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How to cite

BISIO SABARIS, Hugo, SOLDATI-FAVRE, Dominique. Signaling cascades governing entry into and exit from host cells by toxoplasma gondii. In: Annual Review of Microbiology, 2019, vol. 73, p. 579–599. doi: 10.1146/annurev-micro-020518-120235

This publication URL: https://archive-ouverte.unige.ch/unige:123112
Publication DOI: 10.1146/annurev-micro-020518-120235

ANNUAL REVIEWS

Annual Review of Microbiology

Signaling Cascades Governing Entry into and Exit from Host Cells by *Toxoplasma gondii*

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Annu. Rev. Microbiol. 2019. 73:579–99

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-020518-120235

Keywords

Apicomplexa, *Toxoplasma gondii*, motility, egress, microneme, signaling, calcium, cyclic nucleotides, phosphatidic acid, kinase, diacylglycerol kinase, guanylate cyclase

Abstract

The Apicomplexa phylum includes a large group of obligate intracellular protozoan parasites responsible for important diseases in humans and animals. *Toxoplasma gondii* is a widespread parasite with considerable versatility, and it is capable of infecting virtually any warm-blooded animal, including humans. This outstanding success can be attributed at least in part to an efficient and continuous sensing of the environment, with a ready-to-adapt strategy. This review updates the current understanding of the signals governing the lytic cycle of *T. gondii*, with particular focus on egress from infected cells, a key step for balancing survival, multiplication, and spreading in the host. We cover the recent advances in the conceptual framework of regulation of microneme exocytosis that ensures egress, motility, and invasion. Particular emphasis is given to the trigger molecules and signaling cascades regulating exit from host cells.

Contents

INTRODUCTION

Toxoplasma gondii belongs to the phylum Apicomplexa, which includes a large group of obligate intracellular protozoan parasites responsible for important diseases in humans and animals. In the spotlight, *Plasmodium* spp., the etiological agent of malaria, are responsible for half a million deaths per year. *Cryptosporidium*, another member of the phylum, is the second-most important severe-diarrhea-causing agent in children after retroviruses (74). The focus of this review, *T. gondii*, is a medically important opportunistic pathogen responsible for toxoplasmosis encephalitis in immune-compromised patients upon reactivation of chronic infection (40). Moreover, primary infection during pregnancy can lead to abortion or congenital disease including ocular toxoplasmosis, the most common cause of eye inflammation worldwide (138).

Together with the ciliates and dinoflagellates, the apicomplexans belong to the superphylum of Alveolata, which shares the peripheral alveolar membrane system below the plasma membrane (PM), termed the inner membrane complex (IMC), as well as an elaborate apical complex including specialized secretory organelles known as micronemes and rhoptries (46). As an obligate intracellular parasite, *T. gondii* actively invades host cells and resides within a vacuole surrounded by a unique parasitophorous vacuole membrane (PVM), where it replicates safely. This parasite shares a unique mode of substrate-dependent motility crucial for egress and invasion. The exocytosis of the micronemes coupled to the activation of the actomyosin system elicits effective parasite egress from infected cells, whereas reinvasion involves the coordinated discharge of both micronemes and rhoptries (47).

Recent studies on the signaling pathways and the implicated second messengers underpinning egress have facilitated a broader understanding of the interplay between various signals that govern this complex cascade of events. The cyclic GMP (cGMP) and intracellular calcium (Ca^{2+}) acting on protein kinase G (PKG) and calcium-dependent protein kinases (CDPKs), respectively, are key players controlling microneme secretion. Additionally phosphatidic acid (PA) is a lipid mediator that contributes in a coordinating manner to microneme secretion and gliding motility (23).

The aim of this review is to integrate current knowledge about the signaling pathways leading to microneme secretion and egress from infected cells, with particular focus on *T. gondii*.

ROLE OF MICRONEME SECRETION IN EGRESS

Microneme exocytosis crucially participates in three key steps of the parasite lytic cycle (13): egress, gliding motility, and invasion (26). Egress from infected cells depends on multiple highly synchronized processes including conoid (an apical organelle composed of tubulin fibers) protrusion, microneme secretion, actomyosin activation, and separation of parasites at their basal pole (**Figure 1**).

The micronemes are apical specialized organelles that exocytose their content in a tightly regulated fashion, releasing perforins, lipases, proteases, and adhesins that are critical for egress, gliding, and invasion (71, 113). Most notably, perforin-like protein 1 (TgPLP1) ensures the lysis of the PVM and host PM (69). Micronemes also ensure gliding motility via the delivery of adhesins at the PM of parasites that participate in a coordinated fashion with the actomyosin system to generate forward motion. These adhesins are typically termed MICs and exist as complexes that are discharged at the apical tip of the parasite. Some of these complexes bind to host cell receptors or the extracellular matrix and are translocated to the basal pole (64, 112). Most of these MICs undergo pre- and postexocytosis proteolytic cleavage, which includes post-Golgi aspartyl protease 3–mediated maturation (37), cell surface processing by subtilisin protease 1 (78), and shedding after secretion performed by rhomboid proteases (38).

The actomyosin system of *T. gondii* is composed of an essential actin (TgACT1) and 11 myosins that are implicated in various processes including parasite motility (MyoA, MyoC, and MyoH), organelle trafficking and positioning (MyoF), basal pole constriction, and intravacuolar cell-cell communication (MyoI). The substrate-dependent motility of apicomplexan parasites is powered by a phylum-specific molecular machine called the glideosome that lies underneath the PM in the pellicular space and is anchored in the IMC. According to the capping model, gliding relies on the rearward translocation of apically discharged MICs powered by the glideosome in order to propel the parasite forward (15, 118). The components and assembly of the glideosome have been recently reviewed, in Reference 47. According to the model, filamentous actin (F-actin) and the cytoplasmic tails of the adhesins are connected via a recently discovered protein called the glideosome-associated connector (GAC) (66). Concordantly, GAC is indispensable for gliding motility, invasion, and egress and binds both F-actin and the cytoplasmic tail of MIC2.

Successful egress also depends on the fission of the recently identified network that connects intravascular parasites at their basal pole. This cell-cell communication accounts for synchronous division of *T. gondii* during acute infection (48, 107). This process resembles *Plasmodium* blood stage division strategy via schizogony, suggesting its evolutionary conservation. During egress the basal F-actin disassembles and parasites very quickly disconnect and invade new cells as individual entities (48, 68, 107, 127). This coordinated timing implies a concerted signaling event that concomitantly triggers microneme exocytosis and ensures parasite separation. Treatment with cytochalasin D, an inhibitor of actin polymerization that primarily acts on parasite actin (36), or genetic deletion of *MyoI* resulted in asynchronous division and loss of connectivity between intravacuolar parasites (48, 107).

Nondividing extracellular *T. gondii* tachyzoites constitute a distinct biological state compared to nongliding, replicating intracellular parasites. Freshly egressed parasites harbor an extruded conoid, secrete micronemes, and are able to glide for several minutes and survive for a few hours (13). Once a suitable host cell is encountered, extracellular parasites attach and apically reorient. Host cell attachment results in a new burst of microneme secretion, which is immediately followed by the discharge of the rhoptries positioned at the apical tip of the parasites (26, 41). The rhoptries contain membranous material and proteins (6, 16) that are directly injected into the host cytoplasm and allow invasion, PVM formation, and subversion of host cellular functions (7, 55, 71) (**Figure 1**).

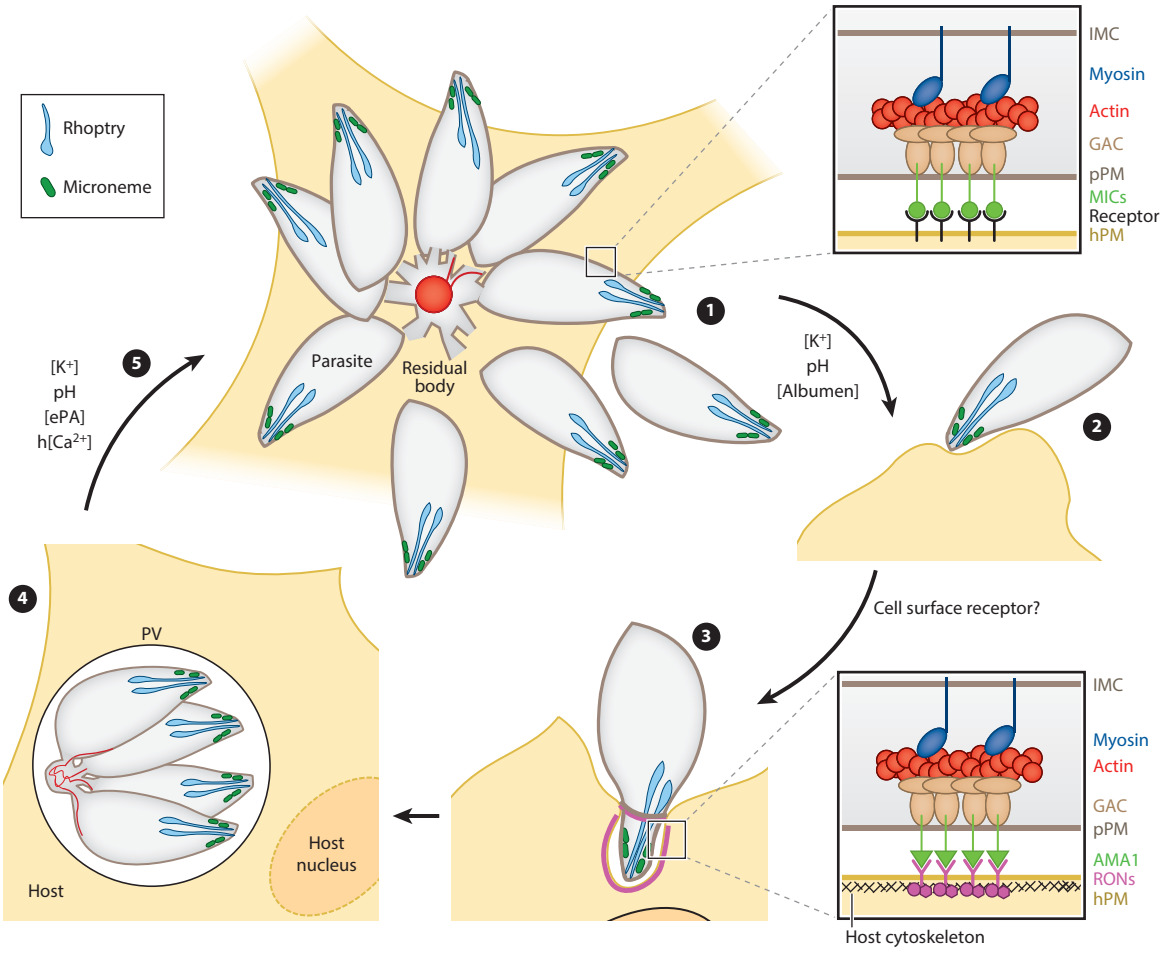


Figure 1

The lytic cycle of attachment, invasion, replication, and egress. Extracellular tachyzoites are capable of movement using gliding motility. (❶) The exocytosis of micronemes (*dark green*) occurs at the apical tip of the parasite. (*Top inset*) As a result of this process, adhesins (MICs) are deposited at the parasite plasma membrane (pPM), where they interact with a host receptor (*black*) at the host plasma membrane (hPM), for example. Rearward translocation of the adhesin by actin (*red*) and myosin machinery (*dark blue*) at the interspace of the inner membrane complex (IMC) and the pPM produces a forward movement of the parasite. The glideosome-associated connector (GAC) connects the actin filaments with the cytosolic tail of the adhesins. Diverse molecules characteristic of extracellular environments (low K^+ levels, pH lower than 7.2, and the presence of serum albumin) trigger gliding motility by providing signals for microneme exocytosis. (❷) Once a suitable cell is detected, parasites attach, reorient by the action of rhomboid proteases, and (❸) eventually discharge micronemes and rhoptries (*light blue*) to initiate invasion. (*Bottom inset*) The nature of the rhoptry discharge signal is still unknown but likely encompasses the recognition of a host cell receptor by a micronemal protein. A similar mechanism is utilized for gliding and the invasion process. One specific adhesin is used for invasion (AMA1), and the host receptor is introduced by the parasite in the hPM (RONs) during the rhoptry discharge process. (❹) Once the invasion process is finished, *T. gondii* resides in the parasitophorous vacuole (PV) composed of host- and rhoptry-derived lipid and proteins, switching into the replicative mode. Parasite division is performed without completion of cytokinesis, resulting in cytosolic-connected daughter cells. This process is dependent on filamentous actin and myosins. (❺) Egress is triggered by a decrease of host K^+ levels or pH, an increase in host calcium levels, or accumulation of vacuolar/extracellular phosphatidic acid (ePA) and requires the coordination of microneme secretion, actomyosin motor activation, and parasite fission. During this process, microneme content destabilizes the hPM and the PV and furnishes the pPM with MICs, which will allow gliding motility. In addition, basal actin filaments collapse during the process of parasite fission and render independent parasites capable of starting a new lytic cycle. Adapted from Reference 10.

SIGNALING CASCADE TRIGGERING MICRONEME SECRETION

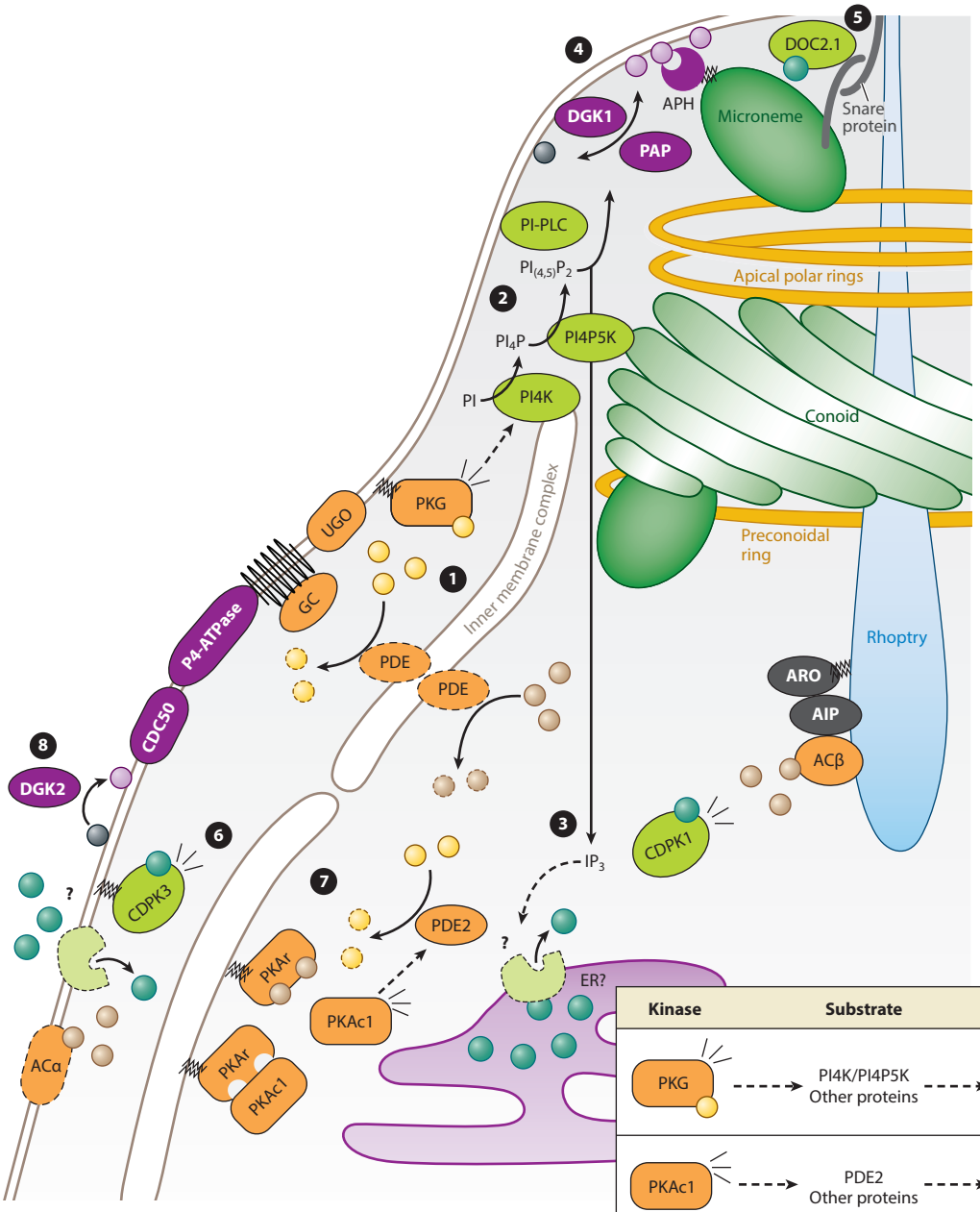
The signaling cascade leading to microneme secretion initiates with the production of cGMP by a large and atypical guanylate cyclase (GC), which is fused to a P4-ATPase domain (10, 21, 140). In turn, induction of the cGMP-dependent protein kinase G (PKG) (19) induces Ca^{2+} mobilization, potentially by phosphorylating and activating key enzymes involved in the formation of phosphatidylinositol (3,4,5) triphosphate [$PI_{(4,5)}P_2$]. $PI_{(4,5)}P_2$ is converted by the PM-associated phosphoinositide phospholipase C (PI-PLC) (18) into inositol triphosphate (IP_3) and diacylglycerol (DAG) (122). In eukaryotic model organisms, IP_3 is known to bind to an IP_3 receptor (IP_3R) that serves as a Ca^{2+} channel, and it releases Ca^{2+} from the endoplasmic reticulum (ER) (53). In *T. gondii*, IP_3 presumably stimulates Ca^{2+} release from an unknown Ca^{2+} store (85). The rise in intracellular Ca^{2+} activates the calcium-dependent protein kinases 1 and 3 (CDPK1 and CDPK3), as well as other members of this family (86, 87).

DAG can be phosphorylated to produce PA through a reversible reaction involving diacylglycerol kinase 1 (DGK1) and phosphatidic acid phosphatases (PAPs/lipins). The accumulation of PA in the inner leaflet of the parasite PM is sensed by an acylated pleckstrin homology domain–containing protein (APH) at the surface of the micronemes (22). Ultimately, a machinery promoting membrane fusion, most likely dependent on SNARE (soluble NSF-attachment protein receptor) proteins, leads to microneme exocytosis (22, 23, 44) (**Figure 2**).

NATURAL EGRESS RESPONDING TO INTRINSIC SIGNALS

Egress is a tightly controlled process, and several extrinsic signals (derived from the host environment) have been shown to act as triggers or mediators of microneme secretion. *T. gondii* atypically divides by endodyogeny and thus remains invasive at almost any point during the intracellular growth phase. This adaptation allows a quick response to insults of the infected host cells by the host immune system or to sudden limitation in nutrients. On the other hand, nonstimulated egress has been termed natural egress (24) and controls the maximum number of division cycles that the parasite undergoes prior to exit. Until recently it was not clear whether natural egress was dictated by an intrinsic signal (derived from the parasite), such as abscisic acid (ABA) (100), or whether the parasites would eventually exit the host cell by mechanical breakage of the PVM and host PM. ABA was proposed to regulate natural egress and conversion to latent stages in *T. gondii* via the production of cADP ribose (100). Although *T. gondii* possesses ADP ribosyl cyclase and hydrolase (31) and a putative ABA-binding G protein–coupled receptor (GPCR) (GPR89, TGGT1_286490), no pathway for the biosynthesis of ABA has thus far been found in apicomplexans. Consequently, the absence of an assigned molecular mechanism behind natural egress has precluded addressing the relevance of this process in vivo.

Recently an alternative and plausible candidate for an intrinsic signal was unveiled. *T. gondii*, as well as the other members of the cyst-forming subgroup of coccidians, encodes a second DGK (DGK2). This protein exhibits a signal peptide, is stored in the dense granules, and is secreted into the PV space, where it accumulates. DGK2 is an active enzyme that produces PA, likely in the outer leaflet of the parasite PM but possibly also on the tubular network and PVM. Of considerable relevance, parasites with deleted *DGK2* harbor a selective defect in natural egress despite complete responsiveness to extrinsic inducers (10). Two other dense granule proteins, GRA41 and GRA22, have also been shown to contribute to natural egress, particularly by affecting Ca^{2+} homeostasis (77, 103), yet the relationship with DGK2 remains to be assessed. Intriguingly, while fast-growing tachyzoites egress after five to six rounds of division, bradyzoites remain trapped in a vacuole that evolves to form a cyst that will grow and persist for much longer periods of time. This difference suggests that stage conversion from tachyzoites to bradyzoites



Kinase	Substrate	Physiological effect
PKG	PI4K/PI4P5K Other proteins	Microneme secretion Parasite fission Actomyosin motor assembly
PKAc1	PDE2 Other proteins	Microneme secretion Parasite replication Stage conversion
CDPK1	Unknown proteins	Microneme secretion Actomyosin motor assembly
CDPK3	Tyrosine transporter Other proteins	Calcium-induced egress Ion homeostasis Metabolism

(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Signaling pathways leading to microneme exocytosis. (❶) Activation of guanylate cyclase (GC) in the presence of two interacting partners, unique GC organizer (UGO) and CDC50.1, at the parasite plasma membrane produces the signaling molecule cyclic guanosine monophosphate (cGMP). The concentration of cGMP depends on production by GC and degradation to GMP by phosphodiesterases (PDEs), particularly the fitness-conferring PDE2. (❷) cGMP activates protein kinase G (PKG), which in turn increases the production of phosphatidylinositol-4,5-bisphosphate [$PI_{(4,5)}P_2$] in *Plasmodium* spp. and potentially in other apicomplexans. $PI_{(4,5)}P_2$ is produced by subsequent reactions of PI4K and PI4P5K. The hydrolysis of $PI_{(4,5)}P_2$ by phosphoinositide phospholipase C (PI-PLC) produces diacylglycerol (DAG) and inositol triphosphate (IP_3). (❸) IP_3 is thought to stimulate release of calcium from the endoplasmic reticulum (ER) by an unknown calcium channel. (❹) On the other hand, DAG is phosphorylated to produce phosphatidic acid (PA) by a reversible reaction catalyzed by the diacylglycerol kinase 1 (DGK1) and phosphatidic acid phosphatases (PAPs). (❺) Plasma membrane PA binds to the acylated pleckstrin homology domain–containing protein (APH) and allows the double C2 domain protein (DOC2.1)-mediated fusion of the micronemes and parasite plasma membrane. This process is likely dependent on a SNARE complex. (❻) Calcium is sensed by a plethora of proteins, including the calcium-dependent protein kinases (CDPKs), particularly CDPK1 and CDPK3. CDPK1 is essential for microneme exocytosis, while CDPK3 likely enhances calcium-dependent signals. (❼) cGMP level is controlled by PDE2 (and possibly other PDEs), which is regulated by the activity of the protein kinase A catalytic 1 domain (PKAc1). PKAc1 is itself tightly controlled by PKA regulatory domain (PKAr), which binds adenylate cyclase (AC)-generated cyclic adenosine monophosphate (cAMP). ACβ is likely the main contributor of cAMP production during tachyzoite stages and localizes to the rhoptry by its binding to armadillo repeats–only protein (ARO) and ARO-interacting protein (AIP). [cAMP] depends also on its degradation to AMP performed by PDEs. (❽) Danger signals like a drop in [K^+], acidification of the parasitophorous vacuole, or a rise in host cytoplasmic [Ca^{2+}] also lead to egress. In the absence of extrinsic signals, DGK2 produces vacuolar PA and restricts intracellular growth timing by triggering egress. Adapted from Reference 68.

also implies a reprogramming of a molecular clock governing egress during the chronic phase of infection. Other reported players associated with egress include the nucleotide-triphosphate-degrading enzymes (NTPases) secreted into the PV. These proteins can be activated by addition of dithiothreitol and are postulated to deplete host cell ATP, leading to a decrease in activity of the Na^+/K^+-ATPase pump, a drop in K^+ levels, and concomitantly, egress (123).

ENVIRONMENTAL SENSING OF EXTRINSIC SIGNALS

T. gondii is ready to escape from an unhealthy host cell or dangerous environment at any point during intracellular replication, and the burning question is what is the parasite able to sense and how? The PVM is permeable to small molecules (119), allowing the diffusion of host metabolites and ions. Leakiness of the host PM leads to an outward flow of K^+ ions; the exposed parasite responds by promoting microneme exocytosis (49, 96). Low K^+ could activate protein kinase A catalytic subunit 1 (PKAc1) (96) or PI-PLC by a GPCR; these are two common targets found in model organisms (56). A GPCR activated by K^+ has recently been identified in *Plasmodium falciparum* (94) that corresponds to TGGT1_262610 in *T. gondii*; however, molecular evidence of its link to cAMP levels or PI-PLC activity still remains to be established.

In extracellular parasites, serum albumin acts as a natural agonist for microneme secretion in a PKG-dependent manner (20). Importantly, sudden exposure to an acidic environment has also been shown to promote parasite gliding (55) and to stimulate microneme exocytosis (114). Intracellular parasites that have undergone approximately five to six rounds of replication experience a rapid drop in pH that appears to be concomitant to egress. Interestingly exposure to an acidic environment can overcome a high-K^+-induced block in microneme secretion (114), in agreement with K^+ and pH being sensed by different receptors. Of relevance, perforin-dependent T cell–mediated cytotoxicity triggers *T. gondii* egress in a caspase-independent manner (108), potentially directly mediating an imbalance in host ion composition.

A rise in intracellular Ca^{2+} originates not only from parasite internal stores but also from the extracellular environment (14). Extracellular Ca^{2+} was shown to enhance egress, invasion, and gliding (105), and hence a still unidentified channel, blocked by nifedipine (105), is responsible

for uptake of extracellular Ca^{2+}. Interestingly, putative voltage-dependent gated Ca^{2+} channels have been identified in *T. gondii* (111) but not functionally characterized to date. Changes in the environmental level of Ca^{2+} detected by the parasite before egress could act as a signal to trigger it (12), and signals known to modulate host cytosolic Ca^{2+} have been shown to impact parasite egress, such as the cell death receptor Fas-Fas ligand that depends on caspase-mediated release of intracellular Ca^{2+} to trigger egress (108). Nitric oxide (NO) was also reported to induce egress in a BAPTA-AM-dependent manner (139). Importantly BAPTA-AM is de-esterified by the host after entry, rendering it unable to cross the PM of the parasite. NO induces heme-dependent soluble GCs in mammalian cells, leading to a rise in the host cytosol Ca^{2+} (35). A host GPCR signaling cascade leading to Ca^{2+} release and calpain-mediated cortical cytoskeleton destabilization has also been involved in efficient parasite egress (93).

Although direct correlation with changes in host Ca^{2+} is missing, attachment of *Toxoplasma*-infected monocytes to endothelial cells is sufficient to trigger egress in a coordinated manner (2), potentially providing a mechanism to minimize plasma exposure before infection of solid organs, including the brain (73). Progesterone and estradiol have also been shown to induce egress with no effect on microneme secretion of extracellular parasites (143), indicating that both hormones act on the host signaling pathways. In contrast, histamine, which is known to raise Ca^{2+} in human foreskin fibroblasts, failed to trigger parasite egress (14), thus either disproving a role of host Ca^{2+} as an egress signal or indicating that the threshold of Ca^{2+} levels needed to trigger egress is not reached by histamine stimulation.

INTEGRATION OF EXTRINSIC AND INTRINSIC SIGNALS

T. gondii possesses a unique GC, while two paralogs are found in *P. falciparum* to produce cGMP. These proteins are predicted to have unusual bifunctional activity, conserved between apicomplexans and ciliates like *Paramecium* and *Tetrahymena* (80), being composed of a fusion between a predicted P-type ATPase-like domain and two GC catalytic domains. The N-terminal P-type ATPase seems indispensable for proper localization of GC to the PM (21, 50), and, though the function of this domain remains elusive, recent genetic data support an essential role of its activity in *T. gondii* (21).

In intracellular parasites, the PA produced by DGK2 might act directly on the flippase-like P4-ATPase domain or indirectly via an unknown receptor, to activate GC. Interestingly, in the gametocyte stages of the rodent malaria parasite *Plasmodium berghei*, xanthurenic acid (XA) has been identified as a potent inducer of exflagellation (8), and the production cGMP could be monitored by crude membrane extracts of gametocytes (98). Although these data suggested that XA could activate GCβ , one of the two GCs present in malaria parasites, XA failed to induce activity of the recombinantly expressed catalytic domains of GCβ (98). Consequently, either the binding for XA is outside of the catalytic domains or modulation of GCβ activity is indirect and mediated by a regulatory protein. Mammalian metabotropic glutamate receptors bind to XA and modulate cAMP levels in the cerebral cortex (45), and similar processes might regulate *Plasmodium* response to XA via GPCR to regulate GC.

Reminiscent of its flippase-like nature, the P4-ATPase domains of both *Plasmodium* and *Toxoplasma* GCs bind to cell division control 50 protein (CDC50), which acts as a chaperone controlling GC stability and localization (10, 50). Of relevance, the conditional knockdown of CDC50.1 in *T. gondii* specifically affects egress from infected cells, indicating that the trafficking of GC and the P-type ATPase activity are essential to sense PV-derived molecules to trigger egress (10). In CDC50.1-depleted parasites, BIPPO [5-benzyl-3-isopropyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one], a phosphodiesterase (PDE) inhibitor that raises cGMP levels by

inhibition of its consumption (22, 62), still induces microneme secretion in extracellular parasites, but it fails to trigger egress. The fact that BIPPO triggers microneme secretion from CDC50.1-depleted extracellular parasites implies they may have higher cGMP levels, relative to intracellular parasites where BIPPO fails to activate egress in the absence of CDC50.1. Since CDC50.1 directly affects the function of the unique enzyme responsible for cGMP production in *T. gondii*, these data strongly suggest that the tonic synthesis of cGMP is higher in extracellular parasites, possibly to ensure a higher rate of microneme exocytosis (10). The carboxyl terminal domain of the GC presents striking structural similarities to that of G protein–dependent adenylate cyclases (ACs) (28). However, despite the absence of biochemical evidence to support GC activity, depletion of the gene in *T. gondii* completely abolishes cGMP production (10) and can be rescued by a cell-permeable cGMP analogue (21). Moreover, heterologously expressed *P. falciparum* GCβ catalytic domains or the whole *Paramecium* enzyme possesses cGMP cycling activity (28, 80).

Remarkably the GC forms a signaling platform at the parasite PM, interacting not only with CDC50.1 but also with a large polytopic protein termed unique GC organizer (UGO) that potentially participates in sensing multiple signals (10). UGO is composed of a series of transmembrane-spanning domains at both the N-terminal and C-terminal sides that are strikingly conserved in the protein across the phylum. In contrast the large, central, and predicted ectodomain is highly divergent between and even within the apicomplexan species. Conditional depletion of UGO causes a mislocalization of the GC and a complete ablation of GC activity, hampering motility, invasion, and egress. The role of the ectodomain remains to be addressed but is likely to be relevant for sensing and integrating signals in extracellular parasites.

CROSS TALK BETWEEN CYCLIC NUCLEOTIDES

The cyclic nucleotides cAMP and cGMP are important second messengers exhibiting pleiotropic roles in eukaryotic cells. cAMP and cGMP are produced from ATP and GTP, by ACs and GCs, respectively. Conversely, hydrolysis via cyclic nucleotide PDEs inactivates these second messengers, establishing a steady state to regulate the threshold levels for activation/repression of downstream components of the signaling cascade (**Supplemental Table 1**).

Supplemental Material >

Distinctive roles have been attributed to cGMP and cAMP in *T. gondii*. The parasite possesses single genes coding for GC and PKG exclusively dedicated to motility, invasion, and egress (10, 19, 21). Remarkably, cGMP signaling via PKG is associated not only with microneme exocytosis but also with separation of daughter cells prior to egress and with activation of the actomyosin system, assessed by the measurement of the apicobasal flux of F-actin (10, 21, 127). In contrast cAMP can potentially be produced by four putative ACs to modulate the activity of three distinct PKA catalytic subunits. *T. gondii* ACα$_1$, ACα$_2$, and ACα$_3$ harbor transmembrane-spanning domains reminiscent of ion channel domains (21). Individual knockouts of each of the ACαs are dispensable, suggesting some redundancy in their function (21, 68). Interestingly, *Plasmodium* encodes only one ACα, which plays a role in microneme exocytosis in *P. berghei* sporozoites, but it does not have a detectable role in the blood stages or subsequent development in the mosquito (104). *T. gondii* ACβ is an ortholog of the bicarbonate-sensitive soluble ACs and localizes to the surface of the rhoptries in complex with the acylated armadillo repeats only protein (ARO) and the interacting partner (AIP) (97). Deletion of ACβ has been shown to induce a strong phenotype (21) as well as to weakly phenocopy the restless phenotype observed upon conditional deletion of PKAc1 (68, 129) (see below). This restless phenotype refers to the parasite's inability to turn off microneme secretion and motility upon infection of a new cell (68, 129). *Plasmodium* also codes for an essential blood stage bicarbonate-sensitive ACβ, which might act as a pH sensor (3, 117). So far, it is not clear whether ACs respond directly to external or internal stimuli, but the essential

role of the PKAc (see below) suggests that cAMP is implicated in multiple processes across the lytic cycle and life stages of *T. gondii* (68, 129) (**Figure 2**).

Cyclic nucleotides are degraded by PDEs, for which there are 18 putative genes in *T. gondii* (62). Two of these PDEs are predicted to confer fitness in *T. gondii* tachyzoites (121) and have been localized but not functionally characterized (68). Importantly, microneme exocytosis and egress (22, 87) can be induced in *T. gondii* by addition of the human PDE5 inhibitor zaprinast (22, 87) or BIPPO (22, 62). Despite their extensive use in the field, the specificity of these chemicals in apicomplexan parasites is not fully assessed. Experiments in *Plasmodium* spp. cell extracts suggest that zaprinast induces a specific rise in cGMP while BIPPO inhibits both cGMP and cAMP degradation, mostly acting on PDEδ, which possesses dual cAMP/cGMP hydrolysis activity (62, 134). *Plasmodium* possesses only 4 PDEs, PDEα–δ, and all of them are predicted to include transmembrane-spanning domains (3). Interestingly, these proteins present stage-specific expression patterns, with PDEα and PDEβ predominantly expressed in late trophozoite and schizont blood stages, PDEγ in sporozoites, and PDEδ in gametocytes (3).

PKG AND PKAs: MASTER REGULATORS OF PARASITE DECISIONS

In Apicomplexa, cGMP universally acts as mediator upstream of the signaling events leading to microneme secretion (22, 25, 88). In *T. gondii*, PKG has been shown to be essential for microneme secretion while dispensable for intracellular growth (10, 19, 21). The apicomplexan PKGs have four cGMP-binding domains; only three of them seem to be functional. Upon binding of cGMP, a conformational change of PKG allows exposure of the active site, enabling the phosphorylation of substrate proteins (76). Interestingly *Toxoplasma* and *Eimeria* produce two isoforms of PKG (19, 39), while only one is detected in *Plasmodium* (3). These two isoforms arise from alternative translation initiation and lead to a cytosolic shorter version of PKG and a dually acylated membrane-associated longer version of the protein (39, 130). Association of PKG with the parasite PM by acylation is both necessary and sufficient to control motility and invasion (19). In contrast, *Plasmodium* PKG seems to associate with the ER membrane but lacks acylation motifs (60).

The inhibition of PKG with either compound 1, trisubstituted pyrrole 4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H-pyrrol-3-yl]pyridine, or compound 2, 4-[7-[(dimethylamino) methyl]-2-(4-fluorophenyl) imidazo[1,2-a] pyridine-3-yl] pyrimidin-2-amine, has been shown to block microneme secretion and egress of *T. gondii* tachyzoites (87, 136). Interestingly, despite the fact that compound 1 and compound 2 have similar specificity in *Plasmodium* (92), compound 2 is less specific in *T. gondii* (68), potentially targeting CDPKs in addition to PKG. New-generation compounds have also been developed to target *Plasmodium* (4) or *T. gondii* (120) PKG and have proved to be attractive potential drugs against apicomplexan parasites.

PKAc is regulated by fluctuating levels of cAMP. The inactive enzyme is composed of a catalytic subunit (PKAc) bound to a regulatory subunit (PKAr) (126) that contains two cAMP-binding domains. Upon cAMP binding to the PKAr subunit, the complex disassembles and leads to the release of an active PKAc. The lack of the N-terminal dimerization domain in apicomplexan PKAr suggests that the holoenzyme is likely composed of one subunit of PKAc and one subunit of PKAr, contrasting with the mammalian relatives that assemble in a tetramer disposition (57, 75).

T. gondii encodes three PKAc subunits (PKAc1–3) and only one PKAr subunit. PKAc1 has recently been characterized in *T. gondii*, and its binding to PKAr has been confirmed (68, 125, 129). PKAc1 localizes to the parasite periphery upon binding of the double dually acylated PKAr by myristoylation and palmitoylation, and it is released soluble and active upon increase in cAMP concentration (68). Previous reports using the PKA-specific inhibitor H89 (82) have suggested

a role of PKA regulating parasite cell division (75). Concordantly, conditional overexpression of PKAc1 or conditional depletion of PKAr leads to a defect in intracellular growth (68). Remarkably, PKAc1 inactivation by gene transcript depletion or overexpression of a cAMP-insensitive mutant PKAr results in acidification-dependent or DGK2-dependent premature egress followed by successive restless invasion events leading to host cell destruction (10, 68, 129). The restless phenotype correlates with the inability of a PKAc1-depleted parasite to dump calcium signaling upon invasion and switch from the motile to the replicative stage. This phenotype does not resemble transmigration since rhoptry discharge and formation of a moving junction occur normally (129). Moreover, a decrease in cAMP production levels mimics the cell destruction phenotype (68). Compound 1 blocks premature egress induced by PKAc1 downregulation, illustrating important cross talk between PKA and PKG signaling pathways (68). Concordantly, phosphoproteomic studies have revealed changes in a putative cGMP-specific PDE upon PKAc1 inactivation, suggesting that PKAc1 could regulate cGMP levels in order to control natural egress (68).

PKAc2 also localizes to the parasite periphery, but it is preferentially expressed during the sexual stages in felids (125). Given the comparable localization with PKAc1, PKAc2 might have similar functions in the definitive host stages of the parasite.

In contrast, PKAc3 localizes to the cytosol, indicating that the spatial control of cAMP levels and PKAc isoforms is crucial to differentially regulating PKAc activity (125). Depletion of PKAc3 leads to an increased conversion of tachyzoites to bradyzoites, indicating that a threshold level of cAMP is central to maintaining tachyzoite commitment (125). Concordantly, treatment of tachyzoites with 3-isobutyl-1methylxanthine, a PDE inhibitor that causes prolonged elevation of cAMP levels (72), causes bradyzoite repression (42). Since transient elevation of cAMP produced by forskolin induces bradyzoite differentiation (72), the level and prolongation of the cAMP signal appears crucial for transition decisions.

Overall, PKA should be seen as a master regulator of parasite transitions, a potential hub to incorporate environmental signals sensed directly or indirectly by ACs to govern important parasite decisions.

CALCIUM HOMEOSTASIS, SIGNALING, AND SENSING

Recent advances in developing genetically encoded Ca^{2+} indicators have offered powerful tools to visualize Ca^{2+} throughout the life cycle of the parasite (14). This technology has been exploited to identify novel compounds modulating Ca^{2+} signaling via a cell-based phenotypic screening (120). PKG activation is associated with Ca^{2+} mobilization and PI-PLC-dependent hydrolysis of $PI_{(4,5)}P_2$ to generate DAG and IP_3. The current model suggests that IP_3 mediates Ca^{2+} release from intracellular stores and regulates diverse functions, among them conoid protrusion (11, 34) and microneme exocytosis (1, 88, 135). PI-PLC acts as a signaling node, involving upstream signaling events related to cyclic nucleotide regulation, environmental cues, DAG/PA interconversion, and IP_3-associated Ca^{2+} release (22, 43). PI-PLC has been localized to the parasite periphery and cytosol in intracellular parasites and accumulates apically in extracellular parasites (22, 43). Recent studies have also shown that PI-PLC colocalizes with centrin 2 at the apical end (61). Intriguingly, despite its clear implication in microneme secretion, conditional depletion of PI-PLC results in fast cell death, likely due to errant lipid signaling (22, 43), implicating a broader role for PI-PLC activity in PM integrity. The same is true for DGK1, for which depletion causes an imbalance between DAG and PA with rapid loss of PM integrity and parasite lysis (22).

Downstream of PI-PLC activity is IP_3 production and the ensuing release of Ca^{2+}, a process sensitive to IP_3R inhibitors (88). IP_3Rs are typically located in the ER and open in response to IP_3 to mobilize Ca^{2+} (5). Alcohols such as ethanol and acetaldehyde, as well as the ryanodine receptor

(RyR) agonists caffeine and ryanodine, efficiently stimulate microneme secretion by elevating intracellular Ca^{2+} in *T. gondii* (22, 25, 88). Despite the pharmacological evidence for their existence, canonical genes encoding either IP_3R or RyR are absent in apicomplexan parasites (101); however, given these data, it is likely that noncanonical IP_3R and RyR genes exist and are awaiting discovery. Following IP_3 production, Ca^{2+} is rapidly mobilized (89, 131) and subsequently quenches generating of waves of cytosolic Ca^{2+} (14), which correlate with multiple rounds of microneme exocytosis (22). These waves of Ca^{2+} potentially ensure an efficient usage of the adhesins pool during gliding motility and provide a mechanism to rapidly extinguish microneme secretion and motility upon successful entry into the host cell (122). Putative Ca^{2+} stores in *T. gondii* include the ER, IMC, Golgi apparatus, mitochondrion, acidocalcisome, and plant-like vacuole (85). While the store utilized during egress is unknown, the ER is the likely Ca^{2+} contributor to triggering microneme secretion (27, 95, 99). Treatment of *T. gondii* parasites with the sarcoplasmic/ER Ca^{2+}-ATPase inhibitor thapsigargin or artemisinin has been shown to prevent reentry of Ca^{2+} in the ER and stimulate microneme secretion, presumably by blocking influx of leaked Ca^{2+} from the ER (27, 95, 99). It should also be noted that the acidocalcisomes may contribute in part to Ca^{2+} release during microneme secretion (81, 90); however, the precise role that this source may play remains incompletely defined. Interestingly, as described above, extracellular Ca^{2+} can act as a stimulator, and the molecular entity is unknown; a channel for Ca^{2+} must exist to uptake extracellular Ca^{2+} and stimulate microneme exocytosis (14).

Apicomplexan parasites lack typical Ca^{2+} effector kinases (PKC/CaMK) and instead utilize phylum-specific CDPKs (reviewed in 9, 85), which contain EF-hand motifs that consist of a helix-loop-helix structural domain binding Ca^{2+} (**Supplemental Table 2**). There are 14 *CDPK* genes in *T. gondii*, and several of these have been implicated in microneme-secretion-dependent events such as motility, invasion, and egress. Specifically, CDPK1 has been shown to be required for Ca^{2+}-regulated microneme secretion, thereby controlling motility, invasion, and egress from host cells (86). Consistent with its role, CDPK1 is important for *T. gondii* dissemination in vivo and reactivation of chronic infections (115). Dynamin-related protein DrpB, which has been implicated in apical organelle biogenesis (17), is a direct substrate of CDPK1, but the importance of its phosphorylation is still unknown (84). Remarkably CDPK1 has recently been shown to control conoid protrusion and activation of the actomyosin system, leading to an apicobasal flux of F-actin independently of the contribution of CDPK1 in microneme secretion (127). Substrate candidates are members of the glideosome, which has been shown to be phosphorylated, but the relevance of these findings in vivo is still unclear (52, 65). Interestingly CDPK1 activity might be regulated by association with the protein deglycase DJ-1 in an oxidation- and Ca^{2+}-dependent manner. Lack of DJ-1 results in an upregulation of CDPK1 and prevents normal exocytosis of micronemes (30).

In contrast, CDPK3 has been shown to impact only Ca^{2+}-ionophore-induced egress, and deletion of the gene does not affect invasion, gliding, or microneme secretion in extracellular conditions (51, 87, 91). Interestingly, egress induced by permeabilization of the host cell using digitonin and low K^+ buffer was significantly lower in parasites lacking CDPK3 but could be increased in the presence of ionophore treatment (91). This led the authors to conclude that CDPK3 might be important to amplify the response to a Ca^{2+} stimulus, rather than being a binary switch from intracellular to extracellular parasites. CDPK3 has also been shown to be implicated in ion homeostasis (128) and metabolism (132) and might be implicated in controlling broader aspects of *T. gondii* cell biology, preparing the cell for intracellular or extracellular conditions. Like PKG, CDPK3 is dually acylated, and its association with the pellicle is essential for its function (51, 87, 91). Overexpression of CDPK1 partially rescued the block in egress experienced by *CDPK3* knockout

Supplemental Material >

parasites, implicating potential cross talk between the kinases (128). A recent study reported that dense granule secretion is negatively regulated by CDPK1 and CDPK3 (70). Logically, this negative regulation might reduce the secretion of proteins that are important during the intracellular phase of the lytic cycle. While the precise targets of the CDPKs and their specific contribution to microneme secretion are yet to be fully described, rapid advances are being made in the field, and identification of specific substrates will likely elucidate the molecular events underpinning Ca^{2+} sensing and signaling leading to microneme secretion.

Although CDPKs have been by far the most-studied Ca^{2+}-regulated proteins in Apicomplexa, *T. gondii* possesses 63 additional EF-hand domain–containing proteins annotated in the genome, many of which have unknown function. Calmodulin (CaM)-like proteins constitute another important branch of 20 proteins harboring EF-hand domains (101, 110). CaM-like proteins undergo conformational changes upon Ca^{2+} binding that affect their interaction with binding partners and function (76). A conserved CaM localized to the apical and basal ring of *T. gondii* tachyzoites acts as a regulator of calcineurin, impacting parasite attachment without affecting microneme secretion (106). CaM and CaM-like proteins can serve as myosin light chains (MLCs) and associate with myosin heavy chains (58). Several MLCs have been characterized to date, with MLC1 and MLC2 associated with MyoA and MyoD, respectively (59, 137). Additional CaM-like proteins are associated with the conoid and potentially bind to MyoH to empower gliding motility, invasion, and egress (54, 83).

Similar to CaM, centrin 1 (CEN1) has been found to be at the centriole, and two other centrins have been localized to the spindle pole and centrosome, where they likely participate in cell division (63). Interestingly, centrin 2 (CEN2) localizes to the preconoidal ring and basal complex and critically participates in microneme secretion by an unknown mechanism (G. Lentini, D.J. Dubois, B. Maco, D. Soldati-Favre, K. Frénal, manuscript under revision). Two more EF-hand-containing proteins have been localized to the PM and rhoptries, while their apparently dispensable function remains unknown (29). Parafusins are also known to play a role in Ca^{2+}-mediated exocytosis (109). *T. gondii* encodes two parafusins that are involved specifically in calcium-ionophore-induced microneme secretion, again by an unknown mechanism (116).

Ultimately, SNAREs and C2-domain-containing (Ferlin-like) proteins mediate vesicle-membrane fusion in diverse cell types in response to Ca^{2+} and membrane curvature. Using a forward-genetics approach, researchers have shown that a conserved C2-domain-containing protein termed DOC2.1 plays a central role in microneme secretion, with a concomitant impact on egress, motility, and invasion in both *T. gondii* and *P. falciparum* (44). Furthermore DOC.2 was shown to bind to Ca^{2+} in *Plasmodium* (67). Although DOC2.1 is yet to be localized in *Toxoplasma*, it has been proposed to mediate microneme release by controlling fusion of SNARE-like proteins/complexes in a Ca^{2+}-dependent manner (44). Three ferlins are encoded in the genome of *T. gondii* (32). Ferlin 2 participates in membrane fusion during rhoptry discharge, likely in a Ca^{2+}-dependent manner (32). Moreover, the homolog of *T. gondii* Ferlin 1 has been shown to be involved in gametocyte egress (102), potentially implicating Ferlin 1 in microneme exocytosis.

Although the signaling events culminating in microneme release are slowly being unraveled, little is known about the actual fusion event at the parasite PM. Membrane fusion involves a universal machinery that includes SNARE and SM (Sec1/Munc18-like) proteins. During fusion, vesicular and target SNARE proteins assemble into an α-helical *trans*-SNARE complex that forces the two membranes tightly together, and SM proteins likely wrap around assembling *trans*-SNARE complexes to catalyze membrane fusion (124). This machinery is controlled by Ca^{2+} via C2-containing proteins, like ferlins and DOC2 (133, 141).

PHOSPHOLIPID HOMEOSTASIS AND PHOSPHATIDIC ACID SIGNALING

The production of IP_3 by PI-PLC and concurrent Ca^{2+} mobilization preceding microneme secretion are linked to the generation of DAG (22). Importantly DAG can be interconverted to PA through a reversible reaction that requires ATP and the action of DGKs and PAPs. As in eukaryotic model organisms, PA is linked to exocytosis (142), and the conversion of DAG to PA at the PM appears essential for microneme secretion in Apicomplexa. Concordantly, blocking PA production either with specific DGK inhibitors or through conditional depletion of the PM-associated DGK1 reduces PA levels and hampers microneme secretion in *T. gondii* (22). Conversely microneme secretion and egress can be induced by treatment of tachyzoites with the PAP inhibitor propranolol, implying that the ensuing buildup of PA facilitates exocytosis (22).

The study that delineated the importance of PA signaling at the parasite PM also revealed the presence of APH, an acylated protein at the surface of the micronemes (22). Of relevance, recombinant APH from both *Plasmodium* and *T. gondii* was shown to bind specifically to PA through both PIP-strip and liposome-binding assays, supporting the view that the protein indeed acts as a PA sensor. Furthermore, structural studies have demonstrated that APH inserts into and clusters multiple phosphate head groups at the bilayer-binding surface (33). A conditional depletion of APH resulted in a selective block in microneme secretion, leading to impairments in parasite motility, invasion, and egress (22). Based on these observations, a plausible model emerged whereby microneme-associated APH binds to PA produced by DGK1 at the inner leaflet of the parasite PM and critically participates in the signaling cascade leading to microneme exocytosis. In this context, DOC2.1 also binds to the membrane, but its lipid specificity is unknown (67) (**Supplemental Table 3**).

Supplemental Material >

PA produced by DGK1 during egress is also postulated to coordinate the concerted action between adhesins and the glideosome. Indeed, GAC possesses a PH domain at the carboxyl terminus of the protein, which has been found to bind to PA and likely contributes to the positioning of GAC to the cytosolic face of the apical PM. The dual binding of GAC to MIC-tails and PA that concentrates at the parasite PM (22) presumably increases selectivity and affinity for secreted adhesins by combining two weak-binding interactions to engender strong binding (79).

FUTURE DIRECTIONS

Recent studies have shed new light on the signaling pathways governing parasite egress from infected cells and provided critical insights into the molecular mechanisms involved. Yet many outstanding questions remain to be addressed. Is the signaling platform composed of the atypical CDC50.1, GC, and UGO, integrating both intrinsic and extrinsic signals, and if yes how? What are the molecular entities responsible for Ca^{2+} homeostasis and acting as Ca^{2+} channels at the parasite PM and the intracellular storage compartments? What are the substrates of the CDPK1 accounting for the essential role of this kinase in microneme secretion, conoid protrusion, and activation of R-actin flux? Is *T. gondii* relying on natural and/or induced egress to spread during chronic infection? What are the signaling events governing rhoptry discharge in coordination with microneme secretion to produce the moving junction and initiate invasion?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank Mathieu Brochet for critical reading of the manuscript. Funding received from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program under grant agreement number 695596 and the Swiss National Foundation to D.S.-F. (FN3100A0-116722).

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Annual Review of Microbiology

Volume 73, 2019

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Errata

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