CLONING AND EXPRESSION OF LEISHMANOLYSIN GENE FROM *LEISHMANIA MAJOR* IN PRIMATE CELL LINES

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Abstract

Leishmanolysin is a worldwide disease that is caused by different species of the genus *Leishmania*. Leishmanolysin, One of the genes expressed by *Leishmania,* appears to be an ideal candidate for genetic vaccination. In this study, a full length sequence, which encodes Leishmanolysin functionally critical regions (amino acids 100-579), was cloned from a *Leishmania* strain endemic to Iran. Analysis by restriction enzyme digestion and DNA sequencing in pUC 19 based T-Vector showed that the cloned gene contained the conserved segments of the Leishmanolysin. The identified segments in predicted protein sequence of our clone contained the important domains that have been known to function at the attachment and internalization steps of the parasite life cycle. The cloned gene was expressed in human transformed muscle (Rhabdomyosarcoma TE671/RD) and African green monkey epithelial (COS-7) cell lines under cytomegalovirus (CMV) promoter, and the expressed protein was detected by enzyme linked immunosorbent assay. Thus the cloned gene may be used as an active component of a naked-DNA vaccine against Leishmaniasis in the geographic areas endemic to this parasite.

Introduction

The World Health Organization (WHO) has estimated that around 350 million people are exposed to the risk of infection by different species if the genus *Leishmania*. *Leishmania* currently affects 12 million people with an annual incidence rate of 2 million new cases. The genus is distributed in over 100 countries and some 100 species of animals could act as their reservoir host [1]. Extracellular flagellated *Leishmania* promastigotes are transmitted to their mammalian hosts through bite of sandflies. Thus, there is a need for

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mechanisms capable of inhibiting the initial survival of at least some of the inoculated parasites.

A critical point in the parasite-host interaction involves the attachment and internalization of promastigotes by macrophages. Numerous studies indicate that surface molecules of the parasite, such as Leishmanolysin (the 63 Kda. glycoprotein protease or gp63) and Lipophosphoglycan (LPG), are critical for parasite attachment, internalization and survival [2-5]. Modifications of these components or failure in their expression induce changes in virulence patterns, as evidence by the reduction in attachment and subsequent internalization [6-7].

In mammalian host, *Leishmania* typically reside within macrophages and dendritic cells, which in addition to their antimicrobial and antigen presenting functions [8] serve as sanctuary reservoirs for the parasite. The parasites are often protected from the toxic and antimicrobial molecules ($O2^{-}$, H2O2 and nitric oxide) present in the host cells. For instance, *L. major* can survive in epidermal Langerhans cells due to the lack of cytokine inducible nitric oxide synthatase (i NOS) in these cells and thus can get transported from infected skin to draining lymph node [9].

Leishmanolysin in *L. major* is encoded by multiple copies of conserved genes, mainly consisting of 1.8 kb coding and 1.3 kb space regions, tandemly arranged on a single chromosome [10]. The predicted protein sequence of *L. major* gp63 is composed of 602 amino acids [11]. Post translational modifications of Leishmanolysin during transport of the protein to the cell surface include removal of the N-terminal 100 amino acids, cleavage of C-terminal peptide by glycosyl-phosphadidylinositol (GPI) anchor attachment and N-linked glycosylation [12-14]. Leishmanolysin is conserved among diverse species of *Leishmania* [11] and is expressed at high levels on the surface of promastigotes [15]. This protein is present in both Extracellular promastigote and intracellular amastigote forms of the parasite [16]. Leishmanolysin exhibits other interesting features including GPI attachment of Leishmanolysin to the surface membrane of *Leishmania* [17] and the enzymatic activity of gp63 as a metalloporoteinase [18].

Several functional domains in gp63 are invoked during internalization of parasite. Among these, a putative adhesive RGD-like motif (SRYD) has been found to be important for cellular attachment [19-21] apparently through the cellular CRI, CR3 and FCγ, R receptors [22-25].

There are two protein core domains that mediate the internalization of promastigotes, one of them encoded by a sequence highly conserved among different *Leishmania* species. One of the these functional core domains includes an adhesive sequence (SRYD) and putative zinc-binding motif (HExxH) and the second domain includes a third histidine residue coordinated with zinc, which determines the structural features of the molecule [26].

We have found that (i) Leishmanolysin active protein contains four main domains that are involved in binding and internalization of parasite to macrophage, (ii) three of these domains are completely conserved between gene 1 and gene 6 copies of *L. major* and relatively conserved between *L. major* and *L. Guyanesis*, (iii) the domains of prepro-region have not significant role in attachment and internalization of the parasite. This clone may be a useful genetic vaccine against the Leishmaniasis.

Materials and Methods

Reagents

The synthetic oligonucleotide primers were synthesized by the abi Applied Bisystems 391 DNA synthesier PCR-MaTE. Restriction and modification enzymes were purchased from Roche molecular biochemicals (Roche Diagnostic Gmbtt, Roche Molecular Biochemicals, D-68298 Mannheim, Germany) or Sigma (Sigma, St. Louis, MO).

Cloning and DNA Manipulations

A pUC19 (Roche molecular biochemicals) and pcDNA3 (Invitrogen) plasmid vectors were used. *E. coli* TOP10 and DH5α component bacterial cells were used for DNA manipulations. Bacteria were propagated in Luria-borth or on Luria-agar(Roche molecular biochemicals) supplemented with ampicillin (50 μg/ml) where appropriate. Plasmids were purified by alkaline lysis of cultures followed by cesium chloride density gradient centrifugation [27]. The map of purified plasmid was confirmed by restriction endonuclease digestion of plasmids and separating products in agarose gels. All DNA manipulations were carried out as instructed by manufacturer as described [27] and modifications are mentioned.

L. major Isolation

L. major strain was originally isolated at the Department of Parasitology, Pasteur Institute of Iran, from a patient with cutaneous lesion. The promastigotes were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (completed RPMI) at 25°C [27].

Isolation of *L. major* Genomic DNA

The promastigotes were obtained by centrifugation at 3000 g at 4°C for 10 min, washed in cold phosphate buffer saline (PBS), pH 7.2. The 10^9 cells were

resuspended in 2-3 ml cell lysis buffer (50 mM NaCl, 50 mM EDTA, 1% SDS, 50 mM Tris-HCl, pH 8.0). Proteinase K was added to a final concentration of 100 μg/ml and the mixture was incubated at 37°C overnight. The DNA was extracted with a phenolphenol: chloroform procedure followed by addition of ethanol precipitation. The precipitated DNA aggregate was removed with pipette tip and then washed with 70% ethanol. The DNA was dissolved in TE (10 nM Tris-HCl, pH 7.2, 1 mM EDTA) and treated with RNase to remove any RNA contaminant. The integrity of genomic DNA was checked by electrophoresing on a 0.7% agarose gel.

Primers and Polymerase Chain Reaction (PCR) Amplification

The following primers were designed for amplification of the Leishmanolysin gene active region (amino acids 100-579). The amplified product was modified to contain a Kozak translational consensus sequence as well as *Hind III* and *BamH1* restriction sites.

Forward primer: 5′-C AGC *AAG CTT* ACC **ATG** GTG CGC GAC GTG AAC TGG GGC GCG C.

Reverse primer: 5′-CGG ACC ACG *GGA TCC* **CTA** CGC CGT GTT GCC GCC GTC CTT GG.

Amplification was carried out in 25 μl of reaction buffer [Tris HCl, pH: 8.8, 20 mM, $(NH_4)_2SO_4$ 10 mM, Gelatin 0.01%, Tween 20 0.2%, $MgCl_2$ 2.5 mM, Forward Primer 25. ηg (~1 μM), Reverse Primer 25. ηg (~1 μM)]. Each dNTP (50 μM), template (100 ng) and Taq DNA Polymerase (1 unit) were added to complete the reaction mixture. Amplification reaction was carried out with hot start procedure. All components were mixed on ice except dNTPs and DNA polymerase. The mixture was heated in thermocycler (Techne, Progene) to 96°C for 7 min and cooled to 70°C, and then dNTP and DNA polymerase were added to the mixture. Amplification program was run 5 cycles 95°C for 60 sec, 5°C for 90 sec, and 72°C for 180 sec, followed by 27 cycles 95°C for 60 sec, and 72°C for 120 sec with 5 sec increment time for each cycle and last final primer extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel.

PUC 19 based T-Vector Preparation

PUC19 (5 μg) plasmid was digested with *Hind III* (5 U/μl) (Roche Molecular Biochemicals) in 110 μl of buffer for 2.5 h at 37°C. The linearized vector was purified by phenol/chloroform extraction and ethanol precipitation.

A single thymidine nucleotide was added to the 3′ends of vector by incubation with 10 units Taq DNA polymerase and 7 μl 100 mM dTTP in 100 μl PCR reaction buffer (Roche Molecular Biochemicals) for 3 h at 70°C. The T-Vector was purified by phenol/ Chloroform extraction by ethanol precipitation and then was stored in frozen aliquots of 250 ηg until use.

DNA Sequencing

DNA sequencing was done by pharmacia LKB A.L.F DNA sequencer and DIG Taq DNA sequencing kit for standard and cycle sequencing with M13/PUC sequencing d (5′-GTAAAACGACGGCCAGT) and M13/PUC reverse sequencing d (5′-CAGGAAACAGCTATGAC) digoxigenine labeled primers (Roche Molecular Biochemicals).

Cells

For expressions of pcDNA3-Leishmanolysin human rhabdomyosarcoma TE671/RD [28] and COS-7 cell lines were used. The cells were maintained in culture in DMEM medium supplemented with 10% fetal calf serum (FCS) and 100 μg/ml of penicillin/streptomycin each at 37°C and cultured in humidified incubator with 5% CO2.

DNA Transfection

Transfection was carried out with Dosper Liposomal Transfection Reagent (Roche Molecular Biochemicals) according to the company instructions: (i) The day before transfecting approximately 2.5×10^5 cells were seeded per well of a 6-well culture plate in 2 ml culture medium. (ii) The cells were incubated at 37°C until they were about 70% confluent. (iii) On the day of transfection, the culture medium was replaced shortly before adding DOSPER/DNA mixture with 1 ml of fresh culture medium without serum. (iv) The mixture of the transfection reagent/nucleic acid was prepared as follows (the first time various amount of Dosper were used "2, 3, 4 and 5 μg" for optimization): Solution A: 1.5 μg DNA (sterile-filtered) was diluted with HEPES buffered saline (HBS; 20 mM HEPES cell culture grade, 150 mM NaCl, pH 7.4) to a final volume of 50 μl (30 μg/ml); Solution B: 4 μl DOSPER was diluted with HBS to a final volume of 50 μl (80 μg/ml). Solution A and B were then combined, mixed gently and incubated at room temperature for 15 min to allow the DOSPER/DNA complex to form. (v) Without removing the culture medium, 100 μl of the DNA/Liposome complex was added dropwise to the culture. Then mixed by gently rocking the culture plate. (vi) The cells were incubated for 6 h at 37°C. (vii) The transfection medium was then replaced and 2-3 ml of fresh complete growth medium. For stable selection of transfectants after 48 h, geneticin (Roche Molecular Biochemicals) was added to growth medium to a final concentration of 400 μg/ml. This medium was replaced every 48-72 h.

Antibody Preparation

Leishmanolysin was purified from a lysate of 2.5×10^{10} promastigotes of *L. major* with Triton X-114 [29], followed by size fractionation using SDS-PAGE with 10% separating gel. The band containing Leishmanolysin was excised from the gel and mixed by equal volume of complete Freund's adjuvant and was used to immunize Newseland white rabbits by three intramuscular injections (i.m.) at two weeks intervals [16].

Antibody Purification

A column was constructed from a 10 ml syringe barrel connected to a short length of rubber tubing, and was closed off by a clip, the bottom of the column as packed with glass wool. 8.5 ml of the protein G coupled sepharose-4B (Sigma) was poured into the column. 5 ml PBS, pH 7.4, was added slowly to the top of the column and displaced fluid was collected into a waste breaker. Then 5 ml of elution buffer (0.05 M diethylamine pH 11.5) was added to the top of the column and displaced fluid was collected again. 5 ml PBS was added to the top of the column and after collection of displaced fluid, 100 µl of antibody was loaded on the top of the column, PBS was passed through the column until all the unbound proteins were washed out (by measuring the OD at 280 nm). Elution buffer was passed through the column to collect bound antibodies, using tubes containing 1 M PBS, pH 6.8 (1/20 collection volume). Eluted antibodies dialyzed against several changes of PBS containing 0.1% sodium azide. The protein concentration was determined by absorbance at 280 nm.

Expression of Leishmanolysin

Rhabdomyosarcoma and COS-7 stable transfectants were harvested and washed two times with PBS. Cells (10^7) were lysed in 1% Nonidet-40, 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.02% NaN3 supplemented with 1 mM phenylmethyl sulfonyl fluoride (PMSF), 2 µg/ml aprotinin, 2 µg/ml Leupeptin and 1 µg/ml pepstatin A) and was sonicated briefly to reduce viscosity [30].

Enzyme-linked Immunosorbant Assay (ELISA)

Purified polyclonal antibody with a concentration of 3 µg/µl (100 µl/well) in bicarbonate buffer (pH 9.6) was placed in an ELISA plate and left overnight at 4°C. The plate was washed three times with PBS, pH 7.4, 200 µl/well 4% skimmed milk powder in PBS and was left for 1 h at room temperature (RT). The plate was washed three times with PBS-T (Tween 20, 0.05% in PBS). 100 µl/well of antigen diluted (1/20) in ELISA buffer (4% skimmed milk powder plus 0.05% Tween 20 in PBS) was added and left for 2 h at RT. The plate was washed three times with PBS-T. 100 µl/well mouse anti-Leishmanolysin antibody diluted in ELISA buffer was added and left for 2 h at RT, and then the plate was washed three time with PBS-T. HRP-labeled anti-mouse antibody, 100 µl/well with optimal concentration (1/1000) in ELISA buffer was added and left for 2 h at RT. Tetramethylbenzidine substrate (0.1 mg TMB in 100 µl DMSO and then added 9.9 ml of 0.1 M sodium acetate pH 6.0 and 3.3 µl of 30% hydrogen peroxide), 100 µl/well was added and left at RT in dark. Once sufficient color has developed in the positive wells, 50 µl/well 12% sulfuric acid was added to stop the reaction and absorbance was measured at A450 nm using ELISA plate reader.

Results

PCR-mediated amplification of the functional region of the Leishmanolysin was carried using genomic DNA preparation from a *Leishmania* strain that was isolated from an endemic region of Iran. To determine specifications of the cloned gene, the PCR product was initially analyzed by restriction enzyme digestion and DNA sequencing. A fragment of 1462 bp was identified. Restriction analysis of the amplified Leishmanolysin gene exhibited the presence of *Eco RV, Nco I* and *Pst I* at 766, 1272 and 1246 sites (Fig. 1).

This full-length product was first ligated to a pUC 19 based T-vector and then the ligation ix was transformed into *E. coli* Top 10 strain. The recombinants were chosen by white-blue selection. Out of 100 colonies, on plates containing IPTG-Xgel, only two were white. After selecting and further purification of these clones, digestion of the corresponding plasmids with *Hind III* and *Bam HI* gave rise to a 1443 bp band related to the Leishmanolysin gene.

After cloning of product in pcDNA3, by Hind III and Bam HI, the recombinant plasmids were further analyzed by digestion with *Cla I, Pst I* and *Eco RV* enzymes. *Cla I* digested the 1668 site of Leishmanolysin preproprotein gene, *Pst I* digested the 1246 site of Leishmanolysin preproprotein gene and 948 and 2333 sites of pcDNA3, and *Eco RV* digested the 766 site of Leishmanolysin preproprotein gene and 951 site of pcDNA3 (Figs. 2 and 3).

The sequenced region of the Leishmanolysin was analyzed further with Blast program no www.ncbi.nlm.nih.gov/blast. The sequenced PCR product was identical to the sequence of *Leishmania major* (*L. major*) Leishmanolysin gene 1 (emb/Y00647/LM GP63), while it showed a considerable variation from the Leishmanolysin gene 6 (gp63-6), (gb/AF039721/AF039721).

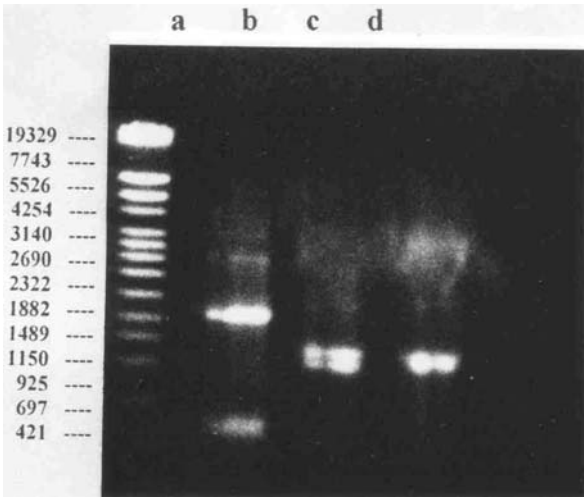

Figure 1. Restriction map of amplified segment. Digestion of product with different restriction enzymes. Lane a, DNA molecular weight marker IV (Roche molecular biochemicals). Lane b *Eco RV*, lane c *Nco I*, lane d *Pst I*.

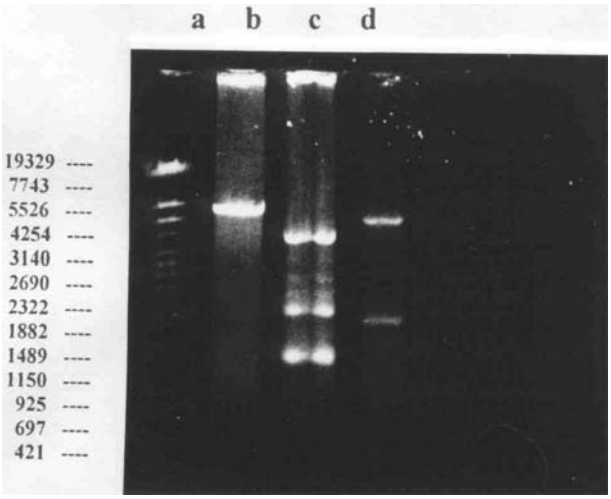

Figure 2. Restriction analysis of recombinants by digestion with different restriction enzymes. Lane a, DNA molecular weight marker IV (Roche molecular biochemicals), lane b *Cla I*, lane c *Pst I* and lane d *Eco RV*.

The predicted amino acids sequence of Leishmanolysin gene 1, Leishmanolysin gene 6 and cloned Leishmanolysin active protein gene products were compared and the results are shown in Figure 4. The cloned sequence was coded for 580 amino acids (100-579). Comparison of this sequence between gene one and six copies of *L. major* showed that 44 amino acids varies between these two copies. From these 44 amino acids, 28 amino acids variation was located between 421-480 amino and other 16 amino acids variation, was distributed among the other sites. The amino acid sequences of 222-277, 297-360 and 374-407 had complete homology to sequences of gene six copy. The sequence of 540-577 varied in 6 amino acids (15.8%) compared to homologe sequence of gene six copy.

The homology of conserved domains involved in macrophage binding and internalization between Leishmanolysin of *L. guyanensis* and cloned Leishmanolysin active protein of *L. major* is shown in Figure 5. In amino acid sequences of 222-277, 297-360 and 374-407 respectively 9, 7 and 8 amino acids (16%, 11% and 23%) were varied with respect to homologe sequence of *L. guyanensis*. The sequence of 540-577 was varied in 12 amino acids (33%).

Discussion

The PCR-mediated amplified product contains initiation and termination codes and the Kozak sequence. Cloning of this product in pcDNA3 leads to the expression of coding sequence for Leishmanolysin active protein (amino acids 100-579 of preproprotein). The expression of this sequence in Rhabdomyosarcoma TE671/RD and COS0-7 cell lines was showed by Enzyme Linked Immunosorbant Assay.

Leishmanolysin active protein is a Zinc proteinase with enzymatic activity. In addition, it contains the domains that are necessary for the binding of the parasite to host macrophage and their internalization. Some domains in the active protein may have a role in one or both of these functions. Such domains are philogenicly coserved and their importance exceeds across species and genuses.

In the Leishmanolysin of *Leishmania guyanensis*, the particular peptide present in the two protein core domains efficiently are involved the internalization of promastigotes in four different of *Leishmania* species. These species are highly conserved among different *Leishmania* species. One of the two functional core domains involves a previously described adhesive sequence (SRYD) and the putative zinc-binding motif (HXxxH). The second functional core domain includes a third histidine residue coordinated with zinc which determines the molecule's structural features [26].

Until now, seven copies of Leishmanolysin gene has been identified in *L. major* [10]. Leishmanolysin genes 1-5, encode a 2.6 Kb transcript in the promastigote stage similar to *L. chagasi* C gene and *L. mexicana* C2/C3 genes. Leishmanolysin gene 6 encodes a 3.5 Kb Transcript in both amastigote and promastigote stages similar to expression pattern of *L. mexicana* C1 and *L. chagasi* C genes. Leishmanolysin gene 7, encodes a 2.6 Kb transcript in stationary phase promastigotes and a 4.0 Kb major transcript in amastigotes.

The divergent 3′ sequence of these genes may contain signals the influence the observed stage-specific

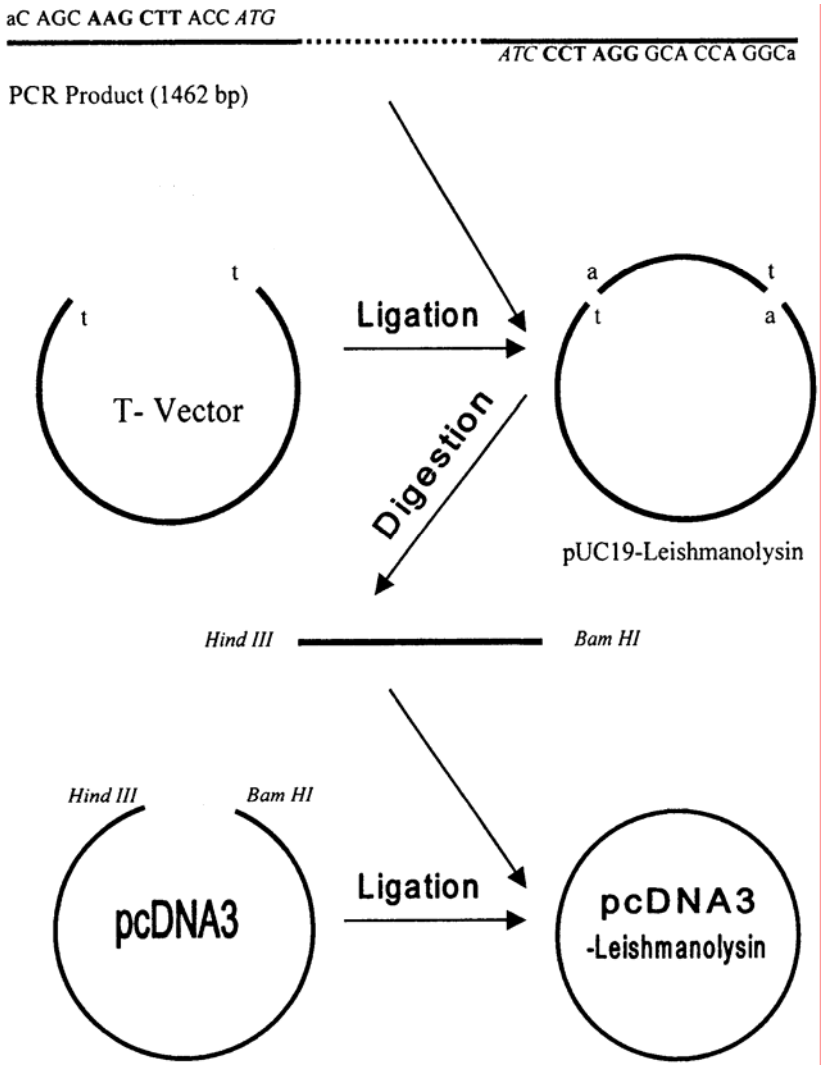

Figure 3. Schematic presentation of cloning steps of Leishmanolysin active protein gene.

(promastigote or amastigote) expression and previous studies have suggested a role for 3´-untranslated region and intergenic region in mediating stage-specific levels of gp63 gene products in *Leishmania* [31].

The predicted amino acid sequences of 222-277, 297-360 and 374-407 were completely conserved in Leishmanolysin of *L. major* gene 1 and 6 copies. The first sequence contains adhesive and zinc binding motif sequences. The second sequence contains the histidine residue coordinated with zinc and the third sequence, zinc binding site. The amino acid sequence of 540-577 varies in 6 amino acids, of which variation in 3 amino acids is related to the anchor attachment site of Leishmanolysin to parasite surface that is not related to the attachment of the parasite to host cells or to enzymatic activity.

When we compared the protein sequence of Leishmanolysin in *L. guyanensis* and *L. major*, it was clear that two most important regions of Leishmanolysin (amino acids sequences of 222-277, 297-360) which is essential for the attachment of the parasite to host macrophage and its internalization, are highly conserved (16% and 11% variation, respectively) in these two species. This conservation is also related to enzymatic activity. Moreover, two less conserved segments were found amino acid positions of 374-407 and 540-577 of preproprotein (23% and 33% variation, respectively). In *L. guyanensis*, one other sequence in active Leishmanolysin is known participate in the attachment and binding of the parasite to macrophages (amino acids of 420-460) but we did not see this sequence in *L. major*.

In *Leishmania guyanensis*, there is another segment which plays a role in binding and internalization of the

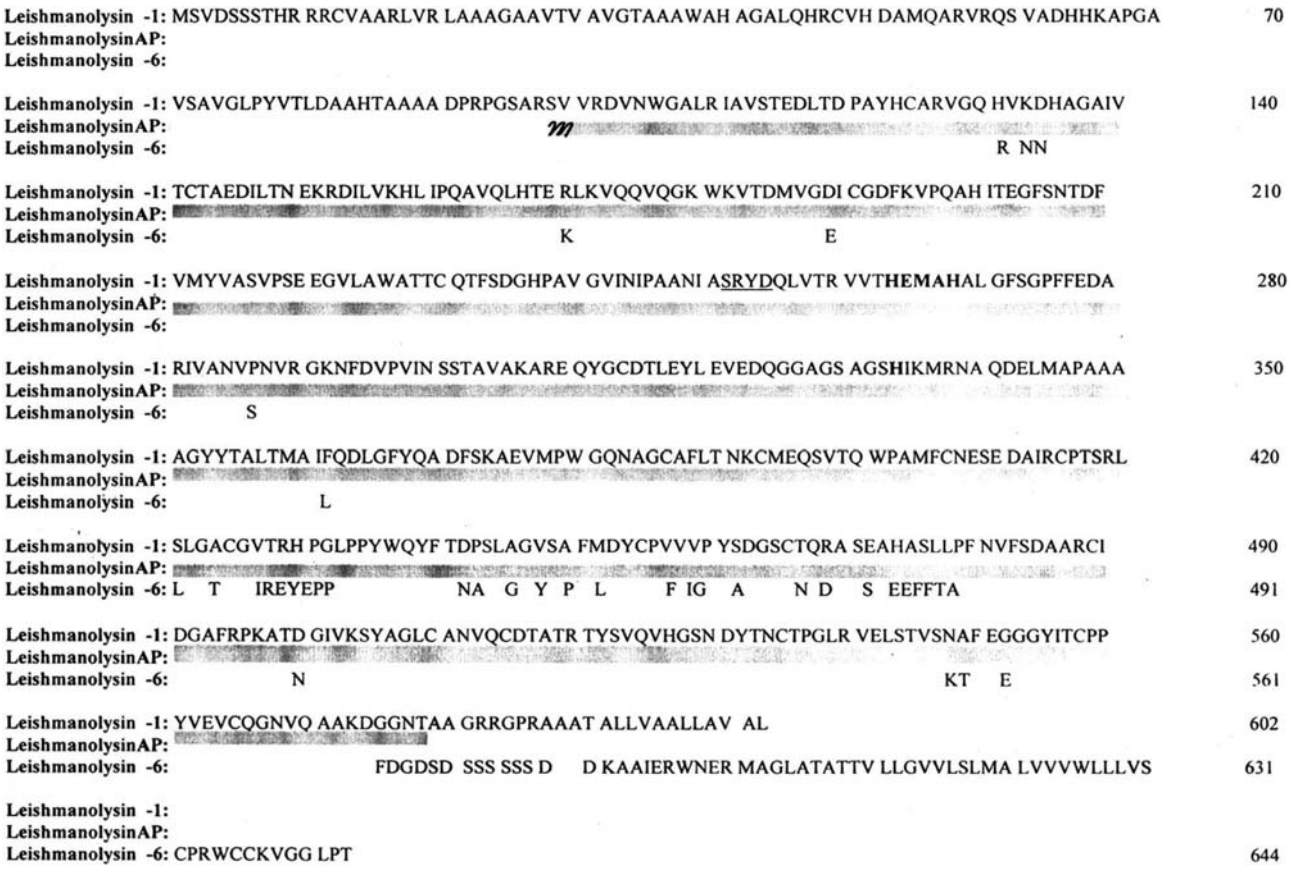

Figure 4. Comparison of the predicted amino acids sequence of Leishmanolysin gene 1, Leishmanolysin gene 6 and cloned Leishmanolysin active protein gene products. Amino acid identities between three sequences are indicated by a blank; differences are indicated by substitutions in Leishmanolysin 6 sequence. Cloned active Leishmanolysin sequence by gray box. Position 260-270 predicted active site. Adhesive sequence (SRYD) is underlined; Zinc-binding motif (HExxH) and third histidine residue coordinated with zinc are represented in boldface.

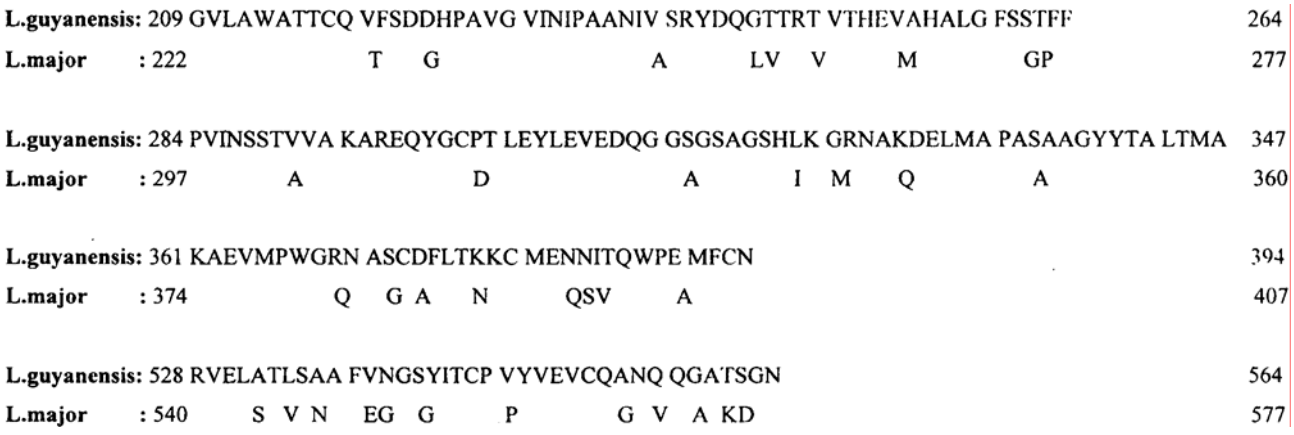

Figure 5. The homology between conserved domains, which have role in binding and internalization of parasite to macrophages in Leishmanolysin of *L. guyanensis* and cloned Leishmanolysin active protein of *L. major*. Amino acid identities between sequences are indicated by a blank; differences are indicated by substitutions in Leishmanolysin active protein of *L. major* sequence.

parasite [26]. This region is located in *L. guyanensis* propeptide but is less conserved in *L. major* complete gene, with a 63% homology among two species (22 amino acids out of 35 amino acids). This segment is located in propeptide and is not a part of our cloned fragment. Thus the role of this segment in binding and internalization of *L. major* should be investigated.

In all experiments on genetic vaccination against *Leishmania major* with Leishmanolysin gene till now, the DNA encoding with signal sequence and pro-region [32-33] for major surface glycoprotein has been used.

The different studies have shown that in genetic immunization, a plasmid with or without signal sequences can generate comparable levels of antigen for B-cell recognition and for uptake by antigen-presenting cells (APC), despite the differential intracellular targeting of the encoded antigen [34]. It can induce Th-1 type T-helper response along with humoral immune response against tetanus [35].

Since the highly conserved domains play a key role in binding of the parasite to the host cells and its internalization, these sequences may contain the most important domains for induction of a protective immune response.

The deletion of the signal peptide presumably instigates the host protective immune response. It seems that other sequences, which are located between the four most important domains either, have suppressive role or no role at all in immune response. Presumably, the deletion of these sequences would increase the protective immune response.

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