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ORIGINAL ARTICLE

An acidic sphingomyelinase Type C activity from *Mycobacterium tuberculosis*

Jorge Castro-Garza[a], Francisco González-Salazar[a,b], Frederick D. Quinn[c], Russell K. Karls[c], Laura Hermila De La Garza-Salinas[d], Francisco J. Guzmán-de la Garza[e], Javier Vargas-Villarreal[a,*]

[a] *Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Monterrey, N.L., México*
[b] *Departamento de Ciencias Básicas, División de Ciencias de la Salud, Universidad de Monterrey, San Pedro Garza García, N.L., México*
[c] *Department of Infectious Diseases, University of Georgia, Athens, GA, USA*
[d] *Coordinación de Planeación y Enlace Institucional, Instituto Mexicano Del Seguro Social, Delegación Nuevo León, Monterrey, N.L., México*
[e] *Unidad de Investigación Epidemiológica y en Servicios de Salud, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Monterrey, N.L., México*

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Abstract Sphingomyelinases (SMases) catalyze the hydrolysis of sphingomyelin to ceramide and phosphorylcholine. Sphingolipids are recognized as diverse and dynamic regulators of a multitude of cellular processes mediating cell cycle control, differentiation, stress response, cell migration, adhesion, and apoptosis. Bacterial SMases are virulence factors for several species of pathogens. Whole cell extracts of *Mycobacterium tuberculosis* strains H37Rv and CDC1551 were assayed using [N-methyl-^{14}C]-sphingomyelin as substrate. Acidic Zn^{2+}-dependent SMase activity was identified in both strains. Peak SMase activity was observed at pH 5.5. Interestingly, overall SMase activity levels from CDC1551 extracts are approximately 1/3 of those of H37Rv. The presence of exogenous SMase produced by *M. tuberculosis* during infection may interfere with the normal host inflammatory response thus allowing the establishment of infection and disease development. This Type C activity is different from previously identified *M. tuberculosis* SMases. Defining the biochemical characteristics of *M. tuberculosis* SMases helps to elucidate the roles that these enzymes play during infection and disease.

* Corresponding author.
E-mail address: jvargas147@yahoo.com.mx (J. Vargas-Villarreal).

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PALABRAS CLAVE
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Factores de virulencia;
México

Actividad de una esfingomielinasa ácida tipo C producida por *Mycobacterium tuberculosis*

Resumen Las esfingomielinasas (SMasas) catalizan la hidrólisis de esfingomielina a ceramida y fosforilcolina. Los esfingolípidos son reconocidos como reguladores diversos y dinámicos de una multitud de procesos celulares que median en el control del ciclo celular, la diferenciación, la respuesta al estrés, la migración celular, la adhesión y la apoptosis. Las esfingomielinasas bacterianas son factores de virulencia reconocidos en varias especies de patógenos. En este trabajo se analizaron los extractos de células enteras de las cepas de *Mycobacterium tuberculosis* H37Rv y CDC1551 utilizando [N-metil-^{14}C]-esfingomielina como sustrato. Se identificó actividad de SMasa-ácida dependiente de zinc en ambas cepas. La actividad máxima se observó a pH 5.5. Curiosamente, los niveles de actividad de SMasa generados a partir de extractos de la cepa CDC1551 son aproximadamente un tercio de los de la cepa H37Rv. La presencia de una SMasa exógena producida por *M. tuberculosis* durante la infección puede interferir con la respuesta inflamatoria del huésped, permitiendo así el establecimiento de la infección y el desarrollo de la enfermedad. Esta actividad tipo C es distinta de las actividades previamente reportadas para *M. tuberculosis*. Definir las características bioquímicas de las esfingomielinasas de *M. tuberculosis* ayudará a dilucidar el papel que desempeñan estas enzimas durante la infección y la enfermedad.

Introduction

Sphingomyelinases (EC 3.1.4.12) (SMases) are enzymes that catalyze the hydrolysis of sphingomyelin to ceramide and phosphorylcholine[5]. Sphingolipids are ubiquitous molecules of eukaryotic cell membranes and constitute a significant portion of membrane lipids[8]. Sphingolipids have been shown to be involved in a variety of physiological functions including regulation of cell growth, differentiation, cell migration, adhesion, inflammation and apoptosis[2,22,24].

SMases are classified into sub-type C and D based on the site of sphingomyelin hydrolysis and on the resulting products. Products from SMase C are ceramide and phosphorylcholine while SMase D degrades sphingomyelin to phosphoryl ceramide and choline[11,19]. Furthermore, SMases are classified by their optimum pH activity and localization into (a) acid SMase (A-SMase), (b) secretory Zn^{2+}-dependent SMase (A-sSMase), (c) neutral Mg^{2+}-dependent SMase (N-SMase), (d) neutral Mg^{2+}-independent SMase (N-iSMase), (e) alkaline SMase, and (f) bacterial SMase-phospholipase C[5]. Several species of bacteria, viruses, and even parasites infect host cells by exploiting either the A-SMase or the N-SMase-ceramide system, or both[3,6,14]. Alternatively, it has been shown that cellular stress produces plasma secretion of A-SMases resulting in the formation of membrane-embedded, ceramide-enriched lipid rafts and the reorganization of receptor complexes leading to signaling events and regulation of a cellular phenotype that protect mice in a sepsis model[9].

Moreover, several pathogenic bacteria produce SMases with direct activity on target host cells. These virulence factors include *Clostridium perfringens* alpha-toxin, possessing phospholipase C-SMase and biological activities causing hemolysis, dermonecrosis and potentially death of the host[20]. Inhibition of *Bacillus cereus* SMase prevented mortality in mice[15]. Phospholipase C/SMase from the opportunistic pathogen *Pseudomonas aeruginosa* has hemolytic activity and is selectively cytotoxic to mammalian endothelial cells[23].

Staphylococcus aureus invades non-professional phagocytes, eventually resides in acidified phago-endosomes and subsequently escapes from this compartment due to a synergistic activity of the cytolytic peptide, staphylococcal δ-toxin and the SMase β-toxin[4]. A similar effect has been reported for the *C. perfringens* SMase activity[25].

We previously reported the identification of alkaline, neutral, and acidic SMase activities from *Mycobacterium tuberculosis*[26]. In the present study, we report a novel acidic mycobacterial Zn^{2+}-dependent SMase activity.

Materials and methods

Mycobacterial whole cell extracts

M. tuberculosis strains H37Rv and CDC1551 were grown in Middlebroook 7H9 medium supplemented with OADC for 10 days at 37 °C. Cultures were harvested by centrifugation at 3000 × *g* for 15 min. An approximate mass of 800 mg (wet weight) in 1 ml of PBS plus 0.5 % Triton X100 was gamma-irradiated for 48 h in a Gammacell 3000 equipment (Elan, Nordion International Inc, Kanata, Ontario, Canada). The gamma-irradiated bacteria were washed twice with PBS and resuspended in 1 ml of sodium acetate-buffer solution 100 mM pH 5.5 (ABS) with 0.5 % Triton X-100 (Sigma Chemical Co., St. Louis, MO) and Boehringer Manheim complete EDTA-free protease inhibitor cocktail (cat. No. 1836-170). Bacterial suspensions were disrupted with the Fast Prep System (MP Biomedicals, Santa Ana, CA) by applying 5 cycles of 1 min at setting 6, with 2 min resting on ice in between

runs. The whole cell extracts were centrifuged twice at 16 000 × *g* for 8 min at room temperature, to remove intact bacterial cells. The preparations were divided into 200 μl aliquots and stored at −70 °C until used. Protein quantification was done by the method proposed by Lowry et al.[13].

Sphingomyelinase activity

The measurement of SMase was based on the method described by Vargas-Villarreal *et al.*[26], with minor modifications. The mycobacterial whole cell extracts were the sources of enzymes for all the described experiments. In brief, 2.5 μCi of [N-methyl-^{14}C]-sphingomyelin (47 mCi/mmol; PerkinElmer Life and Analytical Science, Boston, MA) was mixed with 200 mM sodium acetate (pH 5.5), 2 mM Zn^{2+}, 4 % Triton X-100 and 0.3 mM sphingomyelin. The mixtures were sonicated in an Ultratip Labsonic System (Lab-Line Instrument Inc., Melrose Park IL), applying one pulse of 40 W/min. The assays were performed by mixing 10 μl of the substrate preparation with 10 μl of whole cell extract suspension of *M. tuberculosis* strain H37Rv or CDC-1551 containing 0–2 μg of total protein. The mixture was vortexed for 10 sec and incubated at 37 °C for 60 min. The reaction was stopped by adding 25 μl of 1 mg/ml sphingomyelin, 1 mg/ml phosphorylcholine and 1 mg/ml choline (Sigma Chemical Co., St. Louis, MO) in 5 % trichloroacetic acid in n-butanol. Non-digested sphingomyelin was separated from the SMase hydrolysis products (phosphorylcholine and choline) by thin-layer chromatography (TLC). The assay mixtures were applied (45 μl) on 10 cm × 10 cm silica-gel plates (0.25-mm thickness, 60-mesh; Merck, Germany). The plates were placed in a TLC tank containing chloroform:methanol:water (65:25:4, v/v) as a mobile phase. The tank was loaded 15 min before starting each chromatographic run with the mobile phase-vapors to saturate the internal atmosphere. The spots corresponding to choline and phosphorylcholine (origin), and sphingomyelin (R_f = 0.29) were developed by exposing the TLC plates to iodine vapors[21]. To identify [^{14}C]-sphingomyelin, [^{14}C]-phosphorylcholine and [^{14}C]-choline spots, their respective relative migration coefficients (R_f) were compared with those of their corresponding non-radioactive standards (Sigma Chemical Co., St. Louis, Mo.) obtained in the same run. The two spots corresponding to [^{14}C]-sphingomyelin, and [^{14}C]-phosphorylcholine and [^{14}C]-choline mixed were scraped from the TLC silica gel plates and placed into plastic vials containing 5 ml scintillation liquid (BCS, Biodegradable Counting Scintillation; Amersham International Corporation, Buckinghamshire, England). Radioactivity in each vial was determined with a 1600 Tri-Carb liquid scintillation spectrometer (Packard Instrument Company, Inc., Downers Grove, IL). The device was adjusted to work with unquenched samples at 96 % efficiency. One unit of SMase activity was defined as 1 pmole of [^{14}C]-sphingomyelin hydrolyzed (equivalent to picomoles of [^{14}C]-phosphorylcholine plus [^{14}C]-choline released) in 1 h of incubation. The specific activity was given as the number of units of SMase activity per mg of total mycobacterial proteins. The classification of the SMase type was determined as described previously[26].

Effect of incubation time, dose, pH and cations on sphingomyelinase activity

Time: The rate of hydrolysis of sphingomyelin was determined at various time points over 60 min by mixing 10 μl of whole mycobacterial extracts (containing 2 μg of total proteins) with 2.5 μCi [N-methyl-^{14}C]-sphingomyelin (final concentration 1 mg/ml) dissolved in 20 μl of 200 mM sodium acetate buffer (pH 5.5), 1 mM Zn^{2+}, and 0.2 % Triton X-100.

Dose: Variable amounts of protein from whole cell extracts from strains H37Rv and CDC1551 were assayed by mixing 10 μl of whole mycobacterial extracts (containing various concentrations of protein between 0 and 2 μg) with 2.5 μCi [N-methyl-^{14}C]-sphingomyelin (final concentration 1 mg/ml) dissolved in 20 μl of 200 mM sodium acetate buffer (pH 5.5), 1 mM Zn^{2+}, and 0.2 % Triton X-100 at 37 °C, for 1 h at pH 5.5.

pH: pH values were adjusted with appropriate proportions of sodium acetate (pH 3–6) or Trizma-Base (pH 7–10) buffers to mycobacterial extracts containing 2 μg of total protein and incubated for 1 h at 37 °C prior to running the standard assay as described above.

Cations: Each metal cation was assayed separately. Assay mixtures with 1 or 10 mM $MgCl_2$, $CaCl_2$, $ZnSO_4$, $HgCl_2$, $MnCl_2$, or $CoCl_2$, or with 1 or 10 mM of chelating agent EDTA (Sigma Chemical Co., St. Louis, MO) were incubated for 1 h at 37 °C to run the standard assay as described above.

Statistics

All the experiments were performed by triplicate (n = 9). Slopes of the effect of incubation time and dose of mycobacterial extracts were calculated by linear regression. Statistical significance was determined using ANOVA.

Results

Dependence of mycobacterial sphingomyelinase on incubation time and dose of extracts

The SMase activity of both H37Rv and CDC1551 strains increased linearly between 0 and 60 min of incubation (r^2 = 0.99). After 60 min of incubation 37.89 pmol and 16.1 pmol of [^{14}C]-phosphorylcholine were recovered from chromatography plates of H37Rv and CDC1551, respectively (Fig. 1).

The amount of [^{14}C]-phosphorylcholine released from [^{14}C]-sphingomyelin also increased linearly as a function of extract dose of both strains (r^2 = 0.99). After 60 min incubation with 2 μg of total protein extract 37.94 pmol and 17.65 pmol of [^{14}C]-phosphorylcholine were recovered from strains H37Rv and CDC1551, respectively (Fig. 2). Those values corresponded to a specific SMase activity of 37.9 × 10^6 U/mg/h for H37Rv, which was 2.15 times higher (p < 0.001) than that of CDC1551 (17.6 × 10^6 U/mg/h).

Dependence on pH

Assayed extracts from both strains produced one primary peak of SMase activity at pH 5.5, which corresponds to

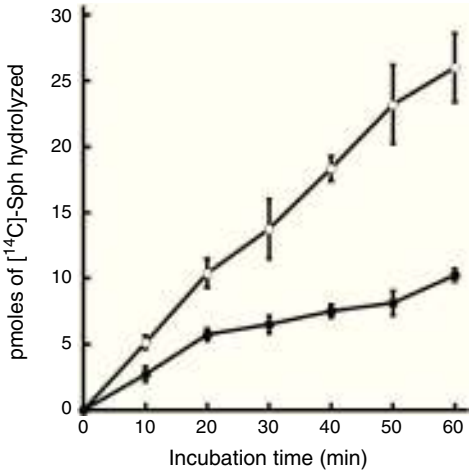

Figure 1 Time-course of mycobacterial SMase activity. Mycobacterial cell fractions (2 μg of total protein) were tested for SMase activity at incubation time points between 0 and 60 min. Symbols represent the mean ± SE of [^{14}C]-phosphorylcholine released by *M. tuberculosis* whole cell extracts of strains H37Rv (○) or CDC1551 (●) in three independent experiments performed in triplicate.

the maximum specific activity. The peak from strain H37Rv extracts was almost three times higher than that of strain CDC1551 (Fig. 3).

Effect of divalent cations

The SMase activity detected in whole cell extracts with no extra reagents added was defined as the basal activity equivalent to 100%. Subsequently, the activity was stimulated at different rates by 1 and 10 mM concentration of cations Co^{2+}, Mn^{2+}, Zn^{2+}, and Mg^{2+} or inhibited by Hg^{2+} and Ca^{2+}. As

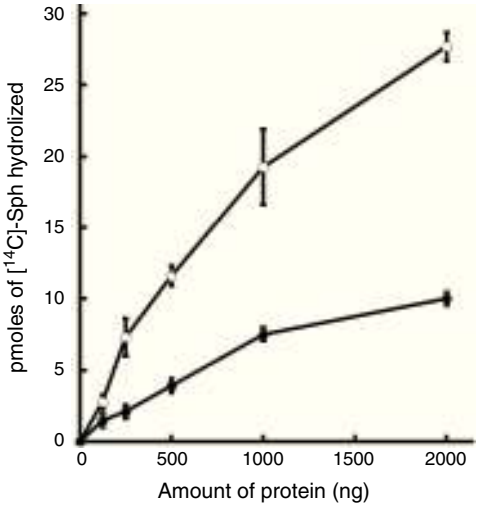

Figure 2 Dose-dependence of *M. tuberculosis* SMase activity. Mycobacterial cell fractions containing 0–2 μg of protein per assay were tested. Symbols represent the mean ± SE of [^{14}C]-phosphorylcholine released [in pmol] by *M. tuberculosis* whole cell extracts of strains H37Rv (○) or CDC1551 (●). All assays were performed at pH 5.5 with 1 h incubation.

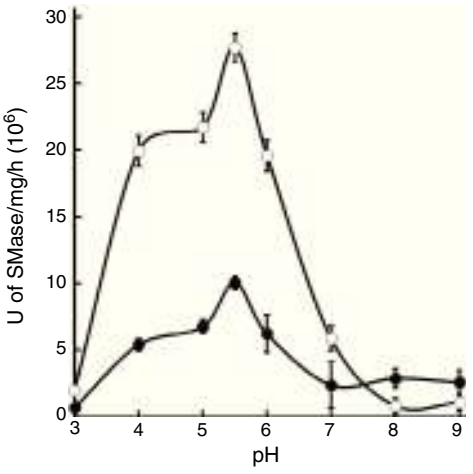

Figure 3 Effect of pH on mycobacterial SMase activity. Mycobacterial cell fractions containing 2 μg of proteins were tested at different pH levels (range 3–9). Symbols represent the means ± SE of SMase specific activity of *M. tuberculosis* whole cell extracts of strains H37Rv (○) or CDC1551 (●).

Table 1 Normalized sphingomyelinase (SMase) activity modulated by cations or EDTA addition

	Mycobacterium tuberculosis strain			
	H37Rv		CDC-1551	
	Concentration (mM)			
	1.0	10.0	1.0	10.0
	Normalized specific SMase activity			
None	1.00	1.00	1.00	1.00
EDTA	0.04	0.01	0.23	0.25
$MgCl_2$	1.02	1.57	1.29	1.74
$MnCl_2$	1.86	1.88	1.75	2.01
$CoCl_2$	2.05	2.23	2.10	2.20
$CaCl_2$	1.02	0.67	1.08	0.77
$HgCl_2$	0.56	0.36	0.82	0.41
$ZnSO_4$	1.32	1.50	1.39	1.87

expected, chelating agent EDTA resulted in the greatest inhibition: 96–99% for strain H37Rv and 75–77% for CDC1551. Results are shown in Table 1.

Discussion

Sphingomyelinases (SMases) are enzymes produced by mammalian cells; their main activity is considered to occur during digestive processes. However, these enzymes and the products derived from the hydrolysis of the substrate sphingomyelin are involved in many other cell regulating functions such as cell growth, differentiation, cell migration, adhesion, inflammation and apoptosis[2,22,24]. A more recent interest in the enzymes has been in their protective roles against pathogens by inhibiting invasion, regulating cytokine responses and inducing apoptosis in infected host cells[7].

Pathogens also produce SMases and they may act as virulence factors as demonstrated with *C. perfringens*[20],

B. cereus[15], *P. aeruginosa*[23], *Listeria spp.*[17] and *S. aureus*[4]. Previously, we reported the identification of acidic, alkaline and neutral SMases activities in *M. tuberculosis*. In that report, neutral SMase had the highest activity and was stimulated by Mg^{2+}, Mn^{2+} and Co^{2+} while acidic and alkaline SMase activities remained uncharacterized[26]. Therefore, in this study, *M. tuberculosis* acidic SMase activity is further characterized. Maximal SMase activity occurs in a pH range from 5 to 6, with optimal activity at pH at 5.5 (Fig. 3). The assay for cation-induced SMase activity at pH 5.5 resulted in elevated activities by Co^{2+}, Mn^{2+}, Zn^{2+}, and to a lesser extent Mg^{2+} (Table 1). Zn^{2+}-inducible activity was not previously reported for *M. tuberculosis*. Thus, it can be classified as Zn^{2+}-dependent acidic SMase activity. By comparing its biochemical characteristics with other SMases, it may correspond to a secretory acidic SMase. In mammalian hosts, this type of SMase regulates immune response[10]. In this instance, the presence of exogenous SMase produced by *M. tuberculosis* during infection may interfere with the normal host inflammatory response thus allowing for the establishment of infection and the development of disease. Recently, Roca and Ramakrishnan[18] observed that the chemical blockade of host acid SMase activity prevents TNF-induced macrophage programmed necroptosis and mycobacterial release from the cell. Thus, *M. tuberculosis* acidic SMase may aid in macrophage escape through the induction of necroptosis by its enzymatic product ceramide.

Presently, *M. tuberculosis* genome annotation in Tuberculist predicts that the protein products from genes *plcA* (*Rv2351c*), *plcB* (*Rv2350c*), *plcC* (*Rv2349c*), and *plcD* (*Rv1755c*, reported as a fragment) have phospholipase and SMase activities and they are noted as virulence factors implicated in the pathogenesis of *M. tuberculosis* at the level of intracellular survival by the alteration of cell signaling events or by direct cytotoxicity[12,16]. Several bacterial phospholipases possess both phospholipase and SMase activities; an example is the alpha-toxin from the pathogen *C. perfringens*[20]. The activity we have identified in this study may correspond to *M. tuberculosis* phospholipase C expressed from gene *plcC* (*Rv2349c*) because the proteins from the *plcA* and *plcB* genes are membrane-associated phospholipases C. Furthermore, another report where *M. smegmatis* was used as an expression system of four *M. tuberculosis plc* genes supports this idea; *plcC* product was found to be the most active of the four recombinant PLCs under acidic conditions[1]. However, a novel *M. tuberculosis* enzyme with SMase activity cannot be ruled out. More research work is needed to make this determination.

Sphingomyelinases (SMases) and their hydrolysis products (ceramide and phosphorylcholine) are regulators of a multitude of cellular processes. Several pathogens produce SMases as virulence factors. In the present study, we report a novel acidic mycobacterial Zn^{2+}-dependent SMase activity. The relevance of this enzyme in tuberculosis pathogenesis remains to be elucidated.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this investigation.

Confidentiality of data. The authors declare that no patient data appears in this article.

Right to privacy and informed consent. The authors declare that no patient data appears in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

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