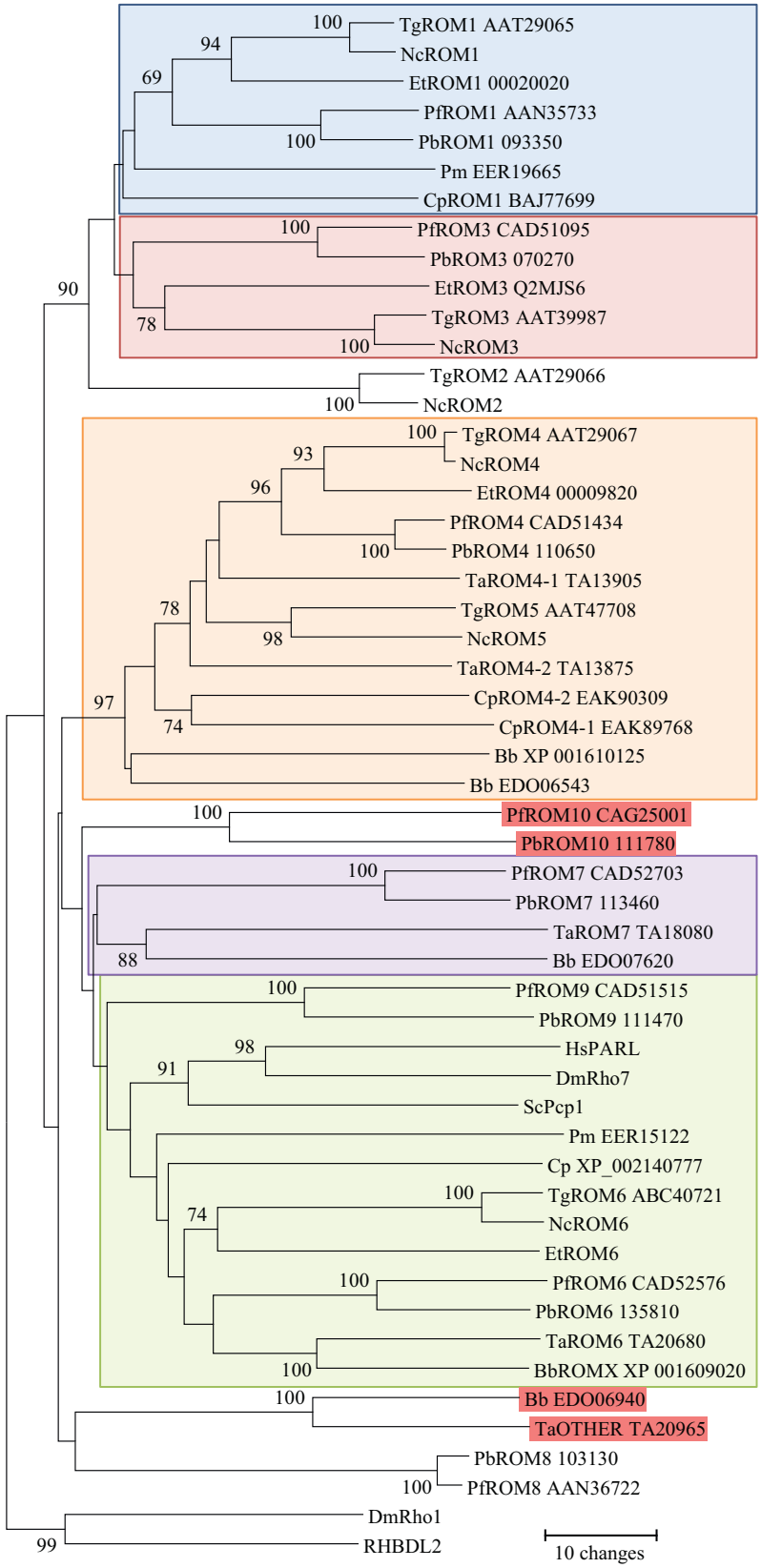

Fig. 1. Phylogenetic analysis of *Apicomplexan* ROMs. All Apicomplexans studies to date possess in common at least one ROM4/5 and one ROM6/9 protease, whereas other ROMs are only found in few species. The tree is based on neighbour-joining-distance analysis. Only nodes supported by a bootstrap value >70 as determined by neighbour-joining analysis are indicated. Bootstrap analysis was performed with 1000 replicates. Protein sequences boxed in red are predicted to be proteolytically inactive rhomboid like proteins according to Urban [76]. Species are represented by two letters preceding each ROM: Bb, *Babesia bovis*; Cp, *Cryptosporidium parvum*; Dm, *Drosophila melanogaster*; Et, *Eimeria tenella*; Nc, *Neospora caninum*; Pb, *Plasmodium berghei*; Pf, *Plasmodium falciparum*; Pm, *Perkinsus marinus*; Ta, *Theileria annulata*; Tg, *Toxoplasma gondii*. The accession or identification number of each protein is shown where available (*P. berghei* identification numbers are given according to the PlasmoDB website). The sequences alignment used to compute the phylogenetic tree is presented in Fig. S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Repertoire of rhomboids like proteins in protozoan parasites with accession numbers.

Apicomplexan								
	T. gondii	*N. caninum*	*E. tenella*	*P. falciparum*	*B. bovis*	*T. annulata*	*C. parvum*	*P. marinus*
ROM1	AAT29065	CBZ54033	contig_00029424	AAN35733			BAJ77699	EER19665
ROM2	AAT29066	CBZ52676						EER01328
ROM3	AAT39987	CBZ51508	Q2MJS6 contig_00030481	CAD51095				EER01784
ROM4	AAT29067	CBZ53994	contig_00015123	CAD51434	EDO06543 XP_001610125	TA13905 TA13875	EAK89768 EAK90309	EER05168
ROM5	AAT47708	CBZ49670	contig_00008282					EER05625
ROM6	ABC40721	CBZ55860	contig_00010048	CAD52576	XP_001609020	TA20680	XP_002140777	EER15122
ROM7				CAD52703	EDO07620	TA18080		EEQ97501
ROM8				AAN36722				EER19266
ROM9				CAD51515				
ROM10				CAG25001				
Other					EDO06940 EDO06560	TA20965 TA15910		

Amoebae			Microsporidia	Kinetoplastida				
E. histolitica	*T. vaginalis*	*N. gruberi*	*E. bieneusi*	*L. major*	*L. infantum*	*L. brasiliensis*	*T. cruzi*	*T. brucei*
EhROM1 EAL46300	TVAG 476950	NAEGRDRAFT 78160	XP_002651740	XP_822242	XP_001462732	XP_001561544	XP_821055 XP_802860	XP_951551
EAL44183	TVAG 037580	NAEGRDRAFT 80985	XP_002649815	XP_888596	LinJ04_V3.0850	XP_001561764	XP_805971	XP_827161
EAL47860	TVAG 112900	NAEGRDRAFT 72906						
EAL49376	TVAG 183030	NAEGRDRAFT 70390						
	TVAG 359500	NAEGRDRAFT 74295						
	TVAG 161010	NAEGRDRAFT 63964						
	TVAG 282180 TVAG 233140	NAEGRDRAFT 66399						
	TVAG 378960	NAEGRDRAFT 70844						
		NAEGRDRAFT 3119						

Only proteins highlighted in the same color and present on the same line are homologous proteins between different species. Predicted proteolytically inactive rhomboid-like proteins are highlighted in red (see also sup. table* 1).

[57]. ROM2 has been found only in the two closely related coccidian parasites *Neospora caninum* and *T. gondii*. This protease is located to the Golgi apparatus and is catalytically active *in vitro* but no information regarding its biological function has yet been reported [53]. A detailed analysis of the trafficking determinants governing the subcellular distributions of TgROM1 and TgROM2 revealed that the N-terminal cytosolic domain plays an important role but additional elements are implicated in the retention of TgROM2 in the Golgi compartment [58]. As expected, the PARL-like rhomboid TgROM6 localizes to the single tubular mitochondrion but the known PARL substrates are absent from the genome of *T. gondii* (Sheiner and Soldati-Favre, unpublished). TgROM6 is likely to perform a distinct function from PARL since there is apparently no evidence for mitochondrion fusion in *T. gondii* [59]. TgROM4 and PfROM4 are large proteins evenly distributed throughout the parasite plasma membrane [48,52,53,58]. TgROM5 also localizes to the plasma membrane of extracellular parasites, but in intracellular parasites its subcellular distribution is less well defined [52,53].

Cell-based cleavage assays have been used to test for the activity and establish the substrate specificity of the apicomplexan rhomboid proteases [48,50,52,53], but, while informative, these studies should be interpreted with caution, as illustrated by the *in vivo* studies of TgROM4 function. This protein was considered inactive in the cell-based cleavage assay [52], but conditional knockout of the *TgROM4* gene revealed that the protease is, in fact, essential for cleavage of TgMIC2, TgAMA1 and possibly also TgMIC8 [60]. Intriguingly, knockdown of TgROM4 did not affect cleavage of TgMIC6 [60] despite an evident sequence similarity with the TgMIC2 cleavage motif [42], strongly suggesting that sequences outside the TMD determine substrate specificity for apicomplexan rhomboids. Consistent with this idea, mutation of a lysine residue positioned eleven residues upstream of the rhomboid cleavage site in TgMIC2 abolishes its cleavage [44]. Although *TgROM4* is an essential gene, the conditional knockout of *TgROM4* in parasites only led to a modest defect in invasion [60]. One plausible explanation for this finding is that residual expression of the inducible copy of *TgROM4* is sufficient to fulfil the vital function of the protease at another step of the parasite lytic cycle. Supporting this idea, a recent study has uncovered an essential role for TgROM4 at the replication step [61]; conditional expression of a catalytically inactive mutant of TgROM4 (ddROM4$_{S\text{-}A}$) did not affect host cell invasion but strongly impaired parasite replication. Expression of ddROM4$_{S\text{-}A}$ presumably acts as a dominant negative mutant and sequesters from the endogenous, active copy of ROM4, the substrates responsible for a signalling cascade leading to parasite growth and division. Strikingly, expression of the cytosolic cleaved form of TgAMA1 but not of TgMIC2 fully reversed the effect caused by ddROM4$_{S\text{-}A}$ [61], suggesting that TgROM4-mediated cleavage of TgAMA1 and possibly other substrates signals *Toxoplasma* parasites to switch from an invasive to a replicative mode. TgAMA1 is an important player in invasion [62] and its involvement also in a mechanism governing division might assure a quick transition from invasion to replication upon entry into the host cell [61]. Although TgAMA1 can signal for replication, it remains to be seen whether other TgROM4 substrates are also implicated in this process, notably during the rounds of replication subsequent to the first division cycle after invasion. Expression of the *P. falciparum* cytosolic cleaved form of AMA1 in *Toxoplasma* parasites expressing ddROM4$_{S\text{-}A}$ also reversed the replication blockage [61], supporting the notion that the signalling mechanism might be conserved across the phylum. Consistent with this, a recent study showed that whereas interference with PfAMA1 cleavage does not impair host cell invasion by *P. falciparum*, it is deleterious for survival of the parasite, indicating that proteolytic processing is necessary for a process other than invasion [63]. Interestingly, *Toxoplasma* and *Plasmodium* rhomboid substrates other than TgAMA1 have been proposed to participate in signalling pathways leading to secretion of the secretory organelles rhoptries [64,65], raising the question

of whether the apicomplexan rhomboids are involved in the regulation of other mechanisms occurring during the parasites life cycle.

Little is known about rhomboid function in Apicomplexans other than *Toxoplasma* or *Plasmodium*. In *Cryptosporidium*, for example, a protease related to TgROM4 and TgROM5 (CpRom) is located at the posterior pole of sporozoites [66], but its function is unknown. In *Eimeria tenella*, vaccination of chickens with a modified virus containing the rhomboid sequence induced an immune response and offered partial protection against *E. tenella* challenge [67].

4.2. Entamoeba

Rhomboid-like proteases have been investigated in detail in *Entamoeba histolytica*, the causative agent of amoebiosis. *E. histolytica* encodes four rhomboid-like proteins but only one – EhROM1 – is catalytically active. Elegant studies have shown that EhROM1 has an essential function in parasite adhesion to host cells and its ability to phagocytose [68]. In resting conditions, EhROM1 is located both at the parasite surface and in internal punctate structures but upon erythrophagocytosis it relocates to internal vesicles [68]. *EhROM1* knockdown leads to a defect in parasite adhesion to healthy cells as well as a generalized phagocytosis defect that are attributed to independent functions of the rhomboid protease, perhaps related to different substrates [69]. Although EhROM1 can cleave *in vitro* the parasite adhesin Gal/GalNAc lectin, there was no change in the expression or localization of this protein in cells depleted of the rhomboid [69].

E. histolytica, like the Microsporidia and Cryptosporidia spp., possess a remnant mitochondrion, called mitosome, which is devoid of mitochondrial DNA and performs only a limited set of functions compared to mitochondria in other species. All these protozoan parasites lack a recognisable rhomboid protease that is phylogenetically related to the mitochondrial DmRho7 or HsPARL.

The current knowledge about rhomboid-like proteases in protozoan parasites with regard to their localization, substrate(s) and function(s) is summarized in Table 2.

4.3. Repertoire in other protozoans

A scrutiny for the presence of rhomboid-like proteases in other free living or parasitic protozoans revealed a broad heterogeneity in gene number and diversity and a predominance of predictably inactive rhomboid-like proteases (Table 1 and Figs. 1 and 2).

Perkinsus marinus, a prevalent pathogen of oysters, belongs to an early branching group of the chromoalveolates, the dinoflagellates, and shares similar organelles with the Apicomplexans. Two of the nine genes encoding rhomboid-like proteins in *P. marinus* have significant phylogenetic similarities with the apicomplexan rhomboids ROM1 and ROM6 and intriguingly early electron microscopy studies revealed the existence of rectilinear micronemes and the presence of a mitochondrion in Perkinsus spp. [70], suggesting that the *P. marinus* ROM1 and ROM6 localize to these organelles, as in *T. gondii*.

The repertoire of rhomboid-like genes in the kinetoplastid species is unexpectedly small, with only two genes in *Leishmania major* and *Trypanosoma brucei* and three genes in *Trypanosoma cruzi* (Table 1). None of these proteins are phylogenetically related to the PARL-like proteases but sequence analysis (http://ihg.gsf.de/ihg/mitoprot.html) predicts the presence of a mitochondrial targeting signal. Interestingly, LmjF.02.0439 and LmjF.04.0850 have been shown to be mitochondrial in *L. major* (D. Tonn and J. Mottram, personal communication).

Naegleria gruberi is a free-living flagellate, member of the Excavate, a major eukaryotic group, including also the pathogenic trypanosomatids. Nine genes encoding for rhomboid-like proteases can be found in the *N. gruberi* genome. Many of these putative rhomboid-like genes lack one or several of the residues involved in nucleophilic catalysis and hence are predicted to be inactive proteases.

Trichomonas vaginalis is an anaerobic flagellated protozoan and the causative agent of the urogenital tract infection trichomoniasis. Strikingly, despite having a genome of approximately 160 megabases and 60,000 predicted protein-coding genes [71], *T. vaginalis* possesses only nine genes encoding rhomboid-like proteases and the majority of them are predicted to be inactive.

Giardia is a binucleate, anaerobic flagellated protozoan that belongs to the phylum of Metamonada. In the *Giardia lamblia* genome, only two genes carry rhomboid-like features (sup. Table 1). These sequences lack some of the rhomboid canonical signature motifs but harbour a putative catalytic Ser residue within the predicted TMD 5. Despite the fact that the *G. lamblia* rhomboid-like proteins are predicted to be inactive, the presence of highly conserved putative rhomboid cleavage sites in the C-terminal transmembrane domain of the Variant Surface Proteins [72] suggests that rhomboid proteases might act as sheddases. This example highlights the degree *f* uncertainity in classifying active versus inactive proteases in absence of experimental evidence.

5. Potential as drug targets

Host cell entry by obligate intracellular apicomplexan parasites is an essential and active process, which utilizes ligand–receptor interactions in concert with the parasite's motility apparatus to rapidly gain entry to cells. *P. falciparum* merozoites use several alternative invasion pathways to infect erythrocytes and this degree of redundancy in the invasion process represents a major hurdle for drug/vaccine approaches interfering with host cell receptor binding [73]. Targeting the activity of PfROM4, the rhomboid-like protease likely responsible for the cleavage of several of the microneme proteins within the transmembrane domain at the end of the host cell invasion process [48,50], may therefore provide a more direct way of interfering with invasion. If, in addition, this protease is implicated in the transmission of the signal for replication, as its counterpart in *T. gondii* [61], disruption of its activity would be a true Achilles' heel for the apicomplexan parasites.

Catalytic activity of many rhomboids is sensitive to the serine protease inhibitors 3,4-dichloroisocoumarin (DCI) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) inhibitors [3] and more knowledge regarding the mechanism of action and substrate specificity is necessary to predict if this family of proteases can be considered as a viable drug target. Drugs that target specifically presenilin activity, an i-CLiP have been obtained (reviewed in [74]) and more recently monocyclic β-lactams have been shown to be selective rhomboid inhibitors [75]. In this study, over 57,000 molecules were tested for their ability to inhibit the AarA rhomboid activity of *P. stuartii*; among others, a monocyclic β-lactam compound was a specific, potent inhibitor. 58 analogues of that compound were tested in gel-based assays for selectivity against the *P. stuartii* AarA and *E. coli* GlpG rhomboids and compared to the classic serine protease chymotrypsin. Different inhibitors showed greater effects against one or the other rhomboid, depending on the compound structures tested. Seven of them were also active *in vivo* when tested against the endogenous *E. coli* GlpG, and *in vitro*, although at a modest level, against the human RHBDL2 rhomboid protease. The inhibitory effect was, however, partially reversible.

Table 2
Localization, substrates and potential functions of rhomboids.

Rhomboid	Localization	Substrates	Function
Toxoplasma gondii			
TgROM1	Micronemes [52,53]	*In vitro:* DmSpitz *In vivo:* unknown	[50,52,53]
TgROM2	Golgi [52,53]	*In vitro:* TgMIC2/TgMIC12 [53] *In vivo:* unknown	Unknown
TgROM4	Plasma membrane [52,53,58]	*In vitro* Not active [52,53] *In vivo:* TgMIC2/TgAMA1/TgMIC8 [60]	Invasion [60] Replication [61]
TgROM5	Plasma membrane (posterior pole of the parasite) [52,53]	*In vitro:* DmSpitz/TgMIC2/TgMIC6/TgMIC12/ PfAMA1/PfEBA175/PfJESEBL/ PfBAEBL/PfRH1/PfRh2A/PfRh2B/PfRh4/PfTRAP/PfMTRAP/PFF0800c/PfMAEBL/PfCTRP [50,52,53] *In vivo:* unknown	Unknown
TgROM6	Mitochondrion (Sheiner and Soldati-Favre, unpublished)	*In vitro:* unknown *In vivo:* unknown	Unknown
Plasmodium spp.			
PbROM1PfROM1PyROM1	Micronemes (PyROM1/PfROM1/PbROM1) [48,56,57] Mononemes (PfROM1) [54]	*In vitro* DmSpitz/PfAMA1/PfRH1/PfRh2B/PfMAEBL/PyUIS4 [50,57] *In vivo:* unknown	Parasitophorous vacuole modification (PyROM1) Parasite development (PbROM1) Unknown (PfROM1)
PfROM4	Plasma membrane [48]	*In vitro:* PfEBA175/PfJESEBL/PfBAEBL/PfRH1/PfRh2A/PfRh2B/PfRh4/PfTRAP/PfMTRAP/PFF0800c/PfMAEBL/PfCTRP [48,50] *In vivo:* Unknown	Unknown
Cryptosporidium spp.			
CpROM	Posterior pole of the sporozoites [66]	*In vitro:* unknown *In vivo:* unknown	Unknown
Entamoeba histolytica			
EhROM1	Parasite surface and internal punctuate structures (resting conditions) Internal vesicles (erythrophagocytosis) [68]	*In vitro:* EhGal/GalNAc lectin [68] *In vivo:* unknown	Parasite adhesion to healthy cells Phagocytosis [69]

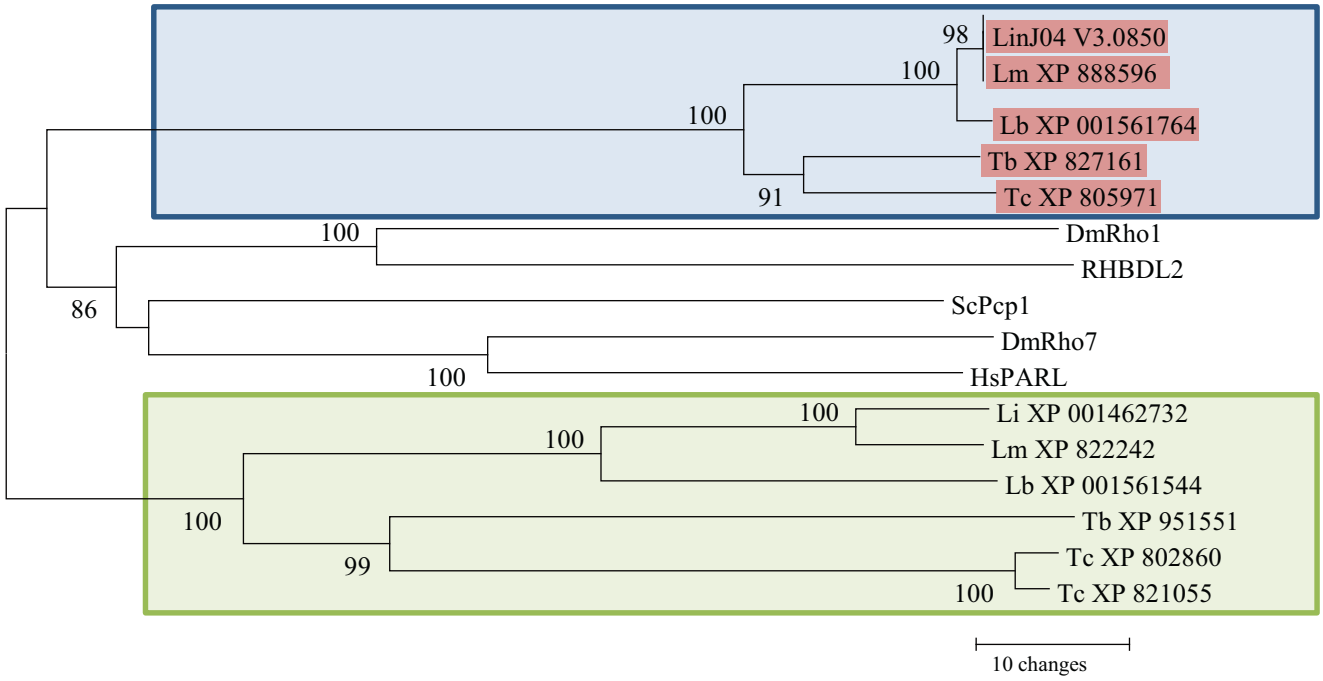

Fig. 2. Phylogenetic analysis of *Kinetoplastidiae* ROMs. The tree is based on neighbour-joining-distance analysis. Only nodes supported by a bootstrap value >85 as determined by neighbour-joining analysis are indicated. Bootstrap analysis was performed with 1000 replicates. Protein sequences boxed in red are predicted to be proteolytically inactive rhomboid like proteins according to Urban [76]. Species are represented by two letters preceding each ROM: Dm, *Drosophila melanogaster*; Li, *Leishmania infantum*; Lb, *Leishmania braziliensi*; Tb, *Trypanosoma brucei*; Tc, *Trypanosoma cruzi*. The accession or identification numbers of each protein are shown where available. The sequences alignment used to compute the phylogenetic tree is presented in Fig. S2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Study of rhomboid-like proteases in a broad range of protozoan parasites is still in its infancy, even if they have been in recent years the centre of attention for researchers working on *T. gondii*, *Plasmodium* spp. and *E. histolytica*. Analysis of the repertoire of rhomboid-like proteases in clinically relevant pathogens has revealed that most predicted genes are unique or shared only between closely related parasites, suggesting that future research on this fascinating family of enzymes will uncover plenty of unexpected functions that may provide new avenues for therapeutic intervention.

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Eimeria tenella sequences were obtained from the Gene3D website (http://www.genedb.org/blast/submitblast/GeneDB_Etenella) with the help of A. Reid from the Sanger institute.

Perkinsus marinus sequences were obtained from the GenBank website (http://www.ncbi.nlm.nih.gov/protein) with the help of N. El-Sayed, E. Caler, J. Inman, P. Amedeo, B. Hass and J. Wortman from the J. Craig Venter Institute.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2011.11.010.

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