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***Toxoplasma* Mechanisms for Delivery of Proteins and Uptake of Nutrients Across the Host-Pathogen Interface**

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Abstract

Many intracellular pathogens, including the protozoan parasite *Toxoplasma gondii*, live inside a vacuole that resides in the host cytosol. Vacuolar residence provides these pathogens with a defined niche for replication and protection from detection by host cytosolic pattern recognition receptors. However, the limiting membrane of the vacuole, which constitutes the host-pathogen interface, is also a barrier for pathogen effectors to reach the host cytosol and for the acquisition of host-derived nutrients. This review provides an update on the specialized secretion and trafficking systems used by *Toxoplasma* to overcome the barrier of the parasitophorous vacuole membrane thereby allowing the delivery of proteins into the host cell and the acquisition of host-derived nutrients.

Keywords

Toxoplasma gondii; translocon; secreted effectors; nutrient acquisition; parasitophorous vacuole; trafficking

Introduction

Toxoplasma gondii is an obligate intracellular parasite that can invade any nucleated cell from probably all warm-blooded animals. Even in immunocompetent hosts, it can cause chronic infections by forming semi-dormant tissue cysts in brain and muscle tissues that can persist for the life of the host. In healthy individuals it can cause mild symptoms and sometimes ocular complications, but it can become life-threatening for infants born to infected mothers and people with weakened immune systems (68). Key to *Toxoplasma*'s success is the coordinated secretion of an arsenal of proteins from three specialized secretory organelles called micronemes, rhoptries and dense granules. Micronemal proteins (MICs) are secreted in a calcium-dependent manner from the parasite's apical end and mediate the attachment to the host cell (15, 40), which provides a signal for the rhoptries to secrete their contents. Proteins from the apical rhoptry neck (RONs) are secreted first and form a

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receptor that is inserted into the host cell plasma membrane thereby providing the handle to which MICs on the parasite plasma membrane (PPM) bind to and which it uses to pull itself inside (10). While it pulls on this handle it makes a transient break in the host cell plasma membrane through which it secretes rhoptry bulb proteins (ROPs) while a screw-like forward motion wraps the host cell plasma membrane around the parasite leading to the formation of the parasitophorous vacuole (PV) in the host cell cytosol (98). The PV is the parasite's replication niche and is surrounded by the PV membrane (PVM), which is the interface between *Toxoplasma* and the host cell. *Toxoplasma* overcomes the barrier of the PVM by secreting dense granule proteins (GRAs) into the PV lumen after which some GRAs traffic to the PVM where they mediate the acquisition of host-derived nutrients while other GRAs form a translocon that delivers GRA effectors into the host cytosol (106). The mechanisms for the delivery of *Toxoplasma*'s secreted proteins to their correct location and the acquisition of host nutrients across the barrier of the PVM are slowly being unraveled and will be reviewed here.

Trafficking pathways to deliver proteins to *Toxoplasma*'s specialized secretory organelles and the positioning of these organelles at secretion sites.

Toxoplasma secretory proteins need to be correctly trafficked to their respective secretory organelle. ROPs and MICs contain motifs that determine their trafficking to the rhoptries or micronemes while trafficking to dense granules is the default pathway of secretory proteins. The trafficking systems and sorting motifs in *Toxoplasma* were recently reviewed (127) and are summarized in Figure 1. The parasite attaches to the host cell with its apical end, which consists of the conoid (a cone-shaped structure of microtubules), two intra-conoid microtubules, and two polar rings (62). Therefore, micronemes and the neck of some rhoptry organelles must be closely appositioned to the apical end to allow fusion with the PPM and the secretion of MICs and RONs that mediate the attachment and invasion of the host cell. The positioning of rhoptries at the apical end of the parasite is mediated by the Armadillo Repeats-Only (*Tg*ARO) protein, which is anchored to the cytosolic face of the rhoptry membrane by the addition of a palmitoyl group by the palmitoyl acyl transferase *Tg*DHHC7 (3, 88). *Tg*ARO interacts with myosin F (*Tg*MyoF) (89), a myosin motor resembling the cargo transporter myosin Va (reviewed in (56)). By interacting with *Tg*ARO, *Tg*MyoF could position rhoptries at the parasite's apical end by walking on the filamentous actin cytoskeleton. At the apical end, the rhoptries are likely crosslinked to the intraconoid microtubules (86) by *Tg*CSCCHAP, a Coccidian Specific CORVET (class C core vacuole/endosome tethering)/HOPS (homotypic fusion and protein sorting) Associated Protein, in cooperation with microtubule associated protein Dynein Light Chain 8a (*Tg*DLC8a) (74). This crosslinking to intraconoid microtubules would allow the membrane of the rhoptry neck to become closely appositioned to the PPM upon extrusion of the conoid. Microneme trafficking to exocytosis sites also depends on *Tg*DLC8a (74).

*Tg*MyoF is also the molecular motor that binds to the surface of dense granules, likely via its WD40 domains, and delivers them to the parasite periphery (58) (Figure 2B). GRA46 has structural homology to myosin motors and was recently shown to localize to the periphery of dense granules and, unlike most other GRAs, GRA46 is not secreted (24). Thus, it is tempting to speculate that GRA46 also plays a role in the movement of dense granules.

Once at the parasite periphery, dense granules are escorted along with Rab11A vesicles and transported along actin filaments to their secretion sites (65). However, contact of dense granules with the PPM is restrained by the inner membrane complex (IMC), a network of stitched alveoli underneath the PPM connected with the cytoskeleton. The diffusive-like motion of dense granules likely enhances their movement in between small gaps of the IMC (41) after which they can start fusion with the PPM (Figure 2B).

Exocytosis of *Toxoplasma's* secretory organelles.

The exocytosis of rhoptries is dependent on the secretion of micronemes (recently reviewed in (40) and not further discussed here). A specialized structure called the moving junction forms the interface between the invading parasite and the host cell. The moving junction consists of a complex between the micronemal adhesin AMA1 present on the parasite surface and RON2, which is secreted into the host cell plasma membrane where its cytosolic part forms a complex with host cytosolic RON4/5/8 (1, 72). Without AMA1 the moving junction is not formed but RONs are still secreted (85). In contrast, parasites that do not express MIC8 do not secrete RONs suggesting that MIC8 might influence rhoptry fusion with the PPM possibly in cooperation with the recently identified claudin-like apicomplexan microneme protein (CLAMP) (67, 120). Two other proteins involved in rhoptry exocytosis are *TgFER2*, which belongs to the Ferlin family of double C2-domain proteins (26), and *TgRASP2*, a member of the recently identified Rhoptry Apical Surface Proteins (*TgRASP1/2/3*) that contain C2 and PH (Pleckstrin Homology) domains (125). C2 and PH domains can mediate interaction with lipids, consistent with *TgRASP2's* ability to bind phosphoinositides (PIPs) and phosphatidic acid (PA). Depletion of *TgRASP2* or absence of its lipid-binding domain completely abolishes rhoptry exocytosis and invasion. *TgRASP2* and *TgFER2* possibly function as part of the machinery that allows the fusion between the rhoptry membrane and the PPM (Figure 2a).

Once *Toxoplasma* resides in the PV, dense granules fuse with the PPM in a process resembling classical exocytosis leading to the secretion of GRAs into the PV lumen, the space between the PPM and the PVM (41, 75). Even though a large GRA secretion event is observed shortly after invasion (17, 28, 41, 75), dense granule exocytosis was considered to be a constitutive calcium-independent process that involved the conserved eukaryotic N-ethylmaleimide-sensitive factor (NSF)/soluble NSF attachment protein (SNAP) receptor (SNARE)/Rab system (23, 65). GRAs can also be spontaneously released from extracellular parasites (73, 119) and this release can be induced by heat-inactivated serum (28) and negatively regulated by calcium (66). Thus, dense granule exocytosis appears to be driven by both constitutive and regulated processes.

Host cell modulation by ROPs.

At the time of invasion, an unknown *Toxoplasma* protein likely induces a transient break in the host cell membrane (126) allowing the secretion of ROPs into the host cell cytosol. Once ROPs are injected into the host cell cytosol, they reside there or are translocated to the host cell nucleus or to the PVM, based on the presence of specific signals. For example, the ROP16 kinase, which can directly phosphorylate host STAT3/6, and the rhoptry protein phosphatase 2 C (PP2C-hn) possess a nuclear localization signal (NLS), that mediates their

translocation to the host nucleus (51, 113). Other ROP kinases or pseudokinases such as ROP17, ROP18 and ROP5 traffic back to the parasite's nascent PVM, which is mediated by an arginine-rich amphipathic helix (RAH) domain (107). ROP5/17/18 form a complex on the PVM that inhibits the immunity-related guanosine triphosphatases (GTPases) (IRGs), which are induced by interferon-gamma (IFN γ) and can vesiculate and destroy the PVM (43).

To aid its migration from the site of infection to distant organs, *Toxoplasma* co-opts host immune cells as Trojan Horses (131). ROP17 enhances the tissue transmigration of infected monocytes which is dependent on the Rho-Rho-associated coiled-coil containing protein kinase (ROCK) pathway, which is generally involved in modulation of the cytoskeleton (39). However, because ROP17's catalytic activity on the PVM also determines translocation of GRA effectors into the host cytosol (95) (discussed below), it is also possible that such a GRA effector modulates the Rho-ROCK pathway. The ROP Toxofilin is injected into the host cytosol upon invasion (76) where it disassembles the host cell cortical actin meshwork at the point of entry thereby facilitating invasion (35). Another cytosolic ROP called *TgWIP* (Wave complex Interacting Protein) was recently identified by an in vivo loss-of-function screen to be important for *Toxoplasma* dissemination. *TgWIP* mediates the disappearance of podosomes, which are actin-rich structures important for the adhesion of the cell to extracellular matrix, in infected dendritic cells and induces a hypermigratory phenotype in these cells (116). Migratory properties of host leukocytes are determined by the dynamic remodeling of the actin cytoskeleton, which can be influenced by the WAVE regulatory complex (WRC) and SHP2 phosphatase both of which are interacting with *TgWIP* (116). The function of most other ROP effectors is unknown.

Host cell modulation by exported GRAs.

A decade ago, GRAs were not considered to be secreted effectors that modulate the host cell. This changed when GRA15, which is secreted onto the PVM, was shown to activate the host nuclear factor- κ B (NF κ B) pathway (111). Since then, multiple PVM GRAs, including GRA6 (79), GRA7 (29), GRA17/GRA23 (52), GRA25 (118), GRA35 (129), and Mitochondrial Association Factor (MAF)1 (100), have been identified as parasite effectors that play an important role in the modulation of host cell signaling pathways, acquisition of host nutrients, or counteracting host innate defense mechanisms. Only few *Toxoplasma* effectors have been identified as exported beyond the PVM. GRA16 was the first GRA identified as exported beyond the PVM after which it localizes to the host cell nucleus, where it binds to host deubiquitinase HAUSP and protein phosphatase (PP)2A and regulates the host cell cycle (9). Subsequently, other exported GRAs that localize to the host nucleus were discovered: GRA24, which triggers and sustains phosphorylation of p38 α Mitogen-activated protein (MAP) kinase and its translocation into host nuclei resulting in enhanced production of proinflammatory cytokines (12); *TgIST*, which blocks STAT1-dependent transcription and thereby counteracts IFN γ -induced parasite clearance (48, 94); TEEGR (*Toxoplasma* E2F4-associated EZH2-inducing gene regulator) (synonymous with inducer of Host Cyclin E (HCE)1), which binds host transcription factors E2F3 and E2F4 resulting in inhibition of host NF κ B signaling and modulation of the host cell cycle (11, 96); and GRA28 with unknown function (90). GRA18 is so far the only GRA exported

beyond the PVM that remains in the host cytosol where it interacts with Glycogen Synthase Kinase (GSK)3/PP2A-B56 leading to β -catenin upregulation and selective modulation of host immune gene expression (59).

***Toxoplasma* exported GRAs are processed in the Golgi.**

Protein export is well studied in *Plasmodium* spp, in which most exported proteins have a signal peptide (SP) followed by a downstream RxLxE/Q/D motif termed the *Plasmodium* export element (PEXEL) (82). PEXEL-containing proteins are post-translationally imported into the ER by a complex consisting of the Sec61 protein translocase, signal peptidase complex (SPC)25 and sometimes Sec62 (80), whereby the PEXEL is recognized and cleaved by the ER-resident aspartyl protease plasmepsin V (PMV), another member of the complex (7, 80, 112). PMV cleaves after the leucine of the PEXEL motif, which reveals a neo N-terminus that is acetylated (Ac-xE/Q/D) in the ER by an unknown N-acetyltransferase permitting a potential cargo selection mechanism by cargo receptors or chaperones for trafficking to the PVM translocon (8, 20). The *Toxoplasma* ortholog of *Plasmodium* PMV is the Golgi-resident Aspartyl Protease 5 (ASP5) (Figure 1) which cleaves the *Toxoplasma* export element (TEXEL) RRL. ASP5-mediated TEXEL cleavage is important for export of GRAs (25, 31, 57) and mutating the TEXEL sequence of exported GRAs impairs their export (25, 31). Unlike the *Plasmodium* PEXEL, the TEXEL cleaved by ASP5 is not always at the N-terminus close to the SP. This suggests that *Toxoplasma* applies another protein export mechanism where the SP directs GRA entry into the ER followed by transport to the Golgi where recognition and cleavage of TEXEL-containing GRAs by ASP5 takes place. Since there is no common feature within the neo N-terminus of ASP5 substrates, it is unclear how ASP5 cleavage would target GRAs for export. One potential model is that one or more chaperone proteins immediately take ASP5-cleaved substrates with them throughout the secretory pathway. Unexpectedly, ASP5 is also required for export of some TEXEL-negative proteins (TNEPs) such as GRA24 and TEEGR (HCE1) (11, 25, 31, 57, 96). The export of TNEPs either relies on TEXEL-containing translocon components for which ASP5 cleavage is needed for their correct function or on ASP5-dependent cleavage of TEXEL-containing chaperones that are needed for the correct trafficking of TNEPs to the translocon (discussed below). In *Toxoplasma* only a subset of ASP5 substrates are exported in contrast to *Plasmodium* where all reported PEXEL proteins are exported. For example, the TEXEL-containing proteins, GRA19, GRA20, and MYR1, are processed by ASP5 but they are not exported and instead localized to the PVM (25, 44, 57, 61). ASP5 also plays a critical role in the correct PV/PVM localization of TEXEL-negative proteins, such as GRA2, GRA3, GRA7 (57), and GRA35 (129). In addition, *ASP5* abolishment results in a defective intravacuolar network (IVN), a network of membranous tubules in the PV lumen which are stabilized by the tubulogenic GRA2 and GRA6 proteins (83), and impairment of host mitochondrial recruitment at the PVM (25, 57). Given that many PV-localized GRAs are TEXEL-negative, ASP5 is likely indirectly influencing the localization of PVM-associated GRAs by affecting the function of proteins that help GRA trafficking to the PVM in a post-secretion event. In contrast to *Plasmodium* PMV, deletion of *ASP5* is not lethal to *Toxoplasma* but leads to reduced in vitro parasite growth, impaired host cell modulation and enhanced susceptibility to immune responses (25, 57), probably due to the cumulative result of defects in protein export and correct localization of proteins at the PV/PVM (57).

Post-secretory trafficking of GRAs to their final destination.

After secretion into the PV lumen, GRAs stay there, associate with membranes of the PVM or the IVN, or are exported beyond the PVM into the host cytosol. Several *Toxoplasma* proteins mediating GRA trafficking and export have been discovered (25, 31, 44, 57, 81, 95) but the trafficking and export mechanisms at a molecular level are still unclear. Except for the exported GRAs, GRA1 (19), and Nucleoside Triphosphate Hydrolase (NTPases) (5, 119), all GRAs described so far are predicted to encode either a single-pass transmembrane domain (TMD) or amphipathic α -helices and associate with membranes of the IVN or PVM. Inside the dense granules, GRAs with a hydrophobic domain do not behave as type I transmembrane proteins and are not inserted in the dense granule membrane but instead are mixed between partially soluble and aggregated states (50). This sorting of GRAs with a single-pass TMD to dense granules only occurs in *Toxoplasma* itself, since when these proteins are expressed in host cells, they sort to the host cell plasma membrane (50). GRAs in the dense granules are partially packed in oligomeric complexes (13), likely their hydrophobic domains are either sequestered directly within the complex or shielded by chaperone-like proteins (Figure 2b), thereby explaining their export as soluble proteins. Once secreted into the PV lumen, a change in the microenvironment might lead to a subsequent conformational change exposing their hydrophobic domain. However, the precise mechanism that subsequently leads to their insertion into the PVM vs. IVN vs. PPM is still unclear. It was shown that the N-terminal domain between the SP and TMD of GRA5 and GRA6 contains an unknown signal that determines correct trafficking to the PVM and exchange of this domain leads to accumulation of these GRAs in the PV lumen (49, 50). How the N-terminal domain of these GRAs promotes a conformational change from a soluble stage to a transmembrane conformation remains to be determined. Possibly specific cofactors, such as protein chaperones delivered to the PV at the same time, recognize sorting motifs within this N-terminal domain and contribute to the post-secretory membrane insertion. Such a model is illustrated for protein trafficking in the cell envelope of gram-negative bacteria. Similar to the spatial structure of the *Toxoplasma* plasma membrane and PVM, the cell envelope of these bacteria consists of the plasma membrane, inner membrane, and outer membrane flanking the periplasm (121). For example in *Escherichia coli*, most non-folded outer membrane proteins after crossing the plasma membrane are assisted by chaperones to stay soluble followed by inserting into the outer membrane (34). A group of periplasmic chaperones called pilotins have been proposed to bind the C-terminus of outer membrane-localized lipoproteins followed by their co-transport to the outer membrane by the Lol system (70, 93).

Little is known about the *Toxoplasma* proteins involved in GRA trafficking after exocytosis into the PV lumen. GRA42 and GRA43, which are localized inside the PV lumen after secretion, mediate the correct trafficking of the PVM-associated GRA35 and GRA17/23 (129), suggesting GRA42/43 function as protein chaperones although they have no homology with any known protein chaperones. ASP5 also influences the PVM localization of GRAs, but GRA42/43 do not contain TEXEL motifs. It is therefore likely that other TEXEL-containing proteins function as regulators of GRA42 and GRA43 or function in the same pathway mediating the correct localization of PVM-associated GRAs. One candidate for such a protein is the kinase WNG1, a novel ASP5 substrate that localizes to the PV

lumen (4, 24) and that is involved in the phosphorylation of several membrane-associated GRAs including GRA35 (4). Parasites without catalytically active WNG1 have reduced membrane association of GRA4, GRA6 and GRA7 as well as impaired IVN morphology, suggesting that WNG1 could directly phosphorylate membrane-associated GRAs as well as provide the energy for correct trafficking of these GRAs to their destination. An alternative hypothesis is that instead of directly phosphorylating the membrane-associated GRAs, WNG phosphorylates a chaperone-like GRA resulting in its disassociation from and release of its cargo leading to membrane insertion of a subset of GRAs where they are phosphorylated by host cytosolic kinases (4). Selective targeting of GRAs to PVM or IVN could further rely on the interactions between the N-terminal domain of GRAs and specific components of these membranes. For example, the lipid composition of the PVM or IVN could affect the localization of GRAs with a hydrophobic domain. Initially, the PVM is almost entirely derived from the host cell plasma membrane and rich in cholesterol and the ganglioside GM1 (21, 30). However, the PVM is depleted of cholesterol over time and parasite proteins are integrated into the PVM. Similarly, the IVN is initially derived from secretion of vesicular material from the parasite's basal end, however late after infection IVN lipids are predominantly host cell derived (16). The lipid environment (thickness, composition, curvature) influences the affinity of proteins for certain membranes (102), which could contribute to GRA targeting. In agreement with this hypothesis, some PVM-localized GRAs, for example GRA7, could have specific affinities for certain lipids (e.g. phosphoinositides) that may be present on the PVM and IVN but not PPM (29). Furthermore, the PVM bilayer (13 nm) is thicker than the IVN bilayer (8 nm) which might affect preferential insertion into PVM vs. IVN (78).

GRA effector translocation across the PVM.

GRA export across the PVM into the host cytosol is only observed once the parasite has invaded, which differs from rhoptry effectors that are discharged into the host cell cytosol upon invasion. A forward genetic screen set up to discover *Toxoplasma* effectors responsible for host c-Myc induction identified MYR1 as the first *Toxoplasma* protein contributing to GRA export beyond the PVM (44). The export of GRA16, GRA18, GRA24, TgIST and TEEGR was absent in the parasites lacking MYR1 (11, 44, 48, 59, 96) whereas the functions of PVM-localized MAF1b and GRA15 were not affected (44). This suggests that MYR1 only affects the export of GRAs that translocate across the PVM but has no effect on the correct localization of GRAs to the PVM. Due to impairment in delivery of GRA effectors into the host cell, *myr1* parasite-infected cells have a dramatically reduced host transcriptional response to *Toxoplasma* infection (91). Subsequently, MYR2 and MYR3, which were identified from the same c-Myc induction mutagenesis screen, were also shown to be involved in export of GRAs across the PVM (81). Notably, both MYR1 and MYR3 localize at the PVM and form a stable interaction whereas MYR2 localizes at the periphery of the PV and did not associate with the other two MYR proteins (81). MYR4 was recently identified as MYR1-interacting protein and involved in GRA16 nuclear translocation (32). Surprisingly, ROP17, discovered from the same screen, was also shown to determine GRA translocation across the PVM (95). Unlike the MYR proteins, the action of ROP17 on GRA translocation takes place on the host cytosolic side of the PVM and depends on its catalytic residues (95). ROP17 is recruited to the PVM after secretion into the host cell

and might phosphorylate and activate a component of the PVM translocation machinery. It should be noted that MYR1/2/3/4 have not been formally shown to form a translocation pore for protein export. It is also possible that they serve a role as chaperoning and/or docking proteins that help exported GRAs reach the protein-conducting pore at the PVM. Recent studies have discovered that the ASP5 substrates GRA44 and GRA45 are necessary for GRA16 and GRA24 export to the host nucleus (6, 32, 130). GRA45 possesses a small heat shock protein (HSP) domain and prevents aggregation of hydrophobic GRAs within the dense granules and influences the correct trafficking of GRAs to the PVM (130). Unlike the *Plasmodium falciparum* PTEX translocon, which contains the unfoldase HSP101 (60), there is no evidence that the *Toxoplasma* translocon contains an unfoldase and all known exported GRAs are intrinsically disordered proteins (55, 99). Fusion of the highly folded murine dihydrofolate reductase (DHFR) domain to highly disordered GRA16 blocks translocation of GRA16 as well as GRA24 to the host cell likely by clogging the translocation pore (31, 81). Taken together, *Toxoplasma* exported GRAs do not seem to require active unfolding for their export. Thus, GRA45 likely functions as a protein chaperone which helps to maintain the proper folding of GRAs destined for the PVM during their trafficking through the secretory system (Figure 2b). GRA45's effect on GRA translocation across the PVM is therefore most likely due to a putative effect on the correct trafficking of translocon components to the PVM. GRA44 contains a putative acid phosphatase domain (6) but it is still unclear how it influences the protein export in *Toxoplasma*.

Transport of small molecules across the PVM.

Toxoplasma's growth is dependent on nutrient scavenging from the host cell as it is auxotrophic for nutrients such as purines, tryptophan, arginine and cholesterol. Additionally the parasite has salvage pathways for amino acids, sphingolipids and pyrimidines (27). The PVM-host cytoplasm is the interface through which parasite proteins mediate the uptake and transport of nutrients into the PV lumen. The subsequent absorbance of these nutrients is via membrane transporters located in the PPM (97, 105). The PVM is selectively permeable allowing the charge-independent diffusion of molecules up to 1.3 kDa (117). The PVM-localized GRA17 and GRA23 play a role in the permeability of the PVM to small molecules (52) (Figure 2c). Parasites without *GRA17* form "bubble" vacuoles that have decreased permeability to small molecules. The expression of *Plasmodium* translocon protein EXP2, a GRA17/23 orthologue, in *gra17* parasites rescued this phenotype, which suggested that EXP2 might have a dual function as a nutrient pore and translocon (52), which was recently confirmed (47). Parasites without *GRA23* display no obvious phenotype, however, when GRA23 is overexpressed in *gra17* parasites it rescues the defect in the PVM permeability to small molecules suggesting it can also form a permeability pore. The simultaneous deletion of *GRA17* and *GRA23* is synthetically lethal, indicating that these proteins function in similar pathways for nutrient transport across the PVM. Because the deletion of GRA17 generates swollen vacuoles it is possible that the GRA17 pore is also involved in the elimination of metabolic waste products and the absence of the pore leads to an osmotic imbalance in the PV lumen, which could explain why 60% of *gra17* bubble vacuoles eventually disrupt, killing the parasites inside. GRA17, GRA23 and EXP2 lack TMDs and their sizes and multiple α -helices suggest a functional analogy to α -pore forming toxins (α PFTs) (52). α PFTs exist as soluble, inactive monomers that, in the context of

their targets, subsequently assemble and multimerize into active complexes that can form large pores in phospholipid membranes (54). A recent Cryo-EM structure of the PTEX complex showed that seven EXP2 monomers form a protein-conducting channel spanning the membrane (60). Super resolution Scanning EM of the *Toxoplasma* PVM revealed pores ranging from 10 to 200 nm and the number of pores in the PVM increased with the length of the intracellular cycle (36), which is consistent with GRAs forming these pores. Whether these pores constitute GRA17/23 nutrient pores and/or pores formed by the translocon mediating GRA export is currently unknown. The presence of pores can explain the traffic of small molecules, however it is unclear how macromolecules larger than 1.3 KDa reach the PV lumen, the only classical transporter identified so far in the PVM is the *Toxoplasma* ADP binding cassette sub-family G (ABCG₁₀₇) likely involved in lipid acquisition (42). The presence of additional transporters in the PVM remains to be determined.

Uptake of host-derived vesicles and host cytosolic proteins through the PVM.

Many intracellular pathogens obtain host nutrients by recruiting host organelles and scavenging essential molecules from them. After *Toxoplasma* invasion, the PV is located to a perinuclear area and recruits the microtubule organizing center (MTOC) by interaction of the PVM with γ -tubulin through an unknown mechanism (128). Recruitment of the MTOC to the PVM likely contributes to the subsequent recruitment of the Golgi complex, ER, and endolysosomal vesicles (108). The conversion of vesicular trafficking towards the PV facilitates membrane fusion events for delivery of host cell nutrients into the PV. Rapidly after invasion ER profiles and Golgi complex stacks are located around the PV (122), possibly to obtain N-glycans and sphingolipids (46, 110). Sec22b is required for recruitment of ER to the PVM. The intricate connections between the ER and PVM also allow *Toxoplasma* antigens to be translocated to the ER and subsequently retrotranslocated via the ERAD system to the host cytosol where they can be loaded onto MHC I for antigen presentation to CD8 T cells (18, 53, 77).

Host mitochondria are recruited to the vicinity of the PV of type I and III, but not type II, strains, which is mediated by the PVM-localized GRA protein MAF1b (100). There is some evidence that *Toxoplasma* recruits mitochondria to scavenge specific lipids: deletion of the Acyl coenzyme A (CoA)-binding protein (ACBP2) in type II parasites results in a defect in cardiolipin metabolism, which can be rescued by expressing type I MAF1b in the type II *achp2* strain (45). On the other hand, mitochondria recruited to the PV can also limit parasite growth by siphoning of fatty acids and degrading them thereby limiting *Toxoplasma*'s access to these fatty acids (101). *Toxoplasma* intercepts and 'ingest' host Rab-derived vesicles loaded with sphingolipids, lipid droplets containing neutral lipids (109), and endolysosomes filled with cholesterol (29), through a phagocytic-like process whereby the PVM surrounds these vesicles after which they pinch off and localize inside the PV lumen (92). The lecithin:cholesterol acyltransferase (*Tg*LCAT), a GRA secreted into the PV lumen, likely degrades the limiting membrane of these vesicles promoting the release of their cargo into the PV lumen (104). *Toxoplasma* can also ingest host cytoplasmic proteins through an unknown mechanism. Because ingested host proteins are rapidly digested inside the VAC, an acidic lysosome-like organelle, the ingestion of host proteins can only be observed in parasites where the VAC's major digestive enzyme Cathepsin L (CPL) is

knocked out (38) or inhibited. *Toxoplasma* might ingest host proteins to acquire amino acids, similar to how *Plasmodium* acquires amino acids from ingested hemoglobin (64). The *Toxoplasma* proteins that mediate the ‘ingestion’ of host proteins or Rab-derived vesicles at the PVM or mediate the transport of Rab-derived vesicle cargo from the PV lumen across the PPM are currently unknown. In *gra2* parasites, which do not have an IVN, the ingestion of host cytosolic proteins, Rab-vesicles, and lipid droplets is significantly reduced, suggesting that the IVN functions as a conduit to provide nutrients to *Toxoplasma* (29, 109, 110). GRA7 is responsible for the membrane constriction that leads to the formation of an intravacuolar membrane and host microtubule bridge named H.O.S.T (Host-Organelle Sequestering Tubulo-structures) that is important for uptake of lysosome-derived vesicles (29). GRA3 is present in the PVM, where it binds specifically to lipids and promotes structural modifications in the PVM that lead to the interaction and retention of the Golgi complex. GRA3 is also involved in the disruption of anterograde traffic to retain host nutrients from the secretory pathway (33). Likely other yet to be identified GRAs are involved in the nutrient uptake mechanisms displayed by *Toxoplasma*.

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Future Issues list:

- What proteins form the membrane pore or channel on the PVM and do GRA44/GRA45, MYR1/2/3/4, and ROP17 play a role in the formation and/or regulation of the translocon or the transport of effectors to the translocon?
- What are the proteins that prevent aggregation of GRAs inside the dense granules, mediate the conformational change of GRAs in the PV lumen, and help GRAs insert into or associate with different membrane fractions in the PV?
- What is the *Toxoplasma* protein(s) recognizing the neo N-terminus of ASP5-cleaved GRAs and how are they and TNEPs subsequently transported to the PVM translocon?
- What is the molecular mechanism of dense granule exocytosis?
- What is the molecular mechanism involved in the host cytosolic protein uptake?
- What is the molecular mechanism of ROP secretion into the host cell cytosol?

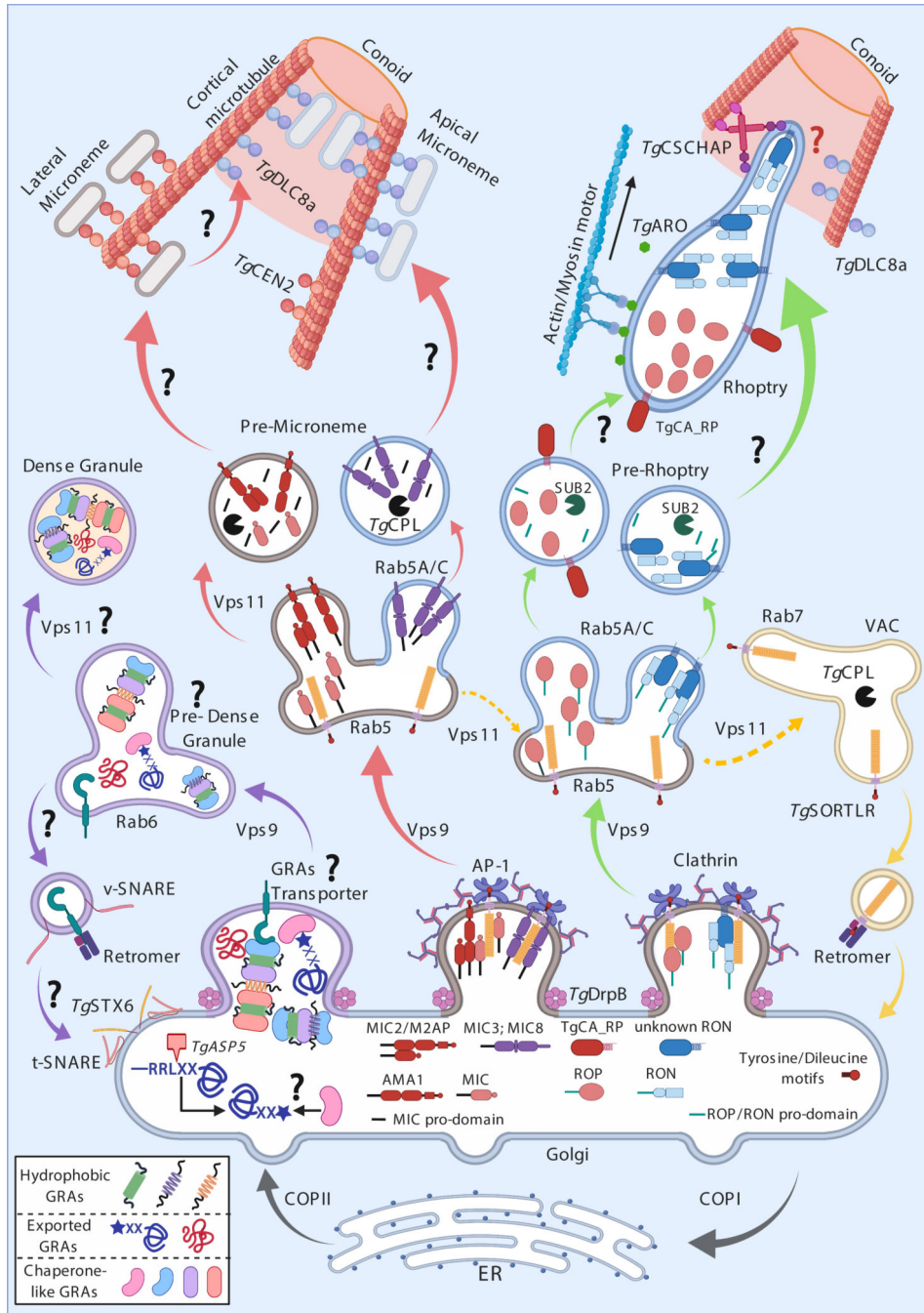


Figure 1. Trafficking of proteins to *Toxoplasma*'s specialized secretory organelles. Vesicles containing GRAs, MICs and RONs/ROPs are trafficked from the ER to the Golgi via the COPII complex (2) while the COPI complex assures retrograde transport (69). At the Golgi, each type of secretory protein localizes to a distinct Golgi region where MICs and RONs/ROPs could bind to the N-terminal domain of the transmembrane Sortilin-Like Receptor (*TgSORTLR*), which binds to the Adaptor Protein (AP)-1 complex with its C-terminal di-leucine motif (LL) (123). Some MICs (e.g., MIC2, AMA1) possess a transmembrane domain and a tyrosine motif (YxxΦ) (127) that could directly interact

with AP1. The hydrophobic domains present in many GRAs are likely kept soluble by a chaperone-like protein, which could interact with an unknown GRA transporter protein. Exported GRAs are cleaved at the TEXEL motif (RRL) by the Golgi-resident ASP5 protease and the resultant N-terminal domain could be acetylated and associate with a chaperone-like protein. The scission of vesicles at the Golgi depends on the dynamin-related protein DrpB (14). MIC- and RON/ROP-containing vesicles are sorted from the Golgi (red and green arrows, respectively) and delivered to Rab5-/Vacuolar Protein Sorting (Vps)9-positive endosomal compartments (114) in a Clathrin/AP1 complex dependent manner (103). It is still unclear how MICs and RONS/ROPs dissociate from *TgSORTLR*. Subsequently, *TgSORTLR* could be transported from a Rab5- to a Rab7/VAC-positive endosomal compartment using the Vps11/HOPs complex (87), and finally recycled back to the Golgi by the retromer complex (115). Apical MICs (e.g., MIC3/8) and RONS/ROPs are delivered to their respective pre-organelles via Rab5A/C-positive vesicles (71) while delivery of lateral MICs (e.g., AMA1, MIC2/M2AP) is dependent on the Vps11/HOPs complex (87). In the pre-micronemes, the pro-domain of MICs could be cleaved by the protease *TgCPL* (37) while in the pro-rhoptries the SUB2 protease could cleave RON/ROP pro-domains (84). It is still unclear how pre-micronemes and pre-rhoptries mature into micronemes and rhoptries. After maturation, lateral and apical micronemes are attached to cortical microtubules using *TgCEN2* and *TgDLC8a*, respectively. The transfer of lateral micronemes to the conoid to get secreted seems to be also dependent on *TgDLC8a* (74). An unknown protein could traffic RON proteins together from the Golgi, thereby promoting the localization of these proteins at the neck of the rhoptry. Subsequently, *TgCA_RP* could mediate the fusion of pre-rhoptries containing ROPs with the neck of the rhoptry (22). The mature rhoptry is then transported to the conoid using actin/myosin motors and *TgARO*. The final attachment of rhoptries to the conoid microtubule depends on *TgCSCHAP* (86) and *TgDLC8a*. The sorting of GRAs from the Golgi compartment (purple arrows) is dependent on Vps9 (114) but independent of Rab5-positive vesicles. Rab6 between the Golgi and endosome could be necessary for the formation of a putative pre-dense granule compartment (124). The retromer complex could also recycle the putative GRA transporter from the pre-dense granules to the Golgi, where syntaxin *TgSTX6* together with t/v-SNARE assures vesicle fusion to the Golgi (63). It is unclear how GRAs are packed into mature dense granules.

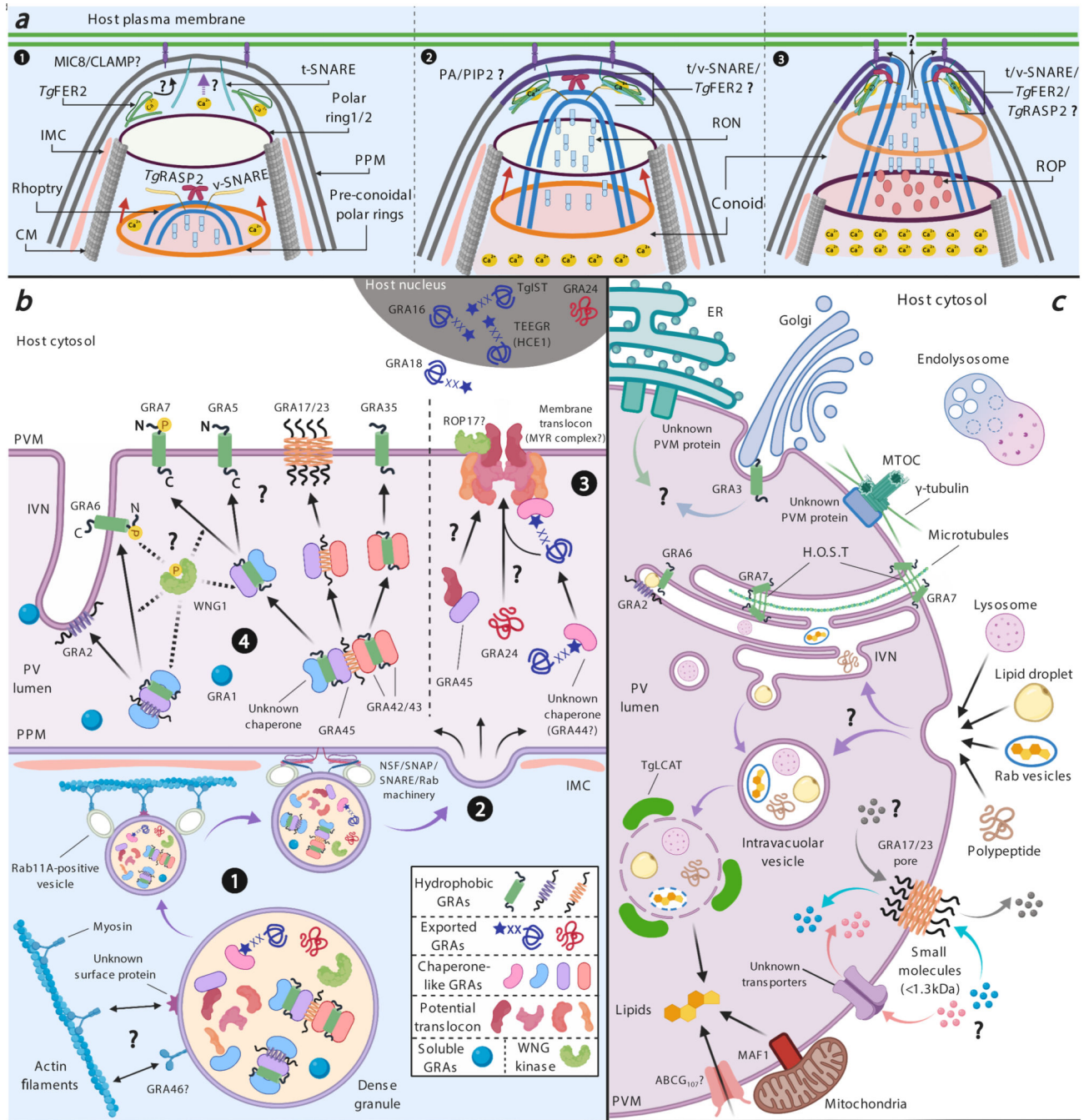


Figure 2. (A) Model for rhoptry secretion.

1) The process of host cell invasion raises the parasite internal $[Ca^{2+}]$ and the upper polar ring and conoid are extended until the base of the conoid protrudes beyond the lower polar ring (62). MICs are secreted, and MIC8/CLAMP could bind to the parasite and host plasma membrane (67, 120). The increase in $[Ca^{2+}]$ or the presence of MICs on the PPM could trigger a signaling cascade that changes the parasite phospholipid composition. The retracted conoid with rhoptry organelle moves through the cortical microtubules towards the apical polar ring. $TgRASP$ and a putative v-SNARE protein are present at the membrane

of the apical end of the rhoptry. A putative t-SNARE and *Tg*FER2 at the PPM could bind to the membrane upon sensing a calcium signal. **2)** When the conoid and the apical end of the rhoptry (containing RON proteins) are close to the apical polar ring, *Tg*FER2 could mediate the interaction of the t/v-SNARE and bring the rhoptry membrane closer to the PPM, which could allow *Tg*RASP2 to interact with PA/PIP2 (Phosphatidic Acid/Phosphatidylinositol (4,5)-bisphosphate) on the PPM. **3)** The conoid ultimately passes through the apical polar ring, and the t/v-SNARE/*Tg*FER2/*Tg*RASP2 complex could mediate the fusion of the rhoptry membrane and the PPM allowing RONs/ROPs to be secreted. Possibly MIC/ROP/RON secretion changes the permeability of the host plasma membrane at the attachment site allowing RONs and ROPs to pass through and reach the cytosol. **(B) Model for trafficking of GRAs from dense granules to their destinations.** **(1)** Inside the dense granules, hydrophobic GRAs are packed as soluble oligomers via shielding of their hydrophobic domains by putative chaperone-like GRAs to avoid fusion with the dense granule membrane. Mature dense granules bind to myosin and/or Rab11A-positive vesicles, which mediate the transport of dense granules via actin tracks to the parasite's periphery where they dock at IMC gaps. **(2)** Docking of dense granules at an IMC gap allows their fusion with the PPM via the NSF/SNAP/SNARE/Rab machinery resulting in exocytosis of GRAs into the PV lumen. **(3)** Once secreted into the PV lumen, ASP5-processed TEXEL-containing exported GRAs with potential N-terminal acylation are escorted by unknown chaperone GRAs to the PVM translocon, of which MYR1/2/3/4 and GRA44 may be components. GRA45 likely functions as a chaperone helping the translocon components traffic to and insert into the PVM. GRA24 is a TEXEL-negative exported protein and traffics to the membrane translocon via an unknown mechanism followed by translocation into the host cell. **(4)** Hydrophobic GRAs (transmembrane, amphipathic helices or α -helices) are escorted by chaperone GRAs to traffic inside the PV lumen and reach their destinations through an unknown mechanism. WNG kinase (WNG1) is involved in the eventual insertion of GRAs into the PVM or IVN via either directly phosphorylating the hydrophobic GRAs or phosphorylating the chaperone GRAs leading to the dissociation of hydrophobic GRA cargo from the chaperone. **(C) Nutrient and small molecule import across the PVM.** Host organelles, such as endoplasmic reticulum (ER), Golgi apparatus and the microtubule organizing center (MTOC), are recruited to the PVM. The relocation of the MTOC leads to the conversion of the microtubules network around the PVM bringing vesicular organelles, such as endolysosomal vesicles, to its vicinity where they can be used as nutrient resources for *Toxoplasma*. Host Golgi is retained in PVM invaginations by GRA3 whereas ER and MTOC are recruited via unknown PVM proteins. Host microtubules are recruited to invaginations of the PVM, mediated by GRA7, where they form a structure named H.O.S.T. responsible for cholesterol uptake derived from host lysosomes. Small nutrients are likely acquired through a membrane pore formed by GRA17/23 and/or other unknown PVM transporters. Host lysosomes, lipid droplets, Rab-positive vesicles derived from the endolysosomal system as well as polypeptides are invaginated into the PV and transiently stored at the IVN and intravacuolar vesicles of which the outer membrane can be digested by *Tg*LCAT resulting in the release of nutrients into the PV lumen. GRA2 and GRA6 are present in the IVN and mediate the uptake of host cytosolic proteins. Host

mitochondria are anchored to the PVM by MAF1. ABCG₁₀₇ is a potential PVM transporter for scavenging host lipids and maintaining lipid homeostasis inside the PV lumen.

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