Calcium Signaling in Excystation of the Early Diverging Eukaryote,
*Giardia lamblia*

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Excystation of *Giardia lamblia*, which initiates infection, is a poorly understood but dramatic differentiation induced by physiological signals from the host. Our data implicate a central role for calcium homeostasis in excystation. Agents that alter cytosolic Ca\(^{2+}\) levels (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, channel blockers, Ca\(^{2+}\)-ionophores, and thapsigargin) strongly inhibit excystation. Treatment of *Giardia* with thapsigargin raised intracellular Ca\(^{2+}\) levels, and peak Ca\(^{2+}\) responses increased with each stage of excystation, consistent with the kinetics of inhibition. Fluorescent thapsigargin localized to a likely Ca\(^{2+}\)-storage compartment in cysts. The ability to sequester ions in membrane-bound compartments is a hallmark of the eukaryotic cell. These studies support the existence of a giardial thapsigargin-sensitive Ca\(^{2+}\) storage compartment resembling the sarcoplasmic/endoplasmic reticulum calcium ATPase pump-leak system and suggest that it is important in regulation of differentiation and appeared early in the evolution of eukaryotic cells. Modulin antagonists also blocked excystation. The divergent giardial calmodulin localized to the eight flagellar basal bodies/centrosomes, like protein kinase A. Inhibitor kinases suggest that protein kinase A signaling triggers excystation, whereas calcium signaling is mainly required later for parasite activation and emergence. Thus, the basal bodies may be a cellular control center to coordinate the resumption of motility and cytokinesis in excystation.

Parasite differentiations are elegant biological adaptations for survival in the environment and transmission (1), yet few life cycles have been completed in vitro (2). The life cycle of *Giardia lamblia* is initiated by excystation, a highly regulated differentiation which is required for transmission (3–5) that may be too rapid to rely entirely on new gene expression (6, 7).

Therefore, we propose that second messengers are central for its regulation. Cell signaling is poorly understood in the lower eukaryotes. Recently, we reported that protein kinase A activity may play a role in initiating excystation (8). Here, we tested the hypothesis that subsequent trophozoite emergence from the cyst wall and cytokinesis may require calcium signaling pathways.

*G. lamblia* is a major cause of water-borne intestinal disease whose basic biology is not well understood, although a genome project is in progress (9–12). The ability of the parasite to undergo complex differentiations in response to signals from the host is key to its pathogenesis. Like most intestinal parasites, *G. lamblia* has a dormant cystic form that persists outside the host and is responsible for transmission when ingested (1, 2, 13). Excystation in response to host gastrointestinal stimuli releases active trophozoites that colonize the small intestine and can cause disease (1, 3, 5).

Although exposure to gastric acid is required to trigger excystation of *G. lamblia* (5), if the cyst wall should open in the stomach, the parasite within would be killed. On the other hand, upon passage into the small intestine, *Giardia* must quickly emerge from the cyst wall, polarize, and re-assemble its adhesive disc and flagella in order to attach and not be carried away by the flow of intestinal fluid. At the same time, the “excyzoite” is becoming metabolically active and dividing into two binucleate trophozoites (7, 14).

Upon ingestion, *G. lamblia* cysts are exposed to extreme changes in osmolarity (from fresh water to isotonic conditions), to temperature increases of up to 33 °C, and to drastic decrease in pH (from ~5 to 6 in fresh water to ~2.0 in the human stomach). After the gastric acid is neutralized, the pH of the small intestine is slightly alkaline (~7.5 to 8.0). Excystation in vitro models the passage of the cyst from fresh cold water through the human stomach and into the small intestine where resumption of trophozoite motility, emergence from the wall, cytokinesis, and attachment occur (3, 15). This paradigm allows dissection of responses to individual signals and crucial triggering and emergence stages.

Although the external stimuli for giardial excystation are identified (5, 15), the mechanism(s) by which the signals are transduced are not known. We showed previously that exposure of cysts to the gastric pH and temperature that trigger excystation (“Stage I”) lead to decreased cytoplasmic pH, mRNA changes, and novel cytoplasmic re-arrangements that may establish cellular polarity (7). Signals modeling the small intestine (“Stage II”) then lead to degradation of cyst wall proteins and increased cytosolic pH (7).

In cells of later diverging eukaryotes, the flow of information between the extra- and intracellular environment is frequently mediated by rapid, transient changes in free intracellular
Calcium Signaling in Giardia Excystation

Ca2+, Ca2+ acts as a powerful second messenger, because its levels are regulated within narrow limits by channels, pumps, and specific Ca2+-binding proteins. Ca2+ is also sequestered within internal storage compartments, mainly the endoplasmic reticulum (ER) (16–19). Later diverging eukaryotes (17, 18) have one or more Ca2+-ATPases (sarco/plasmal/golgi/endoplasmic reticulum calcium ATPase, SERCA), pumps that maintain low cytosolic Ca2+ by pumping it into a membrane compartment, generally the ER in non-muscle cells and the SR in muscle cells. This pump also retrieves Ca2+ that has leaked into the cytosol and is critical for Ca2+ homeostasis and cell function (17).

Many of the actions of Ca2+ in later eukaryotes are mediated by the calcium-binding protein, CaM (16). CaM and its signaling cascades are highly conserved from fungi to mammals, and blocking CaM signaling arrests these cells in the G1 or G2 phase of the cell cycle (20, 21). However, giardial cysts are quadrinucleate, reported to be 16N, and to undergo two rounds of cytokinesis after excystation before re-entering the cell cycle (22). Munoz et al. (22) have purified giardial gCaM and shown that it resembled bovine CaM biochemically. They also reported that CaM antagonists inhibit excystation of fecal Giardia cysts (23). In the present study, we have examined the roles of cytoplasmic Ca2+ homeostasis and CaM in giardial excystation.

EXPERIMENTAL PROCEDURES

G. lamblia Differentiation—G. lamblia trophozoites (strain WB, clone C6, ATCC 50803) were cultured, excysted, and as described (3). Briefly, trophozoites and incomplete cysts were lysed by incubation in double distilled water for 20 min at room temperature. Water-resistant cysts were washed and stored in distilled water at 4 °C overnight (pre-excystation). In Stage I of excystation, cysts were exposed to HCl, pH 4.0, with cysteine and reduced glutathione for 20 min at 37 °C. In Stage II, acid-treated cysts were washed and treated with trypsin in pH 8.0 bicarbonate-buffered Tyrode’s solution for 60 min at 37 °C (7, 15). Cells were then collected by centrifugation and resuspended in TYI-S-33 growth medium at 37 °C for 30 min. Emerged motile trophozoites were enumerated in hemocytometer chambers using differential interference contrast microscopy. The percent excystation was calculated as the sum of the motile trophozoites and partially emerged trophozoites × 100, divided by the initial number of viable cysts with type I morphology (3).

Inhibition of Excystation—Inhibitors were dissolved in double distilled water, ethanol, or Me2SO and diluted into water (pre-excystation) or excystation solutions. Solvent was the control in each case. For initial screening and determination of 50% inhibitory concentrations (IC50) of active compounds, each inhibitor was present for a 1-h preincubation at 4 °C and added again during Stages I and II of excystation, because cysts were pelleted between stages. Inhibitors were not included in the emergence step to avoid possible effects on trophozoites. Viability was determined by trypsin blue exclusion and by differential interference contrast microscopy (3). To determine the specific stage(s) at which each inhibitor acted, cysts were preincubated with the inhibitor (generally at 1 μM) for 30 min at 37 °C in an 8-well chamber coverslip slide. The unattached cells were removed, and PBS was replaced with excystation medium and a coverslip. Live parasites were imaged using a Zeiss LSM 510 laser scanning confocal microscope equipped with an argon-krypton (455/488) laser and appropriate filter set.

Sequenceing the Calmodulin Gene—Preliminary BLASTX annotation of single-pass reads in the Giardia Genome Project data base (www.mbl.edu/Giardia) indicated that clone EJ2820 contained a likely CaM homologue. Complete coding information for the Giardia calmodulin gene was obtained by direct sequencing of EJ2820. We amplified the clone insert using PCR and primers specific to the Giardia sequences, and then sequenced this product completely using a LICOR automated scanner (27). The sequence has since been confirmed by additional random sequence data from the genome project data base and by sequencing constructs. The CaM amino acid alignment was produced using the ClustalW WWW Service at the European Bioinformatics Institute (www.ebi.ac.uk/clustalw). The structure of gCaM was modeled against a template of bovine CaM, crystallized with Ca2+, 1CLL (28), using Swiss-Model and Swiss-PDB Viewer (29).

Constructs and Transfection for Immunolocalization of gCaM—Plasmid pNLoP2-gfetkR was digested by XhoI and self-ligated to generate pNLoP2. The gCaM gene with its 5’-untranslated region was amplified from genomic DNA using PCR and oligonucleotides CM5NF (GGCGCGTAGCGGTAGAATTAAAATTCAGAA) and CMgAUM (GGCGCAATTGTAGTATGCATGATCCTCCTCCTCCTCCGAGATCTTCTTACCGAAG) with NheI and EcoRI and ligated in place of the NheI–EcoRI-excised luciferase gene and 32-bp ran promoter and two copies of a 19-bp tet operator sequence in pLoPo2 (30). The resulting plasmid pNMC contains the gCaM gene controlled by its own promoter with an AU1 tag fused at the C terminus. Cells were transfected with pNMC and selected with G418 as described (31), and stable transfectants were maintained in G418 (600 μg/ml) after 24 h in growth or encystation media with G418, washed in PBS, and attached to glass coverslips (2 × 104 cells/coverslip), then fixed and stained for indirect immunofluorescence assay (8). Cells were reacted with anti-AU1 monoclonal antibodies (Covance, Princeton, NJ, 1:300 in blocking buffer), which was detected with anti-mouse ALEXA 568 (Molecular Probes, Eugene, OR, 1:500 in blocking buffer). Localization of gCaM was visualized for indirect immunofluorescence assay (8). Cells were reacted with anti-AU1 monoclonal antibodies (Covance, Princeton, NJ, 1:300 in blocking buffer), which was detected with anti-mouse ALEXA 568 (Molecular Probes, Eugene, OR, 1:500 in blocking buffer). Localization of gCaM was visualized.

RESULTS

Role of Calcium Homeostasis in Excystation—Our initial findings that excystation was strongly (>70%) inhibited by decreasing free extracellular Ca2+ with EGTA (5 mM) or by buffering intracellular free Ca2+ with 5 mM BAPTA-AM (Calbiochem) suggested a central role for Ca2+ homeostasis. BAPTA-AM crosses the plasmalemma because of its acetoxy group, which is hydrolyzed by cellular esterases. The charged BAPTA molecule is kept within the cytoplasm where it chelates cytosolic free Ca2+ (32).

We tested the hypothesis that Ca2+ homeostasis might be important for excystation and that Giardia may be particularly
susceptible to perturbation of Ca\(^{2+}\) during the cellular activation that occurs later in excystation (Stage II). We found that verapamil, a Ca\(^{2+}\) channel blocker that should lower cytoplasmic Ca\(^{2+}\) concentrations (33), strongly inhibited excystation. The IC\(_{50}\) for verapamil when it was present throughout excystation was 49 \(\mu\)M. No inhibition was observed when verapamil (100 \(\mu\)M) was present only during pre-excystation or Stage I, but it inhibited >75% in Stage II (Fig. 1A). Ionomycin is a carboxylic ionophore (34), and A23187 is a Ca\(^{2+}\)-selective carboxylic ionophore that stimulates Ca\(^{2+}\)-dependent biological reactions without disturbing Na\(^{+}\) or K\(^{+}\) gradients (35). These two Ca\(^{2+}\) ionophores should greatly increase cytoplasmic Ca\(^{2+}\) by allowing influx of Ca\(^{2+}\) from the medium. Ionomycin (1 \(\mu\)M) and A23187 (1 \(\mu\)M) were also most inhibitory in Stage II, although they also inhibited significantly in Stage I (Fig. 1, B and C). When each ionophore was present throughout excystation, the IC\(_{50}\) for ionomycin was 38 nM and for A23187 it was 126 nM. The idea that these inhibitors were acting specifically was supported by \(^{45}\)Ca uptake studies (data not shown). Ionomycin increased the rate of \(^{45}\)Ca uptake by ~2.5-fold, relative to solvent controls. Measurements of intracellular Ca\(^{2+}\) levels (see below) confirmed that ionomycin functions as a Ca\(^{2+}\) ionophore in Giardia cysts and trophozoites, because we calibrated each trace with ionomycin to obtain the \(R_{\text{max}}\) needed to calculate intracellular levels of Ca\(^{2+}\).

**Evidence of a Calcium-pumping ATPase Needed for Excystation**—In most eukaryotic cells, Ca\(^{2+}\) pump activity due to SERCA is specifically inhibited by the sesquiterpene lactone, TG (36). The addition of 5 \(\mu\)M TG (Calbiochem) to Indo-1 loaded trophozoites, cysts, or excysting cells produced an immediate and sustained rise in Indo-1 fluorescence, corresponding to an increase in free intracellular Ca\(^{2+}\) (Fig. 2A). The traces shown in Fig. 2A are qualitative in nature and do not allow for accurate comparisons across all stages, due to differences in calibration values (see “Experimental Procedures”). Therefore, we converted all fluorescence ratio data to nanomolar cytosolic Ca\(^{2+}\) concentration, ranging from 40 to 85 nM (data not shown). The data in Fig. 2B show that the addition of TG caused significant increases in cytosolic Ca\(^{2+}\) levels. The effect of TG did not depend on extracellular Ca\(^{2+}\), because similar data were obtained when Indo-1-labeled cells were resuspended briefly in Ca\(^{2+}\)-free medium containing 0.3 mM EGTA (data not shown). This indicates that the source of the elevated Ca\(^{2+}\) is an intracellular Ca\(^{2+}\) store dependent on a TG-sensitive, presumably SERCA-like pump.

Under the conditions of our experiments, we did not detect significant changes in the basal Ca\(^{2+}\) levels during excystation (data not shown). Excysting cells in all stages exhibited an increase in intracellular Ca\(^{2+}\) in response to TG. Moreover, the magnitude of peak TG-induced Ca\(^{2+}\) release increased with progression through excystation (Fig. 2B). The peak effect of TG was lowest in the dormant cyst form (656 ± 66 nM), intermediate in Stage I (1158 ± 108 nM) and Stage II (1035 ± 92 nM) excysting cells, and greatest in newly excysted cells (1577 ± 116 nM), which were similar to trophozoites (1517 ± 234 nM). The effect of TG on cysts was significantly lower and on trophozoites and excyzoites was higher than in all other stages (\(p < 0.05\)).

TG inhibition of excystation also increased during the course of differentiation. The IC\(_{50}\) for TG when present throughout excystation was 7 \(\mu\)M. TG at 10 \(\mu\)M significantly inhibited giardial excystation only during Stage II (Fig. 3). However, inhibition by TG was greater when it was present in both Stages I and II, in contrast to the other inhibitors that did not show any additive effects (not shown). The lower response of non-induced cysts to TG may be because this is a dormant form or because of lower penetration into the intact cyst wall. However, giardial cysts (Fig. 4) stained with fluorescent TG, which localized to a likely Ca\(^{2+}\) storage compartment, resembling the ER (37), by confocal microscopy. Taken together, our data suggest that function of a TG-sensitive calcium storage compartment resembling the SERCA pump-leak system is important in regulation of giardial excystation.

**Evidence of CaM as a Central Mediator in Excystation**—We found that three specific CaM inhibitors (38) blocked excystation (Fig. 5). The calculated IC\(_{50}\) values are as follows: chlorpromazine 28 \(\mu\)M, trifluoperazine ~15 \(\mu\)M (Fig. 5), and calmidazolium 1 \(\mu\)M (data not shown) (23, 38), when present throughout excystation. These inhibitors may also primarily affect parasite emergence from the cyst wall, because they were most effective during the late stage that models cyst arrival in the host small intestine (Fig. 5).

**Sequence and Structural Analyses of gCaM**—We sequenced a giardial CaM gene (GenBankTM AAK97377) from a genomic DNA clone identified by the Giardia Genome Project (Fig. 6). Searching this data base (www.mbl.edu/Giardia) for additional homologues by BLAST, using bovine CaM as the query sequence, did not reveal other giardial CaM homologues but only previously reported centrin/caltractin genes in the CaM family with \(p\) values \(<10^{-37}\) and 3 \(\times\) \(10^{-37}\) (39). Several A- or A-T-rich regions typical of giardial initiator sequences are upstream of the start of translation of gCaM and a slightly divergent putative giardial polyadenylation signal, GTGATAAT (consensus: AGTRAAY, overlaps with the stop codon (data not shown)), which is not unusual for *Giardia* (10). Interestingly, the gCaM gene has a completely overlapping ORF, with no known homologues, on the opposite strand. Both the gCaM gene and the
antisense ORF are transcribed during growth and differentiation (not shown). This is also not unusual for *Giardia* (40). Southern analyses also showed that *Giardia* CaM is a single copy gene (data not shown). This is important because the gCaM we cloned differs in amino acid composition from CaM purified from *Giardia* extracts by Munoz et al. (22). The amino acid composition determined by Munoz et al. (22) for bovine CaM also differed from the published bovine CaM sequence (28). We cannot explain these differences, but we have confirmed our sequence, and gCaM expressed in *Escherichia coli* was active in stimulating cAMP phosphodiesterase activity (data not shown).

CaM senses calcium by binding up to four Ca\(^{2+}\) ions with four conserved 12-residue helix-loop-helix "EF hands" (41, 42). Because gCaM is so divergent overall, we modeled its three-dimensional structure using bovine CaM (1CLL, crystallized with Ca\(^{2+}\) at 1.7 Å, Ref. 28) as a template. Despite the differences in sequence, the backbone carbon atoms could be superimposed. Moreover, the portions of each sequence predicted to have helical secondary structure were identical. In each EF hand, positions 1 (Asp), 6 (Gly), and 12 (Glu) are the most highly conserved (43), and gCaM differs only in having Asp instead of Glu-12 in the fourth EF hand (Fig. 6, data not shown).

**Cellular Location of gCaM**—In trophozoites (Fig. 7) and encysting cells (not shown), gCaM expressed with either an N-terminal or C-terminal AU-1 epitope tag localized to the flagellar basal bodies or centrosomes that are between and slightly anterior to the two nuclei. A variable amount of gCaM also localized to the cytosol (Fig. 7). gCaM also localized to the basal bodies in water-resistant cysts.2

2 S. Shah, personal communication.

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**Fig. 2.** TG-induced increases in intracellular Ca\(^{2+}\) in *Giardia* during the course of excystation. A, Indo-1/AM fluorescence was monitored continuously, and TG (5 μM) was added at the time indicated by the arrow. Qualitative elevations in [Ca\(^{2+}\)]\(_i\) were evident at each life cycle stage. These raw qualitative traces (which cannot be compared directly) are representative of at least three similar experiments. B, conversion of Indo-1/AM fluorescence ratio to quantitative [Ca\(^{2+}\)]\(_i\) levels. Peak changes in [Ca\(^{2+}\)]\(_i\), in response to TG (as in A) were calculated as described under "Experimental Procedures." Data plotted are mean ± S.E., *n* = 4. *, cysts had a significantly lower peak response to TG compared with that of all other stages tested, *p* < 0.05. **, stages I and II were significantly different from cysts and excyzoites/trophozoites *p* < 0.05. +, excyzoites and trophozoites had significantly higher peak responses than other stages, *p* < 0.05.
Understanding of their biological roles.

Many genes in signaling pathways (10) signaling via second messengers may be especially important in giardial differentiation. Although the giardial genome contains many genes in signaling pathways (10–12), there is little understanding of their biological roles.

The inhibition of excystation by agents that affect cytoplasmic Ca\(^{2+}\) levels by very diverse mechanisms strongly supports the importance of regulation by Ca\(^{2+}\) signaling. The Ca\(^{2+}\) channel blocker verapamil, which should decrease cytoplasmic Ca\(^{2+}\) levels, inhibited excystation, as did EGTA and BAPTA-AM. Similarly, the Ca\(^{2+}\) ionophores, A23187 and ionomycin, which elevated intracellular Ca\(^{2+}\), also inhibited excystation. These ionophores create channels in the bilayer lipid membrane that are selectively permeable to Ca\(^{2+}\). We found that ionomycin increased cytosolic Ca\(^{2+}\) and abolished the gradient across the giardial plasma membrane in all stages. Taken together, these data suggest that cytosolic Ca\(^{2+}\) levels must be closely controlled, especially in Stage II, for excystation to proceed.

In studies with inhibitors, it is crucial to determine the specificity of the effect or target, especially in an organism as divergent as Giardia. Importantly, the targets of the inhibitors used in these studies have been identified at the biochemical and/or molecular level in G. lamblia. Another criterion is whether an inhibitor is active in the concentration range where its effects are specific. In general, the inhibitors used in these studies were effective within the concentration ranges used in mammalian cells, despite the need for them to penetrate or act across the cyst wall. A third criterion is absence of nonspecific toxicity. None of the inhibitors used in these studies killed cysts, and the lower numbers of trophozoites that emerged after exposure to the inhibitors had normal morphology and motility (see Ref. 23 and this work).

The divergence between prokaryotes and eukaryotes is the most significant of evolutionary discontinuities. G. lamblia may straddle this boundary, as it is a true eukaryotic cell that has important prokaryotic properties (40, 44, 45). In evolutionary terms, the divergence of Giardia is reported to be at least twice as ancient as the common ancestor of yeast and man (46). Thus, Giardia may be a valuable model for study of the evolutionary appearance of Ca\(^{2+}\) signaling pathways because this key function diverges so greatly between prokaryotic and eukaryotic cells.

Unlike the bacteria and the archaea, which actively extrude Ca\(^{2+}\) but do not appear to use it commonly for intracellular signaling, eukaryotes sequester this ion in endomembrane-bounded compartments. In contrast, cyclic AMP functions as a second messenger in both bacteria and eukaryotic cells (47, 48). Similar to this work, modulators of cytosolic Ca\(^{2+}\) blocked ookinet differentiation in Plasmodium (49). This ion is also important in invasion of host cells by Leishmania mexicana (50) and in cellular aggression by Entamoeba histolytica (51). Ca\(^{2+}\) ionophores also trigger rupture of the parasite vacuole and escape of Toxoplasma gondii from host cells (52). Trypanosomatis and malaria parasites sequester Ca\(^{2+}\) in novel acidic organelles (53).

Our studies with TG suggest that the giardial ER may sequester Ca\(^{2+}\), a key function of the ER in higher eukaryotic cells. TG increases cytoplasmic Ca\(^{2+}\) levels by inhibiting its re-uptake into the ER. The rapid and robust increase in giardial [Ca\(^{2+}\)] (see Ref. 23 and this work), in response to TG suggests that this storage system may have a large capacity to store Ca\(^{2+}\) and that the Ca\(^{2+}\) cycles into and out of the storage compartment rapidly. Thus, kinetically, the TG-sensitive Ca\(^{2+}\) storage compartment in Giardia resembles the SERCA pump-leak system found in many mammalian cells (18, 36). The ability of G. lamblia to release Ca\(^{2+}\) into the cytoplasm in response to TG was evident throughout the life cycle. Further studies are needed to characterize the exact mechanisms and sites of Ca\(^{2+}\) pumping and sequestration in Giardia and to characterize gene(s) encoding putative Ca\(^{2+}\)-transporting ATPase.

Giardia excystation may also be an important model for study of Ca\(^{2+}\) signaling because of its relative simplicity. Giardia lacks mitochondria, which act as a high capacity, low affinity Ca\(^{2+}\) segregation system (9, 19). Genomic and molecular analyses to date have also revealed fewer genes or isoforms of many proteins in the calcium/CaM signaling pathway. There is no biological or genomic (www.mbl.edu/Giardia) (12) evidence for the existence of a guanylyl cyclase homologue or nitric-oxide synthase pathway to generate cyclic GMP or NO as

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**Fig. 3.** Kinetics of excystation inhibition by TG. 10 μM TG was present only at the stage or stages indicated. *, p < 0.05, significant inhibition compared with solvent control.

**DISCUSSION**

Giardia excystation is an excellent model for studies of cellular activation from dormant states (44). Unlike many other parasites, the G. lamblia life cycle can be completed in vitro (3). Because both the external milieu and host gastrointestinal tract are extremely variable, Giardia must constantly monitor and react to stimuli from its environment. Therefore, cell signaling via second messengers may be especially important in giardial differentiation. Although the giardial genome contains many genes in signaling pathways (10–12), there is little understanding of their biological roles.

In studies with inhibitors, it is crucial to determine the specificity of the effect or target, especially in an organism as divergent as Giardia. Importantly, the targets of the inhibitors used in these studies have been identified at the biochemical and/or molecular level in G. lamblia. Another criterion is whether an inhibitor is active in the concentration range where its effects are specific. In general, the inhibitors used in these studies were effective within the concentration ranges used in mammalian cells, despite the need for them to penetrate or act across the cyst wall. A third criterion is absence of nonspecific toxicity. None of the inhibitors used in these studies killed cysts, and the lower numbers of trophozoites that emerged after exposure to the inhibitors had normal morphology and motility (see Ref. 23 and this work).

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potential second messengers, suggesting that certain eukaryotic mediator pathways may not function in Giardia. Alternatively, these genes may be too divergent to be recognized readily by search programs.

The two major arms of Ca\(^{2+}\) signaling are mediated by CaM and protein kinase C (48). A potential protein kinase C homologue is in the Genome Project Data base (www.mbl.edu/Giardia), but specific inhibitors did not affect excystation (see Ref. 23 and this work). Thus, there appears to be specificity in Ca\(^{2+}\) signaling in excystation, and Ca\(^{2+}\) signaling in Giardia may reflect an ancestral or early diverging state. Moreover, blocking excystation by CaM inhibitors does not appear to be due to cell cycle arrest, as in higher organisms, because giardial cysts undergo two rounds of cytokinesis before they re-enter the cell cycle (14). In the long run, our studies of excystation may reveal novel functions for CaM signaling that are not readily accessible in other cell systems.

Although CaM is among the most conserved proteins known (16, 54, 55), gCaM appears to be highly divergent. The identity of numerous CaM sequences from animals, plants, and fungi ranged from 63 to 67% and similarity ranged from 83 to 87% based on multiple sequence alignment. For example, gCaM has 63% identity and 86% similarity to bovine CaM and 67% identity and 83% similarity to *P. falciparum* CaM (54). In contrast, malaria CaM is 87% identical and 97% similar to bovine CaM. Compared with a 1998 analysis of conserved residues (55), gCaM differed from the consensus of all available sequences in 10 positions. Therefore, gCaM may give valuable insights into CaM evolution and structural requirements.

Although it has no enzyme activity, CaM can bind to and modulate the activities of many enzymes (56, 57). CaM has nine conserved methionine residues that have been shown to be important for interaction with target proteins (58, 59). Single mutations of most of these methionines to glutamine had profound effects on CaM binding to target proteins or peptides (58). However, mutation of four methionines to leucine had little, if any, effect (59, 60). A striking divergence of giardial CaM is that four of the canonical Met positions are occupied by leucine, one by isoleucine, and one by serine. In addition, unlike most CaMs that are devoid of cysteine, gCaM has a cysteine residue near its C terminus (Fig. 6).

Our analyses support the hypothesis that because of its highly divergent amino acid sequence, gCaM will provide a valuable model for probing CaM structure/function in an early eukaryotic cell. Thus, gCaM is a “natural mutant” for investigation of structure/function relationships in this key regulatory protein. It is consistent with findings with site-specific mutants showing that leucine can substitute for methionine (60). Study of gCaM and identification of its downstream targets may help

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3 D. S. Reiner, H. G. Morrison, and F. D. Gillin, unpublished observations.
understand why this substitution has rarely happened in nature.

Earlier, we found that the myristoylated PKA inhibitor, amide 14-22, inhibited excystation and was most effective at the early stages, suggesting that PKA activity is needed for triggering excystation (8). Cysts have a very low rate of glucose uptake and metabolism (61). Because cysts are filled with glycogen (37), glycogenolysis stimulated by PKA may be important to provide initial energy for excystation. In contrast, CaM and Ca\(^{2+}\) homeostasis appear to be more important later, during the cellular activation and emergence phase. CaM may activate one of its downstream effectors, calcineurin (protein phosphatase PP2B), to decrease PKA activity, which is needed early in excystation. This could reflect a change from early dominance of gPKA to gCaM signaling later in excystation. PKA regulatory subunit is a favored substrate of PP2B (62). Moreover, dephosphorylation of cyst wall proteins was reported to be important in excystation (68), although the possibility that this may be due to degradation by protease (7) was not ruled out.

In the case of signaling proteins with many potential downstream effectors, functional specificity can be attained by proximity to cellular structures. PKA localizes to specific cellular sites by interacting with corresponding anchoring proteins (8). CaM is cytosolic in many cells but localizes to the bull sperm midpiece (64) and to the parflagellar rod in trypanosomes (65). The association of CaM with different flagellar structures may suggest roles for CaM in regulating the motility of diverse flagellated cells. Interestingly, we found that both gCaM (Fig. 7) and gPKA (8) localize to the flagellar basal bodies between midpiece (64) and to the paraflagellar rod in trypanosomes (65). This is consistent with changes we have observed during mitosis, when it was also recruited to the centrosomes (9). During encystation, trophozoites, which are shaped like a half-pair, round up and lose the ability to re-attach because their flagella and adhesive disc are curled up inside the cyst wall (68). A major challenge of excystation in the duodenum is for the excyste to emerge from the cyst wall, recover motility, undergo its first round of cytokinesis, and attach before being carried away by intestinal flow. The location of gCaM, especially in proximity to PKA, suggests that the basal bodies may be important cellular control centers that help coordinate the assembly of new flagella with the location of the cleavage furrow for equal distribution of nuclei and cytokinetic structures to each daughter cell.

Ca\(^{2+}\) strongly influences cell mobility, in part by effects on microtubule stability, and microtubules originate at the basal bodies. We found that taxol, which binds to microtubules and stabilizes their polymerization (48), also inhibited excystation (IC\(_{50}\) = 10 \(\mu\)M) at any stage but most strongly in Stage II (data not shown). This is consistent with changes we have observed in the giardial cytoskeleton throughout excystation (7). In contrast, cytocalasin (100 \(\mu\)M), which is a microfilament inhibitor and inhibits trophozoite attachment (11), did not affect excystation (not shown). Most microtubule-mediated cellular functions require remodeling of microtubules, which is inhibited by taxol (48). Thus, taxol likely acts at a number of steps in giardial excystation that require microtubules.

Taken together, these data support the idea that Ca\(^{2+}\) is a key second messenger in regulating excystation of G. lamblia. Many other medically important protozoan parasites, e.g. Cryptosporidium parvum, E. histolytica, and T. gondii, several tapeworms, and nematodes, are transmitted as resistant cyst or oocyst forms. Infection of a new host depends upon the ability to excyst after exposure to gastric and intestinal stimuli (1). Giardia may be a valuable model for understanding parasite differentiation in the intestinal tract. On a basic level, giardial excystation is also a unique model of cellular awakening from dormancy in response to environmental signals. Therefore, it provides valuable opportunities to understand the evolutionary appearance of eukaryotic signaling mechanisms. Conservation of pathways from Giardia to man supports universal function and importance for eukaryotic cells, whereas differences may reflect either early divergence or adaptation to a parasitic life style.

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