Leishmania major parasites express cyclophilin isoforms with an unusual interaction with calcineurin

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The immunosuppressive effects of the fungal metabolite cyclosporin A (CsA) are mediated primarily by binding to cyclophilins (Cyps). The resulting CsA–Cyp complex inhibits the Ca^{2+}-regulated protein phosphatase calcineurin and down-regulates signal transduction events. Previously we reported that CsA is a potent inhibitor of infections transmitted by the human pathogenic protozoan parasite *Leishmania major in vitro* and *in vivo*, but does not effect the extracellular growth of *L. major* itself. It is unknown how *L. major* exerts this resistance to CsA. Here we report that a major Cyp, besides additional isoforms with the same N-terminal amino acid sequence, was expressed in *L. major*. The cloned and sequenced gene encodes a putative 174-residue protein called *L. major* Cyp 19 (LmCyp19). The recombinant LmCyp19 exhibits peptidyl-prolyl *cis/trans* isomerase activity with a substrate specificity and an inhibition by CsA that are characteristic of other eukaryotic Cyps. To determine whether calcineurin is involved in the discrimination of the effects of CsA we also examined the presence of a parasitic calcineurin and tested the interaction with Cyps. Despite the expression of functionally active calcineurin by *L. major*, neither LmCyp19 nor other *L. major* Cyps bound to its own or mammalian calcineurin. The amino acid sequence of most Cyps includes an essential arginine residue around the calcineurin-docking side. In LmCyp19 this is replaced by an asparagine residue. This exchange and additional charged residues are apparently responsible for the lack of LmCyp19 interaction with calcineurin. These observations indicate that resistance of *L. major* to CsA *in vitro* is mediated by the lack of complex formation with calcineurin despite CsA binding by parasitic Cyp.

INTRODUCTION

Leishmania sp. are intracellular protozoa that infect macrophages in the vertebrate host and cause a broad spectrum of diseases, the leishmaniases, in man ([1], vol. 1, pp. 122–168). After inoculation into the mammalian host, the parasites are taken up in the phagolysosome of host macrophages. Here the parasite has developed several escape and survival strategies by mechanisms that are largely unknown [2–5]. We and others have shown that a cyclic undecapeptide, cyclosporin A (CsA), interferes with these mechanisms, resulting in an activation of macrophages for the killing of parasites. In contrast, the extracellular growth of *L. major* is not inhibited by CsA [6–9]. The same compound is, however, markedly toxic to other protozoa like *Plasmodium falciparum* or *Toxoplasma gondii* [10,11].

The anti-parasitic effect of CsA or the resistance of parasites is not yet understood. It is hypothesized that the decision on whether a parasite is affected by CsA or not is dependent on its interaction with high-affinity binding proteins of CsA, collectively termed cyclophilins (Cyps) [10–14]. Biochemical studies have shown that Cyps are peptidyl-prolyl *cis/trans* isomerases lacking other enzymic and chaperone effects (PPIase; EC 5.2.1.8) [15,16]. Different isoforms of single-domain Cyps (e.g. CypA, CypB, CypC, CypD and Cyp40) have been described [17,18]. They can catalyse rate-limiting *cis/trans* peptidyl-prolyl isomerization in protein folding or regulate protein–protein interactions through conformational changes [19–21].

The interaction of CsA with cell functions is frequently not merely a simple result of the interaction with the major Cyp, CypA, but rather the CypA–CsA complex forms a composite surface that inhibits the activity of a third protein, the calcium/calmodulin-dependent Ser/Thr phosphatase calcineurin [22]. By inhibiting the activity of calcineurin, CsA blocks transcription factors responsible for the enhanced expression of many genes involved in T-cell activation [23]. Recent studies reveal that the mechanisms of immunosuppression and the known antifungal action of immunosuppressive drugs are remarkably similar. In addition to CsA, FK506, a structurally unrelated macrolide, diffuses into pathogenic fungi such as *Cryptococcus neoformans* and *Neurospora crassa*, and binds to their receptor proteins, the FK506-binding proteins (FKBP). The FKBP–drug complex then inhibits fungal calcineurin, resulting in arrest of the cell cycle, in enhanced sensitivity to stress and in inviability [24–26]. Toxicity of CsA and FK506 to *C. neoformans* was manifested at 37 °C but not at 22 °C, which suggests that calcineurin might be required for pathogenicity [27]. The association of temperature-resistant growth and virulence is not limited to pathogenic fungi. The heat shock response is also thought to be involved in the pathogenesis and differentiation of *L. major* [28]. After transmission from the insect vectors at 25 °C, the promastigote form of *L. major* is adapted to, differentiates and survives as an amastigote in mammalian hosts at 37 °C. Remarkably, CsA is not directly toxic to either promatigotes or amastigotes of *L. major* [6–8].

In contrast, the molecular mechanisms mediating CsA resistance in some parasitic protozoa have not yet been elucidated. On the basis that CsA binding by Cyps and the formation of a complex with the phosphatase calcineurin might represent important steps in the molecular mechanism of CsA action, we

Abbreviations used: CsA, cyclosporin A; Cyp, cyclophilin; LmCyp1, *Leishmania major* cyclophilin 19; PPIase, peptidyl-prolyl *cis/trans* isomerase; Suc, succinyl.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number Y13576.

decided to define the molecular structure of Cyps in *L. major*, its affinity for CsA and interaction with calcineurin. We assumed that either *L. major* parasites are not affected by CsA because they express Cyps that do not bind CsA with sufficient affinity or the CsA/Cyp complex does not inhibit the calcineurin cascade.

We showed that various Cyps are expressed in *L. major* and the major Cyp isoform, LmCyp19, possesses PPIase activity towards different peptide substrates. Further, we found that LmCyp19 forms a complex with CsA at nanomolar concentrations, but without detectable affinity for parasitic or mammalian calcineurin. These findings support the prediction that parasites, as masters at manipulating the structures and pathways of the host cell for their own nefarious purpose, have their own Cyps adapted to ligands other than calcineurin.

MATERIALS AND METHODS

Leishmania strains and culture methods

L. major promastigotes (WHO MHOM/IL/81/FEBNI) were cultured *in vitro* in 96-well microtitre plates as described [6].

Isolation of CsA-binding proteins

L. major promastigotes or cells of the macrophage cell line J774 (ATCC) were harvested by centrifugation, washed once with ice-cold PBS, suspended at 4 °C in buffer A [20 mM Tris/HCl (pH 7.8)/100 mM NaCl/1 mM PMSF/0.1 % (v/v) Tween] and sonicated. The lysate was cleared by centrifugation (13000 ***g*** for 10 min) and the supernatant (hereafter referred to as *L. major* or macrophage lysate) was processed as described below. Characterization and purification of *L. major* Cyps were done as previously described [9]. For peptide sequence analysis, the eluted Cyps were further purified on a HPLC chromatography system with a C_4 reverse-phase column (Pharmacia, Freiburg, Germany). Purified Cyps were subjected to amino acid sequence determination at Toplab (Munich, Germany) on a Beckman System Little Foot 3600 TD® protein sequencer equipped with an on-line phenylthiohydantoin derivatives system by standard procedures.

Assay of PPIase activity, substrate specificity and inhibitory studies

The PPIase activity of *L. major* Cyps was determined by measuring the *cis-trans* isomerization of the substrate Suc-Ala-Xaa-Pro-Phe-4-nitroanilide (Suc = succinyl; Xaa = Ala, Phe, Leu, His, Lys, Glu or Gly) by the method of Fischer et al. [16]. The kinetic was initiated by adding 6 μl of the substrate solution to the reaction mixture containing Hepes buffer, chymotrypsin and either test or control samples. First-order rate kinetics were observed with a rate constant of $k_{obs} = k_0 + k_{enz}$ and $k_{obs} = k_0 + k_{cat}/K_m[E_0]$ respectively, where k_0 is the rate constant of the uncatalysed *cis–trans* interconversion, and k_{obs} is the observed first-order rate constant for PPIase catalysis. The value of the specific constant k_{cat}/K_m was calculated from the relationship $k_{enz}/[E_0] = k_{cat}/K_m$, in which $[E_0] = [PPIase]_{total}$. This value was determined from the total enzyme protein concentration on the assumption that all PPIase molecules are enzymically active. For comparing PPIase activities of crude protein fractions, the arbitrary unit, $AU = (k_{obs}/k_0) - 1$ was used. The inhibition of LmCyp19 PPIase activity by CsA was estimated by using the model of a competitive tightly binding inhibition as described in [29]. Stock solutions of CsA and substrates were prepared in DMSO. The stability of enzyme activity was checked by adding the solvents without inhibitory molecules.

Generation of DNA probes by PCR

Degenerated primers corresponding to the derived N-terminal peptide sequence YTPHY (5′-TACACSCCSCACTACCC-3′, sense sequence) of parasitic CsA-binding proteins and the conserved internal consensus sequence QGGDFT (5′-SGTRA-ARTCRCCRCCCTG-3′, anti-sense sequence) were synthesized by TIB Molbiol (Berlin, Germany).

Promastigotes were lysed by 1 % (v/v) SDS/20 mM EDTA/0.5 mg/ml proteinase K in PBS for 3 h at 55 °C to prepare the genomic DNA of *L. major*. The DNA was isolated further by using standard protocols [30]. Approx. 500 ng of genomic DNA was used as a template for the PCR reaction performed in a Gene Amp PCR System 2400 (Perkin Elmer, Applied Biosystems). The total volume was 50 μl, containing 50 pmol of degenerate primers, 10 nmol of dNTPs, 2.5 units of *Taq* DNA polymerase (Promega, Heidelberg, Germany), 5 % (v/v) DMSO and 5 μl of 10 × PCR buffer (Promega). The following thermal cycler protocol was used: 28 cycles of denaturing at 95 °C (1 min)/annealing at 55 °C (2 min)/extension at 72 °C (2 min). Unique PCR products were separated on a preparative 1 % (w/v) low-melting-point agarose gel (Appligene Oncor, Heidelberg, Germany) and cloned into pCRII vector (InVitrogen, Leek, Netherlands). Transformed recombinant *Escherichia coli* strain M15 (pREP4) cells were identified by blue–white screening. Plasmid DNA was isolated with standard procedures [30] and sequenced (Replicon, Berlin, Germany).

Screening of an *L. major* cDNA library/cloning and sequencing

Poly(A)$^+$ RNA from promastigotes was isolated by Oligotex Direct mRNA Kit (Qiagen, Hilden, Germany) and an lZAPII expression library was prepared from the corresponding cDNA (Stratagene). Screening of the library was performed by a PCR method with primers corresponding to the derived DNA sequence from the N-terminal cloning step (5′-TGTCGTTGAATCCA-ACCC-3′, sense; 5′-GCACATGAAATCCGGGAT-3′, anti-sense). The PCR reaction was performed in a total volume of 50 μl. Other PCR conditions were the same as those described above. cDNA species from positive plaques were subcloned into pBluescript SK(+/−) via excision *in vivo* (Stratagene, Heidelberg, Germany). The complete 2.2 kb cDNA fragment was sequenced by Replicon (Berlin, Germany).

Overexpression of LmCyp19 with an N-terminal histidine tag

The coding sequence was amplified by PCR and cloned in pQE30 (Qiagen), resulting in isopropyl β-D-thiogalactoside-inducible expression of the LmCyp19 as a histidine-tagged fusion protein. The 5′ PCR primer included a *Bam*HI recognition side and had the sequence 5′-GGGGATCCCCTTACACGCCGCAC-3′; the 3′ primer included a *Pst*I recognition side and had the sequence 5′-GGCTGCAGTTAAAGCTGCCCGCAG-3′. PCR amplification was performed with 10 ng of pBluescript DNA as template and the following thermal cycler protocol: 30 cycles of denaturing at 95 °C (1 min)/annealing at 48 °C (1 min)/extension at 72 °C (2 min). PCR products were digested with *Bam*HI and *Pst*I, cloned into pQE30 vector (Qiagen) and transformed the *E. coli* strain M15 (pREP4).

Purification of recombinant LmCyp19 from *E. coli*

E. coli M15 cells harbouring plasmids pREP4 and pQE30 were grown at 37 °C to a D_{600} of 0.8 and treated with 1 mM isopropyl β-D-thiogalactoside to induce the expression of LmCyp19. After growth for a further 2 h the cells were harvested by centrifugation (20 min at 5000 ***g***), resuspended in 30 ml of HKP buffer [25 mM

Hepes (pH 7.5)/25 mM NaCl/5 mM $MgCl_2$/1 mM EDTA/2 mM $CaCl_2$/10 mM NaF/1 mM 2-mercaptoethanol/5 % (w/v) sucrose/30 % (v/v) glycerol] and lysed by sonication. The supernatant after centrifugation (10000 ***g*** for 30 min) was loaded on an Ni^{2+}-nitrilotriacetate–agarose column (1 cm × 5 cm), equilibrated with HKP buffer. Non-specifically bound protein was eluted with NEB buffer [25 mM Hepes (pH 7.0)/500 mM NaCl/5 mM $MgCl_2$/10 mM NaF/1 mM 2-mercaptoethanol/10 % (v/v) glycerol, 0.1 % (v/v) Nonidet P40]. Recombinant His-tagged LmCyp19 was eluted by a 50 ml linear gradient of 40–250 mM imidazole in NEB buffer and purified to homogeneity by FPLC (Pharmacia, Uppsala, Sweden) with a phenyl-Superose resin (Pharmacia). The dialysed protein was applied to the column in 50 mM phosphate buffer, pH 7.5, with 20 %-satd. $(NH_4)_2SO_4$ and eluted with a linear gradient of 20–0 % $(NH_4)_2SO_4$ in 50 mM Hepes buffer, pH 7.5, at a flow rate of 0.5 ml/min. The homogeneous LmCyp19-containing fractions after SDS/PAGE were pooled, dialysed against 50 mM Hepes (pH 7.5)/10 % (v/v) glycerol and stored at −20 °C.

Production of anti-(*L. major* Cyp) antiserum

Polyclonal anti-peptide antibodies against a conserved Cyp motif (amino acid sequence of hCyp18, residues M^{60}CQGGDFTRHNA^{70} [18]) were described previously [31]. A peptide corresponding to the N-terminal sequence of LmCyp19 (residues P^1YTPHYPVVES^{11}) was synthesized commercially by the Merrifield solid-phase technique (Research Genetics, Huntsville, AL, U.S.A.). A solution of 10 mg/ml LmCyp19 peptide was coupled with 0.2 % glutaraldehyde to keyhole limpet haemocyanin (1 mg/ml in PBS) as described [31]. New Zealand White rabbits were injected intramuscularly with 200 μg of the immunogen conjugate in 1 ml of Freund's complete adjuvant. On days 14 and 42 the rabbits were again injected subcutaneously with 200 μg of immunogen in Freund's complete adjuvant at five to ten sites. On day 60 the animals were bled, boosted and 10 days later bled again. This boosting and bleeding schedule was continued three times to obtain an adequate supply of antiserum. Serum was generated by clotting overnight; the serum was stored at −20 °C.

SDS/PAGE and Western blot analysis

SDS/PAGE [12.5 % or 15 % (w/v) gel] and immunoblot analysis were performed as described [31], except that the nitrocellulose membrane were blocked with 3 % blocking reagent (Boehringer Mannheim GmbH, Mannheim, Germany) in PBS. Antisera were diluted 1:750 for polyclonal anti-(conserved motif Cyp) [31] and 1:1000 for anti-LmCyp19 and anti-(calcineurin B) antibodies (ABR Affinity Bioreagents, Grünberg, Germany). Filters were then incubated with horseradish peroxidase-conjugated goat anti-(rabbit IgG) (Dianova, Hamberg, Germany) diluted 1:3000 in 3 % blocking reagent/PBS for 2 h at room temperature. After being washed with PBS/0.05 % (v/v) Tween 20, proteins were revealed with chloronaphthol staining or the enhanced chemiluminescence system (Amersham, Little Chalfont, Bucks., U.K.).

Assay of Cyp–CsA–calcineurin complex formation

Assay of complex formation was performed by the procedure of Foor et al. [32]. In brief, incubations (total volume 500 μl) were for 30 min on ice and contained various combinations of the following components: 5 μg of purified recombinant LmCyp19, 150000 c.p.m. of [^{3}H]CsA (Amersham), 250 μg of protein from *L. major* or J774 macrophage lysate (described above). The incubation buffer contained 20 mM K_2HPO_4, pH 7.0, 100 mM Na_2SO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM EDTA and 0.2 mM 2-mercaptoethanol. The reaction mixture (100 μl) was subjected to gel filtration on a Superose 12 HR 10/30 column (Pharmacia) at a flow rate of 0.5 ml/min; 1 ml fractions were collected. A portion (500 μl) of fractions was counted and reported as c.p.m. per mg of protein. The second part of the fractions was subjected to SDS/PAGE [12.5 % (w/v) gel] and Western-blot analysis was performed as described above.

RII peptide dephosphorylation

The assay was performed as described [33]. ^{32}P-RII phosphopeptide (DLDVPIPGRFDRRSVAAE; Biomol, Hamburg, Germany), labelled with cAMP-dependent protein kinase catalytic subunit (Sigma) to approx. 600 c.p.m./pmol and purified by HPLC, was incubated with 100 μl of calcineurin-containing fraction (fraction 10) from the above gel filtration at 30 °C in 50 μl of assay buffer [100 mM Tris/HCl (pH 7.5)/0.3 M NaCl/15 mM $MgCl_2$/0.3 mM $CaCl_2$/0.3 mg/ml BSA/1.5 mM dithiothreitol] and 0.1 μM calmodulin (Sigma). Where indicated, the following ordered additions were made for the assay: 5 mM EGTA, 10 μM okadaic acid, 100 nM to 5 μM LmCyp19, 100 nM to 1 μM human Cyp18, and 100 nM to 1 μM CsA. The reaction was initiated by the addition of ^{32}P-RII (5 μM). After 30 min at 30 °C, the extent of ^{32}P-RII dephosphorylation was analysed after addition of 0.5 ml of 0.1 M potassium phosphate with 5 % (w/v) trichloroacetic acid. Free P_i was isolated by Dowex cation-exchange chromatography (AG50W-X4, H^+ form, 400 mesh; Bio-Rad) and quantified by liquid scintillation. Assays were performed in duplicate and the radioactivity from blanks lacking gel-filtration fractions was subtracted.

RESULTS

Purification of various *L. major* Cyps

Pilot studies revealed the occurrence of members of Cyps and FK506-binding proteins in *L. major* lysates [9]. As shown in Figure 1(A), the predominant Cyp had an approximate molecular mass of 19 kDa. In accordance with the nomenclature for PPIases, the enzyme was named cytoplasmic LmCyp19 [15]. Additional Cyps of 18 and 22 kDa were detected by staining with Coomassie Blue (Figure 1A, lane 7) and in Western blotting experiments with anti-Cyp antibodies (Figure 2). The proteins were specifically associated with the CsA-affinity matrix as they could not be eluted by high-salt buffer (Figure 1A, lanes 5 and 6). The eluted 66 kDa protein seemed to be non-specifically associated with the affinity matrix because it could be eluted by buffer without CsA (Figure 1A, lane 5). The various Cyps were purified further and subjected to amino acid sequence analysis. N-terminal sequencing of LmCyp19 and the 22 kDa protein revealed that the latter has the same amino acid sequence as identified for LmCyp19 (PYTPHPVVES), unrelated to any known sequences in the protein database (Figure 3).

Glycosylation as a reason for the gel-mobility shift of the various Cyps was excluded by culturing *L. major* in the presence of tunicamycin or treatment of parasite lysates with glycosidases before application to the CsA affinity column (results not shown).

Rabbit anti-peptide antibodies were developed to confirm the N-terminal sequences of these proteins and their characterization as Cyps. The two peptides included residues 1–10 of the N-terminus of LmCyp19 (PYTPHPVVES) and a sequence spanning the conserved motif of Cyps (residues 60–70 in hCyp18; MCQGGDFTRHN) respectively [18]. Western blots were performed with *L. major* lysates and proteins eluted from the CsA matrix (Figure 2). Both antisera reacted with a protein band cor-

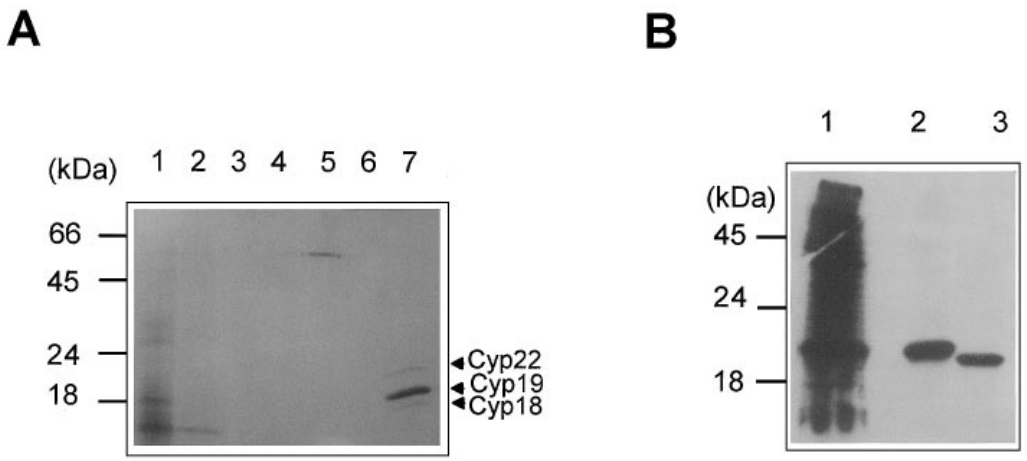

Figure 1 Purification of different Cyps from *L. major* and overexpressed LmCyp19

(**A**) Lysates of parasites (lane 1) were loaded on a CsA-affinity column, extensively washed with low-salt (lanes 2-4) or high-salt buffer (lanes 5 and 6) and eluted with CsA (lane 7). The eluted proteins were analysed by SDS/PAGE and staining with Coomassie Blue. The molecular masses of standard proteins are indicated at the left. (**B**) Purification of recombinant LmCyp19. Denatured samples of various purification steps were subjected to SDS/PAGE and proteins were detected by staining with Coomassie Brilliant Blue. Lane 1, *E. coli* crude lysate; lane 2, eluate from the Ni^{2+}-NTA–agarose column; lane 3, LmCyp19 purified from the cytosol of *L. major*.

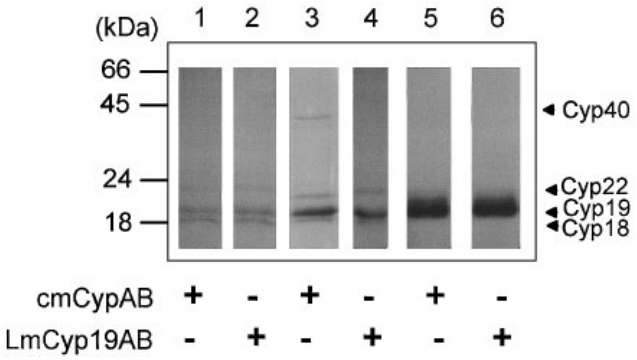

Figure 2 Anti-peptide antibodies recognize the different Cyp isoforms of *L. major*

Parasitic homogenate (50 μg of protein, lanes 1 and 2), eluted fraction from a CsA-affinity column (lanes 3 and 4) and recombinant LmCyp19 (lanes 5 and 6) were separated by SDS/PAGE [12.5% (w/v) gel], blotted on nitrocellulose membrane and developed with antibodies directed against a conserved motif (MCQGGDFTRHN) of Cyps (cmCypAB) (lanes 2, 4 and 6) or against the PYTPHYPVVES peptide that recognizes the N-terminus of LmCyp19 (LmCyp19AB) (lanes 1, 3 and 5). Protein molecular masses are indicated at the left.

responding to LmCyp19. Additional proteins migrating at 18 and 22 kDa were also detected in lysates and affinity matrix-eluted fractions. A 40 kDa protein band reacted with both antibodies only after enrichment on the affinity matrix.

cDNA cloning and deduced amino acid sequences of the dominant LmCyp19

Full amino acid sequences were derived from a PCR-based method for screening DNA libraries. The main limitation of this technique was the need for precise sequence information from the target gene for the design of specific and efficient PCR primers. Therefore degenerate PCR primers corresponding to the N-terminal amino acid sequence of *L. major* CsA-binding proteins and the conserved Cyp region QGGGDFT were designed first to allow the amplification of a 230 bp PCR product from the genomic DNA of *L. major*. The 230 bp PCR product was cloned into pCRII; 30 independent clones were isolated and sequenced. Sequence analysis revealed 76 amino acids corresponding to the N-terminus of an *L. major* Cyp (results not shown).

The full Cyp amino acid sequence was derived from an amplified λZAPII *L. major* cDNA library, screened by PCR with primers corresponding to sequence data from the genomic clone of the Cyp N-terminus (see above). The inserts from 30 independent clones were subcloned; the sequences obtained were all identical. The LmCyp19-encoding region of 528 bp corresponded to a translation product of 176 residues with a calculated molecular mass of 18.693 kDa (Figure 3) and was in good agreement with the molecular mass determined from SDS/PAGE analysis (Figure 1A). Translation of the two alternative frames yielded several stop codons; none of the peptide sequences derived from these translated cDNA frames matched any identified protein. The initial proline residue identified by N-terminal sequencing of LmCyp19 was located exactly downstream of the presumed initiation methionine residue. A homology search of the major protein databases revealed the highly conserved central amino acid region of Cyps in LmCyp19, in contrast with the first 11 amino acids at the N-terminus, which were specific for this parasite. Sequence alignment of LmCyp19 with various Cyps revealed 77% similarity to a parasitic Cyp from *Trypanosoma brucei* and 70% with human Cyp18. In addition, its relation to Cyp18 from the parasite *Toxoplasma gondii* was 67% (Figure 3).

Overexpression of *L. major* Cyp19 in *E. coli*

The 176-residue Cyp was overexpressed in *E. coli* with an N-terminal histidine tag in the pQE30 vector, resulting in a recombinant LmCyp19 with a larger molecular mass than the translation product of the corresponding LmCyp19 gene. Ni^{2+}-NTA–agarose affinity chromatography was used to separate recombinant His-tagged LmCyp19 from *E. coli* Cyps. The approx. 20 kDa recombinant *L. major* Cyp (Figure 1B) was subsequently purified from contaminating LmCyp19 proteolytic

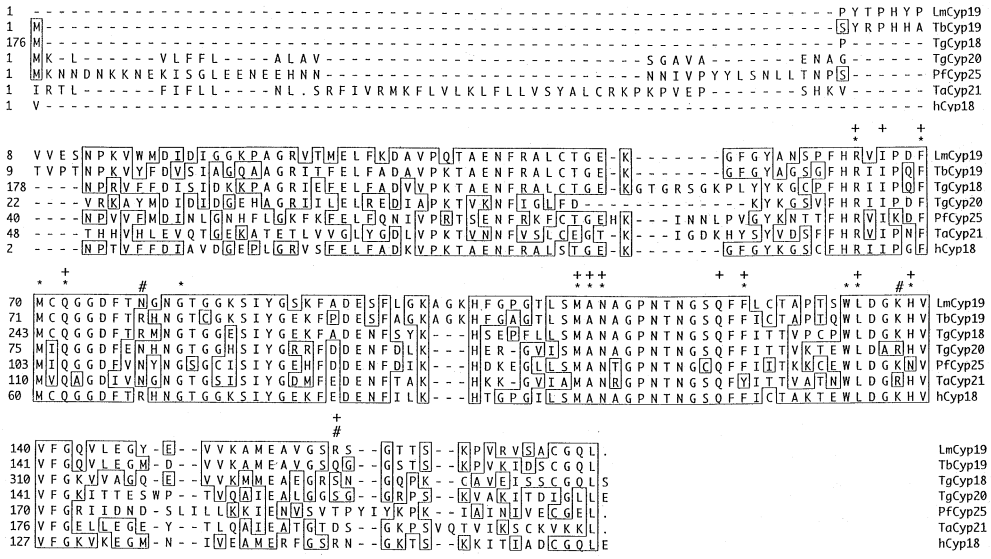

Figure 3 Sequence alignment of deduced amino acid sequences of LmCyp19 with human and parasite Cyps

The amino acid sequence of the human Cyp 18 are denoted hCyp18 (accession number X52851); *Trypanosoma brucei* (U68270), TbCyp19; *Toxoplasma gondii* (U04633), TgCyp18; U04634, TgCyp20; *Theileria annulata* (U55771), TaCyp21; *Plasmodium falciparum* (U10322), PfCyp25. Amino acid residues involved in CsA (*), substrate (+) and calcineurin (#) binding of the human Cyp are indicated [40–42]. Amino acids conserved in all listed enzymes are boxed.

Table 1 Subsite specificity of LmCyp19

The ratios k_{cat}/K_m are shown by setting the value of the substrate Suc-Ala-Xaa-Pro-Phe-4-nitroanilide to 100% for Xaa = Ala (k_{cat}/K_m of LmCyp19 = 1.5×10^6 $M^{-1} \cdot s^{-1}$). Values of k_{cat}/K_m were calculated from the expression $k_{cat}/K_m = (k_{obs-}k_u)/[E]$, where k_{obs} is the first-order rate constant and k_u is the rate constant for isomerization in the absence of enzyme. Data for a host cyclophilin, the human recombinant Cyp18, are taken from [29]; data for the *E. coli* Cyp18 are from [47]. Abbreviation: n.d., not determined.

	Relative k_{cat}/K_m (%)		
Amino acid, Xaa	LmCyp19	Human Cyp18	*E. coli* Cyp18cy
Gly	45	37	38
Ala	100	100	100
Leu	112	85	48
Phe	68	n.d.	25
His	39	19	7
Lys	56	29	8
Gln	71	n.d.	n.d.
Glu	74	67	16

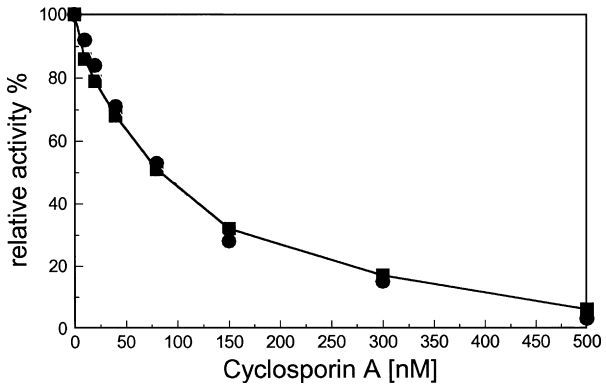

Figure 4 Inhibition of LmCyp19 by CsA

Recombinant (●) or wild-type (■) LmCyp19s were preincubated with increasing concentrations of CsA and the remaining PPIase activity was analysed with the substrate Suc-Ala-Ala-Pro-Phe-4-nitroanilide. PPIase inhibition assays were performed in two replicates; the symbols represent the average values.

fragments by hydrophobic-interaction chromatography. The cross-reactivity of the purified protein with the LmCyp19 N-terminal specific anti-peptide antibody confirms the expression of the recombinant Cyp (Figure 2).

Kinetic parameters and inhibition of *L. major* Cyp19

To define the relationship of the purified LmCyp19 to the protein family of Cyps, the role of the P1 subsite of substrates (for subsite nomenclature see [34]) was examined. The specific constant k_{cat}/K_m for the *cis–trans* interconversion of the -Xaa-Pro-bond in Suc-Ala-Ala-Pro-Phe-4-nitroanilide was 1.5×10^6 $M^{-1} \cdot s^{-1}$. Results obtained from the substrate type Suc-Ala-Xaa-Pro-Phe-4-nitroanilide, with variations of the amino acid in the Xaa position, are shown in Table 1, together with the data for recombinant human Cyp18 and the *E. coli* cytoplasmic Cyp18. The pattern of specificity of LmCyp19 is reminiscent of human cytoplasmic Cyp, with a remarkable exclusion of histidine preceding proline in the substrate. LmCyp19 shows the highest PPIase activity towards substrates with small aliphatic side chains. These results agree with other characterized eukaryotic Cyps [29]. The k_{cat}/K_m for LmCyp19 is clearly different from that for *E. coli* Cyp, so that the possibility of contamination with the prokaryotic PPIase during protein purification can be excluded.

The binding capacity of recombinant and wild-type LmCyp19 to CsA was analysed by inhibition of its PPIase activity in the presence of CsA (Figure 4). The results for inactivation of recombinant LmCyp19 at different concentrations of CsA fitted

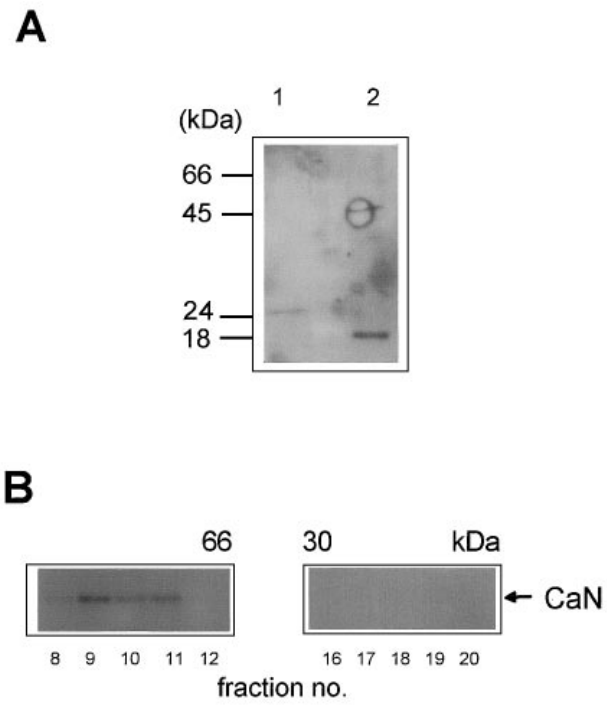

Figure 5 Presence of calcineurin B in a larger complex demonstrated by Western blot

(**A**) Whole parasite (lane 1) or macrophage (lane 2) extract was separated by SDS/PAGE; calcineurin was detected by Western-blot analysis. (**B**) Calcineurin (CaN) B subunit from *L. major* homogenates was eluted in a 100 kDa complex after gel-filtration on Superose 12 as described in the Materials and methods section. Fractions were concentrated by acid precipitation and separated by SDS/PAGE; calcineurin B was detected by Western blot analysis with rabbit antiserum against mammalian calcineurin. The positions of BSA (66 kDa) and myoglobin (30 kDa) as molecular mass standards are indicated.

the equation of competitive tight-binding inhibition, resulting in a K_i of 5.2 nM.

Detection of an *L. major* calcineurin

Several suggestions for the mode of action of cyclosporin in its anti-parasitic capacity have been made, including the role of calcineurin [11–13,35]. On the assumption that the observed resistance of *L. major* parasite to CsA is based on a low affinity of parasitic Cyps in forming the trimer complex Cyp–CsA–calcineurin, we examined first whether a calcineurin-like protein exists in the parasite. Calcineurin in mammalian cells is a heterodimeric enzyme comprising a 60 kDa catalytic A subunit (calcineurin A) and a 19 kDa Ca^{2+}-binding regulatory subunit (calcineurin B) [33,36]. Parasitic homogenates were subjected to SDS/PAGE and analysed with a polyclonal anti-(calcineurin B) antiserum. A calcineurin B-like protein with a molecular mass of 25 kDa cross-reacted with the antibody (Figure 5A).

To determine the presence of heterodimeric calcineurin in *L. major*, parasitic homogenate was size-fractionated by gel-filtration experiments. *L. major* calcineurin B, comparable to calcineurin B from macrophages, was detected by immunohybridization in fractions 9–11 after separation by FPLC with a Superose 12 column (Figure 5B). The molecular mass of the proteins that were eluted in these fractions was evaluated with protein standards as being approx. 100 kDa, suggesting that the parasitic calcineurin B might form a high-molecular-mass complex with calcineurin A.

The LmCyp19–CsA complex has no affinity for calcineurin

To address the role of CsA in modulating the binding of Cyp and calcineurin, we analysed this protein–protein interaction in *L. major*. In previous reports, an FPLC size-exclusion radiochromatography assay was established that monitored the formation of a complex between Cyp–CsA and calcineurin [32]. In this assay the Cyp–CsA complex is eluted from a sizing column in fractions corresponding to the molecular mass of Cyps (Figure 6, fractions 17–19). In the presence of calcineurin a higher-molecular-mass Cyp–CsA–calcineurin complex is formed, which is eluted in earlier fractions (fractions 9 and 10), distinct from the Cyp–CsA complex. A modification of this assay was used to analyse the binding capacity of parasitic Cyps to calcineurin in the presence of [^{3}H]CsA. Gel-filtration fractions were characterized by determination of radioactivity and by Western blotting with anti-LmCyp19 antibodies, anti-(host cell Cyp18) antibodies or anti-(calcineurin B) antibodies to identify Cyps and calcineurin.

Primarily, the recombinant LmCyp19 was preincubated with [^{3}H]CsA and separated by gel filtration. The [^{3}H]CsA–LmCyp19 complex was detected by radioactivity and by immunohybridization with the anti-LmCyp19 antibody in fractions 17–19 (Figure 6A). When *L. major* homogenates were added to preformed [^{3}H]CsA–LmCyp19, neither radioactivity nor LmCyp19 was shifted to fractions 9 and 10, containing the high-molecular-mass complex of calcineurin. Uncomplexed [^{3}H]CsA–LmCyp19 remained in fractions 17–19 (Figure 6A, Western blot). In parasitic homogenates without additional recombinant LmCyp19, the Cyp was detected by radioactivity and immunohybridization (results not shown) only in fractions 18–20 in the presence of [^{3}H]CsA, indicating a low or deficient affinity of parasitic Cyps for calcineurin (Figure 6A). In contrast, when gel-filtration experiments of protein homogenates from macrophages were performed as a positive control, a significant portion of [^{3}H]CsA was shifted to high-molecular-mass complexes (fractions 9 and 10) (Figure 6B).

If parasitic calcineurin is functionally and structurally comparable to calcineurin from macrophages, it should interact with host Cyp–CsA complexes. As expected, when mixtures of recombinant human Cyp18, preincubated with [^{3}H]CsA and parasitic homogenate, were size fractionated by gel filtration, radioactivity together with human Cyp18 were shifted to fractions 9 and 10 (Figure 6B).

LmCyp19 does not interfere with calcineurin activity of *L. major*

To determine whether *L. major* expresses functional active calcineurin, fractions from the above gel-filtration experiments were examined for their ability to catalyse the dephosphorylation of a well-characterized calcineurin substrate, the RII phosphopeptide. In confirmation of the immunoblot data with an anti-(calcineurin B) antiserum, calcineurin activities were found in fractions 9–11 from the gel filtration of *L. major* (results not shown). The dephosphorylation was considered as calcineurin phosphatase activity by inhibition with a Ca^{2+}-chelating agent (5 mM EGTA) and 10 μM okadaic acid (Table 2). The observed rate was comparable to results from other parasites, but lower than for mammalian enzymes [33,37]. The parasitic (LmCyp19) and human (hCyp18) Cyps were further tested for their effect on the dephosphorylation of the RII phosphopeptide by calcineurin.

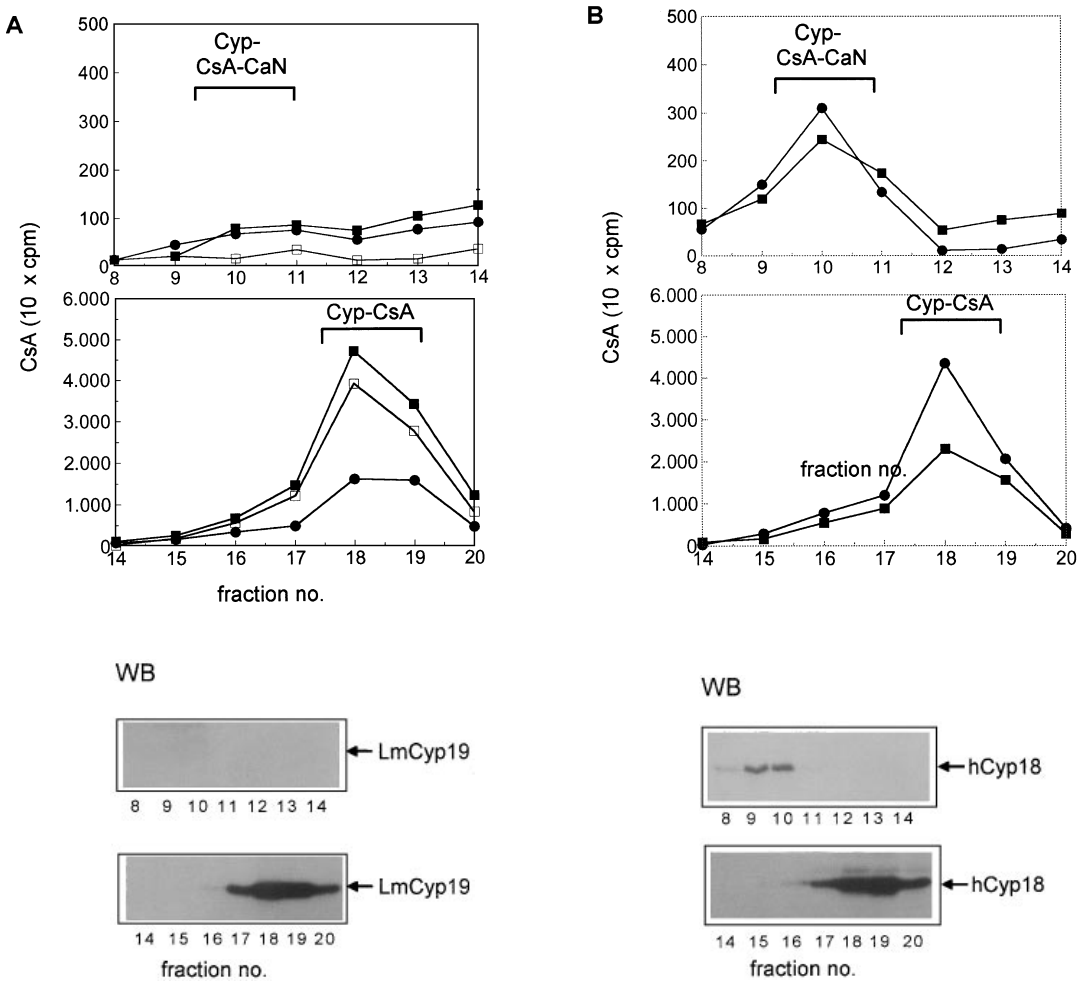

Figure 6 LmCyp19 forms no high-molecular-mass complex with calcineurin (CaN)

Preincubated mixtures were subjected to FPLC gel filtration on Superose 12. Fractions were analysed by β-scintillation and immunohybridization (Western blot; WB) with specific antibodies against LmCyp19 or against a conserved motif of Cyp, also recognizing human Cyp18. (**A**) [^{3}H]CsA was incubated with pure recombinant LmCyp19 (□), *L. major* homogenate only (●) or LmCyp19 plus *L. major* homogenate (■). A representative Western blot of the analysed mixture [^{3}H]CsA–LmCyp19–*L. major* homogenate is shown. (**B**) A Cyp–CsA–calcineurin complex is formed by incubation of *L. major* homogenate with human Cyp18 in the presence of [^{3}H]CsA (●). A representative Western blot is shown. The elution profile is comparable to the pattern of a separated macrophage [^{3}H]CsA mixture, as a positive control (■). Brackets denote the fractions containing the Cyp–CsA and Cyp–CsA–calcineurin complexes. Each column profile presents results from a single experiment. The experiment was repeated with independent cell and protein preparations with essentially the same results.

Table 2 Parasite calcineurin activity is not inhibited by LmCyp19

^{32}P-RII peptide substrate was incubated with fraction 10 from gel filtration (see Figures 5 and 6) as described in the Materials and methods section. The release of ^{32}P was measured without inhibitor or in the presence of the indicated inhibitor concentrations. All activity values are means ± S.D. from two different size-exclusion experiments.

Inhibitor	Calcineurin activity (pmol/min per mg of protein)	Relative activity (%)
None	7.56 ± 0.73	100
5 mM EGTA	0.97 ± 0.36	13
10 μM Okadaic acid	1.09 ± 0.47	14
1 μM CsA	7.34 ± 0.56	97
1 μM hCyp18	7.49 ± 0.67	99
1 μM CsA + 1 μM hCyp18	1.20 ± 0.31	16
1 μM LmCyp19	7.52 ± 0.54	99
5 μM LmCyp19	6.52 ± 0.45	86
5 μM CsA + 5 μM LmCyp19	6.42 ± 0.17	85

Neither hCyp18 or CsA alone inhibited the dephosphorylation. However, calcineurin phosphatase activity of *L. major* was completely inhibited by the hCyp18–CsA complex. In contrast, the LmCyp19–CsA complex did not inhibit dephosphorylation of RII phosphopeptide when used in comparable concentrations. The LmCyp19–CsA complex was only minimally inhibitory (15%) at 5 μM (Table 2), but also with LmCyp19 alone at this concentration; it therefore seems to be non-specific.

DISCUSSION

Our original aim was to test whether *L. major* parasites modulate their CsA-binding proteins so that they are insensitive to the action of CsA. The approach was to use a CsA-affinity matrix previously shown to be efficient for the isolation of cylophilins in *T. gondii* [35] and to determine whether these Cyps are modified structurally or functionally. Unexpectedly, we found that *L.*

major expresses Cyps with a high affinity for CsA. This prompted us to re-examine the original hypothesis that the insensitivity of parasites to CsA results from a changed expression of Cyp or a decreased affinity of these proteins for CsA. In a reciprocal approach we tested whether or not the biochemical properties of Cyps or the capacity to interact with calcineurin discriminates parasites in CsA-sensitive organisms.

Here we report the characterization of the major cyclophilin from *L. major*, LmCyp19. It is highly similar to all Cyps previously isolated and is active as a PPIase that can be inhibited by CsA at nanomolar concentrations. Comparison of the sequences of LmCyp19 with the major host Cyp and with various protozoan Cyps demonstrates that LmCyp19 shares in the core region a 65–85 % similarity (residues 40–144) with Cyps from *Trypanosoma brucei*, *Toxoplasma gondii*, *Theileria annulata* and *Plasmodium falciparum* [35,38,39]. The residues of human Cyp18 that have been shown by NMR and X-ray crystallographic analysis to be involved in CsA and substrate binding are present in LmCyp19, including a tryptophan residue known to be essential for CsA binding at position 133 of LmCyp19 [40–42]. Accordingly, the subsite specificity for tetrapeptides of LmCyp19 with respect to the P1 position and the sensitivity to CsA are only slightly different from those of the other eukaryotic Cyps [29]. Outside the core region, the first 12 residues are unique in sequence but similar in the overall size to the N-termini of other parasitic Cyps. The C-terminus is also unique, excluding the last four residues, CGQL, with an evident similarity to characterized Cyps [18]. These dissimilar domains and the amino acid replacements (see below) might function simply to increase the specific activity of the PPIase by providing specificity for selected substrates, larger than the analysed small peptides.

Like the major human Cyp18, LmCyp19 is highly expressed in promastigotes of *L. major*, but three additional Cyps with molecular masses of 18, 22 and 40 kDa could be identified in the cytosol by their CsA-binding activities. Clustered bands of Cyps were reported in earlier studies from other species to be CypA (18 kDa), CypB (21 kDa), CypC or Cyp40 (40 kDa), with different N-terminal sequences [15,18]. In contrast, each of the sequenced Cyps from *L. major* has the identical PYTPHYPVV N-terminal sequence to that of the major LmCyp19. We tried to clone the genes of Cyp isoforms by using different combinations of techniques. Neither PCR on genomic DNA nor cDNA with degenerate primers designed to hybridize to highly conserved regions of all Cyps or a combination with primers to the N-terminal sequence could detect more than one gene coding for LmCyp19. It has recently been shown that the 11 Cyp isoforms of the nematode *Caenorhabditis elegans* have similar N-terminal sequences to each other but not to Cyps from other species [43]. Extending these experiments by searching the *L. major* expression sequence tags (EST) database and searching the genomic DNA sequences generated by the *L. major* genome project identified larger Cyps but with different N-termini (A. Pahl, unpublished work). The presence of a single gene encoding two Cyps, one with a signal sequence, has been previously noted in *Neurospora crassa*, in which one gene encodes both the cytosolic and mitochondrial Cyps [44]. However, such a mechanism seems to be unlikely in *L. major*. Alternatively, the similar molecular masses of the dominant 19 kDa and the minor 22 kDa CsA-binding proteins lead us to speculate that one of the two proteins might be modified post-translationally. Efforts to prove our hypothesis and their biological relevance continue.

Our results that Cyps exist in *L. major* and LmCyp19 has a high CsA-binding capacity exclude the possibility that the resistance of *L. major* to CsA might be explained by either the lack of Cyps or the failure to bind the drug.

Our alternative assumption proposes that the resistance of *L. major* is mediated by the lack of complex formation with the phosphatase calcineurin. Calcineurin is prevalent in many species and acts as a functional phosphatase involved in diverse cellular functions such as signal transduction [22,45]. In this study we demonstrate that calcineurin exists in the parasite, but *L. major* Cyps are unable to interact with calcineurin in the presence of the drug CsA. We found that the calcineurin B subunit is present in crude extracts of *L. major* with a slightly larger molecular mass than mammalian calcineurin B. By size-exclusion chromatography we showed that calcineurin is a component of a 100 kDa protein complex, probably consisting of calcineurin A and calcineurin B. Furthermore, when a specific synthetic peptide is used as substrate, this complex shows the specific calcineurin phosphatase activity described for the protein from different sources [33,37]. Although calcineurin and Cyps are present in *L. major*, neither the proteins alone nor the proteins in the presence of CsA bound to calcineurin interfere with the phosphatase activity *in vitro*. The lack of interaction of the LmCyp19–CsA complex with parasitic calcineurin was mirrored by the lack of an interaction with mammalian calcineurin. In addition, mammalian Cyp binds parasitic calcineurin and inhibits its activity but LmCyp19 does not, suggesting that parasitic calcineurin is functionally identical with mammalian calcineurin but that LmCyp19 is different from its mammalian counterpart. It is noteworthy that similar observations have been made for helminthic parasites, which also lack a CsA-mediated inhibition of calcineurin [37].

Why does the Cyp–calcineurin complex not interact in *L. major*? First, given the highly conserved protein–protein interaction surface of Cyp and calcineurin, one can imagine structural reasons. In fact, three surface residues surrounding the drug pocket of human CypA with positively charged side chains, Arg-69, Lys-125 and Arg-148, have been suggested as potential sites of electronic interaction with calcineurin [46,47]. If each of these three amino acids is changed to a residue containing either negatively charged or uncharged amino acids, the human CypA then reveals a decreased ability to interact with calcineurin, although the CsA-binding affinities are hardly influenced by these mutations. According to these results, Arg-69 in LmCyp19 is replaced by Asn-69 and no association with calcineurin could be detected in our experiments. Characterization of Cyps from *P. falciparum*, *T. gondii*, *T. annulata* and *S. mansoni* revealed an identical Arg-69 → Asn replacement, suggesting a general mechanism of parasitic Cyps to decrease their calcineurin affinity.

The results presented here and previous studies *in vivo* [9] provide a model for the role of Cyps in mediating the antiparasitic effects of CsA: besides its highly conserved PPIase activity, Cyps from *L. major* have developed specific structural modifications, resulting in the lack of calcineurin binding. We hypothesize that LmCyp19 has developed a specific composite surface to interact with putative protein ligands different from the mammalian counterpart. The binding pocket of LmCyp19 might regulate the physiological function of these as yet unknown ligands and might manipulate cellular processes of the host cell in this way. It seems plausible that these protein–protein interactions are either inhibited or stabilized by endogenous CsA. More detailed research is in progress to determine whether this hypothesis is correct.

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