Acta Tropica 145 (2015) 8–16

Contents lists available at ScienceDirect

Acta Tropica

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

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

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ARTICLE INFO

*Article history:*
Received 18 November 2014
Received in revised form 29 January 2015
Accepted 30 January 2015
Available online 7 February 2015

*Keywords:*
*Dirofilaria immitis*
Glyceraldehyde 3-phosphate dehydrogenase
Galectin
Plasminogen
Fibrinolysis

ABSTRACT

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.



## 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.

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http://dx.doi.org/10.1016/j.actatropica.2015.01.010
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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).

### 2.4. Bioinformatic analyses

The deduced amino-acid sequence of rDiGAPDH and rDiGAL were analyzed using the following bioinformatic tools: BLAST searching of the homologous sequences in the NCBI and Swissprot/Uniprot databases (http://www.ncbi.nlm.nih.gov/, http://www.uniprot.org/); analysis of conserved domains with SMART (http://smart.embl-heidelberg.de); theoretical isoelectric point (p*I*) and the molecular weight (MW) calculations (http://www.expasy.org/tools/pi_tool.html); prediction of transmembrane domains with the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0); prediction of signal peptides with SignalP 3.0 (Bendtsen et al., 2004) (http://www.cbs.dtu.dk/services/SignalP); search for glycosyl–phosphatidyl anchors in the sequence with the big-PI Predictor (Eisenhaber et al., 2000) (http://mendel.imp.ac.at/sat/gpi/gpi_server.html); multiple sequence alignment with ClustalW 2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and prediction of the secondary structures and three-dimensional modeling with the Swiss-Model server (Arnold et al., 2006; http://swissmodel.expasy.org/) using as templates the X-ray crystal structure of a GAPDH from *B. malayi* (code pdb: 4K9D) for DiGAPDH (identity of 96.76%) and the crystal structure of *Toxascaris leonine* GAL (code pdb: 4HL0) for DiGAL (identity of 90.11%). The 3-D models were visualized with the RasMol software v. 2.7.5.2.

### 2.5. Plasminogen binding assays

To determine whether the rDiGAPDH and rDiGAL would bind plasminogen an ELISA test was carried out as described previously (González-Miguel et al., 2012). In brief, multiwell microplates (Costar) were coated with 0.5 μg/well of each protein, blocked and then incubated with increasing concentrations (from 0 to 30 μg/ml) of human plasminogen (Acris antibodies). After incubation with the corresponding antibodies and with a chromogen, the OD was measured at 492 nm in an easy reader (Bio-Rad). In parallel, competition assays were performed by including 50 mM of the lysine analogue ϵ-aminocaproic acid (ϵACA) during plasminogen incubation.

### 2.6. Plasminogen activation assays

Plasminogen activation assay was performed in a test volume of 100 μl by measuring the amidolytic activity of generated plasmin (González-Miguel et al., 2012). In each well 2 μg of human plasminogen (Acris antibodies) were incubated in PBS with 3 μg of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) in the presence of 1 μg of rDiGAPDH or rDiGAL. Activation of plasminogen was initiated by addition of 15 ng of tPA (Sigma). In parallel, plasmin generation was also measured in the absence of tPA. Plates were incubated at 37 °C for 2 h and the hydrolysis of the chromogenic substrate was monitored by measuring absorbance at 405 nm every 30 min. Each sample was analyzed in triplicate.

### 2.7. Cell culture and stimulation of endothelial cells

Canine aortic endothelial cells (CnAOEC) (Cell Applications, Inc.) were grown in canine endothelial growth mediums (Cell Applications, Inc.). Plates were precoated with an attachment factor solution (Cell Applications, Inc.) and cells were cultured at 37 °C in a humidified atmosphere in the presence of 5% carbon dioxide and 95% air. Medium was changed every 3 days. Endothelial cells ($10^6$ cells/plate) were plated on 100 mm culture plates and grown for 4 days to obtain confluent cultures and treated with 1 μg/ml of rDiGAPDH or rDiGAL for 24 h. Non-stimulated cells were used as controls under the same conditions.

### 2.8. Cell lysates and Western blot analyses

Western blot analysis was performed as previously described (Morchón et al., 2010) with slightly modifications. Treated and control CnAOEC were lysed in ice-cold lysis buffer (20 mM Tris–HCl (pH 7.5), 140 mM NaCl, 10 mM ethylendiaminetetraacetic acid, 10% glycerol, 1% Igepal CA-630, aprotinin, pepstanin, pepstatin, and leupeptin at 1 μg/ml each, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). Protein samples (10 μg) were separated by SDS–PAGE under reducing conditions and blotted onto polyvinylidine difluoride membranes. Membranes were blocked before incubation with the primary antibodies anti-tPA and anti-uPA (Santa Cruz Biotechnology, Inc.) according to the manufacturer's recommendations. After incubation with HRP-conjugated secondary antibodies, bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Anti-α-tubulin antibody (Oncogene Research Products) was used to confirm loading of comparable amount of protein in each lane. Protein expression was quantified by densitometry using Scion Image Software (Scion).

### 2.9. Generation of an anti-rDiGAPDH and anti-rDiGAL antisera

Antisera against *D. immitis* rGAPDH and rGAL were generated by subcutaneous immunization of four New Zealand female rabbits with three doses of each protein in 0.2% saponin solution. First dose of 1 mg at the beginning of the experiment, plus two doses of 500 μg 7 and 10 days later. Rabbits were bled 20 days after the last dose. Sera were collected, serially diluted and titred by ELISA. The reactivity and specificity of the sera were also assessed by Western blot on rDiGAPDH, rDiGAL or on ES extract from *D. immitis* adult worms (DiES) containing the corresponding native proteins. In brief, recombinant proteins (2 μg each) and DiES (10 μg) were electrophoresed on 12% SDS–PAGE gels, electrotransferred onto nitrocellulose membranes, blocked with 2% BSA and incubated with the antisera against rDiGAPDH, rDiGAL or with a negative control serum at 1/500 dilution. After washing, immunoblots were incubated with 1/2000 diluted peroxidase-labeled anti-rabbit IgG and revealed with 4-chloro naphthol. Images were digitized with the scanner GS-800 Densitometer (Bio-Rad) using the Quantity One Software v.4.6.5 (Bio-Rad).

### 2.10. Immunolocalization of proteins in D. immitis adult worms

Immunolocalization assays were carried out in microtome-cut 5 μm sections after dehydrate and embed in paraffin *D. immitis* adult worms. The sections were placed on microscope slides, deparaffinized in xylene, rehydrated and blocked with 1% BSA in PBS. Then, sections were firstly incubated with the anti-rDiGAPDH or anti-rDiGAL rabbit antisera or with a negative serum (rabbit preinmune serum), all of them diluted 1/50 in blocking buffer. Secondly, samples were incubated with an anti-rabbit IgG antibody conjugated to Alexa Fluor 594 (Invitrogen) diluted 1/400 in blocking buffer containing phalloidin-Alexa Fluor 488 (Invitrogen) diluted 1/200, which binds to actin microfilaments. All incubations were carried out for 1 h at 37 °C in a humid chamber and between each step, three washes of 5 min with PBS containing 0.05% Tween-20 were performed. Finally, the samples were washed four times, mounted in antifade reagent (Prolong Gold, Invitrogen) and analyzed with a Leica TCS-NT confocal microscope.

### 2.11. Statistical analysis

The results from the plasminogen-binding assay, plasminogen activation assay and Western blots for the tPA and uPA expression were analyzed with the Student's *t*-test. The results were expressed

as the mean ± SEM of at least three independent experiments. In all experiments, a significant difference was defined as a $p$-value of <0.05 for a confidence level of 95%.

## 3. Results

### 3.1. Amplification, cloning, sequencing, and expression of D. immitis GAPDH and GAL

Amplification of *D. immitis* GAPDH and GAL cDNA by RT-PCR resulted in a PCR product of around 1000 and 850 bp respectively. These were cloned into the pSC-A vector and fully sequenced. BLAST analysis of the sequence demonstrated its identity as glyceraldehyde 3-phosphate dehydrogenase and galectin. The GAPDH new sequence was deposited in the Gen-Bank under accession number JQ780095.1. The full *D. immitis* GAPDH and GAL cDNA contained 1020 and 846 nucleotides, encoded proteins of 339 and 280 amino acids, with a theoretical molecular weight of 36,179 and 32,085 Da, and p*I* of 7.11 and 6.08 respectively.

The bioinformatics analyses of the deduced amino acid sequences did not reveal a signal peptide, transmembrane helices or glycosyl-phosphatidyl inositol anchors. The percentage identity between DiGAPDH and homologous sequences from other organisms (*O. volvulus*, *S. bovis*, *Candida albicans*, *Streptococcus pyogenes*, *Bacillus anthracis* and *Trichomonas vaginalis*) whose GAPDH had been previously related with plasminogen-binding activities was analyzed using multiple sequence alignment with the ClustalW program (Fig. 1). The analysis revealed a range of identities between the 94.99% of the filaria *O. volvulus* and the 43.07% of the protozoa *T. vaginalis*. Additionally, seven conserved lysine residues in all the sequences were found and highlighted. Amino-acid conservation of DiGAPDH and DiGAL was analyzed by alignment with homologous sequences from other parasites (Figs. S1 and S2). DiGAPDH and DiGAL revealed a strong identity with the homologous sequences from other filarial nematodes (*B. malayi*, *O. volvulus*, *Loa loa* and *Wuchereria bancrofti*) ranging from 97.46% and 94.99% in the case of DiGAPDH (Fig. S1) and from 96.79% and 94.81% in the case of DiGAL (Fig. S2). These sequences also showed high identities in the alignment with proteins from other non-filarial parasitic helminths. DiGAPDH revealed identities of 87.61%, 76.33% and 73.96% with the GAPDH from *Ascaris suum*, *S. mansoni* and *Fasciola hepatica* (Fig. S1); and DiGAL revealed identities of 88.13 and 82.73 with GAL from *A. suum* and *Haemonchus contortus* (Fig. S2). Conserved lysine residues of DiGAL were also highlighted. In silico three-dimensional modeling of the molecules predicted the 3D structures showing in the case of DiGAPDH a homo-tetramer with 15 α-helices and 4 β-sheets (Fig. 2A). Molecular modeling of DiGAL showing a monomer with the presence of 2 α-helices and 26 β-sheets (Fig. 2B). Conserved lysine residues were highlighted and were visualized on the outside of the proteins.

The *D. immitis* GAPDH and GAL cDNA were cloned into the expression vector TOPO/pDEST. After induction of expression in *E. coli*, the hexahistidine-tagged recombinant proteins were purified under denaturing conditions using nickel affinity chromatography. The purified recombinant proteins rDiGAPDH and rDiGAL had molecular weights of 38.6 kDa and 34.6 kDa in polyacrylamide gel.

### 3.2. rDiGAPDH and rDiGAL bind plasminogen

The binding level of plasminogen to rDiGAPDH and rDiGAL was assessed by ELISA (Fig. 3). Analyses showed that both recombinant proteins bind plasminogen and that this binding is directly proportional to the amount of plasminogen. Comparing the results obtained by both recombinant proteins, rDiGAPDH showed higher plasminogen-binding capacity than rDiGAL (Fig. 3). The negative control consisting of wells coated only with BSA showed some non-specific binding activity, but always with values significantly lowers than those obtained by rDiGAPDH and rDiGAL ($p < 0.05$). To determine whether or not lysine residues are involved in binding, a competition experiment including 50 mM ϵACA was carried out. In this case the binding was inhibited about 90% in the case of rDiGAPDH and approximately 70% in the case of rDiGAL, resulting in slightly higher optical densities than the negative control (Fig. 3).

### 3.3. rDiGAPDH and rDiGAL enhance the activation of plasminogen by tPA

In order to assess the ability of rDiGAPDH and rDiGAL to activate plasminogen and generate plasmin on their own, the amidolytic activity of plasmin generated in the presence or absence of tPA was measured. Negative controls replacing each recombinant protein for BSA or tPA were also used. Fig. 4 shows the capacity of rDiGAPDH and rDiGAL to stimulate plasmin generation by tPA obtaining optical densities significantly higher than the negative controls ($p < 0.05$). Both proteins obtained similar results and plasminogen-activation did not occur in the absence of tPA. Furthermore this effect is inhibited by 50 mM ϵACA, indicating the involvement of lysine residues in the process.

### 3.4. rDiGAPDH and rDiGAL enhance the expression of uPA and not of tPA in canine endothelial cells

To study the possible effect of rDiGAPDH and rDiGAL on the expression of the main activators of fibrinolysis (tPA and uPA), the parasitic proteins were employed to stimulate CnAOEC in culture. Proteins from rDiGAPDH/rDiGAL-treated or untreated vascular endothelial cell extracts were separated by SDS–PAGE and analyzed by Western blotting using anti-tPA or anti-uPA antibodies. As shown in Fig. 5, both proteins induced a significant increase in uPA protein expression after 24 h of stimulation ($p < 0.01$), being this increase higher in the case of rDiGAPDH stimulation. None of the proteins led to significant differences in the protein expression of tPA.

### 3.5. Immunolocalization of DiGAPDH and DiGAL

In a first step, antisera against rDiGAPDH and rDiGAL were generated. The reactivity and specificity of these antisera were tested in ELISA and Western blot prior to their use in the immunolocalization studies. The antibody titers of these antisera were higher than 1/500, with an OD of 1.12 and 1.07, respectively, while a negative serum showed an OD of 0.12 and 0.16 at the same dilution. The anti-rDiGAPDH and anti-rDiGAL antisera reacted strongly and specifically with the recombinant proteins and with the native GAPDH and GAL proteins in the DiES extract in the Western blot analyses. The negative sera showed no reactivity with any of the proteins tested (data not shown).

The anatomical localization of DiGAPDH and DiGAL was carried out in histological sections of *D. immitis* adult worms by immunofluorescence using the rabbit polyclonal antisera previously generated. As shown in Fig. 6, all sections showed green fluorescence throughout the soma of the parasite, as a result of the binding of phalloidin-Alexa Fluor 488, actin ligand which serves as a positive control of the technique. Sections incubated with the anti-rDiGAPDH or anti-rDiGAL antisera showed, in addition, specific reactivity (in red) against the parasitic GAPDH and GAL, respectively. Both proteins are located scattered throughout all the soma, being especially abundant in the cuticle (reflected by an orange color in the overlay of Phalloidin-Alexa Fluor 488 + Alexa Fluor 594

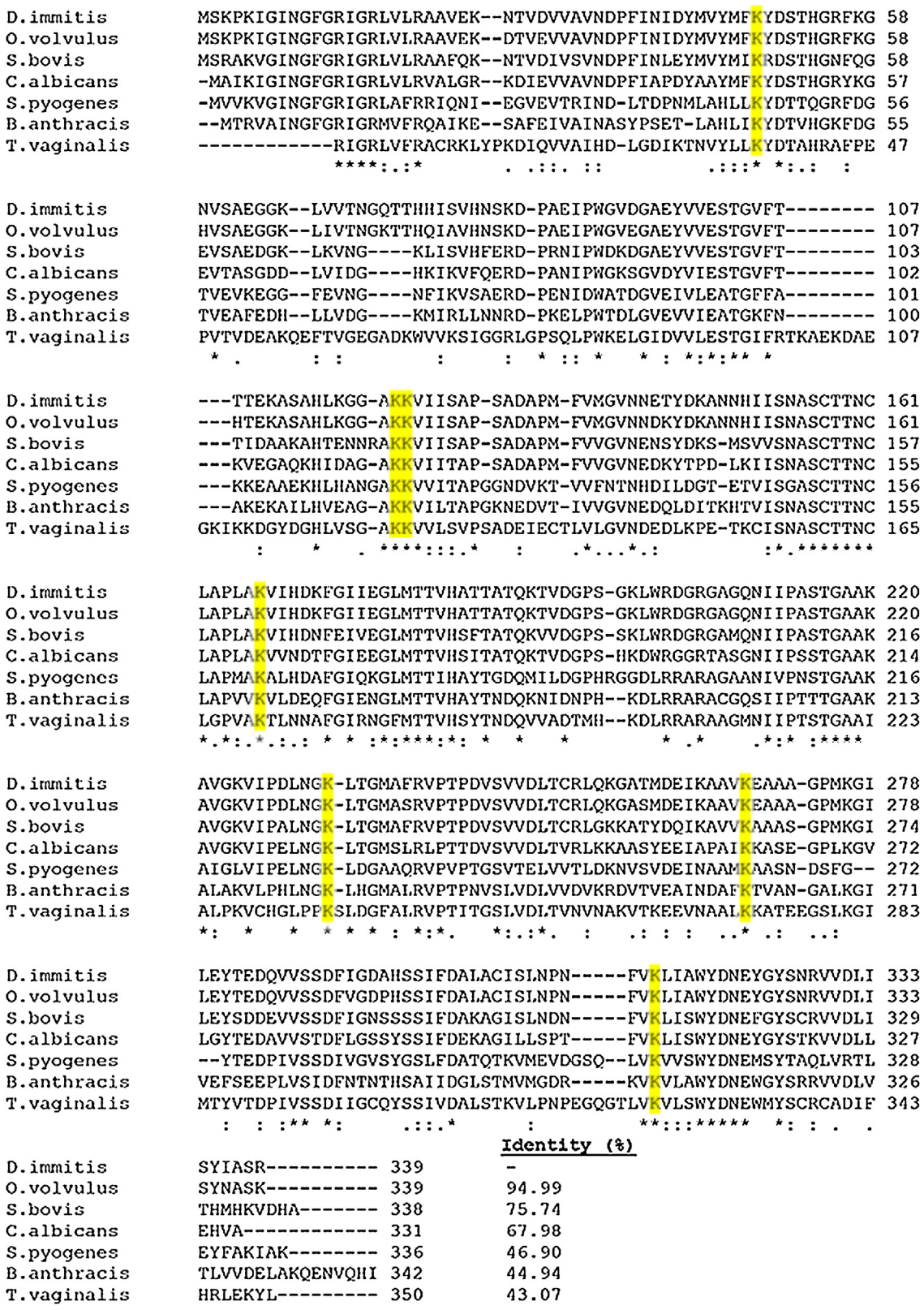


**Fig. 1.** Alignment of the *D. immitis* GAPDH sequence (AFL46382) with the GAPDH from *O. volvulus* (CAA70607), *S. bovis* (ACC78613), *C. albicans* (AAC49800), *S. pyogenes* (AAK33348), *B. anthracis* (AIF58743) and *T. vaginalis* (AAA30325), which have previously been associated with plasminogen-binding activities. The percentage of sequence identity between *D. immitis* sequence and the others is indicated. The amino acids conserved in all the sequences are labeled with asterisks, and conservative and semiconservative substitutions are labeled with two and one point, respectively. Conserved lysine residues are shaded in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

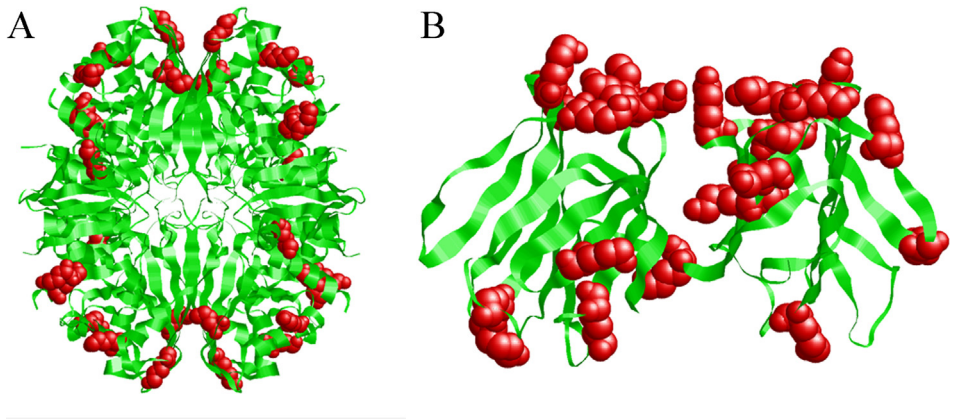


**Fig. 2.** Molecular modeling of *D. immitis* GAPDH (A) and GAL (B). The secondary structure of the proteins were predicted with the Swiss-Model web server (http://swissmodel.expasy.org/) by analogy with the X-ray crystallography available models. The three-dimensional models of the molecules were visualized with the RasMol application v. 2.7.5.2. Conserved lysine residues of proteins were highlighted as red balls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

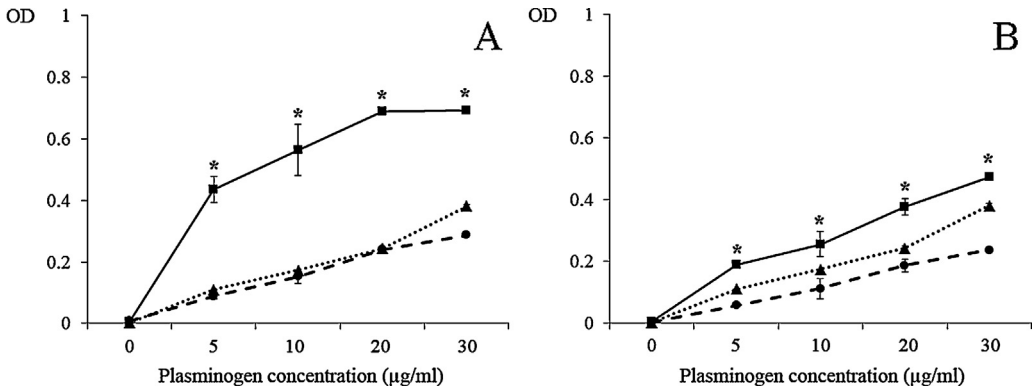


**Fig. 3.** Plasminogen binding to 0.5 μg of rDiGAPDH (A) or rDiGAL (B) measured over a range of plasminogen concentrations using a microtiter plate method. (■) Incubation with increasing concentrations of plasminogen, 0–30 μg/ml. (▲) Competition assay with 50 mM ϵACA included during plasminogen incubation. (●) Negative control consisted of wells coated only with BSA. Each point is the mean of three replicates ± SD. The asterisk (*) designates significant ($p < 0.05$) differences.

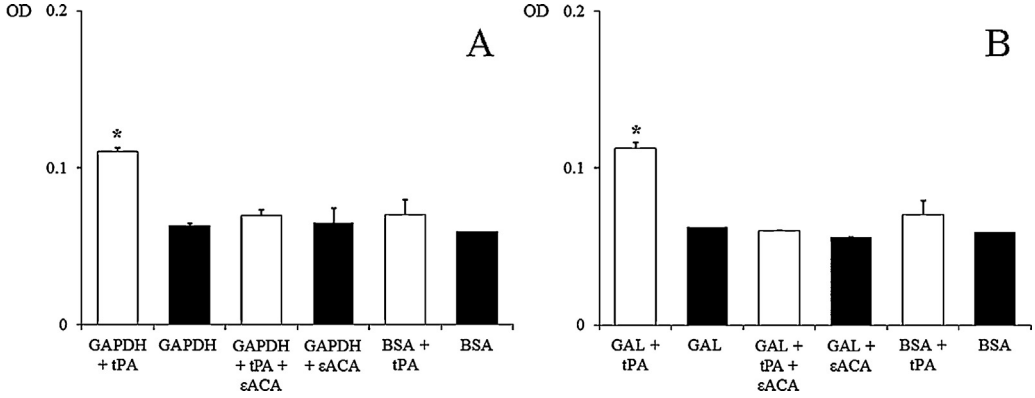


**Fig. 4.** Plasminogen activation and plasmin generation by rDiGAPDH (A) and rDiGAL (B). (□) 15 ng of tPA was added to mixtures containing 2 μg of human plasminogen, 3 μg of D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) and 1 μg of each recombinant protein (or BSA as negative control) in the presence or absence of 50 mM of ϵACA in a test volume of 100 μl. (■) No tPA was added to reaction mixtures. Optical densities measured at 405 nm after 120 min of incubation. Each point is the mean of three replicates ± SD. The asterisk (*) designates significant ($p < 0.05$) differences.

images). Sections incubated with a rabbit negative serum showed no specific red fluorescence from recombinant proteins.

## 4. Discussion

Two recent in vitro studies demonstrated the participation of the ES and surface antigens of *D. immitis* in the activation of the fibrinolytic system. In addition, some of the antigens responsible for this enhancement were identified (González-Miguel et al., 2012, 2013). Taking into account these previous data and the importance of the anticlotting mechanisms for *D. immitis*, a parasite that survives for years in the pulmonary arteries of its host, the objective of this work was to investigate the participation of the *D. immitis* GAPDH and GAL in the fibrinolytic system activation using recombinant forms of both proteins. This involves knowing whether or not these proteins are able to bind plasminogen, stimulate plasmin

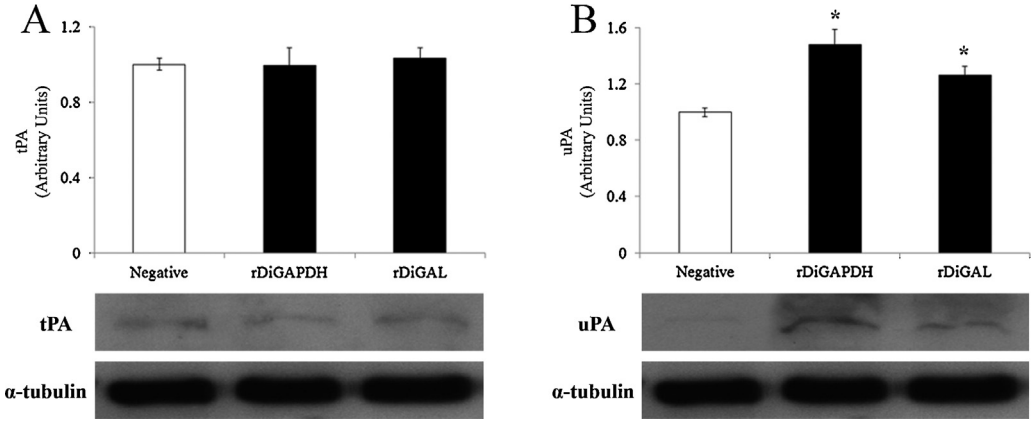


**Fig. 5.** Effect of rDiGAPDH and rDiGAL on the expression of tPA and uPA in canine vascular endothelial cells. Protein extracts from lysed rDiGAPDH or rDiGAL treated or untreated confluent cell cultures were analyzed by Western blot for tPA and uPA. α-Tubulin served as a protein control. Results were expressed as the mean ± SEM of at least three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences from control cells. (■) Stimulated endothelial cells with 1 μg/ml of rDiGAPDH or rDiGAL. (□) Non-treated control cells.

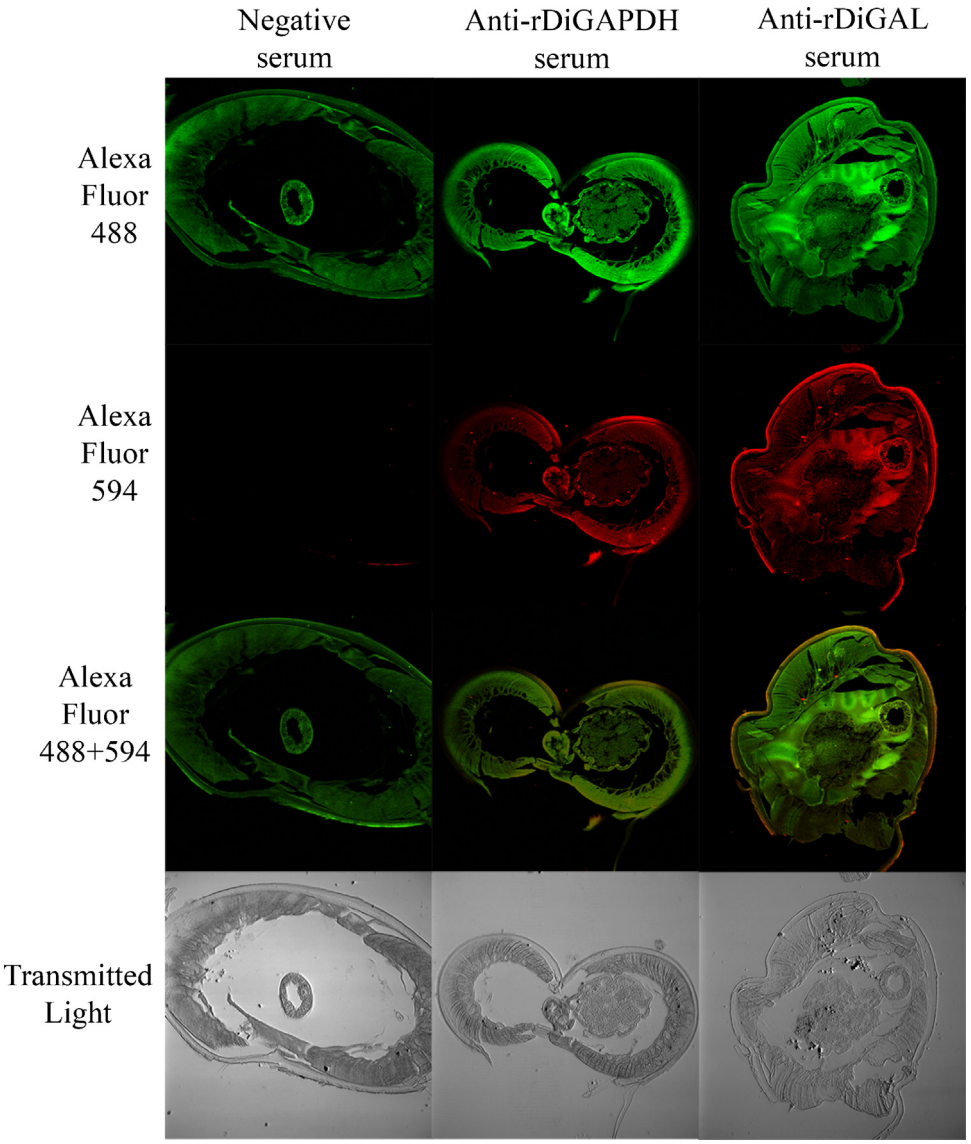


**Fig. 6.** Immunolocalization of DiGAPDH and DiGAL in sections from *D. immitis* adult worms. Images of parasite sections incubated with phalloidin-Alexa Fluor 488 (in green, specific binding to Actin) plus the negative or the anti-rDiGAPDH or anti-rDiGAL rabbit sera and an anti-rabbit IgG-Alexa Fluor 594 (in red). Corresponding transmitted light images are also adressed. Magnification 4×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

generation, interact with the expression of the main fibrinolytic activators and express in an antigenic compartment of the parasite in contact with the blood of the host.

Two peptide sequences of 339 and 280 amino acids were respectively obtained by cloning and sequencing of the *D. immitis* GAPDH and GAL cDNAs. The subsequent bioinformatics analysis have highlighted the high degree of evolutionary conservation of these proteins, both in the structural characteristics of their 3D models, and in the multiple sequence alignments carried out with homologous proteins from other helminth parasite species. On the other hand, none of the two proteins showed structural motifs for their transport or expression on the cell surface (signal peptide, transmembrane motifs or GPI anchors). However, both proteins have been identified by immunoproteomic techniques in the ES and surface extracts of *D. immitis* (González-Miguel et al., 2012, 2013). This may be related to unconventional mechanisms of protein transport, as for example with the association of these proteins with exosome-like secretion vesicles. This has been postulated as an extracellular transport mechanism both for glycolytic enzymes from several groups of parasites (Gómez-Arreaza et al., 2014) and for galectins (Nickel, 2003).

Both the rDiGAPDH and rDiGAL showed ability to bind plasminogen (higher in the case of rDiGAPDH) and to stimulate plasmin generation in an in vitro system. Plasminogen activation occurred only in the presence of the tPA, as observed previously using the ES and surface antigens from *D. immitis* (González-Miguel et al., 2012, 2013) or GAPDH from different bacteria, fungi or parasites species (reviewed by Figuera et al., 2013). In addition, to our knowledge, this is the first time that GAL is proposed as a physiological receptor of plasminogen. Competition assays with the $\epsilon$-ACA acid revealed the involvement of lysine residues from both proteins in the binding of plasminogen. The interaction between plasminogen and their receptors has been related to the presence of carboxyl-terminal lysine residues (Plow et al., 1995). DiGAPDH alignment with homologous sequences from other organisms that have been related to plasminogen-binding activities (Figuera et al., 2013) shows that the carboxyl-terminal domain of these proteins are not highly conserved, and that in some cases domains lack lysines (see Fig. 1). However, there are highly conserved internal lysine residues in the amino acid sequences of DiGAPDH and DiGAL. Therefore, it is possible that conserved internal lysine residues are involved in the binding of plasminogen to these proteins as it has been postulated for the enolase of *Streptococcus pneumoniae* (Ehinger et al., 2004). In addition, after viewing the spatial location of the conserved internal lysine residues of the DiGAPDH and DiGAL in their 3D models, these residues seem to be located externally in these molecules, which would facilitate the accessibility of plasminogen.

rDiGAPDH and rDiGAL did not cause a stimulation of basal tPA production in canine endothelial cell cultures. However, it has been shown that whole *D. immitis* ES antigens are able to produce an overexpression of this fibrinolytic activator in human endothelial cells in culture (González-Miguel et al., 2012). This suggests that other molecules of *D. immitis* are responsible for this process. On the other hand, rDiGAPDH and rDiGAL produced a significant stimulation of the basal uPA production in canine endothelial cells in culture. To our knowledge, this is the first time that the relationship between a parasitic antigen and the overproduction of an activator of fibrinolysis in an in vitro system has been demonstrated. This could have particular relevance since uPA, in addition to its role as activator of fibrinolysis, plays a key role in tissue remodeling inducing proliferation and cell migration (Nicholl et al., 2005a), and high levels in its expression are related to cardiovascular disease (Fuhrman, 2012).

In order to ascertain whether the ability of rDiGAPDH and rDiGAL to bind plasminogen may have relevance in vivo, it is necessary that these proteins are expressed and/or located in tissues of the parasite in close contact with the host blood (Hawley et al., 2000). Immunofluorescence study indicates that DiGAPDH and DiGAL are especially abundant on the surface of *D. immitis*, in addition to have an intracellular localization which is expected for a glycolytic enzyme and a lectin. In the case of the GAPDH, this is consistent with the results of a recent study in which it was identified as one of the five most abundant proteins in the cuticle of *D. immitis* (Morchón et al., 2014).

The ability of these molecules to enhance the activation of fibrinolysis could be an important survival mechanism for *D. immitis* in its intravascular environment. However, overstimulation of the plasminogen/plasmin system has been related to cellular invasion and intra-organic migration in different pathogens (Jong et al., 2003; Bernal et al., 2004). In addition, the overproduction of plasmin has been linked with the proliferation and migration of vascular cells and with the degradation of extracellular matrices in humans (Nicholl et al., 2005b Yang et al., 2005; Roth et al., 2006; Hayashi et al., 2009). This suggests a possible involvement of the over-activation of the fibrinolytic system by *D. immitis* antigens in the long-term development of the pathogenic mechanisms that occur during cardiopulmonary dirofilariosis.

In conclusion, we have shown that *D. immitis* GAPDH and GAL are able to bind plasminogen and enhance plasmin generation by tPA with the involvement of lysine residues. In addition, these proteins stimulate the expression of the fibrinolytic activator uPA on canine endothelial cells in culture and they are expressed on the surface of the worms. Therefore, DiGAPDH and DiGAL could be used by *D. immitis* to stimulate the activation of the fibrinolytic system through the plasminogen binding to its surface or excreted molecules, as a mechanism to avoid blood clot formation in its close environment.

## Acknowledgements

This research was supported by Agencia de Desarrollo Económico de Castilla y León (cofinanced with FEDER funds), Junta de Castilla y León (grant no. SA090/A09), Spain.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.01.010.

## References

Arnold, K.M., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201.

Astorgues-Xerri, L., Riveiro, M.E., Tijeras-Raballand, A., Serova, M., Neuzillet, C., Albert, S., Raymond, E., Faivre, S., 2014. Unraveling galectin-1 as a novel therapeutic target for cancer. Cancer Treat. Rev. 40, 307–319.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.

Bernal, D., de la Rubia, J.E., Carrasco-Abad, A.M., Toledo, R., Mas-Coma, S., Marcilla, A., 2004. Identification of enolase as a plasminogen-binding protein in excretory–secretory products of *Fasciola hepatica*. FEBS Lett. 563, 203–206.

Bhattacharya, S., Ploplis, V.A., Castellino, F.J., 2012. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J. Biomed. Biotechnol. 2012, 482096.

Cesarman-Maus, G., Hajjar, K.A., 2005. Molecular mechanisms of fibrinolysis. Br. J. Haematol. 129, 307–321.

Crowe, J.D., Sievwright, I.K., Auld, G.C., Moore, N.R., Gow, N.A., Booth, N.A., 2003. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. Mol. Microbiol. 47, 1637–1651.

de la Torre-Escudero, E., Manzano-Román, R., Pérez-Sánchez, R., Siles-Lucas, M., Oleaga, A., 2010. Cloning and characterization of a plasminogen-binding surface-associated enolase from *Schistosoma bovis*. Vet. Parasitol. 173, 76–84.

Ehinger, S., Schubert, W.D., Bergmann, S., Hammerschmidt, S., Heinz, D.W., 2004. Plasmin(ogen)-binding alpha-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. J. Mol. Biol. 343 (4), 997–1005.

Eisenhaber, B., Bork, P., Yuan, Y., Loeffler, G., Eisenhaber, F., 2000. Automated annotation of GPI anchor sites: case study *C. elegans*. Trends Biochem. Sci. 25, 340–341.

Erttmann, K.D., Kleensang, A., Schneider, E., Hammerschmidt, S., Büttner, D.W., Gallin, M., 2005. Cloning, characterization and DNA immunization of an *Onchocerca volvulus* glyceraldehyde-3-phosphate dehydrogenase (Ov-GAPDH). Biochim. Biophys. Acta 1741, 85–94.

Figuera, L., Gómez-Arreaza, A., Avilán, L., 2013. Parasitism in optima forma: exploiting the host fibrinolytic system for invasion. Acta Trop. 128, 116–123.

Fuhrman, B., 2012. The urokinase system in the pathogenesis of atherosclerosis. Atherosclerosis 222, 8–14.

Genchi, C., Kramer, L.H., Prieto, G., 2001. Epidemiology of canine and feline dirofilariasis: a global view. In: Simón, F., Genchi, C. (Eds.), Heartworm infection in humans and animals. Ediciones Universidad de Salamanca, Salamanca, Spain, pp. 121–134.

Gómez-Arreaza, A., Acosta, H., Quiñones, W., Concepción, J.L., Michels, P.A., Avilán, L., 2014. Extracellular functions of glycolytic enzymes of parasites: unpredicted use of ancient proteins. Mol. Biochem. Parasitol. 193, 75–81.

González-Miguel, J., Morchón, R., Mellado, I., Carretón, E., Montoya-Alonso, J.A., Simón, F., 2012. Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium. Mol. Biochem. Parasitol. 181, 134–140.

González-Miguel, J., Morchón, R., Carretón, E., Montoya-Alonso, J.A., Simón, F., 2013. Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system. Vet. Parasitol. 196, 235–240.

Hayashi, M., Matsuzaki, Y., Shimonaka, M., 2009. Impact of plasminogen on an in vitro wound healing model based on a perfusion cell culture system. Mol. Cell. Biochem. 322, 1–13.

Hawley, S.B., Green, M.A., Miles, L.A., 2000. Discriminating between cell surface and intracellular plasminogen-binding proteins: heterogeneity in profibrinolytic plasminogen-binding proteins on monocytoid cells. Thromb. Haemost. 84, 882–890.

Jolodar, A., Fischer, P., Bergmann, S., Büttner, D.W., Hammerschmidt, S., Brattig, N.W., 2003. Molecular cloning of an alpha-enolase from the human filarial parasite *Onchocerca volvulus* that binds human plasminogen. Biochim. Biophys. Acta 1627, 111–120.

Jong, A.Y., Chen, S.H., Stins, M.F., Kim, K.S., Tuan, T.L., Huang, S.H., 2003. Binding of *Candida albicans* enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. J. Med. Microbiol. 52, 615–622.

Joseph, G.T., Huima, T., Klion, A., Lustigman, S., 2000. A novel developmentally regulated galectin of *Onchocerca volvulus*. Mol. Biochem. Parasitol. 106, 187–195.

Lama, A., Kucknoor, A., Mundodi, V., Alderete, J.F., 2009. Glyceraldehyde-3- phosphate dehydrogenase is a surface-associated: fibronectin-binding protein of *Trichomonas vaginalis*. Infect. Immun. 77, 2703–2711.

Mebius, M.M., van Genderen, P.J., Urbanus, R.T., Tielens, A.G., de Groot, P.G., van Hellemond, J.J., 2013. Interference with the host haemostatic system by schistosomes. PLoS Pathog. 9, e1003781.

Morchón, R., González-Miguel, J., Mellado, I., Velasco, S., Rodríguez-Barbero, A., Simón, F., 2010. Adult *Dirofilaria immitis* excretory/secretory antigens upregulate the production of prostaglandin E2 and downregulate monocyte transmigration in an in vitro model of vascular endothelial cell cultures. Vet. Parasitol. 170, 331–335.

Morchón, R., González-Miguel, J., Carretón, E., Kramer, L.H., Valero, L., Montoya-Alonso, J.A., Simón, F., Siles-Lucas, M., 2014. Proteomic analysis of the somatic and surface compartments from *Dirofilaria immitis* adult worms. Vet. Parasitol. 203, 144–152.

Nicholl, S.M., Roztocil, E., Davies, M.G., 2005a. Urokinase-induced smooth muscle cell responses require distinct signaling pathways: a role for the epidermal growth factor receptor. J. Vasc. Surg. 41, 672–681.

Nicholl, S.M., Roztocil, E., Galaria, I.I., Davies, M.G., 2005b. Plasmin induces smooth muscle cell proliferation. J. Surg. Res. 127, 39–45.

Nickel, W., 2003. The mystery of nonclassical protein secretion. A current view on cargoproteins and potential export routes. Eur. J. Biochem. 270, 2109–2119.

Plow, E.F., Herren, T., Redlitz, A., Miles, L.A., Hoover-Plow, J.L., 1995. The cell biology of the plasminogen system. FASEB J. 9 (10), 939–945.

Ramajo-Hernández, A., Pérez-Sánchez, R., Ramajo-Martín, V., Oleaga, A., 2007. Schistosoma bovis: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. Exp. Parasitol. 115, 83–91.

Roda, O., Ortiz-Zapater, E., Martínez-Bosch, N., Gutiérrez-Gallego, R., Vila-Perelló, M., Ampurdanés, C., Gabius, H.J., André, S., Andreu, D., Real, F.X., Navarro, P., 2009. Galectin-1 is a novel functional receptor for tissue plasminogen activator in pancreatic cancer. Gastroenterology 136, 1379–1390.

Roth, D., Piekarek, M., Paulsson, M., Christ, H., Bloch, W., Krieg, T., Davidson, J.M., Eming, S.A., 2006. Plasmin modulates vascular endothelial growth factor-A-mediated angiogenesis during wound repair. Am. J. Pathol. 168, 670–684.

Tristan, C., Shahani, N., Sedlak, T.W., Sawa, A., 2011. The diverse functions of GAPDH: views from different subcellular compartments. Cell Signal. 23, 317–323.

Venco, L., 2007. Heartworm (*Dirofilaria immitis*) disease in dogs. In: Genchi, C., Ronaldi, L., Cringoli, G. (Eds.), *Dirofilaria immitis* and *D. repens* in Dog and Cat and Human Infections. Rolando Editore, Italia, pp. 117–125.

Yang, Z., Eton, D., Zheng, F., Livingstone, A.S., Yu, H., 2005. Effect of tissue plasminogen activator on vascular smooth muscle cells. J. Vasc. Surg. 42, 532–538.