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Importance of enolase in *Giardia lamblia* differentiation

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Abstract

The ability of *Giardia* to differentiate into cysts which survive in the environment and release the virulent trophozoites after ingestion in the small intestine is essential for transmission and disease. We examined the role of enolase, a glycolytic enzyme, in *Giardia* differentiation. The sequence of *Giardia lamblia* enolase (gEno) is most similar to enolases in *Homo sapiens* and *Leishmania mexicana*, and shows the conserved catalytic and metal-binding residues. We used an integration vector to stably express wild type and mutant gEno. In trophozoites, wild type gEno localized to the cell membrane, caudal flagella and cytosol. gEno is present on the wall of mature cysts, but not in encystation secretory vesicles (ESV). The expression of gEno with a deletion of residues G167-K169, or mutations H389Q/R390S significantly inhibited excystation while mutation of residue D257K had no effect. These results suggest a role for enolase in regulation of *Giardia* excystation.

Keywords

Giardia lamblia; enolase; excystation; differentiation

After ingestion, giardial cysts release trophozoites that colonize the small intestine where they can cause disease. However, if they are carried downstream, trophozoites must differentiate into cysts to survive in the environment [1, 2]. In addition to major morphological changes, glycolytic metabolism decreases during encystation and increases during excystation [3]. Both differentiations are essential for transmission and disease, but the roles of metabolic enzymes are not completely understood.

Enolase (2-phosphoglycerate hydrolase) is a metal-ion-activated glycolytic enzyme that catalyzes the reversible elimination of water from 2-phosphoglycerate (2PGA) to form phosphoenolpyruvate. Enolase is one of the most abundantly expressed enzymes in the cytosol of a variety of organisms [4] and its mRNA transcript is highly expressed in *Giardia* trophozoites [5]. Recent evidence describes a role for enolase activity in the pathogenesis and cellular differentiation of several organisms [6–10]. Moreover, despite the absence of an N-terminal secretion signal, enolase has been located on the surface of a variety of parasites [10–14]. Thus, enolase is suggested to be a new surface-associated virulence factor. In *Giardia*, enolase is specifically secreted from trophozoites in the presence of intestinal epithelial cells [15]. Although it is antigenic in natural human and experimental mouse infections [16, 17], it is not an effective vaccine target in mice [18].

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Enolases are highly conserved, and gEno shares a high level of identity with enolase α and γ of *Homo sapiens*, and *Leishmania mexicana* (51%), and 49% and 46% with *Trypanosoma brucei* and *Entamoeba histolytica*, respectively. The alignment also reveals that gEno has the important residues for enolase activity (Fig. 1). In gEno, the five active site catalytic residues correspond to H170, E222, K361, H389 and K412 (marked as stars in Fig. 1). In other cells, mutation of any of these residues significantly reduces enolase activity [12, 19–21]. During the 2PGA binding, enolase undergoes important conformational changes. The loops, V153-F169 and S250-G277 allow the protonation of 2PGA by H159 [4]. In gEno, the first loop is highly conserved. In addition, gEno contains the residues that can bind divalent metal ions (i.e. Mg^{2+} , Zn^{2+} , and Mn^{2+}). Mg^{2+} is the strongest enolase activating metal [4, 22]. Binding of two Mg^{2+} ions is essential: the first Mg^{2+} (“ Mg^{2+} (I)”) is required for the correct conformation of the active site and binds to E306, D257, and D336; the second Mg^{2+} (“ Mg^{2+} (II)”) directly interacts with S41 and 2PGA [23]. *Giardia* is one of the few parasites that can be encysted and excysted *in vitro* and we tested the localization and role of HA-tagged gEno in both differentiations, using a recently developed integrated vector that introduces a C-terminal triple HA tag under the selection marker puromycin [24]. The localization of HA-tagged gEno (Fig. 2D–F) was identical to that of native gEno with a specific antibody (Fig. 2A–C) [15]. The introduction of the epitope tag did not affect the localization of enolase. Despite the absence of an N-terminal secretion signal, gEno localizes to the plasma membrane, as well as the cytoplasm, and caudal flagella of vegetative trophozoites (Fig. 2A, B, D and E). The presence of enolase on the plasma membrane has also been observed in other protozoan parasites, bacteria, helminths and fungi and is suggested to aid in completion of their life cycle and/or tissue invasion [6–14]. Similar to *Entamoeba histolytica*, *Entamoeba invadens* and *Naegleria fowleri* [6–8], gEno associates with the cyst wall in mature water-resistant cysts (Fig. 2G and H). In addition, the HA-tagged gEno expression was analyzed by Western blotting using a specific gEno antibody [15]. Two bands matching the predicted full-length native gEno (48 kDa) and HA-tagged gEno (54 kDa) show the preponderance of the wild-type protein (Fig. 1J).

To identify a specific role of enolase, we used an integrated vector [24] to obtain four independent constructs that each expressed either wild type or one of three mutated forms of gEno and assessed the capacity of the mutant *Giardia* to encyst and excyst. Untransfected *Giardia* (C6) and trophozoites expressing wild type HA-tagged gEno (called “gEno-pKS”) were used as controls. The mutant cell lines are: gEno-del-pKS, a deletion of G167-K169 amino acids involved in 2PGA conversion; gEno-mutI-pKS, in which the first Mg^{2+} binding site D257 was mutated to K257; gEno-mutII-pKS, in which the active site residues H389 and R390 were mutated to Q389 and S390. Mutations of either H159 or H373 (corresponding to H170 and H389, respectively in the gEno sequence) resulted in significant decreases in yeast enolase activity [4, 20, 21]. These gEno mutations and deletion did not affect the growth or localization of HA-tagged enolase in vegetative trophozoites (data not shown). Trophozoites were induced to encyst and ESV were counted at 20 h and cysts at 48 h after induction. The total number of ESV in gEno-del-pKS and gEno-mutII-pKS encysting trophozoites was only slightly, but significantly reduced (Fig. 3A). No significant differences were observed in the number of mature cysts (data not shown). This was surprising since enolase is important in encystation of *E. invadens* and *N. fowleri* [6, 8]. Although the transgene only replaces one of the four copies of enolase genes, allowing for expression of the wild-type enzyme, the gEno-del-pKS and gEno-mutII-pKS mutations significantly impaired the ability of parasites to excyst (43 and 66%, respectively) compared to the controls (Fig. 3B). The fact that two separate mutations, that likely inactivate enolase, cause the same phenotype leads us to conclude that this enzyme has an important role in *Giardia* excystation. In contrast, mutation of D257, one of the Mg^{2+} (I) binding sites (gEno-mutI-pKS) does not have any apparent effect on excystation (Fig. 3B). Since only one of the three Mg^{2+} (I) sites was mutated, it is possible that the overall conformation of the active

site pocket and Mg²⁺ binding are unaffected. Future studies will evaluate the enzymatic activities of these gEno mutants. Our finding that parasites expressing gEno mutants are impaired in excystation indicates that enolase might have crucial roles in cellular activation from dormancy. This opens the door for in depth investigations of the role of other metabolic enzymes in differentiation of *Giardia* and other protozoan parasites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

gEno	<i>Giardia lamblia</i> enolase
CWP	cyst wall protein
ESV	encystation secretory vesicles
HA	hemagglutinin

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Highlights

- The glycolytic enzyme enolase localizes in the cyst wall of *Giardia*
- Exploited gene integration technology to introduce deletions and mutations in *Giardia*
- Deletion and mutation of important enolase sites impair excystation of *Giardia*

Fig. 1. Sequence analyses of *Giardia lamblia* enolase (gEno)

Giardia lamblia enolase (gEno, GL50803_11118; GenBank Accession No.

XP_001709336.1) was aligned with the enolase genes of *Entamoeba histolytica* (Ehist)

(GenBank Accession No. AAA80166.1), *Leishmania mexicana* (Lmex) (GenBank

Accession No. DQ221745), *Trypanosoma brucei* (Tbru) (GenBank Accession No.

AAF73201) and α and γ enolase of *Homo sapiens* (AHomo and GHomo) (GenBank

Accession No. NP_001419.1 and GenBank Accession No. NP_001966.1). Active site

residues are marked with a star and metal-binding residues are labeled with a black circle.

The alignment was made using Vector NTI Advance software, version 9 (Life

Technologies). Black arrows indicate the residues that were deleted or mutated in this study.

The underline identifies the highly conserved first loop involved in the protonation of 2PGA by H159 [4].

Fig. 2. Immunolocalization of enolase in *Giardia lamblia* trophozoites and cysts

Using genomic *Giardia* DNA and the primer pairs Eno-pKS-F and Eno-pKS-R (supplementary Table 1), a 925 bp coding region of gEno was amplified by PCR and cloned into an integration vector (called “gEno-pKS”) that introduces a C-terminal triple HA tag under puromycin selection [24]. Plasmid gEno-pKS was linearized using *Apal* (Promega), and introduced into *Giardia lamblia* WB (clone C6, ATCC 50803). Transfected cells were cultured [25] and encysted as described [26]. gEno localization was analyzed using a fluorescence microscope (Nikon Eclipse E800). Vegetative trophozoites (A–F) and mature water-treated cysts (G–I) were stained with a specific gEno antibody (A, B) [15] and anti-HA-FITC antibody (green; Roche) (D and E, G and H). Mature cysts were also stained with an anti-CWP1-TRITC antibody (red; A300cy3R-1X, Waterborne) [27] (H). DIC images are shown on the right. Nucleic acid staining is shown in blue (DAPI). Arrows indicate trophozoite or cyst surface, and arrowheads show caudal flagella. Transfected cells were harvested and prepared for Western blotting using a specific gEno antibody (J) [15]. Black arrows indicate native and HA-tagged gEno. Bar = 10 μm.

Fig. 3. Enolase participates in *Giardia lamblia* excystation

The overlapping-extension PCR was used to generate three mutant forms of gEno. We designed two flanking primers: Eno-pKS-F and Eno-pKS-R and three pairs of internal primers to generate the desired alterations: Eno-Del-F, Eno-Del-R, Eno-MutI-F, Eno-MutI-R, Eno-MutII-F and Eno-MutII-R (Supplementary Table I). The first pair of internal primers was designed to produce a deletion of G167-K169, the second pair produced a single mutation D257K and the third pair produced a double mutation H389Q/R390S. Fragments were sequenced (EtonBio) using primers pKS-F and pKS-R, and cloned into the integration vector that introduces a C-terminal triple HA tag under puromycin selection [24]. The resulting plasmids, gEno-del-pKS, gEno-mutI-pKS or gEno-mutII-pKS, were linearized by *ApaI* digestion and introduced into *Giardia* trophozoites. Puromycin selected parasites were induced to encyst [26] and excyst [28]. Bars represent the average numbers of ESV/50 cells (A) and the % of inhibition of excystation (B). Data were statistically analyzed by ANOVA (StatPlus® 2009) and P values of 0.05 were considered significantly different. Error bars represent the standard deviation (n=3; *p-value=0.02, **p-value=0.017, ***p-value=0.00011).