Biochemical and Biophysical Research Communications 524 (2020) 135–141

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ceramide synthase 2 knockdown suppresses trophozoite growth, migration, *in vitro* encystment and excystment of *Entamoeba invadens*

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ARTICLE INFO

Article history:
Received 16 December 2019
Accepted 14 January 2020
Available online 21 January 2020

Keywords:
Parasites
Gene silencing
Sphingolipids
PKC
Subcellular localization

ABSTRACT

Entamoeba invadens is the protozoan which causes multiple damages in reptiles and is considered a prototype for the study of the Entamoeba encystment/excystment *in vitro*. Here we report that EinCerS2 knockdown promoted decrease in sphingomyelin (SM) subspecies with long-chain fatty acids (24:0) down to 50% but increase sphingolipids with short-chain fatty acids (16:0) up to three times in both trophozoites and cysts of *E. invadens*. EinCerS2 silencing also resulted in decreased trophozoites' movement, proliferation, cysts formation, and trophozoites hatched after excystment. By immunofluorescence assays, a polyclonal antibody against EinCerS2 detected the enzyme in the cytoplasm of *E. invadens* trophozoites, colocalizing with Endoplasmic Reticulum-resident cognate EiSERCA. Interestingly, EinCerS2 was redistributed close to the plasma membrane during encystation, suggesting that the generation of diacylglycerol (DAG) via synthesis of sphingolipids and the activation protein kinase C might participate in the encystment process of *E. invadens*.

1. Introduction

Sphingolipids represent one of the major classes of eukaryotic lipids essential for cell membranes physiology [1]. Ceramide constitutes the hydrophobic backbone of all complex sphingolipids and structurally consists of a fatty acyl of variable chain length bound to an amino group of a sphingoid base typically, sphingosine. These lipids not only serve structural roles in biomembranes but they also have wide effects on signal transduction and the regulation of cellular functions [2].

Ceramide synthesis is initiated by serine palmitoyl-transferase. The resulting sphingoid base is then acylated by ceramide synthase (CerS), a family of six enzymes that produce an array of chemically diverse dihydroceramides from C14-28 [3]. All CerS conserve a TRAM-LAG-CLN8 domain, where LAG corresponds to the Lag1p motif which has been shown to be essential in the activity of such enzymes [4,5].

Entamoeba invadens, is a pathogenic species that causes an invasive disease in reptiles. This organism encysts efficiently *in vitro* [6], for this reason it has been used as a model system to study Entamoeba differentiation. Using this system, it has been shown that a number of processes including sphingolipids signaling are important during encystment [7].

It has been reported that the proliferation and growth of *E. invadens* requires the generation of diacylglycerol (DAG) via synthesis of sphingolipids [8] necessary for protein kinase C activation involved during encystment of *E. invadens* [7]. Recently we described that *E. invadens* possesses within its genome six loci coding for CerS corresponding to those described in higher eukaryotes. The genes that participate in the synthesis of sphingomyelins with long-chain fatty acids, mainly EinCerS2, play an important role during the encysting process. In addition, when the first enzyme of the biosynthesis of sphingolipids was inhibited *E. invadens* was not able to achieve its phase conversion [7].

Here we show that respective silencing and overexpression of EinCerS2 quantitatively altered the sphingomyelin species expressed in trophozoites and cysts of *E. invadens*. Correspondingly, whereas silencing decreased the proliferation rate, transwell migration, the number of cysts and excystment rate, EinCerS2 overexpression rendered opposite effects. EinCerS2 is recognized by the anti-EinCerS2 antibody raised here, and was detected in the

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https://doi.org/10.1016/j.bbrc.2020.01.093
0006-291X/

cytoplasm of trophozoites, co-localizing with EiSERCA, an endoplasmic reticulum-resident ATPase where ceramide biosynthesis takes place [9]. In cysts, however, it localized at specific sites close to the plasma membrane. These results suggest that CerS2 plays an important role in trophozoite growth and motility as well as encystation/excystation of *E. invadens*.

2. Materials and methods

2.1. E. invadens culture and encystation in vitro

E. invadens trophozoites (IP-1 strain) were axenically grown at 28 °C in TYI-S-33 medium supplemented with 10% bovine serum and harvested as described [10]. To induce encystation, 5×10^6 trophozoites were incubated in medium with its nutrients diluted to 50% [6]. To confirm cyst formation, cells were centrifuged at 1500 rpm for 5 min, the pellet was resuspended in 5 ml of 0.1% sarkosyl (Sigma-Aldrich) and incubated for 30 min at room temperature. Finally, the number of detergent-resistant cysts was determined using a Neubauer chamber and verified with Calcofluor White staining (Sigma-Aldrich). All experiments were initiated with cell suspensions obtained from cultures grown for 72 h.

2.2. PCR and relative-quantitative RT-PCR

Genomic DNA and total RNA were isolated from *E. invadens* trophozoites using the Wizard Genomic DNA purification kit (Promega) and TRIzol® reagent, respectively. cDNA was synthesized using SuperScript™III First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions. PCR amplifications were performed with DNA as template using Veriti™ Applied Biosystems™ Thermal Cycler. Relative-quantitative PCR was carried out using the FastStart DNA Master SYBR Green I kit (Roche Applied; Mannheim, Germany) and amplification was performed on a Real Time PCR CFX96™ (Bio-Rad). Expression values were determined by the $2^{-\Delta\Delta CT}$ method [11] normalized to RNA Pol 2 gene.

2.3. Generation of EinCerS2 knock down trophozoites

The first 606 pb from the 5' end of the EinCerS2 (EIN_046610) gene were PCR-amplified with 5' (CACCCTAGG**ATG**AGAACAACGAAAGTTCG; *Nhel* site underlined, start codon in bold) and 3' (CTAGCTAGCCGAAAAAGTCATCAAAAACACG; *Avrll* site underlined) primers flanked with appropriate sites to substitute the Myc gene in the pEi-ck-Myc plasmid. For EinCerS2 silencing, the PCR amplification was carried out with primers where the restriction sites were inverted (5′ *AvrII* and 3′ *NheI*) and the amplicon was cloned in the same vector as before. Constructs were verified by sequencing. 50 μg of the resulting plasmids, pEick-CerS2, pEick-asCerS2 or the empty vector were transfected into trophozoites through electroporation as described [12]. The transfected parasites were incubated at 28 °C, selected with 3 μg/ml of G-418 (Gibco™) and maintained as stable cell lines. Silencing of EinCerS2 was confirmed by RT-qPCR, Western blot and immunofluorescence assays.

2.4. Cloning and expression of CerS2 gene

The Lag1p motif-containing 606 pb-long fragment of the EinCerS2 gene was PCR amplified as before, except that for its directional cloning the sense primer contained the *BamHI* site before the start codon, and the antisense primer that included the *XhoI* site before the complementary sequence to the stop codon. To avoid mutations during amplification, PCR reaction was carried out using the high-fidelity enzyme KAPA HiFi DNA polymerase (KAPABIOSYSTEMS). Then, EinCerS2 gene was cloned into the pET-28a (+) vector (Novagen) using the *BamHI* and *XhoI* sites, in frame with the His-Tag encoding gene. For EinCerS2 expression, *Escherichia coli* (strain BL21 (DE3)) competent cells were transformed with the pET-EinCerS2 or empty vector used as a negative control. The recombinant protein was induced with 1 mM IPTG (Sigma-Aldrich) for 4 h at 37 °C. Recombinant protein was purified from bacteria lysates as described [13], continued for purification Ni-NTA sepharose affinity chromatography (ThermoFisher) following the manufacturer's recommendations. Induction of the recombinant protein and its purification was examined by SDS-PAGE.

2.5. Generation of α-EinCerS2 polyclonal antibodies

EinCerS2 (50 μg) emulsified in Titer-Max Gold adjuvant (50 μL) (Sigma, St. Louis, MO, USA) was subcutaneously and intramuscular inoculated into Balb/cJ male mice. Two more doses of EinCers2 (25 μg) were injected at 20 days intervals and then, animals were bled to obtain α-EinCerS2 antibody. Pre-immune serum was obtained before immunization.

2.6. Protein extraction and Western blot

For protein extraction, *E. invadens* trophozoites (2×10^6) were centrifuged at 360×g for 5 min and pellet was resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.01% Triton X-100) containing cOmplete™ protease inhibitor cocktail and 1 μM E64 (Sigma-Aldrich). Cell lysates were centrifuged, and the soluble fraction was collected, protein concentration was determined by Bradford method [14].

For Western blot assays, trophozoites lysates (20 μg) were separated in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), were transferred to nitrocellulose membranes (Amersham Hybond® ECL™) [15] and probed with mouse α-EinCerS2 (1:1000), or α-His (1:1000) (Sigma-Aldrich) antibodies. Membranes were washed, incubated with α-mouse HRP-labeled secondary antibody (1:10,000) (Invitrogen), finally reactions were developed by chemiluminescence with Western Lightning® Plus-ECL kit (PerkinElmer).

2.7. Laser confocal microscopy assays

The immunofluorescence assays were performed as previously reported [16]. Briefly, trophozoites were grown on coverslips, fixed with 3.7% paraformaldehyde (PFA) at 37 °C for 20 min, permeabilized with cold acetone for 2 min and blocked with PBS1X-albumin 1% at 37 °C for 1 h. Then, cells were incubated with either mouse α-EinCerS2 (1:100), or rabbit α-EinSERCA (1:50) [9] antibodies at 37 °C for 1 h, followed by incubation for 1 h with α-mouse Alexa Fluor™ 568 or α-rabbit Alexa Fluor™ 488 secondary antibodies (ThermoFisher; 1:100) as appropriate. Finally, nuclei were counterstained by with 4',6-diamidino-2-phenylindole (DAPI; ThermoFisher) and the samples were observed through a confocal microscope (Carl Zeiss LSM 700) using the ZEN 2009 software. To evaluate the co-localization between molecules, Pearson coefficients were obtained from at least 50 confocal independent images (laser sections 0.5 μm) using the ImageJ 1.45v software and the JACoP plugin [17].

The cyst samples were stained as follows. 5×10^6 cysts were fixed with 3.7% PFA for 1 h at room temperature in gentle constant rotation. Subsequently, cysts were centrifuged at 2500 rpm for 5 min at room temperature. The fixed cysts were resuspended in PBS and 3 cycles of freezing in liquid N2 were performed for 1 min and thawing at 37 °C for 2 min to fracture the cyst wall and allow access of the antibodies. Immediately cysts were permeabilized

with Triton X-100 0.1% for 5 min with gentle rotation. Then cysts were treated with the cold Methanol-Acetone mixture in a 1:1 ratio and incubated for 5 min at room temperature in gentle rotation. The cysts were then blocked with PBS 1X-Albumin for 1 h at room temperature and were incubated with the antibodies mentioned above, the samples were assembled, and their analysis was carried out as previously described. As a positive control, BODIPY FL C_5 was used a sphingolipid marker dye.

2.8. Lipid extraction and quantification

Lipids were extracted and quantified using the methanol stop procedure as previously reported [7].

2.9. Migration assays

Serum-starved (3 h) trophozoites (7.5×10^4) were placed in the higher chamber of transwell inserts (5 μm ø pore size, Costar) and 600 μl of adult bovine serum were added to the lower chamber. Trophozoites were then incubated for 3 h at 28 °C. At the end of the incubation, the inserts and the serum were removed, and trophozoite migration was determined by counting the number of trophozoites that were attached to the lower chamber of the well.

2.10. Statistical analysis

Values of all assays were expressed as mean ± standard error of at least three independent experiments. Statistical analyzes were performed using the GraphPad Prism V 6.0 software by Anova or Student's test.

2.11. Ethic Statements

The Institutional Animal Care and Use Committee (IACUC) ethics committee reviewed and approved the animal care and use of mice to produce antibodies (Protocol Number 0208-16) by the document CICUAL 001, in which is specified that our institute fulfils the NOM-062-ZOO-1999 that deals with the Technical Specifications for Production, Care and Use of Laboratory Animals given by the General Direction of Animal Health of the Minister of Agriculture (SAGARPA), that verify the fulfil of the international regulations/guidelines for the use and care of animals used in laboratory and has verified and approved the animal care at CINVESTAV (Verification Approval Number: BOO.02.03.02.01.908).

3. Results

3.1. CerS2 knockdown changes CerS2 mRNA levels in E. invadens

We previously showed that CerS2 is overexpressed in both stages of the life cycle of *E. invadens* [7]. To partially characterize EiCerS2, specific primers were designed to PCR-amplify a 606 bp fragment, corresponding to the amino-terminus of EinCerS2 harboring the Lag1p domain involved in Ceramide Synthase catalysis. The fragment was cloned into the *E. invadens* pEi-ck-Myc expression plasmid, both in sense and antisense for overexpression and silencing purposes (Fig. 1A). Sequencing of the fragment revealed an open reading frame without introns, 100% identical to the gene annotated in the AmoebaDB. After amoeba transformants were established, RT-PCR assays of evidenced a product of the expected size, indicating that EinCerS2 gene is transcribed (Fig. 1B). Intriguingly, RT-qPCR assays showed that overexpression (Oe) of EinCerS2 was three-fold higher compared to empty vector transfectants (V), whereas silencing of EinCerS2 (Si) resulted in only 50% reduction versus V transfectants (Fig. 1C).

3.2. CerS2 downregulation changed SMs species expression in both stages of the life cycle of E. invadens

Because CerS2 was the most abundant CerS in both *E. invadens* trophozoites and cysts [7], we expected that its downregulation would cause dramatic changes in sphingolipids. Mass spectrometry (ESI/SM) assays were used to analyze the qualitative and quantitative changes of the different SM subspecies in trophozoites and cysts of *E. invadens*. CerS2 knockdown decreased the main SM subspecies (24:0) down to 50% (*m*/*z* 815.9) versus control (Fig. 2A). Interestingly the SMs with short-chain fatty acids, mainly 703.9 (18:1/16:0) and 705 (18:0/16:0), respectively increased eight and three times compared to the subspecies of SM identified in the control trophozoites (Fig. 2A). Conversely, overexpression of EinCerS2 resulted in an increase of all the subspecies of SM that make up the membranes of *E. invadens*, mainly of SM 815.9 (18:1/24:0) with

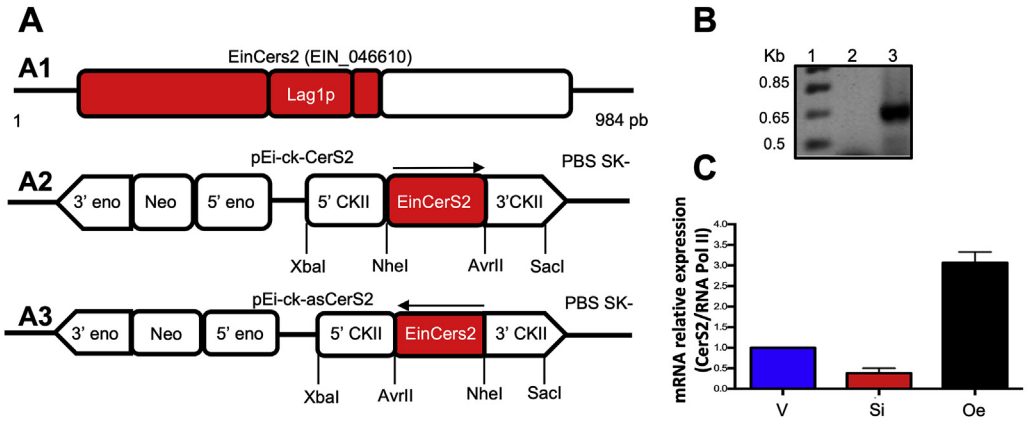

Fig. 1. EiCerS2 gene amplification (1–606) and constructs. (A) Diagram of the protein domains coded in the EinCerS2 gene, locus EIN_046610 (A1). The first 606 pb of the gene (in red) was amplified and used to substitute the Myc gene in the pEi-ck-Myc plasmid in the same (pEi-ck-EinCerS2; A2) and the opposite orientation (pEi-ck-asEinCerS2; A3) for overexpression and silencing purposes. **(B)** Amoeba transformants were established with each plasmid and transcription of the pEi-ck-EinCerS2 was verified by RT-PCR and analysis in 1% agarose gels. Lane 1, base pairs markers. Lane 2, minus RT PCR reaction. Lane 3, 606 bp CerS2 fragment. **(C)** CerS2 relative expression in amoeba transformants carrying empty vector (V), silencing (Si) and overexpression (Oe) plasmids was measured by RT-qPCR normalized to the RNA Polymerase ll gene expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

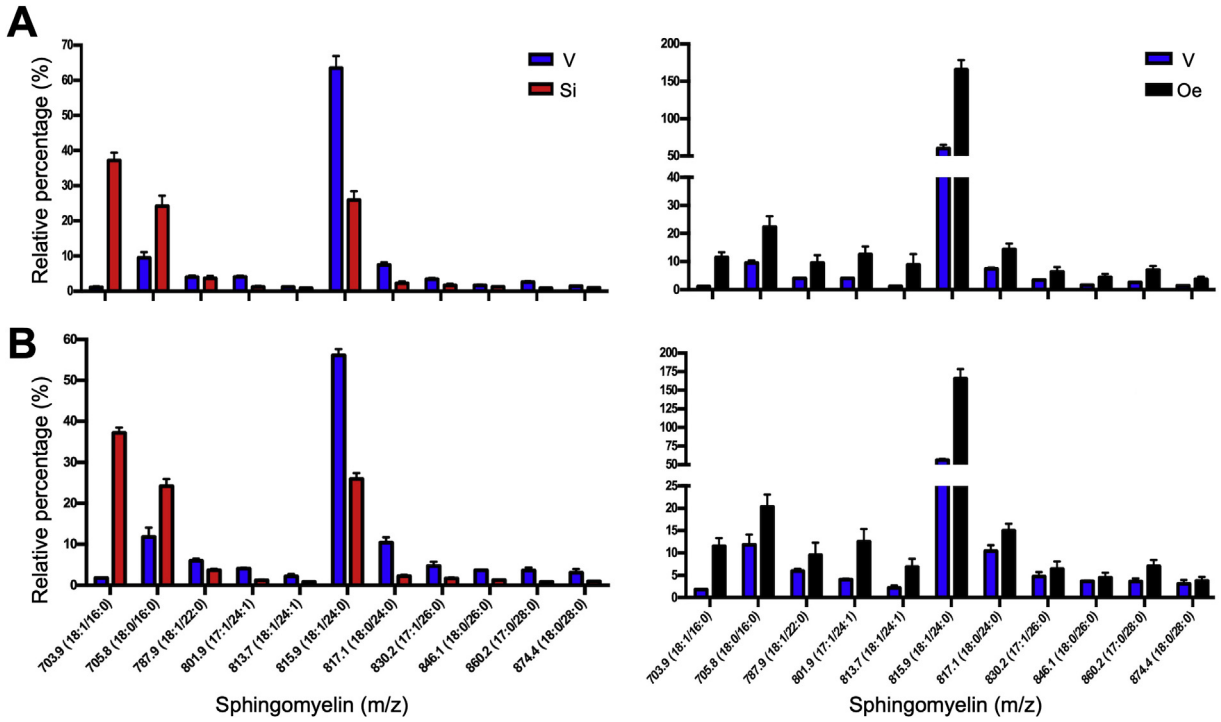

Fig. 2. Mass spectrometry analysis of the different species of SM present in trophozoites and cysts of *E. invadens* transformants. **(A)** The subspecies of SM were obtained by alkaline hydrolysis of 72 h cultures of *E. invadens* vector-transfected trophozoites. **(B)** Transfected trophozoites were incubated in encystment medium for 96 h and the subspecies of SM in cysts were obtained by alkaline hydrolysis. The data shown represent the mean ± the standard error of at least three independent experiments.

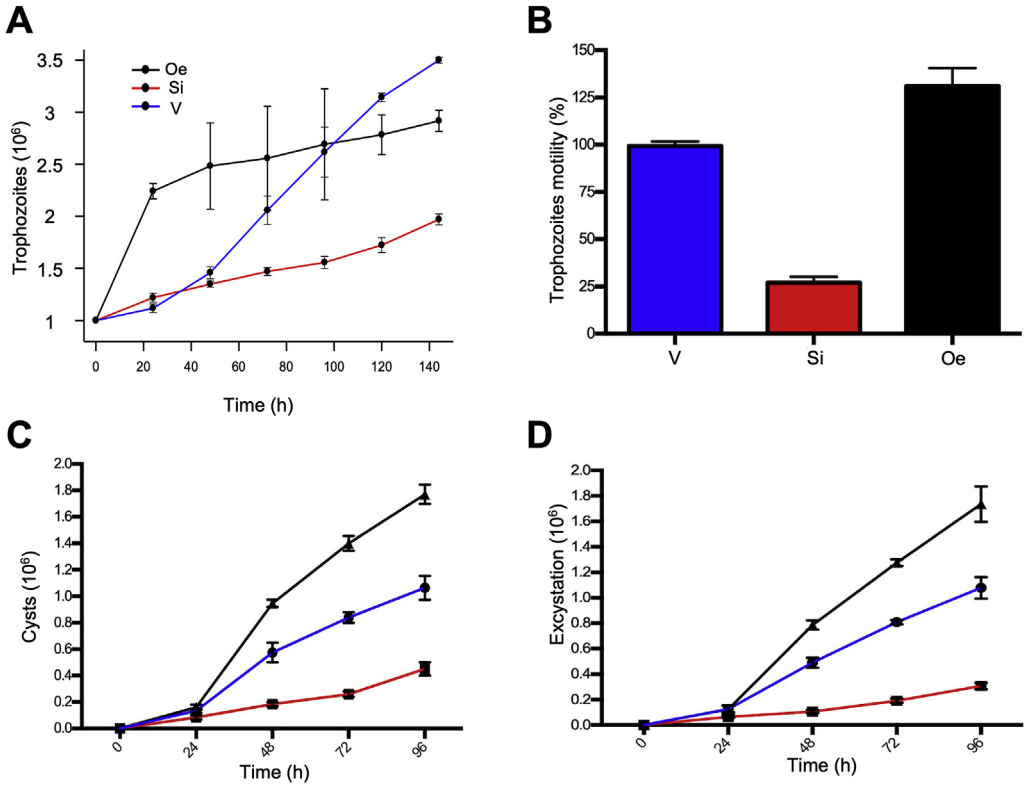

Fig. 3. Alteration in the proliferation, mobility, encystation and excystation of trophozoites of *E. invadens* due to the silencing of EinCerS2. Proliferation rate **(A)**, migration **(B)**, encystment **(C)** and excystment **(D)** of empty vector (V), EinCerS2 silenced (Si) and EinCerS2 overexpressor (Oe) amoeba transformants.

over 300% increase. The same trends were observed for the different SM in the cysts (Fig. 2B).

3.3. Knock down of EinCerS2 gene affects viability processes in E. invadens

We also analyzed the effect of EiCerS2 silencing and overexpression on proliferation. Both control trophozoites and EiCerS2 overexpressor trophozoites were able to proliferate two-fold faster than knockdown trophozoites (Fig. 3A).

To evaluate the importance of EinCerS2 protein on motility, we placed the transfected trophozoites (V, Si, Oe) in the upper chamber of transwell inserts and counted the number of trophozoites that were able to move toward the lower chamber, following a serum stimulus. Interestingly, after 3 h incubation, knockdown EinCerS2 trophozoites remained in the upper chamber, showing low migration capacity, whereas trophozoites with CerS2 overexpression showed higher motility than the control (Fig. 3B).

To confirm the role of EinCerS2 on the *in vitro* encystation and excystation of *E. invadens,* we tested the effect of silencing of EinCerS2 gene in these processes. The knockdown trophozoites reached 4.5 times less encystment in comparison with trophozoites under control conditions. In the case of trophozoites with the CerS2 overexpression, after 96 h, the number of encysted cells was 0.8 times more versus control trophozoites (Fig. 3C). On the other hand, the number of cysts hatched reached was higher in control and overexpression conditions compared to knockdown cysts (Fig. 3D).

3.4. Differential localization of EinCerS2 in trophozoites and cysts

We generated a recombinant (r) EinCerS2 to raise antibodies against it, and to initiate the characterization of EinCers2. As expected from the *E. invadens* genome database (http://www.amoebadb.org), the antibodies detected a single band of approximately 41 kDa (Fig. 4A), which was not detected by the preimmune serum. The purified rEinCerS2 was analyzed in SDS-PAGE gels (Fig. S1). His-Tag detection showed bands of approximately 19 kDa, the expected molecular weight for rEinCerS2 (16 kDa of EinCerS2 and 3 kDa of the His-Tag) (Fig. 4A). Specificity of α-EinCerS2 antibody was tested by Western blot assays on membranes containing total extracts of trophozoites using purified rEinCerS2 as control (Fig. 4B). Densitometric analyses of the gels in 4B show that EinCerS2 expression corresponded to empty vector, overexpressor, and silenced transformants, respectively (Fig. 4C). In mammalian cells, CerS is located in the endoplasmic reticulum (ER) from where they distribute to different organelles. Confocal images of trophozoites in steady state conditions showed that EinCerS2 co-localized with EiSERCA throughout the cytoplasm (Fig. 4D), a possible analogous site equivalent to an ER in this parasite. On the other hand, in knockdown trophozoites a decrease of EinCerS2 signals was observed, along with EiSERCA signals (Fig. 4D). In the case of cysts EinCerS2 was located at specific sites close to the plasma membrane (Fig. 4D). In addition, sphingolipids were identified using BODIPY FL C_5 (Fig. 4E), and as a negative control for the α-EinCerS2 antibody, preimmune serum plus DAPI were used (Fig. 4F).

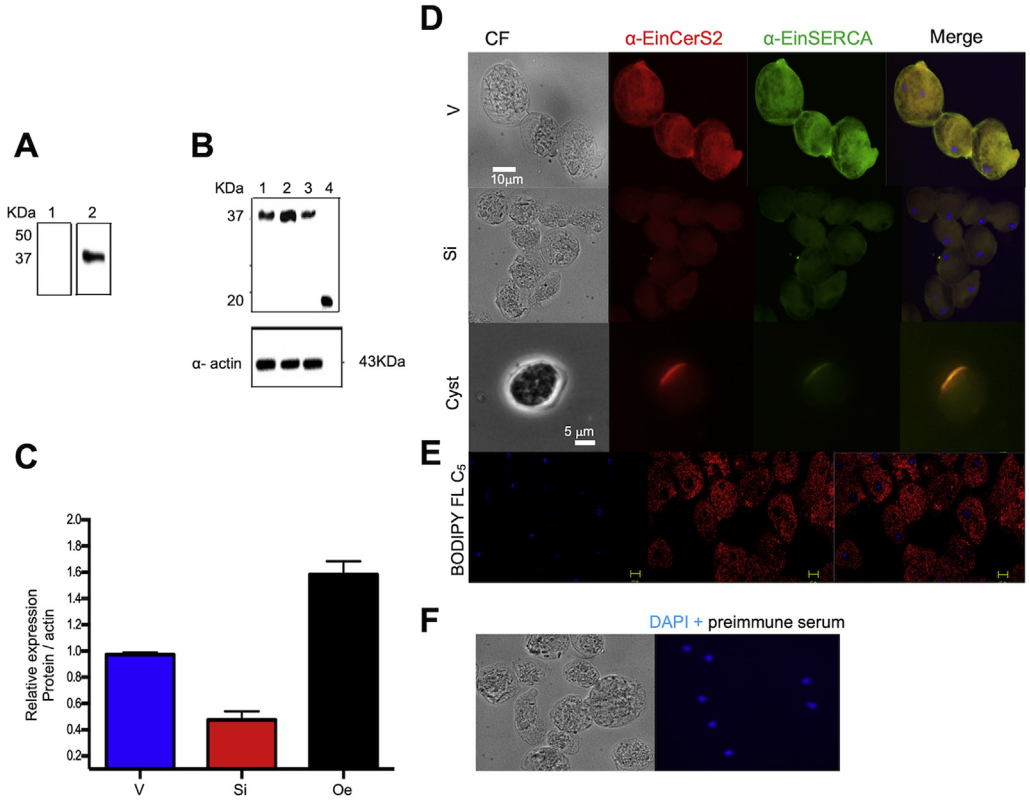

Fig. 4. Immunodetection of EinCerS2 and EinSERCA in trophozoites and cysts of *E. invadens*. **(A)** Protein extracts of *E. invadens* trophozoites were used in Western blot assays. Lane 1: proteins detected by pre-immune serum. Lane 2: anti-EinCerS2 antibody. **(B)** Protein extracts of trophozoites transfected with empty vector (V; lane 1), pEi-ck-EinCerS2 (Oe; lane 2), pEi-ck-asEinCerS2 (Si; lane 3), and purified rEinCerS2 were used in western blots probed with the anti-EinCerS2 antibody. Blots were stripped and re-probed with an anti-actin antibody. **(C)** Densitometric analysis of the bands observed in B normalized against actin. Data are presented as the mean ± the standard error. **(D)** Wild-type and EinCerS2-silenced trophozoites and cysts were fixed on coverslips and labeled with the α- EinCerS2 (red) and α-EinSERCA (green), CF = phase contrast, DAPI (blue) antibodies, for analysis by confocal microscopy. Negative control, using pre-immune serum and secondary antibody Pearson's correlation coefficient = 0.98. **(E)** Trophozoites of *E. invadens* were marked with BODIPY dye as positive control. **(F)** Pre-immune and DAPI incubation of wild type trophozoites. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Encystation of Entamoeba is crucial for transmission of amoebiasis, therefore impairing the ability of the trophozoites to form cysts can block the spread of this fatal disease. With the limited knowledge in the field of encystation biology, earlier reports attempted to characterize the biomolecules that form the cyst wall [18–23], but the underlying signaling mechanisms remain unveiled. Hence, there is an urgent need to identify novel encystation specific signaling molecules and decipher the signaling cascades involved. Recently we reported that synthesis of sphingolipids is required for *E. invadens* encystment. In addition, the suppression of DAG source impacts the processes in which PKC intervenes, among them cellular proliferation, protein phosphorylation, and encystment of *E. invadens* [7].

It has been reported that inactivation of CerS2 catalytic activity in mice affects transcription of genes involved in lipid metabolism and cell division [24]. In this study, we demonstrated that CerS2 knockdown resulted in specific reduction of an individual CerS expression. An increase in SM with short-chain fatty acids in response to CerS2 silencing has been reported *in vitro* [25] and *in vivo* [24] assays, suggesting a complex cross-regulation between the different CerS thus intricately regulating sphingolipids biosynthesis. Silencing of CerS2 negatively impacted trophozoites proliferation and motility, probably due to membrane neoformation, as well as encystment and excystment. These results suggest that the SM with long-chain fatty acids are necessary for these vital processes to take place.

The generation of a polyclonal antibody against EiCerS2 allowed us to suggest that the main organelles where sphingolipid synthesis is carried out are distributed in the cytoplasm of the parasite, which are redistributed during encystment. The location of EinCerS2 in the cytoplasm correlated with a SERCA-type calcium ATPase [9], suggesting that both proteins are resident of the Endoplasmic Reticulum. Interestingly, SERCA signals were diminished in EinCerS2 silenced trophozoites, suggesting that the second messengers generated by sphingolipid biosynthesis (ceramide and/or sphingosine 1-phosphate) could also regulate the expression of SERCA in *E. invadens*.

We previously showed that myriocin impaired *E. invadens* proliferation and encystment. Both functions were reconstituted by the addition with D-erythrosphingosine, a metabolite that is not synthesized in the presence of the inhibitor, indicating that the production of DAG via sphingolipid synthesis is essential for the activation of PKC involved in these processes [7]. Recently it was described that the activity of Ezrin, Radixin and Moesin (ERM) family of proteins is regulated by sphingolipid phosphorylation [26]. The structural ERM proteins are able to bind both to the actin cytoskeleton and the plasma membrane conforming a scaffold for signaling pathways that are used for cell proliferation, migration, cell division [26]. Sphingolipid regulation of ERM function must play an important role in the rearrangement of the actin cytoskeleton during *E. invadens* encystment. In addition, an encystment-specific PKC has been described which transcriptionally regulate chitin synthesis pathway and glycogen phosphorylase genes [27]. We postulate that such PCK could be activated by the DAG produced during *de novo* synthesis of sphingolipids thus regulating chitin synthesis.

We conclude that altogether, our results demonstrate that *E. invadens* possesses the Ceramide Synthases and accompanying enzymatic machinery required to carry out the synthesis of sphingolipids, especially long-chain fatty acids SMs essential for growth, proliferation, encystment and excystation of *E. invadens*.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgments

We are grateful to I.B.T. Lorena Ramirez-Reyes (Unidad de Proteómica, Genómica y Metabolómica, CINVESTAV-IPN) for SM analysis, to PhD Upinder Singh, and PhD Gretchen Ehrenkaufer for kindly providing the pEi-CK-myc plasmid. This work was supported by scholarships 261717 to J.M.J.W. and 278075 to R.A.G. and grants 127557-M and 236104 to J.V. from Consejo Nacional de Ciencia y Tecnología, México.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.01.093.

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