Optogenetic modulation of an adenylate cyclase in Toxoplasma gondii demonstrates a requirement of the parasite cAMP for host-cell invasion and stage differentiation

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Running head: Optogenetic control of cAMP in asexual stages of Toxoplasma

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Background: cAMP research in intracellular parasites remains underappreciated, and requires a specific method for cyclic nucleotide regulation.

Results: Optogenetic induction of cAMP in T. gondii affects host-cell invasion, stage-specific expression and parasite differentiation. The underlying method allows a versatile control of parasite cAMP.

Conclusions: Optogenetic parasite strains offer valuable tools for dissecting cAMP-mediated processes.

Significance: The method is applicable to other gene-tractable intertwined systems.

ABSTRACT

Successful infection and transmission of the obligate intracellular parasite Toxoplasma gondii depends on its ability to switch between fast-replicating tachyzoite (acute) and quiescent bradyzoite (chronic) stages. An induction of cAMP in the parasitized host cells has been proposed to influence parasite differentiation. It is unknown whether the parasite or host cAMP is required to drive this phenomenon. An unequivocal research on cAMP-mediated signaling in such intertwined systems also requires a method for an efficient and spatial control of the cAMP pool in the pathogen or in the enclosing host cell. We have resolved these critical concerns by expressing a photo-activated adenylate cyclase that allows a light-sensitive control of the cytosolic cAMP in T. gondii, or in its human host cells. Using this method, we reveal multiple roles of the parasite-derived cAMP in host-cell invasion, stage-specific expression and asexual differentiation. An optogenetic method provides many desired advantages such as, it (i) allows rapid, transient and efficient induction of cAMP in the extracellular/intracellular and acute/chronic stages; (ii) circumvents difficulties often faced in cultures \textit{i.e.,} poor diffusion, premature...
degradation, steady activation and/or pleiotropic effects of cAMP agonists and antagonists; (iii) involves genetically-encoded enzyme expression, hence, inheritable to the progeny; and (iv) allows conditional and spatiotemporal control of cAMP. Importantly, a successful optogenetic application in Toxoplasma also illustrates its wider utility to many other genetically amenable two-organism systems such as, symbiotic and pathogen-host interactions.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite of nearly all vertebrates. Other related parasites of medical and veterinary importance include Plasmodium, Eimeria, Cryptosporidium, Neospora, Babesia and Theileria. It causes ocular and cerebral toxoplasmosis in individuals with immune dysfunction, and in developing fetuses and neonates. The parasite also inflicts spontaneous abortions in animals, thus imposes an economic burden (1). In addition, T. gondii serves as a widely used model to understand pathogen-host interactions and protozoan development. Its type-I strains exist mainly as fast-replicating tachyzoite stage, and cause tissue necrosis (acute infection); while, type-II strains can also form tissue-dwelling bradyzoite cysts, which persist for the entire life of the host (chronic infection). Successful infection and transmission of Toxoplasma relies on multiplication, persistence and inter-conversion of these two asexual stages (2).

Cyclic nucleotides, cAMP and cGMP, are universal regulators of cell signaling. They are generated from ATP or GTP by the catalytic action of adenylate cyclase or guanylate cyclase, respectively. The adenylate cyclases involved in cellular signaling belong to the class III; some are membrane-bound, and others are cytosolic in metazoans (3). The membrane-bound isoforms are commonly regulated by G-proteins in response to external stimuli, whereas the soluble ones respond to intracellular signals, such as calcium and bicarbonate levels (3). The most prominent examples of cAMP-activated proteins include protein kinase A (PKA), transcription factors (e.g., CREB-1) and cAMP-gated ion channels (4-6). Upon activation, PKA, for instance, can phosphorylate its target proteins, and exerts numerous effects such as, gene modulation and ion conductance. Phosphodiesterases degrade cAMP to counter-regulate cAMP-mediated signaling (7). The affinities of such cAMP signaling-associated proteins e.g., PKA and phosphodiesterase for cAMP range in nM to μM amounts, and its cellular levels are strictly regulated (4-8). It has been shown that an activator of adenylate cyclase, forskolin, can exert a transient rise in cAMP levels of Toxoplasma-infected cells, and induces bradyzoite formation (9,10). In contrast, a membrane-permeable and non-hydrolysable analog of cAMP and phosphodiesterase inhibitors can cause a persistent activation of cAMP-regulated host/parasite signaling, and
reduce bradyzoite differentiation (9,10). It is however debatable whether the parasite or host cAMP determines the stage switching in *T. gondii*. In addition, other possible roles of cAMP described in *Plasmodium* e.g., host-cell invasion (11,12) have not been identified in *T. gondii* yet.

Typically, the study of cAMP-mediated pathways depends on chemical activators and inhibitors affecting protein kinases and phosphodiesterases, as well as on membrane-permeable analogs of cyclic nucleotides. The use of such commercial modulators, targeting primarily the mammalian proteins, is not appropriate to examine cAMP signaling in intracellular pathogens. A concomitant and pleiotropic action of these drugs on the host cell and on the enclosed pathogen often obscures the interpretation of results. An unambiguous and comprehensive research on cAMP signaling in two-organism systems, such as pathogen-infected host cells or in symbiotic models therefore requires specific, efficient and spatiotemporal control of the cellular cAMP levels within individual partners. Non-invasive control of biological processes by photo-activated molecules has become a common method in recent years (13). Optogenetic tools have gained a significant momentum following the discovery of channelrhodopsin that allows modulation of the membrane voltage, a general parameter applicable to essentially all cells (14). Its wide application has stimulated the demand for the photo-regulatable proteins to modulate general regulators such as, cyclic nucleotides and inositides (15,16). Recently, a photo-regulated and soluble adenylate cyclase from a lithotrophic bacterium *Beggiatoa* has been reported (17,18). We utilized this bacterial adenylate cyclase to appreciate the roles of cAMP in the asexual stages of *T. gondii*, and to establish its application in a model intracellular pathogen.

**MATERIALS AND METHODS**

*Biological reagents*

All cell culture media and additives were purchased from PAA and Sigma-Aldrich (Germany). The XL-1b strain of *E. coli* (Stratagene) was used for molecular cloning and vector amplification. RNA isolation, cDNA synthesis and plasmid preparations were performed using commercial kits (Life Technologies and Analytik Jena). DNA-modifying enzymes and oligonucleotides were obtained from NEB and Invitrogen. The primary (anti-Myc, anti-GFP) and secondary (Alexa488/Alexa594-conjugated) antibodies were from Sigma-Aldrich and Life Technologies. Anti-*Tg*Gap45 and anti-*Tg*Actin antibodies and the *TaTi* (type-I) strain (19) of *T. gondii* were donated by Dominique Soldati-Favre (University of Geneva, Switzerland). Anti-*Tg*Sag1 antibody was a donation of Jean-François Dubremetz (University of Montpellier, France). The bradyzoite reporter type-II strain (*Pru-*Δ*ku80*-Δhxgprt/*pTg*LDH2-GFP) was provided by Louis Weiss (Albert Einstein College of Medicine, US) and David J. Bzik.
The pTKO vector was a donation of John Boothroyd (Stanford University, US).

Parasite culture and tachyzoite assays
Human foreskin fibroblast (HFF) cells were cultivated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, MEM nonessential amino acids, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator (37°C, 5% CO₂). Type-I and type-II strains of T. gondii were used to infect confluent HFF monolayers at a multiplicity of infection of 3-4, and passaged every 2-3 days, unless stated otherwise. For invasion assay, syringe-released parasites (40 hrs of infection) were used to infect HFF cells grown on a coverslip (MOI, 10; 37°C; 1 hr). Samples were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde (2 min), and neutralized in 0.1 M glycine/PBS (5 min). They were blocked in 3% BSA/PBS and stained with α-TgSag1 (1:1500, 1 hr) antibody. Samples were washed 3x with PBS, permeabilized in 0.2% Triton-X100/PBS (20 min) and stained with α-TgGap45 (1:3000, 1 hr). They were washed again, and incubated with Alexa488-/Alexa594-conjugated antibodies (1:3000, 1 hr). The fraction of invaded parasites was determined by counting the parasites labeled with α-TgGap45/Alexa594 (red) but not with α-TgSag1/Alexa488 (green) antibodies (22).

Molecular cloning and genetic manipulation of T. gondii

The full-length ORF of bPAC was amplified from a pET28a expression plasmid using PfuUltraII fusion polymerase and indicated primers (Table 1). The bPAC-GFP under the control of the TgGRA2 regulatory elements was cloned at the NsiI restriction site into the pTKO-DHFR-TS-GFP plasmid. To generate the ddFKBP-bPAC-Myc construct, bPAC-Myc was cloned (NsiI/Pacl) into the pTKO-DHFR-TS vector. In a second step, the ddFKBP fragment was inserted at the NsiI and PstI restriction sites. The PCR primers, cloning strategy and restriction sites are described in Table 1. Fresh extracellular parasites (~10⁷) of the indicated strains were transfected with the expression construct (50 μg) dissolved in cytomix solution using a BTX630 instrument (1.7 kV, 50 Ohm, 25 μF, 250 μs). The transfected parasites were selected for their resistance to 1 μM pyrimethamine (23), and cloned by limiting dilution in 96-well plates.

Indirect immuno fluorescence analysis
The parasitized HFF monolayers grown on glass coverslips for 24-72 hrs were washed with PBS and fixed with 4% paraformaldehyde for 20 min, followed by neutralization in 0.1 M glycine/PBS (5 min). Cells were permeabilized with 0.2% Triton-X-100/PBS (20 min), and nonspecific binding was blocked with 2% BSA in 0.2% Triton-X-100/PBS for 30 min. Samples were then stained using anti-Myc (1:1000, mouse or rabbit), anti-GFP (1:5000, rabbit), anti-TgActin (1:1000, mouse), anti–TgGap45 (1:3000, rabbit),
or anti-TgSag1 (1:1500, mouse) antibodies for 1 hr. Alexa488- or 594-conjugated fluorescent antibodies (anti-rabbit or anti-mouse, 1:3000) were used as secondary antibodies for 45 min. Samples were mounted with Fluoromount G, and imaged by a Zeiss Apotome microscope (Zeiss, Germany).

*Photo-activation and cyclic AMP measurements*

Tachyzoite-infected HFF monolayers (MOI, 1-3) were scraped 24-72 hrs post-infection, and the parasites were mechanically released by passing the samples through 22G and 27G needles. The host-free parasites were washed 2x with intracellular-type medium (ICM; 400g for 10 min). The ICM (pH 7.4) contained 20 mM HEPES, 140 mM KCl, 10 mM NaCl, 2.5 mM MgCl₂, 5 mM glucose, 0.1 µM CaCl₂, 1 mM sodium pyruvate, MEM vitamins, MEM amino acids and nonessential amino acids. A defined number of host-free parasites (0.5-5 x 10⁵) was suspended in 100-125 µl of ICM containing 1 mM ATP and incubated at 37°C. Photo-activated samples were exposed to 455 nm light (LED Royal Blue, 100 mW/mm²) for the indicated duration. For cAMP assays with intracellular parasites, infected monolayers (MOI, 1; 24-72 hrs infection) were washed 2x with ICM, and the cells were covered with 0.5 ml of pre-warmed ICM containing ATP (1 mM) prior to light exposure. To measure cAMP, extracellular parasites or the parasitized cells were lysed by addition of 96% ethanol (final concentration, 65%) and stored on ice for 5-10 min. The extracts were centrifuged at 2000g for 15 min at 4°C, and the supernatants were lyophilized, and stored at -20°C. The cAMP levels were quantified using a kit and internal standards (cAMP Biotrak Enzyme immunoassay, GE Healthcare). The measurements of biological replicates in a given assay were executed simultaneously with suitable controls and standards to minimize plate-to-plate deviation. The cAMP variations between individual assays are most likely due to the parasite strains, state of culture (extracellular, intracellular), multiplicity of infection and infection periods.

*Stage differentiation in type-II T. gondii*

To assess bradyzoites, we stained the cyst wall containing glycoproteins (such as, CST1) with biotinylated *Dolichos biflorus* lectin (1:500; Vector Labs, US) and Alexa594/Alexa488-conjugated streptavidin (24). We also scored bradyzoite-specific (pLDH2-regulated) GFP fluorescence in the type-II reporter strain of *T. gondii* (20,21). No apparent differences in bradyzoite assessment were observed when using lectin or GFP staining. The parasites (0.5-1x10⁵) were allowed to infect HFF monolayers (on the coverslips placed in 24-well plates) for 3 days prior to fixation and immuno-staining. Tachyzoites were visualized by anti-TgSag1 staining. *Dolichos biflorus* lectin or anti-GFP antibody was used to detect bradyzoites. The parasite vacuoles with dual fluorescence (transition stages) were considered as differentiating bradyzoites.
Bradyzoite differentiation in bPAC-expressing human fibroblasts

Confluent monolayers were transfected with a bPAC-mCherry fusion construct (pCMV-bPAC-mCherry) using Lipofectamine 2000 (Life Technologies; transfection efficiency ~25%). Total cAMP was extracted 28 hours post-transfection (+/-light, 455 nm, 2 min) and assayed, as described above. To test the influence of host-derived cAMP on the parasite differentiation, transgenic host cells (24 hrs post-transfection) were grown on coverslips in 24-well plate and infected with a type-II strain of T. gondii (5000 parasites/well). The parasitized cells were exposed to light (455 nm, 2 min) 4 hrs post-infection. Samples were incubated for an additional 48 hrs to allow stage conversion, and then fixed with 4% paraformaldehyde. Bradyzoites were stained with Dolichos biflorus lectin, and quantified in the mCherry-positive transgenic host cells.

RESULTS

Expression of bPAC in T. gondii allows an optogenetic induction of parasite cAMP

To examine whether a photo-activated adenylate cyclase from Beggiatoa (bPAC) can be functionally and stably expressed in T. gondii tachyzoites, we transfected a type-I strain with the pTgGRA2-bPAC-GFP construct expressing a fusion enzyme with green fluorescent protein (bPAC-GFP). The bPAC protein was detected in the parasite cytosol, as deduced by immunolocalization with a known cytosolic marker, actin (25) (Fig 1A). We tested these parasites for their ability to elevate the cAMP level in response to light. To this end, we first analyzed fresh extracellular parasites of the transgenic and parental strains to avoid the extraneous background of host-cell cAMP. We measured a temporal effect of light exposure (455 nm) on the cAMP levels in the transgenic and parental parasites, which revealed an exposure time-dependent increase of cAMP pool in the bPAC-expressing strain (Fig 1B). In comparison to the parental parasites, the transgenic strain showed about 4.6-fold higher amount of cAMP (Fig 1C), indicating the presence of a functional enzyme even in the absence of light (dark activity; also see below). A 2-min exposure of the transgenic parasites to blue light caused ~3.7-fold increase in the cAMP level. Collectively, these data provided a proof of principle for an optogenetic control of the cytosolic cAMP in tachyzoites of T. gondii.

Conditional expression of bPAC provides a quantitative control of parasite cAMP

The challenges of the dark activity and a modest cAMP induction prompted us to optimize and enhance the efficacy of our optogenetic model. Based on the assumption that a constitutive expression of bPAC would have allowed the parasite to adjust its regulatory machinery, we generated a conditional transgenic strain harboring bPAC fused with a Shield1-regulatable destabilization domain (ddFKBP)
Moreover, we implemented a rapid and reversible stabilization of bPAC by ddFKBP when desired, to circumvent its inadvertent expression and *dark activity* in routine cultures. A ddFKBP-bPAC-Myc expression construct driven by a constitutive *TgGRA2* promoter was generated, which encoded a bPAC fusion protein flanked with a ddFKBP domain and Myc-tag at its N- and C-terminus, respectively (Fig 2). This construct was expressed in type-I tachyzoites. Immuno-fluorescence demonstrated a cytosolic expression of the fusion protein. In addition, the bPAC expression was Shield1-dependent, because no staining was detectable without the ligand (Fig 2A). Immunoblot assays validated the integrity and ligand-regulation of the bPAC protein, where the addition of Shield1 caused the appearance of a 53-kDa band, detected by anti-Myc antibody (Fig 2B). A comparison of the parental and transgenic parasites (-Shield1/light off state) showed similar levels of cAMP in the two strains, indicating the absence of an apparent *dark activity* (Fig 2C).

The basal level of cAMP in extracellular transgenic tachyzoites was amplified by 4- and 3-fold upon light or Shield1 exposure alone, respectively (Fig 2C). A total increase of 47-fold was obtained when both stimuli were provided together. As expected, induction of cAMP level by Shield1 alone reflects the *dark activity* of the ddFKBP-bPAC-Myc protein. The results also confirm that the aforementioned *dark activity* of bPAC-GFP is likely not due to activation by GFP, because its appearance is irrespective of the type of epitope-tag. The induction of cAMP level by light in the absence of Shield1 is due to photo-activation of the nascent enzyme prior to its proteasomal degradation (also see below in intracellular parasites; Fig 4). The degradation kinetics of the photo-induced cAMP levels in host-free transgenic parasites demonstrated that the cAMP pool declined very rapidly to about 40% within 30 sec, and stabilized over the next 15 min (Fig 2D). A maximum of ~70% cAMP was degraded during the assay. In a nutshell, we achieved a high and largely reversible cAMP induction, and restricted the *dark activity* in normal (off/basal state) cultures. A regulatable expression resolved the major concerns associated with a constitutive expression of bPAC. It also offered a two-step (Shield1 and light) and temporal (transient, persistent) control of parasite cAMP.

**Elevation of the cAMP levels in type-I strain affects tachyzoite invasion**

It has been reported that hepatocyte and erythrocyte invasion by *Plasmodium* sporozoites and merozoites depend on cAMP signaling, which regulates the parasite exocytosis (11,12). To confirm the occurrence of this phenomenon in *T. gondii* tachyzoites, we executed secretion/invasion assays using our optogenetic strain (Fig 3A). Intracellular tachyzoites were incubated with Shield1 to stabilize bPAC before isolating extracellular parasites. Host-free tachyzoites were photo-activated to induce their
cAMP level, incubated for secretion, and then allowed to invade HFF cells. Samples were immuno-stained to distinguish between the invaded or intracellular (red) and extracellular (red/green) tachyzoites (22) (Fig 3B). The parental and transgenic strains exhibited a similar 50-60% invasion efficiency under the control conditions (-Shield1 or -light). As expected, Shield1 and light exposures, alone or together, did not affect the parental parasites (Fig 3C). In contrast, the Shield1-treated transgenic strain invaded rather poorly (26%) when compared to the untreated transgenic and parental controls. A further induction of cAMP in the transgenic strain by light pulse did not accentuate the invasion defect irrespective of Shield1 exposure (Fig 3C). Unfortunately, our efforts to detect and quantify the parasite secretion were futile; thus, further research is required to link cAMP induction with secretion and invasion events. We presume that an elevation of cAMP by Shield1-mediated stabilization of bPAC induces parasite secretion prior to host-cell infection, which leads to an impaired invasion rate of the transgenic parasites in our assays.

Expression of bPAC in a type-II strain allows investigation on the role of cAMP in stage differentiation

The successful expression of bPAC in type-I strain motivated us to optogenetically manipulate a type-II strain, and define the roles of cAMP in the stage-specific expression and parasite differentiation. The Pru-Aku80-Δhxgprt/pTgLDH2-GFP strain was transfected with the pTgGRA2-ddFKBP-bPAC-Myc construct. This reporter strain expresses GFP under the control of bradyzoite-specific TgLDH2 elements (21). The ddFKBP-bPAC-Myc protein was expressed in bradyzoites as well as in tachyzoites (Fig 4A). As expected, bradyzoites expressed GFP, while tachyzoites were GFP-negative. The bPAC fusion protein was also regulatable by Shield1 in both stages (Fig 4A). Immunoblots revealed an expected 53-kDa band in response to Shield1 treatment confirming the integrity and ligand-regulation (Fig 4B). The enzyme was stabilized within 2 to 4 hrs of Shield1 incubation. The extended incubations with Shield1 (8 hrs, 72 hrs) showed further increases in the bPAC expression (Fig 4B). The cAMP assays confirmed bPAC activity in the transgenic type-II strain replicating within their host cells (Fig 4C).

The cAMP level in host cells infected with the transgenic strain in its off state (-Shield1/-light) was barely above the uninfected cells, indicating only a minor contribution of the parasite-derived basal cAMP level (Fig 4C). In other words, host-derived cAMP accounted for a major fraction in the parasitized cells. Our quantitative deductions estimated about 4000-7000 fmol of cAMP per million HFF cells. To calculate the approximate fold inductions in the parasite-derived cAMP levels while replicating in their host cells, we subtracted the host cAMP from the parasitized
cultures for individual treatments (paired subtractions). We estimated that Shield1 and light alone exerted ~3 and ~10-fold induction of the basal cAMP level in the parasite, respectively. A collective exposure to both stimuli amplified the parasite-derived cAMP level by 60-fold. These data on intracellular parasites are in agreement with those observed in type-I extracellular parasites (Fig 2C). We quantified a maximum of 180,000 fmol of cAMP per million parasitized HFF cells (+Shield1/+light) (Fig 4C). It is plausible that cellular resources within the parasite (ATP etc.) may have become limited, and restricted a maximal induction of cAMP level in the optogenetic strain.

**Transient induction of the parasite cAMP is required for the bradyzoite-specific gene expression**

It is known that forskolin, a chemical activator of adenylate cyclase, can rapidly increase the host and/or parasite cAMP, and induce bradyzoite formation (9,10). To determine a putative role of parasite-derived cAMP in the process, we performed stage differentiation assays using our optogenetic type-II strain (Fig 5). Bradyzoite (green) and tachyzoite (red) were identified by their stage-specific expression of GFP and Sag1 proteins, respectively (Fig 5A) (20,21,27). Such a dual staining allowed us to assess the stage switch in response to elevation of cAMP. We first exerted a transient increase in the cAMP levels of optogenetic type-II parasites by stabilizing and activating bPAC with Shield1 (4 hrs) and light (2 min). The bradyzoite and tachyzoite stages were allowed to develop for 3 days in the absence of any stimuli, and then quantified microscopically. As shown (Fig 5B), we quantified a 3 to 4-fold decrease in tachyzoite to bradyzoite ratio following treatment of the transgenic and the parental strains with sodium nitro-prusside (SNP), which is a known inducer of bradyzoites and cAMP/cGMP signaling (9,10). Notably, we determined a significant 2-fold decrease in tachyzoite to bradyzoite ratio in the transgenic cultures following an induction of cAMP (Shield1/+light) when compared to the equivalent parental cultures, as well as to the untreated cultures of the two strains (Fig 5B). In accord, the bradyzoite percentage in the Shield1- and light-exposed transgenic cultures was also increased (Fig 5C). These data together emphasize the specificity of the stage conversion process. The SNP treatment yielded a maximal differentiation, which is likely due to concurrent activation of multiple pathways, including cAMP and cGMP-signaling in the parasitized cells (9,10). Collectively, we show that induction of parasite-derived cAMP can modulate stage-specific expression in *T. gondii*.

**A minimum threshold level of parasite cAMP is required to promote bradyzoite differentiation**

To determine the threshold cAMP levels and the duration required by optogenetic parasite to commit stage conversion, we utilized variable exposure times from 0.5-8 min in presence or
absence of Shield1 (Fig 6). These assays also included lectin staining to ascertain the authenticity of bradyzoite cysts and its positive correlation with stage-specific GFP expression (Fig 6A). We first tested the kinetics of cAMP stimulation in parasitized host cells (Fig 6B). The cAMP level in parasitized host cells ($10^6$) was increased to 30,000 and 160,000 fmol within 30 sec of light exposure in the absence and presence of Shield1, respectively, and saturated thereafter. Interestingly, an increase in bradyzoite formation above the *basal* level was observed only in Shield1-treated and light-exposed cultures (Fig 6C). A 30 sec pulse was clearly sufficient to induce the stage conversion in Shield1-treated, but not in untreated cultures. Consistently, longer photo-activation periods up to 8 min effectively induced the parasite differentiation only in Shield1-treated samples, though the increment was not linear. Our comparisons of the cAMP levels and differentiation assays revealed that an elevation of cAMP up to 50,000 fmol was clearly not adequate to induce a stage switching, and that a substantial induction up to $1 \times 10^5$ fmol was required (Fig 6B, 6C). Moreover, even though the cAMP pool stabilized within 30 sec, its sustained higher level (achieved by extended photo-activation) moderately but consistently augmented the bradyzoite formation. These data are consistent with the degradation kinetics of cAMP in host-free parasites (Fig 2D), which showed a rapid decline in photo-induced levels of cAMP (down to 40% in 30 sec). In other words, a concurrent synthesis and degradation of cAMP demands a high threshold induction to commit stage switching, which could only be achieved by the protein stabilization and photo-activation. Both stimuli together maintain an otherwise rapidly declining cAMP level above its threshold, indicating the importance of signal strength as well as duration for the parasite differentiation.

*Host-derived cAMP is not a likely determinant of parasite differentiation*

We next assessed a putative role of host cAMP in the parasite differentiation using human fibroblast cells in line with other assays. We transfected the bPAC-mCherry construct into host cells, infected them 24 hrs later with the type-II parental strain for 4 hrs, photo-activated bPAC for 30 sec, and then incubated culture for an additional 48 hrs to allow the stage differentiation. Samples were scored by lectin/mCherry staining to estimate the bradyzoites in the transgenic host cells (Fig 7A). Despite a vast induction of host cAMP level to $5 \times 10^5$ fmol, bradyzoite formation remained unchanged, when compared to control dark samples with 40,000 fmol of cAMP (Fig 7B). Transfected HFF cells displayed about 6x higher than usual cAMP (~40,000 vs. ~7000 fmol; *dark activity*), though it was well below the bradyzoite-inducing threshold levels within the parasite itself (Fig 6B). It is worth mentioning that we observed a higher *basal* stage conversion in the transfected HFF cells than non-transfected...
cells (30% vs. 20%), which is likely due to transfection-associated stress. In brief, our data do not support a role of host cAMP in the parasite differentiation. The generation of a stable and conditional host cell line (other than primary cells, such as HFF) devoid of the bPAC dark activity is required to consolidate these findings.

A persistently higher level of the parasite cAMP represses bradyzoite differentiation

A sustained activation of cAMP-dependent signaling in host and/or parasite by a non-hydrolysable cAMP analog or by phosphodiesterase inhibition was shown to reduce bradyzoite formation (9,10). The transgenic strain expressing ddFKBP-bPAC-Myc exhibited ~7-fold increase in the basal cAMP level after 72 hrs of culture with Shield1 in comparison to the untreated transgenic or to the parental strain (Fig 8A). We, therefore, exerted a moderate but persistent induction of cAMP in intracellular parasites by culturing them with Shield1 during the differentiation process. As anticipated, the exposure to Shield1 did not affect the parental strain. In contrast, we scored 2-fold increase in tachyzoite to bradyzoite ratio in the transgenic strain (Fig 8B), which was due to reduced bradyzoite counts (Fig 8C). These results agree with the former finding, and further emphasize a need of parasite-derived cAMP in the process of stage differentiation.

**DISCUSSION**

We have generated transgenic *Toxoplasma* strains expressing a photo-activated adenylate cyclase from a lithotropic bacterium. The enzyme is cytosolic and exerts a sizeable induction of the parasite cAMP level. A hierarchical control of cAMP by enzyme expression and photo-activation offers a flexible modulation of cyclic nucleotide in diverse setups, such as during the infective-extracellular or replicative-intracellular stages. A conditional expression of bPAC in type-I and type-II strains allows analysis of cAMP-mediated signaling in acute and dormant stages of *T. gondii*. The advantages of the new method are: (a) specific and quantitative cAMP regulation within the parasite or host cell; (b) bPAC is gene-encoded *i.e.*, inheritable to the progeny; (c) it circumvents routine problems in culture *e.g.*, poor diffusion, premature degradation and sustained activation; (d) a transient, persistent and reversible control on cAMP can be achieved to satisfy versatile experimental setups; (e) a functional bPAC can be expressed irrespective of the N- and/or C-terminal fusion, or epitope size (GFP, mCherry, Myc, ddFKBP), and thus suitable for a spatial control of different cAMP pools by organelle-specific targeting. Although useful for a modest cAMP induction, the dark activity of bPAC may pose a drawback (18). This problem can however be resolved by conditional expression, which also avoids the undesired expression of a foreign protein in routine cultures. A much higher and more dynamic cAMP induction further advocates the use of a conditional expression in our model. We are now extending...
the optogenetic application to the mutagenized isoform of bPAC to regulate the cGMP levels in *T. gondii* (17). Notably, this method is also applicable to virtually all gene-tractable intertwined models such as, symbiotic and pathogen-host interactions/relationships.

Cyclic nucleotide-mediated signaling is known to regulate multiple processes in the protozoan parasites and other pathogens (28,29). Much of our knowledge is derived from *Plasmodium* species, where cAMP signaling is required for exocytosis, host-cell invasion (11,12), and for the formation of gametocytes (30). Consistent with *Plasmodium*, our results suggest a role of cAMP in host-cell invasion by *Toxoplasma* tachyzoites that remains to be ascertained by parasite secretion studies. Interestingly, a moderate induction of cAMP (achieved by Shield1) appears to be sufficient in regulating the invasion/secretion events, as a light exposure did not increase the observed invasion defect in type-I tachyzoites. In contrast, a much higher threshold of cAMP was required to induce bradyzoite formation in type-II parasites, which could only be achieved by photo-induction. It is established that physicochemical (pH, temperature, SNP) stress can induce tachyzoite-to-bradyzoite conversion (2,31; this work), and that cyclic nucleotides are general mediators of stress signaling and differentiation in many other microbes (28,29). Our work illustrates the importance of parasite cAMP in differentiation, and suggests a conserved cAMP-mediated stress signaling in *T. gondii*. Consistently, the parasite genome encodes all imperative factors required for a generic cAMP signaling such as, adenylate cyclases, phosphodiesterases, protein kinases, regulatory subunits and cAMP-binding proteins (www.ToxoDB.org). In other models, the PKA-dependent as well as PKA-independent pathways are known to control the gene expression (32). The availability of the optogenetic strain allows a dissection of such downstream events in *T. gondii*.

Bradyzoites and tachyzoites differ in their stress tolerance, metabolism and growth rates, which depend on a rewiring of the gene expression (2,31). Tachyzoites express a unique set of proteins including Sag1, Eno2 and Ldh1, whereas bradyzoites show a distinct expression of other proteins e.g., Cst1, Bag1, Enol1 and Ldh2 (33,34). Using our method, we reveal the requirement of the parasite cAMP for stage-specific expression (pLDH2, pCST1, pSAG1). A family of cAMP-responsive nuclear factors controls transcription by binding to the cAMP-response elements in other eukaryotes (32). While it is known that transcription of the bradyzoite genes is directed by autonomous promoter elements (35), their binding factors remain to be characterized. In this regard, the plant-like AP2 transcription factors are currently the most promising candidates (36). One of such AP2 factor has recently been shown to govern the stage-specific expression and differentiation (37). The activity of AP2 factors is likely...
regulated by phosphorylation (38) that may be controlled by cAMP signaling. Notably, our optogenetic strain can be utilized to examine the cAMP-mediated epigenetic coding and gene modulations during/upon stage differentiation, as well as to identify the mediators of cAMP signaling. For instance, genome-wide analyses of the gene expression and histone codes in response to low/high cAMP levels could be performed to identify a common set of stage-specific and cAMP-regulated genes. In addition, a follow-up promoter mapping of such genes could be used to classify the signature elements and their binding factors. Along the line, future research should also reveal how a transient or persistent cAMP induction exerts a reciprocal effect on parasite differentiation, which may depend on differential activation of transcriptional regulators.

REFERENCES


**FOOTNOTES**

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*Abbreviations*: ddFKBP, ligand-regulatable destabilization domain FK506 binding protein; DHFR-TS, dihydrofolate reductase thymidylate synthase; GRA2, granule 2 protein; HXGPRT, hypoxanthine guanine phosphoribosyl-transferase; ICM, intracellular-type medium; LDH2, lactate dehydrogenase 2; PAC, photo-activated adenylate cyclase; PKA, protein kinase A; SAG1, surface antigen 1.

**FIGURE LEGENDS**
**Fig 1**: Transgenic expression of a photo-activated adenylate cyclase in *T. gondii* tachyzoites permits the elevation of cytosolic cAMP in the parasite. (A-C) Schematics of the construct designed to express a photo-activated adenylate cyclase of *Beggiatoa* (bPAC) fused with green fluorescent protein (GFP) at its C-terminus under the control of the *TgGRA2* regulatory elements (5'- and 3'-UTR) in type-I tachyzoites. The p*TgGRA2*-bPAC-GFP construct encoding the bPAC-GFP fusion protein was transfected into *TaTi* tachyzoites, and selected with pyrimethamine (1 µM) to produce clonal lines (A) Immuno-staining of intracellular parasites (24 hrs post-infection) was performed using anti-*TgActin* (1:1000, mouse) and Alexa594-conjugated antibodies along with GFP imaging. The merge image shows co-localization of GFP with the parasite actin. A representative vacuole harboring four crescent-shaped tachyzoites is shown. The scale bar in differential interference contrast (DIC) image equals to 5 µm. (B) The amounts of cAMP in extracellular parental and transgenic tachyzoites (0.5-5 x 10⁶) in response to light. (C) Induction of cAMP levels of the transgenic strain following a 2-min light exposure (455 nm). The error bars in panel B and C show the mean±SEM of three independent assays.

**Fig 2**: Shield1-regulatable conditional expression of photo-activated adenylate cyclase in *T. gondii* allows a hierarchical control of cAMP. (A-D) Scheme of the construct designed to express bPAC fused with the Shield1-regulatable destabilization domain (ddFKBP) at the N-terminus and a Myc-tag at the C-terminus. The expression of ddFKBP-bPAC-Myc protein was driven by the *TgGRA2* regulatory elements in the type-I (*TaTi*) strain. Transgenic pyrimethamine (1 µM)-resistant parasites were used to make clonal lines. (A) Immuno-fluorescence analysis of the intracellular parasites (30 hrs post-infection) was performed using anti-Myc (1:1000) and anti-*TgSag1* (1:1500), and Alexa594-/Alexa488-conjugated antibodies. The scale bars correspond to 5 µm. (B) Immunoblots of transgenic parasite protein (ddFKBP-bPAC-Myc) and actin loading control were performed using anti-Myc (1:1000) and anti-*TgActin* (1:1000), and HRP-conjugated antibodies. (C) The cAMP levels in extracellular tachyzoites (2.5 x 10⁶) of the transgenic (*pTgGRA2-ddFKBP-bPAC-Myc*) or parental strains in the absence or presence of Shield1 and light (455 nm, 2 min). (D) Kinetics of the cAMP degradation in the transgenic strain. Extracellular tachyzoites from panel C were analyzed for their cyclic nucleotide decay after 2 min photo-induction. Shield1 (0.5-1 µM) treatments in panel A-D were performed in culture for the indicated durations. The error bars in panel C and D show the mean±SEM of three independent assays.

**Fig 3**: Induction of cAMP by Shield-mediated stabilization of bPAC activity affects the parasite invasion. (A-C) Construct expressing ddFKBP-bPAC-Myc fusion protein in type-I strain of *T. gondii* (as in Fig 2). (A) Scheme of the assay depicting bPAC stabilization in parasites, photo-activation, incubation in DMEM, and host-cell invasion. (B) Two-color invasion assay with differential staining of intracellular (invaded, red) and extracellular (red and green) tachyzoites. (C) The numbers of invaded parasites were
calculated following immuno-staining with anti-\( Tg\text{Sag1} \) (1:1500) and anti-\( Tg\text{Gap45} \) (1:3000) antibodies. In total, 100 parasites per assay were scored from two to four independent experiments. The statistics was done using the Student’s \( t \)-test with respect to the untreated basal control of individual strains (*)\( , p<0.05 \).

**Fig 4**: Conditional expression of a photo-activated adenylate cyclase in type-II strain of \( T. gondii \) allows studies on parasite differentiation in response to cAMP modulation. (A-C) Schematics of the \( pTg\text{GRA2-ddFKBP-bPAC-Myc} \) construct transfected in the \( Pru-\Delta ku80-\Delta hxgprt/pTgL\text{DH2-GFP} \) strain of \( T. gondii \). Pyrimethamine-resistant stable transgenic parasites were used to make clonal lines. (A) Immuno-staining of intracellular parasites (24 hrs post-infection) was performed using anti-Myc (1:1000) and anti-GFP (1:5000), and Alexa488-/Alexa594-conjugated antibodies. The vacuoles containing bradyzoites (left) or tachyzoites (right) are shown. Tachyzoites show Shield1-regulatable Myc staining but lack the bradyzoite-specific GFP staining. The scale bars equal to 5 \( \mu \)m. (B) ImmunobLOTS of the transgenic parasite protein (ddFKBP-bPAC-Myc) and actin loading control using anti-myc (1:1000) and anti-\( Tg\text{Actin} \) (1:1000), and HRP-conjugated antibodies. Shield1 (1 \( \mu \)M) treatment was performed in cultures for the indicated times. (C) Measurements of cAMP using uninfected and infected host fibroblast cells (10\(^6\)). Cells were infected with the transgenic strain (MOI, 1). Shield1 (1 \( \mu \)M, 4 hrs) and/or light (455 nm, 30 sec) treatments were performed in cultures 52 hrs post-infection. The error bars in panel C shows the mean±SEM of three independent assays.

**Fig 5**: A transient elevation of cAMP promotes bradyzoite formation in the optogenetic type-II strain. (A-C) The \( pTg\text{GRA2-ddFKBP-bPAC-Myc} \) construct was expressed in the \( Pru-\Delta ku80-\Delta hxgprt/pTgL\text{DH2-GFP} \) strain. (A) Bradyzoite and tachyzoite staining of the transgenic strain (\( pTg\text{GRA2-ddFKBP-bPAC-Myc} \)). Intracellular parasites were immuno-stained using anti-\( Tg\text{Sag1} \) (1:1500) and anti-GFP (1:5000) antibodies (72 hrs infection). Tachyzoite (red) and bradyzoite (green) stages were visualized by staining of the Sag1 and GFP proteins, respectively. The vacuole with a dual staining in top panel indicates an asynchronous differentiation. The scale bar equals to 5 \( \mu \)m. About 100-200 vacuoles per assay were scored to estimate the stage ratio (panel B) and bradyzoite percentage (panel C). (B, C) The parental or transgenic parasites (10\(^6\)) were allowed to infect fibroblast cells (MOI, 1; 72 hrs) in the absence or presence of the indicated reagents (Shield1, 1 \( \mu \)M, 4 hrs; sodium nitro-prusside, 100 \( \mu \)M, 72 hrs), and/or light exposure (455 nm, 2 min). Samples were immuno-stained to visualize the parasite stages as shown in panel A. The error bars specify the mean±SEM of three (parental) to four (transgenic) independent assays. The statistics in panel B and C (**, \( p<0.01 \); *** , \( p<0.001 \)) was performed using the Student’s \( t \)-test with respect to the untreated basal control of individual strains.
Fig 6: A minimum threshold of parasite cAMP is required to induce bradyzoite differentiation. (A-C) The pTgGRA2-ddFKBP-bPAC-Myc construct was expressed in the Pru-Δku80-Δhxgprt/pTgLDH2-GFP strain. (A) Bradyzoite cyst wall (red) and stage-specific GFP (green) expression in the transgenic strain were detected by immuno-staining with biotinylated Dolichos biflorus lectin/Alexa594 and anti-GFP/Alexa488 antibodies 72 hrs post-infection. (B) Measurements of cAMP in the fibroblast cells (10^6) infected with the transgenic strain (MOI, 1; 52 hrs post-infection). Shield1 (1 µM, 4 hrs) and/or light (0-8 min) treatments were performed in the cell culture prior to cAMP extraction. The grey bar shows the minimum threshold range of cAMP required to induce bradyzoite differentiation. (C) The transgenic parasites (0.5-1.0 x10^4) were allowed to infect fibroblast cells (MOI, 0.1) in the absence or presence of Shield1 (1 µM) for 4 hrs followed by photo-activation (0-8 min). The parasites were immuno-stained 72 hrs of infection, and a total of 100-200 vacuoles per assay were scored for lectin staining to estimate the frequency of bradyzoites. The error bars show the mean±SEM of three independent assays. The statistics was performed using the ANOVA and Bonferroni tests between all paired samples (**, p<0.01; ***, p<0.001).

Fig 7: Photo-induction of host cAMP levels in human fibroblasts transiently expressing bPAC-mCherry does not alter bradyzoite differentiation of a type-II strain. (A-B) The pCMV-bPAC-mCherry construct was expressed in human foreskin fibroblasts. Cells were infected with the parental type-II (Pru-Δku80-Δhxgprt/pTgLDH2-GFP) strain of T. gondii. (A) A representative bradyzoite-infected host cell expressing bPAC-mCherry is shown (24 hrs post-infection). Bradyzoites and transgenic host cells were visualized by lectin staining and mCherry florescence, respectively. The scale bar corresponds to 10 µm. (B) Host cells were transfected, and infected with the parental type-II strain (MOI, 0.1; 4 hrs) 24 hrs post-transfection. The infected host cells were kept in the dark or exposed to light (455 nm, 2 min) 28 hrs post-transfection followed by incubation for 48 hrs to allow the parasite differentiation. Samples were stained by lectin to score bradyzoites in bPAC-mCherry transgenic fibroblasts. The host cAMP levels were measured 28 hrs post-transfection without or with photo-activation (455 nm, 2 min). The error bars show the mean±SEM of two independent assays.

Fig 8: Persistent induction of cAMP decreases bradyzoite formation in type-II strain expressing ddFKBP-bPAC-Myc protein. (A-C) The pTgGRA2-ddFKBP-bPAC-Myc construct was expressed in the Pru-Δku80-Δhxgprt/pTgLDH2-GFP strain. (A) The cAMP levels in the human fibroblast cells infected with the transgenic or the parental strain (10^6 cells; MOI, 1; 72 hrs) were determined after culturing in the absence or presence of Shield1 (no photo-activation). The indicated fold-change was estimated by subtracting the cAMP levels of uninfected host cells. (B) Effect of persistent cAMP elevation on tachyzoite to bradyzoite ratio in the transgenic strain compared to the parental control. The assessment of bradyzoite-
tachyzoite-specific GFP and Sag1 expression was performed as shown in Fig 5. A total of 100-200 parasite vacuoles per assay were scored. (C) Percentage of bradyzoite vacuoles from the assay described in panel B. The error bars show mean±SEM of three (parental) to five (transgenic) independent assays. The statistics in panel B and C were performed using the Student’s t-test with respect to the untreated basal control of individual strains (**, p<0.01; ***, p<0.001).

Table 1: Oligonucleotide sequences used in this study

<table>
<thead>
<tr>
<th>Primer Name (restriction site)</th>
<th>Primer Sequence (restriction site underlined)</th>
<th>Cloning Vector (research objective)</th>
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<tr>
<td><strong>Making of pTgGRA2-bPAC-GFP Construct</strong></td>
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<td>bPAC-F1 (NsiI)</td>
<td>CTCATCATGCAATATGATGAAACGCCTGGTGTA</td>
<td>Cloning in pTKO-DHFR/TS-GFP vector for the C-terminal GFP-tagging of bPAC</td>
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<td><strong>Making of pTgGRA2-ddFKBP-bPAC-Myc Construct</strong></td>
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<td>ddFKBP-F1 (NsiI)</td>
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<td>bPAC-Myc-R1 (PacI)</td>
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</tbody>
</table>
Fig 1

A

GFP α-TgActin Merge DIC

B

![Graph showing cAMP induction](image)

C

![Bar chart showing cAMP induction](image)
**Fig 2**

**A**

<table>
<thead>
<tr>
<th>Shield1 (+)</th>
<th>Shield1 (-)</th>
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<tr>
<td>(+) Shield1</td>
<td>(-) Shield1</td>
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**B**

**Shield1 Exposure**

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<th>48 h</th>
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<td>α-Myc</td>
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<tr>
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<td>α-TgActin</td>
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</table>

**C**

![Graph showing cAMP levels](image)

**D**

![Graph showing cAMP levels over time](image)
**Fig 3**

**A**

- **pTgGRA2** → **ddFKBP** → **bPAC** → **Myc**

- **HFF Infection (MOI=1, 24 h)**
- **Incubation with Shield1 (0.25 µM, 16 h)**
- **+/- Light Pulse (455 nm, 30 sec)**
- **Incubation in DMEM (1 h, 37 °C)**
- **HFF Infection (MOI=10, 1 h, 37 °C)**

**B**

- **α-Sag1**
- **α-Gap45**
- **Merge**
- **DIC**

- Extracellular
- Invaded

**C**

- **Parasites Invaded (%)**

<table>
<thead>
<tr>
<th></th>
<th>Parental Control</th>
<th>ddFKBP-bPAC-Myc (Type-I)</th>
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<td>- Shield1/- Light</td>
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</tr>
<tr>
<td>- Shield1/+ Light</td>
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<td></td>
</tr>
<tr>
<td>+ Shield1/- Light</td>
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<td>+ Shield1/+ Light</td>
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</tr>
</tbody>
</table>
Fig 4

A

(+)

Shield1

α-Myc

α-GFP

α-Myc

α-GFP

(-)

Shield1

Bradyzoites

Tachyzoites

Shield1 Exposure

0 h

2 h

4 h

8 h

72 h

53-kDa

α-Myc

42-kDa

α-TgActin

B

C

Uninfected HFF

Parasitized HFF

(ddFKBP-bPAC-Myc, Type-II)
Fig 7

A

Lectin  mCherry  Merge  DIC

B

Transgenic HFF (bPAC-mCherry)

light (2 min)  (-)  (+)  (-)  (+)

f mol cAMP/10⁶ HFF Cells

Bradyzoite Differentiation (%)
Optogenetic modulation of an adenylate cyclase in Toxoplasma gondii demonstrates a requirement of the parasite cAMP for host-cell invasion and stage differentiation

Anne Hartmann, Ruben Dario Arroyo-Olarte, Katharina Imkeller, Peter Hegemann, Richard Lucius and Nishith Gupta

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