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How Apicomplexa Parasites Secrete and Build Their Invasion Machinery

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Keywords

Apicomplexa, invasion, exocytosis, microneme, rhoptry, moving junction

Abstract

Apicomplexa are obligatory intracellular parasites that sense and actively invade host cells. Invasion is a conserved process that relies on the timely and spatially controlled exocytosis of unique specialized secretory organelles termed micronemes and rhoptries. Microneme exocytosis starts first and controls the intricate mechanism of rhoptry secretion. To assemble the invasion machinery, micronemal proteins—associated with the surface of the parasite—interact and form complexes with rhoptry proteins, which in turn are targeted into the host cell. This review covers the molecular advances regarding microneme and rhoptry exocytosis and focuses on how the proteins discharged from these two compartments work in synergy to drive a successful invasion event. Particular emphasis is given to the structure and molecular components of the rhoptry secretion apparatus, and to the current conceptual framework of rhoptry exocytosis that may constitute an unconventional eukaryotic secretory machinery closely related to the one described in ciliates.

INTRODUCTION

Apicomplexa parasites such as *Plasmodium*, *Toxoplasma*, and *Cryptosporidium* prevail as pathogens with major health and socioeconomic importance worldwide, especially in developing countries. Every year, *Plasmodium* parasites account for more than 400,000 deaths due to malaria (161). *Toxoplasma gondii* is one of the most widespread human pathogens, infecting up to one-third of the population. Although toxoplasmosis is not a life-threatening disease in healthy adults, it is clinically relevant in pregnant women and immunocompromised patients (117) and induces serious ocular consequences even in healthy individuals. Cryptosporidiosis, caused by *Cryptosporidium*, is one of the major causes of devastating diarrhea and malnutrition in children (137).

The apical complex is the essential armament of Apicomplexa enabling their obligate intracellular lifestyle. It comprises a unique set of secretory organelles—micronemes, rhoptries, and dense granules—involved in host cell attachment and invasion and formation of an intracellular secluded environment, namely the parasitophorous vacuole (PV), which separates the parasite from the host cell cytoplasm to facilitate its intracellular development (80). Invasion is an essential and unique mechanism shared by many apicomplexans and occurs in less than 30 s (52, 93). A hallmark of the Apicomplexa invasion process is an electron-dense structure between the parasite and host cell membranes that is referred to as the moving junction (MJ) (1). The MJ serves as a firm anchor to the host cell, allowing it to endure shear forces during invasion, and also provides a support for the gliding machinery, an actomyosin-dependent process that propels the parasite inside the host (48). Its formation requires coordinated assembly of an intricate protein complex arising from the discharge of both microneme and rhoptry

proteins (3, 17, 81, 136).

TWO TIMELY REGULATED SECRETION EVENTS TO BUILDUP THE INVASION MACHINERY

During invasion, the parasite exquisitely orchestrates the sequential exocytosis of micronemes, followed by that of rhoptries (27, 113). Not only is the timing of secretion tightly regulated, but the location where it takes place is also restricted to the apical tip of the parasite. Microneme secretion initiates prior to the exit (egress) of the parasite from the host cell and persists during gliding and attachment of the parasite to a new host cell (20, 42). Rhoptry discharge is likely triggered upon contact of the apex of the parasite with the host cell just before the beginning of invasion (43), and it is completed by the time the nascent PV is formed (113). Upon release, micronemal (MIC) proteins relocate either in the PV or to the surface of the parasite, where they facilitate disruption of the cell membrane for egress and cell traversal during tissue passage (122) and allow motility (48) and recognition and attachment to the host cell (28, 38, 100). In contrast, rhoptry proteins are injected into the host cell, where they reach different locations: the host plasma membrane (HPM), the cytosol, the nucleus, the PV membrane (PVM), or the PV lumen. Rhoptry proteins are involved in various cellular processes during the lytic cycle of Apicomplexa, notably invasion, PV formation, nutrient import, and control of cell-autonomous defenses (15, 51, 59, 72, 80, 94). When targeted to the HPM, at the attachment site of the parasite, rhoptry proteins associate with MICs to form cooperative complexes that contribute to the elaboration of the MJ [apical membrane antigen 1 (AMA1)–rhoptry neck protein (RON) complex] and possibly to the delivery of rhoptry proteins within the host (Rh5-Ripr-CyRPA complex), at least in *Plasmodium*, as detailed below (see the section titled Crossing the Host Cell Membrane Barrier). The assembly of these essential complexes at the host-parasite interface in such a short time frame (a few seconds)

must be finely controlled in time and space to result in productive invasion. One ingenious yet simple way to coordinate these two sequential exocytic processes is to ensure that rhoptry discharge is triggered only following the interaction between surface-translocated MICs and host cell receptors. For instance, the binding of *Plasmodium falciparum* merozoite micronemal protein EBA175 to glycophorin A on the red blood cell (RBC) correlates with rhoptry secretion (132). Similarly, *Plasmodium berghei* and *Plasmodium yoelii* p36 and p52, two micronemal proteins of the 6-cys protein family (7, 89), and *Toxoplasma* MIC8 (73) are also implicated in triggering rhoptry secretion.

The sensing of external cues, i.e., a drop in pH or a rise in phosphatidic acid (PA), by intracellular parasites sets the start of microneme secretion. A complex signaling pathway is then initiated in the parasite cytoplasm, primarily relayed through the modulation of cyclic GMP (cGMP) and intracellular Ca^{2+} concentrations. These second messengers in turn will eventually lead to the release of micronemes (for a compelling review see Reference 20). Then, the parasite stops microneme exocytosis to prevent further motility and escape from the newly formed vacuole. This switch-off mechanism is controlled by cyclic AMP (cAMP) signaling and its downstream effector protein kinase A (PKA), which suppress cytosolic Ca^{2+} level elevation (69, 151). This feedback mechanism is likely activated before the parasite begins its internalization, as, with the exception of AMA1 (65), MICs are not found in the lumen of the vacuole nor associated with the surface of the intracellular part of the parasite. In contrast to micronemes, the signaling pathways governing rhoptry secretion and the interconnection with the binding of MICs to host cell receptors are currently unknown. However, in recent years, the characterization of some key molecular actors has shed light on fusion events of the parasite plasma membrane (PPM) with both micronemes and rhoptries—a process referred to as exocytosis.

MICRONEME AND RHOPTRY EXOCYTOSIS: A SNARE-INDEPENDENT MECHANISM?

Exocytosis is classically divided into three steps: docking of vesicles at specialized sites, priming that renders them competent for exocytosis, and fusion with the plasma membrane. Some components involved in these steps are highly conserved in eukaryotes and are the core machinery for fusion. These include *N*-ethyl maleimide sensitive factor (NSF), soluble NSF adaptor proteins (SNAPs), SNAP receptors (SNAREs), the Sec1/Munc18 (SM) protein Munc18–1, and Munc13s (116). Apicomplexa genomes encode some of these factors, but recent studies showed that they are involved in organelle trafficking rather than exocytosis (8–10, 18, 26). Consequently, regulated exocytosis of apicomplexan secretory organelles might rely on different molecular effectors. Besides the aforementioned core machinery, specialized factors like synaptotagmins bring additional regulation to the exocytic process, usually via an ancestral calcium-binding domain known as the C2 domain, which also confers lipid- and protein-binding properties (116). Interestingly, some C2-containing proteins are conserved in Apicomplexa and have been shown to control secretory organelle exocytosis. For instance, the double C2 (DOC2) protein homolog in Apicomplexa, while very divergent from its mammalian counterpart, fulfills similar calcium-dependent exocytosis activation. *Plasmodium* DOC2 binds to Ca^{2+} when associated with membranes (68), and both *Toxoplasma* and *Plasmodium* DOC2 mutants are affected in microneme secretion (46). Mammalian DOC2 is a vesicular protein that interacts with many exocytosis-related proteins— including Munc13 and SNAREs—and regulates neurotransmitter release (105). In *Toxoplasma*, DOC2 may act as a broad calcium-dependent exocytosis activator, as depletion of *Tg*DOC2 also affects secretion by rhoptries and dense granules (143). Two additional C2-containing proteins, FER1 and FER2, are also involved in microneme exocytosis and rhoptry

exocytosis, respectively (35, 142). They are members of the conserved family of ferlins, which typically contain five to seven C2 domains, but are part of a unique clade that originated independently from mammalian ferlins (143). Although ferlins are absent from the conventional model of calcium-dependent exocytosis established in neurons, they can play a direct role in exocytosis in other cell types, as demonstrated for otoferlin. Otoferlin is present at a specialized synaptic zone of the inner ear cells and controls the fusion of vesicles upon mechanical stimulation by sound (120). Surprisingly, otoferlin-mediated exocytosis does not require typical SNARE proteins (96), thereby supporting an alternative model of exocytosis independent of SNAREs. At present, it is still unknown whether microneme and rhoptry release may also follow this unconventional route, but the description of the rhoptry secretion machinery below fits well with this model.

MICRONEME EXOCYTOSIS

Micronemes are small, rod-like organelles clustered at the apex of apicomplexans. Distinct populations have been proposed in both *Toxoplasma* and *Plasmodium* that may correlate with different biological functions. These populations, their abundance, and their morphology have been recently covered by comprehensive reviews (6, 42). In *Toxoplasma*, micronemes accumulate beneath the conoid—an apical truncated-cone-shaped structure (99)—or at the lateral periphery. A few micronemes are also visible engaged inside the conoid (42, 57, 88, 125) and one microneme vesicle has been recently shown by cryo-electron tomography (cryo-ET) (57) to be docked at the exocytic site, close to but distinct from the rhoptry exocytic site described below (see the section titled Structural Elements of the Rhoptry Secretion Apparatus Pinpointed by Cryo-ET). Microneme exocytosis is likely driven by a fusion process during which the vesicles completely merge with the PPM. Few molecular players of microneme exocytosis have been identified. *Toxoplasma* FER1 controls both the traffic and exocytosis of micronemes (142).

Concordant with this dual function, it localizes to several compartments of the parasite's secretory pathway as well as to an apical spot of the conoid that may correspond to the microneme exocytosis site. Interestingly, the ortholog of *Tg*FER1 in the rodent malaria model, *P. berghei* ferlin-like protein (*Pb*FLP), is crucial for gamete egress (98), a process relying on the exocytosis of microneme-like vesicles called osmiophilic bodies and egress vesicles. Thus, the phenotype of a *Pb*FLP mutant supports a broad and conserved function of FER1 in microneme and microneme-like organelle exocytosis. Transporters have also been reported as required for microneme secretion, although their precise function during this process remains elusive (60, 71). Two pleckstrin-homology (PH) domain-containing proteins conserved in Apicomplexa are crucial for microneme exocytosis, namely APH and PH2. *Tg*APH decorates the surface of micronemes, where it is ideally positioned to sense and bind PA produced at the inner leaflet of the PPM (23). By doing so, APH clusters PA molecules at the lipid bilayer, facilitating the close apposition of micronemes with the PPM and ultimately providing a stable molecular scaffold for the subsequent recruitment of priming and fusion molecular players (41), such as FER1 and DOC2 (46, 142). *Pf*PH2 is an apically localized protein of the merozoite with a phosphoinositide-binding property, which controls exocytosis of only a subset of micronemes containing *Pf*EBA proteins (45). Interestingly, in the rodent malaria parasite *P. yoelii*, APH controls the secretion not of EBA proteins but rather other MIC proteins, including AMA1 and MTRAP (29). This suggests that in *Plasmodium*, APH and PH2 might have a mutually exclusive role to regulate exocytosis of different sets of microneme vesicles. Apart from PA, other lipids are important for microneme secretion. In *T. gondii*, maintenance of the phosphatidylserine (PS) asymmetry by the flippase ATP2A (a P type-IV ATPase also named P4-ATPase1 in Reference 31) is crucial for efficient microneme exocytosis (19). The protein candidates binding PS enriched at the inner leaflet of the plasma membrane remain to be identified. To

conclude, while the core components of exocytosis described in mammals are not shared within Apicomplexa, the molecular players regulating microneme exocytosis identified so far support a similar lipid- and calcium-dependent process.

A UNIQUE ALVEOLATA SECRETION MACHINERY PROMOTES INJECTION OF RHOPTRY PROTEINS INTO THE HOST CELL

Apicomplexa Rhoptries: A Unique Shape

The number of rhoptries varies among apicomplexan parasites and even between different developmental stages within a given species. *T. gondii* tachyzoites possess 8 to 12 clustered together at the apex (43), *P. falciparum* merozoites have 2, and sporozoites have 2 to 4 (77, 124), while ookinetes, a noninvasive stage, are devoid of them. *Cryptosporidium parvum* sporozoites have a single rhoptry (88). However, rhoptries are morphologically similar, with a unique club shape comprising a narrow tubular duct or neck that opens at the anterior pole of the parasite and a posterior sac-like body or bulb (43, 61). These delimited rhoptry regions define two classes of luminal proteins, the rhoptry neck (RON) proteins and the rhoptry bulb (ROP) proteins (22, 80, 86, 119). These proteins are released sequentially upon exocytosis to fulfill distinct functions. RONS are secreted first and mainly used for invasion, whereas ROPs contribute to postinvasion processes (37, 38, 43, 51, 59, 80, 94). This subcompartmentation of RONS/ROPs is clearly visible in *Toxoplasma* tachyzoites and *Plasmodium* merozoites but is less obvious for *Plasmodium* sporozoites, which may reflect distinct protein secretion mechanisms (145). How rhoptries maintain their shape and dimorphism is currently unknown, but mutants that lose this neck/bulb organization (30, 60, 129) fail to invade host cells, supporting a crucial link between rhoptry structure and exocytosis. Despite the lack of physical separation between the neck and the bulb, EM reveals a region of intermediate density that connects the electron-dense neck to the less electron-dense bulb (82). Freeze-fracture studies of *Toxoplasma* rhoptries also revealed pore-like structures

along the bulb and particles arranged in a parallel row along the entire surface of the organelle (82). Recently, cryo-ET of both *Toxoplasma* and *Cryptosporidium* rhoptries uncovered another layer of structural complexity specifically in the neck region. It revealed a short anterior tapering tip followed by two types of membrane-associated helical filaments that delineate anterior and posterior neck regions (88) (**Figure 1**), as also shown by expansion microscopy (83). These filamentous arrangements may contribute to the proper allocation of the rhoptry proteins to the different subcompartments of the organelle.

Structural Elements of the Rhoptry Secretion Apparatus Pinpointed by Cryo-ET

Unlike micronemes, rhoptries are predocked at the PPM (88). In *Toxoplasma*, *Cryptosporidium*, and *Plasmodium*, cryo-ET showed that rhoptries are not in direct apposition with the PPM but in contact with an intermediate vesicle called the apical vesicle (AV) (5, 88, 90), previously identified in *Sarcocystis* (107), *Eimeria* (44), and *Toxoplasma* (108). A pair of rhoptries in *T. gondii* and *P. falciparum*, and the single rhoptry in *C. parvum*, are linked to the AV. Via this AV, rhoptries are connected to a rosette of intramembranous particles embedded in the PPM that represent the docking site for rhoptry exocytosis (5, 88, 90) (**Figure 1**). This unique arrangement requires two fusion events: the rhoptries with the AV, and the AV with the PPM, as discussed below. The AV has a characteristic shape and size that differ in *Plasmodium*, *Cryptosporidium*, and *T. gondii*, where it is circular, teardrop-shaped, and nearly ellipsoid, respectively (88, 90). The docking of the AV to the PPM is sustained by an elaborate multicomponent structure named the rhoptry secretory apparatus (RSA), conserved in Apicomplexa (88, 90). The particular differences observed between the RSA structures of *Toxoplasma*, *Plasmodium*, and *Cryptosporidium* (90) may reflect adaptations in the molecular mechanisms for their specific parasitic activities. **Figure 1b,c** details the RSA and the possible

functions of its different elements. The RSA is a prime candidate to exert the mechanical force needed to bring opposing membranes (the AV membrane and PPM) in proximity, allowing their fusion and thus rhoptry exocytosis. It defines a new class of secretion machinery in pathogenic eukaryotes that is likely conserved within Alveolata, a large group of unicellular eukaryotes including Apicomplexa, Ciliata, Dinoflagellata, Chromerida, and Perkinsozoa.

Rhoptry Exocytosis Relies on an Alveolata Conserved Machinery

The rosette was first described by freeze-fracture in the late 1970s in both Ciliata (*Paramecium* and *Tetrahymena*) and Apicomplexa (*Toxoplasma*, *Eimeria*, *Plasmodium*, and *Sarcocystis*) (reviewed in 134). This structure is essential for exocytosis of secretory organelles in Ciliata (trichocysts in *Paramecium* and mucocysts in *Tetrahymena*). The genes required for rosette assembly or for exocytosis per se were named *nd*, for “non-discharged,” in *Paramecium*. Interestingly, comparative genomic analysis evidenced the conservation of some *nd* genes within Alveolata, particularly *nd6* and *nd9*, and reverse-genetics studies of these *nd* genes in *Toxoplasma* and *P. falciparum* demonstrated that the rosette is the site for rhoptry exocytosis in Apicomplexa (5). *Nd9* depletion led to a strong decrease in the number of rosettes visible at the apex of *T. gondii* tachyzoites (5) and to drastic structural defects of both RSA and AV anchoring (88). This finding establishes the link between the formation of the rosette and the exocytosis of the rhoptries, highlighting the importance of the RSA and AV docking to the PPM in this process. In *Toxoplasma*, *Nd6* and *Nd9* are part of a molecular complex comprising other factors: *NdP1* (*Nd* partner 1), *NdP2* (*Nd* partner 2), and *FER2*. *NdP1* and *NdP2* are conserved and unique to Alveolata. They are equally important for exocytosis of mucocysts in *Tetrahymena* and rhoptries in *Toxoplasma* (5), thus supporting the existence of a conserved Alveolata secretory machinery.

The bewildering complexity of the RSA poses new challenges in terms of

understanding its assembly, composition, and how it assists the membrane fusion event between the AV and PPM. Nd6 and NdP2 are the only proteins detected at the site of exocytosis, raising the question as to whether the others might be only transiently associated or present in such a low amount that they cannot be observed by microscopy. Nd6 may have a direct role in exocytosis, as a *Toxoplasma* Nd6 mutant is compromised in rhoptry secretion while the rosette appears normal (5). However, a subtle defect in the RSA structure is not excluded since Nd6 lacks transmembrane domains and is thus likely associated with the cytosolic part of the RSA. Nd6 may regulate fusion events through its predicted RCC1-like domain, which could act as a guanine exchange factor (GEF) toward small GTPases (58). Intriguingly, Nd9, NdP1, and NdP2 also lack transmembrane domains, making them unlikely to be structural components of the rosette, yet the rosette is disassembled in the corresponding mutants (5). Consequently, they might assist RSA assembly or stabilization. The presence in Nd9 and NdP1 of armadillo-like and leucine-rich repeats, two domains involved in protein-protein interactions, would support this hypothesis. NdP2 (5) and FER2 (35) may act as Ca^{2+} sensors through their C2 domains. In contrast to the other members of the Nd complex, FER2 contains a transmembrane domain. However, it does not accumulate at the site of exocytosis in *Toxoplasma* but is detected along the cytosolic face of the inner-membrane complex in intracellular tachyzoites and relocates to the cytosolic face of the rhoptry neck in extracellular parasites (35). The functional significance of these observations and the role of FER2 (if any) in rosette and RSA assembly require further investigation.

The conservation of the secretion process between Apicomplexa and Alveolata is not only restricted to the membrane fusion event; it also extends to the signaling events leading to exocytosis. Recent studies in *T. gondii* described membrane-bound proteins, named CRMPs (cysteine repeat modular proteins), as part of a large complex essential for rhoptry secretion and invasion (109, 131, 133). CRMPs are

conserved in *Tetrahymena* and required for mucocyst release (133). Unlike Nd proteins, *T. gondii* CRMPs are not required for assembly of the RSA and are only transiently associated with the exocytic site prior to invasion (133). CRMPs and their partners contain putative host cell-binding domains, suggesting that they may act as molecular host sensors to ensure timely coordination of host cell contact and rhoptry exocytosis.

Rhoptry Exocytosis: More Than One Fusion Event

The bulk of the rhoptry organelle membrane does not become integrated into the PM upon exocytosis, as electron-lucent rhoptries, or empty rhoptries, are still visible upon invasion and connected by their apical end to the PPM (2, 24, 61, 95, 101) (**Figure 2a**). The mechanism for their post-discharge disassembly or recycling is unknown. In *Plasmodium*, there are two rhoptries that dock and likely fuse to the AV (2, 61, 90). This event might also occur in *Toxoplasma* (95), as suggested by the merge between two rhoptries during invasion and by the docking of two rhoptries with the AV (88).

In *Cryptosporidium*, the single rhoptry can be imaged either in a prefusion state with the AV in the absence of any exocytosis trigger or not fused at all (88). In *Toxoplasma*, the AV shape changes upon calcium ionophore treatment, which might suggest a prefusion state (125). In *Plasmodium* merozoites, two additional states have been observed by cryo-ET (90), both with the AV absent. In the first one, the two rhoptries are distinct, with their necks often twisted around each other, and only one of the two rhoptry tips is docked to the RSA. In the second state, the two rhoptries are fused together and their common tip is directly docked to the RSA. In these different organizations, the RSA adopts different conformations, including the opening of a gate-like density in the rhoptry-fused state (90). These observations support the existence of a dynamic process of rhoptry docking/fusion to the AV preceding exocytosis at the PPM. Due to its location

and function in *Toxoplasma*, RASP2 (rhoptry apical secretion protein 2) is a prime candidate to regulate the fusion event of the rhoptry to the AV (138) (**Figure 1c**). This protein, unique and conserved among Apicomplexa, caps the extremity of the rhoptry neck on its cytosolic face, where it interacts with RASP1 and RASP3. Together they might correspond to the distinct density detected at the tip of the rhoptry by cryo-ET (88). RASP2 depletion compromises rhoptry secretion, further leading to invasion defects in both *Plasmodium* and *Toxoplasma* (138). Through its C2-like (Ca^{2+} -dependent lipid-binding) and PH-like domains, *TgRASP2* binds to PA and phosphatidylinositol 4,5-bisphosphate (PIP_2)—two lipids known to participate in membrane fusion events—thereby supporting a function in rhoptry docking/fusion. However, the interaction of RASP2 with lipids is calcium independent, as its C2 domain is divergent and has lost its ability to bind calcium (138). Further studies are required to generalize the function of RASP2, as a recent study in *Plasmodium* showed that *PfRASP2* [named cytosolically exposed rhoptry-leaflet-interacting protein 1 (CERLI1)] localizes exclusively to the rhoptry bulb, a location that does not fit well with a function in rhoptry exocytosis (87).

The last identified factor for rhoptry secretion is *TgPL3*, a patatin-like phospholipase present at the apical cap but also associated with the tip of the parasite in extracellular tachyzoites (159). It is mechanistically unclear how *TgPL3* is involved in rhoptry secretion, but its function is dependent on its phospholipase A2 (PLA2) domain. The use of a PLA2 inhibitor also impairs rhoptry discharge, further supporting a role of phospholipase in rhoptry exocytosis, although the target of this compound needs to be verified (103).

A Reload Machinery for Successive Rhoptry Discharge?

During invasion, *Toxoplasma* discharges only a subset of its rhoptries (108). Concordantly, only two are docked at the AV, while several proximal free rhoptries

appear to wait for successive rounds of secretion (88). Once invasion is completed, nondischarged rhoptries are still visible at the parasite apex (95, 108). In contrast, in *Plasmodium* merozoites, no intracellular rhoptry labeling is detected by immunofluorescence following RBC invasion (113), and the rhoptries are not detected by cryo-ET (21), advocating for the simultaneous discharge of the two rhoptries.

Why would some parasite species exhibit more than two rhoptries? They might permit successive attempts of invasion in case of a failed attempt and/or may be used to inject rhoptry proteins into uninfected cells, as shown for *Toxoplasma* (75), to restrict the magnitude of parasite-specific T cell responses (32). In the case of *Plasmodium* sporozoites, the parasites undergo two successive invasion steps: The midgut-derived sporozoites first enter the mosquito salivary glands, and then upon transmission to the host, they invade hepatocytes. Both invasion processes are dependent on rhoptry secretion (47, 66, 115). Interestingly, midgut-derived sporozoites have four rhoptries (124), whereas only two are reported in sporozoites in salivary glands (77) and none are reported in hepatocytes after infection (115), suggesting that sporozoites successively discharge two rhoptries at a time.

In *T. gondii*, three to six vesicles accumulate within the conoid in close association with a pair of intraconoidal microtubules (ICMTs) (57, 88, 101, 125) (**Figure 2a**). Referred to as microtubule-associated vesicles (MVs), they are similar in shape and size to the AV and are coated with a discontinuous layer of protein density, similar to the one observed on the AV (88). An emerging model proposes that MVs function as new AVs in subsequent rounds of rhoptry docking and exocytosis. They would move along the microtubules to reach the apical tip of the parasite, where their docking to the PPM would induce assembly of a new RSA, a process already described in *Paramecium* when secretory organelles dock to the plasma membrane (see review in 134). The involvement of microtubules in transporting MVs needs to be experimentally demonstrated, yet the requirement for the microtubule dynein

motor DLC8a (dynein light chain 8a) in the proper apical docking of the rhoptries supports this model (84). This reloading process might exist in other coccidian parasites, e.g., *Eimeria* sporozoites that display several rhoptries, similar vesicles, and ICMTs (24).

Crossing the Host Cell Membrane Barrier

It is widely reported in the literature that upon exocytosis, the rhoptry content accesses the host cytosol via the formation of a pore in the HPM, but the data supporting this assumption are very scarce. A few images have captured an opening between the apical rhoptry and the HPM (24, 61, 95, 140). An ~40-nm pore has been observed by freeze-fracture in the PVM (initially HPM) over the apical tip of the parasite during *T. gondii* invasion (43). Finally, a transient spike of HPM conductance was measured by electrophysiology when the apical end of the parasite attached to the cell, before penetration (140). This result has been interpreted as the formation of a transient opening in the HPM to allow injection of the rhoptry material inside the host.

In *P. falciparum*, a tripartite complex is speculated to be important for the formation of this pore (**Figure 2a**). This complex results from assembly of the rhoptry neck protein Rh5 (reticulocyte-binding protein homolog 5) (63, 118) with two MICs, namely Ripr (Rh5-interacting protein) (33) and CyRPA (cysteine-rich protective antigen) (112). *PfRh5*, *PfCyRPA*, and *PfRipr* colocalize at the site of interaction between the merozoite and the RBC. *PfRh5* binds to an RBC via its direct interaction with basigin (BSG) (40). This ligand-receptor interface is essential for invasion, and Rh5 is one of the most promising target for a next-generation blood-stage vaccine against malaria (111). The resolution of an Rh5 crystal structure alone or in complex with BSG revealed a novel protein fold resembling a flat, kite-shaped architecture that exposes binding sites for BSG (34, 162). Independent functional analysis of Rh5, Ripr, and CyRPA revealed that the proteins are required for a

calcium flux within the erythrocyte. This calcium signal is itself correlated with RBC echinocytosis upon invasion, a dramatic dehydration-type morphology of the RBC presumably linked to rhoptry secretion (4, 152, 157). A recent study regarding the structure and biophysical properties of the Rh5-CyRPA-Ripr complex supports their insertion into membranes (160). This study places CyRPA as a critical mediator of complex assembly and proposes that following binding to BSG, the dissociation of Rh5 and Ripr from CyRPA allows membrane insertion of the Rh5-Ripr complex and formation of a pore in the HPM (160). In addition, the Rh5 coiled-coil domain has some structural homology with the coiled-coil domain of SipB, a bacterial protein that forms part of the *Salmonella* type III secretion system, and is thus responsible for transport of bacterial effectors across the HPM (64). These observations led to the proposal that Rh5-CyRPA-Ripr might have a role in the establishment of a pore between the merozoite and the HPM, through which rhoptry proteins could be transferred. However, there are conflicting data regarding the capacity of a recombinant Rh5 protein alone or in complex to trigger calcium release within the erythrocyte (4, 160), and the biochemical properties of *Pf*Rh5 do not fit with membrane insertion and pore formation (34, 162). If the Rh5-CyRPA-Ripr complex is responsible for the formation of a pore to inject the rhoptry content in *P. falciparum*, its function cannot be extended to Apicomplexa in general nor to all *Plasmodium* species, as genes encoding Rh5 protein are restricted to the *Laverania* subgenus (49). Alternatively, the RSA might have a direct role in pore formation, with the anterior central channel (**Figure 1b,c**) being inserted into the HPM to provide a transmembrane route for rhoptry delivery (88). In line with this idea, the AV, which organizes this putative pore, is absent from *Paramecium* and *Tetrahymena*, which discharge the content of their secretory organelles into the extracellular milieu, and present in Apicomplexa that contain rhoptries that inject proteins into host cells.

BUILDING THE MOVING JUNCTION: AN APICOMPLEXA-SPECIFIC DEVICE

Moving Junction: Self-Assembly of Parasite Proteins at the Parasite-Host Interface

The MJ corresponds to an intimate attachment between the host and parasite PMs. It is restricted to the tip of the parasite at the beginning of invasion and evolves into a ring shape encircling the parasite as it progresses into the host cell (1). Once the parasite is fully internalized, the MJ eventually disassembles at the posterior end of the parasite when the PV pinches off from the HPM. First described at the molecular level in *Toxoplasma*, this unique invasion structure is shaped by the micronemal protein AMA1 and several rhoptry neck proteins (RON2, RON4, and RON5) (3, 81, 136) (**Figure 2**). Remarkably, apicomplexan parasites that use an MJ-dependent invasion rely on this same protein complex, which is highly conserved within Apicomplexa (for reviews, see 16, 128). Additional RON components of the MJ are restricted to Coccidia, e.g., RON4_{L1} and RON8 (56, 136). Upon microneme secretion, the transmembrane protein AMA1 is translocated at the parasite surface, while the subsequent discharge of rhoptries targets the RON2/RON4/RON5 proteins to the HPM (17) (**Figure 2b**). RON2 is the only transmembrane protein of the RON complex. Its extracellular C terminus directly binds AMA1 (17, 78, 135, 150), while its large N-terminal portion localizes to the cytosolic face of the HPM, where it interacts with the other RONs (16, 56, 136).

Importantly, disrupting the AMA1-RON2 interaction using antibodies (or peptides), or by reverse genetics, stressed the crucial role of this complex to the invasion process (for reviews, see 16, 128). *Toxoplasma* AMA1 or RON2 mutants are motile and successfully attach to the host cell—likely via MIC-receptor interactions—but eventually detach from the host cell owing to a strong defect in firm MJ-dependent attachment to the host cell (79, 91). Strategic point mutations

within the *Tg*RON2 binding site for *Tg*AMA1 mimic the phenotype of a RON2 knockdown, supporting the view that the AMA1-RON2 interface accounts for the structural integrity of the MJ (79). Despite being initially controversial in *Plasmodium* (review in 13, 62, 128), the role of AMA1 in establishment of the merozoite MJ is now well accepted. The loss of *Pf*AMA1 leads to a complete block in RBC invasion without preventing the merozoites from extensively interacting with the RBC membrane (36). Its functions in sporozoites' invasion of the salivary glands and hepatocytes have been recently clarified (47). AMA1 interacts with RON2 and RON4, and these proteins are required for sporozoite invasion in both the mosquito and mammalian hosts (47, 53, 66, 130, 163), supporting the view that AMA1 and RONs facilitate host cell invasion across *Plasmodium* invasive stages.

Toxoplasma gondii tachyzoites use AMA1/RON2, but interestingly, this parasite can deploy three additional AMA-RON complexes: AMA2/RON2 (79), AMA3/RON2_{L2} (110), and AMA4/RON2_{L1} (79, 103). The latter two are highly expressed and functional at the sporozoite stage (103, 110). Remarkably, the loss of AMA1 in tachyzoites leads to parasite adaptation, with upregulation and use of the AMA2/RON2 and AMA4 complexes (79, 103). It is still unclear why the parasite would require distinct MJ complexes for different stages and even several pairs for a given stage, but importantly, these results illustrate the diversity and flexibility of the MJ components to fulfill the invasion process. Altogether, the organization of the MJ clearly highlights a self-reliant mechanism in which the parasite provides both the ligand (AMA) and the receptor (RON) to build up a key structural feature of invasion.

Structural Details of the AMA1-RON2 Interaction Reveal an Exceptionally Stable Structure and the Separate Coevolution of the AMA1-RON2 Pairs in Apicomplexa

The co-crystal X-ray structures of AMA1 in complex with its interacting RON2 peptide (RON2_{sp}) revealed a conserved binding paradigm in *Toxoplasma* and *Plasmodium*. In its apo form, AMA1 displays a flexible loop (DII loop) intimately associated with the base of a large hydrophobic groove (39, 106). This loop can be readily displaced upon RON2 binding, exposing the full functional binding surface of the AMA1 cleft, a prerequisite for the proper insertion of RON2 in a U-shaped conformation (147, 154). The RON2 N-terminal helix is anchored on one side of the groove and extends to a disulfide-closed β -hairpin loop (cystine loop) at the opposite side (**Figure 2c**), resulting in a very high-affinity interaction in the nanomolar range and representing one of the widest surfaces of interface reported so far for a binary ligand-receptor interaction bridging a pathogen to a host cell (147, 154). Globally, the co-crystal structures of other AMA-RON pairs follow the same archetype, with AMA4-RON2_{L1} being the most divergent (103, 110). The AMA1-binding site of RON2 corresponds to the most conserved region between apicomplexan RON2 orthologs. Despite this apparent conservation, there is no cross-reactivity between AMA1 and RON2 from different Apicomplexa (78, 110, 146) or with only low affinity, as observed between *P. falciparum* and *Plasmodium vivax* (153). Functional mutagenesis studies coupled to the comparison of several AMA1-RON2 binding interfaces identified the RON2 cystine loop as a major driver for the separate coevolution of AMA1-RON2 pairs within Apicomplexa (11, 147, 154). Moreover, the DII loop of AMA1 acts as a structural gatekeeper to govern RON2 selectivity to the AMA1 apical groove in *T. gondii* (102). Overall, the structural insights into AMA1-RON2 interaction revealed an exceptionally stable structure that could offset the mechanical stresses encountered by the parasite to pass through the constricted MJ ring.

AMA1-RON2 Complex: Connecting Host and Parasite Cytosolic components

Apicomplexa gliding motility provides the mechanical force required to actively penetrate host cells (48). In this mechanism, the MJ not only needs to be firmly attached to the host cell to withstand the sheer mechanical forces associated with the parasite movement but also serves as an anchoring point to engage the parasite motility machinery. The transmembrane AMA1 and RON2 proteins expose domains to the cytosolic face of the parasite and host cell, respectively, and are therefore ideally positioned to mediate such functions. Supporting this model, accumulation of F-actin is detected on the parasite (114, 148) and host cell (54) sides of the MJ.

It has been demonstrated that the cytoplasmic tail of AMA1 is essential for invasion, although its precise function remains enigmatic (126, 149). Despite being quite divergent among Apicomplexa, AMA1 tails are functionally interchangeable between parasite species (e.g., *P. falciparum* with *P. berghei* or *P. vivax*) (149). Two conserved aromatic residues close to the C terminus of the protein (*Pf*AMA1-FW^{602–603} and *Tg*AMA1-FW^{519–520}) are necessary for AMA1 function, as mutation of both into alanine abrogates invasion (126, 149) without affecting attachment or MJ formation in *Toxoplasma* (79). These results suggest that AMA1 also contributes to the invasion process through interactions with parasite cytosolic proteins. One such appealing partner would be the glideosome-associated connector (GAC), a central player in the gliding machinery that connects transmembrane MICs to actin filaments (67). Interestingly, the AMA1 cytoplasmic tail is also subject to posttranslational modifications. In *Plasmodium*, phosphorylation of Ser-610 by PKA induces structural changes of the tail (104) and is required to promote efficient invasion (85, 149). Correspondingly, disrupting PKA activity abrogates merozoite invasion and proteolytic shedding of AMA1 (see below) (104, 158), which may be at least partly explained by the lack of AMA1 phosphorylation. Although the significance of this phosphorylation is not yet clear, it may have a role in

signaling and/or in recruiting the gliding machinery. In *Toxoplasma*, dephosphorylation of Ser-527 is induced upon binding of RON2 to AMA1, a prerequisite to achieve fully efficient invasion (76). Altogether, these results highlight the fact that AMA1 is not a simple tether but likely also exerts important functions related to invasion, such as linking the MJ to the gliding machinery or relaying crucial downstream parasite signaling. Paradoxically, while serving as a firm anchor to the host cell, transmembrane MIC proteins, including AMA1, are constitutively shed by parasite surface proteases belonging to the rhomboid family (ROM4 and ROM5) (12, 65, 121, 127) or the subtilisin family (SUB2) (36), thereby releasing their ectodomains into the extracellular milieu. One way to reconcile these two processes is based on the observation that the binding of *Tg*RON2 to *Tg*AMA1 reduces *Tg*AMA1 rhomboid-mediated cleavage (76). The parasite-host cell connection would be specifically maintained at the MJ where it could engage with the parasite traction force to drive internalization.

On the host cell side, the RON components of the MJ are exposed to the cytosolic face of the HPM. Most of them are essential for invasion, but understanding of their respective contribution has been hampered by their intermingling as a protein complex (14, 55, 56, 78, 97, 155). Their function might depend on their phosphorylation status, as in *Toxoplasma*, RON13 phosphorylates RON4, among other rhoptry substrates, and is required for MJ assembly (83). Studies in *T. gondii* revealed direct interaction of RON4 with host cellular β -tubulin (144) and also evidenced a direct role of RONs in recruiting host proteins, namely ALIX (apoptosis-linked gene 2-interacting protein X), CIN85 (Cbl-interacting protein of 85 kDa), CD2AP (CD2-associated protein), and TSG101 (tumor susceptibility gene 101) (55) (**Figure 2**). Their accumulation at the MJ is strictly dependent on their capacity to interact with specific motifs displayed on RON proteins. Importantly, parasite mutants unable to recruit ALIX, TSG101, and CIN85/CD2AP exhibit a decrease in their invasion efficiency (55). These

host factors are known to interact with each other and to form cargo-specific intermediates involved in various biological processes. Of relevance are their interaction with actin and tubulin—both reported to accumulate at the MJ (54, 141)—and their role in connecting membrane proteins to the underlying cytoskeleton to establish and maintain intercellular junctions (25, 70, 123). Therefore, they might physically anchor the RON complex to the host cortical actin cytoskeleton, and by doing so provide the necessary tensile strength required to withstand forces exerted by the parasite during host cell penetration. Furthermore, the proteins of the complex exposed to the host cell cytosol (RON4, RON4_{LI}, RON5, and RON8) might gate the access of host PM molecules to the PVM, working as a selective and protective barrier against fusion of the PV with the endolysosomal system (92).

MOVING JUNCTION FORMATION AND RHOPTRY SECRETION: ONE CANNOT BE EFFECTIVE WITHOUT THE OTHER

MJ formation requires prior exocytosis of the rhoptries to first inject the RON complex in the host cell to properly bridge the parasite to the host cell. Intriguingly, there is some evidence indicating that the reverse might also be true. In *Plasmodium*, antibodies (or peptides) targeting the AMA1-RON2 interface or the disruption of AMA1 do not prevent RBC echinocytosis, suggesting that rhoptry secretion is likely taking place (50, 129). However, during these defective invasions, ROP proteins remain associated with the surface of the RBC (50, 74, 113). Similarly, in *Toxoplasma*, conditional AMA1 and RON5 mutants show improper targeting of the rhoptry content inside the host (3, 83, 91). By maintaining a tight apposition between the PPM and HPM, the MJ may secure the continuum between the rhoptry lumen and the host cytoplasm, ensuring injection of the rhoptry content at the desired target site—the host cell. One notable observation is that upon contact with its host, *P. falciparum* AMA1 knockout merozoites trigger RBC lysis

(36). This suggests a role for the MJ in the safeguarding the pore formed in the HPM and highlights the intricate dependence between the rhoptry secretion machinery and the MJ structure.

FUTURE DIRECTIONS

Recent technical breakthroughs have shed light on the structure of the RSA (88) but also more broadly on the organization of the apex of the parasites (21, 24, 57, 125, 139, 156). Some molecular players of the rhoptry exocytosis process have been identified, but further mapping of these factors onto the in situ structure of the RSA will be necessary to provide an insightful understanding of this unique eukaryotic secretion machinery. Despite these recent advances, many important questions remain. How does the tiny space of the parasite apex accommodate both microneme and rhoptry fusion events? Following invasion, how are the empty rhoptry saccules disassembled and/or recycled? How do successive rounds of rhoptry exocytosis take place in coccidian parasites? What are the signaling events that coordinate microneme and rhoptry release? Why does the rhoptry secretion machinery display an additional layer of complexity with an AV? What is the molecular composition of the AV, and does it play a role in breaching the HPM? To what extent is the rhoptry secretion machinery conserved in Ciliata?

Beyond these burning questions, another challenge will be to directly visualize the injection apparatus using in situ cryo-EM on invading parasites. Data will not only elucidate the fold of the RSA upon exocytosis and possibly evidence of the formation of the pore within the host but also contribute to our understanding of how the MJ is organized and integrated with these structures.

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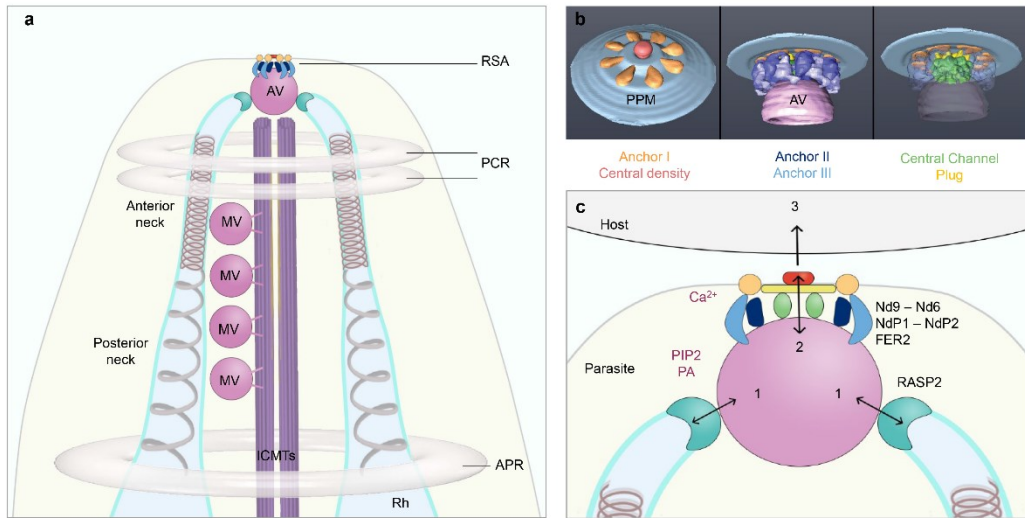


Figure 1. A multifaceted secretion machinery supports rhoptry protein exocytosis. (a) Scheme of *Toxoplasma gondii* apex with a focus on the rhoptry neck and the rhoptry secretion machinery structures. The anterior and posterior regions of two rhoptry necks are represented with their respective membrane-associated filaments. The anterior tapering tips of the two rhoptries are in close contact with an AV via rhoptry tip densities (*teal*). The AV is anchored to the PPM via the RSA. Three to five vesicles, similar to AVs, are associated with a pair of ICMTs. These MVs could serve as new AVs by moving along the ICMTs, allowing the reloading of a new RSA for subsequent rounds of rhoptry secretion. (b) 3D display of the PPM, RSA, and AV in *T. gondii*. The RSA is a complex ultrastructure that comprises a central density, three anchors (anchors I, II, and III), and an anterior central channel between the AV and the PPM. The central density is exposed on

the extracellular side, where it sits on top of a cytoplasmic structure with radiating spokes (*yellow plug*), and on an anterior channel (*green*) located between the AV and the plug. The central density and anchor I correspond to the apical rosette previously spotted by freeze-fracture. The central density may be involved in the establishment of a transient pore within the PPM. Anchor I might have a structural role, as it physically connects the PPM to the AV via anchors II and III. The peripheral contact of anchors II and III with the AV may have a double function: rigid anchoring of the AV and prevention of spontaneous membrane fusion. Upon signaling, the radial dispersion or torque motion of anchor I in the RSA could exaggerate the twist existing in anchors II and III, thereby pulling the AV closer to the PPM for fusion. (c) Close-up view of the molecular actors involved in the mechanism of rhoptry secretion. RASP2, associated with the tip of the neck, binds to PA and PIP₂. These signaling lipids might trigger fusion between the rhoptry necks and the AV (1). An Alveolata-specific complex (Nd9, Nd6, NdP1, NdP2, FER2) is involved in assembly of the RSA and fusion of the AV with the PPM (2). Given the presence of calcium lipid-binding domains on NdP2 and Fer2, calcium may act as a trigger for the fusion step. The molecular players responsible for the export of the rhoptry content into the host are unknown (3). Abbreviations: APR, apical polar ring; AV, apical vesicle; ICMT, intraconoidal microtubule; MV, microtubule-associated vesicle; Nd6, nondischarged 6; NdP1, Nd partner 1; PA, phosphatidic acid; PCR, preconoidal ring; PIP₂, phosphatidylinositol 4,5-bisphosphate; PPM, parasite plasma membrane; RASP2, rhoptry apical secretion protein 2; RSA, rhoptry secretion apparatus. Panel *b* adapted with permission from Reference 88.

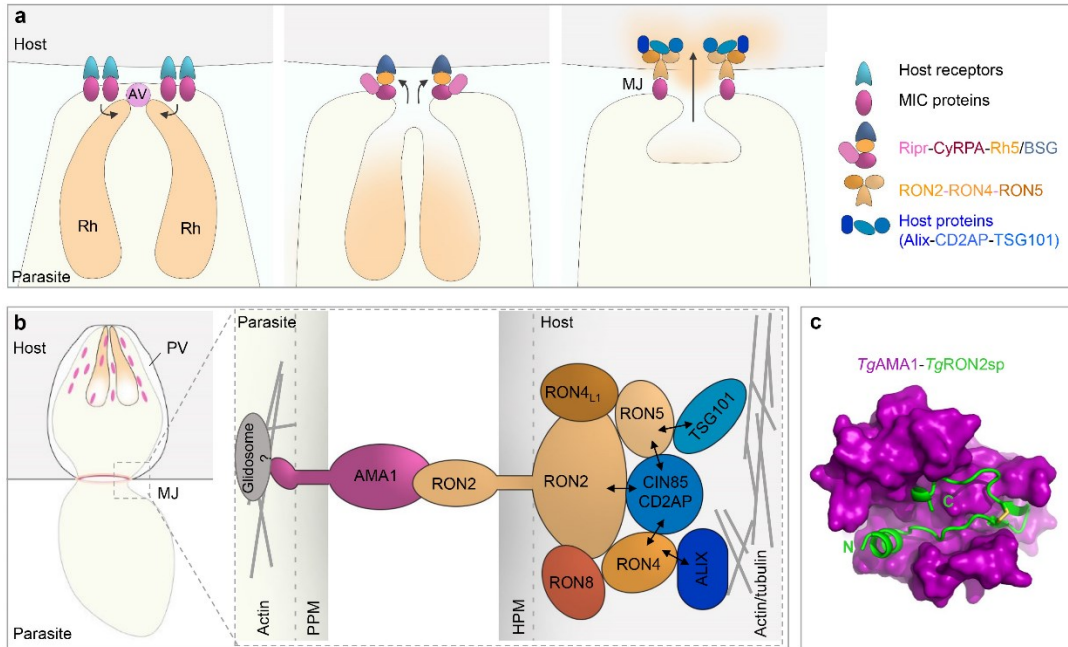


Figure 2. Cooperative roles of microneme and rhoptry proteins for invasion.

(a) Rhoptry secretion relies on host cell attachment mediated by recognition of host cell receptors by MICs (*left*). The two rhoptry necks fuse with the AV and with the PPM. Rhoptry neck proteins are then released and establish complexes with MICs to initiate the formation of a fusion pore in the HPM (*middle*) and then to build the MJ (*right*). In *Plasmodium falciparum*, pore formation may be dependent on formation of a stable complex between two microneme proteins, Ripr and CyRPA, and the rhoptry neck protein Rh5, which assemble at the host-parasite interface to interact with the host receptor basigin. This in turn allows delivery of the rhoptry neck proteins RON2, RON4, and RON5 into the host cell and intimate interaction of the extracellular part of the transmembrane protein RON2 with the micronemal protein AMA1. In *Toxoplasma*, the RON proteins recruit cytosolic host proteins such as ALIX, TSG101, CD2AP, and CIN85. (b) Close-up of the MJ complex during invasion. The MJ connects the PPM and HPM via the interaction between the ectodomain of AMA1 and the C-terminal region of RON2. On the parasite side, the cytoplasmic tail of AMA1 might be connected

to the glideosome machinery. On the host side, actin and tubulin accumulate at the MJ and RONS subvert host protein networks. RON4 possesses two YP(x)nL motifs that specifically recruit two ALIX proteins. RON5 has one PPPY and two P(T/S)AP motifs that account for accumulation of TSG101 at the MJ. Moreover, RON2, RON4, and RON5 contain multiple Px(P/A)xPR motifs that bind to CIN85 and CD2AP and massively recruit CIN85/CD2AP to the MJ. (c) Top view of the cocrystal structures of *Toxoplasma gondii* AMA1 (purple) with its RON2-interacting peptide (RON2sp) (green). TgRON2sp is anchored into the apical groove of TgAMA1 in a disulfide-anchored U-shaped conformation. Abbreviations: ALIX, apoptosis-linked gene 2-interacting protein X; AMA1, apical membrane antigen 1; AV, apical vesicle; BSG, basigin; CD2AP, CD2-associated protein; CIN85, Cbl-interacting protein of 85 kDa; CyRPA, cysteine-rich protective antigen; HPM, host plasma membrane; MIC, micronemal; MJ, moving junction; PPM, parasite plasma membrane; PV, parasitophorous vacuole; Rh5, reticulocyte-binding protein homolog 5; Ripr, Rh5-interacting protein; RON2, rhoptry neck protein 2; TSG101, tumor susceptibility gene 101. Figure adapted with permission from Reference 147.

