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Giardia lamblia: Intracellular localization of alpha8-giardin

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ABSTRACT

Alpha8-giardin (α8-giardin) is a member of the multi-gene α-giardin family in the intestinal parasitic protozoan, *Giardia lamblia*. This gene family shares an ancestry with the annexin super family, whose common characteristic is calcium dependent binding to membranes that contain acidic phospholipids. In the present study, the antigenicity, hydrophilicity, flexibility, surface probability, and secondary structure of α8-giardin amino acids were predicted by bioinformatics applications. A specific anti-peptide antiserum, anti-P3, was used to determine the intracellular location of α8-giardin with confocal immunofluorescence microscopy and immunoelectron microscopy. The results indicated that α8-giardin was located on the plasma membrane and flagella, but not on the ventral disk. Reduction of α8-giardin transcript levels by ribozyme-mediated cleavage decreased trophozoite motility and growth rate, indicating the functional importance of α8-giardin to *Giardia* trophozoite biology.

1. Introduction

Giardia lamblia (syn. *G. intestinalis*, *G. duodenalis*) is a parasitic flagellated intestinal protozoan, which is found worldwide and causes diarrhea in humans and other mammals (Hunter and Thompson, 2005). The life cycle includes the infective, immotile cyst, which by virtue of its tough cell wall is able to survive the inhospitable conditions of the environment and the host's stomach, and the vegetative, motile trophozoite, which causes diarrhea and malabsorption in the infected individual (Gillin et al., 1996). After the cysts pass through the stomach, cysts transform into flagellated trophozoites, which adhere to the intestinal epithelium of their host by means of their ventral disk, a concave cytoskeletal structure surrounded by the plasma membrane (Elmendorf et al., 2003). Motility and attachment to the intestinal wall are mediated by cytoskeletal structures, so the pathogenesis of Giardia depends directly on the cytoskeleton. Three major classes of giardins, alpha (α), beta (β), and gamma (γ), have been identified as components of the Giardia cytoskeleton (Nohria et al., 1992; Peattie, 1990; Peattie et al., 1989). The α-giardins form a large class of annexin-like molecules (Baker et al., 1988; Bauer et al., 1999; Weiland et al., 2005; Wenman et al., 1993) encoded by 21 different genes numbered from α1 to α19-giardin (α7-giardin appears as three variants), all having significant sequence similarities.

Prior studies attempting to identify the intracellular locations of α-giardins using rabbit polyclonal antisera were limited by cross-reaction with other proteins. These limitations have been overcome by the use of epitope tagged α-giardins (Weiland et al., 2003) However, four giardins (α4, α7.1, α8, and α11) could not be localized since the transfection experiments were lethal to the parasite. Therefore, we have utilized an immunocytochemical approach to determine the intracellular localization of α8-giardin. In addition, ribozyme-mediated cleavage of the transcript was used to evaluate the importance of α8-giardin.

2. Materials and methods

2.1. Organisms

The *G. lamblia* isolate utilized in these studies (C2), was derived from a patient in southwest China (Lu et al., 1996). This isolate had been called a Genotype B isolate. However, the α8-giardin sequence from C2 had only two nucleotide differences from the WB sequence (Genotype A1), but was only 79% identical to the GS sequence (Genotype B). Therefore, we compared the pyruvate kinase (PK) and pyruvate,phosphate dikinase (PPDK) sequences of C2 with those of WB and GS. The PK sequence was identical to that of WB and 85% identical to that of GS, while the PPDK sequence was 99.6% identical to that of WB and 84% identical to that of GS. Therefore, the C2 isolate belongs to Genotype A. Trophozoites were cultivated axenically with modified TYI-S-33 medium (Keister, 1983).

2.2. Synthesis of dominant epitopes on α8-giardin amino acids and preparation of corresponding antisera

The antigenicity, hydrophilicity, flexibility, surface probability, and secondary structures of α8-giardin were predicted by bioinfor-

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matic approaches. Three antigen peptides P1 (aa 7–17; AYVVAQDLHNA), P2 (aa 30–40; TGRYVSGYREK), and P3 (aa 296–306; AYDIGKILAEK) were designed on the basis of the above analyses. These three peptides were synthesized, conjugated to keyhole limpethemocyanin (KLH), and injected into rabbits to prepare corresponding antisera. Indirect ELISA was performed to analyze the titers of anti-P1, anti-P2, and anti-P3, and Western blotting was used to identify the specificity of the antisera.

2.3. Preparation of cytoskeletons

G. lamblia cytoskeletons were isolated as described (Crossley and Holberton, 1983). Briefly, the organisms were harvested at 1600*g* for 20 min at 4 °C, resuspended in a small volume (<1 ml) of either 10 mM Tris–HCl (pH 8.3), or morpholino propane sulfonic acid (pH 8.3), containing 2 mM EDTA, 2 mM DTT, 1 mM ATP, 2 mM MgSO4, and 150 mM KCl(TEDAMP or MEDAMP buffer). The trophozoites were counted with a hemacytometer, and then extracted for 5 min at room temperature in TEDAMP (or MEDAMP) buffer plus 0.5% Triton X-100 at a concentration of 107 Giardia/ml. The cytoskeletons were pelleted at 15,000*g* for 15 min at 4 °C and were used immediately or stored at −70 °C. Freshly prepared cytoskeletons were used for immunofluorescence and transmission electron microscopy.

2.4. Immunofluorescence (IF) staining of G. lamblia trophozoites

Washed trophozoites were allowed to attach to coverslips at 37 °C, then fixed with methanol for 7 min and permeabilized with acetone for 10 min, both at −0 °C. After rehydrating with TBS for 10 min at room temperature, the cells/cytoskeletons were incubated with blocking buffer (3% bovine serum albumin, BSA) in TBS for 10 min, followed by incubation with rabbit anti-α8-giardin antiserum (1:800 in TBS) or a monoclonal antibody against tubulin (1:1000 in TBS) for 1 h. After washing three times with TBS, the cells were incubated for 1 h in the dark with DYE-light488 anti-rabbit IgG, from sheep (1:100 in TBS) as secondary antibodies. The coverslips were then rinsed in TBS before being mounted with DAPI (4′,6′-diamidino-2-phenylindole; Invitrogen, Carlsbad, CA, USA).

Alternatively, isolated cytoskeletons were allowed to attach to coverslips at 37 °C, fixed for 30 min with 3% paraformaldehyde (PAF) and incubated with blocking buffer for 30 min. After washing three times with TBS, the cytoskeletons were immunodecorated in the same way and were incubated for 1 h in the dark with DYE-light488 anti-rabbit IgG, from sheep (1:100 in TBS) or DYE-light488 goat anti-mouse IgG, (1:100 in TBS), respectively, as secondary antibodies. After another three washes with TBS, cells and cytoskeletons were observed using a confocal laser scanning microscope (Leica DM 5500 B).

2.5. Immunoelectron microscopy

Cell suspensions of trophozoites were centrifuged for 5 min at 600*g*. The cell pellets were fixed with 0.25% glutaraldehyde and 4% PAF overnight at 4 °C, resuspended in 4% PAF in a phosphate buffer (0.135 M PO4, pH 7.6), and incubated at 4 °C for 8 h. Fixed cell samples were centrifuged for 5 min at 600*g* and then three 10 min washes in phosphate buffer. Subsequently, cells were pelleted and cryoprotected in a 30% sucrose in phosphate buffer. After that, the cells were washed twice in PBS-Tween 20 and then embedded into a 0.5% NaBH4 solution. Subsequently, the pellet was blocked in 6% FCS, floated overnight on a 1:100 dilution of primary rabbit anti-α8-giardin peptide antibody, incubated for 2 h in secondary goat anti-rabbit 5 min, washed with phosphate buffer and embedded in SE-EM enhancer for 2 h. After three 10 min washes in phosphate buffer, the samples were post-fixed in 1% osmium acid (OsO_4) for 2 h at 4 °C while being protected from light. They were then washed three times in double distilled water for 10 min each time, followed by dehydration in progressive increases in ethanol concentration (50%, 70%, 90%, and 100%) for 10 min each, then three 10 min washes in 100% acetone. The samples were embedded in VCD4206 medium and 100% acetone (1:1 saturated solution) for 2 h at room temperature, and then embedded in Epon 812 for 24 h at 37 °C after warming up to 60 °C for 48–72 h, to allow polymerization. Ultra-thin sections (70–80 nm) were stained with uranyl acetate for 20 min, lead nitrate for 5 min, and observed in a JEOL transmission electron-microscope.

2.6. Construction of the hammerhead ribozyme expression vector

The GUC trinucleotide at 600–602 bp of the open reading frame of the gene from Giardia α8-giardin (GenBank database Accession Number AY781323) was chosen as the ribozyme cleavage point. A 72-base oligonucleotide (KH8R), 5′-GTCGACCATGCAGATAGCTGT CTCCAAGCTGATGAGTCCGTGAGGAC-GAAACTTGTTTGTGTGCTCCTCGTAAG-3′ was used as the reverse primer. The underlined portion is the core structure of a hammerhead ribozyme sequence and is flanked by 22 and a 24-nucleotide (nt) fragments complementary to the α8-giardin cDNA sequences 603–626 and 578–599, respectively. The forward primer 5′-GGA TCCTGTGATTCTGTGCAGCACAGATGACT-3′ is from bases 300 to 325 of the α8-giardin ORF and provides a BamH I restriction site for cloning. The 358 bp PCR fragment was cloned into pMD18-T (Takara, Japan). Plasmid pGCV634/GFP/GCV2174 was used as the template for amplifying the necessary components of the Giardia lamblia virus (GLV) transfection using the primer pair, 5′-CTGC AGTAATACGACTCACTATAGGAAGAAGTGCCAGGCCATTACC-3′ and 5′-GTCGACCGCTGCCGCCCAGCGCGTGATTATC-3′ for GCV634, and the primer pair, 5′-GGATCCGTCGCGGGGTACCCAGCTAGAATGATG-3′ and 5′-CCCGGGCGACCCCCTCGTACGCTGCCTCCTAC-3′ for GCV 1423. These products were cloned into the vector containing the ribozyme with the α8-giardin sequence, one on each side, resulting in the transfection vector, pGCV634/KH8/GCV1423 (KH8). Sequence analysis was used to confirm the correct inserts.

For a negative control, another 76-base oligonucleotide (KHpk) was synthesized: 5′GTCGACGATTAGGATGTGAGGGGTGCTATGC TGATGAGTCCGTGAGGACGAAACGACAGCTTAGATGGAATGCGCA-3′. Its flanking sequences were complementary to Giardia PK cDNA sequences 274–299 bp and 597–648 bp (GenBank database accession number XM_764552), respectively. Using this oligomer as a template, PCR was performed with the primer pair, 5′-GGATC CGACCAGAAAAAGCACATCAGCATTAT-3′ and 5′-GTCGACGATTAG GATGTGAGGGGTGCTATGCTGATGAGTCCGTGAGGACGAAACGACAG CTTAGATGGAATGCGCA-3′ to provide BamH I/Sal I restriction sites on each sides of the KHpk cDNA.

To make another negative control, anti-α8, PCR was performed with primer pair 5′-GGATCCGACGCATTGGTGGACCTTGT3′ and GTC GACGAGTGGGATGGCGGTGTTAT-3′, using genomic DNA of *G. lamblia* as templates. This PCR product was the α8-giardin cDNA without the inserted hammerhead ribozyme sequence, but with BamH I/Sal I restriction site on the ends. These two PCR fragments were digested with BamH I/Sal I and inserted into pGCV634/GFP/GCV2174 to create recombinant plasmids pGCV634-KHpk, and pGCV634-anti-α8, respectively.

2.7. Extracellular cleavage of α8-giardin mRNA by ribozyme

The vector pGCV634/KH8/GCV1423 was linearized by SmaI at the 3′end of the template for in vitro transcription using T7 RiboMAXTM Express Large Scale RNA Production System (Promega) according to the manufacturer's instructions.

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The in vitro ribozyme cleavage assays were performed as previously described (Dan et al., 2000; Dan and Wang, 2000), with a 1:1 M ratio of total Giardia RNA and the chimeric ribozyme transcripts. RNA annealing and integrity was confirmed by agarose gel electrophoresis. RNA cleavage was confirmed by real-time PCR.

2.8. Intracellular cleavage of α8-giardin mRNA by ribozyme

Log-phase trophozoites were transfected by the chimeric GCV-ribozyme transcripts via electroporation as previously described (Wang et al., 1995). The transfected cells were transferred to fresh Giardia culture medium and incubated for 24, 48, 72, and 96 h.

2.9. Real-time PCR

Total RNA was extracted with Trizol (Invitrogen) from the hammerhead ribozyme-transfected trophozoites (as mentioned above) at different time points to examine the presence of introduced ribozyme by RT-PCR. Random primer 9 was used for synthesis of the first cDNA strand, followed by PCR using primers RQH.

The sensitivity of the PCR was tested by relative real-time quantitative (RQ) RT-PCR, using 10-fold serial dilutions triplicate, ranging from 101 to 109 copies. For RQ RT-PCR, random 9-base oligonucleotides were used for synthesis of the first strand cDNA, followed by RQ RT-PCR using primers (RQH1: 5′-TAGAGGGA GGCAATGGAGC-3′) and (RQH2: 5′-GCCTTGCTTGGCTCACAG-3′), which spanned the cleavage site of the ribozyme. For RQ RT-PCR, the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (GenBank database Accession Number M88062) was used as the internal standard, using primers 5′-CAGG TCGCTTTACAACGAAGA-3′ and 5′-TACAACCGCAGACGAACATAG-3′, which yielded a 112 bp product. Reactions were performed for 15 min at 95 °C followed by 40 cycles consisting of 1 min at 94 °C, 30 s at 60 °C and 30 s at 70 °C. All tests were performed in triplicate in the same run. Amplification reactions were run on a Roche LightCycler 2.0 (Applied Biosystems) and data analysis was carried out with Roche LightCycler 2.0 software, version 1.2.3.

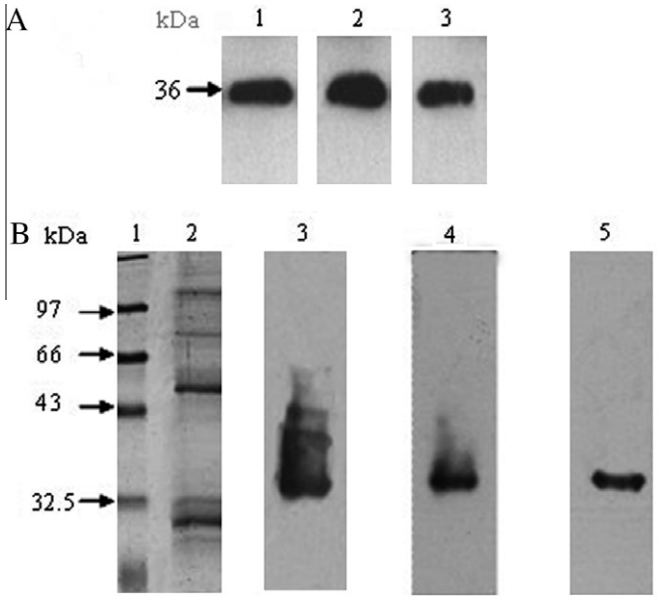

Fig. 1. An immunoblot analysis of *G. lamblia* cytoskeletal proteins by three epitope antibodies demonstrates specificity of anti-P3 for α8-giardin. (A) Immunoblot of *G. lamblia* recombinant α8-giardin protein shows that anti-epitope antibodies, anti-p1, anti-p2, anti-p3, all recognize the 36 kDa recombinant α8-giardin protein. (B) Western blot of *G. lamblia* cytoskeletal proteins separated by SDS–PAGE (12% acrylamide). (1) marker proteins; (2) Coomassie stain of total cytoskeletal proteins; (3) anti-P1 and (4) anti-P2 show reactivity with several proteins ranging in size from 34 to 44 kDa in size, while (5) shows specific reactivity of anti-P3 with a 34 kDa band with cytoskeletal protein.

2.10. Western blot

At 24 h post transfection, total protein was extracted with the M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific; Waltham, MA, USA) from the hammerhead ribozyme-transfected trophozoites (as mentioned above) to examine the relative expression of α8-giardin. The total protein concentrations were determined by BCA assays, and equally loaded for Western blotting using the affinity purified peptide antibody, anti-p3, at a 1:1000 dilution. Antibody to α-tubulin and HRP anti-rabbit IgG were products from Beyotime (Beijing, China).

2.11. Giardia growth and α8-giardin localization after ribozyme transfection

In order to understand the effect of α8-giardin on parasite growth, trophozoites were transfected with ribozyme PGCV-KH8 were compared with untransfected trophozoites and trophozoites transfected with PGCV-GFP. They were placed on ice for 15 min to detach all the cells from the wall of the culture tube, then

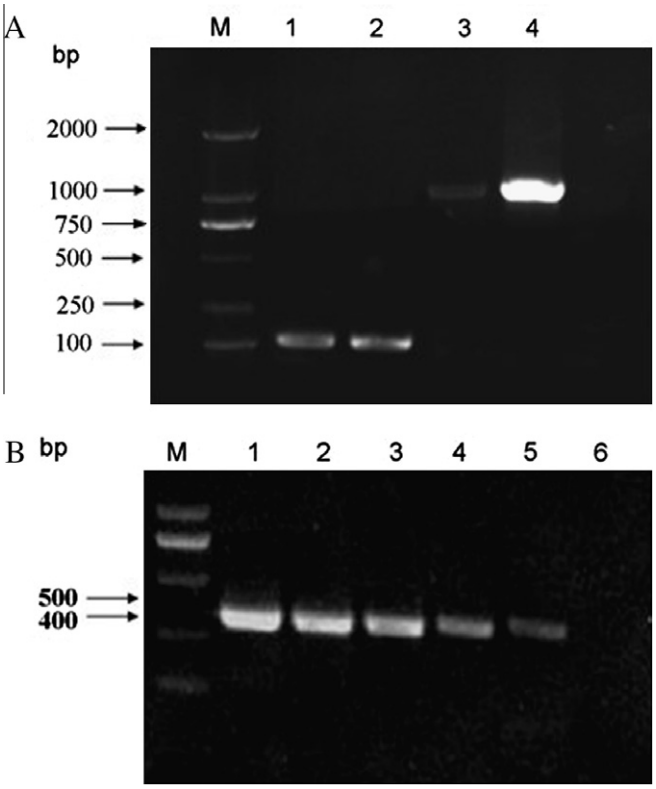

Fig. 2. Quantitative real-time PCR demonstrates reduction of α8-giardin transcript levels in transfected trophozoites. (A) Quantitative real-time PCR demonstrates the effect of ribozyme cleavage of Giardia α8-giardin mRNA. Lane M, DNA size markers; (1) (RQ) RT-PCR product of a 116 bp portion of GAPDH in transfected trophozoites; (2) (RQ) RT-PCR product of GAPDH in untransfected trophozoites; (3) (RQ) RT-PCR product of the α8-giardin transcript (ORF size is 936 bp) in transfected trophozoites; (4) (RQ) RT-PCR product of the α8-giardin transcript in untransfected trophozoites. (B) Quantitative real-time PCR evaluation of the levels of the α8-giardin antisense transcript produced by KH8 transfected trophozoites. Lane M, DNA maker; (1) α8-giardin antisense transcript levels 24 h after electroporation; (2) α8-giardin antisense transcript levels 36 h after electroporation; (3) α8-giardin antisense transcript levels 48 h after electroporation; (4) α8-giardin antisense transcript levels 72 h after electroporation; (5) α8-giardin antisense transcript levels 96 h after electroporation; and (6) α8-giardin antisense transcript levels in untransfected trophozoites.

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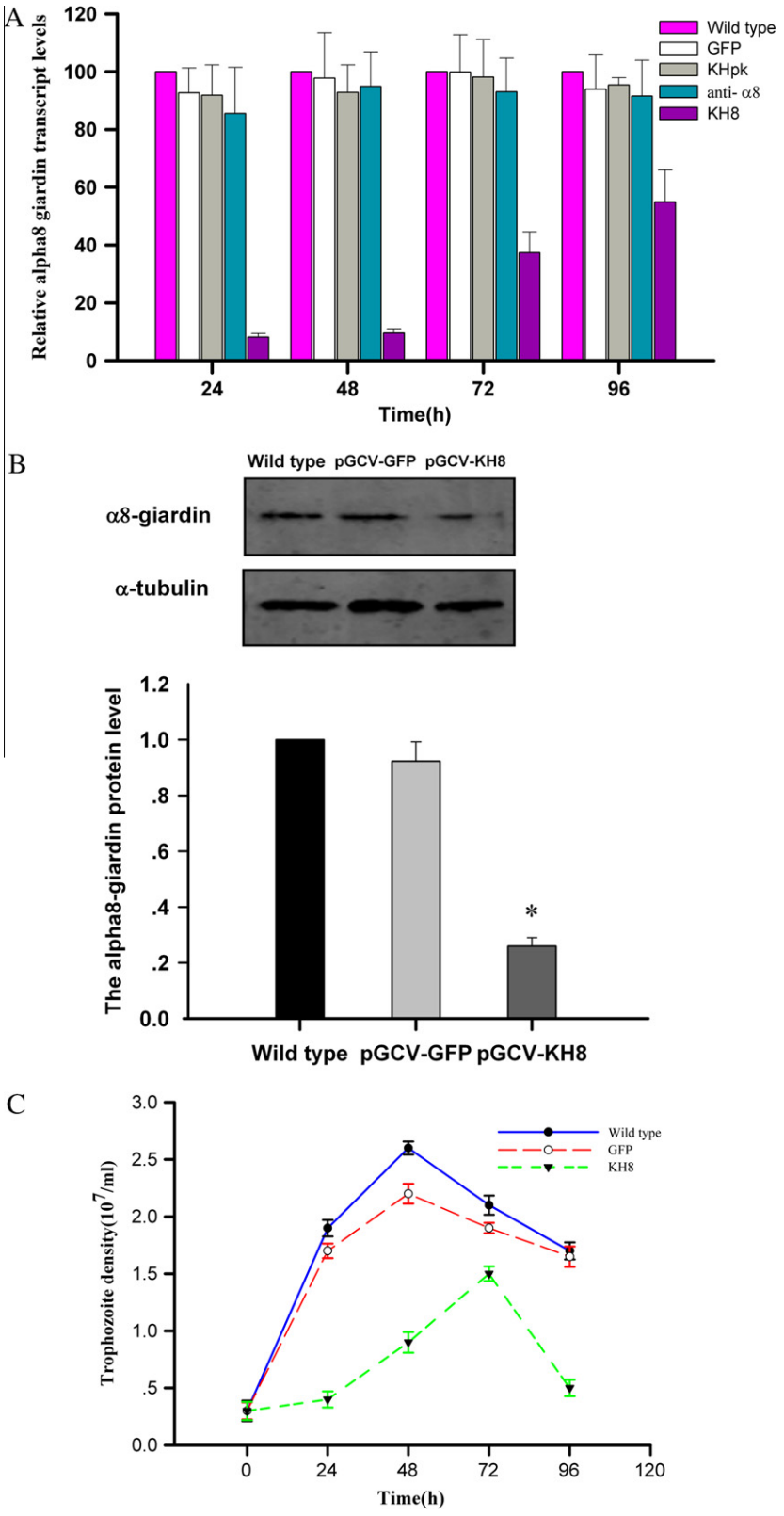

Fig. 3. Effect of transfection with a knockdown vector on transcript levels of α8-giardin in comparison with other genes (mean ± S.E). The transcript levels of α8-giardin in knockdown transfectants and untransfected controls were determined by quantitative RT-PCR at 24–96 h, and expressed as the ratio between α8-giardin mRNA and GAPDH mRNA in the samples. The amplification plots and standard curve were obtained with the in vitro transcript from α8-giardin. Serial 10-fold dilutions with 7 × 108–7 × 102 pg per reaction well were made in EASY Dilution (Takara). Amplification was repeated three times for each dilution. The level of α8-giardin mRNA in KH8 transfected *G. lamblia* trophozoites was 8.52% on 24 h and the level went back up to 54.9% on 96 h. (B) Western blotting demonstrates that α8-giardin levels are reduced in knockdown transfectants. α8-giardin protein levels were measured using immunoblot assay with antibody anti-p3. α-tubulin was used as a control for equal loading of proteins. The ratio of α8-giardin was evaluated by densitometric analysis and data were expressed as multiples of the control value. The α8-giardin levels were significantly decreased in the PGCV-KH8 transfected cells compared with both PGCV-GFP transfected and untransfected cells. (*indicates reduction in comparison with controls, $P < 0.01$). (c) The growth rate of *G. lamblia* trophozoites transfected with KH8 is slower than that of mock transfectants. The hammerhead ribozyme transfected cells grew at a slower rate than that of the control and GFP groups. The results were showed as mean ± S.E of three independent experiments.

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harvested and counted with a hemacytometer 24, 48, 72, and 96 h after transfection. The IF assay was used to confirm the cellular localization of α8-giardin before and after transfection. The fluorescence intensity levels were analyzed by Leica LAS AF Lite 1.8.9.

2.12. Statistical analyses

Quantitative data were statistically analyzed by independent sample *t*-tests and ANOVA. $P < 0.05$ was regarded as significant. Quantified results were presented as mean ± SD. N indicates the number of independent experiments ($N = 3–5$).

3. Results

3.1. Identification of α8-giardin immunodominant domain

The immunodominant epitopes of α8-giardin were predicted by using several bioinformatics approaches, and resulted in the identification of three candidate antigens, P1 (7–17 aa), P2 (30–40 aa) and P3 (296–306 aa) (Korber et al., 2006; Saha and Raghava, 2006). The highest titer of each antiserum was 1:120,000, 1:80,000, and 1:120,000, respectively. These candidate antisera were reacted with recombinant α8-giardin expressed in Escherichia coli as a poly-histidine fusion protein, and with cytoskeletal protein in Western blots. All three antisera recognized a 34 kDa band of both recombinant α8-giardin and cytoskeletal protein (Fig. 1). Anti-P1 and anti-P2 cross-reacted with other protein bands in the 34–44 kDa size range, but anti-P3 was specific to a 34 kDa protein band (Fig. 1).

3.2. Extracellular cleavage of α8-giardin mRNA

A 22 nt hammerhead ribozyme flanked by portions of the α8-giardin sequence was used to transcribe antisense RNA that was designed to cleave the α8-giardin mRNA of Giardia between nucleotides 600 and 602 of the coding sequence. Transcripts from

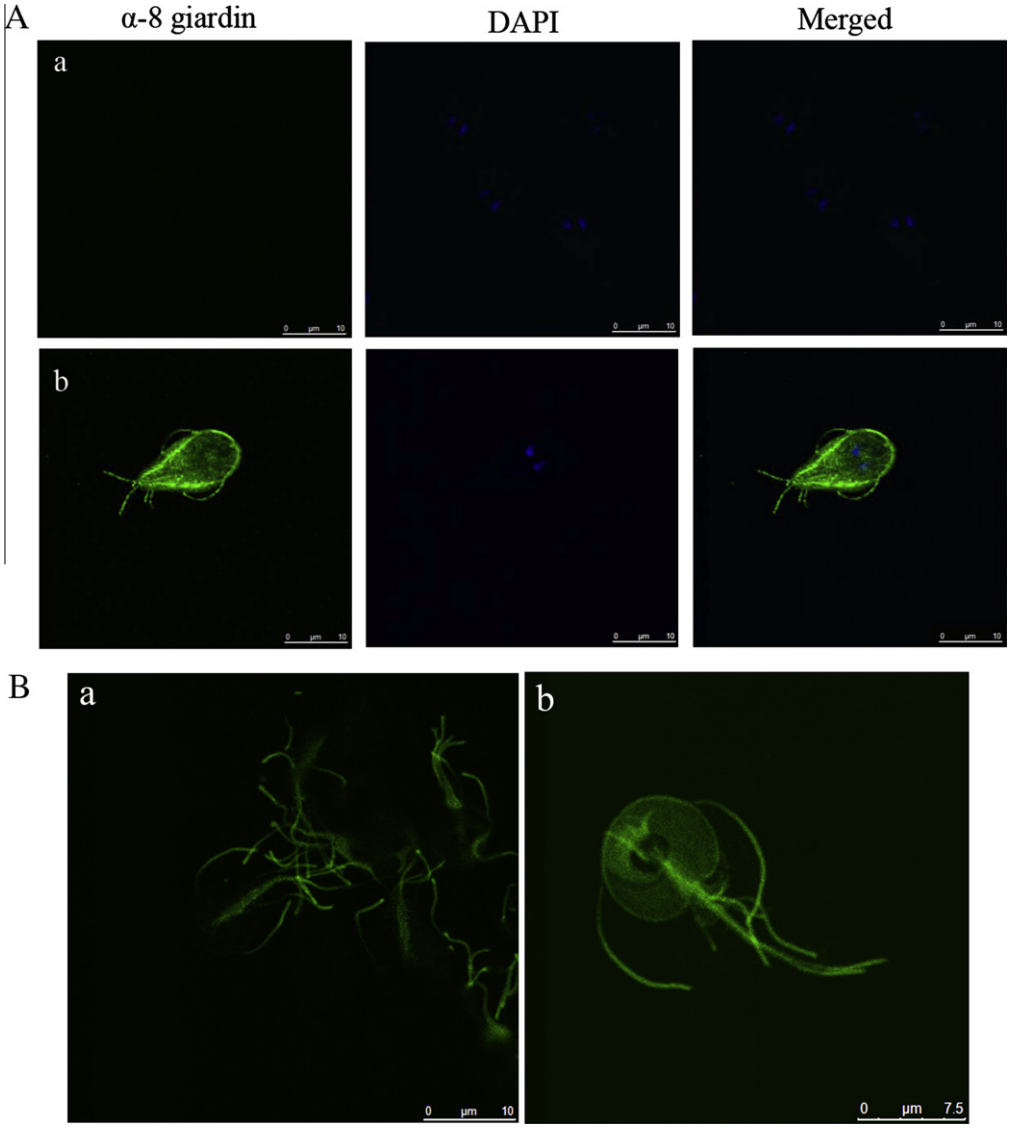

Fig. 4. Immunofluorescent localization of α8-giardin to the plasma membrane and flagella. (A) Intracellular localization of α8-giardin identified by CIEM in *G. lamblia* trophozoites. (a) control with pre-immune serum. (b) fixed and permeabilized trophozoites were incubated with an affinity purified peptide antibody against alpha8-giardin (green). Secondary antibodies used were DYE-light488 anti-rabbit IgG from sheep. Note that the fluorescence significantly arranged near the plasma membrane the flagella. Bar is 10 μm. (B) Fluorescent antibody staining of the *G. lamblia* cytoskeleton. (a) Isolated Giardia cytoskeletons stained with antibody anti-p3. (b) Cytoskeletons stained with sheep anti-tubulin IgG. Note that the anti-giardin antibodies in A stain the flagellae, which appear as fibers structures, but do not stain the ventral disks. The anti-tubulin antibodies in B react both with disks and flagella. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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the recombinant vector were synthesized with the T7 RiboMAXTM Express Large Scale RNA Production System and tested for cleavage of α8-giardin mRNA. The substrate was cleaved by the pGCV634/KH8/GCV1423 transcript, and no cleavage was detected with control Giardia total RNA that did not contain the ribozyme motif (Fig. 2A).

3.3. Expression of the ribozyme and α8-giardin gene interruption

After transfection of pGCV634/KH8/GCV1423, the presence of the ribozyme was monitored with RT-PCR at different time intervals (Fig. 2B). The transcript levels of α8-giardin as determined by quantitative RT-PCR are shown in comparison to untransfected controls and two negative controls and expressed as the ratio between α8-giardin mRNA and GAPDH mRNA in the samples (Fig. 3A). These studies were done in the absence of drug selection, so that if there was a difference in growth rate, untransfected trophozoites could overtake the transfected organisms in a population. The ribozyme level was the highest at 24 h and steadily declined up to 96 h, indicating a gradual increase in the proportion of untransfected cells in the population. The knockdown transfection also decreased protein levels as determined by densitometric comparison of knockdown transfectants with wild type and parasites transfected only with GFP (Fig. 3B).

The level of α8-giardin mRNA in KH8-transfected Giardia trophozoites was 8.52% of the wild type value at 24 h and increased to 54.9% at 96 h. in contrast, the level of α8-giardin mRNA in the negative control KHpk group and GFP group showed no difference in comparison with untransfected trophozoites. The anti-α8 group, which did not have the ribozyme motif, showed some inhibitive activity.

3.4. Intracellular localization of α8-giardin identified by confocal immunofluorescence microscopy (CIFM) and immunoelectron microscopy (IEM)

Anti-P3 was used to identify the intracellular localization of α8-giardin by CIFM and IEM. When intact trophozoites were observed by CIFM, α8-giardin was found on the plasma membrane and the flagella (Fig. 4A). The antibody reacted with the flagella but not with the ventral disk in isolated cytoskeletons (Fig. 4B-a). In contrast, the control anti-tubulin antibody (mouse anti-tubulin) reacted with both the disk and the flagella in isolated cytoskeletons (Fig. 4B-b). The IEM studies showed the arrangement of gold particles on the plasma membrane and flagella (Fig. 5).

3.5. Comparison of Giardia reproduction and α8-giardin localization before and after hammerhead ribozyme transfection

The growth curve shows that the density of PGCV-KH8 transfected cells reached a peak at 72 h after transfection, while the densities of both PGCV-GFP transfected and untransfected cells peaked at 48 h. After 48 h of cultivation, the wild type and GFP trophozoites peaked at 8.67 and 7.33 (GFP) times the initial concentration. In contrast, the PGCV-KH8 reached only 1.33 and 2.9 times its original concentration at 24 and 48 h after transfection, respectively, with a peak of a 3.1-fold increase at 72 h after transfection (Fig. 3B), indicating a delayed peak and reduced maximum density of organisms with the anti-α8-giardin knockdown.

Immunofluorescent staining with anti-α8-giardin was done with wild type and hammerhead ribozyme transfected cells to determine the effect of transfection with the α8-giardin knockdown on the intensity and localization of anti-α8-giardin staining.

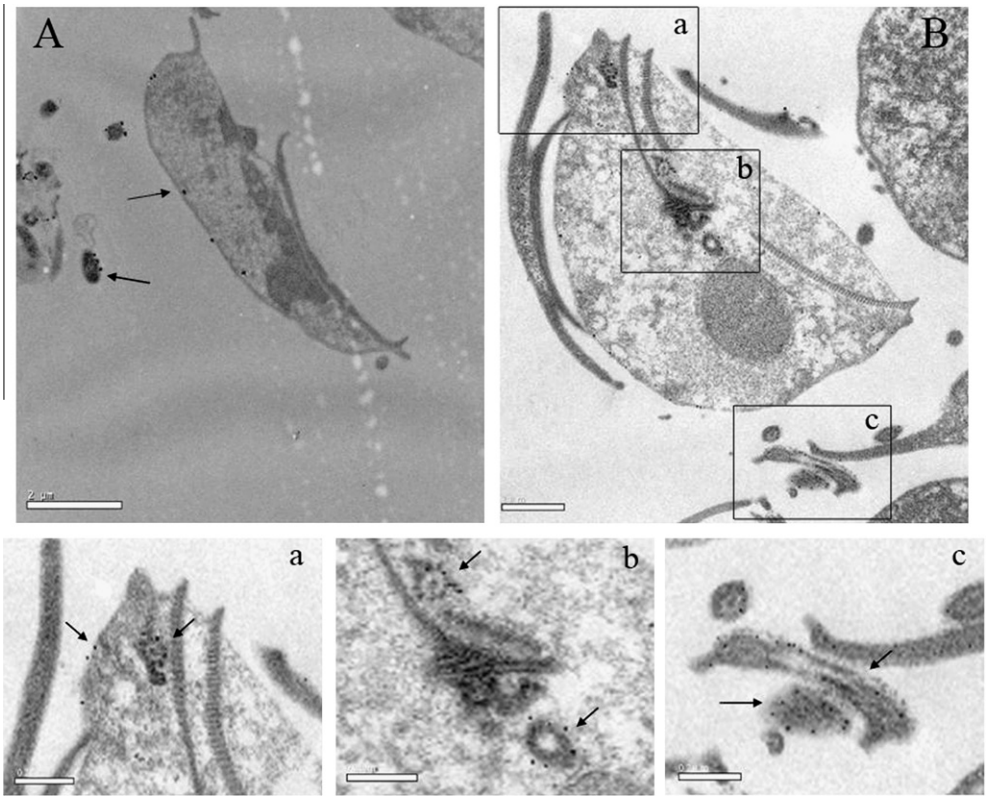

Fig. 5. Immunoelectron microscopy of α8-giardin demonstrates localization to the plasma and flagella in TEM sections. (A) Coronal section of a trophozoite shows gold particles arranged near the plasma membrane and on the flagella. (B) Coronal section and its magnification image (a–c). Gold particles are arranged near the plasma membrane, the base and outer face of the flagella.

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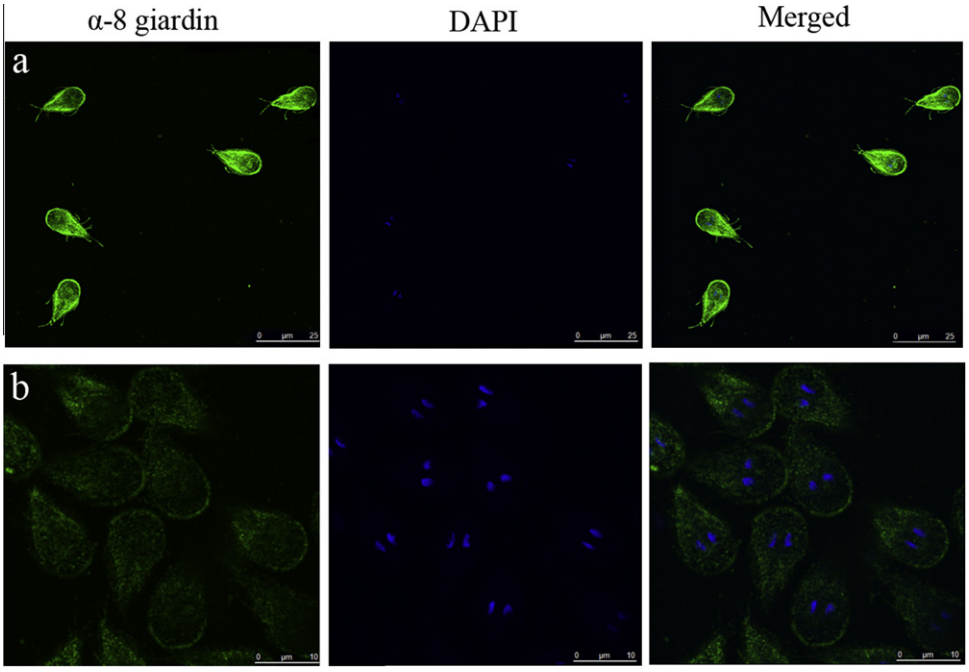

Fig. 6. Immunofluorescence staining intensity is reduced in hammerhead ribozyme transfected cells compared to wild type cells. (a) The average fluorescence intensity of the wild type cells is 95.57. (b) 24 h after transfection, the average fluorescence intensity is 35% of the wild type level.

The average fluorescence intensity of the cells with the α8-giardin knockdown was only 35% that of the wild type cells (Fig. 6). In addition, the membrane and flagellum fluorescence pattern changed markedly. The immunofluorescent stain is much stronger on the membrane and flagella of the control cells, while it is weaker on those of the transfected cells (Fig. 6).

4. Discussion

α-Giardins belong to a large class of annexin-like molecules that are located at the outer edges of the microribbons (Baker et al., 1988; Bauer et al., 1999; Weiland et al., 2005; Wenman et al., 1993). Several studies have shown that α-giardins are common immunoreactive proteins in Giardia (Char et al., 1991; Janoff et al., 1989; Taylor and Wenman, 1987; Wenman et al., 1993). Some studies have attempted to determine the intracellular localization of α-giardins using rabbit polyclonal antisera, but these antibodies were cross-reactive with other proteins (Nohria et al., 1992; Weiland et al., 2003). One approach used to solve the problem of cross-reactivity was the use of epitope tagged (AU-1) constructs (Weiland et al., 2005). This study demonstrated that most α-giardins localized to the plasma membrane and/or the flagella. A few α-giardins (α3-, 5- and 17-giardin) also localized to the adhesive disc. However, transfection experiments with constructs encoding α4, 7.1, 8 and 11 were lethal to the parasite. Therefore, we have used a combination of approaches to study the biologic role of α8-giardin and its cellular localization. First, in order to obtain specific antisera for immunolocalization studies, we used a bioinformatic approach to identify peptides that were most likely to be immunogenic as well as unique to α8-giardin by using algorithms that predict antigenicity, hydrophilicity, flexibility, surface probability and secondary structure. Through these analyses, we identified three candidate antisera. Western-blot results we concluded that the 296–306 aa region used to generate anti-P3 was the most specific. This region was from the long C-terminal extension, which is found in three members of the α-giardin family and is of unknown function. The sizes of the C-terminal extensions are 17 (α8-giardin), 26 (α14-giardin), and 77 amino acids (α19-giardin) (Pathuri et al., 2009). We then used anti-P3 for localization studies by confocal and electron microscopy. Both approaches demonstrated the localization of anti-α8-giardin antibodies to the flagellae and plasma membrane.

In order to study the importance of α8-giardin in Giardia growth, we utilized the GLV hammerhead ribozyme vector, which has been used successfully for a variety of knockdown studies of a variety of Giardia genes (Dan et al., 2000; Dan and Wang, 2000). This approach demonstrated the importance of α8-giardin in the growth and cytoskeletal structure of trophozoites. These studies also demonstrated the specificity of the α8-giardin immunolocalization studies in that localization studies done after a ribozyme knockdown demonstrated the disruption of the structures identified before transfection. Although the precise function of α8-giardin remains unknown, its specific localization to the plasma membrane and the flagella suggests the possibility of its involvement in vesicular transport, which would be similar to the functions of annexins in other organisms (Gerke and Moss, 2002). Alternatively, it may play a more specific flagellar function. (Elmendorf et al., 2003).

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