Toxoplasma gondii

Cyclic AMP-Dependent Protein Kinase Subunit 3 Is Involved in the Switch from Tachyzoite to Bradyzoite Development

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ABSTRACT

Toxoplasma gondii is an obligate intracellular apicomplexan parasite that infects warm-blooded vertebrates, including humans. Asexual reproduction in T. gondii allows it to switch between the rapidly replicating tachyzoite and quiescent bradyzoite life cycle stages. A transient cyclic AMP (cAMP) pulse promotes bradyzoite differentiation, whereas a prolonged elevation of cAMP inhibits this process. We investigated the mechanism(s) by which differential modulation of cAMP exerts a bidirectional effect on parasite differentiation. There are three protein kinase A (PKA) catalytic subunits (TgPKAc1 to -3) expressed in T. gondii. Unlike TgPKAc1 and TgPKAc2, which are conserved in the phylum Apicomplexa, TgPKAc3 appears evolutionarily divergent and specific to coccidian parasites. TgPKAc1 and TgPKAc2 are distributed in the cytomembranes, whereas TgPKAc3 resides in the cytosol. TgPKAc3 was genetically ablated in a type II cyst-forming strain of T. gondii (PruΔku80Δhxgprr) and in a type I strain (RHku80Δhxgprr), which typically does not form cysts. The Δpkac3 mutant exhibited slower growth than the parental and complemented strains, which correlated with a higher basal rate of tachyzoite-to-bradyzoite differentiation. 3-Isobutyl-1-methylxanthine (IBMX) treatment, which elevates cAMP levels, maintained wild-type parasites as tachyzoites un- }

Importance

Toxoplasma gondii is one of the most prevalent eukaryotic parasites in mammals, including humans. Parasites can switch from rapidly replicating tachyzoites responsible for acute infection to slowly replicating bradyzoites that persist as a latent infection. Previous studies have demonstrated that T. gondii cAMP signaling can induce or suppress bradyzoite differentiation, depending on the strength and duration of cAMP signal. Here, we report that TgPKAc3 is responsible for cAMP-dependent tachyzoite maintenance. In addition, the Δpkac3 mutant had a defect in the production of brain cysts in vivo, suggesting that a substrate of TgPKAc3 is probably involved in the persistence of this parasite in the intermediate host animals.

RESEARCH ARTICLE

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T. gondii, a protozoan pathogen that is a member of the phylum Apicomplexa, is found throughout the world with an estimated 30% seroprevalence in humans (1). This parasite can differentiate between rapidly replicating tachyzoites that cause acute infection and slowly growing bradyzoites found in the tissue cysts (2). Consumption of undercooked meats containing tissue cysts is a significant risk factor for transmission of T. gondii (3). There is also a sexual stage, i.e., the oocyst, which develops in cats and that can also transmit infection when it is ingested in contaminated water or food. Primary infection with this parasite during pregnancy can cause congenital infection resulting in spontaneous abortion, stillbirth, or fetopathy (4). Tissue cysts containing bradyzoites persist in the host, causing chronic infection. This latent infection can reactivate, with bradyzoites becoming tachyzoites, leading to encephalitis or other diseases, when the immune system is compromised due to HIV infection, immuno-suppressive medications, or other factors (4). A better understanding of the molecular mechanisms of parasite differentiation is needed to elucidate the pathogenesis of this infection and for the development of new therapeutic approaches to eliminate latency.

Previous reports have shown that physicochemical stress can induce bradyzoite differentiation in tissue culture (5). A shift to high pH (i.e., pH 8.2), which is widely used to induce bradyzoites, causes a short-term upregulation of cyclic AMP (cAMP) levels in parasitized cultures (6). An optogenetically induced short-term elevation of cAMP within the parasite has been demonstrated to promote bradyzoite formation (7). While a transient cAMP pulse induces bradyzoites, a prolonged induction of cAMP results in...
inhibition of differentiation (6, 7), suggesting the presence of bi-directional cAMP-induced regulatory mechanisms that may be differentially responsive to the duration or kinetics of cAMP availability.

In eukaryotic cells, cAMP binds to cAMP-dependent protein kinase A (PKA) regulatory subunits (PKArs), leading to the activation of PKA catalytic subunits (PKAcs) (8). In spite of the similarity among PKAc isoforms in an organism, they are often involved in regulating distinct pathways and responses. For example, the three PKAc isoforms of *Saccharomyces cerevisiae* work distinctly by phosphorylating specific transcription factors during nutrition starvation (9) and in response to various carbon sources (10). Previous work using H89, a small-molecule inhibitor for all of the PKAc isoforms, demonstrated that PKAcs in *T. gondii* play roles in regulating the rate of cell division (11) and bradyzoite differentiation (6, 12). In *Plasmodium falciparum*, PfPKA has been reported to regulate invasion (13), and Toxoplasma invasion has been reported to be affected by PKA signal ablation (7). The PKAc isoforms responsible for these biological functions have not been identified. Furthermore, it remains unclear whether the same PKAc isoform transduces the signal for these distinct biological functions or if different isoforms regulate these biological functions.

To better understand the various functions of the *T. gondii* PKAs, we first identified the PKA catalytic subunits in the *Toxoplasma* genome and then sought to identify catalytic subunit-specific functions in this pathogen.

**RESULTS**

The *Toxoplasma* genome encodes three putative PKA catalytic subunits. Bioinformatic searches identified three distinct PKAc subunits in the *T. gondii* genome ([http://www.ToxoDB.org](http://www.ToxoDB.org)), *Tg*PKAc1 (TGME49_226030), *Tg*PKAc2 (TGME49_228420), and *Tg*PKAc3 (TGME49_286470). Phylogenetic analysis was performed using the PKAc genes from *T. gondii*, *Plasmodium falciparum*, *Babesia bovis*, *Eimeria tenella*, *Neospora caninum*, *Perkinsus marinus*, *Cryptosporidium parvum*, *Et*, *Eimeria tenella*; *Sc*, *Saccharomyces cerevisiae*; *Hs*, *Homo sapiens*). Bootstrap confidence values above 80% are shown in red. (B) RHΔku80Δhxgprt was transfected with C-terminally HA-tagged *Tg*PKAc1, -2, and -3 expression constructs driven by the GRA1 promoter, inoculated into host cells, and incubated for 24 h. Fixed parasites were stained with anti-HA rat MAb 3F10 and anti-IMC1 rabbit antibody followed by detection with Alexa 594-conjugated anti-rat IgG goat secondary antibody or Alexa 488-conjugated anti-rabbit IgG goat secondary antibody. Nuclei were stained with DAPI. Bars, 10 μm.
multiple 6-h time points and extended time series from the David Roos Laboratory.

Next, we checked the localization of each PKA catalytic subunit by expressing hemagglutinin (HA)-tagged PKAc proteins. TgPKAc1-HA and TgPKAc2-HA were primarily detected at the parasite periphery and less prominently in the cytosol and colocalized with inner membrane complex (IMC) marker IMC1, whereas TgPKAc3-HA localized primarily to the parasite cytosol (Fig. 1B). PKA activation is usually regulated by binding of the regulatory subunit of PKA (PKAr) to PKAc. TgPKAr1 localized mainly to the parasite periphery, with increased labeling in the apical end (see Fig. S1 in the supplemental material). When parasites were liberated from host cells, extracellular parasites showed localization patterns similar to those seen with intracellular parasites, i.e., TgPKAc1 and -2 were detected at the periphery of parasites and TgPKAc3 was detected primarily in parasite cytosol (Fig. 2A). When parasites were incubated under bradyzoite culture conditions (pH 8.2, low CO₂), TgPKAc3 was seen at the periphery of parasites in a punctate pattern (Fig. 2B).

HA-tagged TgPKAc1 and a kinase domain of TgPKAc3 (TgPKAc3-Δ120Nterm) were expressed in mammalian (293T) cells to examine whether the predicted PKA catalytic domains had kinase activity. Purified TgPKAc1-HA and TgPKAc3-HA proteins bound to anti-HA columns were used for an in vitro kinase assay. Both TgPKAc1 and TgPKAc3-Δ120Nterm demonstrated cAMP-dependent kinase activity [Fig. 3, lanes “cAMP(+), inhibitor(−)”, and lanes “cAMP(−) · inhibitor(−)”. The kinase activities of both TgPKAc1 and TgPKAc3-Δ120Nterm were inhibited by the PKA-specific inhibitors H89 and protein kinase inhibitor peptide (PKI), but not by a mixture of protein kinase C (PKC)/Ca²⁺/calmodulin-dependent protein kinase (CAMK) inhibitors (Fig. 3).

Overall, these results demonstrate the presence of at least three PKAc subunits in T. gondii, of which TgPKAc3 appears to be distinct from the other two isoforms, based on its phylogenetic divergence and subcellular localization.

Generation of the TgPKAc3 mutant and complementation strains in the cyst-forming type II T. gondii strain PruΔku80ΔhxgpRT. To explore the physiological importance of TgPKAc3 for asexual reproduction and differentiation in T. gondii, we generated a TgPKAc3 null mutant (Fig. 4A) in the cyst-forming type II strain PruΔku80ΔhxgpRT, which expresses a stably integrated copy of green fluorescent protein (GFP) whose expression is driven by the bradyzoite-specific LDH2 promoter, thereby facilitating identification of vacuoles containing bradyzoites (15). Genetic deletion of TgPKAc3 by double homologous recombination-mediated integration of the hypoxanthine xanthine-guanine phosphoribosyltransferase (HXGPRT) selectable marker at the TgPKAc3 locus was confirmed by PCR screening (Fig. 4B). The subsequent knockout strain was designated PruΔku80Δpkac3. This mutant was then complemented with TgPKAc3-3HA under the control of its native promoter. Integration of the TgPKAc3-3HA expression cassette in the complemented strain PruΔku80Δpkac3::PKAc3-3HA was confirmed by PCR (Fig. 4B). The immunofluorescence assay (IFA) was performed using anti-HA antibody, and bradyzoite differentiation was monitored by examining parasites for GFP expression (Fig. 4C). TgPKAc3-3HA was expressed in both tachyzoites (GFP-negative parasites) and bradyzoites (GFP-positive parasites). The majority of the signal was in the parasite cytosol in tachyzoites, and punctate signals in the parasite periphery were observed only in bradyzoites. These distributions are consistent with what was seen with transient expression of these genes in T. gondii (Fig. 1B and 2B).

Immunoblot analysis demonstrated four distinct bands
in the complemented strain PruΔku80Δpkac3::PKAc3-3HA, which migrated close to the expected molecular mass of TgPKAc3-3HA of 60.5 kDa (the 3HA tag is ~3.3 kDa). These bands may represent splicing variants, posttranslational modifications, or proteolysis. In the total protein lysates from each strain, GFP signal was increased in the PruΔku80Δpkac3 strain (Fig. 4D), suggesting that loss of TgPKAc3 results in an increased proportion of bradyzoites under normal in vitro culture conditions (i.e., pH 7.2 and 5% CO2).

The TgPKAc3 mutant demonstrates a growth defect. To measure the effect of TgPKAc3 disruption on parasite growth throughout multiple cycles of replication, we performed plaque assays. Plaques from the PruΔku80Δpkac3 strain were not visible in this assay, and this mutant exhibited a marked growth defect compared to the parental strain (Fig. 5A), which was restored by genetic complementation with PKAc3-3HA in the PruΔku80Δpkac3::PKAc3-3HA strain. PKA signaling is reported to have a role in host cell invasion (7, 8), and therefore, invasion efficiency was measured. There was no significant difference in invasion rates among the parental, null, and complemented strains (Fig. 5B). Next, parasite replication was examined, as PKA signaling has also been reported to regulate replication (6, 16). The parasite number within a parasitophorous vacuole at 10 h was slightly decreased in the Δpkac3 strain and was recovered by complementation (Fig. 5C), whereas at 18 h postinfection (hpi), we could not find any significant differences in replication (Fig. 5D).

This result suggests that the Δpkac3 strain did not have a defect in invasion but might have had a defect in the early initiation of cell division, explaining the decrease in parasite number at the early time point. The cell division speed of Δpkac3 strain was not different from that of the wild type, and thus, the difference between the parental strain and the Δpkac3 strain did not increase over time.

TgPKAc3 knockout affects tachyzoite-to-bradyzoite differentiation in both type I and II T. gondii strains. To determine whether TgPKAc3 was involved in tachyzoite and bradyzoite regulation, we examined the rate of bradyzoite differentiation at 48 h postinfection. PruΔku80Δpkac3 had cyst wall staining even under normal culture conditions (Fig. 6A). The basal rate of bradyzoite formation in the mutant was much higher than the two control strains (80% versus 30%) when cultured under standard cell culture conditions (pH 7.2, 5% CO2) (Fig. 6B). When the CO2 concentration was dropped to 0.5% and the strains were cultured in pH 8.2 medium, the number of cyst wall-positive vacuoles increased in both the parental and mutant strains (Fig. 6B). Because PruΔku80Δpkac3 has a high differentiation rate even under normal culture conditions, it was difficult to tease out if the ablation of TgPKAc3 affected bradyzoite induction by high pH and 0.5% CO2. Therefore, we next examined the effect of Δpkac3 in the RHΔku80hxgprt T. gondii strain, which rarely differentiates into bradyzoites in vitro and would not be expected to have a high rate
of bradyzoite induction under bradyzoite culture conditions (pH 8.2, 0.5% CO₂).

The RHΔku80Δpkac3 strain was established (see Fig. S2A in the supplemental material) using the same strategy used for the PruΔku80Δpkac3 strain. RHΔku80Δpkac3 plaques were detectable (see Fig. S2B), but the plaque sizes were significantly smaller than those of parental RHΔku80Δhxgprt (see Fig. S2B). Similarly to PruΔku80Δpkac3, the RHΔku80Δpkac3 strain did not show any difference in the invasion rate and did not have a difference in its rate of cell division (see Fig. S2C and D). The RHΔku80Δpkac3 strain demonstrated cyst wall staining under normal culture conditions (~14% in RHΔku80Δpkac3), whereas the parental strain had few CST1-positive vacuoles (~1% in parental RHΔku80Δhxgprt) (Fig. 7A). To observe the effect of the bradyzoite induction in the RH strain, we treated cells with high pH without addition of CO₂ (ambient air contains ~0.02% CO₂). When the parasites were cultured under these bradyzoite induction conditions, 72% of RHΔku80Δpkac3 vacuoles were cyst wall positive, significantly greater than that seen in the parental RHΔku80Δhxgprt (13%) (Fig. 7B). These results are consistent with TgPKAc3 functioning as a negative regulator of bradyzoite differentiation under the high-pH, low-CO₂ stress conditions widely used for bradyzoite research. This also demonstrated that the function of TgPKAc3 is independent of strain lineage in T. gondii.

cAMP-dependent tachyzoite maintenance is dependent on a TgPKAc3 signal. According to previous studies, a prolonged elevation of cAMP in T. gondii maintains the growth of tachyzoites and prevents bradyzoite differentiation. Because the TgPKAc3 mutant had a defect in maintaining tachyzoites under normal culture conditions, the involvement of TgPKAc3 in cAMP-dependent tachyzoite maintenance was examined. The various T. gondii parental and mutant strains were treated with 500 μM 3-isobutyl-1-methylxanthine (IBMX), which is a phosphodiesterase inhibitor that causes a prolonged elevation of cAMP levels in T. gondii (6) and can cause bradyzoite repression (12). Consistent with our previous study on IBMX (6), cyst wall-positive vacuoles in wild-type parental T. gondii treated with IBMX decreased compared to dimethyl sulfoxide (DMSO) control-treated parasites. The Δpkac3 strain did not have a change in its differentiation rate.
with IBMX treatment (Fig. 8). The TgPKAc3 complementation restored the bradyzoite repression effect of IBMX (Fig. 8). These data suggest that cAMP-dependent tachyzoite maintenance utilizes TgPKAc3 signaling.

**Effect of the absence of TgPKAc3 on cell cycle regulation.** Bradyzoite differentiation is linked to regulation of the cell cycle in *T. gondii* (17). To further investigate how TgPKAc3 signaling could affect bradyzoite differentiation regulation, we characterized parasite cell cycle regulation in our mutants, and we quantified the population of parasites in the G1 phase during the first (8 h postinvasion) and second (16 h postinvasion) division cycles. We used a centrosome marker to estimate G1 versus S/M/C distributions to analyze asynchronous populations of parental and PKA-knockout strains (18). There was no difference in length of G1 or fractional distribution of M/C stages in the Δpkac3 strain compared to the parental or TgPKAc3-complemented *T. gondii* strain (see Fig. S3 in the supplemental material). Therefore, it appears that bradyzoite differentiation in the Δpkac3 strain is not related to a specific defect in cell cycle regulation in this knockout strain.

**Effects of TgPKAc3 deletion on parasite infection in vivo.** The effects of TgPKAc3 in the acute phase of infection were examined by infecting both RH- and Pru-derived TgPKAc3 mutant strains and performing a survival curve analysis. Under all conditions tested, the absence of TgPKAc3 did not significantly alter the survival curve of infected mice (see Fig. S4A in the supplemental material). To evaluate the effect of TgPKAc3 on in vivo chronic infection, C57BL/6J mice were infected with the Pru-derived TgPKAc3 strains. Brain cysts were quantified in survivor mouse brains at 7 weeks postinfection. The parental PruΔku80Δhxgprt, Δpkac3, and TgPKAc3-complemented strains all produced brain cysts (Fig. 9). Cyst number decreased in the PruΔku80Δpkac3 strain and returned to parental levels in the complemented strain (Fig. 9). These data suggest that ablation of TgPKAc3 reduces the cyst number in the mouse brain during latent infection.

**DISCUSSION**

Activation of PKA by a short-term cAMP pulse induces bradyzoite differentiation, whereas a prolonged cAMP pulse inhibits differentiation (7). It is likely that there are distinct PKA signaling pathways in the tachyzoite with opposing effects on parasite differentiation. Inhibition of PKA signals by treatment with PKA catalytic subunit inhibitor H89 induces bradyzoite differentiation (11), suggesting that PKA catalytic subunit activity may be involved in cAMP-mediated tachyzoite maintenance. The PKA catalytic isoform responsible for this effect could not be identified, as H89 treatment inhibits multiple PKA catalytic subunit isoforms. Our study provides the first insight into the mechanism by which cAMP signaling can regulate both bradyzoite induction and tachyzoite maintenance. The divergent PKA isoform TgPKAc3 has a unique biological role in *T. gondii* in cAMP-dependent tachyzoite maintenance.

Bradyzoite gene expression and cell division regulation are distinct events. Unlike its parental strain, the TgPKAc3 mutant...
Collectively, these data suggest that the Δpkac3 strain lacks the coordination between bradyzoite gene expression and cell division regulation that has been previously described. We propose a new bradyzoite differentiation model in which bradyzoite gene expression and cell cycle regulation are separately, but coordinately, regulated and integrated by specific signals, including the TgPKAc3 signal. Proper cAMP signaling appears to be important for switching between tachyzoites and bradyzoites. The Δpkac3 strain had fewer brain cysts, which could imply that integrated bradyzoite differentiation is also required for efficient production or maintenance of the cyst number in vivo in the mouse brain and other tissues. Stage-specific antigen expression regulation is known to regulate the immunogenicity of the bradyzoite antigens (20). The effects of antigen presentation from early bradyzoite gene expression during dissemination may be involved in the reduction of cyst numbers seen in the Δpkac3 strain. Alternatively, Δpkac3 strains may not readily disseminate to the brain.

Examination of published phosphoproteome data (available on http://www.ToxoDB.org) for proteins with PKA motif [R/K][R/K][X]phos[S/T] [see full gene identifier [ID] list in Table S1 in the supplemental material] reveals several AP2 transcription factor family members with PKA phosphorylation sites, including AP2XI-4, which has been previously described as a regulator of bradyzoite gene expression (21). Enzymes involved in epigenetic modification, such as TgGCN5b (TGGT1_243440) (22) and candidate histone lysine methyltransferases (TGGT1_000420, TGGT1_054420, and TGGT1_087910) also have phosphorylated PKA motifs (see Table S1). These putative PKA substrates suggest that parasite PKA signaling may regulate both transcription and chromatin structure to regulate genes important for maintenance of the tachyzoite state. PKA regulation of developmental transitions is a universal theme in eukaryotic biology, and PKA signaling in the budding yeast transduces the PKA catalytic subunit-specific signals by targeting a transcriptional factor (9).

Possible regulatory mechanisms of PKA in Toxoplasma. Both TgPKAc1 and TgPKAc3 have in vitro cAMP-dependent protein kinase activity. This suggests that both proteins have a capacity to interact with canonical PKAr (i.e., the mammalian PKAr in HEK293T cells which expressed the parasite PKAc). TgPKAc1 expressed in wheat germ cell extract has been reported to be less susceptible to PKI and H89 than bovine PKA expressed in wheat germ extract (11). We were unable to produce active PKAc in bacterial systems. Due to the known issues in expressing fully active recombinant PKA, the in vitro kinase activity of immunoprecipitated TgPKAc complex may differ from recombinant protein expressed in vitro using wheat germ cell extract. While the recombinant protein kinase domain of TgPKAc3 was demonstrated to be active using an in vitro kinase assay, TgPKAc3 has a large N-terminal domain that may be involved in the function of TgPKAc3. Notably, TgPKAc3 demonstrated a change in localization between tachyzoite (primarily diffuse in the cytosol) and bradyzoite (punctate distribution in the periphery of the parasite) conditions. Localization changes in PKA catalytic subunits have been reported, as a regulatory system for PKA activity in mammalian cells and localization of the PKA complex to subcompartments in cells are known to be regulated by diverse members of A kinase anchor protein (AKAP) (23). Although T. gondii lacks obvious AKAP homologs, Apicomplexa-specific atypical scaffolding proteins could act to regulate PKA activity. Overexpression of TgPKAc3 caused a delay in tachyzoite replication (K. Kim and

FIG 6 Disruption of TgPKAc3 induces bradyzoite-specific cyst wall formation. The cyst wall was stained to measure the bradyzoite differentiation status in the parental parasite PruΔku80Δhxgprt (Parent), knockout clone PruΔku80Δpkac3 (PKAc3KO), and complemented clone PruΔku80Δpkac3::PKAc3-3HA (COMP). Infected host cells were grown under normal culture conditions or bradyzoite induction conditions (pH 8.2 and CO2 depletion) for 48 h. Parasites were stained with anti-CST1 antibody (shown in green) or anti-Toxoplasma serum (shown in red). (A) Representative images of vacuoles with cyst walls (center panel, PruΔku80Δpkac3) and without cyst walls (left and right panels, showing PruΔku80Δhxgprt and PruΔku80Δpkac3::PKAc3-3HA, respectively) under tachyzoite culture condition. (B) Cyst wall-positive vacuoles were identified as parasitophorous vacuoles that have anti-CST1 signal associated with the parasitophorous vacuole. Quantitative measurements of the cyst wall-positive vacuole rate are shown. At least 100 total vacuoles per sample were counted, and cyst wall-positive vacuoles per total vacuoles are shown. Mean values and standard deviations from independent triplicate experiments are shown. If one-way analysis of variance detected significant differences among the group, Tukey’s honestly significant difference test results are shown. *, P < 0.05; **, P < 0.01; n.s., nonsignificant.

does not exhibit coregulation of cell division and bradyzoite gene expression. Previous studies with wild-type T. gondii have shown that in vivo tissue cyst and in vitro stress-induced bradyzoites have concurrent bradyzoite gene expression and cell division slowing (16, 19), implying a cell cycle regulation different from that of tachyzoites (17). Although we observed increased expression of bradyzoite differentiation markers (cyst wall and GFP signal driven by bradyzoite-specific LDH2 promoter) under normal tachyzoite growth conditions, the cell division rate of Δpkac3 parasites was not significantly different from that of the parental or complemented strains. Cell cycle analysis also detected no differences between wild type and mutants (see Fig. S3 in the supplemental material) (17). The Δpkac3 strain did not have the clear G2/G1 peak seen in fully mature bradyzoites from in vivo tissue cysts (17).
M. S. Eaton, unpublished data), also suggesting that regulation of the activity of \( \text{TgPKAc3} \) is required for proper cell cycle progression.

**Summary hypothesis.** We hypothesize that \( \text{TgPKAc3} \) functions to prevent bradyzoite gene expression and to keep tachyzoites replicating as tachyzoites. Several bradyzoite repressor genes in *Toxoplasma* have now been reported, including transcription factor AP2IX-9 (24) and *T. gondii* serine protease inhibitor 1 (\( \text{TgSPI1, TGME49_208450} \)) (25). Both of these proteins repress bradyzoite differentiation under high-pH (8.2) conditions; hence, they are expected to function in early bradyzoite stages (22, 23). In contrast, the \( \Delta \text{pkac3} \) strain has high rates of bradyzoite formation even under the conditions that normally promote tachyzoite replication. Therefore, it is probable that \( \text{TgPKAc3} \) inhibits bradyzoite differentiation constitutively by maintaining tachyzoites. Spontaneous tachyzoite-to-bradyzoite differentiation and cell division speed changes have been noted using *in vitro* tissue cultures infected with VEG strain sporozoites (19), suggesting that bradyzoite differentiation is an innate programmed response and that spontaneous differentiation to bradyzoites is based on the number of cell divisions. In this model, the persistence of tachyzoites (i.e., inhibition of bradyzoites) is an active process that requires specific gene regulation and active signaling in the parasite. Further investigations of active tachyzoite maintenance signals are clearly needed to fully understand bradyzoite differentiation and regulation in this important apicomplexan.

**MATERIALS AND METHODS**

**Sequence analysis.** \( \text{TgPKAc1} \) (TGME49_226030), \( \text{TgPKAc2} \) (TGME49_228420), and \( \text{TgPKAc3} \) (TGME49_286470), which were predicted to have

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**FIG 7** \( \text{TgPKAc3} \) affects bradyzoite differentiation in the RH strain. The cyst wall was stained to measure the bradyzoite differentiation status in the parental parasite RH\( \Delta \text{ku80Δhxgprt} \) (Parent) and knockout clone RH\( \Delta \text{ku80Δpkac3} \) (RH \( \Delta \text{pkac3} \)). Infected host cells were grown under normal culture conditions (A) or bradyzoite induction conditions (pH 8.2 and complete CO\(_2\) depletion) (B) for 48 h. Parasites were stained with anti-CST1 antibody and anti-*Toxoplasma* serum. Cyst wall-positive vacuoles were identified as parasitophorous vacuoles that have anti-CST1 signal associated with the parasitophorous vacuole. Quantitative measurements of the cyst wall-positive vacuole rate are shown. At least 100 total vacuoles per sample were counted, and cyst wall-positive vacuoles per total vacuoles are shown. Mean values and standard deviations from independent triplicate experiments are shown. Statistical differences determined by Student’s \( t \) test between parent and \( \text{TgPKAc3KO} \) are shown (**, \( P < 0.01 \)).

**FIG 8** *Long-term elevation of cAMP causes tachyzoite maintenance via \( \text{TgPKAc3} \) signal.* After a 2-h invasion window, parasites were treated with IBMX (500 \( \mu \)M final concentration) or vehicle control DMSO for 48 h. The cyst wall was stained to measure the bradyzoite differentiation status in the parental parasite Pru\( \Delta \text{ku80Δhxgprt} \) (Parent), knockout clone Pru\( \Delta \text{ku80Δpkac3} \) (PKAc3KO), and complemented clone Pru\( \Delta \text{ku80Δpkac3::PKAc3-3HA} \) (COMP). Cyst wall-positive vacuoles were identified as parasitophorous vacuoles that have anti-CST1 signal associated with the parasitophorous vacuole as shown in Fig. 5A. At least 100 total vacuoles per sample were counted, and percentages of cyst wall-positive vacuoles per total vacuoles are shown. Mean values and standard deviations from independent triplicate experiments are shown. Student’s \( t \) test between DMSO and IBMX treatment was performed (*, \( P < 0.05 \); **, \( P < 0.01 \)).

**FIG 9** *In vivo cyst production with \( \text{TgPKAc3} \) mutant parasites.* Six- to 8-week-old female C57BL/6J mice were infected with 10,000 parasites via the intraperitoneal route. Seven weeks after infection, the tissue cyst number in the infected mouse brain was counted. The average numbers of cysts and standard deviations (\( n = 4 \) for wild type [WT], \( n = 3 \) for knockout [KO], and \( n = 4 \) for complemented) are shown. Mean values were statistically analyzed with one-way analysis of variance for detecting a difference in the groups and with a post hoc Tukey honestly significant difference test for pairwise comparison. n.s., \( P > 0.05 \); *, \( P < 0.05 \); **, \( P < 0.01 \).
TABLE 1  Primers used in the present study

| Primer name | Sequence (5′→3′)* | Research objective |
|-------------|-------------------|--------------------|
| TGME49\_286470\_3UTR\_F | TTGGTGAGCCAGCGGTTTCGCCAGTCGAGGCTTTAAACCAGAACTAGACATAAGACAGACGC | KO vector |
| TGME49\_286470\_5UTR\_R | GCCGGTTAGTGCAAGTTGCCTGTGCAACTGCTGGCTGCTCTGCTGACAT | KO vector |
| TGME49\_286470\_3UTR\_R | TTTGCTGAGCTAAGTGACCCGGGTGAGGACTAGTTGAGCGCAAGGTTGATGACAGGCTTCT | KO vector |
| TGME49\_286470\_3UTR\_F | GTGAGCGGATAACATTTACACAGGAAGACCGGCAAGGAGCATACGAGGAGTC | KO vector |
| KO\_F | AGAGAAGACCGCTTCCGCAAG | KO check |
| KO\_R | ACTGGGAACAGACGCAAGAT | KO check |
| WT\_F | AGAGAATAGACGGGAAAGA | WT check |
| WT\_R | GAATGGTTGTGTGATGAGA | WT check |
| COMP\_F | TGATTAGGGCAAGCTCAGGAAAGAGAAGACCTTCCGCAAG | Complement vector, complement check |
| CDS\_R | CGTGCTACGGGTGACATTCAAAATTGTGCAAGAAGGCTGTCG | Complement vector, complement check |
| pLIC-3HA-F | AGGGTACCCGTACGACGTC | Complement vector, complement check |
| pLIC-3HA-R | TTCTGAGCTTGGCGTAATCA | Complement vector, complement check |
| COMP\_R | CACCACTTCTGGTGACTAT | Complement check |

* Underlined sequences were used for ligation-independent cloning.

an STKc-PKA-like domain (cd05580) with the NCBI conserved domain database (26), were retrieved from ToxoDB. Additional protein kinase domains were retrieved using a BLAST search employing TgPKAc1, -2, and -3 as the query sequence. Genes of Toxoplasma, Neospora, and Eimeria were retrieved from ToxoDB. Genes of Homo sapiens and Saccharomyces cerevisiae (strain ATCC 204508/S288c) were retrieved from UniProt. Other genes were retrieved from NCBI GenBank. The protein kinase domain predicted with a conserved domain search (26) was used for alignment and phylogenetic analysis. Alignment and phylogenetic analysis were performed with MEGAS (27). Phylogenetic relationships were then inferred by using the maximum likelihood method based on the Le-Gascuel 2008 model (28). An initial tree(s) for the heuristic search was obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using a JTT model. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 1.4834]). Amino acid positions that are conserved in more than 90% of proteins were used for calculation. There were a total of 287 positions in the final data set. Five thousand bootstrap replications were used to estimate the percentage of trees in which the associated taxa clustered together.

Cell culture. Human foreskin fibroblasts (HFF) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (Life Technologies). The T. gondii RHΔku80Δhxgprt (29) strain was used in the overexpression study, and the PruΔku80Δhxgprt strain, which expresses GFP driven by the bradyzoite-specific LDH2 promoter (15), and RHΔku80Δhxgprt were used in the TgPKAc3 knockout experiments. Parastites were harvested from HFF cells cultured under a CO2-depletion condition for 2 days before fixation. Fixed cells were stained as described in the immunofluorescence assay section with anti-HA monoclonal antibody (MAb) 3F10 (Roche, Basel, Switzerland), rabbit anti-GAP45 antibody (30) (a generous gift from Dominique Soldati) in a 1:3,000 dilution, anti-IMC1 rabbit antibody (a generous gift from Michael White) in a 1:250 dilution, and anti-GFP rabbit antibody (Life Technologies, Carlsbad, CA) in a 1:500 dilution.

Heterologous expression of kinase domain of TgPKAc1 and -3. C-terminally HA-tagged TgPKAc1 coding sequence and the kinase domain of TgPKAc3 (TgPKAc3Δ120) were cloned into the pDNA3 mammalian expression vector. Two micrograms of DNA was used to transfect 293T cells cultured in a 6-well plate with FuGene (Roche). Transfected cells were harvested 48 h later to purify the HA-tagged PKAc. Three wells of transfected 293T cells were lysed in 1.5 ml lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl [pH 8.0], and protease inhibitor cocktail [Roche]) for 30 min on ice. Cells were centrifuged for 10 min at 13,000 rpm, and supernatants were used as lysates. One hundred fifty microliters of anti-HA antibody conjugated to magnetic beads (Miltenyi Biotec, San Diego, CA) was added to cell lysates and incubated on ice for 30 min. Antibody-bound fraction was purified according to the manufacturer’s instruction with the μMACS isolation kit (Miltenyi Biotec). The protein kinase assay was performed with the protein bound to the column directly with a protein kinase A assay kit (Millipore, Billerica, MA). The reaction was carried out in a 25-μl reaction volume at 37°C for 10 min and stopped by spotting the reaction mixture onto P81 phosphocellulose paper. Filter paper was extensively dried for 30 s, washed 3 times with 0.75% H3PO4, and washed once with acetone. Incorporation of radioactive 32P to the phosphorylated PKA substrate peptide Kemptide was measured by scintillation counter.

TgPKAc3 knockout and complementation. The knockout construct was built as previously described (31). Briefly, 1-kbp upstream and downstream genomic DNA sequences of TgPKAc3 (TGME49\_286470) were amplified from the genomic DNA from PruΔku80Δhxgprt. These fragments were concatenated into pRS416 yeast shuttle vector flank the selectable marker hygromycin phosphotransferase (HXGPRT) cassette using S. cerevisiae ATCC 90845 to make a ΔPKAc3 vector. All the primers used in the plasmid construct are listed in Table 1. Toxoplasma gondii PruΔku80Δhxgprt was transplanted with linearized ΔPKAc3 vector as previously described (31) and selected in the presence of 25 μg/ml mycophenolic acid and 30 μg/ml xanthine. Incorporation of HXGPRT selectable marker at the TgPKAc3 locus was verified by PCR, and the parasites were subcloned by limiting dilution. Confirmation of the knockout was done using primer pairs detecting integration sites (KO\_F and KO\_R) and primers detecting the wild-type locus (WT\_F and
WT_R) as depicted in Fig. 4A. For the knockout of TgPKAc3 in RHku80Δhxgprt, the same strategy was used, except that the knock-out plasmid was prepared from the genomic DNA (gDNA) of RHku80Δhxgprt (see Text S1 in the supplemental material).

For the complementation of TgPKAc3-KO strain, a 4-kbp genomic locus of TgPKAc3 including 2,279 bp upstream from the predicted start codon and the whole coding sequence except a stop codon was amplified from the gDNA of PruΔku80Δhxgprt and cloned into the pLIC-3HA-DHFR plasmid (generous gift of Michael White), to produce a C-terminal 3HA fusion, TgPKAc3, which was designated pPKAc3-3HA-DHFR (Fig. 3A). Five micrograms of pPKAc3-3HA-DHFR was linearized with the NotI restriction enzyme and nucleofected as described previously (32). Nucleofected parasites were selected with 1 μM pyrimethamine and subcloned by limiting dilution. The presence of the expression cassette for TgPKAc3-3HA was confirmed with PCR with primers COMP_F and COMP_R.

Immunofluorescence assay (IFA). HFF cells grown on coverslips were infected with T. gondii and incubated for 48 h. Infected host cells were rinsed with ice-cold phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were treated with PBS containing 0.2 M glycine and 0.2% Triton X-100 for 20 min to quench the fixation and to permeabilize the membrane. Cells were washed with PBS three times and blocked with 2% bovine serum albumin in PBS with 0.1% Tween 20 (blocking buffer) for 60 min at 37°C. Antibodies were diluted in the same blocking buffer and incubated for 60 min at 37°C followed by washing three times with PBS with 0.1% Tween 20 (PBS-T). Rat monoclonal antibody 3F10 (Roche), which is a primary antibody for the HA epitope tag, was used at a 1:250 dilution. Alexa 594-conjugated anti-rat IgG goat secondary antibody (Life Technologies) was used at a 1:1,000 dilution. To enhance GFP signal, GFP conjugated with horseradish peroxidase (HRP) (Roche), anti-GRA1 monoclonal antibody (M. White, unpublished data). IMC7 reportedly localizes to mature mother cell inner membrane complex (35). At the same time, anti-GFP antibody was used to label the bradyzoite parasites. At least 100 total parasitophorous vacuoles were counted for one sample, and the number of parasites per vacuole was calculated.

Bradyzoite differentiation assay. Bradyzoite differentiation was measured by staining the cyst wall. Briefly, purified parasites were inoculated onto HFF cells in a 24-well plate at a concentration of 1,000 parasites/well. After a 2-h incubation, the medium was changed to fresh medium and incubated under a 5% CO2 condition (normal) or was changed to induction medium (DMEM supplemented with 1% FBS and 50 mM HEPES, adjusted to pH 8.2 with NaOH) and incubated under an 0.5% CO2 condition (pH 8.2 and CO2 depletion). For the bradyzoite induction with the RH strain, ambient air with a humid box was used; thus, CO2 concentration was around 0.02%. Infected host cells were extensively incubated for 48 h, fixed with 4% paraformaldehyde in PBS, and stained as described for the immunofluorescence assay. Salmon E monoclonal antibody (1:500) for cyst wall protein 1 (CST1) (31) and 1:4,000-diluted rabbit anti-Toxoplasma serum were used as primary antibodies to detect cyst wall and total parasites, respectively. Alexa 488- and 594-conjugated anti-mouse or -rabbit IgG antibody diluted 1:1,000 was used as a secondary antibody. Green fluorescent signal at the parasitophorous vacuole membrane was identified as the cyst wall. At least 100 vacuoles were scored to determine the number of parasitophorous vacuoles with or without cyst walls.

Animal experiments. Ten female 6- to 8-week-old C57BL/6j mice (The Jackson Laboratory, Bar Harbor, ME) per group were infected with purified tachyzoites intraperitoneally. For the brain cyst burden determination, at 7 weeks postinfection surviving mice in each group were euthanized and brains were collected for the brain cyst counting. Brains were cut in half, and one half was homogenized in PBS to a final volume of 600 μl for each brain. Samples of 60 μl were counted, and total cyst counts per brain were calculated.

Ethics statement. All animal experiments were conducted according to the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals. Animals were maintained in an AAALAC-approved facility, and all protocols were approved by the Institutional Care Committee of the Albert Einstein College of Medicine, Bronx, NY (Animal Protocols 20121104, 20121109, and 20121110; Animal Welfare Assurance no. A3312-01). No human samples were used in these experiments. Human foreskin fibroblasts were obtained from the ATCC.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00755-16/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.

Figure S1, TIF file, 1.9 MB.

Figure S2, TIF file, 0.5 MB.

Figure S3, TIF file, 0.1 MB.

Figure S4, TIF file, 0.2 MB.

Table S1, XLSX file, 0.1 MB.
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