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Identification and Characterization of a Novel Calcium-Activated Apyrase from Cryptosporidium Parasites and Its Potential Role in Pathogenesis

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Abstract

Herein, we report the biochemical and functional characterization of a novel Ca2+-activated nucleoside diphosphatase (apyrase), CApy, of the intracellular gut pathogen Cryptosporidium. The purified recombinant CApy protein displayed activity, substrate specificity and calcium dependency strikingly similar to the previously described human apyrase, SCAN-1 (soluble calcium-activated nucleotidase 1). CApy was found to be expressed in both Cryptosporidium parvum oocysts and sporozoites, and displayed a polar localization in the latter, suggesting a possible co-localization with the apical complex of the parasite. In vitro binding experiments revealed that CApy interacts with the host cell in a dose-dependent fashion, implying the presence of an interacting partner on the surface of the host cell. Antibodies directed against CApy block Cryptosporidium parvum sporozoite invasion of HCT-8 cells, suggesting that CApy may play an active role during the early stages of parasite invasion. Sequence analyses revealed that the capy gene shares a high degree of homology with apyrases identified in other organisms, including parasites, insects and humans. Phylogenetic analysis argues that the capy gene is most likely an ancestral feature that has been lost from most apicomplexan genomes except Cryptosporidium, Neospora and Toxoplasma.

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Introduction

Cryptosporidiosis, caused by the obligate intracellular protozoan Cryptosporidium, is a ubiquitous infectious disease that can cause a persistent and chronic diarrhea [1]. Most Cryptosporidium infections in humans are self-limiting, but severe disease may occur in immunodeficient hosts, in particular in AIDS patients [2]. Although Cryptosporidium hominis (C. hominis) is the most prevalent cause of endemic disease in humans, Cryptosporidium parvum (C. parvum) also can cause serious outbreaks when humans are exposed to contaminated water supplies or are otherwise in close contact with the non-human carriers of this parasite. Effective vaccines and safe non-toxic anti-cryptosporidial drugs are not yet available. The cellular and molecular mechanisms of infection are poorly understood, mostly due to difficulties in propagation of the parasite in culture.

A Cryptosporidium infection is initiated when the host ingests oocysts from which invasive sporozoites emerge and infect enterocytes [3]. Clearly, interactions between macromolecules of the parasites and host cells are of critical importance for the establishment of the infection and consequent survival of the parasite. Thus, pathogenic factors such as parasite proteins or macromolecules responsible for attachment or invasion, or factors that block host cell responses, are ideal targets for drug and vaccine development.

Nucleotide mediated signaling plays a central role in maintaining homeostasis in many tissues. Thus, ecto-nucleotidases are major players in the regulation of purinergic signaling, modulate inflammation and immune responses in Langerhans cells [4], and lead to cardioprotection and protective responses to hypoxia/ischemia in mice [5,6]. As signaling molecules, extracellular nucleotides also serve as danger signals induced by pathogen infection as well as cell or tissue injury, triggering various cellular events such as proliferation, differentiation and chemotaxis [7]. Recently, high ecto-nucleotidase activity of several protozoan parasites - including Toxoplasma gondii, Entamoeba histolytica, Leishmania tropica, Leishmania amazonensis, Trypanosoma cruzi, Trypanosoma brucei, and Trichomonas foetus - has been shown to interfere with the extracellular signaling of the host and affect the virulence and pathogenesis of these organisms [8,9,10,11,12,13,14,15,16].
16,17]. Thus, it has been suggested that these enzymes play a role in the pathogenicity of these parasites by controlling the host cell response to infection, specifically by: (i) protecting the parasite from the cytolytic effects of extracellular ATP, (ii) regulating ectokinase substrate concentrations, (iii) preventing activation of signal transduction cascades associated with cellular injury, and (iv) facilitating cellular adhesion [18,19,20,21,22,23,24,25,26,27,28], reviewed in [29].

Among ecto-nucleosidases, Ecto-ATPases, or E-ATPases, are cell-surface enzymes that hydrolyze a range of extracellular nucleoside triphosphates (NTPs) and nucleoside diphosphates (NDPs). Most of the E-ATPases are apyrases (ATP diphosphohydrolases, EC 3.6.1.5), enzymes that were originally defined as those that catalyze the hydrolysis of both adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP) and inorganic phosphate (P_i) [18]. The majority of known apyrases belong, on basis of sequence homology, to the CD39 family. CD39, also known as ENTPD1 (ectonucleoside triphosphate diphosphohydrolase 1), is an integral plasma membrane protein with two transmembrane domains and a large heavily glycosylated extracellular region with nucleoside triphosphate diphosphohydrolase activity [18,29,30]. However, a novel and evolutionarily distinct apyrase, that differs from the CD39 family in amino acid sequence as well as its exclusive calcium-dependent functionality, has been identified in the salivary glands of blood-sucking bed bug Cimex lectularius [31]. A series of homologs to the C. lectularius gene were recently found in other blood-sucking insects, as well as in vertebrates, including humans, indicating that these enzymes represent an evolutionarily widespread family of proteins [31,32,33,34,35,36,37,38].

Herein we describe for the first time the biochemical and functional characterization of an apyrase from C. hominis, designated CApy, its potential role during the infection, and the resulting implications for pathogenesis during cryptosporidiosis.

Materials and Methods

Ethics statement

All mice were housed at the vivarium at Virginia Commonwealth University (VCU) in an AAALAC-accredited facility and experimentation followed VCU Institutional Animal Care and Use Committee approved protocols (VCU IACUC Approved Protocol AM10329, Cryptosporidium Reverse Vaccinology). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).

Mammalian cell culture and C. parvum

Intestinal epithelial HCT-8 cells were obtained from ATCC (CCL-244) and grown in 75-cm² flasks in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal calf serum, in 37°C and 5% CO₂. C. parvum oocysts were obtained from the University of Arizona. Cells were stored at 4°C until use.

Plasmid construction

The sequence encoding the C. hominis apyrase gene (CApy) (Chro. 60194) lacking the N-terminal signal sequence was obtained by PCR amplification from C. hominis genomic DNA and cloned into the pTriEx-1-Ek/LIC vector (Novagen) using the following primers: apyLICF 5′GACGACGGCAAAGATGATAGA-CGAAAGGAGGGTTG and apyLICR 5′GAGGAGAGCG-CGGTTATATAAATTGATCCGCTGATT. The 5′ end of the primers incorporated the LIC (ligation independent cloning) sequences (underlined). The amplified CApy product was ligated into pTriEx-4 after treatment with LIC-qualified T4 DNA polymerase as described by the manufacturer (Novagen). E. coli strain NovaBlue (Novagen) and E. coli strain BL21(DE3) (Novagen) were used for plasmid maintenance and protein expression, respectively.

Expression and purification of rCApy protein

The E. coli strain BL21(DE3) transformed with pTriEx-4/CApy was cultured aerobically in TB medium (Omnipoint ExpressTM Autoinduction System, Novagen) supplemented with ampicillin (100 μg/ml) at 37°C under constant agitation. The CApy protein was purified and expressed in inclusion bodies (not shown). Cell pellets were resuspended in BugBuster protein extraction reagent (Novagen) and incubated for 30 min. The suspension was centrifuged at 39000 x g (Sorvall SS-34 rotor) for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended in BugBuster protein extraction buffer (Novagen) and incubated for 5 min at room temperature. The resulting protein is fused to a His6-S-tag and was expressed in inclusion bodies (not shown). Cell pellets were resuspended in BugBuster protein extraction buffer and added and mixed by vortexing for 1 min. The suspension was centrifuged for 15 min at 39000 x g (Sorvall SS-34 rotor) for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended in BugBuster protein extraction reagent with LysosymeTM solution (Novagen). Following incubation for 5 min at room temperature, 6 volumes of 1:10 diluted BugBuster protein extraction buffer was added and mixed by vortexing for 1 min. The suspension was centrifuged for 15 min at 39000 x g (Sorvall SS-34 rotor) for 4°C and the supernatant was discarded. rCApy inclusion bodies were resuspended in 20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 6 mM guanidine hydrochloride (buffer 1) and vortexed for 5 min. The buffer was adjusted to 20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 4 mM guanidine hydrochloride, 10 mM imidazole (buffer 2) and applied to Ni²⁺ chelate affinity chromatography using a column with a 1 ml bed volume (GE Healthcare). The solubilized rCApy inclusion bodies were loaded onto a Ni²⁺ column in buffer 2 (GE Healthcare). Bound proteins were washed with buffer 3 (buffer 1 with 25 mM imidazole) and eluted with buffer 4 (buffer 1 with 500 mM imidazole). The eluted material was renatured by dialysis overnight against a minimum of 50 volumes of 100 mM Tris, pH 8.0, 1 M arginine, 2 mM EDTA, 1 mM GSH (glutathione reduced), 0.1 mM GSSG (glutathione oxidized), 5% glycerol at 4°C. Finally, after additional dialysis overnight at 4°C against a minimum of 200 volumes of PBS (phosphate buffered saline), pH 7.4, or 20 mM MOPS, pH 7.4, the purity of the CApy protein preparation was examined by SDS-PAGE analysis. Protein concentrations were determined according to Bradford (BioRad).

To purify Nus, the supernatant after bacterial cell lysis as described above was diluted 1:1 with 20 mM sodium phosphate pH 7.4, 500 mM NaCl, 10 mM imidazole (buffer 5) and loaded onto a Ni²⁺ chromatography column (GE Healthcare) equilibrated with buffer 5. Column bound proteins were washed with buffer 6 (buffer 5 with 50 mM imidazole) and eluted with buffer 7 (buffer 5 with 500 mM imidazole). The eluted material was dialyzed overnight against 4°C against a minimum of 200 volumes of PBS, pH 7.4.

Production of antibodies against rCApy

Groups of five female 6 to 8 week old C57BL/6 mice (Jackson Laboratory, MA) were immunized i.p. on day 0 with 20 μg purified rCApy or Nus formulated in Freund’s complete adjuvant.
Mice were boosted i.p. twice on day 21 and 42 with 20 μg purified rCAp or Nus formulated in incomplete Freund’s adjuvant. Blood samples were collected on day 63 and sera were prepared, pooled, and stored at −20°C. The serum against Nus protein was used as an unrelated control serum.

**SDS-PAGE and Western blot analysis**

To obtain sporozoites, 1×10^6 *C. parvum* oocysts were washed three times (5,000×g for 4 minutes at 4°C) with Hanks’ Balanced Salt Solution, transferred to excystation medium (0.75% Sodium Taurocholate and 0.25% Trypsin in Hanks’ medium) and incubated 37°C for one hour.

For preparation of soluble and insoluble fractions of oocysts and sporozoites, parasites were hyed in a NucleiP P-40 based lysis buffer (1% Nonidet P-40, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 20% glycerol, proteinase inhibitor cocktail (Roche)) for 20 min at room temperature. After centrifugation at 16,000×g, the supernatant (soluble fraction) was collected and the pellet (insoluble fraction) was dissolved in Nonidet P-40 based lysis buffer.

To remove N and O-linked oligosaccharides from native CAp, sporozoites obtained as described above were treated with the Enzymatic Carbo Release Kit™ (QBio, San Mateo, CA) as recommended by the manufacturer. In brief, 2×10^6 sporozoites in 35 μl of water were mixed with 55 μl of Carbo Release buffer. After addition of 27.5 μl denaturation buffer, the sample was boiled for 5 min, chilled on ice, and supplemented with 27.5 μl Triton-X. For incubation with various combinations of carbohydrate removing enzymes, the sporozoite extract was distributed into 1.5 ml microfuge tubes (45 μl each), and 1 μl each of the following enzymes were added: PNGase, sialidase, β-galactosidase, glucosaminidase and/or O-glycosidase, as recommended by manufacturer. After incubation at 37°C for 16 hr, protein loading buffer was added, the samples were incubated at 100°C for 5 min, and an equivalent of 2.5×10^6 sporozoites was subjected SDS-PAGE (12% polyacrylamide). Separated proteins were transferred to PVDF nitrocellulose membrane (Millipore) for 90 min at 350 mA and 4°C. Following blocking for 1 h at room temperature with 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween20 (TBST) containing 2% milk powder (blocking buffer); the nitrocellulose membrane was incubated for 1 hour at room temperature with mouse anti-rCAp serum diluted 1:500 in blocking buffer. After 3 washes with TBST, the membrane was further incubated with anti-mouse IgG HRP-conjugate (Sigma) diluted 1:5000 in blocking buffer. The final reaction was revealed by chemiluminescence using ECL Western blotting detection reagent (Pierce) and BioMax light film (Kodak) as recommended by the manufacturers.

**Apyrase activity assays**

Apyrase activity assays were conducted in 96-well microtiter plates at 23°C in a final volume of 80 μl of 20 mM MOPS, pH 7.4, 100 mM NaCl containing 0.5 μM rCAp and 2.5 mM NTPs, NDPs, or NMPs. Additionally, CaCl2, MgCl2 and/or EDTA the solutions to an end concentration of 3 mM. After 10 min, 20 μl of Chan’s reagent (Sensolyte™ MG Phosphate Assay Kit, AnaSpec) was added to determine the amount of inorganic phosphate (P_i) formed. After 5 min of gentle shaking, absorbance was measured at 630 nm. The units used for enzyme activity are μmol of P_i generated per mg of protein per hour.

**HCT-8 cell binding assay**

Cell binding assays were performed similar as previously described [40]. Briefly, HCT-8 cells (1×10^5/well) were seeded in 96-well microtiter plates (Costar) and grown overnight at 37°C. After fixation with 4% paraformaldehyde in PBS, pH 7.4, for 1 h at RT, the cells were washed three times with PBS, pH 7.4, blocked for 1 h at room temperature with PBS, pH 7.4, containing 10% FCS (PBS-FCS), washed three times with PBS, pH 7.4, and incubated with varying concentrations of rCAp or Nus protein as an unrelated control protein, in PBS-FCS. After 1 h at 37°C, the cells were washed four times with PBS, pH 7.4, containing 0.05% Tween20 (PBS-Tween), and incubated for 1 h at 37°C with mouse anti-His6-HRP conjugated antibody 1:10,000 (SantaCruz) diluted in PBS-FCS. After four washes, PBS-Tween substrate buffer containing o-phenylenediamine (SIGMAFAST OPD™) was added, the cells were incubated for 30 min and the OD was measured at 450 nm.

**Cell invasion assay**

Sporozoites (1×10^6) were incubated with 1:10 diluted mouse anti-rCAp serum or non-related control serum (anti-Nus) for 30 min at 37°C, washed three times with PBS, pH 7.4, and added to 24-well plates containing a monolayer of HCT-8 cells in a ratio of 4 parasite:1 cell. Following incubation under culture conditions for 3 hours, extracellular parasites were removed by washing three times with PBS, pH 7.4, and the plates were returned to the incubator. Cells were collected 24 hours post-infection RNA was extracted from infected cells using the RNAqueous system (Ambion) following the manufacturer’s recommendations. RNA samples were stored at −80°C until quantification of *C. parvum* rRNA by RT-PCR.

**Real-time quantitative RT-PCR**

RT-PCR was used to quantify the *in vitro* infection rate of HCT-8 cells by *C. parvum*. Thus, RNA samples from HCT-8 cells infected with *C. parvum* sporozoites pretreated with mouse anti-rCAp serum and the corresponding control (mouse anti-Nus serum) were incubated with TURBO DNA-free DNase (Ambion) following the manufacturer’s instructions and used for Real-Time RT-PCR analysis using TaqMan™ technology. Primers and probes specific for *Cryptosporidium* rRNA and human rRNA were designed using Primer Express® version 2.0 (ABI). For each target, forward and reverse primers and an internal probe were synthesized. Probes were synthesized with 5’ end linked FAM (6-carboxyfluoresceine) and 3’ end fluorescent TAMRA (6-carboxytrytamethylrhodamine) dyes. Amplification and analysis were performed in our ABI7900HT instrument. The infection rate was obtained by calculating the ratio of human rRNA versus *Cryptosporidium* rRNA, with 100% infection represented by the negative control (treated with anti-Nus serum).

**Sequence analysis**

Putative signal peptide cleavage sites and asparagine-linked glycosylation sites of CAp were determined using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). The theoretical molecular weights and isoelectric points were determined with PeptideMass (http://www.expasy.ch/tools/peptide-mass.html).

**Phylogenetic analysis of CAp**

Putative orthologs of CAp were identified by BLAST [41] search of the CAp protein sequence against NCBI GenBank non-redundant protein database (nr), with a threshold of 1E-6 for the expectancy value E. All the identified protein sequences were aligned with ClustalX [42] and manually checked for adequate aligned length (>75% of the protein aligning). To expedite subsequent bootstrap analyses and simplify the final tree, phylogenetic analysis was performed by maximum likelihood using PHYML [43] in two
steps: first, a preliminary analysis (without bootstrap and using less thorough tree space search strategies) identified possible in-paralogs as well as out-paralogs that reflected the same phylogenetic pattern. These sequences were then removed from the alignment, and the sequences were realigned and reanalyzed to check whether the phylogeny remained stable after removal of the paralogs. The second analysis, involving only sequences remaining after the first step, was performed with one hundred bootstrap replicates and the SPR strategy [44] for the search of the optimal trees. The amino acid substitution model used was LG with 4 categories of gamma-distributed substitution rates, as selected by ModelGenerator [45]. Table S1 lists the GenBank accession numbers for the 109 sequences utilized in phylogenetic reconstruction.

Results

Identification and sequence analysis of a C. hominis apyrase (CAPy)

Analysis of the recently sequenced C. hominis and C. parvum genomes identified a single ecto-ATP diphosphohydrolase (apyrase, EC 3.6.1.5) in each sequence that displays high similarity to the human soluble calcium-activated nucleotidase 1 (SCAN-1, Fig. 1A) (GenBank accession numbers EAL36710, and EAK98929). The Cryptosporidium apyrase (CAPy) is a single copy gene with a predicted open reading frame of 1035 bp coding for a 345 amino acid protein. Further characterization of CAPy amino acid protein sequence predicted a signal peptide with a cleavage site between residues 22 and 23. The cleavage results in a protein of 323 amino acids, with a predicted molecular mass of 36,922 Da and an isoelectric point of 5.23 (Fig. 1B). No GPI anchor or other known cell targeting signals were found, suggesting that the protein is either attached to the membrane by a hydrophobic stretch or secreted. In addition, N-linked glycosylation sites were not detected.

Expression, Purification and Refolding of recombinant CAPy

The C. parvum and C. hominis apyrase amino acid sequences are 98% identical. Herein, we describe the cloning, expression and purification of recombinant CAPy derived from C. hominis, designated rCAPy. Thus, the DNA sequence encoding amino acid residues 34–345 excluding the signal peptide of the gene was amplified from C. hominis genomic DNA. The resulting 936-bp fragment was inserted into the pTrEx4 vector (Novagen) C-terminally fused to a His6-tag. As shown in Fig. 2, rCAPy was expressed in E. coli in inclusion bodies (IBs) and was therefore solubilized as described in the Materials and Methods. Subsequent purification by Ni2+- chelate chromatography under denaturing conditions led to a highly purified and homogenous product (Fig. 2, lane 7). During refolding of rCAPy, little or no protein was lost due to precipitation (Fig. 2, lane 8). Nevertheless, in subsequent dialysis against PBS, pH 7.4, or MOPS pH 7.4 (Fig. 2, lanes 9 and 10), a significant fraction of the refolded protein precipitated when protein concentrations exceeded approximately 0.35 mg/mL. A similar dependency on protein concentration for successful refolding was reported for human SCAN-1 [38].

Activity of CAPy requires Ca2+ and is highest against UDP and GDP

We evaluated the nucleotidase activity of rCAPy using a variety of substrates and divalent cations. ATPase and ADPase activities were measured at 2.5 mM total nucleotide concentrations in the absence of divalent cations, as well as in presence of 5 mM CaCl2, or MgCl2 (Fig. 5A). Nucleotidase activity was only detected in presence of calcium ions. To determine enzyme substrate specificity of CAPy, a variety of nucleoside mono-, di-, and triphosphates were used as substrates (2.5 mM final nucleotide concentrations) in the presence of 5 mM CaCl2 (Fig. 5B). The activity for the preferred substrates UDP and GDP is high, whereas activities towards ATP and GTP are lowest. No measurable hydrolysis of any investigated mono-phosphate was detected. These results reflect those expected for human apyrase SCAN-1.

Recombinant CAPy binds to the HCT-8 cells in a dose dependent fashion

To assess the possibility that CAPy may play a role in the attachment of Cryptosporidium sporozoites to enterocytes, we measured the ability of recombinant CAPy to bind HCT-8 cells. Binding assays were performed by adding various concentrations of rCAPy to paraformaldehyde fixed HCT-8 cells as described in the Materials and Methods. As shown in Fig. 6, rCAPy binds to HCT-8 cells in a dose-dependent and saturable manner. No binding was observed when an unrelated protein (bacterial Nus) was used in identical assays.
Antibodies directed against rCApy block invasion of HCT-8 cells by C. parvum

To further gain insight into the role of CApy during parasite invasion, we performed in vitro invasion assays using HCT-8 cells. Thus, sporozoites were pre-incubated with mouse anti-rCApy serum or unrelated control serum and the infection rate was measured using quantitative RT-PCR (Fig. 7). In these experiments, antibodies against rCApy were able to reduce the invasion of enterocytes by sporozoites by over 60%, suggesting that CApy plays a role during the early stages of infection.

Sequence and phylogenetic analysis of apyrase sequences

To perform a reconstruction of the evolutionary history of CApy, we identified putative orthologs in as many genomes as possible (Table S1) and used these sequences to perform a multiple sequence alignment of the apyrase domain of Cryptosporidium sp. with apyrase homologs of their respective hosts.

Figure 1. Schematic outline of CApy and multiple sequence alignment of the apyrase domain of Cryptosporidium sp. with apyrase homologs of their respective hosts. A. Alignment of selected apyrase sequences. Sequence alignment was performed using the CLUSTAL 2W algorithm. The GenBank accession numbers of sequences are given in parentheses. CH, Cryptosporidium hominis (XP_666945); CP, Cryptosporidium parvum (XP_627524); CM, Cryptosporidium muris (XP_002140694); HS, Homo sapiens (NP_620148); MM, Mus musculus (EDL34666), BT, Bos Taurus (XP_596269). The enzymatic activity of the human apyrase has been established by biochemical analysis [56], therefore residues important for nucleotide and Ca$^{2+}$ binding in the human apyrase and the predicted counterparts in corresponding apyrases of other species are indicated by gray shadows and pluses. Residues that differ from the human sequence at positions with potential impact on enzymatic activity are colored red. Cysteine residues are shadowed light blue. B. CApy is a protein comprising 345 aa, including a 22-aa signal peptide (SP), and an apyrase domain spanning residues 26–345.

B

Antibodies directed against rCApy block invasion of HCT-8 cells by C. parvum

To further gain insight into the role of CApy during parasite invasion, we performed in vitro invasion assays using HCT-8 cells. Thus, sporozoites were pre-incubated with mouse anti-rCApy serum or unrelated control serum and the infection rate was measured using quantitative RT-PCR (Fig. 7). In these experiments, antibodies against rCApy were able to reduce the invasion of enterocytes by sporozoites by over 60%, suggesting that CApy plays a role during the early stages of infection.

Sequence and phylogenetic analysis of apyrase sequences

To perform a reconstruction of the evolutionary history of CApy, we identified putative orthologs in as many genomes as possible (Table S1) and used these sequences to perform a
Phylogenetic analysis (Fig. 8, and see Materials and Methods) showed that among the Unikonta [49,50], a CApy ortholog is present in Choanozoa, the Amoebozoa, in one Opisthokonta of uncertain phylogenetic affiliation (Capsaspora), and in several Metazoa, but not in any fungi. Among the Bikonta, CApy orthologs only seem to be present in the Chromalveolata, comprised of Alveolata (which includes apicomplexan parasites) and Stramenopiles, and in one Excavata, Naegleria gruberi, which grouped at a deep divergence, and with low bootstrap support, with the Unikonta. The grouping of Apicomplexa and Stramenopiles has strong bootstrap support, as have the internal groupings of cryptosporidia, diatoms and oomycetes. The branch connecting Toxoplasma and Neospora with Cryptosporidium is relatively well supported, with a bootstrap value of 75 (only values above 50 are shown on Fig. 8). Other known Bikonta (Excavata, Plantae and Rhizaria) genomes seem to lack an ortholog of this protein, with the exception of the excavate Naegleria, as noted above.

Multiple sequence alignment of these orthologs from protozoan, invertebrate, and vertebrate organisms shows that most variations occur in the N-terminus, possibly reflecting differences in the

Figure 2. Expression, Purification and Refolding of recombinant CApy. The electrophoretic analysis (SDS-PAGE, 12% PAA under reducing conditions) shows extracts of non-induced and induced E. coli cultures (lane 1 and 2) bearing the CApy gene in the pTriEx-4 expression vector from samples taken at different steps of protein purification (lanes 3–10). Following bacterial cell lysis the soluble (supernatant, lane 3) and insoluble (pellet, lane 4) fractions show that CApy was mostly found in the insoluble fraction in form of inclusion bodies (IBs). The solubilized IBs (see Materials and Methods) were loaded onto a Ni²⁺ chelate column and purified under denaturing conditions, flow-through (lane 5), wash (lane 6), and elution (lane 7) fractions were collected. The eluate containing the purified CApy was refolded by dialysis against folding buffer (lane 8), which was subsequently dialysed against PBS, pH 7.4 (lane 9) or 20 mM MOPS, pH 7.4 (lane 10).

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Figure 3. Expression of CApy in oocysts and sporozoites. CApy is expressed in oocysts and sporozoites. Aliquots of NP-40 soluble (S) or insoluble (I) C. parvum oocyst and sporozoite fractions were resolved by 12% SDS-PAGE, transferred to nitrocellulose and probed with mouse anti-CApy serum as described in the Materials and Methods.

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Figure 4. Glycosylation of CApy in sporozoites. C. parvum sporozoites were treated as recommended in the Enzymatic Carbo Release Kit™ (QAbio, San Mateo, CA) for identification of glycosylation. In brief, sporozoites were suspended in Carbo Release buffer, and denaturation buffer was added. After incubation at 100°C for 5 min, samples were chilled on ice and Triton-X was added. The enzymes PNGase, Sialidase, β-Galactosidase, Glucosaminidase, O-Glycosidase were added alone or in combinations. Following incubation at 37°C for 16 hours, protein loading buffer was added and samples were incubated again at 100°C for 5 min. Proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose and probed with mouse anti-CApy serum.

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secretory systems, as well as possible different localizations of the protein, depending on the specific function in the respective organism. Nevertheless the apyrase domain, and in particular the structurally important regions, seem to be highly conserved (data not shown). Surprisingly, orthologs occur in various infectious disease causing eukaryotic parasites, as well as their respective hosts, yet they are apparently absent in yeast and bacteria. Overall, our analysis argues that CApy was present in the progenitors of Cryptosporidium, but was lost in other apicomplexans except Toxoplasma and Neospora, or, less likely, that the gene was picked up by the parasites from more distantly related organisms by lateral gene transfer.

Discussion

In this study, we have identified and characterized a C. hominis gene, CApy, which encodes an ecto-apyrase homolog of a group of calcium-dependent apyrases that catalyze hydrolysis of both nucleoside triphosphate (NTP) and nucleosine diphosphate (NDP) to nucleoside monophosphate (NMP) and inorganic phosphate (P). These enzymes are highly conserved among the
infection with Cryptosporidium during the onset of immune responses, suggests other possible roles which generally serve as danger signals that are rapidly produced from neutrophil attack. Our recombinant CApy functions as a soluble apyrase with exclusive calcium-dependent activity, consistent with its human homolog ortholog SCAN-1 [34,38], as well as various insect apyrases from Cimex lectularius [31], Phlebotomus papatasi [32], and Lutzomyia longipalpis [35]. As observed for SCAN-1, UDP and GDP, rather than ADP or ATP, are the preferred substrates of CApy [34,35]. Several indications suggest that CApy could be involved in processes involving human enterocytes by Cryptosporidium. Thus, CApy seems to be found on the apical surface of sporozoites, binds to the host cell in a dose-dependent manner suggesting the presence of a potential receptor on the host cell surface, and elicits antibodies that significantly block invasion. Our observations support previous reports that indicate that ecto-apyrases play various roles during host pathogen interaction [31]. Recently it was reported, that antibodies directed against an ecto-NTPDase from Trypanosoma cruzi and Leishmania amazonensis are able to significantly reduce the rate of infection of these parasites [10,22]. Furthermore chemical inhibition of the ecto-NTPDase led to a reduction of the virulence of T. cruzi in in vivo experiments [12]. Moreover, Bissagio et al. 2003 [10] suggested that Mg²⁺-dependent ecto-ATPase activity on the surface of T. cruzi could stimulate the adherence of the parasite to the host cell and protect from neutrophil attack.

The ability of CApy to inactivate extracellular nucleotides, which generally serve as danger signals that are rapidly produced during the onset of immune responses, suggests other possible roles of CApy during parasite pathogenesis. Moderate to severe infection with Cryptosporidium is characterized by mucosal inflammation with neutrophils and macrophages in the lamina propria underlying intestinal epithelial cells, as well as the presence of intraepithelial neutrophils (53), reviewed in [54]. Cell and tissue damage, hypoxia, leukocyte activation, decreased pH and other stress factors previously described to be caused by this pathogen (7,55) may lead to the release of large amounts of extracellular nucleotides into the intestinal lumen at the site of inflammation. Our in vitro experiments indicate that CApy may interfere with the interaction of ADP, ATP, UDP, and UTP with different subsets of purinergic receptors, thereby possibly impacting various signaling pathways normally activated during inflammation resulting in modulation of cellular immune responses. In addition to its potential role in attachment, an apyrase expressed on the surface or secreted by the parasite may be an effective counter measure to the release of extracellular nucleotides rapidly secreted at local sites of infection which would otherwise display potent innate immune-enhancing activities underpinning successful parasite propagation. Further studies are necessary to test this hypothesis.

Apyrases are broadly distributed among the tree of life and are present in many pathogenic parasites [28]. To gain insight into the evolution of this pathogenic factor we performed phylogenetic analysis of CApy and all orthologs that we could identify from available sequences. Our analysis, using recent multi-gene phylogenies of eukaryotes as a benchmark [49], suggests that CApy was present in the apicomplexan progenitor, but the gene was lost from apicomplexans other than Cryptosporidium, Neospora, and Toxoplasma. Moreover, the phylogenetic analysis indicates that all Chromalveolata sequences form a monophyletic group, suggesting that CApy might have been an ancestral feature of the group that was later lost in other Apicomplexa (e.g., Plasmodium, Theileria) and the Ciliata (e.g. Tetrahymena and Paramaecium). While Toxoplasma, Neospora and Cryptosporidium branch together, they do so with moderate bootstrap support of 75. This is likely due to the greater sequence divergence of the Toxoplasma and Neospora apyrases, which can lead to lower phylogenetic signal and more homoplasy with other sequences. Assuming that the phylogenetic groups Bikonta and Unikonta are natural, the absence of this apyrase in other bikonts (all Excavata but Naegleria, all Plantae, and possibly Rhizaria, for which no complete genomes were available at analysis time) suggests that either the CApy gene is ancient but was lost in these bikont lineages, or that this gene is novel and was transferred from unikonts to Chromalveolata (or vice-versa) early in evolution of these groups. Our current taxonomic sampling of this gene does not allow a definitive conclusion, emphasizing the need for further genomic studies of more diverse taxa among the unikonts, Chromalveolata, Rhizaria, as well as basal Plantae, e.g. rhodophytes and glaucophytes.

In summary, herein we described a new potential pathogenic factor, the CApy apyrase, in Cryptosporidium. CApy may play multiple roles during the infection, including an active participation in the attachment and invasion of Cryptosporidium to the host cells. In addition, we provide indirect evidence suggesting that CApy could potentially interfere with extracellular nucleotide signaling responsible for triggering an inflammatory response in injured tissues, possibly delaying it and providing an opportunity for the parasite to successfully establish the infection. Phylogenetic evidence suggests that either this gene was acquired very early in evolution but lost in many of the nearest relatives of Cryptosporidium, explaining its otherwise broad distribution among the tree of life and among pathogenic parasites, or, less likely, that Cryptosporidium, Toxoplasma, and Neospora may have acquired the gene by lateral gene transfer. Finally the evidence provided by this study indicates that CApy might be a potential drug target and/or vaccine candidate against cryptosporidiosis.

Supporting Information

Table S1 Database accession number of sequences used in phylogenetic reconstruction.

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Author Contributions
Conceived and designed the experiments: PAM UW. Performed the experiments: PAM UW AML FT. Analyzed the data: PAM UW JMA.

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