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Phospholipase A_1: A novel virulence factor in *Trypanosoma cruzi*

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ABSTRACT

Phospholipase A_1 (PLA_1) has been described in the infective stages of *Trypanosoma cruzi* as a membrane-bound/secreted enzyme that significantly modified host cell lipid profile with generation of second lipid messengers and concomitant activation of protein kinase C. In the present work we determined higher levels of PLA_1 expression in the infective amastigotes and trypomastigotes than in the non-infective epimastigotes of lethal RA strain. In addition, we found similar expression patterns but distinct PLA_1 activity levels in bloodstream trypomastigotes from Cvd and RA (lethal) and K98 (non-lethal) *T. cruzi* strains, obtained at their corresponding parasitemia peaks. This fact was likely due to the presence of different levels of anti-*T. cruzi* PLA_1 antibodies in sera of infected mice, that modulated the enzyme activity. Moreover, these antibodies significantly reduced *in vitro* parasite invasion indicating the participation of *T. cruzi* PLA_1 in the early events of parasite–host cell interaction. We also demonstrated the presence of lysophospholipase activity in live infective stages that could account for self-protection against the toxic lysophospholipids generated by *T. cruzi* PLA_1 action. At the genome level, we identified at least eight putative genes that codify for *T. cruzi* PLA_1 with high amino acid sequence variability in their amino and carboxy-terminal regions; a putative PLA_1 selected gene was cloned and expressed as a recombinant protein that possessed PLA_1 activity. Collectively, the results presented here point out at *T. cruzi* PLA_1 as a novel virulence factor implicated in parasite invasion.

1. Introduction

Chagas disease constitutes a major medical and economical problem throughout America. Its etiological agent is *Trypanosoma cruzi*, an intracellular protozoan that invades nucleated cells in mammalian hosts and presents a high degree of genetic variability [1]. It is well known that the diverse parasite isolates, recently classified into six distinct discrete typing units, display heterogeneous infectivity rates [2–4]. This association between *T. cruzi* lineages and virulence has been related to the differential expression of several factors involved in invasion, morphogenesis and pathogenesis [4–7]. On the other hand, it has been described that the presence of neutralizing antibodies against relevant antigens in the sera of infected animals modified parasite virulence and modulated the course of infection [8,9].

Phospholipases are lipolytic enzymes widespread in nature that hydrolyze phospholipids and play crucial roles in diverse biochemical and biological processes [10]. In particular, Phospholipases A have been considered virulence factors for many pathogenic bacteria like *Escherichia coli*, *Helicobacter pylori*, *Neisseria spp.* and *Yersinia spp.*, among others [11–15]. As concerns protozoans, in African trypanosomes the involvement of phospholipase A_1 (PLA_1) in pathogenesis has been intensively studied [16–19]. In the case of *T. cruzi*, evidences related to phospholipid degrading enzymes were associated to the inflammatory responses that appear surrounding degenerating amastigote nests in various tissues of Chagas disease patients [20]. Accordingly, we demonstrated that upon parasite autolysis, free fatty acids (FFA) and lysophospholipids were rapidly accumulated as consequence of endogenous lipid degradation, evidencing the action of PLA_1 and lysophospholipase A (LysoPLA) activities [21]. It is well known that these bioactive lipids are harmful for cells and it has been described that these compounds released from lysed *T. cruzi* trypomastigotes, have toxic effects on culture cells and could be involved in the pathogenesis of Chagas

Abbreviations: dpi, days post infection; Ffa, free fatty acids; Lpc, lysophosphatidylcholine; LysoPLA, lysophospholipase activity; Pc, phosphatidylcholine; pla_1, phospholipase A_1; $Tcpla_1r$, recombinant *T. cruzi* phospholipase A_1.

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disease [22]. In previous works we isolated and characterized *T. cruzi* PLA_1, being in the infective amastigotes and trypomastigotes a membrane-bound and secreted activity that was up to 20-fold higher with respect to the non-infective epimastigotes [21,23]. Furthermore, we demonstrated that the enzyme significantly modified the host cell lipid profile with generation of second lipid messengers and concomitant protein kinase C activation, an enzyme that has been implicated in the up regulation of parasite invasion, suggesting that *T. cruzi* PLA_1 is involved in the early events of parasite–host cell interaction that precede parasite invasion [23,24].

This paper analyze the role of *T. cruzi* PLA_1 as a virulence factor by evaluating protein expression and activity levels in lethal and non-lethal strains and by studying the participation of PLA_1 neutralizing antibodies in the modulation of parasite invasion. We also performed the identification of putative genes that codify for *T. cruzi* PLA_1 and carried out the cloning and expression of the recombinant enzyme.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-[1-^{14}C] palmitoyl-phosphatidylcholine, (40–60 mCi/mmol), ECL western blotting detection system and molecular weight markers were obtained from General Electric (Arlington Heights, IL, USA). Soybean phosphatidylcholine type II-S, bovine serum albumin-fraction V, histopaque-1083, lipid standards, EGTA, Triton X-100, phenylmethylsulfonyl fluoride, protease cocktail inhibitor, Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride and dimethyl sulfoxide were from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum was from GIBCO BRL (Rockville, MD, USA). Silica GEL 60 thin layer chromatography plates, organic solvents and the expression vector pET30 were from Merck (Darmstadt, Germany). Centriplus Centrifugal Filter Devices were purchased from Millipore Corporation (Burlington, MA, USA). The anti-α-tubulin and peroxidase-conjugate antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DNeasy Blood & Tissue Kit, QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit were purchased form QIAGEN (GmbH, Hilden, Germany). pGEMT vector system, restriction enzymes, T4 DNA ligase, isopropyl-1-b-D-galactopyranoside and nickel resin were from Promega (Madison, WI, USA). PCR SuperMix High Fidelity and *E. coli* One Shot BL21 (DE3) pLysS competent cells were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Parasites

2.2.1. T. cruzi bloodstream trypomastigotes

T. cruzi bloodstream trypomastigotes from RA and Cvd (*T. cruzi* VI, lethal) and K98 (*T. cruzi* I, non-lethal) strains maintained in CF1 mice were used [4,7,25,26]. Parasites were obtained from whole blood at their corresponding parasitemia peaks: 7 days post infection (dpi) for RA and Cvd and 45 dpi for K98, and purified by density gradient using Histopaque-1083 [27]. Trypomastigotes were then separated from mononuclear cells by centrifugation (3000 × *g*, 10 min, 20 °C), supernatants were centrifuged (10,000 × *g*, 30 min, 20 °C) and the pellets containing the trypomastigotes were stored at −80 °C until used. For PLA_1 activity assays, bloodstream trypomastigotes were purified by Histopaque-1083 and the subsequent steps performed at 4 °C to avoid capping [28]. Local guidance for animal care and experimentation were followed throughout this research according to institutional policies.

2.2.2. T. cruzi culture amastigotes and trypomastigotes

T. cruzi culture amastigotes and trypomastigotes from RA and K98 strains were obtained from Vero cells, previously infected with bloodstream trypomastigotes and grown in 199 medium + 10% fetal bovine serum at 37 °C, 5% CO_2 [29].

2.2.3. T. cruzi epimastigotes

T. cruzi epimastigotes from RA strain were axenically grown in a biphasic medium at 28 °C, as previously described [30].

2.3. Parasite homogenates

The different parasite samples (bloodstream trypomastigotes, culture trypomastigotes/amastigotes and epimastigotes, 1×10^8/ml) were resuspended in 10 mM Tris/HCl, pH 7.4, in the presence of protease inhibitors (1× protease cocktail inhibitor; 2 mM phenylmethanesulfonyl fluoride; 0.5 mM Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride) and disrupted by four cycles of freezing and thawing. Samples were then centrifuged at 27,000 × *g* for 30 min and the resultant supernatants (homogenates) stored at −80 °C until used [23].

2.4. Specific anti-PLA_1 antibodies

Given that no commercial anti-PLA_1 antibodies were available, they were raised against native *T. cruzi* PLA_1 as well as recombinant *T. brucei* PLA_1. BALB/c mice (2 months of age) were injected by intramuscular route with 10 μg/ml protein/mouse of native *T. cruzi* PLA_1 purified from amastigotes [23] or recombinant *Trypanosoma brucei* PLA_1 [31], with complete Freund adjuvant (days 1 and 7) and with incomplete adjuvant (days 14 and 21). Ten days after the last immunizing dose sera were collected and stored at −20 °C until used [32].

2.5. Phospholipase A_1 expression

PLA_1 expression was determined by immunoblot in culture amastigotes/trypomastigotes and in epimastigotes of RA strain, as well as in bloodstream trypomastigotes of RA, Cvd and K98 strains. The specific anti-PLA_1 antibodies described above were used (1:500). The different samples (20–50 μg protein/lane) were resuspended in Laemmli sample buffer [33] + 100 mM dithiothreitol and analyzed on 12% SDS-PAGE. The immunoblot was developed using anti-mouse HRP conjugate (1:2000) and the chemiluminescence ECL detection kit, according to the manufacturer's instructions. Images were acquired with the Storm® Gel and Blot Imaging System (Amersham Biosciences). For loading control detection of α-tubulin was performed [34]: the same membranes were washed with warm stripping buffer (0.2 M glycine, 0.1% SDS, 1% tween 20, pH 2.2) for 10 min, blocked with 1% BSA, 1 h at room temperature and then incubated with rabbit polyclonal anti-mouse α-tubulin (1:10,000) overnight at 4 °C. The immunoblot was developed using goat polyclonal anti-rabbit IgG-HRP (1:5000) and bands were detected as described above. PLA_1 expression levels were determined by densitometry using Gel-Pro® Analyzer 4.0 software and the intensity of the signal of the PLA_1 band was normalized to that corresponding to α-tubulin.

2.6. Phospholipase assays

Phospholipase activity was determined in homogenates of RA, Cvd and K98 bloodstream trypomastigotes and in homogenates of K98 culture trypomastigotes by analyzing the products of hydrolysis of radio labeled phosphatidylcholine (PC) [21]. Briefly, the incubation mixture consisted of 140 μl of enzyme sample (∼10 μg-protein) or corresponding buffer (control), 40 μl of