Published in final edited form as:

Curr Opin Microbiol. 2015 August ; 26: 48–52. doi:10.1016/j.mib.2015.05.001.

The Apicomplexan CDC/MACPF-like pore-forming proteins

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Abstract

Pore-forming proteins (PFPs) encompass a broad family of proteins that are used for virulence or immune defense. Members of the cholesterol-dependent cytolysins (CDCs) and membrane attack complex/perforin (MACPF) family of PFPs form large β -barrel pores in the membrane. The CDC/MACPF proteins contain a characteristic 4-stranded β -sheet that is flanked by two α -helical bundles, which unfold to form two transmembrane β -hairpins. Apicomplexan eukaryotic parasites express CDC/MACPFs termed perforin-like proteins (PLPs). Here we review recent studies that provide key insights into the assembly and regulation of the Apicomplexan PLP (ApiMACPF) molecular pore-forming mechanisms, which are necessary for the osmotically driven rupture of the parasitophorous vacuole and host cell membrane, and cell traversal by these parasites.

Introduction

Pathogens and hosts utilize pore-forming proteins (PFPs) to facilitate pathogen internalization, release or translocation of effector proteins and/or target membrane lysis. Eukaryotic PFPs include complement membrane attack complex (MAC) and perforin (PF) defense proteins collectively referred to as MACPFs. The Apicomplexan phylum of eukaryotic parasites produces proteins that conserve a MACPF-like motif and are termed perforin-like proteins (PLPs). The recent solution of the crystal structures of several MACPF proteins [1–5] revealed a structural fold that was strikingly similar to one in the PFP perfringolysin O (PFO), a bacterial cholesterol-dependent cytolysin (CDC) [6]. This fold forms the CDC and MACPF β -barrel membrane pores (reviewed in [7,8]).

These studies strongly suggested that the related Apicomplexan MACPF-like proteins (ApiMACPFs) form β -barrel pores in a manner similar to the CDC/MACPFs. Herein we review the ApiMACPFs, focusing on pore formation and its contribution to pathogenesis. For more extensive reviews on Apicomplexan life cycles and the ApiMACPFs addressed herein please refer to recent reviews [9,10].

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The distribution of ApiMACPFs in Apicomplexans

Genomic analysis of Apicomplexans over the past decade has revealed that one or more genes encoding putative ApiMACPFs are present in *Toxoplasma*, *Plasmodium*, *Theileria* and *Babesia*. These proteins were identified by a consensus sequence located immediately upstream of the second of two membrane spanning β -hairpins that contribute to β -barrel pore formation in MACPF proteins (described below). The number of known or putative ApiMACPFs in these species varies widely, from two in *Toxoplasma* to up to 5 in *Plasmodium*, which likely reflects the complexity of their lifecycles. The studies of the structure and function of ApiMACPFs have been confined to those from *Toxoplasma* and *Plasmodium*.

Overview of the CDC/MACPF domain and pore formation

Pore formation begins with secretion/release of soluble CDC/MACPFs that utilize their C-terminal domain to bind to the membrane surface. Membrane-bound monomers oligomerize into a ring-like intermediate structure, termed the prepore, which has not yet penetrated the membrane [14,15]. The CDC/MACPF domain consists of a central β -sheet, comprised of four individual β -strands, flanked on either side by two sets of α -helices [2,3,6,11]. These α -helical bundles unfurl into transmembrane β -hairpins (TMHs), which assemble into a β -barrel pore that inserts into the membrane [1,12,13,16]. The pore complex size varies depending on the specific CDC or MACPF: the CDC pore is typically comprised of 35-40 monomers with an estimated pore diameter of 250-300Å (reviewed in [8]), whereas MACPFs contain 13-20 monomers with a pore diameter of 80-200Å [2,14]. The size(s) of the various ApiMACPFs pore is unknown, but gel analysis of oligomeric complexes of the *T. gondii* TgPLP1 suggest >22 monomers comprise its pore complex [17].

A notable difference in the CDC and MACPF pore-forming mechanisms is that the CDC prepore complex undergoes a 40Å vertical collapse, which is necessary to bring the TMHs sufficiently close to the membrane surface to span the bilayer [18]. The MACPFs contain longer TMHs and do not undergo a similar collapse [1,14].

The ApiMACPF pore-forming mechanism

Recently, bacterial and eukaryotic cell expression systems have been used to purify active ApiMACPFs that exhibit a pore-forming mechanism with fundamental similarities to the CDC/MACPFs. Pore formation and hemolytic activity by recombinant TgPLP1 (purified from *Escherichia coli*) was demonstrated recently by Roiko and Carruthers [17]. TgPLP1 exhibited significantly reduced specific activity compared to the CDC listeriolysin O. Without parasite-derived native protein for comparison, it remains unclear whether recombinant TgPLP1 is simply less active on RBCs, which are not a natural substrate, or if the refolding of the protein from inclusion bodies was inefficient. Their work, however, was significant since the recombinant TgPLP1 formed large oligomeric complexes on RBCs; the largest appeared to exceed 2.8 mDa, which corresponds to >22 monomers per oligomeric complex. Both the C- and N-terminal domains that flank the MACPF domain were shown to bind to RBC membranes and it was suggested that both might be necessary for efficiently binding the parasitophorous vacuole (PV) and/or host cell membranes during egress.

The same group recently showed that low pH (5.4) within the PV specifically activated membrane binding of TgPLP1 [**19]. The low pH had opposite effects on the C- and N-terminal domains: C-terminal domain binding increased while binding of the N-terminal domain decreased. This suggests that the N-terminal domain does not participate in binding the PVM during pH-triggered egress. The authors [**19] also proposed that turning off the pore-forming activity of TgPLP1 at neutral pH after PV and cell lysis would be important to limit damage to nearby cells that could serve as new host cells for the parasite. If TgPLP1 pH-induced activity is reversible, however, it would have ramifications for host cell lysis since the cytoplasmic pH is likely near neutrality.

Garg et al. [20] used HEK-293 cells to express *P. falciparum* PfPLP1 and showed that it also forms pores and higher order oligomeric complexes on human RBCs. Membrane binding by PfPLP1 was Ca^{2+} -dependent, similar to perforin, wherein 2 calcium atoms are bound to the C2 binding domain [21]. Although Ca^{2+} is essential to trigger egress of *T. gondii*, the binding function of TgPLP1 does not exhibit a similar Ca^{2+} -dependency (V. Carruthers, personal communication).

Garg et al. [20] also revealed that the purified PfPLP1 MACPF domain alone exhibited pore-forming activity at a level similar to that of the holoprotein. This result is inconsistent with our current understanding of the CDC/MACPF pore-forming mechanism. The C-terminal domain of all characterized CDC and MACPF proteins is required to bind and anchor the monomers to the membrane to initiate pore formation or the oligomeric complexes disengage from the membrane during β -barrel pore insertion [22]. Hence, the formation of a pore complex by PfPLP1 in the absence of the binding domain is difficult to explain.

Generally, these data strongly suggest that the ApiMACPFs form a pore in a manner similar to the CDC/MACPF proteins. Studies have shown that ApiMACPF pore formation contributes to egress and cell traversal but not cell invasion.

ApiMACPFs and cellular egress of the parasite

Kafsack et al. [**23] first showed the involvement of an ApiMACPF, TgPLP1, in egress of *T. gondii*. A second ApiMACPF, TgPLP2, is also encoded but no function is yet known. Parasites that lack TgPLP1 exhibit delayed PVM permeabilization following Ca^{2+} -ionophore induced egress; the eventual egress was attributed to mechanical damage from motile tachyzoites. However, TgPLP1-deficient parasites are also unable to undergo natural, non-ionophore induced egress, a process that does not require motility [24]; rather, osmotic pressure is the primary force that drives rupture of the PV and cell membranes.

The importance of osmotically driven rupture of the membranes is consistent with the observation in *P. falciparum* wherein the osmolyte Tetronic 90R4 prevented RBC lysis by PfPLP2 in gametocytes within the mosquito midgut, and to some extent prevented the rupture of the PVM [**25]. Osmolytes, such as the polyethylene glycols or dextrans, protect cells from pore-induced colloid osmotic lysis if their hydrodynamic radii are larger than the pore, which prevents the influx of water into the PV vacuole and cell cytoplasm. Since Tetronic 90R4 is highly hydrated and has a tendency to aggregate into larger complexes it

likely prevented cell and PV lysis by exceeding the size of the PfPLP2 pore. PfPLP2 is essential for host cell rupture, but not for lysis of the gametocyte PV, yet some osmotic stabilization of the PV was also observed, suggesting its lysis is also a pore-driven process [25,26].

These data indicate that the ApiMACPFs are essential for osmotic rupture of the PV and/or the cell membrane, but the egress process also requires other factors. In both *P. falciparum* and *T. gondii*, one or more proteases are involved, which appear to weaken the host cell cytoskeleton [27-29]. Furthermore, the fact that this process is driven or initiated by osmotic swelling of the PV and host cell shows that the intracellular osmotic pressures must support the influx of water first into the PV and then the host cell. The cytoplasmic concentration of proteins in RBCs and nucleated cells is 300-400 mg/ml [30,31]; thus, to osmotically lyse the PV these high levels of colloids must be offset. The osmotic status of the *P. falciparum* infected RBC is complex and incompletely understood, although degradation of up to 80% of the hemoglobin in the digestive vacuole of *P. falciparum* likely helps offset the colloid imbalance between the PV and RBC cytoplasm [32]. Whether *T. gondii* also degrades host cytoplasmic proteins is unknown. Hence, the Apicomplexans must manipulate the osmotic pressure of the PV and/or the host cell cytoplasm to facilitate ApiMACPF initiated rupture of PV and host cell membranes.

The requirements for egress of male and female gametocytes from the RBC in the midgut of the mosquito are different than that for merozoite escape. Male gametocyte infected RBCs require PfPLP2 for host cell lysis, whereas an unknown system is necessary for PV lysis [25,26]. This same PLP has been implicated in the escape of *P. falciparum*, but not *P. berghei*, female gametocytes [25,26]. However, osmiophilic bodies appear to be involved in egress of female gametocytes from both species and analogs may be present in the male gametocytes [33,34]. The contents of these bodies are released by the female gametocyte immediately before egress and contain a large, abundant hydrophilic protein (Pfg377), which could alter the osmotic pressure of the PV to facilitate its rupture. Whether a similar mechanism might be involved in the male gametocyte escape from the PV is unknown. These observations show that the plasmodia have the capacity to manipulate the osmotic pressures of the PV, but do not explain how the female gametocytes escape without forming pores in the PVM and/or the host cell membrane to facilitate the influx of water and osmotic lysis.

ApiMACPFs and cell traversal

Apicomplexans breach tissue barriers by cell traversal, a process that is less well understood than egress, but is also one in which the ApiMACPFs play an essential role(s). Although *T. gondii* must breach a number of barriers (i.e., intestine, vascular endothelium) there is no evidence for involvement of a PFP. The plasmodia cross the mosquito midgut epithelium, and the epidermis and liver sinusoidal layer in humans. The traversal of the mosquito midgut by *Plasmodium* ookinetes of rodent pathogens requires the ApiMACPF membrane-attack ookinete protein (MOAP) [35], which is related to PfPLP3. Traversal also requires PfPLP5 [36]: knocking out either protein yields the same phenotype wherein ookinetes are trapped within the midgut. Why two distinct ApiMACPFs are required for this process remains

unknown, but may be related to differences in the membrane structure/receptors of the outer and inner surfaces of the midgut epithelial cell membrane [37].

SPECT-2 (sporozoite protein essential for cell traversal) is required by sporozoites to cross the epidermis after injection [38] and the sinusoidal barrier surrounding the liver [39]. It is most closely related to *P. falciparum* PfPLP1, which functions in merozoite escape from RBCs. The cells in the skin and hepatic sinusoidal barrier that are subjected to traversal generally do not survive [40,41], as is probably true for the mosquito midgut traversal process. This is likely due in part to the ApiMACPF pore formation and osmotic lysis of these cells, but the complete process of traversal has other requirements [42].

Perspectives

While it is evident that the PLPs are only part of the complex processes of egress and cell traversal, many intriguing questions remain about their contributions therein. For instance, how they control the sequential formation of pores in the PV and plasma membranes is unclear. Do ApiMACPFs have specific receptors on membranes: is this why both PfPLP3 and PfPLP5 are required for ookinete cell traversal? Why does escape from the RBC in the blood and mosquito midgut require different PLPs? Many PFPs exhibit N-terminal domains that vary in primary structure: do they encode special functions? Finally, several ApiMACPFs from the *Babesia* and *Theileria* are atypically large and show evidence of 2-3 TMH pairs in their primary structures, which is unprecedented in the CDCs and MACPFs. Hence, continued study of the ApiMACPFs will likely reveal new and exciting insights into their pore-forming mechanism(s) and how they facilitate egress and cell traversal in these complex pathogens.

Acknowledgments

This work was supported by grant 1R01 AI037657 from the NIH NIAID to RKT. We appreciate the helpful discussions with V. Carruthers.

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* of special interest

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Highlights

- *Toxoplasma gondii* PLP1 (TgPLP1) exhibits pH-dependent binding.
- *Plasmodium falciparum* PLP1 (PfPLP1) exhibits calcium-dependent lytic activity.
- Recombinant TgPLP1 and PfPLP1 are lytic and form higher order oligomers.
- PLPs mediate the osmotic membrane rupture of the parasitophorous and cell membranes.