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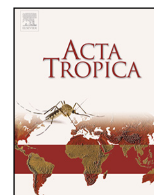

Surface-displayed glyceraldehyde 3-phosphate dehydrogenase and galectin from *Dirofilaria immitis* enhance the activation of the fibrinolytic system of the host

Javier González-Miguel [a,*], Rodrigo Morchón [a], Mar Siles-Lucas [b], Ana Oleaga [b], Fernando Simón [a]

[a] *Faculty of Pharmacy, Laboratory of Parasitology, Institute of Biomedical Research of Salamanca (IBSAL), University of Salamanca, 37007 Salamanca, Spain*
[b] *Laboratory of Parasitology, IRNASA, CSIC, Salamanca, Spain*

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A B S T R A C T

Cardiopulmonary dirofilariosis is a cosmopolitan disease caused by *Dirofilaria immitis*, a filaroid parasite whose adult worms live for years in the vascular system of its host. Previous studies have shown that *D. immitis* can use their excretory/secretory (ES) and surface antigens to enhance fibrinolysis, which could limit the formation of clots in its surrounding environment. Moreover, several isoforms of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and galectin (GAL) were identified in both antigenic extracts as plasminogen-binding proteins. The aim of this work is to study the interaction of the GAPDH and GAL of *D. immitis* with the fibrinolytic system of the host. This study includes the cloning, sequencing and expression of the recombinant forms of the GAPDH and GAL of *D. immitis* (rDiGAPDH and rDiGAL) and the analysis of their capacity as plasminogen-binding proteins. The results indicate that rDiGAPDH and rDiGAL are able to bind plasminogen and stimulate plasmin generation by tissue plasminogen activator (tPA). This interaction needs the involvement of lysine residues, many of which are located externally in both proteins as have been shown by the molecular modeling of their secondary structures. In addition, we show that rDiGAPDH and rDiGAL enhance the expression of the urokinase-type plasminogen activator (uPA) on canine endothelial cells in culture and that both proteins are expressed on the surface of *D. immitis* in close contact with the blood of the host. These data suggest that *D. immitis* could use the associated surface GAPDH and GAL as physiological plasminogen receptors to shift the fibrinolytic balance towards the generation of plasmin, which might constitute a survival mechanism to avoid the clot formation in its intravascular habitat.

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1. Introduction

Fibrinolysis is one of the main anticlotting mechanisms of the hemostatic system. Its key molecule is plasminogen, an abundant component of blood and zymogen of serine protease plasmin, enzyme responsible for degrading fibrin clots. The conversion of plasminogen into plasmin is regulated by binding to receptors via its five kringle domains, which have affinity for lysine residues and plasminogen activators (tPA and uPA) (Cesarman-Maus and Hajjar, 2005).

In order to maintain and propagate in the circulatory system, many bloodborne pathogens not only require adaptations to evade the activity of the host immune system, but also need to prevent blood clotting through interaction with the fibrinolytic system (Mebius et al., 2013). Cardiopulmonary dirofilariosis is a chronic and potentially fatal parasitic disease that affects dogs and cats around the world (Genchi et al., 2001). It is characterized by the presence of *D. immitis* adult worms in the pulmonary arteries and right ventricle of the infected hosts, where they can live for years causing a chronic inflammatory pathology (Venco, 2007). In previous studies, we have demonstrated the ability of *D. immitis* to bind plasminogen, enhancing plasmin generation by tPA by using two antigenic compartments (ES and surface) in an in vitro system.

Abbreviations: ES, excretory/secretory; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GAL, galectin; rDiGAPDH, recombinant form of the GAPDH of *D. immitis*; rDiGAL, recombinant form of the GAL of *D. immitis*; tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator; OP, optical density; ϵACA, lysine analogue ϵ-aminocaproic acid; CnAOEC, canine aortic endothelial cells; DiES, excretory/secretory antigens from *D. immitis* adult worms.

* Corresponding author at: Faculty of Pharmacy, Laboratory of Parasitology, University of Salamanca, C/del Licenciado Méndez Nieto, s/n, 37007 Salamanca, Spain. Tel.: +34 923294535; fax: +34 923294515.
E-mail address: jglez@usal.es (J. González-Miguel).

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We have also observed that the ES antigens are able to induce an overexpression of the fibrinolytic activator tPA in vascular endothelial cells in culture. Additionally, we have respectively identified a total of 10 and 11 plasminogen-binding proteins in the ES and surface extracts of the parasite, which included different isoforms of GAPDH and GAL (González-Miguel et al., 2012, 2013).

GAPDH has historically been regarded as a "housekeeping" protein. However, its involvement in numerous cellular processes in addition to glycolysis has been recently demonstrated. These include DNA repair, tRNA export, membrane fusion and transport, cytoskeletal dynamics and cell death (Tristan et al., 2011). Moreover its relationship with the fibrinolytic system has been widely studied being identified as plasminogen-binding protein in bacteria (Bhattacharya et al., 2012), fungi (Crowe et al., 2003) and parasites (Erttmann et al., 2005; Ramajo-Hernández et al., 2007; Lama et al., 2009). GAPDH is one of the most studied plasminogen receptors in parasites together with enolase, which has been reported as plasminogen-binding protein for the related filaria *Onchocerca volvulus* (Jolodar et al., 2003) or *Schistosoma bovis* (Ramajo-Hernández et al., 2007; de la Torre-Escudero et al., 2010) among others.

Galectins are β-galactoside-binding proteins characterized by its high level of evolutionary conservation, having been identified in many species from nematodes to mammals. Galectins have a wide range of biological functions in different processes including homeostasis, apoptosis, and vascular embryogenesis and in pathological conditions such as pre-eclampsia, inflammation, diabetes, atherosclerosis and cancer (Astorgues-Xerri et al., 2014). Related to filarial worms, onchocercal molting L3 strongly express GAL, being this protein proposed as good target for protective responses (Joseph et al., 2000). The interaction between this molecule and plasminogen has not yet demonstrated. However, the link between GAL-1 expression and cancer cell invasion with the demonstration of a direct interaction between tPA and GAL-1 in pancreatic cancer cells and stromal fibroblasts surrounding the tumor has been recently shown. This interaction enhanced tPA proteolytic activity and increased cell migration and invasion (Roda et al., 2009).

The aim of this study was to perform the molecular and functional characterization of the *D. immitis* GAPDH and GAL showing their capabilities as plasminogen-binding proteins, their relationships with the endothelium-dependent components of the fibrinolytic system and confirming their presence on the surface of the parasite.

2. Materials and methods

2.1. Parasite material

Adult worms of *D. immitis* were obtained from hearts of infected dogs from Gran Canary (Canary Islands, Spain) through the jugular vein using Flexible Alligator Forceps.

2.2. RNA isolation, RT-PCR, and cloning of GAPDH and GAL cDNA

Total RNA from adult worms was extracted using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. First-strand cDNA was synthesized from *D. immtis* adult worms RNA using the first-strand cDNA synthesis kit (Roche) as recommended by the manufacturer. The cDNA sequence of the *D. immitis* GAPDH and GAL were amplified using the following primers:

GAPDHFwd (5′-ATGAGCAAACCAAAGATTGGAATC)
GAPDHRev (5′-TTATCTGCTGGCGATGTAAGAG)
GALFwd (5′-ATGCACCACAACGAATATGAAACGAATTAC)
GALRev (5′-CTAGTGCATTTGAATACCGCTCACTTC)

The primers from GAPDH were designed on the consensus sequence resulting after the alignment of GAPDH cDNA sequences from *O. volvulus* and *Brugia malayi* (GenBank accession numbers U96177.1 and U18137.1 respectively). The primers from GAL were designed on the sequence of GAL cDNA sequences from *D. immitis* (GenBank accession number AF237485.1). PCR was performed in 1 cycle at 94 °C for 5 m, 35 cycles at 94 °C for 1 m, 46 °C for 46 s and 72 °C for 1 min 30 s, and 1 cycle at 72 °C for 5 m. The PCR products were electrophoresed in an agarose gel and the bands were purified from the gel using the StrataPrep DNA Gel Extraction kit (Stratagene) as recommended by the manufacturers. The GAPDH and GAL PCR products were cloned into the pSC-A vector using the StrataClone PCR Cloning kit (Stratagene) following the manufacturer's instructions. Both clones were purified with the Machery-Nagel NucleoSpin Plasmid kit.

2.3. Expression and purification of the rDiGADPH and rDiGAL

PCR products containing the whole rDiGADPH and rDiGAL coding sequences were cloned into the TOPO vector (Gateway System, Invitrogen) following the manufacturer's instructions. The recombinant plasmids were transformed into the *Escherichia coli* XL1B. Transformed cells were grown in LB-agar plates with ampicillin (100 μg/ml) overnight at 37 °C. Three colonies for each molecule were grown in liquid LB plus ampicillin overnight at 37 °C in agitation, and cells were harvested for plasmid extraction. Extracted plasmids were digested with *Eco*RI to check the insert presence. The TOPO vectors containing the fragments of interest were used for a ligation reaction with the pDEST7 vector (Gateway System, Invitrogen) following the manufacturer's instructions. Ligation reaction was transformed into XL1B cells and grown in LB-agar plates with ampicillin (100 μg/ml) overnight at 37 °C. Three colonies for each molecule were grown in liquid LB plus ampicillin overnight at 37 °C in agitation, and cells were harvested for plasmid extraction. Extracted plasmids were digested with *Eco*RI to check the insert presence. Vectors containing the inserts of interest were sequenced with the T7 primer (Sequencing Service of the Salamanca University) to check for the correct reading frame. The vectors containing the molecule of interest in reading frame were used to transform BL-21 expression cells. These were grown in liquid LB plus ampicillin (100 μg/ml) overnight at 37 °C in agitation. Cultures were diluted 1:20 in fresh medium and further growth until reaching an optical density (OD) of 0.5 at 600 nm. Then, expression of the recombinant proteins was induced by adding L-arabinose at a final concentration of 0.2% and further growing at 37 °C for 4 h in agitation. The induced cells were harvested and sonicated in a buffer containing 50 mM Na_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8 for rDiGADPH and 8 M urea, 100 mM NaH_2PO_4 and 10 mM Tris–Cl, pH 7.9 for rDiGAL. After a 20 min centrifugation step at 10,000 × *g*, the supernatant was applied to a HIS-Select® Nickel Affinity Gel (Sigma) for affinity purification of the histidine-tagged rDiGADPH and rDiGAL, according to the manufacturer's instructions. Urea was eliminated for rDiGAL by washing the column with wash buffer (100 mM NaH_2PO_4, and 10 mM Tris–Cl pH 6.3) containing decreasing concentrations of urea (from 6 M to 0 M). Then, the recombinant proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 7.9). The eluted rDiGAPDH and rDiGAL were dialyzed in PBS for 24 h at 4 °C and stored at −80 °C until use. The purity and yield of each protein obtained after purification were assessed in 12% polyacrylamide gels using Coomasie blue staining. The densitometry was calculated with the PDQUEST program (Bio-Rad).

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