An apically located hybrid guanylate cyclase–ATPase is critical for the initiation of Ca\(^{2+}\) signaling and motility in Toxoplasma gondii

Edited by Ronald C. Wek

Protozoan parasites of the phylum Apicomplexa actively move through tissue to initiate and perpetuate infection. The regulation of parasite motility relies on cyclic nucleotide-dependent kinases, but how these kinases are activated remains unknown. Here, using an array of biochemical and cell biology approaches, we show that the apicomplexan parasite Toxoplasma gondii expresses a large guanylate cyclase (TgGC) protein, which contains several upstream ATPase transporter-like domains. We show that TgGC has a dynamic localization, being concentrated at the apical tip in extracellular parasites, which then relocates to a more cytosolic distribution during intracellular replication. Conditional TgGC knockdown revealed that this protein is essential for acute-stage tachyzoite growth, as TgGC-deficient parasites were defective in motility, host cell attachment, invasion, and subsequent host cell egress. We show that TgGC is critical for a rapid rise in cytosolic \([\text{Ca}^{2+}]\) and for secretion of microneme organelles upon stimulation with a cGMP agonist, but these deficiencies can be bypassed by direct activation of signaling by a \([\text{Ca}^{2+}]\) ionophore. Furthermore, we found that TgGC is required for transducing changes in extracellular \(pH\) and \([K^+]\) to activate cytosolic \([\text{Ca}^{2+}]\) flux. Together, the results of our work implicate TgGC as a putative signal transducer that activates \([\text{Ca}^{2+}]\) signaling and motility in Toxoplasma.

The phylum Apicomplexa comprises a group of obligate intracellular eukaryotic parasites of wide medical and agricultural significance. Plasmodium spp. cause malaria, Cryptosporidium spp. induce severe gastrointestinal disease, and Toxoplasma infection leads to toxoplasmosis. Toxoplasma is transmitted by ingestion of oocysts shed from an infected cat or consumption of meat harboring cyst forms. Toxoplasma then differentiates in the gut into the acute tachyzoite forms that traverse the intestinal barrier. Toxoplasma disseminates throughout the body, causing flu-like symptoms in healthy adults; however, infection during pregnancy can lead to massive tissue destruction in the developing fetus, resulting in abortion and neurological defects, or severe disease in immunodeficient individuals. Acute stages can then differentiate into chronic bradyzoite forms, which occurs in the central nervous system and muscle, creating a life-long untreatable reservoir for reactivation later in life. Latent infection of the retina can also lead to progressive blindness, which is particularly prevalent in some countries (1).

Central to transmission and survival of all apicomplexan species in their hosts is their ability to move through tissue and invade host cells. Parasite movement is driven by a unique form of cellular locomotion termed “gliding motility.” Gliding motility is propelled by an actomyosin-based motor, termed the “glideosome,” which lies underneath the plasma membrane (2, 3). The glideosome binds to short cytoplasmic tails of transmembrane adhesins, which then attach to host cell receptors or the extracellular matrix. The current model suggests that the glideosome generates force for motility by dragging transmembrane adhesins through the plane of the membrane to the rear of the parasite, thus driving forward movement (2, 3).

Upon appropriate environmental cues, Toxoplasma, like all apicomplexan parasites, must activate motility. Environmental...
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cues regulate the release of adhesins from the microneme organelles onto the parasite surface and activate the glideosome. In Toxoplasma, gliding motility and microneme secretion can be stimulated upon exposure to lower extracellular [K+] and an acidic (high [H+] environment, and extracellular phosphatidic acid (ePA)2 (4–6). Changes in [K+] are thought to be a marker of perturbations in the electrochemical gradient, as likely experienced upon host cell damage or when exposed to the extracellular milieu. A drop in vacuolar pH, in contrast, has been proposed to be a signal of host lysosomal fusion but also may be a parasite-induced phenomenon to activate perforin-like protein 1 (PLP-1), which is required for egress (6, 7). In recent work, it has been shown that Toxoplasma also produces ePA in the vacuolar space, and this has been proposed to act as a molecular clock triggering “natural” host cell egress (4). Although the parasite receptors that receive these external signals remain unknown, they all converge on inducing an intracellular signaling cascade that leads to microneme secretion and glideosome activation, which is required for motility (4–6).

Irrespective of environmental cue, activation of motility converges on Ca2+ signaling (8). A rise in cytosolic [Ca2+] ([Ca2+]cyt) is temporally linked with activation of host cell egress and motility and is required for release of adhesins from the micronemes (9–11). Across all apicomplexan parasites, translation of [Ca2+]cyt into enzymatic activity is mediated, at least in part, by a family of “plant-like” Ca2+-dependent protein kinases (CDPKs) and calcineurin, a Ca2+-dependent phosphatase (8, 11–18). Substrates of these proteins are presumably activated/deactivated by phosphorylation/dephosphorylation events, which are required for motility to be triggered. Numerous CDPK-dependent phosphorylation sites have been identified, including those found on components of the glideosome (11, 19–23).

In Toxoplasma, cyclic guanosine monophosphate (cGMP) signaling appears to act upstream of Ca2+ signaling and is required to activate motility as well as a range of other developmental transitions (24, 25). In other systems, the magnitude and duration of cGMP signaling are dictated by the balance between production by guanylate cyclases (GCs) and its degradation by phosphodiesterases (PDEs). In mammalian cells, GC activation is often coupled with hormone receptors and is classically known as a central mediator of nitric oxide sensing and transduction. cGMP-binding effectors in Apicomplexa appear to be limited to protein kinase G (PKG), and there is a noticeable lack of cGMP-gated ion channels that provide a link with Ca2+ signaling in mammalian systems (26). In Apicomplexa, inhibition or genetic depletion of PKG blocks egress, invasion, and motility and prevents a rise in [Ca2+]cyt (10, 24, 25, 27–29). Furthermore, PDE inhibitors, which block the breakdown of cGMP, activate motility in a Ca2+-dependent fashion (10, 16, 30). In Toxoplasma and Plasmodium, cGMP signaling also regulates phosphoinositide signaling, which is necessary for the production of phosphatidic acid (PA), which is required for microneme secretion (24, 31). Thus, cGMP appears to be important for both induction of Ca2+ signaling and the production of intracellular pools of phospholipid species, both of which are required to activate motility.

Despite an emerging understanding of signaling modules and their interconnectedness, it remains a mystery how apicomplexan parasites sense their external environment to activate intracellular signaling. Although apicomplexans appear to be devoid of G protein–coupled receptors and receptor tyrosine kinases, they do contain nucleotide cyclases with accessory domains that suggest that production of both cGMP and cyclic AMP (cAMP) might be triggered in cis (26). Of particular note is the presence of ATPase-like domains that are predicted to be involved in ionicsensing/transport. In this regard, GC in Plasmodium, which contains such accessory domains, is required for motility of ookinete stages (32), but the importance of such a protein in Toxoplasma or in the disease-causing asexual stages of other apicomplexan species is not fully explored.

Here, we functionally characterize a putative GC during lytic-stage growth in Toxoplasma. TgGC is a very large transmembrane protein (~477 kDa) and contains multiple accessory domains that could act in environmental sensing. We demonstrate that TgGC has a dynamic positioning, localizing to the apical end in actively motile forms, which is redistributed to a more disperse cytosolic localization during intracellular replication. We demonstrate that TgGC is critically required for Toxoplasma egress, attachment, invasion, motility, and microneme secretion and furthermore is needed for production of PA and activation of a rise in [Ca2+]cyt. Moreover, we determine that TgGC is important for sensing changes in environmental [K+] and pH, thus highlighting the importance of this protein in detecting extracellular signals required for activation of motility.

Results

Toxoplasma gondii encodes a putative guanylate cyclase that has a dynamic localization

We were interested in determining how Toxoplasma senses its external environment to activate motility. Toxoplasma has one predicted guanylate cyclase (TgGC; TGGT1_254370), with 19 transmembrane domains and a predicted size of 477 kDa (Fig. 1A). TgGC includes multiple predicted accessory domains, including (from N to C terminus) an E1–E2-like Ca2+-ATPase domain, a haloacid dehalogenase (HAD)-like hydrolase domain adjoined to another ATPase-like region, and a nucleotide cyclase domain split in two by a large insertion (Fig. 1A). Classic P-type ATPases are composed of two sets of TM domains followed by a group of six TM domains. This feature is shared with the N-terminal ATPase domain in TgGC. The C-terminal cyclase domain structurally resembles mammalian membrane-associated adenylyl cyclases, which are...
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-~110kD ~120kD
-~65kD ~75kD
-~50kD

**TGTT1_254370**

- Cation-transporting ATPase-like
- Haloacid dehalogenase-like
- Split nucleotide cyclase domain

**A**

Diagram showing protein bands with molecular weights and domain annotations.

**B**

A assay with bands for αTy and αHA showing expected molecular weights.

**C**

Western blot results for TgGCty and HA-TgGC.

**D**

Immunofluorescence images for αTy and αTgGAP45 in extracellular and intracellular compartments.

**E**

Immunofluorescence images for αHA and αTgGAP45 in extracellular and intracellular compartments.

**F**

Immunofluorescence images for αTy and αTgGAP45 with DAPI merge.

**G**

Immunofluorescence images for αHA and αTgGAP45 with DAPI merge.

**H**

Immunofluorescence images for αHA and αTgGAP45 with DAPI merge.

**I**

Immunofluorescence images for αHA and αTgGAP45 with DAPI merge.
comprised of two sets of six TM domains and downstream cytosolic cyclase catalytic domains (C1 and C2 domains). The C1 and C2 regions of TgGC are highly conserved but are inverted in order as compared with mammalian adenyl cyclases. In its C2 domain, the two amino acids (Glu-2987 and Ala-3137) that are characteristic of guanylyl cyclases are present, suggesting that this protein is specific for the production of cGMP (33, 34) (Fig. 1A). Orthologues of TgGC have been identified in ciliates and other apicomplexan parasites, which vary somewhat in their domain architecture (35, 36).

To investigate the function and localization of TgGC, we generated two epitope-tagged lines by genetic modification at the endogenous locus. A Ty tag was introduced to the C terminus of TgGC (TgGC-Ty) (Fig. S1), and in a separate line we appended three tandem HA epitope sequences to the N terminus of TgGC as part of generating a conditional knockdown by promoter replacement (HA-TgGC cKD) (Fig. S1). We then performed Western blots on extracellular parasites, staining with antibodies specific to appended epitope tags (Fig. 1, B and C). We observed several species migrating at ~65 (most prominent), ~110, and above 250 kDa in TgGC-Ty (Fig. 1B, panel i), whereas HA-TgGC cKD showed species at ~50, ~80, ~120, and above 250 kDa (Fig. 1B, panel ii). Mapping these putative proteolytic fragments to the predicted domain structure with ToxoDB (http://toxodb.org/toxo)3 (57) shows the ~65- and 110-kDa species detected by TgGC-Ty as both ending between the C1 and C2 catalytic domains (Fig. 1A). Fragments detected using HA immunoblotting of HA-TgGC show that ~50-, ~75-, and ~120-kDa species only contain the most N-terminal ATPase domain as predicted by ToxoDB (Fig. 1A). To investigate further the highest-molecular-weight species, we ran samples on 3–8% Tris acetate gels. Bands at ~460 kDa were identified in TgGC-Ty and HA-TgGC parasite lysates, which matches the theoretical molecular mass of 477 kDa (Fig. 1C), suggesting that at least a proportion of TgGC is at or close to the predicted full-length protein.

We then investigated the localization of TgGC by IFA using epitope-tagged lines. In extracellular tachyzoites, we found, using their respective epitope antibodies, that HA-TgGC and TgGC-Ty localized to the apical end of parasites in a crescent-like shape (Fig. 1, D and E). This staining pattern appears to be largely apical of TgGAP45 (Fig. 1, D, panel i, and E, panel i) and similar to that of TgISP1, a marker of the apical plate of the IMC (37) (Fig. 1, D, panel ii, and E, panel ii). At this resolution, however, given the small distance between the plasma memb.

Figure 1. Toxoplasma GC is a large multidomain protein that has a dynamic localization. A, domain structure of TgGC (TGGT1_254370) as predicted by ToxoDB. Predicted domains are highlighted in different colors with their corresponding names in the same color and position of predicted transmembrane domains shown. Horizontal lines with corresponding molecular masses show approximate sizes of products detected by Western blotting in B. Positions of residues Glu-2987 and Ala-3136 that predict guanylyl cyclase activity are shown. B, Western blotting of TgGC-Ty (panel i) and HA-TgGC (panel ii) showing the presence of multiple products. C, 3–8% Tris acetate gels to resolve larger molecular weight species (smaller species as shown in B are cut off). D, TgGC-Ty localization in extracellular tachyzoites and colocalized with the peripheral marker TgGAP45 (panel i) and the apical IMC marker TgISP1 (panel ii). E, localization of HA-TgGC in extracellular parasites using HA antibodies together with the peripheral marker TgGAP45 (panel i) and the apical IMC marker TgISP1 (panel ii). F, localization of TgGC-Ty using Ty antibodies in intracellular parasites. Panel i, colocalization with the peripheral marker TgGAP45. The white arrowhead points to accumulation of TgGC-Ty in the residual body. Panels ii and iii, colocalization with Tg IMC1 antibodies, respectively, demonstrating the lack of TgGC-Ty in developing daughter cells (white arrows). G, localization of HA-TgGC with HA antibodies in intracellular parasites in both vacuoles containing single (panel i) and multiple (panel ii) tachyzoites. H, stimulation of egress using BIPPO (panel i) and A23187 (panel ii) in HA-TgGC–expressing parasites where HA marks the position of HA-TgGC and TgGAP45 marks the periphery of tachyzoites.

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J. Biol. Chem. (2019) 294(22) 8959 – 8972

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petitive immunoassay. In the parental line, we detected a \( \sim 3.5 \)-fold increase in levels of cGMP upon BIPPO treatment, which upon TgGC depletion was significantly blunted (Fig. 2C). This is consistent with a similar experiment performed by Bisio et al. (4) on a TgGC mutant generated with the auxin-inducible system.

To assess the importance of TgGC during the lytic cycle, we first performed a plaque assay (Fig. 2D). Although we observed a mild decrease in size of plaques upon ATc treatment in parental lines (\( \Delta ku80/TATi \)), we found an almost complete lack of plaques upon depletion of TgGC expression (TgGC cKD + ATc). This severe loss in plaquing suggests that TgGC is important for efficient lytic-stage growth.

We then performed a series of assays to determine at what stage of lytic-stage growth TgGC is required. Host cell attachment was first evaluated on both parental parasites and TgGC cKD mutants in the presence or absence of ATc. We observed that depletion of TgGC by ATc leads to an approximately 50% reduction in their attachment efficiency as compared with controls under the conditions used (Fig. 2E).

We then assessed the efficiency of invasion using differential staining of extracellular and intracellular parasites before and after detergent treatment, respectively (38) (Fig. 2F). We found that although parental tachyzoites invaded at a similar rate with or without ATc preincubation, loss of TgGC expression (+ ATc) resulted in a drastic loss of intracellular parasites, thus suggesting that TgGC is required for efficient invasion (Fig. 2F).

We then monitored whether TgGC regulates parasite replication. Although ATc treatment itself causes a mild delay in replication, we could see no additional effect upon the loss of TgGC at either 18 or 24 h postinvasion (Fig. 2G, panels i and ii).

We then determined the role of TgGC during tachyzoite host cell egress. Parasite egress can be induced with agonists that stimulate both cGMP- and Ca\(^{2+}\)-mediated signaling pathways. BIPPO activates cGMP-dependent TgPKG-mediated host cell egress (30), whereas the Ca\(^{2+}\) ionophore A23187 directly induces Ca\(^{2+}\)-dependent egress (39). In parental tachyzoites, stimulation with 5 \( \mu M \) BIPPO for 5 min in the presence or absence of ATc led to potent induction of host cell egress. However, TgGC depletion with ATc caused egress to drop to \(~40\)% of controls, even at this high agonist concentration and extended incubation time (Fig. 2H, panel i). Interestingly, stimulation with 4 \( \mu M \) A23187 for the same time frame did not result in a deficiency in egress in TgGC-depleted tachyzoites, suggesting that loss of host cell egress in this mutant is somewhat specific to stimulation with a cGMP agonist (Fig. 2H, panel ii).

Overall, this analysis strongly suggests that TgGC participates in efficient host cell attachment, invasion, and cGMP-induced egress but unlikely plays a role in replication.

**TgGC is important for tachyzoite motility and cGMP-induced microneme secretion**

Gliding motility is central to invasion and egress; therefore, we wanted to determine whether TgGC is required to activate this process. To most easily stimulate motility in a synchronous fashion without using a small-molecule agonist (i.e., BIPPO or A23187), we used 0.01% saponin treatment to release tachyzoites from infected host cells. We and others have shown that saponin treatment selectively permeabilizes host, but not parasite, membrane and does not interfere with downstream signaling pathways, allowing for a synchronous burst of motility without the need to artificially activate signaling pathways (10, 40).

As expected, the parental strain (+ ATc) could potently induce motility upon breakdown of host membranes (Movies S1 and S2), although TgGC-depleted tachyzoites were severely stunted in their ability to migrate away from the point of origin despite being released from host cell membranes by the saponin treatment (Movies S3 and S4). From these assays, we quantitated the average speed (Fig. 3A) and maximum speed (Fig. 3B), and in doing so we observed that TgGC-depleted tachyzoites were highly deficient in gliding motility. This is also clear when comparing the instantaneous speed of representative parental (Fig. 3C, panels i and ii) and TgGC-depleted tachyzoites (Fig. 3C, panels iii and iv) over a 300-s filming period.

Micronemes are apical organelles that harbor adhesins and other factors required for host cell egress, motility, and invasion. We therefore wanted to determine whether TgGC was required for the exocytosis of these organelles. To do this, we treated TgGC cKD with or without ATc, stimulated microneme secretion in extracellular tachyzoites using A23187 or BIPPO, and quantitated secretion using Western blotting (Fig. 3D). As expected, TgGC-replete tachyzoites were able to secrete basal levels of the microneme protein PLP-1, which was greatly enhanced upon treatment with either A23187 or BIPPO (Fig. 3D, panels i and ii). Depletion of TgGC with ATc led to a significant blunting of BIPPO-stimulated secretion but did not appear to affect basal levels (Fig. 3D, panels i and ii). Interestingly, TgGC-depleted tachyzoites were able to secrete more PLP-1 into the supernatant upon A23187 treatment than TgGC-replete controls (Fig. 3D, panels i and ii). The reason for this is unknown. Overall, this highlights the importance of TgGC in inducing microneme secretion, which can be bypassed by directly stimulating Ca\(^{2+}\) signaling using A23187, similar to that observed with egress.

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**Figure 2. Functional analysis of Toxoplasma GC during lytic-stage growth.** A. generation of a Tet-Off–based conditional knockdown of TgGC (TgGC cKD) (genetic strategy and validation are shown in Fig. S1). B. Western blot analysis of ATc-treated tachyzoites over 36 and 84 h showing disappearance of HA signal despite equal loading (TgGAP45). C. quantitation of cGMP levels in the parental line and TgGC cKD after treatment with ATc. D. plaque assay of the parental line and TgGC cKD with and without ATc treatment showing loss of plaquing capacity upon selective depletion of TgGC. E. attachment assay of parental and TgGC cKD parasites with and without ATc treatment normalized to parental line per scan area. F. two-color invasion assay of TgGC cKD with and without ATc normalized to parental controls. G. replication assay of parental and TgGC cKD tachyzoites with and without ATc at 18 (panel i) and 24 (panel ii) h. H. egress assay of TgGC cKD with and without ATc treatment normalized to parental controls when stimulated with the PDE inhibitor BIPPO (panel i) or the Ca\(^{2+}\) ionophore A23187 (panel ii). All graphs represent mean \( \pm \) S.E., \( n = 3 \) or more. p values were calculated pairwise using an unpaired two-tailed t test: **, \( p < 0.01 \); ****, \( p < 0.0001 \); ns, not significant. DHFR, dihydrofolate reductase.
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Figure 3. Toxoplasma GC is important for gliding motility and microneme secretion. Motility of parental + ATc and TgGC cKD + ATc parasites was monitored over a period of 5 min (300 s), and average speed (A) and maximum speed (B) were quantitated using ImageJ. C, tracking of instantaneous speed over 300 s of filming of representative parental + ATc (panels i and ii) and TgGC cKD + ATc (panels iii and iv). Graphs represent mean ± S.D. with each parasite tracked representing one data point. p values represent Welch’s t test: ****, <0.0001. D, microneme secretion assay on TgGC cKD ± ATc either unstimulated (basal) or induced with BIPPO or A23187. Panel i, representative Western blot using TgPLP1 antibodies. Panel ii, quantitation of band intensities for PLP1 as normalized to total protein loaded (as marked by REVERT™ total protein stain kit (LI-COR Biosciences)). p values were calculated pairwise using an unpaired two-tailed t test, n = 3: ns, not significant; *, <0.05; **, <0.01.

TgGC is required for BIPPO-dependent changes in cytosolic [Ca^{2+}] and for normal phosphatidic acid levels

A rise in [Ca^{2+}]_{cyt} is functionally required for microneme secretion and is temporally associated with host cell invasion and egress (8–10, 39, 41, 42). We and others have also demonstrated that cGMP and Ca^{2+} signaling are linked (10, 43, 44). Therefore, we wanted to determine whether TgGC is required for a rise in [Ca^{2+}]_{cyt} thus providing evidence as to how this nucleotide cyclase functions to activate motility-dependent processes. Previous studies conducted by our group and others have used the genetically encoded GFP-based Ca^{2+} biosensor GCaMP, which allows for temporal analysis of intracellular [Ca^{2+}], whereby fluorescence levels correlate with the local Ca^{2+} concentration (9, 10, 44). To undertake these
experiments, we introduced a GCaMP6/mCherry plasmid into TgGC cKD by single crossover integration into the uprt locus. We then used a FACS-based assay to monitor cytosolic \([\text{Ca}^{2+}]_{\text{cyt}}\) upon different stimuli at a range of concentrations. We first treated TgGC cKD with or without ATc and measured resting levels of fluorescence. After 20-s acquisition, we then treated with a dose titration of BIPPO (Fig. 4A, panel i), kinetic plots of fluorescence from GCaMP6-expressing extracellular tachyzoites in the presence (−ATc) and absence (+ATc) of TgGC upon addition of a serial dilution of BIPPO at \(t = 20\) s measured for a total of 100 s. Panel ii, corresponding rate of change of fluorescence (\([\text{Ca}^{2+}]_{\text{cyt}}\)) was calculated based on values plotted in panel i. B, panel i, kinetic plots of fluorescence from GCaMP6-expressing extracellular tachyzoites in the presence (−ATc) and absence (+ATc) of TgGC upon addition of a serial dilution of A23187 at \(t = 20\) s measured for a total of 100 s. Panel ii, corresponding rate of change of fluorescence (\([\text{Ca}^{2+}]_{\text{cyt}}\)) was calculated based on values plotted in panel i. C, quantification of total PA levels, normalized to parental controls, in the presence (−ATc) and absence (+ATc) of TgGC. Graphs in A, panel ii; B, panel ii; and C represent mean ± S.E., \(n = 3\) or more. \(p\) values were calculated pairwise using an paired two-tailed \(t\) test: *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\).

Figure 4. Toxoplasma GC is required for cGMP-dependent increase in cytosolic \([\text{Ca}^{2+}]_{\text{cyt}}\) and normal phosphatidic acid levels. A, panel i, kinetic plots of fluorescence from GCaMP6-expressing extracellular tachyzoites in the presence (−ATc) and absence (+ATc) of TgGC upon addition of a serial dilution of BIPPO used (Fig. 3A, panels i and ii). Despite this, TgGC-depleted parasites are still equally capable of responding to A23187, demonstrating that there is no intrinsic problem with the ability to induce changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) (Fig. 3B, panels i and ii).

It has recently emerged that PA generation is important for microneme secretion and likely acts independently of cytosolic \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling (31). To determine whether TgGC activity is also required for the production of this phospholipid species, we again depleted TgGC using ATc treatment and monitored the relative amount of PA produced. We found that loss of TgGC results in a around a 40% decrease in PA, suggesting that cGMP production is important for optimal production of this phospholipid (Fig. 4C).
TgGC acts downstream of sensing changes in extracellular pH

*Toxoplasma* regulates motility upon changes in environmental ionic composition. Two potent extracellular factors that activate motility is a drop in [K⁺], which is thought to be a sensing mechanism for damaged host cells (5), and pH (*i.e.*, a rise in [H⁺]), which could be a marker of immune attack (6). To date, it is not understood how changes in ionic composition are sensed by *Toxoplasma* to activate motility. Due to the presence of ion-sensing/transport-like domains in TgGC, we wondered whether this putative nucleotide cyclase is required for relaying extracellular [K⁺] and [H⁺] to activate motility. To test this, we used GCaMP6-expressing TgGC cKD and our FACS-based assay to monitor changes in cytosolic [Ca²⁺] upon changes in extracellular [K⁺] and [H⁺]. Because a drop in ionic concentration could not be easily performed online (as above during FACS acquisition), we instead performed this by resuspending parasites in “intracellular buffer” (ICB), which has relatively high [K⁺] (142 mM), then quickly-spinning samples, and resuspending them in new buffer followed by immediately putting them under the FACS machine (Fig. 5A). We could see that if tachyzoites are re-exposed to a high extracellular [K⁺] ICB, no rise in GCaMP6 mean fluorescence intensity (MFI) is observed (Fig. 5B). However, resuspension of tachyzoites in extracellular buffer (ECB) (5 mM [K⁺]) leads to a rise in GCaMP6 MFI, which drops to approximately half the maximum level observed over the 100-s acquisition time (Fig. 5B). When TgGC is then depleted by ATc treatment, we could show that a switch to ECB does not lead to a rise in [Ca²⁺]ₘₚ, suggesting that this nucleotide cyclase acts at or downstream of sensing extracellular [K⁺] (Fig. 5B).

We then tested whether TgGC controls activation of Ca²⁺ signaling upon exposure to lower pH (6). It has been shown that a drop in pH can overcome the suppressive effects of high [K⁺] (6). We therefore tested whether a drop in pH, from 7.4 to 5.4, could stimulate a rise in [Ca²⁺]ₘₚ even when kept in high [K⁺]. As above, we tested this by quickly-spinning tachyzoites in ICB, pH 7.4, and resuspending them in ECB, pH 5.4. We found that only when the pH is dropped to 5.4 do we see a rise in cytosolic [Ca²⁺] as measured by GCaMP6 MFI, which also decayed over the 100-s acquisition time (Fig. 5C). Likewise, when we depleted TgGC by ATc treatment, we saw that activation of [Ca²⁺]ₘₚ signaling was ablated (Fig. 5C), suggesting that cGMP production also is required for sensing changes in environmental pH.

**Discussion**

Here, we have functionally characterized a putative guanylate cyclase in *Toxoplasma* (TgGC), interrogated its function during acute-stage lytic growth, and further determined how it integrates into signaling pathways that regulate motility, host cell egress and invasion. Our work complements and extends two other groups’ recent observations, which also demonstrate the importance of TgGC in microneme secretion, motility, invasion, and egress while also providing evidence that this enzyme is required for the production of cGMP (4, 45). In addi-
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Figure 6. A model of TgGC function in Toxoplasma. TgGC directly or indirectly can sense the extracellular environment ([H⁺], [K⁺], and ePA) and activate cGMP production. cGMP binds to and activates TgPKG, which resides at the parasite periphery. Phosphorylation of substrates leads to the activation of PA production in the inner leaflet of the plasma membrane. TgPKG also activates a rise in cytosolic [Ca²⁺], which in turn potentiates plantlike CDPKs, which are required for microneme secretion. Micronemes release adhesins onto the parasite surface, stimulating motility, invasion, or egress.

Our work outlines the importance of TgGC in the production of PA and induction of downstream Ca²⁺ signaling and reveals a role for this nucleotide cyclase in transducing environmental stimuli that are known to activate motility. We propose a model here, which we discuss below (Fig. 6).

One of the most fundamental unanswered questions in apicomplexan biology is how this group of intracellular parasites senses their environment? Toxoplasma, like other apicomplexan parasites, can sense a drop in [K⁺] and pH and have also been shown to sense ePA (4–7, 46). These stimuli activate motility, but how the parasite receives these signals remains enigmatic. We show that TgGC, a multipass transmembrane protein, has several domains that suggest this nucleotide cyclase could potentially sense, or even transport, ions and may allow coupling to production of cGMP, thus activating downstream signaling cascades to activate motility (Fig. 6). Of particular interest in this regard is the P-type ATPase E1–E2-like domain, which in other systems is able to transport a variety of substrates, including (but not limited to) Ca²⁺, H⁺, K⁺, and even lipid species, all of which have been implicated in activating motility. TgGC also contains a HAD-like domain, which is part of a superfamily that serve as the predominant catalysts of metabolic phosphate ester hydrolysis in all three superkingdoms of life. HAD-like phosphatases can have a variety of cellular roles, including in metabolism, regulation of enzyme activity or protein assembly, cell housekeeping, and nutrient uptake (47). HAD-like enzymes can also provide a chemical handle for interactions of metabolites with enzymes. HAD-like domains can be associated with ATPase domains, and this appears to be the case in TgGC. The function of these domains and how they couple to the production of cGMP are the clear next steps, which will be critical to determine whether and how this protein is involved in transducing extracellular signals, such as ionic concentration, into the production of cGMP to trigger intracellular signaling. What is clear is that residues that are predicted to be important for the P-type ATPase or guanylate cyclase activity are critical to the function of TgGC (45).

If TgGC is indeed the sensor for receiving extracellular signals, this huge protein must be exposed to the extracellular milieu (Fig. 6). We show, in extracellular parasites, that TgGC is found at the apical end in a crescent-like pattern in a very similar localization pattern to the known IMC protein TgISP1. TgISP1 is known to be deposited in the apical cap of the IMC, suggesting that TgGC is indeed also found in this location (37); however, we also demonstrate that TgGC is absent from internally developing daughter cells where canonical IMC proteins can be found, challenging this notion and suggesting that TgGC is indeed more likely found in the plasma membrane. Clearly, understanding the topology of TgGC and how it interacts with ions, K⁺, H⁺, and ePA in particular will be an important part of the puzzle in ultimately determining how Toxoplasma, and related apicomplexan parasites that have a similarly structured TgGC, sense their environment to activate motility.

We also demonstrate that TgGC can be detected by Western blotting as multiple proteolytic products and has a dynamic localization. When tracking N-terminal processing (with antibodies against the appended HA epitope tag) we found three major species of ~50, 75, and 120 kDa. Mapping of these fragments to the predicted domain structure suggests that all these fragments harbor the P-type ATPase E1–E3-like region, which is predicted to be involved in the transport of ions or lipids. Most interestingly, we found that the vast majority of HA-tagged protein appear to redistribute, in intracellular replicating parasites, to an apparent cytoplasmic-like localization despite having three predicted transmembrane domains. Also, we demonstrate that upon agonist stimulation, N-terminal fragments of TgGC can be found at the apical end in a punctate-like pattern. This is in contrast to the proteolytic products derived from the C terminus (as tracked using an appended Ty epitope tag). These fragments, both of which appear to result in cleavage of TgGC between the two catalytic domains, seem to be found at the apical cap region in extracellular parasites as well as intracellular replication. This pattern of proteolytic processing is consistent with that found in recent publications (4, 45, 48). Together these results pose interesting questions about the nature of TgGC, showing that during intracellular replication, fragments of this protein do not colocalize and only do so during stages that are motile. Whether these products then reassociate when both are localized to the apical tip, when zoites are extracellular, is yet unknown.

We also demonstrate that TgGC is required for activating a rise in [Ca²⁺]cyt and for the normal production of PA (Fig. 6). Previous work in Plasmodium has shown that the cGMP-effector kinase PKG and a GC orthologue is required for phosphorylation of phosphatidylinositol kinases as well as the production of phosphoinositide species. In other organisms, hydrolysis of phosphatidylinositol 4,5-bisphosphate produces diacylglycerol...
and inositol 1,4,5-trisphosphate, the latter of which stimulates the release of \( \text{Ca}^{2+} \) from the endoplasmic reticulum, leading to a rise in the cytosolic concentration of this cation. Diacylglycerol can then be converted into PA by diacylglycerol kinase, which is then required for microneme secretion (49). Our data suggest that *Toxoplasma* GC, directly or indirectly, is required for both the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) and the production of PA, thus further suggesting that this nucleotide cyclase is a potential master regulator that acts upstream of both these important events that drive motility (Fig. 6).

Together with other recently published work, this study suggests a model of how TgGC functions (Fig. 6) (4, 45). Our work suggests that TgGC acts downstream of environmental ionic cue sensing, which then produces cGMP to activate TgPKG. The activation of gliding motility, invasion, or egress (Fig. 6).

**Experimental procedures**

**Toxoplasma transfection and in vitro culture**

*Toxoplasma* was grown under standard conditions. Briefly, human foreskin fibroblasts (HFFs) were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated Cosmic calf serum (HyClone) in a humidified atmosphere of 10% \( \text{CO}_2 \) to confluence. Upon tachyzoite infection, medium was exchanged to Dulbecco’s modified Eagle’s medium with 1% fetal calf serum (D1 medium). Transfection proceeded using either a Gene Pulser II (Bio-Rad) or a Nucleofector 4D system (Lonza). Gene Pulser II transfection took place using standard procedures using 15 μg of purified DNA. Nucleofection proceeded by using 2 × 10⁶ tachyzoites and 5 μg of DNA in 20 μl of buffer P3 (Lonza) and pulsing using program F1-115.

**Plasmid construction**

All DNA plasmids were designed based on the annotation of TgGC (TGGT1_254370) available in ToxoDB. To generate TgGC cKD tachyzoites, two homologous DNA flanks were generated for ligation to the promoter replacement plasmid pPR2–3HA, one corresponding to a region upstream of the TgGC gene and one corresponding to the start codon. The upstream flank was amplified using the oligonucleotides 5’-TTAATTAAACAATTTGGTATTGACGCCGCGCCGAAAGGGAC-3’ and 5’-CATATGCTAGTGCAGGAAATGAGGAGG-3’ and inserted into pPR2–3HA using NdeI and PacI sites, whereas the downstream flank was amplified using oligonucleotides 5’-CCCGGGATGAAAGAAGACGAGGAAAC-3’ and 5’-GATATCCAAATTGCAGCAGGACCTTCGCTTTGTG-3’ and inserted into pPR2–3HA using Xmal and EcoRV sites. Following transfection, transgenic parasites were selected by pyrimethamine treatment. Introduction of GCaMP6/mCherry plasmid into the *uprt* locus was performed as described previously (21).

**Antibodies**

The following antibodies were used in this study: rat anti-HA (3F10, Roche Applied Science; 1:1000), mouse αTy (BB2; 1:1000), mouse αMIC2 (1:5,000) (50), rabbit αTy (produced in-house) (1:500), rabbit αTgGAP45 (1:2000) (51), αTgSP1 (1:1000), αTgSAG1 (1:10,000) (DG52 (52)), and rabbit αTgPLP1 (1:1000) (53).

**cGMP detection in extracellular parasites**

cGMP concentrations were measured with the cGMP HTRF-based competitive immunoassay (CisBio BioAssays, Bedford, MA) following the manufacturer’s instructions. The detection method utilizes binding of endogenous cGMP to disrupt the fluorescence resonance energy transfer (FRET) between Eu³⁺ cryptate-labeled anti-cGMP and d₄-labeled cGMP. For cGMP assays, parasites were snap frozen following treatment with 5 μM BIPPO or exposure to low \([\text{K}^+]\) or low pH conditions for 20 s. Between 3.5 × 10⁶ and 6 × 10⁵ cells were used per replicate. cGMP concentrations were normalized to cell number and expressed as a ratio to unstimulated parasites.

**Plaque assays**

Plaque assays were performed by inoculating confluent monolayers of HFFs in 6-well plates (Corning) with 200 tachyzoites. Where applicable, ATc was added at 1 μg/ml. The cultures were left undisturbed for 7 days, after which the monolayers were fixed with 80% ethanol and stained with crystal violet (Sigma) to visualize the plaques.

**Immunofluorescence assay**

Samples were fixed with 4% (v/v) formaldehyde in PBS for 10 min followed by permeabilization with 0.1% Triton X-100 in PBS for 10 min and blocking with 3% BSA in PBS. Cells were incubated with primary antibodies diluted in 3% BSA in PBS (Fig. 6). Secondary antibodies were diluted in IFA blocking buffer, incubated for 1 h at room temperature, and washed as above. Coverslips were immersed in PBS containing DAPI for 5 min and subsequently mounted in Vectashield™ (Vector Laboratories). To detect extracellular parasites by IFA, coverslips were immobilized with 0.1% Triton X-100 in PBS. Cells were blocked again in 3% BSA in PBS, incubated with rabbit αTgGAP45 antibody, and washed, and then counterstained with Alexa Fluor.
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488 and 594 secondary antibodies together with DAPI. Triplicate slides were visualized for each of three experiments. Four fields (field size, 1333.8 × 1580.8 μm) on each slide were observed. Parasite invasion was represented by the ratio of intracellular parasites to the sum of intracellular and extracellular parasites. The percentage of invasion was calculated by normalizing to the parental − ATc group. Tachyzoite attachment rate was determined by counting the number of host cell nuclei and calculating the normalized attachment rate relative to the number of parasites initially added.

**Replication assay**

Parasites were pretreated ±1 μg/ml ATc, and 5 × 10^5 parasites were added onto coverslips of HFFs in 24-well plates. Parasites were allowed to invade for 1 h, and extracellular parasites were washed away with PBS. Fresh D1 medium with or without 1 μg/ml ATc was added to wells. After 18 or 24 h of growth at 37 °C, parasites were fixed and stained with αTgGAP45 primary antibodies followed by Alexa Fluor 594 secondary antibodies. The proportions of vacuoles that contained 2, 4, 8, and 16 parasites/vacuole were evaluated. Triplicate slides were quantified for each of three experiments. Nine fields (field size, 1333.8 × 1580.8 μm) on each slide were scanned.

**Egress assay**

Parasites were incubated ±1 μg/ml ATc for 36 h and harvested, and 5 × 10^5 parasites were added onto confluent HFF monolayers on coverslips in 24-well plates. Parasites were allowed to grow for 24 h ± ATc before washing off extracellular parasites. Parasites were treated with 5.5 μM BIPPO or 4 μM A23187 in Ringer’s buffer for 5 min to stimulate egress and fixed by addition of 1 ml of 8% formaldehyde in PBS. Parasites were detected by IFA using αTgGAP45 antibodies. Each experimental condition was performed in technical and biological triplicates where more than 100 vacuoles were scored to evaluate the proportion of vacuoles that underwent egress.

**FACS analysis of parasite Ca^{2+} response**

GCAM6/mCherry plasmid was integrated into the uprt of tachyzoites using FUDR selection as described previously (41). TgGC cKO/GCAM6f parasites were incubated ±1 μg/ml ATc for either 36 or 84 h before scraping and needle-passing to force release from HFFs. Parasites were resuspended in ECB (141.8 mM NaCl, 5.8 mM KCl, 1 mM CaCl_2, 1 mM MgCl_2, 5.6 mM glucose, and 25 mM HEPES, pH 7.2), and 100 μl of parasites was aquired into FACS tubes to which a serial dilution of agonists was added online on a BD LSR II WT flow cytometer (BD). Aliquots were then centrifuged at 350 rpm for 5 min to pellet the parasites before resuspending in 10 ml of D1 medium and centrifuging as above. The parasites were then resuspended in D1 medium and aliquoted into tubes on ice at 4.5 × 10^5 parasites/100 μl. An equal volume of D1 medium containing one of the following conditions was added to the tubes: 1) no additive; to be raised to 37 °C, 2) 16 μM A23187, and 3) 10 μM BIPPO. The tubes were incubated at 37 °C for 20 min (except for the ice-condition tubes) before centrifuging for 3 min at 8000 rpm at 4 °C. The supernatants were removed and mixed with an equal volume of 2X SDS-PAGE sample buffer before performing Western blotting. Microneme secretion was then assayed using the LI-COR Odyssey system, and band intensities quantitated using ImageStudioLite software (LI-COR Biosciences).

**Motility assay**

TgGC cKD and WT parental tachyzoites were incubated with 1 μg/ml ATc for 30 h at 37 °C in T25 flasks before needle-passing through a 27-gauge needle and centrifuging at 350 rpm for 5 min in a Beckman GS-6KR centrifuge to pellet cellular debris. The supernatants were centrifuged at 2000 rpm for 5 min to pellet the parasites before resuspending them in fresh D1 medium. Parasites were added to HFF monolayers in wells of Ibidi 8-well plates at a multiplicity of infection of 1 parasite:5 host cells and incubated in the presence of 1 μg/ml ATc for 40 h at 37 °C. Motility was then triggered by disrupting the host cell PM by adding 0.01% saponin (final concentration) and filming over a 5-min period every 2 s on a Leica SP8 confocal platform equipped with heated stage and resonant scanner. Each parasite was then tracked using the Manual Tracker macro in ImageJ. Values were graphed and statistical tests were performed in Prism.

**Phosphatidic acid extraction and quantification**

PA was extracted and quantified as described previously (31, 54). Briefly, total lipid spiked with 25 nmol of C13:0 fatty acid was extracted by 1:2 chloroform:methanol (v/v) and 2:1 chloroform:methanol (v/v) in the presence of 0.1 M HCl. The pooled organic phase was subjected to biphasic separation by adding 0.1 M HCl and then dried under N_2 gas flux prior to being dissolved in 1-butanol. Total lipid was then separated by 2D high-performance TLC with 1 μg of PA (C17:0/17:0; Avanti Polar Lipids) using 60:35:8 chloroform:methanol:28% NH_3.OH (v/v/v) as the first dimension solvent system and 50:20:10:13:5 chloroform:methanol:acetic acid:water (v/v/v/v/v) as the second dimension solvent system (55). The spot corresponding to PA was identified according to the migration of 7.2. Aliquots of extracellular tachyzoites were then spun down, the supernatant was discarded, 100 μl of either ECB (see above) or low-pH buffer (5 mM NaCl, 142 mM KCl, 2 mM EGTA, 1 mM MgCl_2, 5.6 mM glucose, and 25 mM HEPES, pH 5.2) was added and quickly mixed, and the samples were applied to FACS.

**Microneme secretion assay**

TgGC cKO tachyzoites were incubated ±1 μg/ml ATc for 48 h at 37 °C, after which the parasites were scraped, needle-passed through a 27-gauge needle, and centrifuged at 350 rpm for 5 min to pellet cellular debris. The tachyzoites were then centrifuged at 2000 rpm for 5 min to pellet the parasites before resuspending in 10 ml of D1 medium and centrifuging as above. The parasites were then resuspended in D1 medium and aliquoted into tubes on ice at 4.5 × 10^5 parasites/100 μl. An equal volume of D1 medium containing one of the following conditions was added to the tubes: 1) no additive; to be raised to 37 °C, 2) 16 μM A23187, and 3) 10 μM BIPPO. The tubes were incubated at 37 °C for 20 min (except for the ice-condition tubes) before centrifuging for 3 min at 8000 rpm at 4 °C. The supernatants were removed and mixed with an equal volume of 2X SDS-PAGE sample buffer before performing Western blotting. Microneme secretion was then assayed using the LI-COR Odyssey system, and band intensities quantitated using ImageStudioLite software (LI-COR Biosciences).
authentic PA standard. The PA spot was then extracted for quantification of fatty acids by GC-MS (Agilent 5977A-7890B) after methanolysis (56). Fatty acid methyl esters were identified by their mass spectrum and retention time and quantified by the calibration curve generated with fatty acid methyl ester standards. Then PA content was normalized according to the parasite cell number and a C13:0 internal standard.

Acknowledgments—We are indebted to L. D. Sibley, V. Carruthers, and D. Soldati-Favre for sharing antibodies. We are most appreciative of ongoing support from WEHI’s center of dynamic imaging led by Dr. Kelly Rogers.

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