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Possible Roles of Protein Kinase A in Cell Motility and Excystation of the Early Diverging Eukaryote Giardia lamblia*

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Since little is known of how the primitive protozoan parasite, Giardia lamblia, senses and responds to its changing environment, we characterized a giardial protein kinase A (gPKA) catalytic subunit with unusual subcellular localization. Sequence analysis of the 1080-kilobase pair open reading frame shows 48% amino acid identity with the cyclic AMP-dependent kinase from Euglena gracilis. Northern analysis indicated a 1.28-kilobase pair transcript at relatively constant concentrations during growth and encystation. gPKA is autophosphorylated, although amino acid residues corresponding to Thr-197 and Ser-338 of human protein kinase A (PKA) that are important for autophosphorylation are absent. Kinetic analysis of the recombinant PKA showed that ATP and magnesium are preferred over GTP and manganese. Kinase activity of the native PKA has also been detected in crude extracts using kemptide as a substrate. A myristoylated PKA inhibitor, amide 14-22, inhibited excystation with an IC50 of 3 μM, suggesting an important role of gPKA during differentiation from the dormant cyst form into the active trophozoite. gPKA localizes independently of cell density to the eight flagellar basal bodies between the two nuclei together with centrin, a basal body/centrosome-specific protein. However, localization of gPKA to marginal plates along the intracellular portions of the anterior and caudal pairs of flagella was evident only at low cell density and higher endogenous cAMP concentrations or after refeeding with fresh medium. These data suggest an important role of PKA in trophozoite motility during vegetative growth and the cellular activation of excystation.

Infection with Giardia lamblia is a major global cause of water-borne diarrheal disease (1). Nonetheless, neither its basic biology nor the pathophysiology of infection is well understood. To date, no giardial toxin or conventional virulence factor has been identified by biological (2) or genomic studies (3). Therefore, it is critical to understand how the parasite survives in the external environment and infects and colonizes a new host. G. lamblia has two life cycle stages that are each remarkably well adapted to survival in very different and inhospitable environments. The dormant, quadrinucleate cyst persists for months in fresh cold water (4). Infection is initiated by ingestion of cysts (5). Exposure of cysts to gastric acid during passage through the host stomach triggers the rapid and dramatic differentiation known as excystation. After entry into the small intestine and stimulation by specific factors (6), the parasite emerges and divides into two equivalent binucleate trophozoites that attach to and colonize the human small intestine. The cyst wall must remain intact during passage through the stomach acid; however, once the cyst enters the small intestine, the wall must open rapidly to enable the parasite to emerge and attach to host enterocytes.

In the small intestine, giardial trophozoites are exposed to complex and everchanging concentrations of hydrogen ions and nutrients, as well as bile acids and digestive enzymes (7). As enterocytes migrate to the tip of the villus, where they are sloughed off into the lumen, attached trophozoites must be able to sense and respond rapidly to environmental signals to remain in the small intestine. Since excystation entails such rapid responses to environmental stimuli, we hypothesized that protein kinase-mediated signaling might be very important. Thus, cell-signaling pathways may be crucial to both giardial colonization in the small intestine and to the cellular activation of excystation. Although many protein kinase gene fragments have been identified by large scale genomic sequencing (Ref. 8; see also the web site for the Giardia Genome Project (Marine Biological Laboratory, Woods Hole, MA)), there is little understanding of their potential roles in giardial survival and growth in the small intestine or in regulation of differentiation.

In many eukaryotic cells, cyclic AMP (cAMP)-dependent signaling pathways play a critical role in regulating cell growth, metabolism, and differentiation (10, 11). Protein kinase A (PKA,1 EC 2.7.1.37), the defining enzyme of the cyclic AMP-dependent signaling pathway alters protein activity by phosphorylation at serine/threonine residues within the motif RX(S/T) (12). PKA is one of the simplest protein kinases known because of its dissociative mechanism of activation (13). PKA holenzyme, a catalytically inactive form, is a tetrameric complex containing two identical catalytic (C) subunits bound to a homodimer of two regulatory (R) subunits (14). Cyclic AMP binding to the R subunit results in dissociation of the holoenzyme.

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‡ The abbreviations used are: PKA, protein kinase A; gPKA, giardial protein kinase A; PKAc, protein kinase A C subunit; PDB, Protein Data Bank; R, regulatory; C, catalytic; PCR, polymerase chain reaction; PKI, protein kinase A inhibitor peptide; PBS, phosphate-buffered saline; ORF, open reading frame; kb, kilobase pair(s).
PKA in Cell Motility and Excystation of *G. lamblia*

with release of active C subunit. The activated C subunit of PKA can phosphorylate a number of intracellular proteins, including enzymes, cytoskeletal proteins, ion channels, and transcription factors (15). How the specific physiological effects of cAMP can be mediated by a broad spectrum protein kinase such as PKA is now being elucidated (16, 17). In a number of diverse cell types, PKA is localized in proximity to substrates by A kinase anchoring protein(s) that bind to both the R subunit and to a specific cell structure, often cytoskeletal (18). Targeting PKA to the proximity of phosphate acceptor protein molecules associated with specific cell structures can also achieve rapid responses to signals (16, 19).

Genes encoding three different C subunits and four different R subunits of PKA have been identified in humans (20). In higher eukaryotes, various combinations of R and C subunits display differences in tissue distribution (21–23), which may determine their functional specificity. The unique distribution and subcellular localization of PKA isoforms in higher eukaryotes may represent a functional interaction with nearby structures. In lower eukaryotes, three isoforms of PKA C subunit have been identified from *Drosophila melanogaster* (24), and from *Saccharomyces cerevisiae* (25). However, only one gene encoding PKA C subunit has been reported from *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Leishmania major*, and *Trypanosoma cruzi* (26–29). *Dictyostelium* PKA plays a key role during differentiation and morphogenesis (30). Similarly, PKA plays critical roles in differentiation of *Leishmania* and *Trypanosoma* (28, 29). In contrast, *Saccharomyces* PKA appears to mediate cellular responses to various extracellular stimuli, including nutrients and heat shock (31).

Here we report the cloning and functional characterization of a giardial homologue of a PKA C subunit. Thus far, only a single isoform of *gPKA* C subunit has been identified in large scale sequencing efforts with ~3-fold coverage of the genome (32). Our studies demonstrate that, although the biochemical properties of the recombinant gPKA are largely similar to those of the mammalian enzyme, gPKA has some noteworthy structural differences. We found that cellular distribution of PKA was correlated with cell density and endogenous cAMP levels. At all cell densities, PKA localized to the eight flagellar basal bodies, which correspond to the centrosomes of higher eukaryotic cell (33). However, at low cell inocula and higher endogenous cAMP concentrations, PKA also localized to marginal plates along the intracellular portions of the anterior and caudal flagella. To our knowledge, this is the first indication of a role for cell signaling in flagellar base-plate associated structures. In addition, a PKA inhibitor greatly decreased giardial excystation. Thus, PKA may play a key role in regulating trophic behavior of the motile form, as well as cellular activation during excystation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless specified, all materials were obtained from Sigma, Fisher, and Life Technologies, Inc.

**G. lamblia Cell Culture and Differentiation**—*G. lamblia* (WB clone C6, ATCC no. 50803) were maintained in TYI-S-33 medium with 10% bovine serum and bile (34). Encystation and excystation were induced essentially as described by Meng et al. (35). Briefly, encystation was induced by growing trophozoites for one culture cycle in TYI-S-33 medium without bile (pre-encystation). Bile-deficient medium was poured off along with unattached trophozoites and replaced with encystation medium containing 0.25 mg/ml porcine bile and 10 mM lactic acid, pH 7.8, and incubated at 37 °C for the time specified. Total encysting cultures were harvested at different time points by chilling and centrifugation and subsequently used for RNA and protein extraction. Cysts were harvested at 66 h by washing and incubating in cold double-distilled water to lyse any trophozoites or incomplete cysts (35). Excystation was induced by a two-step method that models cyst passage from the cold hypotonic freshwater external environment into the warm, acidic stomach, pH 4.0, 20 min, 37 °C (stage 1). Stage 2 models cyst passage from the stomach into the small intestine with exposure to slightly alkaline pH (8.0) and protease (1 mg/ml bovine trypsin type II) for 1 h at 37 °C. Cysts were pelleted at 8300 × *g* and resuspended in growth medium for 60 min at 37 °C. Emerged motile trophozoites were pelleted again, resuspended using a homogenizer with the inhibitor present and measured. The percentage of excystation was calculated as the sum of the motile trophozoites and partially emerged trophozoites divided by the initial number of viable cysts.

**Inhibition of Encystation**—Myristoylated PKA inhibitor (amide 14-22, Calbiochem NovoBiochem, San Diego, CA) was dissolved in water and diluted into water (pre-encystation) or excystation solutions. Inhibition was assessed with the inhibitor present for 1 h before incubation at 4 °C and added again during stage 1 and 2 of excystation, since cysts were pelleted between stages. However, inhibitor was not included in the emergence step in growth medium because of possible effects on trophozoites. 50% inhibitory concentration (*I*50) was estimated by interpolation from concentration-inhibition curves. Viability was determined by trypsin blue exclusion (35). To determine the stage(s) at which each inhibitor was effective, cysts were preincubated with inhibitor (at a higher than IC50 concentration, 10 μM) prior to stage 1 in water at 4 °C, or during stage 1 or stage 2 of excystation.

The mean inhibition was normalized to solvent controls for each experiment. All experiments were repeated at least twice. Data shown are mean (±S.D.) for duplicate wells. *p* values were calculated by paired two-tailed Student’s *t* test with the normalized comparison value set at 100% for control excystation (35).

**PCR Amplification**—A 250-base pair DNA fragment of PKA catalytic subunit was obtained by PCR amplification of *Giardia* genomic DNA using degenerate primers (upper primer, 5′-TACT/CATGGAGACT/C(T) TTAA/A/G/C/TC3′; lower primer, 5′-CCXXA/G/C/G/CAT/C/TCCACAA/G/TC/CTG3′, where X is all four nucleotides). Primers were designed on the basis of the consensus sequence in the catalytic subdomains VIB and IX of the PKA catalytic subunit. Designed degeneracy allowed every possible codon for the consensus amino acid residues to maximize the probability of amplification. The amplified PKA fragment was purified, sequenced, and used to screen a *Giardia* cDNA library.

**Screening of a cDNA Library**—A *G. lamblia* WB clone C6 cDNA library was made in AZAP (Stratagene, La Jolla, CA) using a kit (ZAP-cDNA kit, Stratagene) according to the manufacturer’s protocol. The library was screened for the full-length clone of PKA using 32P-labeled probe as described previously (36). Five positive plaques were obtained by screening ~6 × 106 plaque-forming units. Positive plaques containing inserts were plaque-purified and inserts cloned into pBluescript plasmids by *in vitro* excision using ExAssist helper phage (Stratagene). Inserts were confirmed by restriction digestion and cross-hybridization.

**DNA Sequencing and Identity Analysis**—A capillary electrophoresis-based automated DNA sequencer (ABI Prism, 310 Genetic analyzer; ABI, Foster City, CA) was used to sequence positive clones from the cDNA library. A PCR-based reaction kit (ABI Prism DRhodamine Terminator Cycle Sequencing Ready Reaction Kit; ABI Prism 310 Capillary, ABI, Foster City, CA) was used to sequence inserts from positive plaques. DNA from purified inserts was cloned into a T7 polymerase-driven expression vector pET30EK (exo−) (39). The gPKA ORF was cloned into a T7 polymerase-driven expression vector pET30E/LEC (Novagen, Madison, WI) according to the manufacturer’s protocol. Two primers (upper primer, 5′-GAGCGACGACAGATTATATCAAGTA-3′; and lower primer, 5′-GAGGAGACGGGCTTATTTAATCCCAC-3′) were designed to amplify the PKA ORF (1.08 kb) and part of the vector sequence. The PCR product was treated with T4 DNA polymerase and dATP only to use the exonuclease activity of the polymerase.
lymase to generate vector compatible overlaps. Pre-digested vector and insert were annealed and transformed to use BL21 (DE3) Escherichia coli cells. Induction of gPKA overexpression in BL21 (DE3) was performed at 37 °C with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Expressed gPKA as a 47-kDa His tag and S tag fusion protein was purified through His-Bind columns (Novagen) and used as antigen for generation of polyclonal antibodies through a commercial vendor (Co-Calico, Reamfown, PA). The His tag and S tag sequences added 6 kDa of molecular mass to the fusion protein.

**Western Blot and Immunodepletion Experiment—**Trophozoites and encysting cells were harvested, and total proteins (50 μg) were used for the immunodetection of gPKA using the polyclonal antibody made against recombinant protein. A chemiluminescence detection kit (Fierce) was used to detect positive signals recognized by anti-gPKA antibody using anti-rabbit HRP-conjugated secondary antibody.

Immunodepletion was done in a two-step process. Crude trophozoite extracts (500 μg) in 100 mM Tris, pH 7.5 and 5 mM MgCl2, were diluted in TAN buffer (10 mM Tris acetate, pH 8.0, 1% Nonidet P-40, 100 mM NaCl) containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mM n-ethylemaleimide, 1 μg/ml leupeptin, and 2 μg/ml aprotinin. Ten μg of rabbit IgG was added to the extract and incubated for 2 h at 4 °C to remove nonspecific binding of proteins to gPKA IgG. Next, 150 μl of Protein A-agarose beads (50% slurry) was added and incubated overnight at 4 °C to remove rabbit IgG. Protein A beads were separated next by centrifugation at 15,000 × g at 4 °C for 5 min and the supernatant was incubated with 50 μg of gPKA antibody for 6 h at 4 °C followed by 200 μl of Protein A beads for 18 h at 4 °C. Protein A beads were separated by centrifugation, and the supernatant was used for the kinase assay.

**Assay of PKA Activity—**Trophozoites were grown to confluence, harvested at 700 × g, and washed three times with PBS. They were lysed in a medium containing 100 mM Tris, pH 7.5, and 5 mM MgCl2, and a mixture of protease inhibitors (Sigma) using a French press (750 p.s.i.). The lysate was centrifuged at 30,000 × g, and the supernatant was passed through a syringe filter (0.45 μm). The filtrate was used for the kinase assay using kemptide (Calbiochem-Novobiochem Corp) as a substrate. Fifty μg of crude extract or purified recombinant gPKA (5 μg) was used in a reaction mixture containing 100 mM Tris, 5 mM MgCl2, 0.2 mM ATP, 0.2 mM xanthine, and 0.5 mM [γ-32P]ATP. The reaction mix was incubated at 30 °C for 10 min and spotted (40 μl) on phosphocellulose filter discs. Filters were washed four times for 5 min each with 1% phosphoric acid and bound radioactivity counted in a liquid scintillation counter (LKB). kinase asays using recombinant gPKA were conducted in the presence or absence of the myristoylated PKA inhibitor 14-22 amide. The PKA-specific hexamer inhibitor (ROYALGY) (Bachem) at different concentrations. Assays using crude extract were performed in the presence or absence of 4 μM cAMP (Calbiochem). The catalytic parameters were measured under standard assay condition, and the data were graphically fit to the Lineweaver-Burk form of the Michaelis-Menten equation (40).

**Immunofluorescence Analysis—**G. lamblia trophozoites were inoculated in growth medium at 350–2000 cells/ml in 12-well tissue culture plates and allowed to grow and attach to 18-mm glass coverslips for 65–70 h in an AnaeroPack jar (Mitsubishi Gas Chemical Company, Inc., Japan) at 37 °C. Coverslips containing attached trophozoites were directly fixed in 100% chilled MeOH (−20 °C) for 10 min at −20 °C and further permeabilized with PBS containing 0.5% Triton X-100 for 10 min. Permeabilized trophozoites were blocked for 1 h in blocking buffer (5% goat serum, 1% glyceral, 0.1% BSA, 0.1% fish gelatin, and 0.04% sodium azide in PBS), and incubated with rabbit anti-gPKA polyclonal antibodies (1/2500 in blocking buffer) and/or mouse monoclonal anti-centrin antibodies (clone 20H5, 1/500 in blocking buffer) (41) for 1 h. At the end of incubation, cells were washed for 5 min (four times) with PBS and incubated with the secondary antibodies (anti-rabbit ALEXA 488 and/or anti-mouse ALEXA 568. Molecular Probes, Eugene, OR) each diluted in blocking buffer 1/800 for 1 h. Next, cells were washed for 5 min (four times) with PBS, post-fixed for 7 min with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in PBS, rinsed with PBS, and mounted on glass slides in Prolong (Molecular Probes). Localization of gPKA and centrin was observed on a Zeiss LSM 510 confocal microscope equipped with argon/krypton (458/488) and helium/neon (543/633) lasers and appropriate filterset.

**cAMP Measurements—**To measure endogenous cAMP levels in various stages of G. lamblia growth, a competitive cAMP enzyme immunoassay (Biotrack Cellular Communications Assays, Amersham Pharmacia Biotech) was employed. G. lamblia were cultured as described for immunolocalization. Using the nonacetylated protocol 3 method of the Biotrack enzyme immunoassay, parasites were lysed in a 500-μl vol-
gPKA Has a Predicted Additional Loop in the Activation Domain—Using an internet-based protein modeling program (37, 38), we compared the three-dimensional structure of gPKA to the α-catalytic subunit of bovine PKA complexed with the inhibitor staurosporine (PDB accession no. 1STC) and H8 protein kinase inhibitor (PDB accession no. 1YDS) (Fig. 2A). However, this is a predicted three-dimensional structure based on the actual crystal structure of the template complexed with inhibitors, which may contribute some degree of inaccuracy. Nonetheless, the superimposed structure of gPKA indicates two major differences. A small loop (red) is in subdomain XI at the C-terminal end of the kinase domain (Fig. 2, A and B). The interaction of the invariant arginine corresponding to Arg-280 (Fig. 2B, yellow arrow), which stabilizes the large lobe by forming an ion pair with Glu-208 is predicted to be present in gPKA (Arg-254 and Glu-182), but the hydrogen (H) bond between Arg-280 and Asn-283 appears to be missing in gPKA. Asn-283, which acts as a phosphate donor for optimum activity (data not shown). Kinase activity of recombinant gPKA using histone (Fig. 3) and kemptide (see below) as substrates. Kemptide is a synthetic heptapeptide (LRRASLG) and is highly specific for PKA. Recombinant gPKA phosphorylated both substrates (specific activity 6.2 ± 0.2 nmol of phosphate/min/μmol of enzyme) and was also capable of autophosphorylation (Fig. 3, A and B). Purified gPKA autophosphorylated in a concentration dependent manner with a stoichiometry of 1–1.8 mol of phosphate/mol of gPKA. Phosphorylated PKA increased with incubation time and was not observed in the control lane without enzyme. The recombinant PKA band is also absent from the control no extract lane in the protein gel (Fig. 3, A and B). The apparent Km for Mg2+ATP and kemptide are in the micromolar range instead of the millimolar range that has been reported for vertebrate PKAc (42). Additionally, Mg2+ is preferred over Mn2+ as the divalent cation and ATP over GTP as the phosphate donor for optimum activity (data not shown). Kinase activity of recombinant gPKA with histone or kemptide was not inhibited by the 6-22 amide (PKI), which is potent inhibitor of mammalian PKAs (data not shown). However, both the shorter myristoylated peptide inhibitor, 14-22 amide (which inhibited giardial excystation, see below) and a PKA-specific synthetic peptide inhibitor, 14-22 amide (which inhibited the large lobe of P. falciparum PKA) showed no inhibition of recombinant gPKA (data not shown). The shorter myristoylated peptide inhibitor, 14-22 amide (which inhibited giardial excystation, see below) and a PKA-specific synthetic peptide inhibitor, 14-22 amide (which inhibited the large lobe of P. falciparum PKA) showed no inhibition of recombinant gPKA (data not shown).
Increasing color intensity represents increasing degree of mismatches leading to red gel showing equal amounts of histone and gPKA in all lanes except in lane N. N contains histone but no extracts. B, autoradiogram showing phosphorylated histone and gPKA. C, Lineweaver-Burk plot of the phosphorylation of kemptide by gPKA at 30 °C. Each point represents the mean of three separate experiments. Apparent $K_m$ for kemptide, ATP, and Mg$^{2+}$, and specific activity of gPKA are presented in the box. D, inhibition of phosphorylation of kemptide by myristoylated PKA inhibitor 14-22 and by the synthetic hexapeptide inhibitor (RGYALG). Data represented as mean ± S.D. (* indicates $p < 0.03$ (n = 3) for the myristoylated inhibitor and $p < 0.005$ (n = 2) for the hexapeptide; ** indicates $p < 0.04$).

hexapeptide inhibitor (RGYALG) were able to inhibit phosphorylation of kemptide by gPKA with IC$_{50}$ values of 10 μM (Fig. 3D) and 3 mM, respectively. The IC$_{50}$ of the hexapeptide inhibitor was lower than that obtained with the PKA from rabbit skeletal muscle (46).

**Catalytically Active PKA Is Present in Giardia Trophozoites**—We measured the phosphorylating ability of the native PKA in the crude extracts of trophozoites using kemptide as the specific substrate (Fig. 4). PKA activity was monitored in the presence or absence of kemptide (Fig. 4B) to eliminate phosphorylation of endogenous substrates by PKA and other kinases. A 5-fold increase in activity was obtained with kemptide, indicating active gPKA in the trophozoite extracts (specific activity 1.2 ± 0.26 nmol/min/mg of protein). Exogenous cAMP at a concentration (4 μM) slightly higher than that used for the mammalian PKA was able to stimulate gPKA activity in the crude extract. A 2.5-fold increase in kemptide phosphorylation was noted following addition of cAMP in the crude extract ($p < 0.002$ (Fig. 4C). To confirm that the activity was due to native PKA, we immunodepleted gPKA from crude trophozoite extracts by immunoprecipitation using a rabbit polyclonal antibody against recombinant gPKA. The specificity of the gPKA antibody was shown by its reaction with the 47-kDa recombinant gPKA and with a single 41-kDa polypeptide in the crude extract in Western blot at a dilution of 1:7000 (Fig. 4C). The cAMP-stimulated phosphorylation of kemptide in crude extracts was due to gPKA and was also supported by immunodepletion studies. As expected, addition of kemptide did not increase kinase activity of the depleted crude extract (Fig. 4B).

Depletion of the native PKA was confirmed by Western blot analysis, which shows no PKA protein band in the depleted lane (Fig. 4A). The higher band in this lane is the residual PKA IgG that reacted with anti-rabbit secondary antibody.

**Immunolocalization of gPKA and cAMP Measurements in Trophozoites**—The cellular localization of PKA enzyme in *G. lamblia* trophozoites was determined using primary antiserum generated to purified recombinant Giardia PKA (or anti-gPKA) and confocal microscopy. At low cell densities (achieved with inocula of 350–700 cells/ml), distinct signals were seen in dense rods or marginal plates along the intracellular portions (axonemes) associated with the pairs of anterior and caudal flagella, as well as in the eight basal bodies located between the nuclei (Fig. 5A). Localization of gPKA to the flagellar basal bodies was confirmed by colocalization with monoclonal anti-centrin antibody (41) (Fig. 5B). In addition to the basal bodies, anti-centrin localized along the two axonemes associated with the posterior-lateral flagella that were not recognized by gPKA (Fig. 5B). Neither antibody reacted in the region of the ventro-lateral pair of flagella. Localization of gPKA at high cell density (inoculum > 1400 cells/ml) also showed staining of basal bodies. However, there was no localization to axonemes and diffuse staining was observed in the cytosol (Fig. 5C). Refeeding cells from the higher inocula cultures with fresh medium for 10 min, led to the localized patterns (Fig. 5A); however, exposure to exogenous dibutyryl cAMP did not (data not shown). Results from the cAMP analyses showed that those cells having a diffuse staining pattern (Fig. 5C) had significantly ($p < 0.03$ for comparisons of 700 with 1400 and 2000 cells/ml) lower endogenous levels of cAMP compared with those parasites with localized staining (Fig. 5A) to flagellar marginal plates compo.
Detection of active gPKA in trophozoite extracts. A, Western blot. Detection of gPKA in trophozoite extracts before and after immunodepletion of gPKA by PKA antibody. The PKA IgG band in the depleted extract lane is the remaining gPKA antibody recognized by the anti-rabbit secondary antibody. B, kinase activity in crude extracts and immunodepleted extracts using kemptide as the substrate. Data represented as mean ± S.D. (n = 3). C, effect of exogenous cAMP on the kinase activity in the crude extract. Data represented as mean ± S.D. (p < 0.02, n = 4).

FIG. 4. Detection of active gPKA in trophozoite extracts. A, Western blot. Detection of gPKA in trophozoite extracts before and after immunodepletion of gPKA by PKA antibody. The PKA IgG band in the depleted extract lane is the remaining gPKA antibody recognized by the anti-rabbit secondary antibody. B, kinase activity in crude extracts and immunodepleted extracts using kemptide as the substrate. Data represented as mean ± S.D. (n = 3). C, effect of exogenous cAMP on the kinase activity in the crude extract. Data represented as mean ± S.D. (p < 0.02, n = 4).

Discussion

Since PKA regulates critical cellular processes in both higher and lower eukaryotes, we characterized a homologue of a PKA catalytic subunit of Giardia. Sequence and structural predictions showed that most of the kinase subdomains that are conserved in higher eukaryotes, are present in gPKA, and we have also identified a regulatory subunit homologue (data not shown), suggesting a conventional PKA holoenzyme. Despite several structural differences, functional characterization demonstrates that both recombinant and native PKA are catalytically active and phosphorylate a PKA-specific substrate, such as kemptide as well as histone. Structural alignments, substrate specificity, and stimulation of the kinase activity of native PKA by cAMP all suggest that the cloned ORF is indeed a Giardia homologue of the C subunit of PKA.

In the case of a ubiquitous enzyme, such as PKA, it is important to understand how different physiological conditions determine specificity. A prominent level of control is to localize signaling proteins to specific subcellular organelles or compartments. Thus, it is very interesting that, in Giardia, PKA can localize to both basal body/centrosome and specific flagellar structures. In certain cells, protein kinase A-anchoring proteins or AKAPs can bind to a PKA regulatory subunit, which then localizes the catalytic subunit to the proper structure. For example, AKAP450 targets PKA to centrosomes in HeLa cells (47). In bovine and human sperm, mAKAP82 localized PKA, via its RII subunit, to the fibrous sheath (48), between the axoneme and the surrounding mitochondrion. In contrast, PKA of a single-celled flagellated alga, Chlamydomonas, is reported to be localized to the radial spokes of the flagellar structure, where it may be involved in regulating dynein ATPase (49). The location of PKA in giardial flagellar structure appears closer to that of mammalian sperm than to that of Chlamydomonas.

Immunolocalization of gPKA in trophozoites showed a selective accumulation of PKA in the eight flagellar basal bodies at low and high cell densities. Localization of PKA to the basal bodies was confirmed by colocalization with centrin, a basal body-specific Ca$^{2+}$-binding protein. Basal bodies are rodlike structures associated with the origins of the flagellar axonemes and are involved in cell motility. The basal bodies of flagellated cells correspond to the centrosomes of higher eukaryotic cells (50). Centrin, which is found in centrosomes in higher eukaryotes and basal bodies of all flagellated and ciliated cell types including Giardia (51), is also involved in mitotic spindle pole segregation in Saccharomyces (52). Interestingly, sequence analyses have shown that a giardial centrin gene has a motif that predicts phosphorylation by PKA. Indirect immunofluorescence studies revealed that antibody specific for phosphorylated centrin reacted with the basal bodies of dividing cells, but not interphase cells. Thus, centrin in the basal bodies may be a substrate for phosphorylation by PKA during a specific stage of the cell cycle. In contrast, gPKA was associated with the paired anterior and caudal flagella only at low cell densities, which correlated with higher endogenous cAMP levels. This is the first evidence that trophozoites can respond to signals from their environment with changes in cAMP levels.

2 W. L. Lingle and J. L. Salisbury, personal communication.
and altered localization of a protein kinase. Since the flagellar localization was also found after refeeding high density cultures, it is likely a response to growth factors.

Giardia has four pairs of eukaryotic flagella (5) that are responsible for both motility and attachment. Each originates from a basal body between the two nuclei and every pair of flagella traverses part of the cell body and emerges at a specific location. Paraflagellar densities or marginal plates have been observed by electron microscopy, along much of the intracellular portion of all four pairs of flagella, but little is known about the composition or function of the marginal plates (53). Only the extracellular portions of the flagella are covered with plasmalemma.

The ability of trophozoites to alter their flagellar motility in response to external conditions may be critical to their ability to colonize the small intestine where the availability of growth factors is constantly changing. The anterior flagella cross anteriorly from the basal bodies and pass through the cell's ventrolateral flange before exiting the cell body. The ventrolateral flange is the outer margin of the cell body that interdigitates between microvilli of the small intestinal enterocytes (9), along with the ventral adhesive disc in trophozoite attachment. The ventrolateral flagella, stained for centrin, but not for PKA. Both the anterior and ventrolateral flagella are important in forward motion of the trophozoite and continue to beat while the parasite is attached to intestinal enterocytes or to inert substrates. In contrast, the caudal flagella, whose marginal plates also stained for PKA, do not appear to beat during swimming or attachment and have a rigid appearance, very distinct from the helical form of the other flagella (9). The

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**Fig. 5.** Immunolocalization of gPKA and analysis of cAMP levels in *G. lamblia* trophozoites. A, at low cell density, anti-gPKA recognizes the intracellular portions of caudal flagella (CF), anterior flagella (AF), and the basal bodies (BB) located between the nuclei. B, centrin localization to the posteriolateral flagella. Colocalization of centrin and gPKA was observed to the basal bodies shown in yellow and indicated by an arrow. C, gPKA was associated with basal bodies (arrow) and the cytosol, but not the flagella, at high cell densities. D, trophozoites that had localized (L) staining to caudal flagella and anterior flagella had significantly higher (*) levels of endogenous cAMP, compared with trophozoites that appeared to have diffuse (d) staining in the cytosol. Data are represented as mean femtomoles of cAMP/10^5 cells ± S.D. (n = 5).
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caudal flagella are thought to be associated with dorsal flexion of the "tail" of the cell body that is involved in trophozoite detachment. Since *Giardia* attach to enterocytes, which migrate to the tip of the villus and are then sloughed off, trophozoites must be able to detach, swim, and re-attach to younger host cells to remain in the small intestine. Our finding of association of gPKA with anterior and caudal flagella, whereas centrin associates with the posterior-lateral flagella, may help understand differential flagellar function. Localization of gPKA and centrin to structures responsible for both motility and cell division may link those functions in an ancient eukaryote. These data support a role for PKA and cAMP in regulating *Giardia* responses to its environment.

Blocking excystation with the cell permeable PKA inhibitor (amide 14-22) suggests a key role for gPKA in this critical differentiation. The role of PKA appears most critical early in excystation. Cysts are ingested from cold freshwater and are differentiated. The role of PKA appears most critical early in excystation (35) and is first exposed to the highly acidic gastric fluid (pH 2.0 to pH 4.0), which is needed for the induction of excystation (35) and is known to increase cAMP concentrations. Yeast must react to transiently favorable growth conditions that allow stationary phase cells to switch to a growth program. Perhaps *Giardia*’s needs are more complex, as its environment changes drastically during excystation. Moreover, within the small intestine, conditions change rapidly according to whether trophozoites are attached to young or senescent enterocytes, or are in the lumen and according to the nutritional status of the host. Our data suggest key roles for PKA in regulating giardial responses to its environment.

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REFERENCES

1. Marshall, M. M., Naumovitz, D., Ortega, Y., and Sterling, C. R. (1997) *Clin. Microbiol. Rev.* 10, 67–85
2. Smith, P. D., Gillin, F. D., Spira, W. M., and Nash, T. E. (1982) *Gastroenterology* 83, 797–803
3. Adam, R. D. (2000) *J. Parasitol.* 86, 475–484
4. Bingham, A. K., Jarroll, E. L., Meyer, E. A., and Radulescu, S. (1979) *Exp. Parasitol.* 47, 284–291
5. Adam, R. D. (1991) *Microbiol. Rev.* 55, 706–732
6. Rice, E. W., and Schaefer, F. W., III (1981) *J. Clin. Microbiol.* 14, 709–710
7. Davenport, H. W. (1977) *Physiology of the digestive tract*, Year Book Medical Publishers, Chicago.
8. Smith, M. W., Aley, S. B., Sogin, M. L., Gillin, F. D. and Evans, G. (1998) *Mol. Biochem. Parasitol.* 95, 267–280
9. Erlander, S. L., and Feely, D. E. (1984) in *Giardia and Giardiasis: Biology, Pathogenesis and Epidemiology* (Erlandsen, S. L., and Meyer, E., eds) pp. 33–65, Plenum Press, New York.
10. McEwen, G. S. (1991) *Curr. Opin. Cell Biol.* 3, 213–217
11. Tramontano, D., Moses, A. C., and Ingbar, S. H. (1988) *Endocrinology* 122, 338–336
12. Walsh, D. A., Perkins, J. P., and Krebs, E. G. (1968) *J. Biol. Chem.* 243, 3763–3765
13. Francis, S., and Corbin, J. (1994) *Annu. Rev. Physiol.* 56, 237–272
14. Kennelly, P. J., and Krebs, E. G. (1991) *J. Biol. Chem.* 266, 15555–15558
15. Habener, J. F. (1991) in *Endocrinology* (DeGroot, L. J., ed) 3rd Ed., pp. 77–92, W. B. Saunders, Philadelphia
16. Rubin, C. S. (1994) *Biochem. Biophys. Acta* 1242, 467–479
17. Schilieze, R. V., and Scott, J. D. (1999) *J. Clin. Invest.* 103, 761–785
18. Edwards, A. S., and Scott, J. D. (2000) *Curr. Opin. Cell Biol.* 12, 217–221
19. Pavlidis, T., and Scott, J. D. (1997) *Science* 278, 2075–2080
20. Skalhegg, B. S., and Tasken, K. (1997) *Frontiers Biosci.* 2, D331–D342
21. Uhler, M. D., Chriavia, J. C., and McElhinny, G. S. (1986) *J. Biol. Chem.* 261, 15360–15363
22. Advani, S. R., Schwarz, M., Shovers, M. O., Maurer, R. A., and Hemmings, B. A. (1987) *Eur. J. Biochem.* 167, 221–2226
23. Shover, M. O., and Maurer, R. A. (1988) *J. Biol. Chem.* 261, 16288–16291
24. Kaldeman, D., and Rubin, G. M. (1988) *Genes Dev.* 2, 1539–1556
25. Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987) *Cell* 50, 277–287
26. Plowman, G. D., Sudarsanam, S., Bingham, J., Whyte, D., and Hunter, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 13603–13610
27. Burki, E., Anjard, C., Scholder, J. C., and Reymond, C. D. (1991) *Gene (Amst)* 102, 57–65
28. Simian-Tov, M. M., Aly, R., Shapira, M., and Jaffe, C. L. (1996) *Mol. Biochem. Parasitol.* 77, 201–215
29. Vassella, E., Reuner, B., Tutz, B., and Boshart, M. (1997) *J. Cell Sci.* 110, 2661–2671
30. Lasko, P. (1995) *BioEssays* 17, 105–107
31. Toda, T. Cameron, S., Sass, P., Zoller, M., Scott, J. D., Hurwitz, M. M., Krebs, E. G., and Wigler, M. (1987) *Mol. Cell. Biol.* 7, 1371–1377
32. Mc Arthur, A. G., Morrison, H. G., Nixon, J. E. J., Passamanek, N. Q. E., Kim, U., Reich, C. I., Holder, M. E., Hinkle, G., Crocker, M. K., Farr, R., Olsen, G. E., Aley, S. B., Adam, R., Gillin, F. D., and Sogin, M. L. (2000) FEMS Microbial Lett. 189, 271–273
33. Moujou, M., Paintrand, M., Vigues, B., and Bornens, M. (1991) J. Cell Biol. 115, 129–140
34. Keister, D. B. (1983) Trans. R. Soc. Trop. Med. Hyg. 77, 487–488
35. Meng, T. C., Hetsko, M. L., and Gillin, F. D. (1996) Infect. Immun. 64, 2151–2157
36. Chakrabarti, R., McCraken Jr. J. B., Chakrabarti, D., and Souha, W. W. (1995) Gene (Amst.) 153, 163–169
37. Peitsch, M. C. (1996) Biochem. Soc. Trans. 24, 274–279
38. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
39. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
40. Lineweaver, H., and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–666
41. Meng, T-C., Aley, S. B., Svard, S. G., Smith, M. W., Huang, B., Kim, J., and Gillin, F. D. (1996) Mol. Biochem. Parasitol. 79, 103–108
42. Smith, C. M., Radzio-Andzelm, E., Madhusuda, Akamine, P., and Taylor, S. S. (1999) Prog. Biophys. Mol. Biol. 71, 313–341
43. Sogin, M. L. (1989) Science 243, 75–77
44. Rubin, C. S. (1979) J. Biol. Chem. 254, 12439–12449
45. Cobb, C. E., and Corbin, J. D. (1988) Methods Enzymol. 159, 292–298
46. Kemp, B. E., Benjamin, E., and Krebs, E. G. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1038–1043
47. Witzczak, O., Skålhegg, B. S., Keryer, G., Bornens, M., Taskén, K., Jahnson, T., and Ørstavik, S. (1999) EMBO J. 18, 1858–1868
48. Moss, S. B., Turner, R. M., Burkert, K. L., VanScoy, B. H., and Gerton, G. L. (1999) Biol Reprod. 61, 335–342
49. Yang, P., and Sale, W. S. (2000) J. Biol. Chem. 275, 18905–18912
50. Salisbury, J. L. (1995) Curr. Opin. Cell Biol. 7, 39–45
51. Holberton, D. V. (1973) J. Cell Sci. 13, 11–41
52. King, S. (2000) Biochin. Biophys. Acta 1496, 60–75
53. Li, Q., and Bever, C. T., Jr. (1998) Oncol. Rep. 5, 227–233
Possible Roles of Protein Kinase A in Cell Motility and Excystation of the Early Diverging Eukaryote *Giardia lamblia*

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