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A Unique Hexokinase in *Cryptosporidium parvum*, an Apicomplexan Pathogen Lacking the Krebs Cycle and Oxidative Phosphorylation

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Abstract

Cryptosporidium parvum may cause virtually untreatable infections in AIDS patients, and is recently identified as one of the top four diarrheal pathogens in children in developing countries. *Cryptosporidium* differs from other apicomplexans (e.g., *Plasmodium* and *Toxoplasma*) by lacking many metabolic pathways including the Krebs cycle and cytochrome-based respiratory chain, thus relying mainly on glycolysis for ATP production. Here we report the molecular and biochemical characterizations of a hexokinase in *C. parvum* (CpHK). Our phylogenetic reconstructions indicated that apicomplexan hexokinases including CpHK were highly divergent from those of humans and animals (i.e., at the base of the eukaryotic clade). CpHK displays unique kinetic features that differ from those in mammals and *Toxoplasma gondii* (TgHK) in the preference towards various hexoses and its capacity to use ATP and other NTPs. CpHK also displays substrate inhibition by ATP. Moreover, 2-deoxy-D-glucose (2DG) could not only inhibit the CpHK activity, but also the parasite growth in vitro at concentrations nontoxic to host cells (IC₅₀ = 0.54 mM). While the exact action of 2-deoxy-D-glucose on the parasite is subject to further verification, our data suggest that CpHK and the glycolytic pathway may be explored for developing anti-cryptosporidial therapeutics.

Keywords

Apicomplexan; *Cryptosporidium parvum*; Hexokinase; 2-deoxy-D-glucose (2DG); substrate inhibition; drug target

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Introduction

The apicomplexan *Cryptosporidium* has recently been identified to be among the top 4 causes of moderate to severe diarrhea in the Global Enteric Multicenter Study (GEMS) of children under 5-year old in developing countries and a “high risk factor for linear growth faltering and death” (Kotloff et al. 2013). It also causes one of the important opportunistic infections in AIDS patients, for which effective treatments are yet unavailable (Chen et al. 2002; Kelly 2011; Mead 2014; Rossignol 2010). This parasite differs from other apicomplexans by the absence of many important metabolic pathways, and its incapability of de novo synthesizing fatty acids, nucleosides and any amino acids (Abrahamsen et al. 2004; Xu et al. 2004). *Cryptosporidium* possesses a remnant mitochondrion, but lost the cytochrome-based respiratory chain, thus relying mainly (if not solely) on glycolysis to produce ATP (Rider and Zhu 2010). Therefore, glycolytic enzymes can be considered as attractive therapeutic targets in this parasite.

In *Cryptosporidium*, glucose and other sugars have to be scavenged from the host or produced by degrading amylopectin by a glycogen debranching enzyme (GDBE) and a glycogen phosphorylase (GP) (Fig. 1) (Rider and Zhu 2010). The *C. parvum* genome encodes a single hexokinase (CpHK) [EC:2.7.1.1] gene to phosphorylate hexoses before routing them into subsequent glycolysis. In the glycolytic pathway, *C. parvum* uses a pyrophosphate-dependent phosphofructokinase (PPi-PFK) and a bifunctional pyruvate:NADP⁺ oxidoreductase (PNO) that differ from the ATP-PFK and pyruvate dehydrogenase (PDH) complex in other apicomplexans and in humans and animals (Rotte et al. 2001; Thompson et al. 2005). Due to the lack of the Krebs cycle (aka, tricarboxylic acid cycle [TCA cycle]) and oxidative phosphorylation, the glycolysis only leads to the production of trehalose (an anti-stress molecule) (Yu et al. 2010), malonyl-CoA for the elongation of fatty acids or polyketide(s) (Zhu 2004), or three possible organic end products (i.e., lactate, ethanol or acetate) for maintaining the carbon flow and recycling NAD(P)H (Rider and Zhu 2010; Thompson et al. 2005) (Fig. 1).

In the present study, we performed detailed and comprehensive analyses on the molecular features and enzyme kinetics of CpHK. Our phylogenetic analysis indicated that apicomplexan HKs including CpHK formed a monophyletic group that was placed between prokaryotic and eukaryotic clades. We found that CpHK differed from the HKs of humans and other apicomplexans in kinetics towards various substrates, particularly by its ability for utilizing ATP and other NTPs (albeit with less efficiencies) and substrate inhibition towards ATP at physiological concentrations. Additionally, we observed inhibition of 2-deoxy-D-glucose (2DG) on the growth of *C. parvum* in vitro, implying that CpHK and key glycolytic enzymes could be explored as a potential therapeutic target in this parasite.

Results

General Sequence Features and Differential Expression of CpHK Gene

Most apicomplexans possess only a single HK gene (e.g., *Cryptosporidium*, *Eimeria*, *Toxoplasma*, *Plasmodium* and *Babesia*), with the exception of *Theileria* species that may contain 2-3 HK isoforms (detailed annotations can be found at <http://www.EuPathDB.org>).

InterPro-based analysis indicated that all apicomplexan HKs contained the 7 signature motifs involved in interacting with ATP and/or hexoses (Fig. 2A) (Albig and Entian 1988; Griffin et al. 1991; Krishnamurthy et al. 2005). However, a unique 5-aa insertion was observed among apicomplexan HKs next to the residues interacting with ATP at the end of motif 2 (Fig. 2B). CpHKs seemed to be more divergent from those of other apicomplexans by the presence of additional 4 insertions (data now shown).

CpHK gene was expressed in all life cycle stages as determined by quantitative real-time RT-PCR (qRT-PCR) using 18S rRNA as control for normalization (Fig. 3). The oocysts and free sporozoites had similar CpHK mRNA levels, while intracellular parasites tended to have increased levels towards later developmental stages. There was a small peak of expression at 12 h post-infection (pi) time, corresponding to the formation of first generation of meronts. The *CpHK* expression was elevated at 72 h pi, implying that *C. parvum* might be actively utilizing glucose or other hexoses for synthesizing storage amylopectin during the formation of oocysts.

Apicomplexan HKs Including CpHK were Highly Divergent from those in Humans and Animals

By data-mining various protist genome databases (e.g., NCBI and EuPathDB), we observed that among alveolates, HKs were only found in apicomplexans and the dinoflagellate *Perkinsus*, but were absent in ciliates. In early eukaryotic branches, highly divergent HKs are present in kinetoplastids (e.g., *Trypanosoma* and *Leishmania*), a few heterokonts (e.g., *Blastocystis*) and amoebae (e.g., *Entamoeba*), but absent in diplomonads, trichomonads and *Naegleria*. Species lacking HKs usually have glucokinases instead. In our phylogenetic analysis, when those highly divergent HK sequences were included in phylogenetic analyses, the resulting trees could not be well resolved, or were unstable (i.e., they might attach to different nodes under different models that were either unsupported or poorly supported), which was characteristic of long branch attraction (LBA) artifacts as previously described for some protistan hexokinases (Richards et al. 2003). Inter-group distance analysis also showed that these early eukaryotic HKs formed outliers (data not shown), which was one of the known major causes of LBA, indicating they were unsuitable to be included in this dataset for reconstructing phylogeny. Therefore, our phylogenetic analyses were performed from datasets containing prokaryotes, apicomplexans, the dinoflagellate *Perkinsus*, kinetoplastids, plants, fungi and animals using a site-heterogeneous mixture model (CAT), maximum likelihood (ML) and Bayesian Inference (BI) methods.

Our phylogenetic reconstructions indicated that all apicomplexan HKs evolved from a common ancestral gene, as they formed a single clade in CAT, ML and BI trees that was fully supported by bootstrapping and posterior analyses (Fig. 4). *Cryptosporidium* HKs were placed at the base of the apicomplexan clade, followed by the Coccidia and Hematozoa, which was congruent with the general phylogenetic relationship of the Phylum Apicomplexa (Templeton et al. 2010; Zhu et al. 2000). Our data also clearly indicated that apicomplexan HKs were highly divergent from those of humans and animals. In fact, the apicomplexan clade was placed at the base of eukaryotes, and more closely related to the orthologs from

prokaryotes, kinetoplastids, dinoflagellates and plants than to those from fungi and animals (Fig. 4).

Kinetic Properties of CpHK Towards Hexoses and Nucleoside Triphosphates

CpHK was expressed as an MBP-fusion protein and purified to homogeneity for determining its enzyme kinetics (see Fig. 5A inset). Based on an initial PK/LDH-coupled assay using 2 mM hexoses and 2.5 mM ATP, CpHK was capable of using glucose (100% activity), mannose (52.5%) and fructose (8.6%), but unable to utilize galactose and sorbose (Fig. 5A). More detailed analysis indicated that CpHK displayed Michaelis-Menten kinetics towards glucose ($K_m = 0.183$ mM, $V_{max} = 26.67$ U; U = nmol/min/μg protein), mannose ($K_m = 0.109$ mM, $V_{max} = 12.68$ U) and fructose ($K_m = 6.49$ mM, $V_{max} = 11.46$ U), suggesting that glucose and mannose might be utilized by CpHK with similar efficiencies, whereas fructose was less likely an effective substrate (Fig. 5B). The kinetic data on glucose were validated by a G6PDH-coupled assay that produced similar Michaelis-Menten kinetics with a comparable K_m value (0.138 mM, $V_{max} = 11.42$ U) (Fig. 5B dashed line). These features made CpHK differ from that of *T. gondii* (TgHK) that could use fructose at a much higher efficiency (i.e., 79% in comparison with glucose) (Saito et al. 2002), or from humans that could use all three hexoses at similar efficiencies, e.g., 78% - 106% for mannose and 90% - 130% for fructose (Magnani et al. 1988; Stocchi et al. 1982).

For nucleoside triphosphates (NTPs), CpHK preferred to use ATP, but could also use other NTPs (i.e., TTP, UTP, CTP and GTP at 6.5% - 17.1% activities in comparison with ATP) (Fig. 5C). The ability for CpHK to use ATP and other NTPs (albeit with less efficiencies) was also unique, as *T. gondii* and human HKs were virtually incapable or with much less capabilities on other NTPs (i.e., <2% or <5% activity, respectively) (Magnani et al. 1988; Saito et al. 2002). Additionally, ATP displayed substrate inhibition on CpHK at physiological concentrations (i.e., peak activity at 2.5 mM and completely inhibited at 10 mM) (Fig. 5D). Substrate inhibition by ATP has also been observed in HKs from other protozoa (e.g., *P. falciparum* and *T. brucei*) and in the yeast HK PII isoform (Dodson et al. 2011; Harris et al. 2013; Moreno et al. 1986). Based on the Haldane equation for substrate inhibition, we obtained the following kinetic parameters for CpHK towards ATP: $K_m = 0.673$ mM, $V_{max} = 5.09$ U, and $K_i = 7.632$ mM. Other NTPs seemed to also display substrate inhibition on CpHK, but their kinetic parameter could not be well resolved by the Haldane equation. A comparison of kinetic parameters between CpHK, TgHK and human HKs is listed in Table 1.

2-Deoxy-D-glucose (2DG) Competed with Glucose and Inhibited *C. parvum* Growth in Vitro

We evaluated whether CpHK and the glycolytic pathway could serve as a potential drug target in *C. parvum* by testing the effect of 2DG on CpHK activity and on the parasite growth in vitro. Using the G6PDH-coupled assay, we observed that 2DG could inhibit the CpHK enzyme activity in a dose-dependent manner (Fig. 6A). The IC_{50} value was at 5.75 mM, corresponding to a K_i value at 0.340 mM based on a competitive inhibition model (Cheng and Prusoff 1973). 2DG as a competitive inhibitor of glucose was also confirmed by detecting the apparent kinetics, in which the K_i value was at 0.498 mM (Fig. 6C and 6D). 2DG had no effect on G6PDH in the control groups using the same amount of enzyme

(G6PDH) and substrates (G6P and NAD⁺). To avoid potential masking of an inhibitory effect due to the presence of excessive amounts of enzymes and substrates, we performed experiments using smaller amounts of G6PDH (0.04 U) and G6P (0.2 mM), which again confirmed that 2DG (10 mM) acted on CpHK (~56% inhibition) rather than G6PDH (Fig. 6B). A similar result was obtained using a PK/LDH-coupled assay, in which 2DG at 10 mM was similarly effective on CpHK (~57% inhibition), but not on PK and LDH (Fig. 6B).

More importantly, a qRT-PCR-based drug efficacy assay revealed that 2DG could inhibit the growth of *C. parvum* in vitro at sub-millimolar levels (IC₅₀ = 0.54 mM) (Fig. 7A). The assay was validated by the positive control paromomycin that displayed the expected drug efficacy (i.e., 82.6% at 0.12 mM) (Fig. 7A inset). At the tested concentrations (0.11 – 9.0 mM), 2DG displayed no apparent cytotoxicity on the HCT-8 cells as determined by microscopic examination of cell morphology and detachment, as well as by the levels of 18S rRNA in treated host cells (Fig. 7B).

We also tested the glucose dependence in *C. parvum* cultured in vitro and whether higher levels of glucose were capable of competing with 2DG to restore the parasite growth inhibited by 2DG. Our data showed that the glucose level affected the growth of both HCT-8 cells and the parasite (Fig. 7C). However, the effect of glucose levels on host cells were minimal or small at levels between 5.5 – 44 mM, although ~40% reduction was observed without glucose (vs. 11 mM, the amount of glucose in regular RPMI 1640 medium). In contrast, the growth of *C. parvum* was much more dependent on glucose. In the absence of glucose, there was a ~90% reduction in the parasite growth (vs. 11 mM glucose). Increasing the levels of glucose dramatically promoted parasite growth (i.e., 10%, 60%, 100% and 160% at 0, 5.5, 11 and 22 mM of glucose, respectively), although such an effect was less significant at 44 mM than at 22 mM (Fig. 7C). The ability for both host cells and the parasite to grow in the absence of glucose was likely due to the presence of certain carbohydrates and other molecules in the fetal bovine serum (5%) that were needed to maintain minimal growth of cells for up to two days. Furthermore, in the presence of 2DG (2 mM), the addition of higher levels of glucose could restore the *C. parvum* growth more dramatically, i.e., 400% to 600% increases at 22 mM and 44 mM of glucose (vs. 11 mM) (Fig. 7D). These observation confirmed that *C. parvum* relies heavily on glucose as a carbon source and 2DG likely acted on the glycolytic pathway in the parasite.

DISCUSSION

Hexokinases are one of the key glycolytic enzymes responsible for the activation of glucose and other hexoses. In the present study, we have shown that the opportunistic protist *C. parvum* possesses a single HK gene that is actively transcribed during its complex life cycle, and the apicomplexan HKs including CpHK are phylogenetically divergent from their counterparts in humans and animals. CpHK enzyme also displayed unique kinetic features in substrate profiles towards hexoses and NTPs that differ from those of *T. gondii* and mammals. More strikingly is the ability of CpHK to utilize other NTPs besides ATP, which may be of advantage to *Cryptosporidium* that solely relies on glycolysis to produce limited amount of ATP. ATP displayed substrate inhibition on CpHK, which was also observed in *P. falciparum*, *T. brucei* and yeast (Dodson et al. 2011; Harris et al. 2013; Moreno et al.

1986). In the case of yeast hexokinase PII isoform (also known as hexokinase II or HXK2), it could be inhibited by Mg^{2+} -free ATP, and was known to play a unique role in the regulation of carbon metabolism by participating in “glucose repression” via the PII/snf1 pathway (Moreno et al. 1986; Rolland et al. 2001). Similarly, we speculate that CpHK may use this feature to regulate the glycolytic pathway to avoid the production of excessive ATP that is typically present at low millimolar levels in cells.

Glycolysis is essential for the anaerobic organisms (e.g., *Entamoeba*, *Trichomonas* and *C. parvum*) that lack the Krebs cycle to generate energy and carbon intermediates. It is also critical to some parasites that possess the Krebs cycle. For example, *Trypanosoma* and *Leishmania* are featured by “aerobic fermentation”, so that even in the presence of oxygen and a fully functional Krebs cycle in cells, they will still rapidly convert glucose in large amounts to mono- and di-carboxylic acids (e.g., pyruvate, succinate and malate) (Cazzulo 1992; Urbina 1994). In the case of *Plasmodium* species, glycolysis was thought to be critical to the intra-erythrocytic stage as a major source for ATP (Fang et al. 2014; Vaidya and Mather 2009), and in fact, 2-halo derivatives of glucose including 2DG also displayed anti-plasmodial activity (van Schalkwyk et al. 2008). On the other hand, humans and animals are much less sensitive to the inhibition of glycolysis by possessing multiple HK isoforms, a fully functional Krebs cycle and the ability to use alternative energy sources (e.g., amino acids or fatty acids). Similarly, other aerobic organisms including the apicomplexan *T. gondii* might also tolerate the blockage of glycolysis. For example, carbon source uptake was found to be not required for ATP maintenance by extracellular *T. gondii* tachyzoites (Lin et al. 2011), and host-derived glucose and its transporter in this parasite were dispensable by glutaminolysis (Blume et al. 2009). We have also noticed an unpublished observation suggesting that HK might be not essential to *T. gondii*, as the tachyzoites of a *TgHK* knockout mutant (RH KU80 background) are viable in vitro and remain virulent in susceptible mice (see user comment on *TgHK* gene at <http://ToxoDB.org>). Collectively, enzymes within the pathway such as hexokinases may serve as attractive drug targets in pathogens that rely solely or mainly on glycolytic pathway for producing ATP, but may be less suitable as drug targets in pathogens with a fully functional Krebs cycle and the capability of using alternative energy sources. Hexokinases have been explored as drug targets in *Trypanosoma*, *Entamoeba* and *Trichomonas* (Chambers et al. 2008; Henze et al. 2001; Hudock et al. 2006; Saavedra et al. 2007; Sanz-Rodriguez et al. 2007). A number of classes of inhibitors against *Trypanosoma* parasites have also been recently developed (Chambers et al. 2008; Hudock et al. 2006; Sanz-Rodriguez et al. 2007). More recently, small molecule inhibitors in the class of isobenzothiazolines were identified against *P. falciparum* HK (PfHK), and some of those compounds reduced *P. falciparum* growth in vitro at low micromolar levels (Harris et al. 2013).

Here we have shown that 2DG could effectively compete with glucose, thus inhibiting the CpHK enzyme activity. 2DG could also inhibit the growth of *C. parvum* in vitro at concentrations nontoxic to HCT-8 cells, indicating that CpHK and the glycolytic pathway could be targeted for developing therapeutics against cryptosporidiosis in humans and animals. As a glucose analog, 2DG and fluoro-2-deoxy-D-glucose (FDG) can be transported into cells via sugar transporters and phosphorylated to become 2DG-6-phosphate (2DG-6P)

and FDG-6P by various hexokinases and glucokinases (Franzusoff and Cirillo 1982; Jaini and Dadachova 2012; Kang and Hwang 2006; Khan et al. 2011; Youderian et al. 1999). However, 2DG-6P cannot be further metabolized. Therefore, both 2DG and 2DG-6P can inhibit HK activity and intervene with glycolysis. 2DG has been actively under investigation as an antiviral and antitumor agent (Kang and Hwang 2006). Its antiviral activity is attributed to interference with the glycosylation of viral proteins and lipids, and proper penetration of the virus into the target cells. In solid tumors, their hypoxic areas mainly rely on anaerobic glycolysis for producing ATP that can be inhibited by 2DG, whereas non-tumor cells are less sensitive to 2DG as they may use alternative energy sources such as fatty acids and amino acids. In yeasts, 2DG not only inhibits glycolysis, but also represses the metabolism of alternative carbon sources via a mechanism of carbon catabolite repression (Ashokkumar et al. 2004; Cereghino and Scheffler 1996; Kahar et al. 2011; Zimmermann and Scheel 1977).

Although further studies are needed to fully understand the action and fate of 2DG in *C. parvum* cells, it is likely that 2DG could be phosphorylated by CpHK, and the observed anti-cryptosporidial efficacy resulted from the inhibition of CpHK by 2DG and the interference of downstream reactions by 2DG-6P. Whether 2DG could be further explored as an anti-cryptosporidial drug may be debatable, as it was not highly potent in inhibiting parasite growth in vitro (i.e., $IC_{50} = 0.54$ mM). However, both 2DG and FDG are known to be safe to humans and animals, and doses at up to 0.25–0.5 g/kg/d (corresponding roughly to ~1.5–3 mM in body weight) were used in clinical trials and animal experiments for treating various tumors (Cheong et al. 2011; Mohanti et al. 1996; Raez et al. 2013). Currently, there are more than 100 registered entries associated with the keyword “deoxyglucose” at the ClinicalTrials database (<http://clinicaltrials.gov/>), indicating that 2DG and FDG are being actively pursued for various clinical applications. Therefore, it may be worth to test the anti-cryptosporidial efficacy of 2DG in vivo. More importantly, the established spectrophotometry-based assays can be readily adapted into a high-throughput screening assay to discover inhibitors to selectively and more effectively inhibit the CpHK for potential drug development.

Methods

Molecular and bioinformatics analyses

CpHK gene was annotated by the *C. parvum* genome sequencing project (gene ID:cgd6_3800; GenBank:XM_627719), which contained a 1,557 bp intronless open-reading frame (ORF) predicting a 518 aa protein. To define its phylogenetic relationship with other orthologs, we used CpHK and other HK protein sequences as queries to search the NCBI non-redundant protein databases, and retrieved more than one thousand protein sequences with E-values smaller than $1.E-20$. Multiple sequence alignments were performed using MUSCLE v3.8.31 (Edgar 2004a, b). Based on the alignments and NJ trees, partial and identical sequences were removed and phylogenetic trees were first reconstructed using a neighbor-joining (NJ) method with Poisson-correction, and the procedures were repeated until a balanced dataset containing 184 taxa (267 aa positions) representing all major taxonomic groups were identified. The dataset was subjected to phylogenetic reconstructions

using a site-heterogeneous mixture model (CAT-Poisson) using PhyloBayes v3.3e (<http://megasun.bch.umontreal.ca/People/lartillot/www/index.htm>), and two homogeneous models (maximum likelihood [ML] and Bayesian Inference [BI]) using MEGA v5.2 (<http://www.megasoftware.net>) and MrBayes v3.2.1 (<http://mrbayes.sourceforge.net>), respectively (Lartillot et al. 2009; Ronquist et al. 2012; Tamura et al. 2011). CAT analysis was performed in two independent runs under default settings for 10,000 cycles with trees saved in every 10 cycles. Posterior analysis was performed after first 25% of the trees were discarded. Tree reconstructions with ML and BI used WAG amino acid substitution model with the consideration of fraction of invariance and 4-rate gamma ($WAG + F_{inv} + \Gamma$). ML bootstrapping analysis was performed with 200 pseudo-replicated datasets. BI analysis was conducted with 1,000,000 generations of searches with two independent runs, each containing four chains running simultaneously. The current trees were saved every 1,000 generations, and posterior probability values were obtained after first 25% trees were removed. Signature domains in CpHK were identified by searching the InterPro database at the InterProScan 5 server (<http://www.ebi.ac.uk/interpro/>) (Hunter et al. 2012). We also summarized the conserved domains as sequence logos from 24 unique apicomplexan HK protein sequences in bitmap using WebLogo 3 server (<http://weblogo.threeplusone.com>) (Crooks et al. 2004).

Quantitative real-time RT-PCR (qRT-PCR) of CpHK transcripts in *C. parvum*

Total RNA was isolated from different life cycle stages in *C. parvum* including oocysts, free sporozoites and intracellular parasites. The IOWA-1 strain of *C. parvum* isolated from infected calves were purchased from Bunch Grass Farm (Deary, ID). Free sporozoites were prepared by an in vitro excystation procedure by incubating oocysts in 0.25% trypsin and 0.5% taurodeoxycholic acid for 60 min at 37 °C, followed by 5-8 washes with PBS. Intracellular parasites were prepared by infecting HCH-8 cells with *C. parvum* for various times as described. qRT-PCR was performed using primers CpHK-1305F (5' AGC AGC ATC TCT TGT CTC AGC 3') and CpHK-1395R (5' TGA TCC ATC TAT GGC AAT GGT 3') in a CFX98 Touch Real-Time PCR Detection System (Bio-Rad Labs, Hercules, CA), in which 30 min reaction at 50 °C was allowed for reverse transcription, followed by 40 thermal cycles each at 94 °C for 20 sec, 58 °C for 30 sec and 72 °C for 30 sec. The levels of 18S rRNA were assayed and used as internal controls for normalization (Zhang et al. 2012). Threshold cycle (C_T) values were used for computing the relative levels of expression using an empirical formula 2^{-C_T} . Experiments were conducted using two sets of RNA samples. In each set, individual samples contained pooled RNA isolated from 3 independent cell samples. RT-PCR reactions included at least three technical replicates for calculating the standard error of the mean (SEM) values.

Molecular cloning and expression of recombinant CpHK protein

The entire CpHK ORF was amplified from *C. parvum* genomic DNA using the high-fidelity *Pfu* DNA polymerase using primers CpHK-F1BamH1 (5' ttg gat ccA TGG AA GAG GAA AAT CAA GC 3') and CpHK-R1Hind3 (5' cca agc ttC AAT GTG TAT TGA CTG TAG 3') (Note: lower cases indicate linker sequences) following standard PCR protocol. PCR amplicon was double-digested with *Bam*HI and *Hind*III, and ligated into the linearized MBPHT-mCherry2 vector containing sequences encoding maltose-binding protein (MBP)

and His-tag, as well as Factor Xa and tobacco etch virus (TEV) protease cleavage sites (Saraiva et al. 2010). The resulting plasmid (MBPHT-mCheery2-CpHK) was sequenced to confirm its identity and sequence accuracy. The expression and amylose-resin based purification followed standard protocols for MBP proteins (Guo and Zhu 2012). The quality and quantity of recombinant MBP-CpHK protein were evaluated by SDS-PAGE and Bradford protein assay using bovine serum albumin (BSA) as standard.

Biochemical analyses

Two types of assays were used to evaluate the enzyme kinetics of CpHK: 1) the glucose-6-phosphate dehydrogenase (G6PDH)-coupled assay was performed in 200 μ L reactions containing D-glucose (2 mM or as specified), ATP (2.5 mM or as specified), NAD⁺ (0.3 mM), G6PDH (2 U), MgCl₂ (5 mM) in Tris.HCl buffer (50 mM, pH 7.5), and MBP-CpHK (or MBP) (500 ng). In this reaction setting, reactions were started by adding MBP-CpHK (or MBP). Glucose-6P produced by HK was converted into glucono-1,5-lactone 6-phosphate, and its consumption of NAD⁺ was monitored spectrophotometrically at a 340 nm every minute for up to 30 min; and 2) the pyruvate kinase/lactate dehydrogenase (PK/LDH)-coupled assay was performed in reactions containing D-glucose, D-mannose, D-galactose, D-fructose or L-sorbose (0.001 – 10.0 mM), ATP (2.5 mM), phosphoenolpyruvate (PEP, 1 mM), NADH (0.15 mM), PK (5 U), rabbit muscle LDH (10 U), and MBP-CpHK or MBP (500 ng). In this reaction setting, ADP produced after phosphorylation of hexoses was converted back to ATP to produce pyruvate from phosphoenolpyruvate by PK. Pyruvate was converted to lactate and the consumption of NADH was monitored spectrophotometrically at a 340 nm every minute for up to 30 min. The G6PDH-coupled assay was used to determine the CpHK kinetics towards glucose, ATP and other NTPs; while the PK/LDH-coupled assay was used to obtain the kinetics of CpHK towards various hexoses. MBP-tag only was used in all assays as negative control and for background subtraction.

The inhibitory effect of 2DG on CpHK was first performed with G6PDH-coupled assay, in which the negative controls included G6P (2 mM) but excluded CpHK to evaluate the effect of 2DG on G6PDH. To avoid potential masking on the effect of 2DG on G6PDH and further confirm its effect on CpHK, experiments were performed using lowered levels of G6PDH (0.04 U) and G6P (0.2 mM), in which the latter represented the approximate amount of G6P produced in 5 min in a standard assay. Additionally, the effect of 2DG on CpHK and PK/LDH was also evaluated using PK/LDH-coupled assay under similar condition as in the standard assay but using 1 U PK, 0.5 U LDH, 1 mM PEP and 2.5 mM ADP. The activities were calculated from the OD₃₄₀ values against a standard curve derived from a series of diluted NADH in the same reaction solution with MBP-tag. G6PDH, PK and LDH used in these assays were purchased from Sigma-Aldrich (St. Louis, MO). All assays were performed independently at least twice in triplicated reactions and at room temperature (23 °C). Enzyme kinetics were analyzed using GraphPad Prism version 5.0f (<http://www.graphpad.com>). The activity of CpHK towards hexoses followed Michaelis-Menten kinetics. Parameters on ATP that displayed substrate inhibition were calculated using a Haldane equation: $v = [S]V_{\max}/(K_m + [S](1+[S]/K_i))$ [Equation 5.43 in (Copeland 2000)].

In vitro drug testing

To evaluate whether the glycolytic pathway could serve as a potential drug target, we tested the efficacy of 2DG on the growth of *C. parvum* in vitro. In the assay, HCT-8 cells were seeded in 24-well plates in RPMI-1640 medium and 10% fetal bovine serum (FBS) at 37 °C under 5% CO₂ atmosphere for overnight or until they reached to ~80% confluence. Oocysts of *C. parvum* were cleaned with 10% Clorox on ice for 5 min, followed by 5-8 times of washes in PBS before they were added into the monolayers with a parasite:host cell ratio at 1:2 (i.e., 1×10^5 oocysts with $\sim 2 \times 10^5$ host cells per well). Parasites were allowed to invade host cells for 3 h at 37 °C, and free parasites were removed by a medium exchange. Infected parasites were incubated for an additional 41 h until the second generation of meronts were fully developed, but the majority of merozoites were not released. After a centrifugation to ensure all potentially released merozoites were settled down at the bottom of wells, cultured cells were gently washed 3 times with PBS. Lysis buffer was then added into the monolayers, and total RNA was isolated using a Qiagen RNeasy Mini Kit (Valencia, CA). A Qiagen one-step RT-PCR QuantiTect SYBR green RT-PCR kit was employed to evaluate parasite growth by detecting the relative levels of parasite 18S rRNA normalized using human 18S rRNA as a control as described (Cai et al. 2005; Fritzler and Zhu 2012; Zhang et al. 2012). Cytotoxicity of 2DG on host cells was evaluated by the relative levels of 18S rRNA in uninfected HCT-8 cells.

To test the glucose-dependency of host cells and *C. parvum*, cells were cultured in a synthetic RPMI 1640 medium containing glucose at various concentrations (0, 5.5, 11, 22, and 44 mM), in which 11 mM represented the glucose concentration in regular RPMI 1640 medium. The growth of uninfected host cells and the parasite were evaluated by detecting the relative levels of their 18S rRNA by qRT-PCR as described above. We also tested whether the inhibition of parasite growth by 2DG would be restored by the addition of extra amounts of glucose. In this assay, RPMI 1640 medium containing 11, 22 and 44 mM glucose was used in the absence or presence of 2DG at the IC₇₀ concentration (2 mM). The infection of parasite and qRT-PCR-based evaluation of parasite growth were performed using the same protocols described above. All in vitro experiments were performed at least twice independently with 3 biological replicates (independent cell samples) and at least two technical replicates in qRT-PCR analysis. The means of technical replicates for individual biological replicates were calculated before computing SEM values.

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Figure 1.

The role of CphK in the unique glycolytic pathway and major connections in *Cryptosporidium parvum*. Abbreviations: **ACC**, acetyl-CoA carboxylase; **AceCL**, acetic acid-CoA ligase; **ADH1**, alcohol dehydrogenase 1; **adhE**, type E alcohol dehydrogenase (bifunctional); **GDBE**, glycogen debranching enzyme; **GGH**, Glucoside glucohydrolase; **GP**, glycogen phosphorylase; **HK**, hexokinase; **LDH**, lactate dehydrogenase; **PGI**, phosphoglucose isomerase; **PGI_M**, phosphoglucose mutase; **PMI**, phosphomannose isomerase; **PDC**, pyruvate decarboxylase; **PNO**, pyruvate:NADP⁺ oxidoreductase; **PPI-PFK**, pyrophosphate-dependent phosphofructokinase.

Figure 2.

Protein sequence features of CpHK. **A**) Sequence logos showing the signature motifs identified in CpHK and other apicomplexan orthologs (Apicomp). Asterisks marked residues that interact with glucose, while underlines marked those known to interact with ATP. Residues were colored according to chemical properties (i.e., polar in green, neutral in purple, basic in blue, acidic in red, and hydrophobic in black); **B**) A 5-amino acid insertion unique to all apicomplexan hexokinases at the end of signature motif II. Residues interacting with glucose or ATP are marked with asterisks.

Figure 3.

Relative level of expression of *CpHK* gene in different life cycle stages as determined by real-time quantitative RT-PCR. The *CpHK* mRNA levels were first normalized using those of 18S rRNA as controls, and then presented as fold changes related to that in the oocysts. Bar represents standard error of the mean (SEM) derived from triplicated reactions of pooled total RNA samples.

Figure 4.

Phylogenetic reconstructions of hexokinases (HKs) as inferred from protein sequences (184 taxa; 267 aa positions) using a heterogeneous model (CAT-Poisson), and two homogeneous models (maximum likelihood [ML] and Bayesian Inference [BI]). ML and BI analyses used WAG amino acid substitution model with the consideration of fraction of invariance and 4-rate gamma ($WAG + F_{inv} + \Gamma_{(4)}$). All HKs from the Apicomplexa including CPHK formed a monophyletic group that is more closely affiliated with prokaryotic than to the human and animal orthologs. Numbers at the major nodes were supporting values by posterior probability (PP) in CAT/BI or bootstrap proportion (BP) in ML analyses.

Figure 5.

Activity of CpHK towards various substrates. **A**) Relative activities of CpHK on glucose and other hexoses. Inset shows the SDS-PAGE analysis of purified recombinant CpHK protein stained with Coomassie blue R-25. M indicates protein molecular weight markers; **B**) Michaelis-Menten kinetics of CpHK towards glucose, mannose and fructose as determined by glucose-6-phosphate dehydrogenase (G6PDH)-coupled assay (on glucose only) and by pyruvate kinase and lactate dehydrogenase (PK/LDH)-coupled assay (on all three hexoses); **C**) Relative activities of CpHK towards various NTPs at 2.5 mM and 0.625 mM concentrations; and **D**) Substrate inhibition of CpHK by ATP, TTP, UTP, CTP and GTP. Complete inhibition by ATP was observed at concentration of 10 mM. All assays were performed at room temperature (23 °C). Bar represents standard error of the mean (SEM) derived from triplicated reactions.

Figure 6.

Inhibition of CpHK enzyme activity by 2-deoxy-D-glucose (2DG). **A**) Dose-dependent inhibition of CpHK by 2DG as determined by a standard G6PDH-coupled assay; **B**) Effect of 2DG (10 mM) on CpHK, G6PDH and PK/LDH as determined by both G6PDH- and PK/LDH-coupled assays. Substrates and enzymes concentrations were lower than those used in standard assay as described in the Methods. **C**) Apparent kinetics of CpHK in the presence of 2DG fitted with the competitive inhibition model; **D**) Lineweaver-Burk plot of data from B. Bar represents standard error of the mean (SEM) from triplicated reactions.

Figure 7.

Glucose-dependency in and effects of 2-deoxy-D-glucose (2DG) on *Cryptosporidium parvum* and host cells. **A)** Dose-dependent inhibition of 2DG on the growth of *C. parvum* in vitro. Paromomycin at 0.12 mM was used as a positive control (inset); **B)** Effect of 44 h treatment of host HCT-8 cells (ileocecal colorectal adenocarcinoma cell line) by 2DG as determined by measuring the relative levels of human 18S rRNA; **C)** Relative growth of HCT-8 cells and *C. parvum* in a synthetic RPMI 1640 medium containing glucose at various concentrations. Percent growth used 11 mM of glucose as the baseline that was equivalent to the glucose level in a regular RPMI 1640 medium; and **D)** Relative growth of *C. parvum* in the presence of 2DG at 2 mM and glucose at 11, 22 and 44 mM. In all assays, *C. parvum* oocysts were allowed to infect seeded HCT-8 cells for 3 h, followed by 41 h intracellular development (44 h total growth time). Uninfected cells were seeded and allowed to grow in parallel. The relative levels of 18S rRNA from host cells and the parasite were determined by a qRT-PCR-based assay. Bar represent standard error of the mean (SEM) from three biological replicates (independent cell samples).

Table 1

Kinetic parameters of *Cryptosporidium parvum* hexokinase (CpHK) on various hexoses and NTPs in comparison with those from *Toxoplasma gondii* (TgHK) and *Homo sapiens* (HsHKs)

Substrate	CpHK		TgHK		HsHKs	
	K_m (mM)	K_{cat} (/s)	K_m (mM)	K_{cat} (/s)	K_m (mM)	K_{cat} (/s)
D-glucose	0.138 - 0.183	20.5 - 46.4	0.008	3.47	0.032 - 0.76	38 - 101
D-mannose	0.109	12.1			0.07 - 0.1	
D-fructose	6.492	0.02			10.0 - 13.0	
ATP	0.673	9.49	1.05	3.32	0.3 - 1.25	54 - 62.3
Reference *	This study		(Saito et al. 2002)		(Ahn et al. 2009; Aleshin et al. 1999; Antoine et al. 2009; Ardehali et al. 1996; Gloyn et al. 2005; Liu et al. 1991; Magnani et al. 1988; Palma et al. 1996; Stocchi et al. 1982; Tsai 2007)	

* Also see additional data for hexokinases TEC 2.7.1.11 at <http://www.brenda-enzymes.org/>.