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Functional analysis of *Leishmania major* cyclophilin

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Abstract

A potent immunosuppressive drug cyclosporin A (CsA) is known to inhibit human cell infection by the pathogenic protozoan parasite *Leishmania major* both in vitro and in vivo. The proposed mechanism of action involves CsA binding to *Leishmania major*-expressed cyclophilin and subsequent down-regulation of signaling events necessary for establishing productive infection. Recently, we identified a ubiquitously expressed membrane protein, CD147, as a signaling receptor for extracellular cyclophilins in mammalian cells. Here we demonstrate that, while being enzymatically active, the *Leishmania* cyclophilin, unlike its human homologue, does not interact with CD147 on the cell surface of target cells. CD147 facilitates neither *Leishmania* binding nor infection. Primary structure and biochemical analyses revealed that the parasite's cyclophilin is defective in heparan binding, an event required for signaling interaction between CD147 and human cyclophilin. When the heparan-binding motif was reconstituted in *Leishmania* cyclophilin, it regained the CD147-dependent signaling activity. These results underscore a critical role of cyclophilin-heparan interactions in CD147-mediated signaling events and argue against the role of *Leishmania* cyclophilin in parasite binding to target cells.

Keywords: *Leishmania major*; Promastigote; Heparan sulphate; Cyclophilin; CD147

1. Introduction

Leishmania are protozoan parasites which cause a wide spectrum of diseases collectively known as leishmaniasis. Transmission to the vertebrate host, initiated by flagellated metacyclic promastigotes, is via the bite of an infected female sandfly vector. Once within the mammalian host, the parasite enters the macrophage, where it transforms into the replicative amastigote stage. Depending both on the species initiating infection and on the immunological status of the host, disease forms range from simple cutaneous and mucocutaneous to diffuse cutaneous and visceral. *Leishmania major* is a well-studied Old World species that causes cutaneous disease in humans and mice (Farrell, 2002).

Cyclosporin A (CsA) has been reported to inhibit infection by the human pathogenic protozoan parasites in vitro and in vivo (Chappell and Wastling, 1992; Meissner et al., 2003; Bua et al., 2004). Since main targets of CsA are cyclophilins, sensitivity of protozoan infection to CsA suggested an important role that cyclophilins may play in protozoan life cycle. Cyclophilins are a large family of proteins that possess peptidyl–prolyl *cis–trans* isomerase activity and are believed to play an important role in cell physiology as chaperones (Barik, 2006) and regulators of protein folding (Kofron et al., 1991). In addition to their intracellular functions, some cyclophilins can be released by cells in response to various stimuli such as inflammatory mediators, including reactive oxygen species (ROS) and lipopolysaccharide (LPS) (Sherry et al., 1992; Xu

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et al., 1992; Jin et al., 2000; Suzuki et al., 2006). These extracellular cyclophilins exert potent chemotactic activity towards many types of immune cells, which may contribute to the pathogenesis of several inflammatory diseases such as rheumatoid arthritis, acute lung injury or allergic asthma (Arora et al., 2005; Kim et al., 2005; Gwinn et al., 2006; reviewed in Yurchenko et al., 2006). A ubiquitously expressed membrane protein, CD147, has been shown to play a critical role in the signaling responses of mammalian cells to extracellular cyclophilins and to contribute to a variety of cyclophilin-mediated physiological and pathological activities (reviewed in Yurchenko et al., 2006).

Cyclophilins are produced by many protozoan parasites. Interestingly, several trypanosomatids, e.g. *Trypanosoma cruzi*, secrete a cyclophilin-like protein during the host cell infection and antibodies raised against the *T. cruzi* isomerase greatly reduce parasite's infectivity (Moro et al., 1995). These results suggest that parasites might secrete cyclophilin to initiate signaling events in the target cells, thus creating the optimal environment for entry and replication. Three isoforms of *Leishmania major* cyclophilin (LmCyp) with molecular masses of 18, 19 and 22 kDa have been identified (Hoerauf et al., 1997; Rascher et al., 1998). The 19 kDa protein (LmCyp19) is the major isoform of LmCyp; the other two isoforms may result from post-translational modification or degradation of this cyclophilin (Rascher et al., 1998). The role of LmCyp19 in the parasite's life cycle remains uncharacterized. The enzymatic peptidyl–prolyl *cis–trans* isomerase activity of LmCyp isoforms was greatly reduced by CsA, however, unlike human cyclophilin A, the complex of LmCyp19 with CsA did not bind to the Ca-regulated phosphatase calcineurin (Rascher et al., 1998). Surprisingly, pretreatment of parasites with CsA did not diminish the level of macrophage infection (Hoerauf et al., 1997). Structural studies of cyclophilin of another species of *Leishmania*, *Leishmania donovani*, revealed complete structural conservation of the cyclosporin-binding site with respect to the homologous human protein, with deviations primarily in the loop regions (Venugopal et al., 2007). Given that interaction of human cyclophilin with CD147 is sensitive to CsA and involves the enzymatically active site of cyclophilin (Yurchenko et al., 2002), we hypothesized that secreted LmCyp19 may facilitate parasite infection by inducing CD147-dependent signaling in target macrophages.

Here, we investigated whether CD147 interacts with *Leishmania*-secreted LmCyp19 and facilitates infection by the parasite. We demonstrate that, unlike human cyclophilins, LmCyp19 does not interact with human CD147 on the surface of target cells, and CD147 facilitates neither *Leishmania* binding nor infection. Comparative analysis of LmCyp19 and human CypA revealed that the *Leishmania* protein is defective in heparan binding, which is required for CypA-initiated signal transduction. Restoration of the heparan binding capacity of LmCyp19 by mutagenesis also restored CD147-mediated signaling activity.

2. Materials and methods

2.1. Parasites and infection

Promastigotes of *L. major* MHOM/IL/79/LRC-L251, kindly provided by Dr. McMahon-Pratt (Yale School of Public Health), were cultured at 26 °C in Grace's medium (Invitrogen, Carlsbad, CA) supplemented with 15% heat-inactivated FCS and 10 μg/ml of Gentamicin (both Invitrogen) as described previously (Scott et al., 1987). Half-confluent cultures of Chinese hamster ovary (CHO) cells (3×10^5 cells/well) in 6-well plates were exposed to 1.5×10^6 parasites (5:1 ratio) for 6 h in serum-free F-12 medium (Invitrogen) at 33 °C as previously described (Guinet et al., 2000). Cells were washed three times with 1 × PBS buffer and kept in the growth F-12 medium supplemented with 15% heat-inactivated FCS. The level of infection was monitored at 6, 24 and 48 h p.i. by light microscopy.

2.2. Cells and reagents

The wild-type CHO.K-1 and heparan-deficient CHO.pgsB-618 cell lines were purchased from ATCC. CHO.pcDNA, CHO.CD147, CHO.pgsB-618.pcDNA and CHO.pgsB-618.CD147 cell lines have been described previously (Yurchenko et al., 2002). The anti-phospho-p42/44 and anti-p42/44 mitogen-activated protein kinase (MAPK) antibodies were from Cell Signaling Technology (Danvers, MA); anti-CD147 mAb that blocks cyclophilin-induced chemotaxis and signaling (Pushkarsky et al., 2001; Yurchenko et al., 2002) was from Ancell (Bayport, MN). Horseradish peroxidase (HRP)-labeled anti-glutathione-*S*-transferase (GST) mAb was purchased from GE Healthcare, Piscataway, NJ. Heparitinase III, which cleaves heparin at α-*N*-acetylglucosaminide-L-iduronic acid linkage, was purchased from Seikagaku America (Falmouth, MA).

2.3. Cell binding assay

Leishmania major promastigotes were labeled with tritiated uracyl (GE Healthcare) as previously described (Mosser and Edelson, 1985). Parasites were incubated with 5×10^5 cells at various parasite-to-cell ratios (5:1, 2:1 and 1:1) for 2 h at 37 °C, washed three times with 1 × PBS, and retained radioactivity was measured on a LS6500 Multi-purpose scintillation counter (Beckman Coulter, Fullerton, CA).

2.4. Heparan binding assay

Escherichia coli producing GST or GST-tagged CypA or LmCyp19 were lysed with PET lysis buffer (50 mM Tris–HCl, pH 8.0; 5 mM dithiothreitol (DTT); 1 mM EDTA;

0.5% Triton X-100 and 2 mM phenylmethanesulphonylfluoride (PMSF) and incubated with heparan agarose (Pierce Biotechnology, Rockford, IL)) overnight at +4 °C. After extensive washing with 1 × PBS, the heparan-bound proteins were eluted with Laemmli SDS sample buffer (Laemmli, 1970), separated by SDS PAGE and visualized with anti-GST antibodies.

2.5. Plasmids and site-directed mutagenesis

DNA encoding a 169 amino acid-long *L. major* cyclophilin (amino acids 9-177, (Rascher et al., 1998)) was cloned into pET14b (EMD Biosciences, San Diego, CA), pTYB12 (New England Biolabs, Ipswich, MA) and pGEX4T-3 (GE Healthcare) vectors and purified according to the manufacturers' protocols. Cloning details and primers used are available upon request. All three constructs yielded significantly less protein than similar constructs expressing human CypA. The highest level of LmCyp19 expression was achieved with the pGEX4T-3 expression plasmid. Site-directed mutagenesis of the LmCyp19 encoding plasmid was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

2.6. Protein purification

Escherichia coli-produced His- or GST-tagged proteins were purified from the cleared lysates by affinity chromatography on Ni-NTA agarose (Qiagen, Valencia, CA) or Glutathione-Sepharose (GE Healthcare), respectively, according to the manufacturers' protocols. The tag was further cleaved off CypA or LmCyp19 using biotinylated thrombin, which was then removed by streptavidin agarose (EMD Biosciences) according to the manufacturer's protocol.

2.7. Signaling analysis by Western blotting

Serum-starved CHO cells stably transfected with pcDNA or CD147 constructs (Yurchenko et al., 2002) were treated with various concentrations of LmCyp19. Cell lysates were separated on 10% SDS-PAGE and analyzed by Western blotting using anti-p42/44 and anti-phospho-p42/p44 MAP kinase antibodies (New EnglandBiolabs).

2.8. Assay of peptidyl–prolyl cis–trans isomerase activity

The peptidyl–prolyl *cis–trans* isomerase assay was performed in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 8.0, at 10 °C using the substrate peptide Suc-AAPF-pNA, which has the Ala-Pro bond in equilibrium between the *cis* and *trans* conformations (Sherry et al., 1992). P-nitroaniline chromophore release from the all-*trans* peptide was monitored at 390 nm. The rate constants were determined as previously described (Sherry et al., 1992).

3. Results and Discussion

3.1. Analysis of L. major binding and infection

Previous studies have shown that, via interaction with virus-associated cyclophilin, CD147 facilitates entry into the host cell of HIV-1 (Pushkarsky et al., 2001) and coronavirus (Chen et al., 2005). We hypothesized that *Leishmania*-produced LmCyp19 might function in a similar manner by interacting with CD147 and facilitating infection by the parasite. For quantitative analysis of the role of CD147 in *Leishmania* infection, we employed CHO cell lines stably transfected with human CD147 (CHO.CD147) or empty pcDNA3.1 vector (CHO.pcDNA) as negative control. The CHO cells were proposed as an ideal in vitro model for studying host–parasite interactions (Guinet et al., 2000) because they are relatively easy to culture and infect. These cell lines have been characterized previously (Yurchenko et al., 2001, 2002). Fig. 1a shows that both CHO.pcDNA and CHO.CD147 cell lines were equally infected with *L. major*. Although a small difference in infection rate between these two cell lines was noticed, it was not statistically significant. It remained possible that infection by *L. major* was facilitated by the hamster CD147 present on CHO cells. To exclude this possibility, we used the anti-CD147 antibody (clone UM-8D6) that was previously shown to block CD147-dependent CypA-induced signaling events in CHO.CD147 cells (Yurchenko et al., 2002). This experiment did not detect any difference in parasite infection in the presence or absence of the antibody (Fig. 1b). Nevertheless, consistent with previously published observations (Meissner et al., 2003), the presence of CsA during infection reduced the rate of infected cells from approximately 45% in non-treated CHO.CD147 cells to approximately 20% in the presence of 1 μg/ml CsA (Fig. 1b).

To exclude the possibility that CD147 contributes to parasite binding to the cell surface, even though this effect is dampened due to high-level infection of CHO cells, we compared *L. major* binding to CHO.pcDNA and CHO.CD147 cells. *Leishmania major* promastigotes were radiolabeled and incubated with the host cell lines at ratios 1:1, 2:1 and 5:1 for 2 h at 37 °C. After extensive washing, cell-associated radioactivity was measured. Similar results were obtained with all three sets of infection conditions. An example of such an analysis (ratio 2:1) is presented in Fig. 1c, which shows that CHO.pcDNA and CHO.CD147 cells bind to *Leishmania* with similar efficiency. Moreover, pre-treatment of CHO.CD147 cells with the blocking antibody to CD147 did not appreciably change the level of parasite binding. Therefore, CD147 is not essential for *Leishmania* infection.

Proteoglycans on the cell surface were shown to facilitate adhesion of *Leishmania* amastigotes to macrophages in vitro (Love et al., 1993). It has been proposed that parasites express on their surface or secrete a high-affinity heparan-binding protein which can interact with heparan sulfate proteoglycans on mammalian cells (Butcher et al.,

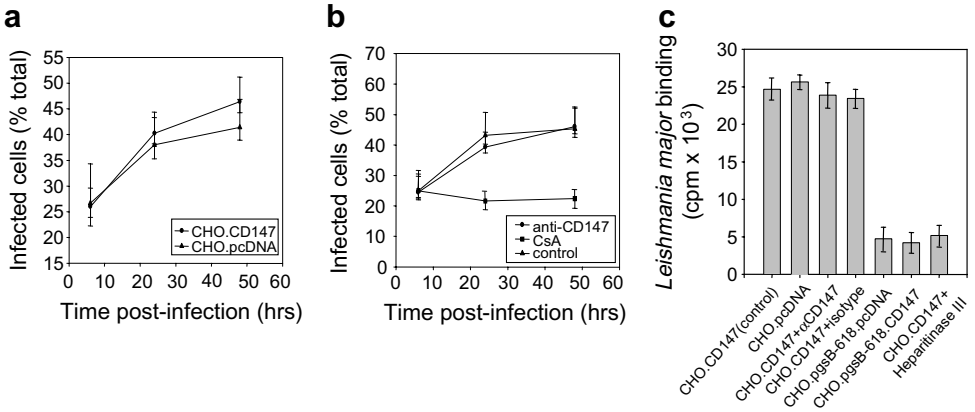

Fig. 1. CD147 does not facilitate *Leishmania major* infection or binding. (a) Half-confluent cultures of CHO.pcDNA (triangles) or CHO.CD147 (circles) cells were exposed to parasites for 6 h in serum-free medium at 33 °C. The level of infection was monitored at indicated times p.i. by light microscopy. Results (mean ± SD of three independent experiments) are presented as percentages of infected cells. The difference in infection rate between CHO.pcDNA and CHO.CD147 cell lines was not statistically significant. (b) CHO.CD147 cells were infected as in panel (a) in the presence of 1 μg/ml of CsA (squares) or 50 μg/ml of the blocking anti-CD147 antibody (circles) added together with the parasite. Nothing was added to control samples (triangles). Results are mean ± SD of three independent experiments. (c) *Leishmania major* promastigotes were radiolabeled and used to assess binding to indicated CHO cell lines. CHO.CD147+αCD147 denotes binding to cells pretreated with 50 μg/ml of anti-CD147 antibody; CHO.CD147+isotype are cells pretreated with 50 μg/ml of IgG1 isotype control antibody; CHO.pgsB-618.pcDNA are heparan-deficient cells transfected with pcDNA3.1; CHO.pgsB-618.CD147 are heparan-deficient cells transfected with *CD147*; and CHO.CD147+Heparitinase III denotes cells pretreated with Heparitinase III to remove heparans. Results are mean ± SD of three independent experiments.

1992). This interaction may represent an important first step in cell-to-cell transmission of *Leishmania* amastigotes. Since CypA binds to heparan sulphates (Saphire et al., 1999), we hypothesized that heparans, rather than CD147, may function as receptors for LmCyp19 to facilitate infection by the parasite. We first tested whether heparan-sulfates are important for promastigote binding. In addition to CHO.pcDNA and CHO.CD147 cell lines, we used two cell lines lacking heparan-sulfates, CHO.pgsB-618.pcDNA and CHO.pgsB-618.CD147. To remove heparans, we also pretreated CHO.CD147 cells with heparitinase III, which cleaves heparans at α-*N*-acetylglucosaminide-L-iduronic acid (Yurchenko et al., 2002). As shown in Fig. 1c, in the absence of proteoglycans the binding of *L. major* promastigotes was greatly diminished indicating that, similar to amastigotes, binding to heparan-sulfates on the cell surface facilitates infection by promastigotes. As has been noted before, this interaction might represent an important initial step in *Leishmania* infection. Again, the presence of human CD147 did not appreciably change *L. major* binding.

Taken together, results presented in Fig. 1 indicate that *L. major* binding and infection do not depend on CD147 but are facilitated by heparan sulphates.

3.2. LmCyp does not stimulate CD147-dependant signaling

We next addressed the question whether LmCyp19, similar to its human homologue, stimulates the downstream signal transduction events. We previously demonstrated that CD147 is an essential component in the cyclophilin-initiated signaling cascade that culminates in extracellular signal-regulated kinases (ERKs) activation (Yurchenko et al., 2001, 2002). Here, we compared human CypA and LmCyp19 in their ability to induce ERKs activation. LmCyp19 and CypA were expressed in *E. coli* and purified close to homogeneity (Fig. 2a) by two-step affinity chromatography (see Section 2). Both proteins were active in the peptidyl–prolyl *cis–trans* isomerization assay, although we noted lower activity of LmCyp (Table 1). Fig. 2b shows that LmCyp19 did not activate the ERK kinases in CHO.CD147 (lane 7) nor in CHO.pcDNA (lane 3) cells, whereas the same concentration of the human CypA stimulated ERKs phosphorylation in the CHO.CD147 cell line (lane 6), but not in CHO.pcDNA cells (lane 2). We also noted that, similar to the human CypA and CypB (Yurchenko et al., 2002), high concentrations of LmCyp19 (~250–500 nM) initiated a CD147-independent signal transduction response (data not shown).

We previously demonstrated the essential role of heparan sulfate proteoglycans in CypA-induced CD147-dependent signaling events (Yurchenko et al., 2002). We reasoned that the inability of LmCyp19 to induce CD147-dependent signaling could be due to its defective interaction with heparan sulfates. LmCyp19 binding to heparans was reduced compared with CypA (Fig. 2c). We estimated the heparan affinity of LmCyp19 to be approximately 1–5% of that of CypA protein.

3.3. Comparison of human and L. major cyclophilins

To understand the molecular mechanisms responsible for the observed differences between LmCyp19 and human CypA, we compared the primary structures of these proteins (Fig. 3). It has previously been shown that F60A

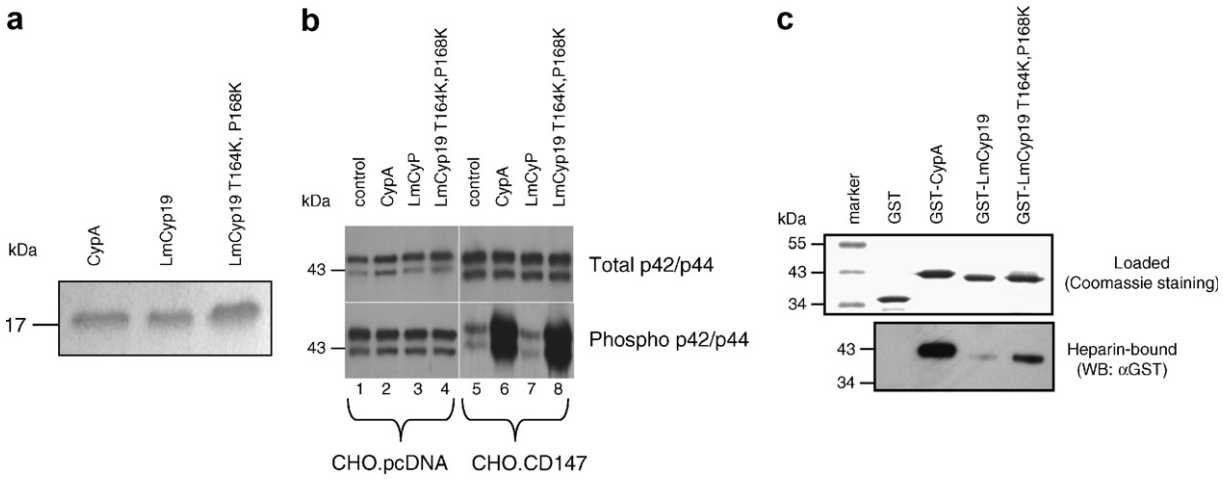

Fig. 2. LmCyp19 does not stimulate extracellular signal-regulated kinases (ERKs) phosphorylation. (a) Gel electrophoresis of recombinant cyclophilins expressed in *Escherichia coli*. (b) Purified cyclophilins (25 nM each) were used to stimulate CHO.CD147 and CHO.pcDNA cells. Control lanes show unstimulated cells. Total and phosphorylated ERKs (p42 and p44) were visualized by Western blotting with anti-p42/p44 (top) or anti-phospho-p42/p44 mAb (bottom), respectively. (c) Equal amounts of glutathione-*S*-transferase (GST), GST-CypA, GST-LmCyp19 or GST-LmCyp19 T164K, P168K were added to Heparin-agarose (5% of the added amount shown in upper panel), washed, eluted in Laemmli SDS sample buffer, separated by PAGE and visualized with anti-GST antibody (bottom panel).

Table 1
Analysis of proline *cis–trans* isomerase activity of LmCyp19

Cyclophilin	k (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})
CypA	0.07248	3.4854×10^6
LmCyp19	0.05524	2.6158×10^6
LmCyp19 T164K,P168K	0.05397	2.4776×10^6

The K_{cat}/K_m values were calculated after subtracting the background isomerization by fitting the data iteratively to a pseudo first-order kinetic equation (Rascher et al., 1998).

and H126A mutations result in an almost complete loss of the rotamase activity, the F113A mutant retains only about 10% of the rotamase activity of the wild-type protein (Zydowsky et al., 1992), and W121 does not affect rotamase activity but is essential for Cyp-CsA interaction (Liu et al., 1991). All amino acids forming the Cyp active site (F60, F113, W121 and H126, numbered according to the human protein) are conserved in LmCyp19 (Fig. 3, shaded). Consistent with this analysis, the isomerase activity of LmCyp19 was only slightly lower than that of CypA (Table 1). In contrast, two out of four basic amino acids (R48, K51, K54 and K55) determining heparan binding activity of human CypA (Yurchenko et al., 2002) are not conserved in LmCyp19, where lysines in positions 164 and 168 (corresponding to K51 and K55 of CypA) are substituted with threonine and proline, respectively (Fig. 3, in bold). To verify that lysine to threonine and lysine to proline substitutions in positions 164 and 168 of LmCyp19 are critical for the observed differences between human and *Leishmania* cyclophilins, we changed these residues back to lysines. The mutant LmCyp19 regained the ability to bind heparans (Fig. 2c). Most importantly, it became capable of initiating CD147-dependent signaling, as it induced ERKs phosphorylation in CHO.CD147 (Fig. 2b, lane 8) but not in CHO.pcDNA cells (Fig. 2b, lane 4). Introduced mutations did not affect isomerase activity of LmCyp19 (Table 1).

For human CypA and CypB, two types of binding sites have been identified (Denys et al., 1998; Yurchenko et al., 2002). The type I sites are the specific functional receptors

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LmCyp19  13 NPKVWMDIDIGGKPAGRVTMELFKDAVPQTAENFRALCTGEKGFGYANSPFHRVIPDFMC  72
               NP V+ DI + G+P GRV+ ELF D VP+TAENFRAL TGEKGFGY  S FHR+IP FMC
CypA      3 NPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHRIIPGFMC  62

LmCyp19P 73 QGGDFTNGNGTGGKSIYGSKFADESFLGKAGKHFGPGTLSMANAGPNTNGSQFFLCTAPT 132
               QGGDFT  NGTGGKSIYG KF DE+F+    KH GPG LSMANAGPNTNGSQFF+CTA T
CypA     63 QGGDFTRHNGTGGKSIYGEKFEDENFI---LKHTGPGILSMANAGPNTNGSQFFICTAKT 119

LmCyp19 133 SWLDGKHVVFGQVLEGYEVVKAMEAVGSRSGTTSKPVRVSACGQL 177
              WLDGKHVVFG+V EG  +V+AME  GSR+G TSK + ++ CGQL
CypA    120 EWLDGKHVVFGKVKEGMNIVEAMERFGSRNGKTSKKITIADCGQL 164
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Fig. 3. Sequence alignment of LmCyp19 and human CypA. Amino acids critical for cyclophilin enzymatic activity are shaded, residues responsible for heparan binding in CypA are in bold and underlined, mutated residues in LmCyp19 responsible for the loss of heparan-binding activity are in bold.

mediating internalization and signaling of bound cyclophilins, while the type II sites are represented by sulfated glycosaminoglycans. Recently, it was demonstrated that syndecan-1, one of the heparan sulfate proteoglycan core proteins, is physically associated with CD147 and is necessary for signaling and chemotactic activity of CypB (Pakula et al., 2007). We proposed that CypA binds first to heparans on the cell surface in order to be presented to its Type I receptor (CD147) for a more efficient interaction (Yurchenko et al., 2002). A similar function of heparans has been described for many chemoattractive agents, such as fibroblast growth factor (FGF) or chemokine regulated on activation normal T cell expressed and secreted (RANTES) (Schonherr and Hausser, 2000). It appears that due to the K164T and K168P substitutions, LmCyp19 is unable to bind heparans as efficiently as its human counterpart, thus its inability to induce signaling *via* CD147. This structural peculiarity of LmCyp19 also argues against its role in heparan-dependent binding of *L. major* to target cells.

Our results identify an important difference between LmCyp19 and its human counterpart, CypA. In contrast to CypA, LmCyp19 does not interact with CD147 and does not induce CD147-dependent signaling. Since cyclophilins mediate inflammatory responses via interaction with CD147 (Yurchenko et al., 2006), this feature of LmCyp19 may help *Leishmania* avoid chemoattracting immune cells to the site of infection. Given high conservation of the rotamase site in LmCyp19, the role of LmCyp19 in the parasite's infection likely concerns chaperone functions, but it does not seem to contribute directly to the parasite's interaction with the target cell. While it remains to be determined whether LmCyp19 is secreted, our results indicate that its extracellular activity is unlikely to be a factor in disease pathogenesis.

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