Comparison of hemolytic activity of the intermediate subunit of *Entamoeba histolytica* and *Entamoeba dispar* lectins

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Abstract

Galactose and N-acetyl-D-galactosamine-inhibitable lectin of *Entamoeba histolytica* has roles in pathogenicity and induction of protective immunity in rodent models of amoebiasis. Recently, the intermediate subunit of the lectin, Igl1, of *E. histolytica* has been shown to have hemolytic activity. However, the corresponding lectin is also expressed in a non-virulent species, *Entamoeba dispar*, and another subunit, Igl2, is expressed in the protozoa. Therefore, in this study, we compared the activities of Igl1 and Igl2 subunits from *E. histolytica* and *E. dispar* using various regions of recombinant Igl proteins expressed in *Escherichia coli*. The recombinant *E. dispar* Igl proteins had comparable hemolytic activities with those of *E. histolytica* Igl proteins. Furthermore, Igl1 gene-silenced *E. histolytica* trophozoites showed less hemolytic activity compared with vector-transfected trophozoites, indicating that the expression level of Igl1 protein influences the activity. These results suggest that the lower hemolytic activity in *E. dispar* compared with *E. histolytica* reflects the lower expression level of Igl1 in the *E. dispar* parasite.

Introduction

Amoebiasis due to infection with *Entamoeba histolytica* (*E. histolytica*) is a problematic parasitic disease in many countries. *E. histolytica* causes an estimated 50 million cases of dysentery, colitis and extraintestinal abscesses, resulting in 40,000 to 100,000 deaths annually [1]. Adherence of *E. histolytica* trophozoites to colonic mucins and host cells is mediated by a galactose (Gal)- and N-acetyl-D-galactosamine (GalNAc)-inhibitable lectin [2]. This lectin is a 260-kDa heterodimer of two glycoproteins: a transmembrane heavy subunit (Hgl) and N-acetyl-D-galactosamine (GalNAc)-inhibitable lectin [2]. This lectin is a 260-kDa heterodimer of two glycoproteins: a transmembrane heavy subunit (Hgl, 170 kDa), one of the key molecules in amebic adherence, and a glycosylphosphatidylinositol (GPI)-anchored light subunit (Igl, 35/31 kDa).

Another GPI-anchored 150-kDa intermediate subunit (Igl, 150 kDa) is non-covalently associated with the Hgl/Lgl dimer in different lipid raft-like domains and also contributes to adherence [3–5]. There are two isoforms of Igl, which are referred to as Igl1 and Igl2, and both
contain multiple CXXC motifs with different localization in *E. histolytica* trophozoites [6, 7]. These two Igls are also found in *Entamoeba dispar* (*E. dispar*), which is morphologically indistinguishable from *E. histolytica*, but is non-pathogenic [8]. The expression level of Igl1 is about twice as high in *E. histolytica* HM-1:IMSS than in *E. dispar* SAW1734RclAR, whereas that of Igl2 is comparable in the two species, suggesting that Igl1 may be associated with the pathogenicity of *E. histolytica* [8]. In fact, Igl1 is recognized by sera from patients with amoebiasis and is also a vaccine candidate [9, 10].

Igl is a parasitic lectin that binds to \( p \)-aminophenyl-\( \beta \)-D-galactopyranoside-Sepharose gel in a Gal-affinity column [5]. *E. histolytica* Igl has also been detected, in addition to Hgl and Lgl, in the protein fraction that binds to GalNAc bovine serum albumin-coated magnetic beads [11]. Recently, while exploring the lectin domain of Igl, we found that Igl1 of *E. histolytica* possesses both hemolytic and cytotoxic activities [12]. However, it is unclear whether Igl1 of *E. dispar* and Igl2 of both species have the same activity. Therefore, in this study, we compared the hemolytic activities of *E. dispar* Igls with those of *E. histolytica* Igls in vitro. We also attenuated expression of Igl1 in *E. histolytica* utilizing a gene-silencing technique and evaluated the effect on hemolytic activity, since *E. dispar* has lower expression of Igl1 compared with *E. histolytica* [8].

**Materials and methods**

Expression and refolding of recombinant Igl proteins and Ni column purification of the proteins

Recombinant EhF-Igls, EdF-Igls, EhNM-Igl1, EhM-Igl1, EhC-Igl1 or EdC-Igl1 proteins with a His-tag at the N-terminus were expressed in *Escherichia coli* BL21 Star(DE3)pLysS cells (Invitrogen) or ECOS™ competent BL21(DE3) cells (Nippon Gene Co.), using the primers shown in Table 1. The proteins were further purified using a Ni column, as described in detail previously [9, 12].

Table 1. Oligonucleotide primers used in the study.

| Primer (for Eh Igls) | Position\(^a\) | Sequence (5’ to 3’)\(^b\) | Ref. |
|---------------------|---------------|---------------------------|------|
| EhIgl-S14           | 40–59         | CCCTCGAGGATTATACTGCTGATAAGCT | [9, 12] |
| EhIgl2-S14          | 40–70         | CCCTCGAGGATTATACTGCTGATAAATCATTAAACC | [7] |
| EhIgl-S294          | 880–898       | CCCTCGAGAAAGAAGAAATAAATGTA | [9, 12] |
| EhIgl-S603          | 1807–1827     | CCCTCGAGAAAGAAGAAATGATAG | [9, 12] |
| EhIgl-AS753         | 2244–2259     | CCCTCGAGTATAGCCTCTGATG | [9, 12] |
| EhIgAS1088\(^c\)    | 3247–3264     | CCCTCGAGTTAAATGCTTATGCTTATT | [9, 12] |

| Primer (for Ed Igls) | Position\(^a\) | Sequence (5’ to 3’)\(^b\) | Ref. |
|---------------------|---------------|---------------------------|------|
| EdIgl-S14           | 40–59         | CCCTCGAGGATTACAAAGCTGATAAATC | [8] |
| EdIgl2-S14          | 40–63         | CCCTCGAGGATTACAAAGCTGATAAATC | [9] |
| EdIgl-S604          | 1810–1830     | CCCTCGAGAAAGAAGAATGATAG | *|
| EdIgl-AS1097\(^c\)  | 3274–3291     | CCCTCGAGTTAAATCCTTATGCTTATT | [8] |

\(^a\) Nucleic acid numbering is based on the *E. histolytica* Igl1 and Igl2 gene sequences (AF337950 and XM_647302) and *E. dispar* Igl1 and Igl2 gene sequences (AB287423 and AB287424).

\(^b\) Nucleotides added for cloning and translation termination are underlined.

\(^c\) EhIgl-AS1088 and EdIgl-AS1097 are common for EhIgl2 and EdIgl2 respectively.

* This study.

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**SDS-PAGE and coomassie brilliant blue staining of purified recombinant proteins**

Recombinant proteins (1 μg each) were mixed with SDS sample buffer (Invitrogen) and subjected to SDS-PAGE. The gel was treated with SimplyBlue Safe stain solution (Invitrogen) and incubated until blue bands appeared on the gel [12].

**Hemolytic assays using recombinant lectins and measurement of released hemoglobin**

Hemolytic assays and quantification of hemolytic activity were conducted as previously described [12]. Briefly, recombinant Igls (2 μM each) were mixed with 50 μl of horseshoe red blood cell (HORBC) solution at room temperature and images were taken at several time points. A Hemoglobin B Test Kit (Wako, Osaka, Japan) was used to measure the concentration of hemoglobin in supernatants of RBCs incubated with recombinant proteins or trophozoites for 8 h or 1 h. The results are expressed as the mean of 5 experiments with SD.

**Culturing Entamoeba trophozoites**

Trophozoites of *E. histolytica* HM-1:IMSS G3 [13] strain were cultivated axenically in Diamond BI-S-33 medium [14] and used for generating Ig gene-silenced trophozoites. Trophozoites of *E. dispar* SAW1734RclAR strain were grown monoxenically with *Pseudomonas aeruginosa* or with *Crithidia fasciculata* and trophozoites of *E. dispar* CYNO9:TPC strain were axenically cultured in YIGADHA-S medium [15].

**Preparation of Igll gene-silenced *Entamoeba histolytica* trophozoites**

Isolation of total RNA and mRNA from trophozoites and cDNA synthesis were performed as previously described [16]. For silencing of the Igll gene, the DNA fragment from 156- to 408-nt (gsIgl1A strain) or from 1- to 466-nt (gsIgl1B strain) in Igll was PCR-amplified from cDNA using Phusion DNA polymerase (New England Biolabs) and specific primer sets (gsIgl1A strain: sense, 5′-CGA GCC CTC ACT GGA AAT AAT AAG ACA TG-3′; antisense, 5′-GTC GGA GCT CAC CAT CAA CAG TAG TAG ACA TC-3′; gsIgl1B strain: sense, 5′-CGA GCC CTC ATG TTT ATT CTT CTT TTA TTC ATA TC-3′; antisense, 5′-GTC GGA GCT CGA CCA ACA CAA TTT TCT GCA TG-3′, containing Stu I and Sac I recognition sites, respectively). The fragments were digested with Stu I and Sac I and ligated into a Stu I/Sac I double-digested psAP-2-Gunma plasmid [17] using a Ligation-Convenience Kit (Nippon Gene Co., Tokyo, Japan). Lipofection of trophozoites and selection and maintenance of transformants were performed as previously described [18].

**Real-time PCR analysis**

Real-time PCR was essentially performed as previously described [8]. Briefly, total RNAs of *E. histolytica* and *E. dispar* trophozoites isolated using a RNeasy Plus Mini Kit (Qiagen) were used for cDNA synthesis with an ExScript™ RT Reagent Kit (Takara). Reaction mixtures for quantitative real-time PCR analysis were prepared using SYBR Premix Ex Taq II (Takara), specific primers, Rox dye, and the cDNAs. Forty cycles of amplification with recording of fluorescence intensity in each cycle were performed using StepOnePlus™ Real-Time PCR System (ABI). Expression levels of Ig1 genes were analyzed using the comparative C_T method with actin as an internal standard. The experiments were repeated 3 times, including the steps of culture and isolation of RNA.
Antibodies

Purified human mAb XEhI-20 (anti-EhIgl1) and XEhI-B5 (anti-EhIgl2) were used for detection of Igls of *E. histolytica* [7]. Mouse mAb ED2-495 against recombinant Ig2 of *E. dispar* was obtained as described previously [8] and characterized before use. Mouse mAb ED2-1 specific for Ig2 of *E. dispar* was used as a control [8]. Pooled ascites rich in mouse mAbs were used in the study.

Dot blot analysis

Recombinant Igls (300 ng) were blotted on a nitrocellulose membrane and then air-dried. Filter strips were blocked with 3% bovine serum albumin in PBS and reacted with ED2-1 or ED2-495 for 30 min. After washing with PBS containing 0.05% Tween-20 (PBST), the strips were incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG antibody (MP Biomedicals) for 30 min. The strips were then washed with PBST and developed with a Konica Immunostaining HRP-1000 kit.

Immunoblot assay

Whole cell lysates (15 or 20 μg protein/well) were applied to a 5–20% gradient polyacrylamide gel (Atto Corp., Tokyo, Japan) and SDS-PAGE was conducted under reducing or non-reducing conditions, respectively. The proteins in the gel were transferred onto an Amersham™ Hybond™ P 0.45 PVDF membrane (GE Healthcare) that was then incubated with 5% skim milk in PBST for blocking. Mouse ascites (ED2-495) against *E. histolytica* Igls was diluted 500 times with PBST containing 5% skim milk. Rabbit antiserum against ATP sulfurylase (AS) of *E. histolytica* was prepared [16] and diluted 500-fold with 5% skim milk in PBST. Anti-mouse and anti-rabbit immunoglobulin F(ab’)2 fragments conjugated with HRP (Amersham) were diluted 3000 times with PBST and used as the secondary antibody. Immunoblot assays with XEhI-20 or XEhI-B5 [7] were performed using SDS-PAGE in a 5–20% gradient gel under non-reducing conditions, with 30 μg of XEhI-20 or XEhI-B5 used as the primary antibody and HRP-labeled goat anti-human IgG antibody (MP Biomedicals) diluted 1000 times with PBST as the secondary antibody. Immobilon™ Western (Millipore) was used as a substrate for visualization of the proteins. Detection of chemiluminescence and quantification of band intensities were performed by Ez-Capture MG and CS Analyzer ver. 3.0 (Atto Corp.), respectively.

Immunofluorescence assay

Sample preparation for IFA was performed as previously described [18, 19]. Briefly, after amoeba transformants were incubated on 5-mm round wells on glass slides, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed four times with PBS, and permeabilized with 0.05% Triton X-100 in PBS for 5 min. After blocking with 3% bovine serum albumin in PBS, samples were reacted with ED2-495 (mouse IgG), XEhI-20 (human IgG), or XEhI-B5 (human IgG) diluted 1:50 in PBS and subsequently reacted with secondary antibody diluted 1:500 (Alexa Fluor® 488 goat anti-mouse or human IgG; Life Technologies) in PBS. Fluorescence images were obtained using a LSM510 Meta confocal Microscope (Zeiss) in lambda emission fingerprinting mode [20, 21].

Hemolytic assay using *Entamoeba histolytica* and *Entamoeba dispar* trophozoites

The assay was conducted as previously described [12, 22] with slight modifications. Briefly, vector-transfected (control) or Ig1 gene-silenced (gsIgl1A or gsIgl1B) *E. histolytica* or wild-
type *E. dispar* (SAW1734Rc1AR strain or CYNO9:TPC strain) trophozoites prepared as described above were harvested and washed with PBS. Then 1x10^5 trophozoites were mixed with 1% HoRBCs in 100 μl PBS and incubated at 37°C for 1 h. The cell suspension was sedimented at 2000 rpm for 5 min and the concentration of hemoglobin in the supernatant was determined as described above.

**Statistical analysis**

Multiple comparisons were performed by ANOVA with a Dunn test, with P < 0.05 considered significant.

**Results**

**Recombinant Igls**

Full-length (EhF-Igl1: aa 14 to 1088 of *E. histolytica* Iggl1, EhF-Igl2: aa 14 to 1092 of *E. histolytica* Iggl2, EdF-Igl1: aa 14 to 1097 of *E. dispar* Iggl1, EdF-Igl2: aa 14 to 1093 of *E. dispar* Iggl2), N-terminal and middle (NM-Igl: aa 14 to 753 of *E. histolytica* Iggl1), middle (M-Igl: aa 294 to 753 of *E. histolytica* Iggl1), and C-terminal (C-Igl: aa 603 to 1088 of *E. histolytica* Iggl1 and aa 604 to 1097 of *E. dispar* Iggl1) regions of *E. histolytica* (Eh) and *E. dispar* (Ed) Igls with a His-tag at the N-terminus (Fig 1) were expressed in *E. coli* [12]. Recombinant proteins were purified using Ni columns, the buffer was changed to PBST, and purities were confirmed by SDS-PAGE (Figs 2A and 3A). The recombinant proteins were then used in further studies.

**Hemolytic activities of recombinant proteins against horse red blood cells (HoRBCs)**

We recently showed that EhF-Igl1 has hemolytic activity [12]. EhF-Igl1 and EhF-Igl2 have 83–84% amino acid sequence identity. EdF-Igl1 has 75–76% amino acid sequence identity with EhF-Igl1, and EdF-Igl2 has 73–74% amino acid sequence identity with EhF-Igl2 [8]. To evaluate whether EhF-Igl2, EdF-Igl1 and EdF-Igl2 also have hemolytic activity, HoRBCs (2% v/v) in PBS were mixed with EhF-Igl1, EhF-Igl2, EdF-Igl1, EdF-Igl2 or EhNM-Igl1 (Fig 2B). EhNM-Igl1 was used as a low activity control because it has less hemolytic activity than EhC-Igl1, but a similar molecular weight to EhF-Igls and EdF-Igls [12]. Samples were mixed in U-bottom 96-well plates and incubated at room temperature for up to 8 h to evaluate the hemolytic activities (Fig 2B) based on the concentration of released hemoglobin [Hb] in the supernatant after 8 h (Fig 2C). EhF-Igls and EdF-Igls had significantly higher hemolytic activities than PBST ([Hb] 0.085±0.0086 g dL^{-1}) and EhNM-Igl1 (0.11±0.0086 g dL^{-1}).

The hemolytic activity of EhF-Igl1 resides in the C-terminus of the protein [12]. To assess whether the C-terminus of EdF-Igl1 also has this activity, we conducted an assay of EhC-Igl1 and EdC-Igl1 (Fig 3), using EhM-Igl1 as a weakly active control. EdC-Igl1 (0.15±0.022 g dL^{-1}) and EhC-Igl1 (0.16±0.0069 g dL^{-1}) had equivalent activity (Fig 3C), and EhM-Igl1 showed slightly higher activity (0.082±0.0040 g dL^{-1}) than PBST (0.047±0.024 g dL^{-1}) in an ANOVA test. These results show that EdF-Igl1 has similar hemolytic activity to that of EhF-Igl1 and that the C-terminus of EdF-Igl1 has a role in this activity.

**Hemolytic activities of *Entamoeba dispar* strains**

We have reported that *E. histolytica* trophozoites have hemolytic activity and that the activity can be blocked by an antibody recognizing M/C-Igl of EhIgl1 [12]. To assess whether *E. dispar* trophozoites also have this activity, the trophozoites were incubated with HoRBCs for 1 hr. The trophozoites of *E. dispar* SAW1734Rc1AR strain were cultured with *Pseudomonas*
**Fig 1. Recombinant Igl proteins used in the study.** Recombinant Igl proteins were constructed with a His-tag at the N-terminus. Full length (F-Igl), N-terminus and middle (NM-Igl), middle (M-Igl), and C-terminus (C-Igl) Igl1 and Igl2 of *Entamoeba histolytica* (Eh) and *Entamoeba dispar* (Ed) were expressed in *E. coli* and purified using Ni columns. Estimated molecular weights of each protein including the His-tag are shown [ExPASy Compute pi/Mw tool (http://web.expasy.org/compute_p/)].

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*aeruginosa* or with *Crithidia fasciculata* monoxenically because they were difficult to culture axenically. Trophozoites of another *E. dispar* strain, CYNO9:TPC, were able to be cultured axenically and the hemolytic activity of this strain was also assessed. As shown in Fig 4, SAW1734RclAR trophozoites cultured with *Pseudomonas aeruginosa* had hemolytic activity (Fig 4A and 4B) while trophozoites of the same strain cultured with *Crithidia fasciculata* and CYNO9:TPC strain did not have activity under the test conditions. The activity in SAW1734R-clAR trophozoites cultured with *Pseudomonas aeruginosa* is due to contamination of hemolysin expressed in *Pseudomonas aeruginosa* [23].

**Hemolytic activity of Igl1 gene-silenced Entamoeba histolytica**

Recombinant Igl proteins from *E. histolytica* and *E. dispar* showed similar hemolytic activities. However, expression of Igl1 in trophozoites of *E. histolytica* is about twice as high as that in *E. dispar* trophozoites [8]. At the same time, *E. dispar* trophozoites did not have hemolytic activity (Fig 4). To evaluate whether the expression level of Igl1 affects hemolytic activity, we generated Igl1 gene-silenced (gsIgl1) *E. histolytica* strains and conducted the hemolytic assay.
Fig 2. Time-course of hemolytic activities of EhF-Igl1, EhF-Igl2, EdF-Igl1, EdF-Igl2 and EhNM-Igl1 proteins. Recombinant Igl proteins (2 μM, 50 μl) were incubated with 50 μl of 2% (v/v) HoRBCs in PBS for the indicated periods. A. Protein purity and amount were confirmed by SDS-PAGE using NuPAGE Novex Bis-Tris (4–12% gradient) gels with 1 μg of each protein. B. HoRBCs were incubated in a 96-well plate after the indicated periods with Igls. Representative images of 5 independent studies are shown. C. Concentrations of hemoglobin (Hb) released in the supernatant of samples incubated for 8 h. Data are the mean ± SD from 5 independent experiments. EhF-Igls and EdF-Igls showed significantly (**p< 0.01 by ANOVA with Dunn test) higher hemolytic activities than EhNM-Igl1 and PBST.

EhF1: EhF-Igl1, EhF2: EhF-Igl2, EdF1: EdF-Igl1, EdF2: EdF-Igl2, NM: EhNM-Igl1.

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Expression levels of Igl1 and Igl2 in the gene-silenced E. histolytica trophozoites were evaluated quantitatively by real-time PCR (Fig 5). E. dispar trophozoites expressed about a half the level of Igl1 compared with E. histolytica trophozoites (Cont), as described previously [8]. Both Igl1 gene-silenced E. histolytica strains (gsIgl1A and gsIgl1B) showed significantly lower expression of Igl1, but not Igl2, compared with vector control trophozoites (Fig 5). We could not generate an Igl2 gene-silenced E. histolytica strain for unknown reason. Interestingly, Igl2 expressions in Igl1-silenced E. histolytica strains were rather high compared with that in vector control trophozoites (Fig 5).

Downregulation of Igl1 was further confirmed by Western blotting and cell staining (Fig 6). Western blotting by mAb ED2-495, which recognizes both Igl1 and Igl2 of E. histolytica and E. dispar (Fig 6A), showed 50–80% reduction of Igl expression in gsIgl1A and gsIgl1B strains, with this reduction due mainly to downregulation of Igl1 (Fig 6B and 6C). Expression levels of Igl2 in gsIgl1A and gsIgl1B strains were higher than in vector control trophozoites, in agreement with the real-time PCR results shown in Fig 5. Downregulation of Igl1 in gsIgl1 strains was also confirmed using an immunofluorescence assay (Fig 6D). These results indicate that establishment of the gsIgl1 strains was successful.
After incubation of vector control or gsIgl1A trophozoites with HoRBCs for 1 h, the rim of the accumulated samples became vague in treatment with control trophozoites compared with gsIgl1A-treated samples (Fig 7A). For quantitative evaluation, supernatants of the incubated samples were collected and assayed for released Hb (Fig 7B). The hemolytic activity of gsIgl1A trophozoites (0.024±0.0066 g dL⁻¹) was significantly lower than that of control trophozoites (0.060±0.0093 g dL⁻¹). PBST treatment gave a [Hb] of 0.012±0.0065 g dL⁻¹. E. histolytica gsIgl1B trophozoites also had a lower hemolytic activity than control trophozoites (Fig 7C and 7D), indicating that lower hemolytic activity reflects lower IgI1 expression in the parasites.
The *E. histolytica* lectin consists of three subunits, Hgl, Lgl and Igl, of which Hgl and Igl have lectin activities [24]. Recently, we found that EhIgl1 had hemolytic and cytotoxic activities [12]. Since *E. dispar*, a non-virulent species, also has an Igl1 subunit homologue, it is of interest to determine whether EdIgl1 has these activities. In this study, we showed that EdIgl1 has similar hemolytic activity to that of EhIgl1, with the site of this activity residing at the C-terminus in both proteins. There are two isoforms of Igl, and therefore, we also evaluated the hemolytic activities of EhIgl2 and EdIgl2. All EhIgls and EdIgls had hemolytic activity in our assay. This is the first study to show that *E. dispar* Igls and *E. histolytica* Igl2 lectins have hemolytic activities.

Factors related to the virulence of *Entamoeba* spp. remain unclear, despite several detailed studies [25–28]. Among the potential factors, both Hgl and Lgl lectin subunit expression are lower in *E. dispar* compared with *E. histolytica* [29]. Low expression of Lgl1 was also found in an avirulent *E. histolytica* Rahman strain compared with the highly virulent *E. histolytica* HM-1:1IMSS strain [30]. Expression of dominant negative Hgl or Lgl in the *E. histolytica* HM-1:IMSS strain and antisense inhibition of expression of Lgl in the same strain gave a less virulent strain [30–32]. Antisense inhibition of expression of EhCP5, an amoebic cysteine protease, in
Fig 6. Establishment of Igl-attenuated Entamoeba histolytica trophozoites. A. Dot blot analysis of reactivity of ED2-495 against E. histolytica and E. dispar Igl1s. B. Western blot of Igl1s in control, gene-silenced E. histolytica and E. dispar trophozoites. C. Relative quantification of Igl1s expressed in control, gene-silenced E. histolytica and E. dispar trophozoites. D. Suppression of Igl1 protein expression by gene-silencing confirmed by IFA. Control: vector transfected E. histolytica trophozoite, gsgl1A: Igl1 gene-silenced E. histolytica trophozoite, AS: ATP sulfurylase, DIC: differential interference contrast. Amino acid sequence alignment of ATP sulfurylase between E. histolytica and E. dispar is shown in S1 Fig.

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the HM-1: IMSS strain resulted in reduced virulence [33–35]. EhCP5 is missing in *E. dispar* [36] and is expressed at a lower level in the Rahman strain compared to the HM-1:IMSS strain [25]. Antisense inhibition of amoebapore expression in the HM-1:IMSS strain also decreases amoebic virulence [37]. Thus, many virulence-related molecules have been identified, but there may be additional factors related to amoebic virulence [38].

The Igl subunit also has vital roles in the pathogenicity of the parasite, including attachment to host cells and killing activities [12, 39, 40]. EhF-Igl1 and EhF-Igl2 have 83–84% amino acid sequence identity, while the amino acid sequence identity of EhF-Igl1 and EdF-Igl1 is 75–76% and that of EhF-Igl2 and EdF-Igl2 is 73–74% [8]. EhC-Igl1 and EdC-Igl1 have 76–77% amino acid sequence identity, with conserved cysteine residues. The Igl families also have different
expression levels in each species, with Igl1 having higher expression in *E. histolytica* than in *E. dispar*, but Igl2 having similar expression in the two species [8]. To assess whether the difference in level of Igl1 proteins affects the hemolytic activity of *E. histolytica*, we generated Igl1 gene-silenced *E. histolytica* trophozoites and compared the activity with vector-transfected *E. histolytica* trophozoites. Interestingly, a 40% reduction of Igl1 protein expression led to a significant decrease in hemolytic activity.

This observation correlates with the weaker cytotoxicity of non-virulent *E. dispar* in a Gal/GalNAc lectin-mediated manner in vitro [41]. Erythrophagocytosis of *E. dispar* was observed in another study [42]. More importantly, *E. dispar* is pathogenic in experimental animals [43–46] and in humans [47, 48]. These effects have recently been reviewed [49]. The mechanism of the pathogenicity to *E. dispar* is still unclear, but it is possible that the incidence of infection by *Entamoeba* spp. is related to the expression levels of Igl1 and other related proteins. Further studies are needed to evaluate this possibility.

One of the interesting observations in this study was increased expression of Igl2 in both of Igl1-silenced strains. By contrast, decreased level of Igl2 expression has been observed by short hairpin RNA-mediated knockdown of Igl1 [50]. The discrepancy may be due to the difference of methods used to prepare transfectants.

Supporting information

S1 Fig. Amino acid sequence alignment of ATP sulfurylase between *E. histolytica* (XP_653570) and *E. dispar* (XP_001738584). Asterisks indicate the same amino acids. (TIF)

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References

1. Stanley SL Jr. Amoebiasis. Lancet. 2003; 361: 1025–1034. https://doi.org/10.1016/S0140-6736(03)12830-9 PMID: 12660071

2. Petri WA Jr, Haque R, Mann BJ. The bittersweet interface of parasite and host: lectin-carbohydrate interactions during human invasion by the parasite Entamoeba histolytica. Annu Rev Microbiol. 2002; 56: 39–64. https://doi.org/10.1146/annurev.micro.56.012302.160959 PMID: 12142490

3. Laughlin RC, McGugan GC, Powell RR, Welter BH, Temesvari LA. Involvement of Raft-Like Plasma Membrane Domains of Entamoeba histolytica in Pinocytosis and Adhesion. Infect Immun. 2004; 72: 5349–5357. https://doi.org/10.1128/IAI.72.9.5349-5357.2004 PMID: 15322032

4. Welter BH, Goldston AM, Temesvari LA. Localisation to lipid rafts correlates with increased function of the Gal/GalNAc lectin in the human protozoan parasite. Entamoeba histolytica, Int J Parasitol. 2011; 41: 1409–1419. https://doi.org/10.1016/j.ijpara.2011.10.003 PMID: 22085647

5. Cheng XJ, Tsukamoto H, Kaneda Y, Tachibana H. Identification of the 150-kDa surface antigen of Entamoeba histolytica as a galactose- and N-acetyl-D-galactosamine-inhibitable lectin. Parasitol Res. 1998; 84: 632–639. PMID: 9747935

6. Cheng XJ, Hughes MA, Huston CD, Loftus B, Gilchrist CA, Lockhart LA, et al. Intermediate subunit of the Gal/GalNAc lectin of Entamoeba histolytica is a member of a gene family containing multiple CXXC sequence motifs. Infect Immun. 2001; 69: 5892–5898 https://doi.org/10.1128/IAI.69.9.5892-5898.2001 PMID: 11500468

7. Tachibana H, Cheng XJ, Tsukamoto H, Itoh J. Characterization of Entamoeba histolytica intermediate subunit lectin-specific human monoclonal antibodies generated in transgenic mice expressing human immunoglobulin loci. Infect Immun. 2005; 77: 549–556. https://doi.org/10.1128/IAI.01002-08 PMID: 19001071

8. Tachibana H, Cheng XJ, Kobayashi S, Okada Y, Itoh J, Takeuchi T. Primary structure, expression and localization of two intermediate subunit lectins of Entamoeba dispar that contain multiple CXXC motifs. Parasitology. 2004; 134: 1989–1999. https://doi.org/10.1017/S0031182007003459 PMID: 17603840

9. Tachibana H, Cheng XJ, Masuda G, Horiki N, Takeuchi T. Evaluation of recombinant fragments of Entamoeba histolytica Gal/GalNAc lectin intermediate subunit for serodiagnosis of amebiasis. J Clin Microbiol. 2004; 42: 1069–1074. https://doi.org/10.1128/JCM.42.3.1069-1074.2004 PMID: 15004055

10. Min X, Feng M, Guan Y, Man S, Fu Y, Cheng X, et al. Evaluation of the C-Terminal Fragment of Entamoeba histolytica Gal/GalNAc Lecnt Intermediate Subunit as a Vaccine Candidate against Amebic Liver Abscess. PLoS Negl Trop Dis. 2016; 10: e0004419. https://doi.org/10.1371/journal.pntd.0004419 PMID: 26824828

11. McCoy JJ, Mann BJ. Proteomic analysis of Gal/GalNAc lectin-associated proteins in Entamoeba histolytica. Exp Parasitol. 2005; 110: 220–225. https://doi.org/10.1016/j.exppara.2005.02.013 PMID: 15955316

12. Kato K, Yahata K, Dhoubhadel BG, Fujii Y, Tachibana H. Novel hemagglutinating, hemolytic and cytotoxic activities of the intermediate subunit of Entamoeba histolytica lectin. Sci Rep. 2015; 5: e13901.

13. Bracha R, Nuchamowitz Y, Anbar M, Mirelman D. Transcriptional silencing of multiple genes in trophozoites of Entamoeba histolytica. PLoS Pathog. 2006; 2: e48. https://doi.org/10.1371/journal.ppat.0020048 PMID: 16733544

14. Diamond LS, Harlow DR, Cunnick CC. A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Trans R Soc Trop Med Hyg. 1978; 72: 431–432. PMID: 212851

15. Kobayashi S, Imai E, Haghighi A, Khalilfa SA, Tachibana H, Takeuchi T. Axenic cultivation of Entamoeba dispar in newly designed yeast extract-iron-gluconic acid-di-hydroxyacetone-serum medium. J Parasitol. 2005; 91: 1–4. https://doi.org/10.1645/GE-3386 PMID: 15856863

16. Makiuchi T, Mi-ichi F, Nakada-Tsukui K, Nozaki T. Novel TPR-containing subunit of TOM complex functions as cytosolic receptor for Entamoeba mitosomal transport. Sci Rep. 2013; 3: e1129.

17. Mi-ichi F, Makiuchi T, Furukawa A, Sato D, Nozaki T. Sul fate activation in mitosomes plays an important role in the proliferation of Entamoeba histolytica. PLoS Negl Trop Dis. 2011; 5: e1263. https://doi.org/10.1371/journal.pntd.0001263 PMID: 21829746

18. Mi-ichi F, Abu Yousuf M, Nakada-Tsukui K, Nozaki T. Mitosomes in Entamoeba histolytica contain a sulfate activation pathway. Proc Natl Acad Sci USA. 2008; 105: 21731–21736. https://doi.org/10.1073/pnas.0807106105 PMID: 19959067

19. Nakada-Tsukui K, Saito-Nakano Y, Ali V, Nozaki T. A retromerlike complex is a novel Rab7 effector that is involved in the transport of the virulence factor cysteine protease in the enteric protozoan parasite.
Entamoeba histolytica. Mol Biol Cell. 2005; 16: 5294–5303. https://doi.org/10.1091/mbc.E05-04-0283 PMID: 16120649

20. Jackson KA, Snyder DS, Goodell MA. Skeletal muscle fiber-specific green autofluorescence: potential for stem cell engraftment artifacts. Stem Cells. 2004; 22: 180–187. https://doi.org/10.1634/stemcells.22-2-180 PMID: 14990857

21. Inomoto C, Umemura S, Egashira N, Minematsu T, Takekoshi S, Itoh Y, et al. Granulogenesis in non-neuroendocrine COS-7 cells induced by EGFP-tagged chromogranin A gene transfection: identical and distinct distribution of CgA and EGFP. J Histochem Cytochem. 2007; 55: 487–493. https://doi.org/10.1369/jhc.6A7110.2007 PMID: 17242462

22. Tovy A, Hertz R, Siman-Tov R, Syan S, Faust D, Guillian N, et al. Glucose starvation boosts Entamoeba histolytica virulence. PLoS Negl Trop Dis. 2011; 5: e1247. https://doi.org/10.1371/journal.pntd.0001247 PMID: 21829737

23. Ostroff RM, Vasil AI, Vasil ML. Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from Pseudomonas aeruginosa. J Bacteriol. 1990; 172: 5915–5923. PMID: 2120196

24. Frederick JR, Petri WA Jr. Roles for the galactose-/N-acetylglucosamine-binding lectin of Entamoeba in parasite virulence and differentiation. Glycobiology. 2005; 15: 53R–59R. https://doi.org/10.1093/glycob/cwj007 PMID: 16037494

25. Thibeaux R, Weber C, Hon CC, Dillies MA, Avé P, Coppée JY, et al. Identification of the virulence landscape essential for Entamoeba histolytica invasion of the human colon. PLoS Pathog. 2013; 9: e1003824. https://doi.org/10.1371/journal.ppat.1003824 PMID: 24385905

26. Mortimer L, Chadee K. The immunopathogenesis of Entamoeba histolytica. Exp Parasitol. 2010; 126: 366–380. https://doi.org/10.1016/j.exppara.2010.03.005 PMID: 20303955

27. Gilchrist CA, Petri WA Jr. Using differential gene expression to study Entamoeba histolytica pathogenesis. Trends Parasitol. 2009; 25: 124–131. https://doi.org/10.1016/j.pt.2008.12.007 PMID: 19217826

28. MacFarlane RC, Singh U. Identification of differentially expressed genes in virulent and nonviral Entamoeba species: Potential implications for amebic pathogenesis. Infect Immun. 2006; 74: 340–351. https://doi.org/10.1128/IAI.74.1.340-351.2006 PMID: 16368989

29. Pillai DR, Kobayashi S, Kain KC. Entamoeba dispar: Molecular characterization of the galactose/N-acetyl-D-galactosamine lectin. Exp Parasitol. 2001; 99: 226–234. https://doi.org/10.1006/expr.2001.4672 PMID: 11888250

30. Anki S, Padilla-Vaca F, Stolarsky T, Koole L, Katz U, Mirelman D. Antisense inhibition of expression of the light subunit (35 kDa) of the Gal/GalNac lectin complex inhibits Entamoeba histolytica virulence. Mol Microbiol. 1999; 33: 327–337. PMID: 10411749

31. Vines RR, Ramakrishnan G, Rogers JB, Lockhart LA, Mann BJ, Petri WA Jr. Regulation of adherence and virulence by the Entamoeba histolytica lectin cytoplasmic domain, which contains a β2 integrin motif. Mol Biol Cell. 1998; 9: 2069–2079. PMID: 9693367

32. Katz U, Anki S, Stolarsky T, Nuchamowitz Y, Mirelman D. Entamoeba histolytica expressing a dominant negative N-truncated light subunit of its gal-lectin are less virulent. Mol Biol Cell. 2002; 13: 4256–4265. https://doi.org/10.1091/mbc.E02-06-0344 PMID: 12475950

33. Anki S, Stolarsky T, Mirelman D. Antisense inhibition of expression of cysteine proteinases does not affect Entamoeba histolytica cytopathic or haemolytic activity but inhibits phagocytosis. Mol Microbiol. 1998; 28: 777–785. PMID: 9643545

34. Anki S, Stolarsky T, Bracha R, Padilla-Vaca F, Mirelman D. Antisense inhibition of expression of cysteine proteinases affects Entamoeba histolytica-induced formation of liver abscess in hamsters. Infect Immun. 1999; 67: 421–422. PMID: 9864246

35. Moncada D, Keller K, Anki S, Mirelman D, Chadee K. Antisense inhibition of Entamoeba histolytica cysteine proteases inhibits colonic mucus degradation. Gastroenterology. 2006; 130: 721–730. https://doi.org/10.1053/j.gastro.2005.11.012 PMID: 16530514

36. Jacobs T, Bruchhaus I, Dandekar T, Tannich E, Leippe M. Isolation and molecular characterization of a surface-bound proteinase of Entamoeba histolytica. Mol Microbiol. 1998; 27: 269–276. PMID: 9484883

37. Bracha R, Nuchamowitz Y, Leippe M, Mirelman D. Antisense inhibition of amoebapore expression in Entamoeba histolytica causes a decrease in amoebic virulence. Mol Microbiol. 1999; 34: 463–472. PMID: 10564488

38. MacFarlane RC, Singh U. Identification of an Entamoeba histolytica serine-, threonine-, and isoleucine-rich protein with roles in adhesion and cytotoxicity. Eukaryot Cell. 2007; 6: 2139–2146. https://doi.org/10.1128/EC.00174-07 PMID: 17827347

39. Cheng XJ, Tachibana H, Kaneda Y. Protection of hamsters from amebic liver abscess formation by a monoclonal antibody to a 150-kDa surface lectin of Entamoeba histolytica. Parasitol Res. 1999; 85: 78–80. PMID: 9950232
40. Cheng XJ, Kaneda Y, Tachibana H. A monoclonal antibody against the 150 kDa surface antigen of *Entamoeba histolytica* inhibits adherence and cytotoxicity to mammalian cells. Med Sci Res. 1997; 25: 159–161.

41. Dodson JM, Clark CG, Lockhart LA, Leo BM, Schroeder JW, Mann BJ. Comparison of adherence, cytotoxicity, and Gal/GalNAc lectin gene structure in *Entamoeba histolytica* and *Entamoeba dispar*. Parasitol Int. 1997; 46: 225–235.

42. Talamás-Lara D, Chávez-Munguía B, González-Robles A, Talamás-Rohana P, Salazar-Villatoro L, Durán-Díaz A, et al. Erythrophagocytosis in *Entamoeba histolytica* and *Entamoeba dispar*: a comparative study. Biomed Res Int. 2014; 2014: 626259. https://doi.org/10.1155/2014/626259 PMID: 25003123

43. Gomes MA, Melo MN, Macedo AM, Pena GP, Callari MV, Silva EF. Characterization of *Entamoeba histolytica* and *Entamoeba dispar* by biological, biochemical, and molecular parameters. Arch Med Res. 2000; 31: S249–250. PMID: 11070303

44. Costa AO, Gomes MA, Rocha OA, Silva EF. Pathogenicity of *Entamoeba dispar* under xenic and monoxenic cultivation compared to a virulent *E. histolytica*. Rev Inst Med Trop S Paulo. 2006; 48: 245–250. PMID: 17086310

45. Dolabella SS, Serrano-Luna J, Navarro-García F, Cerritos R, Ximénez C, Galván-Moroyoqui J.M., et al. Amoebic liver abscess production by *Entamoeba dispar*. Ann Hepatol. 2012; 11: 107–117. PMID: 22166569

46. Guzmán-Silva MA, Santos HL, Peralta RS, Peralta JM, de Macedo HW. Experimental amoebic liver abscess in hamsters caused by trophozoites of a Brazilian strain of *Entamoeba dispar*. Exp Parasitol. 2013; 134: 39–47. https://doi.org/10.1016/j.exppara.2013.01.015 PMID: 23380037

47. de Martínez AM, Gomes MA, Viana JdaC, Romanha AJ, Silva EF. Isoenzyme profile as parameter to differentiate pathogenic strains of *Entamoeba histolytica* in Brazil. Rev Inst Med Trop S Paulo. 1996; 38: 407–412. PMID: 9293086

48. Graffeo R, Archibusacci CM, Soldini S, Romano L, Masucci L. *Entamoeba dispar*: A rare case of enteritis in a patient living in a nonendemic area. Case Rep Gastrointest Med. 2014; 2014: 498058. https://doi.org/10.1155/2014/498058 PMID: 24851190

49. Oliveira FM, Neumann E, Gomes MA, Callari MV. *Entamoeba dispar*: Could it be pathogenic. Trop Parasitol. 2015; 5: 9–14. https://doi.org/10.4103/2229-5070.149887 PMID: 25709947

50. Linford AS, Moreno H, Good KR, Zhang H, Singh U, Petri WA Jr. Short hairpin RNA-mediated knockdown of protein expression in *Entamoeba histolytica*. BMC Microbiol. 2009; 9: 38. https://doi.org/10.1186/1471-2180-9-38 PMID: 19222852