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De novo synthesis of sphingolipids plays an important role during in vitro encystment of Entamoeba invadens

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ABSTRACT

Entamoeba invadens is a protozoan, which causes multiple damages in reptiles and is considered a prototype for the study of the Entamoeba encystment in vitro. Here we report for the first time the role of the *de novo* synthesis pathway of sphingolipids during the encystment of *E. invadens*. *In silico* analysis showed that this parasite has six putative genes coding for ceramide synthases (CerS), all of them coding for proteins containing the Lag1p motif, a region conserved in the ceramide synthases of multiple organisms, suggesting that they might be *bona fide* CerS. The six genes of *E. invadens* are differentially expressed at different time intervals in both stages trophozoite and cyst, based on the results obtained through qRT-PCR assays, the genes involved in the synthesis of sphingolipids with long-chain fatty acids CerS 2,3,4 (EIN_046610, EIN_097030, EIN_130350) have maximum points of relative expression in both stages of the *E. invadens* life cycle, which strongly suggest that the signaling exerted from the synthesis pathway of sphingolipids is essential for the encystment of *E. invadens*, since the generation of the more abundant sphingomyelin (SM) subspecies with long-chain fatty acids are fundamental for the parasite to reach its conversion from trophozoite to cyst. When myriocin was used as an inhibitor of serine palmitoyl CoA transferase (SPT), first enzyme in the *de novo* biosynthesis of sphingolipids, the trophozoites of *E. invadens* were unable to reach the encystment. Since the effect of myriocin was reversed with exogenous D-erythrosphingosine (DHS), it was demonstrated that the inhibition was specific and it was confirmed that the synthesis of sphingolipids play an essential role during the encystment process of *E. invadens*.

1. Introduction

Entamoeba invadens causes amebiasis in reptiles that result in nearly absolute mortality [1]. The two forms in its life cycle, trophozoite and cyst, are morphologically similar to the human parasite *Entamoeba histolytica*, responsible for dysentery and liver abscess. *E. invadens* encystment is readily induced in vitro in response to nutrient reduction [2], for this reason it has been used as model system to study encystation for the genus *Entamoeba.*

The encystation process of *E. invadens* involves continuous changes of the mobile trophozoite, continued by the synthesis and formation of a cyst wall (chitin fibril, a small set of chitin-binding lectins: Jacob, Jessie and chitinase) [3], which protects the encysted cell from adverse environmental conditions outside the host. Previously, it was reported that the encystment of *E. invadens* undergoes the rearrangement of the cytoskeleton of actin playing a decisive role in the determination of changes in cell morphology and helping with the transport of cell wall components to the cell surface during encystation process [4].

It has been reported that the growth and proliferation of *E. invadens* requires the generation of diacylglycerol (DAG) via synthesis of sphingolipids [5]. DAG activates protein kinase C (PCK), necessary for transit through cell cycle [6,7]. Myriocin-mediated inhibition of this pathway affects the proliferation of the parasite [5]. Also, it was reported that inhibiting DAG-dependent PKCs affects the growth and encystment of *E. invadens* [8], suggesting that PKC plays a very important role in both stages of the protozoan life cycle. To date, however, the participation of the sphingolipid

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synthesis pathway in the encystment process of *E. invadens* remains unknown, since only a few studies have focused on the identification of the sphingolipids that make up the membranes of the genus Entamoeba [9,10].

Because the role of PKC in encystment depends on the DAG generated in sphingolipids biosynthesis, and such synthesis pathway is carried out in organelles apparently absent in *E. invadens* (Endoplasmic Reticulum, Golgi Apparatus), the aim of the present work was to link the first step of ceramide synthesis and encystment using the specific inhibitor of SPT myriocin, the first committed step in sphingolipid synthesis. Encystment-induced *E. invadens* cultures treated with myriocin decreased the number of cysts, and encystment was recovered upon addition of D-erithrosphingosine. *In silico* identification and in vitro partial characterization of the genes that code for the putative enzymes involved in the synthesis of sphingolipids, particularly ceramide synthases (CerS) showed that putative CerS2 (EIN_046610) is more abundantly expressed both in trophozoites and during encystment. In addition, by mass spectrometry, we identified the pattern of sphingomyelins (SM) at both stages of the cycle of *E. invadens*. We observed that putative CerS2 might impact the SM expression differences observed between the two cell stages. These results suggest that sphingolipid synthesis plays an important role in *Entamoeba* encystation.

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 [11]. To induce encystation, 1×10^6 trophozoites harvested at 72 h, were resuspended in medium with its nutrients diluted to 50% [2]. Then, cells were collected at 24, 48 and 72 h after exposure to encystation medium. To confirm cyst formation, cells were centrifuged at 1500 rpm for 5 min, the pellet was resuspended in 1 ml of 0.1% sarkosyl and incubated for 30 min at room temperature. Finally, the number of detergent-resistant cyst was determined using a hemocytometer and verified with Calcofluor White dye. All experiments were initiated with cell suspensions obtained from cultures grown for 72 h.

2.2. In silico identification and characterization of E. invadens CerS

Homo sapiens CerS2 (access number Q96G23) and *S. cerevisiae* (access number P38703) protein sequences retrieved from the Uniprot database (http://www.uniprot.org) were used as query to search putative *E. invadens* EiCerS1-6 proteins and their orthologues. Predicted proteins from the EIN_046610, EIN_097030, EIN_130350, EIN_194360, EIN_429040, EIN_222110 gene sequences of *E. invadens* (http://amoebadb.org) were characterized *in silico* using the software deposited in the Expasy Bioinformatics Resource Portal (http://expasy.org). 3D structure modeling the amino acid sequences of EiCerS1-6 were analyzed using the RaptorX server (http://raptorx.uchicago.edu/) and topology were obtained via the Protter (http://wlab.ethz.ch/protter/start/). Phylogenetic analysis was performed using UPGMA implemented in the MEGA 5.05 software package. Bootstrapping was performed for 1000 replicates.

2.3. Relative-quantitative RT-PCR

Cellular RNA was isolated with TRIzol® from the cellular cultures at different time intervals during the trophozoite stage and encystation. Two micrograms of total RNA were transcribed into cDNA using SuperScript™III First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions. 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 LightCycler® II Carousel-Based System. Specific primers for each gene are listed in Table S1. Expression values were determined by the $2^{-\Delta\Delta CT}$ method [12] with RNA Pol 2 gene as the normalizing factor.

2.4. Lipid extraction

Lipids were extracted using the methanol stop procedure as previously reported [13]. In brief, the cell pellets (trophozoites or cyst) suspended in methanol were heated at 55 °C during 20 min, after cooling the tubes at room temperature, chloroform was added 2/1 methanol (v/v). Subsequently the samples were subjected to alkaline hydrolysis (0.1 M KOH in methanol) for 2 h on ice and neutralized with glacial acetic acid to remove glycerophospholipids. Sphingolipids were extracted with chloroform/methanol (2/1) and dried under nitrogen flow. To normalize the samples, the nmoles of Pi present in the chloroform/methanol phase (phospholipids phosphorous) were determined by the method of Ames and Dubin [14], and samples were subjected to mass spectrometry analysis in positive mode, using apparatus ESI ionization 3200 Q-TRAP LC/MS/MS AB SYSTEM. A scan of precursors for *m/z* 184 was used to detect the subspecies of SM and scans of the precursors for *m/z* 264 (d18:1 backbone) and 266 (d18:0 backbone) collision energies were performed (DP 70, EP 10, CE 50, CXP 3.3). The results were analyzed following the methods previously described [15].

2.5. Statistical analysis

Data were analyzed with SigmaPlot (Systat Software Inc., San Jose, CA, USA), and data were expressed as the mean ± SE of at least three experiments. Statistical analysis was performed with Student's *t*-test. $P < 0.03$ was considered statistically significant.

3. Results

3.1. The effect of myriocin and D-erithrosphingosine on the encystment of E. invadens

To analyze the importance of biosynthesis of sphingolipids on cyst formation, the effect of myriocin an inhibitor of SPT [16], the first enzyme of biosynthesis of sphingolipids was analyzed. We found that a concentration of 1.5 μM of inhibitor caused 90% reduction of encystment (Fig. 1A), and the addition of 2.5 μM D-erithrosphingosine to the encystment medium restored encystment to an 85% (Fig. 1B), demonstrating that SPT inhibition was specific. Higher concentrations of D-erithrosphingosine were less efficient in encystment restoration, in agreement with reports showing that exogenously added sphingoid bases inhibit PKC [17]. The importance of PKC during encystation was demonstrated when employing specific inhibitors to this enzyme (staurosporine, chelerythrine, calphostin, sphingosine), which inhibit the encystment process [8].

3.2. Six putative ceramide synthase genes in E. invadens

Our analysis of Amoeba DB (http://amoebadb.org/), revealed the presence of six putative CerS genes, three of them annotated as putative longevity assurance factors (EIN_046610, EIN_097030, and EIN_222110) and the remaining three annotated as putative ASC1 proteins (EIN_194360, EIN_429040, and EIN_130350). They

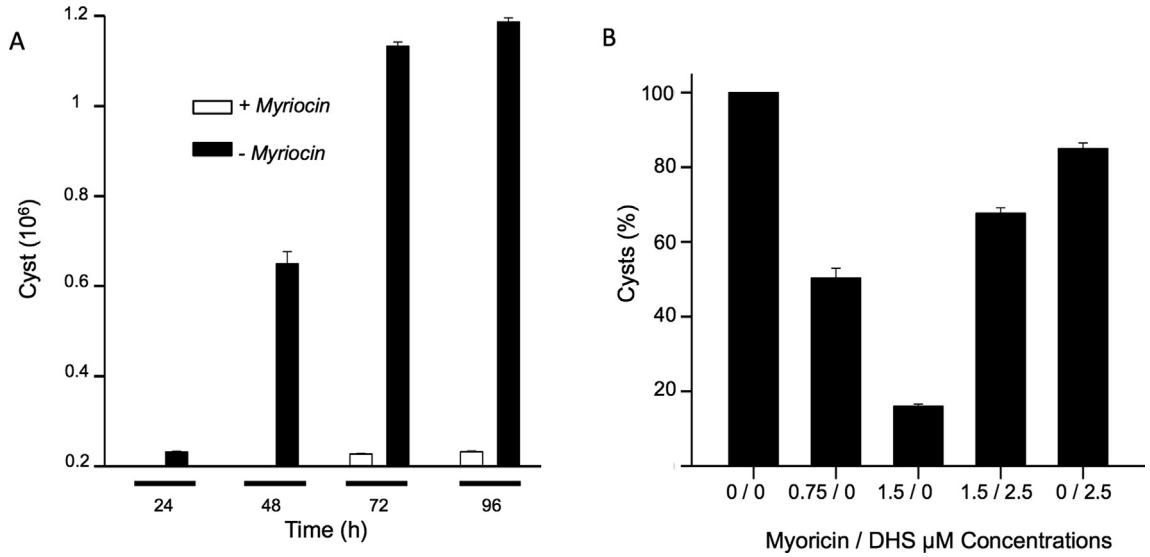

Fig. 1. Myriocin-induced sphingolipid synthesis inhibition affects *E. invadens* encystment. (**A**) Effect of myriocin on *E. invadens* encystment. *E. invadens* cultures were induced to encystment by incubation in diamond medium diluted to 50% at 28 °C, in the absence (black bars) or the presence of 1.5 µM myriocin (white bars). (**B**) D-Erythrosphingosine restores proliferation of myriocin-inhibited amoebas. *E. invadens* cells were seeded at a density of 2×10^3 cells/ml in Diamond's medium in the absence (control 0/0) or presence of myriocin (0.75–1.5 µM) plus D-erythrosphingosine (0–2.5 µM). Every 24 h, cells were counted in a hemocytometer. The data shows the mean ± the standard error or three experiments.

conserve protein sequence similarity and identity with respect to human and *S. cerevisiae* orthologs (Tables S3A–B) and show strong similarity (39.5–57.4%) among them (Table S2). The six putative *E. invadens* CerS proteins (EinCerS1-6) (Fig. 2B) contain a classic TLC domain (Tram, Lag1p, CLN8), which have been described in humans, yeasts, and worms; within the TLC domain, the Lag1p motif includes two contiguous His residues involved in CerS activity [18,19].

According to the predictions obtained from the analysis *in silico* of EinCerS1-6, these proteins possess sites of *N*-myristoylation, *N*-glycosylation, sites of union ATP/GTP and multiple sites of phosphorylation through PKC, CKII and TyrK (Table S4) which could be regulating the activity of these enzymes. Also, similar to the human CerS1 ortholog, all *E. invadens* CerS lack the HOX domain at their NH-terminus; such domain might have a structural function since it has been ruled out as necessary for CerS activity [20].

The full length amino acids sequences of each one of the six EinCerS were aligned with the CerS from multiple organism to obtain a phylogenetic tree using the Mega 5.05 software. Interestingly the six isoforms of EinCerS and the five isoforms of the EhiCerS were grouped closer to each other and far from the CerS from human, yeasts, plants, insects, worms, and other protozoans such as *Leishmania major* y *Trypanosoma cruzi*, that appear to have a more recent descent (Fig. 2A).

In order to obtain some evidence about the structures of each one of the six isoforms of EinCerS1-6, their 3D models were obtained using RaptorX software.

The predicted 3D structures are conformed mostly by alpha helices and 5–8 transmembrane passes (tp), in this way those of even tp number their NH_2 and COOH terminal ends remain oriented towards the same side of the membrane, for example 8 tp include loci EIN_046610, EIN_097030 and EIN_429040; or in opposite directions in odd number such as 5 or 7 tp including loci EIN_194360, EIN_130350 and EIN_222110. Interestingly, in all cases, the COOH terminal is oriented to the inner side of the membrane of the possible Endoplastic Reticulum. The topology was confirmed using the Protter software (Fig. S1). In all EinCerS, however, the last amino acids of the Lag1p motif face the cytoplasm. A local alignment among the CLN8 motifs of the CerS *H. sapiens* and *E. invadens* allowed us to determine the possible region probably involved in acyl chain selectivity in this parasite. Except EIN_097030, the aforementioned regions are topologically located in the last loop of amoebic proteins (Fig. S1).

3.3. The genes EinCerS1-6 are expressed in both cell stages

In order to confirm the *bona fide* expression of the putative genes identified *in silico*, specific oligos were designed (Table S1) to perform RT-qPCR assays. We observed that during the trophozoite stage, comprising 72 h incubation period (Fig. 3A, continuous line), all six genes display maximum expression in the first 6–24 h of incubation, indicating that the main synthesis of sphingolipids occurs early in trophozoite cultures. Interestingly, EinCerS6 (EIN_194360) has a bimodal expression with an additional expression 24 h later. The process of encystment (Fig. 3A, discontinuous line), comprises the 96 h of postinduction. EinCerS2 (EIN_046610), EinCerS3 (EIN_130350), and EinCerS4 (EIN_097030) reached of maximum expression 48 h postinduction, suggesting that *de novo* sphingolipids biosynthesis is necessary for parasites to reach cyst conversion. Interestingly, SM differential expression was observed between cell stages (see next section).

3.4. Identification of the different subspecies of SM in both stages of the life cycle of E. invadens

Qualitative and quantitative comparisons of the different SM subspecies in both stages of the life cycle of *E. invadens* were made through mass spectrometry (ESI/SM). We observed a wide distribution of SM, from those with fatty acids of 16 carbon atoms, that correspond the mass (*m*/*z*) 703 and 705 (representing 2–10% of the total mass), to those SM with fatty acids of 28 carbon atoms (also amounting 2% of the total). The main SM subspecies possesses a *m*/*z* of 815.9 with fatty acid of 24 carbon atoms (amounting 60% of the total) (Fig. 3B). Considering all of those subspecies that possess a fatty acid with of 24 carbon atoms as a whole, they amount to 80% of the total SM. This distribution of the different SM is maintained in the cyst stage (Fig. 3B). Interestingly, the sphingomyelin pattern in higher eukaryotes is dominated by sphingomyelin subspecies

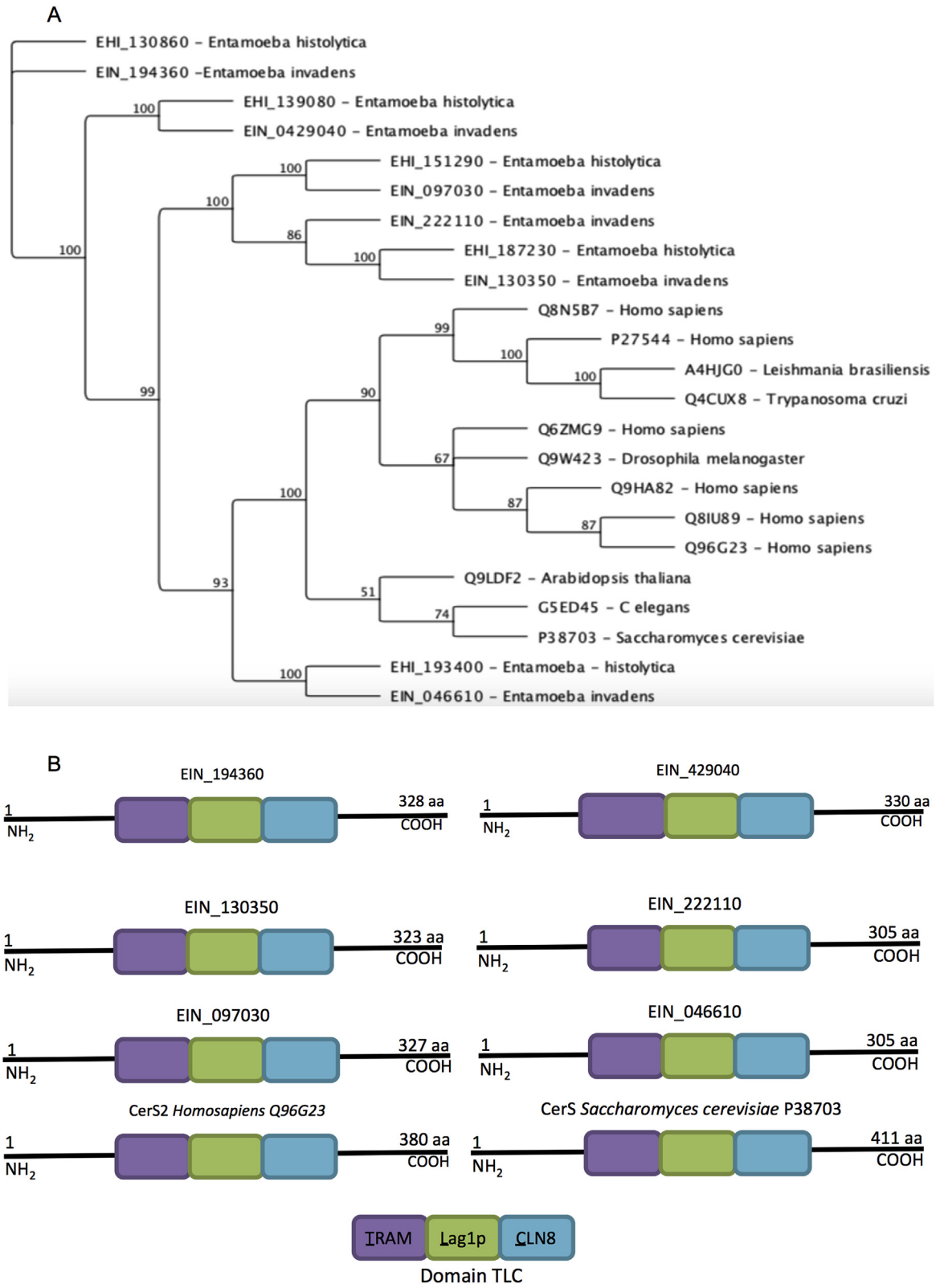

Fig. 2. Phylogenetic tree and structural domains of EinCerS1-6. (A) Phylogenetic tree indicating the position of *E. invadens* CerS proteins among different species. Numbers on horizontal lines in the trees indicate the confidence percentages of the tree topology from bootstrap analysis of 1000 replicates. (B) Schematic representation of the main structural *E. invadens* CerS domains and its *H. sapiens* and *S. cerevisiae* orthologues. TRAM-Lag1p-CLN8 (TLC) domains are related with the synthesis of ceramide. Numbers to the right correspond to the amino acid length of the proteins.

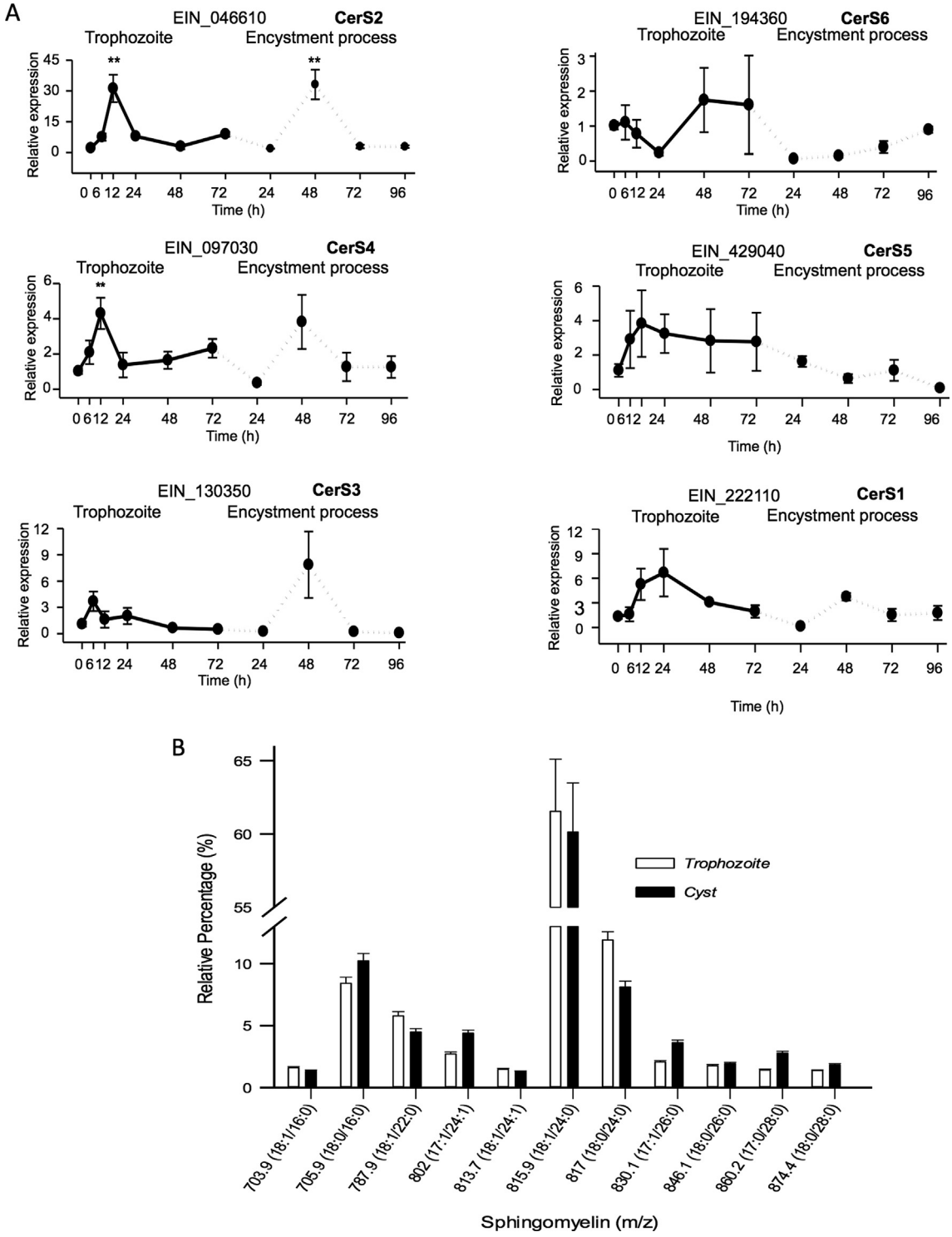

Fig. 3. Expression kinetics of EinCerS 1–6 genes products and sphingomyelin subspecies in *E. invadens* trophozoites and cysts. (**A**) *E. invadens* trophozoites cultures and encystment induced cells were collected at different time intervals and gene expression was evaluated by qRT-PCR. The data are presented as the mean ± the standard error. **P < 0.03 compared to the housekeeping transcript control. (**B**) SM subspecies were obtained by alkaline hydrolysis of total phospholipids from 72 h-long trophozoite cultures (white bars), and 96 h-encystment induced cells (black bars). The data show the mean ± the standard error of each SM of at least three experiments.

with short-chain fatty acids, whereas in *E. invadens* sphingomyelins with long-chain fatty acids prevail, this should be associated with the peculiar plasticity, osmotic shock resistance and locomotion of this parasite. Surprisingly, we observed that *E. invadens* is able to synthesize subspecies of sphingomyelin with sphingoid bases of 17 carbon atoms (*m*/*z* 802, 830 and 860), similar to human cells where sphingosine or sphinganine of 18 carbon atoms also predominates [21].

4. Discussion

It has been demonstrated that PKC activation is carried out by

DAG, which in turn results from degradation of glycerophospholipids by their corresponding phospholipases [22]. However, an alternative and perhaps more important source of DAG, that results from the synthesis of sphingolipids, has also been considered for PKC activation [5–7].

Cerbon and co-workers [5–7] demonstrated that synthesis of sphingolipids occurs during the first hours of incubation, resulting in major DAG production which results in highest activation of PKC αβ, required for G1 to S cell cycle transit, and proliferation. Here we found that synthesis of sphingolipids is also required for *E. invadens* encystment, since the addition of myriocin blocked the process, and further addition of D-erythrosphingosine restored encystment. This shows that *de novo* SM biosynthesis is key during *E. invadens* encystment.

Some protozoa mainly make up their membranes inositol phosphoryl ceramide (IPC) species [23]. In contrast, *E. invadens* produces sphingomyelin as the primary sphingolipid complex via CerS. We identified the SM subspecies characteristic of trophozoites and cysts of *E. invadens*. Surprisingly in both cell stages the same species of SM predominate; with fatty acids of variable length and insaturations, that might account for the membrane plasticity and locomotion which are so peculiar to the parasite. *E. invadens* possess a wide distribution of SM (C16-C28). In higher eukaryotes such distribution is achieved by six ceramide synthases CerS1-6 [24]. Searching AmoebaDB we identified six putative CerS genes that appear to have early descent. Notably all CerS putative gene products conserve the TLC domain, including the catalytic Lag1p motif [18]. Interestingly, the eleven amino acids-long fatty acid-affinity motif CLN8 [25] is absent in all six CerS of *E. invadens*. Altogether, our results show that this organism possesses the enzymatic machinery necessary to generate the different subspecies of SM.

The qRT-PCR expression assays of the six CerS genes showed a different expression profile for each one of the genes at the different time intervals in both stages of the protozoan life cycle, suggesting that participation of sphingolipids is fundamental during *E. invadens* proliferation [5], and encystment.

Until now, a great relevance has been given PKC activation by DAG originated from the hydrolysis of glycerophospholipids. However, much less attention has been given to such activation by DAG originated from sphingolipids biosynthesis. This work, along with our previous report [5], show that the suppression of the later source of DAG affects the processes in which PKC intervenes, among them cellular proliferation, protein phosphorylation, and in this case, the encystment of *E. invadens*. In spite of this underestimation of PKC activation by sphingolipids synthesis-driven DAG, previous reports show that such source of DAG is not exclusive of this parasite, for example, it has been demonstrated that the synthesis of sphingolipids is necessary for the encystment of *G. lamblia* [26]. In addition, previous works carried out in MDCK cells [[6], yeast [7], and rat uterus epithelial cells [27] demonstrated the participation of sphingolipid biosynthesis-driven DAG in PKC activation.

Altogether our results demonstrate that synthesis of sphingolipids is required for *E. invadens* encystment. Considering the scarce evidence on the presence of the organelles involved the biosynthesis of sphingolipids, namely Endoplasmatic Reticulum and Golgi Apparatus in the genus Entamoeba [28], it remains to identify the subcellular compartments in which such biosynthesis takes place, as well as the transformation and the degradation of this lipid species.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.12.005.

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