

Research Article

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Protein phosphatase PP2C in the flagellum of *Leishmania major*: cloning and characterization

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Abstract

The main goal of this work consisted in cloning, purifying and characterizing a protein phosphatase 2C (PP2C) from promastigotes of *Leishmania major*. The gene was cloned and amplified by PCR using specific oligonucleotides and the recombinant protein was purified by affinity chromatography. The peak with maximal protein concentration was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and revealed a protein of 44.9 kDa with PP2C activity. This activity was dependent on divalent cations (Mg^{+2} and Mn^{+2}) and was optimal at pH of 8.5, using phosphothreonine as the substrate. Sanguinarine inhibited the activity of the recombinant *LmPP2C*, while protein tyrosine phosphatase inhibitors had no effect. The recombinant *LmPP2C* was used to generate polyclonal antibodies. These antibodies recognized a protein of 44.9 kDa in different *Leishmania* species; the *LmPP2C* was localized in the flagellar pocket and the flagellum of promastigotes.

Introduction

The parasite *Leishmania major* is the causative agent of cutaneous leishmaniasis in the Old World. *Leishmania* survives and replicates within host macrophages and evades numerous host defences by inhibition of important cell functions, including the modulation of host cell signalling pathways through phosphorylation and dephosphorylation mechanisms. Protein phosphorylation and dephosphorylation processes are regulated by protein kinases and phosphatases, respectively, which generally occur on tyrosine, serine or threonine residues (Cohen, 2002; Wang *et al.* 2008). Protein phosphatases are classified in three families based on substrate specificity, sequence alignment, structure and their catalytic mechanism, which are: PPP (phosphoprotein phosphatases), PPM (metal-dependent protein phosphatases) and PTP (protein tyrosine phosphatases) (Moorhead *et al.* 2007). The PPP family consists of the phosphatases PP1, PP2A and PP2B. The PPM family contains type 2C protein phosphatases (PP2C) and pyruvate dehydrogenase phosphatase (PDP). The PP2C is a monomeric enzyme that requires the metal cations Mg^{+2} or Mn^{+2} for enzymatic activity (Das *et al.* 1996; Andreeva and Kutuzov, 2004; Schweighofer *et al.* 2004; Lammers and Lavi, 2007; Moorhead *et al.* 2007). In the human genome, 16 genes have been identified which generate 22 isoforms by a process of alternative splicing; each of these enzymes regulates distinct signalling pathways (Das *et al.* 1996). The roles of PP2C in eukaryotic cells are diverse and have been described in different organisms such as the plant *Arabidopsis thaliana*, in which 76 candidates of PP2C-type phosphatases were identified in its genome (Schweighofer *et al.* 2004). Different PP2C from *A. thaliana* participate in various signalling pathways, such as the one regulated by abscisic acid and the signalling pathways activated by mitogen protein (Mitula *et al.* 2015). In protozoan parasites such as *Plasmodium falciparum*, an unusual PP2C phosphatase (*PfPP2C*) was identified. It has two catalytic sites, one with serine and the other with threonine, with an identity between them of about 9% (Mamoun *et al.* 1998). Sporozoites of *Plasmodium yoelli* and *Plasmodium berguei*, incubated with increasing concentrations of potassium, enhanced the infectivity of the parasites and the copy number of the transcripts for PP2C was increased, when compared with parasites not exposed to potassium in which the expression of the enzyme was kept at basal levels (Kumar *et al.* 2007). *Toxoplasma gondii* also has a protein phosphatase PP2C (*TgPP2C*), which is secreted from the rhoptries, a group of secretory organelles involved in host cell invasion. Once inside the parasitophorous vacuole, *TgPP2C* is transported to the nucleus of the host cell. In contrast, a PP2C knockout strain had reduced intracellular growth, which was reversed by complementation with the wild-type gene for PP2C (Gilbert *et al.* 2007). PP2C was cloned in the parasite

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Leishmania chagasi (LcPP2C); however, there are no reports of its function in the parasite or in the host cell (Burns *et al.* 1993). The genome analysis of *L. major* predicted the existence of 45 phosphatases of the PPP family and 15 of the PPM family; however, the biochemical characterization and localization of these enzymes in this parasite have not yet been reported. We previously identified a membrane-bound PTP in *L. major* that differs in expression, activity and ultrastructural localization between the procyclic and metacyclic stages of the parasite life cycle (Aguirre-García *et al.* 2006). We now report the cloning, expression, purification and biochemical characterization of PP2C from *L. major*. The enzyme was identified in different *Leishmania* species and its ultrastructural localization is shown in *L. major* promastigotes.

Materials and methods

Culture of *Leishmania spp* promastigotes

Leishmania major MHOM/SU/73/5-ASKH (a generous gift from Dr H. Moll, University of Würzburg, Germany), *Leishmania (L.) mexicana* MNYC/BZ/62/M379, *Leishmania (L.) venezuelensis* MHOM/VE/80/PMH3, *Leishmania (L.) amazonensis* IFLA/BR/67/PH8, *Leishmania (V.) panamensis* MHOM/PA/71/LS94, *Leishmania (V.) braziliensis* MHOM/BR/75/M2903, *Leishmania (V.) donovani*, [a generous gift from Dr Nancy Sarabia, Centro Internacional de Entrenamiento e Investigación Médica in Cali, Colombia (CIDEIM)], promastigotes were cultivated in RPMI 1640 medium, which contained penicillin (100 units mL⁻¹), streptomycin (100 µg mL⁻¹) and 10% fetal bovine serum, at 26 °C (all reagents were from Gibco, Invitrogen Corporation Carlsbad, CA, USA). The cultured promastigotes were washed twice with phosphate-buffered saline (PBS) at pH 7.5, centrifuged for 10 min at 2500 × g at room temperature (RT).

Extraction of DNA and amplification of the gene for PP2C from *L. major*

Total DNA was extracted from *L. major* promastigotes using TRIZOL. The gene was amplified using the forward oligonucleotide (Fw) 5'ATGGGCATTCCACTTCCGA3' and reverse oligonucleotide (Rv) 5'TCACTGCGTCTGCTCACC3'. The PCR was performed using 100 µL of the following reaction mixture: 1.5 mM MgCl₂; 0.2 mM deoxynucleoside triphosphates; 100 ng of the corresponding oligonucleotides; 10 ng genomic DNA and one unit of Taq DNA polymerase (Invitrogen) using 40 cycles for 5 min at 94 °C, 30 s, 1 min at 50.7 °C, 30 s and 48 s at 72 °C, and a final extension of 10 min at 72 °C. The gene was cloned into pET-23b expression plasmid using the *Nde* I and *Hind* III restriction sites. The gene was completely sequenced and transformed into BL21 (DE3) pLysS cells (Novagen, Madison, WI, USA). Bacteria containing the plasmid with the gene of protein phosphatase PP2C of *L. major* were grown in Luria-Bertani medium and selected for ampicillin resistance (100 µg mL⁻¹) at 37 °C allowing them to grow until the OD of the culture at 600 nm reached 0.8–1.0. Expression of the recombinant protein was induced with 0.4 mM isopropyl-thio-β-D-galactopyranoside (IPTG) for 3 h. After incubation, bacteria were harvested by centrifugation and processed immediately, or frozen at –70 °C until use.

Purification of recombinant PP2C from *L. major* (LmPP2C)

Cell cultures in volumes of 100 mL were centrifuged and suspended in 50 mL lysis buffer (50 mM Tris HCl, pH 8; 300 mM NaCl; 1 mM benzamidine, 100 µM leupeptin, 2 µg mL⁻¹ aprotinin

and 5 mM imidazole). The suspension was sonicated five times for 40 s with resting periods of 2 min between each interval and 32% amplitude, using a ModelVCX 650 Ultrasonic processor (Ultrasonics, Inc.). The homogenate was centrifuged at 21 000 × g for 1 h at 4 °C to obtain the supernatant with the soluble protein. The supernatant was loaded onto a Ni-charged column previously equilibrated with binding buffer (50 mM Tris-HCl, pH 8; 300 mM NaCl and 5 mM imidazole). The recombinant protein (LmPP2C) was purified and eluted with an elution buffer (50 mM Tris-HCl, pH 8; 300 mM NaCl and 50–500 mM imidazole) and the protein concentration was quantified using the Bradford method (Bradford, 1976).

Production of polyclonal antiserum

Anti-LmPP2C antibodies were generated in rabbits as previously described by Montfort and collaborators (Montfort *et al.* 1994). Before the immunization, the rabbits were bled, and pre-immune serum was collected. Briefly, rabbits were injected intramuscularly with 150 µg of recombinant LmPP2C emulsified in complete Freund's adjuvant; the same procedure was repeated 2 weeks later without adjuvant. The immunization was done with two weekly intramuscular injections, after which the animals were bled and antiserum was separated by centrifugation and stored at –20 °C. The pre-immune and immune sera were used for Western blot analysis. Rabbits were housed at the animal facilities of the Unidad de Investigación en Medicina Experimental de la Facultad de Medicina, UNAM, and their handling was done following the National Ethical Guidelines for Animal Health NOM-062-ZOO-1999 and the guidelines recommended for animal care by the Ethical Committee of the Medical School of the UNAM.

Phosphatase activity assays

p-NPP substrate

Acid phosphatase activity was determined as described by Dissing *et al.* (1979). Briefly, 0.7 µg of recombinant LmPP2C was incubated in the following buffers: 50 mM MES, pH 5–6.5; 50 mM MOPS, pH 7–7.5; 50 mM HEPES, pH 8; 50 mM TRIS, pH 8.5–9 and 50 mM CAPS, pH 10–11 and added to 10 mM MgCl₂ and 10 mM *p*-nitrophenyl phosphate [*p*-NPP] in a final volume of 100 µL for 60 min at 37 °C. Afterwards, the reaction was stopped with 20 µL of 2 N NaOH. The absorbance was read at 405 nm using a micro titre plate reader. Divalent cations (10 mM MgCl₂ or MnCl₂) were added in 50 mM HEPES, pH 8.

Phosphopeptides

Tyrosine and serine/threonine phosphatase activity was assayed using Promega's non-radioactive tyrosine phosphatase assay system. The release of inorganic phosphate (*P_i*) was monitored by measuring the absorbance of the molybdate-malachite green-phosphate complex. A 0.7 µg of recombinant LmPP2C was incubated in a total volume of 100 µL of assay buffer containing 50 mM HEPES pH 8.0 plus 10 mM MgCl₂. The reaction was started by adding 50 µM Tyr phosphopeptide-1 substrate [END (pY) INASL], 50 µM Thr [RRA (pT)VA] during 30 min at RT and stopped with 50 µL molybdate dye/additive mixture. The optical density of the samples was read at 630 nm, using a curve of phosphates as standard (Aguirre-García *et al.* 2006).

Effect of inhibitors of phosphatases on recombinant LmPP2C

The activity of recombinant LmPP2C (0.7 µg) was analysed in the presence of specific PTP inhibitors such as 200 µM sodium orthovanadate, 200 µM ammonium molybdate and 50 µM sodium

pervanadate. Additionally, serine/threonine phosphatase inhibitors were tested such as 5 nM calyculin and 1 μ M okadaic acid.

Sanguinarine, a specific inhibitor for PP2C, was used at 0.1, 0.5, 1, 5, 10 and 20 μ M. For the inhibition assays, 100 μ L of the reaction mixture was pre-incubated for 15 min at RT before adding the *p*-NPP substrate (all reagents from Sigma-Aldrich, St. Louis, MO, USA) and were incubated thereafter for another 60 min at 37 °C. Afterwards, the reaction was stopped with 20 μ L of 2 N NaOH. The absorbance at 405 nm was read using a micro titre plate reader. Recombinant PTP from *Yersinia enterocolitica* (YePTP) was used as a control of PTP activity (purchased from Calbiochem, La Jolla, CA, USA).

SDS-PAGE and Western blot

Identification of the His-Tag in recombinant LmPP2C

Total extract (TE) of bacteria was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels and then electro transferred to nitrocellulose membranes. The blots were incubated with a polyclonal HRP anti-His₆-Tag antibody at a 1:1000 dilution in TBS-T (200 mM Tris-HCl, 150 mM NaCl, 0.005% Tween-20) and washed four times every 10 min with TBS-T.

Identification of PP2C in TEs of *L. (L.) mexicana*, *L. (N.) panamensis*, *L. (N.) braziliensis*, *L. (L.) venezuelensis*, *L. (N.) donovani*, *L. (L.) amazonensis*, *L. major* promastigotes

All promastigotes were harvested by centrifugation at 2000 \times g for 10 min and then washed three times with PBS. The pellet containing the parasites was suspended in cold lysis buffer (10 mM imidazole pH 7.2, 2 μ g mL⁻¹ leupeptin, 10 μ g mL⁻¹ aprotinin, 2 mM benzamidine) and then sonicated (TE). Afterwards, 10 μ g of TE from different *Leishmania* species and were analysed by SDS-PAGE in 10% acrylamide gel and electro transferred onto nitrocellulose membranes. The PP2C protein was used as a control. These were washed with TBS-T and blocked by treatment with 5% milk in TBS-T for 1 h; then they were incubated with antibodies against LmPP2C using a dilution of 1:1000 in 1% milk in TBS-T overnight at 4 °C. After this incubation, the membranes were washed with TBS-T and incubated with the secondary HRP-goat anti-rabbit IgG antibody (Biomedica, Foster City, CA, USA) at a dilution of 1:5000 in TBS-T with 3% of milk. The membranes were finally washed 10 times in TBS-T.

Bands were detected using enhanced chemiluminescent substrate (Super-Signal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA), following the manufacturer's instructions.

Fluorescence microscopy

Leishmania major promastigotes were washed with PBS and allowed to adhere to poly-L-lysine-coated cover slides for 90 min. They were then fixed with 2% paraformaldehyde in PBS for 30 min at RT and washed three times with PBS. Afterwards, these cells were permeabilized with 5% triton X-100 and blocked with 5% BSA for 30 min. They were then incubated overnight at 4 °C with antibodies against the recombinant LmPP2C using a 1:100 dilution. The parasites were washed with PBS after the incubation and stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG secondary antibodies at a 1:200 dilution. Cells were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) at a 1:1000 dilution in PBS. Cover slides were mounted with vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and observed in a fluorescence microscope (Axioskop 2 mot plus, Carl Zeiss, Germany). The images were recorded in an AxioCam HR digital

camera and processed with the Axiovision software (Carl Zeiss, Version 4.8), (Gomez de Leon *et al.* 2014).

Immunolocalization of PP2C by immune electron microscopy (IEM)

Detection of PP2C in *L. major* promastigotes was achieved by electron microscopy (Gomez de Leon *et al.* 2014). Briefly, promastigotes were washed with PBS and then fixed with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS for 1 h at RT. Once washed, the promastigotes were gradually dehydrated in ethanol and embedded in LR White resin (London Resin, Polysciences, Inc., Warrington, PA, USA). Promastigotes in resin were deposited in plastic moulds and were polymerized overnight, under ultraviolet light, at 4 °C. Thin sections of the blocks were obtained in an Ultracut E ultra-microtome (Reichert Jung, Austria) and then mounted on Formvar-covered nickel grids. Immunogold labelling was carried out by flotation of the mounted sections on drops of the respective solutions; non-specific labelling was diminished by incubation with 1% skim milk and PBS-T (PBS and 0.05% Tween-20) for 30 min. Grids with the sections were incubated with the antibody against LmPP2C at a 1:10 dilution in PBS-T for 1 h at RT and overnight at 4 °C. Grids were washed with PBS-T and then incubated with a goat anti-rabbit polyclonal antibody coupled to 10 nm gold particles for 2 h at RT (Zymed, Thermo Scientific, PA, USA) (1:40 dilution in PBS-T). After thorough washings in PBS and distilled water, sections were contrasted with 2% uranyl acetate and a saturated solution of lead citrate and then examined with a transmission electron microscope (TEM, JEOL 1400x, JEOL Ltd, Japan). As negative control, sections were incubated with pre-immune rabbit serum diluted in PBS-T and then with the secondary antibody coupled to gold particles.

The identification of the subcellular structures positive for PP2C was performed using thin sections of parasites that were processed to preserve the ultrastructure. (Gomez de Leon *et al.* 2014). Briefly, promastigotes were fixed for 1 h in 2.5% glutaraldehyde. After thorough rinsing in PBS, parasites were post-fixed for 1 h in 1% OsO₄ at 4 °C, rinsed, gradually dehydrated in ethanol, and finally embedded in Spurr's resin. Thin sections were obtained with an Ultracut E ultramicrotome and stained with uranyl acetate and lead citrate. Copper grids with the sections were examined in the TEM at 80 keV. Digital images were obtained and processed with Adobe Photoshop software (USA).

Bioinformatic analysis

Homology modelling was performed using the SWISS-MODEL server (<http://swissmodel.expasy.org/interactive>); the model was validated with the Mod Eval software version master.e8f19a7 (<https://modbase.compbio.ucsf.edu/evaluation/>), and the structure obtained was edited using the Swiss-PDBViewer programme version 4.1 (<http://www.expasy.org/spdbv/>). Secondary structure prediction was carried out using PSIPRED Protein Sequence Analysis Workbench version 3.3 (<http://bioinf.cs.ucl.ac.uk/psipred/>) (Melo *et al.* 2002; Arnold *et al.* 2006; Shen and Sali, 2006; Bordoli *et al.* 2009; Guex *et al.* 2009; Buchan *et al.* 2013; Biasini *et al.* 2014).

Phylogenetic analysis of *L. major* PP2Cs

The non-rooted phylogenetic tree was constructed using the MEGA 6 programme from the alignment of 15 amino acid sequences PP2Cs present in the *L. major* genome with the protein sequence of the LmxM.25.0.750 gene of *L. mexicana* and the protein sequence of the human PP2C (PPM1A, NP_066283.1), which was used as seed.

Results

Cloning, expression and purification of PP2C of *L. major*

In order to determine the primary structure of PP2C homologue from *L. major*, forward and reverse primers were constructed from the sequence of the LmjF.25.0750 gene in TriTrypDB, the database of Trypanosomatids (tritrypdb.org). A fragment of 1200 bp was amplified from *L. major* DNA using PCR. The pET-23b (+) expression vector containing a C-terminal His₆ Tag was transformed into BL21 strain of *Escherichia coli* for expression and purification of the PP2C fusion protein. The transformed BL21 cells were grown and induced with 1 mM IPTG for 3 h at 30 °C, the recombinant protein was observed in gels stained with Coomassie blue (Fig. 1A, lanes 2 and 3), in contrast to cells in which the expression of the protein was not induced (Fig. 1A, lane 1). The presence of the histidine tag in the recombinant protein was verified by Western blot with specific antibodies that recognized the C-terminal His₆ Tag (Fig. 1B, lanes 2 and 3), which was negative in non-induced cells (Fig. 1B, lane 1). TE of

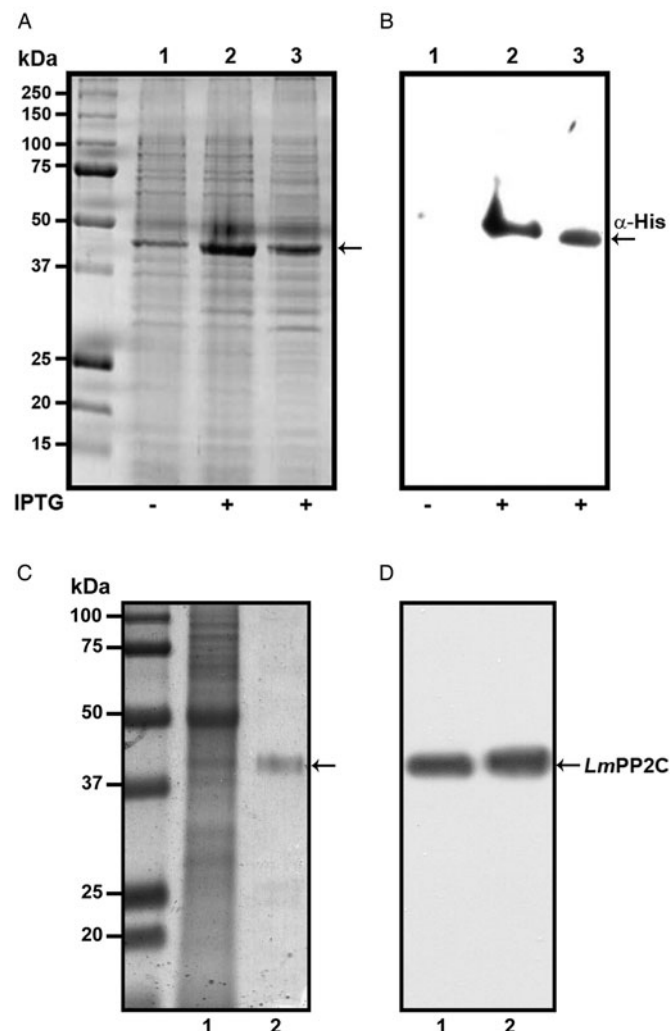


Fig. 1. Expression, purification and identification of PP2C of *Leishmania major* (*Lm* PP2C). (A) Expression of *Lm* PP2C in different fractions of *Escherichia coli* cells. Crude extract prepared from non-induced *E. coli* cells BL21 (1), *E. coli* cells induced with IPTG (2) and supernatant (3). SDS-PAGE gel was stained with Coomassie-blue dye. (B) Western blot analysis of the same sample used above: crude extract prepared from non-induced *E. coli* cells BL21 (1), *E. coli* cells induced with IPTG (2) and supernatant (3). The C-terminal His-Tag was detected using a monoclonal antibody. (C) SDS-PAGE gel of TE of *L. major* (1) and *Lm* PP2C purified (2) were stained with Coomassie Blue. (D) Western blot of TE of *L. major* (1), and purified *Lm* PP2C (2), using polyclonal antibodies against *Lm* PP2C.

L. major promastigotes and recombinant protein were analysed by SDS-PAGE and stained with Coomassie blue (Fig. 1C, lanes 1 and 2). This result showed that the purified protein had the expected molecular mass of 44.9 kDa. The anti-*Lm*PP2C antibodies were checked by Western blot using TE of *L. major* and purified *Lm*PP2C. These antibodies recognized a 44.9 kDa protein in both samples (Fig. 1D, lanes 1 and 2).

Multiple sequence alignment

Using Clustal W multiple-sequence alignment, the sequence of the cloned *Lm*PP2C revealed that it has 98% similarity with the sequence of PP2C of *L. chagasi*. It also demonstrated that the complete *Lm*PP2C open reading frame shares 11 distinct conserved motifs in the enzymes belonging to this family (Fig. 2A).

Furthermore, both *Hs*PP2C and *Lm*PP2C have conserved amino acids for catalytic activity such as: the aspartic acid residues involved in the coordination of Mg⁺² and Mn⁺² cations. The catalytic site of PP2C is localized in a cleft between two central β sheets formed by aspartic acids D38, D55, D221, D265, arginine R33 and glutamic acid E37 (Fig. 2B). Identical residues were identified at these positions in all aligned sequences (Fig. 2A). These residues were confirmed to be present in *Lm*PP2C.

Phylogenetic tree analysis

To compare the protein LmjF.25.0750 analysed in this work with the members of the PP2C in *L. major* family and for the identification of conserved residues, a multiple-sequence alignment was constructed using the 15 *L. major* PP2C, LmxM.25.0750 and the human PP2C (PPM1A). Similarity relationships among the amino acids sequences were obtained and we identified characteristic motifs preserved by evolution. A total of 11 conserved signature motifs were identified that seem to correspond to structural elements and exposed functional residues of PP2C proteins (data not shown). To investigate the phylogenetic relationships of PP2C genes between *L. major* phosphatases, *L. mexicana* PP2C and the human PPM1A, we constructed a phylogenetic tree based on the alignments of PP2C domains using the maximum likelihood method. The phylogenetic analyses indicated that the 15 *Lm*PP2C proteins were divided into four groups (Fig. 2C, I–IV). The LmjF 25.0750 recombinant protein clustered together with LmxM.25.0750 (Fig. 2C, III), while the nine Lmj phosphatases formed an independent group (Fig. 2C, IV). There was a cluster of three proteins in group I, whereas LmjF 36.1230 was in an independent branch (Fig. 2C, II).

Biochemical characterization of *Lm*PP2C

Cation dependence

Phosphatases PP2C are usually monomeric enzymes that require metal cations such as Mg⁺² or Mn⁺² for their enzymatic activity. We analysed the activity of *Lm*PP2C with the substrate *p*-NPP in the presence or absence of divalent cations. We observed that *Lm*PP2C showed higher phosphatase activity in the presence of MnCl₂, compared with MgCl₂ (Fig. 3A).

Substrate specificity

Substrate specificity was tested by dephosphorylation assays using peptides phosphorylated in tyrosine and threonine residues. It was observed that *Lm*PP2C dephosphorylate preferentially the threonine substrate (Fig. 3B), whereas the tyrosine substrate was not dephosphorylated.

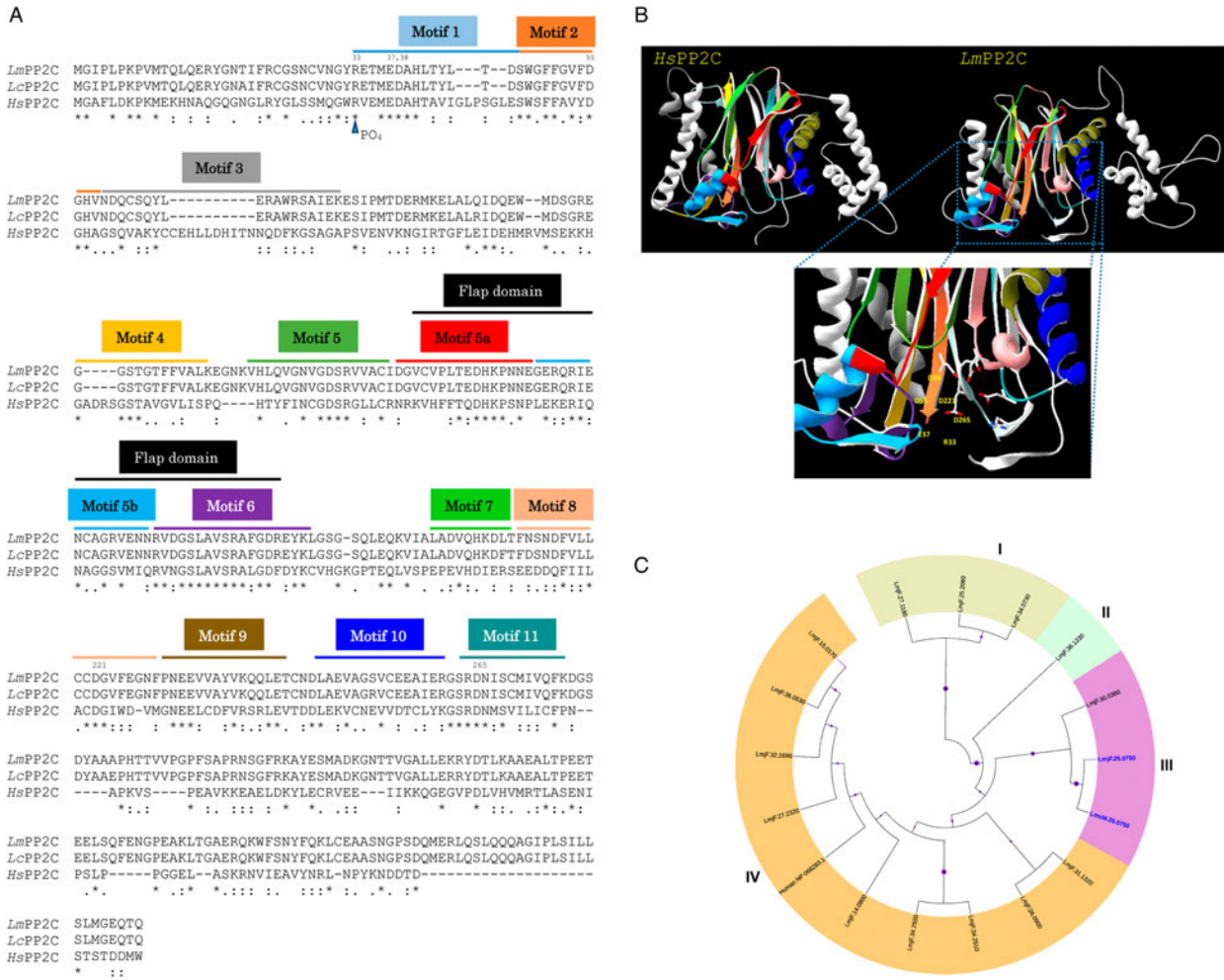


Fig. 2. Multiple sequence alignments of human, *Leishmania major* and *Leishmania chagasi* PP2C and phylogenetic analysis of *L. major* PP2Cs. (A) Multiple sequence alignment of Human PP2C (HsPP2C), *L. major* (LmPP2C) and *L. chagasi* (LcPP2C). Alignment of the catalytic domain sequences was performed using the programme CLUSTAL W. The most important residues of the three PP2C are indicated with numbers and motifs with colours: motif 1 (blue line), motif 2 (orange line), motif 3 (grey line), motif 4 (yellow line), motif 5 (dark green line), the Flap domain is indicated by a black line. Motifs 5a and 5b are shown with a red and dark blue line respectively, motif 6 (purple line), motif 7 (clear green line), motif 8 (pink line), motif 9 (brown line), motif 10 (blue line) and motif 11 (water green line). (B) Ribbon diagram of HsPP2C and LmPP2C. The catalytic site of LmPP2C was amplified, showing the important amino acids. (C) Phylogenetic analysis of *L. major* PP2Cs. The non-rooted phylogenetic tree was constructed in the MEGA 6 programme from the alignment of 15 amino acid sequences PP2Cs present in the *L. major* genome with the protein sequence of the LmxM.25.0.750 gene of *Leishmania mexicana* and the protein sequence of the human PPM1A (NP_066283.1). Seventeen proteins were grouped into four groups and designated with different colour (I–IV). The evolutionary history was inferred using the maximum probability, method based on the Le_Gascuel_2008 model.

Optimum pH

The influence of pH on the enzymatic activity for recombinant LmPP2C was evaluated using two different substrates: *p*-NPP and the phosphothreonine peptide [RRA (pT) VA] in different buffers. The results showed that the optimal activity of PP2C phosphatase reached at pH 8.5 using *p*-NPP as substrate, while the phosphothreonine substrate showed the maximum at pH 7 (Fig. 3C).

Effect of various inhibitors of phosphatases on the activities of LmPP2C

In order to characterize if the phosphatase activity is due to PTP or PP2C activity, we used different phosphatase inhibitors: orthovanadate, molybdate and pervanadate for PTP and calyculin, okadaic acid and sanguinarine for serine/threonine phosphatases. LmPP2C was not inhibited by the specific PTP inhibitors (Fig. 4A), which contrasts with the YePTP that was completely inhibited by these compounds (Fig. 4A). Calyculin and okadaic acid did not inhibit LmPP2C or YePTP (Fig. 4A). However, sanguinarine, the specific inhibitor of PP2C activity, inhibited

LmPP2C in a dose-dependent manner (0–20 µM), with a complete inhibition at a concentration of 10 µM (Fig. 4B).

Western blot reactivity of recombinant LmPP2C

Antibodies were prepared against the recombinant LmPP2C (α-LmPP2C) and used to identify the PP2C protein in TE promastigotes from different *Leishmania* species: *L. (L.) mexicana*, *L. (V.) panamensis*, *L. (V.) braziliensis*, *L. (L.) venezuelensis*, *L. (V.) donovani*, *L. (L.) amazonensis*, *L. major* and LmPP2C. In all *Leishmania* species, a 44.9 kDa protein was identified (Fig. 5A, lanes 1–7) which had the same molecular mass as LmPP2C (Fig. 5A, lane 8). Tubulin, as a loading control, was included in the same experiment.

Immunolocalization of PP2C in *L. major* promastigotes by immunofluorescence

The analysis of the distribution of the PP2C in promastigotes of *L. major* was performed by indirect immunofluorescence. Parasites were observed using phase contrast microscopy (Fig. 5B, panel a).

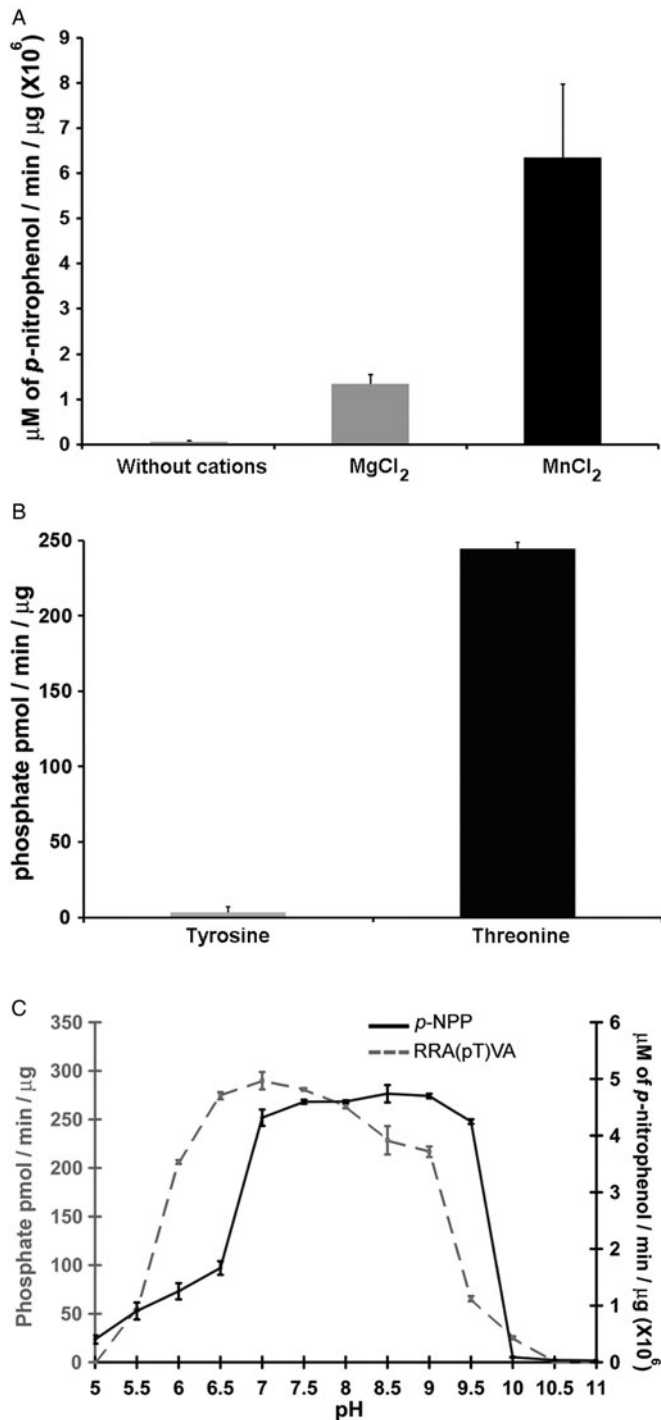


Fig. 3. Biochemical characterization of *LmPP2C*. (A) The effect of MgCl₂ and MnCl₂ on *LmPP2C* activity was measured under standard conditions using *p*-NPP 10 mM as a substrate. (B) Hydrolysis of [END (pY) INASL] and Thr [RRA (pT) VA] by *LmPP2C*. (C) Determination of optimum pH for the activity of *LmPP2C*. *PP2C* was incubated with the appropriate buffers at different pH (5–11) in the presence of 10 mM MgCl₂ and different substrates: *p*-NPP and RRA(pT) VA.

The nucleus was stained with DAPI (Fig. 5B, panel b). In several parasites, the distribution of PP2C showed a fluorescent pattern confined to the flagellum and to the flagellar pocket (big arrows). Interestingly, some parasites exhibited a three-band pattern located in the cell body (small arrows) without staining of the flagella (Fig. 5B, panel c). The merge of both: nuclei and PP2C labelling, is shown in panel d (Fig. 5B). The negative control with pre-immune serum showed no labelling of the parasite (data not shown).

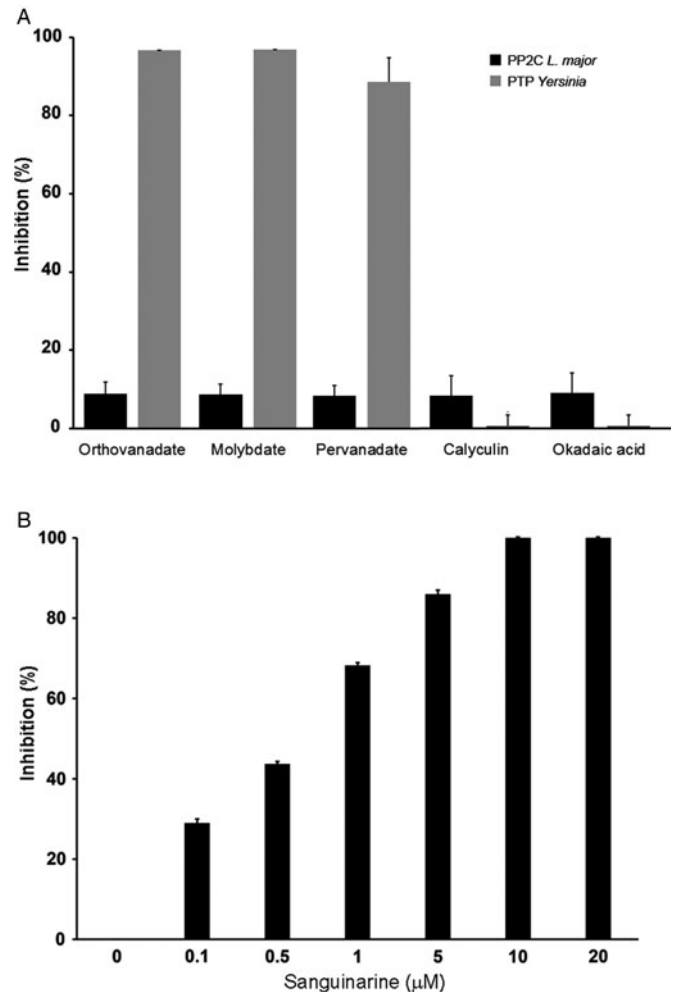


Fig. 4. Effect of different inhibitors on *LmPP2C* and *YePTP* activities. (A) Inhibitors of PTP (orthovanadate, molybdate and pervanadate) and inhibitors of serine threonine phosphatases (calyculin and okadaic acid) were incubated with *LmPP2C*, and *YePTP* and phosphatase activity was analysed by *p*-NPP. (B) An inhibitor of the PPM family (sanguinarine) was incubated at different concentrations with *LmPP2C*. The bars represent the mean values of the per cent relative activity obtained separately in three different experiments.

Ultrastructural localization of PP2C in promastigotes of *L. major*

The distribution of PP2C in promastigotes of *L. major*, studied by IEM using α -*LmPP2C*, showed labelling of the flagellum and the flagellar pocket (Fig. 6C–E). In order to determine precise location of the immune gold labelling, fine structure images of the flagellar pocket and the flagellum were done in longitudinal and transverse sections (Fig. 6A and B). Negative controls with pre-immune serum showed no labelling of any structure of the parasite (data not shown).

Discussion

In recent years, the number of PP2C family members has grown steadily, and more interesting features, phenotypes and potential clinical applications have been described for the family of phosphatases. Orthologs of human PP2C can be found in virtually all organisms, ranging from mammals, insects, plants, bacteria and yeasts to parasites. This conservation throughout evolution indicates that these enzymes probably play important roles in regulating key cellular signalling events (Su and Forchhammer, 2013). The genome of *L. major* was reported in 2005 (Ivens *et al.*, 2005), and 2 years later, the first analysis of catalytic domains of

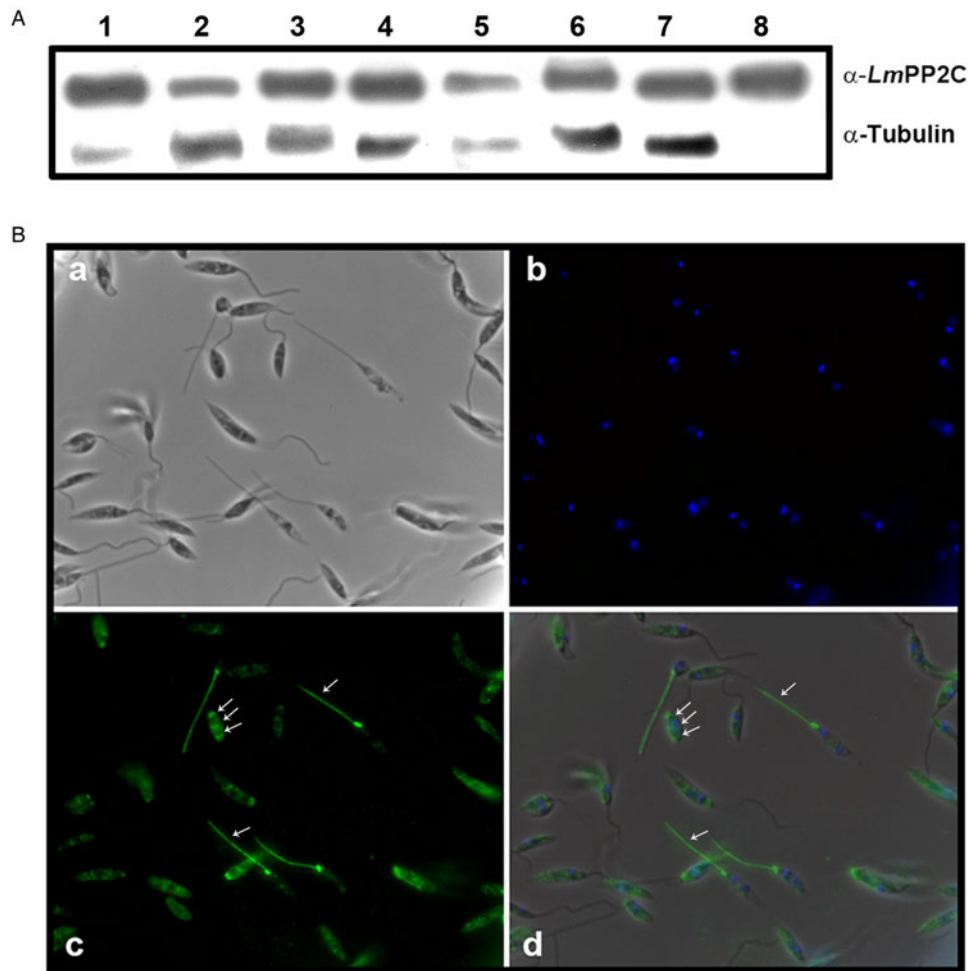


Fig. 5. PP2C: immunodetection in TE promastigotes from different *Leishmania* species and immunolocalization in promastigotes. (A) Immunodetection of PP2C protein in TE promastigotes from *Leishmania (L.) mexicana* (1), *Leishmania (V.) panamensis* (2), *Leishmania (V.) braziliensis* (3), *Leishmania (L.) venezuelensis* (4), *Leishmania (V.) donovani* (5), *Leishmania (L.) amazonensis* (6), *Leishmania major* (7), promastigotes *Lm* PP2C recombinant was used as control (lane 8). Tubulin as a loading control. (B) Immunolocalization of PP2C in *L. major* promastigotes by immunofluorescence microscopy. Parasites were observed in phase contrast (panel a), the nucleus was stained with DAPI (panel b). Distribution of *Lm* PP2C was determined by using an antibody against PP2C and then evidenced with a secondary antibody conjugated to FITC (panel c). Inset d represents the merge of the different staining conditions. Scale bar = 5 μ m.

protein phosphatases in Trypanosomatids was also reported. The authors identified 86 phosphatases in *Trypanosoma cruzi*, 78 in *Trypanosoma brucei* and 88 in *L. major*. In the case of *L. major*, 58 serine/threonine phosphatases were reported, of which 30 were from the PPP family, 13 were phosphatases that dephosphorylate the carboxy-terminal domain of RNA polymerase II (F-cell production eukaryotic-like phosphatases) and 15 were PP2C eukaryotic-like phosphatases (Brenchley *et al.* 2007). Currently, the function of each of the 15 different PP2C from *L. major* is unknown.

In the present work, we cloned PP2C from *L. major* and generated a recombinant His-Tag PP2C fusion protein. *Lm*PP2C was expressed in BL21 *E. coli* cells and purified. The biochemical characterization of *Lm*PP2C showed it had a molecular mass of 44.9 kDa, an activity optimum at alkaline pH, that it could specifically dephosphorylate a threonine substrate and had a dependence on divalent cations (Mg^{+2} and Mn^{+2}) for enzymatic activity; all of which are also biochemical characteristics of PP2C from mammalian cells. In all the PP2C, the catalytic domains are important because they include conserved motifs that contain invariant residues involved in the binding of the two metal cations (D42, 66, 202 and 240) and on the phosphate group of the substrate (R37) (Das *et al.* 1996). Alignment of the phosphatase domains of *T. gondii* with other PP2C proteins showed an absence of two conserved aspartic acid residues that are important for metal binding

and typical PP2C activity (Gilbert *et al.* 2007). The absence of these residues caused that the recombinant PP2C protein showed low metal-dependent phosphatase activity.

Examination of the primary sequence of *Lm*PP2C shows homology to other PP2C proteins. Additionally, the conservation of the essential catalytic residues between human PP2C (*Hs*PP2C) and *Lm*PP2C suggests that the latter is a PP2C from the PPM family. The analysis of sequences for the PPM phosphatase proteins has identified 11 motifs, which are also conserved between the different phosphatases. The sequence alignment of *Lm*PP2C with the consensus sequences of *Hs*PP2C and *Lc*PP2C phosphatases indicated that *Lm*PP2C contains also 11 conserved motifs characteristic of PP2C. In other microorganisms, such as bacteria, it has been observed that motifs 5a and 5b (Flap domain) of the PP2C protein are absent, thus affecting the enzymatic activity (Schlicker *et al.* 2008). On the other hand, PP2C α has a Flap domain, which is inserted between strands β 8 and β 9 of the catalytic domain (Das *et al.* 1996; Pullen *et al.* 2004; Schlicker *et al.* 2008). This domain, with conserved motifs 5a and 5b, is found in PP2C phosphatases, like the mitochondrial PDP and the phosphatase domains of fungal adenylate cyclases, but is absent in bacterial SpoIIE-type phosphatases (Kennelly, 2001; Pullen *et al.* 2004). The function of the Flap domain is uncertain, but appears to be involved in substrate recognition (Schlicker *et al.* 2008; Su *et al.* 2011).

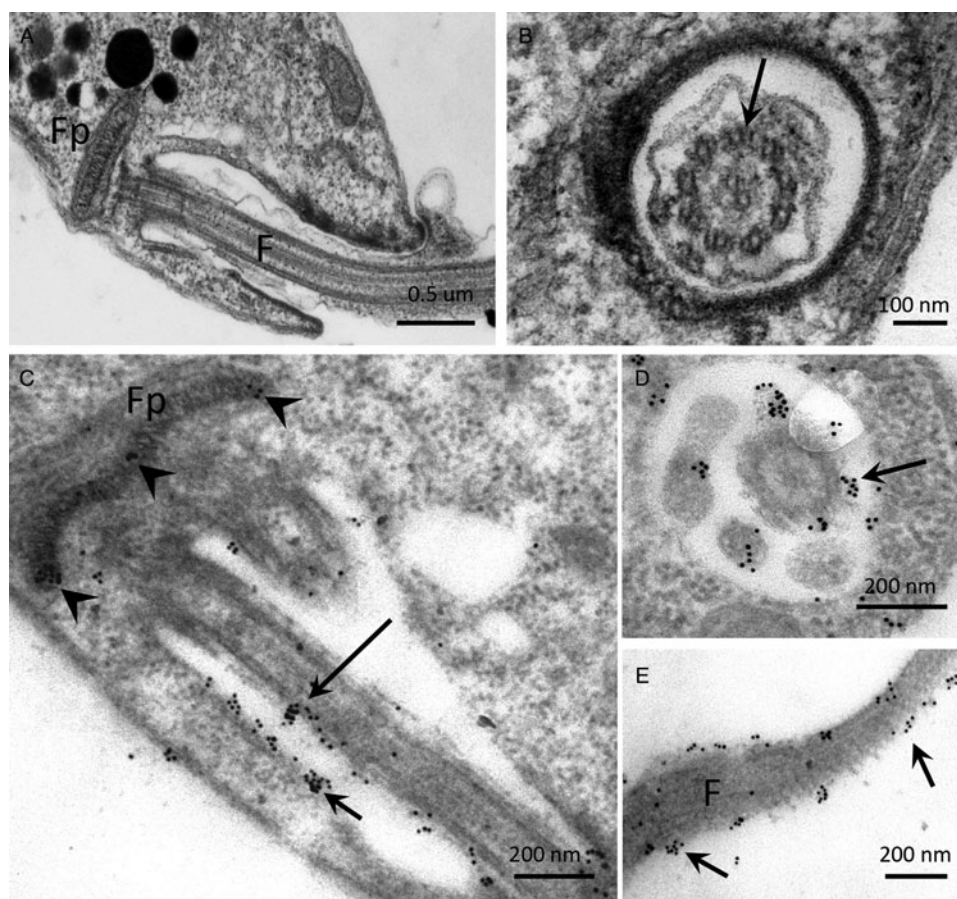


Fig. 6. IEM localization of *LmPP2C* in promastigotes. Insets (A) and (B) correspond to controls of ultrastructure of the flagellar pocket of promastigotes seen in longitudinal and transverse thin sections, respectively. Insets (D, E) correspond to the immuno-gold labelling of the *LmPP2C* in magnified zones of the promastigote in longitudinal (inset C) and transverse (inset D) views of the flagellar pocket. Inset (E) corresponds to the labelling of *LmPP2C* in a magnified zone of the flagellum. Scale bar = 200 nm.

The phylogenetic analysis showed that the 15 *L. major* PP2C are clustered in four groups. Surprisingly, the recombinant protein LmjF.25.0750 and the different species LmxM.25.0750 are grouped in the same cluster suggesting that they share a common ancestor, and, more distant in the same cluster, is LmjF.30.0380. The groups in the phylogenetic tree of PP2C represent proteins with highly similar sequences; however, to the best of our knowledge, there are no reports of their structure, localization in the parasite or functional results.

Using a general substrate for phosphatases, *LmPP2C* showed a strong preference for Mn^{+2} , in comparison to Mg^{+2} . The preference for this cation has also been observed in other PP2C enzymes like the STP1 phosphatases of *Streptococcus agalactiae* and *Pseudomonas aeruginosa* (Mukhopadhyay *et al.* 1999; Rajagopal *et al.* 2003). *In vitro* assays using *p*-NPP as a substrate showed that the highest specific activity of *LmPP2C* was at pH 8.5 in the presence of Mg^{+2} ; however, when using phosphotreonine, the optimal pH was observed at 7.0. It is well known that PP2C of pathogenic microorganisms show alkaline optimal pH, as has been observed in Prp C from *Bacillus subtilis* and PPC6803 from *Synechocystid* (Obuchowski *et al.* 2000; Ruppert *et al.* 2002). For *LmPP2C*, both divalent cations (Mg^{+2} and Mn^{+2}) are important for catalytic activity at alkaline pH.

We show novel data on the ultrastructural localization of PP2C in *L. major* promastigotes, which was found in the flagellum, the flagellar pocket and in the micro axoneme. The heterogeneous staining pattern of the parasites may be due to different development stages of the parasite, since the culture was not synchronized. In other Trypanosomatid parasites, such as *Trypanosoma*

rangeli, a PTP was reported to be associated with the parasite flagellum, yet the function remains unknown (Prestes *et al.* 2012). In most of these cells, the PP2C enzymes are mainly localized in the cytosol, and in the case of *T. gondii*, the parasite also uses secretion from the rhoptries during invasion, in order to deliver a parasite-derived PP2C into the host cell and target it to the host nucleus (Gilbert *et al.* 2007).

The localization of PP2C in the flagellum of *L. major*, an important structure for differentiation in Trypanosomatids, is noteworthy since the phosphatase could potentially exert a regulatory function within this organelle. Yet the role of *L. major* PP2C remains to be established and functional tests are warranted to determine its biological role in the parasite.

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