Biochemical and Biophysical Research Communications 522 (2020) 574–579

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

The metabolic pathway of sphingolipids biosynthesis and signaling in *Entamoeba histolytica*

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ARTICLE INFO

Article history:
Received 9 November 2019
Accepted 18 November 2019
Available online 27 November 2019

Keywords:
Entamoeba histolytica
Ceramide
Sphingolipid
Diacylglycerol
Protein kinase C

ABSTRACT

Sphingolipids (SLs) synthesis involves a complex metabolic pathway occurring between the endoplasmic reticulum (ER) and Golgi apparatus, generating ceramide synthesis and complex lipids, respectively. Here we show that *E. histolytica*, apparently lacking cellular organelles (ER and Golgi apparatus), synthesizes a wide variety of sphingolipid subspecies, being particularly abundant those of long-chain fatty acids. *In silico* analysis showed five putative genes coding for ceramide synthases (CerS), all of them coding for proteins containing the TLC domain, a region conserved in CerS of multiple organisms. These genes are abundantly expressed in different growth phases. Silencing and overexpression of CerS C4M4U4 (the closest homolog of human CerS 2 and 3) demonstrated its involvement in the synthesis of ceramide. Additionally, we identify C4M4U4, SMS2 and PKC (α, βII) proteins and their subcellular localization of *E. histolytica,* suggesting that these subcellular compartments might be involved in the biosynthesis and signaling pathway of sphingolipids, and evidencing different sphingolipid synthesis pathways in *Entamoeba.*

1. Introduction

Sphingolipids (SLs) represent 20–30% of total membrane lipids. Besides influencing membrane structure and properties, SLs are involved in various signaling pathway mechanisms, for this reason their study has increased recently [1-4]. Their presence has been demonstrated in eukaryotic cells, including animals, plants, fungi, protozoa and prokaryotes [5-8]. The biosynthetic pathway of SLs involves a highly complex metabolic pathway which is carried out between endoplasmic reticulum (ER) and Golgi apparatus, generating ceramide synthesis and complex lipids respectively. Diverse studies suggest that ceramide is synthesized in the ER and transported to the Golgi, through vesicular or non-vesicular trafficking [11,12].

Ceramide is the building block of SLs. It is formed by a long chain base either sphinganine (18:0) or sphingosine (18:1) in eukaryotic cells, or phytosphingosine in yeast, and a fatty acid of variable length, which binds to the main chain by an amide bond, through a reaction of N-acylation [13]. In mammals this process is performed by a family of six enzymes, ceramide synthases (CerS) located in the ER. CerS display specificity towards a fatty acyl of chain length defined [5,14], generating a variety of ceramide subspecies, which differ in fatty acid chain length, the status of hydroxylation and/or the number of insaturations in any of its hydrophobic chains, thus leading to a large number subspecies of SLs.

Evolutionarily, these enzymes conserve the TRAM-LAG-CLN8 (TLC) domain, including the Lag1p motif, an essential region credited of being the active site [17,18]. Various studies are focused on elucidating the specificity of each CerS towards determined fatty acid chain [10,19], as well as the mechanism(s) involved in the regulation of these enzymes [20,21]. The enzymes involved in the synthesis of sphingomyelins corresponds to sphingomyelin synthases (SMS), located in the Golgi apparatus [37].

Entamoeba histolytica is the organism responsible of amebiasis in humans, for this reason several studies have focused on the mechanisms involved in virulence and pathogenicity that affect the host cell [24,25]. Structurally, this is a simple eukaryote, lacking well-defined subcellular regions present in other organisms such as ER and Golgi apparatus [26], organelles involved in SLs

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https://doi.org/10.1016/j.bbrc.2019.11.116
0006-291X/

biosynthesis, hence its importance as a study model. Previous studies show the different lipid species present in different strains within this genus, including SLs species [27]. Likewise, it has been shown the major role of SLs in signaling mechanisms, regulating growth and cell proliferation in *Entamoeba* [3].

Here, using mass spectrometry we report the structural characterization of sphingomyelin subspecies present in *E. histolytica* trophozoites. We also identified *in silico* and amplified by RT-PCR five putative CerS genes. C4M4U4 protein possess highest homology to the human ortholog CerS 2–3. Amoeba transfectants harboring C4M4U4 silencing and overexpression constructs were established, and sphingomyelin subspecies were assessed. Respectively, we found diminished and enhanced long-chain (24–26 C) sphingomyelin species, suggesting that CerS C4M4U4 is involved in their synthesis. Moreover, antibodies used against C4M4U4 protein co-localized the protein with *EhSERCA* to the cytoplasmic vesicular network [36].

Our results show that despite the apparent absence of cellular organelles the enzymatic machinery involved in ceramide and complex lipids synthesis (SLs) is present in this organism, particularly C4M4U4 which might be responsible of long chain sphingomyelins.

2. Materials and methods

2.1. Cell culture

E. histolytica trophozoites from (HM-1: IMSS strain) were axenically grown at 37 °C in the TYI-S-33 medium as described by Diamond [28]. For lipid analysis, *E. histolytica* trophozoites were cultured for 72 h. For RT-PCR assays, RNA from *E. histolytica* trophozoites harvested at 6, 12, 24, 48, and 72 h was used.

2.2. Lipid extraction and mass spectrometry analysis

The extraction of lipids, mass spectrometry analyses, and *m/z* data analysis were carried out as previously described [4].

2.3. Bioinformatic analysis

To perform search of sequences Involved in the synthesis of ceramides and sphingomyelins in *E. histolytica* were considered various sequences of CerS reported in other organisms, such as Human and yeast. A BLAST-p analysis was performed for each of these sequences in the database AmoebaDB. The alignments shown as well as the determination of the percentages of similarity were obtained through the web server Clustal Omega [29]. Phylogenetic analyzes were carried out by the neighbor-joining methods using the program MEGA 6.0 [30] were performed for 10,000 bootstrap replicates. The transmembrane topology analysis was performed using the web server Phobius [31]. To create the schematic representations, the web server Protter was used [32]. The identification of domains and motifs as well as the regions with post-translational modification possible were obtained using the web server: scanprosite [33].

2.4. C4M4U4 plasmid constructs and amoeba transfectants

The EHI_193400 gene was amplified by PCR using primers flanked with the Sma I and Xho I restriction sites, EHI_193400 primers (Supplementary Table 6) at the 5′ and 3' ends, respectively. PCR products were cloned in the plasmid pKT3M 04-trigger [34] and pEhExHA [15].

Constructs were verified by sequencing. 10 μg of the resulting plasmids, pKT193400, PEhHA193400 and of the empty vectors were transfected into trophozoites with Superfect reagent (Qiagen) as suggested by the provider. Transfectants were stepwise selected with 10 μg/ml of G418.

2.5. RT-PCR and relative qRT-PCR assays

For RT-PCR assays, total RNA from trophozoites was extracted with TRIZOL reagent (Gibco) according to the manufacturer's instructions, and the samples were treated with DNAse A (Roche). cDNA was synthesized using 1 μg of total RNA, oligo-dT primers and the superscript II reverse transcriptase.

PCR amplifications were carried out using primers specific to the corresponding genes encoding each putative CerS (Supplementary Table 6). Amplicons were visualized in agarose gels and confirmed by sequencing. 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 EHI_193400 primers were used. Expression values were determined by the $2^{-\Delta\Delta CT}$ method [35] with RNA Pol 2 gene as the normalizing factor.

2.6. Immunofluorescence microscopy

6 and 72 h culture trophozoites were harvested and transferred to 8 mm round wells on glass slides, fixed with 3.7% paraformaldehyde, permeabilized with 0.2% Triton x-100/PBS, blocked with PBS-1% BSA and were incubated with primary antibody at the specified dilution: p-PKC α, βII (Thr 638/641) 1:200 (#9375 Cell Signaling Technology, Inc); or mouse monoclonal anti-HA tag 1:300 (ab130275); or rabbit polyclonal *EhSERCA* 1:50 (a kind gift from MA Rodríguez-Rodríguez); our rabbit Polyclonal to SMS 2 1:200 (ab103060); As negative control, primary antibody was omitted. After 3x washing with PBS, samples were incubated for 1 h at room temperature with the second anti-mousse IgG antibody tagged with Alexa Fluor® 488 goat anti mouse IgG (Invitrogen A11001) 1:1000; or Alexa Fluor® 594 goat anti-Rabbit (Invitrogen) 1:1000. After rinsing with PBS, cover slips were mounted with Vecta Shield-DAPI 1:10000 for nuclei staining. Samples were viewed in a Zeiss LSM700 confocal microscope.

2.7. Statistical analysis

Sphingolipid subspecies data were analyzed with SigmaPlot (Systat Software Inc., San Jose, CA, USA), and were expressed as the mean ± SE, of at least four independent experiments.

3. Results

3.1. Sphingomyelin subspecies in E. histolytica

The sphingomyelin subspecies identified by mass spectrometry in *E. histolytica* trophozoites comprise two sphingoid bases, sphinganine (18:0) and sphingosine (18:1), and a variety in fatty acid (16–26) carbon atoms (Fig. 1A). The most abundant subspecies correspond to sphingomyelins with long-chain fatty acids (24–26 C). For example, whereas subspecies of *m/z* 815.9 corresponds to sphingomyelin (18:0/24:1), and those with short-length fatty acids, as subspecies of *m/z* 703.9 and 705.9 correspond to sphingomyelins (18:1/16:0) and (18:0/16:0), respectively.

3.2. Identification of candidates for CerS in E. histolytica

Supplementary table 1 show the five putative CerS proteins identified *in silico*. All CerS sequences contain the TLC domain conserved in this enzyme family, but lack the Hox motif, identifying

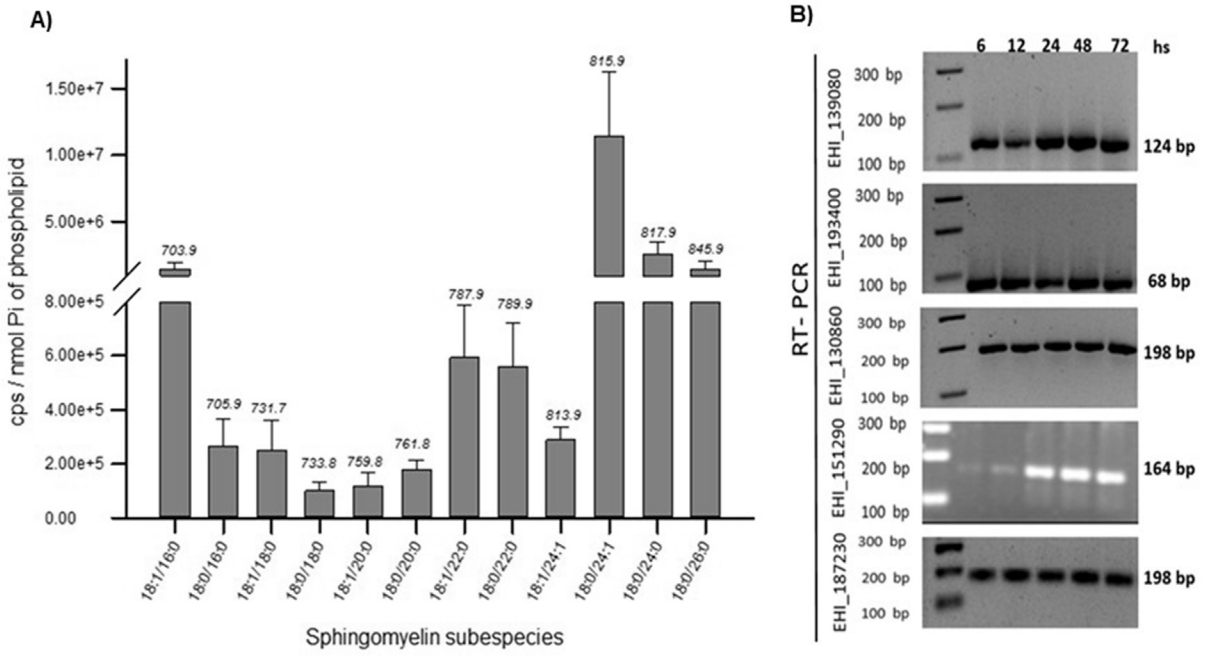

Fig. 1. Sphingomyelin subspecies identified in *E. histolytica* trophozoites. A) Data shows average measurements from 72 h cultures of wild type *E. histolytica* trophozoites (n = 4 independent experiments±SD). **B)** Expression of the five putative genes of CerS identified in *E. histolytica* at different proliferation times.

these as protozoa CerS [23]. The phylogenetic analysis showed that the *Entamoeba* CerS, like those of trypanosomatids and yeast, arose later than human, insects and plants CerS (Fig. 2A). Supplementary Table 2 shows the positions of the TLC domain, the Lag1p motif, putative serine (Ser) and threonine (Thr) phosphorylation sites, and putative N-glycosylation and miristoylation sites. Protein C4M4U4

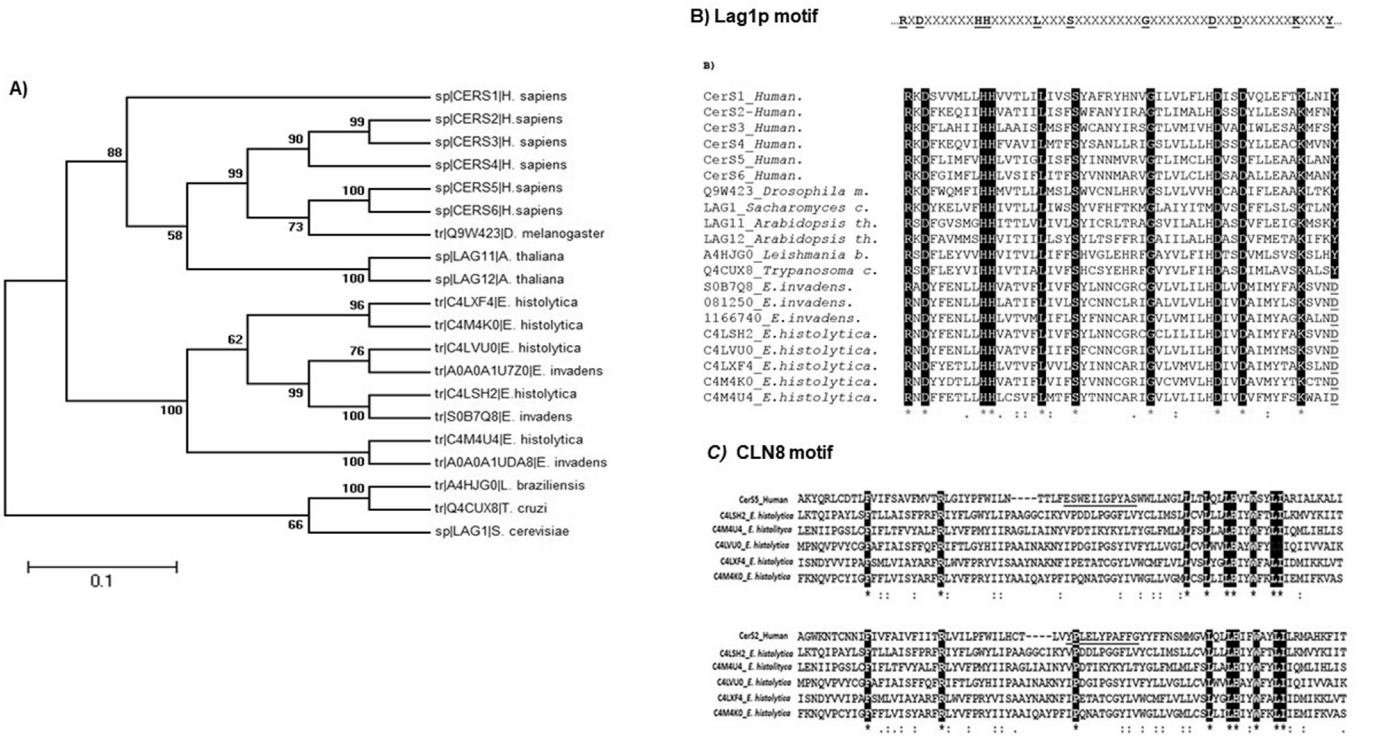

Fig. 2. Analysis *in silico* of *E. histolytica* CerS.A) Phylogenetic tree of *E. histolytica* CerS. The amino acid sequences include *S. cerevisiae* LAG1, *Drosophila melanogaster* Q9W423, *Leishmania braziliensis* A4HJG0, *Trypanosoma cruzi* Q4CUX8, putative *E. invadens* CerS loci: EIN_116740, EIN_133810, EIN_081250, and EIN_060030; CERS1, CERS2, CERS3, CERS4, CERS5, and CERS6; *Arabidopsis thaliana* LAG11, and LAG12; , and putative *E. histolytica* CerS: C4LSH2, C4LVU0, C4LXF4, C4M4K0, and C4M4U4. Numbers represent bootstrap values. The bar represents the percentage of substitutions. **B and C)** Alignments of CerS Lag1p and CLN8 motifs, respectively. Asterisks indicate positions that have a single, fully conserved residue among Lag1p homologues. Colon and period marks indicate that stronger-and weaker-score groups are fully conserved among Lag1p homologues, respectively.

has a bipartite nuclear localization signal, and protein C4M4K0 has an endoplasmic reticulum (KNEL) retaining sequence.

Several studies dilucidated the specificity of each human CerS (I-VI) towards a determined fatty acid chain [5,14]. Global alignments between the human and amoeba CerS sequences were performed to clarify a similar specificity towards fatty acid chain. Our results showed that it is difficult to attribute univocal homology of any human CerS to those identified in *E. histolytica* (Supplementary Table 3). However, local alignments using the CLN8 motif of the six human CerS allowed us to attribute homology to each one of the five amebic putative (Supplementary Table 4) which might reflect the possible specificity that each amoeba CerS towards a defined fatty acyl chain. There is a significant similarity (~43–56%) amongst the TLC domains of the *Entamoeba* CerS (Supplementary Table 5). Interestingly, from the 11 residues that constitute the Lag1p signature [18], the last Tyr (Y) residue is substituted by an Asp (D) residue in *Entamoeba*; however, it should be mentioned that in position 45 within this motif, amoeba sequences only present a tyrosine, which maybe fulfilling the function of the non-conserved residue in position 52 (Fig. 2B). Recently, the CLN8 motifs of human CerS5 and CerS2 confer specificity of a fatty acid chain length [10]. Such motifs of CerS5 and 2 are well conserved in the five putative *E. histolytica* CerS (Fig. 2C).

Like in other organisms, the topologies of amoeba CerS were predicted to contain between VI-VIII transmembrane regions, in which Lag1p motifs are immersed in the membrane. CLN8 motifs are also shown (Supplementary Fig. 1). RT-PCR experiments show that *E. histolytica* trophozoites express all putative CerS at different proliferation times (Fig. 1 B).

3.3. Silencing and overexpression of locus EHI_193400

In humans, CerS2 and CerS3 synthesize long-chain ceramides, which correspond to the most abundant ceramide subspecies in *E. histolytica*. By homology, is possible that one *Entamoeba* CerS2 or CerS3 ortholog might be coded in the locus EHI_193400 corresponding to the protein C4M4U4.

To explore the implication of enzyme C4M4U4 in ceramide synthesis, we silenced and overexpressed the enzyme using pKT3M 04-trigger and pEhExHA plasmids, respectively. After transfectants were established, silencing and over expression efficiency was assessed by RT-PCR and qRT-PCR. Fig. 3A and B shows, respectively, a decrease and increase of EHI_193400 normalized gene transcription, compared to empty vector controls.

Sphingomyelin subspecies profiles were carried out in the C4M4U4 silenced and overexpression transfectants (Fig. 3C and D). As expected, C4M4U4 silencing resulted in 55% decrease of long-chain fatty acid sphingomyelins, and discrete changes of short-chain fatty acid sphingomyelins. Surprisingly, C4M4U4 overexpression produced a five-fold increase in long-chain sphingomyelins (24–26 C). These results suggest that locus EHI_193400 codes for a *bona fide* CerS.

3.4. Subcellular localization of C4M4U4, SMS2 and p-PKC (α, β II) in Entamoeba histolytica

Fig. 4c, d and e show the localization of the C4M4U4, SMS2 and EhSERCA proteins respectively. Fig. 4d shows that C4M4U4 CerS protein co-localizes with *EhSERCA* to the cytoplasmic vesicular network, Also, since DAG results from sphingolipids biosynthesis, we explored the cellular localization of DAG-mediated p-PKC (α, βII) activation during the first 6 h of incubation, a time in which the highest levels of DAG and activation of PKC are reached [3]. Fig. 4 l shows that active PKC localizes at the outer nuclear surface, suggesting a nuclear signaling function. Fig. 4 b, g and k shown nuclei staining with DAPI. Fig. 4 a, f and j shown negative controls.

4. Discussion

As in humans and yeast, *E. histolytica* can synthesize a wide range of ceramides with fatty acids from 16 to 26 carbon atoms suggesting that, in order to achieve this, several CerS are required. This feature is of great interest since most sphingomyelins reported in higher eukaryotic cells correspond to short-chain subspecies (C 16) with *m/z* 703.5/705.9, whereas longer acyl chain length (C 24), such as *m/z* 815.9 are much less represented [5] being a completely different sphingolipid subspecies distribution in the membranes of *E. histolytica* could explain the differential behaviors related to locomotion, fluidity and membrane resistance.

In *E. histolytica* we identified five genes encoding putative ceramide synthases whose function can be attributed to the presence of the TLC domain, including the Lag1p motif, as has been proposed in other CerS enzymes [18]. The reptilian parasite *E. invadens* have sphingomyelin subspecies like those reported in this work, of which the long chain fatty acid sphingomyelin with *m/z* 815.9 is highly represented [4]. However, whereas in *E. histolytica* only five CerS were identified, six were identified in *E. invadens*, indicating that like humans, *E. histolytica* is able to synthesize a wide variety of sphingomyelins, with fewer CerS. The human ortholog of CerS 1 appears to be absent in *E. histolytica* (Supplementary Table 4). As reported, human CerS 1 and 4 generate ceramides with fatty acids of 18 carbons [5], therefore the putative C4M4K0 could fulfill the activity in the absence of CerS 1 in *E. histolytica*. The expression profiles of the five putative CerS genes at different times of *E. histolytica* proliferation demonstrate the also differential role of these enzymes in the biology of amoebas, highlighting the importance of sphingolipid synthesis in cell proliferation and growth of this organism.[3].

Silencing and overexpression of the EHI_193400 locus resulted in both its mRNA reduction and increase, and changes in the sphingomyelin subspecies profile, which are part of the final product of the sphingolipid biosynthetic pathway, demonstrating that enzyme C4M4U4 is involved in ceramide synthesis in *E. histolytica*.

In mammals, yeasts and other protozoa, there are multiple enzymes and mechanisms described in the biosynthesis of SLs, involving a compartmentalized system between ER and Golgi [9,11]. It is widely accepted that no defined ER and Golgi apparatus are present in *E. histolytica*. However, ER-resident proteins have been reported [26]. In addition, we observed that the putative ceramide synthase C4M4K0 contains a RE retention motif, and all putative *Entamoeba* CerS contain the ER-resident *Lag1p* motif [17,22], suggesting the existence of similar subcellular organelles in this parasite. Nonetheless, key components of the sphingolipid biosynthesis have not been identified in *Entamoeba*, namely, the proteins involved in ceramide transport (CERT), as well as the mammalian and yeast CerS regulatory proteins (TOR Complex, Casein Kinase 2) [20,21]. These data pose the question of whether in this genus sphingomyelin biosynthesis is carried out in the same way as has been reported in other organisms. It is possible that a different sphingolipid synthesis pathway occurs in amoebae.

Cerbón et al. (2009) demonstrated that the DAG generated during sphingolipid synthesis was necessary for the activation of PKC, with maximum PKC activation/DAG levels observed in 5–6 h of incubation, conditions required for the G1 to S cell cycle transit, and cell proliferation [3]. Recently we demonstrated that the DAG obtained by sphingolipids biosynthesis is necessary to activate the PKC involved in *E. invadens* encystation [4]. PKC activation requires Ser/Thr phosphorylation, and it has been reported the existence of diverse putative Ser/Thr kinases (PPK) in *E. histolytica* [16].

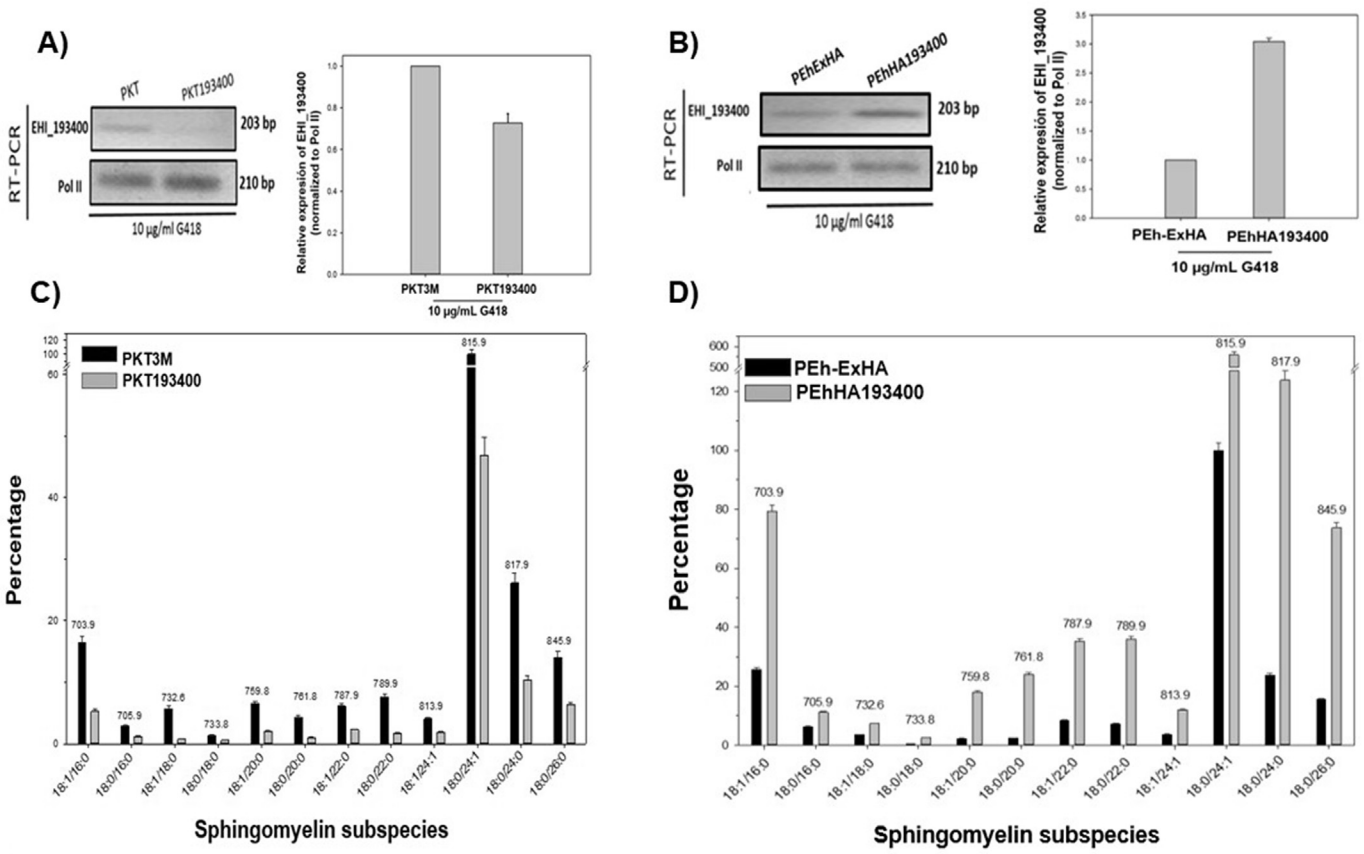

Fig. 3. Silencing and overexpression of the EHI_193400 mRNA (C4M4U4**) induced changes of sphingomyelin subspecies. A and B)** Respective qualitative (RT-PCR) and quantitative (qRT-PCR) expression of EHI_193400 gene transcription, in silence and overexpression transfectants, compared to an empty vector. **C and D)** Sphingomyelins subspecies profiles identified in the C4M4U4 silenced and overexpression transfectants.

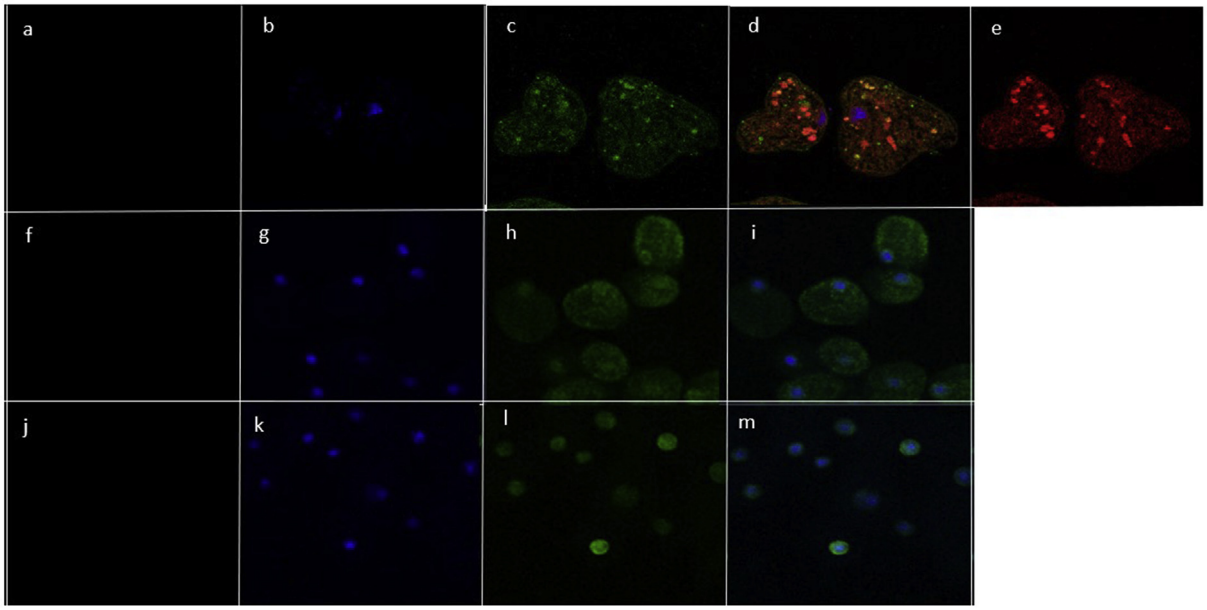

Fig. 4. - Subcellular localization of C4M4U4, SMS2 and p-PKC (α, β II) in trophozoites. Fig. 4c, d and e show the localized of the C4M4U4, SMS2 and EhSERCA proteins respectively. Fig. 4d shows that C4M4U4 CerS protein co-localizes with *EhSERCA* to the cytoplasmic vesicular network. Fig. 4 l shown that active PKC localizes at the outer nuclear surface. Fig. 4 b, g and k shown nuclei staining with DAPI. Fig. 4 a, f and j shown negative controls. Fig. 4 d, I and m shown the MERGE.

Considering the human protein kinase C (α, βII) sequences, a putative protein kinase 2 was identified in AmoebaDB (EHI_053130).

In this work we have shown that the parasite *E. histolytica*, despite the apparent absence of cellular organelles possess the enzymatic machinery involved in ceramide and complex lipids synthesis (SLs). The subcellular localization of C4M4U4, SMS2 and PKC (α, βII) in *E. histolytica* suggests that these compartments might be involved in the biosynthesis and signaling pathway of sphingolipids, interestingly there is a nuclear signaling.

Declaration of competing interest

Authors declare no conflict of interests.

Acknowledgements

We thank Lorena Ramirez Reyes (Unidad de Proteómica, Genómica y Metabolómica, CINVESTAV-IPN) for SM analysis. This work was supported by scholarships 278075 to RAG and 261717 to JMJW, grants 127557-M and 236104 to JV, and 238566 to JTAS, all from Consejo Nacional de Ciencia y Tecnología, Mxico.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.11.116.

References

[1] D.E Vance, J.E. Vance. Biochemistry of Lipids, Lipoproteins and Membranes, 5th ed., Elsevier, Amsterdam ; Boston, 2008.
[2] J. Cerbon, N. Baranda-Avila, A. Falcon-Munoz, I. Camacho-Arroyo, M. Cerbon, Sphingolipid synthesis and role in uterine epithelia proliferation, Reproduction 156 (2018) 173–183.
[3] J. Cerbon, T. Olguin, P.R. Alvarez-Grave, R.C. Lopez-Sanchez, *Entamoeba* invadens: sphingolipids metabolic regulation is the main component of a PKC signaling pathway in controlling cell growth and proliferation, Exp. Parasitol. 122 (2009) 106–111.
[4] J.M. Jáuregui-Wade, J. Valdés, J.T. Ayala-Sumuano, R. Ávila-García, Cerbón-Solorzano, De novo synthesis of sphingolipids plays an important role during in vitro encystment of *Entamoeba invadens*, J. Biochem Biophys Res Commun. 508 (4) (2019 Jan 22) 1031–1037.
[5] H. Park, C.A. Haynes, A.V. Nairn, M. Kulik, S. Dalton, K. Moremen, A.H. Merrill Jr., Transcript profiling and lipidomic analysis of ceramide subspecies in mouse embryonic stem cells and embryoid bodies, J. Lipid Res. 51 (2010) 480–489.
[6] S. Schorling, B. Vallee, W.P. Barz, H. Riezman, D. Oesterhelt, Lag1p and Lac1p are essential for the acyl-CoA-dependent ceramide synthase reaction in *Saccharomyces cerevisae*, Mol. Biol. Cell 12 (2001) 3417–3427.
[7] N. Azzouz, B. Rauscher, P. Gerold, M.F. Cesbron-Delauw, J.F. Dubremetz, R.T. Schwarz, Evidence for de novo sphingolipid biosynthesis in *Toxoplasma gondii*, Int. J. Parasitol. 32 (2002) 677–684.
[8] A. Yamamoto, I. Yano, M. Masui, E. Yabuuchi, Isolation of a novel sphingoglycolipid containing glucuronic acid and 2-hydroxy fatty-acid from flavobacterium-devorans atcc 10829, J. Biochem.Tokyo 83 (1978) 1213–1216.
[9] A.H. Futerman, B. Stieger, A.L. Hubbard, R.E. Pagano, Sphingomyelin synthesis in rat-liver occurs predominantly at the cis and medial cisternae of the golgi-apparatus, J. Biol. Chem. 265 (1990) 8650–8657.
[10] R. Tidhar, I.D. Zelnik, G. Volpert, S. Ben-Dor, S. Kelly, A.H. Merrill, A.H. Futerman, Eleven residues determine the acyl chain specificity of ceramide synthases, J. Biol. Chem. 293 (2018) 9912–9921.
[11] K. Funato, H. Riezman, Vesicular and nonvesicular transport of ceramide from ER to the Golgi apparatus in yeast, J. Cell Biol. 155 (2001) 949–959.
[12] K. Hanada, K. Kumagai, N. Tomishige, T. Yamaji, CERT-mediated trafficking of ceramide, Bba-Mol Cell Biol L 1791 (2009) 684–691.
[13] S.T. Pruett, A. Bushnev, K. Hagedorn, M. Adiga, C.A. Haynes, M.C. Sullards, D.C. Liotta, A.H. Merrill Jr., Biodiversity of sphingoid bases ("sphingosines") and related amino alcohols, J. Lipid Res. 49 (2008) 1621–1639.
[14] M. Levy, A.H. Futerman, Mammalian ceramide synthases, IUBMB Life 62 (2010) 347–356.
[15] Y. Saito-Nakano, T. Yasuda, K. Nakada-Tsukui, M. Leippe, T. Nozaki, Rab5-associated vacuoles play a unique role in phagocytosis of the enteric protozoan parasite *Entamoeba histolytica*, J. Biol. Chem. 279 (2004) 49497–49507.
[16] K. Anamika, A. Bhattacharya, N. Srinivasan, Analysis of the protein kinome of Entamoeba histolytica, Proteins 71 (2008) 995–1006.
[17] N. Kageyama-Yahara, H. Riezman, Transmembrane topology of ceramide synthase in yeast, Biochem. J. 398 (2006) 585–593.
[18] S. Spassieva, J.G. Seo, J.C. Jiang, J. Bielawski, F. Alvarez-Vasquez, S.M. Jazwinski, Y.A. Hannun, L.M. Obeid, Necessary role for the Lag1p motif in (dihydro)ceramide synthase activity, J. Biol. Chem. 281 (2006) 33931–33938.
[19] R. Tidhar, S. Ben-Dor, E. Wang, S. Kelly, A.H. Merrill, A.H. Futerman, Acyl chain specificity of ceramide synthases is determined within a region of 150 residues in the tram-lag-CLN8 (TLC) domain, J. Biol. Chem. 287 (2012) 3197–3206.
[20] S. Aronova, K. Wedaman, P.A. Aronov, K. Fontes, K. Ramos, B.D. Hammock, T. Powers, Regulation of ceramide biosynthesis by TOR complex 2, Cell Metabol. 7 (2008) 148–158.
[21] T. Fresques, B. Niles, S. Aronova, H. Mogri, T. Rakhshandehroo, T. Powers, Regulation of ceramide synthase by casein kinase 2-dependent phosphorylation in Saccharomyces cerevisiae, J. Biol. Chem. 290 (2015) 1395–1403.
[22] D.A. Andres, I.M. Dickerson, J.E. Dixon, Variants of the carboxyl-terminal KDEL sequence direct intracellular retention, J. Biol. Chem. 265 (1990) 5952–5955.
[23] A. Mesika, S. Ben-Dor, E.L. Laviad, A.H. Futerman, New functional motif in Hox domain-containing ceramide Synthases - identification of a novel region flanking the Hox and TLC domains essential for activity, J. Biol. Chem. 282 (2007) 27366–27373.
[24] E. Orozco, M.A. Rodríguez, F.C. Hernández, The role of phagocytosis in the pathogenic mechanism of Entamoeba histolytica, in: J.I. Ravdin (Ed.), Amebiasis: Human Infection by Entamoeba Histolytica, vol. 10158, John Wiley and Sons, New York, New York, 1988, pp. 314–325, 605 Third Avenue.
[25] M.A. Rodriguez, E. Orozco, R. Arroyo, Proteins of Entamoeba histolytica trophozoites involved in the adhesion to target cells, Arch. Med. Res. 25 (1994) 205–210.
[26] A. Mazzuco, M. Benchimol, W. De Souza, Endoplasmic reticulum and golgi-like elements in Entamoeba, Micron 28 (1997) 241–247.
[27] J. Cerbon, J. Flores, Phospholipid-composition and turnover of pathogenic amebas, Comp. Biochem. Physiol. B 69 (1981) 487–492.
[28] L.S. Diamond, D.R. Harlow, C.C. Cunnick, New medium for axenic cultivation of entamoeba-histolytica and other Entamoeba, Trans. Roy. Soc. Trop. Med. H 72 (1978) 431–432.
[29] M. Goujon, H. McWilliam, W.Z. Li, F. Valentin, S. Squizzato, J. Paern, R. Lopez, A new bioinformatics analysis tools framework at EMBL-EBI, Nucleic Acids Res. 38 (2010) W695–W699.
[30] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[31] L. Kall, A. Krogh, E.L.L. Sonnhammer, Advantages of combined transmembrane topology and signal peptide prediction - the Phobius web server, Nucleic Acids Res. 35 (2007) W429–W432.
[32] U. Omasits, C.H. Ahrens, S. Muller, B. Wollscheid, Protter: interactive protein feature visualization and integration with experimental proteomic data, Bioinformatics 30 (2014) 884–886.
[33] E. de Castro, C.J.A. Sigrist, A. Gattiker, V. Bulliard, P.S. Langendijk-Genevaux, E. Gasteiger, A. Bairoch, N. Hulo, ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins, Nucleic Acids Res. 34 (2006) W362–W365.
[34] L. Morf, R.J. Pearson, A.S. Wang, U. Singh, Robust gene silencing mediated by antisense small RNAs in the pathogenic protist Entamoeba histolytica, Nucleic Acids Res. 41 (2013) 9424–9437.
[35] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[36] A. Martinez-Higuera, A. Salas-Casas, M. Calixto-Galvez, B. Chavez-Munguia, D.G. Perez-Ishiwara, C. Ximenez, M.A. Rodriguez, Identification of calcium-transporting ATPases of *Entamoeba histolytica* and cellular localization of the putative SERCA, Exp. Parasitol. 135 (2013) 79–86.
[37] A.H. Futerman, B. Stieger, A.L. Hubbard, R.E. Pagano, Sphingomyelin synthesis in rat liver occurs predominantly at the cis and medial cisternae of the Golgi apparatus, J. Biol. Chem. 265 (1990) 8650–8657.