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Translocation of effector proteins into host cells by *Toxoplasma gondii*

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Abstract

The Apicomplexan parasite, *Toxoplasma gondii*, is an obligate intracellular organism that must co-opt its host cell to survive. To this end, *Toxoplasma* parasites introduce a suite of effector proteins from two secretory compartments called rhoptries and dense granules into the host cells. Once inside, these effectors extensively modify the host cell to facilitate parasite penetration, replication and persistence. In this review, we summarize the most recent advances in current understanding of effector translocation from *Toxoplasma*'s rhoptry and dense granule organelles into the host cell, with comparisons to *Plasmodium spp*. for broader context.

Introduction

The *Apicomplexa* are a phylum of intriguing, single-celled eukaryotes that all require invasion into another eukaryotic cell for growth and survival. This large, diverse group of parasites includes many that are important for human and animal health, but herein we focus on *Toxoplasma gondii*, a causative agent of potentially devastating disease in the developing human fetus and in immunocompromised human adults [1]. *Toxoplasma gondii*'s complex life cycle involves sexual development within its feline definitive host, as well as asexual development within another eukaryotic host cell [2], where individual parasites undergo several replicative cycles within their intracellular niche until the host cell lyses and releases multiple daughter parasites referred to as tachyzoites for their rapid growth (*tachos* means *speed* in Greek). The asexual arm of the lifecycle is remarkable for the parasites' broad host range, which encompasses virtually any nucleated cell type from almost any warm-blooded animal, including humans. Therefore, *Toxoplasma*'s intracellular lifestyle necessitates communication with and co-opting of a vast array of host cell types, each of which is defined by its own species-and cell type-specific biology.

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The best-studied *Toxoplasma*-host cell relationship is that between tachyzoites and nonphagocytic, fibroblastic mammalian cell lines. This relationship begins with tachyzoite invasion into the host cell, an active process mediated largely by the parasites themselves [3–5]. During invasion, the host cell's plasma membrane is invaginated, ultimately completely encompassing the parasites within a parasitophorous vacuole (PV) within which the parasites divide (Fig. 1). Invasion is mediated by two sets of secretory organelles at the apical ends of the parasites – numerous small, rice-shaped micronemes and the less numerous but much larger, club-shaped rhoptries (*rhoptry* means *club* in Greek). The rhoptry proteins are introduced into the host cell during invasion and play an active role in invasion itself, as well as in the establishment of a replicative niche within the host cell and concomitant co-opting of host cell functions [5–7].

During or soon after invasion, the parasites secrete a further set of secretory proteins from spherical secretory organelles called dense granules, which are found distributed throughout the parasites (Fig. 1, GRA Secretion and PVM Maturation). The transit of these proteins across the plasma membrane of the dividing parasites is thought to be relatively conventional for eukaryotic secretion. The intriguing and remarkable part of their journey involves the translocation of a subset of these proteins across the PV membrane (PVM), into the host cytosol [8] or nucleus [9*–14]; in both cases, the effect on the host cell is profound. In this review, we summarize our current understanding of how effector proteins from the rhoptries and dense granules of *Toxoplasma gondii* are introduced into the host cell, with particular emphasis on advances made in the last four years, and with comparison to what is known in *Plasmodium* where relevant.

Rhoptry Effector Translocation into Host Cells:

Final Destinations and Activities of Rhoptry Effectors: Each rhoptry organelle contains an armament of effector proteins that collectively manipulate host cell biology. Each rhoptry organelle can be partitioned into a narrow, apical neck region, which houses rhoptry neck proteins (RONs), and a wide basal bulb region, which contains rhoptry bulb proteins (ROPs). At or around the time of invasion, the rhoptries introduce RONs and ROPs into the host cell, a key event for the parasites' survival and virulence.

After rhoptry protein release, the RONs form an essential, highly conserved protein complex in the host cell membrane that serves as a receptor for the microneme-derived surface ligand, AMA1 [15–19]. When RON2, the transmembrane component of the complex, binds AMA1 [20,21], they and the other RONs form a tight junction called the moving junction, named for its movement down the length of the parasite as it invades into the host cell (Fig. 1). This structure is thought to grant the parasites purchase as they actively pull themselves into the host. In contrast to the RONs, ROPs do not appear to perform an essential role in invasion and are poorly conserved between *Toxoplasma* and *Plasmodium* (reviewed in [22]). In *Toxoplasma*, the ROPs include effectors that localize to various niches within the host cell once introduced. Some decorate the host-cytosolic side of the PVM, (various ROP2 family members [23]), where they can serve various functions including neutralizing host innate immune responses (e.g., ROP5/17/18 [24–27]). Others, such as toxofilin, remain cytosolic and can remodel the host's cortical actin cytoskeleton [28–30], while still others reach the

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host nucleus, where they can impinge on the STAT activation pathway (i.e., ROP16 [31–33]) and other transcriptional processes (e.g., PP2C-hn [34]; see Fig. 1 for depictions of final destinations of ROPs). In addition to these known ROP effectors, genetic, proteomic, and bioinformatic screens have identified many additional putative ROP effectors, but the majority of these have yet to be validated as true rhoptry proteins. Note, however, that a few so-called "ROP" proteins, that were presumptively named such for their homology to the ROP2 family, have recently been demonstrated to actually be released predominantly, if not exclusively, from other secretory organelles such as the dense granules [35–37] and constitutive secretory vesicles [38].

Mechanism of Rhoptry Effector Secretion: Despite the importance of RONs and ROPs to *Toxoplasma* invasion and growth, the triggers leading to their release and the mechanism of their introduction into a host cell are not yet clear. Not surprisingly, at least a part of the trigger involves contact with the host cell; e.g., host plasma membrane cholesterol appears to be required for rhoptry secretion in *Toxoplasma* tachyzoites [39]. Additional factors include the micronemal protein MIC8, which is required for RON4 secretion [40], and the calcium sensing protein, FER2 [41]. The exact roles for these proteins and pathways are not yet known but multiple calcium-dependent protein kinases have been identified in these parasites (reviewed in [42]), and one or more of these could play a key role in the signaling.

Regarding the mechanics and machinery of injection itself, ultrastructural studies depict shortening and emptying of the rhoptries over the course of a few seconds [43,44] (depiction in Fig. 1). It has also been demonstrated that only a minority of the rhoptries discharge at any one time [45]. With the exception of RON2, which somehow ends up spanning the host plasma membrane, all known rhoptry effectors are soluble proteins that must traverse three lipid bilayers (i.e., the rhoptry membrane, the parasite cell membrane, and the host cell membrane), an unusual and nontrivial topological problem seldom encountered in eukaryotic systems. The solution in prokaryotes is triple-membrane spanning complexes such as type III, type IV, and type VI secretion systems (reviewed in [46–48]); however, apicomplexans possess no apparent orthologs to these systems.

Early work using scanning EM revealed a rosette of small depressions on the apex of *Toxoplasma* and *Sarcocystis* tachyzoites [49], which was interpreted as possible fusion events between the rhoptry necks and the plasma membrane. However, subsequent ultrastructural studies of *Toxoplasma* tachyzoites and *Plasmodium* merozoites actively invading their respective host cells and imaged by transmission EM of ultrathin sections revealed a ~50 nm opening between the parasite and host cell membranes [43,50]. Although this suggested that a possible channel links at least the parasite and host cytosols, the rhoptry membrane near this possible channel in such images is indistinct, leaving open the question of whether there is a continuous path or channel that stretches all the way from the rhoptry lumen and across the parasite and host plasma membranes. Supporting such a model is the fact that rhoptry discharge is accompanied by a transient disruption in the host membrane [51], although the relationship of this to rhoptry protein injection has not been further investigated. Also in support of this model are freeze-fracture images of tachyzoites and merozoites in the midst of invasion which show a tantalizing indentation within the nascent

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PVM at the location of the extreme apical tip of the parasite [45,52]. It is tempting to speculate that this is a view of the rhoptry pore or channel from inside the host cell. As yet, however, no molecules within this structure have been identified and its precise function remains unknown, although in a more recent ultrastructural study of *Toxoplasma* tachyzoites, Paredes-Santos et al proposed that small vesicles located within the parasite's apex are the source of the putative pore and therefore dubbed them "porosomes" [44]. Their small number (~4–6), size (~50 nm), and placement make this an appealing model but, as yet, there are no data to directly indicate such a role.

Dense Granule Effector Translocation:

Final Destinations and Activities of Dense Granule Effectors: Following invasion and establishment of the nascent PV, *Toxoplasma* secretes dense granule proteins (GRAs) to modify its PV to create a hospitable niche for replication within the host cell. Several critical PV modifications include: establishment of the intravacuolar network (IVN), an elaborate network of membranous nanotubules whose lumens are topologically contiguous with the host cytosol and that appear to bud from the PVM into the PV space [53–55]; rearrangement of the host cytoskeleton and recruitment of host organelles to the PV (reviewed in [56]); establishment of PV pores to function as a molecular sieves and to scavenge essential nutrients from the host [57,58]; and modulation of the host response to optimize parasite growth and avoid host defenses (reviewed in [6]). These PV modifications are dependent on the secretion of GRAs that ultimately reside within the PV space, integral to or associated with the PVM, or fully within the host cell cytosol or nucleus.

GRAs are thought to be constitutively secreted from the parasite [59], although a large burst of secretion resembling classical exocytosis is observed shortly after invasion [60–63] and appears to be negatively regulated by calcium [64] (Fig 1, GRA Secretion and PVM Maturation). Interestingly, some GRAs can be observed within the host cell even prior to the formation of a PV, when parasite invasion is inhibited by the actin inhibitor cytochalasin D (e.g., GRA7 and GRA15 [65,66]). Under these conditions the GRA proteins are found near the site of parasite attachment localized to membranous structures termed evacuoles (because they are "empty" of parasites), which also include many of the rhoptry proteins described above. Although the genesis of evacuoles is thought to be associated with rhoptry discharge, the exact basis of their formation is not yet clear, and neither is the mechanism by which ROP and GRA effectors associate with them, or even the topology of the effectors associated with these structures [43,50,67,68]. An intriguing possibility is that they reflect the action of membrane-scavenging ROPs and GRAs acting at or near the site of invasion.

The ultimate destination of secreted GRAs varies; some localize to the PV lumen (e.g., GRA1 [53,69]), while others decorate the PVM (e.g., GRA17/23 [58] and MAF1 [70]) and/or the IVN (e.g., GRA2 and GRA6 [71]) (see Fig. 1 for illustration of final destinations of GRAs). Those exposed to the host cytosol can interact with various host proteins and processes (recently reviewed in [72]). One such example is a GRA that mediates the recruitment of host mitochondria to the PVM in some strains and was therefore dubbed MAF1 for mitochondrial association factor 1 [70,73]. Additionally, some GRAs can also be found in what appear to be vesicular-like, membranous extensions of the PV in the host

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cytosol. These structures have been dubbed BOAS because of their "beads-on-a-string"-like appearance (e.g., GRA3, GRA7, and GRA14 [65,74,75]) (depicted in Fig. 1). The function of BOAS is unknown, although they frequently appear to run between multiple PVs in the same host cell, or between a PV and the host cell nucleus [74,75].

Perhaps the most interesting set of GRA proteins are the ones that are somehow translocated as soluble proteins across the PVM and into the host cell cytosol or nucleus, where they can affect host transcription. Several such have been identified in the past few years, including GRA16, GRA24, GRA18, TgIST, GRA28, and HCE1/TEEGR [8–14]. Our current understanding of how these proteins are translocated across the PVM in *Toxoplasma*-infected cells is heavily influenced by work done in the more extensively studied *Plasmodium falciparum* (*P. falciparum*) blood stage parasites, which will be briefly summarized here (and was recently reviewed in [76]).

Role of Proteolytic Cleavage in Translocation of Dense Granule Effectors Beyond the PV

In *P. falciparum*, dense granule proteins exported into the red blood cell can be classified into two groups. The first includes proteins that contain a host-targeting/*Plasmodium* export element (HT/PEXEL) [77,78]. This motif, generally comprised of the amino acid sequence "RxLxE/Q/D," is cleaved by an ER-resident aspartyl protease, plasmepsin V, which appears to license such proteins for export across the PVM [79,80]. The second group of exported proteins lack an HT/PEXEL, and are dubbed PEXEL-negative exported proteins, or PNEPs [81,82]. While not themselves processed by plasmepsin V, some PNEPs, such as PfEMP1, require several PEXEL-containing targets of plasmepsin V, as well as plasmepsin V activity, for proper trafficking through the erythrocyte to the red blood cell surface [83–85].

Toxoplasma also contains proteins with HT/PEXEL-like motifs, dubbed TEXELs for *Toxoplasma* export elements, which have the simpler amino acid consensus sequence of "RRL" [86*,87]. Of the proteins identified thus far that translocate beyond the *Toxoplasma* PVM, only GRA16, TgIST, and GRA18 contain TEXELs. The remaining are TEXEL negative exported proteins or TNEPs (reviewed in [88]). TEXEL-containing proteins are cleaved by the *Toxoplasma* homolog of plasmepsin V, ASP5, a Golgi-resident protease [86*, 89*,90*]. In contrast to *Plasmodium*, however, *Toxoplasma* has several TEXEL-containing GRAs that are processed by ASP5, yet do not appear to be exported beyond the PVM [36,87,89*]. Therefore, unlike *Plasmodium*, the presence of a TEXEL does not necessarily license a protein for export beyond the PVM in *Toxoplasma*. An active ASP5, however, appears to be required for the export of all exported *Toxoplasma* GRAs studied so far, whether or not they contain a cleavable TEXEL motif [8,11,86*,89*,90*]. It is possible that this requirement for ASP5 relates to its role in processing a component of the translocation machinery through which all of the exported proteins pass, rather than the effectors themselves. Alternatively, export of TNEPs might rely on ASP5-dependent cleavage of other TEXEL-containing proteins that act as chaperones or translocation partners.

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Machinery for Effector Translocation from the PV to the Host Cell

In *Plasmodium*, a complex known as the *Plasmodium* translocon of exported proteins, or PTEX, is responsible for the translocation of dense granule proteins into the host cell [91–94]. Recent structural data show that the core complex includes the proteins, HSP101, PTEX150, and EXP2, which associate to form a PV membrane-spanning translocon [95**]. HSP101 is a ClpB-like AAA+ ATPase which is thought to facilitate the translocation process, and EXP2 is the protein-conducting channel of the complex [91–93,95**,96].

Protein unfolding is a necessary step for export of effectors into the host cell in *Plasmodium* [97], and the requirement for this appears to hold true in *Toxoplasma* as well. This is based on the observation that fusing GRA16 to the highly folded and structured murine dihydrofolate reductase (DHFR) domain prevents translocation of the fusion, in addition to a non-engineered exported effector, GRA24, consistent with the GRA16-DHFR fusion "blocking" a protein translocon [90*,98**]. Also in support of this hypothesis, structural analysis of known exported GRA proteins predicts them to be highly disordered and thus akin to "unfolded" proteins [99,100]. Thus, unlike for *Plasmodium*, *Toxoplasma* GRAs may not require active unfolding due to their inherent lack of structure (further reviewed in [6]).

While homologs to some of the PTEX components exist in *Toxoplasma*, such as GRA17 and GRA23 (homologs of EXP2) and TgClpB1 and TgClpB2 (homologs of HSP101), they do not appear to play a role in protein translocation beyond the *Toxoplasma* PV [58,101]. Instead, GRA17 and GRA23 appear to form pores within the PV to facilitate small molecule/nutrient acquisition [58], as also recently described for EXP2 in *Plasmodium* [94]. It is tempting to speculate that nutrient acquisition may be the original, ancestral function of EXP2 in vacuolar-dwelling apicomplexans, and blood stage malaria parasites may have further adapted EXP2 to function in protein translocation [94]. Nevertheless, the data would currently indicate that the protein-translocation function of the PTEX machinery is not conserved across the phylum.

So, if the PTEX machinery is not serving this function in *Toxoplasma*, what is? The answer came from genetic screens for *Toxoplasma* mutants incapable of activating certain host processes that are dependent on translocated GRA effectors. This led to the identification of a set of parasite loci necessary for host c-Myc regulation and therefore dubbed "*MYR*" loci based on this phenotype. Four such loci have so far been identified, *MYR1, MYR2, MYR3* [98**,102*] and recently, *ROP17*, which encodes a rhoptry protein kinase [25,103]. In the case of MYR1, it was shown that a majority of the host transcriptomic response to *Toxoplasma* infection in HFFs is lost in *myr1* mutants [104*], consistent with the observation that the translocation of all GRAs known to cross the PVM as soluble proteins and so far tested depends on MYR1 [8,13,14,98**,102*]. Interestingly, the processes mediated by the PVM-associated effectors MAF1 (mitochondrial association) and GRA15 (NF-KB activation) do not appear to be dependent on MYR1 [102*], suggesting that the proper localization of these proteins to the PVM occurs through a different mechanism [102*], perhaps the presence of some intrinsic, membrane-associating domain (both have strongly predicted transmembrane domains).

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Little is known about the precise, mechanistic function of the MYR proteins and ROP17 in the translocation of effectors across the PVM. All four localize to the PVM and appear to be membrane-associated [25,98**,102*,103], with MYR1 and MYR3, at least, forming a stable complex [98**]. Interestingly, MYR1 is processed by ASP5 to yield stable N- and C-terminal fragments that are disulfide-bonded [98**]. This processing is not, however, necessary for protein export, as unprocessed, full length MYR1 harboring a mutated ASP5 cleavage site can still promote the translocation of the effector GRA24 to the host nucleus [104*]. This result, combined with the fact that MYR2 and MYR3 have no TEXEL site and are thus unlikely to be substrates for ASP5, suggests that if the requirement for an active ASP5 in moving GRAs across the PVM is due to ASP5's cleavage of a component of the translocation machinery, it is likely a protein other than MYR1/2/3. Additionally, given that ROP17 is an established protein kinase [25], and its function in translocation depends on being catalytically active [103], it is very probable that ROP17 functions to phosphorylate some component of the translocation machinery. Definitive word will come when all the components of the *Toxoplasma* effector translocation machinery have been identified and tested for ASP5-dependent processing and ROP17-dependent phosphorylation.

The four known MYR proteins have somewhat conserved orthologs (35–57% amino acid identity) in the closely related genus *Neospora caninum* [98**,102*,103]. *Neospora* parasites have a different definitive host (canines) but display asexual growth that is almost indistinguishable from that of *Toxoplasma* tachyzoites within the cells of its intermediate mammalian hosts. Assuming these orthologs function in *Neospora* as they do in *Toxoplasma*, a possibility not yet tested, their divergence suggests some co-evolution with their GRA cargo. Indeed, of the known *Toxoplasma* GRAs, *Neospora* has clear orthologs for only GRA16 and GRA18, consistent with a rapid evolutionary pressure presumably related to their different life cycles and preferred hosts. Consistent with this, there are no apparent MYR homologues within the genomes of the more distantly related *Sarcocystis* or *Plasmodium*, suggesting a function specific to only a subset of the tissue-dwelling coccidia.

Conclusion

In this review, we have briefly summarized the current state of knowledge for how effector proteins are introduced into cells infected with *Toxoplasma* tachyzoites. The available data for rhoptry protein translocation are limited to phenomenological observations and/or tantalizing EM images, leaving the transit of rhoptry proteins from the parasite into the host cytosol an intriguing and wide-open question. Knowledge of the machinery involved in translocation of dense granule proteins beyond the PVM is further advanced; however, much work remains to determine the complete machinery, the structures of the component parts, the mechanism by which cargo proteins are recognized for export and how the entire process is regulated. It is perhaps surprising that the components of the machinery that moves proteins across the PVM has apparently diverged among the *Apicomplexa* but until we understand all the nuanced details of their operation, and the cargo they translocate, the reasons for this will remain a mystery.

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Highlights

- *Toxoplasma* introduces ROP and GRA effectors into host cells to coopt host functions
- ROP effector secretion occurs during invasion via an unknown mechanism
- MYR1–3, ROP17, and ASP5 are required for transit of GRAs across *Toxoplasma*'s PVM
- *Plasmodium* employs different machinery (PTEX) to transport GRAs across the PVM

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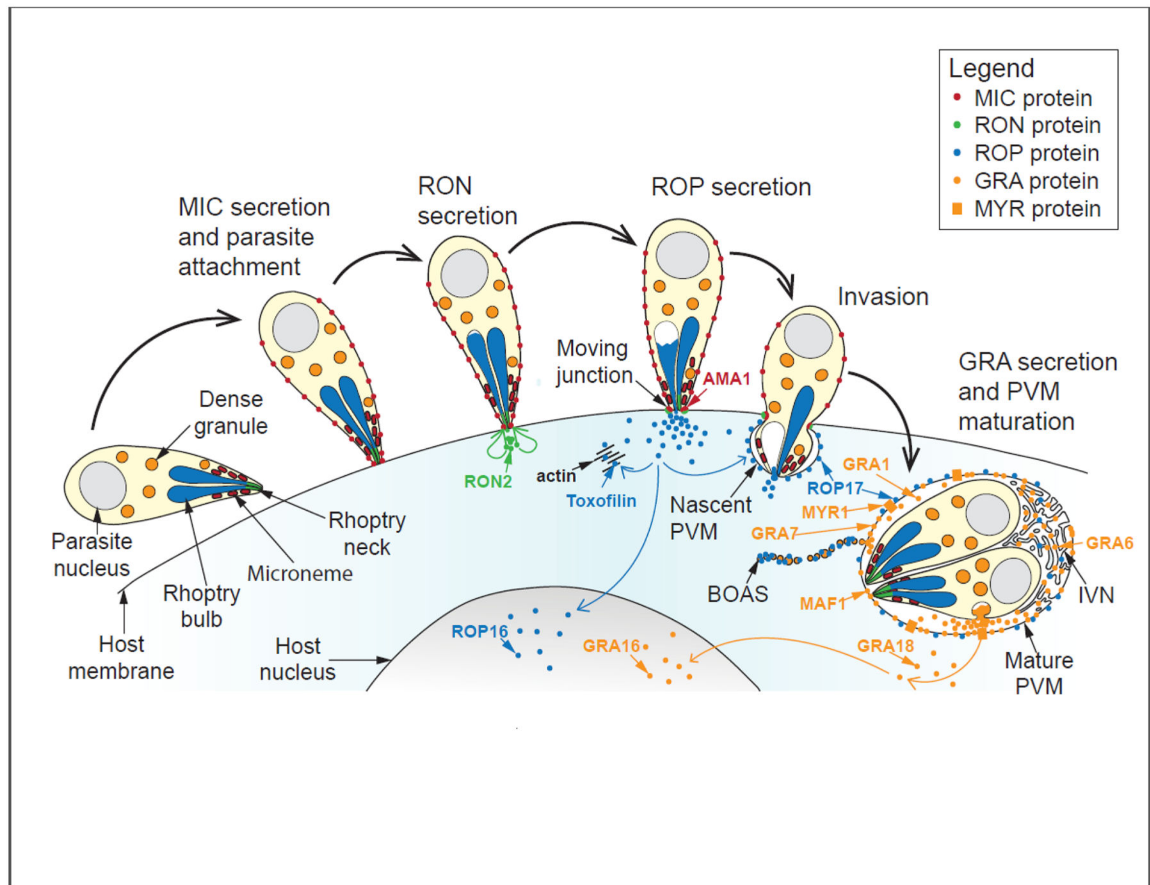

Figure 1:
An integrated model for effector protein secretion by *Toxoplasma gondii* tachyzoites into the host cell, illustrated in the context of *Toxoplasma* invasion. First, the micronemes secrete micronemal proteins (MICs) that are anchored in the parasite plasma membrane and mediate parasite attachment to the host cell (**MIC secretion and parasite attachment**). Upon strong parasite attachment to the host cell, the rhoptry organelle discharges in a manner simultaneous with parasite invasion, first introducing rhoptry neck proteins into the host cell (**RON secretion**) that go on to establish the moving junction, and subsequently rhoptry bulb proteins (**ROP secretion**) that either translocate to the host nucleus, associate with the nascent or fully formed parasitophorous vacuole membrane (PVM), or associate with cytosolic actin (i.e., toxofilin). Secretion of dense granule proteins (GRAs) into the host may also begin as early as during parasite **invasion**. Once the parasites have fully penetrated the host cell, PVM maturation occurs concomitantly with continued GRA secretion (**GRA secretion and PVM maturation**), and GRAs are either deposited into the PV space, PVM, or intravacuoloar network (IVN), or they are translocated across the PVM in a fashion dependent on several MYR proteins (orange rectangles), and end their journey in either the host cytosol or the host nucleus. Note that ROPs and GRAs are also found in structures called beads on a string (BOAS), vesicular-like entities of unknown function that may originate by blebbing off of the PVM. Selected parasite effector proteins that are examples of the different classes of proteins mentioned above are shown; i.e., the rhoptry proteins,

RON2 (host plasma membrane), ROP16 (host nucleus), toxofilin (host cytosol), and ROP17 (PVM); and the dense granule proteins, GRA1 (PV space), GRA6 (IVN), GRA18 (host cytosol), GRA16 (host nucleus), GRA7 (PVM), and MAF1 and MYR1 (PVM).

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