Reports of Biochemistry & Molecular Biology
Vol.7, No.1, Oct 2018

Original article

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Construction of a Novel DNA Vaccine Candidate encoding LmSTI1-PpSP42 Fusion Protein from *Leishmania major* and *Phlebotomus papatasi* Against Cutaneous Leishmaniasis

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Abstract

Background: Cutaneous leishmaniasis (CL) is a serious public health problem in many tropical countries. The infection is caused by a protozoan parasite of *Leishmania* genus transmitted by Phlebotominae sandflies. In the present study, we constructed a eukaryotic expression vector to produce a fusion protein containing LmSTI1 from *Leishmania major* (*L. major*) and PpSP42 from *Phlebotomus papatasi (Ph. papatasi)*. In future studies we will test this construct as a DNA vaccine against zoonotic CL.

Methods: The nucleotide sequences encoding the LmSTI1 protein and a fragment encoding 79% of PpSP42 were amplified using *L. major* and *Ph. papatasi* genomic DNA, respectively. The amplicons were cloned into the pcDNA3.1(+) eukaryotic expression vector. The recombinant plasmid pcDNA-LmSTI1Pp42 was propagated in *Escherichia coli* (*E. coli*) and used to transfect HEK-293T cells. The expressed fusion protein was analyzed by Western blotting using anti-LmSTI1 mouse serum.

Results: Sequences encoding LmSTI1 and partial PpSP42 were cloned into pcDNA3.1(+). Production of the recombinant LmSTI1Pp42 fusion protein was confirmed by Western blotting.

Conclusions: An LmSTI1Pp42 fusion protein was expressed in HEK-293T cells. This construct may be an effective DNA vaccine against CL.

Keywords: Cloning, DNA vaccine, *Leishmania major*, LmSTI1, PpSP42.

Introduction

Leishmaniases are a group of important worldwide parasitic infectious diseases caused by obligatory intracellular protozoans of the genus *Leishmania*. These are transmitted to their vertebrate hosts, including humans, by the bite of infected female sandflies during blood feedings (1-3). Infections caused by *Leishmania* parasites manifest in a wide range of clinical forms in humans (4), including asymptomatic, cutaneous, diffuse cutaneous, mucosal cutaneous, visceral, and post-kala-azar dermal leishmaniases (5). Cutaneous leishmaniasis (CL) is the most common form of the disease, caused by propagation of the parasite inside the host's dermal macrophages. Two-thirds of all new CL cases occur in Afghanistan, Algeria, Brazil, Colombia, Iran, and Syria (1). Cutaneous leishmaniasis is highly prevalent in Iran; in 2012–2013, 37,001 cases were reported with the incidence estimated at 25/100,000 people (6).

Despite efforts by researchers worldwide, CL remains an important public health problem in many parts of Asia, North Africa, and Central and South Americas. Chemotherapeutics against the disease are currently available; however, due to toxicity, side effects, and high costs, they cannot be readily used. In addition, no effective vaccine has yet been

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Received: Dec 12, 2017; Accepted: Jan 15, 2018

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authorized against CL (7-9). In recent years, the complex relationship between the vector, the parasite, and the vertebrate host has been recognized. It has been shown that components in the vector's saliva contribute to the infection (10-12).

The induction of cellular immune responses, and more specifically a CD4+ Th1 response, is essential to control the infection. A proper response against CL involves activation of the infected macrophages by secretion of Th1 cytokines including IFN-γ. The parasites inside the phagolysosomes of the activated macrophages are then destroyed by cytotoxic reagents including hydrogen peroxide and nitric oxide (13). A protective CL vaccine should induce sufficient cellular immunity and cell responses to overcome the infection (14).

In lieu of safe, efficient, and affordable anti-leishmanial vaccines or drugs, new modes of prophylactics and therapies are essential to combat CL (9). In this regard, DNA vaccines have been shown to enhance long-lived humoral and cellular immune responses *in vivo* in a variety of animal models (15). Moreover, vaccination with plasmid DNA has been shown to induce protective immunity through both MHC class I- and class II-restricted T cell responses in a variety of experimental infection models, while in general, conventional protein vaccinations have been shown to induce only MHC class II-restricted responses (16). A potent immune response against CL is unlikely to be induced by only one antigen; therefore, different antigenic compounds need to be studied (17). Incorporation of such candidate antigens in plasmids encoding more than one antigen may enhance DNA vaccine immunogenicity (18). As the first step of an ongoing project with the aim of developing a DNA vaccine against CL, a recombinant plasmid with one antigen from the parasite and another from its vector was constructed. From *Leishmania major* (*L. major*), we used LmSTI1, a homologue of yeast stress-inducible protein STI1, which contains six copies of tetratricopeptide (TPR) consensus motif and is related to a highly-conserved family of stress-inducible proteins (19). Webb and colleagues in 1996 identified the full-length *L. major* LmSTI1 gene, which is expressed in a temperature-dependent manner in both *L. major* promastigotes and amastigotes by a 26-37 °C shift in the culture temperature. Vaccination experiments with recombinant LmSTI1 protein plus either IL-12 or LmSTI1 DNA resulted in a mixed cellular response that was skewed toward a Th1 phenotype and protected BALB/c mice (19-22). We also used exon 2 of a gene that encodes 79% of Ph. papatasi salivary gland protein 42 (PpSP42) in the construct.

The sandfly's saliva contains proteins that modulate the mammalian host's immunological and physiological responses to the bites to accommodate parasitic invasion and establishment of the infection (23, 24). Different sandfly salivas differ in composition (25), and the induced immune responses are species-specific (26). PpSP42 is a homolog of LJM11 salivary gland protein of *Lutzomyia* (*Lu.*) *longipalpis*, the *L. brasiliensis* vector in the New World which its long-term protection against CL has been recently shown (27). Among the interesting attributes of this protein are its lack of homology with mammalian proteins, its capability to induce antibodies in humans and dogs, and its ability to be over-produced in prokaryotic expression systems (28, 29). PpSP42 was identified by Valenzuela and colleagues in 2001; however, its function remains to be characterized (23). Here, following construction of a plasmid to express LmSTI1Pp42, its production in mammalian cells was demonstrated using human embryonic kidney 293 (HEK) cells.

Materials and methods

Parasite

L. major promastigotes MRHO/IR/75/ER were cultured in RPMI 1640 medium (Biosera, France) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biosera, France), 100 U/ml penicillin, and 100 µg/ml streptomycin, and incubated at 24 ˚C. The promastigotes were harvested in mid-logarithmic phase at a density of $2x10^6$/ml.

Bioinformatics analyses

Nucleotide sequences were verified by Chromas Lite 2.01 (Technelysium Pty Ltd., Australia) and Sequencher 4.8 (Gene Codes, USA). The nucleotide-to-amino acid translations and the theoretical protein molecular weight estimations

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were performed by ExPASy Bioinformatics Resource Portal < http://web.expasy.org/>.

Genomic DNA extraction

Genomic *L. major* DNA was extracted from the promastigotes by phenol/chloroform method. Briefly, 2×10^7 promastigotes were pelleted by centrifugation for 10 min at 800 x *g*, washed with phosphate buffered saline (PBS), and lysed in 350 µl lysis buffer containing 0.1 M Tris-HCl, pH 8.0, 1% sodium dodecyl sulfate (SDS), 0.1 M NaCl, l0 mM EDTA, and 3.5 µl of proteinase K (100 µg/ml) at 55 °C for 2 h. The lysate was added to an equal volume of phenol/chloroform (450 µl) to remove proteins. This mixture was centrifuged at 13,400 x *g* at 4 °C for 15 min and an equal volume of chloroform was added to the supernatant, which was then re-centrifuged as above. The supernatant was mixed with 1/10 volume of 3 M sodium acetate and two volumes of 100% ethanol to precipitate the DNA, and centrifuged as above for 10 min. The DNA pellet was washed with 70% ethanol, dissolved in 100 µl of sterile distilled water, and stored at -20 °C until use. Genomic DNA from a female *Ph. papatasi* sandfly trapped in Kaleibar region of East Azerbaijan (Iran) and isolated by Dr. Parviz Parvizi, Department of Parasitology, Pasteur Institute of Iran, was obtained as a gift. The DNA concentrations and their quality were assessed by spectrophotometry on a NanoDrop 1000a (Thermo Scientific, USA) and electrophoresis on 1% agarose gels.

PCR amplifications and sub-cloning of LmSTI1 and PpSP42

Promastigote genomic DNA was used as the template to PCR amplify the 1641-bp full-length LmSTI1 gene encoding a 62.1 kDa protein. Forward and reverse primers were designed based on DNA sequences of LmSTI1 available in GenBank by accession number U73845 (Table 1).

Table 1. PCR primers. The underlined segments depict the incorporated restriction sites. The bold letters on LmSTI1 F and PpSP42 R show a Kozak and a stop codon, respectively.

Gene	Primer (5'- 3')
LmSTI1 F	ATCTGGTACC**CACCATGGCG**GACGCAACTGAGCTGAAGAAC
LmSTI1 R	GGTCGAATTCCTGACCAAAACGAATGATGCCAGCTG
PpSP42 F	GCTCGAATTCGCTTACGATTCAGGAAATATTGTACCAAC
PpSP42 R	ATCACTCGAG**TCA**CATAATGTCTGTGCCAAAATTGAAG

The forward primer contained a *Kpn*I (GGTACC) restriction site and a Kozak translation initiation sequence consensus (CACCATGGCG). The reverse primer contained an *Eco*RI (GAATTC) restriction site. To amplify PpSP42, *Ph. papatasi* genomic DNA was used as the template along with primers, designed using DNA sequences available in GenBank by accession number KX611849.1 (Table 1). The lack of introns between the primers and the exons was verified by comparing *Ph. papatasi* sp42 mRNA (GenBank accession number AF335491.1) with the *Ph. Papatasi* genome sequence (NCBI PRJNA20293; Fig. 1).

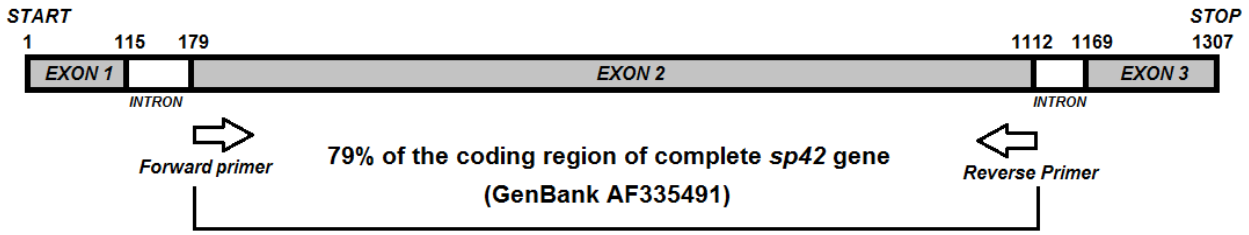

Fig. 1. Schematic view of *Ph. Papatasi* PpSP42 gene (GenBank accession number AF335491) exons and introns, depicting the locations of the primers on exon 2.

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The forward and reverse primers, containing *EcoR*I (GAATTC) and *Xho*I (CTCGAG) restriction sites, respectively, and a stop codon (TCA) in in the reverse primer, were used to amplify a 945-bp amplicon. The amplifications were performed in 25 µl volumes containing 1 µl of genomic DNA as the template, 10 pmol of each primer, 1.5 mM $MgCl_2$, and ExPrime Taq™ DNA Polymerase (Genet Bio, Republic of Korea). The thermocycling program was 94 ºC for 10 min initial denaturation, followed by 30 cycles of 94 º C for 1 min, 60 ºC for 1 min, and 72º C for 2 min, with a final extension at 72 ºC for 10 min. The amplicons were electrophoresed on 1% agarose gels, stained with DNA Green viewer (Pars Tous, Iran), and visualized on a UV transilluminator. The size markers used to estimate PCR products were 100-bp and 1-kbp DNA ladders (SinaClon, Iran).

The LmSTI1 and Pp42 amplicons were purified with a PrimePrep™ PCR Purification Kit (GeNet Bio, South Korea) and ligated into the pTZ57R/T cloning vector (InsTAclone™ PCR product cloning kit, Thermo Scientific, USA) following the manufacturer's protocol. The resulting colonies were screened by colony-touch PCR using the above primer sets specific to each amplicon (Table 1). Extracted plasmids were prepared from the TA-cloned plasmids using a TIANprep kit (Tiangen, China). The integrities of pT-LmSTI1 and pT-PpSP42 plasmids were verified by nucleotide sequencing using M13F and M13R universal primers from Gen Fanavaran Co. (Iran).

Construction of eukaryotic expression vector for the fusion protein

LmSTI1 was cloned into pcNDA3.1 (+) eukaryotic expression vector to produce pCLmSTI1 as follows: the TA-cloned pT-LmSTI1 plasmid and pcDNA3.1 (+) were double-digested separately with *Kpn*I and *EcoR*I (Fermentas, Germany) in 1X Tango buffer. The double-digestion reactions were performed in two steps. First, in 40 µl volumes, 200 ng of pT-LmSTI1 or pcDNA3.1 (+) were incubated with 4 µl of *Kpn*I at 37 °C for 2 h. Second, 1 µl of *Eco*RI was added to each reaction and the mixtures were incubated at 37 °C for an additional 2 h. The digested products were analyzed by electrophoresis on 1% agarose gels as described above. The bands belonging to the double-digested LmSTI1 and the vector fragments were extracted from the agarose gel with an AccuPrep® Gel Purification Kit (Bioneer, Germany). The fragments were then ligated in a 1:1 molar ratio using T4 DNA ligase (Vivantis, Malaysia). *Escherichia coli* Top10 cells were transformed with the preliminary recombinant plasmid, named pCLmSTI1. Colonies grown on agar plates containing 50 µg/ml of ampicillin were screened by colony-touch PCR using LmSTI1-specific primers. The expected amplicon size was 1641 bp. A selected colony was grown overnight and pCLmSTI1 was extracted as described above. The plasmids were analyzed by *EcoR*I single- and *Eco*R1/*Kpn*1-doouble-digestions. The expected linear plasmid sizes from the single digestion were 7068 bp, and for the *EcoR*I/*Kpn*I double-digestion, 1640 bp for the LmSTI1 insert and 5428 bp for pcDNA3.1(+). The integrity of the construct and the lack of deletions or mutations were verified by nucleotide sequencing (Gen Fanavaran Co., Iran).

Next, PpSP42 was added to pCLmSTI1 to produce pCLmSTI1Pp42 as follows: the recombinant plasmids PpSP42 and pCLmSTI1 were double-digested at 37 °C for 2 h with 1 µl each of *Xho*I and *EcoR*I (Fermentas, Germany) in separate 20 µl volumes containing 2 µg of DNA and 1X Tango buffer. The digestion products of expected fragment sizes of 939 bp for PpSP42 and 7064 bp for pCLmSTI1 were gel-extracted and ligated as above. The recombinant fusion plasmid, named pCLmSTI1Pp42, was used to transform competent *E. coli* Top10 cells. Colonies were selected as above and recombinants were identified by *EcoR*I digestion with expected linear plasmid size of 7950 bp and *EcoR*I/*Xho*I double digestion with expected fragment sizes of 2595 bp for the LmSTI1Pp42 insert and 5428 bp for pcDNA3.1(+), followed by DNA sequencing as described above.

Transfection of HEK-293T eukaryotic cells with the recombinant plasmid

HEK-293T cells were transfected with pCLmSTI1Pp42 using X-tremeGENE HP DNA Transfection Reagent (Roche, Germany). After two passages the cells were seeded at 4×10^5

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cells/2 ml medium/well in two wells of a six-well plate with 9.4 cm^2 diameter wells, and grown in RPMI 1640 medium (Biosera, France) supplemented with 10% FCS (Biosera, France), 1% glutamine, and 0.1% penicillin/streptomycin in aseptic conditions, and incubated at 37 °C in 5% CO_2 with 70% humidity for 48 h. The transfection reagents for each well contained 200 µl of serum-free RPMI medium, 6 µl of transfection reagent (1:3 ratio), and 4 µg of either pCLmSTI1Pp42 or pcDNA3.1 (+) as a control. After mixing, the plate was incubated for 15 min at room temperature and the transfection complexes were added dropwise to the 60-70% confluent cells with gentle shaking to ensure even distribution of the ingredients in each well. The plate was incubated for 72 h and protein expression was assayed.

Extraction of recombinant proteins, SDS-PAGE, and Western-blotting

Following incubation with the transfection reagents, the cells were harvested and washed three times with sterile PBS and centrifugation at 13,400 x g at 4 °C for 1 min. The cells were then lysed with 300 µl of NP40 non-denaturing lysis buffer containing 150 mM NaCl, 50mM Tris-HCl, pH 8.0, 1% NP-40, and protease inhibitors, and incubated for 30 min at room temperature. The cell lysates were vigorously vortexed for 10 min and then centrifuged at 13,400 x g at 4 °C for 20 min. The clear cell lysate supernatants were collected and stored at -80 °C until use.

Proteins were separated by 8% SDS-PAGE using 100 µl of the clear lysates along with pre-stained protein ladder SM7002 (SinaClon, Iran) at 40 V for 2 h. The gel was stained overnight with Coomassie Blue staining solution. An additional SDS-PAGE was performed and the polypeptides from the gel were transferred to a PVDF membrane (Millipore, USA) at 15 V for 30 min. The membrane was blocked with PBS containing 1% BSA (Sigma, Germany) for 2 h and then incubated overnight at 4 °C with a polyclonal murine anti-LmSTI1 antibody diluted 1/100. After washing, the membrane was incubated for 2 h with anti-mouse IgG-HRP (Sigma, Germany). The proteins were detected with DAB (Bio-Rad, Germany) staining.

Results

Construction of the DNA vaccine

A 1641-bp *L. major* LmSTI1 amplicon and a 930-bp *Ph. papatasi* PpSP42 amplicon were obtained by PCR (Fig. 2). The predicted sizes of the cloned fragments were verified by double-digestions of pCLmSTI1Pp42 with *Kpn*1/*Eco*R1 and *Kpn*1/*Xho*1 (Fig. 3), and the integrities of pCLmSTI1 and pCLmSTI1Pp42 were verified by DNA sequencing. Based on the sequencing results, the expressed pCLmSTI1Pp42 fusion protein contains 859 amino acids (Fig. 4).

Expression and detection of LmSTI1Pp42 fusion protein in eukaryotic cells

HEK-293T cells were transfected with pCLmSTI1Pp42 and expression of the fusion protein with an approximate Mw of 100 kDa (theoretical Mw: 97.1 kDa) was verified by SDS-PAGE and Western blotting with a polyclonal anti-LMSTI1 antibody (Fig. 5).

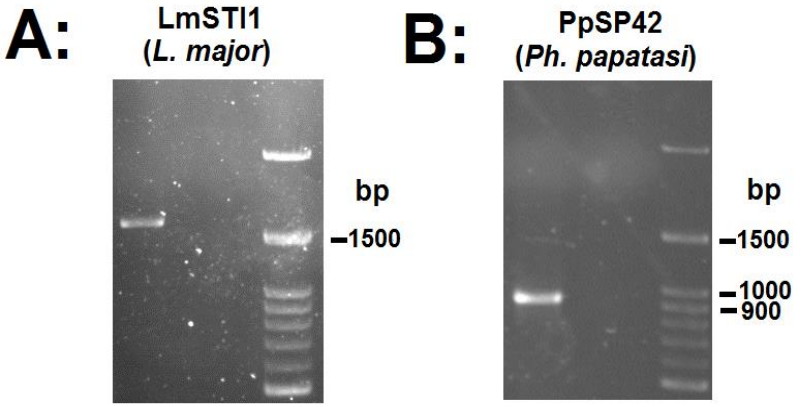

Fig. 2. A: Agarose gel electrophoresis of A: the full length *L. major* LmSTI1gene fragment (expected size of amplicon: 1641bp), B: *Ph. papatasi* partial PpSP42 fragment (expected size of amplicon: 930 bp).

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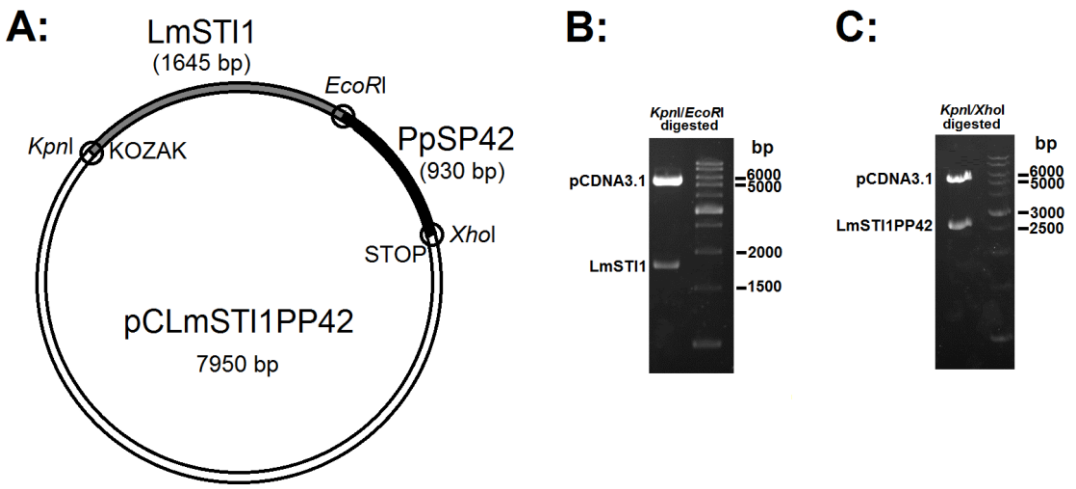

Fig. 3. A: Schematic diagram of pCLmSTI1Pp42 construct composed of pcDNA3.1(+) eukaryotic expression vector and in-frame *L. major* LMSTI1 and *Ph. Papatasi* SP42 genes. B: pCLmSTI1 double-digested with *Kpn*I and *EcoR*I (expected digested fragments: ~5400 bp and ~1600 bp). C: pCLmSTI1Pp42 double-digested with *Kpn*I and *Xho*I (expected digested fragments: ~5400 bp and ~2500 bp).

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  1 MADATELKNK GNEEFSAGRY VEAVNYFSKA IQLDEQNSVL YSNRSACFAA MQKYKDALDD
 61 ADKCISIKPN WAKGYVRRGA ALHGMRRYDD AIAAYEKGLK VDPSNSGCAQ GVKDVQVAKA
121 REARDPIARV FTPEAFRKIQ ENPKLSLLML QPDYVKMVDT VIRDPSQGRL YMEDQRFALT
181 LMYLSGMKIP NDGDGEEEER PSAKAAETAK PKEEKPLTDN EKEALALKEE GNKLYLSKKF
241 EEALTKYQEA QVKDPNNTLY ILNVSAVYFE QGDYDKCIAE CEHGIEHGRE NHCDYTIIAK
301 LMTRNALCLQ RQRKYEAAID LYKRALVEWR NPDTLKKLTE CEKEHQKAVE EAYIDPEIAK
361 QKKDEGNQYF KEDKFPEAVA AYTEAIKRNP AEHTSYSNRA AAYIKLGAFN DALKDAEKCI
421 ELKPDFVKGY ARKGHGYFWT KQYNRALQAY DEGLKVDPSN ADCKDGRYRT IMKIQEMASG
481 QSADGDEAAR RAMDDPEIAA IMQDSYMQLV LKEMQNDPTR IQEYMKDSGI SSKINKLISA
541 GIIRFGQEFA YDSGNIVPTG VAYDAASKML FFGIPRKYSK VPITLAQLST RSYNSAEIPN
601 PPLDKFSGKS KQPLSSVYQP VIDDCRRLWV LDVGIVENEA ERKTYPIIKP SLIAFDLTKS
661 NYPEIHRYEL TGEAGKNPLG YGGFAADVVN PKRCSDKNEK TYIYIANFDE NSLIVYDKKK
721 GEAWSLKDDS FKPEGVTTFT LNGKEHKFKA GIFGIALGDR NKEGNRPAYY LAGSSTKLYR
781 LDTKLLKKKS SKLEPKLIGD RGFKTEAIAL AHDPETKVLF FAEADSRQVS CWNIKHELKP
841 ENVGVIYTNP NFNFGTDIM
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Fig. 4. Predicted amino acid sequence of the pCLmSTI1Pp42 fusion protein. The bold residues represent LmSTI1 and the underlined residues represent the partial PpSP42 protein sequence. The highlighted grey residues are encoded by pcDNA3.1(+).

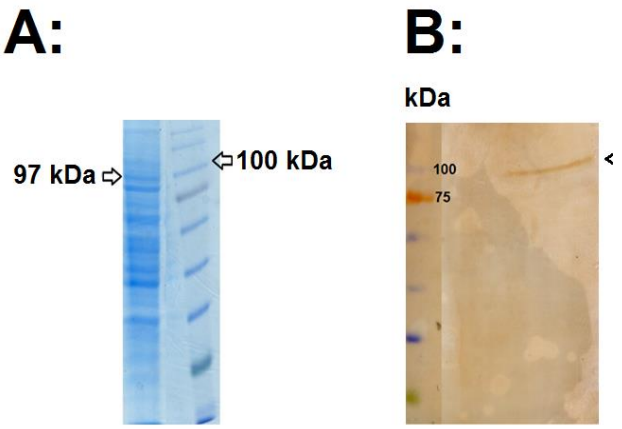

Fig. 5. A: SDS-PAGE of clear lysate of pCLmSTI1Pp42-transfected HEK-293T cells, after 72 h of incubation. B: Western blot of clear lysate of pCLmSTI1Pp42-transfected HEK-293T cells after 72 h of incubation. Protein was detected by polyclonal murine anti-LmSTI1 antibody

Discussion

Leishmaniasis remains a major health problem in many countries worldwide and the infection rate has remained steady in recent decades. Although limiting the reservoirs and vectors as well as identifying new drugs offers promise, particularly in view of the emerging drug resistances, development of safe and effective vaccines remains the best hope for achieving a definitive control of leishmaniasis (30). The major steps to increase the efficiency of a new vaccine against CL are to identify suitable new antigens and adjuvants and recognize the type of immune response generated by the protected hosts (31). Achieving an effective vaccine against CL should therefore trigger Th1 immune responses, which could potentially be due

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to recognition of specific antigens from both the vector and parasite.

One of the main strategies to control CL is to develop a DNA vaccine. The immune responses against DNA vaccines can induce both CD4+ and CD8+ T cells, which leads to polarized cellular immunity, manifested by activated Th1 cell signaling, similar to an adjuvant (32). In contrast, production of a Th2 immune response is associated with susceptibility to, and intensification of, the disease (33). In this regard, various antigens including TSA, KMPII, TRYP, LACK, GP63, KMP-11, CPA, CPB, PSA2, H1, Leish-111f, and P4 have been evaluated as parts of potential DNA or recombinant vaccines against leishmaniasis (22, 34-41). Among the antigens of interest, LmSTI1, which is constitutively expressed in both *L. major* promastigotes and amastigotes, has been proposed as a vaccine candidate against cutaneous and visceral leishmaniasis (20, 22). Moreover, several *Ph. papatasi* and *Lu. longipalpis* salivary proteins have been characterized. Of these, SP15 and LJM19 have been used in DNA vaccine assays with various degrees of success (23, 42-44).

Here, we expressed a recombinant eukaryotic plasmid containing the *L. major* LmSTI1 gene in conjunction with exon 2 of the *Ph. papatasi* PpSP42 gene in HEK-293T eukaryotic cells. The potential protection by this vaccine candidate against experimental *L. major* infection will be evaluated in our laboratory in an animal model.

Acknowledgment

This project was funded by research grant # IPI-726 from the Pasteur Institute of Iran. The authors thank Dr. P. Parvizi, Department of Parasitology, Pasteur Institute of Iran, for providing *Ph. papatasi* genomic DNA. We also thank Dr. F Riazi-Rad, Department of Immunology, Pasteur Institute of Iran, for his technical assistance. This article has been extracted from the Ph.D. thesis written by T. Miandoabi (registration no. 219) from the Department of Medical Parasitology and Mycology, School of Medicine at Shahid Beheshti University of Medical Sciences.

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